npg

Original Article

Development of β-amino-carbonyl compounds as androgen receptor antagonists

Zhi-yun ZHANG[1, #], Yan-hui ZHU[1, #], Cai-hong ZHOU[1], Qing LIU[1], Hui-li LU[1], Yun-jun GE[1], Ming-wei WANG[1, 2, *]

[1]The National Center for Drug Screening and the CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (CAS), Shanghai 201203, China; [2]School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, China

Aim: Androgen receptor (AR) antagonists have proven to be useful in the early control of prostate cancer. The aim of this study was to identify and characterize a novel β-amino-carbonyl-based androgen receptor antagonist.
Methods: Different isomers of the β-amino-carbonyl compounds were obtained by chiral separation. The bioactivities of the isomers were evaluated by AR nuclear translocation, mammalian two-hybrid, competitive receptor binding and cell proliferation assays. The expression of genes downstream of AR was analyzed with real-time PCR. The therapeutic effects on tumor growth *in vivo* were observed in male SCID mice bearing LNCaP xenografts.
Results: Compound **21** was previously identified as an AR modulator by the high-throughput screening of a diverse compound library. In the present study, the two isomers of compound **21**, termed compounds **21–1** and **21–2**, were characterized as partial AR agonists in terms of androgen-induced AR nuclear translocation, prostate-specific antigen expression and cell proliferation. Further structural modifications led to the discovery of a androgen receptor antagonist (compound **6012**), which blocked androgen receptor nuclear translocation, androgen-responsive gene expression and androgen-dependent LNCaP cell proliferation. Four stereoisomers of compound **6012** were isolated, and their bioactivities were assessed. The pharmacological effects of **6012**, including AR binding, androgen-induced AR translocation, NH$_2$- and COOH-terminal interaction, growth inhibition of LNCaP cells *in vitro* and LNCaP xenograft growth in nude mice, were mainly restricted to isomer **6012–4** (1R, 3S).
Conclusion: Compound **6012–4** was determined to be a novel androgen receptor antagonist with prostate cancer inhibitory activities comparable to bicalutamide both *in vitro* and *in vivo*.

Keywords: prostate cancer; structural modification; androgen receptor; antagonist; molecular docking; β-amino-carbonyl compound

Introduction

Prostate cancer represents 29% of all cancer cases diagnosed and 9% of cancer deaths in the United States in 2012[1]. Prostate cancer develops slowly and starts with abnormal hyperplasia caused by the stimulation of androgens. However, there are also cases of aggressive prostate cancer. Conventional treatment involves surgical removal and radioactive and hormone deprivation therapies; the latter is achieved either by reducing circulating androgen levels or by suppressing the action of endogenous androgen at its receptor.

The commercially available androgen receptor (AR) antagonists can be divided into two classes: steroidal (such as cyproterone acetate) and non-steroidal (such as bicalutamide, nilu-

tamide and flutamide). Bicalutamide, which is marketed as Casodex, is an orally available agent widely used in the treatment of prostate cancer[2-4] and hirsutism[5]. The drug was first introduced in 1995 as part of a combination therapy (with surgical or medical castration) for advanced prostate cancer and was subsequently employed as monotherapy for earlier stages of the disease[4]. Although bicalutamide is effective in reducing prostate specific antigen (PSA) levels and arresting tumor growth, prolonged dosing could lead to a paradoxical clinical phenomenon called "anti-androgen withdrawal syndrome", which is characterized as decreases in blood PSA and cancer remission after withdrawal of the drug[6]. Another phenomenon relates to castration therapy, whereby the cancer becomes androgen independent years after the surgery and advances to a stage called "androgen-refractory prostate cancer". These unexpected outcomes have resulted in doubts regarding AR as a drug target for prostate cancer. Nonetheless, recent studies suggest that AR is associated with both androgen-depen-

---

dent and androgen-independent prostate cancers[7]. Second-generation anti-androgens (such as MDV3100) are able to suppress the growth of both types of prostate cancer[8].

The activation of AR involves the binding of an androgen to cytoplasmic AR, followed by the translocation of the androgen-receptor complex from the cytoskeleton to the nucleus. In the nucleus, the AR complex interacts with androgen-response elements and recruits co-regulators to regulate the expression of target genes, such as PSA and kallikrein-related peptidase (KLK2), leading to cell proliferation. The NH_2- and COOH-terminal (NC) interaction, specifically the interaction between the N-terminal FXXLF motif of the AR N-terminal domain (NTD) with the active function 2 (AF2) region of its ligand binding domain (LBD), is important for the full function of AR[9]. Although bicalutamide only blocks the NC interaction, MDV3100 inhibits both nuclear translocation and NC interaction[8].

In the search for novel non-steroidal AR antagonists, we previously screened 16000 small molecules and discovered β-amino-carbonyl compounds as AR modulators with high binding affinity[10]. As a follow-up study, here we describe the pharmacological properties of compound **21** as an AR modulator. Further structural modifications identified compound **6012** as an AR antagonist. Its stereoisomer, isomer **4** (1R, 3S), displays bioactivities comparable to bicalutamide.

Materials and methods
Reagents
Dihydrotestosterone (DHT) was purchased from Sigma Chemical Co (St Louis, MO, USA). Casodex (bicalutamide) was provided by Prof Lian-zhi CHEN of China Pharmaceutical University (Nanjing, China). Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium were procured from Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) and charcoal/dextran-treated FBS (CDT-FBS) were purchased from Hyclone (Logan, UT, USA). Fugene HD transfection reagent was purchased from Roche (Indianapolis, IN, USA). [3H]DHT was obtained from PerkinElmer (Boston, MA, USA). The nuclear receptors of human origin used in the binding assays were produced according to the method reported previously[10]. The human AR (pSVAR0) plasmid was kindly provided by Dr AO BRINNKMANN of Erasmus University Medical Center Rotterdam (Rotterdam, The Netherlands). The CheckMate Mammalian Two-Hybrid System was obtained from Promega (Madison, WI, USA). The plasmid pEGFP-C1-AR was purchased from Addgene (Cambridge, MA, USA). The anti-androgen receptor antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo China Co, Ltd (Shanghai, China).

Cell culture
Human embryonic kidney 293 cells (HEK293) and human prostate carcinoma cell lines LNCaP and DU 145 were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA). The human prostate carcinoma cell line

PC-3M was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). LNCaP cells were maintained in RPMI-1640 medium with 4.5 g/L glucose, supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL streptomycin. The cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere. HEK293 cells were cultured in DMEM containing 10% FBS. DU 145 and PC-3M cells were cultivated in RPMI-1640 supplemented with 10% FBS.

