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Cell-based assays for screening androgen receptor ligands

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Abstract

The androgen receptor (AR, *NR3C4*), mediates the majority of androgen effects on target cells. The AR is activated following ligand binding that result in activation of target gene transcription. Several cell based model systems have been developed that allow sensitive detection and monitoring of steroids or other compounds with AR bioactivity. Most cell based AR reporter models use transgenic gene constructs that include an androgen response element (ARE) that controls reporter gene expression. The DNA cis-regulatory elements that respond to AR share sequence similarity with cis-regulatory elements for glucocorticoid (GR, *NR3C1*), mineralocorticoid (MR, *NR3C2*) and progesterone (PGR, *NR3C3*) receptors, which has compromised AR selectivity for some models. In recent years, the sensitivity and selectivity of AR bioassays have been significantly improved through careful selection of cell models, utilization of improved reporter genes and the use of yeast two hybrid AR systems. This review summarizes and compares the currently available androgen-responsive cell model systems.

Keywords

Androgen receptor; luciferase; GFP; *in vitro* bioassay; reporter gene assays

INTRODUCTION

Androgens represent a broad group of steroid hormones that mediate their effects through binding and activation of the androgen receptor (AR, *NR3C4*). The AR is expressed in a variety of tissues, including the heart, pituitary, skeletal muscle, uterus, and thyroid, with the highest expression level observed in the prostate, adipocyte and liver.[1] The AR can be activated by several physiologic ligands (mainly testosterone and dihydrotestosterone, DHT) that bind the AR with different affinities and bioactivity.[2,3] DHT is the most active

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physiologic androgen with a tenfold higher androgen receptor bioactivity than testosterone. [4,5]

Binding of androgen to the cytosolic AR[6] results in a conformational change in the receptor that causes dissociation of heat shock proteins, transport from the cytosol into the cell nucleus and dimerization of the androgen-AR complexes.[7,8] The AR dimer then binds to a specific sequence of DNA known as the androgen response element (ARE) that enhances transcription of AR-responsive genes.[9–18] AREs are identified by the presence of six-nucleotide half-site consensus sequences spaced by three random nucleotides in the promoter region of target genes: 5'-TGTTCT-3.[19,20] Conversely, anti-androgens like casodex or hydroxyflutamide, bind to the AR and cause nuclear translocation but no transcriptional activation.[17,18] AR has a characteristic structure: two activation functions (AF1 and AF5) in the N-terminal domain (NTD), a DNA-binding domain (DBD) which contains the dimerization domain, a nuclear localization signal (NLS), a hinge region, and a carboxy-terminal ligand-binding domain (LBD) which contains a third activation function domain (AF2). All the AR regions are highly conserved except the NTD which is important in transcriptional regulation.

AR cell based models have been applied to a variety of discovery-based projects. Many focus on defining novel androgens that might play a role in human diseases of androgen excess (premature adrenarche or polycystic ovary syndrome).[21,22] In addition to endogenous steroid hormones, AR cell based assays have been used to define androgenic activity in legumes, soybeans, yams and industrial chemicals with concerns of their ability to act as endocrine disruptors and/or toxicants. [23] Finally, AR bioassays have become an alternative method for the detection of designer androgens in laboratories testing serum for sports doping. [24]

Historically, androgens have been measured as individual steroids using selective immunoassays. While these assays perform relatively well and provide a degree of high throughput, such assays can be flawed by cross-reactivity with steroids of similar structure. In addition, as there are a number of different steroids that can activate the androgen receptor, the immunoassay approach of measuring one steroid at a time does not provide a broad view of the circulating androgen milieu. Over the past ten years there has been an expansion in the use of gas chromatography-mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) for measurement of natural and synthetic androgens. These methods have an improved specificity over most antibody based immunoassays. In addition, these methods allow a broader analysis of multiple steroid hormones and may be important for disease diagnosis. However, these methods are not useful in the identification of unknown synthetic or naturally occurring androgenic steroids or other substances; for these types of studies, investigators have relied on *in vitro* cell-based AR bioassays.

Cell-based steroid receptor reporter assays have become an important resource for compound profiling and drug discovery because of their ability to provide quantitative and functional information within a short time span. The cells used for developing cellular AR assays have two specific requirements: abundant expression of the AR and a reporter system

driven by an ARE. The principles of the reporter gene assays are quite simple and rely on AR ligand entry into the cells, binding to the cytoplasmic AR, translocation of the AR complex into the nucleus, binding to the ARE, resulting in an increase in reporter gene expression. Importantly, the activity of a ligand can be elucidated in samples without the need to have any information on chemical structure. A variety of reporter genes have been used for model development, including β -galactosidase, luciferase, lactamase, and green fluorescent protein (GFP). In this review we discuss the cell-based AR bioassays currently available for detection of androgenic and anti-androgenic activity (Figure 1).

