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SCREENING FOR ANTI-HORMONES BY RECEPTOR STUDIES

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SUMMARY

The class of steroidal anti-hormones which exert their effect by interaction with the target cell cytosol receptor has been studied with a view to developing a method of screening for highly specific anti-hormonal agents. It has been shown that compounds with moderate affinity for the receptor may be classified as either true agonists or impeded agonists with possible antagonistic activity, whereas compounds with weak affinity are weak agonists, but also likely antagonists. These studies have been carried out on the androstanolone, estradiol, aldosterone and progesterone receptors and are illustrated by the results obtained with a potent anti-androgen (R 2956), anti-estrogen (RU 16117), anti-aldosterone (spironolactone) and anti-progesterone (R 2323). The primary advantage of running studies in parallel on several receptors resides in the possibility of classifying the anti-hormones according to whether they possess affinity for a given receptor, thus providing a criterion for the selection of highly specific compounds virtually devoid of hormonal side-effects. Moreover, since similar hormonal receptors with the same physico-chemical parameters and specificity exist in various species including man, animal receptor data may be extrapolated to human studies, allowance being made in the evaluation of activity for variations in pharmacokinetics and metabolism.

INTRODUCTION

Studies over the last decade have firmly established that cytosol from hormone-sensitive tissues contains hormone-specific receptors [1–6]. Moreover, for any particular hormone, this cytosol receptor has the same physicochemical properties in different species including man, as for instance has been lately demonstrated in the case of the progestin receptor [7–10]. The search for potent hormonal steroids has therefore been focussed on the determination of the basic structural requirements for high affinity steroid–receptor interactions [10–13] in order to construct the kernel, which having undergone the appropriate chemical modifications to ensure good pharmacokinetics and metabolism in man, will give the ideal clinically active form. Until now, however, all these studies have considered compounds in relation to a single receptor only and no attention has been given to the elaboration of the hormonal profile of a compound *via* receptor binding studies. Such a study might be of special interest to deduce the requirements for dissociation of basic hormonal activities and for possible hormone antagonism. Moreover, these requirements might lead to the conception of a compound which interacts highly specifically, but weakly, with the receptor and thus prevents it from undergoing the conformational changes necessary to induce the biological response [14].

The following study on several potent anti-hormones was performed in order to judge the value of receptor binding studies carried out in parallel under standardized conditions for the screening of hormonal antagonists acting at the cytosol receptor level.

EXPERIMENTAL RESULTS

Hormone-specific binders in cytosol from hormone-sensitive tissue

Figures 1 to 4 illustrate the existence of hormone-specific binding components in hormone-sensitive tissue cytosol from a variety of species. They show the sedimentation in sucrose gradients of complexes formed between labelled natural hormone (estradiol, androstanolone, progesterone and aldosterone) and cytosol receptor. In each case, addition of excess radioinert hormone suppresses the labelled peak, thus demonstrating the saturability of the cytosol receptor.

The choice of tissue in these examples was governed either by their use in classic tests for the measurement of biological activity or by the possible clinical implications of drugs acting on these tissues.

Estradiol binds to a cytosol receptor in the mouse uterus (Fig. 1a) which is widely used for routine evaluation of uterotrophic and anti-estrogenic activities [15]. It also binds specifically to the anterior and, to a lesser extent, middle regions of the rat hypothalamus [16, 17], but as expected does not bind to nervous tissue which does not respond to a hormonal stimulus (cortex) (Fig. 1b). The action of estradiol, both at a peripheral and central level, is therefore mediated by interaction with a specific cytosol receptor.

Androstanolone is bound in rat prostate cytosol (Fig. 2a)—rat prostate is the classic material for determination of androgenic and anti-androgenic activities [18]—and also by the sebaceous gland of the hamster (Fig. 2b) [19], a model used for the study of anti-androgens in the treatment of acne [20].

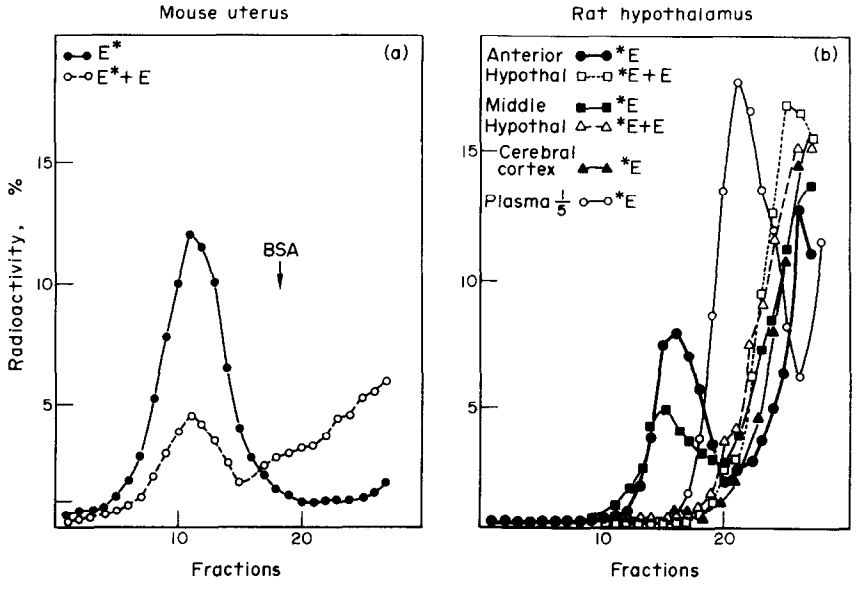

Fig. 1. *In vitro* binding of labelled estradiol to uterine cytosol from immature mice (a) and to rat hypothalamus and cerebral cortex cytosol (b).

