The Synthetic Androgen Methyltrienolone (R1881) Acts as a Potent Antagonist of the Mineralocorticoid Receptor

ARMELLE-NATSUO TAKEDA*, GREGORY M. PINON*, MARCELLE BENS, JEROME FAGART, MARIE-EDITH RAFESTIN-OBLIN* and ALAIN VANDEWALLE*

INSERM, U773, Centre de Recherche Biomédicale Bichat-Beaujon CRB3, BP 416, F-75018, Paris, France; Université Paris 7 - Denis Diderot, site Bichat, F-75870, Paris, France.
a) Running title page: Antimineralocorticoid effects of androgens

b) Corresponding authors
Alain Vandewalle and Marie Edith Rafestin-Oblin
INSERM U773, Centre de Recherche Biomédicale Bichat-Beaujon (CRB3)
BP 416, 16 rue Henri Huchard
F-75870, Paris, France.
E-mail: vandewal@bichat.inserm.fr
       oblin@bichat.inserm.fr

c) number of text pages: 30
number of Figures: 8
Number of references: 40
Abstract: 250 words
Introduction: 813 words
Discussion: 1342 words

d) List of nonstandard abbreviations used
Aldo, Aldosterone; Am, amiloride; Ams ISc, amiloride-sensitive short-circuit current; AR, androgen receptor; CCD, cortical collecting duct; DHT, dihydrotestosterone; DM, defined medium; DMEM, Dulbecco’s minimal essential medium; EGF, epidermal growth factor; ENaC, epithelial sodium channel; FCS, fetal calf serum; GR, glucocorticoid receptor; HF, hydroxyflutamide; HFM, hormone-free defined medium; Isc, short-circuit current; LBD, ligand-binding domain; MMTV, mouse mammary tumor virus; MR, mineralocorticoid receptor; 18OVP, 18-oxo-18-vinylprogesterone; T, testosterone; 19-nor T, 19-nor-testosterone; 17α-T, 17α-methyl testosterone.
ABSTRACT

Aldosterone binds to the mineralocorticoid receptor (MR), and exerts fine control over Na\(^+\) absorption in renal collecting duct cells (CCDs). Many natural and synthetic steroids can also bind to the MR to produce agonist or antagonist effects. Here we investigate whether androgenic hormones act as MR agonist or antagonist ligands in CCDs. Testosterone (T), dihydrotestosterone (DHT) and methyltrienolone (R1881), a synthetic androgen agonist, all bind to the MR. R1881 displayed the same affinity for MR as aldosterone. Androgens did not activate the MR transiently expressed in human embryonic kidney HEK 293T cells, but did antagonize aldosterone-induced MR trans-activation activity (R1881＞DHT＞T). Short-circuit current (I\(_{sc}\)) experiments, used to measure transepithelial Na\(^+\) transport, revealed that 10\(^{-5}\) M T and DHT or R1881 prevented the rise in the amiloride-sensitive component of I\(_{sc}\) caused by aldosterone in mouse mpkCCD\(_{cl4}\) collecting duct cells partially and totally, respectively. In contrast, androgens had no effect on stimulated-I\(_{sc}\) elicited by the specific glucocorticoid agonist RU26988. Docking of steroids within the crystal structure of the ligand-binding domain of MR, together with trans-activation studies, revealed that the contacts between the 17β-hydroxyl group of androgens and the Asn770, Cys942 and Thr945 residues of the ligand-binding cavity stabilize ligand-binding complexes, but are not strong enough to keep the receptor in its active state. Altogether, these findings indicate that androgen ligands, particularly R1881, act as MR antagonists in aldosterone target cells, and provide new insights into the requirements for MR activation to occur, as well as for the designing of new selective MR antagonists.
Introduction

Aldosterone, the major mineralocorticoid hormone, is involved in the control of water and sodium homeostasis and regulation of blood pressure. The effects of aldosterone are mediated by the mineralocorticoid receptor (MR), a transcription factor present in sodium-transporting epithelia, such as those of the distal renal tubules, distal colon, and sweat and salivary glands (Pearce et al., 2003). In the kidney, the fine regulation of Na⁺ reabsorption takes place in the distal nephron. In the principal cortical collecting duct (CCD) cells, Na⁺ is reabsorbed via the apical ENaC, which constitutes the rate-limiting step for Na⁺ entry, and is extruded from the cells by the basolaterally-located Na⁺,K⁺-ATPase (Rossier and Palmer, 1992).

The human MR is a member of the superfamily of nuclear receptors that are organized into three functional domains (Arriza et al., 1987). The N-terminal domain harbors a ligand-independent activation function (AF-1). This domain is important for interactions with transcriptional coactivators (Kitagawa et al., 2002; Pascual-Le Tallec et al., 2003) and with the ligand-binding domain (LBD) (Rogerson and Fuller, 2003). The centrally-located DNA-binding domain is involved in DNA binding and in receptor homo- and heterodimerization. The LBD is located in the C-terminal region, and contributes to nuclear localization. The LBD is involved in homo- and/or hetero-dimerization, and in the interaction with the heat-shock protein, hsp90. It is also characterized by a ligand-induced trans-activation function (AF-2) (Arriza et al., 1988; Mangelsdorf et al., 1995; Ribeiro et al., 1995). The crystal structures of the LBD of human MRs carrying the S810L mutation (Fagart et al., 2005) and/or the C808S mutation (Li et al., 2005; Bledsoe et al., 2005) and forming a complex with an agonist ligand have been solved. Consistent with sequence homologies between MR and the glucocorticoid receptor (GR), the progesterone receptor (PR) and the androgen receptor (AR), the structure of the MR closely resembles the agonist-bound structures of these oxosteroid
receptors (Bledsoe et al., 2002; Williams and Sigler, 1998; Matias et al., 2000). The structure is composed of 11α helices and four small β-strands that are folded to form a three layered structure surrounding the ligand cavity.

