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Report

Transformation of estrone and estradiol in hormone-dependent and hormone-independent human breast cancer cells

Effects of the antiestrogen ICI 164,384, danazol, and promegestone (R-5020)

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Key words: breast cancer cells, estrone, extradiol estrogen metabolism, Danazol, 17β-hydroxysteroid dehydrogenase, Promegestone

Summary

Using different hormone-dependent (MCF-7, T-47D) and hormone-independent (MDA-MB-231, Hs-578S, MDA-MB-436) human breast cancer cells, the interconversion estrone (E_1) ⇌ estradiol (E_2) was explored. The data show very clearly that in the hormone-dependent cells the tendency is to form E_2 after incubation with E_1, whereas after incubation with E_2 most of this estrogen remains unchanged. In the hormone-independent cells, in contrast most of E_1 remains E_1, while E_2 is converted into E_1. The tendency of the reductive ⇌ oxidative direction is supported by the analysis of estrogens in the culture medium. To explore the possible action of different drugs on the 17β-hydroxysteroid dehydrogenase (17β-HSD) activity, it was observed that the potent antiestrogen ICI 164,384 inhibits the conversion of E_1 to E_2, while a lesser effect is observed with Danazol and only weak inhibition is obtained with the progestagen Promegestone (R-5020). It is concluded that the orientation of 17β-HSD activity for the interconversion $E_1 \rightleftharpoons E_2$ in hormone-dependent and -independent cells is related to the hormonal status of the cells.

Introduction

There is substantial information that breast cancer tissue contains the necessary enzyme systems to synthesize estradiol, the hormone which plays an important role in the origin and evolution of this disease [1–3]. Two principal pathways are implicated in the formation of estradiol in breast cancer tissues: the 'aromatase pathway' which transforms androgens into estrogens [4–6], and the 'sulfatase pathway' which converts estrone sulfate into estrone [7–11]; this estrogen is then transformed into estradiol by the 17β-hydroxysteroid dehydrogenase (17β-HSD) activity [12–14]. It was observed that the 17β-HSD activity in breast cancer depends on the experimental conditions: *in vitro* studies using human tumor homogenates indicated that the predominant 17β-HSD activity was oxidative rather than reductive [13]. However, *in vivo* studies after isotopic infusion of estrogens to post-menopausal breast cancer patients have shown that the reductive direction ($E_1 \rightarrow E_2$) was greater than the oxidative ($E_2 \rightarrow E_1$) [15]. The knowledge of mechanisms and factors regulating 17β-HSD activity in different tissues is limited. It was demonstrated in the endometrium that progestins can control this activity in the oxidative direction ($E_2 \rightarrow E_1$) [16, 17]; however, using breast cancer cell lines the data are contradictory. Coldham and James [18] show that the progestin medroxyprogesterone acetate

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(MPA), stimulates the reductive activity of the 17β-HSD and the authors suggested that this is the way in which progestins may increase cell proliferation *in vivo*. On the other hand, Couture *et al.* [19] observed that in the treatment of ZR-75-1 breast cancer cells with MPA, the oxidative ($E_2 \rightarrow E_1$) direction is predominant. In the present paper, using different hormone-dependent (MCF-7, T-47D) and hormone-independent (MDA-MB-231, MDA-MB-436, Hs 578S) human breast cancer cell lines, the interconversion estrone ⇌ estradiol was explored. The effects of the antiestrogen ICI 164,384, Danazol, and the progestin Promegestone (R-5020) in this transformation are also included.

Materials and methods

Chemicals

[^{3}H]-Estrone (^{3}H-E_1) (S.A.: 48 Ci/mmol), [^{3}H]-estradiol (^{3}H-E_2) (S.A.: 48 Ci/mmol), [4-^{14}C]-estrone (^{14}C-E_1) (S.A.: 51 mCi/mmol), and [4-^{14}C]-estradiol (^{14}C-E_2) (S.A.: 57 mCi/mmol) were obtained from New England Nuclear Division (Dupont de Nemours, Les Ulis, France). Promegestone (R-5020: 17α,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione) was a gift from Cassenne Laboratories (Puteaux, France). Tamoxifen (TAM) (ICI 46,474: trans-1-(p-dimethylaminoethoxyphenyl)-1,2-trans-diphenylbut-1-ene); 4-hydroxy-tamoxifen (4-OH-TAM) (ICI 79,280: 1-(p-dimethylaminoethoxyphenyl)-1-(4-hydroxyphenyl)-2-phenylbut-1-ene); and ICI 164,384 [N-n-butyl-N-methyl-11-(3,17β-dihydroxyestra-1,3,5(10)-trien-7α-yl)-undecanamide], were a gift from Dr A.E. Wakeling (ICI Ltd, Macclesfield, England). Danazol [17β-hydroxy-17α-pregna-2,4-dien-20-yne-(2,3α-isoxazol)] was a gift from Sterling-Winthrop Laboratories (New York, USA).