Nuclear translocation analysis
LNCaP cells were seeded into poly-L-lysine-pretreated 96-well plates with RPMI-1640 medium containing 10% FBS and cultivated for two days. Then, the medium was changed to phenol red-free RPMI-1640 containing 10% CDT-FBS, and the cells were cultured for one day. The cells were then treated with different concentrations of DHT or test compounds alone (agonist mode) or test compounds together with DHT (antagonist mode) for 3 h before immunofluorescence staining was performed. Briefly, the LNCaP cells were washed with PBS and fixed with 30 μL 4% paraformaldehyde for 10 min. After washing with PBS, the cells were blocked with 30 μL blocking buffer (10% goat serum, 1% BSA, 0.25% Triton X-100 in PBS, pH=7.4) for 1 h. The cells were washed with PBST (0.25% Triton X-100 in PBS, pH=7.4) three times and then treated with 30 μL anti-androgen receptor antibody (PG-21, 1:100) at 37 °C for 2 h. The cells were washed with PBST; 30 μL of Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) was added to the cells and incubated for 1 h in the dark. The cells were washed with PBS and stained with DAPI. The plates were evaluated using a Cellomics ArrayScan (Thermo Scientific, MA, USA) with DAPI in channel 1 and Alexa 488 in channel 2. The intensity difference of the Alexa 488 signal between the nucleus and cytoplasm (MEAN-CircRingAvgIntenDiffCh2) of the cells was used as a parameter for the measurement of nuclear translocation. The sample preparation for confocal microscopy was similar to the protocol described above, except that the LNCaP cells were seeded onto glass slides, and images were captured with a confocal microscope FV10i (Olympus, Tokyo, Japan).

To assess AR nuclear translocation in transiently transfected cells, HEK293 cells were seeded (at a density of 1.2×10^4 cells per well) onto a glass slide placed at the bottom of a 24-well plate in phenol red-free DMEM containing 10% CDT-FBS. The cells were cultured for 2 d. The pEGFP-C1-AR plasmid was transfected into the cells for two days using Fugene HD. The cells were treated with test compounds for 3 h, followed by fixation with 4% paraformaldehyde for 10 min. The cells were then washed twice with PBS and stained with DAPI (15 μmol/L) for 10 min before a final wash with PBS. Images were obtained with an FV10i confocal microscope.

Cell cycle analysis
LNCaP cells were treated with phenol red-free RPMI-1640 supplemented with 10% CDT-FBS for one day. The cells were then detached and re-suspended in phenol red-free RPMI-1640 medium containing 10% CDT-FBS and seeded into 6-well

plates at a density of 3×10^5 cells per well and cultivated for 2 d. After compound treatment for 2 d, the cells were dissociated with trypsin-EDTA and collected by centrifugation. The cell pellet was washed with 5 mL cold PBS, and the cells were resuspended in 0.5 mL of cold PBS and fixed with 5 mL of 70% ethanol (vortex briefly). The cells were centrifuged again, washed with 5 mL cold PBS, briefly vortexed, resuspended with 100 µL RNase A (2 mg/mL), 150 µL PBS and 250 µL propidium iodide (PI) (0.1 mg/mL in 0.6% Triton X-100 in PBS) and stored in the dark for 45 min. Before measurement, the cells were filtered to eliminate any clumps. The DNA content of the labeled cells was measured using the Accuri C6 flow cytometry system (BD Biosciences, NJ, USA), and 2×10^4 data points were collected in the gate for each sample. The cell cycle was analyzed with FlowJo flow cytometry analysis software (Tree Star, Inc, OR, USA).

Cell proliferation assay

LNCaP cells were cultured for two days in RPMI-1640 medium containing 10% FBS at a density of $1–2\times10^6$ cells per dish. On d 3, the culture medium was removed, and the cells were washed once with PBS. The cells were incubated with phenol red-free RPMI-1640 medium containing 10% CDT-FBS and cultivated for 1 d. The cells were then detached with trypsin-EDTA and collected by centrifugation at 900 rounds per minute for 3 min. The cells were seeded into 96-well plates at a density of 1×10^5 cells per well in phenol red-free RPMI-1640 medium containing 10% CDT-FBS. The cells were cultivated for 2 d and treated with different concentrations of test compounds for 5 d. Cell viability was measured with the CCK-8 kit (Dojindo).

Cytotoxicity assay

DU 145 and PC-3M cells were cultivated with RPMI-1640 medium containing 10% FBS at a density of $1–2\times10^6$ cell per dish for 2 d. The cells were detached using trypsin-EDTA and collected by centrifugation at 900 rounds per minute for 3 min. The cells were then resuspended in RPMI-1640 medium containing 10% FBS and seeded into 96-well plates at a density of 1×10^5 cells per well in the same medium. The cells were cultivated for one day and treated with test compounds for an additional 3 d. Cell viability was measured with the CCK-8 kit (Dojindo).

Mammalian two-hybrid assay

The mammalian two-hybrid system from Promega was used in this study. The cDNAs encoding the N-terminal domain (NTD, amino acid 1-505) of AR were subcloned from pSVAR0 into the pACT vector between the *Bam*H I and *Xba* I sites, and the cDNAs encoding the ligand-binding domain (LBD, amino acid 604-910) of AR were subcloned into the pBIND vector between the *Bam*H I and *Xba* I sites. HEK293 cells were seeded into 96-well plates in DMEM medium containing 10% FBS (2×10^4 cells/well) and cultivated for 1 d. The cells were then co-transfected with pACT-NTD, pBIND-LBD and pGL4.31 using Fugene HD and incubated for 24 h. Then, different con-

centrations of test compounds were added to the cells with or without 10 nmol/L DHT. After incubating the cells for one day, luciferase activity was measured using Steady-Glo (Promega) and an Envision plate reader (PerkinElmer).

PSA and KLK2 expression analysis

LNCaP cells were cultivated in phenol red-free RPMI-1640 medium containing 10% CDT-FBS in 10-cm dishes for one day, and the cells were seeded into 12-well plates at a density of 3×10^5 cells/well. After cultivation for two days, the cells were treated with test compounds for 8 h, and total RNA was extracted using a total RNA extraction kit (TianGen, Beijing, China). cDNAs were synthesized using a high-capacity cDNA synthesis kit from Life Technologies (Carlsbad, CA, USA). A real-time PCR analysis was performed using 2× SYBR Green master mix (TaKaRa, Dalian, China) and an ABI 7300 instrument (Life Technologies). The PCR primers were as follows: *β-actin* forward, 5′-AGCGAGCATCCCCCAAAGTT-3′, and reverse, 5′-GGGCACGAAGGCTCATCATT-3′; *PSA* forward, 5′-AAGCACCTGCTCGGGTGA-3′, and reverse, 5′-CCACTTC-CGGTAATGCACCA-3′; *KLK2* forward, 5′-CACAGCT-GCCCATTGCCTAAAGAA-3′, and reverse, 5′-GGCCTGT-GTCTTCAGGCTCAAA-3′. The samples were analyzed in duplicate, and the gene expression was normalized to the housekeeping gene *β-actin*. Single bands of the correct size were verified by a dissociation curve analysis and gel electrophoresis of the PCR products.

Receptor binding assay

Baculovirus-derived AR extract protein (approximately 60 mg/L) was loaded into each well of an IsoPlate (PerkinElmer) containing the assay buffer [10% glycerol (v/v), NaH_2PO_4 25 mmol/L, $NaMoO_4$ 10 mmol/L, KF 10 mmol/L, dithiothreitol 1.6 mmol/L, EDTA 1.5 mmol/L, CHAPS 2 mmol/L, 1× protease inhibitor cocktail]. Then, [^{3}H]DHT (110 Ci/mmol, 5 nmol/L) and various concentrations of test compounds or DHT (2.5 µL) were added to obtain a final volume of 100 µL per well. The plates were sealed and incubated overnight at 4 °C. Hydroxyapatite [HA, 25% (v/v), 25 µL] was added to each well, and the plates were gently agitated twice for 5 min each. Following centrifugation at 2500 rounds per minute for 3 min at 4 °C, the supernatant was decanted, and 100 µL assay buffer was added to each well. This washing procedure was repeated twice before adding 150 µL scintillation liquid (PerkinElmer). The plates were gently agitated to resuspend HA, and the radioactivity was measured with a MicroBeta counter (PerkinElmer). Nuclear receptor cross-reactivity binding assays including glucocorticoid receptor, estrogen receptor-α and β were performed as described previously[11].

