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Development of a novel cell based androgen screening model

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Abstract

The androgen receptor (AR) mediates the majority of androgen effects on target cells. The DNA cis-regulatory elements that respond to AR share sequence similarity with cis-regulatory elements for glucocorticoid, mineralocorticoid and progesterone receptors (GR, MR and PR respectively). As a result, many of the current AR screening models are complicated by inaccurate activation of reporters by one of these receptor pathways. Identification of more selective androgen testing systems would be beneficial for clinical, pharmacological and toxicologic screening of AR activators. The present study describes the development of a selective androgen-responsive reporter cell line that expresses AR but does not express GR, MR and PR. CV1 cells were stably transduced to express human AR and an androgen-responsive *gaussia luciferase* gene. Clonal populations of AR expressing cells were isolated. Quantitative RT-PCR (qPCR) and western analysis confirmed stable integration of AR in the most responsive clonal line which was named 'CV1-ARluc'. Stimulation of CV1AR-luc with androgenic ligands (testosterone and 5α-dihydrotestosterone) for 18 h caused an increase in luciferase activity in a dose-dependent manner. Other steroid hormones including aldosterone, cortisol, and progesterone did not stimulate luciferase response. The CV1-ARluc also increased luciferase activity when treated with human serum extracts. In conclusion, the CV1-ARluc cells provide a novel model system for screening of new AR agonists and antagonists and can determine the androgenic activity of human serum samples.

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Keywords

Androgen activity; luciferase; stable *in vitro* bioassay; human serum; AR selectivity

1. INTRODUCTION

Androgens are hormones that play an essential role in the differentiation and maintenance of primary and secondary male sexual characteristics[1]. The two main human androgens are testosterone (T), which is involved in the initial virilization phases of the human male embryo, and 5α-dihydrotestosterone (DHT), which is the active hormone in most androgen target tissues [2]. T is mainly synthesized by the testicular Leydig cells, in peripheral tissues, as well as to a lesser degree in ovaries and adrenals. T is converted to DHT by 5α-reductases and also can be converted to estradiol by aromatase. DHT is the most active physiologic androgen, inducing tenfold higher androgen receptor (AR, *NR3C4*) bioactivity than T [3, 4]. In addition, other endogenously produced steroids exhibit various degrees of androgenic activity [5, 6]. Several synthetic androgen-related compounds (AR agonists and antagonists) have also been developed to modulate androgen signaling in therapeutic settings [7, 8].

Androgens mediate their effects through binding and activation of the AR. AR is a member of the steroid nuclear receptor superfamily [9] and acts as a ligand-dependent transcription factor [10]. Among this family, five steroid receptors are known: estrogen (ESR, *NR3A1*), progesterone (PR, *NR2C3*), androgen, mineralocorticoid (MR, *NR3C2*) and glucocorticoid (GR, *NR3C1*) receptors. AR activates a wide range of target genes that encode proteins and noncoding RNAs, including regulatory microRNA species [11].

Similar to the other steroid receptors, unbound AR is located in the cytoplasm. Upon ligand binding, AR goes through a series of conformational changes, dimerization and translocation to the nucleus, which is mediated by a nuclear localization signal. Translocated AR binds to androgen response elements (ARE). These ARE are characterized by a consensus (or near consensus) sequence 5′-TGTTCT-3′, which is located in the promoter or enhancer regions of AR gene targets. The DNA cis-regulatory elements that respond to AR share sequence similarity with cis-regulatory elements for GR, MR and PR. The similarity of the response element for AR and the other steroid receptors, and particularly the wide-spread expression of the GR, has been problematic in the development of selective receptor screening assays.

The determination of androgen levels or the discoveries of new androgenic compounds are key elements for the diagnosis of a number of diseases in children and adults. Assays that detect bioactive serum androgens in a sensitive and selective manner benefit the diagnosis and treatment of several pediatric endocrine disorders, such as precocious puberty and ambiguous genitalia. In addition, androgen bioassays provide a screening tool for androgen abuse and endocrine disruptors [12]. Over the past 10 years, several bioassays were developed using different methods [13]. One of the first assays developed relied on a chloramphenicol acetyltransferase (CAT) reporter model [14]. This system was limited by experimental variation due to the transient nature of transgene expression. A luciferase reporter bioassay, using MDA-MB453 cells, was developed by Wilson et al [15]. The major caveat of this assay was that it responds to AR as well as to GR agonists. Other androgen-

reporter cell lines were developed but most of them were transiently transfected [16–18]. Transient transfection assays [19] can provide similar information with stable assays but may not reflect endogenous levels of receptor. A stable expression of AR in the cells can eliminate the need for repetitious transient transfections, reduce the variability associated with these transient assays and moreover be utilized for high-throughput studies. Until now, a selective androgen-responsive transcriptional activation assay has not been widely available.

