Journal of Steroid Biochemistry, Vol. 9, pp. 461–466

0022-4731/78/0501-0461$02.00/0

RECEPTORS AND BREAST CANCER: DO WE KNOW IT ALL?

WILLIAM L. MCGUIRE, DAVID T. ZAVA, KATHRYN B. HORWITZ, ROBERTO E. GAROLA and GARY C. CHAMNESS
Department of Medicine, University of Texas Health Science Center, San Antonio, TX 78284, U.S.A.

SUMMARY

The use of cytoplasmic estrogen receptor to predict endocrine responsiveness in breast cancer patients is now well established. It is our contention that this pertinent clinical application is only the first of many contributions from receptor studies to the clinic. Efforts are now underway to examine the distribution of receptors within tumor cells, and in particular the significance of receptors in tumor nuclei in the absence of hormone. Also, the demonstration of a direct stimulation of progesterone receptor synthesis by estradiol in cultured breast tumor cells now permits a careful dissection of the complete estrogen response system which should provide clues to the mechanism of antiestrogen action in causing tumor regression. Finally, it is now evident that pharmacologic effects of steroids in breast cancer may be mediated through different pathways than expected from studies of normal physiology.

INTRODUCTION

We have previously published several general reviews dealing with hormones, receptors, and breast cancer [1–3] and also the correlation of cytoplasmic estrogen receptor (ER) with the response to endocrine therapy in breast cancer patients [4–7]. It is our intention in this report to highlight some of our recent findings, which in some instances represent a departure from previous efforts.

QUANTITATIVE CYTOPLASMIC ESTROGEN RECEPTOR IN METASTATIC BREAST TISSUE AND RESPONSE TO ENDOCRINE THERAPY

Several years ago we proposed that most metastatic breast tumors were heterogeneous and that a given tumor probably contained a mixture of cells which did or did not contain ER [8]. It was suggested that those tumors containing a high level of ER had more cells containing ER than tumors with low ER levels, and that tumors with high ER values might show better response (regression) following endocrine therapies. In an earlier report this seemed to be true but too few cases were available to arrive at definite conclusions [6]. Now that we have more than 100 metastatic biopsies and clinical correlations, we have re-examined this question and find that the response rate to endocrine therapy is indeed correlated with the amount as well as the presence of ER in the tumor (Table 1). Tumors containing very high ER values have a response rate nearly double that of tumors with low or intermediate ER values. This has been confirmed in a smaller series of patients by Heuson *et al.*[9]. We therefore suggest that quantitative analysis of cytoplasmic ER permits identification of patient subgroups having very low (< 3 fmol ER/mg), intermediate (3–100 fmol ER/mg), or very high (> 100 fmol ER/mg) response rates.

ESTROGEN RECEPTOR IN TUMOR CELL NUCLEI

According to the current operational hypothesis, estrogen enters a dependent tumor cell and binds to receptor molecules, which then undergo a transformation and enter the nucleus to promote tumor growth. If the source of estrogen is removed by ovariectomy and adrenalectomy, or if its access to ER is blocked by antiestrogens, the chain of action is broken and the tumor regresses. Thus, a favorable response to any of these treatments can only be expected if the tumor has available both ER itself and sufficient estrogen to translocate at least some ER to cell nuclei. In other words, the presence of nuclear ER in a tumor specimen is evidence that a functional receptor was present in the cytoplasm at the time of biopsy and furthermore that estrogen in the environment was entering the cell, binding the receptor, and stimulating cell growth. Thus, nuclear ER should be a prerequisite finding in tumors that will respond to ablative procedures designed to reduce endogenous estrogens.

This becomes important because some tumors may have defects such that ER can bind estrogen but cannot enter the nucleus. There is precedent for this from studies of glucocorticoid-sensitive lymphoma cell lines; though most glucocorticoid-resistant sublines are found to lack glucocorticoid receptor, about 10% have a defective receptor which binds the hormone

Table 1. ER level and clinical response with metastatic biopsy

ER Level (fmol/mg cytosol protein)	Tumor response rate
< 3	2/33 = 6%
3–10	10/22 = 45%
11–100	14/30 = 46%
101–1,000	21/26 = 81%

but cannot be translocated into the nucleus. If some ER+ tumors carried such defects, no ER would be found in their cell nuclei even if abundant estrogen were present. The net result would be an ER+ tumor failing to respond to therapy, which is a frequent finding. Measurements of nuclear ER might help identify such tumors in advance.

