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RESEARCH PAPER

Discovery and biological characterization of a novel series of androgen receptor modulators

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Background and purpose: Selective androgen receptor modulators are of great value in the treatment of prostate cancer. The purpose of this study was to provide a preliminary characterization of a new class of non-steroidal androgen receptor modulators discovered in a high-throughput screening campaign.
Experimental approach: Competitive receptor binding, luciferase-based reporter methods, cell proliferation and *in vivo* assays were employed to evaluate an initial set of compounds from chemistry efforts.
Key results: Forty-nine analogues from the chemistry efforts showed high affinity binding to androgen receptors, agonist and/or antagonist activities in both CV-1 and MDA-MB-453 transfection assays. A proliferation assay in LNCaP cells also exhibited this profile. A representative of these non-steroidal compounds (compound 21) was devoid of activity at other nuclear receptors (oestrogen, progesterone, glucocorticoid and mineralocorticoid receptors) in the CV-1 co-transfection assay. At the same time, in an immature castrated rat model, it behaved as an androgen receptor antagonist against the growth of prostate, seminal vesicles and levator ani induced by exogenous androgen. Separation of compound 21 into its enantiomers showed that nearly all the androgen receptor modulating activity and binding resided in the dextrorotatory compound (23) while the laevorotatory isomer (22) possessed weak or little effect depending on the cell type studied.
Conclusions and implications: These non-steroidal compounds may represent a new class of androgen receptor modulators for the treatment of not only prostate cancer but other clinical conditions where androgens and androgen receptors are involved in the pathological processes.
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Keywords: nuclear receptor; androgen; prostate cancer

Abbreviations: compound 21, 1-(4-chlorophenyl)-3-(furan-2-yl)-3-(4-nitrophenylamino)-propan-1-one; DHT, 5α-dihydrotestosterone; FBS, fetal bovine serum; MMTV, mouse mammary tumour virus; T, testosterone

Introduction

Prostate cancer, benign prostate hyperplasia, hirsutism, alopaecia, anorexia nervosa, breast cancer and acne are well known to be sensitive to androgens and to respond to androgen receptor antagonist therapy (Liao, 1994; Shaw, 2002; Zouboulis and Degitz, 2004; Gao *et al*., 2006). Prostate cancer is currently the most commonly diagnosed non-dermatologic cancer among male subjects in the United States. The American Cancer Society estimated that 218 890 men in the United States would be diagnosed with prostate cancer and 27 050 men would die of it in 2007 (Jemal *et al*., 2007). The androgen receptor is a ligand-regulated transcription factor in the nuclear receptor superfamily and is a key molecular target not only for the normal function of the prostate but also in the growth and progression of prostate cancer (Tsai and O'Malley, 1994; Zhou *et al*., 1994). Testosterone and 5α-dihydrotestosterone (DHT) are the two predominant naturally occurring androgens that bind to androgen receptors and subsequently activate androgen-responsive genes. This activation can be blocked by androgen receptor antagonists through competitive inhibition of androgen binding to the receptor (Tindall *et al*., 1984; Moguilewsky and Bouton, 1988; Singh *et al*., 2000).

There are two types of androgen receptor antagonists, steroidal and non-steroidal. Non-steroidal agents, such as flutamide (Eulexin) and bicalutamide (Casodex) (Figure 1a; 1 and 2), are preferred, because steroidal antiandrogens, for example, cyproterone acetate (Figure 1a; 3), in general,

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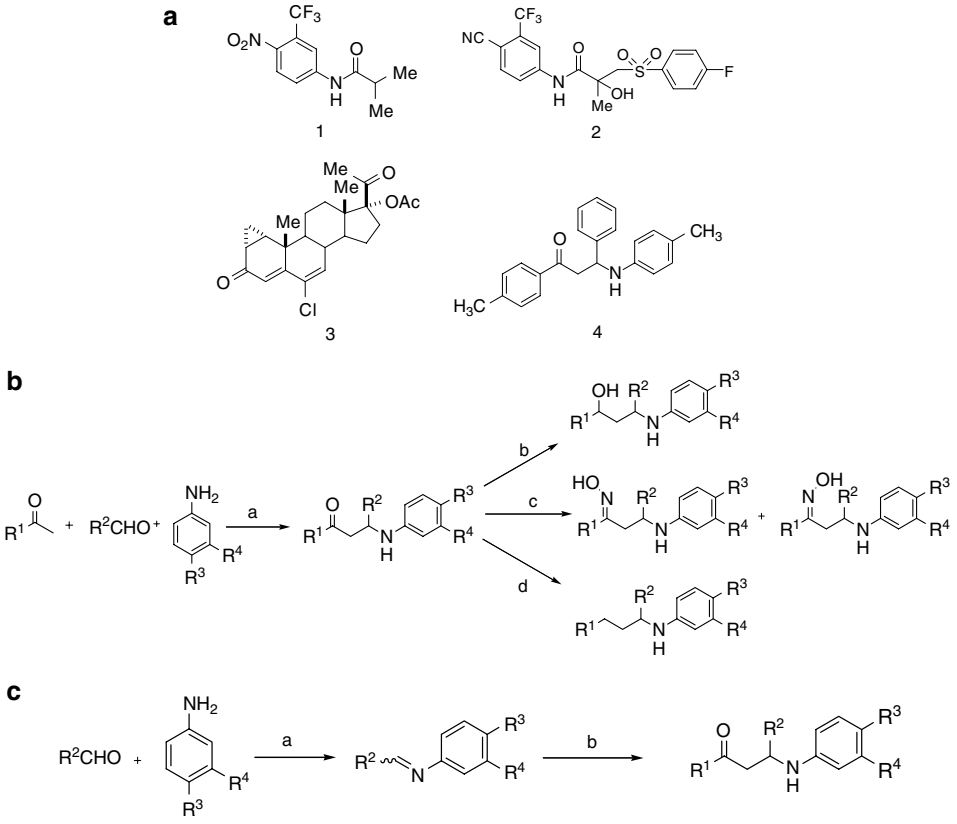


Figure 1 (**a**) Structures of various androgen receptor antagonists and an initial high-throughput screening hit. (**b**) Reagents and conditions: a, conc. HCl, EtOH, 0−50°C, overnight; b, $NaBH_4$, MeOH, 0°C to room temperature, 2 h; c, $NH_2OH.HCl$, pyridine, room temperature, overnight; d, H_2NNH_2, KOH, H_2O, 140–160°C, 1 h. (**c**) Reagents and conditions: a, toluene, reflux, 32 h; b, 4-nitroacetophenone (3-nitroacetophenone), conc. HCl, EtOH, 70°C, 30 min.

induce adverse effects owing to crossreactivity at other steroid hormone receptors (Hamann *et al*., 1995). Of the androgen receptor antagonists currently marketed or undergoing clinical trials, none achieves effective therapeutic results without efficacy-limiting side effects (Haddad, 2006). In addition, severe gynaecomastia, nausea, diarrhoea and liver toxicity have been observed in many patients, and the majority of prostate cancer patients receiving antiandrogen therapy ultimately become resistant. Indeed, this resistance is characteristic of what is known as the 'antiandrogen withdrawal syndrome (AWS)' where cessation of treatment results in a dramatic drop in blood prostate-specific antigen levels. In this case, resistance manifests itself as antiandrogens acting as agonists on androgen receptors (Kelly and Scher, 1993; Kelly *et al*., 1997; Paul and Breul, 2000). Clearly, there is an unmet medical need for the treatment of prostate cancer. Thus, we set out to find novel, non-steroidal androgen receptor antagonists that may lead to more effective therapies.