The aim of this study was to develop a stable cell-based *in vitro* bioassay that expresses the human AR (hAR) gene with sensitive and selective reporter readout. For this purpose, a stable cell line was made with CV1 cells stably transduced with hAR and an MMTV promoter-driven *luciferase* reporter gene. The resulting model is selective for androgens and does not exhibit reporter activation by other steroid receptors. In addition the model appears useful to determine circulating androgenic bioactivity in human serum samples.

2. MATERIALS AND METHODS

2.1 Materials

T, DHT, cortisol (Cort), progesterone (Prog), aldosterone (Aldo), androstenedione (AD4), hydroxyflutamide (OHF) and the 11-keto and 11-hydroxy forms of androstenedione and T were purchased from Sigma (Missouri, USA). Coelenterazine used for the luciferase assay was purchased from Promega (Wisconsin, USA). Penicillin, streptomycin, hygromycin, geneticin (G418) and DMEM/F12 medium were purchased from Life technologies (New York, USA).

2.2 Cell line

The CV1 monkey kidney cell line was obtained from the American Type Culture Collection (ATCC). The cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) from GE Healthcare Life Science (Utah, USA) and antibiotics including 1% penicillin/streptomycin. The cells were incubated under a humid atmosphere of 5% CO_2, at 37°C, and the medium was changed every 3 days. CV1 cells were plated at a density of 20,000 cells/well (48 well dish) in growth medium and grown to 60% confluence after which they were treated for steroids activity.

CV1-ARluc cells were plated in a 48 wells culture plate in 500 μl of growth medium (10% FBS/DMEM-F12, G418 and Hygromycin). The cells were incubated under a humid atmosphere of 5% CO_2, at 37°C. All treatments were performed with charcoal-stripped FBS serum to eliminate contaminating steroids.

2.3 Stable Transduction

CV1 cells (20,000 cells/well) were plated about 18 h before transduction in a 48 well-dish. The lentivirus pBM14-MMTV with the *Gaussia Luciferase* gene was diluted 1:10 in DMEM/F12 medium and added to the flask with 8 μg/mL of polybrene. The flask was centrifuged at 1200 rpm for 80 min and after 4 h in a humidified 5% CO_2 incubator the cells were supplemented with 1 ml of DMEM/F12 containing 10% FBS without any antibiotics.

After 48 h, the cells were selected in medium containing 1200 μg/ml of G418. The medium was changed three times a week. The obtained cells, named CV1-luc, were transduced with a lentivirus containing the hAR gene and the hygromycin selective gene. The stable transfection was performed as described above, using a multiplicity of infection (MOI) of 10 and 8 μg/mL of polybrene. 50 clones were obtained after 14 days of dual antibiotic (G418 and Hygromycin) selection. The clones were isolated using cloning rings (Sigma, Missouri, USA) and re-seeded and grown in a 48-well dish. After reaching 60% confluence, the cells were treated in DMEM/F12 containing 10% charcoal-stripped FBS and 10 nM of testosterone. After 24 h the treated cells were assayed for luciferase activity using the appropriate luminescence kit (Coelenterazine, Promega). The clone with the largest T induced luciferase activity was named CV1-ARluc and was used for further studies.

2.4 Isolation of RNA and qPCR analysis

The cells (25.000 cells/well) were grown for 24 h in 48 well culture. Total RNA was isolated from the cells previously plated using an RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quantity and purity were assessed by a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). For cDNA generation, 100 ng of total RNA was reverse transcribed using the High Capacity Kit (Applied Biosystems, Foster City, CA, USA). For qPCR, 12 ng of prepared cDNA was mixed with Fast Universal PCR Master Mix (Applied Biosystems). AR and peptidylprolyn isomerase A (PPIA) primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). PPIA was used as the housekeeping control gene.

2.5 Protein extraction and protein assay

Cells were lysed in 200 μL Mammalian Protein Extraction Reagent (Pierce Chemical Co., Illinois, USA), and the protein content was estimated by the bicinchoninic acid (BCA) protein assay using the BCA protocol (Thermo Scientific, Illinois, USA).

