11β-Alkyl-Δ^9-19-Nortestosterone Derivatives: High-Affinity Ligands and Potent Partial Agonists of the Androgen Receptor

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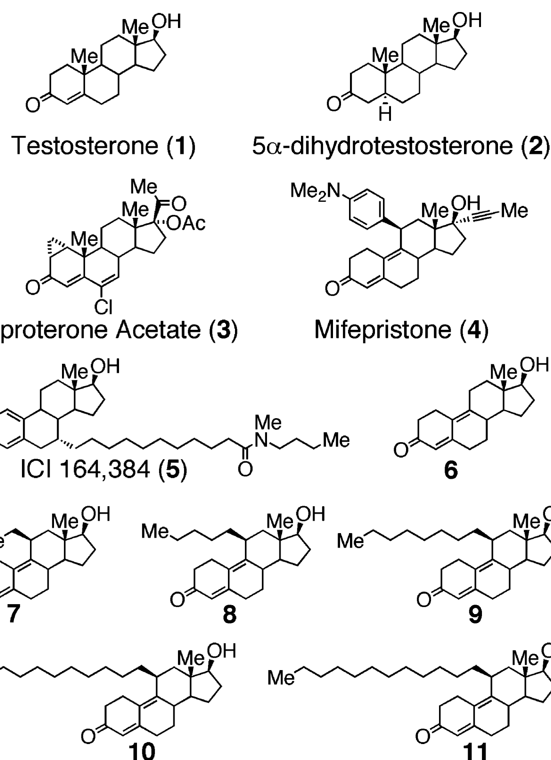
Abstract: We report the synthesis of novel steroidal androgen receptor ligands comprising 11β-alkyl-Δ^9-derivatives of 19-nortestosterone. These compounds are structurally related to the antiprogestin, antiglucocorticoid, and antiandrogen drug mifepristone (RU486). Nortestosterone analogues bearing 11β-octyl and 11β-decyl side-chains bind tightly to recombinant AR protein (IC_{50} = 6.6 nM and IC_{50} = 0.8 nM), block AR dimerization, exhibit activity against LNCaP prostate cancer cells, and comprise partial AR agonists with low antiglucocorticoid activity.



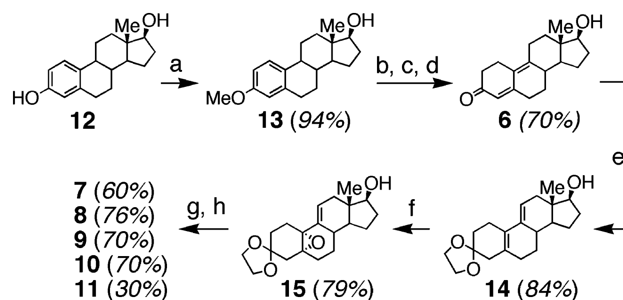
The androgen receptor (AR) is a member of the nuclear receptor superfamily of ligand-regulated transcription factors.[1] This steroid hormone receptor comprises a major drug target involved in prostate cancer, acne, hirsutism, male pattern baldness, and androgen insensitivity syndrome (AIS).[2−5] Binding of the steroid hormones testosterone (**1**) and the more potent 5α-dihydrotestosterone (**2**) to the AR initiates a complex series of events that result in translocation of the AR into the nucleus, binding to specific DNA sites, recruitment of components of the transcriptional machinery, and activation of the expression of specific genes.[1] In many prostate cancers, these androgen-dependent processes are required for cellular proliferation.[6] Hence, androgen antagonists such as cyproterone acetate (CPA, **3**) that block AR-mediated gene expression are often used as first-line therapeutics against prostate cancer. However, many clinically employed antiandrogens are limited by low relative binding affinities, low selectivity across the nuclear hormone receptor superfamily, or agonist activity toward AR mutants such as T877A that can emerge in advanced prostate cancers.[6,7] As a consequence of these limitations, novel small molecule antiandrogens are desired as improved prostate cancer therapeutics.[8]

