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Gonadotropin Releasing Hormone Binding Sites in Human Epithelial Ovarian Carcinomata

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Abstract—*As a first step to investigate whether gonadotropin releasing hormone (GnRH) analogs might be able to modulate directly the proliferation of human epithelial ovarian carcinomata, we checked if binding sites for GnRH are present in these malignancies. Specific binding of* [^{125}I][D-*Ala*^{6}-*des Gly*^{10}]-*GnRH-ethylamide (GnRH agonist = GnRH-A) could be demonstrated in plasma membranes from* 32 *out of* 40 *ovarian carcinomata tested. This binding was dependent on temperature, time and plasma membrane concentration. Mathematical analysis of the binding data showed that the interaction of GnRH-A with the binding sites was consistent with a single class of low affinity, high capacity binding sites* (K_a = 1.42 ± 0.14 × 10^5 M^{-1}; *range*: 0.3–3.8 × 10^5 M^{-1}; R = 209 ± 69 × 10^{-12} M/mg *membrane protein; range* 16–400 × 10^{-12} M/mg *MP*; $\bar{x}$ ± *S.E.*, n = 32). *Native GnRH and the GnRH antagonist* [D-p-*Glu*^{1}, D-*Phe*^{2}, D-*Trp*^{3,6}]-*GnRH had* K_a *values comparable to those of the GnRH-A used.* [^{125}I]*GnRH-A binding could not be displaced by oxytocin, thyrotropin releasing hormone and corticotropin releasing factor in concentrations up to* 10^{-4} M. *Somatostatin cross-reacted with binding sites from some carcinomata, while it did not displace GnRH-A binding in membranes from others. Though the functional role of this specific binding site for GnRH in human epithelial ovarian carcinomata is still obscure, it might be part of an autocrine regulatory system and provide a possible point of attack for therapeutic approaches using GnRH analogs in this malignancy.*

INTRODUCTION

IN CONTRAST to other gynecological malignancies, no satisfactory endocrine therapies have so far been developed for ovarian carcinomata (for review see [1]). In rats, gonadotropin releasing hormone (GnRH) has been described as a modulator for ovarian function (for review see [2, 3]). In this species high affinity, low capacity receptors have been demonstrated in the ovaries comparable to pituitary GnRH receptors (for review see [2, 3]). Also in the human, direct inhibitory effects of a GnRH agonist on a hormone-producing ovarian tumor (arrhenoblastoma) [4] and on granulosa cell progesterone secretion [5] have been demonstrated. High affinity, low capacity GnRH receptors, however, could not be found in human ovarian tissue [6]. In later studies, low affinity, high capacity GnRH binding sites have been characterized in human corpora lutea [7, 8]. As a first step in the direction of a possible clinical use of GnRH analogs in the treatment of ovarian neoplasia, we investigated whether or not specific GnRH binding sites are present in human ovarian epithelial carcinoma.

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MATERIALS AND METHODS

Chemicals

[D-Ala^6-des Gly^{10}]-GnRH-ethylamide, GnRH-acetate, oxytocin, somatostatin, thyrotropin releasing hormone, corticotropin releasing factor, [D-*p*-Glu^1, D-Phe^2, D-Trp^{3,6}]-GnRH and lactoperoxidase were purchased from Sigma, Munich, F.R.G. On HPLC (ODS-Hypersil 3μm; C_{18};

60 × 4.6 mm column; solvent 18% acetonitrile in 0.2 M triethylammoniumphosphate buffer pH 2) the [D-Ala^6-des Gly^{10}]-GnRH-EA was 99.7% pure. All other chemicals were obtained from E. Merck, Darmstadt, F.R.G., and were of analytical grade.

Iodination of GnRH agonist

The GnRH agonist analog [D-Ala^6-des Gly^{10}]-GnRH-ethylamide (GnRH-A) was labelled with ^{125}I using the lactoperoxidase method according to Clayton *et al.* [9] and purified on a 50 × 2.5 cm column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) using 0.1 M acetic acid in water as eluent containing 0.25% of bovine serum albumin. The specific radioactivity of the labelled peptide, determined by self-displacement in a rat pituitary membrane receptor assay was between 1 and 2 mCi/μg. Maximal tracer bindability, determined with an excess of rat pituitary membranes was 40–50%. Rechromatography of aliquots of this tracer in two different thin-layer systems on silica gel (Sil G/UV 254 plates; solvent system 1: butanol:acetic acid:water, 4:1:5; solvent system 2: butanol:acetic acid:water:ethylacetate, 1:1:1:1) revealed no significant impurities.

