New Approaches to Target the Androgen Receptor and STAT3 for Prostate Cancer Treatments

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Abstract: Prostate cancer (PCa) is a common cause of death in men and remains incurable in the androgen-refractory phase. Growing evidence has shown that the androgen receptor (AR) and signal transducers and activators of transcription 3 (STAT3) could be effective targets for androgen-refractory PCa therapy. Many strategies have been reported to inhibit the AR or STAT3 activities. In this review, we focus on the AR N-terminal domain and AR chaperones, as well as small molecule inhibitors to STAT3 with which we discuss some new approaches to target the AR and STAT3 as potential treatments for androgen-refractory PCa.

Key Words: Androgen receptor, STAT3, Domain, AR chaperones, Small molecule inhibitor.

INTRODUCTION

The central role of androgen receptor (AR) in the PCa progression has been well established. AR remains active in all stages of PCa and becomes more sensitive in hormone-refractory PCa (HRPC). Some cytokines, growth factors, and even anti-androgens can activate the AR. Moreover, alterations of its co-factors also promote the AR activation [1-3]. Eventually, these factors will act on the AR to enhance proliferation of PCa cells at very low concentrations or absence of androgens in HRPC. Some approaches targeting AR signal for PCa treatments as shown in Table **1** have been proposed [4]. They are currently in phase I/II clinical trials for HRPC. Furthermore, additional novel therapeutic approaches to target the AR signaling are being investigated *in vitro* and in animal models *in vivo* [5].

Another potentially important mechanism underlying the development of HRPC is the up-regulation of the interleukine-6 receptor ((IL-6R)/JAK/STAT3 cascade [6]. STAT3, a member of JAK-STAT signaling pathway, is a latent transcription factor which can be activated by many cytokines and growth factors. The circulating concentrations of IL-6 were increased in the serum of patients [7]. Increased IL-6 can also enhance AR function through activation of STAT3 in an androgen-independent manner. *In vitro* studies have demonstrated that IL-6-dependent activation of the JAK/STAT3 pathway is accompanied by transition from androgen-dependent to androgen-independent PCa cell growth [8]. Androgen-dependent PCa cells, normally undergo apoptosis when androgens are withdrawn, however treatment with IL-6 or transfection with constitutively active STAT3 results in protection of the cells from apoptosis and therefore resistance to androgen deprivation [9]. The hypothesis that STAT3 is involved in the progression of HRPC is further supported by the recent observation that adenoviral gene delivery of wild-type Stat3 (AdWTStat3) to HRPC cell line DU145 increased the number of lung metastases by 33-fold in an experimental metastasis assay compared with controls [10]. STAT3 activation can promote cell growth and survival in HRPC independent of the AR. Tam *et al.* have investigated both the expression levels and activation of the IL-6R/JAK/STAT3 pathway in matched hormone-sensitive and hormone-refractory tumors from the same patients, the results showed that STAT3 is crucial for the transition to androgen-refractory PCa [11]. STAT3 has also been demonstrated to play a critical role in facilitating immune evasion by negatively regulating cellular and innate immune responses [12], and it can induce the expression of CD46, one of the complement-regulatory proteins, and protects PCa cells from complement-dependent cytotoxicity. Recent study showed that STAT3 up-regulated the expression of microRNA-21, which is over expressed in numerous cancers, and inhibit the tumor cell apoptosis [13]. All of these studies suggested that STAT3 could be a potential therapeutic target for PCa therapy.

AR and STAT3 pathways frequently co-exist and cross-talk in HRPC [14]. Recently, it is shown that androgen pretreatment increased STAT3 protein levels in an IL-6 autocrine/paracrine dependent manner in the androgen-dependent prostate cancer cell line LNCaP. STAT3 overexpression promotes AR–STAT3 complex formation and increases the AR response to IL-6 and EGF. Moreover, the sensitization of the AR to EGF and IL-6 is dependent on STAT3. Therefore, it is suggested a positive feedback loop existed within cells, and this feedback loop not only ensures maintenance of the AR signal but also increases AR activation by other signals such as EGF and IL-6 [15]. However, presently it is not clear if this positive feedback loop is operative *in vivo*. Understanding interactions and changes in signal networks related to AR and STAT3 pathways *in vitro* and *in vivo* is important, and can help us to develop more effective methods to treat HRPC or PCa with potential to become HRPC. In this article, we will discuss some novel approaches to target AR and STAT3 in PCa cells.

