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Binding of [^{3}H]-Methyltrienolone (R1881) by Human Breast Cancers

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Abstract—*The synthetic radioligand* [^{3}H]-*R*1881 *binds to both androgen and progestogen receptors; these two types of receptor activity can be separated by competition experiments with radioinert steroids of defined biological activity. Using two standard tissues, rat prostate and human uterus, which are sources of androgen and progestogen receptors respectively, the optimal conditions for the determination of each type of activity were established. For the purposes of routine assay, androgen receptors were quantified after saturation of progestogen receptor sites with* 125 nM *radioinert* R5020 *using* [^{3}H]-*R*1881 *and increasing concentrations of radioinert* R1881. *Progestogen receptor activity could be identified using the same radioligand and competition with radioinert progesterone or* R5020, *though for routine purposes, progestogen receptors were quantified using the more specific radioligand,* [^{3}H]-*R*5020. *The binding of* [^{3}H]-*R*1881 *to tumour cytosol was examined in* 122 *human breast cancers. Seventy-two tumours* (59%) *showed binding. Androgen receptor activity alone was present in* 16 *tumours, progestogen receptor activity alone in* 30 *tumours and both types in* 26 *tumours. Tumours containing progestogen receptor activity also showed binding to the progestogen* [^{3}H]-*R*5020, *whilst those containing androgen receptors alone did not. Androgen receptor concentration varied from* 17 *to* 210 fmol *binding sites/*mg *cytosol protein (mean value* 68) *and the mean* K_d *was* 2.15×10^{-9}M. *Progestogen receptor concentration varied from* 25 *to* 1350 fmol *binding sites/*mg *cytosol protein (mean value* 410) *and the mean* K_d *was* 1.35×10^{-9}M. *The biological significance of the presence of these types of receptor in human breast cancers is currently being assessed from clinical follow-up.*

INTRODUCTION

THE SYNTHETIC androgen, methyltrienolone (R1881), forms a useful ligand for the measurement of androgen receptor activity. Unlike the natural androgen, 5α-dihydrotestosterone, R1881 does not bind with high affinity to serum proteins such as sex hormone binding globulin and is not readily metabolised by target tissues [1]. This may be of particular value in human breast cancers which actively metabolise steroid hormones [2] and may be contaminated with serum-derived material. However, R1881 also binds to progestogen receptors [3, 4], which are often present in breast tumours [5]; this is a complicating factor in using the ligand for the measurement of androgen receptors in human breast cancer. The present study describes the characterization of the binding of tritiated R1881 in cytosol according to whether competition occurs with androgen, progestogen or both types of steroid, the determination of the optimal conditions needed for the assay of each steroid receptor and the application of these methods to human breast cancers.

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MATERIALS AND METHODS

Chemicals

Radioinert methyltrienolone (R1881), promegestone (R5020), [^{3}H]-R1881 (58.2 Ci/mmol) and [^{3}H]-R5020 (56.5 Ci/mmol) were gifts from Dr J. P. Raynaud (Roussel-Uclaf, France). Radioinert 5α-dihydrotestosterone (5α-DHT), progesterone and triamcinolone acetonide were obtained from Sigma (Poole, U.K.).

Tissues

Prostate was obtained from male Sprague Dawley rats 24 hr after bilateral orchidectomy.

Human uterus was obtained from patients undergoing hysterectomy.

Breast cancers were obtained at mastectomy or biopsy from patients with histologically proven disease.

All material was transported on ice to a cold room and immediately processed.

Cytosol preparation

Tissues were dissected free from surrounding fat and connective tissue, finely cut with scissors and homogenised at 0°C at a tissue: buffer ratio of 1:5 (w/v) in Tris buffer (10 mM Tris, 0.25 M sucrose and 1.5 mM EDTA to which 7.8 mg dithiothreitol, i.e. 0.5 mM, was added per 100 ml buffer immediately before use) employing a Silverson homogenizer at minimum speed for 3 × 15 sec with 1-min intervals for cooling. The homogenate was centrifuged at 105,000 *g* for 1 hr in an MSE Superspeed 50 centrifuge and the resulting supernatant was used as cytosol.