Non-steroidal pure antiandrogens have been used in the clinic for a number of years, with flutamide and bicalutamide being the best-known examples. Recently, there have been attempts to apply what has been discovered with selective oestrogen receptor modulators to the area of androgen-based therapeutics resulting in selective androgen receptor modulators (Rosen and Negro-Vilar, 2002; Kearbey *et al*., 2007). The idea here is that the binding of a ligand to androgen receptors will be different from the binding of the natural hormone such that the structural changes occurring within the receptor are somewhat different, leading to altered cofactor recruitment patterns that will be dependent upon the cell type (Rosen and Negro-Vilar, 2002). As a result, certain desirable activities will be retained whereas undesirable ones (side effects) will be reduced or eliminated. This approach has been well documented with oestrogen receptor modulators where undesirable mammary gland effects have been removed (Swaby *et al*., 2007). Fulvestrant (Faslodex) that induces the degradation of oestrogen receptors (Reid *et al*., 2003; Buzdar, 2004) is a typical example of such an endeavour.

There have been a number of recent reports on the synthesis of novel, non-steroidal androgen receptor agonists that possessed reduced activities on the prostate and associated tissues (for example, seminal vesicles) while maintaining other desirable effects such as bone-building properties (Higuchi *et al*., 1999; Kong *et al*., 2000; Rosen and Negro-Vilar, 2002; Higuchi *et al*., 2007; Kearbey *et al*., 2007). Thus the precedent to apply what has been learned in the oestrogen receptor modulators area to androgen receptor modulators seems secure.

In this paper, we report the high-throughput screening (HTS) of 16 000 synthetic or natural compounds from a diverse chemical library, the discovery, preliminary structural modifications and biological characterization, both *in vitro*

and *in vivo*, of a novel series of androgen receptor modulators based on the core structure of the initial HTS hit, 3-(phenylamino)-propan-1-one pharmacaphore (Figure 1a; 4).

Methods

Receptor-binding assay

Baculovirus-derived androgen receptor extract protein ($\sim 60\,mg\,L^{-1}$) was loaded into each well of Isoplate (Perkin Elmer, Boston, MA, USA) containing the assay buffer (10% glycerol (v/v), NaH_2PO_4 25 mM, $NaMoO_4$ 10 mM, KF 10 mM, dithiothrietol 1.6 mM, EDTA 1.5 mM, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate 2 mM, phenylmethanesulphonylfluoride 1 mM, aprotinin $5\,mg\,L^{-1}$ and leupeptin $5\,mg\,L^{-1}$), followed by [^{3}H]DHT ($110\,Ci\,mM^{-1}$, 5 nM) and various concentrations of test compounds or DHT (2.5 μL), to give 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 the next morning and the plates were gently agitated twice for 5 min each. Following centrifugation at 2500 r.p.m. 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 radioactivity was measured by a MicroBeta counter (PerkinElmer). Nuclear receptor crossreactivity binding assays, including oestrogen receptor-α, progesterone receptor, glucocorticoid receptor and mineralocorticoid receptor, were performed as described previously (Ning *et al*., 2007).

High-throughput screening procedure

To apply the HA binding assay to HTS under the optimal androgen receptor concentration, both maximal binding (MB; dimethyl sulphoxide (DMSO)) and nonspecific binding (1 μM DHT) were assessed and the Z' factor was calculated according to the literature (Zhang *et al*., 1999).

The compound library used for screening consists of 16 000 pure synthetic compounds and natural products. A 10-compound pool per well mix was applied to the primary screening (HA assay), with an average concentration of 8 μM for each compound dissolved in 100% DMSO solution. This X–Y matrix system maximizes the advantage of HTS and allows duplicate screening of each compound for internal confirmation of a potential hit. In each 96-well Isoplate, 16 wells of rows 1 and 12 were used as negative (DMSO) and positive (DHT) controls, respectively. Samples showing greater than 85% inhibition were considered 'hits'. Positive compounds were re-screened and confirmed 'hits' were further studied by determining binding dose–response curves.

Reporter gene assay

Transient co-transfection assay was performed in monkey kidney fibroblast CV-1 cells according to the method described previously (Ning *et al*., 2007). Briefly, CV-1 cells were seeded in 6-cm dish (6×10^5 cells per dish) in the presence of Dulbecco's modified Eagle's medium supplemented with 10% charcoal/dextran-treated fetal bovine serum for 24 h before being transfected with Fugene 6. Two micrograms of the reporter plasmid (MMTV-Luc) and 0.4 μg of pSVAR0 were introduced simultaneously into cells and incubated for 8 h. Cells were then harvested with 0.05% trypsin and 0.02% EDTA prior to reseeding onto a 96-well microtitre plate (8000 cells per well). They were incubated for 24 h with or without various concentrations of control or test compounds. For antagonist assay, test samples were added 30 min ahead of DHT. Cell extracts were prepared and the expressed luciferase activity was determined in a Wallac 1420 multilabel counter (VICTOR^2, PerkinElmer) using a Steady-Glo luciferase kit from Promega (Madison, WI, USA). To detect potential cytotoxicity of compounds, treated cells were reacted with AlamarBlue (United States Biological, Swampscott, MA, USA) for 4 h and the fluorescence is monitored at 540 nm excitation wavelength and 590 nm emission wavelength on a FlexStation 384^{II} (Molecular Devices, Sunnyvale, CA, USA) prior to luciferase activity measurement.

The human breast carcinoma cell line MDA-MB-453 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). For the construction of reporter plasmid stably transfected cell line, 2 μg of MMTV-Luc and 0.2 μg of pSFFV.neo vector were transfected into 1×10^5 MDA-MB-453 cells in a 6-cm cell-culture dish. On the second day, the cells were split into three 100-mm cell-culture plates and $400\,\mu g\,mL^{-1}$ geneticin (Merck KGaA, Darmstadt, Germany) was added to the cell culture on the third day. The selection medium (Leibovitz's L-15) containing 10% FBS in the presence of $800\,\mu g\,mL^{-1}$ geneticin was changed every 3 days until colonies were formed. The single colony was picked up and the best responder selected based on its significant reactions upon stimulation by different concentrations of DHT. The stable MDA-MB-453 cell line expressing MMTV-Luc reporter gene was used to evaluate the androgen receptor transactivation activity with a similar procedure to that described above except that cells were seeded onto 96-well plates at a density of 2×10^4 cells per well.