The aldosterone-dependent activation of gene transcription is thought to be a multistep process. In its ligand-free state, MR is found predominantly in the cell cytoplasm as a hetero-oligomeric complex in association with the heat shock protein hsp90 (Rafestin-Oblin et al., 1989). The MR-hsp90 interaction appears to be required to maintain the receptor in a non-functional state, and to fold the MR-LBD in a ligand-binding competent state (Couette et al., 1998). Aldosterone binding induces a change in receptor conformation that allows the dissociation of the associated proteins, transfer into the nucleus and the subsequent recruitment of transcriptional coactivators (Trapp et al., 1995; Couette et al., 1996; Fejes-Toth et al., 1998; Hellal-Levy et al., 2000; Hultman et al., 2005). The aldosterone-associated receptor binds as a dimer to hormone response elements in the promoter region of target genes, and initiates hormone-mediated transcription by specific interactions with the transcriptional machinery. Site-directed mutagenesis experiments based on three-dimensional homology models have shown that the contact between the Asn770 residue of the MR-LBD and the 21-hydroxyl function, present in all natural steroids with agonist properties, plays a key role in maintaining MR in its active conformation (Fagart et al., 1998).

Many natural and synthetic steroids bind to MR, and depending on the nature of their substituents, may exhibit MR-antagonist properties. This is the case, for instance, of progesterone, the antagonist activity of which has been linked to its inability to establish contact with the Asn770 residue of the MR-LBD (Fagart et al., 1998). Two progesterone derivatives, 17α- and 20α-hydroxyprogesterone (but not 11β-hydroxyprogesterone), act as potent MR antagonists, and inhibit the aldosterone-stimulated absorption of Na⁺ in cultured mouse mpkCCD\textsubscript{34} collecting duct principal cells (Rafestin-Oblin et al., 2002). Rossier et al.
(1980) first reported that testosterone (T) specifically displaces [3H]-aldosterone from its nuclear and cytoplasmic binding sites, and antagonizes the stimulating action of aldosterone on Na⁺ transport in the toad bladder. However, the ability of natural and synthetic androgens to activate or inactivate MR, and to stimulate or inhibit Na⁺ absorption has not been yet investigated in appropriate models of mammalian collecting duct cells.

In this study, the ability of the two main natural androgens, T and its active metabolite dihydrotestosterone (DHT), and the potent synthetic steroidal AR agonist methyltrienolone (R1881) to modulate Na⁺ absorption was analyzed using the short-circuit current (Isc) method in cultured mpkCCDcl4 collecting duct cells, which had retained the main features of intact CCDs, including a Na⁺ transport mechanism that was stimulated by aldosterone (Bens et al., 1999; Rafestin-Oblin et al., 2002). The ability of T, DHT, and R1881 to activate or inactivate MR was investigated in cis-trans cotransfection assays in HEK 293T and COS-7 human embryonic kidney cells. The antagonist properties of androgens are discussed in the light of docking experiments performed using the structure of the MR-LBD (Bledsoe et al., 2005).

**Materials and Methods**

Chemicals. Aldosterone (4-pregnen-11β,21-diol-18-al-3,20-dione; Aldo), testosterone (17β-hydroxy-4-androsten-3-one; T), 19-nortestosterone (17β-hydroxy-19-norandrost-4-en-3-one; 19-nor T), 17α-methyl testosterone (17β-hydroxy-17α-methyl-4-androsten-3-one; 17-met T) and dihydrotestosterone (5α-androstan-17β-ol-3-one; DHT) were from Sigma (St Louis, MO, USA). 18-oxo-18-vinyl progesterone (18-vinyl-4-pregnen-3,18,20-trione; 18OVP) was a gift from A. Marquet (Paris, France). Spironolactone [7α-(acetylthio)-17β-hydroxy-3-oxopregn-4-en-21-carboxylic acid γ-lactone; Spiro] was a gift from Pharmacia/Pfizer Laboratories (Chicago, IL). R1881 (17β-hydroxy-17α-methyl-19-norandrost-4,9,11-trien-3-one) was purchased from Perkin Elmer (Courtaboeuf, France). Mifepristone [11β-(4-
dimethylaminophenyl)-17β-hydroxy-17α-(1-propynyl)-19-norandrost-4,9-dien-3-one; RU486], RU26752 [7α-(propyl)-17α-hydroxy-3-oxopregn-4-en-21-carboxylic acid γ-lactone] and RU26988 [11β,17β-dihydroxy-17α-(1-propynyl)androst-1,4,6-trien-3-one] were kindly provided by Aventis Laboratories (Paris, France). Hydroxyflutamide [2-hydroxy-2-methyl-n-(4-nitro-3-(trifluoromethyl) phenyl)propanamide; HF] as from Merk, Theramex (Monaco). [1,2-3H]-aldosterone (40-60 Ci/mmol) was purchased from GE Heathcare (Orsay, France). The ligands used in this study are depicted in Fig. 1. All other hormones and reagents were from Sigma.

**Expression Vectors and Reporter Constructs.** The expression plasmids pchMR, pchMR<sub>A770</sub> and pchMR<sub>A945</sub> contain the entire coding sequence for the wild-type human MR and of mutant MRs in which Ala is substituted for Asn at position 770 (MR<sub>A770</sub>) or for Thr at position 945 (MR<sub>A945</sub>) (Fagart et al., 1998). The C942A mutation was created on the recombinant pchMR using the QuickChange site-directed mutagenesis kit (Stratagene, Lajolla, CA). The oligonucleotide sense primer, 5’-CTGCTGGAATTCTTGCTAC-3’, was used together with the corresponding antisense oligonucleotide. The desired mutation was identified by direct sequencing (MWG Biotech, Roissy CDG, France). After identifying the mutated clones, MR fragments were excised with BpU1102i/AflIII and subcloned into a new pchMR vector. The expression plasmid, pchGR, contains the entire coding sequence for the wild-type human GR (Hellal-Levy et al., 1999). The plasmid pFC31Luc, which contains the mouse mammary tumor virus (MMTV) promoter driving the luciferase gene, was kindly provided by H. Richard-Foy (LMBE, Toulouse, France). The plasmid pcβgal encodes β-galactosidase.
**Coupled Cell-free Transcription and Translation.** The plasmid pchMR (1 µg), containing cDNA encoding the wild-type MR, was transcribed for 90 min at 30°C using T7 RNA polymerase and translated into the rabbit reticulocyte lysate system purchased from Promega according to the Manufacturer’s instructions (Promega, Charbonnières, France).

