Pergamon

J. Steroid Biochem. Molec Biol. Vol 48, No. 1, pp. 111–119, 1994

0960-0760/94 $6.00 + 0.00

Non-steroidal Antiandrogens: Synthesis and Biological Profile of High-affinity Ligands for the Androgen Receptor*

G. Teutsch, F. Goubet, T. Battmann, A. Bonfils, F. Bouchoux, E. Cerede, D. Gofflo, M. Gaillard-Kelly and D. Philibert†

Centre de Recherches Roussel UCLAF, 102 route de Noisy, 93230 Romainville, France

New *N*-substituted arylthiohydantoin antiandrogens were synthesized. These compounds presented exceptionnally high relative binding affinities (RBAs) for the rat androgen receptor (AR): up to 3 times that of testosterone (T) and 100 times the RBAs of non-steroidal antiandrogens such as flutamide, Casodex and Anandron. Furthermore, unlike available markers for AR, they were totally devoid of any binding to the other steroid receptors. RU 59063, the molecule with the highest RBA, was tritiated. When it was compared to [^{3}H]T for the assay of rat, mouse, hamster and human AR, it gave rise to the same number of binding sites but its K_a ($6 \times 10^9\,M^{-1}$) for rat and human AR were, respectively 3 and 8 times higher than that of T. Moreover RU 59063, unlike T, was devoid of any specific binding to human plasma. *In vivo*, these compounds displayed antiandrogenic activity while being devoid of any agonistic effect. Thus, RU 56187, given orally in castrated male animals, prevented in a dose-dependent manner the effects of 3 mg/kg testosterone propionate (TP) on mouse renal ornithine decarboxylase (acute test) and of 0.5 mg/kg TP on rat prostate weight (chronic test). In these two models, its ED_{50} was 0.6 and 1 mg/kg, respectively. In the intact rat, when given alone, it inhibited dose-dependently the effect of endogenous androgens on the seminal vesicles ($ED_{50} \approx 1$ mg/kg) and prostate ($ED_{50} \approx 3$ mg/kg) weights. These results suggest that these new compounds may be useful as specific markers for the androgen receptor as well as for the treatment of androgen-dependent diseases or disorders such as prostate cancer, acne, hirsutism and male pattern baldness.

J. Steroid Biochem. Molec. Biol., Vol. 48, No. 1, pp. 111-119, 1994

INTRODUCTION

Antiandrogens are substances which antagonize the biological responses induced by endogenous or exogenous androgens, by inhibiting competitively their binding to the receptor. The only pure antiandrogens known up to now, are non-steroidal compounds which bind exclusively to the androgen receptor [1]. This characteristic distinguishes them from steroidal antiandrogens such as cyproterone acetate [2, 3], the first such a drug to have been used in therapeutics [4], which also interacts with the progestin and glucocorticoid receptors [1, 5]. The pure antiandrogens flutamide [6] and Anandron [7] are used in the treatment of prostate cancer [8–11]. A more recent compound, Casodex [12, 13], is in clinical development for the same indication [14, 15]. The common feature of these pure antiandrogens is their very weak relative binding affinity (RBA) for the androgen receptor (AR), 50 to 100 times less than that of testosterone [1, 13, 16].

Structure–affinity considerations with different Anandron analogues suggested that an improvement of the affinity for the AR might be achievable. To this end, a series of *N*-substituted arylthiohydantoins was synthesized. The compounds were submitted to an oriented 3-step screening: RBA for the five classical steroid receptors [17] coupled to two *in vivo* bioassays performed in male castrated animals supplemented with testosterone propionate (TP): renal ornithine decarboxylase (ODC) activity in mice (acute test) [18],

*This paper is dedicated to Dr Edouard Sakiz, in appreciation of his 40 years of commitment to research in endocrinology.

†Correspondence to D. Philibert.

Received 16 July 1993; accepted 23 Sep. 1993.

Abbreviations: Testosterone (T), 17β-hydroxy-4-androstene-3-one; DHT, 5α-dihydrotestosterone; triamcinolone acetonide, 9α-fluoro-11β, 16α, 17α, 21-tetrahydroxy-1-4, pregnadiene-3, 20-dione-16, 17 acetonide; RU 28362, 11β, 17β-dihydroxy-6, 21-dimethyl-17α-pregna-1, 4, 6-trien-20 yn-3 one; R 5020, 17α, dimethyl-19-nor-pregna-4,9-dione-3,20-dione; dexamethasone, 9α-fluoro-11β, 17α, 21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione.

and accessory sex organ weights in rats (chronic test) [19]. This paper presents the results obtained with 3 compounds, which were selected on the basis of their high RBAs for the AR. Their biological profiles were compared to those of known non-steroidal antiandrogens. Two compounds were investigated more extensively: RU 59063 which, in its tritiated form, is a good candidate as a specific, high-affinity AR marker, and RU 56187 which displayed potent antiandrogenic activities *in vivo*.

EXPERIMENTAL

Synthesis of N-substituted arylthiohydantoins

Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Spectral data were recorded on the following spectrometers: IR, Nicolet 5 SX, in chloroform solution unless stated otherwise; NMR, Bruker AC or AM in $CDCl_3$ with TMS as an internal standard; UV, Perkin Elmer Lambda 9 in ethanol. Microanalyses were in agreement with calculated values ($\pm 0.3\%$) for the elements cited. Chromatographic purifications were performed using 50–100 parts (w/w) of Merck 60 silica gel (0.04–0.063 mm). All reactions were conducted under a nitrogen atmosphere.

2-Alkylamino-2-cyano-propanes **1a–1c**

These compounds were prepared according to the general procedure of Exner *et al.* [20] by slow addition of the relevant amine to neat acetone cyanohydrin. They were not isolated, but best used directly for the transformation 3→4.