Homogenization of tissue (0·33 g/ml (a) or 0·66 g/ml (b)) in an ice-cooled Teflon–glass homogenizer in 0·01 M Tris–HCl (pH 7·4), 1·5 mM EDTA buffer (a) containing 12 mM thioglycerol (b); centrifugation at 105,000 *g* for 60 min; incubation of 1 ml cytosol with 2 nM (a) or 0·2 nM (b) [6,7-^{3}H]-estradiol (58 Ci/mmol) either alone or in the presence of 200 nM (a) or 20 nM (b) radioinert estradiol for 60 min (a) or 30 min (b) at 0°C; layering (0·3 ml) on a 6·5–20% sucrose gradient; centrifugation at 42,000 rev./min (a) or 47,000 rev./min (b) for 18 h (a) or 16 h (b) at 4°C in a Spinco $L_2$65B (SW 50·1 rotor); radioactivity count on 2-drop fractions.

Progesterone is known to bind to a specific uterine cytosol receptor common to various species [10] as illustrated in Fig. 3 for human endometrium and myometrium. A potent synthetic progestin R 5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione), which is not bound by the CBG-like receptor which interacts with progesterone, but which is bound by the "7-8S" progesterone receptor, was used to bring this binding into evidence. Competition by the potent progestin, norgestrel (13-ethyl-17-hydroxy-18,19-dinor-17α-4-pregnen-20-yn-3-one) [21], and by the impeded progestin, R 2323 (13-ethyl-17-hydroxy-18,19-dinor-17α-4,9,11-pregnatrien-20-yn-3-one) [22–24], has established the progestin-specificity of this receptor.

Aldosterone, the only hormone considered here which is not a sex-hormone, binds to different classes of specific cytosol receptors present in the kidney [25] and is active in stimulating transepithelial Na^+ transport. The transfer of electrolytes is commonly studied on the frog skin [26] and, as shown in Fig. 4b, an aldosterone-specific receptor has been identified in this tissue.

Methodology to track the hormone-specific receptor implicated in a pure biological response

Cytosol contains various binding components, other than the hormone-specific receptor chosen for study, the influence of which has to be eliminated if the true competitive effect of the hormone is to be determined. These components are:

Non-specific binding whether of tissular or plasmatic (contamination) origin. This may be minimized by carefully washing the tissue or by diluting. For instance, non-specific binding is negligible in very dilute

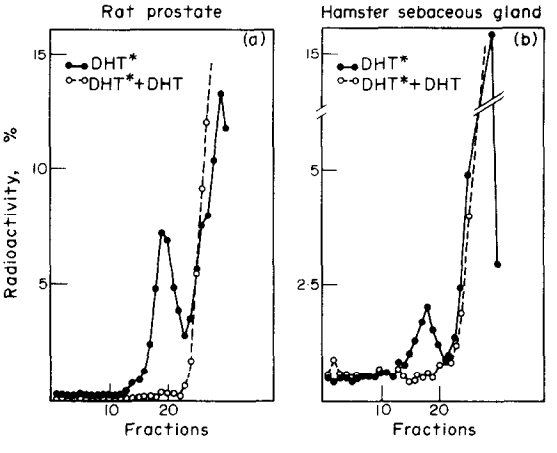

Fig. 2. *In vitro* binding of labelled androstanolone to ventral prostate cytosol from castrated rats (a) and to sebaceous gland cytosol from castrated hamsters (b).

Incubation of minces (500 mg (a) or 80 mg (b)) in 5 ml Krebs–Ringer Phosphate buffer with 10 nM [1α-^{3}H]-androstanolone (24 Ci/mmol) either alone or in the presence of 5 μM (a) or 1 μM (b) radioinert androstanolone for 30 min at 0°C; homogenization in an ice-cooled Teflon–glass homogenizer in 4 ml (a) or 1·5 ml (b) 0·01 M Tris–HCl (pH 7·4), 1·5 mM EDTA, 2 mM mercaptoethanol buffer; centrifugation at 105,000 *g* for 60 min; layering (0·8 ml) on a 5–35% glycerol gradient; centrifugation at 40,000 rev./min for 18 h at 4°C in a Spinco $L_2$65B (SW 41 rotor); radioactivity count on 4-drop fractions.

