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RECEPTOR BINDING OF ALLYLESTRENOL, A PROGESTAGEN OF THE 19-NORTESTOSTERONE SERIES WITHOUT ANDROGENIC PROPERTIES

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Summary—Allylestrenol (17α-allyl-17β-hydroxy-4-estren) is an orally active progestagen of the 19-nortestosterone series resembling progesterone since it has no detectable androgenic activity in animal studies and in the human. In the present study, the affinity of its 3-keto metabolite for the transformed progesterone receptor in intact MCF-7 cells was about twice that of progesterone and cyproterone acetate and about 2–3 times less than that of medroxyprogesterone acetate and norethisterone, reflecting the known progestational activity of allylestrenol. The affinity of 3-ketoallylestrenol for the transformed androgen receptor in intact MCF-7 cells was weak (like other progestagens lacking androgenic activity or possessing anti-androgenic activity) and lower than that of weakly androgenic progestagens. On the other hand, the relatively high affinity of 3-keto-allylestrenol for the non-transformed androgen receptor at 4°C in the cytosol fraction did not reflect the known lack of androgenic activity of allylestrenol. Thus competitive studies carried out with transformed receptor complexes in intact cells at 37°C and non-transformed complexes in cytosol distinguish progestagen with weak androgenic activity (e.g. norethisterone) from those displaying no androgenic activity or possessing anti-androgenic activity (e.g. 3-keto-allylestrenol, progesterone, cyproterone acetate and spironolactone).

INTRODUCTION

Allylestrenol is an orally active progestagen of the 19-nortestosterone series [1] which is used clinically in the treatment of threatened abortion [2–4]. Treatment during early pregnancy results in a reduction in the number of miscarriages [2, 3] probably due to stimulation of placental function [4–6]. Allylestrenol (3 × 5 mg daily) has a progestational effect on vaginal cytology in women [6]. Clinical studies also suggest that allylestrenol intake at therapeutic dosage does not increase the incidence of hypospadia in the foetus [7, 8]; this is in agreement with its lack of androgenic activity found in animal studies [1]. Allylestrenol is different in this respect from other progestagens derived from 19-nortestosterone which, in addition to their progestational activity, also display at least some androgenic activity in pharmacological and teratological studies. Allylestrenol is also devoid of oestrogenic and glucocorticoid activity and is considered to be a pure oral progestagen [1].

In order to explain its hormonal properties at the molecular level, we analysed the binding of allylestrenol, its 3-keto metabolite and a number of reference compounds to progesterone, androgen and oestrogen receptors in MCF-7 cells, glucocorticoid receptors in IM-9 cells and human SHBG, an important carrier protein for androgens.

EXPERIMENTAL

Labelled ligands

Org 2058: 16α-ethyl-21-hydroxy-19-nor-4-pregnene-3,20-dione; Estradiol: 1,3,5(ω)-estratriene-3,17β-diol; 5α-Dihydrotestosterone: 17β-hydroxy-5α-androstan-3-one; Dexamethasone:9-fluoro-11β, 17,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione; Testosterone: 17β-hydroxy-4-androstene-3-one.

Competitors

Progesterone: 4-pregnene-3,20-dione; Allylestrenol:17α-allyl-17β-hydroxy-4-estren; 3-Keto-metabolite of allylestrenol: 17α-allyl-17β-hydroxy-4-estren-3-one; Cortisol: 11β,17,21-trihydroxy-4-pregnene-3,10-dione; Norethisterone: 17-hydroxy-19-nor-17α-pregn-4-en-10-yn-3-one; Medroxyprogesterone acetate (MPA): 17-hydroxy-6α-methyl-4-pregnene-3,20-dione 17-acetate; Cyproterone acetate: 6-chloro-1β,2β-dihydro-17-hydroxy-3′H-cyclopropa [1,2]-1,4,6-pregnatriene-3,20-dione 17-acetate; Metribolone (R1881): 17β-hydroxy-17α-methyl-4,9,11-estratriene-3-one; Spironolactone: 17-hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactone 7-acetate.

