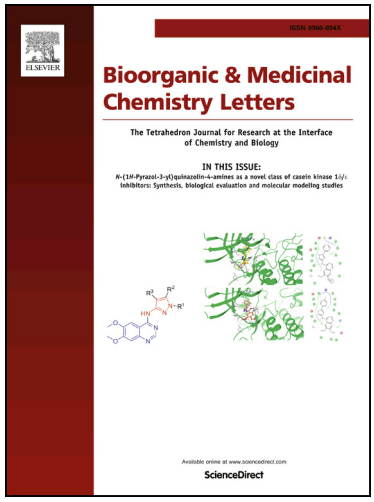

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Design, Synthesis and Biological Evaluation of Novel 3-oxo-4-oxa-5α-androst-17β-amide Derivatives as Dual 5α-reductase Inhibitors and Androgen Receptor Antagonists

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Abstract

Prostate cancer (PCa) is the second leading cause of death in men. Recently, some researches have showed that 5α-reductase inhibitors were beneficial in PCa treatment as well. In this study, a series of novel 3-oxo-4-oxa-5α-androst-17β-amide derivatives have been designed and synthesized in a more simple and convenient method. Most of the synthesized compounds displayed good 5α-reductase inhibitory activities and androgen receptor binding affinities. Their anti-proliferation activities in PC-3 and LNCaP cell lines were also evaluated and the results indicated that most of the synthesized compounds exhibited potent anti-proliferative activities. It is obvious that the androgen-dependent cell line LNCaP was much more sensitive than the androgen-independent cell line PC-3. Among all the synthesized compounds, **11d** and **11k** displayed the best inhibition activity with 4-fold more sensitive toward LNCaP than PC-3, which was consistent with their high affinities observed in AR binding assay. Molecular modeling studies suggested that **11k** could bind to AR in a manner similar to the binding of dihydrotestosterone to AR. Compared to the finasteride , **11k** showed a longer plasma half-life (4 h) and a better bioavailability. Overall, based on biological activities data, compound **11d** and **11k** can be identified as potential dual 5α-reductase inhibitors and AR antagonists which might be of therapeutic importance for prostate cancer treatment.

Key words
Prostate cancer; AR antagonists; 5α-Reductase inhibitors; Synthesis; Steroids

Prostate cancer (PCa) is the second leading cause of death in men only less than lung cancer. It was estimated that around 220,800 cases were diagnosed in the United States in 2015 alone [1]. In China, that number was 60,300 and has increased rapidly over the last 10 years [2]. Androgens, including testosterone (T) and dihydrotestosterone (DHT), and androgen receptor (AR) signaling

are necessary for prostate development and homeostasis [3]. T is synthesized in testicles and adrenal glands, and can be further converted to more potent DHT by 5α-reductase in prostate [4]. Activated via the binding of androgens, AR are crucial in the development, maintenance, and regulation of the male phenotype and reproductive biology. In the absence of hormone ligands, AR is located in the cytoplasm and sequestered into heat shock protein (HSP) complexes. Upon binding of hormones, the HSPs dissociate from the complex and the AR dimerizes and translocates to the nucleus. AR dimers bind to AR-response elements (AREs) in the DNA, recruit coactivator complexes that modify chromatin structure, recruit RNA polymerase II and induce transcription [5,6]. Huggins et al. introduced androgen deprivation as a therapy for advanced and metastatic PCA in 1941 [7]. Thereafter, androgen ablation therapy has been shown to produce the most beneficial responses in multiple settings in PCA patients [8].

5α-reductases, which are NADPH-dependent enzymes, are responsible for the reduction of 4-ene-3-oxosteroids to the corresponding 5α-3-oxosteroids. The 5α-reductase family is composed of three isozymes, with the types 1 and 2 being the most known. Type 1 5α-reductase (5αR-1) is mainly expressed in the skin and liver at an optimal pH range of 6.0~8.5, while the type 2 5α-reductase (5αR-2) at an optimal pH 5.5, is mainly found in prostate and other genital tissues [9]. More recently, type 3 isozyme was identified in castration-resistant prostate cancer cells as well as in other tissues such as the pancreas, brain, skin and adipose tissues [10, 11]. 5α-Reductase inhibitors, like finasteride (**1**) and dutasteride (**2**), were used in the clinic for the treatment of BPH and were also proposed for chemoprevention and treatment of prostate cancer. It is found that progressive castration resistant prostate cancer is characterized by increased 5αR-1 and decreased 5αR-2 levels. Dutasteride blocks both type 1and type 2 isoenzymes and has an inhibitory effect in prostate cancer [12]. It is currently being studied as a chemopreventive agent and in combination with other second-line androgen-reducing agents in PCA treatment [13]. Unfortunately, different clinical trials have demonstrated that these 5α-reductase inhibitors increased the risk of high-grade prostate cancer, which prevent FDA approval of finasteride and dutasteride usage in prostate cancer treatment [14]. This warrants the investigation of other potent and specific molecules with fewerside effects.