THE CELL BASED ANDROGEN ASSAYS

YEAST-BASED SYSTEMS USING A β -GALACTOSIDASE REPORTER

In yeast cells, steroid bioactivity of substances can be determined without the presence of any other mammalian proteins/pathways influencing the AR activity. These cells have the advantages of easy handling, fast growth, inexpensive media components and robustness towards toxic effects of the tested chemicals or solvents[25]. These attributes make the yeast AR screen a fast and easy tool. Some disadvantages of yeast assays include laborious pre-assay cell preparation and complex cell lysis steps. Using yeast assays to express mammalian proteins also raise concerns regarding phosphorylation, glycosylation, folding and post translational modifications.

β -galactosidase (β -gal) is encoded in *E. coli* by the *lacZ* gene of the *lac* operon. The enzyme function in bacteria is to cleave lactose to form glucose and galactose. Chlorophenol red- β -D-galactopyranoside (CPRG), a chromogenic substrate, described by Seeber and Boothroyd, [26] and the synthetic compound, o-nitrophenyl- β -D-galactoside (ONPG), described by Li *et al.*[27] are used for spectrometric detection of β -gal. Both substrates are colorless but became colored once hydrolyzed by β -gal. For the ONPG/ β -gal assay the time required for yeast exposure to the tested compounds is 6 h. The cells are then lysed and an aliquot of the extract is mixed with the β -gal reaction substrate in a buffer containing sodium phosphate and magnesium chloride. The assay ends with the spectrophotometric measurement of the yellow reaction product (o-nitrophenol). The production of o-nitrophenol, per unit time, is proportional to the concentration of β -gal, allowing the intensity of the yellow color produced to determine the enzyme concentration.[27] The use of XGal (5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside) for β -gal detection requires at least 16 h of yeast exposure to the test compounds.[28,29] XGal, a chromogenic substrate for β -gal, produces a blue color that can be detected visually over background. Using the XGal substrate, β -gal assays provide a more sensitive reporter for activity, but XGal is not as quantitative as the β -gal assay.[30]

As shown in Table 1, for most of the β -gal assays examined, as exposure times increase, EC₅₀ values decrease. In 1991, Purvis *et al.* [31] developed an androgen-inducible expression system for *Saccharomyces cerevisiae*.³¹ The PGKare-lacZ (PGK promoter followed by ARE and lacZ sequence) was integrated into the *S. cerevisiae* genome at the *ura3-52* locus. The resulting strain was then stably transfected with human AR (hAR) expression plasmids. The transfected cells were incubated in the presence of different concentrations of DHT and assayed for β -gal activity. EC₅₀ was 1nM for DHT treatment

with a steroid exposure time of 40 h. A similar AR assay with comparable steroid exposure time and EC₅₀ was developed by Sohoni P. and Supter PJ.[32] Based on the hypothesis that one chemical may activate multiple steroid receptors, they used two recombinant yeast strains: one containing a gene for the human estrogen receptor (also containing a plasmid carrying an estrogen responsive element regulated lacZ reporter) and the other yeast strain expressing the human AR (also containing an ARE regulated lacZ reporter). When an active ligand bound to either receptor, lacZ was transcribed/translated and then secreted into the medium. The medium could then be used for the chromogenic substrate CPRG. They confirmed previously reported anti-androgenic and estrogenic activity of vinclozolin and p,p'-1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE)[33,34] and they found estrogenic activity in several reported anti-androgenic compounds namely o,p'-1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), bisphenol A and butyl benzyl phthalate.

Chatterjee S. *et al.* [35] constructed a yeast-based AR bioassay to evaluate the androgenic activity of endocrine disruptors from pulp and paper mill effluents. The system consisted of hAR and ARE -driven *lacZ* transformed in *S. cerevisiae*. Production of *lacZ* was shown to be driven by the CYC1 yeast promoter and β -gal activity was detected using XGal. The assay detection required at least 16 h of exposure to the tested chemicals; EC₅₀ was 16 nM for testosterone and 4 nM for DHT, which was consistent with the performance of other, previously constructed, assays.[36,37]

A recently reported AR cell bioassay, more selective than those previously described, was developed by Lee JH *et al.*[38] The group developed a detection system for androgenic and anti-androgenic compounds, which was based on yeast two-hybrid protein interactions. A yeast strain, ARhLBD-ASC1, was established by co-transformation of yeast cells harboring *lacZ* reporter plasmid. ARhLBD-ASC1 is a dual vector expressing system containing the LexA fused hinge–ligand binding domain (hLBD) of the human AR, and B42 fused to ASC-1 that interacts with the AR-hLBD in an androgen-dependent manner. In this yeast strain, androgens, but not other hormones, stimulated β -gal activity. β -gal activity was measured as a colorimetric reaction following 16 h of incubation. This system allowed relatively high throughput and could be done in 96 well dishes.

