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Partial agonist/antagonist properties of androstenedione and 4-androsten-3β,17β-diol

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Abstract

Androgens play important endocrine roles in development and physiology. Here, we characterize activities of two "Andro" prohormones, androstenedione (A-dione) and 4-androsten-3β,17β-diol (A-diol) in MDA-MB-453 (MDA) and LNCaP cells. A-dione and A-diol, like cyproterone acetate, were partial agonists of transfected mouse mammary tumor virus (MMTV) and endogenous prostate-specific antigen (PSA) promoters. Different from bicalutamide but similar to CPA, both are inducers of LNCaP cell proliferation with only mild suppression of 5α-dihydrotestosterone (DHT)-enhanced cell growth. Like bicalutamide and cyproterone acetate, A-dione and A-diol significantly antagonized DHT/R1881-induced PSA expression by up to 30% in LNCaP cells. Meanwhile, in MDA cells, EC_{50}s for the MMTV promoter were between 10 and 100 nM. Co-factor studies showed GRIP1 as most active for endogenous androgen receptor (AR), increasing MMTV transcription by up to five-fold, without substantially altering EC_{50}s of DHT, A-dione or A-diol. Consistent with their transcriptional activities, A-dione and A-diol bound full-length endogenous AR from MDA or LNCaP cells with affinities of 30–70 nM, although binding to expressed ligand-binding domain (LBD) was ≥20-fold weaker. In contrast, DHT, R1881, and bicalutamide bound similarly to LBD or aporeceptor. Together, these data suggest that A-dione and A-diol are ligands for AR with partial agonist/antagonist activities in cell-based transcription assays. Binding affinities for both are most accurately assessed by AR aporeceptor complex. In addition to being testosterone precursors in vivo, either may impart its own transcriptional regulation of AR.

Keywords: Endogenous androgen receptor binding; Partial AR agonist/antagonist; MDA-MB-453 cells; LNCaP cells; AR K_d

1. Introduction

Androgens, also termed rejuvenating hormones or male hormones, are primarily involved in male phenotype development and maintenance of male physiology [1]. The two most important natural androgens playing roles on reproductive and non-reproductive target tissues are testosterone (T) and 5α-dihydrotestosterone (DHT) [2]. Androstenedione (4-androsten 3,17-dione; hereafter A-dione) is an immediate precursor for T, acting as a substrate for 17β-hydroxysteroid dehydrogenase [3]. Androgen signaling during embryogenesis is required in the development of the male phenotype for animals with XY chromosomes. Androgens also support physiological functions in men through all stages of life, including childhood, puberty, adulthood, and aging [4]. Increasing evidence has also demonstrated that despite the lower serum testosterone levels in women, androgens also play important roles in female physiological functions and maintenance [5].

The androgens, T and DHT, function mainly via a single 917 amino acid androgen receptor (AR). Both AR protein and mRNA are ubiquitously expressed throughout the body [6]. As a member of the steroid receptor subfamily of the nuclear receptor superfamily, the AR is a ligand-inducible transcription factor [7]. Upon ligand binding, the AR dissociates from its chaperones, e.g. heat shock protein 90 (Hsp90), and is converted into an active state ready to bind to its response

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elements, recruit co-activators and initiate transcription [8–10]. The liganded receptor can also interact with other transcription factors via protein/protein interaction and function more like a ligand-regulated co-factor [11,12].

Androgen deficiency in men at any age reduces libido, bone mineral density (BMD), muscle mass, and strength. Androgen replacement has been widely prescribed to improve these conditions. However, considering the side effects of current androgen therapies, such as acne, hirsutism, and prostate growth, their use must be carefully weighed in terms of risk versus benefit. Problems in the delivery methods and in the ability to achieve steady physiological hormone levels also hamper the broad utilization of androgen therapy [2,13,14]. Sub-physiological levels of androgens in women also result in clinical symptoms similar to those in men, such as reduced BMD, libido, and lean body mass. Current androgen supplementation to women has the similar risks and benefits as seen in men [5].

In recent years, progress in the development of therapies targeting nuclear receptors has directed much focus on the androgen receptor in an attempt to improve current androgen therapy. Promising progress has been made in this field based on published information and patents. Compounds demonstrating anabolic activity on muscle and/or bone without significant androgenic action on prostate and/or skin have been developed in both steroid and non-steroid categories [14–17]. This latest development and progress have provided the proof of principle for the generation of better selective androgen receptor modulators (SARMs) that could be safely used in the clinic. From our preliminary knowledge on SARMs, it is clear that these agents behave neither like androgen agonists, such as DHT or R1881, nor like antagonists, such as bicalutamide. In in vivo models, it has been reported that prototype SARMs can display agonist activity in some target tissues, such as bone and/or muscle, and antagonist activity on other organs, such as skin and/or prostate [16,18]. DHT and its synthetic analog R1881 have been shown to be AR agonists in all the target tissues. Bicalutamide on the other hand has been shown to act as AR antagonists to targets [19]. Cyproterone acetate (CPA), originally intended as an AR antagonist, also exhibits partial agonist activity [20,21]. Preliminary data from the ORX mouse in vivo model implied that CPA could be a prototype SARM, which prevented ORX-induced bone loss without increasing seminal vesicle weight of ORX mice [18].

Androstenedione and 4-androsten-3β,17β-diol (A-diol) are both commonly referred as “Andro” prohormones. Both, A-dione and A-diol can elevate urinary excretion of testosterone [22], suggesting metabolism to T after oral ingestion by men. Usage of prohormones has been generally associated with some beneficial effects, such as improving serum testosterone concentrations, increasing muscular strength and muscle mass, reducing body fat and enhancing mood [23].

In this report, we characterized transcriptional and proliferative activities and binding properties of A-dione and A-diol in comparison to DHT, R1881, bicaalutamide, and CPA. Two endogenous AR sources from MDA-MB-453 (MDA) and LNCaP cells were examined in this study. The gene regulation activity of the above AR ligands was assessed in MDA/mouse mammary tumor virus (MMTV) semi-endogenous receptor/reporter assays in the absence or presence of co-factors. Similar to CPA, A-dione and A-diol behaved like partial agonists but with greater potency. Their partial agonism in inducing prostate-specific antigen (PSA) production in LNCaP cells was also demonstrated in an agonist mode. As with CPA and bicalutamide, A-dione and A-diol also suppressed DHT or R1881 induction of PSA in LNCaP cells by 20–30% in an antagonist mode. Interestingly, they, like CPA, did induce LNCaP cell growth with only minor inhibition of DHT-induced growth. Finally, their binding affinity to AR was measured using aporeceptors from MDA and LNCaP cells in comparison to rhesus ARLBD expressed in yeast or bacteria. Both ligands, like CPA, preferentially bound to the AR aporeceptor complex with high affinity, while binding to the ligand-binding domain (LBD) was somewhat weak. The collective data demonstrated that A-dione and A-diol are AR ligands displaying neither DHT- nor bicalutamide-like profiles. Most similar to CPA, A-dione and A-diol maintain a further distinction in that they display more agonist than antagonist activity.

