

# Antimineralocorticoid 11 $\beta$ -Substituted Spirolactones Exhibit Androgen Receptor Agonistic Activity: A Structure Function Study

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## ABSTRACT

In humans, spironolactone and mesprenone are well known antimineralocorticoids without C-11 $\beta$  substituents. These compounds display antagonist properties by acting through the human androgen receptor (hAR). In contrast, we demonstrate here that synthetic mineralocorticoid antagonists bearing hydrophobic C-11 $\beta$  substituents and C-17 $\gamma$ -lactone are potent hAR agonists in vitro. The three-dimensional construction of both the ligand binding domain (LBD) of the hAR and the human mineralocorticoid receptor (hMR), based on the crystal structure of the LBD of the human progesterone receptor, revealed not only that the interactions with the steroidal A- and D-rings seemed to be crucial for stabilization of active hMR or hAR conformation, but that other steroidal substitutions could influence the agonist versus antagonist activity of ligands. The docking of synthetic compounds bearing C-11 $\beta$  hydrophobic

substituents within the ligand binding pocket of hAR demonstrated that precise positions of the steroid, such as C-11 and C-17, are in close contact with some residues on the receptor, C-11 with Gly 708 and C-17 with Asn705 and Thr877. These contacts are crucial for the stabilization of the active receptor conformation. Mutation of Asn705 by alanine altered the 11 $\beta$ -substituted spirolactone-mediated *trans*-activation function of hAR, suggesting an anchoring of the C-17-lactone carbonyl group (C-22) with this residue. The stabilizing effect of the H12 helix in its active conformation is also induced by hydrophobic contacts between the Gly708 and C-11 $\beta$  substituents, as recently observed with the A773G-hMR mutant in the presence of similar drugs. The study of the role of these substituents suggests efficient new directions for the drug design of selective androgen agonists.

Mineralocorticoid and androgen receptors are members of the nuclear receptor superfamily of ligand-responsive transcription factors. They have marked structural and functional similarities (Evans, 1988) and are characterized by three basic functional domains: the amino-terminal A/B domain, which has a ligand-independent *trans*-activation function; the DNA binding domain, which allows DNA binding and receptor dimerization; and the ligand binding domain (LBD), involved in chaperone protein interaction, dimerization, and hormone-dependent *trans*-activation. The carboxy-terminal regions of these two receptors are relatively well conserved (50% homology). Aldosterone, a natural hormone, exerts its effect through the MR. The action of aldosterone is

inhibited by spironolactone and progesterone, which act as competitive inhibitors by forming inactive ligand-receptor complexes. The antimineralocorticoid spirolactones have been used for the past 30 years in the treatment of sodium-retaining states and as antihypertensive agents (Corvol et al., 1981). However, the well known aldosterone antagonist, spironolactone, also displayed such side effects as antiandrogenic activity in rats and humans (Corvol et al., 1975). New antimineralocorticoids have been developed (Singh et al., 2000) in an attempt to obtain derivatives with antagonistic activity higher than that of spironolactone and devoid of the side effects exhibited by this compound (i.e., gynecomastia, decreased libido, and impotence in male patients) due to its antiandrogenic properties (Losert et al., 1986). The actions of the two predominant natural androgens, testosterone and dihydrotestosterone, are mediated through the AR. Compounds like cyproterone acetate or hydroxyflutamide that block the action of androgens have been proved useful in the

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**ABBREVIATIONS:** LBD, ligand binding domain; MR, mineralocorticoid receptor; AR, androgen receptor; hMR, human mineralocorticoid receptor; hAR, human androgen receptor; hPR, human progesterone receptor; LBP, ligand binding pocket; PALM, PC-3 androgen luciferase; MMTV, mouse mammary tumor virus; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; WT, wild-type.

treatment of benign prostate hypertrophy and prostate cancer in men, or hirsutism in women.

To investigate how agonists and antagonists interact with the human MR (hMR) and/or the human AR (hAR), three-dimensional models of the hMR and hAR-LBD, have been recently drawn (Fagart et al., 1998; Poujol et al., 2000). They were based on the crystal structure of the holo human retinoic acid receptor- $\gamma$ -LBD (Renaud et al., 1995), human estrogen receptor- $\alpha$  (Brzozowski et al., 1997) and hPR (Williams and Sigler, 1998). The crystal structure of hAR-LBD in complex with the agonist ligand R1881 (methyltrienolone) has recently been determined (Matias et al., 2000) and confirms our model (Poujol et al., 2000). These hMR and hAR-LBD models revealed LBPs, which were further analyzed to identify the residues, involved in the specific recognition of mineralocorticoid or androgen ligands or their antimineralocorticoid or antiandrogen counterparts.