In order to study the effect of endocrine disruptors on AR, Nishikawa J *et al.* [39] developed a yeast model system with short exposure time (4 h) but relatively low sensitivity (EC₅₀ around 10 nM DHT). The major goal of the study was to develop a novel screening method to examine chemical effects on several steroid receptors. Y190 yeast cells were transformed with the pGBT9–LBD of the estrogen and androgen receptors, GAL4-receptor DBD and GAL4AD–coactivator fusion proteins. The major goal of the study was to develop a novel screening method to examine chemical effects on several steroid receptors. Because the yeast strain Y190 harbors a GAL4 binding site upstream of a *lacZ* reporter gene, GAL4DBD-ER binds to the regulatory region of the *lacZ* gene. If GAL4DBD-ER interacts with GAL4AD-coactivator, GAL4AD recruits the basal transcriptional machinery to the promoter region of the *lacZ* gene resulting in β -gal production. The system was adapted for other receptors by exchanging the ER portion of GAL4DBD fusion with other receptors. In addition, the models were improved by including mammalian nuclear receptor co-factors. For the development of the AR bioassay, the ER-LBD was changed to AR-LBD and the β -

gal reporter responses were enhanced by adding a vector containing mammalian nuclear receptor coactivators. Based on these studies, the steroid receptor models were most effective using the following co-factors: ER-TIF2, AR-SRC1, PR-TIF2, GR-SRC1 and MR-SRC1.

Different combinations of plasmids in the yeast Y187 were used by Li *et al.* [29] Plasmids used were pGBT9, the AR-LBD and pGAD424 GRIP1/FL (described by Doesburg *et al.* [40]) or pGBT9 ERR γ and pGAD424 GRIP1/FL. This model has a low compound exposure time (2 h) but limited sensitivity (EC₅₀ around 13 nM DHT). These investigators developed the models to study endocrine disruptors in pesticides which were suspected of modulating the endocrine systems in humans. The endocrine disruptors examined for their ability to interact with the ER, AR, PR or ERR γ included p, p'-dichlorodiphenylethane (p, p'-DDE), p, p'-dichlorodiphenyltrichloroethane (p,p'-DDT), hexachlorobenzene (HCB) and r-hexachlorocyclohexane (r-HCH). The results showed that p,p'-DDE was an ER agonist and an AR and PR antagonist (PR > AR), while p,p'-DDT was an ER agonist and AR antagonist. HCB and r-HCH were antagonists for AR and ERR, while r-HCH was a PR antagonist and a weak antagonist of ERR; the endocrine disruptor, r-HCH was able to reverse the ERR inhibition induced by 4-hydroxytamoxifen.

Yeast-based assays assessing chemical interactions with the estrogen, androgen, and progesterone receptor were developed by Gaido *et al.* [41] For the AR bioassay, the EC₅₀ was around 3.5 nM DHT with an exposure time of 18 h. The yeast contained two separate plasmids: an expression plasmid containing the CUP1 metallothionein promoter fused to the human nuclear receptor cDNA, and a reporter plasmid carrying two ARE upstream of the structural gene for β -gal; this system overexpressed two proteins (RSP5 or SPT3) in yeast containing either the progesterone or the androgen receptor, respectively.

One of the earliest reporter gene assays, chloramphenicol acetyltransferase (CAT reporter), has also been used for AR bioassay development. The CAT enzyme is normally found in prokaryotes but not eukaryotes. It transfers the acetyl group from the acetyl CoA molecule to chloramphenicol, causing its detoxification. Xu *et al.* used this reporter system to develop a hAR reporter assay using the CV-1 cell line (African Monkey kidney cell line). [42] The CV-1 cell line was transiently transfected with an ARE driven reporter gene plasmid (pMMTV-CAT) and a hAR expression plasmid AR/pcDNA3.1. An EC₅₀ of 0.39 nM was observed for DHT following an incubation period of 24 h. Using this AR reporter model the group investigated Bisphenol A (BPA), 4-octylphenol, 4-nonylphenol and a number of pesticides for agonistic and antagonistic activities. The caveat of this system is its experimental variation due to the transient nature of transgene expression.

β -LACTAMASE REPORTER MODEL

The β -lactamase (BLA) reporter system, which can be used for studying gene expression in living cells, uses the bacterial enzyme TEM-1 BLA which lacks the periplasmic secretory signal sequence. BLA is encoded by the ampicillin resistance gene, a 29- kDa enzyme, and is active either as a monomer or when fused N or C terminally to a heterologous protein. [43] It can cleave β -lactam-containing molecules with simple kinetics and high catalytic efficiency. Overexpression of BLA does not show toxicity in eukaryotic cells. CCF2 and

CCF4 (coumarin cephalosporin fluorescein), the β -lactamase fluorescent substrates, can be detected by fluorescence resonance energy transfer (FRET). In the intact molecule, excitation of coumarin at 408 nm leads to efficient FRET to the fluorescein derivative and produces green fluorescence. Cleavage of CCF2/4 by β -lactamase separates the two fluorophores, causing loss of FRET and excitation at 408 nm that result in blue fluorescence detectable at 460 nm. Thus, based on the change in the fluorescence emission signal, live cells expressing BLA can be distinguished by epifluorescence microscopy, fluorescent plate reader, or flow cytometry.