2-Trifluoromethyl-4-isothiocyanato-benzonitrile 3

The aniline 2 [21] (2.23 g, 12 mmol) was added over 10 min to a well-stirred heterogeneous mixture of thiophosgene (1 ml, 13 mmol) in water (22 ml) at room temperature (RT). The stirring was continued for 1 h, leading to a progressive decolourization of the medium. The reaction mixture was extracted with chloroform (3 × 10 ml), dried over magnesium sulphate and evaporated to dryness under reduced pressure. The crude desired compound was obtained as a brownish syrup (3 g, >100%) and was used as such for the next step. IR: 2017 cm^{-1} (N = C = S), 2224 and 2210 cm^{-1} (CN), 1609, 1562 and 1497 cm^{-1} (aromatics). An analytical sample can be obtained by chromatography (toluene–cyclohexane, 1:1): m.p. 40°C; UV: λ max = 235 nm (ϵ = 15100) and 309 nM (ϵ = 29300); analysis: C, H, F, N, S.

4-(5-Imino-2-thioxo-3,4,4-trimethyl-1-imidazolidinyl)-2-trifluoromethyl-benzonitrile **4a**

To a solution of the crude isothiocyanate 3 (3 g, 12 mmol) in THF (23 ml) and triethylamine (0.23 ml) was added 2-methylamino-2-cyanopropane (1.15 g, 11.7 mmol). The reaction mixture was refluxed for 40 min and then evaporated to dryness under reduced pressure. The residue was purified by chromatography (CH_2Cl_2–acetone, 95:5) and recrystallized from isopropanol, yielding 2.629 g (66%) of the desired imine **4a**. m.p.: 173–174°C; UV: λ max = 233 nm (ϵ = 20500), 256 nm (ϵ = 24100); ^{1}H-NMR: δ = 1.59 (s, 6H, gem-diMe), 3.29 (s, 3H, NMe), 7.76 (d, 1H, H-5), 7.88 (d, 1H, H-3) and 7.98 (d, 1H, H-6); analysis: C, H, F, N, S.

The following compounds were prepared by the same procedure using the relevant cyanoamines: *4-[4,4-dimethyl-3-(2-hydroxyethyl)-5-imino-2-thioxo-1-imidazolidinyl)-2-trifluoromethyl-benzonitrile* **4b**. Yield = 75%; m.p.: 181°C; UV: λ max = 233 nm (ϵ = 20300), 259 nm (ϵ = 23700); TLC (SiO_2, Merck F_{254}, CH_2Cl_2–acetone, 6:4) Rf = 0.27; analysis: C, H, F, N, S. *4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-imino-2thioxo-1-imidazolidinyl)-2-trifluoromethyl-benzonitrile* **4c**. Yield = 61.5%; amorphous solid; TLC (SiO_2, Merck F_{254}, CH_2Cl_2–acetone, 6:4) R_f = 0.25; ^{1}H-NMR: δ = 1.59 (s, 6H, gem-diMe), 1.65 and 1.94 (m, 2 × 2H, the protons on C-2 and C-3 of the butyl chain), 3.72 (m, 4H, protons on C-1 and C-4 of the butyl chain)

4-(5-Oxo-2-thioxo-3,4,4-trimethyl-1-imidazolidinyl)-2-trifluoromethyl-benzonitrile **5a**

The imine **4a** (2.207 g, 6.76 mmol) was suspended in 6N aqueous HCl (44 ml) and heated to reflux temperature for 1 h. The reaction mixture was poured onto ice–water (1:1, 20 g) and extracted with methylene chloride (4 × 10 ml). The combined organic extracts were dried over magnesium sulphate, evaporated to dryness and recrystallized from isopropanol, affording 2.10 g (95%) of **5a**. m.p.: 171°C; UV: λ max = 231 nm (ϵ = 18600), 252 nm (ϵ = 23800); ^{1}H-NMR: δ = 1.58 (s, 6H, gem-diMe), 3.32 (s, 3H, NMe), 7.78 (d, d, 1H, H-5), 7.91 (d, 1H, 3-H) and 7.96 (d, 1H, H-6); analysis: C, H, F, N, S.

4-[4,4-Dimethyl-3-(2-hydroxyethyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-trifluoromethyl-benzonitrile **5b**

2N aqueous hydrochloric acid (10 ml, 20 mmol) was added to a solution of the imine **4b** (4.6 g, 12.7 mmol) in methanol (65 ml) and refluxed for 1 h. The reaction mixture was cooled to RT, poured into cold water (300 ml) and extracted with ethyl acetate (3 × 30 ml). The organic fraction was dried over magnesium sulphate and evaporated under reduced pressure. The residue was purified by chromatography (cyclohexane–ethyl acetate, 1:1) and recrystallized from CH_2Cl_2–cyclohexane, affording 4.37 g (95%) of **5b**. m.p.: 130°C; UV: λ max = 232 nm (ϵ = 18700), 254 nm (ϵ = 22700); 1H-NMR: δ = 1.61 (s, 6H, gem-diMe), 2.16 (s, 1H, OH), 3.94 (m, 2H, N-CH_2), 4.05 (m, 2H, CH_2-O); analysis: C, H, F, N, S.

4-[4,4-Dimethyl-3-(4-hydroxybutyl)-5-oxo-2-thioxo-1-imidazolidinyl]-2-trifluoromethyl-benzonitrile **5c**

Proceeding as for the preparation of **5b**, compound 5c was obtained in 78.7% yield. m.p.: 78–79°C; UV: λ max = 232 nm (ϵ = 19500), 254 nm (ϵ = 24000);

^{1}H-NMR: δ = 1.60 (s, 6H), 1.67 (m, 2H), 1.96 (m, 2H), 3.72 (m, 4H), 7.78 (d, 1H), 7.90 (m, 1H) and 7.96 (m, 1H); analysis: C, H, F, N, S.

Animals

Male Sprague–Dawley rats and Swiss mice were purchased from Iffa Credo (France). Male golden Syrian hamsters were obtained from Charles River (U.S.A.) and female New Zealand rabbits from ESD (France).

