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A Multifunctional Androgen Receptor Screening (MARS) Assay Using the High-Throughput HyperCyt® Flow Cytometry System

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Abstract

Background—The androgen receptor (AR) is a steroid hormone receptor which regulates transcription of androgen-sensitive genes and is responsible for the development and maintenance of male secondary sexual characteristics. Chemicals that interfere with AR activity may lead to pathological conditions in androgen-sensitive tissues. A variety of reporter systems have been developed, driven by androgen sensitive promoters, which screen for chemicals that modulate androgenic activity. We have developed a flexible, high-throughput AR transcriptional activation assay, designated the Multifunctional Androgen Receptor Screening (MARS) assay, to facilitate the identification of novel modulators of AR transcriptional activity using flow cytometry.

Methods—Androgen-independent human prostate cancer-derived PC3 cells were transiently co-transfected with an expression vector for the wild-type human AR and an androgen-sensitive promoter regulating the expression of destabilized enhanced GFP (dsEGFP). The transfected cells were stimulated with established androgenic and antiandrogenic compounds and assessed for increased or decreased dsEGFP expression. To screen for antagonists of AR transcription, the AR agonist R1881 was co-administered at sub-maximal concentrations with potential AR antagonists. The assay was formatted for high throughput screening using the HyperCyt® flow cytometry system.

Results—Agents with established androgenic and antiandrogenic activity were used for validation of the MARS assay. AR agonists were found to potently induce dsEGFP. Furthermore, AR agonists induced dsEGFP expression in a dose-dependent manner. Alternatively, AR antagonists blocked dsEGFP expression when co-administered with low-dose R1881, which also occurred in a dose-dependent manner.

Conclusions—Modulators of AR transcriptional activity can be successfully identified by the MARS assay, utilizing a rapid, flexible, sensitive, and high-throughput format. Dose-response

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curves can be successfully generated for these compounds, allowing for an assessment of potency. Due to its simplicity and high-throughput compatibility, the MARS assay and HyperCyt® system combined with flow cytometric analysis represents a valuable and novel addition to the current repertoire of AR transcriptional activation screening assays.

Keywords

Androgen receptor assay; androgens; antiandrogens; flow cytometry; HyperCyt®; biomolecular screening

INTRODUCTION

Endogenous androgens such as testosterone and dihydrotestosterone (DHT) are necessary for the normal development and maintenance of male sex accessory organs, such as the prostate gland (1). Disruption of androgenic activity is of increasing concern as a mediator of disease in males (2,3). With a growing awareness that endocrine disrupting chemicals influence the activity of steroid hormone receptors, it is important to identify and determine the potency of the broad range of compounds that have the ability to modulate androgenic activity (4). Therefore, assays that screen for endocrine disrupting compounds are proving to be invaluable for identifying and characterizing agents that alter androgenic activity, which may contribute to endocrinopathies.

The action of androgens is mediated by the androgen receptor (AR), an approximately 110 kilodalton protein residing in the cytoplasm that, upon binding androgen, undergoes a conformational rearrangement and translocates to the nucleus, dimerizes, and alters the transcriptional regulation of androgen-responsive genes (5,6). In addition to endogenous AR ligands, a substantial number of synthetic steroidal and non-steroidal AR ligands have been described (7). These include steroidal compounds such as methyltrienolone (R1881), a synthetic AR agonist, cyproterone acetate (CPA), a synthetic anti-androgen, and non-steroidal anti-androgens such as bicalutamide (BC), flutamide, and nilutamide. Antagonists of the AR block AR transcriptional activity through at least one of two mechanisms. For example, AR antagonists may block the conformational change of the AR which occurs upon agonist binding, preventing nuclear translocation of the AR and dimerization of receptors, and thus inhibiting AR-mediated gene transcription (8). Alternatively, AR antagonists may bind the AR resulting in translocation of the AR into the nucleus; however, a functional AR transcriptional complex does not develop, thus inhibiting AR activation (9). In either instance, the critical activity is the inhibition of a functional AR complex that modulates transcriptional activity, which is not discerned by *in vitro* assays that screen only for AR competitors or that screen for receptor nuclear translocation without assessing transcriptional activation.

Induction of gene expression by a wide array of transcription factors, including steroid hormone receptors such as the AR, has been studied using a variety of reporter gene assays. These assays are typically comprised of a reporter plasmid that is either transiently or stably transfected into an appropriate cell line either concurrent with a receptor expression vector or into a cell line expressing the receptor of interest. The reporter plasmid contains response elements specific for the receptor of interest regulating a promoter which in turn drives expression of the reporter gene; expression of this reporter gene can be measured by specific assays for the reporter of interest. Commonly used reporter genes in these assays include the enzymes luciferase, β-galactosidase, and chloramphenicol acetyltransferase (CAT) which require analysis based on the entire cell population rather than transcriptional activation on a single cell level (10). Additionally, in order to measure activity of luciferase, β-galactosidase, or CAT, an exogenous substrate must be added to the assays to develop the

signal. Thus, reporter gene assays are available that allow some flexibility in the development of assays for gene expression regulation by transcription factors, such as the AR.

Green fluorescent protein (GFP) is an alternative reporter which has several distinct advantages to other reporter proteins; GFP based assays are unique from most other reporter assays in that they allow analysis of intact cells on a single cell basis and they do not require addition of reagents to cells in order to detect assay signal. The commonly used form of GFP, termed enhanced green fluorescent protein (EGFP), is relatively stable when expressed in mammalian cell lines. A destabilized form of green fluorescent protein (dsEGFP) has been generated by fusing a PEST sequence to the C-terminus of EGFP, effectively reducing the half life of dsEGFP to 2 h while retaining the spectral properties of EGFP (11). The dsEGFP has been shown to be an effective reporter in a range of assays, including those for activation of c-fos (12), IL-8 induced transcription (13), NF-κB activation (11,14), proteosome inhibition (15) and, perhaps most relevant to steroid hormone receptor transcriptional assays, for activation of transcription by glucocorticoid receptor (16). Several of these reports compare EGFP and dsEGFP as transcriptional reporters (12-14) and demonstrate that dsEGFP is a more dynamic reporter than EGFP which also provides more reproducible results.

In this study, we establish the utility of a multifunctional androgen receptor screening assay (designated the MARS assay) using PC3 prostate cancer cells (17) transiently cotransfected with expression vectors for the human AR (hAR) and a reporter plasmid consisting of the androgen-responsive MMTV promoter driving expression of dsEGFP. The activities of the potent synthetic androgen R1881 and the therapeutically employed AR antagonist bicalutamide were initially established in the MARS assay system prior to extension of the assay to a small test set of compounds with known abilities to modulate AR transcriptional function. We show the effectiveness of the MARS assay in screening this set of compounds and the dynamic range of the assay, which allows for delineation of dose-response activities for both AR agonists and AR antagonists. Due to the cell based nature of this assay, it was naturally incorporated with the HyperCyt® system allowing for high-throughput flow cytometric analysis of compounds that modulate AR activity.

MATERIALS & METHODS

CHEMICALS

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. R1881 (i.e., methyltrienolone) was from PerkinElmer (Waltham, MA). Bicalutamide was from ZereneX Molecular (Manchester, UK). Cyproterone acetate and 4-androstenedione were from Steraloids (Newport, RI).