Wilkinson *et al.*[44] developed a panel of steroid hormone receptor bioassays by stably engineering expression of Gal4-DBD, with specific nuclear receptor LBD, using the HEK293 cell line with stable insertion of a GAL4 promoter driven β -lactamase reporter. Plated cells were incubated for 16 h with ligands or test compounds. Lactamase substrate was then added and fluorescence signal read using a fluorescent plate reader. After subtracting the average fluorescence intensity from the cell-free controls, the 460 nm/530 nm emission ratio was calculated. The response ratio corresponds to the 460 nm/530 nm emission ratio of the stimulated wells divided by the 460 nm/530 nm emission ratio of the unstimulated wells. The AR lactamase bioassay exhibits high sensitivity to DHT with an EC_{50} of 1 nM. The particular utility of this assay is its potential for high throughput screening and a high degree of selectivity for the AR.

LUCIFERASE REPORTER MODEL

Firefly luciferase is one of several bioluminescent reporters that have achieved broad use for molecular biology studies. Compared to the tests previously discussed, some of the luciferase AR models have higher sensitivities than those with lactamase, particularly with the mammalian cell models that can detect picomolar levels of DHT. The details, including the sensitivity of these AR-cell based assays, are reviewed in Table 2. The most commonly used luciferase is from the firefly *Photinus pyralis*. This gene encodes a 61 kDa enzyme that oxidizes D-luciferin in the presence of oxygen, ATP and Mg^{2+} ; the fluorescent product of the reaction can be quantified by measuring the released light using a luminometer. The assay is rapid, simple, relatively inexpensive, sensitive, and possesses a broad linear range. Cells transfected with a luciferase reporter plasmid are lysed using a detergent-containing buffer. The substrate can be mixed with the lysate; some luminometers directly inject the reagents into the lysate and the fluorescence is read at a defined time after mixing. The luciferase reporter is most often used as a read-out of gene expression to study transcriptional control mechanisms (promoter studies) or to study activity of transcription factors (as is the case for the AR models). Both yeast and mammalian cell line AR-driven luciferase reporter models have been developed[38].

Yeast-AR luciferase models—Yeast systems are inexpensive and do not contain all the mammalian enzymes, activators and coregulators and hence may not support maximal transcriptional activity for all receptors. However, the low costs and quick cell expansion capabilities make it a good choice for experimental goals.

Michellini *et al.* developed a bioluminescent yeast-based bioassay for androgens.[37] The bioassay is based on *S. cerevisiae* cells, modified to express hAR, and contain ARE sequences to regulate expression of luciferase. The bioassay responds to testosterone in a concentration-dependent manner from 0.05 to 1000 nM. The EC₅₀ of DHT is 10 nM. This assay is also able to respond to progesterone and 17 β -estradiol, with an EC₅₀ of 20 and 50 nM respectively, apparently via an AR mechanism. An *S. cerevisiae* strain, expressing hAR, estrogen receptor α or estrogen receptor β , with luciferase controlled by the receptors' respective hormone responsive elements, was developed by Leskinen *et al.*[36] These investigators describe the construction and use of a set of bioluminescent yeast strains for the detection of compounds that regulate androgen or estrogen receptor mediated hormonal signaling. The luciferase coding sequence was inserted into the vector pRS316/GPD-PGK, [45] between the GPD promoter and PGK terminator yielding pRS316luc. Sample analysis can be performed in one day and there is no requirement for cell lyses or centrifugation. Yeast cells were incubated with test compounds or complex samples for 2.5 h, resulting in an EC₅₀ value of 5.5 nM DHT.

Another yeast AR bioassay, using a bacterial luciferase reporter, was developed by Eldridge *et al.*[46] An EC₅₀ of 9.7 nM was observed for DHT using a *S. cerevisiae* strain engineered to respond to androgenic chemicals. The strain contained stable expression of the hAR and a reporter controlled by an ARE between two promoters (GPD and ADH1). Co-transformation of this plasmid with a second plasmid (pUTK404), containing the genes required for aldehyde synthesis (luxCDE) and FMN reduction (frp), yielded a bioluminescent reporter system that is responsive to a wide variety of bioactive androgens.

Mammalian cell AR luciferase models—Mammalian cell-based bioassays have been developed in immortalized cell lines which are relatively easy to culture, maintain, and show higher sensitivity than the yeast system. However, a careful characterization of model systems is necessary. It is imperative that the parent cell line selected for the development of the bioassay does not contain steroid-metabolizing enzymes since that could give inaccurate luciferase response results. Secondly, parent cells containing other steroid receptors could pose a problem since the ARE consensus DNA sequence has almost 80% similarity to cis-regulatory elements of glucocorticoid (GR, *NR3C1*), mineralocorticoid (MR, *NR3C2*) and progesterone receptors (PGR, *NR3C3*).[5,16] In these models, reporter gene expression can be activated by hormone ligands leading to false positives. Numerous mammalian cell lines, including prostate carcinoma cells (LNCaP, 22Rv1, PC3 and DU-145)[47] or other cells (HepG2, CV-1, COS-1, COS-7 and CHO) have been engineered to develop androgen reporter assays.[48,49]