2. Materials and methods

2.1. Reagents

MDA, COS-1, and LNCaP cell lines were purchased from ATCC (Manassas, VA). LipofectAmine 2000 reagent, phenol red free DMEM (Gibco 11054-020), RPMI1640 (Gibco 11835-055), Gentamicin (Gibco # 15710-072), L-glutamine, HEPES, trypsin, regular and charcoal-stripped fetal bovine serum (FBS) were all from GIBCO BRL/Invitrogen (Carlsbad, CA). Hydroxylapatite (# 391947) was from Calbiochem (La Jolla, CA). Human Insulin (I-0259), poly-ethyl-imine, sodium molybdate, dexamethasone, CPA (C3412), Triamcinolone acetonide (TAC), DHT were all products of Sigma (St. Louis, MO), while A-dione and A-diol were from Steraloids (Newport, RI 603-654-9509). R1881 and [^{3}H]R1881 were purchased from NEN (Boston, MA). Proteinase inhibitor and FuGENE6 transfection reagent were from Roche Molecular Biochemicals (Indianapolis, IN). Dual-Glo luciferase assay system and phRL-TK vector were purchased from Promega (Madison, WI). pESP-1 yeast protein expression system/culture media and Quick Change Multi Site-Directed Mutagenesis Kit were from Stratagene (La Jolla, CA). Unifilter-96 GF/B and MICROSCINT were from Packard (Boston, MA). PSA ELISA kit was from Diagnostic Systems Laboratories (Webster, TX). Cytostar 96-well scintillating microplates RPNQ0162 and [methyl-^{14}C]thymidine CFA532 were from Amersham (Piscataway, NJ).

2.2. Expression of GST-rhesus ARLBD (rhARLBD)

Full-length rhARLBD cDNA (WO02090529 GenBank accession no. AY526325) was generated from rhesus monkey prostate mRNA. The ligand-binding domain region corresponding to 622 T and 917 Q of the human AR (hAR) clone with 917 amino acid residues was PCR amplified and inserted in frame with GST at the SmaI site of pESP-1 or at the BamHI/XhoI sites of pGEX-4T-1 (Pharmacia). GST-rhARLBD was expressed according to the manufacturer's protocols. Yeast or bacteria pellets were washed twice in TEGM/PI (10 mM Tris–HCl, 1 mM EDTA, 10% glycerol, 1 mM beta-mercaptoethanol, 10 mM sodium molybdate pH 7.2, one pellet of PI in 50 ml of buffer). Crude yeast or bacteria lysate containing GST-rhARLBD was prepared by disrupting cells via vortexing in the presence of glass beads in cold TEGM/PI buffer. The whole process was kept cold by six cycles of alternating vortexing for 30 s then placing on ice for 2 min. Cleared supernatant was collect after centrifugation at 10,000 × *g* at 4 °C for 10 min. The lysate containing GST-rhARLBD was used for ligand displacement assays.

2.3. Validating the effect of mutations in hAR from MDA Cells

Mutations present in human AR from MDA cells at position F723 to L, L808 to M, and Q865 to H (GenBank accession no. AF162704) were studied using rhAR cDNA as a template. Triple mutations in the corresponding sites were engineered using a Quick Change Multi Site-Directed Mutagenesis Kit. Clones with the expected mutations were selected through sequence analysis. Both wild-type (wt) and mutant rhARs were expressed in COS-1 cells via transient transfection using LipofectAmine 2000 reagent. Cell lysates were prepared by rinsing the cells 2× in TEGM/PI buffer and scraping the cells off of the plate in TEGM/PI. The final cell concentration was adjusted to 10^7 cells/ml. The cells were snap frozen in an ethanol/dry ice bath and stored at −80 °C for subsequent AR binding assays. Saturation binding assays were performed on both wild and mutant rhARs. K_d values were calculated by using the KELL analysis program.

2.4. Preparation of hAR from MDA and LNCaP Cells

MDA cells were cultured in RPMI 1640 containing 20 mM Hepes, 4 mM L-glu, 10 μg/ml of human insulin, 10% FBS, and 20 μg/ml of Gentamicin. LNCaP cells were cultured in RPMI 1640 medium containing 10% FBS serum. Two to three days after seeding, cells reached 70–85% confluence, and were detached using trypsin. Cells were collected in complete RPMI1640 media and centrifuged at 10,000 × *g* for 10 min at 4 °C. The cell pellet was washed twice in TEGM. The final cell pellet was resuspended in TEGM at 10^7 cells/ml, then snap-frozen and stored at −80 °C.

2.5. Hydroxylapatite (HAP)-based AR binding assays

AR binding was carried out in 150 μl of TEGM binding buffer. Test compounds were serial diluted and combined with 20 nM TAC and ∼0.5 nM [^{3}H]R1881. This mixture was then mixed with lysate containing AR or ARLBD and incubated at 4 °C overnight. The following day, 75 μl of a 50% (v/v) hydroxyapatite slurry was added to each sample, vortexed, and incubated on ice for ∼10 min. The samples in 96-well format were then washed in washing buffer (40 mM Tris, pH 7.5, 100 mM KCl, 1 mM EDTA, and 1 mM EGTA) using The FilterMate™ Universal Harvester (Packard). The washing process transfers the HAP pellet containing ligand-bound receptor onto the Unifilter-96 GF/B, which was pre-wetted in 0.5% poly-ethyl-imine. The HAP pellet on the filter plate was then incubated with 50 μl of MICROSCINT20 for 2 h before being counted using a TopCount (Packard). IC_{50} values for each compound were generated using MRLCalc. IC_{50} was further converted to equilibrium binding constant K_i using $K_i = K_d \times IC_{50}/(K_d + L)$, where K_d was the equilibrium dissociation constant of [^{3}H]R1881 to MDA hAR (0.45 nM), LNCaP hAR (0.56 nM), and rhARLBD (0.31 nM), L was the concentration of [^{3}H]R1881 used in the experiment.

2.6. MDA–MB453 endogenous hAR and transfected MMTVluc reporter assays

For each assay plate, ∼2 million MDA cells were transfected with 3 μg of MMTVluc reporter and 1 μg of carrier DNA or 3 μg MMTVluc with 1 μg of co-factor using FuGene6. For assays without co-factors, 6 μg of MMTVluc per plate was used. Renilla luciferase reporter phRL-TK at 100 ng/96-well plate was also included in each transfection for signal normalization. On the following day, cell media containing transfection agents were replaced by fresh OptiMEM medium containing the test compounds. Luciferase and Renilla luciferase activities were measured ∼24 h after treatment following manufacturer's protocols using a 1450 MicrobetaJet (Wallac). Cells were treated with serially diluted compounds.