EXPRESSION VECTORS

The pIRES2-DsRedExpress plasmid was obtained from Clontech (Mountain View, CA). The human AR was PCR amplified using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and directionally subcloned into the XhoI/SacII sites of pIRES2-DsRedExpress to construct pDsRedhAR. The pMMTVdsEGFP plasmid was constructed by replacing the luciferase gene from pMMTVlux (18) as an Xho1/Not1 fragment with the destabilized EGFP from pd2EGFP-1 (Clontech).

CELL CULTURE and TRANSFECTION

PC3 human prostate carcinoma cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) + 5% fetal bovine serum (FBS, Invitrogen,

Carlsbad, CA). For the MARS assay, PC3 cells were plated 72 h prior to transfection in 96-well, flat-bottomed tissue culture plates at 4,000 cells per well in DMEM containing 4% charcoal-stripped FBS (CSS) and 1% FBS. Cells were transfected with pDsRedhAR and pMMTVdsEGFP at a ratio of 1:20 using FuGENE 6 according to the manufacturers' instructions (Roche Diagnostics, Indianapolis, IN). In addition, to test for non-specific MMTV promoter activation (i.e., not mediated by the AR), cells were transfected with pCMVβ in place of pDsRedhAR and pMMTVdsEGFP at a ratio of 1:20 using FuGENE 6, as described above. For confocal microscopic analysis, cells were seeded at a concentration of 2×10^4 cells per well onto 12-mm glass coverslips 72 h prior to transfection.

TRANSCRIPTIONAL ACTIVATION ASSAYS

Twenty-four h after transfection, test compounds or vehicle were added to cells in DMEM containing 4% CSS and 1% FBS. For antagonist assays, potential AR antagonist compounds or vehicle were added to cells in DMEM containing 1pM R1881, 4% CSS and 1% FBS. To control for non-specific activation of the MMTV promoter, cells cotransfected with the pIRES2-DsRedExpress and pMMTVdsEGFP vectors were treated identically to plates in the agonist assay as described above and induction of dsEGFP expression in the absence of the AR was noted. Cells were incubated for 24 h following transfection before analysis by microscopy or using the HyperCyt® system.

MICROSCOPIC ANALYSIS

For confocal microscopic analysis of dsEGFP fluorescence, treated coverslips were fixed overnight in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate-buffered saline (PBS) at 4°C. Coverslips were washed 3× with PBS and mounted using VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) containing 6μM 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO). Confocal images were collected using a Zeiss LSM510 microscope and a 20× objective. For fluorescence microscopy, the number of dsEGFP positive cells per treatment was determined using an Olympus IX70 inverted microscope with a 4× objective. Images from each well were captured using an Olympus Digital Camera with MagnaFire 2.1A Digital Microimaging software as 8-bit TIFF images and the number of dsEGFP positive cells per an image were counted using ImageJ 1.38× software (19). Four wells were examined for each treatment and each experiment was performed in duplicate. Consistency of transfection over a 96-well plate was established by examining for equivalence of DsRed expression between wells from either pIRES2-DsRedExpress or pDsRedhAR using fluorescence microscopy prior to sample treatment.

HyperCyt® ANALYSIS

Twenty-four h after treatment, medium was removed from each well cells in 96-well plates were trypsinized with 25μL 0.25% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) for 3 min. Trypsin was neutralized by addition of 75μL DMEM containing 5% FBS and cells were transferred to 96-well V bottom PCR plates. Cells were pelleted by centrifugation at 1,200×g and gently resuspended in 25μL DMEM. Plates were rotated at approximately 12rpm at 4°C for up to 30 min prior to analysis. The HyperCyt® autosampling system (20,21) was used to sample individual wells for 1.2 s per well (approximately 2.5μL sample pickup). Samples were acquired using a CyAn™ ADP flow cytometer (Dako, Inc., Fort Collins, CO). Data analysis was completed using IDLQuery software written by Dr. Bruce Edwards (22).

RESULTS

The ability of PC3 cells transiently cotransfected with pDsRedhAR and pMMTVdsEGFP to respond to androgenic stimuli was initially established by microscopic examination of low power fields for dsEGFP positive cells following treatment (Fig. 1A-D) prior to formatting the assay for HyperCyt® high-throughput flow cytometry (Fig. 1E). To verify AR-dependent dsEGFP expression in response to androgen stimulation, PC3 cells seeded and cotransfected on glass coverslips were stimulated with either vehicle (i.e., DMSO) or 10pM R1881. The expression of dsEGFP was dependent on androgen stimulation, as dsEGFP expression was induced in R1881-treated cells, while unstimulated cells displayed no background dsEGFP expression (Fig. 1A-B). Additionally, dsEGFP expression in this system was dependent on expression of the AR, as cells cotransfected with pIRES2-DsRedExpress and pMMTVdsEGFP showed no activation of the MMTV promoter upon stimulation with androgen (data not shown). To insure uniform transfection across each well of a 96-well plate, the levels of DsRed expressed from either pIRES2-DsRedExpress or pDsRedhAR were assessed by fluorescence microscopy prior to treatment. The dynamic range of the MARS assay was illustrated by stimulation of cells with sub-maximal concentrations of R1881 (e.g., 1pM), resulting in dsEGFP expression in a lower number of cells (Fig. 1C) than observed with maximal stimulation. The use of R1881 concentrations in the linear dose-response range allows for the investigation of compounds with AR antagonist activity in that coadministration of low-dose R1881 enables the identification of potential antagonists. Figure 1D illustrates the effect of a well-characterized AR antagonist, bicalutamide, on low-dose R1881 induction of dsEGFP (Fig. 1C).

A time-course study was performed using the MARS assay in order to establish the optimal time point after treatment for the screening assay. For both cytometric and microscopic analyses, a time at which maximal dsEGFP expression was induced provides the greatest dynamic range for evaluation of transcription modulation in the MARS assay. PC3 cells in 96-well plates were cotransfected with pDsRedhAR and pMMTVdsEGFP and stimulated with R1881 in the absence or presence of bicalutamide. At 6, 12, 24, 48 and 72 h after treatment, the wells were imaged and the number of dsEGFP expressing cells determined (Fig. 2A). This microscopic analysis established that 24 h post treatment was the optimal time for analysis of the MARS assay, as the difference in dsEGFP expression between background and 1nM R1881 treated cells was found to be maximally induced at this time point. Additionally, dsEGFP expression in wells treated with 1pM R1881 was maximal at 24 h after treatment, and this dsEGFP expression could be efficiently inhibited by the presence of 1μM bicalutamide at 24 h.

In order to determine the dynamic properties of the MARS assay, an androgen dose-response using PC3 cells cotransfected with pDsRedhAR and pMMTVdsEGFP was performed. Cells stimulated with doses of R1881 ranging from 10^{-15} to 10^{-9} M were analyzed by counting the number of dsEGFP expressing cells in 4 independent wells and the percent of dsEGFP positive cells were compared to the maximum number of cells expressing dsEGFP in wells treated with 10nM R1881 (Fig. 2). A three parameter logistics curve analysis of the R1881 dose-response gives an EC50 for R1881 of 7.81×10^{-13} M (Fig. 2). Importantly, treatment of cells with varying doses of R1881 in the presence of 1μM of the AR antagonist bicalutamide shifted the dose response to the right, with an EC50 of 4.73×10^{-12} M (Fig. 2).