Cell culture

The human breast cancer hormone-dependent (MCF-7, T-47D) and hormone-independent (MDA-MB-231, MDA-MB-436, Hs-578S) cell lines were kindly provided by Drs M.E. Lippman and R.B. Dickson (Georgetown University, Washington DC, USA). The cells were grown in Eagle's Minimum Essential Medium (MEM) containing 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 10 mM Hepes (A.T.G.C., Noisy-Le-Grand, France) supplemented by 10% fetal calf serum (FCS) (D.A.P., Vogelgrun, France) for MCF-7 cells, and 5% FCS for the others, incubated at 37° C in a humidified atmosphere of 5% CO_2. Media were changed twice a week. The cells were passed every 8–10 days and replated at $\sim 3 \times 10^6$ cells/75 cm^2 plastic flasks (A.T.G.C.).

Transformation of [^{3}H]-estradiol or [^{3}H]-estrone by human mammary cancer cells

After 3 days of culture in 5% steroid-depleted FCS, prepared by mixing the dextran-coated charcoal (DCC, 0.1–1% w/v DCC-FCS), preconfluent cells were incubated for 24 h at 37° C in MEM containing 5×10^{-9} M of [^{3}H]-E_1 or [^{3}H]-E_2 in the absence or presence of the antiestrogen ICI 164,384, Danazol, or the progestagen Promegestone (R-5020). At the end of incubation, the medium was removed, the cells washed twice with ice-cold HBSS (Hank's balanced salt solution), harvested by scraping, and centrifuged. The radioactive material in the pellet of the cells and in the culture medium was extracted with ethanol 80% for 24 h at – 20° C. DNA content in the remaining pellet of the cells was evaluated according to Burton [20]. The qualitative analysis and the quantitative evaluation of estrone and estradiol were carried out after isolation by thin layer chromatography on silica gel (Merck F254), which was developed with chloroform/ethyl acetate (4:1 v/v). [^{14}C]-E_1 or [^{14}C]-E_2 were used to evaluate losses.

Determination of 17β-hydroxysteroid dehydrogenase activity

Preconfluent cells were cultivated in MEM containing 5% DCC-FCS for 4 days, washed twice with ice-cold HBSS (calcium-magnesium-free), harvested by scraping, and centrifuged at 800 × g for 10 min.

The pellet was resuspended in 2 volumes of 0.05 M Tris-HCl buffer (pH 8.0) or NADPH (1.0 mM). Cells were disrupted in a Teflon-glass Potter-Elvehjem homogenizer and centrifuged at 1,000 × g for 10 min. The supernatant is referred to as the 'enzyme sample'. All procedures were carried out at 0–4° C. The enzyme assay was performed using 400 µl of enzyme sample (0.10–0.80 mg protein) and 1.2–30 × 10^4 dpm of [^{3}H]-estrone plus 0.8–20 nmols of the non-radioactive estrogens (final S.A. 15,000 dpm/nmol). The reaction was initiated by adding the enzyme sample pre-incubated for 5 min in the presence or absence of the various drugs, dissolved in ethanol. The final concentration of ethanol in the assays was < 0.5% v/v. After incubation for 30 min at 37° C, the reaction was stopped by the addition of 0.5 ml methanol containing 4,000 dpm of [^{14}C]-E_2 to evaluate the losses. Unlabeled estrone and estradiol carriers (100 µg) were used as reference indicators. After centrifugation at 2,500 × g for 10 min, the supernatant was evaporated and the residue redissolved in 50 µl of ethanol. Estrone and estradiol were separated by TLC using chloroform/ethyl acetate (4:1, v/v) system. Each steroid area was transferred to a liquid scintillation vial and extracted with 0.5 ml of ethanol; 3 ml of Opti-fluor (Packard, Rungis, France) were added and the vials analyzed for [^{3}H]- and [^{14}C]-content with quench corrections by external standardization. Proteins were determined by the Coomassie brilliant blue dye method [21], and the apparent kinetic parameters of the 17β-HSD were determined using the Lineweaver-Burk plot [22].