We recently showed on hMR that C-11-substituted steroids with a C-17 $\gamma$ -lactonic ring displayed antagonist properties, but acted as potent agonists when Ala773 was substituted by Gly (Auzou et al., 2000). Moreover, hAR has a glycine at the corresponding position (Gly708). This residue, when mutated by an alanine, is associated to a partial androgen insensitivity syndrome (Albers et al., 1997). In the present study, the same C-11-substituted steroids with a C-17 $\gamma$ -lactonic ring (Fig. 1) were assayed for their capacity to bind and stimulate transcription of hAR. As for Ala773Gly in hMR (Auzou et al., 2000), we determined the specific role of the C-11 substituents facing the 708 glycine residue in the hAR-LBP. We observed that the introduction of an unsaturated hydrophobic group located on the C-11 $\beta$  position of the C-ring does not modify the affinity of these compounds for the hAR and induces potent hAR transcriptional activity in two cellular models: the naive cell line CV-1 or the prostate cancer cell line PC-3 (Térouanne et al.,

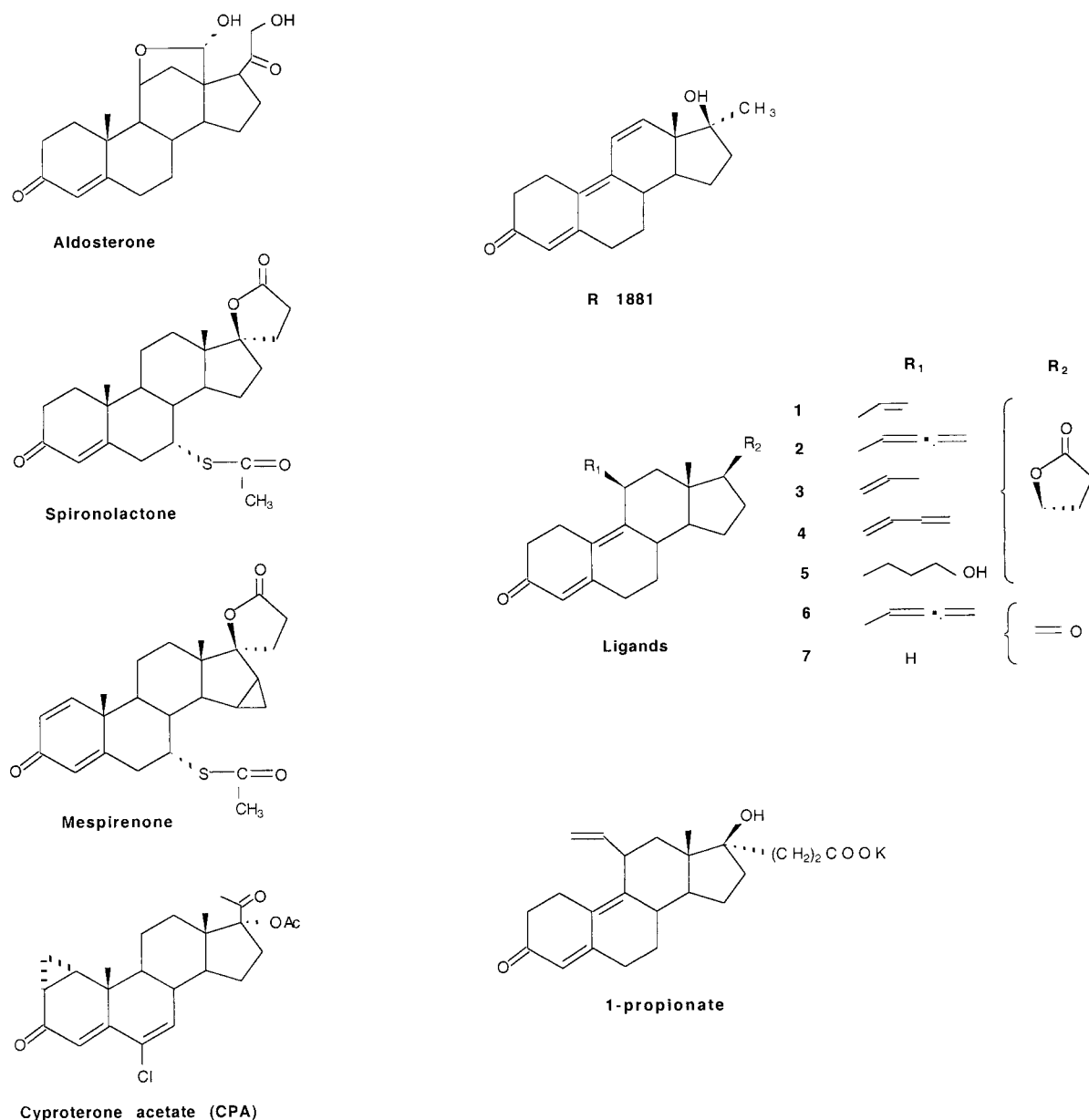


Fig. 1. Chemical structures of steroidal ligands.

2000). We showed that Asn705 is specifically involved in anchoring of the C-22 carbonyl group and Thr877 in anchoring of the 17 $\beta$ -oxygen of the  $\gamma$ -lactonic ring. This study clearly demonstrated that this novel family of mineralocorticoid antagonists bearing hydrophobic C-11 substituents and C-17 $\gamma$ -lactone acted as potent androgen agonists.

## Materials and Methods

**Chemicals.** Spironolactone (SC9420) was obtained from Searle Laboratories (Chicago, IL). Mesprenone was from Schering Laboratories (Kenilworth, NJ). [ $^3$ H]R1881 (87 Ci/mmol) and unlabeled R1881 were purchased from NEN Life Science Products (Paris, France). 11 $\beta$ -Vinyl-3-oxo-19-nor-17 $\alpha$ -pregna-4,9-diene-21,17-carbolactone (**1**) (Nickisch et al., 1985), 11 $\beta$ -allenyl-3-oxo-19-nor-17 $\alpha$ -pregna-4,9-diene-21,17-carbolactone (**2**), 11-ethylidene-3-oxo-19-nor-17 $\alpha$ -pregna-4,9-diene-21,17-carbolactone (**3**), 11-(3-propenylidene)-3-oxo-19-nor-17 $\alpha$ -pregna-4,9-diene-21,17-carbolactone (**4**), 11 $\beta$ -(3-hydroxypropyl)-3-oxo-19-nor-17 $\alpha$ -pregna-4,9-diene-21,17-carbolactone (**5**), 11 $\beta$ -allenyl-3-oxo-19-nor-17 $\alpha$ -pregna-4,9-diene-3,17-dione (**6**), androsta-4,9-diene-3,17-dione (**7**), and potassium 3-(17 $\beta$ -hydroxy-3-oxo-19-nor-4,9-diene-17 $\alpha$ -yl)-propionate (**1**-propionate) were synthesized in our laboratory (Faraj et al., 1990; Claire et al., 1993). Structures and abbreviations of the steroids are given in Fig. 1.

