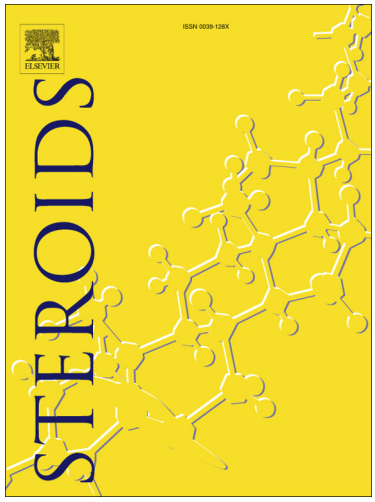

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Design and synthesis of novel steroidal imidazoles as dual inhibitors of AR/CYP17 for the treatment of prostate cancer

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Abstract

Both AR and CYP17 are important targets for blocking androgen signaling, and it has been accepted that multifunctional drugs have a low risk of drug resistance in the treatment of cancer. Thus, herein a series of steroidal imidazoles were designed, synthesized and evaluated as dual AR/CYP17 ligands. Several compounds displayed good biological profiles in both enzymatic and cellular assays. SAR studies showed that introducing oximino at the C-3 position of steriodal scaffold is beneficial to the enhancement of AR antagonistic activity. Among these compounds, the most potent compound **13a** exhibited the best AR inhibition (IC_{50} = 0.5 μM) that was 27-fold increase compared with the hit compound **5** as well as comparable CYP17 inhibition (IC_{50} =11 μM). Additionally, **13a** displayed promising anti-proliferative effects on LNCap cell lines with the IC_{50} value of 23 μM which was superior to positive control **Flutamide** (IC_{50} =28 μM). Furthermore, the docking results of **13a** revealed that the oxygen atom at the position of C-3 connected to the heme of CYP17, which may be helpful for its satisfactory dual-target inhibition. In summary, this study provides an efficient strategy for multi-targeting drug discovery in the treatment of prostate cancer.

Keywords: AR antagonists, CYP17 inhibitors, prostate cancer, dual target

1. Introduction

Prostate cancer (PC), one of the most common malignancies in men, is the second leading cause of cancer-related death among males after the lung cancer in Europe and US [1]. The ligand-dependent transcription factor androgen receptor (AR), which can be activated via binding of androgens, plays a central role in promoting the growth and progression of PC. AR is a member of nuclear receptor consisting of N-terminal domain (NTD), DNA binding domain (DBD), Hinge region and Ligand binding domain (LBD) [2]. Endogenous androgen such as testosterone and dihydrotestosterone (DHT) exhibit their biological actions by binding to the LBD of AR [3]. With no androgens binding, inactive AR is located in cytoplasm and binds to heat shock protein 90 (HSP90). Androgens binding leads to Hsp90 release and the following formation of homo-dimeric AR complex which subsequently translocates to the nucleus and initiates AR-mediated responses such as the synthesis of specific proteins like prostate-specific antigen (PSA) and triggering of cell proliferation [4]. Thus, blocking the AR transcription or reducing androgen level could be useful therapeutic regimens in the treatment of PC, for which androgen deprivation therapy (ADT) has been regarded as the first-line treatment.

AR antagonists are a group of ligands that can potently prevent endogenous androgens from activating AR through binding to the same site as androgens do. A number of small molecular AR antagonists, such as Flutamide, Bicalutamide, and Enzalutamide have been so far approved for the treatment of PC [5-7]. Although these antiandrogens have made great achievements in the treatment of PC, undesired resistance is still an unsolved problem which has severely limited their clinical application [8]. Several mechanisms have been proposed for contributing to the development of castration-resistant prostate cancer (CRPC), including AR aberrations, AR point mutations, AR gene amplification, AR overexpression, the synthesis of intratumoral androgens and the emergence of AR splice variants that lack of LBD [9-12]. In view of accumulating evidence showing that AR continues to be the key molecular driver in CRPC [13], blocking androgen signaling axis remains the mainstay treatment of CRPC. To reduce the risk of resistance and improve the therapeutic effects, combinational drug therapy [14, 15] or development of AR-based multi-targeting antitumor agents [16, 17] offered new opportunities.

On the other hand, CYP17 (17α-hydroxylase-17, 20-lyase) has been another promising target for the treatment of PC [18, 19]. CYP17 is a monooxygenase and distributed in adrenal gland, tests and prostate cancer cells [20]. The enzyme has a pivotal role for the synthesis of androgens by catalyzing the conversion of pregnenolone and pregesterone into the corresponding androgens dehydroepiandrosterone and androstenedione respectively [21]. Therefore, suppressing CYP17 can attenuate the generation of androgens in PC cells, which is supposed to be an effective therapy for AR-dependent PC. Abiraterone acetate (**1**) is the first approved CYP17 inhibitor of its class for the treatment of CRPC by US Food and Drug Administration (FDA) [22]. Since then, a variety of next generation of CYP17 inhibitors have been successively discovered, e.g. TAK-700 (**2)**, VT-464 (**3**) and so on (Fig. 1A). Most encouraging is the recent results reported in clinical trial of a multifunctional agent TOK-001 (**4**), acting as both an androgen receptor antagonist and a CYP17 inhibitor, suggesting the clinical potential of designing dual AR/CYP17 inhibitors for the treatment of CRPC [23-25].

Moreover, a single multitargeting molecule might enhance clinical benefit and result in increased compliance, more predictable pharmacokinetic (PK) profiles and reduced risk of drug–drug interactions compared with combined regimens [26, 27].

In our ongoing efforts to develop multitarget drug candidates in hormonal therapy area [28-31], we reported herein the synthesis and biological evaluations of novel steroidal imidazoles as dual AR/CYP17 inhibitors through structure modification of a previously discovered androstane-17-β-carboxanilide-based AR-antagonist **5** (AR antagonistic activity of 17 μM) (**Figure 1B**). Docking results of compound **5** within AR (PDB: 4oea) revealed no specific interactions formed by the carboxanilide moiety, suggesting it as an amendable site for optimizing AR antagonistic activity (**Figure 1B**). To achieve dual inhibition toward both targets, we therefore replaced the carboxanilide with various imidazole-substituted alcohols based on the fact that nitrogen-containing heterocycle moieties have been proven to be responsible for CYP17 inhibitory activity due to their ability of coordinating with the iron at the reactive center of CYP17 [32]. Finally, we further investigated minor varieties at C-3 position and double bond toleration in steroid ring to expand structure-activity relationship (SAR) (**Figure 1B**). Preliminary biological evaluation on both CYP17 and AR proteins identified several compounds with significantly improved AR antagonistic activity as well as excellent CYP17 inhibition.