2.7. PSA induction and cell proliferation of LNCaP cells

Freshly prepared LNCaP cells were suspended in RPMI 1640 medium containing 10% charcoal-stripped serum at 2 × 10^4 cells/ml for PSA assay and at 1 × 10^4 cells/ml for proliferation assay. Cells in 100 μl of medium were seeded into each well of 96-well plates. For plates used in the proliferation study, 0.5 μCi/ml of [methyl-^{14}C]thymidine was included in the medium. Cells were treated with compounds diluted in 25 μl of medium 2–4 h after seeding. Cells were treated with compounds either in the presence (antagonist mode) or absence (agonist mode) of 0.5 nM of DHT or R1881. PSA levels in the medium were measured 24 h after compound treatment using a PSA ELISA kit. Cell proliferation was measured using a 1450 MicrobetaJet (Wallac) at 24, 48, and 72 h.

2.8. Statistics

Statistical analyses were performed by Fisher's Protected Least Significant Difference followed by ANOVA, using Statview for Windows software (version 5.0.1, SAS Institute Inc., Cary, NC).

3. Results

3.1. Identification of endogenous AR sources for the characterization of AR ligands

A mature AR aporeceptor complex, including AR, Hsp90, Hsp70, and other chaperones, was suggested to be critical in maintaining the high binding affinity of AR to its ligands [8,24]. It was also observed that some of the AR ligands exhibited higher binding affinity to AR from stably transfected cells than to over expressed AR or ARLBD [25] (and our unpublished data). Therefore, it was important to search for an endogenous human AR source that retained high binding affinity to various AR ligands in addition to DHT or R1881. The MDA cell line expresses high levels of AR (∼73,000 receptors per cell), with undetectable levels of estrogen receptor (ER) and progesterone receptor (PR) [26]. The hAR cDNA sequence from MDA cells deposited in GenBank indicates the existence of three mutations in its ligand-binding domain: F723L, L808M, and Q865H. To test the impact of these mutations on ligand-binding activity in comparison to wild-type AR, we generated equivalent (F723L, L808M, and Q865H) mutations in rhesus AR, which shares identical amino acid sequences to hAR in the LBD. Both wild-type and mutant rhARs were examined for saturation binding using [^{3}H]R1881 as the test ligand. Saturation-binding curves and Scatchard analysis showed that [^{3}H]R1881 bound with essentially identical affinity to either the wild-type or the mutant rhAR (Fig. 1). The K_d for R1881 binding to wt and mutant rhAR were 0.14 (±0.02) and 0.25 (±0.03) nM, respectively. In parallel studies, saturation binding of [^{3}H]R1881 to endogenous hAR from MDA cells revealed a K_d value of 0.45 (±0.07) nM. These values are consistent with the published K_ds for R1881 binding to hAR [27], suggesting that the endogenous hAR from MDA cells binds to ligand with equal affinity versus that seen with the wild-type receptor. Separate analyses with an unrelated synthetic AR ligand also suggested that the mutations found in hAR from MDA cells do not impact ligand-binding affinity (data not shown).

We also examined the binding affinity of [^{3}H]R1881 to hAR found in LNCaP cells, where expression is relatively high. LNCaP cells do not express GR, PR, or ER [28,29]. Like MDA cells, the hAR sequence found in LNCaP cells encodes a mutation (T877A) [21]. Saturation binding and Scatchard analyses showed that [^{3}H]R1881 bound to the endogenous hAR with a K_d of 0.56 (±0.07) nM, which is essentially identical to the value obtained using MDA hAR or the wild-type hAR, as noted above. This validates AR from both MDA and LNCaP cells for the characterization of various AR ligands.

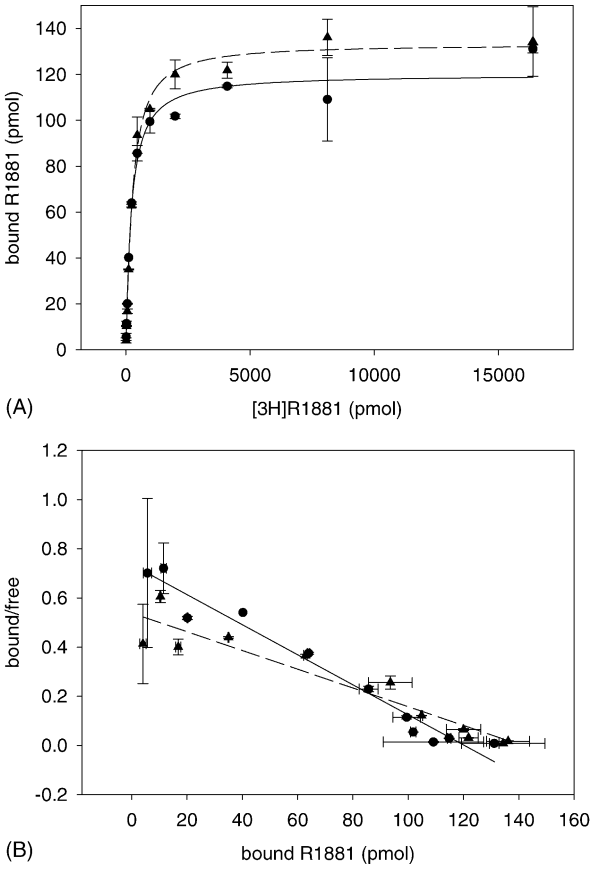

Fig. 1. Saturation binding study on wild-type (wt) and MDA version mutant type (mt) of rhesus monkey AR (rhAR) using [^{3}H]R1881. Curves show wt (solid line, •) and mt (dashed line, ▲.) (A) Plot of specifically bound [^{3}H]R1881 (*y*-axis) vs. concentration of added ligand (*x*-axis). Both wt and mt show typical saturation binding curves and curves are essentially overlapping. (B) Standard Scatchard analysis of binding data. Concentration of specifically bound steroid is shown on the *x*-axis and the ratio of specifically bound steroid to free steroid is shown on the *y*-axis. The two lines show similar slopes.