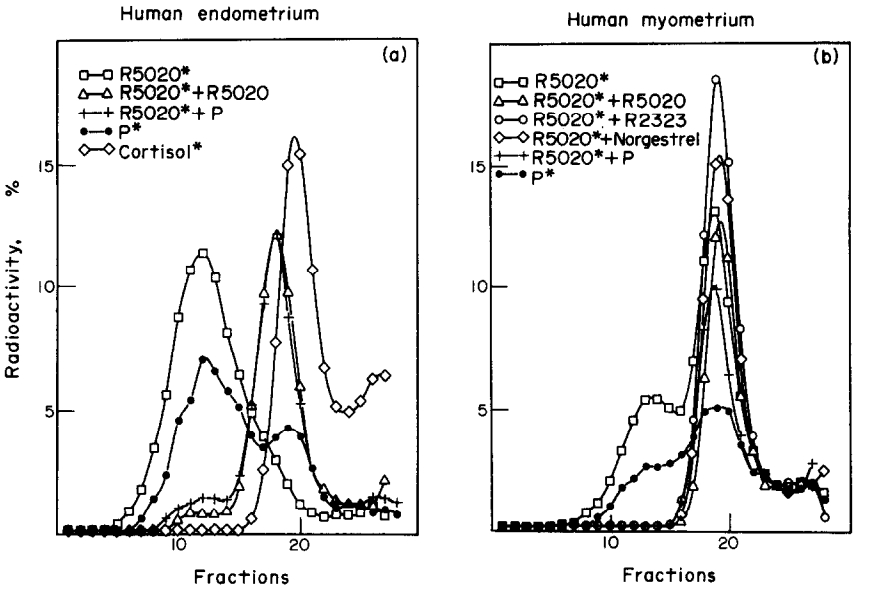

Fig. 3. *In vitro* binding of labelled progestin to human endometrial (a) and myometrial (b) cytosol. Homogenization of 0·33 g/ml tissue from estrogen-treated women in an ice-cooled Teflon–glass homogenizer in 0·01 M Tris–HCl (pH 7·4), 1·5 mM EDTA, 12 mM thioglycerol, 10% (v/v) glycerol buffer; centrifugation at 105,000 *g* for 60 min; incubation of 1 ml cytosol with 6 nM labelled hormone ([6,7-^{3}H] R 5020 (51 Ci/mmol), [1-^{3}H]-progesterone (27 Ci/mmol)) either alone or in the presence of 100 nM radioinert steroid for 60 min at 0°C; layering (0·3 ml) on a 5–20% sucrose gradient prepared in homogenization buffer; centrifugation at 45,000 rev./min for 16 h at 4°C in a Spinco $L_2$65B (SW 50·1 rotor); radioactivity count on 2-drop fractions. R 5020 = 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; R 2323 = 13-ethyl-17-hydroxy-18,19-dinor-17α-4,9,11-pregnatrien-20-yn-3-one; Norgestrel = 13-ethyl-17-hydroxy-18,19-dinor-17α-4-pregnen-20-yn-3-one.

media as used for radioimmunoassay [27]. Otherwise, two techniques are particularly useful: differential dissociation of specific and non-specific binding by the use of Dextran-coated charcoal [28] and representation of data in a proportion graph for subsequent mathematical analysis of the binding curve [29, 30].

Enzymes. By the use of cytosol at 0°C and also by choosing the active metabolite for binding studies, the effect of enzymatic activity can be obviated. Testosterone is primarily active *via* 5α-reduction to androstanolone which binds to the rat prostate cytosol receptor with an intrinsic association constant of $1 \times 10^9\,M^{-1}$, although as yet there is no unequivocal evidence that testosterone is inactive *per se*. There is no 5α-reductase activity in cytosol [31], but on the contrary, enzymes reducing androstanolone to the diols are present [32]. The use of androstanolone with cytosol at 0°C therefore meets both requirements of the use of the active metabolite with high affinity for the receptor and reduction of undesirable enzymatic activity.

Binding to a specific plasma protein. The presence of cytosol-contaminating specific plasma binding proteins, such as estradiol binding protein (EBP) in the immature rat [33], sex steroid binding protein (SBP) which binds estradiol and testosterone in man [34] and progesterone binding globulin (PBG) in the pregnant guinea-pig [35, 36] restricts the use of these species for the study of estradiol, testosterone and progesterone binding respectively. In some instances, however, the plasma protein is present in all species, e.g. corticosteroid binding globulin (CBG) [37], and methods for eliminating the binding have to be devised. For instance, in uterine cytosol, progesterone exhibits saturable-binding not only to a "7-8S" progestin-specific receptor but also to a CBG-like component [7, 8, 38, 39]. Several techniques have already been developed to track the progestin-specific receptor, namely, pooling the fractions corresponding to a single peak of a sucrose gradient [8], precipitation of total cytosol with 40% ammonium sulfate [9], use of an excess of a ligand specific to the interfering binder or of immunoadsorption by an antibody. The technique, which, however, in our laboratories has given the most spectacular results, is the use of a tag such as the highly potent progestin, R 5020, which is specifically bound by the "7-8S" but not "4-5S" progesterone receptor [7–10].

Binding to a specific tissue receptor other than the receptor under study. In this case, methods such as separation of a gradient into pooled fractions and precipitation by ammonium sulfate cannot be used. A synthetic tag bound by the receptor under study only would distinguish between several receptors, but is not always available. Such a tag is for instance lacking for the study of aldosterone which exhibits two kinds of specific binding and low non-specific binding in rat kidney cytosol. According to our studies (Fig. 5), aldosterone binds to the low affinity site (the so-called "glucocorticoid receptor") with an intrinsic association constant (K_s) of $8 \times 10^7\,M^{-1}$

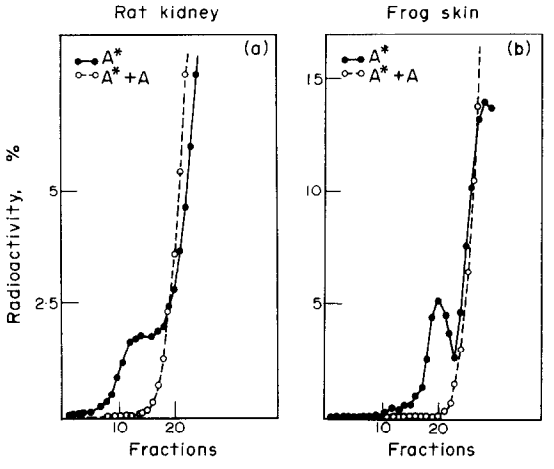

Fig. 4. *In vitro* binding of labelled aldosterone to kidney cytosol from adrenalectomized rats (a) and to skin cytosol from frogs maintained for 3 days in 6‰ NaCl (b).

Kidney cytosol: Preparation of slices from kidneys perfused with 50 ml 0·01 M Tris–HCl (pH 7·4), 1·5 mM EDTA, 12 mM thioglycerol, 10% (v/v) glycerol buffer; incubation of slices (500 mg) in 5 ml of Krebs–Ringer Phosphate buffer with 5 nM [1,2-^{3}H]-aldosterone (53·2 Ci/mmol) either alone or in the presence of 500 nM radioinert aldosterone for 30 min at 25°C; homogenization of incubated slices in an ice-cooled Teflon–glass homogenizer in above glycerol buffer; centrifugation at 105,000 *g* for 60 min; layering (0·3 ml) on a 5–20% sucrose gradient; centrifugation at 42,000 rev./min for 17 h in a Spinco $L_2$65B (SW 50·1 rotor); radioactivity count on 2-drop fractions.

