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Identification of Novel Steroidal Androgen Receptor Degrading Agents Inspired by Galeterone 3β-Imidazole Carbamate

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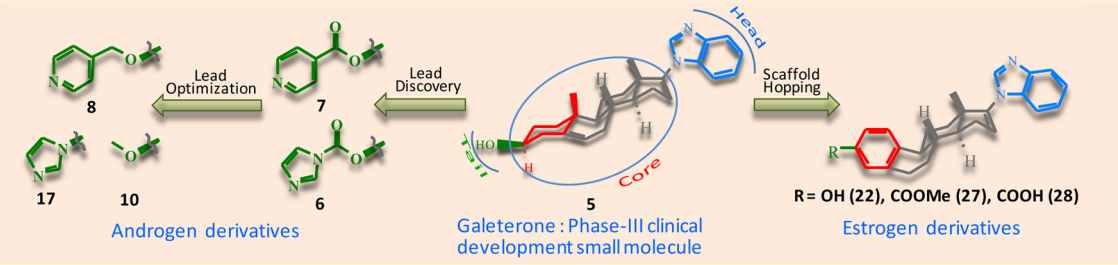
Supporting Information



ABSTRACT: Degradation of all forms of androgen receptors (ARs) is emerging as an advantageous therapeutic paradigm for the effective treatment of prostate cancer. In continuation of our program to identify and develop improved efficacious novel small-molecule agents designed to disrupt AR signaling through enhanced AR degradation, we have designed, synthesized, and evaluated novel C-3 modified analogues of our phase 3 clinical agent, galeterone (**5**). Concerns of potential *in vivo* stability of our recently discovered more efficacious galeterone 3β-imidazole carbamate (**6**) led to the design and synthesis of new steroidal compounds. Two of the 11 compounds, 3β-pyridyl ether (**8**) and 3β-imidazole (**17**) with antiproliferative GI_{50} values of 3.24 and 2.54 μM against CWR22Rv1 prostate cancer cell, are 2.75- and 3.5-fold superior to **5**. In addition, compounds **8** and **17** possess improved (~4-fold) AR-V7 degrading activities. Importantly, these two compounds are expected to be metabolically stable, making them suitable for further development as new therapeutics against all forms of prostate cancer.

KEYWORDS: *Androgen receptor (AR), splice variant AR (AR-V7), androgen receptor degrading agents (ARDAs), prostate cancer*

Globally, prostate cancer (PC) is the second most commonly diagnosed human cancer in men, accounting for ~260,000 deaths yearly.[1] Almost 80% of cases are diagnosed as localized disease, and radiation or surgery can be curative. However, despite current treatment options, there is still a relapse rate of 30−60%.[1]

Androgens and androgen receptor (AR) play crucial roles in the development and progression of PC.[2,3] As a consequence, for locally advanced or metastatic disease, hormonal treatment with androgen deprivation therapy, which blocks the production (CYP17 inhibitors: abiraterone; **1**)[4] and/or activity of androgen (antiandrogens, bicalutamide; **2**)[5] (Figure 1), is a standard approach for the majority of patients, but, for most cases, the duration of response is limited to 12−24 months, and the disease will become castration-resistant prostate cancer (CRPC) with no treatment options.[6] Approximately 85% of CRPC patients die within 5 years and docetaxel (**3**) is currently the only treatment shown to provide even minimal survival benefit.[7]

In castration-resistant environment, aberrant AR reactivation is implicated through numerous mechanisms, which leads to overexpression of mutated AR, AR amplification, and local androgen synthesis.[8−10] Recently, multiple alternative spliced

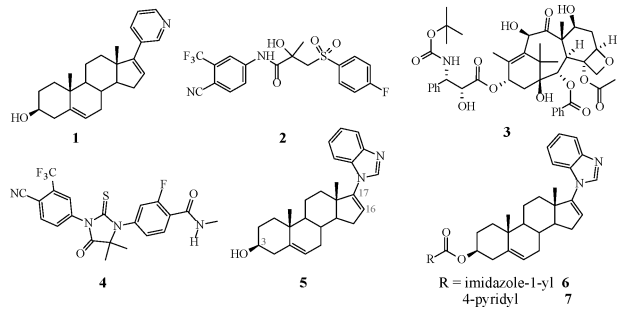


Figure 1. Chemical structures of compounds **1**−**5** (clinical anti-PC agents) and selective ARDAs (**6**−**7**).