**Expression and Reporter Constructs.** pFC31Luc, which contains the MMTV promoter driving the luciferase gene, was obtained from H. Richard-Foy (LBME, Toulouse, France). Expression vectors pSG5hAR and pSG5hARN705A were a gift from Dr. Nicolas Poujol. For transient transfection, all plasmids were purified with nucleobond-AX cartridges (Macherey-Nagel, Hoerd, France).

**Ligand Binding Specificity.** Binding specificities were measured in the human prostate adenocarcinoma PC3-cells, stably transfected with hAR and an AR-responsive luminescent reporter gene. This cell line was named PALM for PC-3 androgen luciferase MMTV (T  rouanne et al., 2000). It expresses hAR at the concentration of 1000 fmol/mg of protein, and it provides a good tool for studying androgen binding specificities in the whole cell binding assay.

PALM cells were seeded in 12-well plates ( $3 \times 10^5$  cells per well) and maintained for 16 h in Ham's F-12 medium containing 3% dextran-coated charcoal-fetal calf serum. Cells were labeled for 2 h with 3 nM [ $^3$ H]R1881 in the presence or absence of increasing concentrations of compounds in 1 ml of serum-free Ham's F-12 medium. Each dilution was performed in triplicate. Plates were put on ice for 15 min, and labeling medium was removed. Cells were washed three times with 2 ml of cold phosphate-buffered saline and harvested in 300  $\mu$ l of lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM CDTA, 10% glycerol, and 1% Triton X-100). Radioactivity was determined on 100  $\mu$ l of lysate.

**Transfection and Luciferase Assay in CV1 Cells.** Monkey kidney CV1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) and supplemented with 10% heat-inactivated fetal bovine serum. CV1 cells were seeded in 12-well plates ( $2 \times 10^5$  cells per well) and transfected 8 h after using calcium phosphate with 100 ng human AR expression vector pSG5hAR or pSG5hARN705A, 1  $\mu$ g of MMTV-luciferase reporter vector (pFC31), and 250 ng of pCMV- $\beta$ -galactosidase as the internal control. After 16 h of transfection, cells were treated with either R1881 or tested compounds for 30 h in serum-free DMEM. Luciferase activity was assayed with a LKB luminometer following the protocol provided by Promega (Charbonn  res, France). Cells were lysed directly in the plates by 300  $\mu$ l of lysis buffer (25 mM Tris phosphate, pH 7.8, 2 mM CDTA, 10% glycerol, and 1% Triton X-100). Luciferase activity was measured on 100  $\mu$ l of lysate aliquots for 10 s after injection with 100  $\mu$ l of luciferase detection solution [20 mM Tricine, pH 7.8, 1.07 mM (MgCO<sub>3</sub>)<sub>4</sub> Mg(OH)<sub>2</sub> · 5H<sub>2</sub>O, 2.67 mM MgSO<sub>4</sub>, 1 mM EDTA, 0.53 mM ATP, 0.47 mM luciferin, and 0.27 mM coenzyme A]. The induction of luciferase activity is indicated in

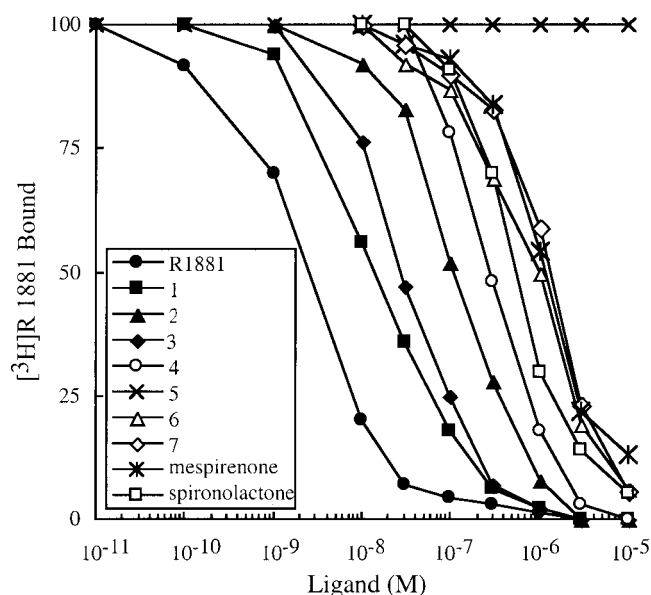
arbitrary units, corrected by  $\beta$ -galactosidase activity and the values obtained for nontransfected cells. At least three independent assays were performed in duplicate.