Skin cytosol: Homogenization of mucosal cells from ventral skin (250 mg) in an ice-cooled Teflon–glass homogenizer in 1 ml of 0·1 M Tris–HCl (pH 7·4), 3 mM $CaCl_2$ buffer; incubation of 2 ml homogenate with 0·5 nM [1,2-^{3}H]-aldosterone (53·2 Ci/mmol) either alone or in the presence of 50 nM radioinert aldosterone for 30 min at 0°C; centrifugation at 105,000 *g* for 60 min; layering (0·8 ml) on a 5–35% glycerol gradient; centrifugation at 40,000 rev./min for 18 h in a Spinco $L_2$65B (SW 41 rotor); radioactivity count on 4-drop fractions.

(number of binding sites (N_s) = 2×10^{-9} M), a result in agreement with published values [40], and to the high affinity site (the so-called "mineralocorticoid receptor") with a K_s of 1×10^{10} M^{-1} ($N_s = 0{\cdot}02 \times 10^{-9}$ M). This latter result differs from previous estimates [40], the discrepancy probably being due to the higher precision of the mathematical analysis used for the interpretation of a proportion graph.

Attempts have been made to eliminate the influence of the low affinity receptor by saturation with dexamethasone [13], but this compound is in fact bound by both specific binders [40] and the extent to which it interferes with each binder cannot be evaluated with precision. In the present study, only competition for total aldosterone binding has been measured.

Screening of four potent anti-hormones on single hormone-specific receptors

Having eliminated all undesirable binding components by the choice of suitable systems, species and tags, we are now in a position to study the interaction between various ligands and a well-defined specific cytosol hormone receptor. In the present study, interest has been focussed on the use of such receptors to further characterize the class of anti-hormones, which act at the cytosol receptor level. In order to evaluate the merits of this system, four potent anti-hormones, known to bind to a specific cytosol receptor, have been chosen. Their structures, as compared to the natural hormones, are given in Fig. 6. RU 16117 [17], the 11α isomer of the very potent estrogen, R 2858 [11, 17, 33], is a highly active anti-estrogen with very weak uterotrophic activity. R 2956 [41–43], a dimethyl derivative of an extremely potent androgen, R 1881 [44], is a powerful testosterone antagonist with very low androgenic activity. R 2323 [22–24], a triene otherwise analogous to a potent progestin, norgestrel, is an anti-progesterone interfering with gestation and exhibiting moderate progestomimetic activity. Spironolactone [45, 46] is a well-known anti-aldosterone.

Various aspects of the binding and biological activity of these compounds have been analyzed individually in Tables 1 and 2. Full data are given in Table 3 which also succinctly describes the experimental conditions used for biological activity determination and competition studies. Preference was given to standardizing experimental conditions in order to obtain entirely comparable results, rather

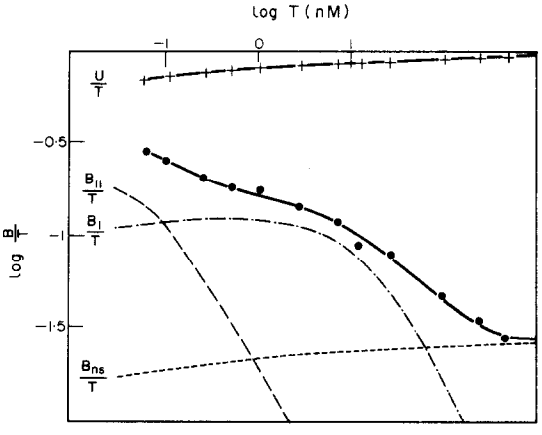

Fig. 5. Proportion graph of aldosterone binding to rat kidney cytosol.

Kidneys from rats adrenalectomized 8 days previously were perfused with 50 ml of ice-cold 10 mM Tris–HCl (pH 7·4), 1·5 mM EDTA, 0·25 M sucrose buffer, minced, homogenized in 1/3 (w/v) of the same buffer in a Teflon–glass homogenizer and then centrifuged at 105,000 *g* for 1 h. The high-speed supernatant (cytosol) (12 mg protein/ml) was then incubated, in fractions of 0·5 ml, with 12 different concentrations of aldosterone ($0{\cdot}05 \times 10^{-9}$ to 500×10^{-9} M). Binding was measured in triplicate by the Dextran-coated charcoal adsorption method: 100 μl of cytosol in the presence of 50 μl of charcoal were stirred for 10 min at 0°C then centrifuged at 1500 *g* for 10 min. The radioactivity of 100 μl supernatant samples was measured. Results are represented in a proportion graph in which the log of the fraction bound (B/T) and unbound (U/T) steroid is plotted against the log of the total ligand concentration (T). This S-shaped curve characteristic of two specific binding systems (B_I and B_{II}) in the presence of non-specific binding (B_{ns}) has been decomposed into its constituent elements, thus enabling the computerized determination of binding parameters [29,30].