Binding measurements

All measurements were performed at 0°C. Cytosol (200 μl) was incubated with 500 μl Tris buffer, which contained competitor steroid where appropriate. Studies with [^{3}H]-R1881 were performed in the absence and presence of 125 nM R5020. The only competitor studied in the absence of R5020 was progesterone at a final concentration of 10 nM, and those in the presence of R5020 were R1881 (final concentrations 1, 2, 4, 8 and 1000 nM) and 5α-DHT. For studies with [^{3}H]-R5020, radioinert R5020 was used as competitor at final concentrations of 1, 2, 4, 8 and 1000 nM.

Tubes containing radioinert competitor and cytosol were left for 40 min before either [^{3}H]-R1881 or [^{3}H]-R5020 (final concentrations 0.5 nM) was added and the tubes mixed and incubated overnight. Bound steroid was then separated from free steroid by the addition of 400 μl dextran-coated charcoal (0.0625/0.625% w/v respectively). After 10 min the tubes were centrifuged and the total supernatant (bound fraction) was decanted into a counting vial containing 10 ml of Liquifluor (Packard). The two phases were mixed, incubated at 37°C for 2 hr and the radioactivity measured in a Tricarb liquid scintillation counter.

Cytosol protein

The protein content of each cytosol was determined by the method of Bradford [6], using bovine serum albumin as standard.

RESULTS

Human uterus

All cytosols of human uterus showed binding of [^{3}H]-R1881. Studies performed with radioinert steroids as competitors showed that R1881, R5020 and progesterone all inhibited the binding but that 5α-DHT was ineffective [Table 1(a)]. The effect of increasing concentrations of radioinert R5020 is shown in Fig. 1. Although binding was only completely inhibited with 500 nM R5020, only a trace of radioligand remained bound at concentrations above 100 nM. These results indicate that the binding was to progestogen receptors, a view supported by a high specific binding of [^{3}H]-R5020 observed with the same cytosols (not shown).

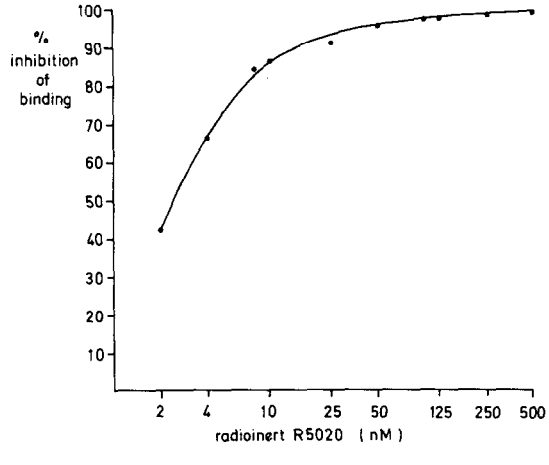

Fig. 1. The effects of increasing concentrations of radioinert R5020 on binding of [^{3}H]-R1881 by human uterus. Percentage inhibition of binding was calculated as described in Table 1. Each point represents the mean of 3 separate estimations. Experimental conditions are as described in Materials and Methods.

Table 1. Characterization of [^{3}H]-R1881 binding to cytosols from human uterus and rat prostate

Tissue	% inhibition of binding by competitor: 5α-DHT (10 nM)	R1881 (10 nM)	Pg (10 nM)	R5020 (10 nM)	R5020 (125 nM)
(a) Human uterus	1.3 ± 0.7	57.7 ± 4.1	43.8 ± 4.0	86.2 ± 1.5	97.4 ± 0.5
(b) Rat prostate	71.2 ± 3.5	81.3 ± 3.6	5.7 ± 2.0	0	5.6 ± 3.2

Values represent the mean ± S.E. of the mean for 4 separate experiments. Percentage inhibition was calculated from the binding in the presence of the appropriate competitor and that in the absence of any competitor, both values being corrected for the non-specific binding found in the presence of 1000 nM radioinert R1881.