AR isoforms (AR-Vs) have been identified in CRPC.[11−14] Unlike full-length AR (fAR), AR-Vs lack the ligand-binding domain (LBD) and are androgen refractory.[12,14] The AR-Vs are expressed at higher levels in various tumors and are 3- to 5-fold more potent than f-AR in transactivating activity.[15] It has

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recently been clearly demonstrated that patients expressing constitutively active AR-Vs do not benefit from antiandrogens and therapies that inhibit androgen biosynthesis.[16,17] In fact, not all CRPC patients respond to novel antiandrogen (enzalutamide; **4**) or CYP17 inhibitors (**1**), and even those who do subsequently relapse within a few months.[18−20] Based on these findings, we envision that effective treatment of CRPC patients will require new drugs that can modulate all forms of AR such as AR degrading/down-regulating agents (ARDAs).[21,22] The substantial anti-PC efficacy of phase 3 development agent galeterone (Figure 1; **5**) in comparison to abiraterone (**1**)[23] or casodex (**2**)[24] may be attributed to galeterone's AR degrading (ARD) activity.

For the development of new ARDAs, we recently showed, using rational structure−activity relationship (SAR) studies that modifications of the C-3 hydroxyl, unlike modifications at C-16 and C-17 of galeterone (**5**), yield novel analogues (e.g., imidazole carbamate, **6** and pyridine carboxylate, **7**) with enhanced PC antiproliferative and AR modulating (AR antagonism and AR/AR-Vs degrading) activities.[22] Mechanistic examination suggests that compounds **5** and **6** induce proteasomal degradation of AR/AR-V7, which involves complex formation with E3 ligases Mdm2 and CHIP (C-terminus of Hsp70-interacting protein) concomitant with enhanced ubiquitination.[25]

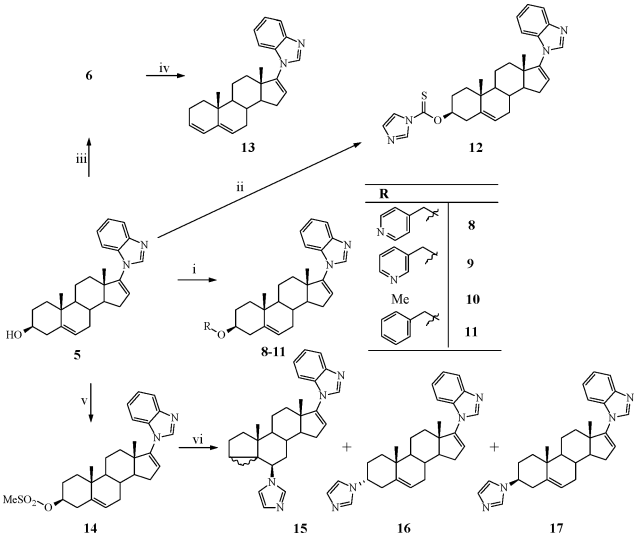
In recent antitumor efficacy studies, we showed that **6** was moderately more efficacious than galeterone (70 versus 60% growth inhibition, respectively, compared to control) in inhibiting the growth of CRPC CWR22Rv1 tumor xenografts.[25] Although we are yet to conduct *in vivo* pharmacokinetics study of these lead ARDAs, the presence of metabolically labile functions such as carbamate and ester groups in compound **6** and **7**, respectively, may be a matter of concern. Therefore, the present study aimed to develop novel ARDAs with metabolically stable chemical moieties tethered at C-3 of androstene and estrogen steroidal cores, but with retention of the C-17 benzimidazole moiety.

