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Specific Interactions of Progestins and Anti-progestins with Progesterone Antibodies, Plasma Binding Proteins and the Human Recombinant Receptor

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This structure–activity study compares the affinity of a series of progestins, progesterone metabolites and anti-progestins for a panel of monoclonal antibodies to progesterone, coypu (*Myocastor coypus*) or guinea pig plasma progesterone-binding proteins (PPBPs) and the human recombinant progesterone receptor A form (PR-A). The compounds tested were progesterone, Promegestone (R5020), Mifepristone (RU486), ZK98,734, Onapristone (ZK98,299), 11α-hydroxyprogesterone, 11α-progesterone hemisuccinate, androsterone, etiocholanolone, 5α- and 5β-pregnane-3,20-diones, and 20α- and 20β-hydroxyprogesterones. The K_i values for these ligands were determined by competitive binding assays using radiolabelled progesterone as the binding site ligand. For anti-progesterone antibodies (e.g. DB3 and 11/32), only progesterone (3.6–8.8 nM), the 11α-derivatives (1.0–5.5 nM) used to prepare the immunogen and the two 5-pregnanediones (20.9–45.1 nM) were bound with high affinity. For PR-A, high affinity binding was found with receptor agonists (K_i = 1.1–6.2 nM), both 5- and 20-reduced metabolites, and antagonists (0.6–28.0 nM), but not with the 11α-derivatives (950 nM–1.0 μM). In contrast, the PPBPs displayed high affinity interactions with progesterone (3.5–4.2 nM) and both 5α- and 20α-reduced metabolites (2.4–3.4 nM). Binding with the β-isomers and R5020 was less pronounced (22–170 nM) and there was no evidence of high affinity binding with PR antagonists (>1.0 μM). Analogs with the 17-keto group did not bind to any of the binders studied. Thus, commonalities among the three types of protein binders were their comparable binding affinities for progesterone (3.5–8.8 nM) and 5-pregnanedione isomers (2.4–330 nM), and a lack of binding for two C17-keto steroids (androsterone and etiocholanolone). The results imply that the tertiary features of the binding domain of these three types of proteins are sufficiently different to result in unique binding structures.

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INTRODUCTION

Specific antibodies directed against progesterone itself and antagonists to the progesterone receptor (PR) have been used to study the biological roles of the ligand in the reproductive process. Anti-progesterone antibodies have been studied for their anti-implantation properties *in vivo* [1, 2], while progesterone antagonists have been investigated for their arrest of the development of experimentally-induced tumors in laboratory animal models (e.g. ZK98,299 [3]) and for pregnancy termination (e.g. RU486 [4]). Antibodies to steroids have also proved to be valuable probes for the study of steroid–protein interactions and in particular the tertiary structure of the antigen combining site. For example, the atomic structure of the progesterone binding site of a specific monoclonal antibody (DB3) directed against 11α-progesterone hemisuccinate has been determined by X-ray crystallography [5], and the molecular basis of cross reactivity of this antibody with related steroids defined [6]. It was found that whereas the binding of progesterone by this antibody resulted in a localized conformational change in the binding site, cross

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reactions with other hydrophobic ligands occurred without any major structural rearrangement. Thus, antigen specificity was displayed through conserved interactions with the steroid D-ring. Some of the cross reactivity was realized by different orientations of the steroid skeleton such that the A-ring was placed into alternative pockets on the surface of the antibody according to the structure of the steroid in question [6]. These findings provide the first detailed definition of a steroid specificity in an antibody and show that steroids with chemical similarities but substantially different conformations can bind in the nanomolar affinity range without any further change in the Fab′ binding pocket.