Rat prostate

All cytosols of rat prostate specifically bound [^{3}H]-R1881. The binding as characterised by competition studies [Table 1(b)] was different from that in human uterus. In the rat prostate system radioinert R1881 and 5α-DHT were effective competitors, whereas R5020 and progesterone had negligible effects. Only at concentrations in excess of 125 nM did R5020 consistently inhibit binding of [^{3}H]-R1881. Data presented in Fig. 2 show the effect of including 125 nM R5020 on the binding of [^{3}H]-R1881 analysed according to Scatchard [7]. At this concentration R5020 had little effect on the dissociation constant of binding (1.3×10^{-9}M) but reduced the concentration of receptor sites by about 5%. These results indicate that the binding of [^{3}H]-R1881 in rat prostatic cytosol was to androgen receptors. Absence of progestogen receptors from rat prostate was confirmed by lack of specific binding of [^{3}H]-R5020 in these cytosols (not shown).

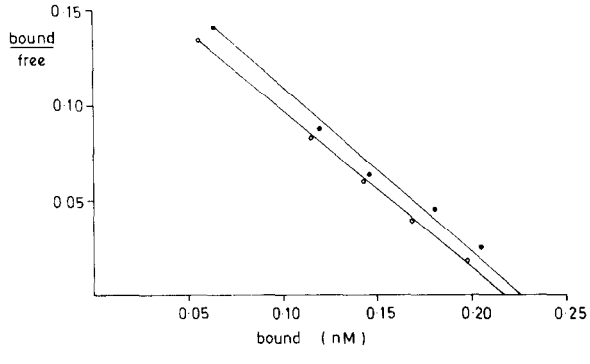

Fig. 2. Scatchard analysis of the binding of [^{3}H]-*R*1881 *in the absence* (●) *and presence* (○) *of* 125 nM *R*5020 *to rat prostatic cytosol. The latter was obtained by pooling prostates from* 5 *animals (protein concentration,* 8.75 mg/ml). *Each point represents the mean of triplicate estimations. Experimental conditions are as described in Materials and Methods.*

Mixture of cytosols from human uterus and rat prostate

In order to determine whether it would be feasible to quantify androgen receptors in the presence of progestogen receptors by blocking the progestogen receptor with excess progestogen, experiments were performed with a mixture of cytosols from rat prostate and human uterus. Cytosols were prepared from rat prostate and human uterus as described in Materials and Methods and aliquots were mixed in equal volumes and assayed with tritiated R1881 and increasing concentrations of radioinert R1881 in the presence and absence of 125 nM R5020. In addition, the prostatic and uterine cytosols were each assayed separately after dilution with an equal volume of buffer. The results are shown in Fig. 3. Scatchard analysis showed that the binding

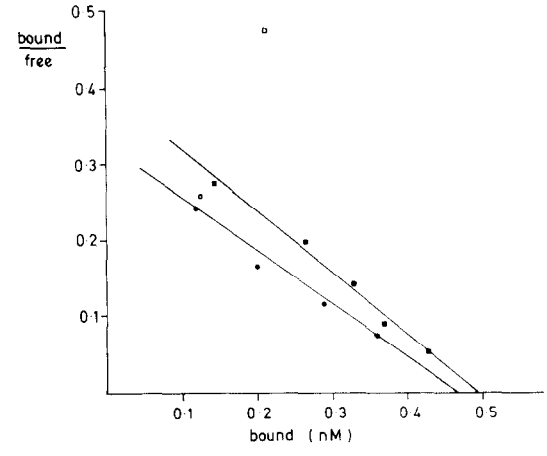

Fig. 3. Scatchard analysis of the binding of [^{3}H]-*R*1881, *in the presence of* 125 nM *R*5020 *to cytosol of rat prostate diluted with an equal volume of buffer* (●), *and to the same cytosol diluted with an equal volume of cytosol from human uterus* (■). *Open symbols represent the binding of each cytosol in the absence of both radioinert steroids R*1881 *and R*5020. *Each point is the mean of triplicate estimations. Prostatic cytosol was obtained by pooling prostates from* 5 *animals (protein concentration* 9.5 mg/ml). *The protein concentration of human uterine cytosol was* 6 mg/ml. *Experimental conditions are as described in Materials and Methods.*