Compounds

[6,7^{3}H]R 1881 (sp. act.: 57 Ci/mmol), [1,2^{3}H]testosterone (sp. act.: 54 Ci/mmol) and [5^{3}H]RU 59063 (sp. act.: 25 Ci/mmol) were prepared by RU radiomolecules Lab. L[2,3^{3}H]ornithine (sp. act.: 46.5 Ci/mmol) was obtained from NEN.

Testosterone, dihydrotestosterone (DHT), R 1881, Anandron (nilutamide = RU 23908), Casodex (ICI 176,334), hydroxyflutamide, cyproterone acetate, triamcinolone acetonide and RU 28362 were synthesized according to described procedures.

Homogenization and assay buffers

Buffer A: 10 mM Tris–HCl, pH 7.4, 0.25 M sucrose; buffer B: buffer A + 2 mM dithiothreitol; buffer C: buffer B + 0.1 mM phenylmethylsulphonyl fluoride and 20 mM sodium molybdate; buffer D: 50 mM Tris–HCl, pH 7.4, 5 mM dithiothreitol, 0.25 mM pyridoxal phosphate, 0.1 mM EDTA.

Dextran coated charcoal (DCC) was prepared by suspending 1.25% Norit A charcoal and 0.625% dextran T80 in the buffer used for tissue homogenization.

RBAs for steroid receptors and human plasma sex hormone binding globulin (SHBG)

The RBAs of test compounds were evaluated in a routine receptor screening described in detail previously [17]. It included the androgen (AR), progestin (PR), glucocorticoid (GR), mineralocorticoid (MR) and estrogen (ER) receptors. AR, PR, GR, MR and ER were prepared respectively from prostate of 1 day castrated rats (180–200 g), uterus of immature estradiol-primed rabbits ($\approx$1 kg), thymus of adrenalectomized (ADX) male rats ($\approx$160 g), kidney of ADX rats (160 g), uterus of 18-day-old mice. All subsequent procedures were carried out at 0–4°C.

Prostates were homogenized in buffer C (1:8, w/v), rabbit uterus (1:50, w/v), mouse uterus (1:25, w/v), kidney (1:3, w/v) in buffer A and thymus (1:10, w/v) in buffer B with a glass–glass Potter.

The homogenates were centrifuged at 209,000 *g* for 30 min. 125 μl aliquots of the supernatant (cytosol) were incubated for 24 h (5 h at 25°C for ER) with 2.5 or 5 nM of the relevant ^{3}H ligand [[^{3}H]testosterone ([^{3}H]T) for AR, [^{3}H]R 5020 for PR, [^{3}H]dexamethasone for GR, [^{3}H]aldosterone with 10^{-6} M RU 28362 [22] for MR and [^{3}H]estradiol for ER] in the presence of increasing concentrations (1–25,000 nM) of cold reference or test compounds.

Plasma was prepared from human female blood, diluted (1:100) with buffer A and 125 μl aliquots were incubated for 24 h with 20 nM of [^{3}H]T in the presence of increasing concentrations of cold T or RU 59063.

Bound ^{3}H ligand measurement by DCC adsorption technique: a 0.1 ml aliquot of incubated cytosol or plasma was stirred for 10 min with 0.1 ml DCC solution in a 96 well microtitre plate and centrifuged for 10 min at 800 *g*. The radioactivity of a 0.1 ml supernatant sample was counted.

RBA calculation: the RBA was defined as the ratio of the concentration of the reference compound over the concentration of the competitor required to inhibit ^{3}H ligand binding by 50% and multiplied by 100. The RBAs of T, progesterone, dexamethasone, aldosterone and estradiol were taken equal to 100.

Binding to AR of various species and to human plasma

AR was prepared from the following target tissues of 1 day castrated animals: cytosols of rat prostate (1:5, w/v), hamster (140–160 g) prostate and flank organs

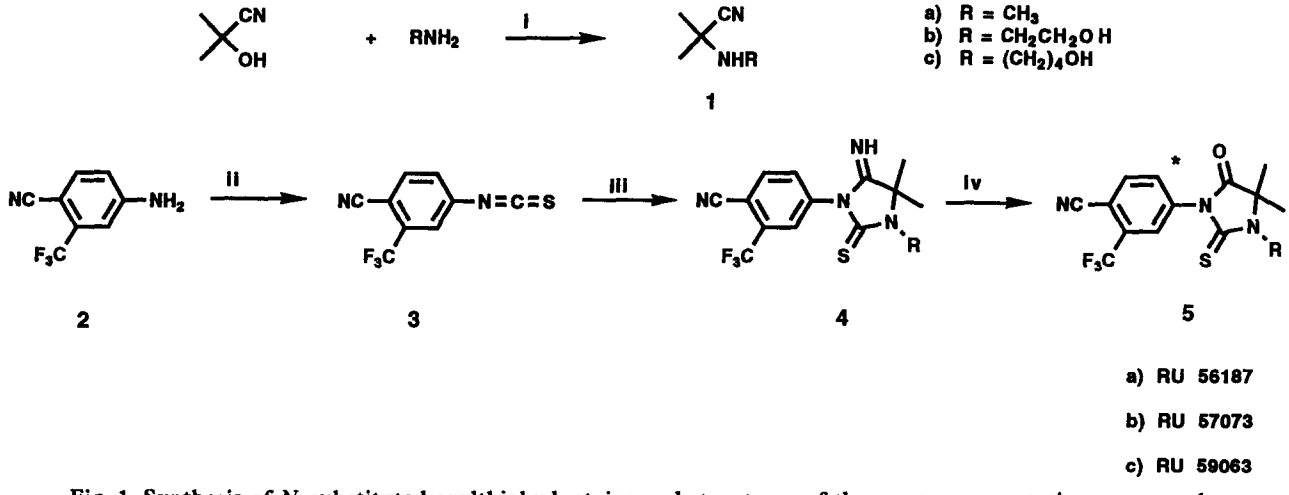
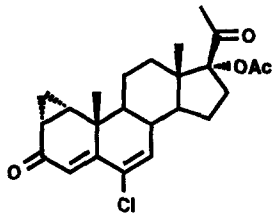
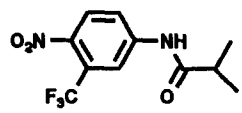


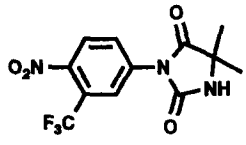
Fig. 1. Synthesis of *N*-substituted arylthiohydantoins and structures of the most representative compounds of this series. (i) Neat, room temperature (RT); (ii) $CSCl_2$, H_2O, RT; (iii) 1, NEt_3, THF, RT; (iv) 2N HCl, MeOH, reflux. *Site of labelling in [^{3}H]RU 59063.