It is important to know whether this is true for other progesterone binders, since the molecular understanding of protein–ligand interactions is crucial for the elucidation of their biological significance. At least three forms of proteins exist with high binding affinities for progesterone, namely specific antibodies artificially raised in animals, nuclear receptors and plasma binding proteins. Although the values for the K_i of these proteins are similar (anti-progesterone antibodies, 1.0–4.0 nM; plasma progesterone-binding proteins (PPBPs), 0.5–2.0 nM; PR, 6.0–10.0 nM) [7–10], their functions are entirely different. A major effect of anti-progesterone antibodies when administered *in vivo* is to bind circulating progesterone leading to ligand withdrawal. PPBPs, as with anti-progesterone antibodies, act as ligand carriers and exert a progesterone-conserving mechanism during pregnancy by reducing the rate of destruction of the ligand to maintain a reservoir of hormone in systemic circulation [11]. Progesterone nuclear receptors bind the ligand after it has been secreted by the corpus luteum or placenta and this precedes the activation or suppression of target genes [12].

The purpose of the present study was to map the extent to which these three types of macromolecules share any of the features of molecular complementarity described recently for five Fab′–steroid complexes [5, 6]. Simultaneous determinations were made of the affinities of anti-progesterone antibodies, coypu and guinea pig PPBPs, and the human recombinant progesterone receptor A form (PR-A). Special consideration was given to the binding of PR agonists, antagonists and biologically inactive metabolites which show substantial differences at the A- and D-ring of the molecule, and of isomeric metabolites of progesterone.

EXPERIMENTAL

Steroid reagents

Progesterone, 11α-hydroxyprogesterone and 20β-hydroxyprogesterone were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Androsterone, etiocholanolone, 5α- and 5β-pregnane-3,20-diones and 20α-hydroxyprogesterone were obtained from Steraloids Inc. (Wilton, NH, U.S.A.). R5020 (Promegestone) and RU486 (Mifepristone) were gifts from Roussel-Uclaf (Romainville, France). ZK98,299 (Onapristone) and ZK98,734 were donations from Schering AG Pharmaceutical Research (Berlin, Germany). The chemical structures and trivial names of these compounds are shown in Fig. 1. The 11α-hemisuccinyl derivative of progesterone (11α-progesterone hemisuccinate) was prepared by reaction of 11α-hydroxyprogesterone with succinic anhydride [2]. [^{3}H]progesterone was purchased from New England Nuclear Research Products (Boston, MA, U.S.A.).

Monoclonal antibodies against progesterone

A panel of monoclonal anti-progesterone antibodies was produced in BALB/c mice and characterized as described previously [2, 7]. The monoclonals used in this study included five IgG_1 (DB3, 10/8, 10/16, 11/32 and 11/34) and one IgM subclasses (11/64). Each antibody was purified from pooled ascites by ion-exchange chromatography [5], dialyzed against phosphate buffered saline (PBS) and stored (1.0 mg/ml) at −20°C.

PPBP

High-affinity PPBP was purified from pregnant coypu (*Myocastor coypus*) and guinea pig plasma according to the method reported previously [8]. The preparation (1.0 mg/ml) was stored at −20°C until use.

Progesterone receptor

Human recombinant PR (A form) was expressed from its cDNA in Sf21 cells infected with baculovirus and extracted as previously described [10, 13]. The receptor preparation was stored at −80°C at a typical protein concentration of 10–15 mg/ml.

Competitive ligand-binding assay

Stock solutions of all compounds were prepared on the day of the assay as 10 mM ethanol solutions at 4°C. The progesterone assay buffer (pH = 7.5) consisted of the following: 10% glycerol, 10 mM Tris, 1.0 mM EDTA, 12 mM monothioglycerol (MTG), 2.0 mM CHAPS (3-[3-cholamidopropyl-dimethyl-ammonio]-1-propane-sulfonate), and 1.0 mM PMSF (phenylmethylsulfonylfluoride). The final assay volume was 500 μl containing 10 μg protein and 2.0–4.0 nM of [^{3}H]progesterone. Depending on the experiment, either variable concentrations of competing ligands or a fixed concentration (500 nM) were used. Incubations were carried out at 4°C for 16 h. Non-specific binding was defined as binding remaining in the presence of 500 nM of progesterone. At the end of the incubation, 400 μl of 7.5% (w/v) dextran-coated charcoal suspension in gelatin phosphate buffer was