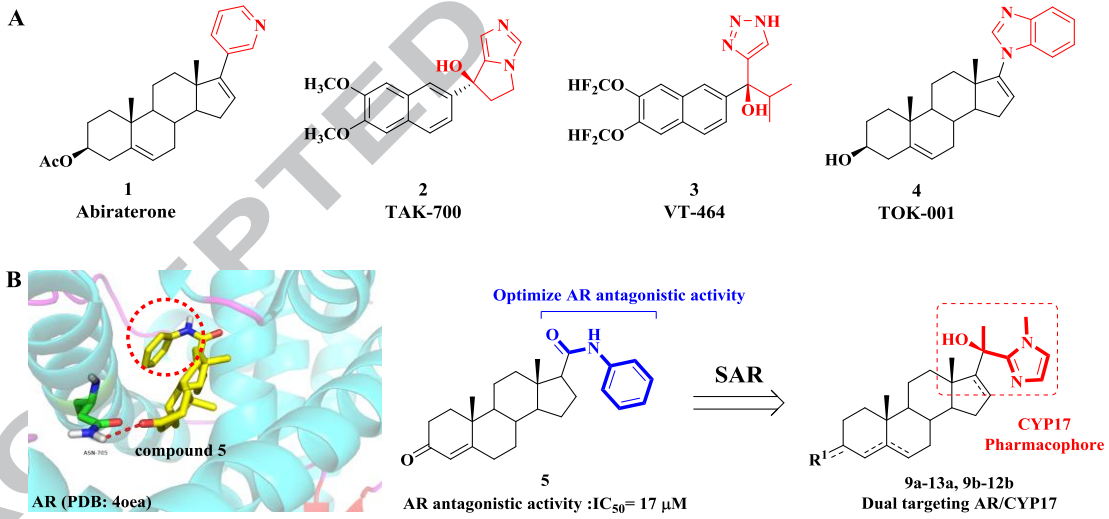


Figure 1. (A) Representative CYP17 inhibitors bearing nitrogen-containing heterocycles. (B) Docking results of compound **5** within AR (PDB: 4oea) and the rational design strategy of dual AR/CYP17 inhibitors.

2. Experimental section

2.1 *Chemistry*

2.1.1. General.

Melting points were determined on XRC-1 a melting point apparatus and are uncorrected. IR was recorded on Nicolet Impact-410. MS spectra were recorded on a Shimadzu GC-MS 2050 (ESI) or an Agilent 1946A-MSD (ESI) Mass Spectrum. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO with a Bruker Avance 300 MHz spectrometer at 300 K. TMS was used as an internal standard and chemical shifts were reported in parts per million (ppm). All coupling constants (J) are in hertz (Hz), and the signals are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Column chromatography was performed with silica gel (100-200 mesh). Most chemicals and solvents were of analytical grade and, when necessary, were purified and dried by standard methods.

*2.1.2. Synthesis of intermediates 3β-hydroxy-pregnane-5-alkene-20-one-3 acetate（**6a**）*

Compound **6b** (6 g, 0.0168 mol) was dissolved in 180ml ethyl acetate and 0.6 g 10% Pd/C was added. Then, H_2 was blown in and the total reaction mixture was stirred at 30 °C for 6 h. The mixture was filtered and the filtrate was concentrated under reduced pressure to obtain the crude product with a yield 98%. ESI-MS *m/z*: 381 $[M+Na]^+$

*2.1.3. General procedure for the synthesis of intermediates 3β-hydroxy-pregnane-5-alkene-20-one (**7a**) and 3β-hydroxy-pregnane-5, 16- diene-20-one (**7b**)*

Intermediate **6a** (5.92 g, 16.5 mmol) was dissolved in 118 mL methanol, and the K_2CO_3 (6.87 g, 49 mmol) was subsequently added. The mixture was stirred at room temperature overnight. Then, 50% acetic acid solution was added to adjust PH 6-7. The mixed solution was concentrated under reduced pressure to remove methanol. The mixture was filtered and washed with water to obtain the white solid **7a** with a yield 93%. ESI-MS *m/z*: 339 $[M+Na]^+$. Compound **7b** was obtained from **6b** according the same procedure. Yield: 95%. ESI-MS *m/z*: 337 $[M+Na]^+$.

*2.1.4. General procedure for the synthesis of intermediates 3β-methoxymethylether-pregnane-5-alkene-20-one (**8a**) and 3β- methoxymethylether-pregnane -5, 16- diene-20-one (**8b**)*

To a solution of **7a** (4.85 g, 15 mmol) in CH_2Cl_2 and N, N-diisopropylethylamine was added. A solution of chloromethyl methyl ether (4.7 mL, 60 mmol) was added dropwise on an ice bath within 30 minutes under stirring. The ice bath was removed and the reaction mixture was stirred at room temperature overnight. The mixture was poured into ice water while stirring. After about 30 min, the mixture was shifted into separating funnel and the organic layer was separated. The aqueous layer was extracted three times with CH_2Cl_2. All organic fractions were washed with saturated saline, dried over anhydrous Na_2SO_4 and evaporated at reduced pressure to obtain light yellow solid **8a** with a yield 98%. ESI-MS *m/z*: 383 $[M+Na]^+$. **8b** was obtained from **7b** in the same way. Yield: 77%. ESI-MS *m/z*: 359 $[M+H]^+$.

*2.1.5. General procedure for the synthesis of final compounds 3β-methoxymethylether-20-(1'-methylimidazol-2-yl)-20-hydroxy-pregnan-5-alkene (**9a**) and 3β-methoxymethylether-20-(1'-methyl imidazol-2-yl)-20-hydroxypregan-5, 16-diene (**9b**)*