Various established steroidal ligands for the AR as well as natural and synthetic non-steroidal compounds possess AR modulating activity. In order to assess the effectiveness of the MARS assay for AR ligands other than R1881 and bicalutamide, a test set of compounds with reported androgenic and anti-androgenic activities was selected (Fig. 3). These

compounds were tested at a single dose of 10μM in a high-throughput screening format using the MARS assay (Fig. 4). PC3 cells cotransfected with pDsRedhAR and pMMTVdsEGFP were seeded in 96-well plates, stimulated with a test compound dose of 10μM and dsEGFP induction was assayed 24 h later (Fig. 4A). Cells were collected for flow cytometric analysis of dsEGFP expression after trypsinization. Analysis of dsEGFP positive cells was performed using the HyperCyt rapid autosampling system with a CyAn ADP flow cytometer and data analysis was performed using IDLQuery software.

Activation of the MMTV promoter was assessed by analysis of wells based on percent cells expressing dsEGFP. The gate for distinguishing GFP-positive response was defined by analyzing a 96-well plate of cells and setting the positive response threshold at a level at which 5% of total events in the plate were above the gate. The percent responding cells in wells containing 10nM R1881 was defined as 100% and all wells were normalized to this response using the formula:

$$\text{la}\ \text{ve to R1881} = \frac{\text{Number of gated events pe}}{\text{Number of gated events with 10}}$$

R1881, DHT, E2, progesterone, bicalutamide, nilutamide and androstenedione all induced significant increases in the percent of cells expressing dsEGFP compared to unstimulated wells (Fig. 4A). The specificity of these compounds in transactivating the MMTV promoter via the AR was confirmed in cells cotransfected with pIRES2DsRedExpress and pMMTVdsEGFP; in the absence AR expression, these compounds did not promote transcription or dsEGFP expression via the MMTV promoter (data not shown).

Compounds with putative AR antagonist activity were also screened in the 96-well HyperCyt® format (Fig. 4B). In this assay, a dose of 1pM R1881 was used in all wells to induce a low level of dsEGFP expression sufficient to allow detection of compounds with AR antagonist activity. Similar to the AR agonist assay, a gate was set such that 5% of total events in the plate were in the GFP positive region, and the percent of GFP positive cells in each well was determined. The percent of GFP positive cells in wells treated with 1pM R1881 alone was defined as 100% and normalization of each well was completed using the formula:

$$\text{ti}\ \text{e to 1pM R1881} = \frac{\text{Number of gated events}}{\text{Number of gated events with}}$$

Compounds with significant antagonist activity at 10μM in this assay included DES, progesterone, bicalutamide, CPA, nilutamide, and flutamide (Fig. 4B). Treatment of wells

with 10μM of the potent AR agonists R1881 and androstenedione resulted in significant increases in dsEGFP positive cells compared to 1pM R1881 controls (Fig. 4B).

In addition to distinguishing AR agonists and antagonists, a viable AR transcriptional assay should address the question of ligand potency. In order to assess the activity of several AR transcriptional agonists and antagonists identified in single-point assays, dose-responses for the various compounds were completed (Fig. 5). Dose response curves were performed for AR agonists including the synthetic androgen agonist R1881 (presented in the analysis of each agonist to facilitate comparison of potency between agonists), as well as naturally occurring hormones including DHT, androstenedione, E2, and progesterone (Fig. 5A-D). Analysis of agonist potency using percent cells responding was completed in a similar manner to the single-point agonist screen. R1881 was the most potent transcriptional agonist of the AR agonists tested, with an EC50 value of 1.34×10^{-12} M (Fig. 5A and Table 1). DHT, an endogenous AR ligand, was found to be the second most potent agonist of AR-mediated transcription (Fig. 5A). Androstenedione, a biosynthetic precursor of DHT and E2, displayed an activity significantly lower than the endogenous AR ligands but slightly better than the ligands of other steroid receptors (Fig. 5B). Ligands for other steroid hormone receptors, estrogen and progesterone, were found to induce AR-mediated transcription only at much higher concentrations compared to DHT or R1881 (Fig. 5C and 5D). A detailed description of the methods used to acquire data for this study is provided as Supplementary Material (Supplementary Fig. 1).

In addition to determining the agonist activity of AR ligands, the anti-androgenic activity of several steroidal and non-steroidal antiandrogens was assessed (Fig. 6A-D). This assay was conducted in a manner similar to the AR agonist dose-responses, except varying concentrations of potential antagonists were determined in the presence of 1pM R1881 and the inhibition of dsEGFP expression relative to the induction of dsEGFP by 1pM R1881 was assessed. The dose-response curve for the well-established antiandrogen bicalutamide was included with each antagonist analysis to allow for comparison between antagonists (Fig. 6A-D). Flutamide was found to be the most effective AR antagonist in this assay, with a calculated IC50 of 1.51×10^{-10}M (Fig. 6B and Table 1). The non-steroidal antiandrogen nilutamide was found to be potent inhibitor of the AR (Fig. 6C). Interestingly, the well-established synthetic estrogen DES was found to be an inhibitor of AR activity using the MARS assay (Fig. 6D). Table 1 shows EC50 and IC50 values determined using the MARS assay for AR agonists and AR antagonists, respectively.

DISCUSSION

In this study, an assay is presented for the assessment of AR transcriptional activity using destabilized EGFP in prostate cells, the HyperCyt® high-throughput screening system, and flow cytometry, which was designated the multifunctional androgen receptor screening assay or MARS assay. The utility of the MARS assay was examined using microscopy-based methods prior to scaling up the assay for high-throughput capabilities using HyperCyt® and flow cytometry. Here, we have validated the MARS assay using a representative set of established natural and synthetic AR agonists and antagonists to determine AR transcriptional modulating activity at a single dose as well as in a dose-response, which demonstrated the sensitivity of the MARS assay and its ability to detect a dynamic range of dsEGFP expression in response to various AR ligands.

The use of dsEGFP as the reporter in this assay was practical for several reasons. GFP-based systems are simpler to perform than most other reporter methods, such as luciferase or CAT based reporter assay methods, since addition of exogenous reagents are not required for development of the reporter signal. Additionally, GFP expression can be verified by

microscopic methods as a very rapid assessment of assay performance. The use of dsEGFP rather than the more stable EGFP confers several additional benefits on the MARS assay. Prolonged EGFP expression can be toxic to cells (23) and this has the potential to significantly influence results. Additionally, the decreased stability of dsEGFP, which has a half-life in living cells of 2 h, allows for increased dynamic range of GFP fluorescence as the rapid turnover of dsEGFP prevents buildup of basally expressed intracellular EGFP which potentially results in a higher level of baseline fluorescence (11). Following initial assay validation by microscopy, we chose to use flow cytometry in later assays for a number of reasons. Microscopic methods allow for analysis of single cells in relatively small numbers, which can be time and resource intensive. Cell homogenization assays such as luciferase- and CAT-based assays allow only for analysis of response on a population level, potentially missing more subtle effects of stimulation on the single cell level. Flow cytometry allows rapid analysis of samples on a single cell basis and provides a convenient method for assaying fluorescence intensity of a population of cells. Additionally, the HyperCyt® autosampling system allows for rapid sampling of treated wells from either a 96- or 384-well plate, further increasing the throughput of the MARS assay by allowing collection of numerous samples in only a few minutes (21).