In both the CV-1 and MDA-MB-453 cell-based assays, the antagonist mode was run in the same manner except that DHT (2 nM for CV-1 cells and 5 nM for MDA-MB-453 cells) was incorporated into all the wells to establish an agonistic baseline against which the compounds were tested. For these assays, the intra-assay coefficient of variation was within 15% and that of inter-assay was below 30%.

Cell proliferation assay

Human prostate carcinoma cell line LNCaP was maintained in RPMI-1640 medium supplemented with 10% FBS. *In vitro* cell proliferation assay was performed as previously described with minor modifications (Culig *et al*., 1999; Chen *et al*., 2004). Briefly, the medium was changed to phenol red-free RPMI-1640 with charcoal/dextran-treated FBS 48 h before assaying. Medium (100 μL) containing 5000 LNCaP cells per well in a 96-well microtitre plate was incubated at 37°C in a humidified atmosphere of 5% CO_2 overnight. Various concentrations of DHT were added to the medium with or

without 10 μM test compounds and incubated for 72 h. Medium containing the same amount of DHT and test compounds was changed once and incubation was continued for an additional 72 h. Absorbance was detected with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Human prostatic carcinoma cell line PC3 was maintained in RPMI-1640 containing 10% FBS. Cells were plated at a density of 5000 per well onto 96-well plates and incubated overnight. Different concentrations of test compounds were introduced to the cells and incubated for 3 days before addition of [^{3}H]thymidine (0.15 μCi per well) followed by incubation for 2 h (Venkateswaran *et al*., 2002). They were then washed with ice-cold PBS once, 5% ice-cold trichloroacetic acid twice, lysed with 0.3 M NaOH (75 μL) for 5 min and terminated by the addition of 0.3 M HCl (75 μL). Fifty microlitres of cell lysate was transferred to a scintillation counting tube and 0.5 mL of Ecoscint A was added to each tube. Incorporation of [^{3}H]thymidine into DNA was measured with a MicroBeta counter (PerkinElmer). Mouse mammary gland tumour cell line SC3 (Minesita and Yamaguchi, 1965) was maintained in minimum essential medium supplemented with 10 nM T and 2% FBS. The medium was changed to serum-free Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA, USA) medium containing 0.1% BSA before plating onto 96-well plates at a density of 2500 cells per well. After an overnight incubation, different concentrations of test compounds were added and incubated with the cells for 3 days. Thereafter, cells were labelled with [^{3}H]thymidine (0.15 μCi per well) and incubated for 2 h. Cell proliferation was assayed as above. IC_{50} values were calculated as the concentrations of compounds required to give half-maximal inhibition on the cell proliferation. Compound efficacy is expressed as the maximum response produced by a test compound in comparison with that elicited by positive controls (100%; hydroxyflutamide for SC3 cells and taxotere for PC3 cells).

Animals and treatment

Three-week-old male Sprague–Dawley rats were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China) and housed at 22°C under a standard light/dark cycle (12:12) without the restriction of food and water. They were allowed to adapt to the new environment for at least 3 days. Castration was carried out with a parallel sham group, which underwent the surgical procedure without removal of testes. Following a 6-week recovery period, rats were randomly divided into six groups (a minimum of eight animals per group): (1) sham-operated group received vehicle alone (Sham); (2) castrated group received vehicle alone (control); (3) castrated animals received s.c. injected testosterone ($0.25\,mg\,kg^{-1}\,day^{-1} \times 10$); (4) castrated animals received s.c. injected testosterone ($0.25\,mg\,kg^{-1}\,day^{-1} \times 10$) and an oral dose of Casodex ($25\,mg\,kg^{-1}\,day^{-1} \times 10$); (5) castrated animals received s.c. injected testosterone ($0.25\,mg\,kg^{-1}\,day^{-1} \times 10$) and i.p. injected 1-(4-chlorophenyl)-3-(furan-2-yl)-3-(4-nitrophenylamino)-propan-1-one (compound 21) ($100\,mg\,kg^{-1}\,day^{-1} \times 10$); (6) castrated animals received s.c. injected testosterone ($0.25\,mg\,kg^{-1}\,day^{-1} \times 10$) and i.p. injected compound 21 ($200\,mg\,kg^{-1}\,day^{-1} \times 10$). Twenty-four hour after the last dosing, the rats were killed and the prostate, seminal vesicles and levator ani were removed. The dry weights of these tissues were determined and comparisons made between the groups.

Chemical synthesis and analysis

The preparation of most 3-(phenylamino)-propan-1-one analogues with the general structure listed in Table 2 is shown in Figure 1b. The target compounds were conveniently synthesized by Mannich reaction between various substituted acetones, aldehydes and anilines via acid catalysis (Lin *et al*., 1991). The total yield for these compounds was between 61 and 92%. Reduction of the 3-(phenylamino) -propan-1-one analogues using different conditions afforded compounds 5, 11, 12, 30 and 49. Reactions between 3-(phenylamino)-propan-1-one analogues and hydroxylamine hydrochloride in the presence of pyridine afforded the compounds 29, 37 and 39. The designed 1-nitrophenyl-3-(phenylamino)-propan-1-one analogues were prepared by reacting the Schiff base derived from aniline with acetophenone derivatives under heating (Figure 1c).

Forty-nine analogues based on the 3-(phenylamino)-propan-1-one pharmacophore (4) were designed and synthesized as above. They were dissolved in DMSO and stored at −20°C before use. All target compounds possessed purity superior to 90%. Nuclear magnetic resonance spectra were obtained on a Varian Mercury-VX300 or 400 MHz apparatus using $CDCl_3$ or DMSO-d_6 solutions as indicated and are reported in p.p.m. relative to internal solvent signal, with coupling constants (*J*) in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Mass spectrometry was carried out on a PerSeptive Biosystems Mariner. Analytical HPLC was performed on an Agilent 1100 series with DAD detector using a Zorbax SB C18 column with an ACN/H_2O solvent. Chiral analysis and separation were performed on the same Agilent system using a chiral CD-PH column, with an ACN/H_2O solvent. Microanalyses were performed with a Elementor Vario-EL elemental analyser for C, H, N, and the results were within ± 0.4% of the theoretical value (Supplementary information 1).

Chiral separation of compound 21

The isolation of two enantiomers of compound 21 was carried out on an Agilent 1100 HPLC system with a chiral CD-PH column (4.6 × 250 mm, 5 μm; Shiseido Fine Chemicals, Tokyo, Japan), using CH_3CN-H_2O (6:4) as solvents. Isomer 22 (2 mg) and isomer 23 (2 mg) were obtained by collecting the eluates at about 20.00 and 20.45 min in repeated HPLC runs. Values of specific rotation of them were measured with a PerkinElmer 342 polarimeter and were −23 (*c* 0.1, $CHCl_3$) for isomer 22 and + 20 (*c* 0.1, $CHCl_3$) for isomer 23, respectively.