When aliquots of the tracer were subjected to HPLC (see above) two additional peaks of radioactivity (~ 10% each of the [^{125}I][D-Ala^6-des Gly^{10}]-GnRH-EA peak) were identified, which were not present in the unlabelled preparation.

Membrane preparation

Ovarian tumors were placed on ice immediately after surgical removal and representative portions were excised by the pathologist preparing the material for histological frozen sections. These tumor samples were snap frozen on dry ice and stored at −72°C. Before the binding assay, the tumor specimens were thawed, weighed, placed on ice and minced thoroughly. Then the minced tissue was homogenized in a 10-fold volume of 10 mM Tris–HCl buffer, pH 7.6, containing 1 mM dithiothreitol and 1 g/l of bovine serum albumin in a Potter homogenizer (glass/Teflon; 600 rpm; 20 strokes). The homogenates were filtered through nylon gauze (100 μm) and centrifuged twice at 1000 ***g*** for 10 min (0–4°C). The supernatants were centrifuged at 20,000 ***g*** for 20 min (0–4°C). The pelleted plasma membranes were resuspended in an appropriate volume of the above Tris–HCl buffer and recentrifuged as described before they were finally suspended in assay buffer. Aliquots of the membrane suspensions were immediately used for the binding studies (see below). Small portions of the suspensions were used for protein determination [10].

Binding assays

Incubations were carried out in polypropylene tubes precoated with 30 g/l BSA. Membrane protein preparations (approx. 1 mg/tube) were incubated with constant amounts of labelled agonist (200,000 cpm) in the presence and absence of unlabelled GnRH analogs or other peptides in the concentrations up to 10^{-4} M in a final volume of 300 μl at 0°C for 6 h. Incubations were terminated by the addition of 0.5 ml of 5 g bovine γ-globulin/l Tris–HCl buffer (0°C) and 1 ml of 180 g polyethylene glycol (8000 molecular weight)/l of Tris–HCl buffer (0°C).

After mixing thoroughly the tubes were centrifuged at 5000 ***g*** for 20 min (0–4°C). After aspiration of the supernatants, the pellets were resuspended in 100 g polyethylene glycol/l Tris–HCl buffer (0°C), and the centrifugation step was repeated before the pellets were measured in a γ-spectrometer. Total binding was measured in triplicate. Non-specific binding was determined in duplicate in the presence of 10^{-4} M unlabelled GnRH analog. Non-specific binding to the tubes, determined in the absence of membranes, was less than 1% of the total radioactivity added. Under these conditions no degradation of [^{125}I][D-Ala^6 des Gly^{10}]-GnRH-EA was detectable after separation of unbound tracer checked by rebinding experiments with rat pituitary homogenates.

Data analysis

The data obtained from the displacement experiments were analyzed using the 'Ligand' program [9], which was kindly provided by the Biomedical Computing Technology Information Center, Vanderbilt Medical Center, Nashville, Tennessee (BCTIC).

RESULTS

Time and temperature dependence of GnRH-A binding

Plasma membranes from a well differentiated serous adenocarcinoma and a poorly differentiated papillary serous carcinoma, which had shown specific binding in pilot experiments were used for this study. The time and temperature dependence of [^{125}I]GnRH-A binding were monitored during incubations at 0 and 20°C for a total period of 12 h. At 0°C nearly maximal specific binding was observed after 6 h. After 8 h specific binding was still a little bit higher, but significantly greater standard errors were observed. Raising the incubation temperature to 20°C resulted in maximal specific binding after 2 h which was, however, lower than that observed at 0°C after 6 h. After 3 h of incubation at 20°C a dramatic fall of specific binding was observed. Figure 1 gives the data from one representative experiment out of two with comparable results. In all subsequent experiments a 6 h