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) values. Student's *t*-test was used to assess the significance of the differences between means; p values ≤ 0.05 were considered significant.

Results

I) Interconversion estrone ⇌ estradiol in breast cancer cells as a function of incubation time

In order to compare the transformation of estrone to estradiol and that of estradiol to estrone, as a function of time, these two estrogens were incubated in physiological concentrations (5 × 10^{-9} M) with a hormone-dependent (MCF-7) and a hormone-independent (MDA-MB-231) human breast cancer cell line, for a period of 2 to 48 h. Figure 1A shows that in the MCF-7 cells estrone is largely converted (70% after 24 h incubation) to estradiol, but when estradiol is incubated, most of the radioactive material (~ 75%) remains as unchanged estradiol (Fig. 1B). On the other hand, in the MDA-MB-231 hormone-independent cells, the conversion of estrone to estradiol is very limited, only 12% after 24 h incubation (Fig. 1C). In contrast to the MCF-7 cells, after incubation of estradiol a great proportion is converted to estrone (Fig. 1D).

II) Metabolism of estrone and estradiol in different hormone-dependent and -independent cell lines

Tables 1 and 2 show, respectively, the transformation of estrone and estradiol in the various cell lines and in the culture medium. The data clearly indicate, and extend the concept, that in the hormone-dependent cells the reaction is predominantly in the reductive direction ($E_1 \rightarrow E_2$), and in the hormone-independent cells the tendency is the oxidative direction ($E_2 \rightarrow E_1$). The very high concentration of estradiol in the culture medium after incubation of estrone is remarkable (Table 1), whereas most of the estradiol remains unchanged after incubation of this estrogen (Table 2) with the hormone-dependent cell lines. Similar data to those obtained using the MDA-MB-231 cells (Figs 1C and 1D), were obtained with the two other hormone-independent cell lines, MDA-MB-436 and Hs 578S. After incubation with these two estrogens (E_1 and E_2) most of the radioactive material is represented by E_1.

III) Effect of the antiestrogen ICI 164,384, Danazol, and Promegestone (R-5020) on estradiol formation in the intact cells and cell homogenates

Previous studies in this laboratory have demon-

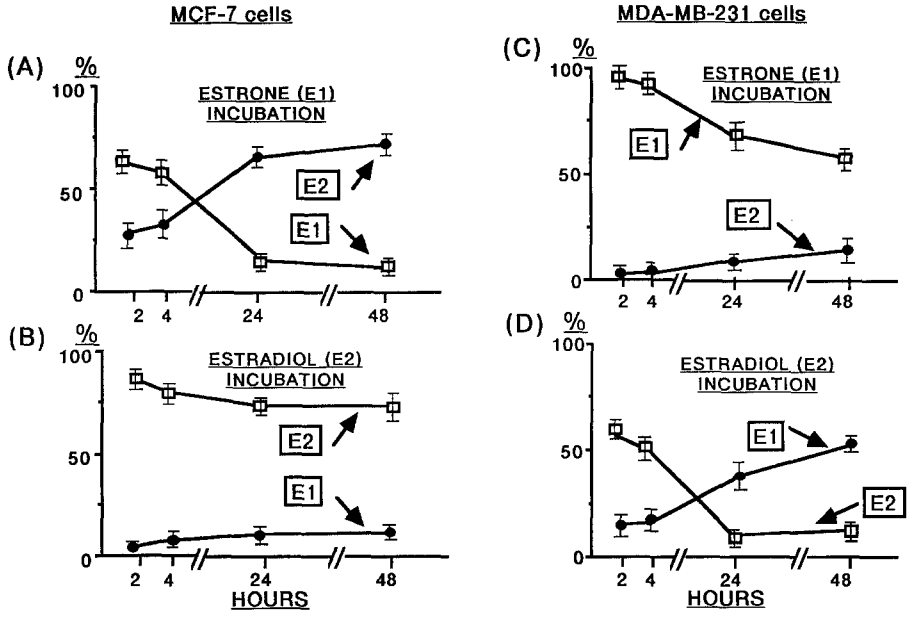

Fig. 1. Time course of the interconversion of estrone ⇌ estradiol in the MCF-7 hormone-dependent and in the MDA-MB-231 hormone-independent cells. Estrone (E_1) or estradiol (E_2) (5×10^{-9} M) were incubated between 2 and 48 h, and E_1 or E_2 in the different cells were isolated and quantified as indicated in Materials and methods. Panel A indicates the conversion after incubation with E_1, and Panel B represents the transformation of E_2 using MCF-7 cells. Panels C and D show the $E_1 \rightleftharpoons E_2$ interconversion in the MDA-MB-231 cells. The values are the average ± SEM of 3 experiments.

