J. Steroid Biochem. Molec. Biol. Vol. 37, No. 3, pp. 343–348, 1990
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ANTIESTROGEN ACTION IN MAMMARY CANCER AND IN FETAL CELLS

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Summary—The present data confirm the very complicity of the response of antiestrogen when this compound is studied in different experimental conditions. The new and potent antiestrogen ICI 164,384, which is considered as a full antagonist in most models studied, concerning the progesterone receptor in the isolated cells of the uterus and vagina of guinea-pig acts as a real agonist. However, this compound antagonizes cell proliferation, progesterone receptor, and decreases the concentration of estradiol in different hormone-dependent mammary cancer cell lines.

Another interesting aspect is the response of the antiestrogen 4-hydroxytamoxifen which in isolated cells of very close tissues such as the uterus and vagina is an antagonist for the former and agonist for the latter concerning the progesterone receptor. In conclusion, the present data added new information in the complicity of the mechanism of action of antiestrogens, but using new models interesting possibilities are opened to understand their responses and their mechanism.

INTRODUCTION

During the last decade it was demonstrated that the utilization of the antiestrogen tamoxifen (in the form of citrate salt, Nolvadex®) provokes a significantly beneficial effect in patients with breast cancer, including postsurgical adjuvant treatment of early breast cancer and treatment of advanced breast cancer. Recently, statistical information by the "Early Breast Cancer Trialists Collaborative Group" [1] on 13,000 patients (women over 50 yr) demonstrated that the utilization of Nolvadex reduced the probability of death by $20 \pm 3\%$ (overview analysis of 5-yr mortality).

The mechanism of the antiestrogen activity is not well established, one of the early hypotheses suggested that such activity is the result of the competitive blockage of the estrogen receptor by the binding of the antiestrogen to this receptor. However, in recent studies, it appears that the biological response of the estrogen and/or the antiestrogen is conditioned by the induction of different conformational structures of the receptor molecule. Webster *et al.* [2] suggested that the antagonistic effect of the antiestrogens is due to their inability to activate gene transcription.

Another possibility is that the antiestrogen action is not mediated by the estrogen receptor. In this concern, it is interesting to mention that tamoxifen can be efficient in 10–15% of estrogen receptor-negative tumours [3]. An anti-proliferative effect of tamoxifen was also demonstrated in estrogen receptor-negative cell lines [4]. Another attractive problem is the relation of antiestrogen with growth factors. It is well established that transforming growth factor-α (TGF-α) or epidermal growth factor (EGF) bind to their receptor in the cell membrane and promote breast cancer cell proliferation [5], the antiestrogen can inhibit the estrogen-mediated secretion of the growth factors [6].

It was also extensively demonstrated that tamoxifen can act as a full or partial estrogen, this is a function of the target organ, experimental conditions and animal species (for a review see Ref. [7]).

This presentation includes recent data on the interaction of antiestrogen–estrogen receptors with various monoclonal antibodies, the effect of the new antiestrogen ICI 164,384 (*N*-n-butyl-*N*-methyl-11-(3,17β-dihydroxyestra-1,3,5,)10-trien-7α-yl) undecanamide [8, 9] in different mammary cancer cell lines and in isolated vaginal and uterine cells of fetal guinea-pig.

Proceedings of the XIV Meeting of the International Study Group for Steroid Hormones, Rome, Italy, 30 November–2 December 1989.

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INTERACTION OF THE ESTROGEN RECEPTOR–ANTIESTROGEN COMPLEX WITH DIFFERENT MONOCLONAL ANTIBODIES (D547 AND H222)

Both estrogens and antiestrogens bind the estrogen receptor with high affinity and there is an interesting correlation between the degree of the affinity and the biological response. Several molecular mechanisms have been proposed to explain differences in the biological response(s) of estrogens and antiestrogens. These include: alterations in the estrogen receptor activation [10]; differences in the receptor form retained in the nucleus [11, 12]; and interaction with specific antiestrogen binding sites [13, 14]. The utilization of monoclonal antibodies against the estrogen receptor [15] opens interesting perspectives for the examination of receptor structure and function. A recent study from this laboratory using three monoclonal antibodies (D547, H222, H226) provided direct evidence for a change in the availability of the specific epitopes recognized by these antibodies as a result of the receptor activation [16], suggesting a change in the exposure of the functional domains of the estrogen receptor during the activation process.