in the combined cytosol was 6.4% higher than that in rat prostatic cytosol alone, although the dissociation constants for the single and combined cytosols were similar. The additional binding in the combined cytosol over that seen in the prostatic cytosol alone may represent incompletely blocked progestogen receptor sites or the presence of small amounts of androgen receptor in the uterine preparation, though Scatchard analysis of the small amount of binding of [^{3}H]-R1881 in the presence of 125 nM R5020 by human uterus failed to yield a satisfactory plot.

Human breast cancer

Binding of [^{3}H]-R1881 was measured in the cytosols from 122 human breast cancers. Tumours were classified as showing specific binding if more than 200 cpm were displaced by the addition of 1000 nM radioinert R1881. This displacement is equivalent to 5 pmol/l (which, on the basis of an average tumour cytosol, would correspond to approximately 7 fmol/mg protein). Using this criterion, 72 tumours bound [^{3}H]-R1881 (Table 2). The binding was characterised by using radioinert 5α-DHT, progesterone and R5020 as competitors. These studies show that in 30 tumours, progesterone or R5020 but not 5α-DHT blocked the binding (progestogen receptor activity); in 16 tumours 5α-DHT but not progesterone or R5020 inhibited binding (androgen receptor activity); and in 26 tumours all 3 steroids acted as competitors (both progestogen and androgen receptor activities).

Table 2. Binding of [³H]-R1881 to cytosols of 122 human breast cancers

Binding	No. of tumours	(% of total)
None	50	(41)
Progestogen receptors*	30	(25)
Progestogen receptors + androgen receptors†	26	(21)
Androgen receptors‡	16	(13)

*Competition with progesterone and R5020.
†Competition with progesterone, R5020 and 5α-dihydrotestosterone.
‡Competition with 5α-dihydrotestosterone.

Parallel studies were performed using [³H]-R5020 as radioligand. These showed that tumours which had bound [³H]-R1881 and in which binding had been blocked by progesterone and R5020 also specifically bound [³H]-R5020. Conversely, tumours which had failed to bind [³H]-R1881, or in which binding to [³H]-R1881 was displaced only by 5α-DHT, failed to bind [³H]-R5020. Thus of the 122 tumours investigated, 56 (46%) contained progestogen receptors and 42 (34%) contained androgen receptors (Table 2).

Quantitation of androgen receptors was performed by measuring the effects of increasing levels of radioinert R1881 on the binding of [³H]-R1881 in the presence of 125 nM R5020. The concentrations of progestogen receptors in the same tumours were obtained by measuring binding of [³H]-R5020 in the presence of varying amounts of radioinert R5020. The results are shown in Table 3. Typical Scatchard plots for the binding of [³H]-R1881 by the cytosols from 2 human breast cancers (I and II), one of which also bound R5020(II), are shown in Fig. 4. The mean dissociation constant of binding of [³H]-R1881 (in the presence of 125 nM R5020) for all the positive tumours was 2.15×10^{-9}M (range, $1.0–3.9 \times 10^{-9}$M) and that for [³H]-R5020 was 1.35×10^{-9}M (range, $0.4–3.2 \times 10^{-9}$M).