2.6 Western analysis

CV1 and CV1-ARluc cell lines were plated at a density of 75,000 cells/well (24 well-dish), in growth medium. Samples were lysed with lysis buffer (2% sodium dodecyl phosphate, 62.5 μM Tris, 0.04% bromophenol blue, 0.5 % dithiothreitol) and heated at 95 °C for 5 min. Proteins were then loaded (20 μg) on 10% Bis-Tris gel and electrophoresed for 1 h before transferring to polyvinylidene difluoride membranes. The membranes were then blocked with 5% BSA for 1 h and incubated with primary antibody (AR, polyclonal rabbit antihuman, 1:1000 BSA, Sigma) and secondary antibody (goat anti-rabbit, 1:5000, Life Technologies).The Pierce ECL Western Blotting Substrate kit (Life technologies) was then used for signal development.

2.7 AR translocation study

CV1-ARluc cells were grown on microscope slides from Globe Scientific (previously treated with 50 μg/ml of Poly D-Lysine at room temperature for 1 h) in 100-mm plates for 24 h in growth medium. Cells were treated with DHT for 18 h and subsequently fixed with methanol at −20 °C for 20 minutes and washed three times with PBS. Slides were then

incubated overnight with a rabbit anti-human AR antibody (Sigma) and then with a secondary goat anti-rabbit antibody (Sigma) for 1h at room temperature. Prolong Gold mounting medium with DAPI was used to visualize the cell nucleus.

2.8 Gaussia Luciferase analysis

CV1 and CV1-ARluc cells were plated at a density of 25,000 cells/well (48 well-dish), in growth medium for 24 h and then treated for indicated time points. The treated medium was collected and 25 μL was mixed with 50 μL of coelenterazine (previously diluted 1:100 in 50 mM Tris, 150 mM NaCl and water). Luminescence was then measured by FLUOstar OPTIMA microplate reader according to the manufacturer's instructions (Life Technologies).

2.9 Sera

Charcoal dextran stripped human serum was obtained by Equitech-Bio. In the present study, we analyzed human serum from 20 healthy adults (10 females and 10 males), age 20–35 years. All samples were collected under protocols approved by the Institutional Review Board (IRB) at the University of Michigan.

2.10 Extraction method

The indicated concentrations of steroids were prepared separately in DMEM/F12 medium with10% charcoal-stripped FBS, and stripped human serum using ethanol as a carrier solvent. Several extraction methods were tested and were found to have variable abilities to disrupt androgen regulation of reporter activity (data not shown). The method that exhibited androgenic activity most similar to unextracted medium was solid phase extraction using Sep-Pak Cartridges column from Waters (Chromatography division Millipore Corporation, MA, USA). The columns were activated with 4 ml of methanol and subsequently washed with 4 ml of deionized water. Standards made separately in DMEM/F12 with 10% charcoal stripped FBS and stripped human serum were dispensed at a volume of 600 μl in the columns, followed by another wash and elution with 2 ml of 100% methanol. Samples were evaporated at 37°C using a thin stream of nitrogen gas and the dried extract was then re-suspended in 300 μl of DMEM/F12 medium with 10% charcoal stripped FBS.

3. RESULTS

3.1. AR expression in transduced CV1 cell line

A double transduction with the lentiviral hAR and Gaussia luciferase constructs was used to obtain a CV1 cell line stably expressing the hAR and the androgen responsive gaussia luciferase gene. A total of 48 geneticin and hygromycin resistant clones were obtained. Out of the androgen responsive clones, to the most responsive to testosterone exhibited an 80-fold induction of reporter gene activity (data not shown). All experiments were conducted using this clone, CV1-ARluc. The untransduced parent CV1 and the transduced CV1-ARluc cell lines were initially tested for human AR expression by qRT-PCR analysis and western blot (Fig. 1). We demonstrate that the CV1-ARluc cell line expresses high levels of AR, whereas AR was found to be absent in the parent CV1 cell line.

To further characterize the cell lines, we analyzed the cytoplasmic and nuclear expression of AR in CV1-ARluc by immunofluorescence (Fig. 2). Since ligand steroid receptors undergo nuclear localization, we traced the fluorescence for AR before and after treatment with DHT, a potent androgen. In the absence of the ligand, AR staining in the CV1-ARluc cells was predominantly located in the cytoplasm. Upon addition of 10 nM DHT for 18 h, 100% of the cells stained positive in the nucleus (Fig. 2). The observed translocation of AR from the cytoplasm to the nucleus in treated cells but not in untreated cells confirmed the specificity of the signal.

3.2 Sensitivity of CV1-ARluc cell line

As shown in Fig. 3 A, the luciferase activity, after treatment with testosterone or DHT (10 nM) was clearly detectable at 3 h and increased progressively when the incubation with the respective ligands was continued up to 24 h. This result suggests that the assay can be used for steroids with androgenic activity even for short exposures.