**Steroid Binding Experiments.** After translation of the wild-type MR, the lysate was diluted (1:3) with ice-cold TEGWM buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20 mM sodium tungstate, 1 mM β-mercaptoethanol, 10% glycerol). The lysate was then incubated for 4 h at 4°C with 1 nM [3H]-aldosterone with or without various concentrations of unlabeled competitors (10^{-11} M - 10^{-5} M). Bound and unbound steroids were separated by the charcoal-dextran method (Fagart et al., 1998). The radioactivity was determined in a LKB liquid scintillation spectrometer after adding 5 ml of Optiphase Hisafe (Perkin Elmer Wallac, Turku, Finland). The apparent dissociation constant (K_{Dapp}) was calculated according to the equation $K_{Dapp} = K_{Daldo} \times [x] / [Aldo]$, where $K_{Daldo}$ is the dissociation constant at equilibrium of aldosterone measured by Scatchard plot analysis (Fagart et al., 1998), [x] and [Aldo] are the concentrations of a compound x and of aldosterone, respectively, required to inhibit 50% of the [3H]-aldosterone binding.

**Cultured Cells.** Experiments were performed using human embryonic renal HEK 293T cells or COS-7 cells (American Type Culture Collection, Manassas, VA), and the mouse renal mpkCCD_{14} collecting duct principal cells previously established in our laboratory (Bens et al., 1999). HEK 293T cells and COS-7 cells were cultured in T75 flasks with high glucose Dulbecco’s modified essential medium (DMEM) (Invitrogen NV Leek, The Netherlands) supplemented with 2 x non-essential amino acids, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES, 10% charcoal-stripped and heat-inactivated fetal
calf serum (FCS) in a 5% CO₂/95% air atmosphere. mpkCCD\textsubscript{cl4} cells were grown until confluent in a modified defined medium [DM: DMEM: Ham’s F12 (1:1 vol/vol), 60 nM sodium selenate, 5 µg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor (EGF), 5 µg/ml insulin, 2% FCS, 20 mM HEPES, pH 7.4] at 37°C in a 5% CO₂-95% air atmosphere. The medium was changed every two days, and all experiments were carried out between the 25\textsuperscript{th} and 36\textsuperscript{th} passages.

**Transfection Procedures.** HEK 293T cells were cultured and transfected in high-glucose containing DMEM supplemented with 10% charcoal-stripped and heat-inactivated FCS, 25 mM HEPES, 2× non-essential amino acids, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂-95% air atmosphere. Transfection was carried out using the phosphate calcium precipitation method. For one 75 cm\textsuperscript{2} flask, the phosphate solution contained 2 µg of one of the receptor expression vectors (pchMR, pchMR\textsubscript{A770}, pchMR\textsubscript{A942}, pchMR\textsubscript{A945} or pchGR), 7 µg of pFC31Luc construct, and 1 µg of pc\textbeta\textsubscript{gal}. Twelve hours after transfection, the cells were rinsed with phosphate buffered saline (PBS), trypsinized and replated in 12-well trays. The steroids to be tested were added to the cells 24 h after seeding and incubation of the steroids with the transfected cells was performed for 24 h at 37°C. Cell extracts were then assayed for luciferase (De Wet et al., 1987) and β-galactosidase (Herbomel et al., 1984). To standardize the transfection efficiency, the relative light units obtained in the luciferase assay were divided by the optical density obtained in the β-galactosidase assay. The transfection protocol used for COS-7 cells was similar to that for HEK 293T cells, but used 4 µg of receptor expression vector, 14 µg of pFC31Luc construct, and 2 µg pc\textbeta\textsubscript{gal}. 
**Short-circuit Current Studies.** mpkCCD cells were seeded on Snapwell filters (insert growth area, 1cm², 0.4 µm pore size, Corning Costar Corp., Cambridge, MA) and grown until confluent in the same modified DM as described above. After 5 days, confluent cells were placed in hormone-free, EGF-free DM, referred to as HFM medium, and supplemented with charcoal-treated steroid-free FCS for 24 h, and then in FCS-free HFM medium (containing 29 mM NaHCO₃) for a further 18 h (Bens et al., 1999; Rafestin-Oblin et al., 2002). Cells were mounted in a modified Ussing-type chamber (Diffusion Chamber System, Costar Corp. Cambridge, MA) connected to a voltage clamp apparatus via glass barrel Micro-Reference Ag/AgCl electrodes. Experiments were always performed using sets of steroid-treated and untreated cells from the same passages to avoid interpassage variations. Cell layers were bathed on both sides (8 ml) with HFM medium warmed to 37°C, continuously gassed with 95% O₂/5% CO₂ with the pH kept at 7.4. The various steroids and compounds to be tested were added to both the apical and basal sides of the filters. Iₛₐₜ (µA.cm⁻²) was measured by clamping the open-circuit transepithelial voltage (Pₒₒ) to 0 mV for 1s. By convention, a positive Iₛₐₜ value corresponded to a flow of positive charges from the apical to the basal compartment.

**Ligand Docking within the hMR Ligand-Binding Domain.** Aldosterone, DHT and R1881 were docked within the crystal structure of the MR-LBD associated with aldosterone (Bledsoe et al., 2005. PDB identification 2AA2) using the O package (Jones et al., 1991). Androgen ligands were manually positioned within the binding pocket in a similar orientation to that of aldosterone. The steroids positioning was refined using the VOIDOO generated probe-accessible and van der Waals volumes as guides (Kleywegt and Jones, 1994).
Statistical Analysis. Results are expressed as means ± S.E.M. from (n) separate experiments. Significant differences between groups were analyzed by Student’s t test. A p value < 0.05 was considered significant.

