Structural Characterization of the Human Androgen Receptor Ligand-binding Domain Complexed with EM5744, a Rationally Designed Steroidal Ligand Bearing a Bulky Chain Directed toward Helix 12*

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Antiandrogens are commonly used to treat androgen-dependent disorders. The currently used drugs unfortunately possess very weak affinity for the human AR (hAR), thus indicating the need to develop new high-affinity steroidal antiandrogens. Our compounds are specially designed to impede repositioning of the mobile carboxyl-terminal helix 12, which blocks the ligand-dependent transactivation function (AF-2) located in the AR ligand-binding domain (ARLBD). Using crystal structures of the hARLBD, we first found that H12 could be directly reached from the ligand-binding pocket (LBP) by a chain positioned on the C18 atom of an androgen steroid nucleus. A set of 5α-dihydrotestosterone-derived molecules bearing various C18 chains were thus synthesized and tested for their capacity to bind hAR and act as antagonists. Although most of those having very high affinity for hAR were agonists, several very potent antagonists were obtained, confirming the structural importance of the C18 chain. To understand the role of the C18 chain in their agonistic/antagonistic properties, the structure of the hARLBD complexed with one of these antagonists, EM5744, was determined at a 1.65-Å resolution. We have identified new interactions involving Gln738, Met742, and His874 that explain both the high affinity of this compound and the inability of its bulky chain to prevent the repositioning of H12. This structural information will be helpful to refine the structure of the chains placed on the C18 atom to obtain efficient H12-directed steroidal antiandrogens.

The human androgen receptor (hAR)5 is a member of the nuclear receptor (NR) superfamily of ligand-activated transcription factors (1). NRs possess a typical modular structure consisting of three main functional domains: a variable NH2-terminal domain, a highly conserved DNA-binding domain, and a conserved ligand-binding domain (LBD) (2). Upon binding of agonist molecules to their ligand-binding pocket (LBP), these receptors undergo an important conformational change that notably affects the position of the carboxyl-terminal α-helix (helix 12, H12) located in the LBD. When bound by an agonist, NRs become active transcriptional factors able to interact directly with DNA at specific response elements (REs) found in the regulatory regions of target genes. These DNA-NR complexes can then recruit coactivators through their ligand-dependent transactivation function (AF-2) formed upon H12 repositioning (3), and hence control transcription of specific genes. It has been shown that AF-2 specifically recognizes and binds the LXXLL motifs usually located in an amphipathic helix found in the coactivator sequences (4–6). The human AR is thus able to bind the LXXLL motifs but its AF-2 preferentially interacts with the FXXLF motifs found in certain hAR coregulatory protein sequences (7, 8). Such an FXXLF motif is also present in the NH2-terminal domain (residues 23–27) of hAR (9) allowing this domain to interact with AF-2. This NH2-terminal domain/LBD interdomain interaction, only observed for hAR, is androgen-dependent (10) and has been shown to be important in regulating a number of androgen-dependent genes.

Through hAR, which mediates their action, the potent androgens testosterone (TESTO) and 5α-dihydrotestosterone (DHT) regulate a wide range of physiological responses, most notably male sexual differentiation and maturation including the development, growth, and maintenance of the normal prostate (11–14). Similarly, androgens and the hAR also play roles in
hARLBD Complexed with an H12-directed Steroidal Ligand

the onset or progression of many androgen-dependent diseases
and disorders, such as polycystic ovarian syndrome (15),
hyperandrogenic syndromes (16), benign prostatic hyperplasia
(17), and prostate cancer (18). Treatment of these disorders
thus require eliminating androgen-induced effects, either by
reducing the concentration of androgens available or by block-
ing access to the hAR with antagonist compounds (antiar-
drogens), able to competitively inhibit the binding of androgens
to the hAR (19, 20). Antiandrogens, despite their efficiency, have
been associated with substantial toxicity (reviewed in Ref. 21).
This could be explained by the fact that all the currently avail-
able pure antiandrogens, flutamide (Efulex), bicalutamide
(Casodex), and nilutamide (Anandron), exhibit very low affinity
for the hAR (10–100-fold lower than that of DHT) and must
consequently be administered in high doses to be efficient. It is
thus of primary importance to develop new higher affinity anti-
androgens to diminish the amount of drug needed to block the
hAR activity while, at the same time, greatly reducing or even
eliminating the side effects of this type of treatment.