strated that after incubation of estrone sulfate (E_1S) with the intact hormone-dependent cancer cells (MCF-7, T-47D, R-27, Ly-2) most of the radioactivity inside the cells is represented by E_2 [8, 9], and it was observed that various substances, including Promegestone (R-5020), Danazol, and the anti-estrogens Tamoxifen, 4-hydroxytamoxifen, and ICI 164,384, can provoke a significant decrease in the conversion of E_1S to E_2 [23, 24]. In another series of studies using cell homogenates, it was demonstrated that Promegestone, Danazol, and different anti-estrogens have an inhibitory and competitive effect

Table 1. Metabolism of estrone (E_1) in the cells and in the culture medium of various hormone-dependent and -independent breast cancer cell lines (data expressed in pmol/mg DNA)

Cell lines	In the cells		In the culture medium	
	E_1	E_2	E_1	E_2
Hormone-dependent				
MCF-7	0.44 ± 0.15	5.20 ± 0.94	14 ± 5.3	70 ± 17
T-47D	0.95 ± 0.42	7.70 ± 2.20	15 ± 7.5	58 ± 13
Hormone-independent				
MDA-MB-231	3.50 ± 1.20	0.37 ± 0.18	59 ± 14	13 ± 6.5
MDA-MB-436	0.70 ± 0.20	0.20 ± 0.10	45 ± 8	25 ± 9
Hs-578	1.50 ± 0.30	0.12 ± 0.06	95 ± 17	32 ± 11

Preconfluent cells were incubated for 24 h with 5×10^{-9} M of E_1; estrone (E_1) and estradiol (E_2) were analyzed and quantified as indicated in Materials and methods. The values are expressed as the mean ± SEM of 6–9 duplicate determinations.

Table 2. Metabolism of estradiol (E_2) in the cells and in the culture medium of various hormone-dependent and -independent breast cancer cell lines (data expressed in pmol/mg DNA)

Cell lines	In the cells		In the culture medium	
	E_1	E_2	E_1	E_2
Hormone-dependent				
MCF-7	0.25 ± 0.04	5.80 ± 2.30	12 ± 5	130 ± 38
T-47D	0.54 ± 0.22	6.10 ± 1.50	12 ± 4.5	70 ± 18
Hormone-independent				
MDA-MB-231	3.65 ± 1.06	0.53 ± 0.20	45 ± 12	38 ± 9
MDA-MB-436	0.80 ± 0.40	0.60 ± 0.10	64 ± 22	48 ± 15
Hs-578	1.40 ± 0.35	0.44 ± 0.13	95 ± 28	65 ± 23

Same as for Table 1. Estrogen incubated: estradiol (5×10^{-9} M). The values are expressed as the mean ± SEM of 6–9 duplicate determinations.

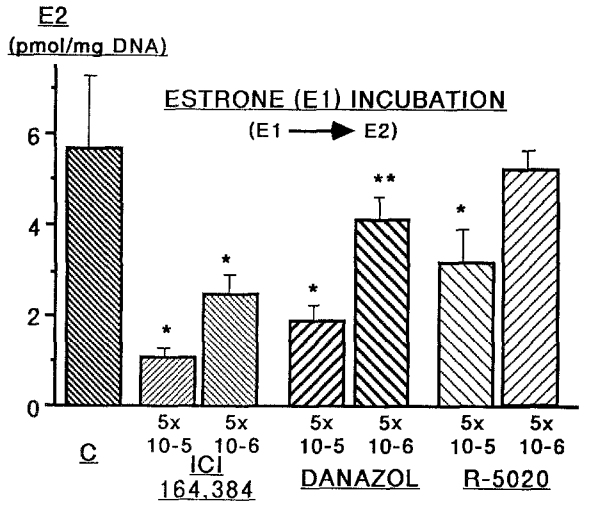

Fig. 2. Effect of the antiestrogen ICI 164,384, Danazol, and Promegestone (R-5020) on the estradiol (E_2) concentration after incubation of estrone with the T-47D cells. Estrone was incubated with the T-47D cells for 24 h at 37° C in the absence (control) or presence of ICI 164,384, Danazol, or Promegestone (R-5020). The calculation of E_2 (in pmols/mg DNA) was carried out after isolation of the hormones as indicated in Materials and methods. The data represent the average ± SEM of 3–4 experiments. * $p < 0.01$ versus control (non-treated cells). ** $p < 0.05$ versus control (non-treated cells).