Mifepristone (RU486, **4**) is under investigation as a potential anticancer agent effective against prostate cancers.[9−12] This drug is a highly potent antiprogestin (IC_{50} = 25 pM)[13] but also exhibits potent antiglucocorticoid (IC_{50} = 2.2 nM)[13] and antiandrogen (IC_{50} = 10 nM)[13] activities. The dimethylaniline substituent at the 11β position of mifepristone is thought to interfere with gene expression by dislodging the activation helix 12 of the progesterone receptor (PR), glucocorticoid receptor (GR), and AR.[14] This active antagonism mechanism is also employed by the antiestrogens raloxifene,[15] tamoxifen,[16] and ICI 164,384 (**5**).[17] Despite the fact that mifepristone is effective against prostate cancer cells in vivo, the use of this drug as a chronically administered anticancer agent may be limited by its potent antiglucocorticoid activity.[18]

In an effort to overcome limitations of mifepristone as an antiandrogen, we synthesized and evaluated the structurally related analogues **6**−**11**. Compounds **8**−**11** bear long aliphatic side chains reminiscent of the antiestrogens ICI 182,780[19] and ICI 164,384 (**5**), but project these substituents from the 11β position analogous to mifepristone. Examination of recent high-resolution X-ray crystal structures of the AR bound to ligands[4,20,21] suggested that these 11β alkyl substituents might similarly disrupt the conformation of the adjacent helix 12 when bound to the AR.

Compounds **6**−**11** were synthesized as shown in Scheme 1. β-Estradiol (**12**) was protected as the methyl

Scheme 1[a]



[a] (a) CH_3I, K_2CO_3, CH_3CN. (b) Na, NH_3, *i*-PrOH, THF. (c) Oxalic acid, H_2O, acetone. (d) Polyvinylpyridinium bromide perbromide, polyvinyl pyridine, pyridine. (e) Ethylene glycol, pyridinium chloride. (f) H_2O_2, hexafluoroacetone, pyridine, CH_2Cl_2. (g) Alkylmagnesium bromide, CuCl, THF. (h) Amberlyst-15 H^+, ethanol.

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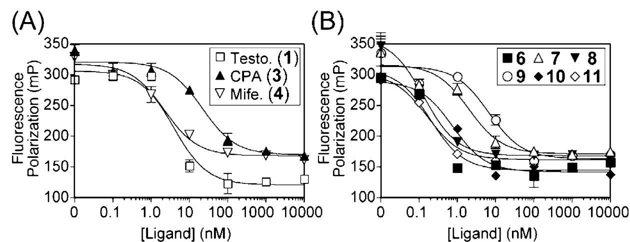

Figure 1. Competition fluorescence polarization binding assays with purified AR protein.

Table 1. Compilation of Data for Synthetic and Control Compounds[a–e]

ligand	AR binding	inhibition of AR dimerization	GR antagonism	LNCaP cytotoxicity
testosterone (**1**)	3.9	–	ND	ND
CPA (**3**)	22	4.7	0.9	61
mifepristone (**4**)	2.2	7.8	0.008	23
6 (11β-H)	0.2	–	133	>100
7 (11β-Et)	2.0	–	1294	55
8 (11β-pentyl)	0.1	0.009	125	17
9 (11β-octyl)	6.6	0.9	979	17
10 (11β-decyl)	0.8	85	1812	17
11 (11β-dodecyl)	0.2	–	870	19

[a] AR binding: IC_{50} values (nM) quantified by in vitro fluorescence polarization assays. [b] Inhibition of AR dimerization and GR antagonism: IC_{50} values (nM) quantified by nonlinear regression analysis of luciferase assay data obtained from transiently transfected CHO−K1 cells. [c] Cytotoxicity: IC_{50} values (μM) determined by quantifying viable LNCaP prostate cancer cells with a sulforhodamine-binding assay[36] after treatment with compounds for 72 h. [d] ND: Not determined. [e] Typical 95% confidence intervals were within 2-fold.