Because most ER molecules carrying hormone are found in the nucleus, failure to account for these could cause a significant underestimate of the number of receptors present in a particular tumor. It is unlikely that the effect would be so extreme as to make an ER+ tumor appear ER−, but the efforts described above to correlate the results of therapy with the *level* of ER will require inclusion of nuclear as well as cytoplasmic ER.

HUMAN BREAST CANCER CELLS IN TISSUE CULTURE

We first approached the question of nuclear receptor in the MCF-7 human breast cancer cell line, in which we have previously demonstrated receptors for all the major classes of steroid hormones [10]. We were surprised to find that the majority of the cellular ER was in the nucleus even in the absence of estrogen [11]. This Rn comprises about 75% of the cell's total population of E binding sites, a subcellular distribution in striking contrast to that found in normal target tissues. Although, to our knowledge, such high levels of Rn have never been reported, we and others have shown that rat uteri quite consistently contain a small amount of Rn, usually about 10% of the total ER. The presence of Rn in MCF-7 cells was first suggested by the experiment of Brooks *et al.*[12] who found that after incubation of cells at 0°C for 1 h, [^{3}H]-estradiol (E*) was located in the nucleus. They suggested, however, that E* had translocated Rc at 0°C, while our data suggest that E* is bound directly to preexisting free nuclear sites.

Rn has many of the same physiological properties ascribed to Rc, although we have detected several minor differences. As others have reported, Rc appears to be extremely unstable at elevated temperatures with nonsaturating doses of E, suggesting either intrinsic instability of Rc or presence of cytoplasmic contaminants which degrade uncharged Rc. We find in contrast, that Rn is stable at 37°C and even slightly increases its affinity for E. In addition, Rc yields different sedimentation values in various concentrations of salt in contrast to Rn, which partially aggregates when diluted to low salt concentrations.

We have attempted to exclude the possibility that the abnormal accumulation of Rn in MCF-7 nuclei is an artifact of tissue preparation. Whole nuclei prepared with Triton X-100 bind E* in a similar fashion to salt extracted, protamine precipitated Rn, and the measured level of Rn is similar by both procedures. There is no specific E* binding in control nuclei from other tissues even while Rc is present. Nor do we find contamination of Rc in the nuclear-myofibrillar pellet of the rat uterus even after vigorous homogenization procedures that completely disrupt nuclei.

In normal target tissue such as the rat uterus, Rc gains entry into the nucleus only after first binding E. Furthermore, nuclear retention of RnE in the rat uterus appears to require the bound ligand. We show, however, that in MCF-7 cells, Rn does not appear to be bound to endogenous ligands that could have facilitated Rn transport into the nucleus. If Rn was originally transported to the nucleus via endogenous ligands, the ligands are no longer present. It also seems unlikely that very low affinity ligands cause translocation of Rn, since it would require enormous concentrations of ligands with affinities much weaker than estradiol to translocate 75% of the estrogen receptor into the nucleus.

When the MCF-7 cells are rapidly dividing in log phase growth, cytoplasmic proteins per cell are stimulated almost 3 fold. By phase microscopy, the ratio of cytoplasmic to nuclear mass is also increased. In concert with increased cytoplasmic protein, we find an almost 2 fold rise in Rc and 4 fold enhancement of Rn. The significance of this 4 fold increase in Rn is not yet known, although one could speculate that since its level is higher during log phase growth, it might play some role in the process of cell growth and division.