The ability of the MARS assay to distinguish AR transcriptional agonists from antagonists was demonstrated in this study. For example, the AR agonists R1881, DHT, E2, progesterone and androstenedione all induced significant increases in the percent of dsEGFP positive cells following 24 h stimulation compared to unstimulated control cells. This same set of AR agonists also significantly increased the level of dsEGFP expression within the cells expressing dsEGFP, as evidenced by an increase in mean fluorescence intensity within this cell population. Note that although 24 h was determined as the optimal time point for MARS assay sensitivity, dsEGFP expression by AR agonists could be detected as early as 6 h after treatment (Fig. 2A). In the single-dose antagonist assay, the known AR antagonists bicalutamide, CPA, nilutamide, and flutamide all significantly reduced the percent of cells expressing dsEGFP stimulated by 1pM R1881. Interestingly, DES, a potent estrogen receptor agonist, was found to act as an AR antagonist, which we speculate could account for some of its therapeutic action in prostate cancer treatment. Although not pursued in the current study, the MARS assay was also capable of detecting AR agonists, such as R1881, DHT, E2 and androstenedione, in the antagonist assay, as these agents were found to significantly increase transcriptional responses when compared to 1pM R1881-stimulated wells. Thus, the MARS assay may prove to have even greater utility as a simultaneous screen for AR agonists and antagonists.

An important facet of the MARS assay is the ability to generate dose-response curves for AR transcriptional agonists as well as antagonists. Our results show the sensitivity of the MARS assay in response to stimulation by a variety of compounds. The EC50 values as determined by the MARS assay are, on the whole, lower than those reported, demonstrating the high sensitivity of the MARS assay. For example, the calculated EC50 for R1881 of 1.34×10^{-12} M determined in this study is lower than typically seen in the literature, but is in agreement with the EC50 value calculated in our initial microscopic analysis of 0.88×10^{-12} M. Values obtained for DHT, E2 and progesterone typically fall into the low end of reported EC50 values for these compounds in AR transcriptional assays. A notable exception to this trend is androstenedione, for which there are few reported EC50 values for AR activation. Our EC50 value for androstenedione was 1.82×10^{-7}M, while others (24,25) have reported EC50 values from 1 to 10nM. There are several significant differences between the MARS assay and many of the other assay systems that have been used which may account for these differences. Notably, the MARS assay uses PC3 cells, a prostate adenocarcinoma-derived cell line which although not natively androgen responsive, is well-characterized for androgen responsiveness following forced AR expression (26-28). Other assay systems have

reported using cell types that do not express the AR and which are not otherwise androgen responsive, including a variety of primate kidney cell lines (Cos7, CV-1) and human cell lines including those derived from hepatic (HepG2), cervical (HeLa), breast (T47D) and kidney (HEK293) cells. In fact, reporter assays in cell lines such as 22Rv-1, another human prostatic cancer cell line, have been shown to be more sensitive to androgen-mediated transcription activation than parallel reporter assays in cell lines such as HepG2 that are not natively androgen responsive (29). The use of dsEGFP as a reporter system used may also influence assay sensitivity. Thus, through dose-response analysis, we show that the MARS assay is exquisitely sensitive for analyzing AR activity and is in general agreement with other assay systems for measurements of AR agonist activity.

In addition to determining EC50 values for AR agonists, the MARS assay can also be used to assess the potency of AR antagonists and to calculate IC50 values for these compounds. Similar to the AR agonists tested in this study, the MARS assay appears to be more sensitive to the effects of antagonists on dsEGFP expression than prior assays, with IC50 values generally lower than those reported in other assays. Notably, several compounds typically considered antiandrogens show partial agonist characteristics when administered at high doses in the MARS assay. All AR antagonists tested except DES and flutamide showed some agonist character at concentrations of 1μM or 10μM, consistent with previously reported observations (e.g., BC (26,30), CPA (29), nilutamide (31)). Consistent with previous reports (24), DES and flutamide did not have background agonist activity and significantly inhibited AR stimulation by androgen in the MARS assay and thus appear to be complete antagonists of AR-mediated transcription.

Analysis of data generated from the HyperCyt® system in the MARS assay using different analytical methods may give subtly different results. For example, analysis on the basis of percent cells expressing dsEGFP over a fixed point allows investigation of how many cells are transcriptionally activated by AR stimulation of the MMTV-controlled reporter dsEGFP. Alternatively, analysis of responding cells on the basis of mean fluorescence allows some delineation of the variable level of gene expression induced by different stimuli. By determining the product of percent cells expressing dsEGFP and the mean fluorescent intensity of these cells, an estimation of total fluorescence allows for concurrent assessment of the number of cells actively transcribing via the MMTV promoter as well as the level of transcriptional activity in these cells. For single high-dose assays as well as agonist dose-response assays, the three different analysis methods give similar results; however, the subtle differences in these analyses are particularly interesting in the context of dose-response curves for antagonistic compounds. Generally, the AR antagonists tested in this assay decrease the percent responding cells at low doses, while higher doses of antagonist were required to decrease dsEGFP expression levels in the remaining responding cells (data not shown). As a product of percent responding cells and mean fluorescence, the total fluorescence of wells treated with AR antagonists also tended to decrease at lower levels of antagonist. Interestingly, treatment with AR agonists such as progesterone, DHT and E2 at 10μM induced expression of dsEGFP in a lower number of cells than R1881; however, in the cells expressing dsEGFP, the level of expression was similar to that induced by R1881, as evidenced by the similarity in mean fluorescence of responding cells, which can only be discerned in assays that perform single cell analysis, such as flow cytometry.

There are many possible applications and extensions of the MARS assay for future use. Because transient transfection is used in MARS assay, the cell type, receptor under forced expression, and the promoter modulated by the receptor under forced expression could be customized for the interests of the assay. This may prove useful for comparing the functionality of the AR between species (e.g., rat to human AR), comparing polymorphic variants of the human AR, or in evaluating the activity of other nuclear hormone receptors.

Furthermore, the rapid, flexible, and sensitive nature of the assay will make it useful for assessing a variety of compounds with the potential to modulate AR transcriptional activity, including environmental contaminants, pesticides and other potential endocrine disrupting substances. The high-throughput design of the MARS assay will allow for the screening of large libraries of compounds for AR transcriptional activity with the potential of discovering novel AR agonists and antagonists. Such agents may prove useful for research and therapeutic purposes. In addition to direct extensions of the MARS assay, the validation of this method, particularly in tandem with the description of a glucocorticoid receptor dsEGFP-based reporter assay (16), suggests the utility of similar reporter gene assays utilizing dsEGFP for other steroid hormone receptors which function as transcription factors upon agonist stimulation. Therefore, we speculate that the HyperCyt system can be used with many different cell types, receptors, and receptor reporters in combination with diverse chemical libraries, providing for numerous high-throughput screening assay options.