Chemistry

3-(4-Methoxy-2-nitrophenylamino)-1-(4-nitrophenyl)-3-phenylpropan-1-one benzaldehyde (38 g, 0.358 mol) was added in a solution of 4-nitroacetophenone (26 g, 0.157 mol) and 4-methoxy-2-nitrobenzenamine (50 g, 0.297 mol) in benzene (500 mL) and the resulted mixture was stirred for 2–3

min. Sulfuric acid (5 d) was added, and the reaction was refluxed in a Dean-Stark apparatus for several hours. The solvent was removed *in vacuo*, and the residue was purified on a silica gel column and eluted with a mixture of ethyl acetate and petroleum ether at a ratio of 5:95 to give the product 3-(4-methoxy-2-nitrophenylamino)-1-(4-nitrophenyl)-3-phenylpropan-1-one (12.1 g, yield 18%).

3-[(4-Methoxy-2-nitrophenyl)amino]-1-(4-nitrophenyl)-3-phenyl-propan-1-ol (compound **6012**)

3-(4-Methoxy-2-nitrophenylamino)-1-(4-nitrophenyl)-3-phenylpropan-1-one (2.5 g, 5.9 mmol) was dissolved in methanol (250 mL), and the resulting solution was cooled to 0 °C. Then, sodium borohydride (1.1 g, 29 mmol) was added in fractions. The reaction was stirred at the room temperature overnight and quenched by addition of water (5 mL). The solvent was removed *in vacuo*, and the residue was extracted with ethyl acetate (3×50 mL). The organic phase was combined and subsequently washed with saturated sodium chloride solution (3×20 mL) and dried over sodium sulfate. After filtration, the solvent was evaporated *in vacuo*, and the product was obtained as the red powder (2.2 g, yield 80%).

Chiral separation

The crude powder of compound **6012** (42.5 g) was separated on a CHIRALPAK AS-H column (0.46 cm ID×15 cm L) with a 2-μL injection at the wavelength of 254 nm. Two fractions (fraction 1, RT=5.5 min; fraction 2, RT=3.4 min) were isolated by eluting with 100% methanol at a flow rate of 1 mL/min. After concentration *in vacuo*, isomer **4** was obtained as red powder (8.61 g, e.e.>98.0%); isomers **1–3** were obtained as a mixture, which was further separated on the same column by eluting with a mixture of methanol and acetonitrile at the ratio of 90:10. Two fractions (1-1 and 1-2) were obtained. Fraction 1-1 (RT=3.6–4.1 min) was the mixture of isomers **1** and **2**; fraction 1-2 (RT=5.5 min) was the solution of isomer **3**. The solution was combined and concentrated *in vacuo* to obtain isomer **3** as red powder (4.98 g, e.e.>98.0%). The mixture from fraction 1-1 was continuously separated on the CHIRALPAK IC column (0.46 cm ID×15 cm L) using a 2-μL injection at the wavelength of 254 nm. Two fractions (1-1-1, 1-1-2) were obtained by eluting with a mixture of hexane, dichloromethane and ethanol at the ratio of 50:48:2 and a flow rate of 1 mL/min. Isomers **1** and **2** were individually obtained from fraction 1-1-1 (RT=5.8 min) and fraction 1-1-2 as red powder (isomer **1**, 9.92 g, e.e.>98.0%; isomer **2**, 4.87 g, e.e.>98.0%).

For ^{1}H NMR (CDCl$_3$) and ^{13}C NMR (CDCl$_3$) data, see Table S3.

Molecular docking

Compound **6012-4** was crystallized in a mixture of ethyl acetate and petroleum ether, and the absolute conformation was determined to be (1R, 3S) 3-[(4-methoxy-2-nitrophenyl)amino]-1-(4-nitrophenyl)-3-phenylpropan-1-ol by an X-ray crystallographic analysis. This compound was docked into the AR ligand binding domain (AR-LBD) [PDB: 2AXA[12]] using the Schrodinger Maestro Glide 4.5 module. Briefly, the crystal structure of the AR-LBD was refined and docked following the user manual in three steps: protein and ligand preparation, receptor grid generation and ligand docking. The reference ligand S1 was used to define the docking box. Docking was performed using the extra precision docking and scoring (XP) module, with the other options set to default. The final result was imaged using the Chimera program.

Animals and treatment

Fifty male SCID mice (21–25 g) were purchased from Shanghai SLAC Laboratory Animal Co, Ltd (Shanghai, China) and housed at 22 °C under a standard light/dark cycle (12:12) with *ad libitum* access to food and water. The animals were allowed to acclimate to their new environment (3 d) before experimentation. Ten million LNCaP cells in 50% Matrigel were subcutaneously injected into the upper shoulder of each mouse. When the tumors reached an average volume of 126 mm^3, the mice were divided into 5 groups of 10 mice per group (vehicle, 2 mg/kg compound **6012-4**, 10 mg/kg compound **6012-4**, 10 mg/kg compound **6012-3** and 10 mg/kg Casodex). The compounds and vehicle were injected intraperitoneally daily for 28 d. All compounds were dissolved in vehicle (5% DMSO, 15% Cremophor EL, 30% PEG-400 and 50% water). The tumor volumes (mm^3) were measured using vernier calipers, and the body weights were measured with an electronic balance twice per week during the treatment. After the experiment was completed, the mice were euthanized by CO$_2$ exposure.

Statistical analysis

A statistical analysis was performed with Student's *t*-test using GraphPad Prism software (GraphPad, San Diego, CA, USA). The data are presented as the mean±SEM. The criterion for significance was a probability of $^bP<0.05$, $^cP<0.01$.

Results

Characterization of the isomers of compound 21

We previously discovered a series of β-amino-carbonyl compounds that function as AR modulators. Compound **21** displayed a high binding affinity and showed AR-modulating bioactivities both *in vitro* and *in vivo*[10]. To explore the underlying molecular mechanism of compound **21**'s bioactivities, we performed nuclear translocation assays with a high content screening system (Cellomics ArrayScan). As shown in Figure 1A, two isomers of compound **21**, (R)-1-(4-chlorophenyl)-3-(furan-2-yl)-3-((4-nitrophenyl)amino)propan-1-one (**21-1**) and (S)-1-(4-chlorophenyl)-3-(furan-2-yl)-3-[(4-nitrophenyl)amino]propan-1-one (**21-2**), stimulated AR nuclear translocation, with EC$_{50}$ values of 14.46 nmol/L and 7.12 nmol/L, respectively. The EC$_{50}$ value for the control ligand DHT was 0.22 nmol/L (Figure 1A). In addition, compound **21-1** also demonstrated an antagonistic activity in the presence of 10 nmol/L DHT (Figure 1B). These results were subsequently confirmed by image analysis with confocal microscopy in LNCaP cells (Supplemental Figure 1).