Kim *et al.* used three prostate cancer cell lines (22Rv1, PC3, and LNCaP) to develop AR-regulated reporter gene assays.[50] While 22Rv1 and LNCaP cell lines have an endogenous AR, the PC3 cell line, reported to be AR negative,[51] was transiently transfected with a hAR expression vector. Among the three cell lines that were transiently transfected with pMMTV-luc, DHT stimulated proliferation only in LNCaP cells. It is important to note that the endogenously expressed AR in LNCaP cells contains a mutation in the ligand binding domain that alters steroid binding selectivity and can lead to activation by a variety of steroids that normally do not activate AR.[52] EC₅₀ values of DHT for 22Rv1, PC3/AR+,

and LNCaP were 0.063 nM, 0.008 nM, and 0.075 nM, respectively. While the sensitivity was good, each line showed endogenous expression of GR and therefore increased luciferase reporter in response to cortisol.

Hartig *et al.*[49] used a human breast carcinoma cell line (MDA-MB-453) and the African green monkey kidney cell line (CV-1). MDA-MB-453 cells were transduced with a luciferase reporter regulated by the MMTV. The MDA-MB-453 cell model expressed endogenous GR and AR. CV-1 cells were transduced as above with MMTV-luc and also a hAR. While the CV-1 exhibited relative selectivity for AR activation, the MDA-MB-453 transduced reporter responded to both glucocorticoids and androgens.

In 2007 Sun *et al.* used CV-1 cells that had been transiently transfected with hAR and MMTV-driven luciferase.[53] These investigators tested the effects of three common pyrethroids (fenvalerate, cypermethrin, permethrin) and their metabolite 3-phenoxybenzoic acid (3-PBA) for anti-androgenic and androgenic activity[53]. The assay displayed appropriate response to known AR agonists (EC₅₀ 3.6 nM with DHT) as well as AR antagonists.

A transient AR reporter assay for detection of anti-androgenic chemicals was used by Vinggaard *et al.*[54] Chinese Hamster Ovary cells (CHO) were co-transfected with vectors containing hAR and MMTV-luc by non-liposomal transfection. Cells were treated for 24 h with the synthetic androgen receptor agonist, R1881 (10 nM), resulting in a 30- to 60-fold induction of luciferase activity. CHO cells were subsequently used to develop a stable cell line.[55] For stable line development, CHO cells were co-transfected with plasmids encoding MMTV-luc, neomycin and hAR. After selection with neomycin and cloning, an active, responsive clone was obtained that stably expressed both the hAR and the luciferase reporter. Stimulation of the cells with androgens for 24 h resulted in about a 15-fold stimulation of luciferase activity, with the minimum effective dose of testosterone being 0.1 nM resulting in an EC₅₀ around 0.5 nM with R1881. Sixty different chemicals (pesticides or their metabolites, and common industrial chemicals) were screened with the cell line for their ability to activate or inhibit reporter as compared to a positive control. The most potent anti-androgenic compounds identified were bisphenol A, α -hexachlorocyclohexane, vinclozolin and 4,4-DDE.

An androgen reporter system that utilizes an endogenously expressed AR was developed by Blankvoort *et al.*[56] The human breast cancer cell line T47D was stably transfected with a luciferase gene under transcriptional control of the PB-ARE-2 promoter. The model system was called AR-LUX (Androgen Receptor-mediated LUCiferase eXpression) and was evaluated for its responsiveness to a number of androgens, anti-androgens, non-androgenic steroids, and to compounds modulating the AR itself. Following 24 h of treatment, an EC₅₀ value of 115nM was determined for DHT. Luciferase responses were also elicited by high concentrations of the steroids progesterone, 17 β -estradiol, aldosterone, and dexamethasone. The ability to selectively examine AR activation was a concern of this model.

The MDA-kb2 cell line, containing an endogenous AR, was developed by Wilson *et al.*[57] Cells were transformed with an androgen-responsive luciferase reporter plasmid driven by

MMTV, selected with geneticin, and cloned. The active clone was chosen and the resulting line termed, MDA-kb2. The MDA-kb2 has been a useful tool for studying the activation of both AR and GR because both receptors are present and both receptors can activate the MMTV promoter. Following 24 h of treatment, an EC₅₀ of 0.14 nM was determined for DHT. This model is relatively easy to use, grows well and is stable but responds to both AR and GR agonists.

The U2OS cell line was used by Sonneveld *et al.*[58] These investigators developed the AR CALUX (Chemically Activated LUCiferase eXpression) bioassay. It contains the human androgen receptor and a luciferase reporter construct containing three AREs coupled to a TATA promoter. The EC₅₀ of DHT was found to be 0.13 nM. The sensitivity of AR CALUX was assessed by measuring the luciferase activity induced by a series of natural steroids (DHT, testosterone and androstenedione).