Starting from previously identified 4-pyridylester (**7**) with potent ARD activity,[22] we decided to keep the pyridyl ring fixed and modify the ester-link to obtain metabolically stable compounds. This modification also serves as probe for the role/significance of the ester moiety toward ARD activity. Considering the relative metabolic stability of ether function over ester, compounds **8** and **9** were designed. Similarly, to probe influence of the pyridyl ring toward ARD activity, the simple methyl (**10**) and benzyl (**11**) ethers were designed (Scheme 1).

These four C-3 ethers (**8**−**11**, Scheme 1) were prepared by following Williamson's etherification method of treating alcohol (**5**) with respective aryl/alkyl halide in the presence of sodium hydride as base in DMF with yields of 12, 43, 77, and 87%, respectively. Considering the potency of **6**, we also prepared the thiocarbamate derivative **12**, 54.5% yield (Scheme 1), by refluxing **5** and 1,1′-thiocarbonyldiimidazole in acetonitrile and dichloromethane. To probe the influence of C3 substituent the $\Delta^{3,5}$-diene (**13**), without any C3 substituent was obtained (13% yield) by neat pyrolysis of **6**[22] at 195 °C (just above its mp).[26]

In our previous investigation, an attempt to synthesize 3β-imidazole derivative of galeterone yielded C-3 imidazole carbamate (**6**).[22] An attempt to synthesize 3β-imidazole derivative by refluxing 3β-mesyl compound **14**,[22] with imidazole under anhydrous condition in toluene resulted in compounds **15** (35%), **16** (3%), and **17** (11%) along with a possible elimination product (R_f value similar to **13**, not isolated) (Scheme 1). These three positional isomers were separated by preparative HPLC (see Supporting Information for method and chromatogram).

Scheme 1. Synthesis of Androstene Derivatives (8−13, 15−17)[a]



[a]Reagents and conditions: (i) NaH, DMF, R-X, rt or heat, 1−12 h; (ii) 1,1′-thiocarbonyldiimidazole, CH_3CN, DCM, reflux, 5 h; (iii) 1,1′-carbonyldiimidazole, CH_3CN, rt, 2 h; (iv) dry heat at 195 °C, 1 h; (v) mesyl chloride, pyridine, ice (5 h), then rt (5 h); (vi) toluene, imidazole, reflux, overnight.

As an androstene core alternate, estrogen was selected, where three derivatives, including C-3 hydroxy (**22**), C-3 methyl-carboxylate (**27**), and C-3 carboxy (**28**) were designed and synthesized (Scheme 2). Synthesis of these compounds was achieved by slightly modifying our routine method for synthesis for galeterone and related compounds.[22]

The synthesis of estrone-3-hydroxy-17-1*H*-benzimidazole compound (**22**, Scheme 2) started with 3-acetylation of estrone with acetic anhydride in pyridine to give **18** in 98% yield. Compound **18** was subjected to Vilsmeier−Haack reaction by treating with phosphorus tribromide in DMF and $CHCl_3$ to give 16-formy-17-bromo derivative **19** in 26% yield. An attempt to append a benzimidazole (BzIm) to C-17 position of **19** following our routine K_2CO_3 in DMF method[22,27] resulted into mixture of four compounds (partial 3-deacetylation with or without BzIm substitution, acetylated BzIm product and substrate). Therefore, we first hydrolyzed 3-acetyl group of **19** by treating with 10% ethanolic-KOH to obtain **20** (28%). Thereafter, the BzIm group was appended using K_2CO_3 in DMF to give **21** in 84% yield and finally 16-deformylation by refluxing with 10% Pd/C in benzonitrile to obtain desired compound **22** in very low isolated 6% yield.