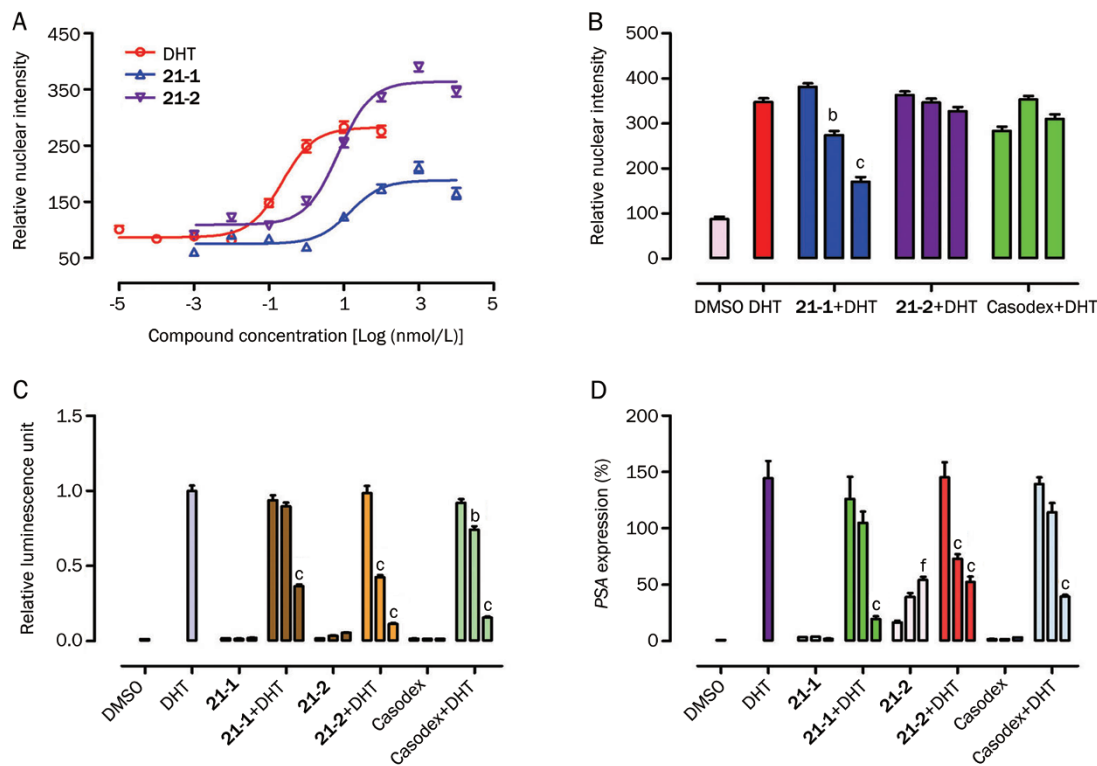To investigate the potency of the two isomers on the NC

Figure 1. Biological activities of compounds **21-1** and **21-2** in the androgen receptor signaling pathway. (A) Compounds **21-1** and **21-2** stimulated the nuclear translocation of androgen receptors. (B) Nuclear translocation of androgen receptors in DHT (dihydrotestosterone)-treated LNCaP cells. Compounds were tested together with 10 nmol/L DHT. (C) Effects of compound **21-2** on androgen receptor NC interaction. (D) *PSA* gene expression. For Figures B, C, and D, the compound concentrations were 100 nmol/L, 1 μmol/L, and 10 μmol/L, from left to right, with or without 10 nmol/L DHT. The data are presented as the mean±SEM of at least three independent experiments. [b]$P<0.05$, [c]$P<0.01$ compared with 10 nmol/L DHT. [f]$P<0.01$ *vs* DMSO control (*t*-test). Casodex: bicalutamide.

interaction of AR, we constructed a mammalian two-hybrid system by fusing the DNA sequence encoding AR-NTD to the gene encoding VP16 and the sequence encoding AR-LBD to the gene encoding Gal 4. The results indicated that both **21-1** and **21-2** possess antagonist activities and block NC interaction of the receptor. The potency of compound **21-2** was comparable to that of bicalutamide (Casodex, Figure 1C).

PSA expression in LNCaP cells was measured after treatment with compounds **21-1** and **21-2**, and both isomers inhibited DHT-induced PSA gene expression at 10 μmol/L. Interestingly, compound **21-2** also behaved as a partial agonist in this assay (Figure 1D), as observed by NC interaction assessment (Figure 1C).

Discovery of the androgen receptor antagonist 6012

As shown above, compound **21** and its isomers modulated the function of AR at several steps in the signaling pathway. However, our efforts in developing novel AR antagonists are limited by the agonist properties of compounds **21** and its isomer with an S configuration (**21-2**). Therefore, we propose that a full AR antagonist should bind to the cognate receptor at a reasonably high affinity and inhibit DHT-induced LNCaP cell growth without obvious cytotoxicity in non-androgen-dependent DU 145 cells. Based on structure-activity relation-

Table 1. Cross-reactivities of the four isomers of compound **6012** with steroid receptors in the receptor binding assay.

Compound	Estrogen receptor α IC$_{50}$ (nmol/L)	Estrogen receptor β IC$_{50}$ (nmol/L)	Glucocorticoid receptor IC$_{50}$ (nmol/L)	Androgen receptor IC$_{50}$ (nmol/L)
Estradiol	0.85	1.03	–	–
Dexamethasone	–	–	1.62	–
DHT	–	–	–	3.13
6012-1	961.2	19000	>50000	16,533
6012-2	>50000	>50000	>50000	16,070
6012-3	>50000	>50000	>50000	23,317
6012-4	>50000	>50000	>50000	93.5

