

Ligand specificity of uterine oestrogen and progesterone receptors in the subadult African elephant, *Loxodonta africana*

M. D. Greyling¹, R. J. van Aarde^{1*} and H. C. Potgieter²

¹Mammal Research Institute and ²Department of Chemical Pathology, University of Pretoria, Pretoria 0002, South Africa

The ligand specificity of progesterone and oestrogen receptors in the uteri of four nonpregnant, nonlactating African elephants, killed during routine culling in the Kruger National Park, were determined. The mean (\pm SEM) K_d values of the oestrogen ($0.18 \pm 0.019 \times 10^{-9}$ mol l⁻¹, $n = 12$) and progesterone ($0.22 \pm 0.025 \times 10^{-9}$ mol l⁻¹, $n = 12$) receptors were essentially similar when [³H]promegestone was used as radioligand in the progesterone receptor assays. However, when [³H]progesterone was used as radioligand, the progesterone receptor exhibited a significantly higher K_d value ($1.03 \pm 0.132 \times 10^{-9}$ mol l⁻¹, $n = 12$) than that of the oestrogen receptor. The use of the different radioligands did not significantly affect the quantitative values obtained for the progesterone receptor. Both the oestrogen and the progesterone receptors displayed a high ligand specificity. The 5 α -reduced metabolites of progesterone exhibited a high relative binding affinity for the progesterone receptor (5 α -pregnane-3,20-dione: relative binding affinity = 43%; 5 α -pregnane-3 α -ol-20-one: relative binding affinity = 20%) but the synthetic antiprogesterin RU 486 did not compete successfully with progesterone in competitive binding studies. However, norethindrone (relative binding affinity = 293%) competed successfully for binding to the progesterone receptor, and may have some potential in the future development of a technique to control reproductive output in the African elephant.

Introduction

Relative high annual rates of population growth in African elephants confined to conservation areas in southern Africa (for example, 7%; Hall-Martin, 1992) may have negative consequences for the maintenance of biological diversity. As a result the densities of some of these populations are reduced artificially through culling operations that are opposed both by some conservationists and the concerned public. There is, therefore, a need for the development of alternative and generally acceptable techniques for inhibiting population growth. In this regard Poole (1993) and Short (1992) suggested that consideration should be given to the use of contraceptives or contragestins, such as an antiprogesterin, like mifepristone (RU 486; Roussel Uclaf, Paris), that may block uterine receptor activity. However, the use of such treatments require detailed knowledge of the reproductive endocrinology in the elephant.

To the best of our knowledge, information on the potential of antiprogesterins to interfere with uterine receptor activity in the African elephant has not been published. Previous studies have documented concentrations of progesterone and oestrogen in the plasma and the corpora lutea of the African elephant (for example, Hanks and Short, 1972; Smith and Buss, 1975; Hodges *et al.*, 1983; McNeilly *et al.*, 1983; Brannian *et al.*, 1988; Plotka *et al.*, 1988; De Villiers *et al.*, 1989; Hodges *et al.*, 1994).

Heistermann *et al.* (1994) documented that plasma concentrations of 5 α -dihydroprogesterone in pregnant elephants are an order of magnitude higher than those of progesterone. They also suggested that plasma concentrations of 5 α -dihydroprogesterone are more closely related to the function of the corpus luteum and reproductive status of African elephants than are plasma concentrations of progesterone. In this regard Hodges *et al.* (1994) illustrated that the 5 α -reduced progesterone metabolites, 5 α -pregnane-3 α -ol-20-one and 5 α -pregnane-3,20-dione (5 α -dihydroprogesterone) are the principal progestins, other than progesterone, produced by the elephant corpus luteum. However, information on the biological activity of the metabolites and on the potential for antiprogesterins to interfere with uterine receptor activity in the elephant has not been published. The present paper thus focuses on the relative binding affinities of uterine progesterone and oestrogen receptors of the elephant for a variety of hormones and their analogues. This study was carried out on material collected from freshly killed subadult cows during routine culling for population management in the Kruger National Park of South Africa.