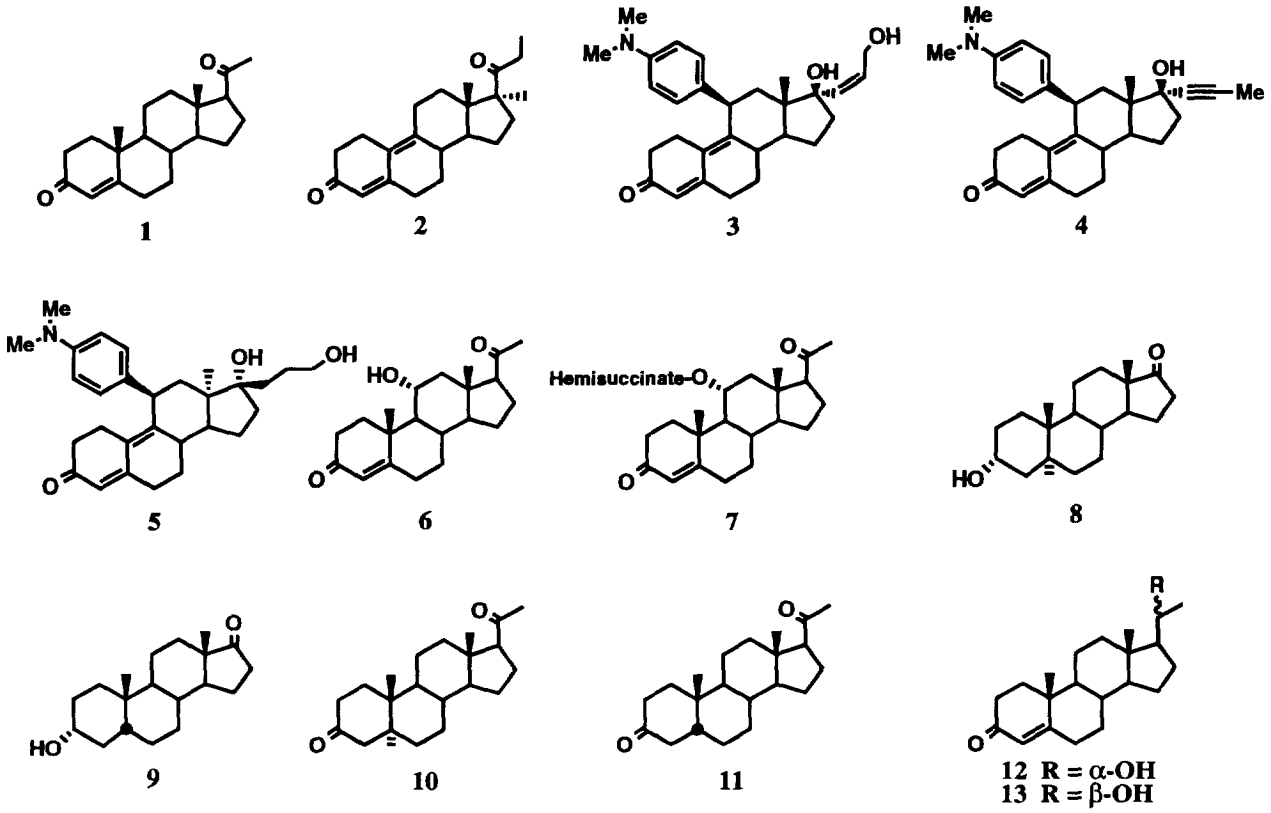

Fig. 1. Chemical structures of the steroids used in this study. 1, progesterone; 2, R5020; 3, ZK98,734; 4, Mifepristone; 5, Onapristone; 6, 11α-hydroxyprogesterone; 7, 11α-hydroxyprogesterone hemisuccinate; 8, Androsterone; 9, Etiocholanolone; 10, 5α-Pregnane-3,20-dione; 11, 5β-Pregnane-3,20-dione; 12, 20α-hydroxyprogesterone; 13, 20β-hydroxyprogesterone.

added [14]. The mixture was vortexed, further incubated for 10 min at 4°C and then centrifuged at 800*g* for 10 min. For PPBPs and antibodies, assay conditions were similar except CHAPS and MTG were excluded from the buffer and the amount of purified protein added to the assay ranged from 100 ng to 1.0 μg, depending upon the particular protein. The amount of bound [^{3}H]progesterone was determined by liquid scintillation counting of an aliquot (700 μl) of the supernatant. The interassay coefficient of variation for the mean K_i value for progesterone was 19%, and any value that was outside the range of ±2 standard deviations of the mean was re-assayed.

