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Inhibitory Effect of 4-Aryl 2-Substituted Aniline-thiazole Analogs on Growth of Human Prostate Cancer LNCap Cells

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Androgen receptor (AR) is ligand-inducible nuclear hormone receptor which has been focused on key molecular target in growth and progression of prostate cancer. We synthesized a series of 4-aryl 2-substituted aniline-thiazole analogs and evaluated their anti-cancer activity in AR-dependent human prostate cancer LNCap cells. Among them, the compound 6 inhibited the tumor growth in LNCap-inoculated xenograft model.

Key Words : Androgen receptor, Antagonist, LNCap cell, Prostate cancer

Introduction

Androgen receptor (AR) is androgen inducible nuclear hormone receptor and a key molecular target in growth and progression of prostate cancer, even at the castration-resistant status.[1,2] Prostate cancer (PCa) is the most common cancer form related to death in men.[3] Nonsteroidal AR antagonists, preventing androgens from binding AR in the prostate, have been extensively developed during the past years.[4,5] The results of research and development are exemplified by several marketed drugs as androgen receptor antagonist including Schering-Plough' flutamide (1, Eulexin®) launched in 1983, Sanofi-Aventis' nilutamide (2, Nilandron®) in 1987 and AstraZeneca' bicalutamide (3, Casodex®) in 1993, as shown in Figure 1. Recent trouble-shooting in AR antagonist development is that reactivation of the AR signaling pathway causes the development of androgen independent prostate cancer. Therefore, we were going to identify new small molecule antagonists of the AR that are more effective than the current AR antagonists. Our primary screening efforts produced a series of thiazole-based compound in AR dependent LNCap cell line, and then we investigated *in vivo* activity.

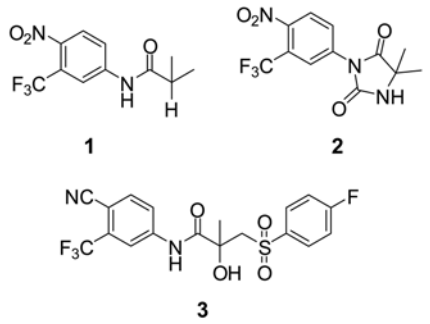


Figure 1. Marketed non-steroidal androgen receptor antagonists in recent.

[a]These authors contributed equally to this work.

Experimental Section

The 4-aryl-2-anilino-thiazole derivative was identified as putative androgen receptor (AR) antagonist for the treatment of prostate cancer through high-throughput screening (HTS) using the chemical library of Korea Chemical Bank (Figure 2). Furthermore, we synthesized its derivatives to address moiety of which could be attributing to their biological activity.

According to the Scheme 1, we synthesized 10 compounds, as follows. All of the 10 compounds were synthesized according to the similar methods described in literatures.[6] The synthetic route of 4-aryl 2-substituted aniline-thiazole analogs is shown in Scheme 1. Commercially available acetophenones **11** was brominated with Br_2 in CH_2Cl_2 to afford -bromo acetophenones **12**. Reaction of -bromoketones **12** with NaSCN in EtOH, followed by condensation with appropriate substituted-anilines **13** under reflux condition gave the desired 2-anilino thiazoles **1-4** in moderate yield. Alternatively, coupling reaction of benzoylisothio-

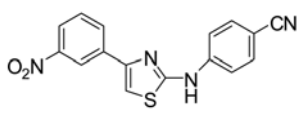
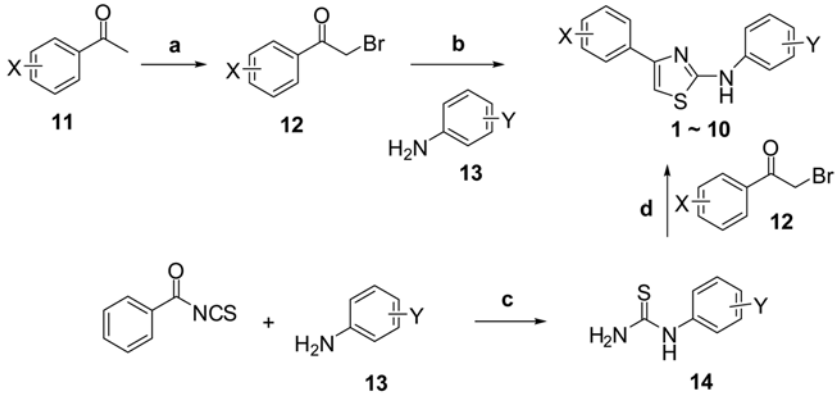


Figure 2. The early hit structure (89% inhibition at 10 uM) obtained from the high-throughput screening using in-house library.