In response to increasing concentrations of T and DHT, there was a sigmoidal increase in reporter gene expression (Fig. 3 B). The first significant response of the reporter gene expression to the androgens was detected at 0.1 nM of DHT and 0.3 nM of T (Fig. 3 B). The lower concentrations of steroids being able to stimulate a significant increase in luciferase expression indicate the high sensitivity of the cells.

3.3. Selectivity of CV1-ARluc cell line to other steroids

Since some members of the nuclear steroid receptors exhibit cross-talk activity via binding of other steroid hormones to their ligand binding domain, we examined the specificity of CV1-ARluc for androgens. This was done by incubating the CV1-ARluc cells with cortisol, progesterone and aldosterone at increasing concentrations. In addition, to insure that GR activity was not present in this model and to determine if high concentrations of cortisol regulate the AR, we incubated cells with concentrations of cortisol up to 1000 nM. As shown in Fig. 4, none of these steroids showed any significant response suggesting a high selectivity of the CV1-ARluc system towards androgenic steroids.

3.4. Treatment of CV1-ARluc cell line with different C_{19} steroids

Several studies suggested that these C_{19} steroids provide a pool of circulating precursors for peripheral conversion to more active androgens. The adrenal glands secrete a variety of C_{19} steroids including androstenedione and the 11-keto and 11-hydroxy forms of androstenedione and T. To better analyze the sensitivity of the CV1-ARluc to weaker different C_{19} steroids, the cell line was treated with T, 11-hydroxytestosterone (11OHT), 11-ketotestosterone (11KT), AD4, 11-hydroxyandrostenedione (11OHAD), 11-ketoandrostenedione (11KAD) at a constant dose of 300 nM. As showed in Fig. 5, the luciferase activity was increased with treatment with all of the previously mentioned steroids with the exception of 11OHAD.

3.5. Effects of a potent anti-androgens on CV1-ARluc

The androgen specificity was also demonstrated by the ability of hydroxyflutamide (OHF), a non-steroidal anti-androgen, to suppress the activity of T (Fig. 6). The effects of OHF were

tested in the CV1-ARluc cells. CV1-ARluc cells were treated with increasing concentrations of OHF, in the presence and absence of the maximally stimulating concentration of T (10 nM). As shown in Fig. 6, OHF alone did not show any significant agonistic activity. However, OHF inhibited T activity in a concentration dependent manner with a significant repression seen for 1–30 μM. The response to testosterone was completely suppressed by 30 μM OHF.

3.6. Serum androgen bioactivity

To examine the androgen activity in human serum, the experiment was performed on extracted samples processed with solid phase extraction. In Fig. 7, a dose response curve was performed using charcoal stripped human serum prepared with different doses of T. Serum androgen bioactivity was also determined from 10 male and female serum samples between ages 20–40 years. These bioassay values indicate the androgen activity in these samples. The range value of the active androgen in female and male were equivalent to 0.9 nM and 15 nM of T, respectively. These data demonstrate that the androgen activity in serum samples can be measured with CV1-ARluc cell line.

4. DISCUSSION

We have developed an *in vitro* assay that is simple, rapid, and quantitative with potential to screen active androgenic compounds including those in human serum. The CV1-ARluc cell line expresses an androgen-responsive luciferase reporter gene and a human AR gene. The CV1-ARluc cell line combines high stability, fast growth, high selectivity, high sensitivity and rapid response to androgens.

Cell-based assays can provide several benefits compared with immunoassay, mass spectrometry and various chromatography methods [3]. Over the past several years, there has been an expansion in the use of gas chromatography-mass and liquid chromatography tandem mass spectrometry for measurements of androgens. These methods are able to analyze multiple steroid hormones but they are not useful in defining the activity of unknown androgenic steroids. Cell-based hormone receptor assays have become an important resource for drug discovery and androgen related diseases. The cells used to develop these assays should have two specific requirements: a reporter system driven by an ARE and an abundant expression of AR. A variety of reporter genes have been used in the past years including β-lactamase [20], β-galactosidase [21–26] and luciferase [15, 18, 27–32] reporter genes. To develop an androgen reporter line superior to the ones available, we chose not to use yeast cells for several reasons. Although yeast-based reporter models [22], have certain advantages (easy handling, rapid growth, inexpensive media components), they require laborious cell preparation and complex cell lysis steps. Importantly, using yeast assays to express mammalian proteins also raises concerns regarding glycosylation, phosphorylation and post translational modifications. Moreover, the estimation of androgenic bioactivity in serum with yeast-based assays has been unsuccessful. In yeast cell lines, anti-androgens have not shown any antagonistic effects, probably due the permeability problem of the cell walls.