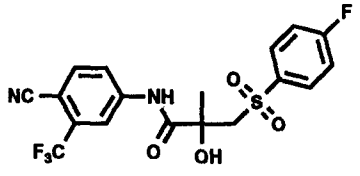
Cyproterone acetate



Flutamide



Anandron



Casodex

Fig. 2. Chemical structures of antiandrogens commonly used in therapeutics.

(1:5, w/v) and male mouse (30 g) kidney (1:3, w/v) were prepared in buffer C, as described above, except that flank organs were frozen in liquid nitrogen and pulverized in a mortar before homogenization.

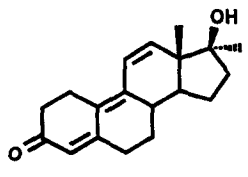
The human AR (hAR) and a truncated hAR (htAR) were obtained as follows: hAR cDNA was cloned from T47D cells and inserted in the eucaryotic expression vector pSG5 (Prof. Chambon, LGME, Strasbourg, unpublished data). The chimaera GAL4-htAR was obtained by inserting in the Xhol site of the expression vector pGAL4M polyII [23] a PCR-amplified fragment containing the D–E regions of hAR (amino acids 625 to 920). These receptors were expressed in COS-1 cells as follows: cells were plated at 2×10^6 cells/9 cm dish in Dulbecco's modified Eagle's medium (Gibco) containing 5% DCC-treated foetal calf serum and transfected with 5 μg plasmid DNA/dish using the calcium phosphate precipitation technique. Cells were harvested about 40 h after transfection and the cytosols were prepared as described above.

0.1 ml aliquots of cytosols or human plasma (1:100) were incubated with concentrations (0.1 to 20 nM) of [^{3}H]T or [^{3}H]RU 59063 for 24 h at 0°C. Non-specific binding was determined in parallel incubations with the ^{3}H ligand in the presence of a 500-fold excess of the corresponding cold compound. Bound radioactivity was determined as described above. Scatchard analysis [24] was used to determine the concentration of binding sites N (fmol/mg protein) and the association constants K_a.

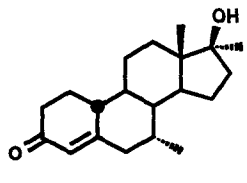
In vivo studies

TP and the test compounds were administered by subcutaneous route (s.c.) in sesame oil containing 5% benzyl alcohol or in corn oil containing 10% ethanol. Orally they were given in an aqueous solution containing 0.5% methylcellulose.

Mouse renal ODC activity. Male mice weighing about 30 g (3 to 6 animals) were castrated. 24 h later, they received simultaneously a single dose of the test compound by oral or s.c. route and 3 mg/kg of TP by s.c. route. 16 h later, the animals were sacrificed, the kidneys removed, pooled and homogenized in buffer D at 4°C. The cytosols were prepared as described above. ODC activity was evaluated according to Djurhuus [25] with slight modifications. Briefly aliquots (triplicate) of 50 μl of cytosol were incubated with 0.025 μCi of [^{3}H]L-ornithine and 10 mM of cold L-ornithine for 1 h at 37°C. Samples were then applied to dry Whatman paper strips and washed 3 times with 0.1 M ammonia in order to eliminate L-ornithine. [^{3}H]Putrescine retained by the paper strip was counted. The results were expressed as fmol of

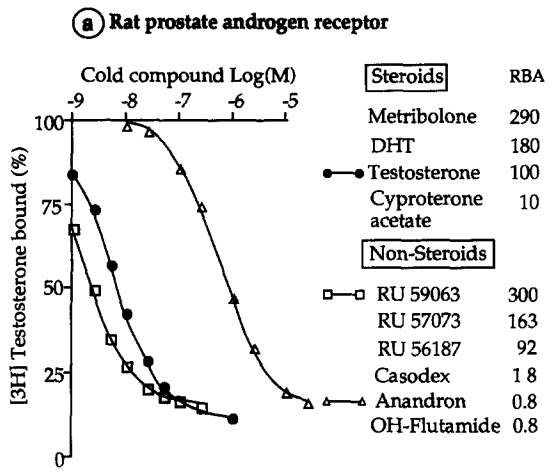


Metribolone (R 1881)



Mibolerone

Fig. 3. Structures of the synthetic androgens widely used as AR markers.



(b) Other steroid receptors

RECEPTORS	Metribolone (R 1881)	RU 59063 RU 57073 RU 56187
	RBA	
PROGESTIN *(Rabbit Uterus)* Progesterone = 100	190	< 0 1
GLUCOCORTICOID *(Rat Thymus)* Dexamethasone = 100	4	< 0.1
MINERALOCORTICOID *(Rat Kidney)* Aldosterone = 100	38	< 0 1
ESTROGEN *(Mouse Uterus)* Estradiol= 100	< 0.1	< 0 1

Fig. 4. RBAs for the 5 classical steroid receptors.

[^{3}H]putrescine formed per mg of protein per hour. The proteins were determined according to the method of Bradford [26].

Accessory sex organ weight in rats. Immature male rats weighing about 100 g were castrated. 24 h later, groups of 5 animals received daily (8 administrations over 10 days, as indicated in Fig. 8) simultaneously 0.5 mg/kg of TP by s.c. route and the test compounds either by oral or s.c. route. 24 h after the last administration the animals were sacrificed, the seminal vesicles and the prostate removed and fixed for 72 h in demineralized water containing 10% formaldehyde. Then the organs were carefully dissected and weighed.