To a solution of N- methylimidazol (6.67 mmol) in dry THF was added dropwise 2.5M n-butyllithium (3.6 mL, 8.33 mmol) at -50 °C with stirring. After 2 h, compound **8a** (0.6 g, 1.67 mmol) was added dropwise and the total reaction mixture was further stirred for 5.5 h at the same temperature. The completion of the reaction was confirmed by TLC and saturated NH_4Cl solution was added to quench the reaction. The organic layer was separated and the aqueous layer was extracted 3 times with ethyl acetate. All organic fractions were pooled, washed with saturated saline, dried over anhydrous Na_2SO_4 and evaporated at reduced pressure to obtain compound **9a**. Light yellow solid; yield: 95%. m.p.: 223-225 ℃; ^{1}H NMR($CDCl_3$, 300MHz), δ(ppm): 6.9(s, 1H, imidazole-4-H), 6.8(s, 1H, imidazole-5-H), 5.4 (d, 1H, 6-H, *J* = 4.7 Hz), 4.7(s, 2H, 3-OCH_2OCH_3), 3.8(s, 3H, N-CH_3), 3.7(m, 1H, 3-H), 3.4(s, 3H,3-OCH_2OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$),δ(ppm): 141.1 (s), 126.4 (s), 125.57 (s), 122.7 (s), 121.27 (s), 94.6 (s), 76.91 (s), 71.6 (s), 58.3(s), 55.1 (s), 50.3 (s), 46.9 (s), 46.9 (d, J = 1.9 Hz), 39.5 (s), 37.1 (s), 36.87 (s), 34.5 (s), 34.3 (s), 31.5 (s), 30.9 (s), 30.3 (s), 29.7 (s) 28.8 (s), 28.6 (s), 20.6 (s), 19.1 (s), 16.7 (s); HRMS (ESI): *m/z* $[M+H]^+$. Calcd for $C_{27}H_{42}N_2O_3$: 442.3195; Found: 442.3198.

9b was synthesized from **8b** according to the same procedure. White solid; yield: 67%. m.p.: 205-207 ℃; ^{1}H NMR($CDCl_3$, 300MHz), δ(ppm): 6.9(d, 1H, imidazole-4-H, J = 1.1 Hz), 6.8(d, 1H, imidazole-5-H, J = 1.0 Hz), 5.8(d, 1H, 16-H, J = 1.7 Hz), 5.4(d, 1H, 6-H, *J* = 4.4 Hz), 4.7(s, 2H, 3-OCH_2OCH_3), 3.8(m, 1H, 3-H), 3.6(s, 3H, N-CH_3), 3.4(s, 3H,3-OCH_2OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$),δ(ppm): 156.8 (s), 150.1 (s), 141.1 (s), 126.3 (s), 125.5 (s), 122.7 (s), 121.3 (s), 94.6 (s), 76.9 (s), 71.7 (s), 58.3 (s), 55.0 – 54.3 (m), 50.3 (s), 46.9 (s), 39.5 (s), 37.1 (s), 36.8 (s), 34.5 (s), 34.3 (s), 31.5 (s), 30.9 (s), 30.3 – 30.2 (m), 28.8 (s), 28.6 (s), 20.6 (s), 19.2 (s), 16.6 (s);HRMS (ESI): *m/z* $[M+H]^+$. Calcd for $C_{27}H_{40}N_2O_3$: 440.3039; Found: 440.3435.

*2.1.6. General procedure for the synthesis of final compounds 20-(1'-methylimidazol-2-yl)-3β, 20-dihydroxy-pregnan-5-alkene (**10a**) and 20-(1'-methylimidazol-2-yl)-3β, 20-dihydroxy-pregnan-5, 16-diene (**10b**)*

To a solution of **9a** (0.5 g, 1.13 mmol) in 10 mL methanol, 1.13 mL 6N HCl was added. The solution was refluxed for 1 h. After cooling, the solution was poured into 100 mL ice-cold water. Then, 15% NaOH was added to adjust PH 7. The mixture was filtered and washed with water to obtain compound **10a**. Beige solid; yield: 69%. m.p.: 230-232℃; ^{1}H NMR(DMSO-d_6, 300MHz), δ(ppm): 7.6(d, 1H, imidazole-4-H, *J* = 1.7 Hz), 7.5(d, 1H, imidazole-5-H, *J* = 1.7 Hz), 6.2(s, 1H, 6-H), 5.3(s, 1H, 20-OH), 3.9(s, 3H, N-CH_3); ^{13}C NMR (75 MHz, DMSO), δ(ppm): 141.7 (s), 129.6 – 125.6 (m), 126.4 – 124.8 (m), 120.7 (s), 118.0 (s), 73.9 (s), 70.4 (s), 58.0 (s), 56.3 (s), 49.9 (s), 42.6 (s), 37.3 (s),

37.1 (s), 36.5 (s), 31.8 (s), 31.6 (s), 31.3 (s), 25.89– 25.3 (m), 23.6 (s), 20.7 (s), 19.5 (s), 13.46 (s);HRMS (ESI): *m/z* $[M+H]^+$. Calcd for $C_{25}H_{38}N_2O_2$: 398.2933; Found: 398.2937.

10b was obtained from **9b** according to the same procedure. White solid; yield 55%. m.p.: 223-225℃; ^{1}H NMR(DMSO-d_6, 300MHz), δ(ppm): 7.7(s, 1H, imidazole-4-H), 7.6(s, 1H, imidazole-5-H), 6.0(s, 1H, 16-H), 5.3(s, 1H, 6-H), 3.8(m, 1H, 3-H), 3.6(s, 3H, N-CH_3); ^{13}C NMR (75 MHz, DMSO), δ(ppm): 158.4 (s), 149.6 (s), 142.0 (s), 126.0 – 125.5 (m), 123.9 (s), 123.0 (s), 120.6 (s), 72.1 (s), 70.4 (s), 58.1 (s), 52.6 (s), 50.3 (s), 46.3 (s), 42.6 (s), 37.2 (s), 36.6 (s), 35.5 (s), 34.7 – 34.5 (m), 31.8 (s), 31.4 (s), 30.4 (s), 29.8 (s), 20.7 (s), 19.4 (s), 15.2 (s), 7.6 (s). HRMS (ESI): *m/z* $[M+H]^+$. Calcd for $C_{25}H_{36}N_2O_2$: 396.2777; Found: 396.2773.

*2.1.7. General procedure for the synthesis of final compounds 20-(1'-methylimidazol-2-yl)-20-hydroxy-pregnan-4-alkene-3-one (**11a**) and 20-(1'-methylimidazol-2-yl)-20-hydroxy-pregnan-4, 16-diene-3-one (**11b**)*