Materials and Methods

The radioligands [1,2,6,7-³H]progesterone (³H-P; specific activity: 95 Ci mmol⁻¹) [2,4,6,7,16,17-³H]oestradiol-17(³H-E₂; specific activity: 157 Ci mmol⁻¹) were obtained from

*Correspondence.

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Amersham International (Amersham, Bucks); [17(-methyl-³H)]promegestone (³H-R5020; specific activity: 86.7 Ci mmol⁻¹) and an unlabelled promegestone were obtained from New England Nuclear (Massachusetts, MA). All other unlabelled steroids, leupeptin and gelatine (Carraggenan Type 1) were purchased from Sigma (St Louis, MO), while SA-Druggists (Port Elizabeth) kindly donated levonorgestrel, ethinyl oestradiol, tamoxifen and danazol. Roussel Uclaf (Paris) kindly supplied RU 486. Activated charcoal was purchased from Merck Chemicals and dextran T 70 from Pharmacia Fine Chemicals.

Animals and tissue collection

Uterine tissue (four or more 1 cm³ blocks of endometrium and myometrium from each animal) was collected from four 8–10-year-old, nonpregnant, nonlactating female African elephants killed during May 1995. Samples collected from the fundus region and the corpus regions of the uterine horn were kept separately. The tissues were placed onto crushed dry ice within 30 min after death. They were wrapped in aluminium foil and immediately snap frozen in liquid nitrogen. Subsequently the samples were transported to the laboratory on dry ice and stored at -70°C until processed. During all dissections, the endometrium was removed first as this tissue was considered to be the most important to this study.

Equilibrium binding assays

Cytosol fractions of the endometrium and myometrium were prepared separately as described by Potgieter *et al.* (1985). The uterine tissue was first cut into thin slices on powdered dry ice. Leupeptin (to a final concentration of 10×10^{-3} mol l⁻¹) was added to 10% (w/v) tissue in TEDAG₁₀ buffer, before homogenization, to inhibit protease activity (Leake and Habib, 1987). The tissue was homogenized in three bursts of 10 s duration, alternated with a 50 s cooling period on crushed ice. A TP Ultra Turrax (Janke and Kunkel, Staufen, Germany) was used during homogenization. The homogenate was centrifuged at 105 000 g for 30 min at 4°C. The protein concentration in the cytosol was determined by the method of Bradford (1976).

Duplicate aliquots (100 µl) of cytosol were incubated with either 50 µl [³H]oestradiol for the oestrogen receptor or [³H]progesterone or [³H]promegestone for the progesterone receptor. The radiolabelled compounds were made up in buffer at seven different concentrations, that ranged from 1 to 12×10^{-9} mol l⁻¹. The selection of this range was based on an initial 15-point saturation curve (0.5 to 64×10^{-9} mol l⁻¹) that indicated that saturation occurred at approximately 16×10^{-9} mol l⁻¹. The buffer (TEDAG₁₀) contained 10×10^{-3} mol Tris-HCl l⁻¹, 1.5×10^{-3} mol EDTA l⁻¹, 1×10^{-3} mol dithiothreitol l⁻¹, 1×10^{-3} mol sodium azide l⁻¹ and 10% (w/v) glycerol with the pH adjusted to 7.4 at 4°C. Nonspecific binding was determined by adding 1000-fold excess of unlabelled diethylstilboestrol (DES), or progesterone or promegestone for the oestrogen and progesterone receptor assays, respectively. All volumes in the tubes were adjusted to a final concentration of 400 µl by the addition of an appropriate volume of buffer. Total count tubes contained 50 µl of the

radioligand and 350 µl buffer. Incubations for the oestrogen receptor lasted 30 min at 20°C. Progesterone receptor assays were incubated on crushed ice for 2 h, after which 100 µl TEDAG₆₀ (60% glycerol (w/v)) was added, followed by an additional incubation on crushed ice for a further 2 h. All reactions were terminated with the addition of 500 µl dextran-coated charcoal. Fresh dextran-coated charcoal solutions were prepared every 2 weeks and consisted of 0.5% (w/v) pre-washed, activated charcoal, 0.1% (w/v) gelatine and 0.5% dextran T70 in TEDAG₁₀ buffer. After the addition of dextran-coated charcoal, the suspension was vortexed, left on ice for 10 min and centrifuged at 2000 g for 15 min. Supernatants (500 µl) were assayed for radioactivity after adding 4 ml scintillation cocktail. The assays for the oestrogen and progesterone receptors were carried out simultaneously and the tissue from each elephant was analysed separately. The processing of frozen duplicates of the same tissue on different days was used to measure the variability in receptor concentrations within and between assays. The interassay coefficients of variation for the progesterone receptor and oestrogen receptor were 16.9% and 11.5%, respectively. The intra-assay coefficients of variation for both receptor types was < 10%.