ship (SAR) information obtained previously[10], we designed and synthesized 72 new β-amino-carbonyl analogs and determined their binding affinities to AR (Supplemental Table 1). There were 30 compounds that displayed relatively high binding affinities were evaluated for their effects on LNCaP cell proliferation (Figures 2A–2C) and cytotoxicity measurements in DU 145 cells. These assays were performed for compounds
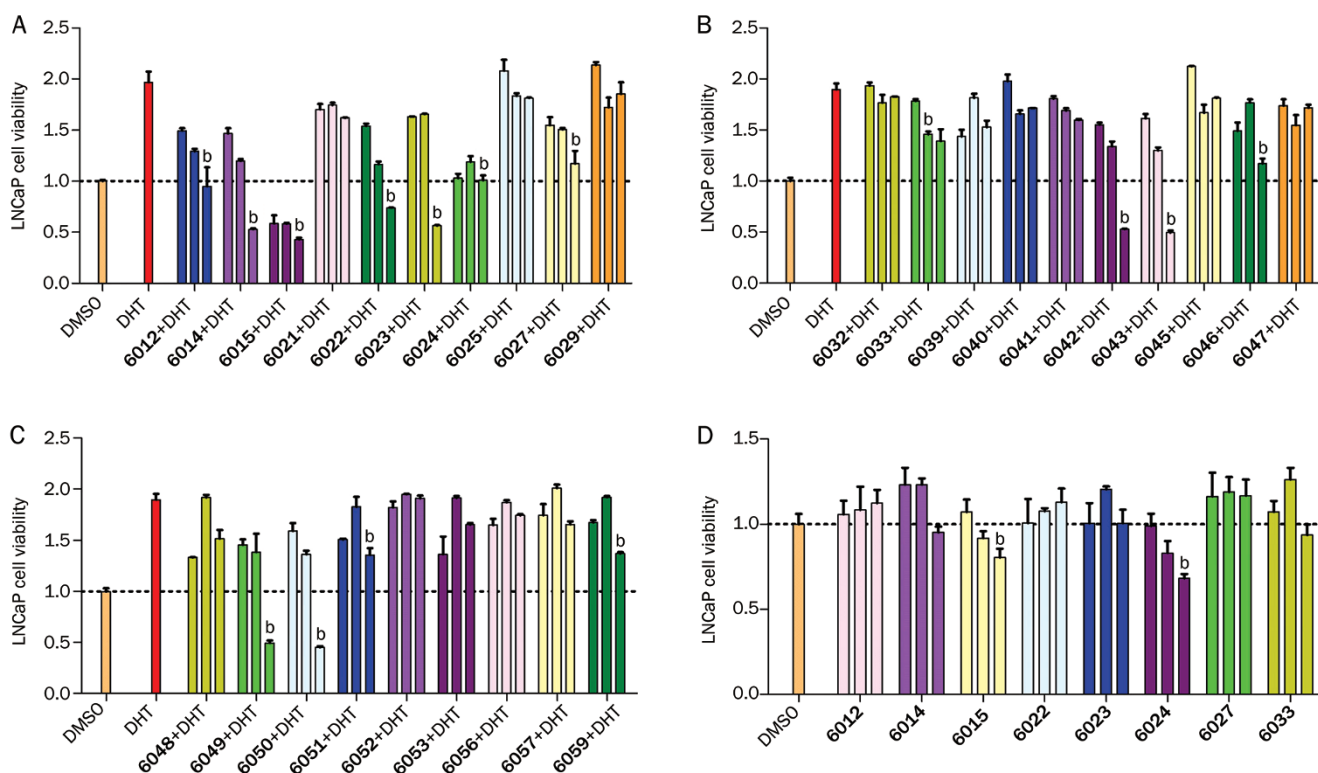
Figure 2. LNCaP cell proliferation and DU 145 cell cytotoxicity assays. (A, B, and C) LNCaP cells were seeded into poly-*L*-lysine-pretreated 96-well plates (1×10^5 cells per well) and incubated for 24 h at 37 °C. Compounds (from left to right: 100 nmol/L, 1 µmol/L, and 10 µmol/L) were added to the cells together with 10 nmol/L DHT, followed by incubation for another 5 d. Cell viability was measured using the CCK-8 kit. (D) DU 145 cells were seeded into 96-well plates (1×10^5 cells per well) and incubated for 48 h at 37 °C. Compounds (from left to right: 100 nmol/L, 1 µmol/L, and 10 µmol/L) were added and incubated with the cells for 3 more days. Cell viability was measured using the CCK-8 kit. The values are the relative cell growth compared with that of the cells treated with DMSO only. The data are representative of at least three independent experiments and are expressed as the mean±SEM. $^bP<0.05$ vs 10 nmol/L DHT (*t*-test). Casodex: bicalutamide.

6012, **6014**, **6015**, **6022**, **6023**, **6024**, **6027**, and **6033** (Figure 2D). DU 145 is a human prostate cancer cell line that lacks measurable AR expression and serves as a control for specificity and potential cytotoxicity. Although compounds **6050**, **6043**, **6042**, and **6049** inhibited DHT-induced LNCaP cell proliferation in a concentration-dependent manner, they all contain a ketoxime group (Figures 2A–2C). Other synthesized compounds with a ketoxime group also showed a similar inhibitory activity with this assay (data not shown), indicating a common cytotoxic nature. Therefore, these compounds were excluded from further studies.

As depicted in Figure 2, compound **6012** appeared to be an AR antagonist. Similar to compounds **21-1** and **21-2**, **6012** had no cytotoxic effect on LNCaP, DU 145, PC-3M, and HEK293 cells, as opposed to the control compound **6024** (Supplemental Figure 2). Follow-up studies identified compound **6012** as a full AR antagonist because it blocked AR nuclear translocation, NC interaction and DHT-induced PSA expression in LNCaP cells with a potency comparable to bicalutamide (Casodex). Compound **6012** did not display any agonist activity at concentrations up to 10 µmol/L. In contrast, compound **6024**, which has a similar structure to **6012**, did not show any AR–modulating activities (Figures 3A–3C).

Characterization of isomer 6012-4

Compound **6012** contains two chiral carbons, suggesting the existence of two groups of enantiomers. We isolated the four stereoisomers and studied their binding affinities for androgen, estrogen and glucocorticoid receptors. As shown in Table 1, isomer **6012-4** demonstrated the highest binding affinity for AR (IC$_{50}$=93.5 nmol/L) and had little cross-reactivity with estrogen receptor α, β and glucocorticoid receptor (>50 µmol/L). Interestingly, isomer **6012-1** displayed some binding capability to estrogen receptor α (IC$_{50}$=961.2 nmol/L), but did not bind androgen or glucocorticoid receptors (Table 1).

