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REVIEW ARTICLE

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Molecules targeting the androgen receptor (AR) signaling axis beyond the AR-Ligand binding domain

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Abstract

Prostate cancer (PCa) is the second most common cause of cancer-related mortality in men in the United States. The androgen receptor (AR) and the physiological pathways it regulates are central to the initiation and progression of PCa. As a member of the nuclear steroid receptor family, it is a transcription factor with three distinct functional domains (ligand-binding domain [LBD], DNA-binding domain [DBD], and transactivation domain [TAD]) in its structure. All clinically approved drugs for PCa ultimately target the AR-LBD. Clinically active drugs that target the DBD and TAD have not yet been developed due to multiple factors. Despite these limitations, the last several years have seen a rise in the discovery of molecules that could successfully target these domains. This review aims to present and comprehensively discuss such molecules that affect AR signaling through direct or indirect interactions with the AR-TAD or the DBD. The compounds discussed here include hairpin polyamides, niclosamide, marine sponge–derived small molecules (eg, EPI compounds), mahanine, VPC compounds, JN compounds, and bromodomain and extraterminal domain inhibitors. We highlight the significant in vitro and in

Abbreviations: ADT, androgen deprivation therapy; Akt, protein kinase B [PKB]; AR, androgen receptor; ARE, androgen response element; AR_{FL}, full-length AR; AR_{SV}, splice variant AR; BET, bromodomain and extraterminal domain; BETi, bromodomain and extraterminal domain protein inhibitor; BPA, bisphenol A; CRPC, castration-resistant prostate cancer; DBD, DNA-binding domain; DHT, dihydrotestosterone; ECM, extracellular matrix; EMT, endothelial mesenchymal transition; ER, estrogen receptor; ERK, extracellular signal–regulated kinases; ETS, erythroblast transformation-specific; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HR, homologous recombination; IDP, intrinsically disordered protein; IL-6, interleukin 6; Im, *N*-methylimidazole; JAK, Janus kinase; Kac, acylated lysine; LBD, ligand-binding domain; LRP6, low-density lipoprotein receptor–related protein 6; MAPK, mitogen-activated protein kinase; mCRPC, metastatic castration-resistant prostate cancer; mTOR, mammalian target of rapamycin; NHR, nuclear hormone receptor; PARP, poly (ADP-ribose) polymerase; PCa, prostate cancer; PI3K, phosphatidylinositol-3 kinase; PPARγ, peroxisome-proliferator-activated receptor-γ; PR, progesterone receptor; PSA, prostate-specific antigen; PTEN, phosphatase and tensin homolog; Py, *N*-methylpyrrole; RNAP2, RNA polymerase II; SAR, structure-activity relationship; SPOP, speckle-type POZ protein; STAT, signal transducer and activator of transcription; TAD, transactivation domain; TMPRSS, transmembrane protease serine 2.

vivo data found for each compound and the apparent limitations and/or potential for further development of these agents as PCa therapies.

KEYWORDS
androgen receptor, androgen receptor degradation, androgen receptor signaling axis, androgen receptor DNA-binding domain, androgen receptor transactivation domain, castration-resistant prostate cancer, prostate cancer

1 | INTRODUCTION

1.1 | Physiologic role and regulation of the androgen receptor

The androgen receptor (AR) is a ligand-activated DNA-binding transcription factor of 110 kDa molecular weight, which facilitates the expression of androgen-dependent gene products (Figure 1).[1,2] As a member of the steroid and nuclear hormone receptor (NHR) super family, it shares many structural and functional features with other receptors such as the glucocorticoid receptor (GR), estrogen receptor (ER), mineralocorticoid receptor, progesterone receptor (PR), and the vitamin D receptor.[3,4] The nuclear steroid receptors consist of three principal domains: (1) the carboxy-terminal ligand-binding domain (LBD), (2) the central DNA-binding domain (DBD), and (3) the *N*-terminal transactivation domain (NTD or TAD; Figure 1). Endogenous androgens, such as testosterone and dihydrotestosterone (DHT) bind the AR-LBD to initiate AR activation. This results in dissociation of heat shock proteins, homodimerization of the AR, translocation to the nucleus, and recognition of and binding to palindromic *cis*-acting elements in target genes, which are known as androgen response elements (AREs; Figure 1). Transcriptional coregulators, including both transcriptional activators and repressors, are corecruited with the AR to ARE sites; the basal transcriptional machinery, including RNA polymerase II (RNAP2) and its cofactors, also form a complex with the AR and its coregulators, the net effect of which is gene regulation. Further comprehensive details on the structure and the function of the different domains of the AR can be found elsewhere.[5]

The principal source of androgens in an adult male is the testes, from where 90% of circulating androgens are derived. Most of the circulating androgens are represented by testosterone, which can be intracellularly converted into the more potent androgen, DHT, by 5α-reductase isoenzymes. Induction of gonadal testosterone synthesis is regulated by production of luteinizing hormone (LH) by the anterior pituitary, which in turn is stimulated by the pulsatile secretion of luteinizing hormone releasing hormone (LHRH) by the hypothalamus (Figure 1). Testosterone has a negative feedback effect on the anterior pituitary and hypothalamus to maintain physiological levels of serum testosterone. Surgical or medical castration thus prevents production of the main source of androgens. However, about 10% of serum androgens are derived from the adrenal glands, which can synthesize the weak androgens, dehydroepiandrosterone and androstenedione, which in turn can be peripherally converted in target tissues, such as prostatic epithelium, to testosterone. Adrenal androgen production is under the regulation of adrenocorticotropin hormone (ACTH) by the anterior pituitary (Figure 1), which in turn is regulated by hypothalamic secretion of corticotropin releasing hormone (CRH). In addition to weak androgens, steroids produced by the adrenals include mineralocorticoids and glucocorticoids, the latter of which results in negative feedback to the anterior pituitary and hypothalamus to control physiologic adrenal steroid production.

1.2 | The role of the AR in prostate cancer

Prostate cancer (PCa) is the second most common cause of cancer-related mortality in men in the United States. The estimated number of new US cases diagnosed for 2018 is 164 690, with an estimated 29 430 deaths due to

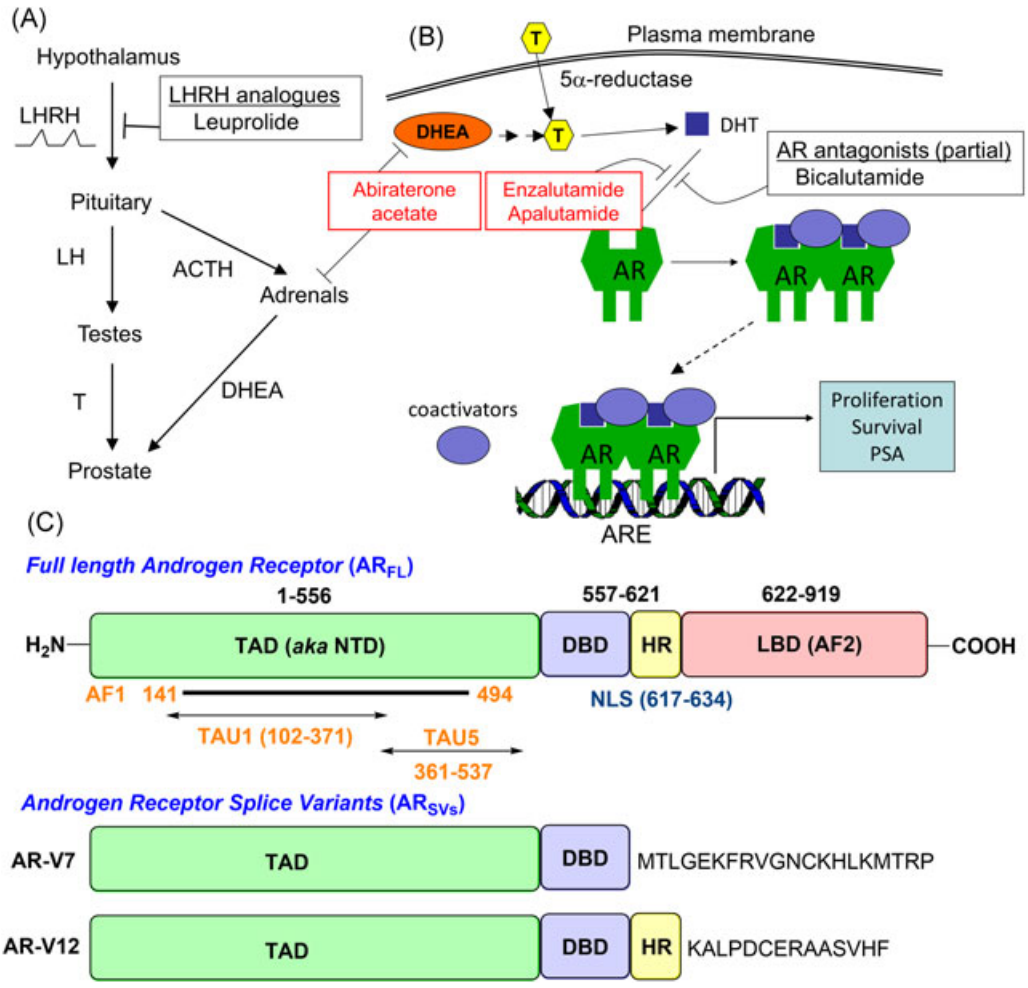


FIGURE 1 A, Hormonal regulation of androgen production by the hypothalamus. B, AR-dependent gene expression and effect of AR-antagonists. C, AR_{FL} and the clinically relevant splice variants (AR_{SVs}) AR-V7 and AR-V12.[1,11] Full-length receptor has three distinct domains (C-terminal ligand binding—LBD, DNA binding—DBD, and N-terminal transactivation—TAD/NTD), while the splice variants lack a functional ligand-binding domain. Most splice variants such as AR-V7 are constitutively active. ACTH, adrenocorticotropin hormone; AR, androgen receptor; AR_{FL}, full-length AR; DBD, DNA-binding domain; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; HR, hinge region; LBD, ligand-binding domain; LH, luteinizing hormone; LHRH, luteinizing hormone releasing hormone; NLS, nuclear localization signal; NTD, N-terminal transactivation domain; PSA, prostate-specific antigen; TAD, transactivation domain [Color figure can be viewed at wileyonlinelibrary.com]

PCa.[6] The AR and the physiological pathways it regulates are central to the initiation and progression of PCa.[5] The binding of androgens to the AR initiates AR-regulated gene expression that drives PCa growth (Figure 1).

PCa is most commonly clinically localized at the time of diagnosis, although about 10% of patients present with advanced, metastatic disease (Figure 2).[8] Surgery and/or radiation therapy (primary local therapy [PLT]) can effectively treat clinically localized disease, although about one-third of patients relapse after PLT. Whether patients present with metastatic disease or it arises in the context of a recurrence after PLT, the mainstay of treatment of metastatic PCa is endocrine therapy aimed at inhibiting the production or action of androgens that engage and activate the AR (Figure 1). Endocrine therapy is most commonly delivered through surgical or medical castration and is termed androgen deprivation therapy (ADT), which effectively inhibits the androgen production

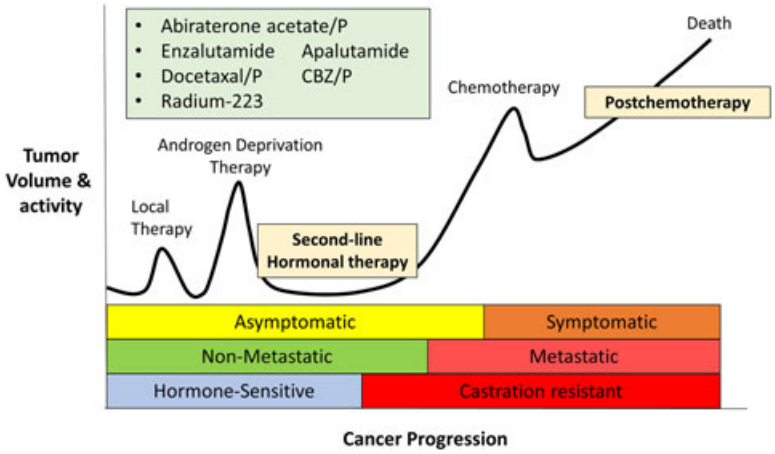


FIGURE 2 Progression and the different stages of prostate cancer. While the currently available therapies (commonly used ones indicated in the figure) are quite responsive at the hormone-sensitive stages, metastatic castration-resistant disease has a poor prognosis. Figure updated and redrawn from reference.[8] P, prednisone; CBZ, cabazitaxel [Color figure can be viewed at wileyonlinelibrary.com]

from the testes. The median duration of response to ADT is 18 to 24 months. Historically, first-generation AR competitive antagonists (eg, flutamide, bicalutamide, and nilutamide) have been combined with ADT (so-called combined androgen blockade [CAB]), although CAB has not yielded clinically meaningful improvements in PCa outcomes.

When PCa progresses despite ADT (Figure 2), it is termed castration-resistant PCa (CRPC), which is the lethal form of the disease. Interestingly, CRPC is most often still dependent upon the activation of the AR for its continued progression.[7,9] Although the AR has nongenotropic effects, reactivation of AR transcriptional activity represents the principal biochemical driving force that is necessary and sufficient for castration resistance. Multiple nonmutually exclusive mechanisms account for ongoing AR transcriptional activity despite castrate levels of serum testosterone: (1) *AR* gene amplification, (2) *AR* mutations that confer agonistic activity of nontraditional ligands (eg, progesterone and corticosteroids), (3) adrenal androgens, (4) intratumoral androgen production, (5) increased ratio of AR transcriptional activators to repressors, (6) somatic mosaicism, and (7) ligand-independent AR activation through posttranslational modification of the AR (eg, phosphorylation). Another important and more recently identified mechanism underlying castration resistance relates to the expression of constitutively active AR variants that lack a functional LBD (Figure 1).[1,2,10,11] These AR variants arise from aberrant splicing of AR messenger RNA (mRNA) and are thus termed AR splice variants (AR_{SVs}). Because the LBD is inhibitory (ie, the LBD is disinhibited upon ligand binding), AR_{SVs} that lack a functional LBD are rendered constitutively active. Furthermore, these AR_{SVs} are constitutively nuclear localized, resulting in a basal level of AR_{SVs} in the nucleus that further enhance ligand-independent AR transcriptional activity.[12–14] In addition to AR-dependent mechanisms of castration resistance, truly AR-independent pathways also exist, although treatments that target these pathways have not yet reached the clinic, and the reader is referred to the reviews on this topic.[15–17]

A particularly important aggressive form of non–AR-dependent CRPC is neuroendocrine prostate cancer (NEPC). NEPC can develop in a de novo pathway, or more commonly, in response to ADT and radiation therapy.[18–20] The 5-year survival rate of these patients are less than 13%.[18,19] Since these tumors are non–AR-dependent for proliferation and survival, they do not benefit from the significant advances in AR-targeted (of any functional domain) therapy achieved during the last decade. Investigation of potential biomarkers and drug targets in NEPC is a prominent avenue of current CRPC research.[18,21,22]

1.3 | Current management of CRPC

Nonendocrine approaches for CRPC have been approved and include cytotoxic chemotherapy such as the taxanes docetaxel and cabazitaxel, systemic radiation in the form of radium-223 (a calcium mimetic that targets the metastases to the bone, the most common site of distant organ involvement), and a cellular vaccine known as Sipuleucel-T. While each of these treatments can improve median overall survival by approximately 2 to 4 months, none is curative and treatment resistance is inevitable (Figure 2).[23]

Based on the pathophysiologic role of continued AR signaling in CRPC, new drugs that target the AR-signaling axis have been brought to the clinic. Abiraterone acetate,[24] an inhibitor of CYP17 and an enzyme that governs androgen production, effectively inhibits androgen production from nongonadal sources including both the adrenals and the tumor tissue itself (Figure 1). These nongonadal sources of androgen can drive AR activation in metastatic CRPC (mCRPC). Based on its ability to prolong progression free and overall survival, abiraterone acetate in combination with the glucocorticoid, prednisone, has received regulatory approval for mCRPC for patients who have undergone chemotherapy or are chemotherapy-naive. More potent, second-generation AR competitive antagonists, including enzalutamide[25] and apalutamide,[26] have likewise received approval for CRPC based on improvements in survival. Despite these clinical advancements for the treatment of CRPC, patients still manifest primary and secondary drug resistance to these therapies.

1.4 | Compounds that target the AR-TAD and DBD

Since the clinical implementation of the aforementioned second-generation endocrine therapies, preclinical models as well as sequencing studies of cohorts of mCRPC patients have demonstrated ongoing AR expression and signaling in post-abiraterone/post-enzalutamide mCRPC.[27] In fact, the AR is the most frequently mutated gene, and an AR-dependent transcriptional program is reactivated in this context.[27] Thus, the AR represents a key driver of castration-resistant growth in both newly developed CRPC and post-abiraterone/post-enzalutamide CRPC.

Importantly, all existing endocrine therapies approved for clinical application to PCa mechanistically function through the LBD.[28,29] Specifically, these therapies either inhibit ligand production (eg, castration or abiraterone acetate) or ligand action (eg, AR competitive antagonists; Figure 1). It has to be noted here that while the above-mentioned therapies target the ligand binding pocket (LBP) of the LBD, there are other surface exposed sites on the LBD that are also of interest to drug development. These sites include activation function 2 (AF2; a hydrophobic cleft serving as a site for coactivator binding) and binding function 3 (BF3; a hydrophobic site allosterically associated with AF2).[5,30–33] Despite the high sequence homology between NHRs for these sites, multiple compounds that target them in the AR-LBD have emerged and are under investigation.[5,30–33] There have also been some exciting recent developments in targeting the AR-signaling axis by the degradation[34] of AR protein with "enzalutamide-like" or "enzalutamide-like molecule conjugated" compounds (eg, proteolysis-targeting chimeras [PROTACs], specific and nongenetic inhibitor of apoptosis protein dependent protein erasers [SNIPER(AR)s]) in the past few years.[35–41]

However, therapies that target other domains of the AR, namely the TAD and DBD (Figure 1) have not yet been developed for clinical application nor extensively researched (compared with the targeting of the LBD). Two principal explanations account for this gap in pharmaceutical development. First, the TAD is an intrinsically disordered protein (IDP) domain, so its crystal structure has not been resolved and therefore structure-based drug design is not currently feasible. Second, the DBD shares extreme homology to that of other NHRs, so specificity of drugs for the AR-DBD has been considered a challenge.[5] Nonetheless, recent drug development projects premised on either actual or in silico drug screens have resulted in potential candidate compounds that can inhibit the AR activity, through either the TAD or DBD. While there have been several reviews published in this area over the last few years, many have not gone into comprehensive detail.[42–46] In the literature review presented here, we discuss the recent developments in molecules that have shown prominent effects toward the AR-signaling axis through direct or indirect interaction with the AR-TAD

or the AR-DBD. We provide an unprecedently comprehensive analysis of the subject area, detailing the associated/impacted biochemical targets/processes, the experimental tools used to probe the targets and present a perspective on the future of targeting the AR-signaling axis beyond the AR-LBD.

2 | HAIRPIN POLYAMIDE ANTAGONISTS OF AR-DNA BINDING

An approach developed by Dervan et al[47] over the last two decades for targeting CRPC is to inhibit the AR-mediated transcription processes at the DNA level with direct antagonism of AR-DNA binding using pyrrole-imidazole containing polyamides. The idea was inspired by the function of the natural product distamycin A, a polyamide DNA minor groove binder, first isolated in 1962 from cultures of *Streptomyces distallicus*.[48] Using the concept of differentiating nucleotide base pairs through specific positions of hydrogen bond donor or acceptor sites and through complementary geometrical flexibility to associate with DNA, these hairpin polyamides have evolved as a highly efficient means of specific recognition of DNA fragments.[49–51] This sequence-specific association of the polyamide disrupts: (1) the association ability of transcription factors, such as the AR, to bind their respective binding site(s) at the DNA; (2) RNAP2 activity; and (3) replicative helicase activity.[47]

2.1 | Binding specificity

The ligand-induced AR homodimers usually function in binding the AREs by identifying specific DNA half-sites (5′-AGAACA-3′) organized as inverted repeats separated by three nucleotides (IR-3 sequences).[4,52] Using this design template, Nickols and Dervan[52] developed a DNA-binding polyamide **PA1** (Figure 3)[53] that targets AREs.