In summary, we have developed an AR transcriptional reporter assay that has the benefits of being rapid, sensitive, and amenable to high-throughput screening, utilizing transiently transfected PC3 prostate cells expressing a dsEGFP reporter plasmid in tandem with an expression vector for the wild-type human AR. In the experiments presented, the MARS assay was shown to be dependent on AR expression in order to drive dsEGFP expression in response to agonist stimulation. AR transcriptional antagonists are capable of inhibiting this dsEGFP expression when coadministered with inhibitors of AR activity. Additionally, AR-driven dsEGFP expression in the MARS assay occurs in a dose-dependent manner, showing the dynamic range of the assay. The use of flow cytometry allows for rapid analysis of dsEGFP expression on a cell-by-cell basis and, in concert with the use of dsEGFP as the reporter, allows for analysis of cells without the need for exogenous reagents to develop the reporter signal. This assay should be useful for a wide variety of future endeavors, including high throughput screening of a variety of potential modulators of AR transcription and extension to other transcriptional reporter assays including those involving other steroid hormone receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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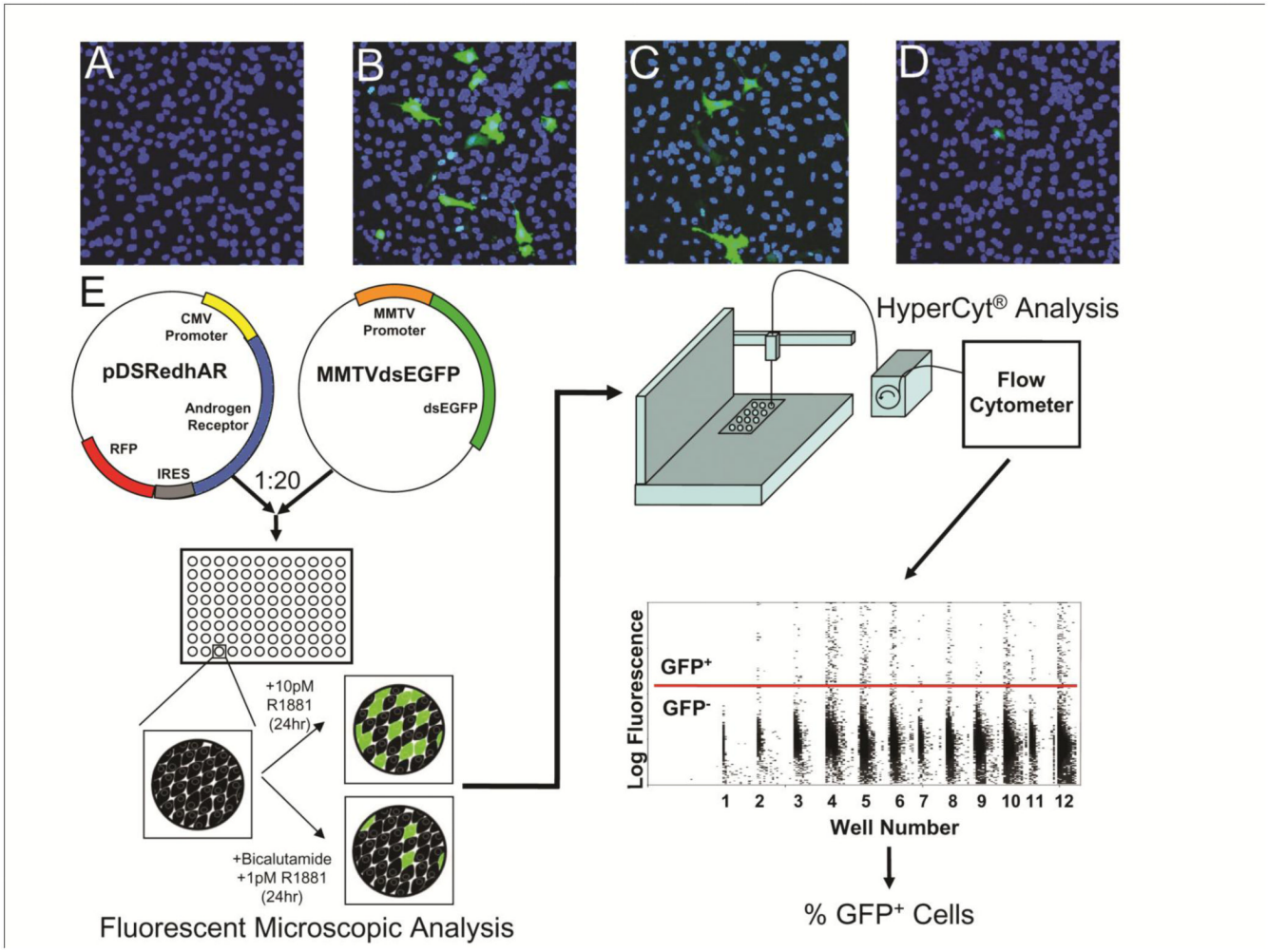

Fig. 1.

Selection of analytical methods using the MARS assay. For each of the analytical methods used, PC3 cells are transfected with pDsRedhAR and pMMTVdsEGFP at a ratio of 1:20. After 24 h, cells were treated and dsEGFP expression was assessed following an additional 24 h incubation. Representative photomicrographs of cells in the MARS assay are shown after treatment with an AR agonist and antagonist analyzed by confocal microscopy (A-D). Cells were seeded on glass coverslips and treated with vehicle (A), 10pM R1881 (B), 1pM R1881 (C), or 1pM R1881 + 10μM bicalutamide (D). Coverslips were fixed following 24 h stimulation and stained with DAPI (blue nuclei); green cells = dsEGFP positive. Images are representative fields using a 20× objective. Schematic of the MARS assay using the HyperCyt® flow cytometry system (E). PC3 cells are seeded in a 96-well tissue culture plate, treated with test compound, and allowed 24 hr to allow response to treatment. Cells are then removed from each well by trysinization and analyzed using HyperCyt® high throughput flow cytometry. The percent of events in each well above a set gate (e.g, red bar) was used to determine the androgenicity or anti-androgenicity of test compounds.