With the aim of designing such new antiandrogens, we
decided to make use of earlier structural findings on the human
estrogen receptor (hER), a receptor structurally related to the
hAR. The hER is unable to interact with coactivator partners
when a ligand bearing a well oriented bulky chain is bound to
its ligand-binding domain (hERLBD) in complex with a natural estrogen (estra-
diol, E2) and a potent antiestrogen (raloxifene), agonist and
antagonist molecules bind at the same site within the LBD.
However, they exhibit different binding modes, inducing a dis-
tinct conformation in the transactivation domain (AF-2) char-
acterized by a different positioning of H12. More precisely, the
size and structure of raloxifene prevent the molecule from
being completely confined within the steroid-binding cavity.
Consequently, its bulky side chain protrudes from the cavity
and impedes H12 from adopting the position found in the
E2-hERLBD complex structure, a conformation essential for
interaction with transcriptional coactivators. Now concerning
hAR, the crystal structures of its LBD (hARLBD) in complex
with the natural androgens DHT and TESTO (23, 24) have
shown that H12 occupies therein the same position as that
observed in the E2-hERLBD structure. Such data suggest that
this helix is essential for the function of the AF-2 of hAR and,
like in the hER, participates in the interaction with coactivators.
This has been confirmed by the structure of the liganded
hARLBD in complex with a peptide derived from physiological
coactivators (25–27).

Using all the available structural information on the hAR,
we then proceeded to molecular modeling studies to find the best
position on an androgen nucleus (here DHT) for introducing a
bulky chain able to reach the site normally occupied by H12.
Finally, the combined data from molecular modeling and struc-
ture/activity relationship studies served as a basis for the design
and improvement of the chain structure, with the aim of max-
imizing the affinity of these steroidal-based compounds for
hAR. This rational approach yielded several different DHT-
based ligands able to bind hAR with high affinity (many folds
over that of DHT). In our in vitro tests, the majority of the
synthesized compounds failed to inhibit the growth of DHT-
stimulated cells or appeared to be potent agonists. However,
a small subgroup proved to be very efficient antagonists of DHT
stimulation, thus indicating that the particular structure of the
bulky chain is of paramount importance for its activity. To
understand the molecular basis of the agonistic and antago-
nisic properties of these different molecules, we attempted to
crystallize a few of these compounds (agonists and antagonists)
in complex with the human androgen receptor ligand-binding
domain. Here we report the crystal structure of one of these
agonist compounds bound to the hARLBD, EM5744 (Fig. 1), a
DHT-based molecule with a strong affinity for the hAR despite
its size, more than 150% that of DHT, and its bulky chain
directed toward H12.

EXPERIMENTAL PROCEDURES

Materials—Chemical products were purchased from Sigma.
Expression vectors and protein chromatography products were obtained from Amersham Biosciences. Protein
concentration was determined with the Bio-Rad Protein
Assay (Bio-Rad Laboratories).