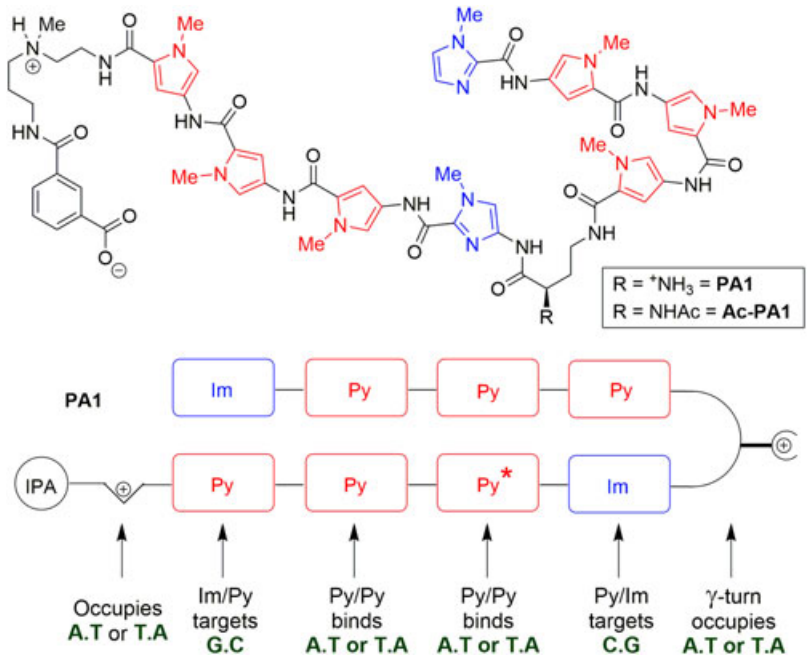


FIGURE 3 Structures of hairpin polyamides **PA1**, **PA2**, and **Ac-PA1**. **PA1** is designed to bind the ARE sequence 5′-AGAACA-3′, while **PA2** has a mismatch (where Py* is substituted by an Im) that should render the binding to be weak to that sequence. Acylation of the γ-turn amino group yields an acetamide (**Ac-PA1**) with an improved in vivo toxicity profile.[53] ARE, androgen response element; Im, *N*-methylimidazole; Py, *N*-methylpyrrole [Color figure can be viewed at wileyonlinelibrary.com]

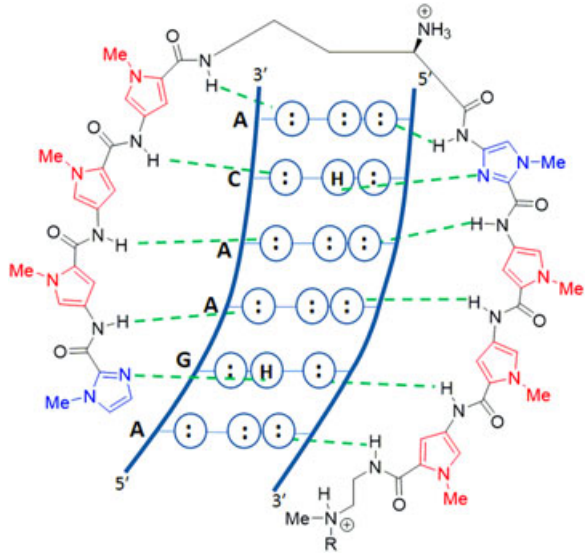


FIGURE 4 Recognition of the ARE DNA half-site by **PA1**. Im/Py pair recognizes G.C, Py/Py pair binds A/T T/A, Py/Im pair recognizes C.G. Figure adapted from reference.[49] ARE, androgen response element; Im, *N*-methylimidazole; Py, *N*-methylpyrrole [Color figure can be viewed at wileyonlinelibrary.com]

PA1 is an *N*-methylimidazole (Im) and *N*-methylpyrrole (Py) derived polyamide that recognize and bind the base pair sequences on the ARE half-sites. The shorter (<6 amino acid pairs) polyamides show the optimal geometrical ability to align with and bind the helical DNA strands. Multiple bifurcated hydrogen bonds between the polyamide backbone's amide hydrogens to the purine N3 and the pyrimidine O2 provide favorable binding affinity.[50,54]

The specificity of binding is established through the different heterocyclic pairs in the polyamide that can recognize specific nuclear base pairs to bind in a complementary manner (Figures 3 and 4). Py/Py pair binds both the A.T and the T.A nucleotide pairs nonselectively via hydrogen bonding interactions. In contrast, the Im/Py pair provides far more specific and directional binding/recognition toward binding its target (Figure 4). The critical interaction that results in such specific recognition lies in the hydrogen bond formation between the lone pair on the imidazole nitrogen and the exocyclic amino group of guanines.[49,54] This interaction brought about by the Im/Py pair while recognizing the G/C base pairs over the A/T base pairs, also specifically distinguishes between G.C vs a C.G pairing. The unfavorable angle to form a thermodynamically efficient[55] hydrogen bond from the cytosine side of a G.C pair makes the imidazole recognize the guanine via hydrogen bonding from the proximal side to the guanine.[49] Hence the Im/Py pair carries a 100-fold greater directional affinity for a G.C base pair than a Py/Im pair.[56]

This binding specificity has been proven via X-ray crystallographic analysis of a polyamide of the structure ImImPyPy-β-Dp (where β is beta alanine, and Dp is dimethylaminopropylamide) bound as a dimer to its target sequence of 5′-WGGCCW-3′ (W = A or T).[54] The antiparallel head-to-tail type binding of this dimer also matched the adjacent DNA strands 5′ to 3′ directionality with respect to its N-terminal to C-terminal orientation within each polyamide.[54] The β-alanine end groups were accommodated in the smooth minor grooves of the A.T and the T.A base pairs flanking the GGCC recognition sequence. The Im/Im pairings are considered to be energetically unfavorable,[56] which prevents the slipped binding modes of the peptides from occurring.

Utilizing the knowledge about these heterocyclic pairs of Im/Py and Py/Py, **PA1** was designed as a cell-permeable hairpin polyamide that targets the gene sequence 5′-WGWWCW-3′ (W = A or T), which is found in the consensus ARE (Figure 4).[49] The antiparallel peptide sequences were connected via a chiral γ-diaminobutyric acid hairpin turn to prevent slipped binding modes and to give improved affinity and selectivity compared with unlinked elements.[57] The γ-turns show preference to occupy A.T base pairs over G.C base pairs, owing to steric

clash with the exocyclic amine of the guanine.[49,57,58] The presence of the isophthalic acid (IPA) moiety in the C-terminal tail facilitates improved nuclear translocation of such polypeptides.[59,60] The aminoalkyl linker connecting the polyamide to the IPA unit also has binding preference toward A/T pairs vs G/C pairs due to steric reasons.[57]

2.2 | In vitro effectiveness

The binding of the polyamide **PA1** to the proximal prostate-specific antigen (PSA) promoter that contains the ARE 5′-AGAACAGCAAGTGCT-3′ was evaluated via quantitative DNAse I footprint titrations using a 5′-^{32}P-labeled polymerase chain reaction (PCR) fragment of pAR-PSA.[52] **PA1** had an association affinity (K_a) of 8.3×10^9 M^{-1} for the ARE consensus half-site 5′-AGAACA-3′. The other half-site sequence 5′AGTGCT-3′ bound **PA1** with a much lower affinity due to the single base pair mismatch in that sequence at the 4th position. Another polyamide, **PA2** (Figure 3), which was designed as a negative control to target the alternative sequence 5′-WGWCGW-3′ did not result in any measurable ($K_a < 10^7$ M^{-1}) binding to either of the ARE half-sites under the conditions utilized. This clearly highlights the ability of these polyamides to bind-specific gene sequences.

PA1 (10 nM) inhibited the binding of factors from nuclear extracts isolated from DHT-stimulated LNCaP cells to the ARE site in the PSA promoter.[52] The binding of **PA1** to the PSA promoter ARE downregulated the androgen-induced expression of PSA, comparable to bicalutamide treatment.[52] This dose-dependent downregulation was measured to be ~70% at concentrations of 10 μM of either compound in comparison to nontreated cells or cells treated with **PA2**. Decreased occupancy of the AR at the PSA promoter and enhancer, as well as the intronic enhancer of the gene FKBP5 in the presence of **PA1** (10 μM) was shown using chromatin immunoprecipitation (ChIP) assays. The inhibition of the AR-induced gene FKBP5 by **PA1** was ~60% at this concentration as compared to ~95% by bicalutamide. Other direct AR target genes such as KLK2, KLK3, and TMPRSS2 were also downregulated (twofold-threefold) by **PA1** as compared with the DHT-induced controls.[52] Evaluation of androgen-induced gene expression using DHT-stimulated LNCaP cells, revealed that **PA1** at a concentration of 10 μM affected the expression of 1053 transcripts by at least twofold compared to the controls.

Using LNCaP cells, Yang et al[61] also linked the antitumor activity of **PA1** to RNAP2 inhibition. This observation is consistent with previous reports that DNA-binding molecules would have significant inhibitory effects on the function of RNAP2.[62] The inhibitory effect was traced to the enhanced degradation of RNAP2 large subunit RPB1, a phenomenon that triggers cellular apoptosis mechanisms.[47,61] This antitumor activity was blocked by the cotreatment with MG132, a 26S proteasome inhibitor. **PA1** activated p53 genes although no significant cellular markers of DNA damage were observed upon extended treatment.[61]

2.3 | In vivo effectiveness and further optimization

PA1 treatment of mice with LNCaP xenografts resulted in up to 64% inhibition of tumor growth at a dose of 1 mg/kg.[61] However, preclinical studies with **PA1** showed significant dose-limiting toxicities.[53,61] Complete removal of the chiral amine unit reduced in vivo toxicity, albeit at the cost of losing therapeutic effectiveness.[47] In later studies, acylation of the α-amino unit at the hairpin turn yielded a derivative (**Ac-PA1**; Figure 3) with prominently less in vivo toxicity, while retaining the activity profile.[53] The differential toxicity could have a relation to the higher liver tissue localization of **PA1** than **Ac-PA1** (33% less), which was measured using radiolabeled (^{14}C) polyamides in a LNCaP xenograft mouse model.[47,63] From the same experiments, the accumulation at the tumor was found to be better with the acylated derivative. With repeated injections (three injections over 7 days) of **Ac-PA1**, ~twofold accumulation was seen at both the tumor and the host-organs. The organ-accumulation is a disturbing factor given the chance for higher levels of toxicity. Interestingly in another study, LNCaP xenograft-bearing mice had greater liver accumulation (and impaired clearance) of **Ac-PA1** than mice with A549 (lung) or U251 (brain) xenografts.[64]

Here, LNCaP xenografts were also found to have much greater localization (up to 5×) of the polyamide than the non-PCa xenografts.

Given the homology between the AR and other NHR DBDs, a polyamide designed to target 5′-WGWWCW-3′ half-site is expected to have promiscuity in binding. Using an enzalutamide-resistant LREX′ PCa cell line, Kurmis et al[65] demonstrated that the acetamide derivative **Ac-PA1** interfered with both AR-driven and GR-driven gene expression. In instances where GR upregulation is the primary resistance mechanism to overcome AR-antagonists, this effect could be beneficial to develop an efficient dual-targeting approach. **Ac-PA1** significantly reduced the growth of VCaP and LREX′ cells in vitro even upon upregulation of AR-driven (by DHT) and GR-driven (by dexamethasone) transcription.[65] Treatment of VCaP xenografts with **Ac-PA1** dose-dependently reduced the tumor volumes up to 70% (5 mg/kg) at 6 weeks. Importantly, enzalutamide-resistant (GR-driven) LREX′ xenografts showed 80% reduction of tumor growth at the cotreatment of enzalutamide and **Ac-PA1**. Authors indicated a 6% weight loss in mice when treated with **Ac-PA1** at 30 mg/kg, which was recovered upon treatment withdrawal (5 days).[65] Arguably, some amount of toxicity would have to be expected (and perhaps accepted depending on the severity of the PCa treatment resistance) in a therapy that has the potential to hit more than one NHR.

2.4 | Outlook

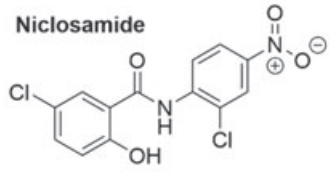
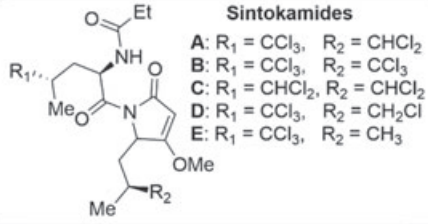
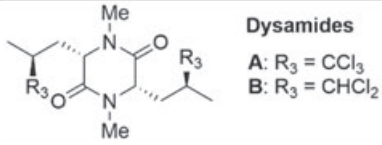
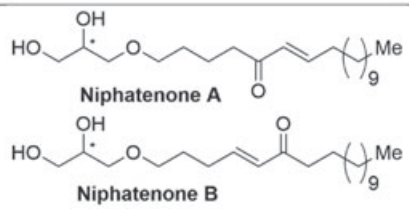
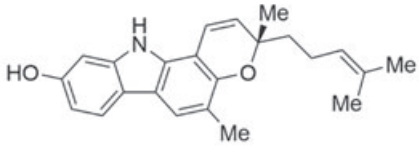
The promising results detailed above has established that hairpin polyamide compounds can be utilized successfully to target ARE (and GRE) in a broader perspective. If specific AR-dependent genes were to be targeted, these polyamides would have to be programmed via changes in sequences/amino acids to bind-specific ARE fragments given the subtle degenerate nature of different AREs. Perhaps incorporating the thiamine-selective recognition element *N*-methyl-3-hydroxypyrrole[49,51] in place of the Py unit could provide increased binding affinities and selectivities in analogs of **PA1**. However, Dervan et al[47] have demonstrated that even though some heterocycle replacements enhance DNA-binding affinity, the ability to permeate the cell or reach the nucleus is compromised by such modification. Padroni et al[66] has shown recently that thiazole derivatives could be used to substitute the imidazole units in **PA1** type hairpin polyamides. Although the double-stranded DNA-binding affinity was demonstrated to be somewhat higher for the 5-alkyl thiazole containing polyamides, G-recognition selectivity was found to be diminished for the thiazole units when compared with the Im units.[66]

Being able to target both AR-driven and GR-driven transcription, **Ac-PA1** derivatives may translate to particularly effective therapeutics against enzalutamide-resistant PCa's that have GR upregulation as the major pathway of resistance. The high molecular weight and the hydrophobic nature of the constructs have made these hairpin polyamides to have poor aqueous solubility.[67] This may hinder an oral drug delivery approach and negatively affect the pharmacokinetic/pharmacodynamic profile moving forward. Besides the efforts to find an optimal formulation strategy[47] minimizing the off-target effects due to DBD homology between NHRs stands as the major challenge ahead for these hairpin polyamides.

3 | NICLOSAMIDE

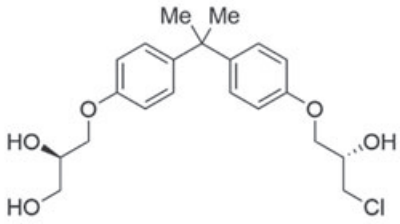
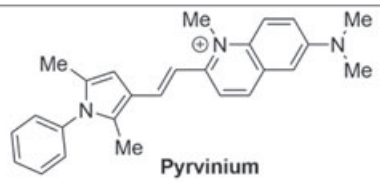
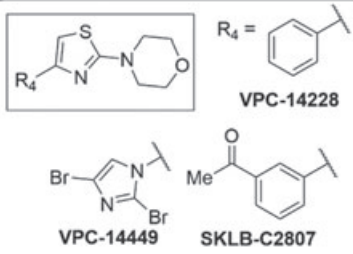
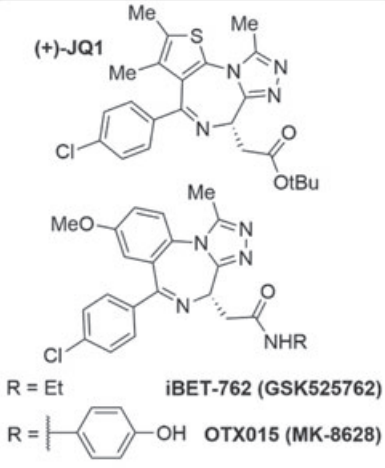
Niclosamide (Table 1) is a Food and Drug Administration (FDA)-approved (1982) anthelminthic drug (niclocide) that has been used for treating tapeworm infections.[68,69] Structurally it is a salicylanilide, which has two aromatic chlorine substituents and an aromatic nitro group. Niclosamide is well-tolerated in humans, which presents a distinct advantage in adapting it for a novel therapeutic use. In fact, utilization of a previously approved drug-like niclosamide provides a rapid path toward clinical trials.[69–71] The mechanism of action of niclosamide against tapeworms involves inhibition of oxidative phosphorylation and the stimulation of adenosine triphosphate (ATP) activity in the mitochondria.[69,72] Since tumor-related malignancies often involve deficits of

TABLE 1 Compounds that affect the AR-signaling axis without interacting with AR-LBD

Compound	Mechanism(s) of action and other details
Hairpin polyamides (see figures 3 and 4 for structure)	• Antagonism of AR-DNA association by binding to DNA androgen response elements (AREs) • Enhanced degradation of RNAP2 large subunit RPB1 triggering cellular apoptosis mechanisms • Disrupts replicative helicase activity • Activates p53 genes
	• FDA approved human anthelminthic • Multipathway (eg, IL6-JAK-STAT, MAPK, and Wnt/β-catenin) inhibitor of cancer • Promotes AR-V7 degradation via a ubiquitin-proteasome pathway • Affects the IL6-STAT3 mediated AR-TAD transactivation, AR nuclear translocation, and AR-DNA–binding activity • Phase I clinical trials with enzalutamide co-treatment on-going (NCT02532114 and NCT03123978)
 	• Peptidic polychlorinated marine natural products • Inhibits AR-TAD transactivation • Analogs with higher degree of chlorination show better activity than less chlorinated analogs • Sintokamide A primarily inhibits AR-AF1 Tau-1 domain
	• Marine natural products • *R*-Niphatenone-B shown to bind the AR-TAD AF1 region • Inhibits AR-TAD transactivation • Further development abandoned due to binding specificity issues
	• Carbazole alkaloid natural product • Multipathway anticancer compound • Inhibits AR transactivation • Induces degradation of full-length and splice variant AR via ubiquitin-proteasome pathway • Reduces AR nuclear translocation

(Continues)

TABLE 1 (Continued)

 **EPI-002** (**EPI-506** = a prodrug of EPI-002)	• AR-TAD inhibitor • Isolated from a marine sponge • Derived from bisphenol A • Covalently binds the AR-TAD-AF1 • Primarily Inhibits AR-AF1 Tau-5 domain • PPARγ modulation effects leading to AR inhibition • Clinical trials (NCT02606123) terminated at end of Phase 1; excessive high pill burden (18 capsules per day) antitumor effects at greater than 2400 mg/kg doses • Further development abandoned
JN compounds (Structures not yet disclosed)	• AR-TAD inhibitors and AR degraders • 10-fold to 30-fold greater potency as compared with EPI-002 in cellular and functional assays • Significant control of tumor growth in xenografts driven by full-length and/or splice variant AR
 **Pyrvinium**	• Quinoline-derived cyanine dye • FDA approved human anthelminthic • AR-DBD inhibitor • Cross-reactivity toward other nuclear hormone receptors
 **VPC-14228** **VPC-14449** **SKLB-C2807**	• Thiazolyl morpholine derivatives initially found through an in silico drug design approach • AR-DBD inhibitors • Key H-bonding interaction of morpholine-O with Tyr-594 of AR-DBD • Does not impede AR nuclear translocation
 **(+)-JQ1** **iBET-762 (GSK525762)** **OTX015 (MK-8628)**	• BET inhibitors (BETi's) • Most BETi are triazolodiazepines • AR-Chromatin binding inhibitors • Direct interaction with AR-TAD shown • In vivo and in vitro activity against PCa • Clinical Trials ongoing for multiple cancers including CRPC (NCT02711956, NCT02607228, NCT02259114)

Abbreviations: AR, androgen receptor; AR-DBD, AR-DNA–binding domain; AR-TAD, AR transactivation domain; BETi, bromodomain and extraterminal domain protein inhibitor; CRPC, castration-resistant prostate cancer; FDA, Food and Drug Administration; PCa, prostate cancer; PPARγ, peroxisome-proliferator-activated receptor-γ; RNAP2, RNA polymerase II

oxidative phosphorylation and decreased availability of ATP in the mitochondrial activity of cancer cells,[73] the effect of niclosamide against such cells became an interesting topic to investigate.