Statistical analysis

Statistical analysis with Student's *t*-test was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA)

and data are presented as mean ± s.e.mean. The criterion for significance was a probability of less than 0.05 or 0.01.

Reagents

Dihydrotestosterone was purchased from Sigma Chemical Co. (St Louis, MO, USA). Casodex was provided by Professor Lianzhi Chen of China Pharmaceutical University (Nanjing, China). Dulbecco's modified Eagle's medium, RPMI-1640 medium and Leibovitz's L-15 medium were procured from Invitrogen Corporation. FBS and charcoal/dextran-treated FBS were bought from Hyclone (Logan, UT, USA). Fugene 6 transfection reagent was the product of Roche (Indianapolis, IN, USA) and Steady-Glo luciferase kit of Promega. Alamar-Blue was obtained from United States Biological. [^{3}H]DHT, [^{3}H]estradiol and [^{3}H]thymidine were purchased from PerkinElmer. [^{3}H]progesterone, [^{3}H]dexamethasone and [^{3}H]aldosterone were supplied by GE Healthcare UK Ltd (Buckinghamshire, UK). Nuclear receptors of human origin used in the binding assays were produced according to the method reported previously (Wu *et al*., 2005). Human androgen receptor (pSVAR0) plasmid was kindly provided by Dr AO Brinnkmann of Erasmus University Medical Center Rotterdam (Rotterdam, The Netherlands) and a luciferase reporter plasmid (MMTV-Luc) by Dr Donald McDonnell of Duke University (Durham, NC, USA). The pSFFV.neo vector was a gift from Dr RD Ye of University of Illinois (Chicago, IL, USA).

Results

Assay validation and HTS parameters

In the present study, we first used various concentrations of androgen receptor protein preparation in the HA androgen receptor-binding assay and the optimal protein concentration was determined as 60 mg L^{-1} (1:30 of the stock solution), which allowed the assay to possess a specific binding window of 2000 c.p.m. (Figure 2a). Under the above optimized assay condition, the IC_{50} value for the natural androgen receptor ligand, DHT, was measured to be approximately 4 nM (Figure 2b). When maximal binding (MB; DMSO) and nonspecific binding (1 μM DHT) were assessed, the assay displayed a signal-to-background ratio of 5. Coefficient of variation (CV) values were 5.4% for MB and 7.9% for nonspecific binding, respectively. The *Z′* factor, which estimates the suitability to HTS, was calculated to be 0.75 (Figure 2c).

High-throughput screening

Of the 16 000 samples initially screened, 130 'hits' (0.81%) showed greater than 85% competitive inhibition on DHT binding to androgen receptor (AR). Secondary (single

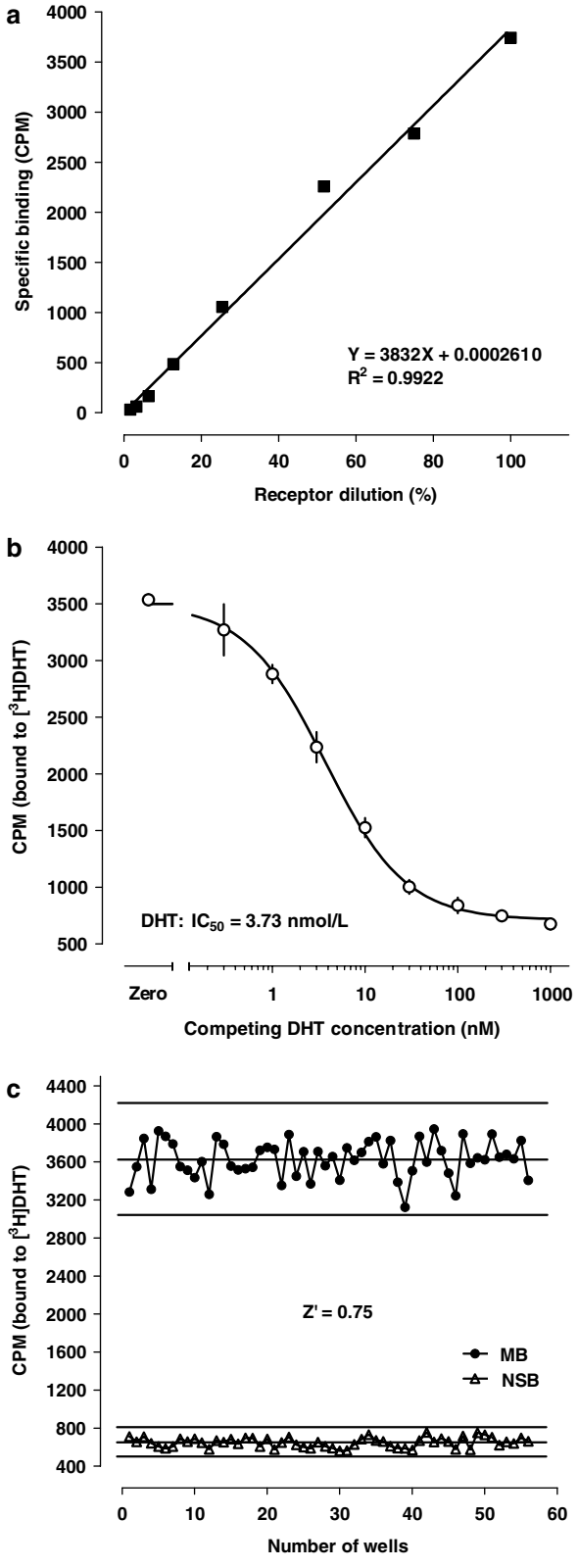


Figure 2 High-throughput screening assay validation. (**a**) Specific-binding ($CPM_{MB}-CPM_{NSB}$) of dihydrotestosterone (DHT) with different amounts of androgen receptor. Serial titration of androgen receptor protein was made to determine the optimal protein concentration. (**b**) Androgen receptor-binding characteristics of DHT measured by the hydroxyapatite (HA) assay with optimal assay conditions, from which IC_{50} value was calculated ($n=3$, mean ± s.e.mean). (**c**) *Z′* factor determination. Assays were performed at the optimized conditions and 56 replicates of specific and background signals were studied. Lines indicate means and means ± 3 × s.d. of the 56 data points.

Table 1 The androgen receptor binding IC_{50} values of the confirmed 'hits'

Compound	*IC_{50} (nM)*	*Compound*	*IC_{50} (nM)*
SH00011659	1632	SH00011867	2959
SH00011635	1540	NPLC0024	809.8
SH00012452	2486	SH00014099	889.0
SH00012299	542.9	SH00017949	1157
SH00110817	1414	SH00000756	172.7
SH00106147	1639	SH00000725	371.8
SH00010288	1717	SH00000642	221.4

compound per well) screening confirmed that 14 of the above 'hits' displayed consistent inhibitory effects with IC_{50} values between 150 and 3000 nM (Table 1). Of these, compound SH00000725 (Figure 1a; 4) was chosen for further chemistry development due to its consistent AR antagonist activities in the CV-1 cell assay.