Synthesis of EM5744—To a mixture of hemiketal (35) (180
mg, 0.55 mmol), 2,6-di-tert-butyl-4-methylpyridine (340 mg,
1.65 mmol) and silver trifluoromethanesulfonate (213 mg, 0.83
mmol) in dry dichloromethane (15 ml) was added 3,5-difluoro-
benzyl bromide (180 μl, 1.38 mmol) at room temperature.
The reaction mixture was stirred overnight at room temperature,
and then concentrated under reduced pressure. Purification
of the crude product by flash chromatography with acetone:hex-
anes (1:1 to 1:9) gave 153 mg (0.313 mmol, 57% yield) of pure
benzyl ether, as a white solid; IR (film): 3432 (OH), 1709 (C
O) cm−1, 1H NMR (acetone-d6) δ: 0.75–0.82 (m, 1H), 0.88 (s, 3H, C-19-
CH3), 0.95–2.10 (m, 22H), 2.44 (dd, 1H, J = 18.5 Hz, and J = 9.2
Hz), 3.35–3.41 and 3.51–3.56 (2m, 2H, -CH2OCOC6H4), 3.87 (s,
4H, C-3-dioxolane), 4.46 (s, 2H, -CH2OCOC6H4), 6.90 (td, 1H,
J = 9.2 Hz and J = 2.2 Hz) and 6.98 (m, 2H). To a solution of
benzyl ether (150 mg, 0.313 mmol) in methanol (15 ml) at room
temperature was added sodium borohydride (37 mg, 0.964
mmol). After 2 h of stirring, the reaction mixture was quenched
by water (15 ml), and then extracted with ethyl acetate. The
organic phase was dried over magnesium sulfate, filtered, and
concentrated. Purification of this crude product by flash
chromatography with acetone:hexanes (1:1 to 1:6) provided
EM5744 as a white solid in 121 mg (0.27 mmol, 49% yield
in 3 steps); IR (film): 3432 (OH), 1709 (C = O) cm−1; 1H NMR
(acetone-d6) δ: 0.75–0.82 (m, 1H), 0.89–1.06 (2m, 3H), 1.09 (s, 3H,
C-19-CH3), 1.24–1.43 (m, 6H), 1.47–1.65 (m, 6H), 1.70–1.75
(m, 3H), 1.91–2.17 (m, 3H), 2.33 (t, 1H, J = 14.2 Hz), 2.44 (td,
1H, J = 14.2 Hz and J = 6.6 Hz), 3.67–3.69 (2m, 2H, -CH2OCH2Ph),
3.87 (s, 4H, C-3-dioxolane), 4.46 (s, 2H, -CH2OCH2Ph), 6.90 (td, 1H,
J = 9.2 Hz and J = 2.2 Hz) and 6.98 (m, 2H). To a solution of
benzyl ether (150 mg, 0.313 mmol) in methanol (15 ml) at room
temperature was added sodium borohydride (37 mg, 0.964
mmol). After 2 h of stirring, the reaction mixture was quenched
by water (15 ml), and then extracted with ethyl acetate. The
organic phase was dried over magnesium sulfate, filtered, and
concentrated under reduced pressure to give the C17 alcohol,
as a crude product. The alcohol in acetone (15 ml) was treated
with a 10% HCl (0.5 ml) and stirred for 1 h. The mixture was
poured into dichloromethane and a 10% aq NaOH, extracted
with CH2Cl2 (4 × 30 ml), dried, and concentrated. Purification
by flash chromatography with acetone:hexanes (1:1 to 1.6)
provided EM5744 as a white solid in 121 mg (0.27 mmol, 49% yield
in 3 steps); IR (film): 3432 (OH), 1709 (C = O) cm−1; 1H NMR
(acetone-d6) δ: 0.75–0.82 (m, 1H), 0.89–1.06 (2m, 3H), 1.09 (s, 3H,
C-19-CH3), 1.24–1.43 (m, 6H), 1.47–1.65 (m, 6H), 1.70–1.75
(m, 3H), 1.91–2.17 (m, 3H), 2.33 (t, 1H, J = 14.2 Hz), 2.44 (td,
1H, J = 14.2 Hz and J = 6.6 Hz), 3.67–3.69 (2m, 2H, 17α-H and
1H of -CH2OCH2Ph), 3.92 (d, 1H, J = 5 Hz, 17β-OH), 3.94–
4.00 (m, 1H, -CH2OCH2Ph), 4.57 (m, 2H, -CH2OCH2Ph), 6.91
(td, 1H, J = 9.3 Hz and J = 2.3 Hz), 7.00–7.04 (m, 2H); MS; m/z,
calculated for C27H36F2O3: 446.26; (M − H) found: 445.3; (M +
H) found: 447.1.
Androgen Receptor Binding Assay in Cell Homogenates—The relative binding affinity (RBA) of each compound (testosterone, EM5744, and R1881) for the hAR was determined as described elsewhere (36) using the hydroxylapatite assay (37). Measurements were made in homogenates of human embryonic kidney (HEK-293) cells stably transfected with human androgen receptor (38). RBA of each compound was calculated as the ratio of concentrations of \(^{3}H\)R1881 and compound required to reduce the specific radioligand binding by 50% (IC\(_{50}\) values). Nonspecific binding of \(^{3}H\)R1881 was assessed by adding a 100-fold molar excess of unlabeled R1881. The RBA value for R1881 was arbitrarily set at 100.

In Vitro Assay of Androgenic/Antiandrogenic Activity in Shionogi Cells—Shionogi mouse mammary carcinoma cells (clone 107) were routinely grown as previously described (29, 30). Cells between passages 37 and 50 were plated in 24-well plates at a density of 18,000 cells/well and allowed to adhere to the surface of the plates for 24 h. Cell proliferation was measured as follows. The medium was replaced with fresh medium containing 2% (v/v) charcoal-stripped fetal bovine serum and the indicated concentrations of compounds to be studied diluted from stock solutions in 99% redistilled ethanol in the presence of DHT (0.3 nM), to evaluate the antiandrogenic activity of these compounds, or in the absence of DHT, to measure their androgenic activity. Control cells received only the ethanolic vehicle. The indicated increasing concentrations of agents were added to triplicate dishes, and cells were grown for 10 days with changes of medium every 2–3 days. The cell number was determined by measurement of DNA content.

Protein Purification—The hAR LBD was expressed and purified as described in Ref. 32. The hAR LBD cDNA (residues 654–919) was cloned in the pGEX 5X-2 vector and expressed as a glutathione S-transferase fusion protein in Escherichia coli strain BL21(DE3) pLysS cells. Expression was carried out at room temperature for 15–18 h in LB broth supplemented with the ligand EM5744 (50 \(\mu\)M) after induction with 100 \(\mu\)M isopropyl-\(\beta\)-D-thiogalactoside. Harvested cells were lysed with several freeze/thaw cycles and sonication in a buffer containing 50 mm Tris (pH 7.3), 150 mm NaCl, 5 mm EDTA, 10% glycerol, 0.5% CHAPS, 10 mm dithiothreitol, 200 \(\mu\)g/ml lysozyme, 1 mm phenylmethysulfonyl fluoride, and 50 \(\mu\)M EM5744. The soluble proteins were loaded onto a glutathione-Sepharose column, washed, and eluted with 15 mm reduced glutathione. The glutathione S-transferase affinity ligand was cleaved with FXa and the protein mixture was loaded onto a DE52 anion exchange column. The eluted hARLBD without glutathione S-transferase was concentrated and further purified on a Superdex 75 size exclusion column using 20 mm HEPES (pH 7.5), 150 mm LiSO\(_4\), 10% glycerol, 0.1% n-octyl-\(\beta\)-glucoside, and 1 mm dithiothreitol. The purified protein was concentrated up to 4 mg/ml. Approximately 0.5 mg of protein were obtained per liter of cell culture.