The utilization of the fetal uterus of guinea-pig is an interesting model because these tissues contain very high concentrations of estrogen receptors [17] and using the monoclonal antibody D547 it was possible to differentiate the activated form of this receptor [18].

Previous studies in our laboratory have shown that the monoclonal antibody D547, whose epitope is between the DNA- and hormone-binding domains, recognizes selectively the activated form of the estrogen receptor from guinea-pig fetal uterus [18]. This interaction was detected by a shift of the receptor sedimentation coefficient in high salt sucrose gradients from 4.5S to 8S. Similarly, the tamoxifen– and 4-hydroxytamoxifen–estrogen receptor complexes, submitted to activating conditions such as heating at 25°C, exposure to high salt concentrations or long incubations, partially bind to the D547 antibody [19, 20] (Fig. 1B and C). However, the fraction of receptor recognized by this antibody is markedly lower when it is bound to both antiestrogens rather than estradiol (Fig. 1A). We also observed that the conditions that induce the binding of the antiestrogen–estrogen receptor complexes to the D547 antibody, simultaneously increase their binding to DNA–cellulose [19]; however, this binding is lower than that of the estradiol–estrogen receptor complex (Table 1). These results show a smaller proportion of activated receptor when it is complexed with both antiestrogens rather than estradiol, suggesting an impaired activation induced by the antiestrogens.

On the other hand, the monoclonal antibody H222, whose epitope is in the hormone-binding domain, recognizes both the activated and non-activated estrogen receptor, shifting its sedimentation coefficient in high salt sucrose gradients from 4.5S to 8S. This antibody interacts similarly with the estrogen receptor complexed with an antiestrogen or with estradiol [20].

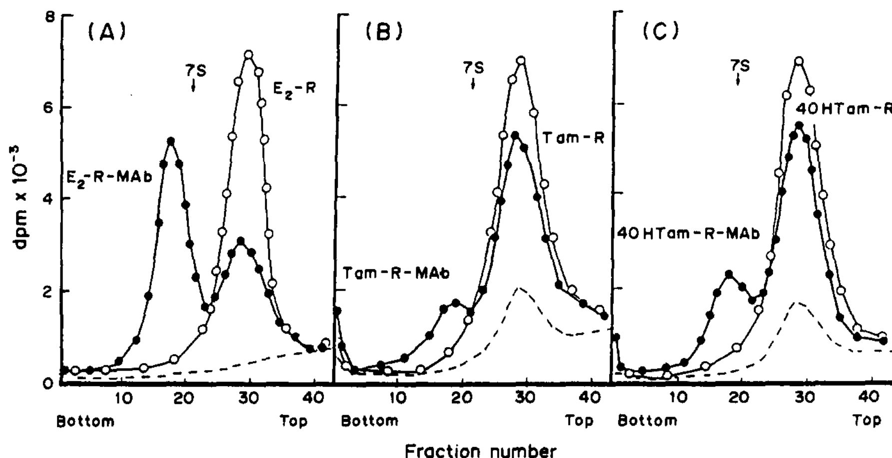

Fig. 1. Interaction of the different ligand–receptor complexes wth the monoclonal antibody D547. Cytosol aliquots were incubated with 10 nM [^{3}H]estradiol (A), 15 nM [^{3}H]tamoxifen (B) or 15 nM [^{3}H]4-hydroxyamoxifen (C) and with (●) or without (○) the monoclonal antibody D547 for 20 h at 4°C. Samples were analyzed in high salt sucrose density gradients as indicated in Experimental. Non-specific binding was determined in parallel cytosol aliquots containing a 100-fold excess of unlabelled estradiol (– – –). Quoted from Ref. [20] (with permission of the *J. Steroid Biochem.*).