The synthesis of 17-benzimibazole estrone-3-carboxlate (**28**, Scheme 2) was initiated by activation of estrone via treatment with triflic anhydride in the presence of organic base (TEA)[28] to give the corresponding triflate **23** in 37.4% yield. Treatment of **23** with Pd-catalyzed carbonylation using $Pd(OAc)_2$ as catalyst, 1,c-bis(diphenylphospheno)propane as the phosphine ligand, in the presence of gaseous carbon monoxide and methanol in DMF[29] gave the corresponding methyl carboxylate **24** in 32% yield. The remaining four steps for the synthesis of desired **28** followed the above-described method for **22**,[22]

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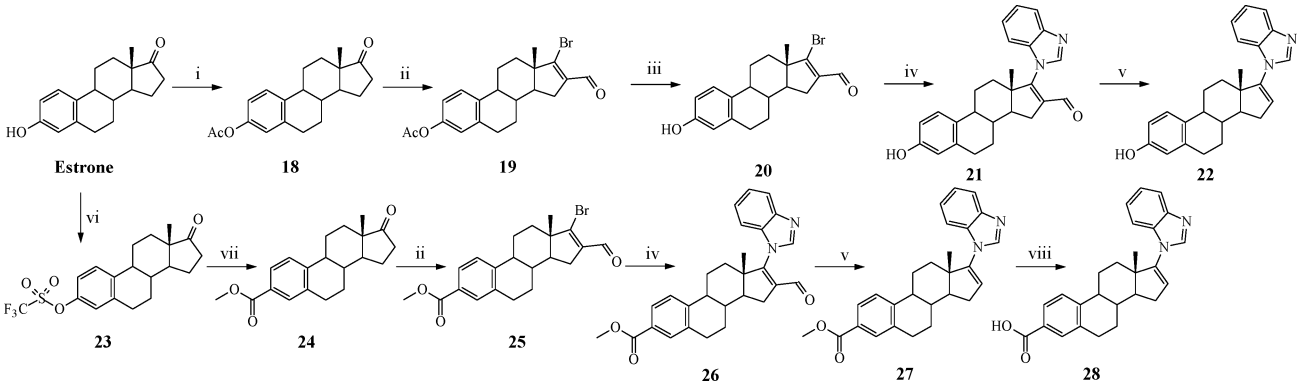
Scheme 2. Synthesis of Estrogen-3-hydroxy (22) and 3-Carboxyl Derivative (28)[a]



[a]Reagents and conditions: (i) pyridine, acetic anhydride, rt, 12 h, (ii) PBr_3, DMF, $CHCl_3$, reflux, 5 h; (iii) ethanol, 10% ethanolic-KOH, rt, 12 h; (iv) BzIm, K_2CO_3, DMF, 80 °C, 1−5 h; (v) benzonitrile, 10% Pd/C, 185 °C, 12 h; (vi) triflic anhydride, TEA, DCM, 0 °C, 1 h; (vii) palladium(II) acetate, dppp, TEA, MeOH, DMF, carbon monoxide, 0 °C, 9 h; (viii) MeOH, 10% methanolic-KOH, reflux, 2 h.