Groups of 5 adult male rats weighing about 200–220 g received daily increasing doses of RU 56187 by oral route according to the scheme of administration described above.

RESULTS AND DISCUSSION

RBAs for the steroid receptors

The RBAs of several *N*-substituted arylthiohydantoins, (see synthetic scheme in Fig. 1), were determined on a routine receptor screening [17] and compared to those of cyproterone acetate (CPA), Casodex, Anandron and hydroxyflutamide, the active metabolite of flutamide (see structures in Fig. 2) and to that of R 1881, one of the two compounds (see structures in Fig. 3) with mibolerone (MB), widely used as AR markers [27–29]. As shown in Fig. 4, RU 59063, RU 57073 and RU 56187 exhibit exceptionally high RBAs for the rat prostate AR. The compound with the highest affinity, RU 59063, inhibits in a concentration-dependent manner the binding of [^{3}H]T to rat AR. Its RBA is similar to that of R 1881, and respectively 1.7, 3, 30, 166 and 375 times higher than those of DHT, T, CPA, Casodex and Anandron or hydroxyflutamide. The RBAs obtained for these latter compounds are in good agreement with those published elsewhere [1, 13, 16].

As shown in the table inserted in Fig. 4, these three new non-steroidal compounds are totally devoid of any binding to other steroid receptors up to 25 μM whereas R 1881 exhibits, respectively strong, moderate and weak RBAs for the rabbit uterus PR, rat kidney MR and rat thymus GR. The latter data are consistent with those published previously [17, 30]. Furthermore MB, has been shown to possess a slightly higher RBA than that of R 1881 for AR and, respectively a 2, 4, and 7 times lower one for PR, MR and GR [31]. The very promising biochemical profile of RU 59063, led us to label it in order to evaluate its binding parameters for the AR of various species.

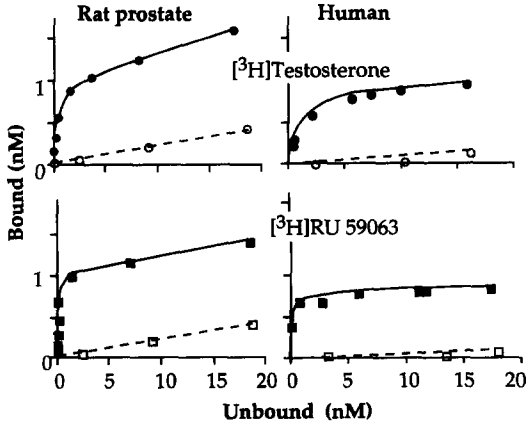


Fig. 5. Saturation curves of [^{3}H]T and [^{3}H]RU 59063 binding to rat prostate and hAR androgen receptors. (●, ■) Total binding; (○, □) non-specific binding.

Binding parameters of [^{3}H]RU 59063 and [^{3}H]T for AR of various species

The binding parameters (number of binding sites, *N*, and association constant, K_a) of RU 59063 and T were determined in the cytosol of target tissues of species commonly used to test androgens or antiandrogens: rat prostate, hamster flank organ and prostate, mouse kidney. They were also evaluated in the cytosol of COS cells in which the full-length (hAR) or a truncated (htAR) human AR were expressed. As illustrated in Fig. 5, incubation with [^{3}H]RU 59063, or [^{3}H]T, show saturable binding of rat prostate and COS

Table 1. Binding parameters (N, K_a) of [^{3}H]T and [^{3}H]RU 59063 for the AR of different species

Binding parameters	Compounds	Rat prostate	Mouse kidney	Hamster		Human	
				Prostate	F.O.[a]	Full length	Truncated
N	Testosterone	219	20	17	132	367	820
(fmol/mg protein)	RU 59063	226	24	15	145	327	920
K_a	Testosterone	1.9	1.2	1.3	0.9	0.8	0.5
(10^9 M^{-1})	RU 59063	6 1	16	4 3	7.2	6.2	6.2

[a]F.O , flank organ.

cells (hAR) cytosols. Furthermore its non-specific binding, like that of [^{3}H]T, is low. This characteristic is a necessary requirement for a receptor marker

the same N: 196, 196 and 176 fmol/mg protein for RU 59063, R 1881 and T, respectively. However, the K_a of RU 59063 (8.4 × 10^9 M^{-1}) was higher than the

N and K_a were deduced from these binding data using the Scatchard analysis and are shown in Table 1. RU 59063 and T gave rise to the same number of binding sites whatever the target tissues. RU 59063 exhibited a very high K_a, from 3 to 13 times higher than that of T, according to the tissue. The reported K_a values (10^9 M^{-1}), are the mean of 3 determinations for rat prostate AR [K_a(RU) = 4–8.4 vs K_a(T) = 1.7–2.1], for hAR [K_a(RU) = 5–7 vs K_a(T) = 0.7–1.2] and for htAR [K_a(RU) = 5–9 vs K_a(T) = 0.4–0.6)]. A single determination was performed for the other tissues. Whereas the K_as of RU 59603 for rat AR, hAR and htAR are identical, those of T are different in spite of the fact that the amino acid sequences of the hormone binding domains of rat and human AR are strictly identical [32]. The origin of this discrepancy is under investigation. Preliminary results seem to indicate that it is not related to 5α-reductase activity.

In addition, the binding parameters of

of R 1881 (6 × 10^9 M^{-1}) and T (2 × 10^9 M^{-1}). Finally, the comparison of the K_a values of RU 59063 reported here with those published for R 1881 and MB, in the rat [30–34], mouse [35] and human [30, 32, 33, 36], confirms that RU 59063 is one of the most potent AR markers known so far and undoubtedly the most selective.