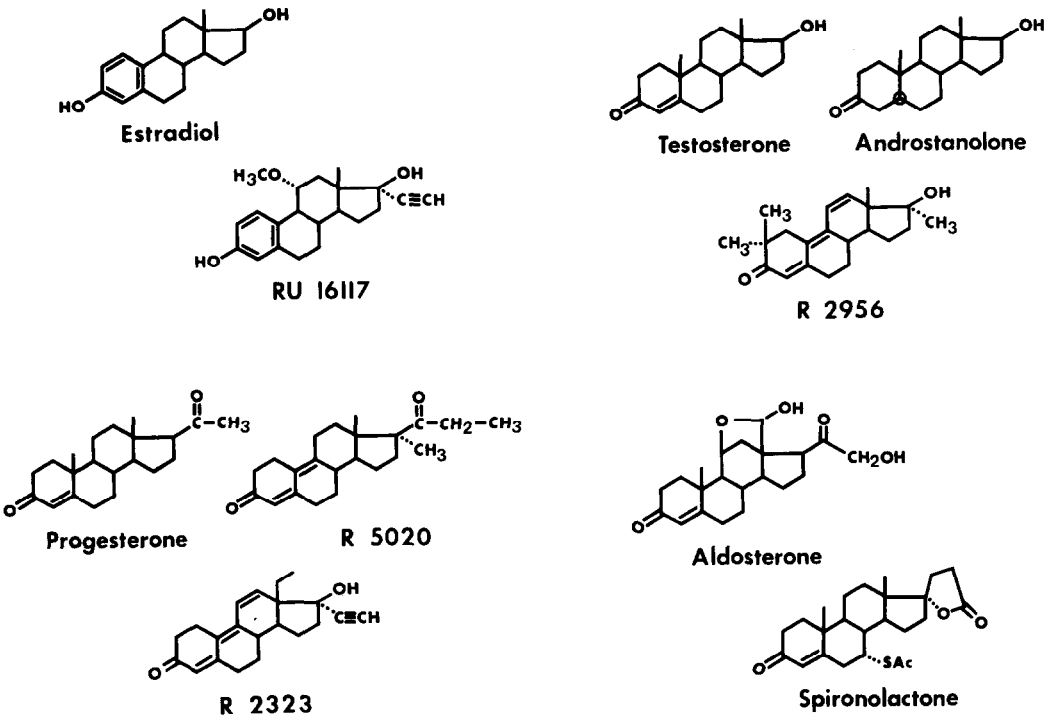

Fig. 6. Test steroids.

Estrogen and anti-estrogen: Estradiol, RU 16117 (11α-methoxy-19-nor-17α-1,3,5(10)-pregnatrien-20-yne-3,17-diol); Progestins and anti-progesterone: Progesterone, R 5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione). R 2323 (13-ethyl-17-hydroxy-18,19-dinor-17α-4,9,11-pregnatrien-20-yn-3-one; Androgens and anti-androgen: Testosterone, Androstanolone (17β-hydroxy-5α-androstan-3-one), R 2956 (17β-hydroxy-2,2,17-trimethyl-4,9,11-estratrien-3-one); Mineralocorticoid and anti-mineralocorticoid: Aldosterone (11β,18-epoxy-18,21-dihydroxy-4-pregnene-3,20-dione), Spironolactone (17-hydroxy-7α-mercapto-3-oxo-17α-4-pregnene-21-carboxylic,γ-lactone,7-acetate). All compounds were synthesized at the Roussel–Uclaf Research Centre and tested for purity (>98%) by thin-layer chromatography.

than to the choice of optimal conditions for each receptor. Binding results have been expressed as the ratio of natural hormone concentration to test-compound concentration required for 50% displacement of labelled compound from specific binding sites. It should be noted that this form of expression does not reflect the true ratio of the intrinsic association constants. Biological activity, measured in classic tests, is given as the ratio of the active natural hormone dose to test-compound dose giving rise to a significant response or, in the case of hormone antagonism, as dose multiples required to counter the effect of an active natural hormone dose.

A pharmacological response measured in the animal varies according to many parameters: species, sex, metabolic biotransformation routes undergone by the test-compound etc.... and can therefore only be used to define, in general terms, the activity profile of a compound. It cannot be equated with the intrinsic ability of this compound to induce a specific response which can only be expressed in molecular terms [10]. For this reason, no attempt has been made to draw quantitative correlations between binding and biological activities.

Results confirm that the chosen anti-hormones compete for the cytosol receptor implicated in the hormonal response they counteract, giving rise to slight hormonal activity (Table 1, top section). Most of the compounds in fact compete for more than one receptor. For instance, R 2323 binds not only to the progestin receptor (0·5) but also to the androgen (0·3) and aldosterone (0·3) receptors, thus giving rise to accessory androgenic (0·02) and, to a much lesser extent, mineralocorticoid (0·00005) activity (Table 1, middle section). Receptor specificity and sensitivity are more marked in the case of the estradiol receptor, which only binds its own class of compounds tightly, than the androstanolone receptor which interacts with most of the natural hormones and with all but one of the chosen antagonists (Table 1, bottom section). The slight uterotrophic activity of androgens at pharmacological doses may in fact be accounted for by binding to a uterine androgen receptor [47].

Table 2 brings to light certain observations regarding binding and activity antagonism. As has already been established in previous investigations, the four chosen test-compounds, known to bind to a specific cytosol receptor, are all hormone antagonists in biological activity tests (Table 2, top section). Some, by interfering with more than one receptor, exert dual activity. Whereas R 2956 is solely anti-androgenic, spironolactone exerts both anti-aldosterone and anti-androgenic activities [48–50] (Table 2, middle section). Certain compounds exert another antagonistic action without interacting with the receptor. For

Table 1. Relative cytosol binding (a) and relative biological activity (b)

	Receptor	Tissue	Binding	Activity
RU 16117	Estrogen	Mouse uterus	0.05	0.01
R 2956	Androgen	Rat prostate	0.2	0.02
R 2323	Progestin	Rabbit uterus	0.5	0.5

	Receptor	Tissue	Binding	Activity
	Androgen	Rat prostate	0.3	0.02
R 2323	Progestin	Rabbit uterus	0.5	0.5
	Aldosterone	Rat kidney	0.3	0.00005

	Estrogen Mouse uterus		Androgen Rat prostate	
	Binding	Activity	Binding	Activity
ESTRADIOL	1	1	0.1	Inactive
RU 16117	0.05	0.01	†	Inactive
ANDROSTANOLONE	†	0.001	1	1
R 2956	†	0.003	0.2	0.02
PROGESTERONE	†	Inactive	0.1	Inactive
R 5020	†	Inactive	0.1	Inactive
R 2323	†	0.001	0.3	0.02

(a) Relative binding = Ratio of concentrations (reference-compound/test-compound) required for 50% displacement of labelled compound from specific binding sites.