After correction for non-specific binding, IC_{50} values were calculated graphically from a log–logit data transformation. The IC_{50} is defined as the concentration of competing ligand needed to reduce specific binding by 50%. Competition curves are plotted as the percentage of bound ^{3}H-labelled ligand ($B/B_0 \times 100\%$) vs the concentration of competitor where B_0 = bound tritiated ligand in the absence of cold competitor, and B = bound tritiated ligand in the presence of cold competitor. K_i values for the compounds were calculated by application of the Cheng–Prusoff equation [15].

RESULTS

Ligand-binding of anti-progesterone antibodies

All six monoclonal anti-progesterone antibodies (DB3, 10/8, 10/16, 11/32, 11/34 and 11/64) displayed a high affinity for progesterone in contrast to their lack of affinity for the receptor agonist, R5020, or antagonists, RU486, ZK98,299 and ZK98,734 (data for two antibodies shown in Table 1). Two representative examples of the binding affinity curves are given in Figs 2(a, b) and 3 and these are typical of all antibodies tested. Among the different progestins and anti-progestins tested, affinity was greatest for the immunogen (11α-hydroxyprogesterone hemisuccinate, 1.0–1.8 nM), 11α-hydroxyprogesterone (4.6–5.5 nM), progesterone (3.6–8.8 nM) and 5α- and 5β-pregnanediones (20.9–45.1 nM; Table 1).

Ligand-binding of PPBPs

Progesterone, 11α-hydroxyprogesterone, 5α- and 5β-pregnanediones, 20α- and 20β-hydroxyprogesterones and R5020 competed effectively with the binding of radiolabelled ligand by high-affinity progesterone binding proteins from coypu and guinea pig plasma [Figs 4(a, b) and 5], though differences existed between the two species. For example, whereas the PPBP

Table 1. Summary of binding affinities (K_i values) of progestins and anti-progestins to progesterone receptor A-form (PR-A), coypu and guinea pig PPBPs and monoclonal anti-progesterone antibodies (DB3 and 11/32)

	Binder				
Compound	PR-A	Coypu PPBP	Guinea pig PPBP	DB3	11/32
Progesterone	6.2 ± 0.7	3.5 ± 0.5	4.2 ± 0.6	8.8 ± 0.9	3.6 ± 0.6
R5020	1.1 ± 0.2	170 ± 21	22 ± 3	>1000	>1000
ZK98,734	0.85 ± 0.09	>1000	>1000	>1000	>1000
Mifepristone	0.58 ± 0.07	>1000	>1000	>1000	>1000
Onapristone	28 ± 4	>1000	>1000	>1000	>1000
11α-hydroxyprogesterone	950 ± 120	280 ± 32	38 ± 5	4.6 ± 1.1	5.5 ± 1.2
11α-Hemisuccinate progesterone	>1000	>1000	>1000	1.8 ± 0.2	1.0 ± 0.2
Androsterone	>1000	>1000	>1000	>1000	>1000
Etiocholanolone	>1000	>1000	>1000	>1000	>1000
5α-Pregnane-3,20-dione	6.9 ± 1.3	108 ± 20	2.4 ± 0.4	20.9 ± 3.8	45.1 ± 5.1
5β-Pregnane-3,20-dione	55 ± 8	330 ± 46	63.2 ± 7.8	26.5 ± 3.2	21.2 ± 5.6
20α-hydroxyprogesterone	58 ± 6	47 ± 6.2	3.4 ± 1.1	>1000	>1000
20β-hydroxyprogesterone	29.8 ± 4.1	178 ± 21	105 ± 15	>1000	>1000