Binding to human plasma SHBG

One major problem in the assay of AR in human tissue with [^{3}H]DHT or [^{3}H]T is the interference by plasma proteins such as SHBG which binds these two hormones with a very high affinity [31]. It was therefore important to assess the binding of RU 59063 to human plasma. As depicted in Fig. 6 (left panel), it does not affect up to 2.5 × 10^{-5} M, the binding of [^{3}H]T to human SHBG whereas cold T reduces it by 50% at a concentration close to 10^{-8} M. The RBA (<0.1%) of RU 59063 for SHBG is similar to the values reported for other non-steroidal antiandrogens [16], but weaker

Table 2. Antiandrogenic activity on renal ODC in Cx mice and prostate weight in Cx immature rats

	Cx mouse Renal ODC Inhibition of the effect of TP %		Cx rat Prostate weight	
Dose: Compounds	1.7 mg/kg s.c.[a]	3 mg/kg p.o.[b]	1 mg/kg s.c.	1 mg/kg p.o.
RU 56187	65	94	69	60
RU 57073	30	67	37	45
RU 59063	64	—	21	23
		80	58	0
Casodex	*3 mg/kg*		*3 mg/kg*	*3 mg/kg*
	46		*68*	*50*
		64	28	0
			3 mg/kg	*3 mg/kg*
Anandron	*3 mg/kg*		*44*	*29*
	46		*10 mg/kg*	*10 mg/kg*
			67	*59*

[a]s.c., subcutaneously; [b]p.o., per orally.

All together, these biochemical studies suggest that RU 59063 is the most suitable ligand known so far for assaying the AR. It presents the following advantages over the existing steroidal ligands: unlike DHT and T, it is devoid of any specific binding to human plasma and unlike R 1881 and MB, it does not interact with other steroid receptors. The latter ligands exhibited a notably strong binding to the PR which is present in androgen-dependent tissues such as human prostate [30, 33]. In order to overcome this drawback, they are commonly used, for assaying the AR, in the presence of a large excess of cold TA or cold DHT [30, 33, 34]. But it has been reported that, in some cases, the inclusion of TA caused underestimation of AR whereas the use of DHT led to an overestimation [30].

In vivo antiandrogenic activity

The antiandrogenic activities of RU 56187, RU 57073, RU 59063, Anandron and Casodex were compared on a preliminary two-step bioassay, performed in male castrated animals supplemented with TP given s.c.: the renal ODC activity in mice (acute test), and the prostate weight assay in rats (chronic test).

In mice, a single injection of 3 mg/kg TP induced, 16 h later, a 50-fold increase in renal ODC activity relatively to vehicle-treated animals (528 ± 41 vs 10 ± 2 fmol putrescine/mg protein/h; $n = 15$). The androgen-specificity of this response was verified by injecting by s.c. route 30 mg/kg of the following compounds: estradiol, the pure glucocorticoid RU 28362 [37], the progestin R 5020 and aldosterone. None of these compounds were able to induce ODC activity (data not shown). In immature rats 8 day's treatment with TP induced an 8-fold increase in prostate weight relative to vehicle-treated animals (167 ± 8 vs 21.2 ± 3 mg; $n = 25$).

In these two models, the compounds were administered concomitantly with TP by s.c. or oral route, and the results obtained were expressed as the percentage of inhibition of the TP effect (TP-treated animals were arbitrarily assigned a value of 0 and vehicle-treated animals a value of 100%). At doses indicated in Table 2, RU 56187 exhibited the most potent antiandrogenic activity whatever the test and the route of administration. On the prostate weight, it proved to be about 3 and 10 times more active than Casodex and Anandron, respectively. This activity is not as high as expected from the differences in RBAs. Preliminary results suggest that, *in vivo*, RU 56187 is rapidly transformed to a low-affinity metabolite.

RU 56187 was selected from this preliminary screening and was studied in more detail by oral route on the 2 models mentionned above as well as in intact rats. As shown in Fig. 7, it antagonized dose-dependently the TP-induced ODC activity in Cx mice. It was fully effective at a dose close to 3 mg/kg while being devoid of agonistic activity when given alone at a dose of 10 mg/kg. On this test, its ED_{50} was about 0.6 mg/kg.

In Cx rats (Fig. 8), it inhibited in a dose-dependent manner the TP-induced accessory sex organ weight increase. It was fully effective on seminal vesicle (SV) and prostate weights at doses of 3 and 10 mg/kg, respectively with ED_{50}s of 0.6 mg/kg (SV) and 1 mg/kg (prostate).

RU 56187 also prevented the effect of endogenous androgens. As shown in Fig. 9, it caused a dose-related reduction of the accessory sex organ weights in intact adult rats with ED_{50}s of 1 mg/kg (SV) and 2.7 mg/kg (prostate). In a similar model, (14 days oral administration) the ED_{50}s reported for Casodex [14] were 2.5 mg/kg (SV) and 7.4 mg/kg (prostate).

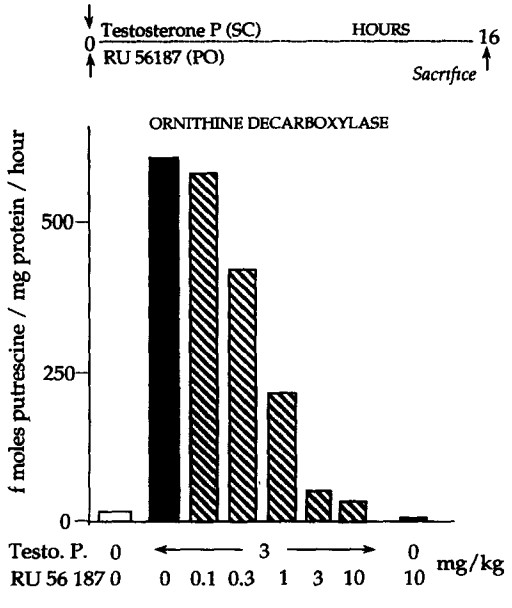


Fig. 7. Oral antiandrogenic activity of RU 56187 on TP-induced renal ODC activity in Cx mice.