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Table 1. Several Compounds Targeting AR Receptor Signal in Clinical Trails for HPRC, HDAC: Histone Deacetylase

Compound	Target	Trial Phase
Lyase inhibitor	androgen synthesis	I-II
5-α-reductase inhibitor	androgen metabolism	III
HDAC inhibitors	AR stability	I-II

AR TARGETING

AR N-Terminal Domain (NTD) and ITS DECOY MOLECULES

AR is located on chromosome Xq11–12 and has 8 exons encoding 4 functional regions. They are the N-terminal domain (NTD) harboring AR transcriptional activation function (AF)-1, the highly conserved central DNA-binding domain (DBD), the hinge region containing a nuclear localization signal, and the COOH-terminal domain (CTD) containing both the ligand-binding domain (LBD) and a ligand-dependent activation function (AF-2) with an co-activator binding surface [16]. Normally, AR activity is strictly ligand-dependent as well as AF-2-dependent in androgen-dependent PCa cells and can thus be blocked by antiandrogens. In fact, the AR CTD is the foremost target of current androgen ablation therapies. For example, castration that lowers circulating and local androgens largely reduce binding of androgens to LBD, therefore diminish AR nuclear translocation and the exposure of AF-2 [17]. Administration of antiandrogens such as bicalutamide that compete the binding of endogenous androgens to the LBD blocks the activity of the AF-2 and recruits co-repressors to the AR in the promoters of AR-regulated genes [18].

Novel approaches to AR inhibition could be based on knowledge surrounding mechanisms of AR activation. In the androgen-refractory PCa cells, AR activity is constitutive, ligand-independent, AF2-independent, and antiandrogen-resistant. One of the potential mechanisms is because of the activation of AR NTD. AR NTD comprises nearly 60% of the coding region and attracts more attention recently [17]. AF-1 embedded within NTD can function as a ligand-independent transcriptional activator, and transactivation unit TAU-1 and TAU-5 located within the AF-1 was important for ligand-independent AR activity in androgen-refractory PCa cells. Further, the AF-1a (101-211) and AF-1b (253-361) domains in the TAU-1 [19] and WHTLF (435-439) motif in the TAU-5 of AR NTD (as shown in Fig **1**) play a key role in mediating the androgen-independent AR transcriptional activity [20]. Calpain, a calcium-dependent proteinase which is highly expressed in PCa cells [21], cleaves the AR into an androgen-independent isoform. *In vitro* and *in vivo* analyses showed that calpain removes the COOH-terminal ligand binding domain generating a constitutively active molecule. Analysis of human prostate tumors indicates that several tumors express higher levels of this truncated AR than in noncancerous prostate tissues [22]. Moreover, the AR can be activated *via* its NTD in response to bone-derived factors and compounds that stimulate the PKA and IL-6 pathways [23, 24]. AR NTD contains numerous phosphorylation sites and interacts with multiple proteins [25]. For instance, TAB2, a sensor for inflammatory signals, has been shown to interact with the AR NTD at residues 179–188 in response to IL-1β and further recruit MEKK1, which in turn mediates dismissal of the N-CoR/HDAC complex and permits derepression of androgen target genes. Finally, IL-1β induces a switch whereby anti-androgens were able to activate the AR through AR NTD [26]. Steroid receptor coactivator-1 was also shown to bind the AR NTD and increase ligand-independent activation of the AR downstream of IL-6 [27]. It is well accepted that the AR NTD would become a potential novel target for the treatment of androgen-refractory PCa.

A possible therapeutic approach is to overexpress an AR NTD peptide to create decoy molecules that competitively bind the interacting proteins required for activation of the

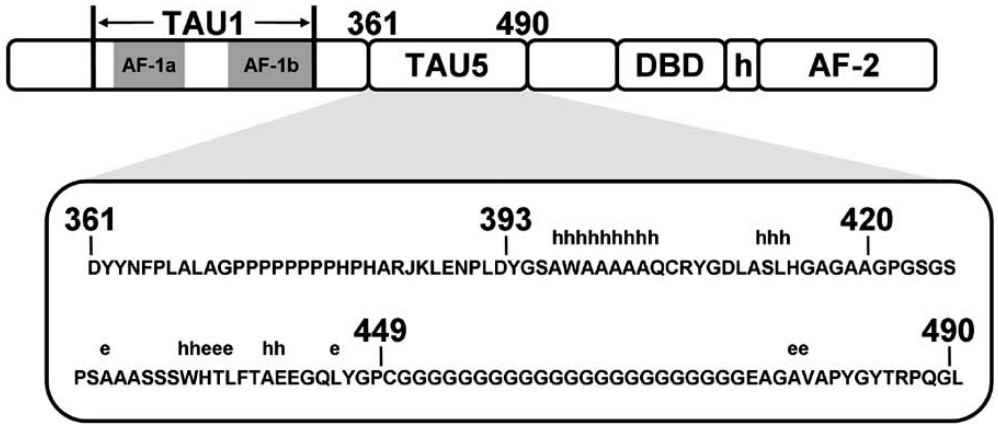

Fig. (1). The structure of TAU1 and amino acid sequence of AR TAU5. Propensity for a-helical (h) or h-sheet (e) secondary structure was determined using the nnpredict algorithm.