AR-binding properties

Forty-nine analogues were designed and synthesized based on the core structure of the initial hit SH00000725 (4). Screening results of these compounds are illustrated in Table 2 showing androgen receptor-binding affinities (IC_{50} values) and their agonist effects in transfected CV-1 and MDA-MB-453 cells. Nine analogues demonstrated better binding potencies than dihydrotestosterone. Clearly, the best results so far are indicated for R^1 = haloaromatic, R^2 = phenyl or one of its isosteres and between R^3 and R^4, and R^3 substitution with an electronegative group (NO_2 for example). In addition, as seen in Table 2, CNOH substitutes for the keto moiety did not significantly alter activities. Among the compounds in Table 2, a number of them were selected for further biological evaluation based on the general criteria described below.

Transcriptional activities on AR

Thirty analogues of compound 4 shown in Table 2 possessed androgen receptor-binding activity (IC_{50}) of less than 200 nM and/or weak or no agonist activity (efficacy $<40\%$) in CV-1 or MDA-MB-453 cell assays. This suggested that they may be acting as antagonists. All of them, including compounds that displayed special structural interest such as the enantiomeric pair 22 and 23 and the diastereomeric pair 11 and 12, were subsequently tested in the antagonist mode with both cell lines and the results are presented in Table 3. DHT was used as the standard steroidal androgen receptor agonist and Casodex as a standard non-steroidal androgen receptor antagonist. Some of these compounds, while exhibiting weak agonist effects, demonstrated reasonable antagonistic effects indicating that subtle changes in the structure could switch between agonist and antagonist activities. For example, changing the R^3 group in compound 7 from NO_2 to CN in compound 8 resulted in the total loss of agonist activity in CV-1 cells. For compounds such as 13, 23, 24, 28, 45 and 46, there was considerable variation in their agonist activities between these two cell lines (Table 2), but some (compounds 13, 23 and 24) displayed noticeable antagonist activities in MDA-MB-453 cells. Of the 30 analogues, eight showed comparable potency to that of benchmark, Casodex. Among these, compound 21 was chosen for further characterization due to its weak agonist (12% efficacy) and moderate antagonist activities (82% inhibition) in MDA-MB-453 cells. As this class of analogues can exist as enantiomers, compound 21 was separated into its D and L isomers and evaluated independently. Nearly all the androgen receptor-modulating activity and binding resided in the dextrorotatory compound (23) whereas the laevorotatory isomer (22) possessed weak or little effect depending on the cell type studied (Figures 3a and b; Tables 2 and 3).

Inhibition of androgen receptor-mediated cell proliferation

SC3 cell is a mouse mammary cell line that responds well to androgens with increased growth (Minesita and Yamaguchi, 1965). PC3 cell is a human prostate cancer cell line that lacks measurable androgen receptors and can serve as a control for specificity and potential cytotoxicity (Venkateswaran *et al*., 2002). Table 4 demonstrates that compounds 21 and 26 behaved as androgen receptor antagonists with full efficacy in SC3 cells whereas their effects on PC3 cell proliferation were very weak with greatly reduced efficacy, indicating little or no toxicity. Compound 26 was selected in this assay as, although structurally similar to all the analogues, it lacked biological activity in transfection assays and could serve as a general cytotoxicity control. Figure 3c depicts the effects of compound 21 and Casodex on androgen-induced proliferation in LNCaP cells. Like DHT, compound 21 significantly increased proliferation of these cells but to a lesser extent (data not shown). In the presence of varying concentrations of DHT, compound 21, like Casodex, was able to markedly block the effect of DHT ($P<0.05$ or 0.01) indicating that it is acting as a partial AR antagonist in this assay. The effect of compound 21 on the expression of prostate-specific antigen in LNCaP cells was also investigated: the compound induced prostate-specific antigen expression when DHT was absent but it significantly reduced prostate-specific antigen levels in the presence of DHT (Supplementary information 2).

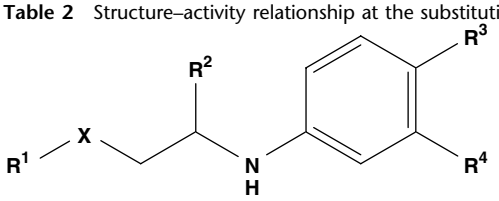
Crossreactivity with other nuclear receptors

The crossreactivity of compound 21 with related nuclear receptors was assessed using human progesterone, oestrogen, glucocorticoid and mineralocorticoid receptor-binding and co-transfection assays. Although weak crossreactivity was detected with progesterone (180.4 nM) and glucocorticoid (531.7 nM) receptor-binding assays, at concentrations up to 10 μM, no agonist or antagonist activity was observed for compound 21 except against androgen receptors in the CV-1 co-transfection assays. This result suggests that 3-(phenylamino)-propan-1-one analogues were highly selective for androgen receptors and were at least to 86-fold more potent on androgen receptors than any other nuclear receptor in receptor-binding assays.

Suppression of androgen actions in vivo

Compound 21 was subsequently tested in castrated immature male rats for its ability to inhibit prostate, seminal

Table 2 Structure–activity relationship at the substitutions of R^1, R^2, R^3 and R^4