Crystallization and Data Collection—Protein crystallization was achieved using the hanging drop vapor diffusion method at room temperature. X-ray quality diffraction crystals of hARLBD-EM5744 complex were obtained using the microseeding technique. The seeding solution was prepared by using a few crystals of hARLBD-DHT complex obtained in 0.1 \(\mu\)M PIPES buffer (pH 7.0) and 1.5 \(\mu\)M MgSO\(_4\) (23) added to 50 \(\mu\)l of this crystallization solution and pulverized with the Seed Bead kit (Hampton Research). hARLBD, freshly purified in the presence of EM5744 and concentrated to 3.9 mg/ml, was used to form crystallization drops in a 0.6:0.3:0.3 (v/v/v) ratio of protein, seeding, and the faces of the steroid labeled \(\alpha\) and \(\beta\). For THG, the extra C17-\(\alpha\)-ethyl and C18-methyl groups are identified. For EM5744, the model shows the C18-[2-(3,5-difluoro-benzoyloxy)-methyl] side chain.

**FIGURE 1. Molecular structures of the hAR ligands used in this study.** DHT, THG, and EM5744 are colored using the standard CPK color set (carbon atoms are depicted in light gray, oxygen atoms in red, and fluorine atoms in light green). For DHT, carbon and oxygen atoms are numbered according to the standard steroid nomenclature, the rings designated by letters and the faces of the steroid labeled \(\alpha\) and \(\beta\). For THG, the extra C17-\(\alpha\)-ethyl and C18-methyl groups are identified. For EM5744, the model shows the C18-[2-(3,5-difluoro-benzoyloxy)-methyl] side chain.
cEDURE (42). After each refinement cycle, the model was manually corrected using O (43) with the $2F_o - F_c$ and $F_o - F_c$ calculated maps. Ligand and water molecules were progressively added to the model. The quality of all models was verified with PROCHECK (44) and the final refinement results and statistics are shown in Table 1. The volume of the cavity occupied by the ligands was calculated with Swiss-Pdb Viewer (45) and all figures were generated with Molscript (46), PYMOL (DeLano Scientific), or Pov-Ray (47). Protein Data Bank accession code for EM5744-hARLBD is 2PNU.

**RESULTS AND DISCUSSION**

**Rational Approach to the Design of H12-directed Antagonist Molecules for hAR**—Before proceeding to the synthesis of antagonist molecules able to efficiently prevent the normal positioning of the hAR COOH-terminal end, we closely examined all the structures we had previously determined for hARLBD (23) to define structural characteristics of this receptor that could be useful for our rational design approach. In the hARLBD-DHT complex structure, the LBP is made of a large nonspecific apolar and closed cavity mainly formed by the side chain of several hydrophobic amino acid residues interacting with the steroid nucleus through van der Waals contacts. The binding site also comprises, at both extremities of the ligand, a few polar residues that firmly tether the steroid molecule via hydrogen-bond networks. Residues delineating the LBP belong to six α-helices (H3, H5/H6, H8, H11, and H12) and to a β-strand located between H5/H6 and H7 (Fig. 2). The last α-helix (H12) acts as a lid closing the supposed entryway by which the steroidal ligand reaches its binding site, ensuring the complete partitioning of the LBP from the external environment. The fact that a residue of H12 (Met^{895}) is located in close proximity of the bound steroid is very interesting because it means that this α-helix may be reached directly from the LBP. This situation is quite similar to that found in the hEρα structure, where Leu^{240}, a residue of H12, delimits the steroid-binding cavity on the β-face of ring C of the estradiol molecule (22). The structure of the hEρα complexed with its selective antagonist raloxifene (22) has revealed that occupation of the position of the side chain of Leu^{240} by a bulky substituent was sufficient to displace H12, the right positioning of which is a prerequisite for transcriptional activation of all NRs and for their interaction with coactivators. Consequently, we have considered the feasibility of designing an hAR ligand bearing a bulky chain, the purpose of which would be to prevent the correct positioning of the COOH-terminal extremity of its LBD, including the orientation H12.

