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RELATIONSHIP BETWEEN PROGESTERONE RECEPTOR BINDING AND PROGESTIN BIOLOGICAL ACTIVITY

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Summary—The binding affinities of a series of steroidal compounds for the hamster uterine progesterone receptor were determined using two sets of incubation conditions. These competitive binding conditions were designed to deduce the relative rates of ligand dissociation from the progesterone receptor. The progestin activity of these compounds was also determined in a bioassay employing the measurement of diamine oxidase in the traumatized hamster uterus. Steroids could be classified into two categories based on either an increase or decrease in relative binding affinity (RBA) with increasing time of competitive incubation. The mean ($\pm$ SEM) progestin biopotency for the compounds having an increase in RBA was 120 ± 18 (progesterone = 100), while the biopotency for compounds having a decrease in RBA was only 44 ± 17. This difference was significant ($P < 0.01$). Linear regression analyses revealed significant correlations between the RBAs and progestin biopotencies. Compounds showing a decrease in RBA with increasing time of incubation did not have antiprogestin activity. Kinetic studies of this type should be useful for selecting compounds with potent agonistic activity, but cannot unequivocally predict anti-hormonal activity.

INTRODUCTION

Binding affinities of synthetic compounds for the progesterone receptor have routinely been determined as an initial appraisal of biological activity [1–4]. These studies provide a base of information regarding the structural requirements for tight binding of ligands to the receptor, and structural modifications that are detrimental to receptor affinity. However, the relative binding affinities (RBAs) determined in typical competitive binding assays do not describe the kinetics of the ligand–receptor interaction; the relative rates of association and dissociation cannot be determined.

Relative rates of dissociation of steroids from receptors can be estimated from competitive binding experiments that are performed under two sets of appropriately chosen conditions [5–7]. Measurements of binding affinities are made after both short-term and long-term incubations. Such experiments permit kinetic determinations to be made without the use of radioactively labeled ligands. These authors [5–7] suggest that steroids having RBAs that increase with incubation time form slowly dissociating complexes with the receptor, while steroids having RBAs that decrease with increasing incubation time form more rapidly dissociating complexes.

Raynaud [5] proposed that an anti-hormone could have a fast rate of dissociation from the receptor relative to the natural hormone. His theory is based on the premise that such a compound would interfere with binding of the endogenous ligand, but would not have agonistic activity because of its rapid dissociation and thus transient interaction with the receptor. Support for this theory is derived from experiments demonstrating that 11α-methoxy-19-nor-1,3,5(10)-pregnatrien-20-yne-3,17-diol, a weak estrogen with anti-estrogenic activity, has a rate of dissociation approx. 20-times faster than that of the corresponding 11β-methoxy steroid, which is a very potent estrogen [8, 9]. In addition, Weichman and Notides [10] have related estrogenic potency to dissociation rate: the slower the rate of dissociation of the ligand from the activated form of the receptor, the greater the biological activity.

Typical competitive binding studies do not distinguish between hormone agonists and antagonists. However, the hypothesis outlined above would be a great aid in the search for anti-hormones. Therefore, we determined the RBAs of compounds for the hamster uterine progesterone receptor under two sets of incubation conditions. These observations were compared to the biological activity of the compounds determined in a hamster progestin bioassay. Our results show that kinetic studies should be useful to select compounds with potent agonistic activity, but are of limited value for predicting anti-hormone activity.

EXPERIMENTAL

Buffers

The buffers were prepared as reported by Leavitt *et al.* [11]. Buffer A contained 50 mM Tris–HCl (Schwarz/Mann, ultrapure), 1 mM EDTA (Sigma) and 12 mM thioglycerol (Sigma), pH 7.5. Buffer A was also prepared with glycerin (Fisher) at a concentration of 30% (v/v, Buffer A/30). Buffer B contained 10 mM Tris–HCl, 1 mM EDTA and 12 mM thio-

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glycerol, pH 7.5. Buffer C contained 10 mM Tris–HCl, 1 mM EDTA and 20% (v/v) glycerin, pH 7.5.

Steroids

Tritiated progesterone (1,2,5,7-^{3}H, 90 Ci/mmol) was purchased from New England Nuclear and purified by thin layer chromatography. All other steroids were obtained from the reference files of The Upjohn Company and were used without purification. The steroids were diluted in Buffer C.

Cytosol preparation

Uteri were obtained between 0800 and 0900 h on the day of proestrus from virgin female hamsters (Engle Laboratory Animals, Inc., Farmersburg, IN), and immediately placed in ice-cold Buffer A. The tissue was trimmed, weighed, minced and homogenized in a 5°C cold room. Tissue was homogenized in Buffer A/30 using a Polytron PT 10St. The homogenates were diluted with Buffer A/30 to a final concentration of 1 g of tissue added to 16 ml of buffer. Cytosol was prepared at 2°C by centrifugation of the homogenate in polyallomer tubes in an OTD-65 ultracentrifuge (Sorvall) using an A-841 fixed angle rotor (Sorvall) at a speed of 40,000 rpm (116,500 ***g***) for 1 h 15 min.