3.2. High binding affinity of A-dione and A-diol to endogenous ARs

Competitive inhibition analyses were used to measure the binding affinities of andro-related prohormones, A-diol and A-dione, for ARs from LNCaP and MDA cells in comparison to full agonists DHT/R1881, the full antagonist bicalutamide and the mixed antagonist CPA. All ligands showed similar binding affinity to the aporeceptors, regardless of whether the AR originated in MDA or LNCaP cells (indicated with ARmda and ARLNCaP in Table 1). The data showed that the equilibrium-binding constant of each ligand was in the following rank order DHT ≈ R1881 > CPA ≈ bicalutamide > A-diol > A-dione. The binding affinities for DHT and R1881 agree well with previous reported values, while CPA and bicalutamide appeared to have higher affinity to endogenous hAR

Table 1
Summary of AR ligand K_is to ARs from different sources and MDA GR

	ARmda	ARLNCaP	rhARLBD	GRmda
DHT	0.14 ± 0.07	0.32 ± 0.07	0.17 ± 0.02	301.97 ± 59.22
R1881	0.25 ± 0.03	0.26 ± 0.03	0.32 ± 0.03	173.20 ± 11.77
CPA	11.05 ± 0.04	5.05 ± 0.28	351.47 ± 1.73	143.63 ± 32.69
4-Androsten-3β,17β-diol	28.04 ± 10.48	23.03 ± 2.91	683.17 ± 151.79	1707.91 ± 125.54
4-Androsten-3,17-dione	60.61 ± 9.52	73.36 ± 3.32	> 1875	> 4355
Bicalutamide	13.20 ± 0.20	14.62 ± 4.71	40.07 ± 26.06	> 4355

as compared to what has been previously reported [27,30]. Furthermore, A-dione and A-diol also exhibited high binding affinity to endogenous ARs with K_is in the low nanomolar ranges (Table 1).

3.3. Transcription activity of A-dione and A-diol on the MMTV promoter in MDA-MB453 cells

Binding assays suggest that the three point mutations (F723L, L808M, and Q865H) found in the hAR aporeceptor from MDA cells had no substantial effect on affinity to the test AR ligands. Because A-dione and A-diol showed significant binding affinity to AR in this system, their transcription activity on the MMTV promoter was further examined in these cells. MDA cells were transfected with an MMTVluc reporter [3], and transcriptional responses of the endogenous hAR to ligands were tested across a broad dosing range (Fig. 2). By these analyses, DHT and R1881 both elicited potent activity with EC_{50}s in the sub-nanomolar range. The Emax for R1881 was approximately 20% less than that for DHT. Both A-dione and A-diol displayed partial agonist activity in this assay. A-diol and A-dione ligands achieved roughly 60–80% of DHT's maximal transcriptional activity, respectively, and the Emax of A-dione approached that of R1881. Interestingly, while A-diol and A-dione fell within ∼2-fold of each other in terms of binding affinity to this hAR aporeceptor, A-diol was approximately 10-fold more potent than A-dione in transcriptional activity. Meanwhile, although CPA displayed two- and five-fold higher affinity to AR than A-diol and A-dione, respectively, its EC_{50} was at least two to three orders of magnitude lower for inducing transcriptional activity. At the top tested concentration of 10 μM, CPA achieved ∼40% of DHT maximal activity. Thus, CPA displayed at least partial agonist activity in the MDA/MMTV transcriptional system, despite its original designation as an AR antagonist [20]. This is consistent with a previous report of partial agonist activity [30]. As expected, bicalutamide elicited no transcriptional activation off of the MMTV promoter at any concentration (Fig. 2). In the MDA/MMTV system, the rank order of potency for transcriptional agonism was: DHT ≥ R1881 > A-diol > A-dione ≫ CPA with bicalutamide being inactive. This suggests that in vitro receptor-binding affinity does not precisely correlate with transcriptional potency in all cases. The data also demonstrate that A-diol and, to a lesser extent, A-dione are partial agonists for AR.

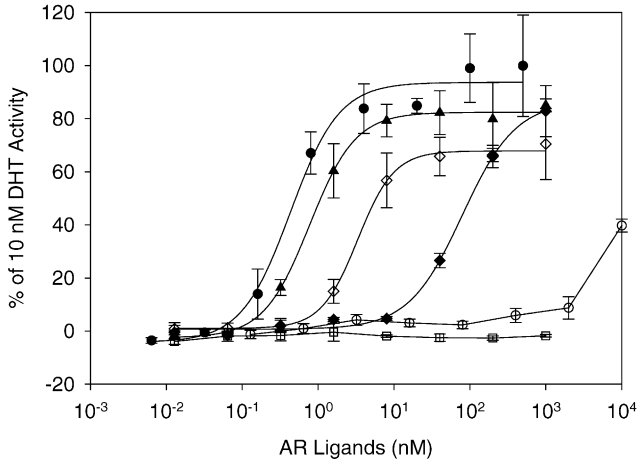

Fig. 2. Regulation of the MMTVluc promoter by endogenous AR and its ligands. MDA cells were transfected with a small amount of MMTVluc and Renilla luciferase vectors, as indicated in Section 2. Cells were treated with compounds at indicated concentrations one-day post transfection. Luciferase activity and renilla luciferase activity were quantitated 24 h after treatment was initiated. Luciferase activity was normalized to renilla activity. The activity at each concentration point is expressed as a percent of the activity of 10 nM DHT. Activity of each AR ligand is plotted vs. the corresponding concentration as mean ± S.D. DHT (●), R1881 (▲), 4-androsten 3,17-dione (♦), 4-androsten 3β,17 β-diol (◊), CPA (○), and bicalutamide (□).

3.4. Co-factor effects on A-dione and A-diol transcription activities

In an effort to search for co-factors that regulate A-dione, A-diol and DHT activities, we surveyed several co-activators and a co-repressor for their ability to influence ligand-induced transcription off of the MMTV promoter. Included were SRC1, GRIP1 (SRC2), AIB1 (SRC3), ARIP3, and the co-repressor, N-CoR [31,32]. Bicalutamide was inactive in the presence of all co-factors, although all other ligands did show responses to some of the co-activators. In preliminary analyses, we observed that neither SRC1 nor N-CoR substantially altered transcription off of the MMTV promoter with any ligand via endogenous AR. Follow-up studies showed a small N-CoR-induced transcriptional repression that was additive to the antagonistic effects of CPA and bicalutamide (data not shown). AIB1-enhanced transcription of A-dione and CPA, while ARIP3 enhanced only CPA-induced transcription. However, for all transcriptionally active AR ligands, GRIP1/SRC2 consistently significantly enhanced transcriptional activity (Fig. 3 and