Scheme 1. Synthesis of 1-10, reagents and conditions; (a) Br_2, CH_2Cl_2, rt, 5 h, 85-95%; (b) i) NaSCN, EtOH, reflux, 1h; ii) appropriate Anilines, EtOH, 16 h, 45-60%; (c) i) acetone, rt, 3 h; ii) 2*N*-NaOH, THF, rt, 4 h, 70-80%; (d) EtOH, reflux, 12 h, 80-95%.

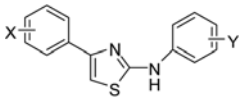


Table 1. *In vitro* activities against LNcap cell

Compound	X	Y	IC_{50} (uM)
			LNCap Cell
1	3-NO_2	3-Cl, 4-CN	36.19 ± 2.60
2	3-NO_2	3-CF_3, 4-CN	19.05 ± 0.31
3	3-NO_2	4-NO_2	13.29 ± 0.49
4	3-NO_2	3-CF_3, 4-NO_2	43.50 ± 2.45
5	**3-NO_2**	**2,4-*di*-Cl**	**8.65 ± 0.02**
6	**3-NO_2**	**2-Cl, 4-NO_2**	**9.55 ± 0.35**
7	H	2,4-*di*-Cl	35.41 ± 0.75
8	3-$NHSO_3Et$	3-CN, 4-CF_3	13.72 ± 0.37
9	4-NO2	4-OH	5.21 ± 1.10
10	4-Cl	4-COOH	40.77 ± 2.42
Bicalutamide			6.46 ± 0.65

cyanate with various anilines **13** was carried out in acetone, and then hydrolyzed with 2*N*-NaOH to afford substituted arylthioureas **14**, which was reacted with **12** in refluxing ethanol to give desired compounds **1-10**, as shown in Table 1, in good yield.[7]

Results and Discussion

The effect of compounds on the viability of human prostate AR-positive LNCap cells were evaluated.[8] Cell viability assay revealed that the compounds **5**, **6**, and **9** showed the potent inhibitory activity in the viability of LNCap cells, having stronger or similar activities to the reference compound, bicalutamide. Before evaluating the therapeutic activity of 4-aryl 2-substituted aniline-thiazole analogs in the AR-positive prostate cancer, we further determined physicochemical properties,[9,10] metabolic stability[11] and CYP inhibition activity[12] of compound **5**, **6**, and **9** that strongly inhibited the viability of LNCap cell. As shown in Table 2, two compounds, **5** and **6** showed appropriate logP values and medium permeability against artificial permeable membrane,

Table 2. Physicochemical properties, metabolic stability, CYP inhibition of thiazole derivatives

Compound	Physicochemical properties		CYP[a]		MS
	logP	PAMPA permeability	3A4 (%)	2D6 (%)	rat, *in vitro*
5	4.71 ± 0.41	−7.0 ± 0.05	13.55	0	65.20%
6	4.22 ± 0.40	−6.5 ± 0.05	25.83	17.25	> 99%
11	2.66 ± 0.80	−5.4 ± 0.02	-	-	-

Inhibition at 10 uM CYP concentration (%)

while compound **11** showed lower logP value though having good activity. Here we decided the compound **6** and **7** with proper logP value that well correlate the solubility and permeability and similar activity to reference compound come into the further study. Additionally, we tested metabolic stability and CYP effect. Both compounds did not show the strong CYP inhibition activity, but in evaluation for metabolic stability in rat, the compound **6**, with 99% of the parent compound remaining after 1 h incubation, showed to be more stable than the compound **5**. Based on the reported data, we chose the compound **6** for further in vivo study.