Further investigating such a suggestion, growth studies have shown that MCF-7 cells are not dependent upon E for growth but are paradoxically inhibited by the antiestrogen Tamoxifen [13]. To confirm the generality of this antiestrogen action, we treated growing MCF-7 cells with the antiestrogen Nafoxidine (Upjohn U-11, 100A) for 48 h in medium with 2% fetal calf serum which had been stripped of steroids by treatment with charcoal. We found that

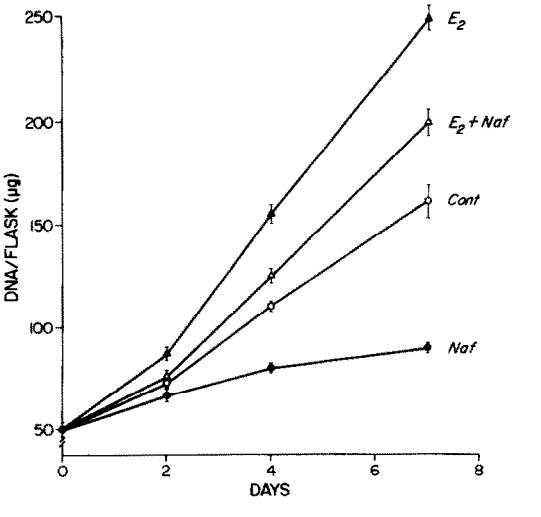

Fig. 1. Effect of estrogen and antiestrogen on the growth of MCF-7 cells. Growth conditions are as in Ref. [11]. E_2, estradiol 10^{-8} M; Naf, Nafoxidine 5×10^{-7} M; Cont, control.

uptake of [^{3}H]-thymidine into DNA was markedly reduced by Nafoxidine (Fig. 1). In spite of the absence of estrogen in the medium, estradiol alone showed only slight stimulation of thymidine uptake, demonstrating again that the hormone is not required for growth. Estradiol did, however, completely reverse the inhibition of thymidine uptake caused by Nafoxidine, as it had also reversed the effect of Tamoxifen. It seemed likely, therefore, that antiestrogens were acting through the estrogen receptor, thus explaining why estradiol countered their inhibitory effects. But why did the cells not appear to require estrogen otherwise?

We suggest the possibility that the uncharged nuclear estrogen receptor is capable of stimulating the growth of MCF-7 cells [14]. The further stimulation of thymidine uptake by estradiol would then be due to the resulting small increase in nuclear receptors translocated from the cytoplasm. If this interpretation is correct, then the binding of antiestrogen molecules must inactivate the receptor, while the reversal of the antiestrogen effect by estradiol results from the blockage of antiestrogen binding and subsequent reactivation of the receptor molecule.

Antiestrogen therapy of human breast cancer is known to cause objective tumor remission in many cases, success being generally correlated with the presence of estrogen receptor in the patient's tumor. If high levels of free nuclear estrogen receptor are present in some patients with breast carcinoma, the MCF-7 results suggest that hormone-ablative therapy might well be less successful since these nuclear receptors might be fully capable of activating cell division even in the absence of hormone. In fact, this phenomenon may explain the resistance of certain receptor-containing breast cancers to hormone-ablative therapy. If so, antiestrogen treatment in these special cases of high nuclear estrogen receptor may very well succeed where other endocrine therapies would fail.

HUMAN BREAST TUMOR BIOPSIES

In order to see if the unusual distribution of ER seen in MCF-7 cells could be found in human breast tumors *in vivo*, we studied biopsy specimens of solid human breast cancers. We had successfully used the protamine exchange assay to measure cytoplasmic and nuclear ER in normal reproductive tissue [15] and in human breast cancer cells in culture [11] but found that in solid human tumor biopsies, nuclear ER was rapidly degraded by proteolytic enzymes in the nuclear extract [16]. This problem could be avoided by adsorbing nuclear ER onto hydroxylapatite prior to performing the exchange assay [17]. Table 2 demonstrates that certain tumors contain only Rc while some tumors contain RnE as would be expected in tumors from patients having circulating estrogens. However, there are also tumors with considerable Rn, as we had previously seen in breast tumor cells in culture.