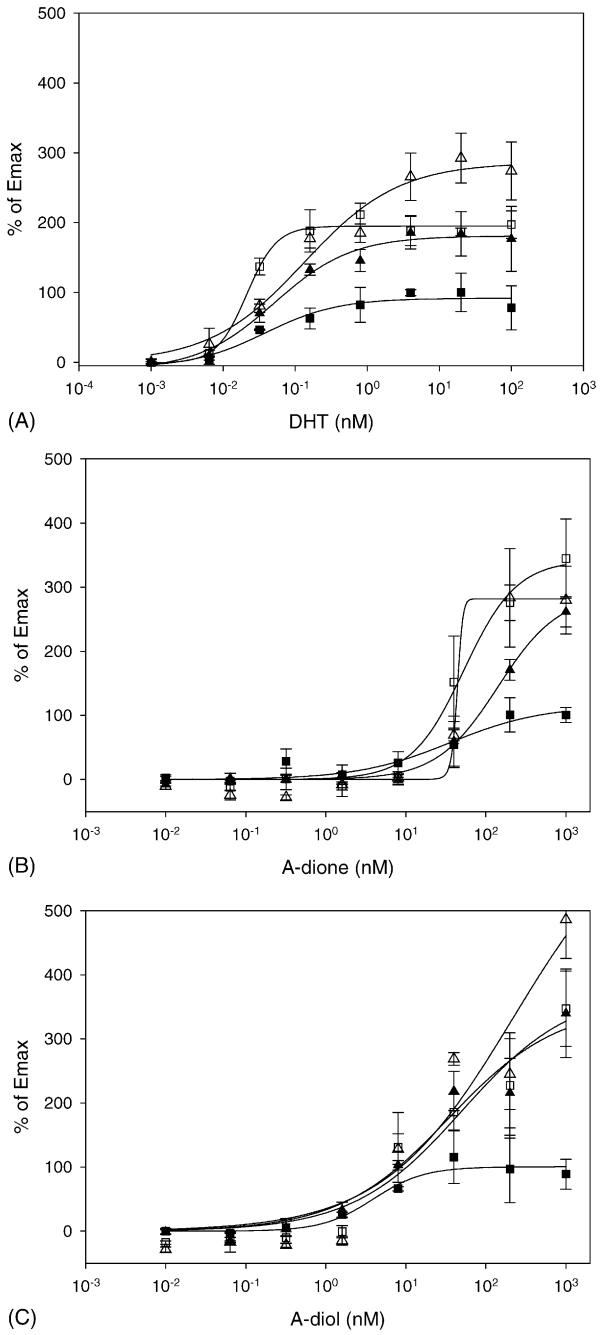

Fig. 3. GRIP1 influence of DHT, A-dione, and A-diol transcription activity on MMTV promoter in MDA cells. MDA/MMTVluc assays were performed in the presence of 0 (■), 0.5 (▲), 1.0 (□), and 1.5 μg (Δ) of GRIP1 per 96-well plate. Cells were treated with increasing concentrations of DHT (A), A-dione (B), and A-diol (C), as indicated. Data (mean ± S.D.) were plotted as a percentage of the Emax for each ligand when measured in the absence of GRIP1. Basal was defined as the activity in the absence of either GRIP1 or ligands.

data not shown). Focusing on A-dione, A-diol, and DHT, we measured GRIP1 transcriptional enhancement using three different levels of transfection (0.5, 1.0, and 1.5 μg), all of which had no effect on basal activity. For each type of GRIP1 transfection, we tested each ligand at seven concentrations as shown in Fig. 3. For A-dione (Fig. 3B) and DHT (Fig. 3A), exogenous expression of GRIP1 increased the transcriptional Emax by up to three-fold at the highest level of expression. For A-diol (Fig. 3C), the enhancement was five-fold, although we did not consider this to be different from the responses to the other ligands. For all three ligands, exogenous GRIP1 showed no consistent, appreciable change to the transcriptional EC_{50}, although slight enhancement was occasionally seen. Together, the data suggest that, of all the tested co-factors, GRIP1 most consistently enhanced AR ligand-induced transcription. We found no evidence that any co-factor selectively altered A-dione- or A-diol-induced transcription differently from effects on DHT or R1881-mediated transcription on MMTV promoter in this system.

3.5. Partial agonist/antagonist properties of A-dione and A-diol in LNCaP PSA induction assays

As demonstrated above, endogenous AR in LNCaP cells also retains its high binding affinity to all tested AR ligands. In addition, glucocorticoid, progesterone, and estradiol receptors are absent in LNCaP cells [28,29] (also our unpublished data). AR ligands can induce endogenous prostate-specific antigen protein production and secretion in these cells, this makes the LNCaP cell an excellent fully endogenous AR receptor/reporter system. LNCaP cells were treated with several concentrations of test ligands to determine optimal concentrations for maximal activity (data not shown). We also examined PSA induction at either 24 or 48 h, with essentially equivalent responses at each time point. After optimization, a single concentration of each ligand that could elicit a maximal response was tested alone (agonist mode; Fig. 4A) or in combination with 0.5 nM R1881 or DHT (antagonist mode; Fig. 4B) at the 24 h time point. DHT and R1881 induced near maximal PSA production at 0.5 nM and, unlike in the MDA cell system, the Emax for both ligands was indistinguishable. Both DHT and R1881 also showed equivalent maximal agonism of the PSA promoter when each ligand was tested at 10 nM (Fig. 4A). As expected, CPA acted as a partial agonist to stimulate PSA production at 1.0 μM, a concentration 10-fold lower than that required to see significant transcription activation in the MDA cell system (Fig. 2). This is consistent with the reported behavior of AR from LNCaP cells, suggesting that the point mutation can broaden the specificity of LNCaP AR for ligands [21,33]. Consistent with its effects in MDA cells, bicalutamide displayed no agonist activity, and indeed it slightly repressed basal PSA levels (inverse agonism) when LNCaP cells were treated with compound alone. In contrast to the reference pure agonists and antagonist, A-dione and A-diol (1.0 μM) elicited partial induction of PSA with activities similar to that of CPA. The "Andro" prohormone ligands displayed 70–75% of the maximal agonism of DHT. These results confirm the partial agonist activities of A-dione and A-diol seen in the MDA cell system.

In further characterize the partial agonism of A-dione and A-diol, we also examined their ability to antagonize agonist-

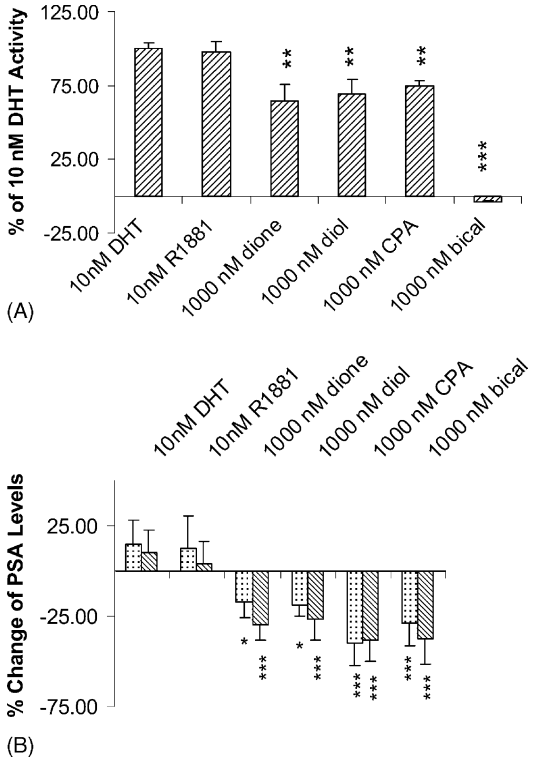

Fig. 4. Regulation of PSA production in LNCaP by AR ligands. (A) Cells were treated with indicated amount of AR ligands. PSA levels in the culture medium were measured 24 h after treatment was initiated. The PSA stimulation activity of each ligand is expressed as percent of 10 nM DHT activity. Basal activity was subtracted from each sample. (B) Cells were treated with the indicated amounts of AR ligands in the presence of 0.5 nM DHT (dots) or R1881 (stripes). PSA was measured as in panel A. PSA level changes were calculated as a percent change vs. the respective 0.5 nM of DHT and R1881 controls. Significance vs. values obtained with DHT or R1881 alone are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Statistic significance was calculated using StatView.