**Transcriptional Activation in PALM Cells.** For transcriptional activity studies, PALM cells were seeded onto 96-well plates, totally opaque (Falcon, Becton Dickinson, Le Pont de Claix, France) in 100  $\mu$ l of Ham's F-12 medium supplemented with 3% of dextran-coated charcoal-fetal calf serum. Cells were then incubated for 30 h with the different compounds in serum-free Ham's F-12 medium. At the end of incubation with the different steroids, culture medium was replaced by 50  $\mu$ l of 0.3 mM luciferin in DMEM without phenol red. Luciferase activity was measured in a MicroBeta TriLux (EG&G Wallac Oy, Turku, Finland) for 2 s per well. The luminescent signal was stable for at least 2 h.

**Model Building.** A model of the hAR LBD has been described previously (Poujol et al., 2000). Briefly, it was generated by homology with hPR using the Modeler package (version 4.0) (Sali and Blundel, 1993) and is based on the sequence alignment using the hPR crystal structure as a template. Ligands were positioned manually in the pocket using the accessible probe and van der Waals volumes as guides. The complexes were energy minimized in 5000 steps with a dielectric constant of 2 using the Powell procedure. The cavity volume of the binding pocket was calculated with VOIDOO (Kleywegt and Jones, 1994), a program for computing molecular volumes and studying cavities in macromolecules such as proteins.

## Results

**Effect of Steroid Substitution on Steroid Binding to the Wild-Type hAR.** The stable transfectant cell line PALM expressed a high concentration of wild-type (WT) hAR compatible with the experimental design. In the whole competitive assay in PALM cells, binding affinity of antimineralocorticoid compounds to AR was assessed using [ $^3$ H]R1881 as a tracer. The displacement curves of these derivatives are shown in Fig. 2 and compared with the reference compound R1881. Except compound **5**, characterized by an 11 $\beta$ -hy-



**Fig. 2.** Competitive inhibition of [ $^3$ H]R1881 binding to hAR in PALM cells by steroidal ligands. PALM cells were incubated 2 h at 37°C with  $3 \times 10^{-9}$  M [ $^3$ H]R1881 alone or in the presence of the indicated concentration of unlabeled compounds (**1** to **7**, mesprenone and spironolactone). Bound [ $^3$ H]R1881 was measured in an aliquot (100  $\mu$ l) of the cell lysate by liquid scintillation. Data are expressed as percent of specific binding observed in the noncompeted control.

droxypropyl substituent, the C-11-substituted spirolactones (**1** to **4**) were highly efficient at inhibiting [ $^3\text{H}$ ]R1881 binding to hAR. The affinity of the 11 $\beta$ -vinyl derivative **1** was the highest ( $K_i = 10^{-8}$  M). Compound **7**, characterized by a 17-ketone function and devoid of any substituent at the C11-position, exhibited much lower affinity ( $K_i = 10^{-6}$  M). Compound **6** (with a C-17-ketone function and a C-11-allenyl substituent) mespirenone, and spironolactone exhibit affinities of the same order. These results suggest that the 11 $\beta$ -substitution/17 $\gamma$ -lactone association is a determinant of the steroid binding to hAR.

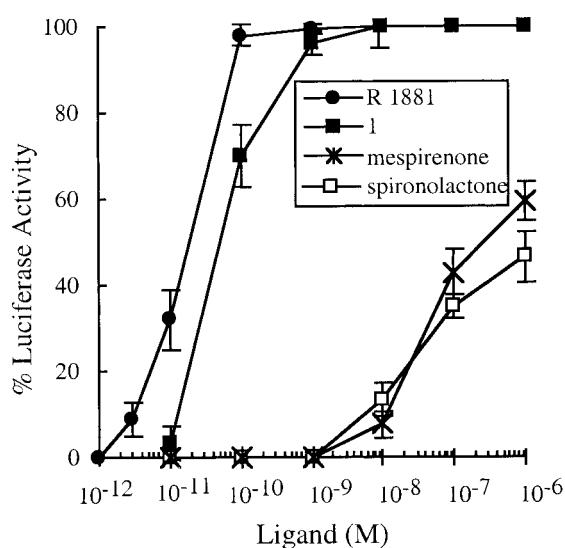
#### trans-Activation Properties of the Wild-Type hAR.

We examined by cotransfection assays (CV1 cells) the ability of the WT hAR to activate transcription in response to R1881, a highly potent androgen, as well as in response to various C11- and/or C-17-substituted mineralocorticoid antagonists with partial agonist activity.

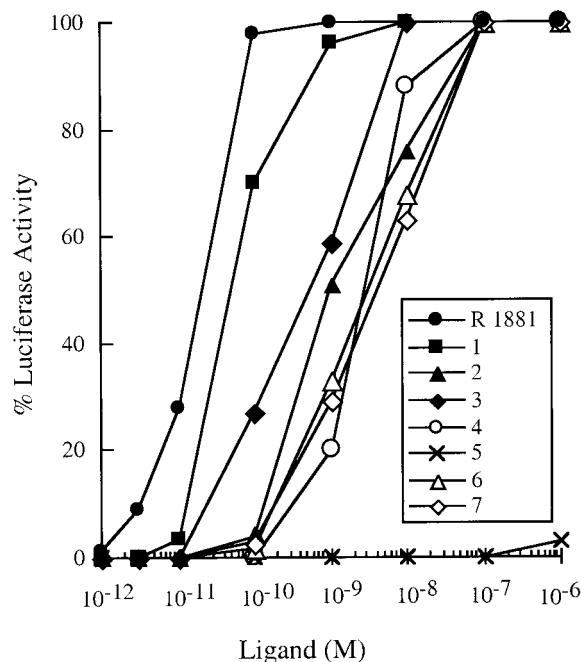
Compound **1** (C-11 $\beta$  vinyl, C-17 $\gamma$ -lactone) induced a right-shifted dose-response curve, and the  $\text{EC}_{50}$  value was quite similar to that of R1881 ( $\text{EC}_{50} = 5.10^{-11}$  M versus  $2.5 \times 10^{-11}$  M) (Fig. 3). Spironolactone and mespirenone, two classic antimineralocorticoid antagonists bearing a 7 $\alpha$ -substitution and devoid of any C-11 substitution, were also tested and induced a large shift toward higher concentrations.