Niclosamide has demonstrated antineoplastic effects against many cancers including myelogenous leukemia,[74,75] lung cancer,[76,77] breast cancer,[78–84] colon cancer,[85–88] ovarian cancer,[89–91] PCa,[80,92–98] adrenocortical carcinoma,[99] hepatocellular carcinoma,[100,101] multiple myeloma,[102] glioblastoma,[103] and osteosarcoma.[104] In these extensive studies, niclosamide has shown remarkable ability to eradicate cancer stem cells, inhibit metastasis, and/or induce/re-establish apoptosis mechanisms. The effects of niclosamide at the cellular level involve multiple signaling pathways that are prominent in cancer progression (Figure 5). It has inhibitory effects toward Wnt/β-catenin, mammalian target of rapamycin complex 1 (mTORC1), signal transducer and activator of transcription 3 (STAT3), nuclear factor κB (NF-κB), and the Notch pathways,[69,105] establishing niclosamide as a multipathway inhibitor of cancer progression.

3.1 | Niclosamide monotherapy

3.1.1 | Effects on the IL6/JAK2/STAT3 pathway

Of the above pathways, "Janus kinase (JAK)–signal transducer and activator of transcription 3 (STAT3)" is a major pathway through which niclosamide is believed to target CRPC.[96–98] Elevated levels of STAT3 has been found in prostatic carcinomas and normal tissues adjacent to such tumors.[106] Hyperactivation of the STAT pathways[107,108] induces cell proliferation and prevents apoptosis mechanisms in human cancer cells through dysregulation of key proteins.[96] STAT3, in particular, regulates the expression of genes that control factors central to cancer progression.[109] STAT3 can be activated by growth factor receptors, nonreceptor tyrosine kinases, or cytokine receptors such as the interleukin 6 (IL-6) receptor.[110] Serum levels of IL-6 are often found to be elevated in advanced PCa patients.[111,112] Upon ligand binding to the IL-6 receptor complex, an intracellular signaling cascade is activated where the receptor-associated JAKs phosphorylate STAT3 at Tyr-705. Dunn et al[113] showed that JAK2, in particular, is responsible for this phosphorylation employing LNCaP cells that lack JAK1 due to epigenetic silencing.[114] The phosphorylation activates the STAT3, which results in dimerization, nuclear translocation, and induction of specific target gene expression. JAK2 can also phosphorylate and activate STAT5, which occurs in 61% of metastatic PCa.[114,115] Importantly, STAT3 signaling can interact with the AR-TAD and thereby facilitate AR transactivation (Figure 5).[116]

In view of the role of the IL6/JAK2/STAT3 pathway on induction of gene expression, growth promotion and activation of the AR, this pathway has been implicated as a major target for PCa treatment.[7,114,117] In an attempt to find nonpeptidic small molecule inhibitors of the STAT3 signaling pathway via high-throughput screening Ren et al[96] found niclosamide as a hit compound. Treatment of DU145 PCa cells that carry constitutively active STAT3 with niclosamide resulted in the dose-dependent inhibition of the STAT3 phosphorylation at Tyr-705.[96] These results are in agreement with the work by Mora et al[118] where the inhibition of constitutively active STAT3 signaling in DU145 cells using antisense STAT3 oligonucleotides induced growth inhibition and apoptosis.

Niclosamide's targeting has shown selectivity toward the STAT3 pathway, without obvious inhibitory effects against the activation of other STAT homologs, STAT1 and STAT5.[96] This effect was deemed not to be exerted by the inhibition of upstream kinase activity of JAKs, since niclosamide did not affect the kinase protein levels over the course of treatment.[96] Using an immunofluorescence assay it was shown that niclosamide (1.0 μM) blocked the EGF-induced nuclear translocation of STAT3 after a 2 hours treatment.[96] Electrophoretic mobility assays revealed that the activity of niclosamide did not result from a direct binding/interaction of niclosamide with the STAT protein's consensus DNA elements.[96] In aggregate, these results indicate that the activity of niclosamide is found in the inhibition of the activation/transactivation and the nuclear translocation of STAT3, although it did not directly bind to the SH2 domain of the STAT3 protein.[96]

Niclosamide strongly inhibited the proliferation and colony formation (half-maximal inhibitory concentration [IC_{50}] = 0.7 and 0.1 μM) of DU145 PCa cells while the effect was not that pronounced in PC3 PCa cells that had a

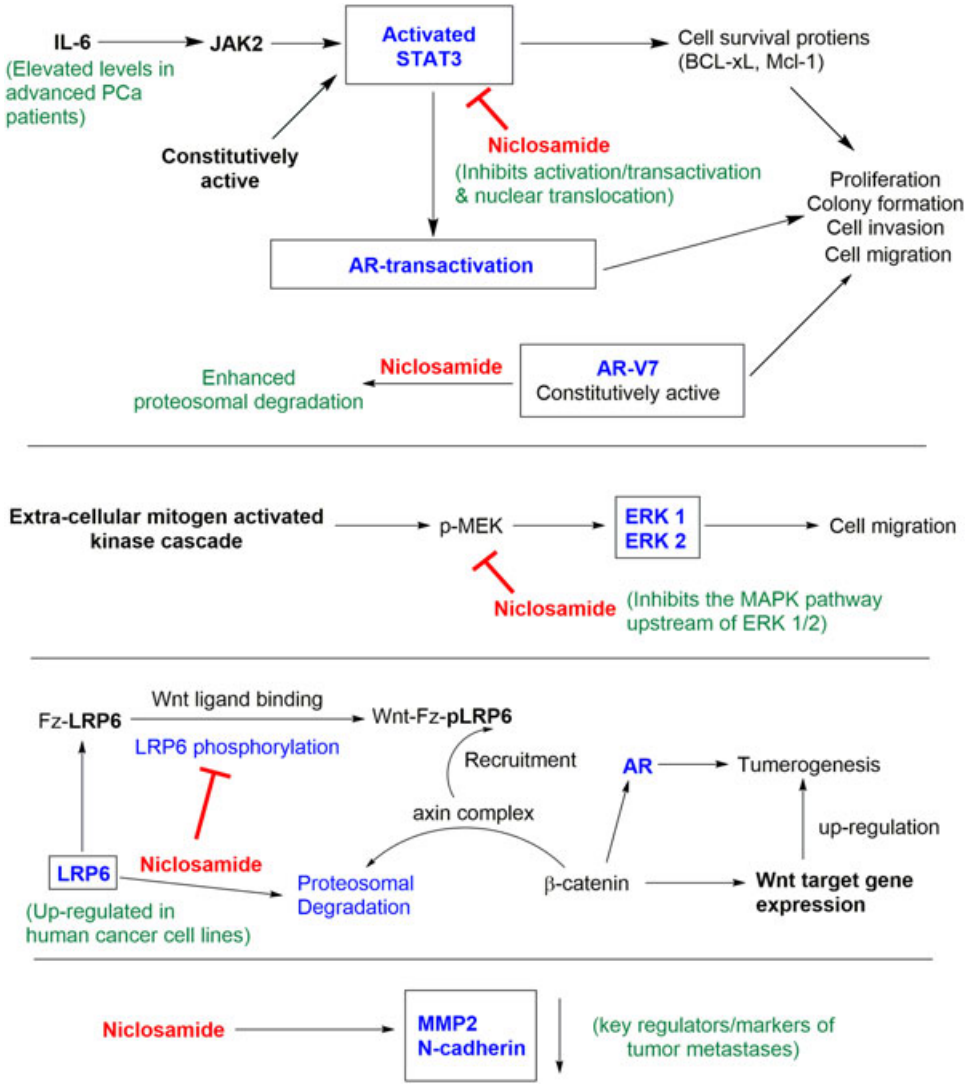


FIGURE 5 Multipathway anticancer effects of niclosamide. Niclosamide has been shown to (1) affect the STAT, Wnt/β-catenin, and the MAPK pathways, (2) to enhance degradation of AR-V7 and LRP6, and (3) significantly lower key regulators/markers of tumor cell metastasis. AR, androgen receptor; ERK, extracellular signal–regulated kinases; JAK, Janus kinase; IL-6, interleukin 6; LRP6, low-density lipoprotein receptor–related protein 6; MAPK, mitogen-activated protein kinase; MMP2, matrix metalloproteinase-2; STAT, signal transducer and activator of transcription 3 [Color figure can be viewed at wileyonlinelibrary.com]

lower level of constitutively active STAT3.[96] Niclosamide-induced G0/G1 phase arrest and the apoptosis of DU145 cells, which may have been a consequence of the downregulation of cell survival Mcl-1 proteins (B-cell lymphoma-extra large [BCL-xL], myeloid cell leukemia [Mcl-1]), and cell-cycle regulators (cyclin D and c-Myc).[96] Similar effects of niclosamide toward the STAT3 inhibition, and the inhibition of STAT3 target genes in LNCaP, C4-2B, and DU145 cells were also shown by Liu et al.[94]

A wound-healing assay (used to measure the migratory properties of cells) using DU145 cells showed niclosamide inhibited wound healing by ~20%, ~60%, ~70% at drug concentrations of 0.2, 1.0, and 5.0 μM, respectively.[97] This inhibition was significantly diminished in cells transfected with STAT3 small interfering RNA (siRNA), indicating the importance of the STAT3 pathway as its mechanism of action.[97] Similar assays conducted by

Liu et al[94] on LNCaP-s17, LNCaP-STAT3, and DU145 cells carrying constitutively active STAT3 has also showed niclosamide-induced dose-dependent wound-healing inhibition (80-90%).

3.1.2 | Effects on the MAPK pathway

Given the possibility for other pathways to be involved in the wound-healing process, the mitogen-activated protein kinase (MAPK) pathway was also probed by Ren et al[97] via monitoring the extracellular signal–regulated kinases 1/2 (ERK 1/2) expression in DU145 cells after niclosamide treatment. Like STAT3, ERK 1/2 activates via initial phosphorylation (pERK) and then exerts downstream effects to promote metastasis. At an initial 4 hours treatment period, niclosamide had no discernable effect on the levels of pERK. However, at 24 hours pronounced inhibition of pERK was seen at concentration of niclosamide >1.0 μM.[97] This delayed response on the ERK-related pathway is in contradistinction to the effects on pSTAT3 levels, which were significantly impacted at similar doses even at the 4-hour mark.[97] Hence the authors postulated that niclosamide might be targeting upstream regulators of the MAPK pathway, which consequently affects the ERK 1/2 activation. To demonstrate this effect of the MAPK pathway on the cancer cell motility the wound-healing assay was conducted in the presence of PD98059, a selective MAPK inhibitor. The niclosamide (1.0 μM) induced inhibition of wound closure was partially recovered in the cells cotreated with PD98059 (43%) compared to the control group (63%).[97] This showed that the downregulation of the MAPK pathway by niclosamide also contributes toward the motility of the DU145 PCa cell line.[97] With the knowledge that MAPK pathway is involved in some AR-independent bypass pathways that drive PCa,[16,119] niclosamide's ability to affect this signaling axis adds to the impact it could have in a cotherapy with an AR antagonist.

3.1.3 | Effects on the Wnt/β-catenin pathway

The Wnt/β-catenin pathway is another cellular pathway that has shown importance in targeting the AR-signaling axis.[120,121] Wnt signaling can interact with the AR-signaling axis and AR gene transcription processes, leading to elevated prostatic tumor growth, cell migration, and invasion properties.[120,122–124] Wnt signaling promotes AR gene transcription while AR signaling is inhibitory toward the Wnt-pathway in hormone-naive PCa cells.[120] However, the two pathways promote each other in CRPC, which leads to androgen-independent PCa progression.[120] In 2011, Lu et al[80] showed that the inhibition of low-density lipoprotein receptor–related protein 6 (LRP6) mediated Wnt/β-catenin activation by niclosamide-induced anticancer effects against prostate and breast cancers. Niclosamide displayed anticancer activity (IC_{s50} < 1 μM) against DU145 and PC3 PCa cells and an ability to induce apoptosis (concentration = 1.2 μM).

The Wnt family of glycoproteins regulates fundamental processes that direct cell proliferation, cell polarity, and cell fate determination during embryonic development and tissue homeostasis.[125] A major component in Wnt signaling associated with the above functions is the transcriptional coactivator β-catenin. With abnormal upregulation, the Wnt/β-catenin pathway can lead to tumorigenesis of multiple types of cancers, including PCa. LRP6 is a coreceptor for Wnt ligands[125] that is expressed and upregulated in human cancer cell lines.[80] Upon Wnt ligand binding to the frizzled (Fz) receptor and its coreceptor LRP6, the LRP6 gets activated and phosphorylated (pLRP6) on the cytosolic side.[125] This Wnt-Fz-pLRP6 complex recruits the axin complex from the cytosol to the receptors. The axin complex in the absence of such Wnt interference is responsible for keeping the β-catenin levels downregulated in the cells via continuous proteasomal degradation of the cytosolic β-catenin.[125] The recruitment of the axin complex to the activated Wnt receptor system disrupts this regulation, and cytosolic β-catenin accumulates. β-Catenin then travels to the nucleus where it functions as a coactivator of multiple transcription factors, including the TCR-LEF complex, which has regulatory effects toward the AR.[125,126] In addition, β-catenin has been shown to perform as a coactivator of ligand-dependent AR function in PCa cells (Figure 5).[127,128]

In experiments conducted by Lu et al[80], treatment of PC3 PCa cells with niclosamide (>0.3 μM) induced significant reduction of free β-catenin levels as evidenced by a GST-E-cadherin binding assay. In PC3 cells transiently transfected with the Wnt/β-catenin signaling reporter TOPFlash luciferase, niclosamide treatment reduced luciferase activity by ~70%.[80] The total cellular levels of axin2 and cyclin D1, which are transcriptional targets of the Wnt/β-catenin pathway, were significantly reduced in PC3 and DU145 PCa cells by niclosamide (<1 μM) treatment.[80] Even more importantly, niclosamide was able to suppress LRP6 expression and phosphorylation at concentrations of 0.3 μM.[80] Treatment with niclosamide of PC3 cells pretreated with cycloheximide (protein synthesis inhibitor) revealed a half-life of 2.3 hours for LRP6, as compared with the control group (without niclosamide) which showed a half-life of 6.9 hours.[80] However, the total LRP6 mRNA levels did not change upon niclosamide treatment as judged by reverse transcription-PCR (RT-PCR). These results indicate that the LRP6 suppression was not at a transcriptional level but rather mediated by enhanced LRP6 degradation.[80] Niclosamide did not significantly affect the levels of cytosolic disheveled-2 (Dvl2), another regulator of the Wnt/β-catenin pathway.[80]

3.1.4 | Effects on cellular markers of tumor metastases

A Boyden chamber assay (used to mimic the in vivo invasion process of cancer cells) by Ren et al[97] using DU145 cells revealed the ability of niclosamide to inhibit the migration of cancer cells through an extracellular matrix (ECM) membrane up to ~90% when treated for 24 hours at drug concentrations up 2.0 μM. Similar results were obtained by Liu et al[94] using LNCaP-STAT3 and DU145 cells carrying constitutively active STAT3 where the invasion was reduced by ~90% at a niclosamide concentration of 0.5 μM. To further explore the ability of niclosamide to inhibit tumor cell metastases, the effect of niclosamide on the levels of key proteins, which are associated with tumor metastasis (MMP2, MMP9, cadherins, and catenins), were evaluated by Ren et al[97] in DU145 cells. MMP2 and MMP9 are key enzymes mediating ECM degradation that promotes metastases, while the cadherins/catenins are key factors in endothelial-mesenchymal transition (EMT).[97] The effect of niclosamide on MMP2 was very pronounced, resulting in almost complete eradication of MMP2 at a drug concentration of 5.0 μM after a 24 hours treatment.[97] While there was also an inhibitory effect toward the level of MMP9, the effect was not as pronounced as for MMP2. The effect of niclosamide on catenins was less distinct as well, a finding that suggests that showing any interference with EMT of DU145 cells is primarily via the regulation of cadherins. In corroboration of this point, the protein level of N-cadherin (a mesenchymal marker) was significantly reduced with the treatment with niclosamide.[97] A repression of the levels of E-cadherin (an epithelial marker), which conventionally is seen as a sign of EMT promotion,[129] was also seen. Some research suggests that the loss of E-cadherin levels alone might not be predictive of EMT.[130]

3.1.5 | Effects on AR degradation

One of the most significant findings related to niclosamide therapy in CRPC models is the downregulation AR_{SVs}.[95] AR splice variant AR-V7, in particular, has been linked to CRPC and resistance to second-generation AR-signaling axis inhibitors such as enzalutamide and abiraterone acetate.[93–95,131] High-throughput screening of HEK293 cells stably transfected with AR-V7 with a PSA-luciferase reporter system identified niclosamide as a possible AR-V7 targeting compound.[95] In LNCaP PCa cells transiently transfected with AR-V7, niclosamide inhibited the non-DHT dependent transcriptional activity (of AR-V7) while enzalutamide could not.[95] DHT-induced transcriptional activity in the same system, however, was knocked down by both niclosamide and enzalutamide. A ChIP assay showed niclosamide significantly reduced the AR-V7 recruitment to the PSA promoter in CR-2 AR-V7 cells, in which enzalutamide had no effect.[95] Treatment of CWR22Rv1 cells with niclosamide inhibited the endogenous AR-V7 expression in a dose-dependent manner. At lower doses (0.5 μM),

the inhibition effect was significantly more prominent toward AR-V7 than the AR_{FL}, showing a preferential inhibition.[95] This effect was found to be due to more rapid AR protein degradation in the presence of niclosamide rather than from a transcriptional level of inhibition.[95] AR-V7 degradation monitored in the presence of cycloheximide (protein synthesis inhibitor) revealed niclosamide enhanced the AR protein degradation.[95] MG132 was able to reduce this AR-V7 degradation, indicating the involvement of the ubiquitin-proteasome pathway for niclosamide-induced AR-V7 degradation.[95] Niclosamide had minimal effects on the expression of full-length AR. Niclosamide (0.5 μM) exerted significant inhibition of C4-2 neo, C4-2 AR-V7, and CWR22Rv1 PCa cell growth and induced cell apoptosis, while not affecting the growth of normal prostate epithelial cells PZ-HPV-7.[95]

3.2 | Niclosamide combination therapies

Given the aforementioned results of niclosamide as a monotherapy, combinations of niclosamide and other compounds have been tested for PCa treatment. In human PCa tissues, AR downregulation induced STAT3 activation leads to the development of PCa stem-like cells.[110] Such activation could result in rapid resistance to the therapies like enzalutamide and result in lethal metastatic disease. As such, a potent STAT3 inhibitor-like niclosamide in combination therapy with a direct or indirect AR-LBD antagonist could result in prolonged treatment effectiveness.