† = A concentration of 2500 nM does not compete.

(b) Relative activity = Ratio of active doses (reference compound/test-compound) giving rise to a significant response.

instance, RU 16117 is anti-estrogenic *via* binding to the estradiol receptor (Table 2, top section), but anti-progestomimetic *via* another mechanism. Similarly, none of the tested progestins bind to the estradiol receptor but all are anti-uterotrophic (Table 2, bottom section).

Table 3, in summarizing all the results, reveals the complexity of action of some of these anti-hormones, in particular of R 2323. This compound is an anti-progesterone [10] *via* binding to the progestin receptor (0·5), but also binds to other receptors (androgen (0·3) and aldosterone (0·3)) without however exerting any marked biological activity. Although slightly androgenic (0·02) and, to an even lesser extent, aldosterone-like (0·00005), it has neither anti-androgen nor anti-aldosterone properties. On the other hand, it exhibits some uterotrophic (0·001) and anti-estrogenic (100) activity without binding to the estrogen receptor. Its uterotrophic activity could be explained by binding to a uterine androgen receptor. Or, depending upon the species under study and its state of maturity, it could be considered as the resultant of biotransformation into an estrogen-like compound and/or of interference with a mechanism regulating the sensitivity of tissues to estrogen. Such an estrogen-like action might potentiate its primary anti-hormonal activity, namely, that of being an anti-progesterone.

CONCLUSIONS

The above results have brought to light two mechanisms for anti-hormonal action, the one acting directly *via* the corresponding cytosol receptor, the other by-passing this receptor. Only the former mechanism, however, can be envisaged as a basis for a systematic study of likely anti-hormones. The present investigation attempts to determine whether it is possible to screen for anti-hormones by receptor studies and, thereby also, define the nature of the anti-hormone obtained.

In vitro receptor studies have indeed revealed that it is possible to determine, by molecular manipulation, the structure which possesses high affinity for a receptor and to equate this affinity with a certain basic hormonal activity, if extraneous factors such as pharmacokinetics and metabolism are taken into account [10, 11]; they have also indicated that it is possible by similar molecular manipulation to dissociate these basic hormonal activities and deduce the structure active at the level of a single receptor only. Thus, the structural requirements necessary for high affinity for a specific hormonal receptor can be defined. Moreover, by careful substitution of the highly specific molecule thus obtained, at points other than those intervening directly in the interaction with the receptor, it should be possible to construct a molecule which will retain some affinity for the receptor, will retain its high specificity towards this receptor, but will either acquire improved pharmacokinetic and metabolic properties as a result of the new substitutions or lose its ability to induce the full hormonal response as a result of "inactivation" of the steroid-receptor complex by steric hindrance.

Table 2. Relative cytosol binding (a) and relative activity antagonism (b)

	Receptor	Tissue	Relative binding	Activity antagonism
RU 16117	Estrogen	Mouse uterus	0.05	10
R 2956	Androgen	Rat prostate	0.2	10
R 2323	Progestin	Rabbit uterus	0.5	10
SPIRONOLACTONE	Aldosterone	Rat kidney	0.5	1000

	Androgen Rat prostate		Aldosterone Rat kidney	
	Relative binding	Activity antagonism	Relative binding	Activity antagonism
R 2956	0.2	10	0.02	Inactive
SPIRONOLACTONE	0.3	20	0.5	1000

	Receptor	Tissue	Relative binding	Activity antagonism
ESTRADIOL	Progestin	Rabbit uterus	0.002	0.025
RU 16117			†	10
PROGESTERONE	Estrogen	Mouse uterus	†	300
R 5020			†	3
R 2323			†	100

(a) Relative binding = Ratio of concentrations (reference-compound/test-compound) required for 50% displacement of labelled compound from specific binding sites.

† = A concentration of 2500 nM does not compete.

(b) Relative activity = Dose multiples required to counter the effect of an active hormone dose (0·27 μg estradiol, 50 μg testosterone propionate, 200 μg progesterone, 1 μg aldosterone).

Table 3. Relative cytosol binding and relative biological activity as compared to reference compounds

	ESTROGEN Mouse uterus			PROGESTIN Rabbit uterus		
	Binding	Weight increase Induct.	Inhib.	Binding	Proliferation Induct.	Inhib.
ESTRADIOL	1	1		0.002	†	0.025
RU 16117	0.05	0.01	10	†	-	10
ANDROSTANOLONE	†	0.001	-	0.002	-	-
R 2956	†	0.003	†	0.001	0.05	-
PROGESTERONE	†	†	300	1	1	
R 5020	†	†	3	3	100	†
R 2323	†	0.001	100	0.5	0.5	10
ALDOSTERONE	0.0005	-	-	0.002	-	-
SPIRONOLACTONE	†	-	-	0.001	†	-