The final assay volume was 500 ml and contained 10 μg protein for PR-A and 2–4 nM of [^{3}H]progesterone and varying concentrations of competing ligands. Incubations were carried out at 4°C for 16 h. Non-specific binding was defined as binding remaining in the presence of 500 nM of progesterone. At the end of the incubation period, bound ligand was separated from free by the dextran-coated charcoal method. For coypu and guinea pig progesterone binding proteins and monoclonal antibodies (DB3 and 11/32), assay conditions were similar except the assay buffer excluded CHAPS and MTG and the amount of purified protein added to the assay was from 100 ng to 1 μg, depending upon the particular protein. K_i values are in nM and are expressed as the mean ± SEM ($n = 3$).

affinity for progesterone was comparable for proteins from both species (3.5 and 4.2 nM, respectively), guinea pig PPBP showed better affinity for R5020 (22.0 nM), 11α-hydroxyprogesterone (38.0 nM) and 5α-pregnanedione (2.4 nM) compared to coypu PPBP (170, 280 and 108 nM, respectively; Table 1). PPBP from both species bound 11α-hydroxyprogesterone but not the hemisuccinate derivative. None of the PR antagonists were bound with an affinity greater than 1000 nM.

Binding characteristics of human PRs

The A-form of the receptor demonstrated a binding affinity that was at least 100-fold greater for progesterone (6.2 nM) and 5α-pregnanedione (6.9 nM), and at least 10-fold greater for 5β-pregnanedione, than for the 11α-substituted derivatives (11α-hydroxyprogesterone, 950 nM; 11α-progesterone hemisuccinate, >1000 nM; Fig. 6). The binding affinity for two of the antagonists was even greater than that of progesterone (RU486, 0.58 nM; ZK98,734, 0.85 nM), though ZK98,299 showed a relatively lower binding affinity (28.0 ± 4.0 nM). Among the other metabolites of progesterone there was significant binding of 5β-pregnanedione, 20α- and 20β-hydroxyprogesterones (55, 58 and 29.8 nM, respectively; Table 1).

DISCUSSION

Mapping contrasting types of three progesterone binding proteins for their ability to bind certain progestins and anti-progestins has revealed that progesterone is the only ligand that is bound at high affinity by all of these macromolecules (3.5–8.8 nM). Progesterone was followed by the two isomeric forms of pregnanedione with binding affinities that varied between 2.4 and 330 nM for all protein binders.

Competitive ligand-binding assay showed that each protein binder has unique properties. Anti-progesterone antibodies showed the greatest discrimination among the compounds studied, notable cross reactivities being found with only five of the steroids tested. The initial screen for these monoclonals selected antibodies with a high affinity for progesterone [2, 6] and the binding of compounds with 11α-substitutions reflects the nature of the original immunogen. It has been proposed from three-dimensional analysis of the antibody binding site that high affinity binding derives from conserved interactions centered on the D-ring of the steroid molecule and hydrogen bonds to C17-keto or C20-keto [6]. The importance of these interactions for high affinity binding is emphasized by the absence of binding when the 20-hydroxyprogesterone metabolites were tested. These compounds were not recognized by the antibodies irrespective of whether they possessed a 20α- or 20β-substitution. In contrast, changes in the A-ring (isomers of 5-pregnanedione) resulted in only a slight reduction in affinity irrespective of whether H5 was in the α- or β-orientation, consistent with the idea that the interactions are centered on the D- rather than the A-ring.

Clearly, the presence of 11β, C17 and side chain substitutions, as in the progestin antagonists, or changes at C17 and/or side chain substitutions, as in