A study by Liu et al[111] corroborated the previously stated findings about the association of PCa and IL-6/JAK/STAT pathway by showing that the inhibition of constitutively active STAT3 reverses the enzalutamide resistance in LNCaP PCa cells. Enzalutamide (20 μM) was able to exert a ~60% inhibition of the growth of LNCaP PCa cells, while the effect was modest (<20% inhibition) for LNCaP-$IL6^{+}$ cells and LNCaP-s17 cells that overexpressed IL-6.[111] These LNCaP-s17 cells were found to carry constitutively active STAT3, and as such had elevated STAT3 signaling resulting in elevated levels of AR, c-Myc, survivin, and BCL-2 proteins compared with the control LNCaP-neo cells.[94,111] The use of AG490 (a JAK2/STAT3 inhibitor) or the use of STAT3-siRNA (knocks down STAT3 expression) reverses the enzalutamide resistance in LNCaP-s17 cells.[94,111] The recruitment of AR to the proximal and the distal enhancer binding sites of the PSA promoter were significantly enhanced in the LNCaP-s17 and LNCaP-STAT3C cells carrying constitutively active STAT3 as compared with the LNCaP-neo control cells.[111] These results were in agreement with previous findings that IL-6 overexpression led to enhanced AR nuclear translocation and AR-ARE DNA-binding activity,[132] and resulted in the upregulation of intracrine androgen levels in the absence of exogenous steroid precursors.[133] Enzalutamide significantly inhibited the recruitment of AR to AREs in the LNCaP-neo cells but failed to have much effect on LNCaP-s17 and LNCaP-STAT3 cells with elevated STAT3 activity.[111] Such results collectively showed that the concurrent use of a STAT3 pathway inhibitor with enzalutamide (or other antiandrogens such as abiraterone) could be beneficial for the treatment of enzalutamide-resistant advanced PCa.

The colony formation activity of an enzalutamide-resistant AR variant expressing C4-2B cell line was dramatically inhibited by enzalutamide (20 μM) and niclosamide (0.25 μM) cotreatment.[95] The success of the combination therapy on anticlonogenic activity was also validated in castration-resistant CWR22Rv1 cells (expressing AR_{SVs} and AR_{FL}) as well as LNCaP-STAT3 and LNCaP-s17 cells.[94,95] Combination treatment of CWR22Rv1 xenografts showed a significant decrease (70% less weight than the control) in tumor weight after 3 weeks of treatment.[95] The synergistic effect was evident in considering that neither the enzalutamide treatment (nonresponsive) nor the niclosamide treatment (~50% less weight compared with the control) alone were able to achieve robust inhibition.[95] The effect of enzalutamide plus niclosamide on the STAT3 downstream target genes were also more pronounced than either of the individual treatments in LNCaP-s17 and LNCaP-STAT3 cells. Combination therapy was superior to individual treatments in inhibiting STAT3

phosphorylation, expression of survivin, and c-Myc, and AR-ARE (LNCaP-s17) recruitment.[94] PSA secretion was downregulated (>50%) by the enzalutamide/niclosamide cotherapy than niclosamide treatment alone. Enzalutamide alone was not able to exert much inhibitory effect on the PSA levels of these cells with constitutively active STAT3. Analysis of the Ki67 (a cellular marker for cell proliferation) protein levels in CWR22Rv1 xenograft tumor samples revealed that while niclosamide moderately inhibited (~30% inhibition) the Ki67 expression, the combination treatment with enzalutamide far more prominently decreased the Ki67 levels (~80% inhibition).[95]

Enzalutamide treatment, while being quite efficient at early PCa treatment, has recently demonstrated prometastatic effects in preclinical models.[94,134,135] The IL-6/STAT3 feed forward loop is a major pathway through which enhanced cell motility and EMT occur in PCa metastasis.[94,136,137] Evaluation of cell migration through a wound-healing assay using LNCaP-s17 and LNCaP-STAT3C cells (both with constitutively active STAT3), revealed a 20 μM concentration of enzalutamide had little effect on wound-healing inhibition (ie, cell migration), whereas total inhibition was achieved when niclosamide (0.25 μM) was used in cotreatment.[94] Cell invasion assays using LNCaP-STAT3 cells showed similar enhancements in the combination treatment (20 μM enzalutamide plus 0.25 μM niclosamide; 90% reduction of invasive cells) as compared with the individual treatments of enzalutamide (no reduction) or niclosamide (50% reduction).[94] These concentrations of enzalutamide are quite high and may not be achievable in vivo.

Similar to the case with enzalutamide cotreatment, niclosamide also resensitizes abiraterone-resistant PCa cells expressing AR-V7 in both in vitro and in vivo experiments.[93] C4-2B AbiR cells expressing significantly high levels of AR-V7 overcame abiraterone (5 μM) resistance in the presence of si-AR-V7 or niclosamide (0.5 μM). Through oral administration niclosamide (500 mg/kg) synergized abiraterone treatment (200 mg/kg) in a CWR22Rv1 xenograft model resulting in dramatically reduced tumor sizes in the cotreated mice.[93] However, these doses are very high and are unlikely to be clinically reproduced without toxicity. Similar demonstrations of cotreatment effectiveness have been done with bicalutamide, a nonsteroidal antiandrogen drug.[138]

3.3 | Clinical trials and outlook

While niclosamide does not have an ideal pharmacokinetic profile based on the anthelminthic treatments,[72,105] the potency with which it inhibits the STAT3 pathway and induces apoptosis of PCa cells made it a promising drug candidate to find a viable treatment toward CRPC.[96] However, the oral bioavailability of niclosamide has been reported to be as low as 10% (rat), with minimal GI tract absorption.[69,139] In fact, this poor absorption has been used as a strategy to employ salicylanilide anthelmintics (including niclosamide) for the treatment of *Clostridium difficile* (gut bacteria) infections.[140] The poor oral bioavailability of niclosamide, that mostly results from the sparingly soluble nature of the compound in aqueous media, could possibly be overcome by the utilization of more water-soluble analogs, preparations, or prodrugs.[105,141] Given that niclosamide affects numerous signaling pathways other than of the AR and can inhibit the growth of AR-null PCa cells as well as non-PCas, it is not clear that niclosamide mediates its anti-PCa effects primarily through the AR (Figure 5). The lack of effect on full-length AR expression and its modest effects on tumor growth as a monotherapy suggest that niclosamide may not serve as a stand-alone treatment of PCa. Moreover, the applicability of niclosamide may be limited as only a minority of CRPCs express AR-V7. As such, major focus has shifted rather toward the development of cotreatments of niclosamide.

The ability of niclosamide to act as an AR splice variant inhibitor, cell invasion/migration inhibitor, and an IL-6/STAT3/AR axis inhibitor while being already an FDA approved the drug, made it an attractive target to pursue as a codrug to resensitize antiandrogen therapies that have succumbed to resistance mechanisms.[142] Two clinical trials (Phase I, NCT02532114 and NCT03123978) were initiated recently to investigate the

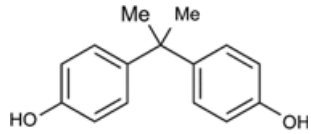
cotreatment of AR splice variant positive mCRPC using enzalutamide and niclosamide. Furthermore, another clinical trial (Phase 1b/II, NCT02807805) to evaluate the side effects of niclosamide treatment in patients with CRPC is now in the recruitment phase for phase II. One of the aforementioned clinical trials, NCT02532114—a dose-escalating study for enzalutamide/niclosamide cotreatment, was concluded recently.[143] The findings from this study appear rather unfavorable for further use of niclosamide in CRPC clinical trials. The minimum effective plasma concentrations relevant to the preclinical response data could not be achieved at the highest tolerable dose (500 mg three-times-a-day [TID]) in this study.[143] While an ideal concentration level above the 82 to 330 ng/mL (0.25-1 μM) range was desired, only a maximum plasma concentration of 35.7 to 82 ng/mL (0.11-0.25 μM) was achievable at 500 mg TID dosing.[143] Although previous use of the drug as an anthelminthic was deemed safe at 2000 mg/day as a single dose (continued for 1-7 days), here in mCRPC patients dose-limiting toxicities were found at 1000 mg TID dosing.[72,143] The toxicity is likely the effect of exceeding a daily tolerable maximum (eg, patient receives 3000 mg of the drug per day with the 1000 mg TID dosing) and/or the reduced ability of CRPC patients to tolerate the drug compared with an otherwise healthy individual with just a helminthic infection. Lack of clinical activity at tolerable doses resulted in the premature termination of this study by its data safety monitoring board.[143] However, these results are in contradiction with the initial results from the phase 1b findings of the trial NCT02807805, where a 1600 mg TID dosing cohort of niclosamide was reported to be safely tolerated.[144] This report claims only a trough serum level of 0.1 μM would be sufficient for anticancer activity, and two patients analyzed (as of the report date) had trough levels of 0.305 and 0.496 μM of niclosamide.[144] It remains to be seen whether final results from NCT02807805 will continue to contradict the findings of the completed study NCT02532114.

While the findings from NCT02532114 are disappointing, the study does establish important groundwork for repurposing of niclosamide as a drug for PCa, as well as other malignancies. The preclinical concerns about the specificity of the effect of niclosamide and the underlying mechanisms that overcome resistance to abiraterone and enzalutamide, seem to agree with the findings of this clinical study. Effective structure-activity relationship (SAR) optimization of niclosamide to improve its oral bioavailability and increased efficacy will be essential for its further development as an antitumor agent.

4 | MARINE SPONGE–DERIVED COMPOUNDS

4.1 | EPI compounds

The EPI compounds (Table 1) developed by Andersen et al[145] are the most well-characterized publicly disclosed AR-TAD inhibitors to date. These compounds inhibit both androgen-dependent and androgen-independent AR activation. They have a structure derived from bisphenol A (BPA).



Bisphenol A (BPA)

BPA, in general, is considered as an endocrine disruptor[146,147] with accumulation potential in mitochondrial membranes that leads to oxidative stress induced cell death/damage.[148] BPA is known to disrupt nuclear hormone receptor signaling, acting as an AR antagonist (IC_{50} = 1-2 μM) and as an ERα agonist (IC_{50} = 10-100 nM).[149,150] BPA is a commonly utilized chemical in industrial processes for the manufacture of polycarbonate plastics, epoxy resins, and other plastic material.[151,152] The annual global production of BPA is close to 4 million tons.[151] As such, environmental accumulation of BPA and its derivatives is inevitable both from industrial waste and from

postconsumer disposal.[153] First isolated from a marine sponge (*Geodia lindgreni*) extract,[154] EPI-001 is likely traceable to BPA derivatives that were present in contaminated (from industrial processes) seawater. On one end of the molecule, the BPA core of EPI compounds is attached to a propane-1,2-diol and on the other end to a chlorohydrin unit via ether linkages (Table 1). The latter functionality provides the ability for EPI compounds to act as covalent binders. EPI-002 (Table 1), synthesized as a single stereoisomer (2R, 20S), has somewhat better performance characteristics in vitro and in vivo than EPI-001, which was a mixture of four diastereomers.[155]

4.1.1 | Initial in vitro and in vivo efficacy

In the initial experiments, EPI-001 inhibited the ligand (R1881 [methyltrienolone]),[156] forskolin, or IL-6 induced activation of the AR to baseline levels.[145] Constitutively active versions of the AR as well as DBD swapped versions were inhibited by EPI-001. These findings established that EPI-001 effects are mediated through the AR-TAD. EPI-001 blocked the androgen-regulated gene expression of some (eg, PSA and TMPRSS2) but not all (eg, BLVRB) genes.[145] Androgen-induced AR interaction at the chromatin level was reduced by EPI-001. This effect was proven not to be a result of decreased levels of AR protein, general prevention of serine phosphorylation of AR, or prevention of AR nuclear translocation.[145] EPI-001 was shown not to affect GR nor PR activity at the concentrations used to inhibit AR.[145] It did not prevent AR ligand binding but inhibited the N/C interaction upon activation.[145] Interaction of EPI-001 at the AR-TAD induced a conformational change as evidenced by steady-state fluorescence spectra. However, no such interaction was observed at the GR-AF1.[145,157] EPI-001 blocked the interaction of the transcriptional coactivator CREB-binding protein (CBP) with the AR-TAD.[145] A similar study with EPI-002 did not inhibit the association of p160 SRC family of coactivators with the AR but showed consistent AR transcriptional inhibition even at elevated SRC levels.[158] EPI-001 inhibited the AR-driven proliferation of LNCaP, PCa2B, and 22Rv1 cells in vitro but did not affect the growth of RKO human colon cancer cells or MG63 osteosarcoma cells.[145]

Intravenous injections of EPI-001 at 50 mg/kg doses to mice demonstrated significant reduction in the weight of the prostates, LNCaP subcutaneous xenografts (start volume = 100 mm^3; 14 days treatment, reduced to 73 mm^3 in EPI treated; 148 mm^3 in control), and serum PSA levels.[145] Intratumoral injections at 20 mg/kg reduced the LNCaP xenograft tumor sizes to 35 mm^3 at 25 days (start = 100 mm^3; control = 436 mm^3). Staining experiments on the harvested xenografts revealed reduced proliferation (Ki67 staining) and increased apoptosis (terminal deoxynucleotidyl transferase dUTP nick-end labeling [TUNEL] screening).[145] Similar demonstrations were made on castrated mice-bearing orthotopic LNCaP xenografts. Conversely, EPI-001 did not affect the growth of AR-null PC3 xenografts. Further experiments of EPI-002 treatment on LNCaP95-derived tumors expressing AR-V7 showed growth attenuation and decreased AR-regulated gene expression.[159] These in vitro and in vivo effects of EPI-001 and its specific stereoisomers against PCa cell lines bearing AR_{FL} and AR_{SVs} were further corroborated by Myung et al.[155]

An independent study by Brand et al[160] showed general agreement to the findings by Sadar et al on EPI-001's ability to affect AR activity, albeit with possible secondary effects (see Section 4.1.4). The multilevel effects of EPI-001 led to the inhibition of transcriptional activation unit 1 (Tau-1) and Tau-5 of the AR-TAD, reduced AR expression, and inhibition of growth of AR-positive and AR-negative PCa cell lines.[160] In vitro domain swap experiments that tethered Gal4DBD to AR-TAD, Tau-1, or Tau-5 proteins showed the ability of EPI-001 to inhibit both the Tau domains.[160] EPI-001 treatment reduced the expression of AR_{FL} (LNCaP, VCaP, LAPC4, and C4-2 cells) and AR_{SVs} (22Rv1 cells), independent of proteasomal degradation. However, AR mRNA and protein expression of CWR-R1 cells were not affected by EPI-001 at the doses utilized.[160] The rate of nascent AR mRNA synthesis in LNCaP cells was reduced by 50 μM treatment of EPI-001. The cell growth inhibition in C4-2 and 22Rv1 cells required greater than 50 μM dosing of EPI-001, while lower concentrations (>5 μM) were sufficient to achieve growth inhibition in LNCaP cells.[160] However, at the higher doses (>50 μM)

growth inhibitory effects were also seen in AR-negative PC3 (PCa), DU145 (PCa), and T47D (breast cancer) cell lines.[160]

Buchanan et al[161] showed that the variable length of the polyglutamine tract within the TAD is known to be inversely associated with the AR transcriptional activity. The inhibition of AR transcriptional activity by EPI-002 was not affected by polymorphic lengths (0, 12, 20, 40, and 49) of the glutamine tract in full-length human AR.[158] EPI-002 was able to inhibit AR isoforms with gain-of-function mutations of the TAD (E255K and W435L) and the LBD (V715M, R761G, H874Y, and T877A). AR-V7 driven expression of UBE2C, CDC20, and AKT1 were significantly reduced upon EPI-002 treatment.[158]

4.1.2 | Stability and covalent binding

The chlorohydrin unit in EPI-001 can be converted to an epoxide species at neutral (pH = 7.4) and basic (pH = 9.4) pH values. However, Brand et al[160] showed that had no significant detrimental effect toward the stability of EPI-001, with 91% and 87% of EPI-001 left after 12 hours in pH = 7.4 and pH = 9.4 media, respectively. Under acidic conditions EPI-001 has excellent stability, while nucleophilic additions at the chlorohydrin could happen depending on the pH of the media and the concentration(s) of the nucleophile(s). At substantially basic conditions (pH = 9.4), EPI-001 formed adducts with glutathione, 2-mercaptoethanol, and cysteamine resulting in 2%, 0%, and 14% of EPI-001 remaining after 12 hours of reaction time with 10 equivalents of nucleophiles.[160] At a more physiologically relevant pH (7.4), 71% (glutathione), 88% (2-mercaptoethanol), and 100% (cysteamine) of EPI-001 was found remaining when exposed to the same nucleophilic substitution conditions.[160] In a separate study by Myung et al,[155] no discernable nucleophilic addition to EPI-001 was found when exposed to 5 equivalents of glutathione or 3 equivalents of 2-mercaptoethanol at pH = 7.4 up to 7 days. Hence it is clear that the thiol alkylating ability of EPI compounds is dependent on the local nucleophile concentration, and that it shows good stability at acidic and neutral pH levels.