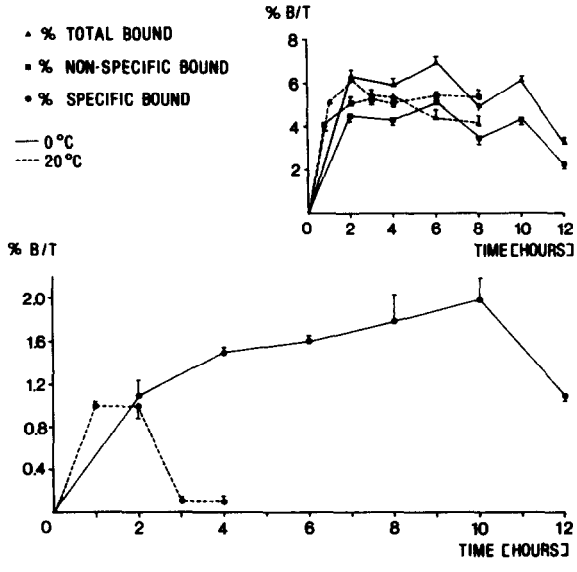

Fig. 1. Time and temperature dependence of binding ($\bar{x} \pm S.E.$) of [^{125}I][D-Ala^6-des Gly^{10}]-GnRH-ethylamide to plasma membranes from a poorly differentiated papillary serous carcinoma. Total binding was measured in triplicates using 88 fmol/tube of labelled GnRH-A at 0 and 20°C. At the indicated time intervals bound GnRH-A was separated from free ligand using the bovine γ-globulin polyethylene-glycol technique (cf. Materials and Methods). Non-specific binding was assessed in duplicate in the presence of 10^{-4} M unlabelled GnRH-A. Comparable results were obtained with the membranes from a well differentiated serous adenocarcinoma.

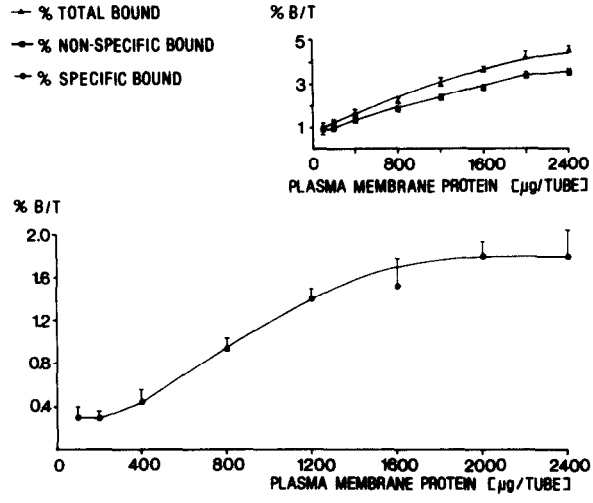

Fig. 2. Dependence of [^{125}I][D-Ala^6-des Gly^{10}]-GnRH-ethylamide binding ($\bar{x} \pm S.E.$) on plasma membrane protein concentration. Membranes were prepared from a well differentiated serous adenocarcinoma. 55 fmol/tube of the labelled analog were incubated in triplicates for 6 h at 0°C with increasing concentrations of plasma membranes in a total volume of 300 µl. Non-specific binding was assessed in duplicates in the presence of 10^{-4} M unlabelled GnRH-A, separation of the bound and free was achieved using the bovine γ-globulin polyethylene-glycol technique (cf. Materials and Methods). Comparable results were obtained with membranes from a poorly differentiated adenocarcinoma.

incubation schedule at 0°C was used. The use of aprotinine (100 kallekrein inhibitor units/ml of buffer) did not increase the binding at 0°C on incubations up to 6 h and was therefore omitted.

Dependence of GnRH-A binding on plasma membrane concentration

GnRH-A binding to tumor membranes (derived from a highly differentiated serous adenocarcinoma and a poorly differentiated adenocarcinoma) was a function of plasma membrane protein concentration. A linear relationship of the binding was observed over a range of 200–1200 µg protein/tube. Data from a representative experiment out of two are given in Fig. 2. The effect of plasma membrane dilution on the binding affinity and the number of binding sites was tested at three dilutions of membranes from two tumors (two highly differentiated serous papillary adenocarcinomata) in the presence of increasing concentrations (10^{-7} to 10^{-4} M) of unlabelled GnRH-A. There was a proportional though not statistically significant increase in the number of binding sites/tube with the increase of the protein concentration with no significant change of the affinity constants (Table 1). The number of binding sites per mg of protein, however, decreased at the high plasma membrane protein concentration of 1560 µg/tube. Membrane protein concentrations between 800 and 1000 µg/tube were used in subsequent studies.