Compound	*R^1*	*R^2*	*X*	*R^3*	*R^4*	*Binding*[a]	*Agonist activity in CV-1*[b]		*Agonist activity in MDA-453*[c]	
						IC_{50} (nM)	*EC_{50} (nM)*	*Efficacy (%)*	*EC_{50} (nM)*	*Efficacy (%)*
DHT						7.3	0.7	100	1.2	100
4	4-CH_3-phenyl	Phenyl	C=O	CH_3	H	1640	NA	NA	NA	NA
5	4-Cl-phenyl	2-furanyl	CHOH	NO_2	H	20.4	NA	NA	383.7	23
6	4-NO_2-phenyl	Phenyl	C=O	CN	H	32.6	1964	27	955.8	21
7	4-Cl-phenyl	Phenyl	C=O	NO_2	H	2.6	21.4	126	589.4	81
8	4-Cl-phenyl	Phenyl	C=O	CN	H	15.8	NA	NA	NA	NA
9	4-NO_2-phenyl	4-Cl-phenyl	C=O	NO_2	H	14.3	28.8	9.0	547.2	10
10	4-NO_2-phenyl	Phenyl	C=O	NO_2	H	21.6	1176	26	NA	NA
11	4-NO_2-phenyl	Phenyl	CHOH	OCH_3	2-NO_2	262.3	NA	NA	NA	NA
12	4-NO_2-phenyl	Phenyl	CHOH	OCH_3	2-NO_2	334.8	2238	11	NA	NA
13	5-Br-2-thienyl	Phenyl	C=O	Cl	NO_2	10.8	2857	60	NA	NA
14	4-Br-phenyl	Phenyl	C=O	NO_2	H	2.8	309.9	89	2126	61
15	4-Cl-phenyl	Phenyl	C=O	NO_2	CF_3	9.5	59.6	119	84.4	46
16	4-Cl-phenyl	Phenyl	C=O	Cl	H	19.0	976.2	50	8335	28
17	4-Cl-phenyl	Phenyl	C=O	Cl	CF_3	13.9	303.2	105	9700	47
18	4-Cl-phenyl	Phenyl	C=O	Cl	NO_2	8.1	14.2	143	3812	82
19	4-Cl-phenyl	4-Cl-phenyl	C=O	NO_2	H	71.0	NA	NA	NA	NA
20	4-Br-phenyl	Phenyl	C=O	Br	CF_3	10.3	41.7	39	NA	NA
21	4-Cl-phenyl	2-furanyl	C=O	NO_2	H	2.9	26.0	96	2591	12
22	4-Cl-phenyl	2-furanyl	C=O	NO_2	H	56.4	286.3	39	NA	NA
23	4-Cl-phenyl	2-furanyl	C=O	NO_2	H	2.1	31.1	164	6753	34
24	4-Br-phenyl	2-furanyl	C=O	NO_2	H	3.6	5132	84	4043	17
25	4-Cl-phenyl	CH_3	C=O	NO_2	H	173.8	NA	NA	NA	NA
26	4-NO_2-phenyl	Phenyl	C=O	F	H	407.9	NA	NA	NA	NA
27	4-F-phenyl	Phenyl	C=O	Cl	NO_2	8.4	26.7	38	NA	NA
28	4-F-phenyl	2-furanyl	C=O	Cl	NO_2	3.2	21.5	49	NA	NA
29	4-Br-phenyl	Phenyl	CNOH	NO_2	H	8.8	27.6	39	61.2	39
30	4-Cl-phenyl	Phenyl	CHOH	NO_2	H	416.6	458.9	17	NA	NA
31	5-Cl-2-thienyl	Phenyl	C=O	NO_2	H	15.1	74.0	87	675.2	54
32	4-Cl-phenyl	2-thienyl	C=O	NO_2	H	4.1	1014	62	3197	17
33	5-Cl-2-thienyl	2-furanyl	C=O	NO_2	H	8.8	166.4	76	2188	77
34	5-Br-2-thienyl	2-thienyl	C=O	Cl	NO_2	2.6	245.8	71	NA	NA
35	5-Cl-2-thienyl	2-thienyl	C=O	NO_2	H	5.0	101.5	112	1524	80
36	5-Cl-2-thienyl	2-thienyl	C=O	Cl	NO_2	10.2	1069	43	2107	58
37	4-Cl-phenyl	2-furanyl	CNOH	NO_2	H	13.6	259.3	38	NA	NA
38	5-Br-2-thienyl	2-thienyl	C=O	NO_2	H	7.4	429.2	28	6511	28
39	4-Cl-phenyl	2-furanyl	CNOH	NO_2	H	9.8	19.4	91	38.5	68
40	4-CH_3-phenyl	Phenyl	C=O	NO_2	H	14.0	146.1	128	479.1	58
41	3-NO_2-phenyl	Phenyl	C=O	NO_2	H	154.9	1800	6	NA	NA
42	4-Cl-phenyl	H	C=O	NO_2	H	169.3	3198	7	NA	NA
43	4-Cl-phenyl	Cyclohexyl	C=O	NO_2	H	73.1	124.5	48	323.4	26
44	Me	Phenyl	C=O	NO_2	H	146.9	1023	22	NA	NA
45	2-thienyl	Phenyl	C=O	NO_2	H	14.8	120.1	40	>10000	70
46	5-Br-2-thienyl	2-furanyl	C=O	Cl	NO_2	7.5	5.2	48	NA	NA
47	4-tert-butyl-phenyl	Phenyl	C=O	NO_2	H	96.6	254.6	21	NA	NA
48	2-naphthyl	Phenyl	C=O	NO_2	H	44.8	23.8	131	271.8	90
49	4-Cl-phenyl	2-furanyl	CH_2	NO_2	H	NA	NA	NA	NA	NA
50	4-NO_2-phenyl	2-thienyl	C=O	H	Cl	197.1	NA	NA	NA	NA
51	4-NO_2-phenyl	2-thienyl	C=O	NO_2	H	16.0	2168	15	3014	22
52	4-Me-phenyl	2-thienyl	C=O	NO_2	H	12.2	507.5	135	4622	85
53	4-Cl-phenyl	2-thienyl	C=O	Cl	H	56.5	4397	77	13352	28

Abbreviations: DHT, 5α-dihydrotestosterone; NA, not active.
Data shown are mean values from one to three independent experiments in triplicate measurements.
[a]Androgen receptor-binding activities of compounds were tested with the hydroxyapatite assay.
[b]CV-1 cells were co-transfected with androgen receptor (pSVAR0) and a luciferase reporter plasmid (MMTV-Luc), and treated with various concentrations of compounds. Efficacy is expressed relative to the response induced by 1 μM DHT (100%).
[c]MDA-MB-453 cells stably transfected with the luciferase reporter plasmid (MMTV-Luc) were treated with various concentrations of compounds. Efficacy is expressed relative to the response induced by 1 μM DHT (100%).

Table 3 Antagonist activities of compounds in CV-1 co-transfection and MDA-MB-453 reporter assays

Compound	*Antagonist activity in CV-1*[a]		*Antagonist activity in MDA-453*[b]	
	IC_{50} (nM)	Max. inhibit. (%)	IC_{50} (nM)	Max. inhibit. (%)
2	96 ± 15	88 ± 2	181 ± 14	87 ± 4
4	1822 ± 582	47 ± 6	NA	NA
5	105 ± 45	87 ± 4	NA	NA
6	NA	NA	NA	NA
8	1536 ± 53	89 ± 3	NA	NA
9	7819 ± 925	59 ± 7	309 ± 118	46 ± 8
10	NA	NA	NA	NA
11	504 ± 76	96 ± 2	3060 ± 218	72 ± 9
12	177 ± 35	98 ± 1	870 ± 109	78 ± 29
13	—[c]	—	2481 ± 689	35 ± 3
19	229 ± 86	97 ± 2	NA	NA
20	4231 ± 944	77 ± 6	2104 ± 798	32 ± 3
21	—	—	716 ± 459	82 ± 11
22	NA	NA	NA	NA
23	—	—	725 ± 103	54 ± 4
24	—	—	820 ± 78	68 ± 7
26	NA	NA	NA	NA
27	2230 ± 537	89 ± 5	572 ± 181	59 ± 5
28	—	—	NA	NA
29	690 ± 137	77 ± 22	NA	NA
30	91 ± 12	86 ± 9	NA	NA
34	—	—	7912 ± 1220	48 ± 8
37	57 ± 18	88 ± 7	4862 ± 1207	84 ± 6
38	—	—	420 ± 72	38 ± 11
39	110 ± 44	30 ± 1	NA	NA
41	1268 ± 285	80 ± 6	1340 ± 72	23 ± 9
42	566 ± 173	82 ± 6	3269 ± 703	34 ± 9
44	33 ± 6	67 ± 5	NA	NA
45	21 ± 7	71 ± 4	NA	NA
46	—	—	NA	NA
47	454 ± 174	93 ± 2	NA	NA
51	5894 ± 484	68 ± 3	NA	NA