Table 2. Cytosol and nuclear estrogen receptor in human breast cancer

	Cytosol	Nuclear extract	
		Free sites	Occupied sites
1	20,670	286	206
2	5,469	195	208
3	4,156	513	70
4	3,526	284	177
5	2,761	411	870
6	2,160	87	14
7	1,781	364	158
8	1,493	450	141
9	1,481	243	174
10	1,161	309	17
11	1,030	533	0
12	899	0	225
13	847	501	242
14	547	0	0
15	294	165	256
16	233	0	0
17	45	0	0
18	36	29	29
19	25	0	35
20	22	81	88
21	14	9	2
22	0	0	0
23	0	0	0
24	0	0	0
25	0	0	0
26	0	0	0
27	0	0	0
28	0	0	0

Values expressed fmol/mg DNA.

We would anticipate that tumors with RnE should respond to ablative endocrine therapy, but the situation is less obvious if the tumor contains appreciable Rn, either alone or along with RnE. If both Rn and RnE were active in stimulating replication and endocrine ablation reduced only RnE, tumor regression might not occur or might be of brief duration since the Rn stimulus would remain. However, antiestrogens which bind and render inoperative both Rc and Rn might cause a more substantial decrease in DNA synthesis and lead to more measurable tumor regression. It must be emphasized that there is no direct evidence that Rn is capable of stimulating replication in the absence of hormone. Additional studies are required to clarify its function.

PROGESTERONE RECEPTORS IN HUMAN BREAST CANCER

In the preceding sections we described our approach for evaluating the function of a tumor's estrogen response system as far as the localization of RnE and Rn in tumor cell nuclei. Here we carry the analysis further by examining tumors for a specific response to estrogen.

We have demonstrated progesterone receptor (PgR) in human breast tumors [18] and have proposed that this receptor, whose synthesis is known to be controlled by estrogen in the uterus, might serve as a

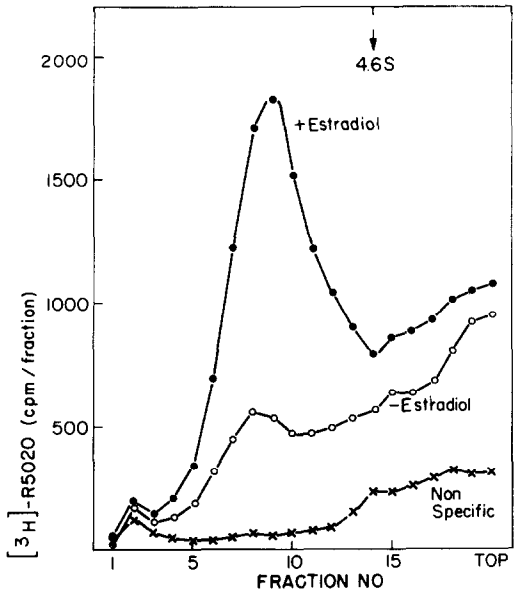

Fig. 2. Sucrose gradient analysis of progesterone receptor in MCF-7 cells in the presence ●——● or absence ○——○ of estradiol.

marker of estrogen action in breast cancer [19, 20]. Thus, the presence of PgR in a tumor would indicate that the entire sequence involving estrogen binding to cytoplasmic receptor, movement of the receptor complex into the nucleus, and stimulation of a specific end product can be achieved in the tumor cell. This would rule out the existence of a defect beyond the binding step.

Though this proposal assumes that PgR is under control of estrogen acting through ER, this inductive effect had not previously been demonstrated in human breast cancer cells, and direct involvement of ER in mediating PgR synthesis had not been described for any tissue. We therefore examined these points in MCF-7 cells. MCF-7 cells have persistently low but nevertheless measurable quantities of progesterone receptor ranging from 50 to 100 fmol/mg cytosol protein (300 to 700 fmol/mg DNA). Figure 2 shows that this basal level is increased 3 fold by 4 day treatment of log phase cells with 10 nM estradiol. The extent of induction varies from 2 to 6 fold among experiments, depending on cell density and growth rate. Although we routinely assay for PgR using 8S binding of the synthetic [^{3}H]-R5020 on sucrose density gradients, 8S binding is also seen using [^{3}H]-progesterone as the ligand.