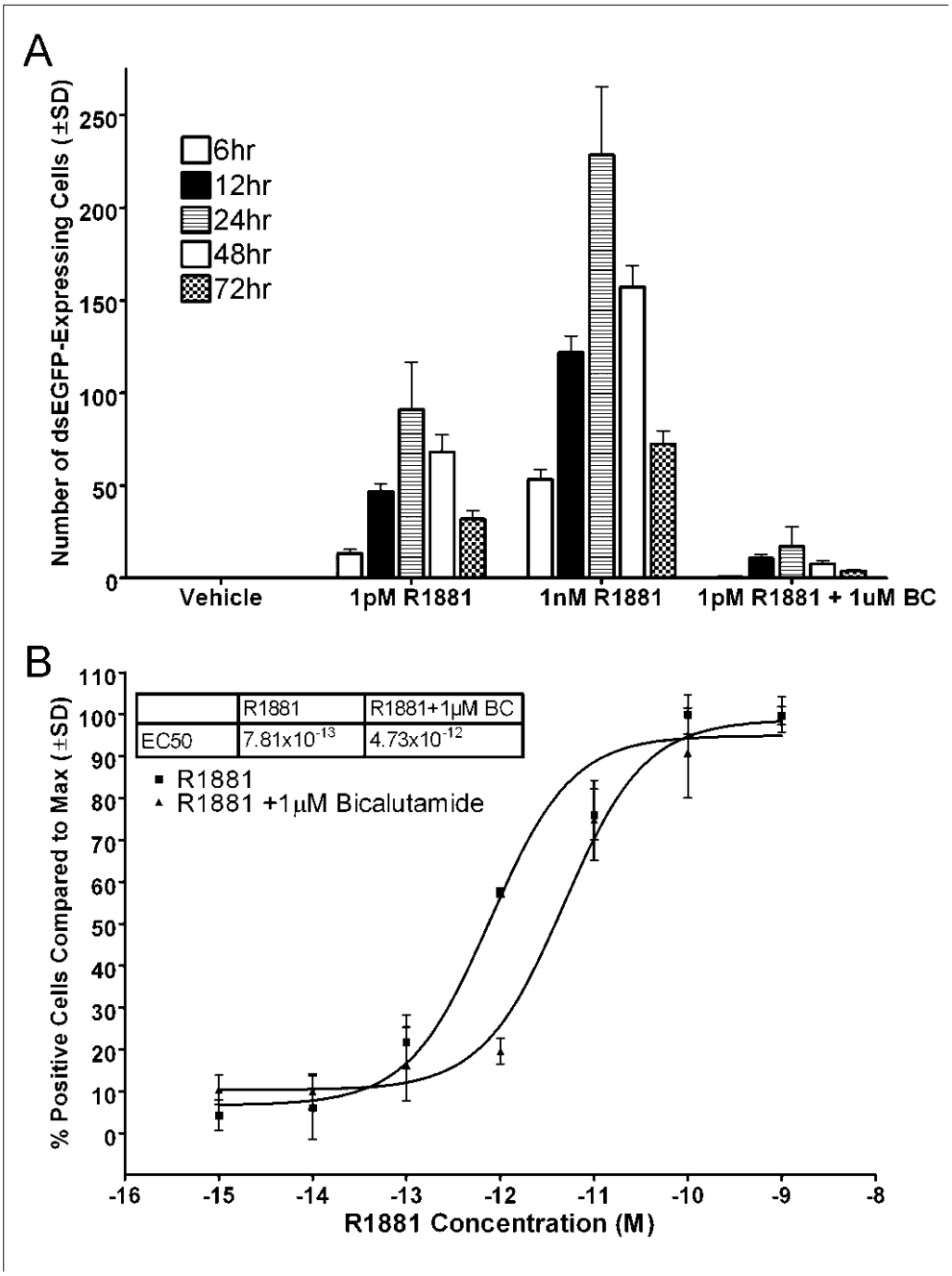

Fig. 2.

MARS assay analyzed by microscopy. Time-course data (A) for dsEGFP expression in PC3 cells seeded in 24-well tissue culture plates and cotransfected with pDsRedhAR and pMMTVdsEGFP. Cells were treated with R1881 with or without bicalutamide (BC) and dsEGFP expression was quantified by microscopy at 6, 12, 24, 48 and 72 h post-treatment. Each time point represents the number of dsEGFP-positive cells per low power field (i.e., 4× objective) in three independent wells. Dose-response data (B) for the MARS assay. PC3 cells seeded in 24-well tissue culture plates and cotransfected with pDsRedhAR and pMMTVdsEGFP were treated with varying concentrations of R1881 in the absence or presence of 1μM bicalutamide. Each point represents 4 independent wells and experiments

were performed in duplicate. Three parameter dose-response curves were analyzed using Graph Pad Prism software.

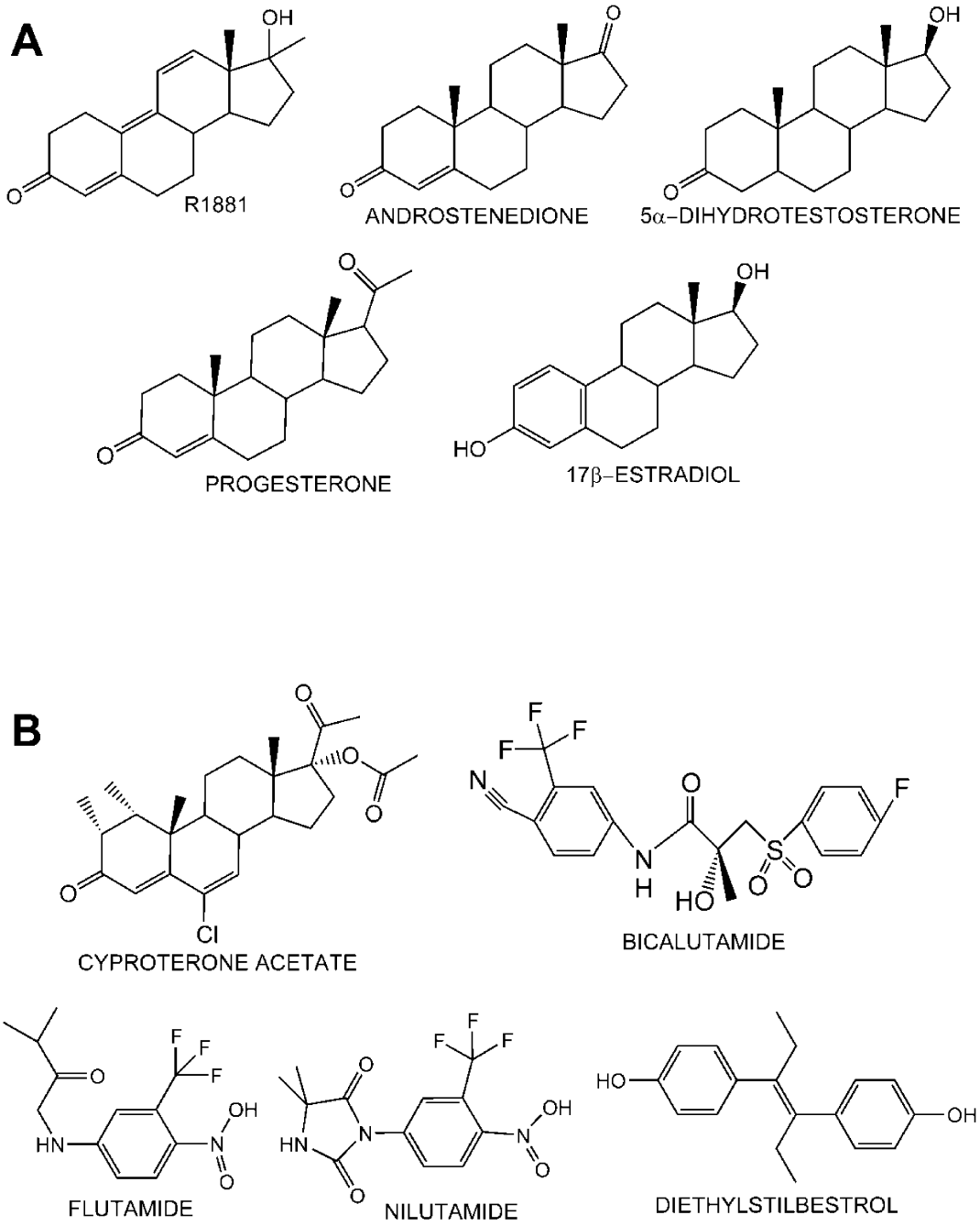

Fig. 3.