Ligand specificity of the oestrogen and progesterone receptors

Endometrial and myometrial tissues were chopped finely with a sharp scalpel while frozen in a Petri dish filled with powdered dry ice. A homogeneous mixture was made of the pieces of both types of tissue and this mixture was subsequently homogenized and assayed for oestrogen and progesterone receptors according to the method of Potgieter *et al.* (1985). Cytosol aliquots (100 µl) were incubated in triplicate for 30 min at 20°C with 50 µl of either [³H]oestradiol (8×10^{-9} mol l⁻¹), or [³H]progesterone (8×10^{-9} mol l⁻¹) in the presence of increasing concentrations (0.3125×10^{-9} mol l⁻¹– 1×10^{-4} mol l⁻¹) of each of the following competitors: ORG 2058, norethindrone, promegestone, levonorgestrel, RU 486, medroxyprogesterone, 5α-pregnane-3,20-dione, 5α-pregnane-3α-ol-20-one, 5α-pregnane-3β-ol-20-one, 5α-pregnane-3β,20α-diol, 5β-pregnane-3,20-dione, 5β-pregnane-3α,20β-diol, 5β-pregnane-3α,20α-diol, 17α-hydroxyprogesterone, 11α-hydroxyprogesterone, oestradiol-17β, oestrone, oestriol, tamoxifen, pregnenolone, 4-pregnen-20α-ol-3-one, hydrocortisone, cortisone, testosterone, dehydrotestosterone and danazol. The reactions were terminated by the addition of dextran-coated charcoal and counted for radioactivity as set out in the previous section.

Each assay included a set of tubes containing a 1000 molar excess of unlabelled DES or progesterone for the oestrogen and progesterone competitive binding assays, respectively; this was used to correct for non-specific binding and to monitor tissue degradation (Wakeling, 1987). If the specific binding did not account for > 60% of the total binding, the tissue was regarded as degraded and the results were not used. Owing to tissue degradation the final analyses are based on seven samples of endometrial (three from the corpus and four from the fundus regions) and five samples of myometrial tissue (three from the corpus region and two from the fundus regions). Calculation of relative binding affinity was determined

Table 1. Binding affinities of the oestrogen and progesterone receptors (mean $K_d \times 10^{-9}$ mol l⁻¹ \pm SEM for the number of individual animals in parentheses) determined by means of 7-point Scatchard plots in nonpregnant, nonlactating, female African elephants

| Receptor type | Tissue type | | | |
|---|-------------------------------|-------------------------------|------------------------------|------------------------------|
| | Endometrial corpus region (3) | Endometrial fundus region (4) | Myometrial corpus region (3) | Myometrial fundus region (2) |
| Oestrogen | 0.140 \pm 0.025 | 0.185 \pm 0.018 | 0.223 \pm 0.038 | 0.190 \pm 0.100 |
| Progesterone (with [³ H]promegestone) | 0.250 \pm 0.056 | 0.235 \pm 0.033 | 0.233 \pm 0.072 | 0.135 \pm 0.025 |
| Progesterone (with [³ H]progesterone) | 0.677 \pm 0.223 | 1.058 \pm 0.263 | 1.470 \pm 0.105 | 0.830 \pm 0.180 |

at the 50% competition level from a displacement curve for each ligand, constructed by plotting the percentage specific binding versus log of the concentration of the competing steroid, followed by the application of Rodbard's equation (1973).