Results

Androgen Ligands Inhibit Aldosterone-Stimulated Na⁺ Absorption. The effects of androgen ligands on Na⁺ transport were investigated in mpkCCD_{cl4} cells using the short-circuit current (I_{sc}) method. Confluent cells grown on filters maintained the typical features of a tight epithelium as assessed by high electrical transepithelial resistance (R_{T}: 3033 ± 246 \Omega \cdot \text{cm}^2, n = 17) and negative transepithelial potential (P_{D}: -65.6 ± 4.7 mV, n = 17). I_{sc} recordings were performed on confluent mpkCCD_{cl4} cells grown on filters and incubated for 3 h without or with steroids added to both the apical and basal side of the filters. Amiloride (10⁻⁵ M), a potent inhibitor of ENaC, was then added to the apical side of the cells to determine the amiloride-sensitive (Ams) component of I_{sc}, which reflects the Na⁺ absorption mediated by ENaC (Bens et al., 1999). As previously reported, 5 x 10⁻⁷ M of aldosterone, a concentration shown to induce maximal Na⁺ absorption in mpkCCD_{cl4} cells (Bens et al., 1999), significantly increased the total and Ams component of I_{sc} by almost 2.4 fold (Fig. 2, A, and inset). T (5 x 10⁻⁷ M) did not have any effect on the total and Ams components of I_{sc} measured in untreated cells (Fig. 2, B, and inset). Similarly, DHT and R1881 (5 x 10⁻⁷ M) did not alter total or Ams I_{sc} (not shown). Compared to basal I_{sc} values measured in untreated cells, an excess of spironolactone (10⁻⁵ M), a classical antagonist of MR, inhibited the rise in total I_{sc} (72%) and Ams I_{sc} (65%) caused by aldosterone (Fig. 2C, and inset). An excess of hydroxyflutamide (10⁻⁵ M), a non steroidal AR antagonist (Singh et al., 2000) had no effect on the rise in total and Ams components of I_{sc} stimulated by aldosterone (Fig. 2 D, and inset).
In contrast, T and DHT partially inhibited the rise in total and Ams \(I_{sc}\) induced by aldosterone by 56% and 51%, respectively (Fig. 2, E and F, and insets), and R1881 almost completely inhibited both total and Ams component of \(I_{sc}\) stimulated by aldosterone (Fig. 2G, and inset). These results suggest that androgens act as MR antagonists in inhibiting the increase in Na\(^+\) transport caused by aldosterone in cultured mpkCCD\(_{cl4}\) collecting duct principal cells.

**Androgen Ligands do not Alter Na\(^+\) Absorption Stimulated by the Selective GR Agonist RU26988.** Aldosterone is known to act on both MR and GR to stimulate sodium absorption. We therefore recorded \(I_{sc}\) to find out whether the inhibitory action of androgen ligands on aldosterone-stimulated Na\(^+\) absorption is mediated via MR and/or GR. We used the synthetic steroid RU26988, a selective GR agonist (Moguilewsky and Raynaud, 1980; Gomez-Sanchez and Gomez-Sanchez, 1983; Rafestin-Oblin et al., 1986). RU26988 (5 x \(10^{-7}\) M) significantly increased the total \(I_{sc}\) by a factor of 2 in confluent mpkCCD\(_{cl4}\) cells grown on filters and RU486, a potent GR antagonist, almost completely inhibited this effect (Fig. 3A). As a result, RU486 significantly inhibited the Ams \(I_{sc}\) caused by RU26988 (Fig. 3A, inset). In contrast, \(10^{-5}\) M of DHT or of R1881 did not impair the rise in total or Ams \(I_{sc}\) elicited by RU26988 in mpkCCD\(_{cl4}\) cells (Figs. 3, B and C and insets). T (\(10^{-5}\) M) also had no inhibitory effect on the RU26988-stimulated \(I_{sc}\) (not shown). These findings indicate that androgen ligands do not inhibit the Na\(^+\) absorption stimulated by the GR agonist RU26988 in cultured mpkCCD\(_{cl4}\) collecting duct cells.

**Androgens do not Activate GR, and do not Inhibit the GR Activity Induced by the Specific GR Ligand RU26988.** To confirm that the inhibitory effect of T, DHT and R1881 on aldosterone-stimulated Na\(^+\) transport does not occur via GR, trans-activation experiments were performed on HEK 293T cells transiently transfected with GR. RU26988 produced a
dose-dependent stimulation (ED$_{50}$: $10^{-9}$ M) of the GR trans-activation activity, as did dexamethasone (Fig. 4A). High concentrations of T, DHT and R1881 did not trans-activate GR, although R1881 did induce a slight stimulation of the GR trans-activation at $10^{-5}$M (Fig. 4A). The GR antagonist activities of androgens were then tested by incubating the transfected HEK 293T cells with $10^{-8}$ M RU26988 alone (100%) or with increasing concentrations of T, DHT, R1881 or RU486 (Fig. 4B). RU486 induced a dose-dependent inhibition of the RU26988-induced trans-activation activity of the GR, whereas T, DHT, and R1881 had only weak inhibitory action on GR activity (Fig. 4B).

**Androgen Ligands Bind to MR.** To check that the effect of androgen ligands on sodium transport does occur as a result of their binding to MR, we investigated the ability of androgen ligands to bind to MR by competition experiments. MR was expressed in vitro using the rabbit reticulocyte lysate expression system, and incubated with $5 \times 10^{-9}$ M $[^3]$H-aldosterone in the absence or presence of unlabeled ligands. A 100-fold excess of unlabeled aldosterone inhibited the binding of $[^3]$H-aldosterone to MR by 90-95% (Fig. 5A). As expected, a 100-fold excess of the two MR antagonists spironolactone and RU26752 (Rafestin-Obblin et al., 1992) have a high efficiency to inhibit $[^3]$H-aldosterone to MR (Fig. 5A). A 100-fold excess of R1881 also displaced 90% of the $[^3]$H-aldosterone bound to the MR, while a 100-fold excess of T or DHT inhibited the binding of $[^3]$H-aldosterone to MR by 36% and 52%, respectively (Fig. 5A). As also shown in Fig. 5B, increasing concentrations of T, DHT, and R1881 ($10^{-10}$ M to $10^{-6}$M) induced dose-dependent displacements of the $[^3]$H-aldosterone bound to MR. The apparent dissociation constant of R1881 for MR ($K_{D_{app}} \sim 0.8 \times 10^{-9}$ M) is very similar to that of aldosterone ($K_{D_{app}} \sim 0.5 \times 10^{-9}$ M). The apparent dissociation constants of T ($K_{D_{app}} \sim 4 \times 10^{-8}$ M) and DHT ($K_{D_{app}} \sim 2.2 \times 10^{-8}$ M) for the MR indicated that their affinities for this receptor were lower than that of R1881. These studies indicate that androgen
ligands have the ability to bind to MR expressed in vitro, and indicate that R1881 displays a high affinity for MR.