Figure 4 shows that other compounds **2** to **6** with 11 $\beta$ -substituents exhibited a right shift in the dose-response curve of nearly 1 to 2 orders of magnitude, corresponding to a slight loss of activity. However, except for compound **5**, which is characterized by a polar 11 $\beta$ -(3-hydroxypropyl) substituent and induced almost undetectable *trans*-activation, all the C-11-substituted spirolactones (**1** to **4**) and compounds **6** and **7**, characterized by a C-17 keto function and (or not) a substituent at the C-11 position, stimulated the hAR activity in a dose-dependent manner. These results reflect an agonist activity of these compounds through the hAR.

We also evaluated a potential partial antagonist activity



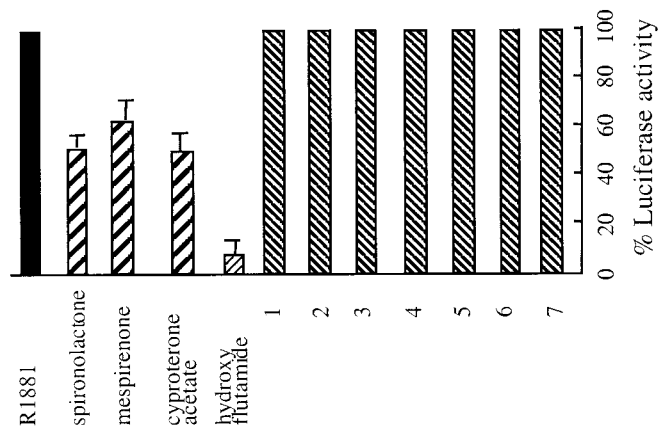
**Fig. 3.** hAR agonist activities of R1881, compound **1**, and antimineralocorticoids (spironolactone and mespirenone) in CV1 cells. The transcriptional activity of transiently transfected AR in CV1 cells was evaluated in the presence of various concentrations of the ligands. *trans*-Activation was determined by luciferase activity normalized to the internal  $\beta$ -galactosidase control and was expressed in arbitrary units. Each point is the mean  $\pm$  S.E.M. of three separate experiments.



**Fig. 4.** Agonist activities of different synthetic 11 $\beta$ -substituted antimineralocorticoids (**1** to **7**). The effects of different ligands were evaluated in CV1 cells. After transfection,  $10^{-12}$  M to  $10^{-6}$  M of R1881 or various antimineralocorticoids were added to the cells. Luciferase activity was measured in cell lysate. The 100% represents the activity obtained with  $10^{-9}$  M R1881. Values represent the means  $\pm$  S.E.M. from three or more experiments.

associated with compounds **1** to **7** and a possible antagonist activity associated with compound **5**. Chemicals **1** to **7** and antiandrogens were tested at  $10^{-6}$  M in the presence of R1881 (10 nM). Figure 5 shows an antagonist activity for the nonsteroidal antagonist hydroxyflutamide and a partial antagonist activity for the steroidal compounds cyproterone acetate, spironolactone, and mespirenone. No antagonist activity was found with any of the derivatives **1** to **7**. No compound possesses a partially antagonist activity when lower concentrations were tested (data not shown).

Transcriptional activity was also measured in the stable cell line PALM (Térouanne et al., 2000).  $\text{EC}_{50}$  values obtained with these two cellular models, CV1 and PALM, are



**Fig. 5.** AR antagonist activity in CV-1 cells. CV-1 cells were cotransfected as described in *Materials and Methods* and incubated in the presence or the absence of  $10^{-10}$  M of R1881 or in the presence of R1881 with  $10^{-6}$  M of the indicated ligands. The top bar shows the activity of R1881 alone (set as 100%). Values represent the means  $\pm$  S.D. of at least three determinations.

TABLE 1

EC<sub>50</sub> values of the hAR in CV-1 and PALM cells

All EC<sub>50</sub> values were determined from full-dose response curves ranging from 10<sup>-12</sup> to 10<sup>-6</sup> M in CV-1 and PALM cells as described under *Materials and Methods*