Abbreviations: DHT, 5α-dihydrotestosterone; Max. inhibit., maximum inhibition; NA, not active.
Data shown are mean ± s.e.mean from at least two independent experiments in triplicate measurements.
[a]CV-1 cells were co-transfected with androgen receptor (pSVAR0) and a luciferase reporter plasmid (MMTV-Luc), and treated with various concentrations of compounds. DHT (2 nM) was added 30 min later to stimulate luciferase activity. IC_{50} values represent concentrations of compounds that inhibited 50% of the response elicited by 2 nM DHT. Percentage inhibition was the maximum suppression produced by a test compound compared to the control response detected in vehicle-treated cells.
[b]MDA-MB-453 cells stably transfected with a luciferase reporter gene plasmid (MMTV-Luc) were treated with various concentrations of compounds and incubated for 24 h. DHT (5 nM) was added 30 min after the addition of compounds. IC_{50} values represent concentrations of compounds that inhibited 50% of the response elicited by 5 nM DHT.
[c]Not determined because of the agonist activity in this cell type.

vesicles and levator ani growth induced by exogenous testosterone. In the castrated control group, there was a significant drop in dry weights of all test tissues compared to the sham group. In the presence of exogenous testosterone, there was a marked increase in the weight of these three tissues as would be expected. Casodex (25 mg kg^{-1}) inhibited this return towards normality as did the two doses (100 and 200 mg kg^{-1}) of compound 21. However, the compound was not as potent as Casodex (Figure 4). In contrast, compound 26, which possesses neither AR agonist nor antagonist properties in CV-1 and MDA-MB-453 cells, was inactive in this model (data not shown).

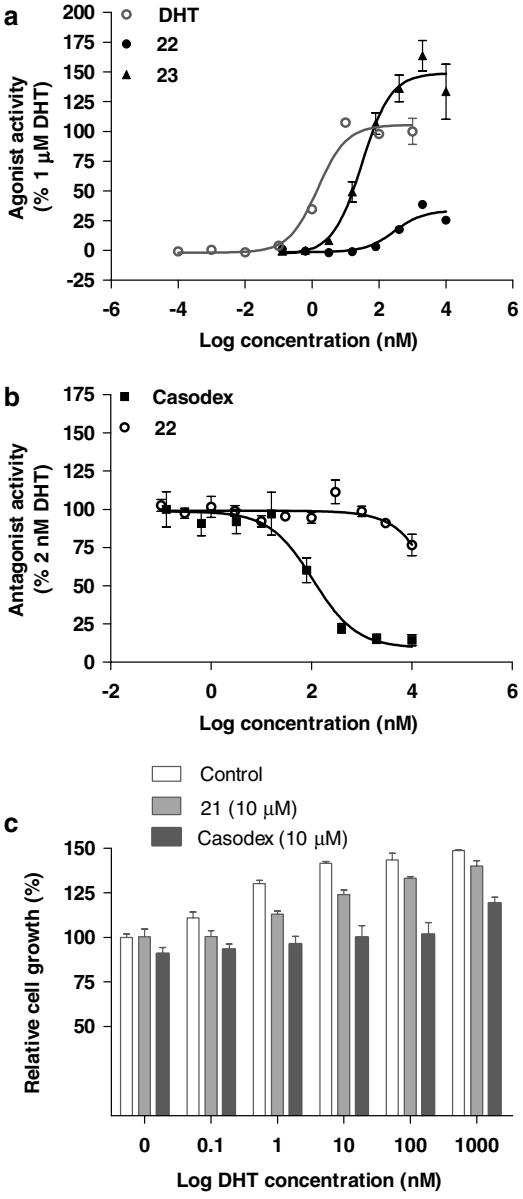


Figure 3 Different bioactivities of compound 21 and its enantiomers. (**a**) CV-1 cells co-transfected with androgen receptor (pSVAR0) and a luciferase reporter gene plasmid (MMTV-Luc) were treated with various concentrations of dihydrotestosterone (DHT), compounds 22 or 23 in the agonist mode. (**b**) Experiment was performed as above in the antagonist mode, except that DHT (2 nM) was added 30 min after the addition of compound 22 or Casodex. (**c**) LNCaP cells were seeded onto 96-well plates and incubated overnight. Different concentrations of DHT and test compounds (10 μM) were added to the cells and incubated for 6 days. Data (mean ± s.e.mean) are representative of three independent experiments.

Discussion

The initial chemical syntheses were primarily guided by a [^{3}H]DHT competitive receptor-binding assay for assessing the androgen receptor-binding affinities of the synthetic ligands. Our preliminary structure–activity relationship (SAR) studies were focused on modifications at the R^3 and R^4 substituents

Table 4 Bioactivities in SC3 and PC3 cell proliferation assays

	SC3 antagonist mode[a]		*PC3 cytotoxicity*[b]	
Compound	*IC_{50} (μM)*	*Inhibition (%)*	*IC_{50} (μM)*	*Inhibition (%)*
Hydroxyflutamide	0.022	100	—	—
Taxotere	—	—	0.001	100
21	1.91	89.2	9.86	51.95
26	0.44	109	5.58	62.38

[a]SC3 cells were seeded onto 96-well plates and incubated for 3 days with different concentrations of test compounds in the presence of 10 nM testosterone. The cells were labelled with [^{3}H]thymidine (0.15 μCi per well) for 2 h thereafter and the incorporation of radioisotope into DNA was counted with a MicroBeta counter.
[b]PC-3 cells were plated onto 96-well plates and incubated with different concentrations of test compounds for 3 days. Cell growth was assessed with [^{3}H]thymidine incorporation as above. IC_{50} values were calculated as the concentrations of compounds required to give half-maximal inhibition on the cell proliferation. Efficacy is expressed as the maximum inhibition produced by a test compound in comparison with that elicited by positive controls (100%; hydroxyflutamide for SC3 cells and taxotere for PC3 cells). Data shown are mean values from two independent experiments in triplicate measurements.