Materials

[7-^{3}H]Org 2058 (1.1×10^{12} Bq/nmol), [1,2,4,5, 6,7-^{3}H]5α-dihydrotestosterone (5.4×10^{12} Bq/mol), [2,4,6,7-^{3}H]estradiol (3.4×10^{12} Bq/mol), [4,5,6,7-^{3}H] dexamethasone (2.6×10^{12} Bq/nmol) and [1,2,6,7-^{3}H]testosterone (4.0×10^{12} Bq/nmol) were manufactured by the Radiochemical Centre, Amersham, U.K. All chemicals were from Baker Co. Activated charcoal No. C-5260, hydroxylapatite and DNA-cellulose (5 mg DNA/g cellulose) were bought from Sigma.

Human breast tumour and human lymphoid cell lines

The MCF-7 human breast tumour cell line and the human IM-9 lymphoid cell line were obtained from Dr Charles M. McGrath (Michigan Cancer Foundation) and Dr Lesniak (National Institutes of Health, Bethesda, U.S.A.) respectively. The conditions for growth of MCF-7 cells were as described before [9]. IM-9 cells were grown as a suspension culture in RPMI-1640 medium (Flow) supplemented with 2.0 g/l $NaHCO_3$, antibiotics and 100 ml/l FCS (Biofluids lot No. 828080) in 4 roller flasks at 5/60 Hz, 27°C. Growth medium was refreshed 3 times a week and the cell concentration was adjusted to between 5×10^5 and 5×10^6 cells/ml. At the end of a growth period cells were harvested by trypsinization, (0.5 g/l trypsin; 15 min, 37°C) and centrifugation (10 min, 2000 N/kg). For whole cell receptor assays (37°C) three identical 96-well tissue culture dishes (Greiner) were filled with 1×10^6 cells/well, in 0.100 ml serum-free culture medium.

Competitive binding studies

The binding studies in cytosol preparations from MCF-cells were carried as described previously [10] for human and rabbit myometrial progesterone receptors, with the exception that the charcoal technique was used for the estimation of all binding affinities and that the TE buffer containing molybdate (10 mmol/l Tris–HCL, 1 mmol/l EDTA, 0.5 mml/l dithioerythritol, 2 g/l NaN_3, 10 mmol/l sodium molybdate, pH7.4 at 4°C) was used for the preparation of the cytosol fraction of MCF-7 cells.

The competitive binding in intact MCF-7 cells and IM-9 cells was performed as described previously for MCF-7 cells [9]. Briefly aliquots (0.100 ml) of cell suspensions (10^7 cells per ml) were added to the wells of microtitration plates and incubated for 45 min at 37°C with the appropriate ligand (15.6 nmol/l[^{3}H]Org 1058, 7.2 nmol/l[^{3}H]dexamethasone, 3.9 nmol[^{3}H] 5 dihydrotestosterone or 3.9 nmol[^{3}H]17β-oestradiol) in 0.100 ml medium minus serum and increasing concentrations of unlabelled competitor. Non-specific binding of tritiated ligand was assessed in the presence of 50-fold excess unlabelled ligand. A freeze-thaw extraction procedure (followed by a treatment with a dextran-coated charcoal suspension) was used for the detection of receptor bound ligand [9].

The estimation of binding affinities for SHBG has been described before [10].

DNA-cellulose and hydroxylapatite binding

The DNA-cellulose and hydroxylapatite binding assays were performed as described by Holbrook *et al.*[11], using separate minicolumns containing only DNA-cellulose and hydroxylapatite respectively.