To validate the antagonist activity of isomer **6012-4**, we transiently transfected GFP-AR into HEK293 cells and stimulated them with isomer **6012-4** in the presence or absence of 10 nmol/L DHT. Isomer **6012-4** inhibited DHT-induced GFP-AR nuclear translocation at 10 µmol/L, without showing obvious agonist activity (Figure 4A). The mammalian two-hybrid assay demonstrated that isomer **6012-4** itself did not stimulate NC interaction (Figure 4B) but blocked 10 nmol/L DHT-induced NC interaction, with an IC$_{50}$ value of 5.36 µmol/L. This result is comparable to the data for bicalutamide (Casodex, IC$_{50}$=2.45 µmol/L). The IC$_{50}$ value of **6012-4** is less potent than that of cyproterone acetate (CPA, IC$_{50}$=88.8 nmol/L),
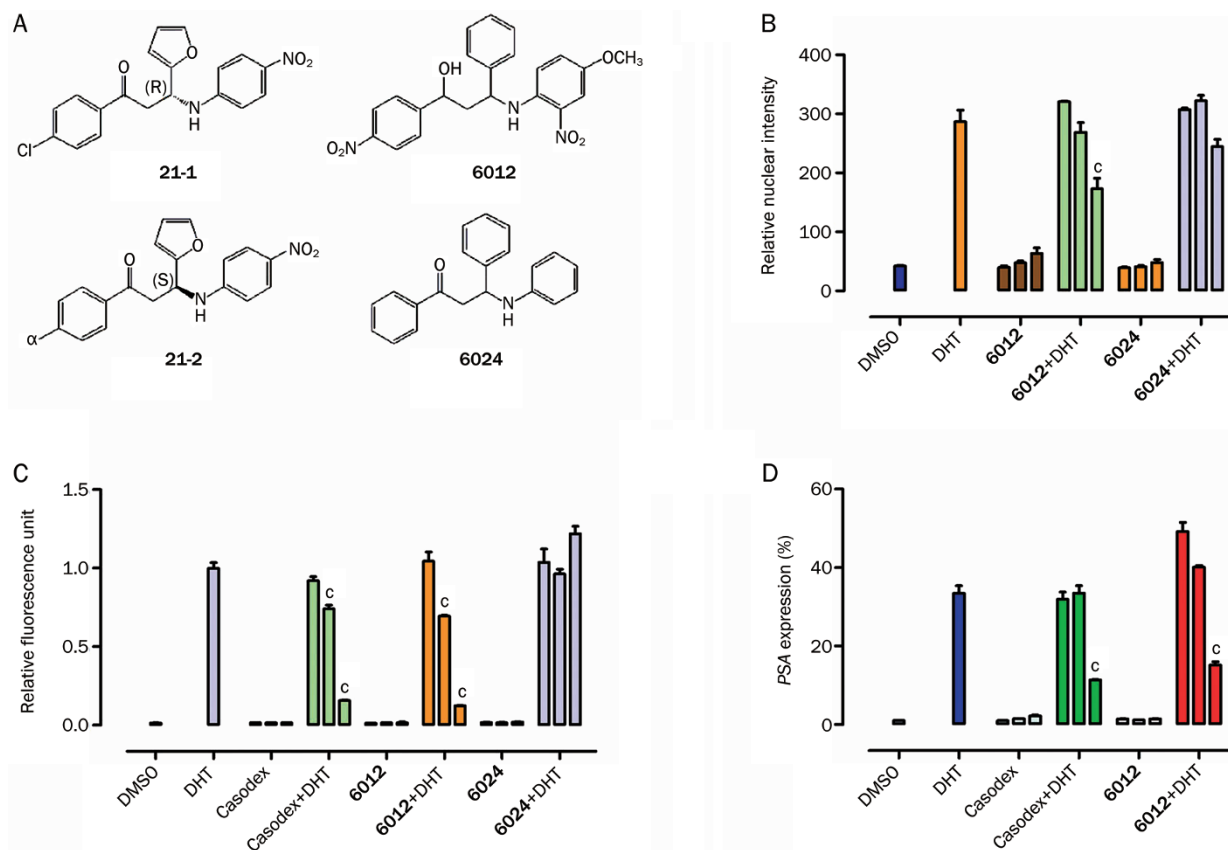
Figure 3. Bioactivities of compound **6012** on the androgen receptor signaling pathway. (A) Structure of compounds **21-1**, **21-2**, and **6012**. (B) Compound **6012** promoted androgen receptor nuclear translocation. (C) Activity of compound **6012** on androgen receptor NH_2- and COOH-terminal (NC) interaction. (D) *PSA* gene expression. For Figures B, C, and D, the compound concentrations were 100 nmol/L, 1 μmol/L, and 10 μmol/L, from left to right, with or without 10 nmol/L DHT. The data are representative of at least three independent experiments and are expressed as the mean±SEM. [c]$P<0.01$ compared with 10 nmol/L DHT (*t*-test). Casodex: bicalutamide.

a steroidal AR antagonist (Figure 4C). A gene expression analysis showed that isomer 4 significantly suppressed DHT-induced *PSA* and *KLK2* expression in LNCaP cells, whereas isomer **6012-3** displayed only a weak antagonistic activity at the same concentration (10 μmol/L; Figures 4D and 4E). In addition, isomer **6012-4** also inhibited DHT-induced LNCaP cell proliferation in a concentration-dependent manner, with an IC_{50} value comparable to that of bicalutamide (Casodex, 23.0 μmol/L *vs* 14.2 μmol/L; Figure 4F). An analysis of cell cycle status indicated that 56.8% of the DHT-treated cells were in G_0/G_1 phase and 29.4% of the cells were in S phase. Treatment with isomer **6012-4** increased the G_0/G_1 cell population from 62% to 72% in a concentration-dependent manner. Additionally, the S phase cell population was decreased from 24.8% to 17.0%. These results suggest that isomer **6012-4** prevented LNCaP cells from entering S phase (Figure 4G).

An *in vivo* study was conducted in SCID mice using daily intraperitoneal injections of isomer **6012-4** at a dose of 10 mg/kg. The treatment was able to suppress the growth of LNCaP xenografts introduced subcutaneously, and the effect was comparable to bicalutamide (Casodex, 10 mg/kg). Isomer

6012-3 did not show any inhibitory activity at the same dose (Supplemental Figure 3).

Configurations of the four isomers of 6012 and molecular modeling

Isomers **6012-1**, **6012-2**, **6012-3**, and **6012-4** were separated on a chiral column, and monocrystals were obtained from the mixed solution of ethyl acetate and petroleum ether. Isomer **6012-2** had NMR data similar to isomer **6012-3**, whereas isomer **6012-1** had data similar to isomer **6012-4**. These results indicate that there are two groups of enantiomers. Finally, the absolute configurations of isomers **6012-2**, **6012-3**, and **6012-4** were assigned as (1R, 3R), (1S, 3S), and (1R, 3S)-3-[(4-methoxy-2-nitrophenyl)amino]-1-(4-nitrophenyl)-3-phenylpropan-1-ol based on X-ray crystallography (Supplemental Figures 4 and 5). All four isomers docked into AR-LBD (2AXA), and isomer **6012-4** interacted with the androgen-binding groove of the receptor in a way similar to that of the reference AR modulator S1 (Figure 5A). In this binding model, the 4-nitrophenyl group on isomer **6012-4** pointed toward LBD active function 2 (AF2).
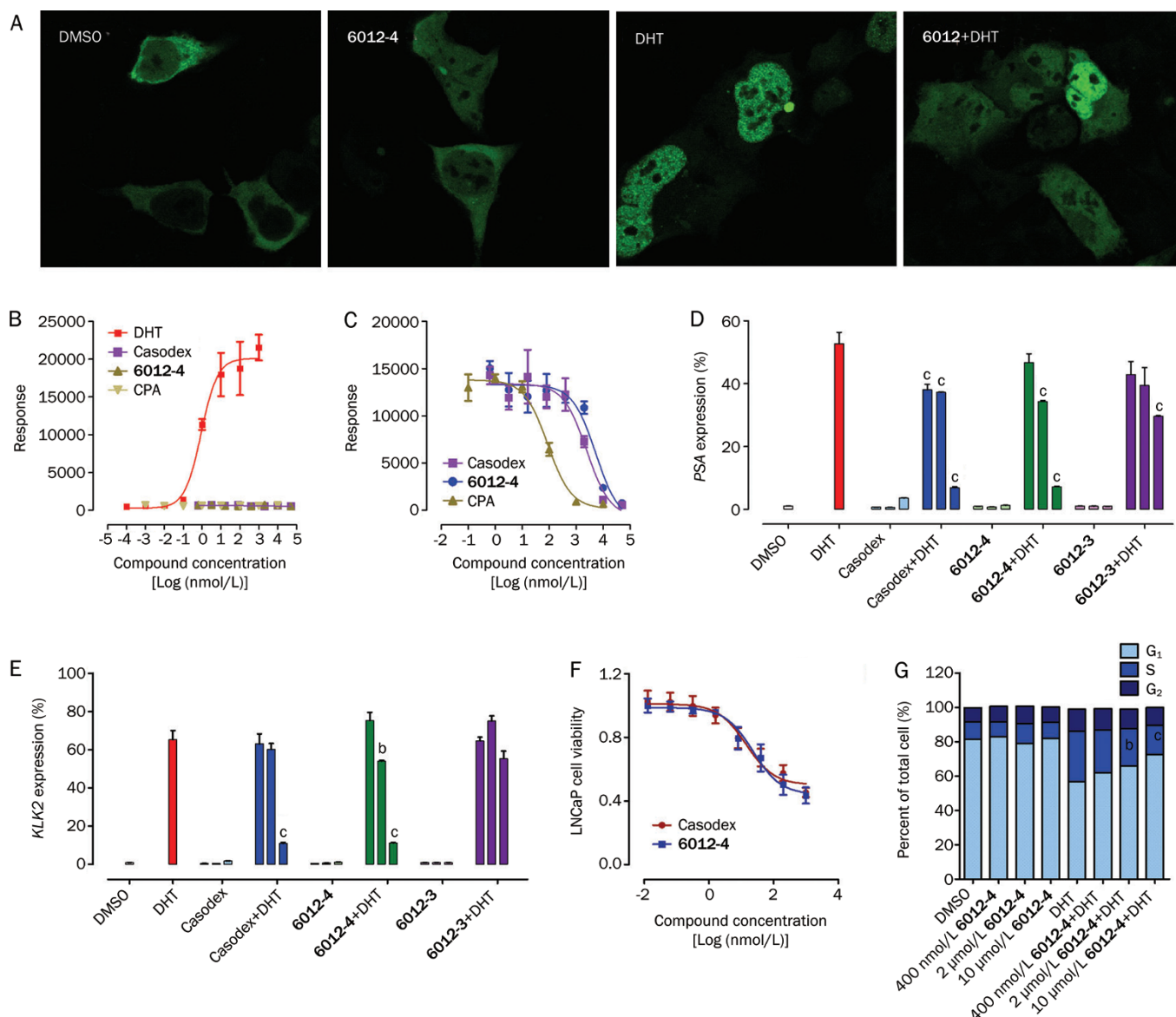
Figure 4. Characterization of isomer **6012-4** as an androgen receptor antagonist. (A) Isomer **6012-4** inhibited DHT-induced androgen receptor nuclear translocation in GFP-AR-transfected HEK293 cells. (B) Isomer **6012-4** showed no agonist activity on androgen receptor NH_2- and COOH-terminal (NC) interaction when tested with the mammalian two-hybrid assay. (C) Isomer **6012-4** antagonized DHT-induced androgen receptor NC interaction. (D) *PSA* expression. (E) *KLK2* expression. (F) Isomer **6012-4** inhibited DHT-induced LNCaP cell proliferation. (G) Cell cycle analysis of compound **6012-4**-treated LNCaP cells. The percentages of G_1 phase cells were compared. For Figures D and E, the compound concentrations were 400 nmol/L, 2 μmol/L, and 10 μmol/L, from left to right, with or without 10 nmol/L DHT. The data are representative of at least three independent experiments and are expressed as the mean±SEM. [b]$P<0.05$, [c]$P<0.01$ *vs* 10 nmol/L DHT (*t*-test). Casodex: bicalutamide.