In the hAR-DHT complex crystal structure (23), the side chain of Met^{895} partly fills a cone-shaped cavity, delimited by atoms of seven residues (Asn^{705}, Gly^{708}, Glu^{708}, Leu^{712}, Trp^{741}, Met^{749}, and Phe^{891}). The fairly narrow tip of this cavity occupied by the Cα atom of Met^{895} opens into the LBP near the C12 and C18 atoms of DHT. Although the passage is quite narrow at this level, our molecular modeling assays had shown it could likely accommodate a chain positioned at C18 of an androgen steroid nucleus, which could then reach the cavity occupied by Met^{895} and prevent correct H12 positioning. The passage through this small opening seemed nonetheless to constitute the major difficulty until we found that the dimension and orientation of this opening were influenced by the structure of the ligand bound. Indeed, study of the hAR complex with THG (23), another highly potent steroid-based androgen (28) (Fig. 1), has revealed that the extra C18-methyl group borne by this steroid compels residue Trp^{741} to modify the position of its indole ring, which contributes to changing the shape of the LBP in this region and, more importantly, to significantly expanding the dimensions of the passage toward H12 (see below). Interestingly, we also noted that a single methyl group at this position (C18) on the steroid nucleus is sufficient to produce an impact on the position of Met^{895}, its Cα being repelled 1.2 Å farther from the ligand nucleus than in the hAR-DHT complex structure (23). The main consequence of this shift is a slight deformation of the NH₂-terminal extremity of H12, which remains, however, and unfortunately for our purposes, well positioned over the LBP, in a position similar to that observed in the complexes with TESTO or DHT.

These observations showing that residues forming the steroid-binding pocket of the androgen receptor are very flexible and allow the passage of a chain able to reach and to disturb the positioning of H12 have convinced us to exploit these structural characteristics for the design of AR antagonists. We have thus designed and synthesized a set of androgen nucleus derivatives bearing substituents of different sizes and lengths at their C18 position. All these molecules were first tested for their capacity to bind the androgen receptor and to inhibit the DHT-stimulated growth of mouse Shionogi mammary carcinoma cells (29, 30). We found that, despite their C18 chain, a large set of them bind the receptor with high affinity, often better than that of DHT, the most potent androgen. Moreover, hAR ligands with a good affinity generally constitute potent agonists. On the
other hand, we have also obtained a number of very potent antagonists with a high affinity for the receptor, thus indicating that the structure of the chain at position C18 is of paramount importance. To understand the molecular basis of the agonistic and antagonistic properties of these different molecules, and to verify that a chain positioned at C18 is really able to pass through the channel and reach H12, we undertook the crystallization of a few of these molecules having the best affinity for the hAR (agonists and antagonists) in complex with the human androgen receptor ligand-binding domain. Among all androgen nucleus derivatives with C18 substituents we have tested, only EM5744 gave us x-ray quality diffraction crystals. Even if this compound acts as an agonist of hAR rather than an antagonist, we analyzed its crystals mainly because it exhibits a very high affinity (see below) despite its long C18-chain substituent and because its structure is very similar to that of others presenting mixed or antagonistic activity. But above all, we were eager to understand why this ligand, the size of which is almost 50% larger than the natural androgens, was able to bind with a so high affinity the hAR without blocking the activity of this receptor in vitro.

**Binding Affinity and Agonist Activity Toward hAR of EM5744**—EM5744 is a DHT-based ligand possessing a strong affinity for the hAR despite its long chain substituent added to the carbon atom at position 18 (see Fig. 1 for its structure). Its in vitro RBA for the wild-type hAR is particularly high (525%) compared with the values obtained for two potent hAR agonists: R1881 (100%) and TESTO (6%) (Fig. 3A; see “Experimental Procedures” for description of the assay). The RBA of EM5744 is even higher than that of the most potent human androgen, DHT (180%), determined under similar conditions (data not shown). Unfortunately, it shows no antagonist activity, that is, it fails to inhibit the DHT-stimulated growth of Shionogi cells when added to the culture medium at a concentration of 10^{-6} M (data not shown). Moreover, it already possesses a significant agonistic activity at 10^{-11} M (Fig. 3B) and can thus be considered to be an agonist of hAR.

**Crystallization and Structure Determination of the hARLBD-EM5744 Complex**—To obtain the EM5744-receptor complex, the hARLBD (residues 654 to 919) was expressed in E. coli cells with a hARLBD::EM5744 fusion protein. The expressed fusion protein was solubilized with digitonin and bound to a column of hAR-expressing affinity chromatography resin. The resin was washed with a buffer containing digitonin and then eluted with a buffer containing 2 M urea. The eluted protein was then dialyzed against a buffer containing 20 mM Tris, pH 8.0, 50 mM NaCl, and 5% glycerol. The dialyzed protein was then concentrated and used for crystallization.

**Figure 3.** Relative binding affinity of EM5744 for the human AR in a cell homogenate and agonistic activity measured in an androgen-sensitive cell line. A, comparison of the capacity of EM5744, TESTO, and methyltrienolone (R1881) to displace [3H]R1881 from the human androgen receptor. The incubation was performed in homogenates of 293 cells transfected with hARLBD with 3 nM [3H]R1881 for 16 h at 0 – 4 °C in the presence or absence of the indicated concentrations of unlabeled compounds. EM5744 and TESTO have relative potencies of 525 and 6% compared with the 100% value set for R1881. Data are expressed as the mean ± S.E. B, effect of increasing concentrations of DHT and EM5744 on the proliferation of the androgen-sensitive Shionogi cells. The cell number was determined by measurement of DNA content. Data are expressed as the mean ± S.E. of triplicate dishes.
and purified in the presence of 50 μM EM5744. The purified protein was finally crystallized in the presence of a 2–4-fold excess of EM5744 (see “Experimental Procedures” for crystallization conditions). The crystals obtained belonged to the P2₁,2₁,2₁ space group and contained one molecule per asymmetric unit. The complex crystal structure was refined to crystallographic R-factor of 18.35% (Rfree = 20.51%) at 1.65-Å resolution (see crystallographic statistics in Table 1).