RESULTS

Binding of tritiated ligands with receptors in intact MCF-7 and IM-9 cells

Increasing concentrations of [^{3}H]Org 2058, [^{3}H]17β-oestradiol, [^{3}H]5α-dihydrotestosterone were incubated with inact MCF-7 cells at 37°C and increasing concentrations of [^{3}H]dexamethasone were incubated with intact IM-9 cells at 37°C. The data were analysed using Scatchard plots. The equilibrium dissocation constants for the tritiated Org 2058-progesterone 17β-oestradiol-oestrogen, 5α-dihydrotestosterone-androgen and dexamethasone–glucocorticoid receptor complexes were 1.9×10^{-9} mol/l, 0.34×10^{-9} mol/l, 0.30×10^{-9} mol/l and 8×10^{-9} mol/l respectively.

Binding of steroid hormone receptor complexes to DNA-cellulose

The cytosol fraction from MCF-7 cells, incubated for 16 h at 4°C with [^{3}H]oestradiol, [^{3}H]Org 2058 or [^{3}H]5α-dihydrotestosterone, contained mostly non-transformed receptor–complexes (80, 60 and 80% respectively) which do not bind to DNA—cellulose as was also found in a previous study [12]. However, when steroid hormone receptor complexes were obtained from MCF-7 cells, incubated for 45 min at 37°C with tritiated steroids, only transformed receptor complexes (the DNA-binding forms) were demonstrated.

Table 1. Relative binding affinity of allylestrenol, its 3-keto metabolite and some reference compounds for the progesterone, oestrogen and glucocorticoid receptors in intact human cells at 37°C

Compound	Relative affinity (intact cells at 37°C) Progesterone receptor (MCF-7 cells)	Oestrogen receptor (MCF-7 cells)	Glucocorticoid receptor (IM-9 cells)
Org 2058	100% (reference)	<0.2	<0.2
Estradiol	<0.2	100% (reference)	<0.2
Dexamethasone	<0.2	<0.2	100% (reference)
Progesterone	12.9 ± 1.5* (4)†	<0.2	9 ± 2 (3)
Cortisol	<0.2	<0.2	40 ± 12 (4)
Norethisterone	41 ± 3 (4)	<0.2	7.7 ± 1.0 (3)
Medroxyprogesterone acetate	66 ± 8 (5)	<0.2	29 ± 2.5 (3)
Allylestrenol	<0.2	<0.2	<0.2
3-Keto-allylestrenol	24 ± 3 (4)	<0.2	9.8 ± 1.0 (3)

*Mean ± SEM.
†Number of individual experiments.

Table 2. Relative binding affinity of allylestrenol, its 3-keto metabolite and reference compounds for the androgen receptor in MCF-7 cells and human SHBG

	Relative affinity %)		
	Androgen receptor (5α-dihydrotestosterone = 100%)		Human SHBG (at 37°C) (testosterone = 100%)
	Cytosol at 4°C	Intact cells at 37°C	
Testosterone	32 ± 4* (8)†	38 ± 2 (6)	100% (reference)
5α-Dihydrotestosterone	100% (reference)	100% (reference)	446 ± 79 (5)
Metribolone (R 1881)	100 ± 4 (3)	110 ± 6 (3)	1 (1)
Norethisterone	17 (1)	7 ± 0.7 (8)	10 ± 0.8 (4)
Medroxyprogesterone acetate	29 ± 2 (3)	8 ± 1.1 (5)	1 (1)
Cyproterone acetate	14.1 ± 1.5 (4)	1.2 + 0.4 (4)	1 (1)
Spironolactone	5.1 (1)	0.5 (1)	
Progesterone	5.8 ± 0.7 (3)	0.5 (1)	1 (1)
Allylestrenol	2.8 ± 0.2 (3)	<0.2	0.9 ± 0.2 (3)
3-Keto-allylestrenol	29 ± 3 (4)	4.5 ± 1 (3)	2.8 ± 0.2 (3)

*Mean ± SEM.
†Number of individual experiments.

Relative affinities for the progesterone, oestrogen and glucocorticoid receptors

The relative affinities of allylestrenol, its 3-keto metabolite and reference compounds for the progesterone and oestrogen receptors in intact MCF-7 cells and the glucocorticoid receptor in intact IM-9 cells are summarized in Table 1.