Statistical analyses

Scatchard and saturation curves with the Rosenthal-correction for nonspecific binding were drawn by means of the COMBICEPT 2000CA software program (Packard Instrument Company, Downers Grove, IL). Dissociation constant (K_d) values are given in 10^{-9} mol l⁻¹ (mean \pm SEM). The Kruskal-Wallis H and Mann-Whitney U test were used to test the differences in binding affinities for the different tissue types. Significance was taken at a 95% level and mean values are followed by one standard error of the mean. Results from Scatchard plots and the competitive binding assays were subjected to least square regression analyses and only data with correlation coefficients of ≥ 0.9 were considered reliable for further analyses.

Results

Estimated mean K_d values for the endometrial and myometrial tissue for the oestrogen receptor differed significantly from those values obtained for the progesterone receptor assay with either [³H]promegestone or [³H]progesterone (Table 1). The mean K_d value for the progesterone receptor assay using [³H]progesterone as radioligand was significantly higher than that calculated from the binding data when [³H]promegestone was used as radioligand. The mean K_d value for the oestrogen receptor proved to be significantly lower than that obtained for the progesterone receptor when [³H]progesterone was employed as radioligand ($P < 0.05$).

The relative binding affinity and apparent K_d values for the various competitors for endometrial progesterone receptors are presented (Table 2). Degradation of the tissue, as discussed previously, precluded estimation of these values for the myometrium, except for the binding of promegestone (relative binding affinity = 199%, $K_d = 0.61 \times 10^{-9}$ mol l⁻¹), RU 486 (relative binding affinity $< 0.1\%$) and oestrone (relative binding affinity $< 0.1\%$). These relative binding affinity values were

Table 2. Relative binding affinities and apparent K_d values of selected potential competitors for the endometrial progesterone receptor in four nonpregnant, nonlactating female African elephants

| Competitor | Apparent K_d ($\times 10^{-9}$ mol l ⁻¹) | Relative binding affinity (%) |
|--|---|-------------------------------|
| ORG 5028 | 0.24 | 380 |
| Norethindrone | 0.30 | 293 |
| Promegestone | 0.65 | 138 |
| Progesterone | 0.89 | 100 |
| 5 α -pregnane-3,20-dione | 2.10 | 43 |
| Levonorgestrel | 3.60 | 24 |
| 5 α -pregnane-3 α -ol-20-one | 4.50 | 20 |
| 5 α -pregnane-3 β -ol-20-one | 27.00 | 3 |
| 5 β -pregnane-3,20-dione | 29.00 | 3 |

The relative binding affinity was $< 0.1\%$ for 5 α -pregnane-3 β ,20 β -diol, 5 β -pregnane-3 α ,20 β -diol, 5 β -pregnane-3 α ,20 α -diol, 17 α -hydroxyprogesterone, 11 α -hydroxyprogesterone, medroxyprogesterone, mifepristone (RU 486), oestradiol, oestrone, oestriol, tamoxifen, pregnenolone, 4-pregnen-20 α -ol-3-one, hydrocortisone, cortisone, testosterone, dehydrotestosterone and danazol.

similar to those obtained for the same competitors when tested with endometrial tissue. The progesterone receptor had a high relative binding affinity for both 5 α -pregnane-3,20-dione (43%) and 5 α -pregnane-3 α -ol-20-one (20%), which are known to be produced in the corpus luteum of the pregnant elephant (Hodges *et al.*, 1994). The two synthetic progestins norethindrone and levonorgestrel, used as oral contraceptives by women, both exhibited a relatively high affinity for the elephant progesterone receptor (norethindrone: relative binding affinity = 293%, $K_d = 0.30 \times 10^{-9}$ mol l⁻¹ and levonorgestrel: relative binding affinity = 24%, $K_d = 3.6 \times 10^{-9}$ mol l⁻¹). However, RU 486, as well as oestrogens, androgens and corticosteroids, had relative binding affinity values $< 0.1\%$. The competitive binding plots for selected ligands are presented (Fig. 1).