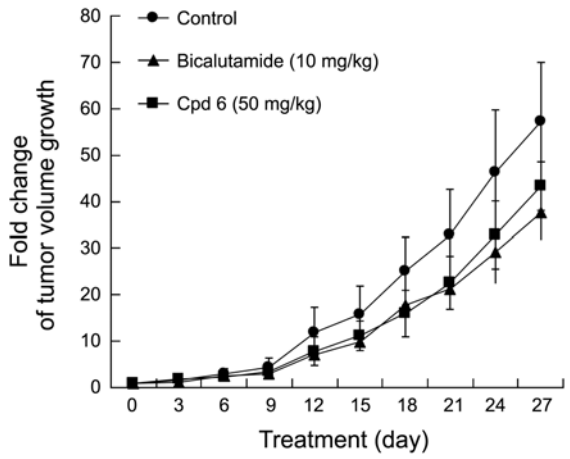


Figure 3. Effect of compound **6** on tumor growth in LNCap-inoculated xenograft model.

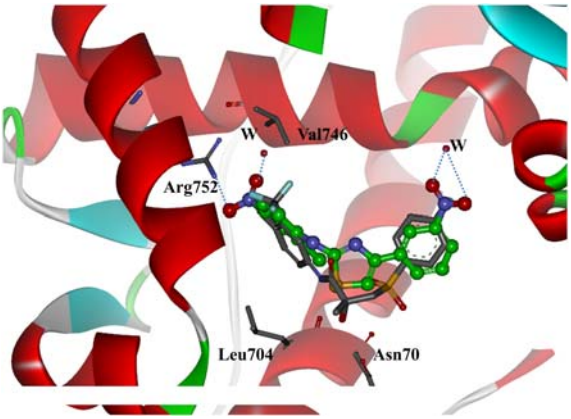


Figure 4. The calculated binding mode for compound **6** based on the bicalutamide complex structure (pdb code; 1Z95). Green element colored molecule, compound **6**, gray element colored molecule, bicalutamide. W represents water molecules. Hydrogen bonding interactions are displayed in blue dashed lines.

Next, the therapeutic activity of the compound **6** was evaluated in LNCap-inoculated xenograft model.[13] As shown in Figure 3, the compound **6** attenuated the tumor growth as observed in the bicalutamide-treated group, suggesting its *in vivo* inhibitory effect on the growth of AR-positive prostate cancer.

Also, we carried out the docking study to confirm the AR binding mode for compound **6**. The AR complex structure as reference was obtained from the x-ray crystal structure of the mutant W741L AR bound to bicalutamide (pdb code: 1Z95),[14] replacing Leu741 in 1Z95 by the wild-type Trp, with adjustment of the conformation of the nearby Met895.

Docking study was carried out using LigandFit[15] module in DiscoveryStudio 3.1 with CHARMM force field. The experimental binding conformation of bicalutamide was well created showing score value of 65.28 with Dock_Score in LigandFit. Compound **6** was well fitted in the binding site with the Dock_Score of 50.91 as shown in Figure 4. The two terminal nitro groups also have H-bonding interactions with Arg752 and two water molecules such as bicalutamide. As docking study, we can confirm compound **6** interacts with AR binding site *in vitro*.

Conclusion

In this paper, we synthesized a series of 4-aryl 2-substituted aniline-thiazole analogs showing the similar binding mode to bicalutamide and evaluated their *anti*-cancer activity in AR-dependent human prostate cancer LNCap cells. Among them, the compound **6** inhibited the tumor growth in LNCap-inoculated xenograft model. Furthermore, we are going to deeply optimize for our compounds to develop the drug for prostate cancer treatment.