Discussion

Our previous investigation led to the discovery of β-amino-carbonyl compounds as AR modulators. Guided by the results of receptor binding and reporter gene assays, a series of medicinal chemistry studies were conducted and resulted in the identification of compound **21** as a selective AR modulator both *in vitro* and *in vivo*[10]. In this study, we followed this lead and characterized the two isomers of compound **21** (compounds **21-1** and **21-2**). Our data indicate that the R configuration (compound **21-1**) is actually a weak AR antagonist, which binds to the LBD to form a conformation that can induce the nuclear translocation of AR but blocks NC interaction and inhibits AR-mediated gene expression. However, the compound **21** with an S configuration (compound **21-2**) is a partial agonist: it stimulated AR nuclear translocation, induced NC interaction and elicited downstream gene expression at a nanomolar level. This inspired us to selectively design and synthesize more β-amino-carbonyl analogs based on the previous SAR information. Of the 72 compounds generated, 30 compounds with relatively higher binding affinities were cho-
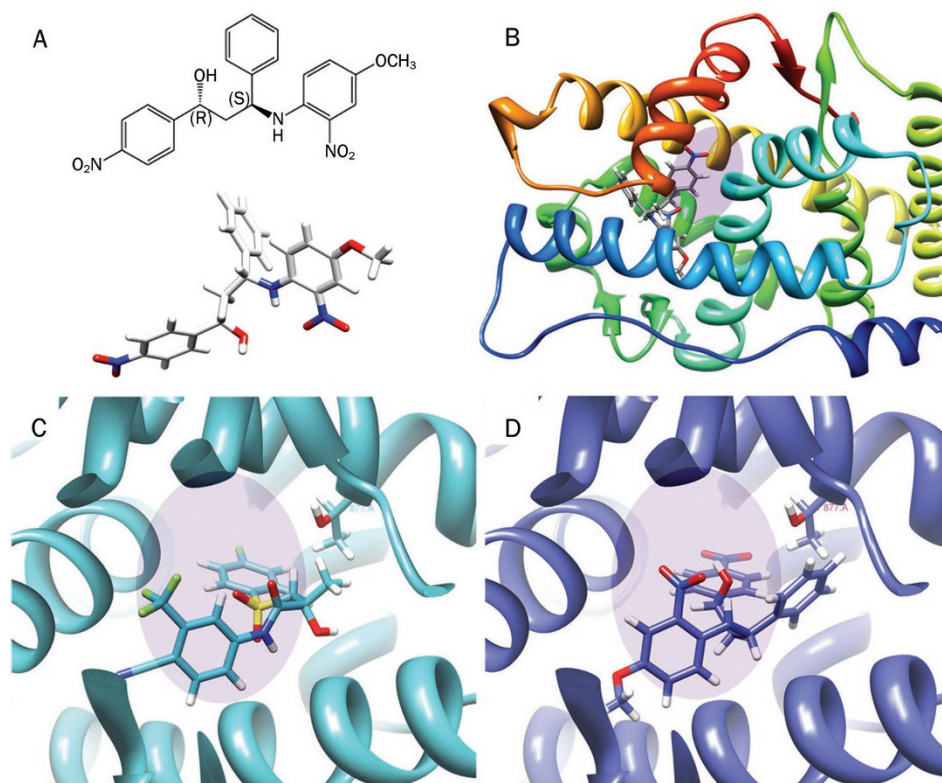
Figure 5. Molecular modeling of isomer **6012-4** to androgen receptor. (A) The structure of isomer **6012-4**. (B) Isomer **6012-4** is docked in the androgen receptor binding domain (2AXA). (C) Binding position of selective androgen receptor modulator S1 in the androgen receptor ligand-binding domain (AR-LBD) (2AXA). (D) Predicted binding position of isomer **6012-4** in AR-LBD. The shaded ellipse indicates the location of the androgen receptor active function 2 (AF2) domain.

sen to study their effects on LNCaP cell proliferation and cytotoxicity in DU 145 cells. LNCaP is an androgen-dependent prostate cancer cell line, and the growth of these cells is stimulated by DHT. In contrast, DU 145 is an androgen-independent cell line; thus, their growth is not influenced by AR modulators. Accordingly, we used DU 145 to assess the cytotoxic effects of the compounds in question. As described above, compound **6012** exhibited a high binding affinity to AR (IC_{50}=93.5 nmol/L), inhibited its nuclear translocation, blocked its NC interaction and suppressed DHT-induced *PSA* expression. This feature was not observed with its analog, compound **6024**, which was used as a control in this study and displayed cytotoxic activities in LNCaP, DU 145 and HEK293 cells.