on the estrone sulfatase activity [25, 26]. As in the conversion of E_1S to E_2 two enzymes are involved, the sulfatase which transforms E_1S to E_1 and the 17β-hydroxysteroid dehydrogenase which converts E_1 to E_2, we explored the effect of various drugs on this last transformation using the intact cells and the cell homogenates. Figure 2 shows that in the hormone-dependent T-47D cells, ICI 164,384 decreases very significantly the E_2 concentration after incubation of E_1, suggesting an inhibitory 17β-HSD activity of this antiestrogen on the reductive direction. The effect is less intense with Danazol, and some inhibition is observed with the progestagen R-5020 at 5×10^{-5} M. No significant effect was observed on the E_1 concentration (data not shown).

The possibility that these substances can act directly on the enzyme itself was explored using the homogenate of T-47D cells. Figure 3 shows a Lineweaver and Burk plot obtained from a range of E_1 concentrations between 2–40 µM. This reciprocal plot was linear, indicating the presence of a single reactive enzyme type and no substrate activation or product inhibition. The maximal 17β-HSD reductive ($E_1 \rightarrow E_2$) activity (Vmax) obtained is 2.75 ± 1.3 nmol/mg protein/h, and the apparent Michaelis constant (Km) is 12.9 ± 2.9 µmol/l.

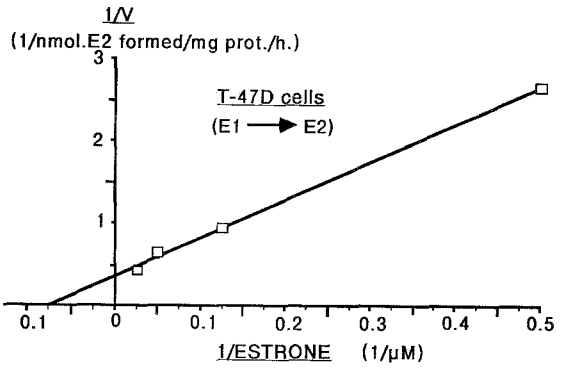

Fig. 3. Lineweaver-Burk plot of the 17β-hydroxysteroid dehydrogenase activity of estrone reduction in the homogenate of T-47D cells. The values are the mean ± SEM of duplicate determinations of 3 experiments.

Figure 4 shows the effect of different drugs on 17β-HSD reductive activity in the cell homogenate of T-47D cells. It is observed that ICI 164,384 inhibits the conversion of $E_1 \rightarrow E_2$ by 37 and 28%, with a respective concentration of 1×10^{-5} and 1×10^{-6} M. The inhibition, as in the intact cell, is also intense with Danazol, while no significant effect is observed with the progestin R-5020.

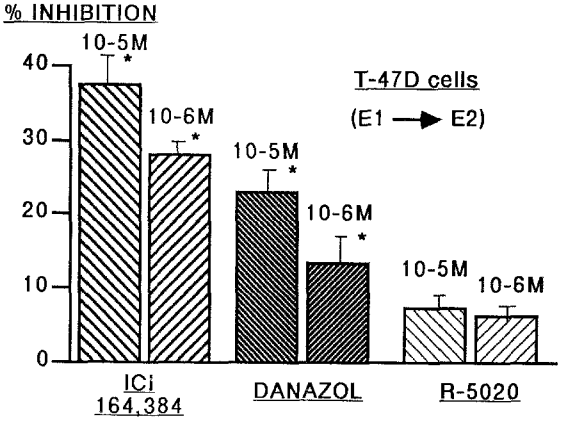

Fig. 4. Effect of ICI 164,384, Danazol, and Promegestone (R-5020) on the 17β-hydroxysteroid dehydrogenase reductive activity in the homogenates of T-47D cells. The percentage of 17β-hydroxysteroid dehydrogenase was determined at 2 µM of estrone and was obtained by calculating the ratio: (Control – Test/Control) ×100. The values are the mean ± SEM of duplicate determinations of 3 experiments. * $p < 0.05$ versus control (non-treated cells).