Binding characteristics

Under the standard conditions described above, the binding of [^{125}I][D-Ala^6-des Gly^{10}]-GnRH-

Table 1. Binding affinity constants and binding capacities at different plasma membrane protein concentrations

Protein per tube (µg)	Affinity constant K_a (10^5 M^{-1})	Number of binding sites per tube (10^{-12} M)	Number of binding sites per mg protein (10^{-12} M)
390	1.39 ± 0.75	26.7 ± 15.6	68.00 ± 40.00
780	1.81 ± 0.43	36.0 ± 9.00	46.80 ± 11.40
1560	1.69 ± 0.72	52.50 ± 23.7	33.60 ± 15.20

Plasma membranes were prepared from a well differentiated serous adenocarcinoma as described in Materials and Methods. 88 fmol/tube of [^{125}I][D-Ala^6-des Gly^{10}]-GnRH-ethylamide were incubated in the presence of increasing concentrations (10^{-7} to 10^{-4} M) of cold agonist. Binding affinity and number of binding sites were determined by the computerized 'Ligand' program.

ethylamide was inhibited in a dose-dependent manner by unlabelled analog (Fig. 3). The interaction of the agonist with the binding sites from human epithelial ovarian carcinoma was consistent with a single class of low affinity ($K_a = 1.42 \pm 0.14 \times 10^5$ M^{-1}, $n = 32$, $\bar{x} \pm$ S.E., range: 0.3–3.8 $\times 10^5$ M^{-1}) and high capacity ($R = 209 \pm 69 \times 10^{-12}$ M/mg membrane protein, range: 16–400 $\times 10^{-12}$ M/mg MP) binding site.

Using a two binding site model for the fitting approach [9], i.e. a low and a high affinity binding site, significantly worse qualities of fit were obtained in comparison with the one binding site model or gave no estimation of binding site characteristics at all.

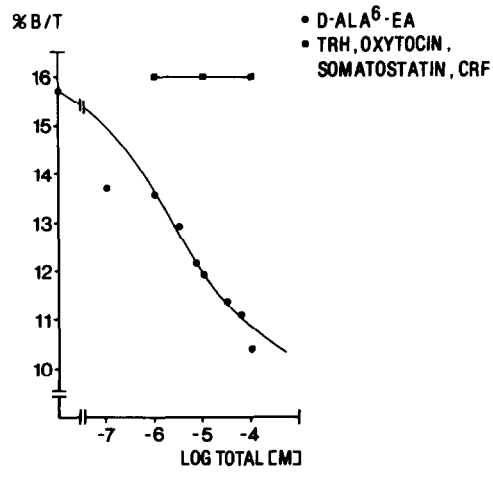

Fig. 3. Displacement of [^{125}I][D-Ala^6-des Gly^{10}]-GnRH-ethylamide from membranes of a well differentiated serous papillary adenocarcinoma by increasing concentrations of cold GnRH analogs and other peptides. Thyrotropin-releasing hormone (TRH), oxytocin, somatostatin and corticotropin-releasing factor (CRF) did not displace the binding. Incubations were carried out under the standard conditions (cf. *Materials and Methods*).

Specificity

The specificity of the GnRH-A binding site was tested using other peptides in concentrations up to 10^{-4} M. Oxytocin, TRH, and CRF did not cause any displacement (Fig. 3). Somatostatin, however, showed cross-reactivity in some of the tumors tested (data not shown), whereas it did not displace [^{125}I]GnRH-A in others (Fig. 3).

Native GnRH and the GnRH antagonist [D-p Glu^1, D-phe^2, D-Trp^{3,6}]-GnRH were bound with nearly the same characteristics as the GnRH-A (*cf.* Table 2).

Of the 40 ovarian epithelial carcinomata tested, GnRH-A binding could be demonstrated in 32. Any tumor giving a specific binding of less than 0.8% of the added radioactivity under standard conditions was classified as negative. No GnRH-agonist binding could be detected in eight epithelial ovarian carcinomata, a squamous bladder carcinoma, in the walls of eight cystadenomas (serous or mucinous), a dermoid cyst, and a carcinoma of the Fallopian tube.

