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Small Molecule Inhibitors Targeting the ‘Achilles’ Heel of Androgen Receptor Activity

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Abstract

Androgen ablation therapy remains the gold standard for the treatment of advanced prostate cancer, but unfortunately, it is not curative and eventually the disease will return as lethal castration-resistant prostate cancer (CRPC). There is mounting evidence supporting the concept that development of CRPC is causally related to continued transactivation of androgen receptor (AR). All current therapies that target the AR are dependent on the presence of its C-terminal ligand-binding domain (LBD). However, it is the N-terminal domain (NTD) of the AR that is the “Achilles Heel” of AR activity, with AF-1 being essential for AR activity regardless of androgen. Recent efforts to develop drugs to the AR NTD have yielded EPI-001 a small molecule, sintokamide peptides, and decoys to the AR NTD with EPI-001 the best characterized and most promising for clinical development based upon specificity, low toxicity, and cytoreductive antitumor activity.

Background

Androgens such as testosterone and dihydrotestosterone mediate their biological effects through the androgen receptor (AR). In adult males, the testes produce the majority of androgen with some contribution from the adrenal glands. Androgens play a role in a wide range of developmental and physiological responses and are involved in male sexual differentiation, maintenance of spermatogenesis, and male gonadotropin regulation. The growth and survival of the prostate is dependent on androgen. When androgens increase in males during puberty there is an increase in growth of the prostate gland, and in adult males when androgens are reduced by castration there is involution of the prostate and apoptosis of prostate epithelial cells. Thus the prostate gland is an androgen-dependent organ where androgens are the predominant mitogenic stimulus. This dependency of the prostate epithelium on androgens provides the underlying rationale for treating advanced prostate cancer with androgen ablation.

Castration-resistant prostate cancer

Prostate cancer is the most frequently diagnosed non-cutaneous tumor in Western men. Primary therapies such as radical prostatectomy and radiation for localized, low grade tumors generally result in low mortality rates. Unfortunately, high grade tumors of Gleason score 7 and above have elevated recurrence rates even when it appears that the tumor has been successfully contained with primary therapy. Approximately 20–40% of prostate cancer patients treated with radical prostatectomy will experience tumor recurrence. Once

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the tumor has recurred, usually manifest by a rise in serum prostate-specific antigen (PSA), androgen ablation therapy is provided to most patients. Androgen ablation therapy is achieved through either orchiectomy (surgical castration) or application of gonadotropin-releasing hormone analogues (chemical castration) which both cause a transient reduction in tumor burden concomitant with a decrease in serum PSA. Unfortunately the malignancy will eventually begin to grow again in the absence of testicular androgens to form castration-resistant disease (CRPC). CRPC is biochemically characterized before the onset of symptoms by a rising titre of serum PSA. Most patients succumb to CRPC within two to three years of biochemical failure. All current therapies directed at AR target its C-terminal ligand-binding domain (LBD) and eventually fail. Treatments not targeting AR (e.g., docetaxel or ProvengeTM) result in an increased life expectancy of 2–4 months.

Androgen receptor in castration resistant prostate cancer

There is mounting evidence supporting the concept that CRPC remains dependent upon AR signaling. Many of the same genes that are increased by androgens in androgen-dependent prostate cancer xenografts become elevated in CRPC, such as PSA. AR protein translocates from the cytoplasm to the nucleus when activated by androgen or alternative signaling pathways (1). Thus the detection of nuclear localization of AR in CRPC supports that the AR may continue to be transcriptionally active in the absence of testicular androgens. Additional support that the AR continues to play a role in CRPC includes: amplification of the AR gene and/or increased expression of AR (2–4); delayed onset of CRPC by altering the timing and sequence of use of antiandrogens and additional responses with CYP17 inhibitors that block the synthesis of androgen (5–6); and that AR expression is essential for proliferation and tumor growth (4,7). Finally, it is known that AR can be activated through its N-terminal domain (NTD) in the absence of androgen by stimulation of the cAMP-dependent protein kinase (PKA) pathway, interleukin-6 (IL-6), and by bone-derived factors (1,8,9).