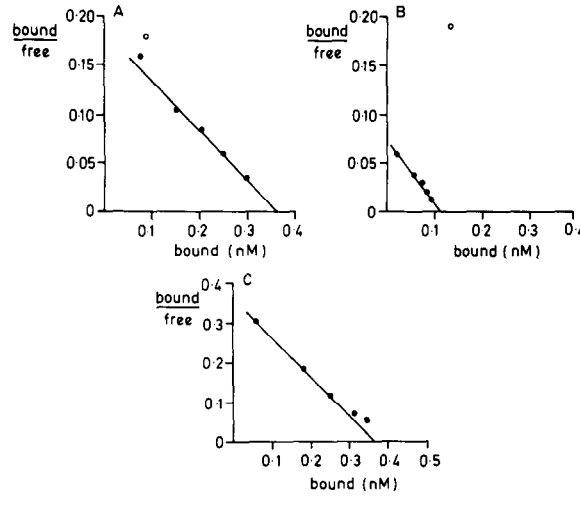

Fig. 4. (A) Scatchard plot for binding of [³H]-R1881 to cytosol of a human breast cancer (I) (protein concentration, 3.1 mg/ml). Solid symbols represent binding in the presence of a fixed concentration of radioinert R5020 (125 nM) and increasing concentrations of radioinert R1881; open symbol represents binding of [³H]-R1881 in the absence of both radioinert R1881 and R5020. This cytosol did not bind [³H]-R5020. (B) Scatchard plot for binding of [³H]-R1881 to cytosol of a human breast cancer (II) (protein concentration, 3.5 mg/ml). Symbols as for (A). This cytosol bound [³H]-R5020, shown in (C). (C) Scatchard plot for binding of [³H]-R5020 to cytosol of human breast cancer II. Each point represents the mean of duplicate estimations. (Assay conditions as described in Materials and Methods.)

DISCUSSION

The use of [³H]-R1881 as a radioligand for the measurement of steroid receptors in human breast cancers suffers from the disadvantage that this synthetic steroid binds to both progestogen and androgen receptors. Since breast cancers may contain one or both of these receptors, it is necessary to characterise the binding of [³H]-R1881 in each tumour. Therefore, in order to quantify receptor activity in tumours with both types of receptor, it is necessary to selectively block binding to one type of receptor while measuring

Table 3. Concentrations of androgen and progestogen receptor binding sites in cytosols of human breast cancers

Tumour type	Concentration of binding sites (mol/mg cytosol protein) [³H]-R1881 binding Mean ± S.E.M. (range)	[³H]-R5020 binding Mean ± S.E.M. (range)
Androgen receptors alone	90 ± 17 (19–217)	— —
Progestogen receptors alone	— —	428 ± 61 (40–1345)
Androgen and progestogen receptors	55 ± 9 (17–210)	370 ± 73 (26–1250)
Total binding	68 ± 9 (17–217)	413 ± 46 (26–1345)

Table 4. Method for routine assay of androgen receptors in human breast cancer using [³H]-R1881

All procedures to be performed at 0°C. 500 mg tumour homogenised in 2.5 ml Tris–sucrose buffer*

Homogenate centrifuged at 105,000 *g* for 1 hr. Resultant cytosol (200 μl) added in duplicate to 500 μl buffer containing 125 nM R5020† in the absence and presence of 1, 2, 4, 8 and 1000 nM R1881† (12 tubes)

Tubes mixed and left for 40 min. 100 μl of [³H]-R1881 (0.5 nM ≃ 20,000 cpm) added, tubes mixed and left overnight

400 μl of dextran-coated charcoal suspension added, tubes mixed, left for 10 min and centrifuged at 3000 rpm for 10 min. Supernatant decanted directly into scintillation vials containing liquifluor (10 ml), mixed and incubated at 37°C for 2 hr

Vials counted by liquid scintillation spectrometry and data analysed according to Scatchard using the 1000-nM tubes to correct for non-specific binding

*If additional material is available, by homogenising 750 mg in 3.75 ml buffer, it is possible to check that the binding of [³H]-R1881 is to androgen receptor by also incubating 200 μl of cytosol with 500 μl of 5α-DHT (10 nM) in the presence of R5020 (125 nM) in duplicate and to assess quantitatively progesterone receptor activity by incubating cytosol in duplicate with 500 μl Tris–sucrose buffer with and without 10 nM progesterone (a further 6 tubes).

†Values refer to the final concentration in a total volume of 0.8 ml.

the concentration of the other. Such a technique has been used successfully to measure androgen receptors by suppressing binding to the progestogen receptor with triamcinolone acetonide [8]. However, under the assay conditions employed in the present study, excess of triamcinolone acetonide did not completely block progestogen receptor activity until very high concentrations, which also blocked androgen receptors, were used. In addition, since an assay for progestogen receptors using [³H]-R5020 was already established in our laboratory, it seemed appropriate to measure androgen receptor activity with [³H]-R1881 plus excess R5020 to block binding to the progestogen receptors.