Azasteroids are widely used as 5α-reductase inhibitors. In recent decades, a number of 4-azasteroids have been synthesized and studied. Interestingly, 4-azasteroids have also been found to be androgen receptor antagonists. Compound **3** (Figure 1) was reported with an IC_{50} of 23μM for human androgen receptor (hAR), and values of 410nM and 15nM for type 1 and type 2 5α-reductase respectively [15]. Considering the fact that -NH- and -O- are bioisosteres, we set out to design and synthesize a series of 3-oxo-4-oxa-5α-androst-17β-amide derivatives as dual 5α-reductase and AR antagonists. The biochemical activity of the synthesized compounds was evaluated for their 5α-reductase inhibitory activities and AR binding affinities. Their anti-proliferative effects of were also investigated in a human prostate cancer cell line LNCaP cells and PC-3 cells.

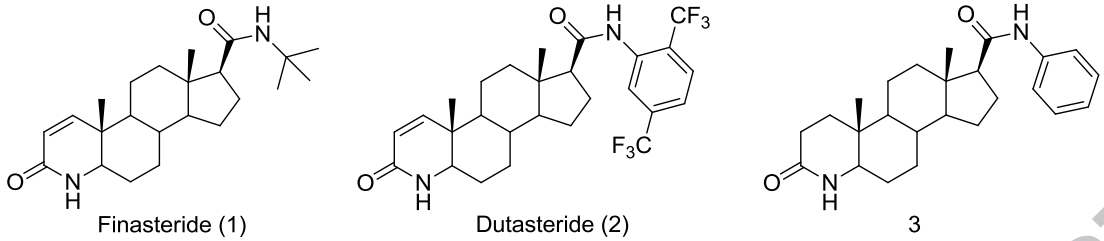
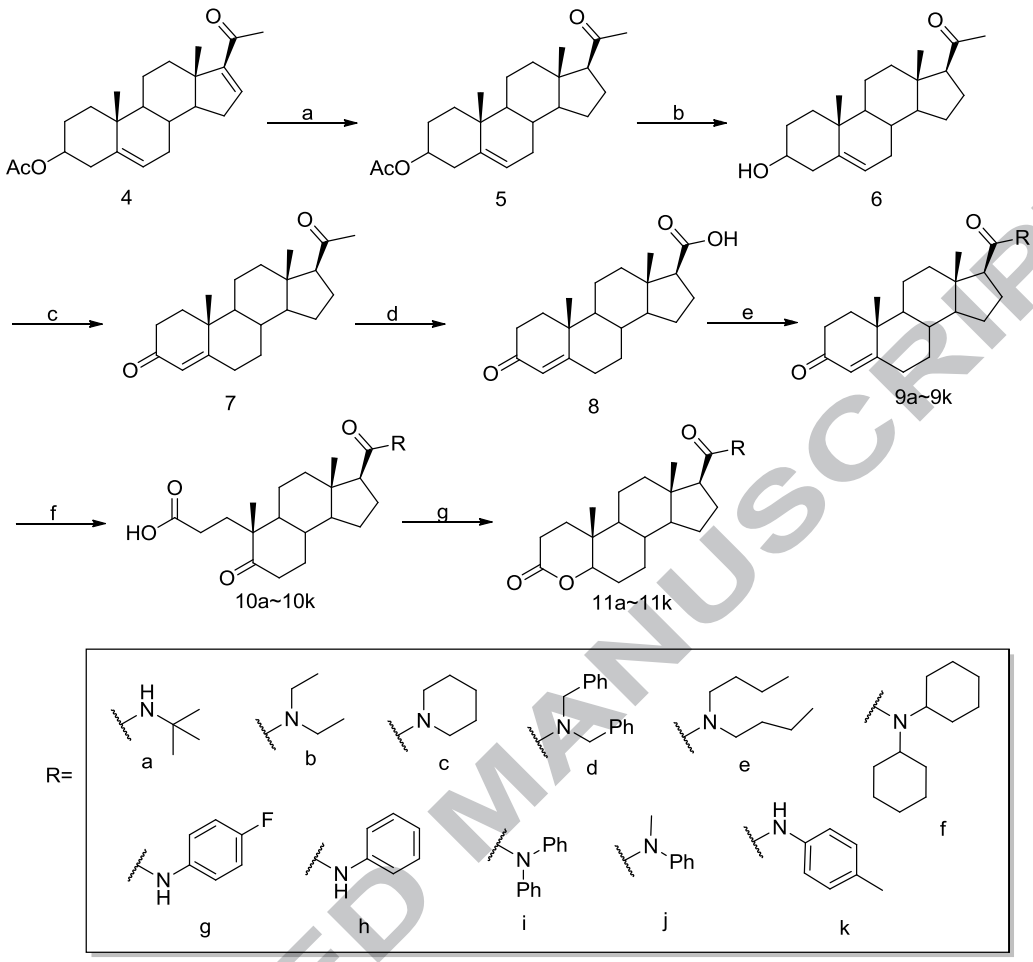