To a solution of **11a** (0.2 g, 0.5 mmol) in 4 mL dry toluene, cyclohexanone (2 mL) and aluminium isopropoxide (0.3 g, 1.5 mmol) was added. The mixture was refluxed for 7 h. After cooling, the reaction mixture was poured into 20 mL cool 15% NaOH with stirring for 20 min. The organic layer was separated and the aqueous layer was extracted 3 times with ethyl acetate. All organic fractions were pooled, washed with water, 10% H_2SO_4 and saturated saline, dried over anhydrous Na2SO4 and evaporated at reduced pressure to obtain **11a**. The crude product was purified by column chromatography (CH_2Cl_2-CH_3OH). White solid; yield: 50%. m.p.: 170-172℃; ^{1}H NMR(DMSO-d_6, 300MHz) δ(ppm): 6.9 (s, 1H, imidazole-4-H), 6.7(d, 1H, imidazole-5-H, J = 0.9 Hz), 5.6(s, 1H, 4-H), 5.0(s, 1H, -OH), 3.8(s, 3H, N-CH_3); ^{13}C NMR (75 MHz, DMSO), δ(ppm): 198.4 (s), 171.5 (s), 152.0 (s), 125.3 (s), 123.5 (s), 123.0 (s), 73.8 (s), 59.1 (s), 55.7 (s), 53.7 (s), 42.1 (s), 38.5 (s), 36.9 (s), 35.4 (s), 35.2 (s), 34.9 (s), 34.0 (s), 32.4 (s), 32.0 (s), 27.0 – 26.7 (m), 23.7 (s), 22.8 (s), 20.6 – 20.5 (m), 17.2 (s), 12.8 (s). HRMS (ESI): *m/z* $[M+H]^+$. Calcd for $C_{25}H_{36}N_2O_2$: 396.2777; Found: 396.2773.

11b was obtained from **10b** in the same way. White solid; yield: 80%. m.p.: 168-170℃; ^{1}H NMR(CDCl3, 300MHz), δ(ppm): 6.9 (s 1H, imidazole-4-H), 6.8(s, 1H, imidazole-5-H), 5.8(s, 1H, 4-H), 5.7(s, 1H, 16-H), 4.5(s, 1H, -OH), 3.6(s, 3H, N-CH_3), 3.2(m, 2H, 2-H); ^{13}C NMR (75 MHz, DMSO),δ(ppm): 197.9 (s), 170.9 (s), 158.0 (s), 149.1 (s), 125.0 (s), 123.2 (s), 123.1 (s), 122.4 (s), 71.6 (s), 56.9 (s), 53.2 (s), 45.8 (s), 38.1 (s), 34.8 (s), 33.9 (s), 33.5 (s), 33.3 (s), 31.9 (s), 31.3 (s), 29.7 (s), 29.5 (s), 20.2 (s), 16.6 (s), 14.7 (s);HRMS (ESI): *m/z* $[M+H]^+$. Calcd for $C_{25}H_{36}N_2O_2$: 397.2773; Found: 394.2617.

*2.1.8. General procedure for the synthesis of final compounds 20-(1'-methylimidazol-2-yl)-3, 20-dihydroxy-pregnan-4-alkene (**12a**) and 20-(1'-methylimidazol-2-yl)-3, 20-dihydroxy-pregnan-4, 16-diene (**12b**)*

$NaBH_4$ (0.0058 g, 0.75 mmol) was added to a solution of compound **17a** (0.1 g, 0.25 mmol) in methanol (4 mL). The mixture was stirred for 3 h at room temperature. 1N HCL was added to adjust PH 7. The mixture was concentrated under reduced pressure. Ice-cool water was added with stirring for 15 min. Next, the mixture was filtered and washed with water to obtain compound **18a**. White solid; yield: 60%. m.p.: 160-162℃; ^{1}H NMR(DMSO-d_6, 300MHz) δ(ppm): 6.9 (s, 1H, imidazole-4-H), 6.7(s, 1H, imidazole-5-H), 5.2(s, 1H, 4-H), 5.0(s, 1H, 20-OH), 4.5(m, 1H, 3-H), 3.9(s, 1H, 3-OH), 3.8(s, 3H, N-CH_3); ^{13}C NMR (75 MHz, $CDCl_3$), δ(ppm): 147.5 (s), 125.3 (s), 125.3 (s), 123.4 (s), 122.7 (s), 74.3 (s), 67.8 – 67.5 (m), 58.3 (s), 56.3 (s), 54.5 (s), 42.9 (s), 39.0 (s), 37.3 (s), 35.3 (s), 35.1 (s), 32.9 (s), 32.1 (s), 29.4 (s), 26.8 (s), 23.6 (s), 22.6 (s), 20.8 (s), 19.1 (d, *J* = 33.6 Hz), 13.27 (s);HRMS (ESI): *m/z* $[M+H]^+$. Calcd for $C_{25}H_{38}N_2O_2$: 398.2933; Found: 398.2937.

12b was obtained from **11b** according to the same procedure. Beige solid; yield: 22%. m.p.: 224-226℃; ^{1}H NMR(DMSO-d_6, 300MHz), δ(ppm): 7.0 (s, 1H, imidazole-4-H), 6.7(s, 1H, imidazole-5-H), 5.7(s, 1H, 16-H), 5.4(s, 1H, 4-H), 5.2(s, 1H, 20-OH), 4.5(m, 1H, 3-H), 3.9(s, 1H, 3-OH), 3.6(s, 3H, N-CH_3); ^{13}C NMR (75 MHz, DMSO), δ(ppm): 158.5 (s), 149.6 (s), 144.6 (s), 125.9 (s), 125.4 (s), 123.6 (s), 122.9 (s), 72.1 (s), 66.4 (s), 57.8 (s), 54.6 (s), 46.4 (s), 37.2 (s), 35.5 (s), 34.4 (s), 34.3 (s), 32.9 (s), 32.0 (s), 30.3 (s), 29.9 (s), 29.4 (s), 20.8 (s), 18.7 (s), 15.2 (s);HRMS (ESI): *m/z* $[M+H]^+$. Calcd for $C_{25}H_{36}N_2O_2$: 396.2777; Found: 396.2773.

*2.1.9. General procedure for the synthesis of final compounds 20-(1'-methylimidazol-2-yl)-20-hydroxy-pregnan-4-alkene-3-oxime(**13a**)*

To a solution of **11a** (0.1 g, 0.25 mmol) in methanol (4 ml), CH_3COONa (0.08 g, 1 mmol) and hydroxylammonium chloride (0.07 g, 1 mmol) was added. The reaction mixture was refluxed with stirring for 2 h. After cooling, the mixture was poured into ice water. The precipitate was collected by filtration and dried to obtain compound **13a**. White solid; yield: 77%. m.p.: 204-206℃; ^{1}H NMR(DMSO-d_6, 300MHz), δ(ppm): 10.5(s, N-OH), 7.1 (s 1H, imidazole-4-H), 6.9 (s, 1H, imidazole-5-H), 5.7 (s, 1H, 4-H), 5.3 (s, 20-OH), 3.8(s, 3H, N-CH_3); ^{13}C NMR (75 MHz, DMSO),δ(ppm): 154.0 (s), 152.5 (s), 151.3 (s), 123.2 (s), 122.4 (s), 117.8 (s), 73.4 (s), 58.2 (s), 55.4 (s), 53.2 (s), 41.9 (s), 37.3 (s), 37.0 (s), 35.3 (s), 34.6 (s), 34.2 (s), 31.7 (s), 31.6 (s), 25.8 (s), 23.4 – 23.3 (m), 22.1 (s), 20.6 (s), 18.4 (s), 17.3 – 17.2 (m), 12.6 (s);. HRMS (ESI): *m/z* $[M+H]^+$. Calcd for $C_{25}H_{37}N_3O_2$: 411.2886; Found: 411.2886.