There are two chiral carbons in compound **6012**, and we isolated its four stereoisomers on a chiral column and tested their binding properties with several steroid hormone receptors. It is evident that isomer **6012-4** is the most active among the four isomers and showed little cross-reactivity with estrogen and glucocorticoid receptors. These results imply that the bioactivities of compound **6012** mainly reside in isomer **6012-4**. LNCaP cells express several mutant ARs, including the well-known AR^{T877A} mutation[13]. Therefore, we transiently transfected GFP-AR, which encoding a wild-type AR, to assess its activity in the mammalian two-hybrid system in HEK293 cells.

Isomer **6012-4** was able to inhibit the nuclear translocation and NC interaction of GFP-AR, suggesting that it specifically blocks the activation of AR. Further validation experiments confirmed that similar to compound **6012**, isomer **6012-4** also suppressed DHT-induced *PSA* and *KLK2* expression in LNCaP cells. *PSA* and *KLK2* are upregulated by AR and are highly expressed in DHT-treated LNCaP cells[14, 15]. The inhibitory effects observed confirm that isomer **6012-4** blocked gene transcription mediated by AR.

The results of our cell proliferation assay demonstrate that isomer **6012-4** inhibited DHT-induced LNCaP cell growth in a concentration-dependent manner, which is a clear indicator of AR antagonism. Arresting the cell cycle prior to S phase suggests a common mechanism of the action for AR antagonists[16, 17]. A preliminary *in vivo* study showed that at a daily intraperitoneal dose of 10 mg/kg, isomer **6012-4** significantly inhibited the growth of subcutaneous LNCaP xenografts. The same regimen for isomer **6012-3** did not have any effect. Therefore, our *in vitro* and *in vivo* data suggest that isomer **6012-4** is an AR antagonist with comparable efficacy to the commercially available AR antagonist bicalutamide (Casodex).

We also solved the configuration of the four enantiomers of compound **6012**. By docking isomer **6012-4** to the LBD of AR, we found that isomer **6012-4** fits the receptor binding groove

and adopted a position similar to S1, a classical AR antagonist. Molecular modeling suggests that the 4-nitrophenyl group on **6012-4** points toward LBD active function 2 (AF2). It is known that AF2 is the binding pocket for the AR N-terminal FXXLF motif, and binding of which is a key process of AR activation. Because the 4-nitrophenyl residue of **6012-4** points toward this particular region of the receptor, it might be responsible for the blockage of NC interaction.

Although there are many anti-androgens reported in the literature, their mechanisms of action differ significantly. Anti-androgens such as Casodex block the interaction between the N-terminus and LBD of the receptor, whereas second-generation anti-androgens, such as MDV3100, inhibit the nuclear translocation and NC interaction of AR. Isomer **6012-4** resembles MDV3100 because it binds to AR and induces a conformation incapable of nuclear translocation and NC interaction.

In summary, we characterized the bioactivities of two isomers of compound **21** and showed that compound **21-1** (R) is a weak AR antagonist while compound **21-2** (S) is a partial agonist. Further structural modifications and functionality analyses led to the discovery of compound **6012** and its four stereoisomers. Isomer **6012-4** displayed desirable *in vitro* and *in vivo* activities as an AR antagonist.

Acknowledgements

Author contribution

Ming-wei WANG, Zhi-yun ZHANG, and Cai-hong ZHOU designed the research; Zhi-yun ZHANG, Yan-hui ZHU, and Yun-jun GE performed the research; Yan-hui ZHU, Hui-li LU, and Qing LIU contributed new reagents or analytic tools; Zhi-yun ZHANG, Cai-hong ZHOU, Qing LIU, and Ming-wei WANG analyzed the data; Zhi-yun ZHANG, Ming-wei WANG, Cai-hong ZHOU, and Qing LIU wrote the paper.

Supplementary information

Supplementary information is available at Acta Pharmacologica Sinica's website.

References

1 Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012; 62: 10–29.

2 Blackledge GR, Cockshott ID, Furr BJ. Casodex (bicalutamide): overview of a new antiandrogen developed for the treatment of prostate cancer. Eur Urol 1997; 31: 30–9.

3 Kolvenbag GJ, Blackledge GR, Gotting-Smith K. Bicalutamide (Casodex) in the treatment of prostate cancer: history of clinical development. Prostate 1998; 34: 61–72.

4 Tyrrell CJ, Kaisary AV, Iversen P, Anderson JB, Baert L, Tammela T, *et al*. A randomised comparison of 'Casodex' (bicalutamide) 150 mg monotherapy versus castration in the treatment of metastatic and locally advanced prostate cancer. Eur Urol 1998; 33: 447–56.

5 Müderris II, Bayram F, Ozçelik B, Güven M. New alternative treatment in hirsutism: bicalutamide 25 mg/day. Gynecol Endocrinol 2002; 16: 63–6.

6 Colabufo NA, Pagliarulo V, Berardi F, Contino M, Inglese C, Niso M, *et al*. Bicalutamide failure in prostate cancer treatment: involvement of Multi Drug Resistance proteins. Eur J Pharmacol 2008; 601: 38–42.

7 Wang Q, Li W, Zhang Y, Yuan X, Xu K, Yu J, *et al*. Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell 2009; 138: 245–56.

8 Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, *et al*. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science 2009; 324: 787–90.

9 Schaufele F, Carbonell X, Guerbadot M, Borngraeber S, Chapman MS, Ma AA, *et al*. The structural basis of androgen receptor activation: intramolecular and intermolecular amino-carboxy interactions. Proc Natl Acad Sci U S A 2005; 102: 9802–7.

10 Zhou C, Wu G, Feng Y, Li Q, Su H, Mais DE, *et al*. Discovery and biological characterization of a novel series of androgen receptor modulators. Br J Pharmacol 2008; 154: 440–50.

11 Ning M, Zhou C, Weng J, Zhang S, Chen D, Yang C, *et al*. Biological activities of a novel selective oestrogen receptor modulator derived from raloxifene (Y134). Br J Pharmacol 2007; 150: 19–28.

12 Bohl CE, Miller DD, Chen J, Bell CE, Dalton JT. Structural basis for accommodation of nonsteroidal ligands in the androgen receptor. J Biol Chem 2005; 280: 37747–54.

13 Sun C, Shi Y, Xu LL, Nageswararao C, Davis LD, Segawa T, *et al*. Androgen receptor mutation (T877A) promotes prostate cancer cell growth and cell survival. Oncogene 2006; 25: 3905–13.

14 Ishiguro H, Ishiguro Y, Kubota Y, Uemura H. Regulation of prostate cancer cell growth and PSA expression by angiotensin II receptor blocker with peroxisome proliferator-activated receptor gamma ligand like action. Prostate 2007; 67: 924–32.

15 Kang Z, Pirskanen A, Jänne OA, Palvimo JJ. Involvement of proteasome in the dynamic assembly of the androgen receptor transcription complex. J Biol Chem 2002; 277: 48366–71.

16 Bai VU, Cifuentes E, Menon M, Barrack ER, Reddy GP. Androgen receptor regulates Cdc6 in synchronized LNCaP cells progressing from G_1 to S phase. J Cell Physiol 2005; 204: 381–7.

17 Zhang J, Hsu B A JC, Kinseth B A MA, Bjeldanes LF, Firestone GL. Indole-3-carbinol induces a G_1 cell cycle arrest and inhibits prostate-specific antigen production in human LNCaP prostate carcinoma cells. Cancer 2003; 98: 2511–20.