Figure 1. The structure of finasteride, dutasteride and lead compound

Taking commercial available compound 16-dehydropregnenolone acetate (**4**) as starting material, the compounds **11a**~**11k** have been easily prepared through 7 steps (Scheme 1). Being treated with Raney Ni, compound **4** could be selective reduced to compound **5**. After hydrolysis in methanol containing 20% K_2CO_3, compound **7** was provided by Oppenauer oxidation. The 17-amide derivatives **9a**~**9k** were obtained by combining different amide with key intermediate compound **8**, which was prepared by haloform reaction. The 17-amide derivatives **9a**~**9k** was than oxidized under the condition of $NaIO_4$/$KMnO_4$ to obtain ring-opening product **10a**~**10k**

The synthetic method of 4-oxasteroids was first reported in 1998 [16]. Being cyclized by Ac_2O, the 4-oxasteroid products could be synthesized by Ru/C catalyzed hydrogenation. Considering the high expense of Ru/C, we developed a new route to obtain 4-oxasteroids. By treated with $NaBH_4$, the ring-opening products **10a**~**10k** can be directly converted to 4-oxasteroid products **11a**~**11k**. The 5α-H isomers have been confirmed by ^{1}H NMR spectrum. 5α-H can form the aa and ae coupling with 6-H and shows the classical dd splitting with the chemical shift (CDCl3, 300MHz) at 3.95~3.98. Compared with the old one, this route is not only much more economical and convenient, but also can get 5α-H isomers simply through column chromatography.

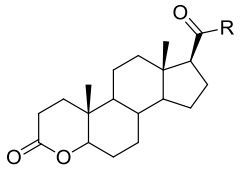


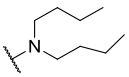
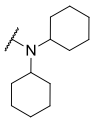
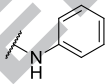
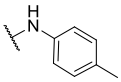
Reagents and conditions: a) Ni, H_2; b) K_2CO_3, CH_3OH; c) cyclohexanone, $Al(O\text{-}i\text{-}Pr)_3$; d) NaOBr, Na_2SO_3; e) $(COCl)_2$, Et_3N, corresponding amine; f)$NaIO_4$, $KMnO_4$; g) $NaBH_4$

Scheme 1. The synthesis of the compound **11a~11k**

All of the eleven synthesized compounds (**11**a-**11k**) were evaluated for their 5α-reductase inhibitory activities and the result was shown in Table 1. Generally speaking, all of the tested compounds exhibited good inhibition towards 5α-reductase at 50μM. In general, aniline derivatives seem to be a bit more active than the rest of the series. Among them, compound **11k** presented the most potent inhibitory activity with the IC_{50} of 0.27μM, better than that of positive control finasteride. Compared with compound **11h**, with an unsubstituted phenyl ring, 4-methylaniline compound **11k** displayed much higher inhibition rate, which indicated that presence of electron-donation substitution on benzene is beneficial for the activity. Moreover, among the aliphatic amine derivatives, piperidine derivative (compound **11c**) is the most potent with the inhibition rate of 89.28%, much better than the small sterically hindered derivatives (compounds **11a** and **11b**). However, the further increase of the steric hindrance of amine derivatives, such as compounds **11d, 11e** and **11f**, led to the decrease of inhibitory activity. This might suggested that cyclic substitutions might be availed to the inhibition activity, but too much steric hindrance reduced the activity.