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7. Analytical data for the compounds (^{1}H NMR 300 MHz); compound (**1**): yellow solid. mp 223-224 °C. ^{1}H NMR (DMSO-d_6) 10.94 (brs, 1H), 8.70 (s, 1H), 8.39 (d, J = 8.1 Hz, 1H), 8.18 (d, J = 8.1 Hz, 1H), 7.88 (d, J = 8.7 Hz, 2H), 7.85 (s, 1H), 7.81 (d, J = 8.7 Hz, 2H), 7.76 (dd, 1H): MS (*m/e*, 70 eV): 322 (M^+); compound (**2**): yellow solid. mp 227-228 °C. ^{1}H NMR (DMSO-d_6) 11.43 (brs, 1H), 8.72 (m, 2H), 8.38 (d, J = 7.8 Hz, 1H), 8.27 (d, J = 9.0 Hz, 1H), 8.20 (d, J = 7.2 Hz, 1H), 7.94 (s, 1H), 7.92 (d, J = 7.2 Hz, 1H), 7.75 (dd, J = 9.0, 7.8 Hz, 1H)): MS (*m/e*, 70 eV): 357 (M^+); compound (**3**): yellow solid. mp 235-236 °C. ^{1}H NMR (DMSO-d_6) 11.35 (brs, 1H), 8.70 (m, 2H), 8.35 (d, J = 7.8 Hz, 1H), 8.18 (d, J = 7.8, 1.8 Hz, 1H), 8.07 (d, J = 8.7 Hz, 1H), 7.91 (s, 1H), 7.84 (dd, J = 8.7, 1.5 Hz, 1H), 7.74 (dd, 1H): MS (*m/e*, 70 eV): 390 (M^+); compound (**4**): yellow solid. mp 216-217 °C. ^{1}H NMR (DMSO-d_6) 11.18 (brs, 1H), 8.72 (s, 1H), 8.44 (d, J = 7.8 Hz, 1H), 8.27 (d, J = 9.3 Hz, 2H), 8.20 (d, J = 8.1 Hz, 1H), 7.93 (d, J = 9.3 Hz, 2H), 7.91 (s, 1H), 7.76 (dd, J = 8.1, 7.8 Hz, 1H): MS (*m/e*, 70 eV): 342 (M^+); compound (**5**): yellow solid. mp 230-232 °C. ^{1}H NMR (DMSO-d_6) 11.15 (brs, 1H), 8.71 (s, 1H), 8.36 (d, J = 7.8 Hz, 1H), 8.25 (d, J = 1.8 Hz, 1H), 8.19 (dd, J = 8.1, 2.1 Hz, 1H), 7.90 (d, J = 8.7 Hz, 1H), 7.89 (s, 1H), 7.76 (dd, 1H), 7.64 (dd, J = 8.7, 1.8 Hz, 1H): MS (*m/e*, 70 eV): 410 (M^+); compound (**6**): yellow solid. mp 258-260 °C. ^{1}H NMR (DMSO-d_6) 10.47 (brs. 1H), 8.91 (d, J = 9.3 Hz, 1H), 8.68 (m, 1H), 8.40 (d, J = 7.8 Hz, 1H), 8.35 (d, J = 2.7 Hz, 1H), 8.27 (dd, J = 9.3, 2.7 Hz, 1H), 8.17 (d, J = 7.8 Hz, 1H), 7.96 (s, 1H), 7.74 (dd, 1H)): MS (*m/e*, 70 eV): 376 (M^+); compound (**7**): yellow solid. mp 197-198 °C. ^{1}H NMR (DMSO-d_6) 9.95 (brs, 1H), 8.64 (s, 1H), 8.47 (d, J = 9.0 Hz, 1H), 8.33 (d, J = 7.8 Hz, 1H), 8.15 (dd, J = 7.8, 2.1 Hz, 1H), 7.76 (s, 1H), 7.71 (dd, 1H), 7.65 (d, J = 2.7 Hz, 1H), 7.46 (dd, J = 9.0, 2.7 Hz, 1H): MS (*m/e*, 70 eV): 366 (M^+); compound (**8**): white solid. mp 193-194 °C. ^{1}H NMR (DMSO-d_6) 9.84 (brs, 1H), 8.57 (d, J = 8.6 Hz, 1H), 7.88 (d, J = 7.2 Hz, 2H), 7.62 (d, J = 2.4 Hz, 1H), 7.41 (m, 4H), 7.30 (m, 1H): MS (*m/e*, 70 eV): 321 (M^+); compound (**9**): yellow solid. mp 205-206 °C. ^{1}H NMR (DMSO-d_6) 9.95 (brs, 1H), 8.52 (d, J = 9.0 Hz, 1H), 8.25 (d, J = 8.7 Hz, 2H), 8.33 (d, J = 8.7 Hz, 1H), 7.79 (s, 1H), 7.63 (d, J = 2.4 Hz, 1H), 7.45 (dd, J = 9.0, 2.4 Hz, 1H)): MS (*m/e*, 70 eV): 366 (M^+).
8. **Cell culture and viability assay.** Human prostate cancer LNCap cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin in humidified atmosphere of 5% CO_2 at 37 °C. The culture medium was changed every 3 days. For viability assay,