Evidence of direct and covalent binding of EPI compounds to the AR-TAD was shown by Myung et al[155] in 2013 through click chemistry experiments. Here, PCa cells were incubated with modified EPI-probes bearing an alkyne functionality. The cells were then lysed, biotin tags were attached to the alkyne functionality using click chemistry, and the subsequent mixtures analyzed using Western blot analysis with antibodies for biotin and AR. All EPI-probes bearing the chlorohydrin moiety covalently bound to the AR regardless of compound chirality.[155] These results indicated that the chlorohydrin unit was essential for the binding at the AR, where compounds that had a hydroxy group in place of the chloro substituent did not show binding activity. Following further experimentation in cell-free conditions using purified recombinant AF1 protein, the binding mechanism of EPI compounds at the AR-AF1 was hypothesized to be: (1) an initial reversible binding (fast) at the binding site, (2) an epoxide formation step (slow) at the chlorohydrin moiety (facilitated by active site amino acids), and (3) covalent binding (fast) to an active site nucleophile with the epoxide.[155]

4.1.3 | Binding site at the AR-TAD

Given that AR-TAD is an IDP domain, it is not trivial to study which subdomains or amino acid residues of it are involved in the covalent binding of EPI compounds. Using solution phase nuclear magnetic resonance (NMR) studies, De Mol et al[162] explored this further to identify regions of AR-AF1 (AA 141-494) that undergo structural changes to facilitate selective binding of the EPI compounds. Two main regions of interest exist in the AF1 that are critical for the transactivation of the AR-TAD and Tau-1 and Tau-5. Tau-1 (AA 102-371) is important for the androgen-dependent activation of AR, while Tau-5 (AA 361-537) has been associated with androgen-independent AR activation mechanisms.[162] Using a predicted model for disorder propensity in the AR-TAD, a 306-residue portion of the IDP that had lower disorder (AF1*, AA 142-448) was constructed and then studied using NMR to reveal partial folding characteristics.[162] Using heteronuclear-multidimensional NMR experiments and a secondary structure prediction algorithm,[163] 50% helical propensity was found at the Tau-1 (185-200) and the Tau-5 (390-410) region residues.[162] This secondary structure formation was independent of the interdomain long-range

interactions. Study of the transverse relaxation rates of the 15N nuclei in the AF1* backbone further corroborated these findings.[162] MS experiments revealed the sufficiently slow reversible interaction of EPI-001 with AR-AF1* before undergoing covalent attachment. Studying this interaction using NMR revealed that distinct 15N chemical shift changes occurred in the Tau-5 residues 354 to 448 upon EPI-001 interaction. In comparison, the effect on the Tau-1 region was minimal. Using smaller polypeptides of Tau-5 interaction region (341-371, 391-414, and 426-446) such interactions could not be demonstrated. Hence it is evident that the entire length of the interaction sequence is necessary, which presumably contributes to the adaptation of a partially folded structure (either naturally occurring or induced by EPI-001) that allows specific drug binding. This binding interaction was nonstereoselective with similar effects seen in the presence of all four diastereomers of EPI-001.[162]

4.1.4 | PPARγ modulation

Nuclear receptor PPARγ (peroxisome-proliferator–activated receptor γ) has important regulatory involvement in multiple disease conditions including cancers, inflammation, and metabolic disorders.[164] Most notable therapeutic use of PPARγ has been in the treatment of type-2 diabetes mellitus, via activation by thiazolidinedione (TZD) drugs. PPARγ agonists have been also generally associated with an ability to reduce tumor progression, including PCa. However, multiple mechanistic studies have found that the observed antiproliferative effects of the PPARγ agonists occur via PPARγ-independent pathways.[165,166] Perhaps the most important of these pathways in PCa is the enhancement of proteasomal degradation of Sp1, a transcriptional factor essential for the expression of multiple genes including the AR.[165–168] Knockdown of Sp1 by siRNA can reduce the mRNA levels of AR and attenuate AR-dependent gene transcription.[166] Furthermore, Sp1 undergoes nontranscriptional inhibition by activated AR.[168,169]

Brand et al[160] hypothesized EPI-001 could modulate the PPARγ function, as a secondary mechanism to exert inhibitory effects on AR expression and activity in PCa. In agreement with this hypothesis, EPI-001 had PPARγ modulation effects similar to PPARγ agonists such as troglitazone.[160] Dose-dependent induction of cyclin-dependent kinase inhibitors p21 and p27 was seen upon EPI-001 treatment, in addition to the inhibitory effects on AR protein expression.[160] In comparison, troglitazone treatment inhibited the activity of AR-GAL4, Gal4-tethered AR-TAD, Tau-1, and Tau-5 analogous to the effects shown by EPI-001.[160] Comparatively, AR-independent, selective PPARγ modulation activity was demonstrated with both EPI-001 and troglitazone, with both inducing mRNA expression of PPARγ target (CIDEC, TXNIP, and PDK4) genes.[160] SiRNA-mediated knockdown of PPARγ activation partially rescued the AR transcriptional inhibition by EPI-001, indicating a possible involvement of PPARγ in EPI-AR inhibition.[160] This knockdown did not affect troglitazone-mediated AR inhibition, which as mentioned before, occurs via PPARγ-independent pathways.[160,166]

Based on the traditional paradigm of PPARγ playing a tumor suppressive role, the above effects of EPI-001 appear beneficial. However, some recent findings suggest PPARγ signaling may actually be positively contributing to the development and progression of PCa.[165] Enhanced expression levels of PPARγ have been found in later stage PCa tissues in recent research,[165,170–172] although there is reason[173] to argue it is also dependent on ethnic/hereditary backgrounds. Inhibition of PPARγ by antagonists such as GW9662 or warfarin can inhibit AR activity.[174] Inverse regulatory effects have also been demonstrated recently by Olokpa et al,[175] where the AR was shown to regulate the expression and the subsequent activity of PPARγ in PCa cells. AR activation by DHT (≥1 nM) reduced the levels and the activity of PPARγ in VCaP and C4-2 CRPC cell lines.[175] Additionally, the use of siRNA to knockdown AR protein resulted in the upregulation of PPARγ activity in CR-2 cells.[175]

With the above findings, it is apparent that there is some contrasting evidence about the role of PPARγ in PCa. The antiproliferative effects of EPI-001 in the study by Brand et al[160] most certainly seem to indicate effects beyond simple inhibition of AR function, supported by the fact that EPI-001 treatment also inhibited the growth of AR-null cell lines. Interestingly, some research shows enhanced PPARγ activity in AR-null (or low) cell lines.[175,176] Hence with the PPARγ agonist functionality of the EPI compounds, they have the ability to exert enhanced PPARγ-dependent (and PPARγ-independent) in vitro effects in such cells.

4.1.5 | Effects on the PI3K-Akt-mTOR pathway

PI3K-Akt-mTOR (phosphatidylinositol-3 kinase [PI3K], protein kinase B [Akt/PKB], and mTOR) signaling pathway has been demonstrated to have importance in PCa biology.[9,177,178] Loss of proper function of the tumor suppressor gene phosphatase and tensin homolog (PTEN) is considered the major upregulation mechanism of Akt signaling in human PCa.[179] PTEN gene is altered in 40% to 60% of advanced PCa cases.[9,180] The inhibition of the PI3K pathway promotes in vitro antiproliferative effects on androgen-induced growth of LNCaP cells, despite the upregulation of AR target gene expression.[177] This upregulation of AR gene expression is linked to the relieving of feedback inhibition of HER kinases.[181] Similar effects were seen in CWR22 PCa xenografts in vivo. Studies with wild-type and mutant AR species showed rapamycin (mTOR inhibitor) mediated upregulation of AR activity required a functional LBD.[177] The cotreatment with bicalutamide and rapamycin led to synergistic, potentiated, and antiproliferative effects on LNCaP cell growth.[177] Marques et al[182] have also demonstrated crosstalk between the PI3K pathway and the AR-signaling axis, where the growth of PC346C xenografts was significantly reduced by PI3K and Akt inhibitors, despite the upregulation of AR target gene expression. This crosstalk between the pathways is reciprocal, given that AR inhibition activates Akt signaling by reducing cellular Akt phosphatase PHLPP levels.[181] Wu et al[183] has shown that this interpathway communication may be dependent on the levels of testosterone. Under low testosterone conditions, AR expression was upregulated in response to subbaseline mTOR activity, and vice versa.[183] Reciprocal communication between AR and the mTOR signaling is also found in other cancers such as breast cancer and hepatocellular carcinoma.[184,185] In hepatocellular carcinoma cells, mTOR signaling reduces AR protein degradation and increases AR nuclear translocation.[185] Using this knowledge, dual inhibition of AR and the PI3K pathways has been validated as an efficient approach for the treatment of PCa in vitro and in vivo.[181]

In an attempt to explore this cotargeting approach in PCa driven by AR_{SVs}, Kato et al[186] evaluated the therapeutic efficacy of a combination of EPI-002 and BEZ235 (PI3K and mTOR inhibitor) in LNCaP95 (enzalutamide-resistant and PTEN-null) CRPC models. In the absence of androgen, BEZ235 increased the expression of AR_{FL} and AR-V7 consistent with the reciprocal feedback mechanism. BEZ235 or everolimus (mTOR inhibitor) both reduced the expression of the AR-V7 regulated gene UBE2C. However, this unexpected effect was seen at longer (48 hours) exposure to the mTOR inhibitors and not so much at 24 hours. EPI-002 was able to reduce the expression of UBE2C by AR-V7 as expected but could not significantly decrease the expression of androgen promoted FKBP5 gene by AR_{FL} at the concentrations (25 μM) used.[186] The latter lack of effectiveness can probably be attributed to the lower potency of the EPI compounds. BEZ235 (15 nM) and EPI-002 both inhibited the phosphorylation of pS6, a ribosomal protein regulated by mTOR signaling. This suggests some cross-reactivity of the covalent inhibitor EPI-002 toward the mTOR pathway. EPI-002 or enzalutamide cotreatment was able to diminish the BEZ235 induced increase of AR_{FL} and AR-V7 in LNCaP cells, and the expression of AR-driven genes in LNCaP95 cells.[186] IL-6 or forskolin-induced AR-TAD activation was lowered by EPI-002, with no further advantage seen by EPI plus BEZ cotherapy. Cotreatment with BEZ235 (5 mg/kg) and EPI-002 (100 mg/kg) showed greater reduction (over 14 days) of LNCaP95 (PTEN-null and enzalutamide-resistant) xenograft volumes than the treatment with each compound alone.[186] While this is a promising in vivo result to establish mTOR and AR dual inhibition is viable in AR-V7-driven PCa,[187] the large amount of EPI compound required to elicit such effect may not be easy to replicate in clinical development.

4.1.6 | Cotreatment with docetaxel

Microtubule targeting taxane drugs, such as docetaxel and cabazitaxel, are the most prominently used treatment at the metastatic castration-resistant stage of PCa. Microtubules play important roles within the cytoskeleton, facilitating intracellular transport functions in the interphase of the cell cycle and in mediating the formation of the mitotic spindle before cell division.[188,189] Taxanes primarily function by binding to the β-tubulin units in cellular

microtubules, stabilizing the polymerized structures, which disrupts the microtubule dynamics required for proper activity.[188,190–192] The inhibition of proper mitotic spindle assembly in the cells activates the "spindle assembly check point", which would eventually lead to apoptosis through the oncosuppressive mechanism called "mitotic catastrophe".[188,190] Apart from this non–AR involved antitumor mechanism of action, taxanes have few other ways they can act against PCa.[193] The ligand-activated AR nuclear translocation is microtubule-network driven with the assistance of microtubule-traversing motor proteins such as dynein.[188] Taxanes effects significantly hinder this process, sequestering AR to the cytoplasm, and hence reducing AR gene transcription.[188,194] Taxanes can drive forkhead box protein O1 (FOXO1; an AR suppressive nuclear transcription factor) nuclear localization, that also results in the suppression of AR-mediated transcription.[188,195] Despite these multipathway inhibitory functions, native or acquired resistance to taxane therapy is seen in mCRPC patients. Among other methods,[188] a primary avenue of resistance is believed to stem from the presence of splice variant forms of the AR.[196,197] In particular, some AR-V7-driven PCa's have marked resistance to docetaxel treatment, both in vitro and in vivo.[196,197] AR-V7, which lacks a hinge region, does not show significant association with microtubules or dynein.[196,197] Although some evidence suggest that the AR-TAD was important for tubulin-AR association,[198] an AR-V7 nuclear translocation mechanism is likely to be independent of the microtubule network, leading to docetaxel resistance.

To evaluate the effectiveness in using an *N*-terminal domain inhibitor of the AR to mitigate this resistance mechanism, Martin et al[199] treated CRPC tumor models with both EPI (EPI-001 or EPI-002) and docetaxel. Docetaxel (1 μM) treatment significantly reduced the 22Rv1 (AR-V7 driven) CRPC cell viability.[199] EPI-002 mono-treatment had a much smaller effect on cell viability despite the 25 μM concentration used. The cotreatment of EPI plus docetaxel was found only to have an additive effect. In 22Rv1 xenografts, a more discernable synergism toward tumor growth suppression was seen at 11 days with docetaxel cotreatment (EPI-001, 200 mg/kg per day; docetaxel, 15 mg/kg per day).[199] EPI-001 treatment alone was unable to suppress this tumor growth despite the relatively high dose utilized. The number of apoptotic cells was (TUNEL assays) far greater in the cotreated xenografts, than in either mono-treatment regimen. Tumor vascularity between the cotreated and the mono-treated groups was not that different.[199]

In agreement with previous findings,[196,197] the cellular distribution of AR_{SVs} was not significantly affected by docetaxel treatment.[199] AR-driven reporter activity of probasin, PSA, and ARR3 genes were all significantly reduced by the cotreatments, though the advantage compared with single-agent treatment was not universally evident.[199] Expression of AR proteins (FL and SVs) was increased in 22Rv1 cells when treated with EPI compound or docetaxel alone. This effect was attenuated by the cotreatment.[199]

In 22Rv1 cells, the expression of N-cadherin (mesenchymal marker) was significantly upregulated by EPI treatment, indicative of EMT initiation. Docetaxel cotreatment attenuated that effect.[199] No significant change in the levels of cellular E-cadherin (epithelial marker) was seen with any treatment combination in vitro.[199] In 22Rv1 tumor xenografts, single-agent treatments increased the levels of E-cadherin and lowered the level of N-cadherin. Unfortunately, cotreatment with EPI and docetaxel reversed this effect, suggesting possible EMT initiation.[199]

Although the suppression of 22Rv1 xenograft growth was promising via the EPI-docetaxel cotreatment, the synergistic effectiveness of this approach is not clear. The use of EPI in this context is further hampered by the need to use a significantly high dosage (200 mg/kg per day) in xenograft studies. A more potent inhibitor of the AR-TAD might be able to exert better synergistic effects to mitigate AR_{SV}-driven taxane-resistance in CRPC.

4.1.7 | EPI compounds as imaging agents

Based on the selective covalent binding ability of the parent compound to the AR-TAD, Sadar and coworkers developed ^{123}I-labeled analogs of EPI-002 as potential tools for the imaging of PCa's that express AR isoforms.[200,201] Iodine substituted (at the carbon-15) EPI-002 was synthesized first as the cold version (I-EPI-002) to test the binding efficacy before moving on to incorporation of the radiolabel. I-EPI-002 inhibited the androgen-induced transcriptional activity of endogenous AR in LNCaP cells at an IC_{50} of 1.17 μM.[200,201] The

authors indicated this to be as potent an effect as enzalutamide treatment and 10 times as potent as EPI-002, with reference to previously reported IC_{50} values for those compounds. The inhibition of AR activity in reporter gene assays was achieved in similar levels (~75%) with the treatment of 25 μM of EPI-002 or 2 μM of I-EPI-002.[200] Luciferase reporter assays demonstrated the iodinated version maintained specificity to the ARE without significant effects on GRE, PRE, nor ERE at the concentrations used.[200,201] I-EPI-002 inhibited the proliferation of LNCaP95 cells (AR-V7 driven) with an IC_{50} of 6.9 μM. Here, EPI analogs caused G0/G1 cell-cycle arrest in the tumor cells.[200] Binding of the radiolabeled probe (^{123}I-EPI-002) to endogenous AR_{FL} in LNCaP95 cells was shown by phosphorimaging. This binding was reduced when the cells were cotreated with EPI-002, reaffirming that they both bind the same target site(s).[200]

In vivo time-dependent biodistribution analysis (with the use of a γ-spectrometer) conducted after the administration (tail-vein) of the radio-probe, revealed broad organ distribution. Highest levels of accumulations were observed in the intestines, gall bladder, and the liver.[200] Authors correlate this effect to the fact that EPI compounds having lipophilic structures (^{123}I-EPI-002 cLog P = 4.2) are expected to be eliminated by the hepatobiliary system.[200] The variation of EPI compound biodistribution in tissues over time (eg, percentage of "injected dose/gram" in the large intestine: 1.2% at 1 hour-66% at 4 hours),[200] may indicate that the binding of EPI compounds at the tissues is sufficiently reversible at physiological conditions. Maximum tissue concentrations (2.2% injected dose/gram) in the LNCaP95 xenograft was found at 2 hours, which could be blocked up to 74% with the coadministration of cold EPI-002 (50 mg/kg).[200] This blocking effect was not seen at PC3 xenografts or muscle tissue, indicating that the blocking was specific to AR containing tissue.[200] Micro-single-photon emission computed tomography/computed tomography (micro-SPECT/CT) imaging at 2 hours was able to corroborate these findings, where the radio-probe was able to specifically visualize the LNCaP95 xenograft vs the PC3 xenograft.[200] While the above observations were quite promising, the stability of the radio-probe was found to be questionable due to the observation of accumulation of radioactivity in the thyroid (2.3% of injected dose/gram). If the ^{123}I dislodging from the probe is a possibility, then further concerns do arise about the reliability of data on the long-term in vivo biodistribution of ^{123}I-EPI-002, as well as about the concomitant toxicological effects. Nevertheless, this study established the first proof-of-concept experiments on using an AR-TAD–targeted compound to visualize AR-driven PCa tissues.

4.1.8 | Clinical trials and outlook

First-in-human phase 1/2 clinical trials (clinicaltrials.gov; NCT02606123) of EPI-506 (Ralaniten acetate) were initiated by ESSA Pharma in 2016-2017. EPI-506 is an acetate prodrug of EPI-002.[45,202] The study was directed to evaluate the safety, pharmacokinetics, maximum tolerated dose, and antitumor activity of EPI-506 in men with end-stage mCRPC who have progressed after prior enzalutamide and/or abiraterone treatment and may have received one prior line of chemotherapy.[203] In the phase 1 study, 28 patients were treated at EPI-506 doses ranging from 80 to 3600 mg/day doses.[203] The drug was found to be generally well-tolerated at doses up to 2400 mg/day. Consistent with the preclinical observations, somewhat higher doses (>2400 mg/day) were required to see any effects on serum PSA levels. Modest lowering of PSA levels (4-29%) were seen in five men at doses greater than or equal to 1280 mg/day. No reductions of PSA by 50% or more, a standard for evaluation of early phase clinical trial activity, was observed. In prioritizing their efforts to develop a different class of AR-TAD inhibitors with increased potency to EPI-506, ESSA Pharma Inc announced in September 2017 that they will discontinue further development efforts toward EPI-506. These newer "Aniten program" compounds are also stated to have structural similarities to the EPI series of compounds.[203]

4.2 | Polychlorinated small peptides: sintokamides and dysamides

Sintokamides (Table 1) are a class of natural products that were isolated from marine sponges *Dysidea* sp. via extraction using methanol (MeOH).[204] Structurally, sintokamides are polychlorinated peptides, capable of

undergoing nucleophilic additions at the enone site and nucleophilic substitution reactions at the chlorinated carbons. Sintokamide A blocked the R1881-induced AR PSA-luciferase reporter activity with no discernable activity against PR-mediated or GR-mediated transcription.[204,205] This effect was found not to be exerted through direct AR-LBD binding, where sintokamide A (0.5-50 μM) was unable to compete off fluoromone binding to the AR-LBD. Similar to EPI-002 treatment, sintokamide A inhibited the proliferation of both LNCaP (AR_{FL} driven) and LNCaP95 (AR-V7 driven) cell lines,[205] but not of AR-negative PC3 PCa cells.[204,206] The inhibition of androgen-induced LNCaP cell proliferation was comparable to the effects of the AR antagonist bicalutamide.[204] Sintokamide A did not affect the AR cellular distribution with or without androgen stimulation.[205]

Sintokamide A (5 μg/ml) inhibited the transactivation of the AR-TAD stimulated by forskolin.[204] Forskolin is an AR-TAD transactivation facilitator which activates the AR through a protein kinase A–mediated pathway.[204,207] Forskolin-induced dephosphorylation of the AR can lead to impaired ligand binding,[208] which suggests the increased activity comes possibly via *N*-terminal transactivation. The forskolin-treated cells significantly increased the luciferase-mediated luminescence indicating AR-TAD transactivation.[204] In comparison, cells that were pretreated with sintokamide A before forskolin treatment had the AR-TAD transactivation significantly diminished.[204,205] However, sintokamide A was less active than EPI-002 in asserting this effect.[205] Furthermore, unlike EPI-002, sintokamide A was not able to inhibit the IL-6–induced AR-TAD transactivation.[205] Use of sintokamide A and EPI-002 in combination had additive effects in inhibiting the AR-mediated gene expression in luciferase reporter assays.[205] With these observations, it was postulated that sintokamide A and EPI-002 likely bind to two different regions in the AR-TAD-AF1.[205]