Competition studies

Aliquots (0.3 ml) of cytosol were incubated at 4°C for 0.5 h and 24 h with 0.1 ml [^{3}H]progesterone (217 pg, 69,000 cpm) and 0.1 ml of various quantities (2.5×10^{-9} to 2.5×10^{-5} M) of unlabeled competing steroids. After incubation the bound and free steroids were separated by adding 0.5 ml dextran–charcoal solution (1.5 g charcoal, Sigma Norit A, plus 0.15 g dextran, Pharmacia T70, in 300 ml of Buffer B). The tubes were mixed for 5s, allowed to stand for 1 min, and then centrifuged for 3 min at 2000 ***g*** at 4°C. Following centrifugation the supernatant was decanted into a scintillation vial and 10 ml of counting fluid (Optisol, Isolab) was added. The RBA of each steroid was determined using the method of Korenman [12]. Progesterone was assigned an RBA of 100.

Progestin bioassay

Hamsters were bilaterally ovariectomized at least 10 days prior to use. Five animals were assigned to each experimental group. Steroids were adminstered once daily for five consecutive days by subcutaneous injection in 0.2 ml of sesame oil. Progesterone was administered to groups of animals at daily doses of 0.1, 0.2, 0.4 and 0.8 mg. All test steroids were initially evaluated at a daily dose of 0.4 mg. If biological activity could not be demonstrated at this dose, the compounds were re-tested at a daily dose of 2 mg. On the fourth day of the assay prior to the fourth steroid treatment a 2-0 silk suture was placed in the entire length of both endometrial cavities. Hamsters were necropsied 96 h after insertion of the uterine sutures. Total uterine weights were obtained and tissue aliquots up to 500 mg were saved for determination of diamine oxidase (DAO) activity. An increase in uterine DAO activity following traumatization of the uterus has previously been shown to be specifically dependent upon progestin administration [13]. Tissue preparation and assay of DAO were performed as described previously [13]. The dose response line for progesterone (μUnits diamine oxidase/mg tissue) was calculated by linear regression. Diamine oxidase values for test steroids were compared to this regression line, and a relative progestin biopotency calculated. Progesterone was assigned a biopotency (BP) of 100.

RESULTS

The steroidal compounds discussed in this report are listed in Table 1 together with their corresponding identification codes. In subsequent tables the compounds are referred to by their code letters.

The relative binding affinities of these 16 compounds for the hamster uterine progesterone receptor are shown in Table 2. The RBAs were determined

Table 1. Identification of steroids tested in progesterone receptor binding assay and progestin bioassay

Compound code	
A	15α-Methyl-4-pregnene-3,20-dione
B	9α-Fluoro-4-pregnene-3,20-dione
C	11β-Fluoro-4-pregnene-3,20-dione
D	21-Fluoro-16α-methyl-4,9(11)-pregnadiene-3,20-dione
E	17-Hydroxy-6α-methyl-4-pregnene-3,20-dione, acetate
F	6α-Fluoro-17-hydroxy-4-pregnene-3,20-dione, acetate
G	17β-Hydroxy-4-estrene-3-one
H	17β-Methoxy-4-estrene-3-one
I	17α-Ethyl-17-methoxy-4-estrene-3-one
J	17α-Ethinyl-17-methoxy-7α-methyl-4-estrene-3-one
K	17α-Ethinyl-17-hydroxy-7α-methyl-4-estrene-3-one, acetate
L	17α-Ethyl-17-methoxy-4,9-estradiene-3-one
M	3-Oxo-4-estrene-17β-carboxylic acid, methyl ester
N	17α-Ethinyl-17-hydroxy-7α-methyl-4-androstene-3-one
O	9-Bromo-11β-fluoro-17β-methoxy-4-androstene-3-one
P	17-Hydroxy-3-oxo-4-androstene-17α-propionic acid, γ-lactone

under two sets of incubation conditions: 4°C, 0.5 h and 4°C, 24 h. The data presented were selected from a larger survey of a total of 61 compounds for which binding affinities were determined using both sets of incubation conditions. The compounds listed in Table 1 are those for which the RBAs were significantly different ($P < 0.05$) between the two experimental conditions for competitive binding assay; RBAs for the remaining steroids were not different when determined after 0.5 or 24 h of incubation. Table 2 also shows the ratios of the 0.5 to 24 h RBAs. These ranged from 0.30 for the steroid that had the largest increase in RBA to 7.00 for the steroid that had the largest decrease in RBA.