cells were seeded in a 96-well plate at 4×10^3 cells/well, cultured for 24 h and then incubated with a compound for 48 h. Then, cell viability was measured in triplicates by the Cell Counting Kit-8 (Dojindo Molecular Technologies, ML) according to the manufacturer's protocol. Absorbance was measured by using Wallac EnVision microplate reader (PerkinElmer, Finland).

9. **logP.** LogP is measured by a pH-metric method, based on a two-phase acid-base titration in a mixture of water and octanol using GLpKa system by Sirius.
10. **Parallel artificial membrane permeability (PAMPA) permeability**. Donor solution (500 μM) was prepared by diluting 1 mM DMSO compound stock solution using the diluted system buffer (pH adjusted to 7.4). Five microliters lipid solution was applied to each filter membrane. Donor solution (150 μL) was added to each well of the filter plate. The filter plate was then put onto a receiver plate with preloaded acceptor sink buffer. The sandwich was incubated at room temperature for 16 h. Samples were taken from both receiver and donor sides at the end of the incubation and analyzed using UV spectrometer. Donor solutions were also analyzed for initial concentration.
11. **Metabolic stability.** Using rat liver microsomes, the amount of parent compound remaining after 1 h incubation. The concentration of the used compound is 5 mM and protein concentration is 1 mg/mL.
12. **CYP3A4 enzyme assay**. The assay was carried out using fluorometric enzyme assays with Vivid CYP3A4 assay kit (PanVera, USA, CA) in a 96-well microtiter plate following the manufacturer's instruction with some modification. The compounds including ketoconazole known as CYP3A4 inhibitor were prepared in acetonitrile to give final concentrations of 10 mM. NADP generating solution (1.0 mM NADPþ, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2 \cdot 6H_2O$, and 0.4 U/mL glucose-6-phosphate dehydrogenase in 10 mM KPO_4, pH 8.0) was added to each well of the microtiter plate followed by the vehicle acetonitrile (control) and the test samples. The plate was covered and then incubated at 37 °C for 20 min. Enzyme reaction was initiated by the addition of enzyme/substrate (E/S) mixture (0.5 pmol recombinant human CYP3A4 enzyme and 5 mM dibenzylfluorescein, DBF). The plate was further incubated for 20 min, followed by the addition of the stop solution to terminate the enzyme activity. Background reading was measured in a similar manner except for the E/S mixture which was added after the enzyme reaction was terminated. The fluorescence of DBF metabolite fluorescein was measured on a fluorescence plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.
13. Animal experiment was in accordance with the guidelines of the Yonsei University Institutional Animal Care and Use Committee at Wonju campus (IACUC approval number YWC-101015-1). Human prostate cancer LNCap cells (1×10^6) were implanted subcutaneously on the left and right flank region of each immunodeficient male mouse (Orientbio Inc., Korea; six-week-old, 17-19 g), respectively. Ten days after transplantation, five mice per cohort were treated orally by gavage (p.o.) five times per week for 4 weeks with the 200 μg or 1,000 μg of test compounds including bicalutamide (AstraZeneca) for a model reference control dissolved in 1% carboxylmethyl cellulose/0.1% Tween-80/5% dimethyl sulfoxide (10 mg or 50 mg per kg body weight). The volume of prostate tumor was quantified repeatedly every three days from the day of treatment through oral route.

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