Biological evaluation of structurally related polychlorinated small molecule peptide analogs called dysamides (Table 1) has shown similar AR inhibitory activity.[206] Like the sintokamides, dysamides were also isolated from marine sponges in the *Dysidea* sp. family.[209] In a comparative activity study, sintokamides A, B, C, and E, as well as dysamide A demonstrated inhibition of a R1881-induced PSA-luciferase signal.[206] Compared to the inhibition effected by bicalutamide, the sintokamides showed inhibition levels of ~60% to 100% of the luciferase signal. Sintokamide B, which has the highest degree of chlorination in its structure, demonstrated the greatest inhibition potency. Dysamide A was also able to reach an inhibition level of ~50%. Interestingly, a nonchlorinated analog of sintokamide was only able to show a modest percentage of inhibition (~20%) as compared with the parent compounds.[206] In a separate study, isopropyl substitutions in place of chlorinated carbon groups in sintokamide A, resulted in a compound (LYP19) with negligible activity (IC_{50} = ~40 μM in an AR reporter assay).[205] Hence these compounds were postulated to also have a mechanism of action similar to EPI-002, involving a nucleophilic attack at the chlorinated carbons, at the AR-TAD binding site. Using click chemistry experiments analogous to those done with EPI compounds,[155] covalent binding of sintokamide A at the AR-TAD was established.[205]

In subcutaneous LNCaP xenograft models, sintokamide A treatment reduced the tumor volume with time.[205,206] However, the metabolic stability of sintokamide A was found to be poor. Following an intravenous dose of 50 mg/kg, a C_{max} (8 μM) lower than the in vitro IC_{50} values was achieved in plasma, with a $t_{1/2}$ of 1.16 hours.[205] Antitumoral effects of sintokamide A (30 mg/kg per day every 3 days) against LNCaP95 xenografts (driven by AR-V7) were demonstrated by the ability to inhibit (~36%) tumor growth up to 15 days.[205] PSA levels and the number of Ki67 (proliferation marker) positive cells in the harvested LNCaP xenografts were also found to be significantly decreased after undergoing sintokamide A treatment.[205]

Unfortunately, the clinical relevance and translation ability of the in vivo data for sintokamide A are uncertain because the compound needs to undergo intratumoral delivery due to its metabolically unstable [205] nature. For any further development of sintokamides a as viable therapy against PCa, SAR studies to improve the in vivo and in vitro qualities of the compound are particularly necessary. To this end, the attempts already made to establish synthetic routes to produce sintokamides via organic synthesis will provide a useful starting-point.[206,210]

4.3 | Niphatenones

Analogous to sintokamides, niphatenones (Table 1) are marine natural products that have been isolated from the marine sponge *Niphates digitalis* through continuous extraction of sponge samples with MeOH.[211] Key structural features of niphatenones include a Michael acceptor enone moiety and an EPI-like glycerol ether moiety along with a long hydrocarbon chain. Naturally occurring niphatenones A and B both carry the stereogenic carbon in the "S" conformation.[211] After initial structure elucidation both stereoisomers of niphatenones A and B were manually synthesized on a larger scale to carry out further analysis.[211] The "R" isomers of both compounds showed somewhat better activity than the naturally occurring "S" isomers, with R-niphatenone-A treatment showing PSA-luciferase activity of ~35%, and R-niphatenone-B treatment reducing activity to 25%. Hence R-niphatenone-B was the best inhibitor of the PSA-luciferase activity that was unveiled through this initial study.[211]

Hydrogenation of the double bond of R-niphatenone-B resulted in loss of half of the activity of the original enone compound. However, this R-dihydroniphatenone-B form still functioned better than S-niphatenone-A, showing that the enone function, while important, was not an absolute necessity for activity. Removing the glycerol moiety (akin to the EPI compounds) from the molecule via other functional group transformations also resulted in similar loss of activity. Shortening the long alkyl chain to a methyl group, resulted in complete loss of activity. Niphatenone-B (S or R) did not affect the proliferation of PC3 (AR-negative) cells, while they did inhibit the AR-dependent proliferation of the LNCaP cells.[211] In another series of experiments involving an AR-driven PB-luciferase reporter assay both the S- and R-niphatenone-B compounds had IC_{50} values around 6 μM toward blocking AR-driven reporter responses.[212]

Transactivation (pretreatment with IL-6 for 24 hours) driven AR-TAD-Gal4-luciferase activity in LNCaP cells was reduced to 76% in the presence of R-niphatenone-B, while S-niphatenone-B reduced it to 50% compared with the control (100%).[212] EPI-002 treatment (positive control) reduced this activity to 40%. The activity of the constitutively active AR splice variant ARvar567 was also lowered to ~65% by both of the enantiomers of niphatenone-B.[212] In assessing the cross-reactivity with other NHRs, Banuelos et al[212] showed that S-niphatenone-B inhibited (lowered to 19%) the steroidal transcriptional activity of full-length AR but not that of the PR. However, here S-niphatenone-B also lowered the transcriptional activity of GR down to 80% as measured using a GRE-luciferase reporter. Using fluorescence polarization assays, S-niphatenone-B was found not to interfere with ligand binding to AR, PR, or GR at concentrations less than 30 μM.[212] S-niphatenone-B significantly reduced (~50%) the N-terminal/C-terminal interaction[213–215] in the AR upon ligand binding.[212] S-niphatenone-B also inhibited the androgen-induced expression of AR-regulated genes PSA (70% inhibition) and KLK2. However, it did not affect the subcellular localization of AR nor the levels of AR protein in LNCaP cells.[212]

Click chemistry experiments by Meimetis et al[211] with R-niphatenone-B (alkyne functionalized analog) showed that it can covalently bind the AR-TAD AF1. Extending this study, Banuelos et al[205] showed S-niphatenone-B covalently bound both AR-AF1 and GR-AF1. Even with the small sequence identity between the two receptor types,[216–218] this result raised questions about the specificity of the niphatenone binding.[212] Alkylation reactions of niphatenones with glutathione reaffirmed the tendency for promiscuous covalent binding.[212] Hence the authors concluded that niphatenone-B was not viable for further development.[212] Analogs with lower reactivity that lack the enone functionality or in which it is replaced by a less reactive homolog may be worth pursuing in the future.

5 | MAHANINE

Mahanine (Table 1) is a carbazole alkaloid present in the leaves and the edible parts of the Thai vegetable *Micromelum minutum*[219,220] and the Southeast-Asian curry leaf plant *Murraya koenigii*.[221] Mahanine exhibits antimicrobial and anti-inflammatory effects.[220–222] It is a potent apoptosis-inducing agent against leukemic cells via

mitochondrial pathways[219,223] and against pancreatic cancer cells via the induction of reactive oxygen species production.[224] Mahanine can restore the activity of epigenetically silenced tumor suppressor gene RASSF1A in human PCa cells resulting in the downregulation of the key cell-cycle regulator cyclin D1.[225]

Exploring the promising activity of mahanine against PCa cells, Amin et al[226] showed it can inhibit ligand-dependent and ligand-independent transactivation of AR, as well as initiate AR degradation and inactivate cyclin-dependent kinase 1 (CDK1) in PCa cells. Within the assay times and the mahanine doses used (up to 10 μM), the cellular AR levels did not significantly decrease. The expression of DHT-induced AR target genes GREB1, NDRG1, PSA, PMEPA1, and SGK1 were also repressed in the presence of mahanine. Mahanine did not affect the luciferase activity of a constitutively active VP16-Gal4DBD vector, showing the Gal4DBD was not involved in the inhibitory effect of mahanine toward the AR-TAD.[226] Forskolin or IL-6 induced luciferase activity via AR-TAD transactivation was reduced by ~80% in the mahanine-treated cells compared with the control.[226]

At a dose of 10 μM, mahanine decreased the levels of AR in LNCaP, VCaP, and 22Rv1 cells over 3 days. Mahanine induced not only the degradation of the full-length AR but the 80-kDa splice variant AR-V7 found in the 22Rv1 cells.[226] Further studies involving the pretreatment of LNCaP cells with cycloheximide to inhibit protein biosynthesis followed by treatment with mahanine, reaffirmed that mahanine induces AR degradation. Such degradation was significantly reduced when mahanine treatment was done in the presence of the proteasome inhibitor MG132, indicating the presence of a ubiquitin-proteasome pathway for the degradation of AR by mahanine. A twofold increase in ubiquitinated-AR was found in LNCaP cells treated with MG132 (5 μM) and mahanine (20 μM) over 12 hours.[226]

The DHT-induced AR nuclear translocation in LNCaP cells was greatly diminished by mahanine as shown by immunofluorescence assays. Similar experiments done on 22Rv1 cells showed that the AR-V7 splice variant was also distributed more in the cytoplasm in the presence of mahanine. Monitoring of the nuclear AR localization over 12 hours showed that the AR content in the nucleus was progressively depleted as the AR migrated into the cytoplasm. By conducting the experiments in the presence of cycloheximide and MG132 the migratory effect was further confirmed.[226] When the LNCaP cells were grown in CS media, mahanine was not able to prevent the DHT-induced nuclear translocation of AR. But the translocated AR was transcriptionally inactive as judged by the PSA expression levels.[226]

Mahanine inhibited the DHT-induced phosphorylation of AR Ser-81 which is considered an important posttranslational modification[227] for AR transcriptional activity.[226] To evaluate a possible pathway by which this inhibition occurs, LNCaP cells were synchronized to the G2-M phase of the cell cycle, where maximal CDK1 activity occurs by treatment with nocodazole (100 ng/mL, 24 hours). CDK1 is known to phosphorylate the AR at the Ser-81 site in an androgen-dependent manner.[228] While the untreated cells showed induction of AR Ser-81 phosphorylation caused by the activation of CDK1 by nocodazole, cells treated with mahanine (10 μM) had significantly decreased phosphorylation.[226] DHT-induced AR Ser-81 phosphorylation in LNCaP cells cotransfected with a constitutively active version of CDK1 (ie, nonandrogen-dependent) was not significantly affected by mahanine. While these data do not indicate the inhibition of AR signaling by mahanine is exclusively dependent on a CDK1-mediated pathway, it is noteworthy since CDK1 activity is commonly elevated in CRPC.[228] Given the ready natural availability and the possibility to devise an efficient total synthesis,[229] mahanine and its derivatives hold some promise to be developed further.[230] Its effects on multiple signaling pathways, however, raise questions about specificity and underlying mechanism(s) of action.

6 | VPC COMPOUNDS, SKLB-C2807, AND PYRVINIUM

6.1 | AR-DBD inhibition at the P-box region

Direct inhibition of the AR-DBD is also a plausible but less explored approach for AR-signaling axis inhibition. Binding of the activated AR (both FL and SVs) to DNA to initiate transcription is achieved through the AR-DBD.

Key interactions in this association are made through the DNA recognition α-helix that consists of a P-box region[231] that inserts into the major groove of the DNA.[232] Unlike the case with the TAD, crystallographic data has been obtained for the DBD (rat, PDB 1R4I) with the use of an AR-DBD dimer bound to a steroid DR3 response element.[3] Based on this structural information, an in silico drug design/screening approach by Li et al established a plausible binding site (Ser-579 to Lys-610) under the P-box region of the AR-DBD.[232–236] Virtual screening of drugs at this site and hit optimization through SAR led to the discovery of thiazolyl morpholine derivatives VPC-14228 and VPC-14449 (Table 1). VPC-14449 showed in vitro inhibition of R1881 stimulated AR activity (IC_{50} = 0.12 μM) and PSA suppression (IC_{50} = 0.17 μM) in LNCaP eGFP cells, comparable to enzalutamide treatment under the same conditions.[232] Activity inhibition, albeit with less potency, was also demonstrated later in enzalutamide-resistant 22Rv1 cells.[14] There was no discernable effect seen on the cell viability of non–AR-driven PC3 cell growth by VPC-14449.[14] Furthermore, it showed no significant effects toward 31 genotoxin-responsive genes (eg, CASP1, XPC, and ATF3), indicating no discernable cytotoxicity.[233] The structure initially reported[232] for VPC-14449 in the 2014 disclosure was later found to be misassigned and subsequently corrected in 2017.[234]

Pyrvinium, a quinoline-derived cyanine dye, was tested along with the VPC compounds due to the reports of its function as an AR-DBD inhibitor.[237,238] While it also inhibited AR transcriptional activity (IC_{50} = 0.194 μM), pyrvinium induced a non-PCa selective strong apoptotic response evidenced by poly (ADP-ribose) polymerase (PARP)-cleaved products.[14,233] Such nonselective PARP cleavage was not seen for VPC-14449.[14] In a study by Lim et al,[237] pyrvinium had significant nonselective inhibition toward other NHRs as well, albeit in prostatic cells. This cross-reactivity could be explained by modeling studies that show the interaction of pyrvinium with the AR-DBD to occur at the conserved area of Lys-610 to Pro-613.[233,237] Nevertheless, there has been much interest in exploring pyrvinium pamoate as an anticancer therapeutic due to the fact it was once used as an anthelminthic drug with FDA approval. Given that it affects a large number of key cellular pathways and cancer types,[239–241] there is a need to alter its structure/function before it can be further developed as a potential therapeutic.

Important coordinating interactions with VPC-14228 at the AR-DBD binding site were found at Ser-579, Val-582, Phe-583, Arg-586, Gln-592, Tyr-594, and Lys-610 through docking studies on a human AR-DBD homology model.[232] Gln-592 (not conserved across other NR) and Tyr-594 in particular were initially proposed to be important in the selective binding to AR-DBD, which has a highly conserved structure with other human NHRs.[232,233] Site-directed mutagenesis experiments (Gln592Asp, Lys593Asp, and Tyr594Asp) confirmed the proposed target site, and further in vitro experiments showed VPC compounds' ability to inhibit the activity of AR_{SVs}.[14,232,233] Y594D mutation (H-bonding site removed) greatly reduced the activity of the VPC compounds in AR_{FL} and AR_{SVs}, while pyrvinium could still strongly inhibit the AR transcriptional activity.[233] Using docking studies, Tyr-594 was shown to form a hydrogen bond with the VPC compound when bound to a human AR-DBD homology model.[232] Later mutagenesis studies (K593D) revealed that Lys-593 was also an essential residue to facilitate VPC compound interaction at the AR-DBD.[14]

VPC compounds, although few folds lower than for AR inhibition, does inhibit ER transcriptional activity at higher doses (>5 μM).[233] Similar selectivity between AR-DBD and GR-DBD was shown using a chimera construct, where the KQKYL sequence of the AR-DBD was replaced by QHNYL.[14] Compared to the wild-type AR (IC_{50} = 0.291 μM), the chimera with GR-DBD (IC_{50} = 11 μM) showed resistance to VPC-14449 inhibition of R1881-induced activity.[14] VPC compounds do not significantly inhibit GR or PR activity at concentrations equal to or less than 25 μM.[233] Using yellow fluorescent protein (YFP) tagged AR_{FL} and AR-V7, Dalal et al[233] showed that the inhibition of the DBD by VPC compounds did not impede the nuclear translocation of the AR. While the chromatin binding of nuclear localized AR_{FL} was suppressed by more than 1 μM concentrations, no significant inhibition was seen for AR-V7 even with the use of 50 μM concentrations of VPC-14449.[14] Similar need for higher concentrations (>10 μM) of VPC-14449 to suppress chromatin-binding interactions were seen for ARv567es in R1-D567 cells and for AR in MR49F (enzalutamide-resistant) and C4-2 (androgen insensitive) cell lines.[14] Association of the translocated AR with PSA and FKBP5 AREs were reduced in the presence of VPC compounds.[233] VPC-14449 also

inhibited the association of ARv567es to FASN and FKBP5 AREs at higher concentrations (>50 μM) but not at lower concentrations (>10 μM).[14]

Dalal et al[14] showed that VPC-14449 could inhibit clinically relevant AR-LBD mutants activity and show additive in vitro effectiveness in cotreatment with enzalutamide. R1881-induced AR-dependent gene expression (TMPRSS2, KLK3, and FKBP5), in cell lines carrying AR with wild-type or mutant LBD, was significantly suppressed by the treatment of 5 μM VPC-14449.[14] A greater concentration (50 μM) of VPC-14449 was required to induce a significant change in AR-V7 driven UBE2C gene expression.[14] Expression of the GR-driven gene FKBP52 was not affected under these conditions.[14] Only a modest inhibitory effect on ARv567es-driven expression of FASN and FKBP5 gene were seen even at 50 μM treatment with VPC-14449.[14] These observations collectively suggest that these compounds do not robustly inhibit AR_{SV}-driven gene expression, contrary to the design principle of the compounds. Dalal et al[14] postulate this may derive from the fact the AR_{SVs} conformational arrangement may differ significantly enough from the AR_{FL} resulting in a different mode of association with the AREs and/or a DBD interacting compound such as VPC-14449. It was also postulated that the AR_{SVs}, which are predominantly nuclear localized, exists in a chromatin-associated form (shown by chromatin fractionation studies) less susceptible to inhibition by the VPC compounds.[14] A higher dosing (intraperitoneal injection, twice daily, 4 weeks) of VPC-14449 (100 mg/kg) was as effective as enzalutamide (10 mg/kg) in blocking androgen signaling in vivo as evidenced by reductions in tumor volume and serum PSA levels in mice with LNCaP tumor xenografts.[233]

Further SAR analysis of VPC-14228 (Table 1) by Xu et al[242] established that the thiazole and the morpholine rings were essential to the activity of the VPC compounds. Introduction of an acyl group at the phenyl ring of VPC-14228 gave rise to a lead compound, SKLB-C2807, which had antiproliferative effects (IC_{50} = 0.38 μM) toward a LNCaP-AR PCa cell line.[242] Docking studies on a human AR-DBD homology model showed SKLB-C2807 forming key H-bonding interactions (between the morpholine-O and Tyr-594, and between the carbonyloxy of the benzene ring with Arg-609) and hydrophobic interactions (benzene ring with Lys-610).[242] The recent studies with SKLB-C2807 corroborated the previous findings with the VPC compounds, and showed selective in vitro activity against AR-positive PCa cell lines and no impediment toward AR nuclear translocation.[242]

The dosing used[14] to obtain significant in vivo effects seems to be quite large when considering the lower in vitro reporter assay IC_{50} (100-200 nM) values generally found for the VPC compounds. Metabolic stability would have to be evaluated more closely to see if there is a correlation. Furthermore, the effectiveness of these compounds against AR_{SV}-chromatin association seems to be minimal. SAR optimization to increase the in vivo efficacy and to increase the ability to affect AR_{SVs} will be critical for future development[243] of these compounds.

6.2 | Targeting the AR-DBD D-box region and AR dimerization

Another key area of extended application for the notion of AR-DBD inhibition is to disrupt the DBD-mediated dimerization of the AR, which is an essential step in the AR activation process.[244] Until recently, AR dimerization (homo and hetero) was thought to exclusively occur in a head-to-tail fashion, facilitated by key intermolecular interactions between the D-box regions of the two associating AR-DBDs.[245,246] However, new findings by Nadal et al[247] do also indicate that the AR-homodimerization mechanism may occur in a head-to-head fashion, facilitated by key intermolecular interactions between the H5 helix regions of the two associating AR-LBD's. Using in silico methods and the former paradigm for AR dimerization, Dalal et al[244] recently found several VPC compounds that disrupt the AR-DBD dimerization interface. The lead compound discovered from this study, VPC-17005, reduced the viability of LNCaP (IC_{50} = 1.5 μM), MR49F (enzalutamide-resistant; IC_{50} = 1.8 μM), and 22Rv1 (enzalutamide-resistant; IC_{50} = 21 μM) cells.[244] PC3 cell viability was not affected up to a concentration of 10 μM (assay limit). These IC_{50} values for VPC-17005 are however either similar (LNCaP and 22Rv1) or lower (about threefold lower in MR49F) than found for VPC-14449 treatment.[244]

VPC-14449 inhibited the transcriptional activity of an AR D-Box site mutant, while VPC-17005 could not. This affirmed that the two VPC compounds possibly impacts two different regions of the DBD.[244] The ligand-induced reporter activity inhibition by VPC-17005 was few-fold selective for AR vs other NHRs. The activities of GR, PR, and ER were not inhibited up to 20 μM (assay limit) VPC-17005 treatment, while significant activity attenuation was seen in AR at a 5 μM concentration.[244] While these results are promising as a proof-of-concept for targeting two different regions of the DBD, it remains to be seen if this selectivity difference (vs other NHRs) is high enough to warrant the development of VPC-17005 as a selective AR-DBD dimerization inhibitor. It would be better to improve the AR-DBD selectivity, possibly via further SAR of VPC-17005. Additionally, future research could also investigate VPC-17005 and VPC-14449 cotreatment to evaluate possible synergistic effects.