Chemical structures of the test set of compounds used for MARS assay validation. Representative agonists (A) and antagonists (B) of AR mediated transcription including the steroidal compounds R1881 and DHT (AR agonists), CPA (AR antagonist), E2 (ER agonist), progesterone (PR agonist) and androstenedione (AR agonist). Also shown are the nonsteroidal antiandrogenic (i.e., AR antagonists) compounds bicalutamide, flutamide, nilutamide, and DES (an established ER agonist).

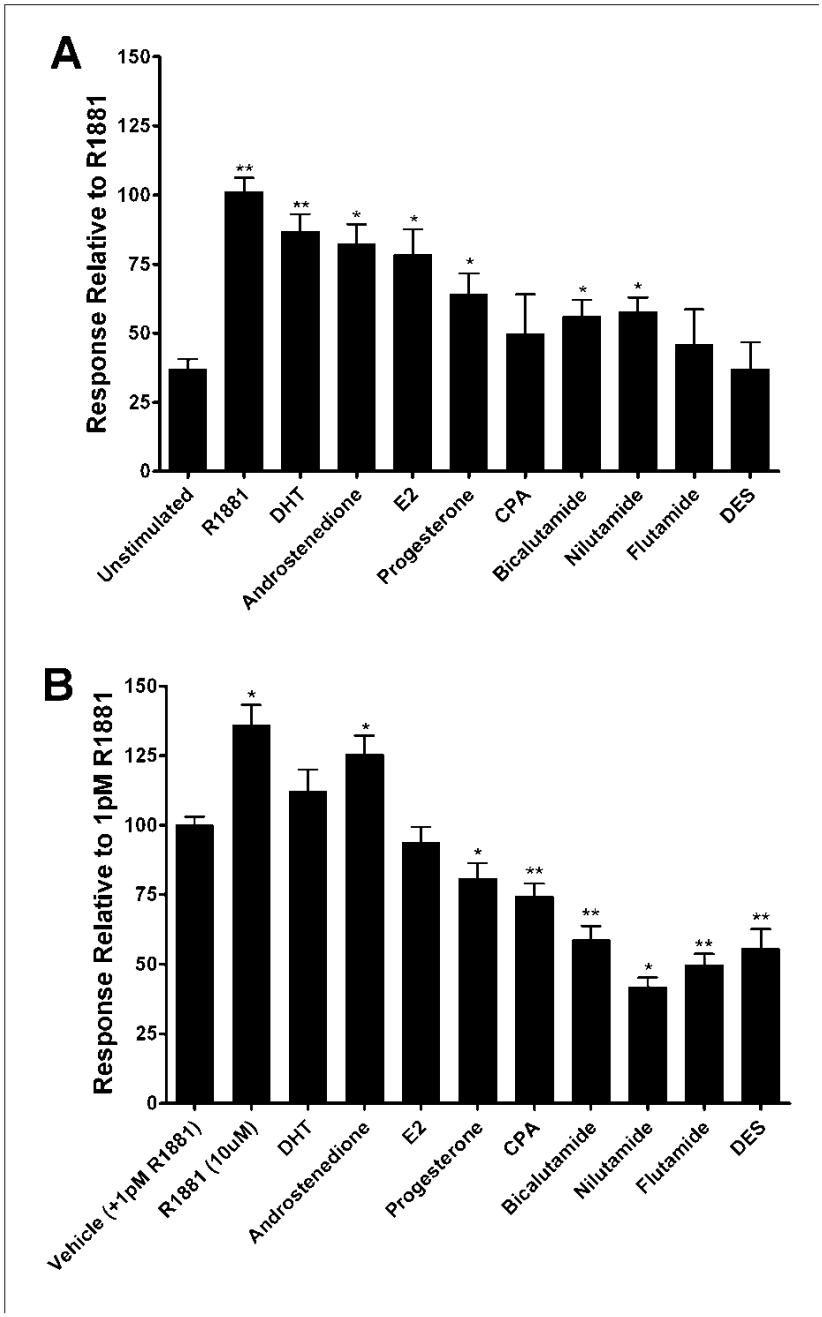

Fig. 4.

Androgenic activity of compounds in a single-dose MARS assay. The androgenic (A) and antiandrogenic (B) activity of a test set of compounds was assessed using the MARS assay and HyperCyt® system. Cells were seeded in 96-well tissue culture plates, cotransfected with pDsRedhAR and pMMTVdsEGFP at a ratio of 1:20, and stimulated 24 h later with compounds, as indicated. Following a 24 h induction of dsEGFP expression, wells were trypsinized and cells analyzed by HyperCyt® high throughput flow cytometry. The percent of GFP^+ cells in each well was normalized to 10μM R1881 treatment in the agonist assay (A) or to vehicle + 1pM R1881 in the antagonist assay (B). ** $p<0.0001$, * $p<0.05$, compared to unstimulated and vehicle (+1pM R1881) in panels (A) and (B), respectively.

Bars represent at least four wells from three independent experiments in (A) and at least ten wells from three independent experiments in (B).