In order to validate our method, we have studied the binding of [³H]-R1881 in rat prostate, reported to be a source of androgen receptor in the absence of significant levels of progestogen receptor, and human uterus, reported to be a source of progestogen receptor in the absence of significant amounts of androgen receptor. Specific binding of [³H]-R1881 to human uterus was readily blocked by progesterone and R5020, confirming that the binding in this tissue was predominantly to progestogen receptors. There was only a small but consistent (about 1%) inhibition of the binding by 5α-DHT; this may represent the presence of trace amounts of androgen receptors within the uterine cytosol. In contrast, the binding of [³H]-R1881 by cytosol of rat prostate was effectively blocked by 5α-DHT, whilst progestogens such as progesterone and R5020 had only minimal effects. The latter may indicate the presence of low levels of progestogen activity within rat prostate or a slight cross-reaction of progestogens with the androgen receptor.

R5020 at a concentration of 125 nM was selected for maximal blocking of progestogen receptor activity with minimal effects on androgen receptor activity. At this concentration R5020 blocked around 97% of the binding of [³H]-R1881 to human uterus but only about 5% of that to rat prostate. Scatchard analysis of [³H]-R1881 binding by rat prostate in the absence and presence of 125 nM R5020 showed similar kinetics, but with a reduction in receptor site concentration of around 5%. Experiments in which cytosols from rat prostate and human uterus were assayed separately and in combination confirmed that androgen receptors could be satisfactorily assayed in the presence of R5020, with only a very slight underestimation of receptor sites. Thus these results suggested that the binding of [³H]-R1881 in these tissues and in human breast cancers could be characterised as being due to either progestogen receptors or androgen receptors, by including competitor studies with radioinert 5α-DHT, R5020 and progesterone. It should be noted, however, that such assays for androgen receptor activity might have two sources of possible inaccuracy: (a) the excess concentration of R5020 used might block a small amount of androgen receptor and tumours possessing only low levels of androgen receptor might thus be misclassified as negative; (b) incomplete blocking of progestogen receptors by R5020 might lead to their being falsely classified as positive for androgen receptors, this being most likely to occur in tumours containing very high levels of progestogen receptors.

Specific binding of [³H]-R1881 was detected in cytosols from 72 of 122 human breast cancers. Binding was characterised as being due to progestogen receptors (competition by progesterone and R5020 but not by 5α-DHT) in 30, androgen receptor (competition by 5α-DHT but not by progesterone or R5020) in 16, and to both androgen and progestogen in 26 tumours (competition by progesterone, R5020 and 5α-DHT). This gives an overall incidence of androgen receptor activity of 34%. The corresponding figure quoted for other workers using different methods varies from 19 to 56% [9–13]. In this study the overall incidence for progestogen receptors was 46%. This figure is identical to that

which we found using [^{3}H]-R5020 as ligand in the same tumours, and is comparable to those reported by others [14, 15].

Quantitation of androgen receptors demonstrated a mean level in the receptor-positive tumours of 68 fmol/mg cytosol protein, with a mean K_d of 2.15×10^{-9} M. These values are comparable to those reported for the androgen receptor by others using different methods [8, 11, 13].

In conclusion, we have defined a method whereby the synthetic radioligand [^{3}H]-R1881 can be used for the simultaneous detection of progestogen and androgen receptors, though in a small number of tumours with very low levels of androgen receptor activity or very high levels of progestogen receptor activity the androgen receptor status may be misclassified. Androgen receptor activity can be quantified by blocking the progestogen receptors with R5020. This method is summarised in Table 4. However, accurate quantitation of progestogen receptors is still best performed with the specific progestogen ligand, [^{3}H]-R5020. The clinical significance of the presence of androgen receptors, or of the simultaneous presence of both androgen and progestogen receptors, remains to be determined.

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