A summary of the relative binding affinity and K_d values of the competitors that were tested on the endometrial oestrogen receptor is presented (Table 3). The displacement curves of the most important competitors are given (Fig. 2). These assays were also conducted on the myometrium but, owing to tissue degradation, estimates could be obtained only for DES

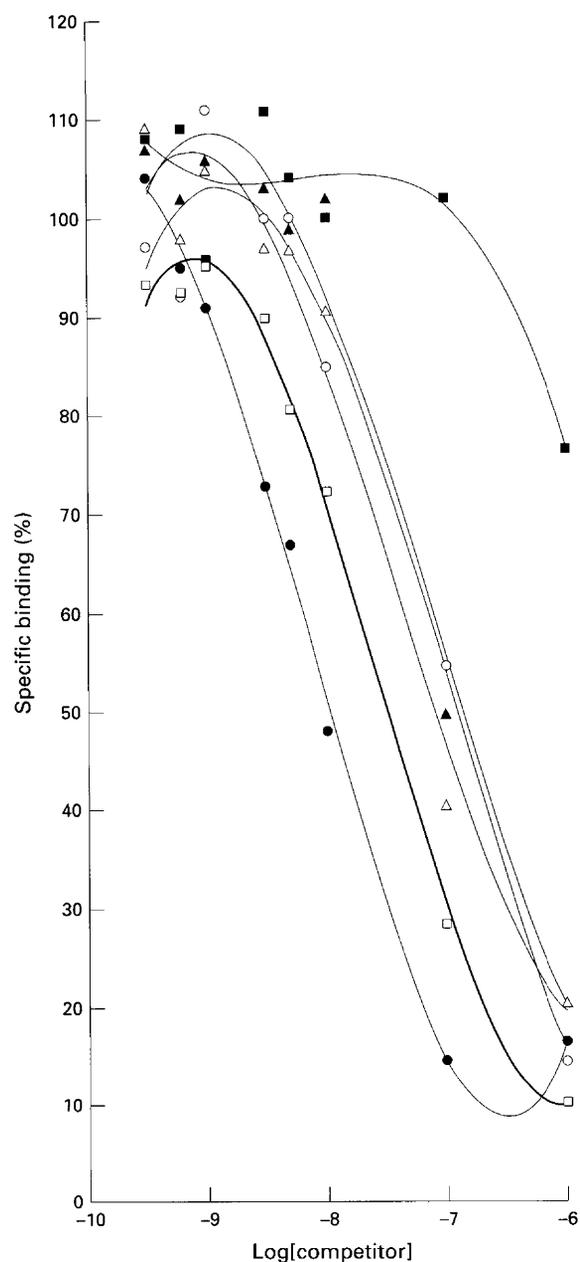


Fig. 1. Competition of various ligands for binding to the endometrial progesterone receptor of African elephants. Norethindrone (●), progesterone (□), 5 α -pregnane-3,20-dione (Δ), levonorgestrel (\blacktriangle), 5 α -pregnane-3 α -ol-20-one (○), RU 486 (■).

(relative binding affinity = 252%, $K_d = 0.083 \times 10^{-9} \text{ mol l}^{-1}$), ethinyl oestradiol (relative binding affinity = 102%, $K_d = 0.20 \times 10^{-9} \text{ mol l}^{-1}$), oestrone (relative binding affinity = 24%, $K_d = 0.88 \times 10^{-9} \text{ mol l}^{-1}$) and oestriol (relative binding affinity = 12%, $K_d = 1.8 \times 10^{-9} \text{ mol l}^{-1}$). These values were similar to those obtained with the endometrium.

Discussion

Antiprogestins could prevent conception by creating a uterine environment hostile to implantation (Cooke *et al.*, 1988) and

Table 3. Relative binding affinities and apparent K_d values of selected potential competitors for the endometrial oestrogen receptor in four nonpregnant, nonlactating female African elephants

| Competitor | Apparent K_d ($\times 10^{-9} \text{ mol l}^{-1}$) | Relative binding affinity (%) |
|--------------------|---|----------------------------------|
| Diethylstilbestrol | 0.14 | 119 |
| Ethinyl oestradiol | 0.15 | 112 |
| Oestradiol | 0.17 | 100 |
| Oestrone | 1.40 | 12 |
| Oestriol | 1.70 | 10 |

The relative binding affinity was < 0.1% for tamoxifen, progesterone, promegestone, norethindrone, mifepristone (RU 486), 5 α -pregnane-3,20-dione, 5 α -pregnane-3 α -ol-20-one, hydrocortisone, cortisone, testosterone and dehydrotestosterone.