Table 1 The inhibition of 5α-ruductase and the affinity of AR by compound **11a~11k**



Compound	R	5α-reductase		hAR	
		I% at 50μM	IC_{50}(μM)	I% at 50μM	IC_{50}(μM)
11a		48.31	-	3.73	-
11b		39.55	-	33.73	-
11c		89.28	6.01	17.35	-
11d		69.79	-	57.06	16.84
11e		36.48	-	11.78	-
11f		33.44	-	11.30	-
11g		46.87	-	18.85	-
11h		67.29	-	7.09	-
11i		15.46	-	3.29	-
11j		84.19	0.89	14.35	-
11k		98.54	0.27	55.50	47.61
Finasteride	-	94.29	0.59	-	-
Mesterolone	-	-	-	-	18.28

The AR binding affinities of synthesized compounds were then assessed by following fluorescence polarization procedure [17] by taking mesterolone as the positive control. As shown in Table 1, the majority compounds displayed moderate affinity rate at the concentrations of 50μM. Compound **11d** and **11k** presented the best potency toward AR, in which compound **11d** exhibited the IC_{50} of 16.84μM, comparable with positive control. Compared with the aliphatic amine derivatives, compounds with aromatic amine substitutions showed better binding affinities.

In vitro anti-proliferative activities of all the synthesized compounds (**11a**~**11k**) was evaluated against 2 human prostate cancer cell lines (LNCaP and PC-3) by taking finasteride and

flutamide as positive control. As shown in Table 2, all the synthesized compounds exhibited potent anti-proliferative activities. It is obvious that the androgen-dependent cell line LNCaP was much more sensitive than the androgen-independent cell line PC-3. It was found compounds **11d**, **11e** and **11k** exhibited the most potent inhibitory activity toward LNCaP with IC_{50} better than the positive control flutamide. Among them, compounds **11d** and **11k** presented about 4-fold more sensitive toward LNCaP than PC-3, which was consisted with their high affinity observed in AR binding assay,

Table 2 The anti-proliferation activities by compound **11a~11k**

Compound	LNCaP		PC-3	
	Inhibition% (80μM)	IC_{50}(μM)	Inhibition% (80μM)	IC_{50}(μM)
11a	37.32		28.10	70.08
11b	42.72		27.87	68.15
11c	12.35		26.23	85.37
11d	81.79	29.78	12.88	127.35
11e	88.94	32.41	89.89	40.13
11f	13.80		32.02	74.34
11g	39.71		46.33	50.03
11h	24.19		31.68	86.17
11i	31.41		35.42	75.17
11j	30.03		35.07	62.19
11k	82.31	26.47	18.06	113.64
Finasteride	NA	NA	0.40	>80
Flutamide	62.98	47.55	21.24	67.34

To further rationalize the prospective activities of 3-oxo-4-oxa-5α-androst-17β-amide derivatives against AR, molecular docking studies were performed using the Discovery Studio 2.5/CDOCKER protocol. The docking orientation and interactions of **11k,** DHT and finasteride within the ligand binding domain (LBD) of AR (4k7a, PDB) are shown in Figure 2. In the docking study, steroidal-core of **11k** was favorably positioned similar to DHT. The 4-O of **11k** played the role of 3-carbonyl of DHT and forms hydrogen bonds with Gln711. **11k** formed a second hydrogen bond with Met780, which was very close to the second hydrogen bond formed between DHT and Asn705. The binding position of finasteride with AR is in an opposite direction to that of DHT with AR. Thus, finasteride is unable to form any hydrogen bonds with AR. This Docking simulation suggested possible basis for the observed activities.