The electronic density map was very well defined and a near complete model, from residues 670 to 919, was finally constructed for the hARLBD-EM5744 complex (Fig. 4). Only the N-terminal end and part of the loop between helices 10 and 11 (residues 845–849) seemed to be disordered because no electron density was observed. Consequently, no model was built for these residues. In addition, the side chains of residues Arg⁷⁷⁶, Arg⁷⁸³, His⁸⁸⁵, and Met⁸⁸⁶ were built into poorly defined electron densities. The final model also contains 201 molecules of water and one molecule of each of these compounds: SO₄, dithiothreitol, and MES. From the very start of the refinement process, a clear and well defined 2Fo – Fc electron density corresponding to the steroidal base of the EM5744 was visible in the LBP with an extension of density outside the cavity. Subsequently, the structure of EM5744 was unambiguously fitted in the Fourier difference density map and then refined with the rest of the model (Fig. 5A).

Structure of EM5744 Bound to the Ligand-binding Domain of hAR—Comparison of our final model with the hARLB-DHT complex structure (RCSB PDB code 2AMA (23)) revealed that the steroid nucleus of both molecules occupies approximately the same position in the LBP and is stabilized at both extremities by hydrogen bonds with the same polar residues. More precisely, their ring A are perfectly superimposed, whereas ring D of EM5744 is slightly shifted toward H3, the O17 atom of EM5744 being 1.0 Å away from the O17 atom of DHT (Fig. 5B). In this position, the steroid nucleus of EM5744 is stabilized by 13 amino acid residues (located at a distance of 4.0 Å or less), whereas the space occupied by its C18 chain is delimited by 6 additional residues. Most of these residues are hydrophobic and interact mainly with the steroid scaffold, whereas a few are polar and form hydrogen bonds with the polar atoms on the ligand (Fig. 5C). The O3 atom of EM5744 is indeed hydrogen-bonded to the Arg⁷⁵² Nη² atom located at a distance of 2.9 Å. There is also a water molecule near the O3 atom (3.2 Å) that is involved in the formation of a hydrogen bond network with Arg⁷⁵²-Nη¹ (2.8 Å), Arg⁷⁵²-Nη¹ (3.2 Å), and Met⁷⁴⁵-O atoms (2.9 Å). The O17 atom of EM5744, although in a different position from that DHT, is stabilized by hydrogen bonds with the same residues (Asn⁷⁰⁹-Oδ¹ at 2.7 Å and Thr⁸⁷⁷-Oγ¹ at 3.0 Å) (Fig. 5, B and C). A slight reorientation of the side chains of these residues maintains the distance separating them from the O17 atom almost the same as for DHT.

### Table 1

| Property                                      | Value          |
|-----------------------------------------------|----------------|
| Resolution (Å)                                | 35.94-1.65 (1.7-1.65) |
| Unit cell parameters                          | a, b, c (Å) 53.65, 66.36, 70.78 |
| α, β, γ (°)                                   | 90             |
| Space group                                   | P2₁,2₁,2₁     |
| Total reflection                              | 265,110 (13,119) |
| Unique reflection                             | 30,302 (2,430) |
| Completeness (%)                              | 97.5 (93.3)    |
| I/σ(I)                                        | 26.5 (5.3)     |
| Rmerge (%)                                    | 5.7 (32.1)     |
| Redundancy                                     | 8.8 (5.4)      |

### Notes

Rmerge = Σ[Ii − ⟨I⟩]/ΣIi, where Ii is an individual intensity measurement and ⟨I⟩ is the average intensity for all measurements of the reflection i.

Rcryst = ∑[Fo − Fe]/|Fo|, where Fo and Fe are the observed and calculated amplitudes of structure factors.