To avoid interference with other nuclear receptors, we selected a cell line with a low background activity of C3 group nuclear receptors and with a good response to androgens when the hAR was stably introduced. We chose to use an MMTV promoter-driven luciferase gene since this approach has been shown to be successful in generation of *in vitro* and *in vivo* models for screening of estrogen compounds [33]. With the present work we show that, this approach can be successfully used to generate a selective androgen reporter cell line, CV1-ARluc. The MMTV promoter is quite selective to AR as well as to GR and PR and, also, contains a number of regulatory sites that can be targeted by other steroids. Wilson et al [34] developed a stable cell line, MDA-kb2, a derivative of a human breast cancer cell line, containing a stably integrated MMTV-luciferase reporter. This cell line strongly responds to glucocorticoids due to endogenous GR. This makes the cell line unsuitable as a selective screening tool. PC3 cells transfected by Kim et al [18], show more androgen specificity obtained by transient transfection of an MMTV promoter and a human AR with high sensitivity. Other bioassays for screening of androgen compounds in human serum have been reported using transient transfection; we have used a stable transduction to develop our cell model because stable expression of AR can eliminate the variability associated with repeated transient transfection. A different cell line, using CHO cells and a stable transfection, was developed by Paris et al [4]. The cell line was stably transfected with hAR but has retained glucocorticoid response due to GR. The T47D cell line was used by Blankvoort et al [31] to develop a new androgen cell-based assay named AR-LUX. T47D expresses an endogenous AR and was stably transfected with a luciferase reporter gene. This assay was used to estimate the levels of some anabolic steroids in the urine of cattle. However, this cell line also responds to added estrogen and progesterone, which may reduce the specificity of the assay.

There is one additional AR bioassay that is highly selective for androgens and there is no reporter response to non-androgenic steroids [20]. Wilkinson et al developed an AR bioassays using the HEK293 cell line. This model is particularly applicable for high throughput screening for AR activation. However, this model makes use of a hybrid AR receptor (GAL4DBD-ARLBD) and therefore may not have all regulatory characteristics of the full length AR.

In the present study, we have developed a new cell line, named CV1-ARluc, which stably express the human AR and an androgen-driven gaussia luciferase reporter. The cell line is able to estimate levels of androgen bioactivity in human serum samples. The androgen specificity of the assay was tested by using high concentrations of different steroids such as cortisol, progesterone and aldosterone, which did not active reporter expression. The low background activity of C3 group nuclear receptors in the CV1 cell line was supported by RT-PCR data, where no GR, ER, AR, PR and MR expression was detected. The specificity of the assay was also showed by checking the inhibitory effects of a synthetic anti-androgen receptor ligand, OHF. OHF anti-androgen effects were also seen in other AR models including CHO [35], DU-145 [36] and PALM [37]. We evaluated androgenic bioactivity in male and female serum samples. In concordance with the data previously reported using a different *in vitro* assay to detect androgen activity in human serum samples by Roy et al [38], our assay shows an androgen bioactivity in female serum samples from 0.8 to 2 nM

and from 10 to 25 nM in normal males. It can be concluded that this assay provides a valid and practical method to analyze serum samples for androgenic activity. The assay can be used to evaluate the physiological androgen levels during androgen-related diseases.

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Abbreviations

AR	androgen receptor protein
ARE	androgen response element
GR	glucocorticoid receptor protein
MR	mineralocorticoid receptor protein
PR	progesterone receptor protein
T	testosterone
DHT	5α-dihydrotestosterone
Cort	cortisol
AD4	androstenedione
Prog	progesterone
11OHT	11-hydroxytestosterone
11KT	11-ketotestosterone
11OHAD	11-hydroxyandrostenedione
11KAD	11-ketoandrostenedione

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HIGHLIGHTS

- We have developed a new cell line, named CV1-ARluc, which stably express the human AR and an androgen-driven gaussia luciferase reporter.
- The androgen specificity of the assay was tested by using high concentrations of different steroids such as cortisol, progesterone and aldosterone, which did not active reporter expression.
- The transactivation assay developed with the help of the cell line is highly sensitive as it detects androgen activity at 0.1 nM of DHT and 0.3 nM of T.
- This assay provides a valid and practical method to analyze male and female serum samples for androgenic activity.