The role of estradiol and ER in PgR induction is indicated by studies in which the levels of the 2 receptors are contrasted after treatment with and withdrawal from estradiol (Figure 3). Within minutes after exposure to estradiol, all ER is in the nuclei in bound form (RnE). Then begins a rapid turnover or processing of RnE so that by 5 hr 70% of RnE has disappeared. This processing step precedes PgR induction, and RnE remains low as long as estrogen is present in the media. If estrogen is removed, processing stops and ER is restored while PgR levels fall to basal values. The reciprocal relationship between PgR levels and ER processing suggests that processing is a necessary intermediate step following RnE binding and preceding synthesis of a specific estrogen regulated protein. The nature of processing is unclear. It may be an active state in which a new equilibrium between receptor degradation and synthesis is achieved, or a redistribution of receptor within nuclear binding sites of differing affinities or specificities, or sequestration of receptor to sites inaccessible to salt extraction. Other experiments not shown suggest that the processing step is saturable, that peak activation occurs when RnE processing is maximal, and that the RnE accumulation in excess of that which is processed may be superfluous [21].

The clinical relevance of estrogen dependent PgR induction in human breast cancer tissue is currently under investigation in many laboratories and clinics. A few preliminary reports have appeared [7, 22] which indicate that patients whose tumors contain both ER and PgR respond to endocrine therapies at nearly double the rate of tumors known to contain ER, but not selected for PgR. These encouraging results need confirmation in longer clinical trials, but they emphasize the fact that basic scientists investigating mechanisms of hormone action in model systems are capable of making important contributions to improved therapeutic strategies in breast cancer.

ANDROGEN DEPENDENCE IN CULTURED HUMAN BREAST CANCER CELLS

Lippman *et al.* have recently reported that MCF-7 cell growth is stimulated by androgens and concluded that this androgen response is mediated by an androgen receptor [23]. However, very high levels of androgen, 10^{-6} M dihydrotestosterone (DHT), were required to elicit the response, suggesting that some other mechanism might be operating. It has been reported that in the rat uterus pharmacological androgens interact not only with androgen receptor

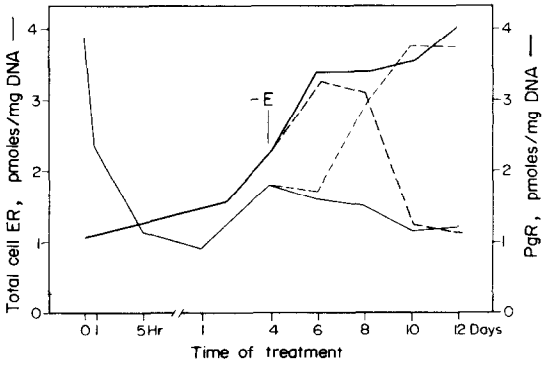

Fig. 3. Effect of estradiol addition and withdrawal on total estrogen receptor content and progesterone receptor synthesis in MCF-7 cells.

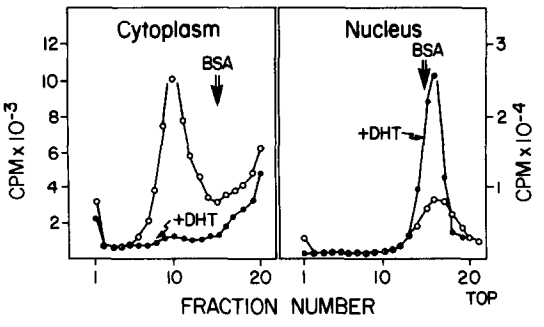

Fig. 4. Translocation of estrogen receptor by 10^{-6} M dihydrotestosterone in MCF-7 cells. Left panel—cytoplasmic depletion, right panel—nuclear accumulation. O——O before hormone addition, ●——● after hormone addition.

but also with estrogen receptor, resulting in translocating the estrogen receptor into the nucleus [24]. We thought perhaps that pharmacological androgens might similarly affect the estrogen receptor in MCF-7 tumor cells, providing a possible mechanism for the observed growth stimulation. We therefore incubated intact cells with physiological (10^{-8} M) and pharmacological (10^{-6} M) DHT, then examined the effect on receptors for estrogen, androgen, progesterone and glucocorticoids. At 10^{-8} M, cytoplasmic androgen receptor is depleted while the other receptors are unaltered, and no growth stimulation occurs. The higher effective dose of DHT, however, depletes not only the androgen receptor but also a significant portion of the estrogen receptor; neither progesterone nor glucocorticoid receptor levels are affected. Figure 4 shows that following cytoplasmic depletion of estrogen receptor with 10^{-6} M DHT, an equal complement of estrogen receptors have entered the nucleus.