**FIGURE 4**. Overall structure of the hARLBD in complex with EM5744. Stereoview showing the binding position of the EM5744 (in standard CPK color set) in the hARLBD structure. NH₂- and COOH-terminal ends of the receptor are indicated by letters N and C. The position of residues (Cys⁸⁶⁴ and Thr⁸⁵⁰) on both side of the missing part of the loop between helices 10 and 11 (see text) is also indicated. β-Strands, α-helices, and coils are, respectively, colored in blue, pink, and orange.
Considering that the contacts (hydrophobic and hydrogen bonds) between the hAR and the steroidal base of EM5744 are very similar to those established with the DHT molecule, the higher affinity of EM5744 for the androgen receptor must be due to additional contacts provided by the long C18 chain substituent of this ligand. In fact, a thorough analysis of our crystal structure reveals that the C18 chain is also very well stabilized, mainly by the establishment of strong interactions with polar or charged amino acids in the immediate vicinity of the fluorine atoms at the extremity of its C18 chain (Fig. 1). Fluorine atoms possess lone pairs of electrons that can act as a hydrogen bond acceptor. It so happens that one of the fluorine atoms of EM5744 establishes an interaction with His874 (N/H925) located at 3.3 Å. In addition, a water molecule found in close proximity (3.0 Å) of this same fluorine could also be involved because it is firmly maintained in place by interactions with the side chain of His874-N/H925 (2.9 Å) and the main chains of Met742-N (3.0 Å) and Gln738-O (2.8 Å) (Fig. 5C). The presence of this third bond with the receptor explains very well the higher affinity of EM5744 for the hAR, as compared with that of DHT or R1881.

Impact of the Binding of EM5744 on the Size and Shape of the LBP—Interestingly and as predicted, the C18 chain of EM5744 juts out of the LBP through the narrow channel previously identified (see above). In addition, as observed in the structure of the hAR complexed with THG (discussed above), the side chain of residue Trp741 is flipped 180° around its C/H925 to accommodate the large C18 substituent. However, because of the presence of the difluoro-substituted phenyl ring that is directed toward its indole ring, Trp741 is compelled to adopt a conformation that is also different from that observed in the hARLBD-THG complex structure (Fig. 6). Some of the other residues forming the ligand cavity also adopt slightly different conformations, a possible consequence of the Trp741 side chain movement. It appears that these subtle conformational changes facilitate the passage of the C18-substituent chain but also allow the steroid nucleus of EM5744 to slightly move in the LBP, as it is the case here, without modifying the strength of the interactions that contribute to maintain it firmly. Structural comparison with other hAR-ligand complexes allowed us to learn how the receptor can modify the volume of its LBP to accommodate ligands with different structures and much larger size by changing the position of the side chain of a very limited number of residues. Indeed, mainly through reorientation of the side chains of Trp741 and Met895 (Fig. 6), the size of the LBP varies significantly (considering only the non-hydrogen atoms: DHT = 582 Å³, THG = 605 Å³, and EM5744 = 853 Å³) proportionally to the volume of the ligand itself (DHT = 249 Å³, THG = 463 Å³, EM5744 = 769 Å³).

FIGURE 5. Position of EM5744 in the androgen receptor LBP and residues of interest. A, view showing the (2F - F) electron density map (blue grid) for the ligand (computed with 1.65-Å resolution data and contoured at 1.00σ level), which allows us to confirm unambiguously the presence of EM5744 bound inside the LBP of the hARLBD. The inset shows the exceptional quality of the electron density map around the steroidal part of EM5744. The EM5744 molecule is colored using standard CPK color set (carbon atoms are depicted in light gray, oxygen atoms in red, and fluorine atoms in light green). B, superposition of DHT (in green, PDB code 2AMA (23)) and EM5744 (standard CPK color set) bound to the hAR. The close-up view shows the shift made by one of the extremities (ring D) of the steroidal part of EM5744 and the resulting movement made by residues Asn705 and Thr877 to maintain their interaction with the O17 atom of the steroidal nucleus. Arg752, the position of which is the same in both complexes, is also represented. Putative H-bonds are shown as dashed green lines. C, representation of the interactions made by EM5744 within the binding cavity. To simplify, only residues involved in hydrogen bonds with EM5744 (directly or via a water (W) molecule) are depicted. Potential H-bonds between ligand, water molecules, and LBP residues are shown as green dashed lines (all possible H-bonds are determined from geometric parameters).
The binding of EM5744 to the hARLBD—As anticipated, the chain of EM5744 juts out of the LBP and partly fills the space normally occupied by the side chain of Met895 (H12) when hARLBD is bound to an agonist molecule (Fig. 6). However, the impact of the binding of EM5744 on the overall structure of the receptor is, against all expectations, somewhat minor. Indeed, a pairwise comparison revealed that the overall structure of the hARLBD-EM5744 complex is very similar to the other structures of agonist-hARLBD complexes published so far (23–27, 31, 32). The bulky C18 substituent of the EM5744 molecule emerges from the LBP, lies down against H5/H6, and slips between H3 and H11 without disturbing the respective position of these α-helices or their three-layered arrangement. In fact, except for the reorientation of side chains of a very few residues (see above), the only noticeable impact of the binding of this very large ligand is a slight displacement of a part of the loop immediately preceding H12 (Asp890–Pro892) together with the NH2-terminal extremity of H12 (Glu893–Ile899). Surprisingly, this displacement is hardly more pronounced than that caused by the presence of THG and its much smaller C18 chain (Fig. 7). Precise comparison of this region (Asp890–Ile899) with that of the hARLBD-DHT complex structure reveals indeed that the presence of EM5744 particularly affects the position of the Ca atom of residues Pro892 (root mean square deviation = 1.0 Å), Glu893 (2.2 Å), Met894 (2.1 Å), and Met895 (1.1 Å). The displacement diminishes very rapidly on both sides of these four residues with the consequence that the major part of H12 occupies a very similar position in the two complexes (Fig. 7). This could be explained by the fact that the difluoro-substituted phenyl ring, which we had intentionally placed at the extremity of the bulky C18 chain of the EM5744 to impede the normal folding of H12 against the LBD surface, is not orientated as predicted. Indeed, although the tip of the C18 chain emerges from the bottom of the hydrophobic groove normally occupied by H12 in the agonist-ligated form of hAR, its difluoro-substituted phenyl ring is almost completely contained inside a small cavity, newly created following the movement of the Trp741 side chain, and maintained there by the strong interaction between one of its fluorine atoms with His874 (see above). With the bulky tip of its C18 chain in this position, EM5744 has a very small impact on the hARLBD structure and above all allows H12 to adopt a fold very similar to that observed in the presence of agonist ligands (Fig. 7).

