J. Steroid Biochem. Molec. Biol. Vol. 37, No. 6, pp. 777–781, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

ANTI-STEROIDAL AND ANTI-GROWTH FACTOR ACTIVITIES OF ANTI-ESTROGENS

GILLES FREISS, CHRISTINE PREBOIS, HENRI ROCHEFORT and FRANÇOISE VIGNON*
INSERM U 148, Unité Hormones et Cancer, 60, Rue de Navacelles, 34090 Montpellier, France

Summary—Both steroid hormones, such as estrogens and progestins acting via nuclear receptors, and growth factors, such as EGF, IGF-I and IGF-II acting via transmembrane receptors, are able to modulate the growth of human breast cancer cells. In addition to its anti-estrogenic action requiring estrogen receptor (ER) and leading to growth arrest, we have previously shown that the anti-hormone tamoxifen (Tam) is able to block EGF, insulin and IGF-I mitogenic activities in total absence of estrogens (BBRC,146,1502,1987). This anti-growth factor activity is observed exclusively in ER+ cells and is rescued by estradiol addition, thus suggesting that it is mediated by accessible ER sites. In the same culture conditions, progestins and anti-progestins do not display such an inhibition, whereas retinoic acid does, thus indicating that this anti-growth factor effect is not restricted to ER ligands. To progress in the understanding of this inhibition, we first analyzed how Tam could affect EGF and IGF-I binding in responsive cells. We have shown that Tam neither affects EGF and IGF-I binding to their respective receptors by direct competition nor modulates their affinities. However, our recent data suggest that Tam pretreatment (6 days) of MCF7 cells, which similarly prevents EGF and IGF-I mitogenic activities, results in opposite effects on the concentrations of their binding sites.

In conclusion, we propose that some steroid antagonists can inhibit not only the action of agonist ligands of the receptors they are binding to, but can also modulate the action of growth factors by decreasing their receptor concentrations or altering their functionalities.

The critical role of estrogens in the promotion and growth of breast tumors has for long been established [1]. The evidence of specific intracellular receptors for estrogens (ER) [2] and progestins (PgR) [3] in biopsies of breast primary tumors or metastases, has rationalized and favored the development of adjuvant hormonal therapies of these tumors since 80% of tumors bearing ER and PgR respond favorably to these treatments [4]. Well-tolerated and efficient estrogen antagonists such as tamoxifen [5] (or Nolvadex®) are widely prescribed to patients [6].

However, the cellular and molecular mechanisms triggering the favorable response or the adverse resistance of tumors to anti-estrogen therapy is yet poorly understood. Moreover, the availability in the past years of numerous human breast cancer cell lines [7, 8], which can be maintained *in vitro* in controlled culture conditions [9], has broadened our view on the many factors that are regulating growth [10, 11]. In addition to steroid hormones and their antagonists, which exert direct stimulatory and inhibitory action on mammary cells [12–14], peptide hormones and growth factors were shown to be potent mitogens on these cells. Insulin [15], epidermal growth factor (EGF) [16] and more recently insulin-like growth factors I and II (IGF-I, IGF-II)[17–19], transforming growth factor (TGFα) [20], acidic and basic fibroblast growth factors (aFGF–bFGF) [18] and the protease cathepsin D (cath-D) [21] were shown to promote breast cancer growth *in vitro*. Estrogens and members of the insulin family were shown to be the most potent mitogens in many cell systems (10-fold stimulation over control) whereas EGF/TGFα, FGFs and cath-D were less stimulatory (2–3-fold over control) (Table 1). The *in vitro* cell systems have therefore allowed to delineate some of the multiple factors which could participate coordinately to the control of breast cancer proliferation *via* endocrine, autocrine/intracrine and paracrine pathways [10, 11].

Proceedings of the 2nd International EORTC Symposium on "Hormonal Manipulation of Cancer: Peptides, Growth Factors and New (Anti-)Steroidal Agents", Rotterdam, The Netherlands, 9–11 April 1990.