Androgen receptor as a therapeutic target

The AR has distinct functional domains that include the C-terminal LBD, a DNA-binding domain (DBD), and an NTD. The AR DBD has been crystallized which would allow for rational drug design, but the high degree of homology of this domain with other steroid hormone receptors may predict poor specificity and toxic side-effects. The transcriptional activity of most steroid hormone receptors is predominantly through the activation function (AF)-2 region in the LBD, the exception being the AR where it is the AF-1 region in the NTD that contributes most of the transcriptional activity (10–12). AR LBD functions independent of the NTD and can still bind ligand even if the AF-1 region is deleted or mutated; however no transcriptional activity can be achieved without the AF-1 region in the NTD.

Currently all conventional therapy has concentrated on androgen-dependent activation of the AR through its C-terminal LBD. These therapies include reduction of androgen that bind to the LBD by chemical or surgical castration and application of antiandrogens (Fig 1A). Unfortunately castration does not completely eliminate levels of androgen in metastatic prostate cancer tissue (13). These residual levels of androgen have raised interest in the roles of adrenal androgens, transport of androgens, and/or *de novo* synthesis of androgen from cholesterol precursors by prostate cancer cells. Thus there is renewed interest in inhibitors of androgen synthesis. These inhibitors include ketoconazole and currently in clinical trials the 17,20-lyase/CYP17 inhibitor abiraterone, TOK-001 and TAK-700. In addition to inhibiting 17,20-lyase activity to reduce synthesis of androgen, ketoconazole and TOK-001 (VN/124-1) also have antiandrogen activity. Antiandrogens competitively bind AR LBD. Antiandrogens used for the clinical management of prostate cancer include bicalutamide,

flutamide, nilutamide, cyproterone acetate, and investigational compounds, MDV3100 and ARN-509. While castration and antiandrogens are effective initially, it is unclear why these approaches eventually fail. Suspected mechanisms include: amplification or overexpression of AR; gain-of-function mutations allowing AR to be activated by steroids or antiandrogens although most of these mutations are considered rare events; ligand-independent activation by growth factors, cytokines, or kinases; overexpression of AR coactivators; intracrine signaling by increased intratumoral androgens; and/or expression of constitutively active splice variants of AR that lack LBD that may be expressed solely or in mixed populations with full-length receptor to form a heterodimer (Fig 1B). Most studies continue to develop antagonists to AR LBD and specifically these include: 1) the allosteric pocket and AF-2 activity (14); 2) *in silico* “drug repurposing” procedure for identification of nonsteroidal antagonists (15); and coactivator or corepressor interactions (16–19).