The cytoplasmic depletion-translocation of estrogen receptor is not limited to DHT only. Testosterone and androstanediol are equally effective, while neither progesterone nor hydrocortisone interact. Thus, androgens appear to have a specific binding site on the estrogen receptor, at which binding causes receptor translocation to the nucleus.

If the translocated estrogen receptor in the nucleus after DHT exposure is functional, then specific products of estrogen action would be expected to result. Progesterone receptor is such a product of estrogen action. Low and high doses of DHT were therefore compared for their ability to induce progesterone receptor synthesis. When intact cells are exposed to 10^{-8} M DHT progesterone receptor levels are unaffected, whereas if cells are exposed to 10^{-6} M DHT progesterone receptor is stimulated three to four-fold, comparable to stimulation by estradiol. Thus the nuclear estrogen receptor–DHT complex must be active at specific gene acceptor sites, inducing products normally considered specific for the action of estrogen.

For these data to be clinically relevant, one would need to demonstrate that pharmacological androgen would be capable of stimulating breast tumor growth under certain conditions. Heise and Gorlich[25] found that physiological concentrations of testosterone propionate had no effect on growth patterns in DMBA tumors. Intermediate pharmacological androgen concentrations induced regession, yet exceedingly high and frequent doses actually enhanced tumor growth. The continuous presence of high dose DHT (10^{-6} M) found to be mitogenic in tissue culture probably more closely simulates the exceedingly high pharmacologic androgen concentrations necessary to enhance DMBA-rat mammary tumor growth.

Although we have shown that 10^{-6} M DHT enhances MCF-7 cell growth through the estrogen receptor, stimulation by the same mechanism in mammary tumors of humans *in vivo* remains only conjecture. Nevertheless, the possibility remains that androgen failure to induce tumor remission in some breast cancer patients may be directly related to the stimulatory action of pharmacologic androgens by mechanisms similar to those seen in the MCF-7 human breast cancer cell line.

Acknowledgements—These studies were supported in part from the National Cancer Institute (CA 11378, CB 23862), the American Cancer Society and the Robert A. Welch Foundation. We thank Dr. J. P. Raynaud of Roussel UCLAF for providing [^{3}H]-R5020. Clinical aspects of these studies were in collaboration with Drs. O. H. Pearson and A. Segaloff.

REFERENCES

1. McGuire W. L., Chamness G. C., Costlow M. E. and Shepherd R. E.: Hormone dependence in breast cancer. *Metabolism* **23** (1974) 75–100.
2. McGuire W. L., Chamness G. C., Costlow M. E. and Horwitz K. B.: Hormone receptors in breast cancer. *Modern Pharmacology–Toxicology*, Vol. 9 (Edited by G. S. Levey). Marcel Dekker, New York (1976) pp. 265–299.
3. McGuire W. L.: Physiological principles underlying endocrine therapy of breast cancer. *Breast Cancer: Advances in Research and Treatment* (Edited by W. L. McGuire). Plenum Press, New York (1977) pp. 217–262.
4. McGuire W. L., Carbone P. P., Sears M. E. and Escher G. C.: Estrogen receptors in human breast cancer—an overview. *Estrogen Receptors in Human Breast Cancer* (Edited by W. L. McGuire, P. P. Carbone and E. P. Vollmer). Raven Press, New York (1975) pp. 1–7.
5. McGuire W. L.: Current status of estrogen receptors in human breast cancer. *Cancer* **36** (1975) 638–644.
6. McGuire W. L., Pearson O. H. and Segaloff A.: Predicting hormone responsiveness in human breast cancer. *Estrogen Receptors in Human Breast Cancer* (Edited by W. L. McGuire, P. P. Carbone and E. P. Vollmer). Raven Press, New York (1975) pp. 17–30.
7. McGuire W. L., Horwitz K. P., Pearson O. H. and Segaloff A.: Current status of estrogen and progesterone receptors in breast cancer. *Cancer* **39** (1977) 2934–2947.
8. McGuire W. L. and Chamness G. C.: Role of estrogen receptor in breast cancer. *Adv. exp. Biol. Med.* **36** (1973) 113–136.