**Androgens Inhibit the Aldosterone-Induced Activity of MR.** To further investigate the properties of the androgen ligands when bound to MR, cis-trans cotransfection assays were performed in HEK 293T cells with pchMR, and a reporter plasmid containing the luciferase gene under the control of the MMTV promoter. Incubating transfected-HEK 293T cells with various concentrations of aldosterone for 24 h led to a dose-dependent increase in MR trans-activation activity, with maximum induction at $10^{-10}$ M aldosterone and an ED$_{50}$ value of $5 \times 10^{-11}$ M (Fig. 6A), a value which is in accordance with previous studies (Arriza et al., 1987, 1988; Hellal-Levy et al., 1999). T and DHT had no effect on the trans-activation activity of the MR (Fig. 6A). At a concentration of $10^{-5}$ M, R1881 increased the trans-activation activity by only 20%. The antagonist activities of androgens were then tested by incubating the transfected HEK 293T cells with $10^{-9}$ M aldosterone alone (100%) or with increasing concentrations ($10^{-9}$ M to $10^{-5}$ M) of T, DHT, or R1881 or with one of the two MR antagonists, spironolactone or RU26752 (Fig. 6B). T, DHT and R1881 all induced dose-dependent inhibition of the aldosterone-induced MR activity (Fig. 6B, left panel). The antagonist potencies of T (IC$_{50}$ $\sim 5 \times 10^{-7}$ M) and DHT ($\sim 10^{-6}$ M) were lower than those of RU26752 (IC$_{50}$ $\sim 3 \times 10^{-8}$ M) and spironolactone (IC$_{50}$ $\sim 8 \times 10^{-8}$ M) (Fig. 6B, left panel). Interestingly, R1881 (IC$_{50}$ $\sim 7 \times 10^{-8}$ M) appeared to be as potent as spironolactone in inhibiting aldosterone-induced MR activity (Fig. 6B, left panel). Transient transfections were also performed using COS-7 cells with no receptor expression: R1881 (IC$_{50}$ $\sim 5 \times 10^{-8}$ M), T (IC$_{50}$ $\sim 4 \times 10^{-6}$ M) and DHT (IC$_{50}$ $\sim 6 \times 10^{-6}$ M) also induced dose-dependent inhibition of the aldosterone-induced MR activity (not shown).
R1881 is a 19-nor steroid, whereas T and DHT have a C19-methyl group. R1881 is characterized by having a 17α-methyl group and by three double bonds (C4-C5, C9-C10, C11-C12), T has a C4-C5 double bond, and DHT has no double bond. The question arises of whether the presence of the 17α-methyl group and/or the lack of the 19-methyl group could be responsible for the potent antagonist activity of R1881. To test this hypothesis, the ability of 17α-methyl testosterone (17-met T) and 19-nortestosterone (19-nor T) to inactivate MR was analyzed. The efficiency of these two molecules in inhibiting aldosterone-induced activity was equivalent to that of testosterone (~ 5 x 10^{-7} M) (Fig. 6B, right panel). This suggests that the efficiency with which R1881 inhibits aldosterone-induced MR activity is not due to the presence of the methyl group at the C17 α position or to the absence of methyl group at the C19 position.

Docking of Androgen Ligands within the Ligand-Binding Cavity of MR. Some of us have previously shown that the contact between the Asn770 residue of the MR-LBD, and the hydroxyl function at the 21 position, common to all MR agonist ligands, is essential to stabilize MR in its active state (Fagart et al., 1998). Conversely, the MR antagonist character of progesterone and spironolactone appears to be attributable to their inability to establish stabilizing contacts with the Asn770 residue (Fagart et al., 1998). To explain the antagonist property of androgen ligands when bound to MR, we examined the positioning of the androgen ligands within the ligand-binding cavity of the crystal structure of the MR-LBD associated with aldosterone (Bledsoe et al., 2005). In this structure, the C3 ketone of aldosterone is hydrogen bound to Gln776 (H3 helix) and Arg817 (H5 helix) (Fig. 7A). The C18 hydroxyl forms a hydrogen bond with the side chain carbonyl of Asn770 and is in position to interact with the Cys942 (Bledsoe et al., 2005). As shown in Fig. 7A, aldosterone is also anchored by two hydrogen bonds between Thr945 and C21 hydroxyl and C20 ketone.
groups. Docking of R1881 in the MR structure revealed that R1881 adopts the same orientation as aldosterone within the ligand-binding cavity (Fig. 7, compare A and B). R1881 is favorably positioned to establish hydrogen bonds with Gln776 and Arg817 (Fig. 7B). The C-17 hydroxyl of R1881 is at a distance of 3.6 Å from Asn770, and of 4.05 Å from Cys942 and Thr945 (Fig. 7B). These distances are too large for hydrogen bonds to be established, but are compatible with van der Waals interactions (Fig. 7, B and C). Moreover, the Δ4, Δ9 and Δ11 double bonds of R1881 are in the vicinity of the aromatic rings of Trp806 and Phe829, allowing interactions to occur (Fig. 7C). The two other androgen ligands, T and DHT, are positioned in a similar way to R1881 within the ligand-binding cavity (data not shown).