ether (**13**) and subjected to Birch reduction[22] to afford the unconjugated diene. Deprotection of the ether under acidic conditions was followed by bromination and dehydrobromination to yield dienone **6**.[23–25] This dienone was protected as cyclic acetal **14**,[23,26–28] followed by epoxidation of the tetrasubstituted alkene to afford **15**. Addition of appropriate Grignard-cuprate reagents to epoxide **15** followed by deprotection of the acetal and dehydration of the 5α-alcohol provided compounds **7**−**11**.[27–29] Two-dimensional COSY and NOESY NMR experiments confirmed that the side-chains projected exclusively from the 11β position of these steroids.

To compare the affinity of 19-nortestosterone derivatives with other known ligands, competition fluorescence polarization assays were employed. These experiments used *E. coli*-expressed AR protein (PanVera Corp) bound to a fluorescent tracer and treated with compounds **1**, **3**, **4**, and **6**−**11**. As shown in Figure 1, equilibrium-binding isotherms were observed with all of these compounds. As listed in Table 1, these data enabled quantification of IC_{50} values by nonlinear regression analysis. Mifepristone (**4**) proved to be the highest affinity of the known ligands ($IC_{50}(4) = 2.2$ nM). Moreover, compounds **6**−**11** bound specifically to the AR with affinities higher than or comparable to mifepristone (e.g. $IC_{50}(\mathbf{8}) = 0.1$ nM; $IC_{50}(\mathbf{9}) = 6.6$ nM; $IC_{50}(\mathbf{10}) = 0.8$ nM), and all of the nortestosterone analogues exhibited higher affinity for the AR than the clinically employed antiandrogen cyproterone acetate (CPA, $IC_{50}(\mathbf{3}) = 22$ nM). Remarkably, increasing the side-chain length from two (**7**) to five (**8**) carbon atoms was found to increase affinity by 20-fold, conferring subnanomolar affinity to **8**. Similarly, the longest side chains of compounds **10** and **11** provided subnanomolar affinity.

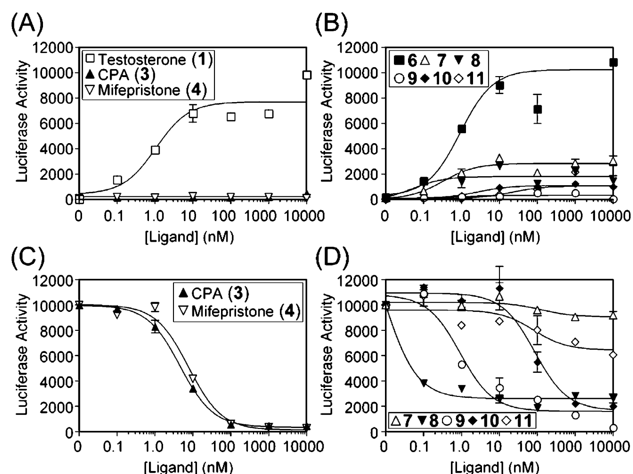

Figure 2. Mammalian two-hybrid assays of ligand-mediated dimerization of the AR. Panels A and B: Dose−response curves that assess agonist potency of control compounds (Panel A) and novel AR ligands (Panel B). Panels C and D: Competition assays in the presence of testosterone (**1**, 1 nM) to identify inhibitors of AR dimerization.

To compare the activities of synthetic compounds **6**−**11** with the androgen testosterone (**1**), the antiandrogen cyproterone acetate (**3**), and the antiandrogen mifepristone (**4**) in living cells, a previously reported[30] mammalian two-hybrid assay in chinese hamster ovary cells (CHO-K1) was employed. These cells were transiently transfected with two expression vectors encoding N-terminal (AR residues 1−660 fused to the VP16 activation domain) and C-terminal (AR residues 624−919 fused to the Gal4 DNA-binding domain) fragments of the human AR that undergo ligand-mediated dimerization.[5,31] These plasmids were cotransfected with a luciferase reporter plasmid and a plasmid constitutively expressing β-galactosidase to control for variations in transfection efficiency.