endogenous full-length AR. It has been reported that AR NTD decoy molecules encoding amino acids 1–558 of the AR NTD ($AR_{1–558}$) reduced tumor incidence and inhibited growth and hormonal progression of PCa tumors that expressed endogenous AR *in vitro* and *in vivo*. In *in vivo* studies, lentivirus technology was used to deliver Decoy AR1–558 into established xenografts. The important feature is that the lentiviral delivery of decoy AR1–558 caused no apparent adverse effects on the host [28]. However, AR NTD decoy molecules also inhibited the transcription activity of progesterone receptor (PR) and estrogen receptor (ER) in PCa cells. Note that AR NTD has three regions that are highly conserved among the steroid receptor family: amino acids 1–30, 224–258, and 500–541 [29]. Further investigation would be required to determine whether there are specific regions in the AR NTD can be effectively used to inhibit AR transactivation while not affecting other steroid receptors.

AR Chaperones and their Inhibitors

Unliganded AR is predominantly maintained in the cytoplasm as an inactive but highly responsive state by a large dynamic heterocomplex composed of heat-shock protein (HSP) 90, 70, 56, and 23, co-chaperones, and tetratricopeptide repeat (TPR)–containing proteins [30]. Ligand binding leads to a conformational change in the AR and dissociation from the large HSP complex. Subsequently, the AR translocates to the nucleus, interacts with coactivators and binds to androgen responsive elements (ARE) in genomic DNA to transactivate target gene expression [31]. During the progression to androgen-refractory PCa, the tightly regulated androgen signaling pathway may be disrupted such that unliganded AR can localize to the nucleus and activate its target genes [32]. Since AR translocation to the nucleus is a prerequisite for its transactivation, dissociation of the AR-chaperone complex is viewed as one of critical regulatory mechanisms of AR signaling [33] and a potential therapeutic target. In addition, chaperones remain important players in the events downstream of AR activation and throughout the life cycle of the AR.

AR contains two nuclear localization signals, NL1 in the DNA-binding domain and hinge region and NL2 in the ligand-binding domain (LBD) [34, 35], and a nuclear export signal (NES) in the LBD that causes cytoplasmic localization of the AR in the absence of ligand [36]. Upon binding androgens, the NES is repressed and AR translocates into the nucleus. Since AR activation can occur independent of androgens [37], changes in the intracellular trafficking of AR leading to ligand-independent nuclear import or impairment of nuclear export could occur in the progression to androgen-independence. Nuclear localization of endogenous AR in androgen-refractory C4-2 cells was increased compared to the parental LNCaP cell line by immunocytochemistry assay. However, nuclear export of AR is not impaired in C4-2 cells, and it was suggested that the less binding of HSP90 resulted in the increased AR nuclear localization [38].

HSP90, one of the key regulators in the AR pathway, is part of a multi-chaperone complex known to stabilize AR [39, 40]. HSP90 protein stabilization and function is dependent on binding and hydrolysis of ATP. Geldanamycin (Fig. **2**), an HSP90 inhibitor, destabilizes AR by competing with ATP for binding to the amino terminus of HSP90 and in-