7 | JN COMPOUNDS

Our own program to develop inhibitors of the AR-TAD have resulted in a series of compounds that selectively inhibit the AR-mediated transcription in AR-positive cells in vitro and attenuate PCa xenograft growth in vivo.[248,249] The initial hit compound (JN018) was identified through a high-throughput screening assay in which reporter gene expression in yeast is dependent upon the constitutive transcriptional activity of an AR_{SV}. Extensive SAR studies led to a series of compounds (termed the "JN series") with over 150 analogs synthesized to date. The active compounds attenuate the growth of AR expressing PCa cells but not AR-null PCa and non-PCa cells. They were shown to directly bind the AR and function through inhibition of the TAD. Interestingly these compounds also have potent and selective degradation effects toward both full-length and splice variant AR. The TAD-inhibitory and the receptor-degradation effects are selective toward the AR, while not affecting the GR. The in vitro antitumor effects of the JN compounds are significantly more specific toward AR-positive cell lines than the in vitro effects recently shown for the AR degradation enhancer ASC-J9®.[250] Furthermore, the active JN series compounds have 10-fold to 30-fold higher potency in comparison to EPI-002 in cell viability and functional assays. While neither the structures nor the specific data of JN series compounds have been disclosed publicly yet, the lead compound along with a few other analogs have moved on to in vivo testing in relevant PCa xenograft models in which oral administration resulted in significant control in tumor growth of VCaP (enzalutamide-resistant), 22Rv1 (enzalutamide-resistant), and LNCaP-AR castration-resistant xenografts, without any apparent toxicity. Based on these promising results, further development of these compounds toward preclinical evaluation is currently underway in partnership with a prominent pharmaceutical company.

8 | BET INHIBITORS

The bromodomain and extraterminal domain (BET) family of proteins have found profound recent interest in being a target for small molecule inhibition. These proteins are characterized by the presence of two tandem bromodomains and one extraterminal protein domain.[251] They are ~110 amino acids containing protein domains that primarily function as readers of lysine acetylation codes in histones and facilitating epigenetic regulation of gene transcription.[251–253] The histone code arising from posttranslational modifications translates to important information that is potentially hereditary and may result in non–DNA-derived phenotypic changes.[254] Enrichment of the H3K27Ac acetylation marker is found at the proximal sites of the AR gene.[255] Several AR cofactors (eg, lysine specific demethylase 1 [LSD1]) are known to control the expression of AR target genes with modifications to the histone proteins.[256]

The primary members of the mammalian BET protein family are BRD2, BRD3, BRD4, and BRDT. Bromodomain-containing proteins are involved in the regulation of oncogenes such as Myc. Overexpression of BRD4, in particular, is found in multiple types of cancer.[257] As such BRD4 has been a subject of extensive research as a drug discovery target in the past decade.[258] BET proteins also serve as key coregulators of other transcription factors such as the AR. Direct association of BRD4 to AR has been demonstrated.[259] Reasonable understanding of the function of BET proteins led to a marked research interest resulting in many pharmaceutical companies attempting to identify BET inhibitors as anticancer therapies.[257,260,261] Availability of crystal structures have amplified this interest due to the ability to adopt in silico design/screening approaches to accelerate hit discovery.[262]

8.1 | BET inhibitor JQ1

Targeting coactivators of AR gene transcription as a method to disrupt AR gene transcription at the chromatin level is an evolving method for targeting CRPC.[263] Out of the BET family of proteins, BRD4, in particular, has shown the ability to interact with the AR-TAD in facilitating AR gene transcription.[264] Building on the reported ability of thienodiazepines to bind bromodomains,[265] JQ1 (Table 1), an efficient cell-permeable small molecule inhibitor of BET proteins, was found through in silico design approaches utilizing the apo crystal structure (PDB: 2OSS) of the first bromodomain of BRD4.[266] It is a pan-BET inhibitor (BETi) due to the highly conserved nature of the BET acylated lysine (Kac)-binding pocket. The S-(+) isomer was the active stereoisomer of the compound with the R-(−) isomer showing no binding ability.[255,266] Chemically JQ1 has a thienotriazolodiazepine core structure. Hence it has structural similarity to allosteric modulators of the $GABA_A$ receptor, such as benzodiazepines (eg, diazepam) and triazolobenzodiazepines (eg, alprazolam). Using an ExpresSProfile assay (with 53 receptor proteins), Filippakopoulos et al[266] showed JQ1 (1 μM) does not affect radioligand binding at the $GABA_A$ receptor benzodiazepine site. Showing further specificity in binding, JQ1 caused partial inhibition of ligand binding only in neurokinin NK2 and adenosine A3 receptors, out of 52 other receptor proteins probed.[266]

JQ1 inhibition of bromodomains is generally specific to the BET family out of all the human bromodomain–containing proteins.[266] This binding specificity is believed to be in part derived from the conserved gatekeeper residue Ile-146 at the bromodomain 1 of BRD proteins 1 to 4.[267] Other bromodomain-containing proteins having a bulkier gatekeeper residue (eg, Tyr in PCAF and GCN5) does not allow for the efficient binding of JQ1-like compounds that have a pendent aryl group.[267] Binding of JQ1 at the BRD3 and BRD4 bromodomains (K_d = 50, 90 nM) was about threefold better than at the BRD2 and BRDT.[266] Luminescence proximity homogeneous assays have established the ability of JQ1 to inhibit the binding of acylated lysines at the BET bromodomains. JQ1 inhibited the binding of a tetraacetylated histone H4 peptide to BRD4 (IC_{50} = 77 [first bromodomain] and 33 nM [second bromodomain]) but not the binding of an acetylated H3 peptide to CREB-binding protein (CREBBP).[266] High-resolution crystal structures and docking studies revealed a perfect fit for the geometrical shape of the (+) isomer at the bromodomain acylated lysine (Kac)-binding sites. In contrast, binding of the (−) isomers to the Kac-binding site in docking studies resulted in high-energy distortions due to steric clashes.[266] Binding of JQ1 stabilizes the Kac-binding site flexibility with significant hydrophobic interactions forming between the ligand and the binding site.[266] JQ1 (500 nM) was able to competitively inhibit BRD4-chromatin association.[266]

A landmark study in 2014 by Asangani et al[264] established that JQ1 selectively inhibited the growth and colony formation of AR-driven VCaP, LNCaP, and 22Rv1 PCa cell lines at 50 to 200 nM IC_{50} values. Knockdown of BRD2-4 proteins by targeted siRNA resulted in similar effects on cell proliferation and invasion, phenocopying JQ1 treatment.[264] JQ1 treatment of AR-positive cell lines further showed G_0-G_1 arrest and apoptosis with a dose-dependent increase in cleaved PARP (apoptosis marker).[264] Similar to the usual BETi effects, JQ1 treatment downregulates the antiapoptotic protein BCL-xl in these cell lines in a dose-dependent manner.[264] AR-regulated target proteins were also downregulated by JQ1 in the AR-positive cell lines. This downregulation was not recovered by cotreatment with a proteasomal inhibitor (bortezomib) indicating the downregulation to be at the transcriptional level.[264]

JQ1 (0.5-2.5 μM) inhibited MYC protein expression in AR-positive PCa cells (VCaP, LNCaP, and 22Rv1) but not in the AR-negative PCa cells (DU145 and PC3).[264] MYC is a critical proto-oncogene that acts as a regulator of cell growth and proliferation. It is upregulated in multiple cancers with its inactivation resulting in tumor regression.[268] Ligand-independent regulation of MYC by the AR occurs in several PCa cell lines.[269] In experiments by Gao et al,[269] treatment with R1881 did not affect the expression levels of c-MYC in 22Rv1 or LNCaP PCa cells. Attenuation of c-MYC levels were seen when treated with JQ1 but not enzalutamide.[269] BET inhibition has generally been linked to the direct knockdown of MYC gene transcription,[268] although contrasting evidence[270] has also emerged in recent years. In support of the lack of a direct link between BET inhibition and MYC regulation, knockdown of MYC by siRNA was unable to attenuate the cell invasion properties of VCaP cells, while JQ1 treatment inhibited cell invasion.[264] Furthermore, exogenous expression of MYC was unable to rescue the cell growth inhibition effects of JQ1 in AR-positive cells.[264]

A study by Chan et al[255] demonstrated the ability of JQ1 to reduce the expression and the androgen-mediated ARE recruitment of AR_{FL} and AR_{SVs}. Dose-dependent reductions of AR_{FL} and AR_{SV} were demonstrated in CRPC cell lines (C4-2, 22Rv1, and VCaP) upon JQ1 (0.1-10 μM) treatment.[255] JQ1 treatment (0.5 μM) inhibited the binding of BRD2 at the proximal H3K27Ac sites of the AR gene in VCaP, R1-AD1, and R1-D567 cell lines.[255] This effect was less prominent (in VCaP cells) toward BRD3 and BRD4 recruitment.[255] Probing this effect further, Chan et al[255] demonstrated that the JQ1-mediated attenuation of AR-chromatin binding was not necessarily dependent on the involvement of H3K27Ac or BET family proteins. JQ1 treatment reduced AR-chromatin binding in BRD4-involved (eg, FASN-ARBSI) and BRD4-noninvolved (eg, intron 5 of FKBP5) sites to a similar extent (~50%) in VCaP cells.[255] These findings suggest that the anticancer effects seen in PCa via BETi treatment are not mediated through the disruption of AR-BET protein interactions alone.

Using gel filtration chromatography experiments, Asangani et al[264] showed the possibility of forming a large functional multiprotein complex with BRD4, AR, and RNAP2. The association ability to AR was also present in BRD proteins 2 and 3. Supporting the notion of JQ1 being a pan-BETi, ChiP-seq assays revealed a 62% to 86% overlap in genome-wide effect on BRD2-4 toward inhibition by JQ1.[264] BRD4 was found to bind the AR-TAD with a K_d of 70 nM.[264] Using halo-tagged AR in in vitro pull-down assays, the interaction with BRD4 (primarily through bromodomain 1) with the AR-TAD was mapped out to occur at the region of amino acids 120 to 160.[264]

Reduction of DHT-induced AR gene expression by JQ1 was comparable to or lower than the effects seen upon enzalutamide treatment in LNCaP and VCaP cells.[264] JQ1 inhibited the DHT-induced recruitment of AR to target loci at an equivalent potency to enzalutamide.[264] Recruitment of AR and BRD4 at shared loci (2031 sites identified) were differentially affected by enzalutamide and JQ1. At these loci, AR-recruitment was better lowered by enzalutamide than JQ1, while JQ1 was able to completely inhibit the DHT-induced BRD4 recruitment at such sites.[264]

TMPRSS-ERG (transmembrane protease serine 2-ETS [erythroblast transformation-specific] related gene) fusion-gene is the most common (~50%) oncogenic genetic alteration found in PCa.[264,271,272] It drives prostatic tumor progression with the expression of PSA as well as ERG (transcriptional regulator) protein. ERG overexpression has been implicated to be involved in developing novel super-enhancer regions, affecting the histone-acetylation code, and thus driving the upregulation of specific genes that could contribute to PCa progression.[273] PSA and the ERG expression in VCaP cells showed significant inhibition (at 48 hours) by the treatment with JQ1 in a dose-dependent manner.[264] These effects were traced to derecruitment of RNAP2 at the ERG gene and AR/BRD4 at the TMPRSS2 promoter/enhancer regions by JQ1.[264]

ChIP-seq analysis revealed that DHT-treatment resulted in increased AR binding at the MYC distal enhancer, while reducing the recruitment of RNAP2 at the gene.[264] Enzalutamide treatment removed this inhibitory effect toward the MYC locus. Hence, Asangani et al[264] postulated that the derepression of MYC gene expression might be a mechanism in enzalutamide resistance in CRPC. JQ1 treatment, in contrast, had no such upregulatory effects on c-MYC expression.[264] Mice-bearing VCaP xenografts showed significantly higher tumor growth inhibition by the treatment of JQ1 (~50%, 50 mg/kg) than by enzalutamide (10 mg/kg).[264] Similar to previous reports, the in vivo treatment by JQ1 also reduced the testis size in the treated mice.[264,274]

Enzalutamide-treated mice had prometastatic (to liver and femur) effects, while JQ1-treated mice showed no discernable metastases.[264]

I-BET762, a triazolobenzodiazepine analog of JQ1, has shown similar in vitro and in vivo BETi functions in models of PCa.[275] Other recent drug development programs have uncovered nondiazepine-type compounds that can still function as BETi's. Y08060, with a reported BRD4 bromodomain 1 inhibiting IC_{50} of 302 nM is an example.[276] This compound is a 2H-benzo[*b*][1,4]oxazin-3(4*H*)-one derivative with a pendent aryl group connected to the core via a sulfonamide linkage.[276] Unfortunately, the cell growth inhibitory ability of this lead compound was still 17-fold to 60-fold less potent than JQ1 in parallel assessment in PCa cell lines (C4-2B, LNCaP, and 22Rv1).[276] Nevertheless the ability to obtain BETi's beyond a diazepine structure may allow one to circumvent off-target effects that may occur at high doses of JQ1-like compounds. A comprehensive review about BRD4 inhibitors with varying structures can be found elsewhere.[258] One of the more interesting of these is the sulfonamide derivative PFI-1, which was found to occupy the Kac-binding site in BRD4 and BRD2.[277,278] A recent study demonstrated the ability of PFI-1 to inhibit the transactivation of constitutively active AR species (AR-V7 and nonsense mutant Q641X) and to attenuate the growth of AR-positive cell lines (LNCaP and 22Rv1).[279] However, at the higher concentrations (20 μM) AR-null PC3 cells also demonstrated growth inhibition similar to LNCaP cells.[279]

Recent evidence has shown AR-overexpression in PCa cells can lead to increased expression of bromodomain-containing proteins, including BRD4.[280] Furthermore, AR-overexpression has also been linked to increased histone acetylation.[281] Together these factors allow for a genome-wide increase in epigenetic DNA accessibility. Such chromatin relaxation leads to abnormally increased transcription factor binding and subsequent gene expression.[280] BETi (JQ1) treatment attenuates this chromatin opening effect particularly in AR-overexpressing cell lines.[280] Experiments by Urbanucci et al[280] has shown that a combination treatment of enzalutamide and JQ1 led to apoptotic effects in an AR-overexpressing VCaP cell line but not in LNCaP cells.

8.2 | Resistance to BET inhibition

Acquired resistance to BETi's in tumors including PCa is an emerging topic of discussion. The mechanism(s) of resistance may likely be tumor-type specific. A study by Power et al[282] indicated that the attenuation of BRD4-chromatin binding can bring about reactivation of AR signaling and a silencing of DNA damage response gene DDR2 (DNA damage repair 2). In BET-resistant cultured LNCaP and 22Rv1 cells, BRD4 inhibition either by the use of JQ1 or a BET-PROTAC degrader conferred no significant antiproliferative effects. Despite the lack of downstream effects BRD4 was present and did bind JQ1, as evidenced by cellular thermal shift assays.[282] Neither JQ1 nor the PROTAC treatments impacted the MYC expression in these BETi-resistant cells.[282] Probing the BETi-resistant cell lines by Gene Set Enrichment Analysis revealed a positive enrichment of AR target genes.[282] Despite the overexpression of multiple canonical AR target genes, the AR transcript levels were not increased in the BETi-resistant cells.[282] The cause of this was traced to increased CDK9 activity and an apparent increase in AR stability.[282] CDK9 is a known facilitator of AR-chromatin binding via the phosphorylation of Ser89 in the AR.[227] This phosphorylation strengthens the AR-chromatin–binding interaction allowing for enhanced AR activity. Inhibition of CDK9 activity led to a significantly larger decrease in cell viability in the BETi-resistant cells than in the sensitive ones.[282] Additionally the response to enzalutamide treatment was enhanced in these cells owing to the upregulation of the AR-mediated transcription.[282]

The levels of DNA damage markers γH2A.X and 53BP1 are elevated in BETi-resistant cells.[282] In the absence of BRD4, signaling of DNA damage is known to be enhanced.[283] However, BRD4 levels are niether linked to the kinetics of repair nor to the generation of DNA damage.[283] COMET assays established further evidence of enhanced DNA damage in the BETi-resistant cells.[282] Additionally, transcriptional silencing was observed in DDR genes.[282] BRD4 recruitment at the DDR genes was reduced in the cells chronically exposed to BET inhibitors.[282] Despite the transcriptional change in DDR genes no significant cell-cycle arrest was observed in the BETi-resistant cells.[282] Downregulation of homologous recombination (HR) genes was also found in these BETi-resistant cells.[282]

In such HR-deficient environments, PARP-mediated DNA repair becomes critical for avoiding DNA damage and promoting cell survival.[284] In response to single-stranded DNA breakage, PARP initiates corrective action through base-excision repair.[284] Supportive of this notion, Pawar et al[282] found that the BETi-resistant cells had high PARP activity, and hence enhanced sensitivity to PARP-inhibition by olaparib. Similar observations in BETi-induced reduction of HR efficiency and increased sensitivity to PARP-inhibitors was recently demonstrated in ovarian cancer by Wilson et al.[285] A phase 2 clinical trial (NCT03047135) to evaluate the efficacy of olaparib in patients with high-risk biochemically recurrent PCa is currently underway. Preliminary efficacy for mCRPC treatment with olaparib has been seen with a 11/49 (ten of whom had mutations in DNA-repair genes) patient PSA response. Due to their impaired DNA-repair ability, the BETi-resistant PCa cell lines have increased sensitivity to cisplatin treatment when compared with the wild-type cell lines. The sensitivity enhancement was twofold in LNCaP cells, while it was 20-fold in 22Rv1 cells.[282]

Another major mechanism of resistance to BETi demonstrated in PCa therapy arises from speckle-type POZ protein (SPOP) mutations.[259,286] This gene is frequently mutated in PCa patients with F133 being the most commonly mutated site.[286] The resistance is believed to occur via increased stability of BET proteins and Akt/mTORK1 activation.[286] SPOP is a cullin (CUL)-based E3 ligase substrate adapter protein involved in the cellular protein degradation machinery.[259] It promotes the ubiquitination of BET proteins in a dose-dependent manner.[259] Gene ontology assays and coimmunoprecipitation assays have revealed BET proteins to be major-binding partners of SPOP.[286] Knockdown or knockout of SPOP in PCa cell lines (C4-2 and 22Rv1) resulted in increased levels of its substrate proteins, including BET proteins and AR, without affecting BET protein mRNA expression.[259] BRD4, in particular, was stabilized by the lack of SPOP activity.[259] Enhanced in vitro colony formation and proliferation was seen in C4-2 cells with SPOP mutations, compared with the cultures with wild-type SPOP.[259,286] Knockdown of BRD4 significantly reduced the SPOP-mutated cell proliferation.[259,286] Short hairpin RNA (shRNA) knockdown of BRD4 reduced tumor growth in mice with 22Rv1 xenografts bearing SPOP mutations.[259] Zhang et al[286] has found that such enhanced stability and the resultant elevated levels of BRD4 leads to an upregulation of cellular Akt-mTORC1 pathway. They also showed the upregulation of several genes in the cholesterol biosynthesis pathway (FDFT1, DHCR24, DHCR7, and MVD) and the Rho GTPase family member RAC1 in SPOP-mutated tumors.[286] PTEN loss and Akt/mTORC activation is a well described major occurrence in PCa cell survival.[287,288] Additionally, caveolin-1 containing cholesterol-rich lipid-rafts have been associated in tumor development and metastases in PCa.[289] Increased levels of RAC1 is associated with increased phosphorylation of Akt. This phosphorylation can be reversed by the knockdown of RAC1, resensitizing the SPOP-mutated cells to JQ1 treatment.[286] Similarly, combined depletion of the cholesterol synthesis genes resulted in attenuation of the Akt/mTORC signaling and resensitized the C4-2 cells to JQ1 treatment.[286]