	ANDROGEN Rat prostate			ALDOSTERONE Rat kidney		
	Binding	Weight increase Induct.	Inhib.	Binding	Na^+/K^+ excretion Induct.	Inhib.
ESTRADIOL	0.1	†	†	0.005	-	†
RU 16117	†	†	†	†	-	-
ANDROSTANOLONE	1	1		†	-	-
R 2956	0.2	0.02	10	0.02	-	†
PROGESTERONE	0.1	†	†	0.3	0.00005	20000
R 5020	0.1	†	†	0.05		
R 2323	0.3	0.02	†	0.3	0.00005	20000
ALDOSTERONE	0.0001	-	-	1	1	
SPIRONOLACTONE	0.3	†	20	0.5	-	1000

Determination of competitive effect under standardized experimental conditions

Determination of competitive effect on estradiol (a), androstanolone (b), progestin (c) and aldosterone (d) receptors in immature mouse uterus (a), castrated rat ventral prostate (b), estradiol-primed rabbit uterus (c) and adrenalectomized rat kidney perfused with 50 ml 10 mM Tris–HCl (pH 7·4), 1·5 mM EDTA, 0·25 M sucrose buffer (d); homogenization of 1 g tissue in 25 ml (a), 5 ml (b), 50 ml (c) and 3 ml (d) of this same buffer; centrifugation at 105,000 *g* for 1 h; incubation of 0·25 ml cytosol with 1 nM [6,7-^{3}H]-estradiol (58 Ci/mmol) (a), [1α-^{3}H]-androstanolone (24 Ci/mmol) (b), [6,7-^{3}H] R 5020 (51·4 Ci/mmol) (c), [1,2-^{3}H]-aldosterone (53·2 Ci/mmol) (d) and 1 to 2500 nM competitor for 2 h (a,b,c) or 3·5 h (d) at 0°C; measurement of bound radioactivity by Dextran-coated charcoal adsorption; analysis of results by proportion graph [29,30]; determination of concentration required for 50% displacement of labelled compound from specific binding sites. Results are expressed as the ratio of reference-compound concentration to test-compound concentration.

Determination of hormonal activity

Estrogenic activity [15]: The uterine weight increase is measured following administration for 3 days to 18-day-old female mice. *Androgenic activity* [18]: The prostate weight increase is measured following administration for 10 days to 3-week-old castrated male rats. *Progestomimetic activity* [51]: The endometrial response is evaluated histologically in McPhail units following administration for 5 days to estradiol-primed immature rabbits. *Diuretic activity* [52]: The Na^+/K^+ ratio is measured on urine collected over 4 h following a single administration to male rats.

Determination of hormonal antagonism

The test-compounds are administered together with 0·27 μg estradiol (total dose) for determination of anti-estrogenic activity [53], with 50 μg testosterone propionate for anti-androgenic activity [54], with 200 μg progesterone for anti-progestomimetic activity [55] and with 1 μg aldosterone to adrenalectomized rats for anti-diuretic activity [56].

† = A 2500 nM test-compound concentration does not compete in binding studies; the test-compound is inactive in biological studies.

— = No results available.

Thus, this approach leads both to the ideal clinically active form and the likely antagonist.

In the present studies, for each natural hormone the interaction with a cytosol receptor has been studied in the hormone-sensitive tissue most frequently used to bring this interaction to light (uterus for estradiol and progesterone, prostate for androstanolone, kidney for aldosterone), but with a standardized technique in order that results be comparable. This standardized technique may, however, also be applied to advantage to single tissues, where several receptors coexist (estradiol, progesterone and androgen receptors in the uterus), thus yielding useful models for the further study of receptor-receptor interrelationships, such as occur in the delicate estradiol-progesterone balance during the cycle. It might also constitute an ideal tool for detecting the presence and amount of different hormone receptors in human breast cancer [57].

REFERENCES

1. Baulieu E. E., Alberga A., Jung I., Lebeau M. C., Mercier-Bodard C., Milgrom E., Raynaud J. P., Raynaud-Jammet C., Rochefort H., Truong H. and Robel P.: *Recent Prog. Horm. Res.* **27** (1971) 351–419.
2. King R. J. B. and Mainwaring W. I. P.: *Steroid–Cell Interactions.* Butterworths, London (1974).
3. Jensen E. V. and DeSombre E. R.: *Ann. Rev. Biochem.* **41** (1972) 203–230.
4. O'Malley B. W. and Means A. R.: *Science* **183** (1974) 610–620.
5. Liao S. and Fang S.: *Vit. Horm.* **27** (1969) 17–90.
6. Edelman I. S. and Fanestil D. D.: *Biochemical Actions of Hormones* (Edited by G. Litwack). Academic Press, New York, Vol. 1 (1970).
7. Philibert D. and Raynaud J. P.: *Steroids* **22** (1973) 89–98.
8. Philibert D. and Raynaud J. P.: *Endocrinology* **94** (1974) 627–632.
9. Philibert D. and Raynaud J. P.: *Contraception* **10** (1974) 457–466.
10. Raynaud J. P., Philibert D. and Azadian-Boulanger G.: In *The Physiology and Genetics of Reproduction* (Edited by E. M. Coutinho and F. Fuchs). Plenum Press, New York, Vol. 1 (1974) p. 143, pp. 147–148.
11. Raynaud J. P., Bouton M. M., Gallet-Bourquin D., Philibert D., Tournemine C. and Azadian-Boulanger G.: *Mol. Pharmac.* **9** (1973) 520–533.
12. Liao S., Liang T., Fang S., Castañeda E. and Shao T. C.: *J. biol. Chem.* **248** (1973) 6154–6162.
13. Funder J. W., Feldman D., Highland E. and Edelman I. S.: *Biochem. Pharmac.* **23** (1974) 1493–1501.
14. Hubinont P. O., Hendeles S. M. and Preumont P. (eds): *Hormones and Antagonists, IVth Inter. Seminar on Reproductive Physiology and Sexual Endocrinology* (Brussels). Karger, Basel (1972).
15. Rubin B. L., Dorfman A. S., Black L. and Dorfman R. I.: *Endocrinology* **49** (1951) 429–439.
16. Kato J.: *Acta endocr., Copenh.* **72** (1973) 663–670.
17. Raynaud J. P.: In *Drug Interactions* (Edited by P. L. Morselli, S. Garattini and S. N. Cohen). Raven Press, New York (1974) p. 151, p. 159.
18. Hershberger L. G., Shipley E. G. and Meyer R. K.: *Proc. Soc. exp. Biol. Med. N.Y.* **83** (1953) 175–180.
19. Adachi K. and Kano M.: *Steroids* **19** (1972) 567–574.
20. Van Wayjen R. G. A.: *Chem. Weekbl.* **69** (1973) 13–14.
21. Edgren R. A., Smith H., Peterson D. L. and Carter D. L.: *Steroids* **2** (1963) 319–335.