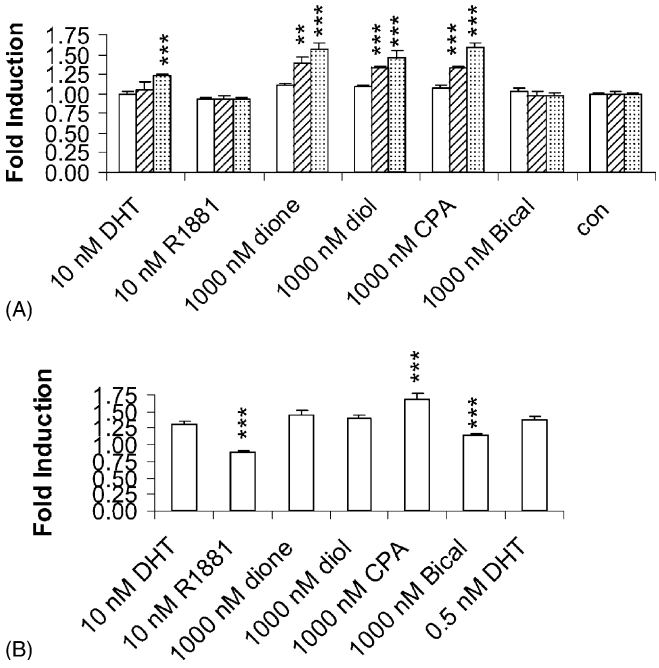

Fig. 5. LNCaP cell proliferation assay. (A) Cells were treated with indicated amount of ligand and cultured in medium containing 0.5 μCi/ml of [methyl-^{14}C]thymidine. Cell growth was measured at 24, 48, and 72 h points. (A) Cell growth was expressed as fold-induction vs. the corresponding control in agonist mode at 24 h (white), 48 h (stripes), and 72 h (stipples). Significance vs. values of the corresponding control was calculated using Fisher's protected least significant difference and ANOVA. (B) Cell growth in the presence of 0.5 nM DHT was measured at 72 h. The growth rate was expressed as fold induction over the 72 h control without any treatment. Significance vs. values of 0.5 nM DHT treatment was obtained as in panel A. All values are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

induced PSA expression. A-dione and A-diol together with other ligands were tested in the presence of 0.5 nM R1881 or DHT (Fig. 4B). As expected, addition of 10 nM DHT or R1881 had a minor additive effect on PSA levels induced by 0.5 nM of DHT or R1881 at 24 h. As a pure antagonist, bicalutamide inhibited DHT and R1881 stimulation of PSA expression activity by 28 and 38%, respectively. CPA also inhibited the agonist activity of DHT and R1881 by 40 and 38%, respectively, confirming its role as a partial agonist/antagonist of hAR in LNCaP cells. Consistent with their partial agonism in PSA expression (Fig. 4A), both A-dione and A-diol antagonized DHT and R1881 PSA induction by approximately 25% (Fig. 4B). This provides clear evidence that A-dione and A-diol, like CPA, behaved as partial agonists/antagonists in this transcription model.

3.6. LNCaP cell proliferation study

The LNCaP growth rate is known to be affected by androgens. To test this response with A-dione and A-diol, we examined LNCaP cell proliferation in both agonist and antagonist modes. No significant differences in growth were observed after 24 h of treatment (Fig. 5A). However, at 48 h, A-dione, A-diol, and CPA stimulated significant growth versus the control, while the remaining ligands were without substantial effect. In this regard, PSA induction (Fig. 4) was more rapid than growth stimulation (Fig. 5A). At 72 h, all ligands except R1881 (10 nM) and bicalutamide significantly enhanced cell proliferation. The observation that A-dione, A-diol, and CPA are strong inducer of LNCaP cell growth agrees with previous reports [29,34]. While 10 nM R1881 had no effect on LNCaP cell growth (Fig. 5A), 0.5 nM R1881 slightly but significantly stimulated cell growth versus the untreated control (data not shown). This is consistent with a previous report that R1881 is growth-stimulatory at low dose and is not stimulatory or even inhibitory at higher concentrations [28]. Again, this differs from the PSA response, in that both concentrations of R1881 were agonistic for PSA expression (Fig. 4).

When LNCaP cells were treated with the same ligands in the presence of 0.5 nM DHT for 72 h, additive cell growth was observed for CPA. However, neither A-dione nor A-diol showed this response (Fig. 5B). In this regard, the growth stimulatory effects of A-diol, A-dione, and CPA differed from

their effects on PSA production after 24 h treatment (Fig. 4B). At a 10-fold higher dose (10 μM), A-dione, but not A-diol, slightly but significantly inhibited DHT-enhanced cell growth (data not shown). Interestingly, R1881 at 10 nM suppressed growth stimulated by 0.5 nM DHT, which is again different from its more agonistic effects on PSA expression (Fig. 4B). As expected, bicalutamide significantly reduced cell proliferation enhanced by 0.5 nM DHT, which was consistent with its effect on PSA production (Fig. 4B and Fig. 5B). Overall, these data suggest an uncoupling of ligand influence on PSA production versus cell growth in LNCaP cells for CPA, R1881, A-dione, and A-diol. Meanwhile, DHT and bicalutamide maintained consistent respective agonistic and antagonistic profiles.