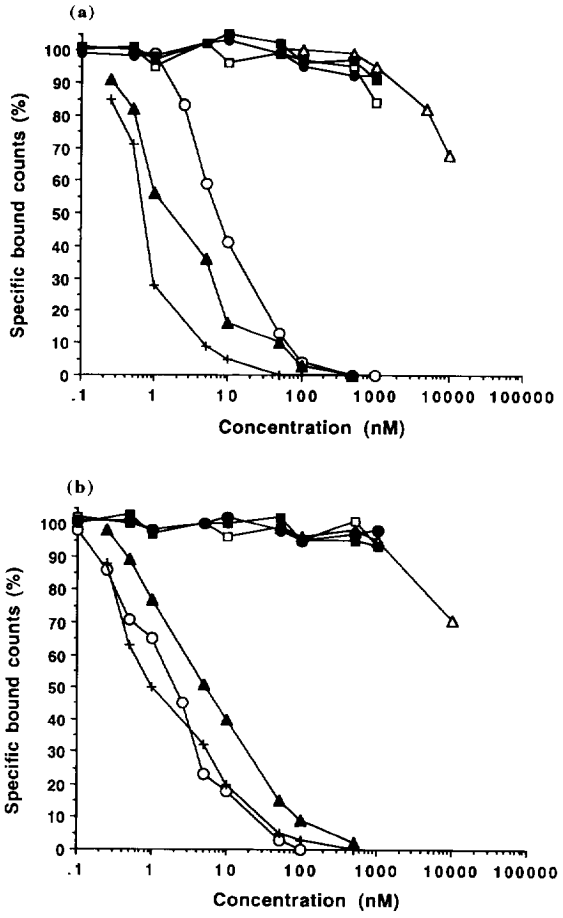

Fig. 2. Binding of progestins and anti-progestins to monoclonal anti-progesterone antibodies DB3 (a) and 11/32 (b). Binding assays were performed as described in the footnote to Table 1, ○, Progesterone; □, Mifepristone; △, R5020; ●, ZK98,734; ■, Onapristone; ▲, 11α-hydroxyprogesterone; +, 11α-hydroxyprogesterone hemisuccinate. Percent of specifically bound radiolabelled progesterone is plotted versus concentration of compound.

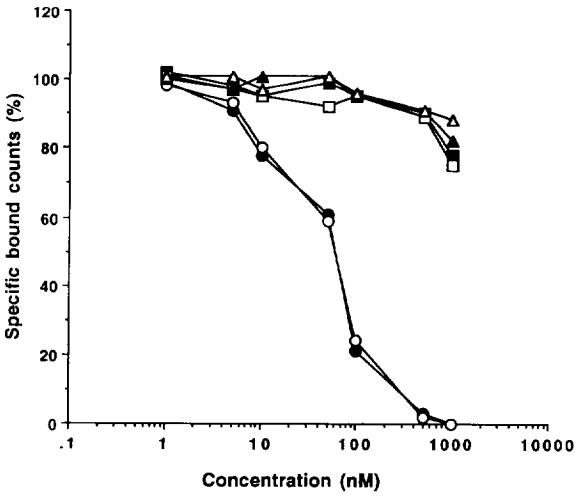

Fig. 3. Binding of various steroids to monoclonal anti-progesterone antibody DB3. Binding assays were performed as described in the footnote to Table 1. ■, Androsterone; □, Etiocholanolone; ●, 5α-Pregnane-3,20-dione; ○, 5β-Pregnane-3,20-dione; ▲, 20α-hydroxyprogesterone; △, 20β-hydroxyprogesterone. Percent of specifically bound radiolabelled progesterone is plotted versus concentration of compound.

R5020, prevent recognition by antibodies presumably because of the lack of critical hydrogen bonding and/or failure to accommodate the modified D-ring substituents in the narrow slot of the binding pocket [5]. It should be noted, however, that the lack of affinity of the anti-progesterone antibodies for androsterone and etiocholanolone in the present study differed from results published previously [5] where higher relative binding was found (progesterone, 1.0 nM; androsterone, 8.0 nM; etiocholanolone, 21 nM). The reason for this discrepancy is not known but it is notable that the technique used in these experiments gave consistently lower IC_{50} relative affinities than earlier reports.

Progesterone receptor (PR-A) bound the agonists and antagonists tested in this study with an affinity similar to that of the wild type PRs (A and B forms) expressed in the human breast cancer cell line, T47D (D. E. Mais, unpublished observations). PR-A only weakly recognized 11α-hydroxyprogesterone, a weak agonist, and its hemisuccinate derivative. Thus, whereas PR-A possesses a large hydrophobic pocket able to accommodate bulky 11β-substitutions such as those found in the antagonists, Mifepristone, Onapristone and ZK98,734 [16], it fails to recognize 11α-substitutions with a high affinity. Similarly, PR-A failed to recognize the C3-hydroxy and C17-keto steroids (androsterone and etiocholanolone) whereas it has significant affinity for the isomeric forms of 5-pregnanedione. This finding suggests that affinity is greatly reduced by the presence of C3-hydroxy and C17-keto structures. The 20-hydroxyprogesterone isomers showed reasonable binding indicating that substitutions at the C20 position reduce, but do not eliminate, receptor recognition of a compound. The findings support the well established observations of others that progestin agonists and antagonists are recognized by the steroid binding domain of PR and that binding affinity resembles or is even greater than that of progesterone itself. This is consistent with the view that antagonist efficacy is related to inhibition at the level of DNA responsive elements [17] either due to the lack of obligatory conformational changes of the receptor and/or inhibition of transcriptional activating factors.