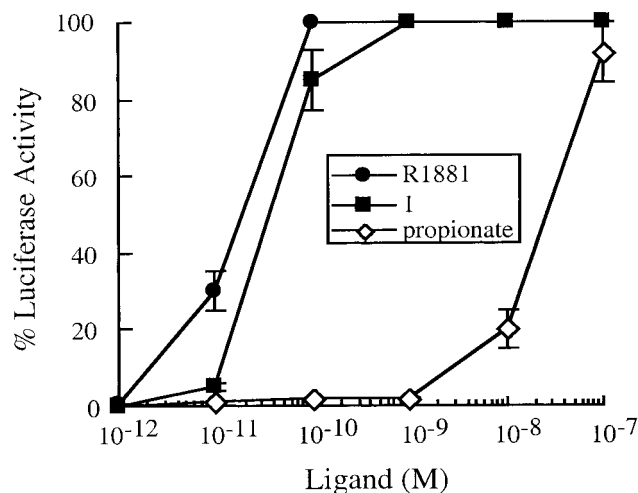
	EC <sub>50</sub> (nM) in CV-1 Cells	EC <sub>50</sub> (nM) in PALM Cells
Agonist R1881	0.03 ± 0.01	0.07 ± 0.02
R <sub>1</sub>	R <sub>2</sub> = Spirolactone	
— Vinyl	0.05 ± 0.02	0.28 ± 0.12
— Ethylidene	0.3 ± 0.3	0.8 ± 0.3
— c = Allenyl	0.8 ± 0.2	3.5 ± 2.0
— Propenylidene	2.8 ± 0.5	17 ± 2
R <sub>1</sub>	R <sub>2</sub> = Ketone	
— c = Allenyl	3.0 ± 1.5	27 ± 8
H	4.0 ± 1.8	40 ± 15

summarized in Table 1. All the values obtained with the PALM cell line were higher, but remained in the same order. In the two cellular models, we observed EC<sub>50</sub> **1** < EC<sub>50</sub> **3** < EC<sub>50</sub> **2** < EC<sub>50</sub> **4** ≤ EC<sub>50</sub> **6** ≤ EC<sub>50</sub> **7**. Therefore, the androgenic transcriptional activities with the spirolactone derivatives are not cell-dependent.

The agonist activity of these compounds could be attributed to their metabolization. One possible metabolite could be the 17β-hydroxy propionate form resulting from a conversion of the 17-spirolactone group already observed for K-canrenoate (Peterfalvi et al., 1980; Losert et al., 1986). The similar 17β-hydroxy opened form derived from compound **1** was synthesized (1-propionate). The tertiary C-17β-hydroxy function of this derivative is able to interact with Asn705, for the agonist R1881 (Poujol et al., 2000). Dose-response curves were generated by adding to the transfected cells increasing concentrations (10<sup>-11</sup>–10<sup>-7</sup> M) of R1881, compound **1**, and its opened form. As shown in Fig. 6, this opened form exhibited a right shift toward higher concentrations (≈10<sup>-7</sup> M), indicating that the agonist activity observed with 11β-substituted 17γ-spirolactones is not due to their metabolization.

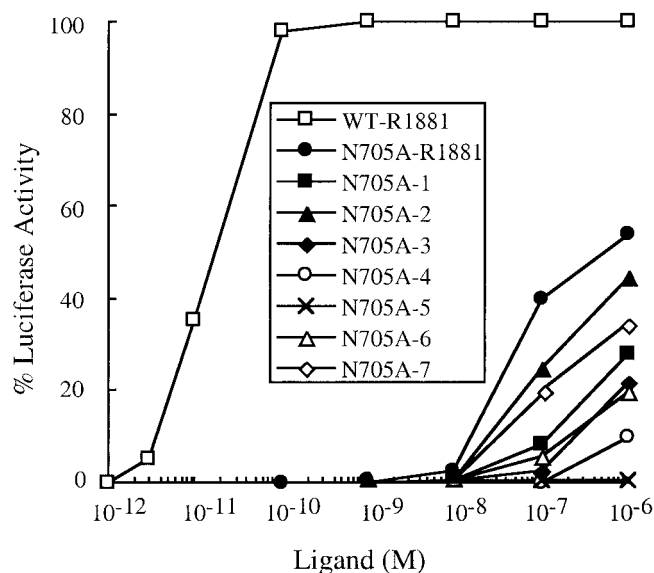
**trans-Activation Properties of the N705A-hAR Mutant.** Recent results showed that the N705A-hAR mutant was unable to bind R1881 with high affinity, resulting in a drastic shift of the *trans*-activation curve (Poujol et al., 2000). In this study, we have tested the ability of compounds **1** to **7** to induce *trans*-activation of the N705A-hAR mutant. Our results (Fig. 7) show that the substitution of Asn705 by alanine induced a larger shift toward higher concentrations (≈10<sup>-6</sup>/10<sup>-5</sup> M) and produced an extremely low *trans*-activation efficiency in response to the different 11β-substituted spirolactone compounds. The relative increase of the effects of 17-oxo compounds **6** and **7** and compound **2**, compared with **1** or **3**, is not significant considering the drastically lower *trans*-activation observed. Altogether, these results demonstrate that the replacement of Asn705 by an alanine residue in the hAR modifies the response to steroids bearing C-17 ketonic or γ-lactonic substituents, which could indicate a crucial interaction between Asn705 and the C-17 (compounds **6** and **7**) or C-22 keto function (compounds **1** to **4**) of these substituents.