Non-PCa cell lines have also shown acquired resistance to BETi's, indicating that this will be a major topic of discussion ahead in the development of BETi's. JQ1-mediated antiproliferative effects in hepatocellular carcinoma were overcome by the cancer cells via the upregulation of Mcl-1.[290] Use of a CDK inhibitor in cotreatment to reduce Mcl-1 expression overcame the JQ1 resistance in HCCLM3 and BEL7402 cell lines.[290]

8.3 | Clinical trials

A BETi currently in clinical trials for CRPC, developed by Zenith Epigenetics, is ZEN-3694.[291] It has concluded a dose escalation and dose confirmation phase 1 clinical trial (NCT02705469) in mCRPC patients. Based on the first clinical trial, the company announced that the drug has a good safety profile and pharmacokinetic properties and that they have identified a maximum tolerable dose.[292] ZEN-3694 binds BET proteins at greater than 20-fold higher potency compared with other human bromodomain–containing proteins.[291] Synergistic antiproliferative effects with enzalutamide/apalutamide were seen in VCaP cells.[291] Submicromolar growth inhibition IC_{s50} were found against several AR_{FL} and AR_{SV} driven PCa cell lines (22Rv1 = 0.19 μM, VCaP = 0.9 μM, LNCaP = 0.40 μM), while having no discernable effect against the AR-null PC3 PCa cell line.[291] GR upregulation in enzalutamide-resistant LNCaP cells was significantly

inhibited by Zen-3694.[291,292] In monotherapy, Zen-3694 (100 mg/kg) had comparable in vivo activity to enzalutamide (10 mg/kg) in VCaP cells.[291] At 2 hours postdosing measurement both PSA and the c-MYC expression were attenuated by ZEN-3694 treatment.[291] Better in vivo tumor efficacy of Zen-3694 than enzalutamide was seen in a 22Rv1 xenograft model at the same doses.[291] With these findings and the results of the first clinical trial, ZEN-3694 has moved on to another phase 1/2 clinical trial (in CRPC patients) for cotreatment with enzalutamide (NCT02711956). Other notable BETis that have progressed to PCa clinical trials[293] include GS-5829 (Gilead Sciences; for mCRPC, NCT02607228) and MK-8628 (Merck; for advanced solid tumors including CRPC, NCT02259114). GS-5829 is being evaluated both as a single-agent and as a cotreatment with enzalutamide. MK-8628 has completed the NCT02259114 phase 1 trial though full information is not yet publicly available.

8.4 | Targeting complexity and outlook

Being key elements to the cellular crosstalk mechanism, BET proteins interact with many cellular-signaling pathways.[257,263,294] Beyond the AR-TAD, the major pathways and factors impacted include MYC, JAK/STAT pathway, PI3K/Akt pathway, p53 acetylation, and the NF-kB pathway.[253,257,258,261,263,295] With the impact on several tumor-related pathways, even the BET inhibitors in clinical trials for a single ailment such as CRPC are also being tested for treatment efficacy in other solid tumors and lymphomas. To date, most reproducible successes found with BET inhibitors lie in the treatment of hematological cancers and as a treatment toward NUT midline carcinoma.[254,260,296] To increase the therapeutic impact cotreatment methods have also been adopted or proposed to combine BET inhibitors with an existing therapeutic (eg, enzalutamide).[257,295] Additionally, PROTAC-like technologies to utilize the cellular protein degradation machinery to degrade BET proteins have emerged as another potential CRPC therapeutic approach.[40,297,298] ARV-771, a pan-BET degrader has led to tumor regression in 22Rv1 mice xenografts (up to 15 days) at a 30 mg/kg dosing.[40]

It is yet unclear why some tumor cell lines do not respond to BETi's despite the generally accepted ability of BETi's to affect the c-MYC expression (and other oncogenic pathways). Even in the absence of AR function one could assume PCa cells such as PC3 and DU145 may be affected by micromolar treatment of a BETi like JQ1. However, this was not the case as demonstrated by Asangani et al,[264] which has led to scrutiny about whether we know enough about the complex associations between the cancer epigenetics, MYC-regulated functions, and the overall effect of BET inhibitors.[299] In regular cells, BET proteins are involved in critical processes, including cytokine gene transcription, T cell differentiation, adipogenesis, insulin production, and suppression of latent viruses.[294] Andrieu et al[300] in a recent study has demonstrated the involvement of BET proteins in the EMT process in breast cancer models. BRD3 and BRD4 inhibited this process, while BRD2 promoted EMT. Additional complexity in BETi treatment is found in the fact that JQ1 interferes the SPOP-mediated proteolytic degradation of BET proteins and increases their half-lives.[286] This could mean a situation where a continued treatment with a BETi would suddenly lead to an opposite-therapeutic effect when the SPOP gene gets mutated, much like how enzalutamide becomes an agonist with AR-LBD mutations.

Being readers of the histone-acetylation code, BET inhibitors may have heretofore unknown effects toward translating the genetic code as well. Given the above factors, major concerns remain about the specificity and the long-term side effects that could occur with BET inhibition despite the promising therapeutic potential. As such, there is some belief that the undertaking of clinical trials of BET inhibitors has been premature.[294]

9 | PERSPECTIVE

Over the last couple of decades, we have learned that the AR remains a critical driver of growth of CRPC. This AR dependence exists at the outset of castration resistance as well as after treatment with novel AR-signaling axis inhibitors like abiraterone and enzalutamide. All of the AR-targeting therapies that have received regulatory approval for clinical use directly or indirectly target the AR through its LBD. It is unlikely that additional compounds

that target the LBD will produce clinically meaningful results given the frequent cross-resistance observed between currently available compounds that target the LBD. Hence the recent developments that have led to the emergence of promising compounds that can affect the AR-signaling axis through effects beyond the AR-LBD is of great interest. This review has focused on discussing the evolution of a subset of those compounds (or compound classes) that have shown interactions with the AR-TAD (EPI compounds, JN compounds, sintokamides, dysamides, niphatenones, niclosamide, mahanine, and BET inhibitors) or the AR-DBD (hairpin polyamides and VPC compounds; Figure 6). Historically, drug development efforts aimed at the N-terminus of the AR have been hampered by lack of structural knowledge of the transactivation domain and the extensive homology of the AR-DBD with that of other nuclear steroid receptors. However, over the last several years, multiple groups have begun to identify compounds that target the AR-TAD or DBD, although poor specificity and/or activity of these compounds may hamper their clinical development. Nonetheless, the AR-TAD and DBD (Figure 1) remain potentially viable drug targets if some of the pitfalls of existing compounds can be overcome. Given the closer homology between the short DBD fragments between nuclear hormone receptors, the AR-TAD is perhaps the more promising of these two target domains to achieve AR selective effects.

A large majority of the reported proof-of-principle work done to target the AR-TAD has used compounds isolated from marine sponge extracts. In this regard, professor Marianne Sadar and her coworkers have established some fundamental experimental techniques to evaluate the binding of small molecule compounds at the AR-TAD. All the marine sponge–derived AR-TAD inhibitors thus far reported function as covalent drugs. Although a number of therapeutics do have covalent warheads, the reactivity of the covalent moiety is an important factor to consider in reducing the off-target effects. For example, the enone moiety in the niphatenones was too reactive to sustain it as a possible therapeutic for further development. The only compound to have progressed into clinical trials thus far from this genre, EPI-506, failed at phase I due to a low clinical response rate. The reported data for the bioactivity of sintokamide A, another marine sponge isolate, suggests that it may also need concentrations similar to EPI-002 for eliciting a clinically relevant effect. Systemic bioavailability of sintokamide A is poor with an elimination half-life of only 1.16 hours. For any further development, further SAR optimization will be necessary to

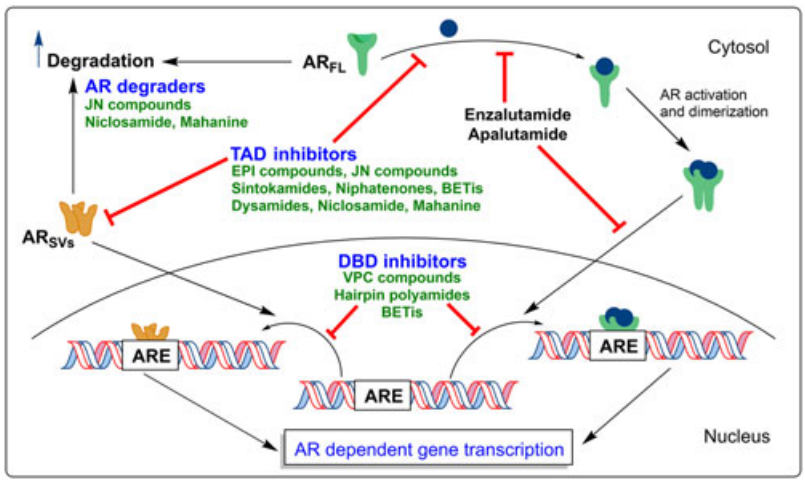


FIGURE 6 The effect on the AR-signaling axis by recently emerged small molecules that have direct or indirect interaction(s) with the AR-TAD or AR-DBD. The anti-PCa activity of some of these compounds (eg, niclosamide, mahanine, BET inhibitors), are not necessarily based on these interactions alone. AR, androgen receptor; ARE, androgen response element; AR_{FL}, full-length AR; AR_{SV}, splice variant AR; BETi, bromodomain and extraterminal domain protein inhibitor; DBD, DNA-binding domain; PCa, prostate cancer; TAD, transactivation domain [Color figure can be viewed at wileyonlinelibrary.com]

improve its in vitro and in vivo characteristics.[205] The poor bioavailability of sintokamides may be correlated to the reactive electrophilic centers in its structure.

A small molecule compound binding at the AR-TAD, in particular, might not have the same binding constraints that an ordered domain such as the LBD would impose. Given the IDP nature of the TAD and the fact that it is inherently designed to interact with multiple binding partners in the cellular environment suggests that the stereochemistry of any chiral centers in a small molecule compound binding the AR-TAD may have less functional significance. This notion is supported by the data for EPI compounds, where all four diastereomers of EPI-001 were found to bind the AR-TAD and produce reasonably similar AR inhibitory effects. In principle, when compared with the LBD and DBD, therapies targeted at the AR-TAD may also have greater tolerance against resistance mechanisms from point mutations. Arguably, the impact of a single-point mutation may have lower impact on its transient binding conformation(s) when compared with a more rigid binding site (LBP, AF2, or BF3) in the AR-LBD. In view of the covalently binding drugs, however, it is of course entirely possible that chronic exposure might still result in a critical point mutation (eg, of a cysteine) at the AR-TAD, which will hinder further covalent binding. Development of multiple mutations at the AR-TAD upon chronic exposure to a drug is also possible. AR-TAD is indeed known to undergo numerous point mutations in PCa, including those that can induce constitutive transactivation.[301,302] Compounds such as the EPI compounds or the JN compounds could be used as effective tools to reach a more definitive understanding of such AR-TAD–related resistance mechanisms. Further study of this IDP domain is critical for raising the effectiveness of future targeted therapeutics of the AR-TAD.

Niclosamide, mahanine, and BET inhibitors, while reported to have direct or indirect interactions with the AR-TAD, do not seem to exert their anti-PCa effects based on such interactions alone. Niclosamide primarily affects the AR gene expression through the inhibition of the MAPK and the IL6/STAT3 pathways, and the enhancement of the degradation of AR-V7. The clinical trials of niclosamide with coadministration of existing AR-antagonists has put greater value on this AR_{SV} degradation effect since substantial in vivo success in reducing AR_{SV} expression was observed with relatively low niclosamide cotreatment doses. However, a major bottleneck for the further development of niclosamide was found in the recently concluded dose escalation study for niclosamide (NCT02532114, in cotreatment with enzalutamide).[143] The general concerns about the specificity of niclosamide's effects due to its multipathway impact and concerns about its poor oral bioavailability were found to be true in the outcome of this study. The concluded NCT02532114 study established the fact that it is essential to do SAR optimization of niclosamide to improve its biological properties before further evaluation, and advices caution against the development of therapies that may have too many multipathway effects. In the use of a drug with poor bioavailability such as niclosamide, the ability for dose escalation would be severely hindered if it has multipathway impact. Mahanine also has AR degradation effects. Inhibition of AR signaling by mahanine seems to be at least partly derived from its inhibition of CDK1 activity.[226] AR degradation effects of mahanine appears to be slow but does affect both the AR_{FL} and AR_{SVs}.[226] Given the involvement of BET proteins in many physiological pathways, BET inhibitors have also demonstrated multiple antitumor effects. BET proteins are possibly important in the androgen-dependent transactivation of the AR and the consequent recruitment at the AREs. However, some data also suggest that the inhibition of AR-ARE recruitment by BETi treatment is not necessarily dependent on the BET proteins.[255] Attenuation of GR upregulation in enzalutamide-resistant cells by the clinical candidate Zen-3694 (a BETi) is also supportive of the notion that the antitumor effects by BETi's in PCa may primarily be founded in processes independent of the AR.[291,292]

Interesting preferential inhibition/degradation effects of the AR_{FL} vs AR_{SVs} has been observed with some of these compounds. Lower doses of niclosamide were shown to enhance the degradation of AR-V7 significantly more than that of AR_{FL}.[95] In spite of its design principle, VPC-14449 has demonstrated significantly less inhibition potency toward AR_{SV}-driven gene expression than what was seen for AR_{FL}.[14] Hence the conformational arrangements of the AR_{FL} and AR_{SVs} may have significant differences in three-dimensional shape as well as in accessibility to different sites that would have to be taken in to account when applying in silico drug design approaches. In this context, NMR studies done on smaller fragments of the AR-TAD to find the binding locations of compounds (EPI-002 and sintokamides) may not present a conclusive determination of binding site or binding

efficacy. The transient, partially folded binding conformations adopted by the AR-TAD are likely to differ between the smaller fragments (AR-AF1, Tau-1, and Tau-5) of the TAD, full-length AR-TAD, and AR_{FL}. Therefore, accompanying biochemical evaluations are essential to complement any receptor-fragment –based NMR finding. Following such analysis, EPI compounds are believed to bind the Tau-5 (AA 102-371) region of the AR-TAD while sintokamides bind Tau-1 region (AA 361-537).[205] Tau-5 in particular is considered necessary for androgen-independent transactivation of the AR. Constitutive activity in AR_{SVs} have significantly low dependence on the amino acid region proximal to the N-terminus of the AR-TAD.[303] This means that the binding of BRD4 at amino acids 120 to 160 of the AR-TAD, as demonstrated by Asangani et al,[264] may have minimal impact toward regulatory functions of the androgen-independent AR transactivation and in PCa driven by AR_{SVs}. BET inhibitors are therefore unlikely to have direct functional consequences toward constitutively active AR isoforms.

Hydrogen bonding is an important factor that governs the intrinsic organization and the intramolecular associations of DNA. Hence it is no surprise that the design principles of the VPC compounds as well as of the DNA-binding hairpin polyamides are strongly rooted in the utilization of H-bonding to provide critical binding interactions as well as directional recognition. Homology between the DBDs of the different NHRs and between the NHRs' association sites at the DNA hamper the utilization of these two therapies to selectively target the AR or AREs. At the current juncture, the practical use of these compounds may be limited to advanced disease conditions where enzalutamide resistance is driven by GR overexpression. While in vitro studies have shown that DBD P-box targeted VPC compounds manifest a few-fold higher binding selectivity for the AR-DBD than that for other NHR DBDs, in vivo work has required higher dosing that may still result in off-target effects when translated to clinical studies. The more recent utilization of VPC compounds to disrupt DBD-mediated AR dimerization is also likely to face similar challenges in target selectivity.

Compounds that promote AR degradation, generally affects the subcellular distribution of the AR protein. The in vitro AR-localization data for mahanine treatment[226] and JN compounds treatment (unpublished) supports this fact, where the nuclear AR fraction is depleted upon drug treatment. It is noteworthy here to mention that such effects were also seen upon galeterone (a CYP17 inhibitor) treatment.[304,305] Galeterone was an AR antagonist that went through several stages of clinical trials.[306] It also enhanced the degradation of AR_{FL} and AR_{SVs} and inhibited AR nuclear translocation in preclinical studies.[304,305] However, similar to the recently concluded clinical trial with niclosamide (NCT02532114), galeterone also failed to translate the preclinical AR degradation response in to viable impact in clinical trials. After reaching phase-III clinical trials, investigation of galeterone as a potential mCRPC treatment of patients expressing AR-V7 (NCT02438007) was abandoned due to insufficient response. The nuclear AR depletion by these small molecule AR degraders could possibly be linked to either a blockage of AR nuclear translocation or/and a promotion of nuclear-efflux of AR protein. The latter effect could simply be equilibrium driven due to the continuous depletion of the AR species in the cytoplasm.

Despite the prominent extension of life expectancy granted to PCa patients by recent development of drugs like enzalutamide and apalutamide, the emergence of inevitable resistance to such therapies raises a need for continued searching for better therapeutics. As outlined in this review, the interplay and crosstalk between multiple oncogenic pathways with the AR-signaling axis makes this an uphill task. Theories that challenge the conventional belief that AR inhibition/degradation represents the most efficacious way to target PCa have also emerged. A recent study has shown that the inhibition of AR signaling in PCa cells results in the desuppression of a PCa-promoting gene, ZBTB46, leading to EMT initiation.[307] Here, Chen et al[307] postulate that simple targeting of AR signaling may predispose PCa to progress to a metastatic castration-resistant state. Similar reports also establish AR as a suppressor of PCa cell invasion via altering miR-4496/β-catenin signaling.[308] Hence, even the complete abolition of AR protein, as promised by the emerging PROTAC-type technologies, might not be an optimal choice for long-term treatment of PCa. Eradication of AR could simply be met by tumor cells in an unforeseen escalation of a reciprocal oncogenic pathway that is suppressed by AR signaling. Hence it is important to continue the study of fundamental processes in PCa to identify not only how to best target the AR-signaling axis but also to unmask novel targets that may arise as a consequence of AR

inhibition. In the search for such targets, Fong et al[309] has recently described the polycomb group protein EZH2 as a novel target in CRPC therapy, inhibition of which can attenuate AR signaling and inhibit PCa cell/xenograft growth via the matricellular protein CCN3. CCN3 has a direct association with the AR-TAD as a part of a negative feedback loop that controls AR activity.[309] Another emerging technique with reasonable efficacy toward PCa growth (both in vitro and in vivo) is in using small molecule spliceosome inhibitors to suppress the alternative splicing derived production of AR-V7.[310] Finding tissue-selective AR modulators has also re-emerged as a potential approach to improve the specificity of AR directed therapies.[311]

The next phase of PCa drug development is likely to have a greater emphasis on accurately identifying resistance mechanisms to current and emerging monotherapies and devising cotreatment options to prolong the effectiveness of the therapeutic. Even with the concerns highlighted in the above paragraph, the AR continues to remain the best target of interest for developing PCa therapies based on the current knowledge of PCa physiology. We believe that compounds with appropriate performance characteristics will make their way to clinical trials, where proof-of-principle studies will be established to show that effective targeting of the AR-TAD or DBD is possible and can lead to clinically relevant improvements in the outcome of patients with mCRPC, the lethal form of PCa.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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