9. Heuson J. C., Longeval E., Mattheiem W. H., DeBoel M. C., Sylvester R. J. and LeClercq G.: Significance of quantitative assessment of estrogen receptors for endocrine therapy in advanced breast cancer. *Cancer* **39** (1977) 1971–1977.
10. Horwitz K. B., Costlow M. E. and McGuire W. L.: MCF-7: A human breast cancer cell line with estrogen, androgen, progesterone, and glucorticoid receptors. *Steroids* **26** (1975) 785–795.
11. Zava D. T. and McGuire W. L.: Estrogen receptor: Unoccupied sites in nuclei of a breast tumor cell line. *J. biol. Chem.* **252** (1977) 3703–3708.
12. Brooks S. C., Locke E. R. and Soule H. D.: Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. *J. biol. Chem.* **248** (1973) 6251–6253.
13. Lippman M. E., Bolan G. and Huff K.: The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. *Cancer Res.* **46** (1976) 4595–4601.
14. Horwitz K. B. and McGuire W. L.: Progesterone and progesterone receptors in experimental breast cancer. *Cancer Res.* **37** (1977) 1733–1738.
15. Zava D. T., Harrington N. Y. and McGuire W. L.: A new exchange assay for nuclear bound estrogen receptor. *Biochemistry* **15** (1976) 4292–4297.
16. Garola R. E. and McGuire W. L.: Estrogen receptor and proteolytic activity in human breast tumor nuclei.. *Cancer Res.* **37** (1977) 3329-3332.
17. Garola R. E. and McGuire W. L.: An improved assay for nuclear estrogen receptor in experimental and human breast cancer. *Cancer Res.* **37** (1977) 3333–3337.
18. Horwitz K. B. and McGuire W. L.: Specific progesterone receptor in human breast cancer. *Steroids* **25** (1975) 497–505.
19. Horwitz K. B., McGuire W. L., Pearson O. H. and Segaloff A.: Predicting response to endocrine therapy in human breast cancer: A hypothesis. *Science* **189** (1975) 726–727.
20. Horwitz K. B. and McGuire W. L.: Estrogen and progesterone: Their relationship in hormone dependent breast cancer. *Progesterone Receptors in Normal and Neoplastic Tissue* (Edited by W. L. McGuire, J. P. Raynaud and E. E. Baulieu). Raven Press, New York (1977) pp. 103–124.
21. Horwitz K. B. and McGuire W. L.: Estrogen control of progesterone receptor in human breast cancer: Correlation with nuclear processing of estrogen receptor. *J. biol. Chem.* **253** (1978) 2223–2228.
22. Bloom N., Tobin, E. and Degenshein G. A.: Clinical correlations of endocrine ablation with estrogen and progesterone receptors in advanced breast cancer. *Progesterone Receptors in Normal and Neoplastic Tissue.* (Edited by W. L. McGuire, J. P. Raynaud and E. E. Baulieu). Raven Press, New York (1977) pp. 125–139.
23. Lippman M. E., Bolan G. and Huff K.: The effects of androgens and antiandrogens on hormone-responsive human breast cancer in long-term tissue culture. *Cancer Res.* **36** (1976) 4610–4618.
24. Rochefort H. and Garcia M.: Androgen on the estrogen receptor I-binding and *in vivo* nuclear translocation. *Steroids* **28** (1976) 549–560.
25. Heise E. and Gorlich M.: Growth and therapy of mammary tumours induced by 7,12-Dimethylbenzanthracene in rats. *Br. J. Cancer* **20** (1966) 539–545.