this makes the hormone receptor the most specific and most accessible target for intervening in hormone action (Baulieu, 1989). However, differences between species in the uptake, binding and transport of steroids and also in the nature of the progesterone receptor interaction (King and Mainwaring, 1974) caution against the use of synthetic steroids without first examining each compound within the particular species of interest and correlating binding affinity with biological activity (Gray and Leavitt, 1987). No information is available presently on uterine steroid hormone receptors in the African elephant and until the relative binding affinities of the oestrogen and progesterone receptors have been screened for a range of antigestagenic steroids, the potential use of antigestagens such as mifepristone (RU 486) to induce abortion, as suggested by Short (1992), cannot be considered seriously as a means of birth control in elephants. Competitive binding studies, such as those used in the present study, should indicate which antigestagens may be of potential use in the development of techniques to control conception rate in elephant populations. However, a high affinity would still have to be correlated with a biological response *in situ*.

The only progestagen that competed successfully for the progesterone receptor in the present ligand-binding studies was norethindrone, which bound to the receptor nearly three times as strongly as progesterone. Norethindrone and norethindrone acetate are used widely to prevent pregnancy in women (Taitel *et al.*, 1995). Low doses of norethindrone and of levonorgestrel (relative binding affinity = 24% in the present study) are considered to have an antifertility effect, while high doses can have an anti-ovulatory effect (Lobo and Stanczyk, 1994). Alternatively, RU 486, which is known to act strongly as an antiprogestin in humans (Gravanis *et al.*, 1985), rabbits and rats (Philibert, 1984), showed a very low affinity for both the progesterone and oestrogen receptors of the uterus of the African elephant. A low relative binding affinity for RU 486 has also been reported in hamsters (Gray and Leavitt, 1987), chickens (Groyer *et al.*, 1985) and tammar wallabies (Fletcher and Blandon, 1988). Gray and Leavitt (1987) found that in hamsters the low binding affinity of the progesterone receptor for RU 486 did not prevent decidualization and normal implantation in the uterus. Should this be the case for

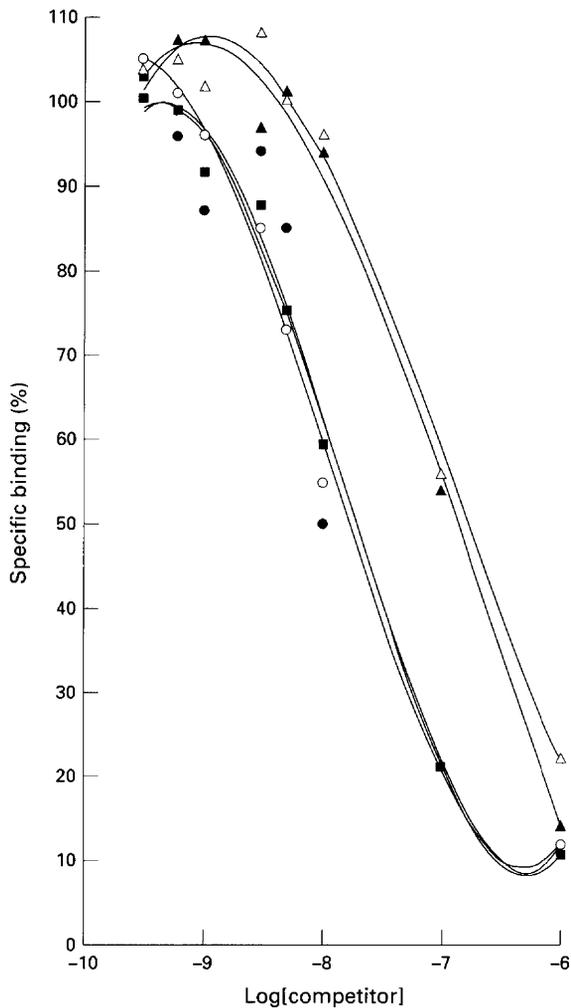


Fig. 2. Competition of various ligands for binding to the endometrial oestrogen receptor of African elephants. Ethinyl oestradiol (●), diethylstilbestrol (○), oestrone (▲), oestriol (△), oestradiol (■).

elephants, RU 486 could no longer be considered to be of potential use to control elephant reproductive output (Short, 1992; Poole, 1993). Although comparative strong binding to the receptor does predict a possible potency as a contraceptive agent, as suggested for norethindrone and levonorgestrel in this present study, a discrepancy in the observed potency may still arise once an agent is applied.