**Ligand Docking.** The hAR-LBP is outlined by helices H3, H5, H7, H11, and H12; the β turn; and loops 6,7 and 11,12. LBP is predicted to be lined by 18 amino acids that are mostly hydrophobic, except for three polar residues: Arg752 and Glu711 at one end of the pocket and Asn705 at the opposite end. R1881 is manually docked by superimposing the ligand on progesterone followed by a few steps of minimization



**Fig. 6.** Comparison of the agonist activities of R1881, compound **1**, and its 17β-hydroxy opened form (1-propionate). Transcriptional activity of hAR in response to the steroidal ligands was evaluated as described previously.

while keeping the Cα carbons of the protein backbone fixed. Gln711 (helix H3) and Arg752 (helix H5) form strong hydrogen bonds (2.5 and 2.33 Å) with the C3-ketone group present in all the agonist and antagonist compounds, and Asn705 (helix H3) forms a hydrogen bond (2.9 Å) with the 17β-hydroxyl group. To better understand the effect of C-11 and C-17 substitutions on the steroid agonist versus antagonist properties, we docked compounds **1** to **7** (Fig. 1) and meprenolone in the hAR-LBP model. In this model, the 17γ-lactonic ring is located in a region delimited by helices H3, H11, and H12 and forms hydrophobic contacts with Leu701 (4 Å), Leu880 (3.5 Å), Val889 (3.6 Å), and Phe891 (4.2 Å) (Fig. 8A). The C-22 keto function and the 17β-oxygen of the γ-lactonic ring make a hydrogen bond with Asn705 (2.4 Å) and Thr877 (2.5 Å), respectively (Fig. 8B). As for A773G-hMR mutant, the hAR-LBP generates a tight hollow delimited by helices H3, H5, and H12 and forms hydrophobic contacts



**Fig. 7.** Transcriptional activation of luciferase activity by the N705A-hAR mutant in the presence of antimineralocorticoids **1** to **7**. CV1 cells were transfected with the mutant N705A-hAR expression vector and treated with R1881 and ligands **1** to **7** from 10<sup>-12</sup> to 10<sup>-6</sup> M.

## Discussion

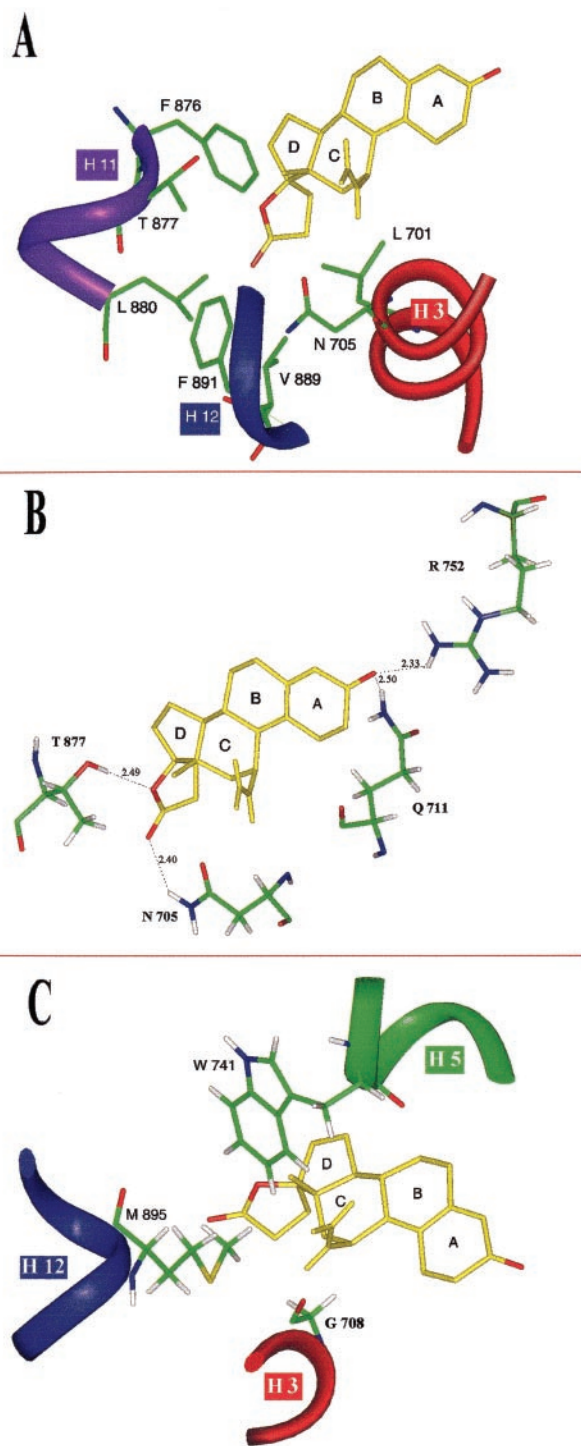
The potential of androgen therapy to address male disorders is rapidly increasing. Significant advances in our understanding of hAR structure and function have provided the basis for selective receptor modulation by different ligands.

We previously showed that 11 $\beta$ -substituted spironolactones are mineralocorticoid antagonists in vitro in transiently transfected cells, and it was demonstrated that the substitution of A773 by Gly was critical for generating mineralocorticoid activity (Auzou et al., 2000). The sequence alignment of hAR and hMR reveals that hAR possesses a glycine (G708) at the corresponding position. In this work, we provide evidence that these 11 $\beta$ -substituted spironolactones bind and activate the WT hAR. To test the transcriptional activities of these products, two cellular models were used in the naive CV-1 cells or the cell line PALM (Terouanne et al., 2000). In these two cases, all the derivatives, except compound **5**, exhibit an agonist activity without antagonist activity. We observed a discrepancy between the EC<sub>50</sub> value of R1881 and the tested products according to the used cells. It is known that transiently transfected genes, when they are overexpressed, can have a great influence on the dose-response curves (Joyeux et al., 1997). Moreover, in transiently cotransfected cells, the receptor gene accessibility to target gene is quite different from that in a chromatin environment, as it is for a stable cell line. So, the *trans*-activation studies in PALM cells reflect most closely physiological conditions.