The steroids are arranged in Table 3 into two groups, those that had either an increase or decrease in RBA from 0.5 to 24 h of incubation. Also shown in Table 3 are the progestin biopotencies for these compounds as determined in the uterine DAO assay. The mean ($\pm$ SEM) BP for the compounds having an increase in RBA was 120 ± 18, while that for the compounds having a decrease in RBA was only 44 ± 17. These groups are significantly different ($P < 0.01$) when compared by Student's t-test. Only two compounds with an increase in RBA had BPs less than that of progesterone (BP = 100), and only one compound with a decrease in RBA had a BP greater than that of progesterone. Linear regression analyses demonstrated significant correlations between the RBAs determined at either 0.5 or 24 h and the BPs for both subsets of data. For those compounds having an increase in RBA the correlation coefficient using the 0.5 or 24 h RBAs as the independent variable were 0.655 ($P < 0.05$) and 0.645 ($P < 0.05$), respectively. Values for compounds having a decrease in RBA were 0.896 ($P < 0.01$) and 0.88 ($P < 0.01$), respectively. There was no significant correlation between the ratio of 0.5 to 24 h RBAs and the BPs.

Table 2. Relative binding affinities of steroids for the hamster uterine progesterone receptor

Compound code*	RBA† at 0.5 h	RBA at 24 h	RBA ratio 0.5 h/24 h
A	67	30	2.23
B	51	130	0.39
C	18	46	0.39
D	29	70	0.41
E	23	13	1.77
F	36	24	1.50
G	21	3	7.00
H	68	38	1.79
I	69	43	1.61
J	21	70	0.30
K	5	13	0.39
L	40	11	3.64
M	31	63	0.49
N	33	12	2.75
O	27	66	0.41
P	10	32	0.31

*See Table 1 for structure identification.
†Progesterone binding affinity = 100.

DISCUSSION

According to the hypothesis outlined in the Introduction, compounds showing an increase in RBA between 0.5 and 24 h of incubation should have a rate of dissociation from the progesterone receptor that is slower than that of progesterone. Therefore, one would expect their biological potencies to be greater than predicted by their affinities for the receptor. Conversely, compounds with RBAs that decrease with incubation time should have rapid rates of dissociation and should have low biological activities. With the exception of Compound P, the compounds showing an increase in RBA had biopotencies that were greater than would be predicted by their RBAs. Compound P may act as a prodrug. The full expression of biological activity could depend upon hydrolysis of the lactone linkage *in vivo*. This would not be expected to occur *in vitro* during the competitive binding assay. Only three compounds (F, G, N) with RBAs that decreased with time had biopotencies that were less than would be expected from their 24 h binding affinities. Four other compounds (A, E, H, I) with decreasing RBAs had biopotencies that were approximately two to three times greater than would be expected. One compound (L) had a biopotency very similar to its RBA.

Raynaud [15] proposed that a compound with a fast rate of dissociation from the receptor should have antihormone activity. The progesterone receptor binding data and progestin bioassay data reported here do not substantiate this hypothesis for the progesterone receptor. Seven of the eight compounds for which a decrease in RBA was observed, and which therefore presumably have a fast rate of dissociation, had measurable progestin activity in the DAO assay. It is possible, however, that the compounds are antagonistic only when adminstered with progesterone and are agonistic when administered

Table 3. Progestin bioactivity of compounds having either an increase or decrease in receptor binding affinity

Compound code*	RBA Ratio 0.5 h/24 h	DAO assay biopotency†
	Compounds with an increase in RBA	
B	0.39	201
C	0.39	121
D	0.41	80
J	0.30	132
K	0.39	126
M	0.49	132
O	0.41	145
P	0.31	24
	Compounds with a decrease in RBA	
A	2.23	96
E	1.77	26
F	1.50	13
G	7.00	0
H	1.79	70
I	1.61	127
L	3.64	14
N	2.75	6

*See Table 1 for structure identification.
†Progesterone biopotency = 100.

alone. While it has been possible to demonstrate a rapid rate of dissociation for one steroidal antiestrogen [8, 9], other investigators have been unable to confirm similar kinetic behavior for androgen, glucocorticoid, progesterone or estrogen receptor antagonists. Wakeling *et al.* [14] studied both steroidal and non-steroidal antiandrogens and were not able to distinguish clearly between agonists and antagonists on the basis of receptor binding behavior. The RBAs decreased with increasing length of incubation for all of the antiandrogens, but the RBAs also decreased for testosterone and 5α-dihydrotestosterone. The dissociation rate constants of antiglucocorticoids also have not been indicative of agonistic or antagonistic activity [15, 16]. For example, the antiglucocorticoid, progesterone, has a dissociation rate that is not different from the glucocorticoid, hydrocortisone [16]. It has also been reported that the RBA of an antiprogestin increases substantially with increasing time of incubation [17]. This suggests that the antihormone has a very slow rate of dissociation from the progesterone receptor. Finally, Rochefort *et al.* [18] reported that the potent antiestrogen, hydroxytamoxifen, dissociates very slowly from the uterine estrogen receptor.

In conclusion, kinetic binding studies of the type reported here cannot be used to unequivocally predict antihormonal activity of compounds. This type of study should be useful, however, to select compounds with potent agonistic activity. Seven of the eight compounds that had an increase in RBA with time of incubation, and presumably a slow rate of dissociation from the receptor, had progestin biopotencies that were greater than would be predicted solely from their affinities for the receptor.

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