*To whom correspondence should be addressed: Dr Françoise Vignon, INSERM U 148, Unité Hormones et Cancer, 60, Rue de Navacelles, 34090 Montpellier, France [*Tel.* (67) 54-13-79; *Fax* (67) 54-05-98].

Table 1. Maximal fold stimulation of MCF7 cell growth (phenol-red free media—1% steroid stripped serum)

E2	(10^{-9} M)	15
IGF-I	(10^{-9} M)	12
IGF-II	(5×10^{-9} M)	12
Insulin	(5×10^{-8} M)	12
EGF	(20 ng/ml)	3
FGFb	(10 ng/ml)	2.5
Cath-D	(5×10^{-9} M)	2.5

In this complex schema of cancer growth regulation which involves steroid hormones associated to nuclear receptors and polypeptide growth factors transducing mitogenic signals *via* transmembrane receptors, we attempted to define whether nonsteroidal anti-estrogens such as tamoxifen were acting exclusively by impeding estrogen action [14, 22] of whether these drugs could also behave as ER-mediated inhibitors [23] acting *via* different mechanisms such as a blockade on the growth factor pathway (Fig. 1).

These studies were made possible *in vitro*, after it was identified that the pH indicator phenol red has an estrogen agonist activity [24]. If the cells are maintained in phenol-red free media, in the absence of serum [25] or in the presence of low concentration of steroid-stripped serum [25, 26], tamoxifen (Tam) or its potent metabolite 4-hydroxytamoxifen (4-OH-Tam) displayed selectively their agonist activities [25, 26]. However, when a mitogen such as insulin or EGF is added, at a concentration which fully stimulates MCF7 cell proliferation, then OH-Tam dose-dependently inhibits their growth [27] (Fig. 2). This indicates that OH-Tam inhibits growth not only by blocking estrogen action but also by impeding growth factor activity. This anti-growth factor activity is not found in the estrogen-receptor negative MDA-MB231 [27] or BT20 cells (unpublished data), which suggests that it required the presence of estrogen receptor. The mediation by ER

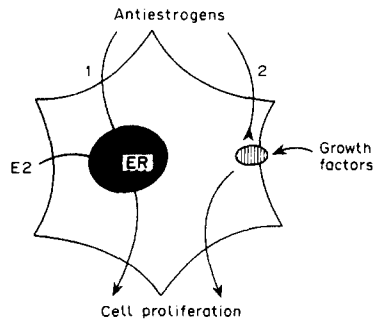

Fig. 1. Possible mechanisms of breast cancer growth inhibition by anti-estrogens.

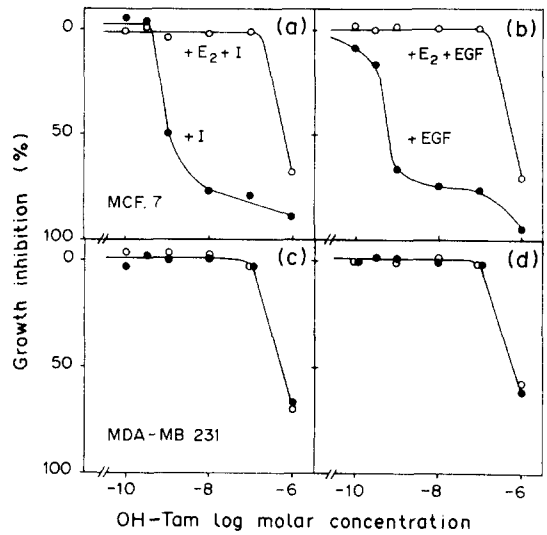

Fig. 2. Anti-growth factor activity of anti-estrogens in human breast cancer cells. (Reprinted with permission from F. Vignon *et al.*, *BBRC*, **146**, 1502–1508, 1987.)