Proteins that bind progesterone with high affinity have been utilized in this study from diverse origins, including antibody-secreting hydridomas, circulating plasma and target cell nuclei, to elucidate further the nature of steroid–protein interactions in the body. The pharmacokinetics of RU486 have demonstrated its prolonged half-life in humans due to binding to the plasma protein, orosomucoid, an α_1-acid glycoprotein

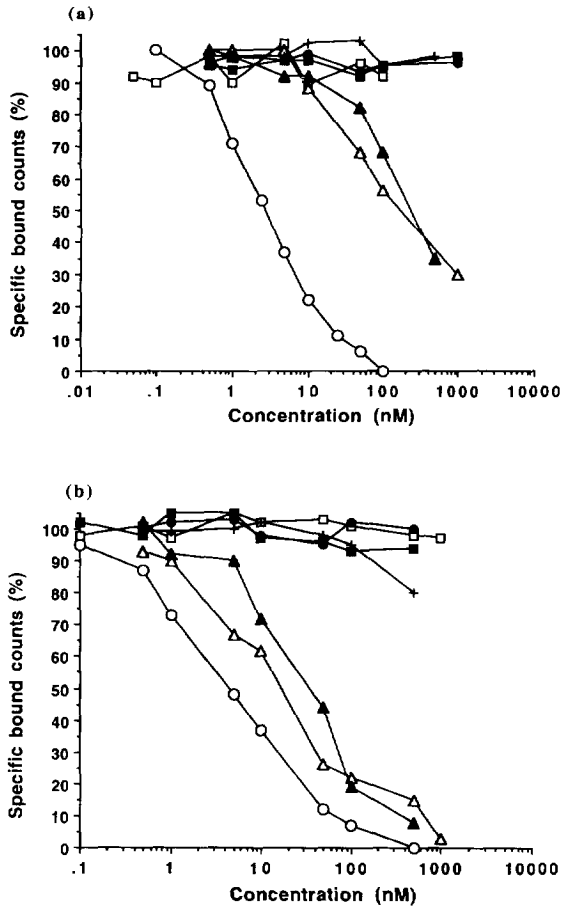

Fig. 4. Binding of progestins and anti-progestins to coypu (a) and guines pig (b) PPBPs. Binding assays were performed as described in the footnote to Table 1. ○, Progesterone; □, Mifepristone; △, R5020; ●, ZK98,734; ■, Onapristone; ▲, 11α-hydroxyprogesterone; +, 11α-hydroxyprogesterone hemisuccinate. Percent of specifically bound radiolabelled progesterone is plotted versus concentration of compound.

[18]. The present studies show that PPBPs bind progesterone, its 11α-hydroxylated metabolite, R5020, and the isomeric forms of 5-pregnanedione and 20-hydroxyprogesterone with high affinity (though less so in the case of the β-oriented steroids). The presence of a bulky group at C11 resulted in a much lower relative affinity, as in 11α-hemisuccinate progesterone and the PR antagonists. Similarly, the occurrence of 3-hydroxy and C17-keto structures reduced affinity. These results show that high affinity plasma binding protein–PR antagonist interactions are very selective, and in the absence of structural data about the binding domain, difficult to predict.

Three common features emerge from these studies. First, all three protein binders showed remarkably high and comparable affinities for progesterone (5.3 ± 0.9 nM; range 3.6–8.8 nM); second, they all bind the isomers of 5-pregnanedione with reasonable affinity; and third, they all lack affinity for the two C17-keto steroids tested. The maps of cross reactivities for the three classes of binders differed substantially.