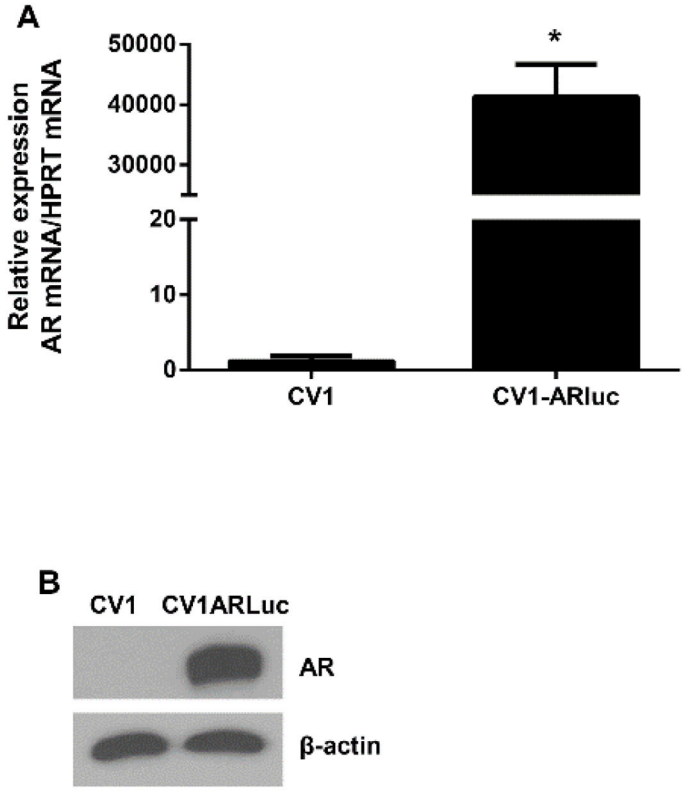

Fig. 1. AR expression in transduced and wild type CV1 cells

CV1 and CV1-ARluc cells were maintained in 10% FBS medium for 24 h and then lysed. (A) RT-PCR analysis of AR expression was tested in both cell lines. AR expression in CV1-ARluc was significantly higher than the CV1 cells. (B) Western analysis of AR was performed on 20 μg of total protein. β-actin was used as a loading control. The untransfected cell line did not show detectable AR, indicating the absence of this protein in this cell line. Figures are representative of three independent experiments with similar results.

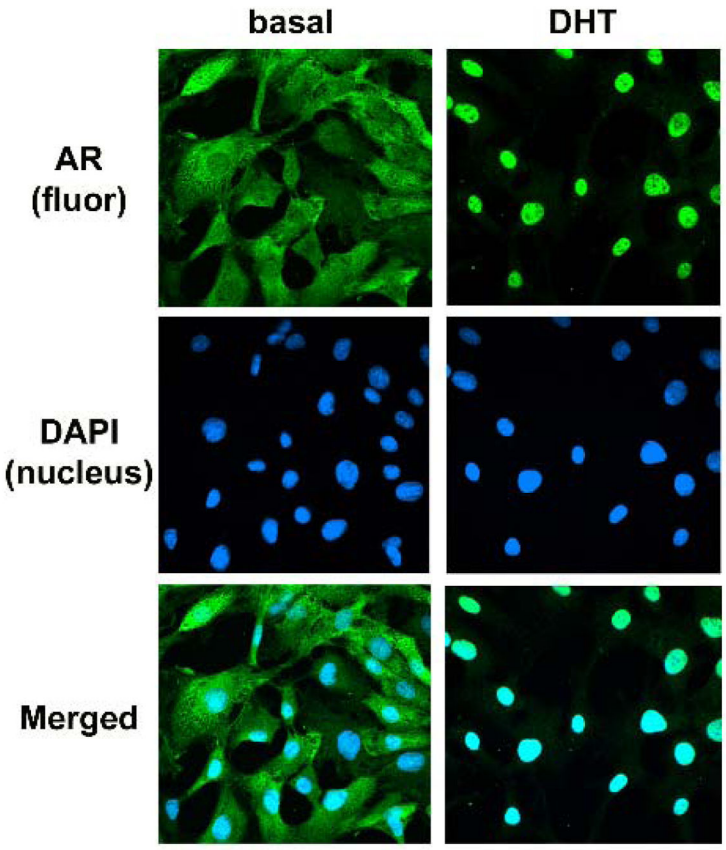

Fig. 2. DHT binding causes AR nuclear translocation

Fluorescence microscopy of CV1-ARluc cells stably transfected with a hAR and treated with or without 10 nM DHT for 18 h. Green fluorescence represents AR immunoreactivity and blue fluorescence is DAPI (nucleus).