In 2011 Sedláčková *et al.* developed two panels of U2OS-based luciferase reporter cell lines using two different reporter formats.[59] In the first model, the activity of the receptor was monitored by a reporter vector containing synthetic promoter with multimerized ARE or the MMTV upstream of the luciferase gene. The second model relied on the chimeric steroid receptor, where the N-terminal part of the receptor containing AF1 and the DBD was replaced by the DBD from the yeast transcription factor Gal4. This construct was co-transfected with reporter vector containing 9 copies of GAL4 response element (used even in HEK293)[60]. The investigators compared the two panels using several ligands and concluded that, in general, both systems generated a similar qualitative response. Both systems (AR/GRE or AR/MMTV) and AR-LBD/9XGal4UAS showed high sensitivity to DHT with an EC₅₀, after 24 h treatment, of 0.01 nM and 0.1 nM, respectively.

GREEN FLUORESCENT REPORTER MODEL

Compared to luciferase assay, fluorescent protein assay offers cheap and faster direct detection using spectrofluorometer or fluorescence microscope. The main advantage of green fluorescent protein (GFP) is that it does not require enzymatic substrates for detection. In addition, the use of different fluorescent proteins enables an investigator to track the expression of two (or more) genes in the same cell (multiplexing). GFP and its genetically enhanced variations are quantitative reporters with high levels of photo-stability and brightness. It is an auto-fluorescent protein initially derived from the Jellyfish *Aequorea Victoria* and can be used for a variety of biotechnological applications.[61] Most of the steroid bioassays that make use of GFP reporter were designed for the determination of estrogenic activity using yeast as the cell model.[41,62–64] The benefit of using GFP is the ability for direct quantification using either a fluorescence microscope or a luminometer. The details, including the sensitivity of these AR-cell based assays are reviewed in Table 3.

Bovee *et al.*[65] constructed recombinant yeast that expresses hAR and a yeast enhanced GFP (γ EGFP), as a measurable reporter protein, in response to androgens. They stably integrated, in the *S. Cerevisiae* genome, the reporter vector and the receptor expression vector. The γ EGFP reporter gene is optimized for yeast expression under control of the CYC1 promoter which contains two ARE sequences. The hAR coding region is constitutively expressed under the control of a GDP promoter. The investigators also

demonstrated that *S. Cerevisiae* did not metabolize test compounds, displayed no crosstalk for non-androgen steroids and had a relative androgenic potency. Androgen activity can be quantified directly in a cytofluorimeter using excitation at 485 nm and measuring fluorescent emission at 530 nm. The EC₅₀ value for testosterone activation of yEGFP expression was 50 nM following 24 h of incubation.

GFP was introduced as an alternative reporter gene in the androgen assay system developed by Beck *et al.*[66] The hAR coding sequence was inserted into expression plasmid YEpbUBi-FLAG1, resulting in the plasmid YEpbUBiFLAG-AR, and the ERE on the reporter vector YRpE2 was substituted with an ARE, resulting in the plasmid YRpE2-ARE. The vector YRpE2-GFP was used as a backbone to create the reporter plasmid YRpE2-GFP-ARE, using GFP as a reporter gene. For evaluation of the reporter system, β -galactosidase, as a primary reporter gene, was added. Several known AR agonistic compounds (5 α -dihydrotestosterone, testosterone, androstenedione, 17 α -methyltestosterone, progesterone, epitestosterone, and norgestrel) were tested to evaluate both reporter systems. The model shows an EC₅₀ of 16nM with testosterone.

In 2008 Dennis *et al.* developed an assay for the assessment of AR transcriptional activity using destabilized enhanced GFP (dsEGFP) in PC3 cells.[67] Confocal images were collected using microscopy and the EGFP quantification was measured by the HyperCyt® high-throughput flow cytometry. PC3 cells were transiently co-transfected with an expression vector for the wild-type hAR (pDsRedhAR) and an MMTV promoter EGFP (pMMTVdsEGFP). Agents with established androgenic and anti-androgenic activity were used for validation of the multifunctional androgen receptor screening assay. HyperCyt analysis requires 24 h treatment with compounds followed by cell centrifugation. A lack of selectivity was demonstrated after treatment with R1881, DHT, E2, progesterone, bicalutamide, nilutamide and androstenedione; all compounds induced significant increases in the percent of cells expressing dsEGFP compared to unstimulated wells. The sensitivity of the assay in response to AR was evaluated on EC₅₀ of R1881 (1.34 pM) and DHT (0.1 nM).

Fluorescent AR translocation bioassay—In recent years several cell-based models have been developed to monitor androgen activity by imaging AR nuclear translocation in response to ligands.[17,18] GFP-tagged AR is the only assay that currently allows detection with fluorescent microscopy and automated image analysis to quantify changes in AR nuclear translocation, intracellular dynamic and solubility in response to compounds and AR mutations. AR transgenes containing GFP or its spectral variants cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP), allow tracking of the dynamic events that occur following ligand binding, using real-time microscopy.