3.7. Reduced binding affinity of A-dione and A-diol towards ARLBD

As noted in Section 3.1, some AR ligands show higher affinity binding to the AR aporeceptor versus over-expressed AR or ARLBD. We therefore examined the affinity of A-diol and A-dione for overexpressed LBD. Rhesus ARLBD, with an identical sequence to that of human ARLBD, was tested. Truncated rhARLBD was expressed as a GST-fusion

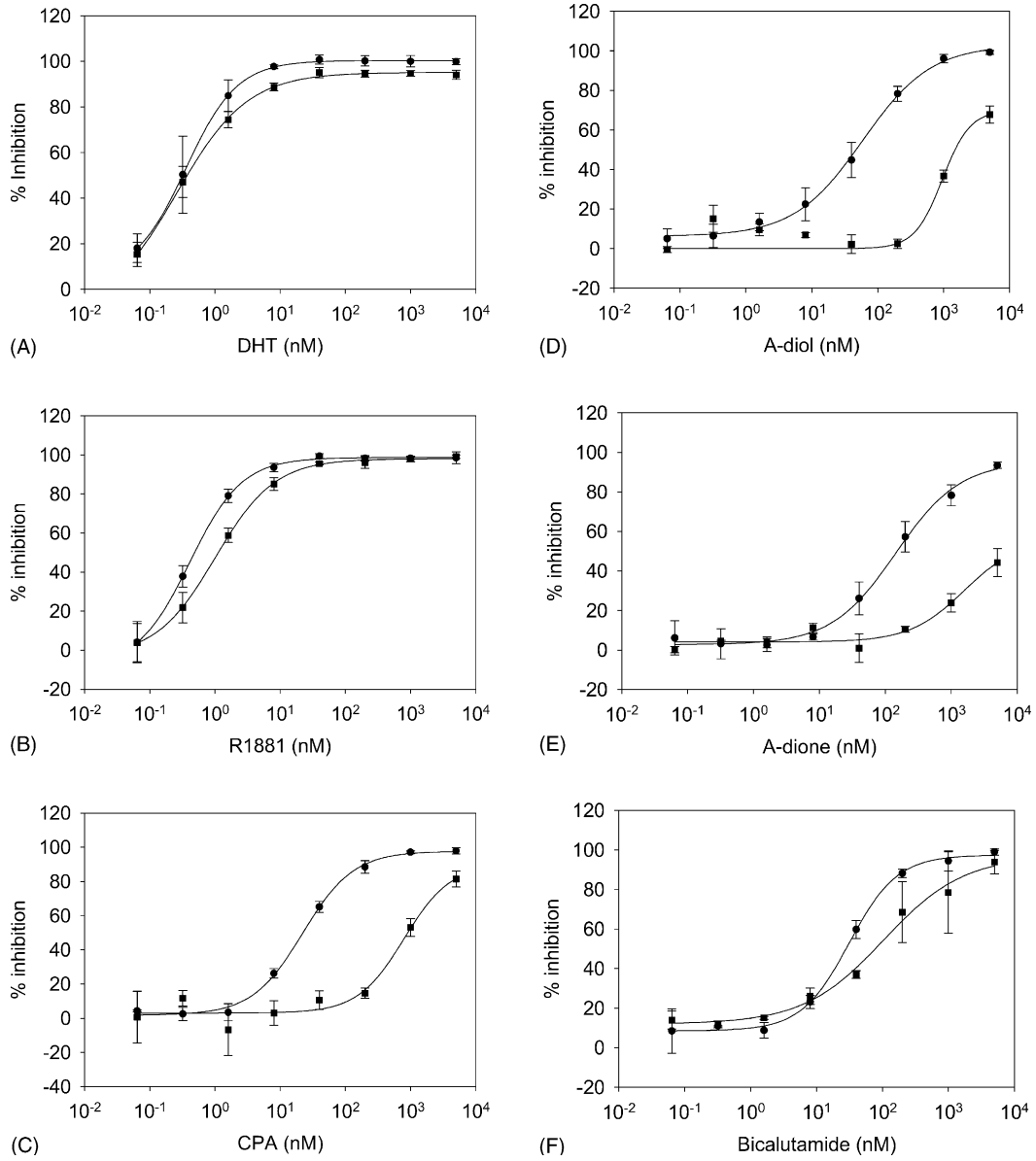

Fig. 6. Ligand displacement assays using AR from MDA-MB453 cells and rhARLBD from yeast or bacteria. Values are plotted using MDA AR (●) and rhARLBD (■). The binding activities from reactions in the absence of unlabeled ligand competition was identified as maximal binding, while the non-specific binding was determined in the presence of >500-fold excess unlabeled ligand. Percent inhibition is plotted vs. concentrations for each ligand, as indicated beneath each *x*-axis. Panels are: A – DHT, B – R1881, C –CPA, D – A-diol, E – A-dione, and F – bicalutamide.

protein using yeast or bacteria as hosts. As shown in Fig. 6A, inhibition-binding curves generated from MDA hAR and rhARLBD were essentially overlapping for the pure agonist DHT. Similar results were obtained using unlabeled R1881 as the competitive ligand (Fig. 6B). Interestingly, when CPA was examined under the same assay conditions, there was a substantial 30-fold reduction in relative-binding affinity for the LBD versus that seen with the endogenous receptor (Fig. 6C). Like with CPA, about 20-fold lower relative-binding affinity was observed with A-diol binding to rhARLBD versus that seen with hAR from MDA or LNCaP cells (Fig. 6D and Table 1). Furthermore, A-dione showed even weaker binding to rhARLBD (Fig. 6E and Table 1), although it's binding affinity to full-length AR was similar to that of A-diol. With bicalutamide, there was a small, three-fold lower binding affinity for the rhARLBD versus that seen with the full-length aporeceptor from either endogenous cell source (Fig. 6F and Table 1). Overall, the data suggest that DHT, R1881, and bicalutamide bind to AR with similar or essentially identical affinities, regardless of whether endogenous AR aporeceptor or overexpressed rhARLBD was tested. Meanwhile, A-dione and A-diol, along with CPA, exhibited significantly reduced binding affinity to ARLBD versus that seen with full-length, endogenous AR. These data suggest that the use of overexpressed LBD, a common approach to examine binding affinities, yields substantially weaker interactions with certain AR ligands. The data suggest that endogenous AR retained high binding affinity to AR ligands with various chemical structures, which again emphasizes that a mature AR aporeceptor complex may be crucial in maintaining high binding affinity to AR ligands with diverse chemical structures. As a consequence, endogenous AR may better predict the potency of various AR ligands in regulating AR target genes.

4. Discussion

In this report, we described the partial agonism/antagonism of "Andro" prohormones, A-diol and A-dione. These ligands were characterized using two endogenous hAR receptor sources validated via characterization of known AR ligands. Whereas the endogenous receptors in these cells do encode certain missense mutations, binding assays show that these mutations do not substantially impact-binding affinity. Meanwhile, binding assays using the endogenous ARs versus expressed ARLBD in yeast or bacteria demonstrated that the identified partial agonists/antagonists required the mature aporeceptor complex for high affinity binding. The unique binding property of those compounds, favoring the endogenous AR over the expressed ARLBD suggest that the aporeceptor can yield more accurate binding results with certain ligands. With clear evidence for receptor binding in the low to mid nanomolar range, we investigated potential transcriptional activities. Indeed in LNCaP cells, PSA expression in both agonist and antagonist modes revealed the mixed activities of A-dione and A-diol as compared with CPA. However, in the LNCaP proliferation assay, all three ligands were strong growth inducers and did not repress DHT-induced cell proliferation. Transcriptional activation of the MMTV promoter in MDA cells yielded the expected levels of agonist activities of tested reference AR ligands and also suggested partial agonism of A-dione and A-diol. Both model transcription systems offer the distinct advantage that they assess transcriptional responses with the endogenous receptor, a property that seems important given the substantially reduced binding affinity for partial agonist/antagonist to rhARLBD, or even transiently overexpressed full-length AR, which could have a less than perfect chaperone composition because of possible receptor titration effects.