2.2 Biological evaluation

2.2.1. AR antagonistic activity *Assay*.

Hela/AR-AREs-luc cells were cultured in DMEM medium (containing 10% (v/v) FBS, (v/v) 0.2% Hygromycin B, 0.6% (v/v) G418 sulfate, 100 U/mL Penicillin and 100 mg/mL Streptomycin) in a 5%

CO_2-humidified atmosphere at 37°C. Cells were trypsinized and seeded at a density of 1 x 10^5/mL into a 96-well plate (100 mL/well) and incubated at 37°C, 5% CO_2 atmosphere for 12 h. Then, removing the medium and every well was treated with 100 μL RT-1640 medium (containing 10% (v/v) FBS, 100 U/mL Penicillin and 100 mg/mL Streptomycin) containing test compounds which had been pre-prepared to provide the concentration range of 2 x 10^{-5} mol/L, 1 x 10^{-5} mol/L, 1 x 10^{-6} mol/L, 1 x 10^{-7} mol/L and 1 x 10^{-8} mol/L in the presence of 0.5 nM testosterone, and they were re-incubated for a further 36 h. Control wells were added the equivalent volume of medium containing 0.2% (v/v) DMSO. The culture medium was removed and every well was washed twice with PBS. Then, cell lysis buffer 20 μL/well was added and oscillated for 5 minutes. Subsequently, the medium was shifted into a new 96-well plate (15 μL / well) which was non-transparent to perform fluorescence detection.

2.2.2. CYP17 preparation and assay

Tests were obtained from male rats that weighed 200-250 g (age of 8-week-old). The tests were minced and homogenized in 0.25 M source (1:4, W/V) then centrifuged at 1000 g for 30 min at 4 °C. The resulting supernatants were further centrifuged at 105000 g for 1 h at 4 °C. Then, the pellets were re-suspended in 20% glycerinum – PBS buffer. The microsomal fraction was stored at -70 °C. The microsomal protein concentrations were determined by the BCA Protein Assay Method.

The activity of CYP17 lase was determined by measuring the conversion of progesterone into 17-hydroxyl progesterone. A solution of 20 μmol / L progesterone (20 μmol / L in methanol), 80 μL PBS and 100 μL lase (1 mg/mL) were mixed. The mixed solution was shaken for 1 min. Then, the mixed solution, NADPH and the sample solution containing test compounds were preheated for 5 min at 37 °C. Next, 20μL NADPH and 2 μL sample solution containing test compounds which had been pre-prepared to provide the concentration range of 2 x 10^{-5} mol/L, 1 x 10^{-5} mol/L, 1 x 10^{-6} mol/L, 1 x 10^{-7} mol/L was added to the mixed solution. They were further incubated at 37 °C for 30min. After centrifuging, the supernatants were detected by HPLC.

2.2.3. MTT assay for anti-proliferative activities

LNCap cells were cultured in RPMI-1640 medium (containing 10% (v/v) FBS, 100 U/mL Penicillin and 100 mg/mL Streptomycin) at 37°C in a 5% CO_2-humidified incubator. All experiments were performed in triplicate. All cells were trypsinized and diluted to a density of 1 x 10^5/mL with RPMI-1640 supplemented with 10% (v/v) FBS. The cells were seeded into 96-well plates at a volume of 100 μL/well and incubated at 37°C, 5% CO_2 atmosphere for 24 h. After this time they were treated with 100 μL/well drug solution supplemented with serial dilutions of test compounds to provide the concentration range of 8 x 10^{-5} mol/L, 4x 10^{-5} mol/L, 1 x 10^{-5} mol/L, 4x 10^{-6} mol/L and 1 x 10^{-6} mol/. Control wells were added the equivalent volume of medium containing 1% (v/v) DMSO. Then, the

plates were re-incubated for a further 48 h. 20 μL MTT (5 mg/mL) was added to every well and cells continued to incubate in darkness at 37°C for 4 h. Carefully removed the culture medium and 150 mL DMSO was then added. The cells were maintained at room temperature in darkness for 20 min to ensure thorough color diffusion before reading the absorbance. The absorbance values were read at 490 nm for determination of IC_{50} values.

2.2.4. Real-Time Polymerase Chain Reaction (RT-PCR).

The LNCap cells were incubated with the corresponding compounds for 24h at 37°C .RNA samples were reverse transcribed to cDNA and the PCR reactions were performed using TaKaRa SYBR Green Master Mix (Code. no. 638320) carried out in StepOnePlus™ Real-Time PCR instrument (4376600, Life Technologies). The program for amplification was 1 cycle of 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 95°C for 10 s. Real- time RT-PCR experiments were performed in triplicate. The primers we used were as follows:

PSA,

forward 5′-GCAGCATTGAACCAGAGGAGTT-3′,

reverse 5′-CACGTCATTGGAAATAACATGGA-3′;

18S rRNA,

forward 5′-AGTCCCTGCCCTTTGTACACA-3′,

reverse 5′-CGATCCGAGGGCCTCACTA-3′.