Testosterone (**1**), the unsubstituted compound **6**, and the ethyl-appended compound **7** were agonists in this whole-cell assay, promoting AR dimerization with EC_{50} values of 0.3 nM (**7**), 0.9 nM (**6**), and 1.1 nM (**1**) (Figure 2). In contrast, the pentyl side chain of **8** conferred only weak agonist activity, and the longer analogues **9**−**11** did not appreciably enable AR dimerization. The two known AR antagonists cyproterone acetate (**3**) and mifepristone (**4**) did not activate reporter gene expression in this assay (Figure 2).

Compounds were evaluated in a competition assay format to assess inhibition of AR dimerization promoted by testosterone (1 nM) in CHO-K1 cells (Figure 2). In this assay, the control antagonists cyproterone acetate (**3**) and mifepristone (**4**) fully blocked reporter gene expression with IC_{50} values of 4.7 nM and 7.8 nM (Table 1). The pentyl-appended compound **8** was a highly potent dimerization inhibitor ($IC_{50} = 0.009$ nM) whereas the octyl-appended compound **9** was similar but less potent ($IC_{50} = 0.9$ nM). Despite their high affinities for the AR, the decyl (**10**) and dodecyl (**11**)-substituted compounds exhibited weaker ($IC_{50}(\mathbf{10}) = 85$ nM) or insignificant (**11**) inhibition of AR dimerization (Figure 2 and Table 1).

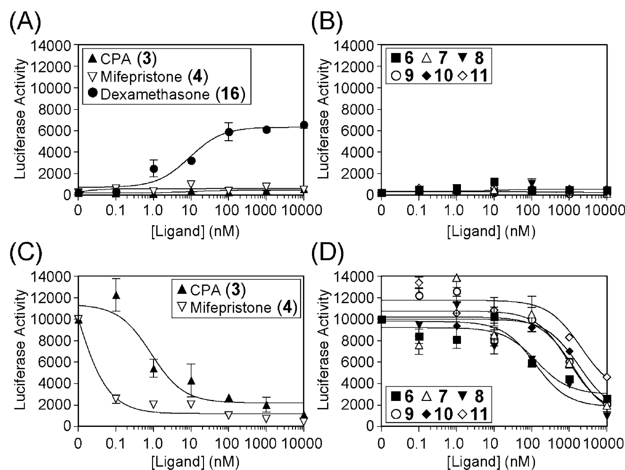

Figure 3. Effects of compounds on GR-mediated gene expression in transiently transfected CHO-K1 cells. Panels A and B: Dose–response curves of control compounds **3**, **4**, and dexamethasone (**16**, Panel A) compared with synthetic compounds **6**–**11** (Panel B). Panels C and D: Competition assays in the presence of dexamethasone (**16**, 100 nM) to quantify GR antagonism.