is further supported by the exclusive rescue of such inhibited cells by high doses of estrogen [22, 27] and by the good correlation between the efficient doses of different compounds and their relative affinities for the estrogen receptor [14, 27] (Fig. 3). OH-Tam and the new ICI compound (ICI 164,384) which is strictly antagonist [28], displayed the same inhibitory pattern ($ED_{50} = 0.5$ nM) whereas Tam is much less potent ($ED_{50} = 0.1\ \mu$M). This anti-growth factor effect is not restricted to ligand of estrogen receptor since retinoic acid (RA), at concentrations in relation to its affinity for its receptors [29] is also inhibitory ($ED_{50} = 0.5\ \mu$M). However, neither the anti-progestin RU 38,486 (RU486) (Fig. 3), nor the synthetic progestin

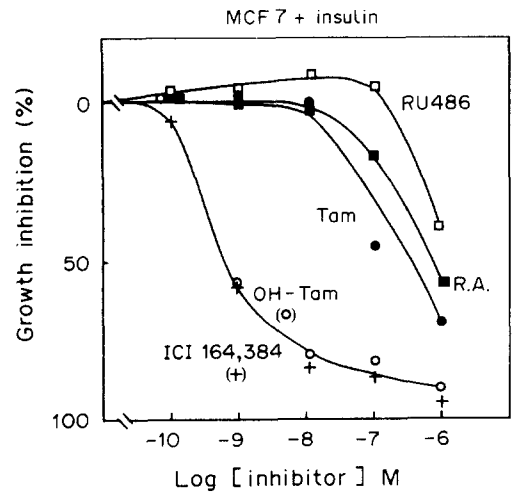

Fig. 3. Comparative anti-insulin activities of anti-estrogens (OH-Tam, ICI 164,384, Tam), of retinoic acid (RA) and of an anti-progestin (RU486).

R5020 (unpublished data) were able to significantly inhibit the proliferation of cells stimulated by IGF-I or EGF though they were both inhibiting the estrogen-stimulated growth in this concentration range (0.1 nM–0.1 μM) [13, 30].

Our original observation that anti-estrogens can completely inhibit proliferation induced by peptide hormones (insulin) and growth factors (IGF-I, EGF) has been confirmed by several groups in MCF7 cells [31, 32] and extended to other cell lines (T47D, ZR75-1) [33, 34] when estrogens were truly absent from the culture media. Similarly, in these culture conditions, it was confirmed that insulin prevented progestin inhibitory action [34]. Failures to reproduce a complete inhibition of EGF- or insulin-stimulated growth by OH-Tam or similar compounds were observed when the culture conditions were either including plain fetal calf serum [35, 36] or low suboptimal insulin concentration [37]. In the first case, the presence of endogenous steroid in the serum, favored the anti-estrogenic action (no EGF or insulin stimulation was seen in these experiments) [35, 36]. In the latter case [37], the presence of low insulin concentration amplified the agonist component of the anti-estrogens as shown in previous experiments [25, 26].

Moreover, it has been shown that PgR expression, as other estrogen-induced responses [38, 39], can also be stimulated by growth factors [25, 40]. In two different systems (fetal uterine cells and breast cancer cells), OH-Tam was able to inhibit the induction of PgR by EGF or IGF-I. This indicates that the anti-growth factor activity of OH-Tam can also affect the regulation of gene expression by growth factors.

The question raised by these results is: How a ligand of a nuclear steroid receptor can inhibit the mitogenic activity which is triggered by a transmembrane receptor? We are currently evaluating different possible levels of alteration in the growth factor pathway, namely ligand binding, concentration and affinity of binding sites and intrinsic receptor tyrosine kinase activity (Fig. 4). Our recent data suggest that EGF binding is not competed by OH-Tam on its specific receptor. However, pre-treatment of MCF7 cells (or T47D cells) for 6 days by OH-Tam, which similarly prevents EGF and IGF-I mitogenic activities, displays opposite effects on the concentration of their binding sites (unpublished data).