Discussion

The present data clearly indicate that 17β-hydroxysteroid dehydrogenase (17β-HSD) activity is oriented in the reductive pathway in hormone-dependent breast cancer cells (MCF-7, T-47D) while in the hormone-independent cells (MDA-MB-231, MDA-MB-436, Hs 578S) the oxidative direction is predominant. Since in these experiments using the intact cells, no exogenous co-factors [e.g.: NAD(P)/NAD(P)H] were added to the culture medium, we can suggest that the predominant enzymatic direction found corresponds to the natural preference of the 17β-HSD activity in the different types of cell. This information extends and confirms a postulated hypothesis in a previous publication [9]. The mechanism of this opposite 17β-HSD activity according to the hormone dependence of the mammary cancer cells is not well understood. We can assume that the reductive or oxidative preference is a function of different 17β-HSD enzyme forms and/or that specific effectors modulate the orientation of the enzyme activity. In this connection, recent data have demonstrated the presence of two human 17β-HSD genes [27, 28] and variations in the expression of 17β-HSD among the human breast cancer cell lines [29]. Related to the present results, it is interesting to mention that in another hormone-dependent breast cancer cell line, ZR-75-1, Couture *et al.* [19] found that the reductive 17β-HSD activity was 3–4 times higher than the corresponding oxidative activity ($E_2 \rightarrow E_1$). In other studies, Singh and Reed [30] found that the conversion of E_2 to E_1 was 27–70 times greater than the reductive reaction in MDA-MB-231 cells, whereas in MCF-7 cells both interconversions had identical activities. Malet *et al.* [31] obtained high conversion of E_1 to E_2 (85%) in the T-47D cells; the oxidative activity represents 15% but can increase to 35% by exposure to exogenous NAD. An interesting aspect of the present results is the analysis in the culture medium, which shows after incubation of E_1 a very high concentration of E_2 for the hormone-dependent breast cancer cell lines.

Despite conflicting data in the literature between *in vitro* and *in vivo* experiments [15], it emerges clearly that in human breast the conversion of E_1 to E_2 (reductive pathway) is the preferential direction and is more intense in the tumor than in normal tissue [33, 34].

Several different drugs were investigated in order to analyze a possible effect on this enzyme. Among the substances tested, the potent antiestrogen ICI 164,384 is the most active, followed by Danazol, while the activity of Promegestone (R-5020) is limited, particularly in the study using cell homogenates.

Using experimental rat tumors and breast cancer tissue, Santner and Santen [35] also observed a potent inhibitory effect of ICI 164,384 on reductive 17β-HSD activity. It was also reported that Danazol at the concentration of 10 μM can inhibit the reductive 17β-HSD activity by 30% in normal human breast tissue [36].

Concerning the activity of the progestins, significant contradictions are observed by different authors. The effect of these drugs is a function of the experimental conditions, the nature of the tissues or cells, and the concentrations and type of molecules studied. For instance, in the endometrium, progestins clearly stimulate the oxidative direction of the enzyme, while in breast tissue models the action is more complex. Mann *et al.* [36] observed that the progestin medroxyprogesterone acetate (MPA) can inhibit the reductive 17β-HSD activity by 61% in normal breast tissue. In MCF-7 cells, Coldham and James [18] found that progestin increases the reductive direction, and in T-47D cells Malet *et al.* [31] demonstrated that after 5 days of incubation R-5020, alone or associated with E_2, was able to stimulate the oxidative pathway 2–3 fold, whereas no effect was detected in the reductive direction. Mehta and Das Gupta [37] observed in UISO-BCA-1 breast cancer cells that R-5020 alone (10^{-8} M) had no effect on 17β-HSD (oxidative or reductive) but when associated with E_2 these authors noted a stimulation of both directions.

It is concluded that in breast cancer, for the 17β-HSD activity, the reductive direction ($E_1 \rightarrow E_2$) is predominant in hormone-dependent and the oxidative direction ($E_2 \rightarrow E_1$) in hormone-independent cells. The antiestrogen ICI 164,384 and Danazol can inhibit the conversion of $E_1 \rightarrow E_2$. The use of 17β-hydroxysteroid dehydrogenase activity as a marker

and the clinical applications in breast cancer of these findings are to be explored.

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