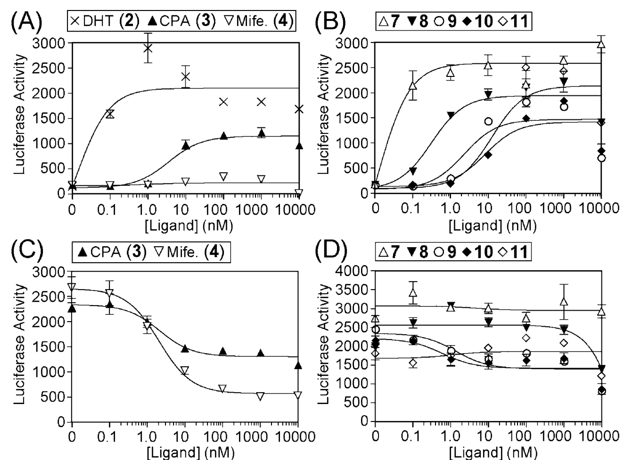

Figure 4. Effects of compounds on AR-mediated gene expression from an MMTV reporter vector in transiently transfected CV-1 cells. Panels A and B: Dose–response curves of control compounds **2**–**4** (Panel A) compared with synthetic compounds **7**–**11** (Panel B). Panels C and D: Competition assays in the presence of DHT (**2**, 0.1 nM).

Although mifepristone (**4**) is a potent AR antagonist, this drug may be limited as a chronically administered antiandrogen by its potent antagonism of the glucocorticoid receptor.[18] High-resolution X-ray crystal structures have demonstrated that this antagonism results from the active displacement of the GR helix 12 by the bulky 11β-dimethylaniline side chain.[14] The clinically employed antiandrogen cyproterone acetate (**3**) is a somewhat less potent antiglucocorticoid that is thought to antagonize the GR through a passive antagonism mechanism.[18] To investigate whether the more flexible 11β-alkyl side chains of compounds **7**–**11** might reduce this undesirable cross reactivity with the GR, these compounds were investigated in CHO-K1 cells transiently transfected with a full-length GR expression vector[32] and a glucocorticoid responsive luciferase reporter vector.[33] As shown in Figure 3, control experiments demonstrated that both cyproterone acetate and mifepristone (**4**) were potent GR antagonists (IC_{50}(**3**) = 0.9 nM; IC_{50}(**4**) = 0.008 nM). In contrast, compounds **6**–**11** were only weak GR antagonists (Figure 3 and Table 1).

To examine the ability of compounds **7**–**11** to affect gene expression driven by full-length AR from an androgen response element (ARE), these compounds were compared with cyproterone acetate (**3**) and mifepristone (**4**) in CV-1 cells transfected with an AR expression vector and an MMTV-luciferase reporter vector. These experiments (Figure 4) revealed that the octyl- and decyl-substituted compounds **9** and **10** comprise potent partial agonists (EC_{50}(**9**) = 2.2 nM; IC_{50}(**9**) = 1.3 nM; EC_{50}(**10**) = 8.4 nM; IC_{50}(**10**) = 0.6 nM) comparable to cyproterone acetate (**3**, EC_{50}(**3**) = 3.6 nM; IC_{50}(**3**) = 2.1 nM). In contrast, the pentyl-substituted compound **8** exhibited greater agonist activity (EC_{50}(**8**) = 0.3 nM; IC_{50}(**8**) = 72 μM). Control experiments confirmed that these compounds exhibit relatively low toxicity to CHO-K1 and CV-1 cells at the highest concentration studied (data shown in the Supporting Information).

Compounds **3**, **4**, **6**–**11** were further investigated for their ability to halt the growth of lymph node carcinoma of the prostate (LNCaP) cells. These cells express a mutant AR (T877A) that recognizes the clinically important antiandrogens hydroxyflutamide and cyproterone acetate (**3**) as agonists.[12,34] In contrast, the antiandrogen mifepristone (**4**) is an antagonist of this mutant AR.[12,35] Cell death curves were constructed from cell density measurements in the presence of compounds, and calculated IC_{50} values are shown in Table 1. Compounds **8**–**11** exhibited IC_{50} values similar to mifepristone (**4**) with 4-fold greater potency than cyproterone acetate (**3**).

These results indicate that 11β-alkyl-Δ^9-19-nortestosterone derivatives bind tightly to the AR LBD and exhibit minimal antiglucocorticoid activity. Some of these compounds potently inhibit AR dimerization, exhibit partial AR agonist activity, and provide interesting candidates for studies in animal models of human prostate cancer.

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Supporting Information Available: Experimental procedures and characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org

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