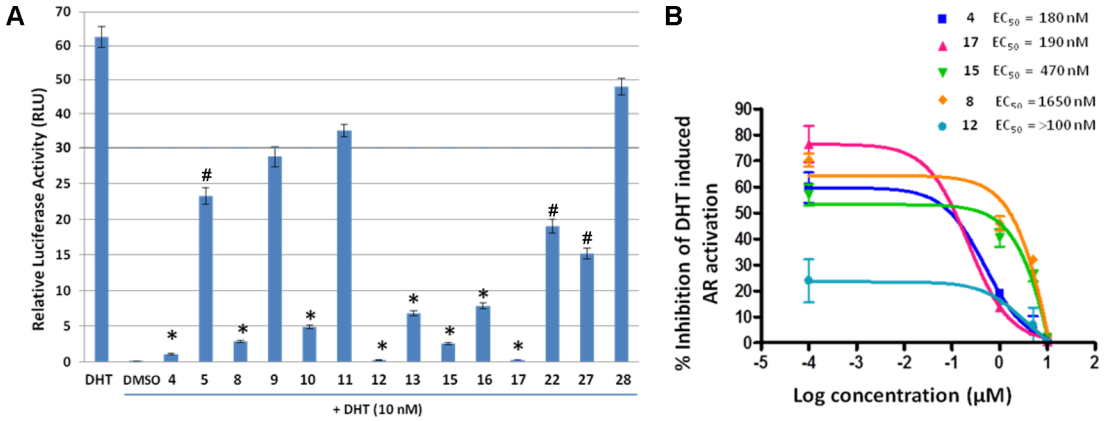


Figure 2. (A) Effects of compounds at 10 μM on dihydrotestosterone (DHT)-stimulated transcription of AR. LNCaP cells were transfected with the AAR2 reporter construct + the *Renilla* luciferase reporting vector pRL-null and treated with novel compounds for 24 h in the presence of 10 nM dihydrotestosterone (DHT). Control, baseline activity without androgen stimulation. Androgen-stimulated luciferase activity (luminescence) was measured in a Victor 1420 plate reader. The results are presented as the fold induction (i.e., the relative luciferase activity of the treated cells divided by that of the control) normalized to that of the *Renilla* luciferase activity. *, $P < 0.01$; #, $P < 0.05$ compared with DHT treated cells. (B) Inhibitory effects of ARDAs/MDV3100 on DHT induced AR transcriptional activity in LNCaP cells. EC_{50} = the concentration of inhibitor (ARDAs/MDV3100) required to inhibit the DHT-induced AR transcriptional activity by 50%. EC_{50} values of the compounds were determined from dose−response curves. Points, mean of replicates from three independent experiments; bars, SE. Solid line, best-fit sigmoidal dose response (variable slope).

involving three intermediate steps: formation of 16-formyl-17-bromo derivative **25** (52%) by Vilsmeier−Haack reaction, then C-17 BzIm condensation to give **26** (95%), followed by Pd catalyzed 16-deformylation to give **27** (57%) and finally basic hydrolysis of methyl ester group to obtain the desired compound **28** in 93% yield.

Structural integrity of all the compounds in the study were characterized and confirmed by ^1H and ^{13}C NMR and HRMS spectroscopy. Purity of the all the compounds used for biological studies are analyzed by UPLC method and are >93% pure (see Supporting Information). Synthesis and purity check for the compounds **6**−**7** and **14** was reported previously.[22]

To determine whether our new compounds modulate f-AR transcriptional activation, we performed a luciferase experiment utilizing LNCaP cells dual-transfected with the probasin luciferase reporter construct AAR2-luc and the *Renilla* luciferase reporting vector pRL-null as we previously described[22,27] and reported in the Supporting Information. As shown in Figure 2A, luciferase expression was significantly increased after 10 nM DHT treatment for 24 h. The ability of the novel compounds (10 μM, each) to modulate DHT-induced AR transactivation was assessed, using enzalutamide (**4**) and galeterone (**5**) as positive controls. Gratifyingly, nine of the 12 new compounds tested were more potent than galeterone and compounds **12** and **17** were equipotent to enzalutamide. The order of potency for the new analogues was **12**, **17** > **8**, **15** > **10** > **13** > **16** > **27** > **22** > **9**, **11** > **28**. Based on these results, we selected the top four compounds and determined the effective concentrations that caused 50% inhibition of DHT-induced AR transactivation (EC_{50} values) from dose−response curves (Figure 2B).

As expected, these compounds exhibited impressive EC_{50} values ranging from <100 to 1650 nM. The EC_{50} of compound **17** (190 nM) was comparable to that of enzalutamide (180 nM), while that of compound **12** (<100 nM; determined via extrapolation) was superior to enzalutamide.

To determine whether our compounds induce AR degradation, LNCaP cells were treated with each of the compounds (**5**, **6**, **8**, **10**, **12**, **13**, **15**−**17**, **27**, and **28**) of interest (15 μM each) for 24 h, followed by Western blotting analysis.