Allylestrenol displayed no detectable affinity for oestrogen, progesterone and glucocorticoid receptors. The affinity of 3-keto-allylestrenol for the progesterone receptor in intact MCF-7 cells was twice that of progesterone and about half that of norethisterone. 3-Keto-allylestrenol displayed no measurable interaction with the oestrogen receptor. The binding of 3-keto-allylestrenol to the glucocorticoid receptor in IM-9 cells was similar to that of progesterone and norethisterone, one-third that of medroxyprogesterone acetate and one-fourth that of cortisol.

Relative affinities for the androgen receptor and human SHBG

The relative binding affinities of allylestrenol, its 3-keto metabolite and reference compounds for the androgen receptor in the cytosol fraction (4°C) and in intact MCF-7 cells (37°C) and for human SHBG are given in Table 2. At 4°C, the binding of 3-keto-allylestrenol to the androgen receptor was the same as that of medroxyprogesterone acetate and about 1.5 times stronger than that of norethisterone; at 37°C the binding affinity of 3-keto-allylestrenol was half that of norethisterone and medroxyprogesterone acetate. 3-Keto-allylestrenol, allylestrenol and reference compounds which did not have androgenic activity (progesterone) or had anti-androgenic activity (cyproterone acetate and spironolactone) displayed 7–12 times lower affinities for the androgen receptor in intact cells than in cytosol whereas compounds with androgenic activity (R 1881 and testosterone) displayed similar or slightly lower relative affinity for the androgen receptor in intact cells than in cytosol. The affinity of allylestrenol and its 3-keto metabolite for human SHBG was found to be very low.

DISCUSSION

In this study we have estimated the relative binding affinities of allylestrenol (which has been described as a pure oral progestagen [1]), its 3-keto metabolite and reference compounds for progesterone, oestrogen, androgen and glucocorticoid receptors and human SHBG. The 3-keto-allylestrenol metabolite displayed much stronger affinity for progesterone, androgen and glucocorticoid receptors and SHBG than allylestrenol showing that addition of the 3-keto group enhances the corresponding biological activities at the cellular level and binding to SHBG. Allylestrenol and 3-keto-allylestrenol display unmeasurably low affinity for the oestrogen receptor confirming the lack of oestrogenic activity found in animal studies [1]. The binding of 3-keto-allylestrenol to the glucocorticoid receptor was also relatively low and comparable to that of progesterone and norethisterone; this explains the lack of adrenal-inhibiting properties of allylestrenol [1]. Our data on the binding of 3-keto-allylestrenol to the progesterone receptor confirm that allylestrenol (in the form of its 3-keto metabolite) is a progestational compound as found in clinical studies [6] and animal studies [1]; in the latter its activity was half that of norethisterone [1]. With regard to the androgen receptor in intact human cells 3-keto-allylestrenol bound weaker than the progestagens norethisterone and medroxyprogesterone acetate which may explain its apparent lack of androgenic activity on the anogenital distance in foetal rats (personal communication, Dr J. van der Vies), in the Hershberger test [1] and in normal adult rats [13]. The results obtained at 4°C do not correlate with the results in biological studies. This confirms that binding studies with androgen receptors in the cytosol fraction cannot be used for differentiating androgenic and anti-androgenic properties of progestagens, [14, 15]. The combination of investigations carried out in the present study in intact cells at 37°C and in a cytosol preparation of these cells distinguishes compounds with androgenic (testosterone) and weakly androgenic activity (norethisterone and medroxyprogesterone acetate) from progestagens which lack androgenic activity or have anti-

androgenic activity (allylestrenol, progesterone, cyproterone acetate and spironolactone). The natural androgens testosterone and 5α-dihydrotestosterone binds very strongly to SHBG. Allylestrenol and its 3-keto metabolite bind weakly and this suggests that androgenic effects due to displacement of endogenous androgens from SHBG [16] will not occur.

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