AR NTD as a target for drug development

AR NTD contains several repeat regions: polyglutamine between residues 58–89; polyproline between residues 371–381; and polyglycine between residues 449–472. The transcriptional activity of the AR in response to ligand requires the NTD AF-1 (Tau1) which is between residues 101 and 370 with core sequence ¹⁷⁸LKDIL¹⁸² and does not play a significant role in ligand-independent activation of the AR (8). Tau5 is between residues 360–485 with core sequence ⁴³⁵WHTLF⁴³⁹ and is considered to be important for activity in the absence of androgen (20). The AR NTD is flexible with a high degree of intrinsic disorder making it impossible to be used for structure-based drug design. The AR AF-1 region has characteristics of collapsed disorder meaning that it is predicted to have some proportion of secondary structure, but not a stable tertiary structure. Induced folding of the AR NTD is thought to require interactions with other proteins including bridging factors and the basal transcriptional machinery to result in active transcription. It is a recent development that intrinsically disordered proteins and regions have been accepted as attractive drug targets to prevent protein-protein interactions. An example is the nutlins that target the intrinsically disordered region of p53. When Mdm2 binds to the intrinsically disordered region (amino acid residues 13–19) of p53, a helical structure is formed that binds into the deep groove on the surface of Mdm2. As discussed by Uversky, interaction between Mdm2 and p53 involves a disorder-to-order transition upon binding which would thermodynamically favor blocking by small molecule competitors (21). Other examples of therapeutics targeting protein-protein interactions include: GX015-070 (Obatoclox) that interacts with the hydrophobic groove of bcl2 proteins to prevent binding of BH3 peptide domain of proapoptotic BCL2 members; inhibitors to XIAP-Diablo interactions and Grb2 SH2 domains; sulindac to prevent RAS-RAF interactions; and inhibitors of WNT pathway targets frizzled-dishevelled (Fz9 inhibitor) and β -catenin-CBP (ICG-001). For most of these interactions, crystal structures of at least one of the proteins have aided in the development of drug leads by permitting for docking and virtual screening. AR NTD has not been crystallized and is intrinsically disordered making drug development labor intensive and requiring assays that test each drug empirically. However, in spite of these difficulties, three separate classes of inhibitors of the AR NTD have recently been described, EPI-001 a small molecule (22), sintokamides which are peptides isolated from marine sponge *Dysidea sp.* (23), and a decoy peptide to the AR NTD (24).

Mechanism of Action of Small Molecule Inhibitors of AR NTD

Deletion experiments have shown that the NTD is essential for transcriptional activity of the AR in response to ligand as well as in the absence of ligand (10). Thus the AR NTD is the “Achilles Heel” of the AR transcriptional activity. Indeed, EPI-001 inhibited AR activity induced by androgen (Tau1) and in the absence of androgen (Tau5) under serum-free conditions (no cholesterol precursors that are required for de novo synthesis of androgens)

by forskolin (FSK), which stimulates PKA activity, interleukin-6 (IL-6), and by factors secreted by osteoblasts (bone-derived factors) (22). EPI-001 also inhibited constitutively active AR devoid of a LBD (22). This implies that the mechanism of action of EPI-001 involves a critical aspect of AR transcriptional activity and is not dependent on the presence of the LBD. Consistent with EPI-001 not binding to the LBD, EPI-001 did not compete with androgen in a competitive ligand-binding assay (22). EPI-001 does not reduce levels of AR protein nor does it prevent nuclear translocation of the AR in response to androgen (22). EPI-001 was specific for inhibiting AR and had no effect on the activities of related human steroid receptors, progesterone receptor (PR) and glucocorticoid receptor (GR) (22). The LBDs and DBDs of these steroid receptors have considerable homology to AR LBD and DBD, but their NTDs have little sequence similarity (<15%). Fluorescence emission spectroscopy reveals that EPI-001 alters the folding of AR AF-1 while having no effect on GR AF-1 (22). In addition, many steroid receptors interact with the same coactivators and bridging factors that are required for AR activity. Just a couple examples are SRC1-3 and CREB-binding protein (CBP) which interact with the AR NTD and their levels of expression are increased in CRPC. EPI-001 inhibits interaction between CBP and AR in response to both androgen and in the absence of androgen, by IL-6 (22). CBP is essential for transcriptional activity regardless of whether the AR is activated by androgen or IL-6. The fact that EPI-001 had no effect on the activities of PR and GR, which also require CBP for activity, supports that EPI-001 interacts with AR NTD rather than CBP to block the interaction. In vitro experiments with recombinant proteins, revealed that EPI-001 inhibits CBP interaction with the AF-1 region of the AR NTD (22). AR interaction with bridging factors like CBP stabilize AR on AREs. Indeed EPI-001 inhibited androgen-induced expression of *PSA* and *TMPRSS2* androgen-responsive genes with well-characterized AREs by a mechanism that involves reduced AR-ARE interaction (22). EPI-001 also inhibits N/C interaction (22) that is required for antiparallel dimer formation and is essential for ligand-dependent activity of the AR. However, bicalutamide also prevents N/C interaction yet AR bound to bicalutamide still can bind to AREs. Currently it is unclear which protein interactions with the AR are essential for AR-ARE binding and stabilization. AR interacts with 169 different proteins, thereby complicating the identification of protein interactions blocked by EPI-001 without application of high throughput approaches. Whether sintokamides inhibit interactions between the AR and a similar group of proteins to that of EPI-001 is currently under intense investigation.