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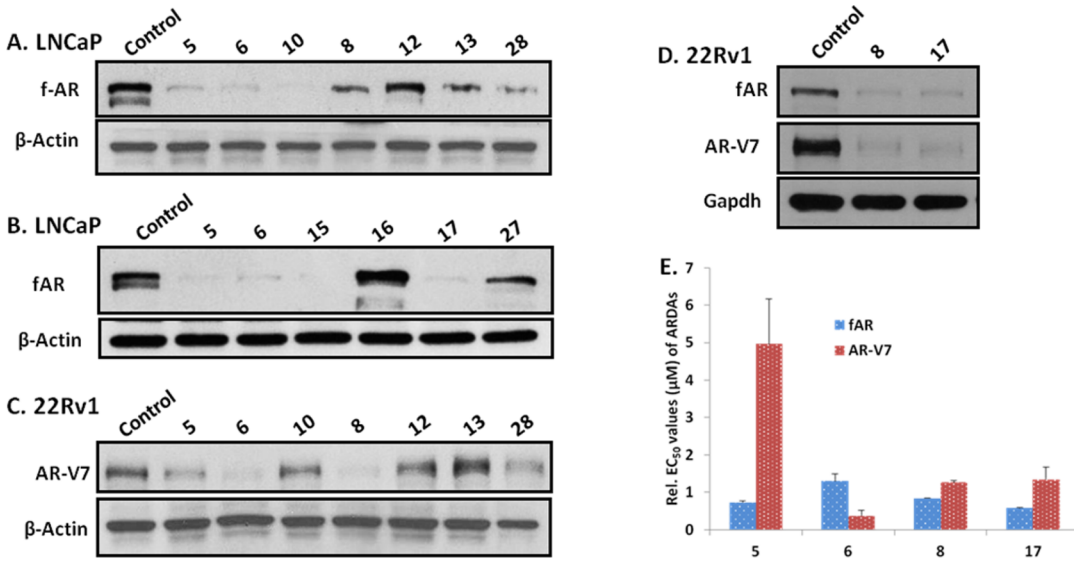

Figure 3. (A–D) Differential effect of compounds on suppressing AR expression in LNCaP and CWR22Rv1 prostate cancer cells: Western blot analysis of f-AR/AR-V7 expression in LNCaP or CWR22Rv1 cells treated with various compounds. Cells were exposed to individual compounds (15 μM) for 24 h, and the protein lysates were subjected to Western blot analysis. (E) EC_{50} values (for fAR and AR-V7) for compounds were determined from dose–response curves following compound treatments (0 → 7.5 μM) of CWR22Rv1 cells for 72 h followed by Western blot analysis of lysates.

As depicted in Figure 3A, B, most of the new compounds significantly caused AR degradation in LNCaP cells, comparable to or better than galeterone (**5**). We note that two compounds **12** and **16** were ineffective. It is also unclear why compound **28** caused significant AR degradation when it showed no effect on inhibition of AR transactivation as depicted in Figure 2A.

Based on our previous reports, which show that AR-Vs drive the progression of CRPC, we next determined the effect of our compounds on the down-regulation of AR-V7 (also called AR-3). As depicted in Figure 3C, we observed that compound **5** and some of our new compounds **6**, **8**, and **28** tested in this assay (CWR22Rv1, prostate cancer cell line) caused significant down-regulation AR-V7. Figure 3D shows f-AR and AR-V7 depletion caused by compounds **8** and **17**. Whereas their EC_{50} values for f-AR are similar, compounds **6**, **8**, and **17** exhibited >4-fold increased efficacy in AR-V7 degradation activity as compared to galeterone (**5**) (Figure 3E).

In response to some concerns of an astute reviewer, we note the following: (i) most of the AR biology studies reported here were conducted in LNCaP prostate cancer cells (expressing T877A mutant AR-FL), the dominant model system in the field that is used widely to study the general biology of AR, to screen for novel AR antagonists;[30,31] (ii) we did not conduct AR ligand binding studies, based on our previous findings that this class of compounds does not have to bind to the AR ligand binding domain to cause modulation/degradation of AR. It is important to note that this class of compounds also causes degradation of AR splice variants that are devoid of the LBD;[22,25] (iii) for the compounds tested (see Figure 2A), agonistic activities were not observed; (iv) we have previously reported on the selectivity of galeterone (**5**) and compound **6**, where we clearly showed that these AR degrading agents do not affect other nuclear receptors,[25] which is likely to be the case with these related new analogues.