In conclusion, we have reviewed data showing that *some steroid antagonists can block not only the action of their agonist ligands*, but *can also inhibit the mitogenic effect of some growth factors*. We propose that *this anti-growth factor action might involve modulation of their receptor concentrations* or *alteration of their receptor functionalities*.

REFERENCES

1. Beatson G. T.: On the treatment of inoperable cases of the carcinoma of the mamma. Suggestion for a new method of treatment with illustrative cases. *Lancet* **ii** (1896) 104–162.

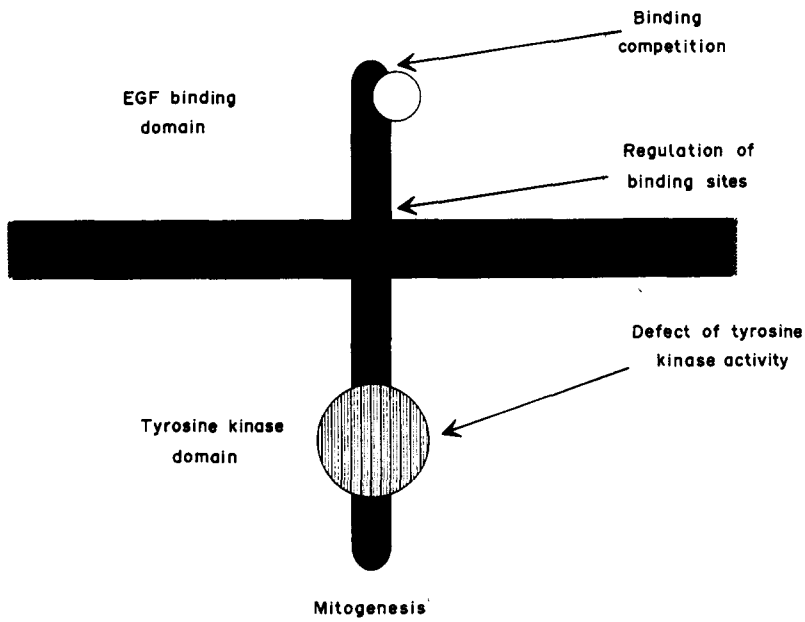

Fig. 4. Potential levels of inhibition of growth factor action by anti-estrogens.