2.2.5. Molecular docking

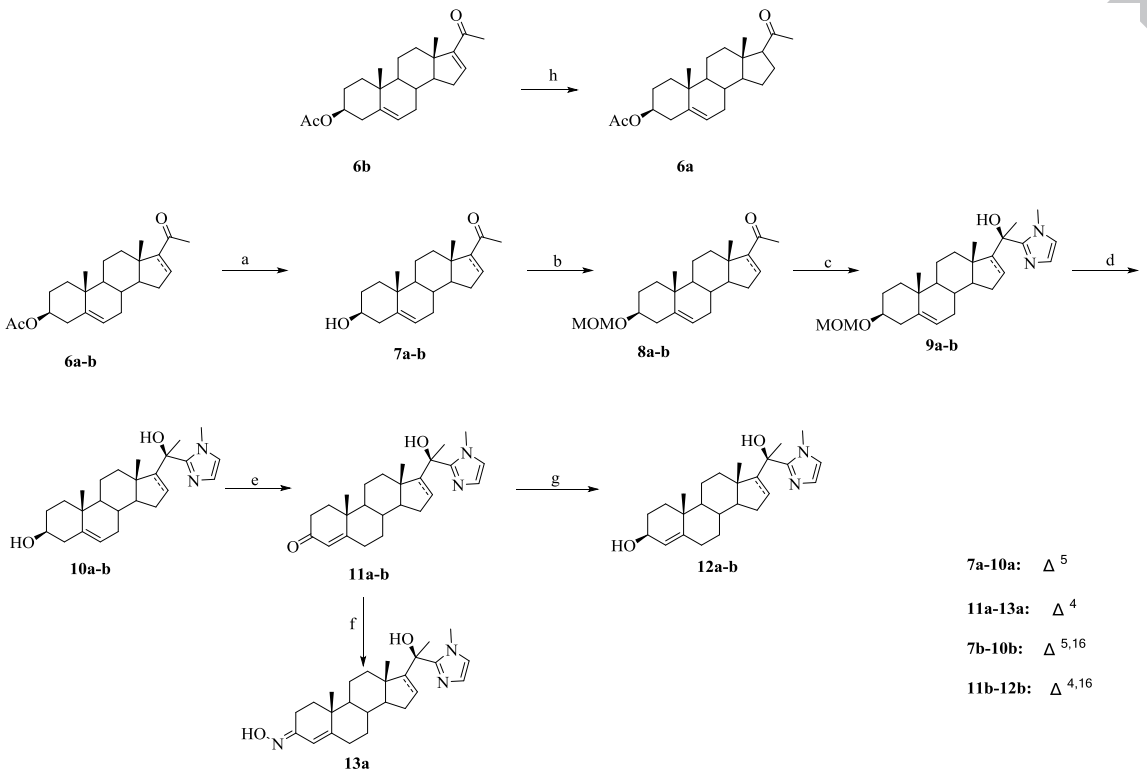
Docking studies were performed using the software Discovery Studio.3.0/CDOCK protocol (*Accelrys Software Inc*.). The crystal structures of AR (PDB code: 4oea) and CYP17 (PDB ID code: 3ruk) were downloaded from Protein Data Bank. The protein and ligand were optimized and charged to perform docking. Up to 10 conformations were retained, and binding modes presented graphically are representative of the highest-scored conformations.

3. Results and Discussion

3.1 Chemistry.

The synthesis of **9a-13a** and **9b-12b** began from **6a** and **6b** respectively (Scheme 1). Hydrolysis of **6a-b** using potassium carbonate afforded the key intermediates **7a-b**, whose hydroxy were then protected by MOMO to yield **8a-b.** According to previous report [33], nucleophilic substitution of **8a-b** with *N*-methylimidazole in presence of n-butyllithium afforded the final compounds **9a-b.** Literature survey revealed that nucleophilic addition resulted into the one major isomer [34, 35]. Also, the products **9a-b** are stable and can be purified by column chromatography over silica gel in good

yield. **10a-b** were synthesized via deprotection of **9a-b** with hydrochloric acid. Subsequently, **10a-b** underwent Oppenauer Oxidation to result in compounds **11a-b**, which were then converted into **12a-b** and **13a** via reduction and oximation respectively. According to previous report, the oximation reduction offered one major isomer [36].



Scheme 1. Synthetic routes of **9a-13a** and **9b-12b**. Regents and conditions: (a) K_2CO_3, CH_3OH, rt, overnight; (b) MOMCl, DIEA, CH_2Cl_2, rt, overnight;c) n-butyllithium, *N*-methylimidazole, THF ,-50 ^oC, 7.5 h; (d) 6N HCl, CH_3OH, reflux, 1 h;(e) cyclohexanone, aluminium iso-propoxide, toluene, reflux, 7 h; (f) AcONa, $NH_3OH.HCl$, CH_3OH, reflux, 1h; (g) $NaBH_4$, THF, rt, 3 h. (h) Pd/C, H_2, EtOAc, 30 ^oC, 6 h.

3.2 Biological evaluation

3.2.1. Antagonistic activity *assays of AR and studies of Structure−activity relationship (SAR)*

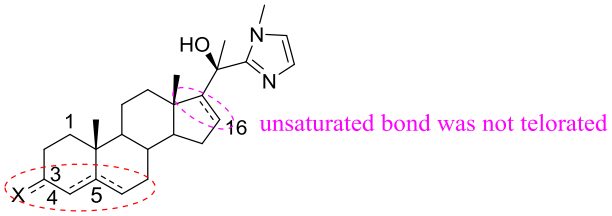
Firstly, we investigated the AR antagonistic activity of synthesized compounds by using luciferase reporter gene assays in Hela/AR-AREs-luc cell lines [37]. Hela/AR-AREs-luc, which contains AR gene, AREs gene and luciferase gene regulated by AREs, is a class of AR-dependent monoclonal cell. When agonist testosterone was added to the cells, the AR was activited, triggering the expression of luciferase gene. Addition of AR antagonists coupling with testosterone would block the transactivation of AR, leading to a decreased luciferase activity, which is in direct proportion to the inhibitory activity of the antiandrogens [38].

As is shown in **Table 1**, compounds **11a**, **13a** showed the best AR antagonistic activity with the IC_{50} value of 3 μM and 0.5 μM, respectively. Moreover, **13a** demonstrated better antagonistic effect than positive control **Fultamide**. Compared with other compounds, **11a** and **13a** bear conjugated

structure in A ring, which may be crucial for AR inhibitory activity. Meanwhile, in contrast to **11a, 13a** contain an oximino at the position of C-3, which revealed that 3-oximino could enhance the antagonistic efficacy. Additionally, by analyzing these data, we found that compound **10-12a** exerted antagonistic behavior stronger than **10-12b.** This result confirmed that unsaturated bond at the position of C- 16, 17 is adverse for AR inhibitory activity. Based on the result, we speculated that the unsaturated bond might weaken the flexibility of the side chain at the position of C-17, resulting in difficult binding of the C-20 hydroxyl with LBD of AR. In addition, from the negligible activity of compound **9a-b**，we can know that introducing long-chain alkanes at the position of C-3 is negative. To sum up, the SAR was obtained (**Table 1**).

Table 1.

The AR antagonistic activity assay and SAR analysis of synthesized compounds.