**Anchoring of Androgen Ligands within the Ligand Binding Cavity of MR.** To find out whether the Asn770, Thr945 and Cys942 residues are involved in the stability of the ligand-receptor complex we used three mutant MRs, MR\textsubscript{A770}, MR\textsubscript{A942} and MR\textsubscript{A945}, in which Ala was substituted for Asn770, Cys942 and Thr945, respectively. Aldosterone is unable to bind to MR following N770A mutation, and so we used the synthetic steroid 18OVP as the agonist ligand (Souque et al., 1995; Fagart et al., 1998). T, DHT and R1881 all inhibited the 18OVP-induced trans-activation activity of MR\textsubscript{WT} and MR\textsubscript{A770}, although their inhibitory efficiencies were lower for MR\textsubscript{A770} than for MR\textsubscript{WT} (Fig. 8, A and B). A 10-fold excess of T, DHT or R1881 inhibited the 18OVP-induced trans-activation activity of MR\textsubscript{A770} by only 20 to 50%, compared to 50% to 85% for MR\textsubscript{WT}. These results suggest that the contact between Asn770 and the C17β-hydroxyl of the androgen ligands contributes to stabilizing the androgen-MR complex, but are not strong enough to maintain MR in its activate state.

Aldosterone is still able to trans-activate MR following the C942A and T945A mutations. However the efficiency with which it trans-activates the two mutant receptors (MR\textsubscript{A942} : ED\textsubscript{50} \( \approx 5 \times 10^{-7} \) M and MR\textsubscript{A945} : ED\textsubscript{50} \( \approx 5 \times 10^{-8} \) M) is lower than that observed with MR\textsubscript{WT} (ED\textsubscript{50} \( \approx 5 \times 10^{-8} \) M).
5 x 10^{-11}$ M). This indicates that the hydrogen bonds between aldosterone and Cys942 and Thr945 residues stabilize the aldosterone-MR complex, but do not seem to be essential for aldosterone-induced MR activation. T, DHT and R1881 still act as MR antagonists following the C942A and T945A mutations (Fig. 8, C and D). However, T and DHT produced less inhibition of the aldosterone-induced activity of MR_{A942} and MR_{A945} than of that of MR_{WT} (compare Fig. 8, C and D and Fig. 6B). These findings suggest that the interaction between T or DHT and the Thr945 and Cys942 residues contribute to the stability of the complex. In contrast to T and DHT, R1881 displays the same efficiency in inhibiting the trans-activation activity of MR_{WT}, MR_{A942} and MR_{A945} (compare Fig. 8, C and D and Fig. 6B). In addition, the partial agonist activity of R1881 (10^{-5}$M) with MR_{WT} (Fig. 6B) was no longer observed with the mutant MRs in which the Cys942 or Thr945 residues had been replaced by an alanine (Fig. 8, C and D). These results indicate that the contacts between the C17β− hydroxyl of R1881 and the residues Thr945 and Cys942 contribute to its partial agonist activity.

**Discussion**

This study shows that the androgen ligands T, DHT and R1881 display antagonist properties when bound to the human MR, and inhibit Na^+ absorption stimulated by aldosterone in renal collecting duct cells. The MR antagonist properties of androgen ligands were demonstrated by testing their ability to inhibit the aldosterone-induced trans-activation activity of the wild-type MR on the MMTV-luciferase reporter gene in HEK 293T cells expressing low levels of endogenous MR, or in COS-7 cells, which have no steroid receptors. T, DHT and R1881 did not exert any agonist activity in either cell line, although R1881 displayed a slight capacity to activate MR at high concentrations. In contrast, the androgen ligands T, DHT and R1881 all inhibited the aldosterone-induced activity of the transiently expressed MR. Trans-activation experiments performed in HEK 293T cells transiently
expressing GR revealed that androgen ligands have no effect on GR activity. They also show that androgen ligands exert almost no inhibitory action on the GR activity induced by RU26988. Thus, our MR and GR trans-activity studies have provided evidence that androgen ligands act as MR antagonists.

The functional consequence of the MR-antagonist properties of androgen ligands was further investigated in renal collecting duct cells by means of short-circuit current measurements. We used immortalized mpkCCDC4 cells that have retained the main functions of the cortical collecting duct cells from which they have been derived, in particular that of possessing Na⁺ absorption which is stimulated by aldosterone (Bens et al., 1999). The fact that spironolactone produced 72% inhibition in the increase in Na⁺ absorption caused by 5 × 10⁻⁷ M aldosterone indicates that the increase in Na⁺ absorption caused by aldosterone is in part attributable to MR occupancy (Rafestin-Oblin et al., 2002). Consistent with their MR antagonist features, T, DHT and R1881 also prevented the rise in Na⁺ absorption elicited by aldosterone in mpkCCDC4 cells. This effect appears to be dependent on MR, since androgens failed to inhibit the increase in Na⁺ absorption caused by the specific GR agonist RU26988. Interestingly, the Isc recordings also show that spironolactone exerted only a weak inhibitory effect on RU26988- stimulated Na⁺ absorption, which contrasted with its potent inhibitory effect on the aldosterone-stimulated Na⁺ current (see Fig. 7). Thus, the results from Isc experiments indicate that aldosterone stimulates Na⁺ absorption via the occupancy of both MR and GR, but that androgen ligands have almost no impact on GR-mediated Na⁺ transport. In accordance with the data from transactivation studies, Isc recordings show that T, DHT and R1881 all inhibited both aldosterone-stimulated MR activity and aldosterone-stimulated Na⁺ absorption.

R1881 has an affinity for MR (0.75 nM) that is similar to that of aldosterone, and much higher than the affinities of T and DHT (40 and 22 nM, respectively). In accordance with
these findings, R1881 appears to exert a more potent antagonist activity than T and DHT. These differences could be explained if more contacts are involved in the interaction between the MR and R1881 than in those with T or DHT. It is unlikely that the presence of the C-17α-methyl group and the absence of the C19-methyl group that characterize R1881 are responsible for its high activity. Indeed, 17α-methyl testosterone and 19-nortestosterone (which lacks the C19-methyl group) are as effective as testosterone in inhibiting aldosterone-induced MR activity. However the interactions between the aromatic rings of Trp806 and Phe829 and the Δ4, Δ9 and Δ11 unsaturations of R1881 are sufficient to stabilize R1881 in the binding pocket of MR, and thereby to improve its binding affinity as compared to that of T or DHT, which lack Δ9 and Δ11 or Δ4, Δ19 and Δ11 insaturations, respectively.