2. Jensen E. V. and Jacobson A. I.: Fate of steroid estrogens in target tissues. In *Biological Activities of Steroids in Relation to Cancer* (Edited by G. Pincus and E. P. Vollmer). Academic Press, New York (1960) pp. 161–178.
3. Horwitz K. B. and McGuire W. L.: Specific progesterone receptors in human breast cancer. *Steroids* **25** (1975) 497–505.
4. Osborne C. K. and McGuire W. L.: The use of steroid hormone receptors in the treatment of human breast cancer: a review. *Bull. Cancer* **66** (1979) 203–210.
5. Furr B. J., Patterson J. S. and Richardson D. N. *et al.*: Tamoxifen. In *Pharmacological and Biochemical Properties of Drug Substances* (Edited by M. E. Goldberg). American Pharmaceutical Association, Washington, Vol. 2 (1979) pp. 355–399.
6. Furr B. J. and Jordan V. C.: The pharmacology and clinical uses of tamoxifen. *Pharmacol. Ther.* **25** (1984) 127–205.
7. Soule H. D., Vazquez J., Long A., Albert S. and Brennan M. A.: A human cancer cell line from a pleural effusion derived from a breast carcinoma. *J. Natl Cancer Inst.* **51** (1973) 1409–1413.
8. Lasfargues E. Y. and Ozzello L.: Cultivation of human breast carcinoma. *J. Natl Cancer Inst.* **21** (1958) 1131–1147.
9. Barnes D. and Sato G.: Methods for growth of cultured cells in serum-free medium. *Anal. Biochem.* **102** (1980) 255–270.
10. Vignon F. and Rochefort H.: Autocrine regulation of breast cancer cell growth by estrogen-induced secreted proteins and peptides. In *Recent Advances in Steroid Hormone Action* (Edited by V. K. Moudgil). Walter de Gruyter & Co., Berlin (1987) pp. 405–425.
11. Lippman M. E., Dickson R. B., Bates S., Knabbe C., Huff K., Swain S., McManaway M., Bronzert D., Kasid A. and Gelmann E. P.: Autocrine and paracrine growth regulation of human breast cancer. *Breast Cancer Res. Treat.* **1** (1986) 59–70.
12. Lippman M. E., Bolan G. and Huff K.: The effects of estrogens and anti-estrogens on hormone responsive human breast cancer in long-term tissue culture. *Cancer Res.* **36** (1976) 4595–4601.
13. Vignon F., Bardon S., Chalbos D. and Rochefort H.: Antiestrogenic effect of R5020, a synthetic progestin in human breast cancer cells in culture. *J. Clin. Endocrinol. Metab.* **56** (1983) 1124–1130.
14. Coezy E., Borgna J. L. and Rochefort H.: Tamoxifen and metabolites in MCF7 cells: correlation between binding to estrogen receptor and inhibition of cell growth. *Cancer Res.* **42** (1982) 317–323.
15. Osborne C. K., Bolan G., Monaco M. and Lippman M. E.: Human responsive breast cancer cell in long term tissue culture: effect of insulin. *Proc. Natl Acad. Sci. U.S.A.* **73** (1976) 4536–4540.
16. Osborne C. K., Hamilton B. and Nover M.: Receptor binding and processing of epidermal growth factor by human breast cancer cells. *J. Clin. Endocrinol. Metab.* **55** (1982) 86–93.
17. Huff K. K., Kaufman D., Gabbay K. H., Spencer E. M., Lippman M. E. and Dickson R. B.: Secretion of an insulin-like growth factor I related protein by human breast cancer cells. *Cancer Res.* **46** (1986) 4613–4619.
18. Karey K. P. and Sirbasku D. A.: Differential responsiveness of human breast cancer cell lines MCF7 and T47D to growth factors and 17β estradiol. *Cancer Res.* **48** (1988) 4083–4092.
19. Osborne C. K., Coronado E. B., Kitten L. J., Arteaga C. L., Fuqua S. A. W., Ramasharma K., Marshall M. and Li C. H.: Insulin-like growth factor-II (IGF-II): a potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor. *Mol. Endocrinol.* **3** (1989) 1701–1709.
20. Dickson R. B., Huff K. K., Spencer E. M. and Lippman M. E.: Induction of epidermal growth factor-related polypeptides by 17β-estradiol in MCF7 human breast cancer cells. *Endocrinology* **118** (1986) 138–142.
21. Vignon F., Capony F., Chambon M., Freiss G., Garcia M. and Rochefort H.: Autocrine growth stimulation of the MCF7 breast cancer cells by the estrogen-regulated 52K protein. *Endocrinology* **118** (1986) 1537–1545.