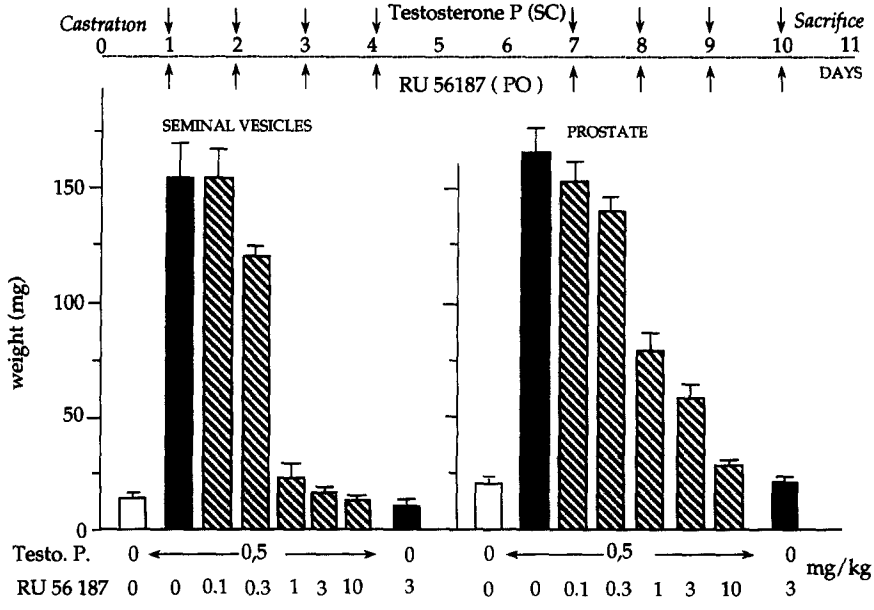


Fig. 8. Oral antiandrogenic activity of RU 56187 on TP-induced sex accessory organ weight in Cx immature rats.

CONCLUSION

The new arylthiohydantoin antiandrogens are characterized by exceptionnally high RBAs for AR: higher than or close to those of the natural androgens T and DHT and 100 times higher than those of previously known non-steroidal antiandrogens. One of them, RU 59063, in its tritiated form, fulfills most of the criteria required for an ideal AR marker. Its K_a for the AR is at least equal to those of R 1881 (metribolone) and MB (mibolerone) and according to species, 3 to 13 times higher than that of T. Unlike existing steroid markers (R 1881, MB), it binds exclusively to AR and unlike T and DHT, it does not interact with SHBG. *In vivo*, another analogue RU 56187 has proved to be the most potent pure antiandrogen known so far. In rats, when given orally, it is, respectively 3 and 10 times more active than Casodex and Anandron. Thus, this compound may be of potential use in the treatment of androgen-dependent diseases such as prostate cancer.

Further work is in progress to extend the usefulness of this new series of compounds, for instance in the area of γ and positron-emitting agents [38] and in the investigation of the mode of action of non-steroidal antiandrogens.

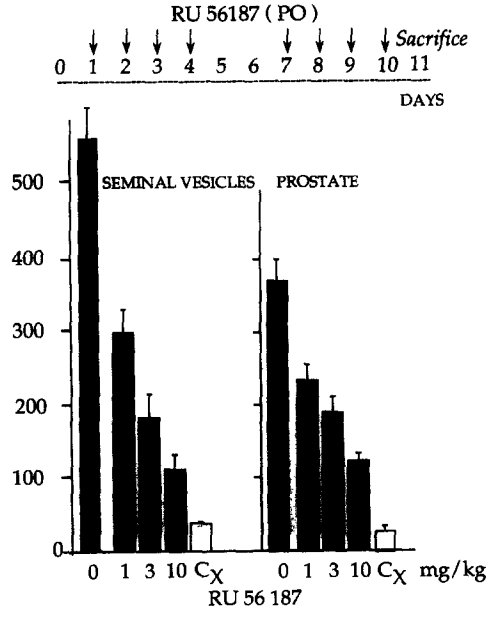


Fig. 9. Oral antiandrogenic activity of RU 56187 on sex accessory organ weight in adult intact rats.

REFERENCES

1 Mogulewsky M and Bouton M. M. How the study of the biological activities of antiandrogens can be oriented towards the clinic. *J. Steroid Biochem.* 31 (1988) 699–710.
2 Neumann F.: Pharmacology and potential use of cyproterone acetate *Horm. Metab Res.* 9 (1977) 1–13
3 Neumann F. and Topert M.: Pharmacology of antiandrogens *J. Steroid Biochem.* 25 (1986) 885–895
4 Neumann F. Pharmacology and clinical use of cyproterone acetate In *Pharmacology and Clinical Uses of Inhibitors of Hormone Secretion and Action* (Edited by B. J A Furr and A. E Wakeling). Bailliere Tindall, London (1987) pp. 132–159
5. Mowszowicz I., Bieber D E., Chung K W., Bullock L. P. and Bardin C. W Synandrogenic and antiandrogenic effect of progestins: comparison with non-progestational antiandrogens. *Endocrinology* 95 (1974) 1589–1599.
6 Neri R., Peets E. and Watnick A.: Anti-androgenicity of flutamide and its metabolite Sch 16423. *Biochem Soc Trans.* 7 (1979) 565–569.
7 Raynaud J. P., Bonne C., Mogulewsky M., Lefebvre F A, Bélanger A. and Labrie F.: The pure antiandrogen RU 23 908 (Anandron)®, a candidate of choice for the combined antihormonal treatment of prostatic cancer a review *The Prostate* 5 (1984) 299–311.
8 Neri R. and Kassem N.: Pharmacology and clinical uses of flutamide In *Pharmacology and Clinical Uses of Inhibitors of Hormone Secretion and Action* (Edited by B J. A. Furr and A E Wakeling) Bailliere Tindall, London (1987) pp 160–169
9. Mogulewsky M, Bertagna C. and Hucher M. Pharmacological and clinical studies of the antiandrogen Anandron *J Steroid Biochem* 27 (1987) 871–875.