Δ ^{4}-3-oximio＞Δ ^{4}-3-ketone＞Δ ^{4}-3-hydroxy

compound	X	Double bond in ring	AR antagonistic activity	
			Inh%[a] at 20 μM	IC_{50} (μM)[b]
9a	-OMOM	Δ^{5}	＜10%	ND[c]
10a	-OH	Δ^{5}	50%	ND
11a	=O	Δ^{4}	91%	3
12a	-OH	Δ^{4}	68.3%	ND
13a	=NOH	Δ^{4}	99%	0.5
9b	-OMOM	$\Delta^{5,16}$	＜10%	ND
10b	-OH	$\Delta^{5,16}$	0.2%	ND
11b	=O	$\Delta^{4,16}$	37%	ND

12b	-OH	$\Delta^{4,16}$	20%	ND
Flutamide	-	-	82%	0.7

[a]Inh%=(the fluorescence value of blank control – the fluorescence value of compound)/ the fluorescence value of blank control.
[b]The IC_{50} value was calculated when the Inh% at 20 μM is > 90%.
[c]ND means not determined.

3.3.2 .CYP17 inhibitory activity evaluation and cell proliferation assay

After preliminary AR antagonistic activity assay, CYP17 inhibitory activity and the potency to inhibit the proliferation of human prostate cancer cell line LNCap were further evaluated. CYP17 inhibitory activity was determined through carrying out the CYP17 biochemical assay according to the procedures previously reported. The CYP17 enzyme was sourced form rat tests microsomes. The enzymatic activity of CYP17 was calculated based on the conversion of progesterone into androstenedione. As is shown in **Table 2**, most compounds showed considerable CYP17 inhibitory activity at the concentration of 20 μM compared with positive control Abiraterone. Compounds **12a, 12b, 13a** manifested the best CYP17 inhibitory potency. Growth inhibitory studies showed that compound **10a** (IC_{50}=12 μM)**, 11a** (IC_{50}=23 μM) and **13a** (IC_{50}=23 μM) exerted the most promising anti-proliferative effects compared with **Flutamide** (IC_{50}=28 μM) and **Abiraterone** (IC_{50}=19 μM) in the presence of 0.1 μM testosterone (T). Interestingly, compound **10a** displayed surprising anti-proliferative activity with the IC_{50} value of 12.01 μM though its unsatisfactory AR and CYP17 inhibition, which meant that the compound may suppress cellular proliferation via other signaling pathways.

Table 2.

CYP17 inhibitory activity and anti-proliferative effects on LNCap cell lines

compound	CYP17 inhibitory activity[a]		Cytotoxicity (IC_{50}, μM)
	Inh% at 20 μM	IC_{50} (μM)	LNCap [c](0.1μM T)
9a	27 %	ND[b]	>80
10a	53%	ND	12
11a	52%	ND	23
12a	86%	3	46

13a	72%	11	23
9b	-	ND	34
10b	61%	ND	>80
11b	63%	ND	>80
12b	87%	3	67
Abiraterone	100%	ND	19
Fultamide	-	-	28

[a]Inh% means that the inhibition of each compound on the CYP17 lase.
[b]ND means not determined.
[c](0.1 μM T) means that the antiproliferative assay was performed in the presence of 0.1 μM testosterone.

To summarize, we found that compound **13a** displayed the best AR antagonistic activity (IC_{50}=0.5 μM) and excellent CYP17 inhibitory activity (IC_{50}=11 μM), meanwhile，it also showed remarkable growth inhibitory effects on LNCap cells (IC_{50}=23 μM) which is better than **Flutamide**. Thus, compound **13a** was elected for further biological assessment.

*3.3.3. Effect of **13a** on the expression of PSA.*

PSA gene was regulated by androgen receptor. Therefore, we adopted quantitative real-time polymerase chain reaction (RT-PCR) to examine the ability of compound **13a** to lower the expression of PSA. The results were shown in **Figure 2**. In keeping with the anti-androgen activity of **13a**, we can observe the obvious decrease of PSA mRNA expression induced by 0.1μM testosterone (**T**), which is superior to Fultamide under the same conditions. This result again confirmed that compound **13a** was a potent antiandrogenic agent.

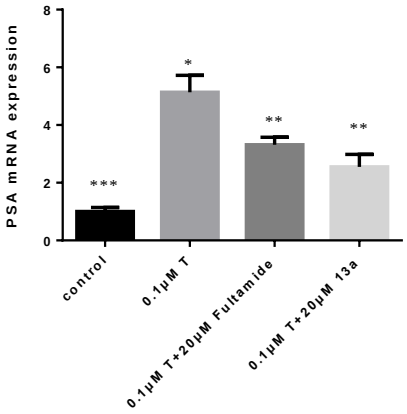


Figure 2. The increased mRNA expression of PSA induced by testosterone was reversed by **13a** in LNCap cells. The mRNA expression of PSA was examined by Real-time PCR. Values are mean ±SD (n=3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. 0.1 μM T group.

3. 4. Molecular docking studies.

In order to explore the binding mode of the designed inhibitors in AR (PDB: 4oea) and CYP17 proteins (PDB: 3ruk), molecular docking studies on the compound **13a** were implemented by CDOCK in Discovery studio 3.0. As we could see from **Figure 3A**, the compound was perfectly located into the ligand binding domain (LBD) of AR. Moreover, the detailed docking analysis (**Figure 3B**) showed that hydroxyimino group at the position of C-3 established hydrogen bonds with Gln-711 (1.8 Å), Met745 (2.2 Å) and Arg752 (1.7 Å) when the hydroxy group picked up hydrogen bonds with Asn705 (3.3Å) and Thr877 (2.3Å). The result just verified our initial design idea and made the potent AR antagonistic activity rational.