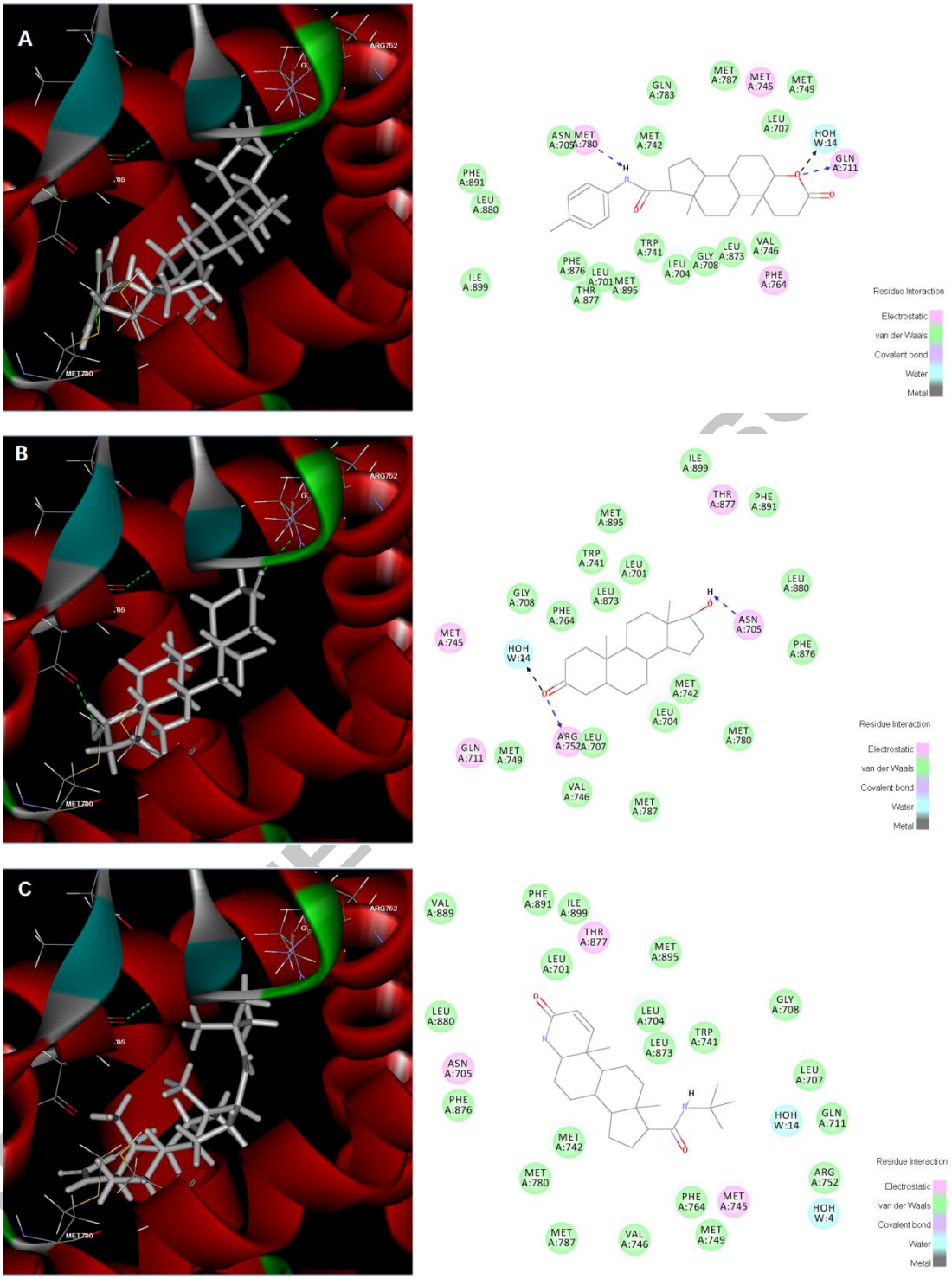


Figure 2. Molecular docking mood of compounds DHT (A) , 11k(B)and finasteride (C) with AR. 2D diagram (D) of DHT (left) and 11k (right) showing hydrogen bond interactions with key amino acids; Asn705, Arg752, Gln711, and Thr780.

The in vivo evaluation of compound **11k**, the determination of pharmacokinetic properties was performed in male SD rats using finasteride as reference compound. The plasma concentration-time curves after oral administration (0.2 mg/kg) to male SD mice are shown in Figure 3. After administration, the observed plasma concentration in mice reached peak levels 4h

post dose and was not detected 6 h after administration. The calculated pharmacokinetic parameters based on the plasma concentration profile following oral administration are shown in Table 3. Despite the concern that lactone might be more susceptible to hydrolysis than lactam, compound **11k** exhibited a plasma half-life of 4.74h, slightly better than finasteride. The superiority of compound **11k** observed when comparing the AUCs of the two compounds, leading to the conclusion that the bioavailability of compound **11k** is better than finasteride.