Crystallographic and in-vitro studies of numerous nuclear receptors have suggested that, in the absence of ligand, the ligand-binding domain that surrounds the ligand-binding cavity is rather dynamic and exhibits some of the properties of a molten globule (Nagy et al., 2004). Binding a ligand compacts the LBD by establishing many polar and hydrophobic contacts. Some of these are involved only in the stability of the ligand-receptor complex, but some others are required to stabilize the ligand-binding domain in its active conformation. The active conformation can be stabilized in two ways. First, the ligand itself may make direct contact with residues in the helix 12, thereby promoting its active position. Second, the ligand may stabilize the lower part of the LBD, so that helix H12 tends to adopt its active position (Nagy et al., 2004). The crystal structure of the MR-LBD associated with aldosterone has revealed that the ligand D-ring substituents are in position to make hydrogen bonds with Asn770, a residue of helix 3, and with Cys942 and Thr945, two residues of the helix 11 (Bledsoe et al., 2005). The mutagenesis results presented in this study, together with previous analyses (Fagart et al., 1998), show that aldosterone retains its ability to activate mutant receptors carrying T945A and C942A mutations. However, the aldosterone efficiency
reflected by the ED$_{50}$ values is much more lower for the two mutant receptors than for the wild type MR. Taken together, these findings suggest that the hydrogen bonds between aldosterone and the Cys942 and Thr945 residues stabilize the aldosterone-MR complex. They probably contribute to maintaining the receptor in its active conformation after aldosterone binding, but seem not to be essential in the activation process. In sharp contrast, the accommodation of aldosterone within the ligand binding cavity, and the subsequent MR activation requires Asn770, a residue of helix 3 that simultaneously acts as a hydrogen bond acceptor from the ligand and a hydrogen bond donor to the backbone carbonyl of Glu955 residue located in the loop H11-H12 (Fagart et al., 1998; Hellal-Levy et al., 2000, Bledsoe et al., 2005).

The androgen ligands T, DHT and R1881, which all have a $17\beta$-hydroxyl, display either no MR agonist activity (T and DHT) or only very weak activity (R1881). The fact that the agonist activity displayed by MR$_{WT}$ and MR$_{A770}$ is no longer observed in MR$_{A942}$ and MR$_{A945}$ strongly suggests that the contacts between R1881 and Cys942 and Thr945 residues are required for MR agonist activity. Stabilization of MR in its active state independently of Asn770 has been already reported for the synthetic compound 18OVP (Fagart et al., 1998). In this case, MR activation is thought to be due to an interaction between Cys942 and the C18 enone oxygen that provides a stabilizing interaction between the ligand and helix 11 (Bledsoe et al., 2005).

The results from the docking experiments and trans-activation studies lead us to propose that the antagonist character of the androgen ligands T, DHT and R1881 is due to the inability of their $17\beta$-hydroxyl groups to form strong hydrogen bonds with the Asn770, Cys942 and Thr945 residues of the MR ligand-binding cavity. The MR antagonist character of T, DHT and R1881 contrasts with the ability of these ligands to activate the androgen receptor (AR) (Kemppainen et al., 1992). The crystal structure of the AR-LBD in association with R1881
revealed that the 17β-hydroxyl group of R1881 forms two strong hydrogen bonds with Asn705, corresponding to Asn770 in MR, and with Thr877, corresponding to Cys942 in MR, and also establishes a van der Waals contact with Leu880, corresponding to Thr945 in MR (Matias et al., 2000). Mutation of AR-Thr877, and to a greater extent mutation of AR-Asn705 interferes with ligand recognition, and reduces the transactivation efficiency (Poujol et al., 2000). Nevertheless the two corresponding mutant receptors, AR_{C877} and AR_{A705}, retain their capacity to be activated by R1881, suggesting that either one of the two contacts is sufficient and strong enough to maintain the receptor in its active state. The bulky side chain of the leucine residue at position 880 in AR reduces the volume of the cavity, as compared to that of the MR cavity, and along with the presence of a threonine at position 877 in AR, this allows the strong hydrogen bonds to form that stabilize R1881-AR in its active state. The inability of R1881 to form strong hydrogen bonds with the corresponding residues in the MR ligand-binding cavity could explain, at least in part, its antagonist properties.
REFERENCES


Footnote

A.-M. Takeda, G. M. Pinon, M.-E. Rafestin-Oblin and A. Vandewalle made equal contributions to the study. This work was supported by the Institut National de la Santé et de la Recherche Médicale. Armelle Takeda present address: Armelle Natsuo-Takeda, Departement of Pharmacology and Toxicology, Lausanne University, Bugnon, 27, CH-1005 Lausanne, Switzerland.
Legends for figures

**Fig. 1.** Structural formulae of the natural and synthetic steroids.

**Fig. 2.** Effects of androgens on Na\(^+\) absorption stimulated by aldosterone. A, \(I_{sc}\) was measured on sets of confluent mpkCCD\(_{cl4}\) cells grown on filters and incubated for 48 h in steroid-free and hormone-free medium as described in Materials and Methods. A-G, after the traces has stabilized (open symbols), cells were incubated for 3 h without (○) or with 5 x 10\(^{-7}\) M aldosterone (●), testosterone (◇), or with aldosterone 5 x 10\(^{-7}\) M plus 10\(^{-5}\) M spironolactone (Spiro, △), 10\(^{-5}\) M hydroxyflutamide (HF, □), spironolactone (Spiro, △), T, DHT or R1881 (▲). All steroids were added to both the apical and basal side of the cells for 3 h, and 10\(^{-5}\) M amiloride (Am, ■) was added for 10 min to the apical side of the cells. A-G, insets, the bars represent the amiloride-sensitive component of \(I_{sc}\) (Ams \(I_{sc}\)) measured in cells incubated for 3 h without (-, open bar) or with aldosterone alone (-, black bars) or T alone (+, open bar) and with aldosterone plus T, Spiro, HF, T, DHT or R1881 (+, hatched bars). Values are means ± S.E.M. from 5 to 6 individual recordings for each steroid tested. *, \(p < 0.05\) **, \(p < 0.01\) between groups.