A. **B.**

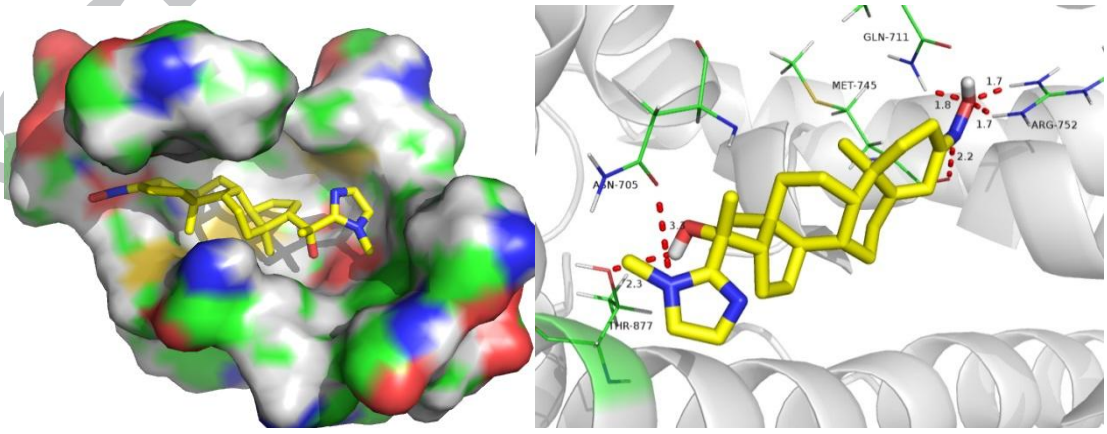
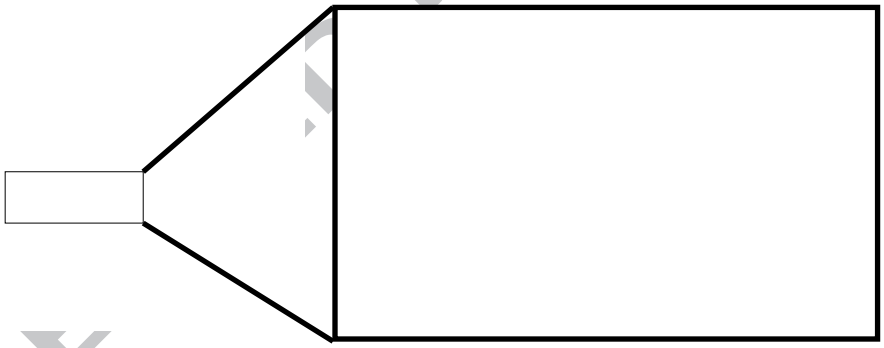


Figure 3.(A) The binding mode of compound **13a** in the cavity of AR protein.(B) The detailed docking result of compound **13a** with AR. The hydrogen bonds were indicated by red dotted lines .

The further docking study of compound **13a** with CYP17 also provided evidence for its promising CYP17 inhibitory activity. **Figure 4A** showed that **13a** was positioned into the active site of CYP17. It was obvious to note that the N atom of *N*-methylimidazole formed a hydrogen bond with Arg239 amino acid residue (1.9 Å). In addition, the O atom at the position of C-3 exactly connected to the heme of CYP17, which was essential to inhibit the activity of CYP17. The overlap of **13a** with Abiraterone was also further performed. As depicted in **Figure 4B**, the binding mode of **13a** was just the opposite of Abiraterone' and the O atom at the C-3 position of **13a** was located into the same site with N atom of Abiterone. Thus, rather than through N atom of *N*-methylimidazole interacting with heme like Abiraterone, **13a** coordinated with heme by the O atom at the C-3 position. Although the unexpected binding mode ran counter to our original idea of design, the final result was not bad. Instead, the new binding mode could indicate another way for discovering novel steroid-based dual AR/CYP17 inhibitors.

A.



B.

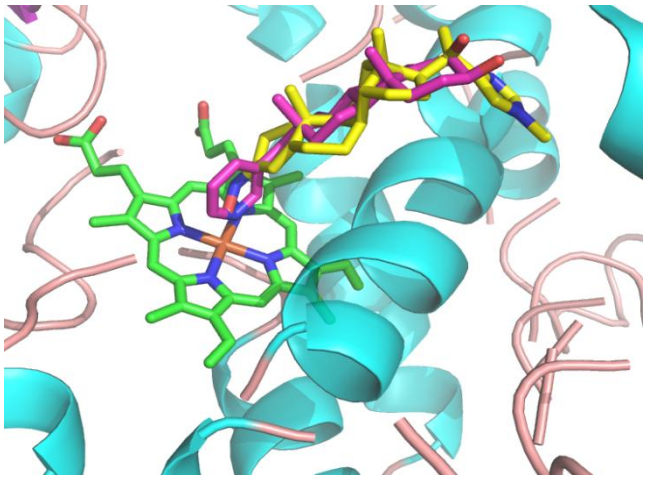


Figure 4. (A) The binding mode of **13a** docked into the active site of CYP17. Red imaginary line represented hydrogen bond. (B) The overlap of **13a** with Abiraterone. Yellow for **13a**, pink for Abiraterone, green for heme.

4. Conclusions

In summary, a series of steroidal imidazoles targeting both AR and CYP17 were rationally designed and synthesized as potent antiandrogenic agents that might have a low risk of drug resistance. A series of biological evaluation *in vitro* and SARs were also investigated. Compound **13a** which exerted simultaneously excellent AR and CYP17 inhibitory activities was finally identified. Meanwhile, it could also significantly reduce PSA expression induced by testosterone, which was more potent than **Flutamide**. These promising pharmacological profiles of **13a** indicated that it could serve as a lead compound for further studies. In addition, the further docking study of compound **13a** with CYP17 revealed a new binding mode, which might open a new avenue for discovering novel steroid-based multifunctional AR and CYP17 inhibitors.

Supplementary data

Supplementary data (the ^{1}H NMR, ^{13}C NMR and HRMS spectra of target compounds associated with this article can be found, in the online version, at.

Acknowledgements

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Abbreviations

AR, androgen receptor; PSA, prostate specific antigen; CYP17, 17α-hydroxylase-17, 20-lyase; RT-PCR, real-time polymerase chain reaction; T, testosterone; g, gram.

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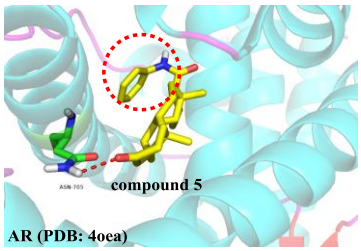
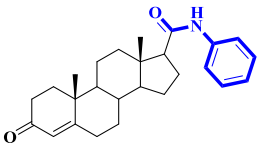


AR (PDB: 4oea)

Optimize AR antagonistic activity



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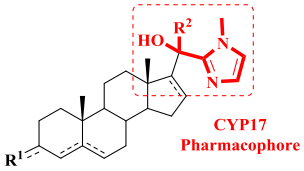
AR antagonistic activity: IC_{50} = 16.84 μM

SAR



Dual targeting AR/CYP17

- Simultaneously inhibiting AR and CYP17 benefits to the treatment of prostate cancer
- A series of new steroidal imidazoles were optimized from hit AR antagonist.
- **13a** exhibited growth inhibitory effects on prostate cancer cells by inhibiting AR and CYP17
- **13a** could dramatically reduce PSA expression induced by testosterone, which was comparable with Abiraterone
- Docking result of **13a** with CYP17 revealed a distinct binding mode compared with Abiraterone, which might open a new avenue for discovery of novel dual AR and CYP17 inhibitors