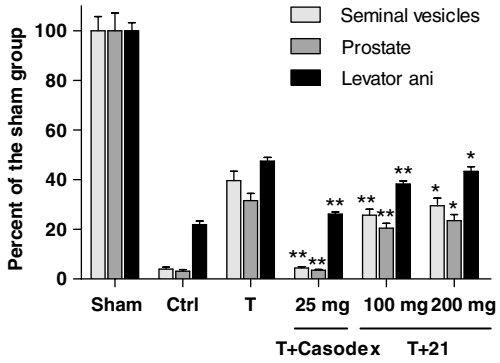


Figure 4 Effects of compound 21 on ventral prostate, seminal vesicles and levator muscle (dry weight) in castrated immature rats. Three-week-old male Sprague–Dawley rats were castrated and allowed to recover for 6 weeks. Rats were randomly divided into six groups ($n \geqslant 8$ per group): sham-operated group received vehicle alone (Sham); castrated group received vehicle alone (Ctrl); castrated animals received s.c. testosterone alone (T); castrated animals received s.c. testosterone and an oral dose of Casodex (T + Casodex); castrated animals received s.c. testosterone and i.p. compound 21 (100 or 200 mg; T + 21). The dry weights (corrected for the body weight of each individual animal) of prostate, seminal vesicles and levator ani were recorded following 10 days of treatment. *$P<0.05$ and **$P<0.01$ compared to testosterone-treated group (T). Data shown are mean ± s.e.mean.

of the phenyl ring, as shown in Table 2. Most modifications, including replacements with Cl, CN, NO_2 or CF_3, led to an increased activity and as the electron-withdrawing capacity diminished, so did activity (compare 7, 8 and 16). This indicates that an electron-withdrawing group on the phenyl ring was crucial for high-affinity binding with the receptor. Moreover, strong electron-withdrawing groups at R^3 position seemed to promote such an interaction more than any other groups. This is a common feature of androgen receptor modulators where strong electron-withdrawing groups have the greatest effect on activity as seen in the structures of flutamide and Casodex (Figure 1a).

Analysis of the three-dimensional structures of compound 21 and Casodex points to a significant similarity between the two (Figure 5). The only difference is the presence of the phenyl ring at what one may consider the 11-position on a steroid nucleus. The stereochemistry at this position is important as revealed by compounds 22 and 23 where the two stereoisomers have been separated. The cell-based assay results suggest that compound 23 possessed both androgen receptor agonistic and antagonistic activities.

We next turned our attention to modifications of R^1, as shown in Table 2. On the basis of the structure of compound 7, R^1 was modified by various replacements based on steric bulkiness or functionality to define the SAR at this site. The aromatic or heterocyclic ring was an important requirement for strong binding affinity, especially for a Cl at the *para*-phenyl position. Attempts to reduce or increase the steric bulkiness of R^1 group resulted in diminished agonist activities (compounds 44 and 47), whereas these two analogues both exhibited strong androgen receptor antagonist profiles in the CV-1 assay. The results indicate that R^1 group was important for the antagonistic effects observed.

With the SAR of the R^1 group being better understood, we then investigated the effect of the R^2 group. As seen in Table 2, good androgen receptor-binding activity was found among the phenyl, furanyl and thiophene analogues. However, compared with compound 7, the furanyl (21) and thiophene (32) analogues exhibited less cytotoxicity in HeLa cells (data not shown). Switching R^2 from aromatic and heterocyclic ring to H, CH_3 or cyclohexyl (compounds 42, 25 and 43) resulted in partial loss of activity. These modifications often led to a transformation from agonist to antagonist. This could prove useful as the SAR develops because the activity profile could be altered with relatively easy structural changes.

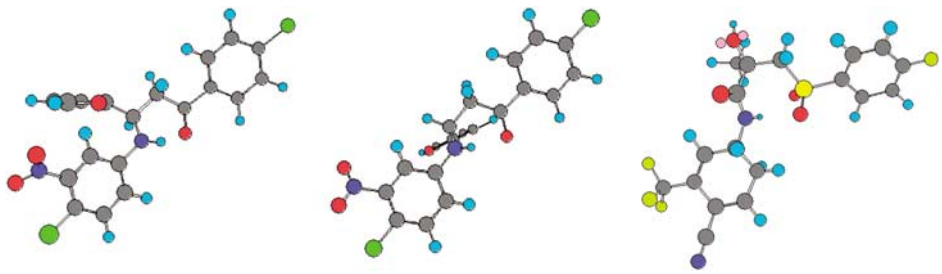


Figure 5 Three-dimensional structures of the two enantiomers of compound 21 (left and middle panel) and Casodex (right panel).

The final group of compounds tested comprised modifications of carbonyl group (X), as shown in Table 2 along the X column. The modifications in the X-linkage suggest that oxime analogues also possess good activity *in vitro* (comparing compound 21 to Z and E isomers 37 and 39). Reduction of the carbonyl to an alcohol led to a drastic decrease in androgen receptor binding (comparing compounds 7–30 and compounds 5–21). However, such modifications also increased the antagonistic activities. At this early stage of SAR, it is premature to guess as to the importance of this linkage area to the overall androgen receptor-modulating property.

There are some discrepancies relating to the potency values measured by receptor binding compared to those obtained by transfection (Tables 2 and 3; Figures 3a and b) and proliferation assays (Table 4; Figure 3c). Ideally, the results from receptor-binding measurement should accord with those from cell-based assays. Nevertheless, the former is a molecular level assay that is more stable and less affected by experimental conditions. In contrast, the outcome of cell-based assays can be influenced by many factors. As a result, discrepancies in potency measurements between these two assay systems often exist (Gaido *et al*., 1999; Legler *et al*., 1999) and, in the present study, the differences are in general within the same log concentration range.

Towards the end of this preliminary SAR study, we separated the enantiomers of one of the compounds, 21. The first set of experiments with this pair of isomers showed that most if not all of the agonist activity in CV-1 cells resided in one of the isomers, compound 23, whereas the other one, 22, possessed weak or little effect depending on the cell type examined. This same agonist enantiomer also had the greatest binding affinity of the pair. However, in the MDA-MB-453 assays, 23 was the only isomer demonstrating antagonist activity (Table 3). Our data point to the difficulty inherent in comparing CV-1 results with those obtained from MDA-MB-453, SC3 and LNCaP cells. The latter three cell types are utilizing endogenous androgen receptor present in the cells whereas the former lacks androgen receptor and must be transfected into the cell. These observations are important for this series of compounds and clearly demonstrate that much (if not all) of the discrepancies observed in the assay panels shown in this paper resulted from potential mixtures of agonist plus antagonist activities. At this early stage of the SAR, due to the difficulty in purifying the two isomers, we did not have enough quantity of the isolated isomers to re-evaluate in the *in vivo* assay and clearly, the SAR is moving towards optimization.

In summary, we have developed and characterized a novel series of 3-(phenylamino)-propan-1-one analogues as androgen receptor modulators. These compounds were evaluated by androgen receptor binding, cell-based reporter and proliferation assays, and the castrated immature rat experiment, respectively. Preliminary SAR analysis revealed that some common structural features are required for the *in vitro* bioactivities. Several compounds were found to be potent agonists and/or antagonists of androgen receptors. In particular, compound 21 was moderately efficacious both *in vitro* and *in vivo*. Further investigations will focus on the discovery of analogues that possess greater pharmacological efficacy and easy isolation or synthetic schemes to produce enantiomerically pure compounds.

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Conflict of interest

The authors state no conflict of interest.

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