Table 1. DNA–cellulose binding of the tamoxifen–estrogen–receptor complex

Experimental conditions	DNA–cellulose binding (% of total receptor)			
	Tamoxifen–receptor complex		Estradiol–receptor complex	
1. 4°C 2 h	6.9 ± 1.5	$n = 8$	9.2 ± 1.0	$n = 7$*
2. 4°C 2 h + 25°C 30 min	17.9 ± 3.3	$n = 4$	25.9 ± 3.2	$n = 4$*
3. 4°C 20 h	13.2 ± 1.1	$n = 3$	18.8 ± 2.7	$n = 5$*
4. 4°C 2 h + 0.4 M KCl 60 min	40.0 ± 2.8	$n = 4$	60.3 ± 4.0	$n = 5$**

Aliquots of cytosol were incubated with 15 nM [^{3}H]tamoxifen or10 mM [^{3}H]estradiol for 2 h at 4°C as indicated above. Sample 2 was warmed at 25°C for 30 min. Sample 3 was incubated at 4°C for a further 18 h. Sample 4 was incubated with 0.4 M KCl at 4°C for 60 min. Samples were assayed for DNA–cellulose binding. The data are expressed as means ± SE of n experiments. *$P < 0.01$; **$P < 0.001$.

Quoted from Ref.[19] (with the permission of *Biochim. Biophys. Acta*).

BIOLOGICAL RESPONSES OF THE ANTIESTROGEN ICI 164,384 ON VARIOUS BREAST CANCER CELL LINES

Extensive information has indicated that the antiestrogen ICI 164,384 has full antiestrogenic properties [8]. The hormone-dependent (MCF-7 [21], T-47D [22]) and hormone-independent (MDA-MB-231 and MDA-MB-436 [23]) mammary cancer cell lines utilized are interesting models to explore the biological responses of new drugs. The effect of ICI 164,384 on cell proliferation, progesterone receptor levels, penetration of estrone sulfate and estradiol concentrations in these cell lines are summarized.

Effect on cell proliferation

The various breast cancer cells plated in 24-well dishes were cultured in the presence of increasing concentrations of ICI 164,384 and after 6 days of treatment the DNA content was determined. The percentage of growth inhibition obtained in the different cell lines is shown in Fig. 2. As observed, the effect on growth inhibition of either MCF-7 or T-47D was dose-dependent, with a higher sensitivity for the MCF-7 cells [24].

The data obtained with the MCF-7 cells is in agreement with that of Wakeling and Bowler [8]. It is to be remarked that a concentration of 100–1000 times of tamoxifen is necessary to provoke a similar effect [24, 25]. On the other hand, the cell growth of the hormone-independent cell line MDA-436 was not affected by this antiestrogen up to a 10^{-6} M concentration which provoked a growth inhibition of 30%.

Effect on progesterone receptor concentrations

In the basic culture conditions, progesterone receptor (PR) levels are 0.5–1.2 pmol/mg DNA in MCF-7 cells and 11.2–21.2 pmol/mg DNA in T-47D cells. After 6 days of treatment with 10^{-10} M estradiol (a concentration sufficient to provoke a maximal induction of PR) either in MCF-7 or in T-47D, this basal level was increased 4–7-fold in MCF-7 cells and to a lesser extent in T-47D (1.6-fold) [24]. In MCF-7 cells, the PR induction was already significantly decreased from a concentration of 10^{-9} M of ICI 164,384 and completely blocked at 10^{-8} M of this antiestrogen. In T-47D cells, a concentration of 10^{-8} M of the antiestrogen was necessary to provoke a significant decrease of the PR induction [24] (see Fig. 3).

Effect in cellular radioactivity uptake after incubation with [^{3}H]estrone sulfate and in estradiol concentration

As indicated in Table 2, the incubation of a physiological concentration of [^{3}H]estrone sulfate (5 nM) showed significant differences in the radioactivity uptake by the various cell lines. The highest cellular uptake of the radioactivity was obtained with the hormone-dependent cell