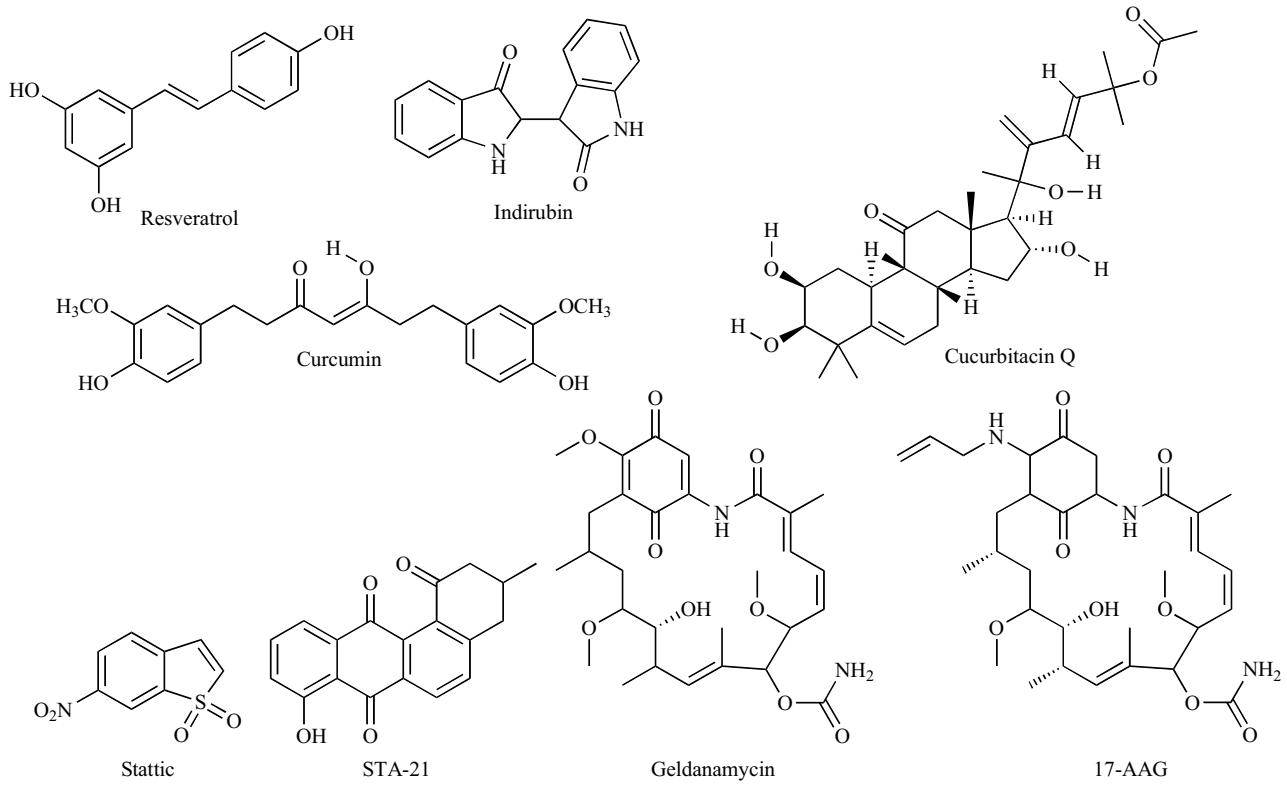

Fig. (2). The chemical structures.

creases AR proteasomal degradation, thereby decreasing the expression of AR-regulated genes [41]. 17-Allylamino-17-demethoxygeldanamycin (17-AAG, Fig. **2**), another specific HSP90 inhibitor derived from geldanamycin, prevents ligand-independent nuclear localization of AR, decreases AR stability, and inhibits basal PSA expression in androgen-refractory C4-2 cells [37]. 17-AAG is currently in clinical testing [42-44]. Phase I testing demonstrated that the effects of 17-AAG were dose dependent and schedule-dependent, and recommended doses are currently in phase II testing.

HSP27, another chaperon of AR, is ATP-independent chaperone that is phosphoactivated by cell stress to prevent aggregation and/or regulate activity/degradation of certain client proteins. Over-expression of HSP27 in LNCaP cells suppressed castration-induced apoptosis and confers androgen resistance [45], whereas HSP27 knockdown using antisense oligonucleotide decreases HSP27 levels, increases caspase-3 cleavage and apoptosis, enhances paclitaxel chemosensitivity, and delays tumor progression *in vivo* [45, 46]. In the classic model of androgen action, in response to androgens, AR dissociates from HSP90 and then associates with the HSP27 to translocate into the nucleus [47]. It has been shown that the AR coactivators STAT3 and ARA55 also interact with HSP27 [48, 49]. Hence, HSP27 and AR may complex with either ARA55 or STAT3 to cooperatively promote AR translocation and transactivation. It was reported that specific HSP27 knocking-down with the antisense drug OGX-427 destabilizes AR by inducing the dissociation of the AR/HSP90 heterocomplex, increasing AR association with the E3 ligase MDM2, and resulting in ubiquitin-proteasome–mediated AR degradation [50]. Taken together, these studies suggest that further investigation on targeting the AR chaperones may be worthwhile for developing effective novel PCa therapy.