Table 3 gives the binding characteristics of 10 representative epithelial ovarian carcinomata analyzed.

DISCUSSION

Our data demonstrate for the first time the presence of a specific low affinity, high capacity binding site for GnRH in human epithelial ovarian carcinoma. It might be argued that impurities in the tracer preparation could account for the observed binding and that the displacement by the unlabelled analog is due to the presence of the same impurities in this material. As the two additional peaks observed on HPLC of our tracer occurred only after iodination and were not present in the original

Table 2. Binding affinities of GnRH-A, GnRH and GnRH-antagonist to membranes of some of the ovarian carcinomata

Material	GnRH agonist	K_a(10^5 M^{-1}) GnRH	GnRH antagonist
Poorly differentiated solid adenocarcinoma	2.32 ± 0.40	—	1.03 ± 0.71
Well differentiated serous adenocarcinoma	1.09 ± 0.50	0.70 ± 0.20	0.30 ± 0.10
Partly papillary, partly solid adenocarcinoma	0.35 ± 0.11	0.21 ± 0.10	0.54 ± 0.08
Differentiated adenopapillary carcinoma	0.71 ± 0.20	1.07 ± 0.40	—
Serous papillary cystadenocarcinoma	0.58 ± 0.28	0.65 ± 0.07	—
$\bar{x}$ ± S.E.	1.0 ± 0.31	0.65 ± 1.5	0.62 ± 0.21

Binding affinities were calculated by the computerized 'Ligand' program.

Table 3. GnRH-binding sites in membranes from a selection of human ovarian carcinomata

Material	Binding sites (10^{-12} M)/mg protein	K_a (10^5 M^{-1})
Highly differentiated serous cystadenocarcinoma	76	1.4
Poorly differentiated solid adenocarcinoma	16	2.3
Poorly differentiated serous adenocarcinoma	212	0.8
Highly differentiated serous papillary adenocarcinoma	148	1.5
Poorly differentiated serous papillary adenocarcinoma	177	1.4
Poorly differentiated adenopapillary carcinoma	97	1.5
Highly differentiated serous papillary adenocarcinoma	301	1.7
Partly differentiated endometroid adenocarcinoma	330	1.0
Poorly differentiated adenocarcinoma	n.d.	n.d.
Partly differentiated partly mucinous adenocarcinoma	n.d.	n.d.

Binding assays were performed and analyzed as described in Materials and Methods.

highly pure [D-Ala^6-des Gly^{10}]-GnRH-EA material, we think that they were formed during the labelling procedure. Most probably they represent iodinated GnRH analog with minor damage to the molecule. Even if they were bound to the ovarian cancer binding site, the fact that the displacement is achieved with the highly pure unlabelled analog speaks against a relevant role of impurities contributing to the binding and displacement. Also native GnRH and a GnRH antagonist (see Table 2) are able to displace the tracer. Preliminary studies using other GnRH-superagonists and other, 5-fold substituted GnRH antagonists (data not shown), resulted in a similar displacement of the tracer as observed with the D-Ala^6 analog. Thus we think that impurities of the tracer preparation and of the unlabelled analogs do not account for the observed binding and displacement.

We are fully aware of the fact that the rather great variance in the binding data, which is due to the low affinity of the binding site, is not satisfactory. Therefore we took care to measure at least complete displacement curves for each tumor and to analyze the binding data by the rather 'objective' 'Ligand' program. Single point determinations or graphical Scatchard analyses are in our eyes absolutely inappropriate for the assessment of these low affinity binding sites. We hope to be able to increase the precision of the binding assay by using analogs with higher affinity. Another approach to improve the quantitation of the binding sites, which we are working on is the generation of monoclonal antibodies.