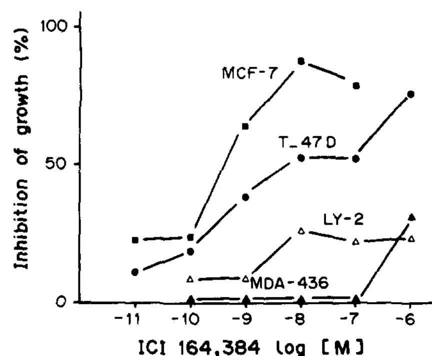

Fig. 2. Inhibition of the growth of various breast cancer cells by the antiestrogen ICI 164,384. MCF-7 (■—■), T-47D (●—●), MDA-231 (△—△) or MDA-436 (▲—▲) cells were plated in 24-well dishes in MEM + 5% DCC-FCS. Increasing concentrations of ICI 164,384 were added at 0 time. The DNA concentrations (in μg/well) were determined after 6 days of cell culture. Growth inhibition was calculated as follows:

$$\% \text{ inhibition} = 1 - \frac{\text{drug treated} - \text{Day 0 control}}{\text{Day 5 control} - \text{Day 0 control}} \times 100.$$

Each point represents the mean of 6 determinations of two separate experiments.

Table 2. Effect of the antiestrogen ICI 164,384 on the radioactivity uptake after incubation of [^{3}H]estrone sulfate (E_1S) with various breast cancer cells

	Uptake (pmol [^{3}H]/mg DNA)			
	Hormone-dependent		Hormone-independent	
	MCF-7	T-47D	MDA-231	MDA-436
[^{3}H]E_1S alone	0.5 ± 0.08	1.9 ± 0.12	0.24 ± 0.05	0.08 ± 0.01
[^{3}H]E_1S + ICI 164,384	0.1 ± 0.02	1.3 ± 0.27	0.41 ± 0.10	0.11 ± 0.01

Preconfluent cells were incubated 24 h with 5 nM [^{3}H]E_1S with or without 10^{-6} M ICI 164,384.
Results are expressed as the mean $\pm$ SE of 3–6 determinations.

lines, MCF-7 (0.5 pmol/mg DNA) and still more in T-47D (2 pmol/mg DNA) cells. The addition of the antiestrogen ICI 164,384 (10^{-6} M) provoked an important decrease of the radioactivity uptake: a diminution of 80% in MCF-7 and of 30% in T-47D cells. The values obtained with the hormone-independent cells are significantly lower, with no significant effect by the addition of the antiestrogen ICI 164,384.

ICI 164,384 also provokes a significant decrease in intracellular estradiol concentration after incubation of estrone sulfate with both MCF-7 and T-47D cells [26, 27].

EFFECT OF THE ANTIESTROGEN ICI 164,384 ON THE PROGESTERONE RECEPTOR OF THE ISOLATED CELLS OF VAGINA AND UTERUS OF FETAL GUINEA-PIG

It was extensively demonstrated that during the perinatal life (fetus and newborns) of guinea-pigs, tamoxifen and other triphenylethylene derivatives act as real estrogens in the target organs (uterus, vagina) [28, 29] of this animal species. The agonistic effect was shown for both growth and progesterone receptor. However, there are no significant quantitative differences in the response when these two tissues are compared [30]. In another series of studies in this laboratory, fetal uterine cells of guinea-pig were isolated and maintained in culture for a long period. In these cells, despite the fact that oestrogen receptors are not detectable, they respond to the hormone in the stimulation of the progesterone receptor, but not in cell proliferation [31]. It was also demonstrated in opposition to the *in vivo* experiments that tamoxifen and 4-hydroxytamoxifen can decrease the progesterone receptor and antagonize the stimulatory effect provoked by estradiol [32]. More recently fetal vaginal cells of this animal species were isolated and maintained in culture for various months [33]. In the present study we explore the effect of the antiestrogen ICI 164,384 in this isolated fetal vagina cell on the progesterone receptor and comparatively to the effect provoked in isolated fetal uterine cells.

Figure 4 shows that in this response ICI 164,384 increases PR in both vaginal and uterine cells. The data are of interest because in these two models, in opposition to all other models experimented, ICI 164,384 can act as a real agonist. Another attractive aspect is the response to 4-hydroxytamoxifen, which in the vaginal cells acts as an agonist, but in the uterine cells as an antagonist.