22. Sakiz E. and Azadian-Boulanger G.: In *Proc. III Inter. Cong. Hormonal Steroids* (Edited by V. H. T. James and L. Martini). Excerpta Med., (Amst) Inter. Congr. Ser. **219** (1971) 865–871.
23. Azadian-Boulanger G., Secchi J. and Sakiz E.: In *Proc. VII World Cong. Fertility and Sterility* (Edited by M. Hayashi and T. Hasegawa). Excerpta Med., (Amst) Inter. Congr. Ser. **278** (1973) 129–133.
24. Sakiz E., Azadian-Boulanger G. and Raynaud J. P.: In *Proc. IV Inter. Cong. Endocrinology* (Edited by R. O. Scour). Excerpta Med., (Amst) Inter. Congr. Ser. **273** (1974) 988–994.
25. Funder J. W., Feldman D. and Edelman I. S.: *Endocrinology* **92** (1973) 994–1004.
26. Ussing H. H. and Zerahn K.: *Acta physiol. scand.* **23** (suppl. 80) (1951) 110–127.
27. Raynaud J. P., Azadian-Boulanger G. and Bucourt R.: *J. Pharmac., Paris* **5** (1974) 27–40.
28. Baulieu E. E., Raynaud J. P. and Milgrom E.: In *Karolinska Symposia on Research Methods in Reproductive Endocrinology, 2nd Symposium: Steroid Assay by Protein Binding* (Edited by E. Diczfalusy) (1970) p. 104, pp. 112–113.
29. Baulieu E. E. and Raynaud J. P.: *Eur. J. Biochem.* **13** (1970) 293–304.
30. Raynaud J. P.: *Computer Programs in Biomedicine* **3** (1973) 63–78.
31. Roy A. B.: *Biochimie* **53** (1971) 1031–1040.
32. Unhjem O.: *Acta endocr., Copenh.* **65** (1970) 525–532.
33. Raynaud J. P.: *Steroids* **21** (1973) 249–258.
34. Lebeau M. C., Mercier-Bodard C., Olds J., Bourquin D., Brécy T., Raynaud J. P. and Baulieu E. E.: *Ann. Endocr., Paris* **30** (1969) 183–197.
35. Milgrom E., Atger M. and Baulieu E. E.: *Nature* **228** (1970) 1205–1206.
36. Burton R. M., Harding G. B., Rust N. and Westphal U.: *Steroids* **17** (1971) 1–16.
37. Westphal U.: *Monographs on Endocrinology.* Springer Verlag, Berlin, Vol. 4 (1971).
38. McGuire J. L. and Bariso C. D.: *Endocrinology* **90** (1972) 496–506.
39. Milgrom E. and Baulieu E. E.: *Endocrinology* **87** (1970) 276–287.
40. Rousseau G., Baxter J. D., Funder J. W., Edelman I. S. and Tomkins G. M.: *J. steroid Biochem.* **3** (1972) 219–227.
41. Azadian-Boulanger G., Bonne C., Secchi J. and Raynaud J. P.: *J. Pharmac., Paris* **5** (1974) 509–520.
42. Azadian-Boulanger G. and Bonne C.: *J. steroid Biochem.* **5** (1974) 352.
43. Bonne C. and Raynaud J. P.: *J. Pharmac., Paris* **5** (1974) 521–532.
44. Feyel-Cabanes T.: *C.R. Soc. Biol., Paris* **157** (1963) 1428–1429.
45. Marver D., Stewart J., Funder J. W., Feldman D. and Edelman I. S.: *Proc. natn. Acad. Sci., U.S.A.* **71** (1974) 1431–1435.
46. Fanestil D. D.: *Biochem. Pharmac.* **17** (1968) 2240–2242.
47. Giannopoulos G.: *J. biol. Chem.* **248** (1973) 1004–1010.
48. Bonne C. and Raynaud J. P.: *Mol. Cell. Endocr.* **2** (1974) 59–67.
49. Steelman S. L., Brooks J. R., Morgan E. R. and Patanelli D. J.: *Steroids* **14** (1969) 449–450.
50. Basinger G. T. and Gittes R. F.: *J. Urol.* **111** (1974) 77–80.
51. Clauberg C.: *Zbl. Gyn.* **54** (1930) 2757–2770.
52. Marcus F., Romanoff L. P. and Pincus G.: *Endocrinology* **50** (1952) 286–293.
53. Dorfman R. I., Kincl F. A. and Ringold H. J.: *Endocrinology* **68** (1961) 17–24.
54. Dorfman R. I.: *Methods in Hormone Research.* Academic Press, New York (1969) 319.
55. Pincus G.: *The Control of Fertility.* Academic Press, New York (1965) 128.
56. Kagawa C. M.: *Proc. Soc. exp. Biol. Med.* **99** (1958) 705–707.
57. McGuire W. L., Chamness G. C. and Shepherd R. E.: *Life Sci.* **14** (1974) 19–33.