Inhibitors to the AR NTD as a therapy for prostate cancer

The NTD has been shown to be a viable target for in vivo intervention as first indicated by application of decoy molecules encoding residues 1–558 of the AR NTD (AR_{1–558}) (24) and then recently using EPI-001 (22). Decoy AR_{1–558} inhibits full-length AR and blocks both androgen-dependent and CRPC tumor growth most probably by a mechanism of mopping-up essential proteins required for transcriptional activity (24). Development of shorter decoy peptides (~100 amino acids in length) to the AR NTD that retain specificity for AR and still have anti-tumor activity has been difficult due to multiple factors including peptide lability and the possible requirement of multiple, nonlinear regions of the AR NTD necessary for protein-protein interactions. Systemic delivery of AR NTD decoys to target intracellular full-length AR in a clinical setting also could be a significant challenge. Small molecule inhibitors such as EPI-001 or potentially sintokamides appear to overcome many of these hurdles for therapeutic development for AR NTD decoys.

Consistent with inhibiting AR activity, EPI-001 blocks AR-dependent proliferation in human prostate cancer cells that express AR and has no effect on the proliferation of cells that do not express functional AR or do not rely on the AR for growth and survival (22). Consistent with EPI-001 blocking the androgen-AR axis, intravenous injection of EPI-001

significantly reduced the weight of benign prostates from non-castrated mature mice compared to control-treated animals (22). EPI-001 also blocked the growth of prostate cancer xenografts in the presence of androgen (non-castrated mature male mice) and most importantly causes tumor regression of CRPC. Male mice bearing LNCaP xenografts treated with EPI-001 by intravenous injection had tumors that were less than half the size of controls after just 2 weeks of treatment (22). Consistent with EPI-001 having specificity for AR, EPI-001 had no effect on PC3 human prostate cancer xenografts that are insensitive to androgen and do not express functional AR (22). These data support that EPI-001 is specific to the AR and does not affect cells that do not depend on functional AR for growth and survival. No toxicity was observed in animals treated systemically with EPI-001 as determined by no loss of body weight, no changes in behaviour, and no pathologic changes in the histology of internal organs (22). Pharmacokinetic studies show that EPI-001 has 86% oral bioavailability. Therefore longer treatment periods can be obtained by oral dosing rather than intravenous and should provide an indication if tumors that express AR will completely regress in response to EPI-001.