DISCUSSION AND CONCLUSION

The present data confirm the complexity of the mechanism of action of antiestrogens and add new experimental models in which it is demonstrated that these substances can act either as antagonists or as agonists. This is particularly interesting for the new antiestrogen ICI 164,384 considered with full antagonistic

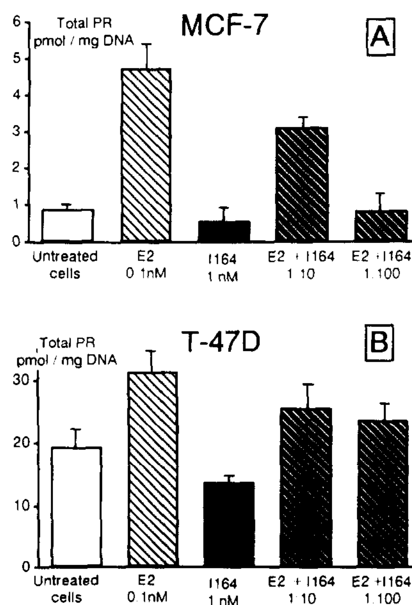

Fig. 3. Effect of the antiestrogen ICI 164,384 on progesterone receptor (PR) concentrations in the MCF-7 and T-47D human breast cancer cells. 5 days after plating in MEM + 5% DCC-FCS, the MCF-7 (A) or T-47D (B) cells were treated for 6 days with 0.1 nM estradiol (E_2) with or without increasing concentrations of ICI 164,384 (I 164) or with 1 nM ICI 164,384 alone. The results are expressed as the mean (total PR = cytosol + nuclear extract) $\pm$ SE of 4–6 determinations [24].

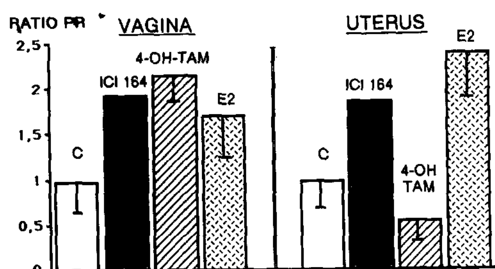

Fig. 4. Effect of the antiestrogens ICI 164,384 and 4-hydoxytamoxifen on the progesterone receptor on isolated vagina and uterine cells of fetal guinea-pig. Fetal vagina or uterine isolated cells were cultured for 6 days in DMEM + 5% FCS-DCC in the presence or absence (C, control) of ICI 164,384 (10^{-8} M), 4-hydroxytamoxifen (4-OH-TAM, 10^{-7} M) or estradiol (E_2, 10^{-8} M). Progesterone receptor (PR) concentrations (in pmol/mg DNA) were measured in the cytosol and in the 0.6 M KCl nuclear extracts using [^{3}H]R5020 as specific ligand. The results indicate total PR (cytosol + nuclear) and are expressed as the ratio of the PR levels in the presence of the compound tested to untreated control levels assigned the value 1. The data represent the average of 3–4 experiments.

properties in all the models already studied [8, 24, 25]. However, in isolated cells of the vagina and uterus of fetal guinea-pig, concerning the progesterone receptor, this compound acts as a real agonist.

Another attractive aspect is the opposition of the response of the antiestrogen 4-hydroxytamoxifen in very close organs such as the vagina and uterus. In effect, for the progesterone receptor, 4-hydroxytamoxifen is stimulatory in isolated cells of the vagina but has an inhibitory effect in the uterine cells. What is the explanation for these differences of response? At the moment there is no clear answer to this question, but if it is generally accepted that the mechanism of action of the antiestrogen is through the estrogen receptor, it can be speculated that there are differences (qualitative and/or quantitative) in the factor(s) which modulate the activation and the binding of the antiestrogen to the estrogen receptor molecule. Complementary information is needed to obtain a clearer explanation.

In the mammary cancer cells MCF-7 or T-47D, the antiestrogen ICI 164,384 has an inhibitory effect on PR and can also block the stimulatory effect produced by estradiol [24]. This is another example of the opposite effect of an antiestrogen for the same parameter, but using different models.

Finally, it is to be remarked that the antiestrogen ICI 164,384 inhibits the penetration of estrone sulfate as well as the cellular concentration of estradiol originated from this sulfate, and the effect is significantly more efficient than that obtained with tamoxifen [26, 27].

Acknowledgements—Part of the expenses of this work were defrayed by the 'Fondation pour la Recherche Médicale Française', and the 'Centre National de la Recherche Scientifique', France (C.N.R.S. URA-548).

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