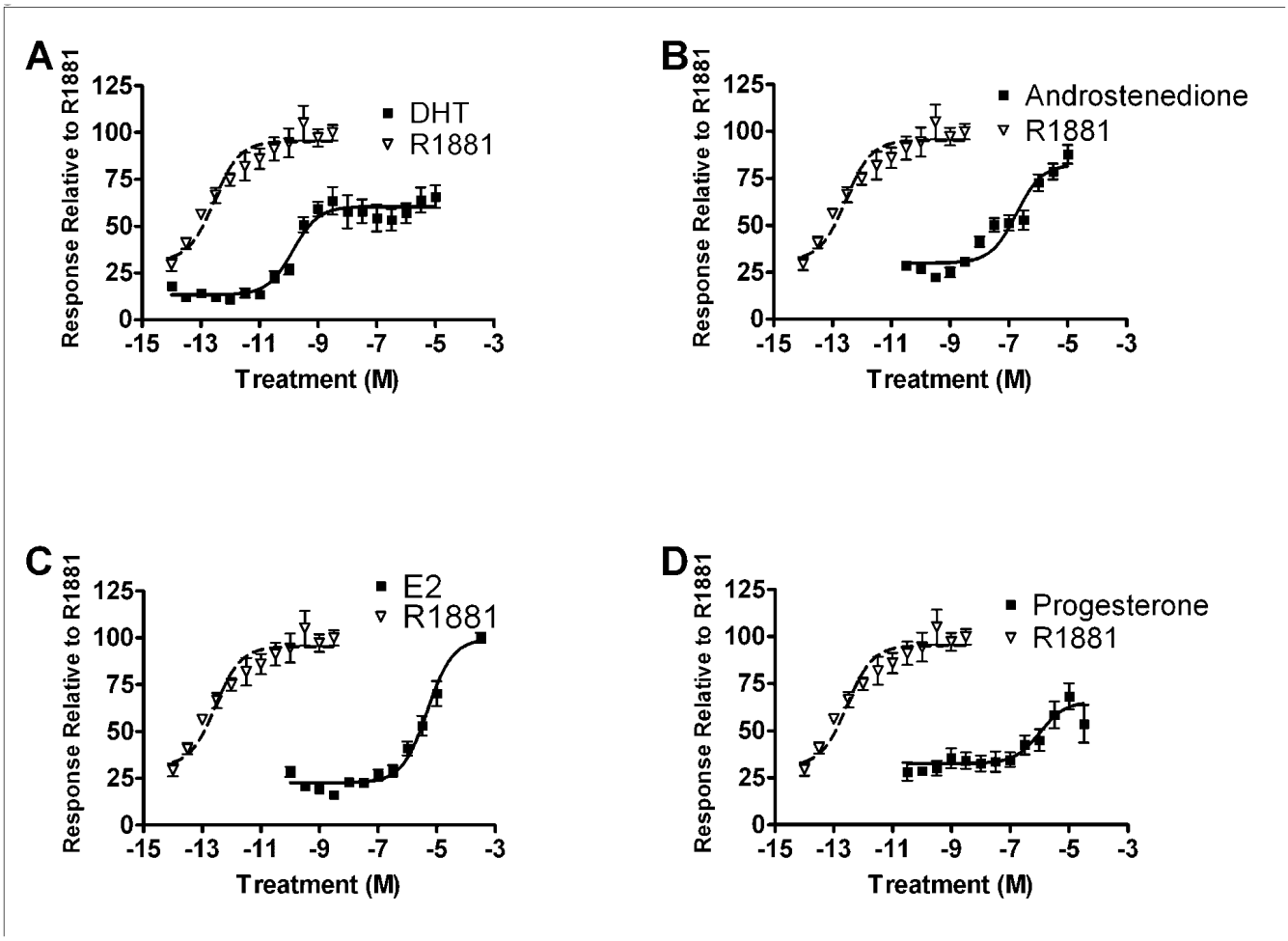

Fig. 5.

Dose-response curves for AR agonists in the MARS assay. The potency of select compounds with observed androgenic (A-D) activity in the single-dose MARS assay was assessed using serial dilutions of the indicated compound to stimulate cells in the MARS assay (A-D). Each point is representative of at least four wells in each of two independent experiments.

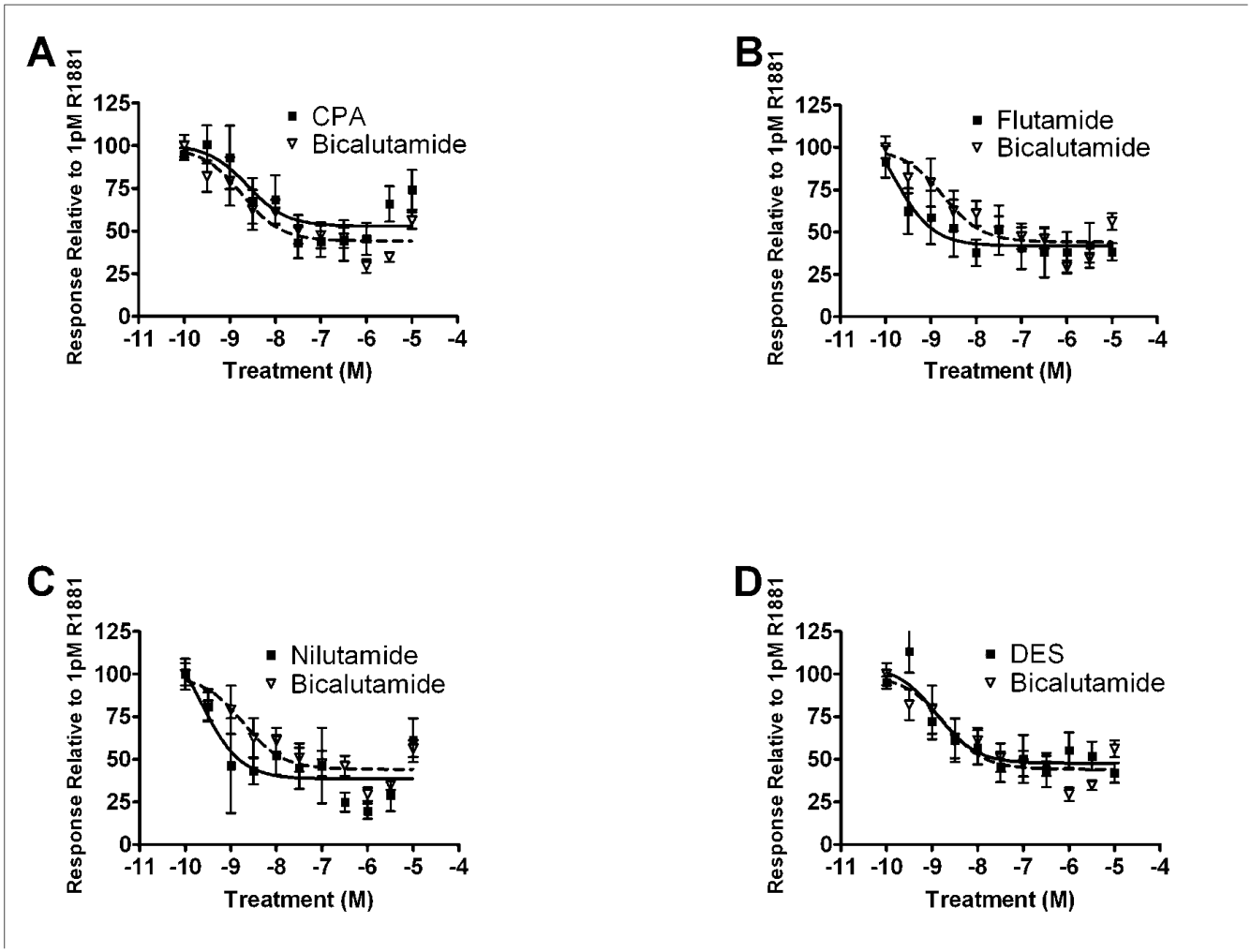

Fig. 6.

Dose-response curves for AR antagonists in the MARS assay. The potency of select compounds with observed antiandrogenic (A-D) activity in the single-dose MARS assay was assessed using serial dilutions of the indicated compound to stimulate cells in the MARS assay in the presence (A-D) of 1pM R1881. Each point is representative of at least four wells in each of two independent experiments.

Table 1

EC50 and IC50 Values Determined Using the MARS Assay*

Agonist[a]	EC50 (M)	Antagonist[b]	IC50 (M)
R1881[c]	1.34×10^{-12}	Flutamide	1.51×10^{-10}
Dihydrotestosterone	1.38×10^{-10}	Nilutamide	2.77×10^{-10}
Androstenedione	1.08×10^{-7}	Diethylstilbesterol	1.27×10^{-9}
Progesterone	1.01×10^{-6}	Bicalutamide	1.85×10^{-9}
Estrogen	4.88×10^{-6}	Cyproterone acetate	2.41×10^{-9}

*Multifunctional androgen receptor screening assay performed as described in the Materials and Methods using the HyperCyt® System for analysis.

[a]Agonist activity determined using a dose range from 10^{-14} M to 10^{-5} M of agonist.

[b]Antagonist activity determined in the presence of 1pM R1881 using antagonist concentrations from 10^{-10} M to 10^{-5} M.

[c]Methyltrienolone