Since these compounds are analogues of galeterone (**5**), we evaluated their ability to inhibit CYP17 enzyme (Table 1). As expected, (except for **17**, IC_{50} = 0.48 μM) the compounds were weak inhibitors of CYP17 (IC_{50} = 2.59 to >15 μM), buttressing established SAR data for potent steroidal CYP17 inhibitors.[22]

Finally, we assessed the antiproliferative activities of our novel compounds in two human prostate cancer cell lines (LNCaP and CWR22Rv1). The inhibitory concentrations that caused 50% growth inhibition of cell proliferation (GI_{50} values)

Table 1. IC_{50} of Select Compounds for Inhibition of CYP17 (17α-Hydroxylase Activity) and GI_{50} Values of Novel ARDA Compounds

	IC_{50} (μM)[a]	GI_{50} values (μM)[b]	
compd	CYP17	LNCaP	CWR22Rv1
8	>15	2.45	3.24
9		4.26	0.81
10	39	8.71	7.50
11		5.12	1.2
12		9.57	7.52
13			
15		6.61	12.30
16		8.91	9.88
17	0.480	1.64	2.54
22	25.9	3.89	0.50
27	>15	6.91	8.74
28			
for comparison			
1	0.048		
4		4.85	
5	0.140	3.93	8.91

[a]IC_{50} value is the concentration of inhibitor that inhibits the CYP17 enzyme activity by 50% each in duplicate. IC_{50} values were each determined from dose–response curve. [b]The GI_{50} were determined from dose–response curves (by nonlinear regression analysis using GraphPad Prism) compiled from at least three independent experiments using LNCaP cells, SEM < 10%, and represents the compound concentration required to inhibit cell growth by 50%. Blank cells = not tested.

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were determined from dose−response curves using MTT assays as we have previously described,[22] presented in Table 1. Although there is a modest correlation between the antiproliferative activities GI_{50} values and inhibition of AR-transactivations, AR degrading activities, compounds **8**, **9**, **11**, **17**, and **22**, are 2.4- (*for LNCaP*) to 18-fold (*for CWR22Rv1*) more potent than lead compound **5**, representing a significant improvement. Their full potential may become evident following comparative *in vivo* antitumor efficacy assessments. It is important to note here that we have previously reported that galeterone and its analogues also inhibit the growth of PC-3 and DU-145 prostate cancer cells,[32] which may also be the case for these new analogues.

In conclusion, a small library of galeterone analogues was designed and synthesized with modifications of substituents at C3 and C6 and the architecture of ring A. The substituent at C3 could be varied somewhat (see compounds **8** and **17**), but a basic heterocycle seems important for bioactivity. Because the yields for these promising compounds (**8** and **17**) are low, we have embarked on new studies to develop facile and robust synthesis for these compounds. This will enable biological studies to assess their efficacies in clinically relevant models of prostate cancer, *in vitro* and *in vivo*. Importantly, the most effective compounds are predicted to be metabolically more stable (by virtues of their C-3 moieties) than compound **6**, making them suitable for further development as new therapeutics against prostate cancer.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00137.

Synthetic experimental details, analytical and further biological data of compounds and biological assay protocols. UPLC chromatograms and high resolution mass spectral data for final compound (PDF)

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Notes

The authors declare the following competing financial interest(s): V.C.O.N. is the lead inventor of galeterone and new analogues, patents, and technologies thereof owned by the University of Maryland, Baltimore, and licensed to Tokai Pharmaceuticals, Inc. A.K.K.-A. and P.P. are co-inventors of some related compounds. A patent application to protect these novel compounds has been filed.

■ ABBREVIATIONS

AR, androgen receptor; ARD, AR down-regulation; ARDAs, AR down-regulating agents; CRPC, castration resistant prostate cancer; gal, galeterone; GI_{50}, compound concentration required to inhibit cell growth by 50%; IC_{50}, compound concentration required to inhibit cell growth by 50%; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; PC, prostate cancer; Pd, palladium; TEA, triethylamine; TLC, thin layer chromatography

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