**Fig. 3.** Effects of androgens on Na\(^+\) absorption stimulated by RU26988. A-C, \(I_{sc}\) was measured on sets of confluent mpkCCD\(_{cl4}\) cells grown on filters as described in the Legend of Fig. 2. After the traces has stabilized (open symbols), cells were incubated for 3 h with 5 x 10\(^{-7}\) M RU26988 alone (○) or with RU26988 plus 10\(^{-5}\) M RU486, DHT or R1881 (▲). All steroids were added to both the apical and basal side of the cells for 3 h and 10\(^{-5}\) M amiloride (Am, ■) was added for 10 min to the apical side of the cells. A-C, insets, the bars represent the amiloride-sensitive component of \(I_{sc}\) (Ams \(I_{sc}\)) measured in cells incubated for 3 h with RU26988 alone (-, open bars) or with RU26988 plus RU486, DHT or R1881 (+, black bars).
Values are means ± S.E.M. from 3 to 5 individual recordings for each steroid tested. $p < 0.01$ versus - RU488 values.

**Fig. 4.** Effects of androgens on the transactivation properties of wild-type GR. HEK 293T cells were transfected with the wild-type GR expression vector, the pFC31Luc reporter plasmid and the pcβgal vector containing the gene coding for the β-galactosidase enzyme. A, the steroid agonist activity was tested by incubating cells with increasing concentrations ($10^{-10}$ M to $10^{-5}$ M) of dexamethasone (Dex), RU26988, T, DHT or R1881 for 24 h. The GR trans-activation activity was determined by luciferase activity, normalized versus the internal β-galactosidase control, and expressed as a percentage of the maximum aldosterone-induced MR activity (not shown on the graph). Values are means ± S.E.M. from three separate experiments. B, the steroid antagonist activity was tested by incubating cells with $10^{-8}$ M RU26988 alone, and with RU26988 plus increasing concentrations ($10^{-8}$ M to $10^{-5}$ M) of T, DHT, R1881 or RU486 for 24 h. The GR trans-activation activity was determined as described above, and expressed as the percentage of the GR activity measured in the presence of RU26988 alone (100%). Values are means ± S.E.M. from three separate experiments.

**Fig. 5.** Steroid binding to the wild-type MR synthesized *in vitro* in rabbit reticulocyte lysate. A, the lysate was diluted 1:3 in TEGWM buffer and incubated with $5 \times 10^{-9}$ M [3H]-aldosterone alone or with a 100-fold excess of unlabeled aldosterone (Aldo), spironolactone (Spiro), RU26752, T, DHT or R1881. The hatched bar represents the binding of aldosterone to the non-relevant protein β-galactosidase. B, the diluted lysate was incubated with $5 \times 10^{-9}$ M [3H]-aldosterone alone, or with increasing concentrations ($10^{-10}$ M to $10^{-6}$ M) of unlabeled Aldo, T, DHT or R1881. In all cases, bound and unbound steroids were separated.
by the charcoal-dextran method. The results are expressed as the percentage of [3H]-
aldosterone binding measured in the absence of any competitors (100%). Values are means ±
S.E.M. from three to six separate experiments.

**Fig. 6.** Effects of androgens on the trans-activation properties of wild-type MR. HEK 293T
cells were transfected with the wild-type MR expression vector, the pFC31Luc reporter
plasmid and the pcβgal vector. A, the steroid agonist activity was tested by incubating the
transfected cells with increasing concentrations (10^{-11} M to 10^{-5} M) of aldosterone (Aldo), T,
DHT or R1881 for 24 h. The MR trans-activation activity was determined by luciferase
activity, normalized in terms of internal β-galactosidase control and expressed as the
percentage of the maximum aldosterone-induced MR activity. Values are means ± S.E.M.
from three separate experiments. B, the steroid antagonist activity was tested by incubating
the transfected cells with 10^{-9} M aldosterone alone (100%) or with aldosterone plus various
concentrations (10^{-9} M to 10^{-5} M) of T, DHT, R1881, RU26752, spironolactone (Spiro),
19-nor testosterone (19-nor T) or 17α-methyl testosterone (19-met T) for 24 h. The MR
activity was determined as described above, and expressed as the percentage of the MR
activity found in the presence of aldosterone alone (100%). Values are means ± S.E.M. from
three separate experiments.

**Fig. 7.** Anchoring of aldosterone and R1881 within the ligand-binding cavity of MR.
A, crystallographic structure of the ligand-binding domain of MR complexed with aldosterone
according to Bledsoe et al., 2005. B–C, positioning of R1881 within the structure of the
ligand-binding domain of MR. The networks of hydrogen and van der Waals contacts
involved in the anchoring of the ligands are depicted as green and grey dots, respectively.
**Fig. 8.** Effects of androgens on the *trans*-activation properties of the mutant MRs. HEK 293T cells were transfected with an expression vector of MR<sub>WT</sub> (A) or MR<sub>A770</sub> (B), MR<sub>A942</sub> (C) and MR<sub>A945</sub> (D) together with the pFC31Luc reporter plasmid and the pcβgal. Before harvesting, cells were incubated with 10<sup>-7</sup> M 18OVP (A, B) or aldosterone (C, D) in the absence or presence of 10<sup>-6</sup> M (x10) or 10<sup>-5</sup> M (x100) T, DHT or R1881 for 24 h. The *trans*-activation activity of the wild type and mutant MRs was determined by luciferase activity, normalized versus the internal β-galactosidase control, and expressed as percentage of the maximum aldosterone or 18OVP-induced MR activity. Values are means ± S.E.M. from three separate experiments.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 8