Using the above binding assay, binding sites of similar characteristics were found by us in human term placentae ($K_a = 5.2 \pm 1.2 \times 10^5$ M^{-1}), human corpora lutea ($K_a = 3.1 \pm 1.5 \times 10^5$ M^{-1}), and human granulosa cells ($K_a = 3.8 \pm 1.4 \times 10^5$ M^{-1}) (Emons *et al.*, unpublished results). With a similar binding assay, as used in the present study, Iwashita *et al.* [11] demonstrated a comparable binding site in human chorionic villi and term placentae (K_a for [D-Ala^6-des Gly^{10}]-GnRH-ethylamide; 5.4×10^5 M^{-1}). Currie *et al.* [12] found a higher K_a for both native GnRH ($K_a = 6.2 \times 10^7$ M^{-1}) and [D-Ser-t-bu^6 des Gly^{10}]-GnRH-ethylamide ($K_a = 5.5 \times 10^7$ M^{-1}) in membranes from term placentae. Bramley *et al.* [8] from the same laboratory described a similar GnRH-A binding site in human corpora lutea ($K_a = 3 \times 10^7$ M^{-1}). The reason for the lower affinities observed in our system and by Iwashita *et al.* [11] as compared to those described by Currie *et al.* [12] and Bramley *et al.* [8] are not clear. Based on our data, however, we assume that the GnRH binding site in human epithelial ovarian carcinomata is the same as the one in human granulosa cells, human corpora lutea and human placental tissue.

A similar low affinity and high capacity binding site for GnRH with a K_a in the order 10^6 M^{-1} has been described by Eidne *et al.* [13] in human breast cancer cells. These authors stress the point that the breast cancer binding site has similar affinities for native GnRH, GnRH superagonists and GnRH antagonist, while pituitary GnRH receptors have a significantly higher affinity for the analogs than for native GnRH [13]. The same observation was made with the binding site in human placental tissue [11]. As the binding site found by us in ovarian carcinomata has also similar affinities for native GnRH, GnRH agonist and GnRH antagonist, we assume that it has comparable characteristics as the one described in human placenta and human breast cancer.

The low affinity of the binding site in ovarian carcinomata for GnRH-A raises the question of possible interactions with degradative enzymes or non-specific proteins in the membrane preparations used. However, the extent of tracer binding in tumor membranes and the specificity of this process for GnRH and its analogs speak in favor of a receptor-like interaction. Especially the fact that a highly substituted GnRH antagonist has a similar affinity for the binding site as GnRH itself demonstrates the specificity of the ovarian carcinoma binding site

for peptides with conformation capable of binding to GnRH receptors. Also the experiments showing that no degradation of [^{125}I]GnRH-A occurs, speak against the theory that the binding site is a degrading enzyme.

The observation that somatostatin was able to displace labelled GnRH-A in some of the tumors remains to be checked in more tumor samples. Maybe a relation to the histological type of the tumor can be found.

At present the functional importance of the GnRH binding sites in human ovarian carcinomata is obscure. In breast cancer and placental tissue these low affinity GnRH binding sites have been interpreted as receptors for GnRH [13] or GnRH-related peptides [14] and direct effects of GnRH analogs on these tisues have been demonstrated (for reviews see [11, 13, 15]). However, the properties of the placental and breast cancer GnRH binding sites are clearly distinct from those of pituitary GnRH receptor, possibly reflecting the need for a low affinity interaction when the regulatory ligand is produced in abundance at close proximity to its receptor sites. Thus, direct effects of GnRH antagonists on breast cancer or human placental cells have only been achieved with rather high (10^{-6} to 10^{-5} M) concentrations [13, 15]. Another theory might be that the natural ligand, produced at close proximity to the binding site might have a much higher affinity to its receptor than pituitary GnRH and its analogs.

The question whether a GnRH-like peptide is produced in the human ovary and in ovarian cancer cells as a part of an autocrine regulatory mechanism remains to be elucidated. A first step is the demonstration of a functional role of the described binding site. This could be done by studying the effects of GnRH agonists and antagonists on ovarian cancer cell proliferation and whether or not second messenger mechanisms are activated in the cells by GnRH and its analogs. These studies are presently being performed in our laboratory.

It cannot be ruled out that the GnRH binding sites in ovarian carcinomata represent only a phylogenetic residue without functional importance. But even in this case it should be interesting to look for a correlation between GnRH binding site density, the histology of the tumors and the clinical course of the disease. At the moment, no such correlations can be found (*cf.* Table 3), but a prospective study looking for such relations is in progress. Also a further biochemical characterization of the binding site is necessary and is presently being performed in our laboratory. As the overall results of surgical, cytotoxic and the present endocrine therapies of ovarian cancer are still disappointing, we hope that the demonstration of specific GnRH binding sites in these tumors might be a first step towards an effective hormonal treatment of this fatal disease.

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