EPI-001 inhibits constitutively active AR lacking the LBD

A potential mechanism for resistance to antiandrogens and castration may involve expression of constitutively active splice variants of AR that lack LBD. Increased expression of constitutively active AR splice variants that are devoid of LBD have recently been described and are associated with earlier disease recurrence and death (25,26). Expression of constitutively active AR occurs with high frequency in CRPC and expression of these variants increase in response to reduced tissue levels of androgen (27). This suggests that the androgen environment plays a critical role in the expression of these variants. Further reduction of prostate tissue levels of androgen by CYP17 inhibitors may result in elevated levels of expression of constitutively active variant leading to CRPC and failure to treatments. To date, two different variants have been detected in clinical tissue, ARv567es and ARV7/AR3 (25–27). Expression of ARv567es is most commonly detected in approximately 43% of 46 metastases that express AR that were obtained from patients that succumbed to CRPC (27). Transfection of ARv567es cDNA yields tumors that are resistant to castration; however, the ratio of expression of full-length AR to variant appears to be an important indicator of tumor response to castration (27). MDV3100, an antiandrogen in clinical trials, that binds to the AR LBD was tested in LNCaP xenografts transfected with AR-V7 such that these tumors should represent a mixed population of full-length AR and variant (28). Unfortunately it was unclear whether there was indeed maintained expression of the variant in vivo since none of the growth advantage in response to castration was observed as reported by others with expression of the variant. Western blot analysis using an antibody to the NTD can not distinguish degraded full-length AR from variant in the harvested tumors and unfortunately non-transfected tumors were not analysed. Difficulty forcing expression of significant levels of AR-V7 protein appears to be a challenge thereby complicating interpretation of the in vivo MDV3100 data in transfected LNCaP cells. Xenograft models using human tumors that naturally express predominantly ARv567es such as LuCaP 86.2 (27) should provide a more physiologically relevant approach for determining the effects of MDV3100 and other AR antagonists on CRPC expressing AR variant lacking the LBD. Since EPI-001 inhibits the AR NTD and prevents essential N/C interaction of AR it should directly block the activity of these variants as well as prevent interaction of the variant with the full-length AR. Indeed, EPI-001 was effective at blocking the activity of a constitutively active deletion mutant, AR1-653, that contains the NTD, DBD and hinge region, but not the LBD (22). It will be of interest to determine if EPI-001 also inhibits naturally occurring splice variants and mixtures of variant and full-length AR as predicted from its mechanism of action. If so EPI-001 may be the first inhibitor available for tumors that solely express variants which occurs in 20% of metastases (27). Combination

therapies using an antiandrogen such as MDV3100 combined with EPI-001 may yield synergistic or additive responses in patients that generally have multiple tumors with varying ratios of full-length AR and variants.

Future directions

Although the discovery of androgen ablation therapy for the treatment of prostate cancer was more than 50 years ago, there is still continued drug development targeted at the AR and androgen axis. Resurgence of interest to developing selective CYP17 inhibitors and more potent antiandrogens is the result of recent impressive clinical data being obtained from abiraterone and MDV3100. However, these inhibitors that ultimately interfere with AR LBD by either reducing ligand or direct interaction also seem to eventually fail and AR transactivation is resumed. The discovery of significant expression of constitutively active splice variants of the AR lacking the LBD in CRPC tissue emphasizes the urgency to develop inhibitors of the AR NTD. EPI-001, sintokamides, and decoy AR₁₋₅₅₈ each target the AR NTD and have each shown significant inhibition of AR with antitumor activity. Developing inhibitors to the intrinsically disordered NTD provides a novel concept in the field of steroid hormone receptor therapy that has previously concentrated on targeting the C-terminus LBD.

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Figure 1. Therapeutic approaches to block the androgen receptor

A. EPI-001 interacts with the AR NTD to block CBP interaction and inhibit AR transcriptional activity. Inhibitors of the LBD include androgen ablation and antiandrogens (AA). CBP, CREB-binding protein; GnRH, Gonadotropin-releasing hormone (GnRH), also known as Luteinizing-hormone-releasing hormone (LHRH); DHT, dihydrotestosterone; NTD, N-terminal domain; DBD, DNA-binding domain; LBD, ligand-binding domain. **B.** Androgen dependent prostate cancer responds to androgen ablation and antiandrogens through the AR LBD. However, inhibition of the NTD with EPI-001 also inhibits androgen-dependent tumor growth. Castration resistant prostate cancer may involve: residual androgens that bind to the LBD; growth factor, cytokines, or kinase signal transduction pathways that target the NTD; constitutively active splice variants that lack the LBD that may be expressed solely or in mixed populations with full-length AR to form a heterodimer; and/or gain-of-function mutations. To date, EPI-001 inhibits all of these mechanisms with the possible exceptions of the heterodimer and the gain-of-function mutations which both have yet to be examined.