To investigate the cellular translocation of GFP-AR after treatment with agonists and antagonists, Marcelli *et al.*[68] utilized an AR (A619Y) containing a mutation in the DNA binding domain[69] that inactivates the transcriptional activity of the receptor. A619Y is able to form distinct foci upon exposure to active compounds. The model relies on transient transfection of HeLa cells with tagged AR. This investigative group has used GFP-AR as well as CFP-AR that allows for dual examination with proteins tagged with YGF.

Incubation of cells for 2 h with ligand (R1881, Casodex, Flutamide, and Estradiol) was sufficient to allow the quantification of AR nuclear translocation (EC_{50} 0.08 of R1881). A high throughput microscopy (HTM) system was used to automate fluorescent image acquisition and analysis of AR nuclear translocation and nuclear foci formation, while the CytoShop software was utilized to quantify the translocation. The results demonstrated that agonist addition resulted in a translocation of the receptor from the cytoplasm to the nucleus where it became organized into stable foci. Interestingly, AR antagonist also caused some nuclear translocation but without the resultant focal distribution (also called hyperspeckling). Fluorescence recovery after photobleaching (FRAP) also revealed that agonist-bound GFP-AR exhibited reduced mobility relative to un-liganded or antagonist-bound GFP-AR.

A different high throughput (HT) image-based assay that quantifies AR subcellular and subnuclear distribution and transcriptional reporter gene activity on a cell-by-cell basis was developed by Szafran *et al.* [70]. This assay permitted the analysis of cell cycle dependent changes in AR function in unsynchronized cell populations, allowing for the determination of cell cycle position with simultaneous analysis of DNA. HeLa cell lines were generated to stably express wild type (GFP-AR), mutant GFP-ART877A (LNCaP mutation) [71] or GFP-ARF764L (AIS mutation) [72]. R1881, mibolerone, and DHT were tested to demonstrate the utility of the AR bioassay. All three compounds induced GFP-AR nuclear translocation in a dose-dependent manner. Using R1881, the calculated EC_{50} concentration for nuclear translocation was 0.96 nM. The AR agonists DHT and mibolerone demonstrated similar effects when compared to R1881. An automated microscope was used to capture the images, and CytoShop and Pipeline Pilot image analysis software was used to quantify.

CONCLUSIONS

There is broad interest in the use of cell based androgen screening assays to assess androgenic activity of chemical compounds as well as to test human sera for novel androgens. Over the past 10 years there have been significant improvements in the available AR cell-based assays which now provide rapid, inexpensive and sensitive androgen screening tools. Improved aspects of the models include greater selectivity for AR and improved reporter gene responses. These assays provide important resource for the discovery of novel androgens that cause human diseases of androgen excess, the detection of prohibited androgenic compounds in athletes, as well as methods for defining environmental contaminants that act as AR endocrine disrupters.

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Abbreviations

AR	androgen receptor protein
NR3C4	Nuclear Receptor Subfamily 3 Group C Member 4 (androgen receptor gene)

ARE	androgen response element
GR	glucocorticoid receptor protein
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1 (glucocorticoid receptor gene)
MR	mineralocorticoid receptor protein
NR3C2	Nuclear Receptor Subfamily 3 Group C Member 2 (mineralocorticoid receptor gene)
PGR	progesterone receptor protein
NR3C3	Nuclear Receptor Subfamily 3 Group C Member 3 (progesterone receptor gene)
LBD	ligand binding domain
DBD	DNA binding domain
CTD	C-terminal domain
NTD	N-terminal domain
DHT	dihydrotestosterone
β-gal	β-galactosidase
hAR	human AR
BLA	β-lactamase

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Figure 1. Cell-based bioassays for the study of AR activity

(A) Models using native androgen receptor rely on ligand/antagonist effects on AR regulation of reporter gene transcription. AR binding causes translocation of the cytosolic AR into the nucleus and its binding to androgen responsive elements that drive a variety of reporters. Models are available that use GFP, luciferase or β -galactosidase. (B) Models using yeast-two-hybrid system for determining androgen activity and activation of reporter gene transcription. Androgen binding causes the translocation of cytosolic AR receptor hybrid, [GAL4 DNA binding domain (DBD)/AR ligand binding domain (LBD)] into the nucleus and its binding and activation of GAL4 promoter-driven luciferase, β -galactosidase or β -lactamase reporter systems.