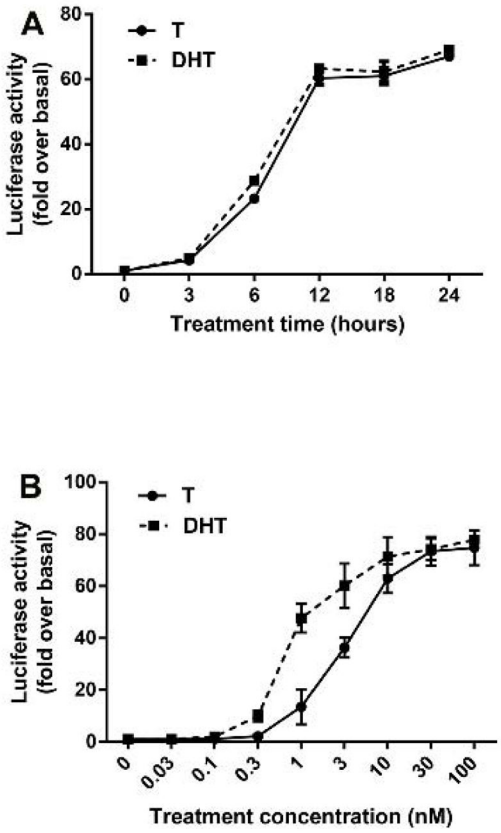

Fig. 3. Time course for AR activation and CV1-ARluc response to known androgens

(A) The cells were incubated with testosterone and DHT 10 nM for 0–24 h. The results are expressed as fold increase over basal of luciferase activity. The results represent the mean ± S.E. of similar experiments performed in triplicate. (B) CV1-ARluc cells were incubated with increasing concentrations of two known androgens: Testosterone and DHT. The values represent the mean ± S.E. of similar experiments performed in triplicate.

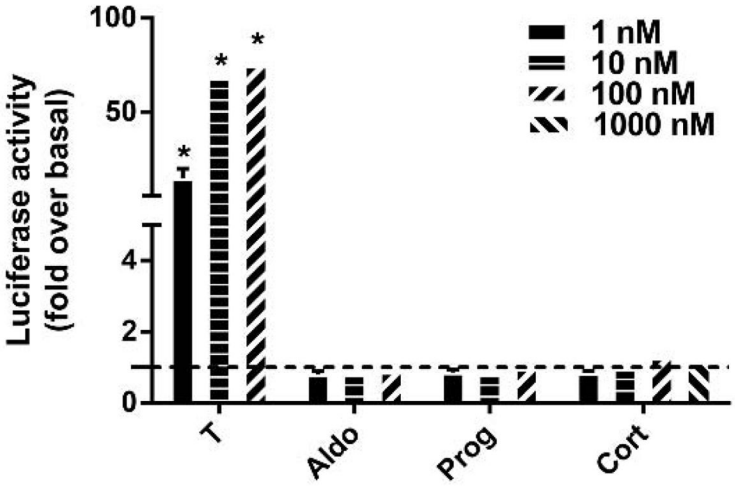

Fig. 4. Reporter gene regulation in response to androgenic and non-androgenic steroids

CV1-ARluc cells were incubated with 1, 10 and 100 nM of aldosterone and progesterone and 1, 10, 100 and 1000 nM of cortisol. The dotted line represents basal conditions. The data represent the mean ± S.E. of three independent experiments, each performed in triplicate (*$p < 0.05$ versus basal).

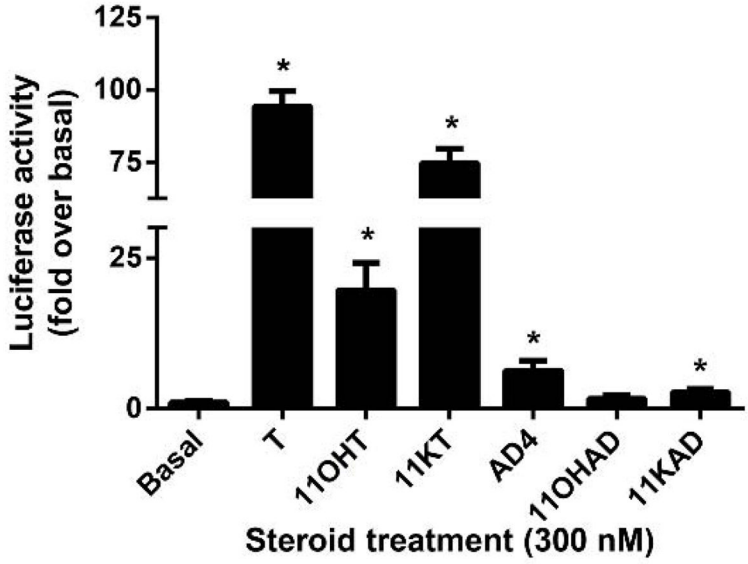

Fig. 5. CV1-ARluc cell line treatment with different C_{19} steroids

CV1-ARluc cells were incubated with 300 nM of T, 11OHT, 11KT, AD4, 11OHAD and 11KAD. The data represent the mean ± S.E. of three independent experiments performed in triplicate (*$p < 0.05$ versus basal).