A given compound must significantly affect the positioning of H12 to block the normal functioning of an NR (for example, in the hERLBD-raloxifene complex structure, H12 is rotated by 130° and over 10 Å further than in the agonist-induced conformation (22)). It is thus not surprising that EM5744 is unable to act as an antagonist of the activity of AR, at least as indicated by the in vitro assays with the Shionogi cell line. EM5744 does not induce a displacement of H12 important enough to prevent the hAR from interacting with nuclear coregulatory factors through its ligand-dependent transactivation function (AF-2). This seems even more obvious when we look at the residues of AF-2 directly involved in the interaction with the amphipathic helix structure containing the LXXLL/FFXL motifs of coactiv-
hARLBD Complexed with an H12-directed Steroidal Ligand

vator binding. The AF-2 of hAR in its agonist-ligated form appears as a hydrophobic cleft in which the hydrophobic residues (Leu or Phe) of the LXXL or FXLF motifs reside when bound to the hARLBD. This cleft is flanked by clusters of residues having opposite charges (33), among which Lys\(^{720}\) (H3) and Glu\(^{897}\) (H12) act as charge clamps establishing H bonds with the backbone atoms of residues immediately flanking the LXXL or FXLF motifs (26). Comparison of the hARLBD-EM5744 and hARLBD-DHT structures shows that side chains of these two residues (Lys\(^{720}\) and Glu\(^{897}\)) are almost superimposed indicating that, despite the slight deformation of its AF-2 caused by the bulky chain of the EM5744, hAR is very likely still able to interact with protein partners through their LXXL/FXLF motifs.

Concluding Remarks—Analysis of the hARLBD-EM5744 crystal structure has yielded some crucial information that has improved our rational design procedure for the production of potent H12-directed steroidal antagonist molecules. First, this analysis gave us the certitude that the large C18 chain does not prevent the steroidal portion from binding the LBP in a position that appears very similar to that of DHT alone. This, in addition to insuring affinity for the hAR, guarantees that the C18 chain of our compounds will be well oriented toward H12. Structural comparison with other hAR-ligand complexes allowed us to learn how the receptor can modify the volume of its LBP to accommodate ligands with a different structure and a much larger size. Also, we obtained strong evidence that a chain on the C18 atom of a steroid nucleus can pass through the existing small opening in the LBP and is well oriented to reach the hydrophobic groove that is normally occupied by H12 in the hAR-agonist conformation. We can thus envisage that an appropriate C18 chain could effectively interfere with positioning of residues of H12, therefore preventing its correct folding and, therefore, blocking the formation of the mature AF-2 binding surface. More importantly, this hARLBD-EM5744 structure gave us precious information concerning the structure of the C18 chains that could efficiently prevent the folding of H12, as well as on the nature of the reactive groups that could establish new and strong interactions with specific residues, notably those located in the vicinity of the LBP. Using this structural information, several new compounds with improved C18 chains have since been synthesized and tested (to be published later). These compounds not only show a very high affinity for the hAR, but, in vitro, several of them exert strong and often pure antiandrogenic activity.

EM5744 (or another DHT-based molecule bearing a bulky C18 chain already synthesized and demonstrating a very high affinity and a pure agonist activity) could prove useful for certain specific therapeutic purposes. Indeed, this new class of synthetic steroidal molecules opens new perspectives for clinical management of disorders resulting from androgen deficiency (34). For example, these compounds could be tested in androgen replacement therapy to treat a variety of disorders including delayed puberty in boys, anemia, primary osteoporosis, hereditary angioneurotic edema, and muscle wasting. In this case, the structural information accumulated throughout the present project could prove very useful if it became necessary to enhance certain properties of these compounds (for example, to improve receptor selectivity or to optimize physicochemical, pharmacokinetic, and pharmacological properties).

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