10. Moguilewsky M., Cotard M., Proulx L., Tournemine C. and Raynaud J. P.: What is an antiandrogen and what is the physiological and pharmacological rationale for combined "castration" + antiandrogen" therapy. In *Prostate Cancer, Part A: Research, Endocrine Treatment, and Histopathology Progress in Clinical and Biological Research* (Edited by G. Murphy

S. Khoury, R. Kuss, C. Chatelain and L. Denis). Alan Liss, New York, Vol. 243A (1987) pp. 315–340

11. Harris M. G., Coleman S. G., Faulds D. and Chrisp P.· Nilutamide. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in prostate cancer *Drugs Aging* 3 (1993) 9–25
12. Furr B. J. A., Curry V. B., Woodburn J. R., Chesterson R. and Tucker H : ICI 176, 334: a novel nonsteroidal peripherally selective antiandrogen. *J. Endocr.* 113 (1987) R7–R9.
13. Furr B. J. A.: "Casodex" (ICI 176,334)—A new, pure, peripherally-selective antiandrogen. preclinical studies. *Horm. Res.* 32 (1989) 69–76.
14. Tucker H.: Non-steroidal anti-androgens in the treatment of prostate cancer. *Drugs Future* 15 (1990) (3) 255–265
15. Mahler C. and Denis L . Clinical profile of a new non-steroidal antiandrogen. *J. Steroid Biochem Molec. Biol* 37 (1990) 921–924.
16. Ayub M. and Levell M. J.: The effect of ketoconazole related imidazole drugs and antiandrogens on [^{3}H] R1881 binding to the prostatic androgen receptor and [^{3}H] 5α-dihydrotestosterone and [^{3}H]cortisol binding to plasma proteins *J. Steroid Biochem.* 33 (1989) 251–255.
17. Ojasoo T and Raynaud J. P.· Unique steroid congeners for receptor studies. *Cancer Res.* 38 (1976) 4186–4198.
18. Bullock L. P.: Androgen and progestin stimulation of ornithine decarboxylase activity in the mouse kidney. *Endocrinology* 112 (1983) 1903–1909.
19. Dorfman R. I.: Biological activity of antiandrogens. *Br. J. Dermat* 82 (1970) 3–8.
20. Exner L. J., Luskin L. S. and De Benneville P. L.: α-(*N*-Alkylamino)-nitriles. *J. Am Chem. Soc.* 75 (1953) 4841–4842.
21 Alastair T. G. Amide derivatives, process for their manufacture, pharmaceutical or veterinary compositions containing them and a method of producing an antiandrogenic effect in a warm-blooded animal. European Patent, EP 0 002 892 (1979).
22. Ojasoo T., Moguilewsky M., Philibert D. and Raynaud J. P.: Study and assay of steroid hormone receptors. *Proc. Symp. Analysis of Steroids*, Sopron, Hungary (1987) 17–44.
23. Tora V., White J., Brou C., Tasset D., Webster V., Scheer E. and Chambon P.: The human estrogen receptor has two

25. Djurhuus R.: Ornithine decarboxylase (EC 4.1.1.17) assay based upon the retention of putrescine by a strong cation-exchange paper. *Analyt. Biochem.* 113 (1981) 352–355.
26. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* 72 (1976) 248–254

27 Bonne C. and Raynaud J. P.: Methyltrienolone, a specific ligand for cellular androgen receptors. *Steroids* 26 (1975) 227–232.
28 Asselin J., Melancon R., Gourdeau Y., Labrie F., Bonne C. and Raynaud J. P.: Specific binding of [^{3}H]methyltrienelone to both progestin and androgen binding components in human benign prostatic hypertrophy. *J. Steroid Biochem.* 10 (1979) 483–486
29. Schilling K. and Liao S.· The use of radioactive 7α,17α-dimethyl-19-nortestosterone (mibolerone) in the assay of androgen receptor. *The Prostate* 5 (1984) 581–588.
30. Stebbings W. S. L., Anderson E , Puddefoot J. R., Farthing M. J. G. and Vinson G. P : A hybrid ligand method for androgen receptor measurement. *J. Steroid Biochem.* 31 (1988) 181–186.
31 Carlson K. E and Katzenellenbogen J. A : A comparative study of the selectivity and efficiency of target tissue uptake of five tritium-labeled androgens in rat. *J. Steroid Biochem.* 36 (1990) 549–561
32. Chang C., Kokontis J and Liao S.: Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc. Natn. Acad. Sci U.S A.* 85 (1988) 7211–7215.
33 Traish A. M., Muller R E. and Wotiz H H.: Binding of 7α, 17α-dimethyl-19-nortestosterone (Mibolerone) to androgen and progesterone receptors in human and animal tissues. *Endocrinology* 118 (1986) 1327–1333
34. Bannister P , Sheridan P. and Losowsky M. S.: Use of new radioactive ligand, 7α, 17α-dimethyl [17α-methyl ^{3}H] 19-nortesterone for the estimation of androgen receptors in rat liver cytosol *J. Steroid Biochem* 23 (1985) 121–123.
35. Kemp C. J. and Drinkwater N R · Genetic variation in liver tumor susceptibility, plasma testosterone levels and androgen receptor binding in six inbred strains of mice. *Cancer Res* 49 (1989) 5044–5047.
36. Young C. Y F., Qiu S., Prescott J. L. and Tindall D. J.: Overexpression of a partial human androgen receptor in E. coli: characterization of steroid binding, DNA binding, and immunological properties. *Molec. Endocr.* 4 (1990) 1841–1849
37. Teutsch G., Costerousse G., Deraedt R., Benzoni J., Fortin M. and Philibert D 17α-alkynyl-11β, 17-dihydroxyandrostane derivatives: a new class of potent glucocorticoids. *Steroids* 38 (1981) 651–665.
38. Liu A., Dence C. S., Welch M. J. and Katzenellenbogen J A :