STAT3 TARGETING

Small-Molecule Inhibitors

Inhibition of STAT3 signaling by a dominant-negative STAT3 mutant [51], antisense approaches [52, 53], decoy oligonucleotides [54-56], small interference RNA (siRNA) [57, 58], or G-quartet oligonucleotides [59] has been demonstrated to suppress tumor growth and induce apoptosis in cancer cells. While numerous small molecules have been reported to inhibit STAT3 signaling, the vast majorities of them are natural product and act on targets other than STAT3 itself. Curcumin (Fig. **2**), an indirect natural product inhibitor of STAT3 signaling, has also been identified as an inhibitor of numerous different signaling pathways [60]. Cucurbitacin (Fig. **2**), a steroidal natural small-molecular inhibitor, reduces STAT3 phosphorylation, but does not directly bind to STAT3 [61]. Indirubin (FIg. **2**), a constituent of a Chinese herbal prescription used for treatment of chronic myelogenous leukemia [62] and a known inhibitor of cyclin-dependent kinases [63], was shown to inhibit STAT3 signaling in breast cancer cells by inhibiting upstream kinase activity, presumably that of c-Src [64]. A similar mechanism of action has been suggested for the natural product, resveratrol (Fig. **2**) [65]. A distinction should be made between a STAT3 pathway inhibitor and a STAT3 inhibitor. The former might have multiple targets. The latter just inhibits STAT3 by direct binding. Direct inhibition of STAT3 itself is less likely to result in unintentional inhibition of additional signaling pathways than the targeting of its signal pathway.

STAT3 contains a DNA binding, a transactivation, and an SH2 domain, and is activated by tyrosine phosphorylation which results in dimer formation through SH2–phosphotyrosyl interaction [66] and transcriptional regulation. Obviously, the SH2 domain, the DNA binding domain, or the transactivation domain can be used as a potential target to inhibit the STAT3 activity. Recently virtual screening system by computer analysis was used to select the small molecular compounds which can directly interact with a specific domain of STAT3 to inhibit its activity. Since the SH2 domain is required for both tyrosine-phosphorylation and dimerization of STAT3, the most logical approach toward inhibition of STAT3 would be impairing the function of its SH2 domain [67]. This should not only inhibit STAT3 activation but also prevent dimerization of any STAT3 molecules that escape inhibition of activation. Song *et al.* have performed a virtual screen against the SH2 domain of STAT3 and discovered that STA-21 (Fig. **2**), a nonpeptidic small molecule, inhibited STAT3 DNA binding activity, STAT3 dimerization and STAT3-dependent luciferase activity in breast cancer cells [68]. However it is not clear if STA-21 can inhibit the function of the SH2 domain of phosphorylated STAT3 *in vitro*. Another nonpeptidic small-molecular STAT3 inhibitor Stattic (Fig. **2**) was found to inhibit the function of the STAT3 SH2 domain regardless of the STAT3 phosphorylation state *in vitro*. Stattic selectively inhibits activation, dimerization, and nuclear translocation of STAT3 and induces apoptosis in STAT3-dependent cancer cell lines [69]. It is easy for the nonpeptidic small molecule inhibitor to enter into the cells, but more studies would be required to investigate if the small molecular inhibitors can inhibit the STAT3 function *in vivo*.

SUMMARY

AR and STAT3 signal pathway stimulate each other in PCa cells. There are many approaches to inhibit their function, some herbal medicine not only inhibit the AR activity but also the STAT3 activation with little side effects, such as curcumin, quercetin and resveratrol [60, 70-72]. But, herbal medicine should just be an alternative therapy along with the traditional androgen ablation. HSP90 inhibitors also can suppress the AR and STAT3 activity [38, 73]. Based on the mechanisms involved in their specific inhibition, combination of inhibitors of the transcriptional functions of STAT3 and AR may have enhanced therapeutic effects for PCa. However, it might be difficult to completely eliminate AR and STAT3 from all PCa cells. Recently, it is reported that the prostatic stem cell-like cells can transform into PCa stem/progenitor cells, these cells does not express AR, but they can differentiate into the cells expressing AR. On the other hand, STAT3 can be constitutively activated in high malignant potential PCa cell lines, such as DU145 and TSU cells. Recently activated STAT3 pathway was observed in the breast cancer stem-like cells [74]. More studies would be required to figure out what PCa stem-like cells are and how we can target these PCa cells to completely eliminate the AR pathway, maybe also STAT3 pathway. Meanwhile, under

conditions of low tumor burden, targeting the AR and STAT3 for specific knockdown may be a viable strategy for delaying or preventing progression of PCa to androgen refractory state.

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