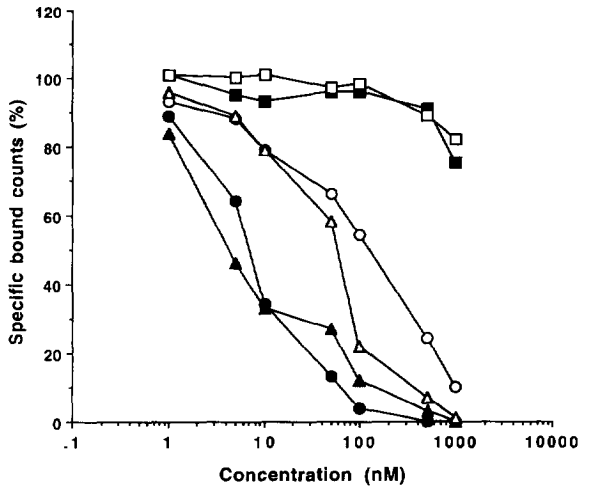

Fig. 5. Binding of various steroids to guinea pig PPBP. Binding assays were performed as described in the footnote to Table 1. ■, Androsterone; □, Etiocholanolone; ●, 5α-Pregnane-3,20-dione; ○, 5β-Pregnane-3,20-dione; ▲, 20α-hydroxyprogesterone; △, 20β-hydroxyprogesterone. Percent of specifically bound radiolabelled progesterone is plotted versus concentration of compound.

Approximate ranking shows that monoclonal antibodies to progesterone bound 5 of 13 compounds tested with a K_i of 1.0–45.1 nM; PPBPs up to 6 compounds with a K_i of 2.4–63.2 nM; and PR-A up to 9 compounds with a K_i of 0.58–58 nM. Duax and Griffin [19] suggested that progesterone antagonists may be expected to have the A-ring composition and conformation necessary for receptor binding and to lack D-ring conformational features and functional groups that induce or stabilize receptor functions. With the

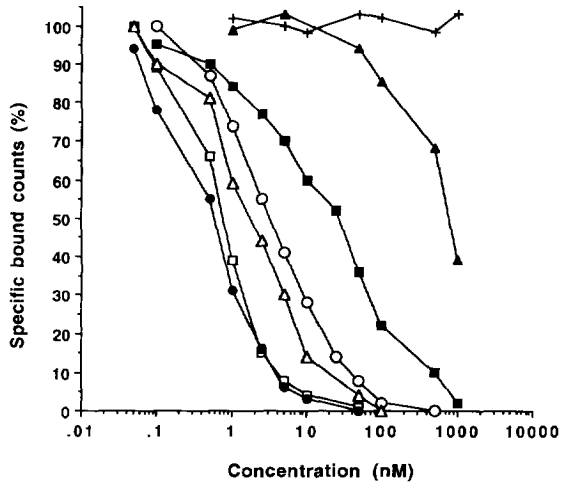

Fig. 6. Binding of progestins and anti-progestins to PR-A. PR-A was expressed in a baculovirus expression system in Sf21 insect cells. Binding assays were performed on crude whole cell extracts as described in the footnote to Table 1. ○, Progesterone; □, Mifepristone; △, R5020; ●, ZK98,734; ■, Onapristone; ▲, 11α-hydroxyprogesterone; +, 11α-hydroxyprogesterone hemisuccinate. Percent of specifically bound radiolabelled progesterone is plotted versus concentration of compound.

exception of 11α-hydroxy substitution in the progesterone molecule which decreased binding affinity substantially, preservation of the A-ring composition and conformation was consistent with receptor binding among the compounds studied. With antibodies, D-ring composition, conformation and structural groups were confirmed as necessary for binding. With PPBPs, there was evidence for a more complex requirement because of the prevention of binding by the presence of bulky groups at C11. The limited commonality among the binders, confined largely to the binding of progesterone, 5-pregnanedione isomers, and the lack of binding for the two C17-keto steroids, implies that each of the high affinity binding domains is unique. Thus, the solution of the three-dimensional structure of the steroid binding domain of PR remains a major priority.

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