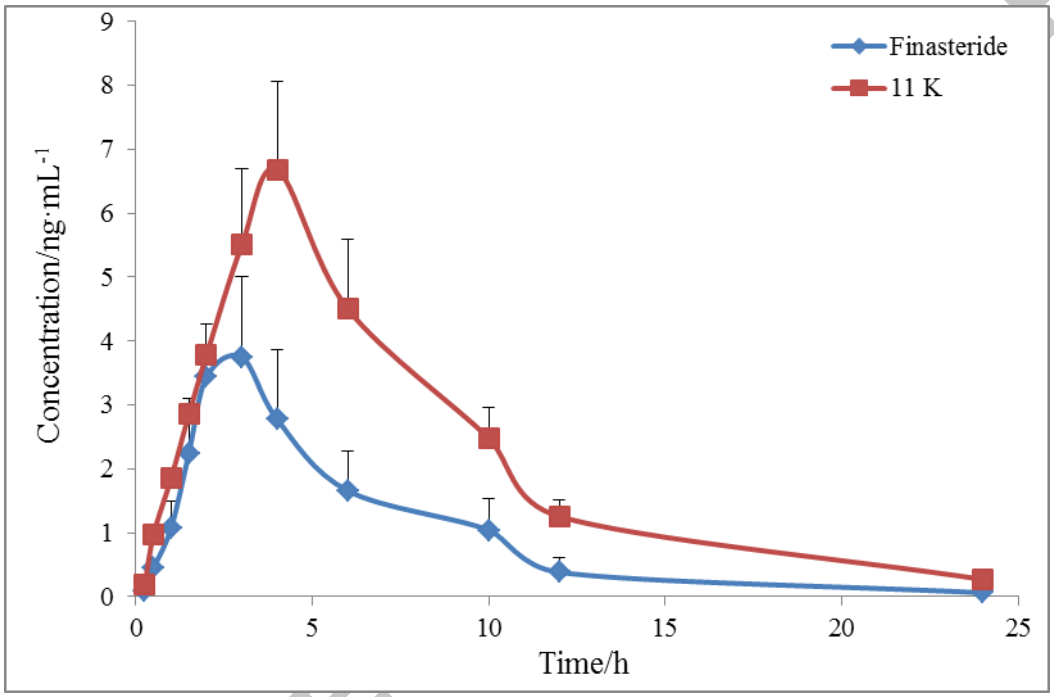


Figure 3. Concentration curve of finasteride and 11k in plasma versus time. Error bars show the value of SD (n=6).

Table 3. Pharmacokinetic Parameters for **11 k** and Finasteride in Rats after po Administration[a]

	$T_{1/2\beta}$(h) [b]	T_{max}(h) [b]	C_{max}(ug/L) [b]	$AUC_{(0\text{-}tn)}$(ug/L*h) [b]	$AUC_{(0\text{-}\infty)}$(ug/L*h) [b]
11k	4.74±0.83	3.83±0.41	6.89±1.22	52.49±9.20	54.11±9.45
finasteride	3.91±0.55	2.67±0.52	4.19±0.91	23.48±8.11	23.79±8.29

[a] Compound 11k and finasteride was administrated at a dose of 0.2mg/kg body weight. Six intact adult male rats were employed for each treatment group; each sample was tested for three times.

[b] $T_{1/2\beta}$, terminal half-life; T_{max}, time of maximal concentration; C_{max}, maximal concentration; AUC, area under the curve;

In this study, a series of novel 3-oxo-4-oxa-5α-androst-17β-amide derivatives have been designed and synthesized in a simple and convenient route avoiding the usage of expensive catalyst. Biological evaluations were performed on their 5α-reductase inhibitory activities and AR binding affinities. Compared with the aliphatic amine derivatives, compounds with aromatic amine substitutions showed higher inhibition rate to 5α-reductase as well as better binding affinities to AR. Results of anti-proliferation effects toward LNCaP and PC-3 cell lines reveled that most of the synthesized compounds exhibited potent anti-proliferative activities. It is obvious

that the androgen-dependent cell line LNCaP was observed to be much more sensitive than the androgen-independent cell line PC-3. Among them, compounds **11d** and **11k** presented about 4-fold more sensitive toward LNCaP than PC-3, which was consistent with their high affinity observed in AR binding assay. Molecular modelling studies suggested that **11k** could binding with AR in a similar mood with DHT. Moreover, compound **11k** showed a long plasma half-life and a high bioavailability. Overall, based on biological activities data, compound **11d** and **11k** can be identified as a potential dual 5α-reductase inhibitors and AR antagonists lead molecule which might be of therapeutic importance for prostate cancer treatment.

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