22. Bardon S., Vignon F., Derocq D. and Rochefort H.: The antiproliferative effect of Tamoxifen in breast cancer cells: mediation by the estrogen receptor. *Mol. Cell. Endocrinol.* **35** (1984) 89–96.
23. Rochefort H.: Do antiestrogens and antiprogestins act as hormone antagonists or receptor-targeted drugs in breast cancer? *Trends Pharmac. Sci.* **8** (1987) 126–128.
24. Berthois Y., Katzenellenbogen J. A. and Katzenellenbogen B. S.: Phenol red in tissue culture media is a weak estrogen: implication concerning the study of estrogen-responsive cells in culture. *Proc. Natl Acad. Sci. U.S.A.* **83** (1986) 2496–2500.
25. Katzenellenbogen B. S. and Norman M. J.: Multihormonal regulation of the progesterone receptor in MCF-7 human breast cancer cells: interrelationships among insulin/insulin-like growth factor-I, serum, and estrogen. *Endocrinology* **126** (1990) 891–898,
26. Van der Burg B., Rutteman G. R., Blankenstein M. A, De Laat S. W. and Van Zoelen E. J. J.: Mitogenic stimulation of human breast cancer cells in a growth factor-defined medium: synergistic action of insulin and estrogen. *J. Cell. Physiol.* **134** (1988) 101–108.
27. Vignon F., Bouton M. M. and Rochefort H.: Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. *Biochem. Biophys. Res. Commun.* **146** (1987) 1502–1508.
28. Wakeling A. E. and Bowler J.: Steroidal pure antioestrogens. *J. Endocrinol.* **112** (1987) 7–10.
29. Petkovic M., Brand N., Krust A. and Chambon P.: A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* **330** (1987) 444–450.
30. Bardon S., Vignon F., Chalbos D. and Rochefort H.: RU486, a progestin and glucocorticoid antagonist inhibits the growth of breast cancer cells via the progesterone receptor. *J. Clin. Endocrinol. Metab.* **60** (1985) 692–697.
31. Berthois Y., Dong X. F. and Martin P. M.: Regulation of epidermal growth factor receptor by estrogen and antiestrogen in the human breast cancer cell line MCF-7. *Biochem. Biophys. Res. Commun.* **159** (1989) 126–131.
32. Wakeling A. E., Newboult E. and Peters S. W.: Effects of antioestrogens on the proliferation of MCF-7 human breast cancer cells. *J. Mol. Endocrinol.* **2** (1989) 225–234.
33. Murphy L. C. and Dotzlaw H.: Endogenous growth factor expression in T47D human breast cancer cells associated with reduced sensitivity to antiproliferative effects of progestins and antiestrogens. *Cancer Res.* **49** (1989) 599–604.
34. Poulin R., Dufour J. M. and Labrie F.: Progestin inhibition of estrogen-dependent proliferation in ZR 75-1 human breast cancer cells: antagonism by insulin. *Breast Cancer Res. Treat.* **13** (1989) 265–276.
35. Koga M. and Sutherland R. L.: Epidermal growth factor partially reverses the inhibitory effects of antiestrogens on T47-D human breast cancer growth. *Biochem. Biophys. Res. Commun.* **146** (1987) 739–745.
36. Koga M., Musgrove E. A. and Sutherland R. L.: Modulation of the growth-inhibitory effects of progestins and the antiestrogen hydroxyclomiphene on human breast cancer cells by epidermal growth factor and insulin. *Cancer Res.* **49** (1989) 112–116.
37. Cormier E. M. and Jordan V. C.: Contrasting ability of antiestrogens to inhibit MCF-7 growth stimulated by

estradiol or epidermal growth factor. *Eur. J. Cancer Clin. Oncol.* **25** (1989) 57–63.
38. Cavailles V., Augereau P., Garcia M. and Rochefort H.: Estrogens and growth factors induce the mRNA of the 52K pro-cathepsin D secreted by breast cancer cells. *Nucleic Acids Res.* **16** (1988) 1903–1919.
39. Nunez A. M., Berry M., Imler J. L. and Chambon P.: The 5′ flanking region of the pS2 gene contains a complex enhancer region responsive to oestrogens, epidermal growth factor, a tumour promoter (TPA), the c-Ha-ras oncoprotein and the c-jun protein. *EMBO J.* **8** (1989) 823–829.
40. Sumida C. and Pasqualini J. R.: Antiestrogens antagonize the stimulatory effect of epidermal growth factor on the induction of progesterone receptor in fetal uterine cells in culture. *Endocrinology* **124** (1989) 591–597.