Androgen Receptor Bioassays using the β -Galactosidase Reporter

Table 1

AR type	Promoter	Exposure time	EC ₅₀ value	Assay cells	Reference
hAR ^{2a}	ARE	40 hours	1 nM DHT	<i>S. Cerevisiae</i>	Purvis et al. 1990 ³¹
hAR ^{2a}	ARE	40 hours	1 nM DHT	<i>S. Cerevisiae</i>	Sohoni and Supler 1998 ³²
hAR ^{2a}	ARE	Overnight	3.5 nM DHT	<i>S. Cerevisiae</i>	Gaido et al. 1996 ⁴¹
hAR ^{2a}	ARE	16 hours	4 nM DHT	<i>S. Cerevisiae</i>	Chatterjee et al. 2007 ³⁵
AR Yeast two-hybrid protein based models					
hAR-LBD	GAL4	16 hours	4.8 nM DHT	EGY48	Lee et al. 2003 ³⁸
GAL4DBD-ARLBD	GAL4	4 hours	10 nM DHT	Y190	Nishikawa et al. 1998 ³⁹
GAL4DBD-ARLBD	GAL4	2 hours	13 nM DHT	Y187	Li et al. 2008 ²⁹

¹ endogenous,
² stable,
³ transient
^a clonal,
^b mixed population

Table 2

Androgen Receptor Bioassays using a Luciferase Reporter

AR type	Promoter	Reporter	Exposure time	EC ₅₀ value	Assay cells	Reference
Yeast cell -based AR Models						
hAR ²	ARE	Firefly Luciferase	3 hours	10 nM DHT	<i>S. Cerevisiae</i>	Michelini et al. 2004 ³⁷
hAR ²	ARE	Firefly Luciferase	2.5 hours	5.5 nM DHT	<i>S. Cerevisiae</i>	Leskinen et al. 2005 ³⁶
hAR ²	ARE	Bacterial Luciferase	3–4 hours	9.7 nM DHT	<i>S. Cerevisiae</i>	Eldridge et al. 2007 ⁴⁶
Mammalian cell-based AR Models						
hAR ¹	MMTV	Firefly Luciferase	24 hours	0.063 nM DHT	22Rv1	Kim et al. 2006 ⁵⁰
hAR ³	MMTV	Firefly Luciferase	24 hours	0.008 nM DHT	PC3	Kim et al. 2006 ⁵⁰
hAR ¹	MMTV	Firefly Luciferase	24 hours	0.075 nM DHT	LNCaP	Kim et al. 2006 ⁵⁰
hAR ^{1a}	MMTV	Firefly Luciferase	48 hours	~0.2 nM DHT	MDA-MB-453	Hartig et al. 2002 ⁴⁹
hAR ^{2a}	MMTV	Firefly Luciferase	48 hours	~0.2 nM DHT	CV-1	Hartig et al. 2002 ⁴⁹
hAR ³	MMTV	Firefly Luciferase	24 hours	3.6 nM DHT	CV-1	Sun et al. 2007 ⁵³
hAR ³	MMTV	Firefly Luciferase	24 hours	~0.5 nM R1881	CHO	Vinggaard et al. 1998 ⁵⁴
hAR ^{2a}	MMTV	Firefly Luciferase	24 hours	~0.5 nM R1881	CHO	Roy et al. 2003 ⁵⁵
hAR ^{1a}	ARE	Firefly Luciferase	24 hours	115 nM DHT	T47D	Blankvoort et al. 2001 ⁵⁶
hAR ^{1a}	MMTV	Firefly Luciferase	overnight	0.14 nM DHT	MDA-kb2	Wilson et al. 2001 ⁵⁷
hAR ^{2a}	ARE	Firefly Luciferase	24 hours	0.13 nM DHT	U2OS	Sonneveld et al. 2004 ⁵⁸
hAR ^{2b}	MMTV	Firefly Luciferase	24 hours	0.01 nM DHT	U2OS	Sedláč et al. 2011 ⁵⁹
hAR ^{2b}	GRE	Firefly Luciferase	24 hours	0.01 nM DHT	U2OS	Sedláč et al. 2011 ⁵⁹
AR yeast two-hybrid protein models						
GAL4DBD-AR LBD	GAL4	Firefly Luciferase	24 hours	0.1 nM DHT	U2OS	Sedláč et al. 2011 ⁵⁹

¹ endogenous,

² stable,

b mixed population

a clonal,

c transient

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Table 3

Androgen Receptor Bioassays using Fluorescent Proteins as a Reporter

AR type	Promoter	Reporter	Exposure time	EC ₅₀ value	Assay cells	Reference
Yeast cell AR bioassay model						
hAR ^{2a}	ARE	yEGFP	24 hours	50 nM T	<i>S. Cerevisiae</i>	Bovee et al. 2008 ⁶⁵
hAR ^{2a}	ARE	GFP	24 hours	16 nM T	<i>S. Cerevisiae</i>	Beck et al. 2008 ⁶⁶
Mammalian cell AR bioassay model						
hAR ^{2b}	MMTV ^b	dsEGFP	24 hours	0.1 nM DHT	PC-3	Dennis et al. 2008 ⁶⁷
Mammalian cell AR nuclear translocation model						
hAR ^{l/b}	NA	Nuclear AR-GFP	2 hours	0.08 nM R1881 [*]	HeLa	Marcelli et al. 2006 ⁶⁸
hAR ^{l/a}	NA	Nuclear AR-GFP	2 hours	0.96 nM R1881 [*]	HeLa	Szafran et al. 2008 ⁷⁰

^l mutated,

²_{WT}

^a stable,

^b transient transfection

^{*} EC₅₀ nuclear translocation

NA= not applicable