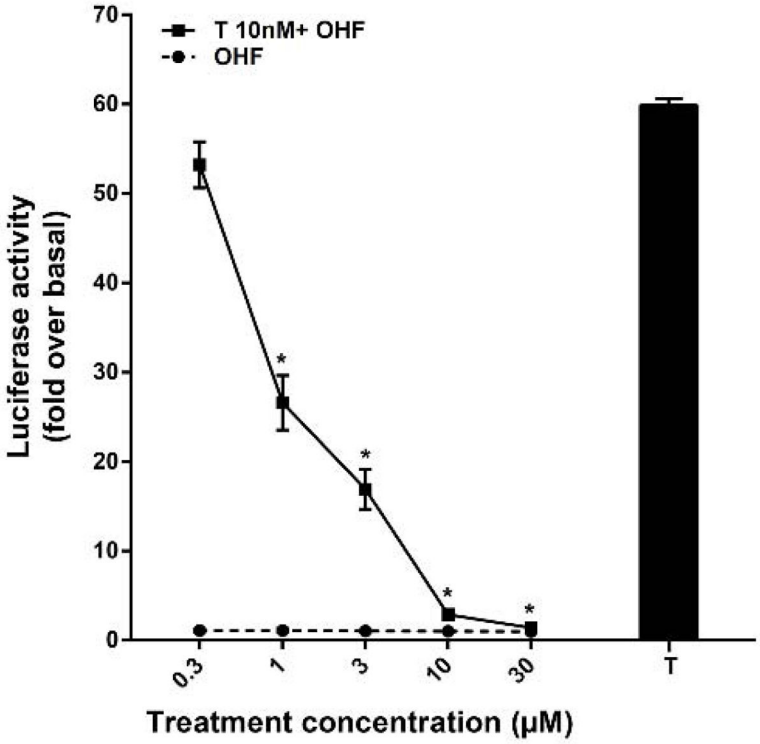

Fig. 6. Antagonistic activity of hydroxyflutamide in CV1-ARluc

Cells were incubated with 10 nM T alone as well as increasing concentration of OHF alone or in the presence of 10 nM testosterone. The values represent the mean ± S.E. of similar experiments performed in triplicate (*$p < 0.05$ versus 10 nM T alone).

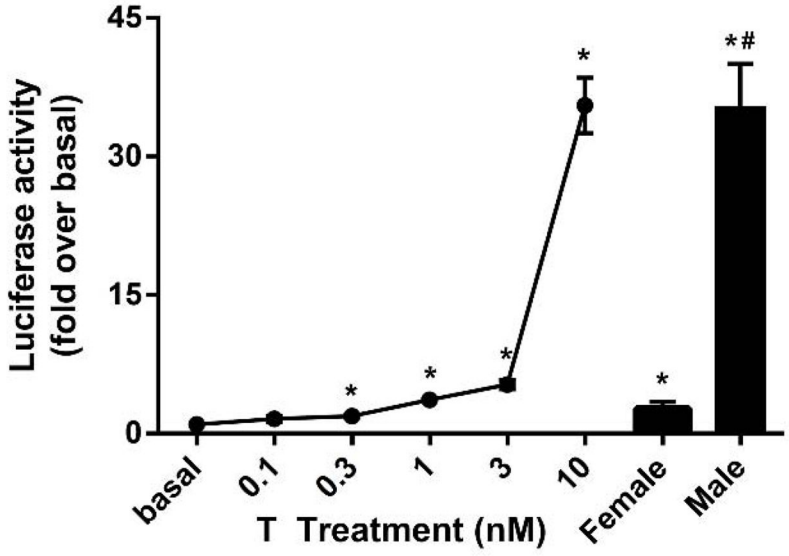

Fig. 7. Comparison of androgen bioactivity levels in human serum samples

Testosterone (0.3–30 nM) and human serum extract (10 male and 10 female) effects on reporter activity. Cells were treated with extracted samples for 18 h. The experiment was performed in triplicate (*$p < 0.05$ versus basal and #$p < 0.05$ versus female).