Neither of the two 5α -reduced metabolites of progesterone (5α -pregnane-3,20-dione and 5α -pregnane-3 α -ol-20-one), known to be produced in the corpus luteum of elephants (Hodges *et al.*, 1994), had a stronger binding affinity for the progesterone receptor than for progesterone. However, affinity is not the only criterion that determines biological efficiency. Despite a low affinity, a low metabolic clearance rate could ensure sustained concentrations in the blood and thereby increase efficiency markedly (Sutherland and Jordan, 1981). Biological potency therefore depends on a combination of receptor affinity and persistence in target tissues (Katzenellenbogen, 1980). The relatively high binding affinity of the progesterone receptor in the elephant for 5α -dihydroprogesterone (43%) which occurs at a 10–20 fold

higher concentration than progesterone in the plasma of pregnant animals (Heistermann *et al.*, 1994), suggests that this metabolite may have some biological activity in this species. The metabolic clearance rate of the 5α -reduced metabolites of progesterone, and the binding affinities of carrier proteins for these substances in the plasma, have yet to be determined for the African elephant. Until these studies are undertaken it remains inappropriate to speculate further on the biological significance of 5α -dihydroprogesterone in this species. However, it is worth noting that progesterone metabolites can act as intracellular mediators of progesterone action in the endometrium (Armstrong and King, 1971).

In the present study, 5β -isomers of progesterone displayed a very low binding affinity for the progesterone receptor when compared to 5α -reduced metabolites. This finding is in agreement with King and Mainwaring (1974), who found that 5α -reduced compounds usually retain some of the biological properties of the parent compound, whereas 5β -isomers lose or change the properties completely.

In the African elephant, both the oestrogen and progesterone receptors proved to be highly specific. Oestradiol had nearly the same affinity for the oestrogen receptor as ethinyl oestradiol and DES, while tamoxifen had a very low affinity for the receptor. This low affinity may have been caused by the hydrophobic nature of tamoxifen resulting in the adsorption to the glass or plastic apparatus, thereby leading to significant errors at low concentrations (Wakeling, 1987). It is more likely, however, that the 4-hydroxytamoxifen metabolite of tamoxifen would have had a stronger affinity for the oestrogen receptor than that of tamoxifen since Rochefort *et al.* (1979) found that this metabolite is ten times more potent than its parent compound.

The fact that the progesterone receptor assays using [3 H]promegestone had a significantly lower K_d value than the assays using [3 H]progesterone, indicates that this receptor has a higher affinity for promegestone than for progesterone. This higher affinity can be explained by the knowledge that promegestone has a high progestin selectivity (Ojasoo, 1995), a nanomolar K_d value for the progesterone receptor and an inability to recognise corticosteroid-binding globulin (Milgrom *et al.*, 1973). The K_d values for the endometrial and myometrial oestrogen and progesterone receptors were similar, suggesting that the receptors of these tissues may have similar molecular characteristics. This possibility is further supported by the observed similarity in relative binding affinity values of competitors obtained for receptors in both the endometrium and the myometrium. This finding is also in agreement with the observation of Grossman and Leavitt (1974) who showed that progesterone receptors in the endometrium of hamsters have the same physiochemical properties as the receptors in the myometrium. However, the myometrial tissue did prove to be more labile than the endometrial tissue, probably as a result of a time-delay in preservation during field collection.

On the basis of the present study we conclude that, in the African elephant, the 5α -reduced progesterone metabolites are of some biological importance, since these substances bound to the progesterone receptor with a relatively high affinity. Furthermore, RU 486, with its low affinity for the progesterone receptor, appears to be unsuitable as a potential antifertility agent in the African elephant. In this regard, the high relative

binding affinity of norethindrone for the progesterone receptor deserves further investigation. Both oestrogen and progesterone receptors proved to be highly specific, which necessitates the screening of all anti-gestagenic substances before they can be applied to reduce the reproductive output in elephants.

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