Molecular chaperones, such as Hsp90and Hsp70, have indeed been shown to play a crucial role in maintaining steroid receptors in a high affinity hormone binding conformation and subsequently their high transcription activities [8,24]. Consistent with this, it has been reported that the Hsp90 inhibitor, geldanamycin, suppresses AR transcriptional activation [35,36], and we have found that geldanamycin abolished binding of ligand to AR (our unpublished observation). Exogenously overexpressed AR or ARLBD has a certain probability of titrating out the endogenous chaperones, depending on expression levels. With sufficiently high receptor expression, this can lead to the formation of an imperfect aporeceptor complex, which we suggest can affect the receptor binding affinity, especially to mixed agonists. It has been observed in the present study that endogenously expressed AR from MDA cells displays consistency in its high binding affinities to agonist, antagonist and partial agonist/antagonist. In contrast to aporeceptor binding, exogenously expressed ARLBD or even full-length AR can have varied and reduced binding affinities to test compounds, depending on expression conditions. In this study, the expressed ARLBD retained its typical binding affinity for DHT and R1881. Nonetheless, it displayed a substantial rightward shift for the partial agonist/antagonist ligands (A-dione, A-diol, and CPA). The observation clearly demonstrates that these ligands are more sensitive to the minor conformation changes of the receptor ligand-binding pocket that are likely supported by chaperones.

In identifying partial agonist/antagonist activities of the ligands described here, and in consideration of the binding properties of the ligands to AR aporeceptor versus expressed LBD, we chose to utilize endogenously expressed AR rather than employing an overexpression system. Nuclear receptor expression levels can indeed alter agonist or antagonist potency in a receptor/reporter transcription assay. To avoid such variable responses, ligands were tested in two model systems with relatively high intrinsic expression levels of hAR. LNCaP, a prostate cancer cell line, made an attractive natural receptor/reporter transcription system, since PSA production is specifically regulated by AR. Although a single missense mutation (T877A) was reported to expand the responsiveness of AR to include estradiol and progesterone, it does not change its response to DHT or bicalutamide [33,37]. Our

binding assays also showed that AR from LNCaP retained similar affinities to those derived from MDA cells and from wild-type AR. A distinct advantage of using the LNCaP/PSA assay system in both agonist and antagonist modes is that it can more precisely identify three types of compound activities. As such, we characterized (a) full agonist activities for DHT and R1881, (b) partial agonist/antagonist activities for CPA, A-dione, and A-diol, and (c) full antagonist activities for bicalutamide. By examining both agonist and antagonist activities, this method provided the most definitive evidence that A-dione and A-diol could display both partial agonist and antagonist activity. It has been previously demonstrated that A-dione could induce a unique AR target gene expression pattern distinct from DHT [3]. Genetic evidence also showed that A-dione itself might play a role in male sexual differentiation in XY patients with 17β-hydroxysteroid dehydrogenase deficiency [38]. Our data further suggests that A-dione and A-diol could function as AR ligands in addition to their roles as andro-related prohormones [23].

In addition to the LNCaP system, we also examined transcriptional responses to the various ligands in MDA cells, whereby the endogenous hAR was used to induce transcription off of the transfected MMTV promoter. In dose response analyses, CPA showed ∼10% of 10 nM DHT activity at 1 μM, although ∼40% of 10 nM DHT activity was observed at 10 μM. This could be attributed to agonism of the glucocorticoid receptor in these cells, to which CPA binds with moderate affinity (Table 1). Nonetheless a response to CPA was also seen in LNCaP cells, whereby partial agonism was seen at lower concentrations, displacing ∼75% of 10 nM DHT activity at 1 μM. Since LNCaP cells do not express detectable levels of GR, PR, or ER [28], the agonist activity of CPA can be attributed, to a great extent, to AR binding. We also note that the point mutation in LNCaP AR may allow CPA, like progesterone and estradiol, a better opportunity to activate AR regulation [21,37]. This mutation also makes R1881 indistinguishable from DHT in the LNCaP PSA assay, in contrast to its somewhat lower activity in the MDA MMTV system. The MMTV promoter also responds to GR regulation, and MDA, cells do express GR, as noted above. CPA, R1881, and DHT all exhibited some binding affinity to GR in the high nanomolar range (Table 1). As noted above, the possibility that GR plays a role in transcriptional activation of the PSA promoter is negligible in the LNCaP PSA reporter system. For R1881 and DHT, a GR contribution is also likely to be negligible in the MDA system with the lower concentration ranges used in the assays. A-dione showed 75–80% agonism to 10 nM DHT at its top tested concentration of 1 μM with no appreciable GR binding affinity. Meanwhile, A-diol clearly reached plateau at 60% agonism with all tested concentrations ≥10 nM. For both of these Andro prohormones, a contribution of GR to the transcriptional response seems unlikely.

In summary, we used several approaches to characterize A-dione and A-diol in comparison to known ligands with full agonist, mixed antagonist, and full antagonist activities. In the process, we validated endogenous hAR from MDA and LNCaP cell lines as behaving much like the wild-type hAR in their binding affinities for known ligands. Transcriptional responses to known ligands, DHT and R1881, were also essentially normal in these cell lines. The composite in vitro hAR binding data using hAR from either cell type versus that from expressed rhARLBD suggested that A-dione and A-diol, like CPA, preferentially bind to the full-length, chaperone-complexed AR aporeceptor. This may suggest that the true nature of these ligands is best assessed in cell-based transcription assays employing endogenous AR aporeceptor or binding assays using endogenous AR. In such a system (LNCaP), both of these ligands displayed clear partial agonist/antagonist activity on the PSA promoter. However, like CPA, both stimulated LNCaP cell growth with little or nor inhibition of DHT-induced cell growth. Transcription assays using MDA cells transfected with reporter and co-factors further profiled A-dione and A-diol, suggesting partial agonistic behavior in this cell system as well. Clearly, GRIP1 served as the greatest potentiator of AR-mediated transcription, with equal effects when combined with full or partial agonists, but not full antagonists. In this semi-endogenous transcription system, the effects of other tested co-factors were not very robust. N-CoR was additive in its repression of DHT-stimulated MMTV activity with CPA or bicalutamide treatments. Based on the composite profile of these ligands in these various in vitro and cell-based systems, we conclude that “Andro” prohormones A-dione and A-diol are AR partial agonists/antagonists that prefer a mature aporeceptor for high affinity binding to AR.

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