The results reported in the present study highlight the importance of hydrophobic 11 $\beta$ -substituents for inducing binding and agonist androgenic activity of these 17-spirolactones. In contrast, spironolactone, the well known spiro lactone with 7 $\alpha$ -substitution, but without any substituent on the C-11 $\beta$  position, exhibits antimineralocorticoid properties but also displays antiandrogenic activity (Corvol et al., 1975). The role of this 7 $\alpha$ -substitution is not well defined today. It has been recently shown (Fagart et al., 1998) that the 7 $\alpha$ -propyl substituent of RU26752, another spiro lactone drug, could be accommodated in a crevice observed in the probe-accessible volume delimited by different hydrophobic residues. Nevertheless, canrenone, a spiro lactone resulting in the metabolism of spironolactone is deprived of 7 $\alpha$ -substituent and retains the same antimineralocorticoid and antiandrogenic activity as spironolactone or RU26752 (Losert et al., 1986).

The 11 $\beta$ -vinyl derivative **1** was the most interesting compound of this series. The docking of compound **1** within the hAR model suggested that the C-11 vinyl group can be accommodated in the tight hollow delimited by helices H3, H5, and H12. It seems that stabilization of the holo conformation is achieved through interactions between the vinyl side chain into the cavity delimited by Gly708, Trp741, and Met895. The decreased binding or the agonist activity is reflected by: **1** > **3** > **2** > **4** > **6** > **7**. The same observation already shown for hMR A773G (Auzou et al., 2000) can be formulated: the nature of the C11 chain or the C11-carbon hybridization (sp<sub>3</sub> or sp<sub>2</sub>) revealed the role played by the size and flexibility of these substituents on their accommodation in the cavity. Nevertheless, docking of the same 11 $\beta$ -substituted spiro lactones (**1** to **4**) in the hMR-LBD model suggested that mineralocorticoid antagonist properties could be explained by constraints observed between the 11 $\beta$ -substituents and Trp806

between the C-11 $\beta$  substituent and the residues Gly708 (3.5 Å), Trp741 (3.9 Å), and Met895 (3.7 Å) (Fig. 8C). The polar and bulky hydroxypropyl group of compound **5** cannot be accommodated in this tight cavity. In contrast, the C-11 substituents of compounds **1** to **4** and **6** fit well.



**Fig. 8.** Ligand docking in the hAR-ligand binding pocket. A, close-up view of the D-ring interaction of compound **1** in site II with Leu701, Leu880, Val889, and Phe891. B, close-up view of the 3-keto anchoring in site I with Gln711 and Arg752 and the C-22-carbonyl group with Asn705 and the 17 $\beta$ -oxygen with Thr877. C, contacts of the 11 $\beta$ -vinyl chain with Gly708, Trp741, and Met895.

in helix H5 leading to the expulsion of the hMR helix H12 from its active position. In the present hAR-LBD model, without constraints with Trp741, H12 is stabilized in an active conformation induced by hydrophobic contacts that would allow coactivators to bind WT hAR.

In this model, the 17 $\gamma$ -lactonic ring of compounds **1** to **4** forms hydrophobic contacts with Leu701, Phe876, Leu880, and Phe891. The C-22 ketone and the 17 $\beta$ -oxygen of the  $\gamma$ -lactonic ring make a hydrogen bond with Asn705 and Thr877, respectively. The mutation of Asn705 by Ala induces a large shift in the *trans*-activation curves compared with R1881. This reveals the importance of the contacts of N705 not only with the 17 $\beta$ -hydroxy function of R1881, but also with the C-22 ketone of the 17 $\gamma$ -lactone of compounds **1** to **4**. Stabilization of helix H12 and the agonist activity of these compounds are probably due to these additional contacts that induce an active receptor conformation.

One of the possible metabolites of compound **1**, the 17 $\beta$ -hydroxy opened form (**1**-propionate), could make a hydrogen bond with Asn705, which would explain the agonist activity of the different 11 $\beta$ -substituted spirolactones on hAR. The right shift toward higher concentration of the *trans*-activation curve of this compound rules out the possibility of this metabolite effect.

Interestingly, the substitution of compound **6** at the C-11 position by an allenyl group is sufficient to induce binding to the hAR (Fig. 2). Compounds **6** and **7**, contrary to their antagonist effect observed with hMRA773G due to a loss of anchoring of the D-ring (Auzou et al., 2000), establish close contacts with Asn705 through their 17-keto function and act as weak agonists ( $EC_{50} \approx 3\text{--}4 \times 10^{-9}$  nM in CV-1 cells). In the case of spironolactone and mesprenone showing an antiandrogenic activity (Losert et al., 1986), the hydrophobic contacts between the  $\gamma$ -lactonic ring and the LBD of hAR are not sufficient to balance the lack of essential contacts with some residues in the C-11 area and to induce stabilized agonist conformation. In conclusion, the results described here point out the role of both the C-11 and the C-17 substituents on spirolactones in stabilizing an active hAR conformation. As recently described for Ala773 when mutated in Gly for hMR, the role of the corresponding Gly708 in hAR is well demonstrated, revealing the crucial role of this residue in the anchoring of androgen agonists. Despite the fact that there is no general rule for transforming a steroid agonist into an antagonist, this study shows that one of the chemical approaches in the androgen series involves structural modifications of the steroid skeleton around the 3-keto, 11 $\beta$ -R<sub>1</sub>, and 17-R<sub>2</sub> groups. These three regions seemed to be crucial

for the stabilization of the active hAR conformation, but the nature of the steroid substitution could influence the agonist/antagonist activity.

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