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Dydrogesterone (Duphaston®) and its 20-Dihydro-derivative as Selective Estrogen Enzyme Modulators in Human Breast Cancer Cell Lines. Effect on Sulfatase and on 17β-Hydroxysteroid Dehydrogenase (17β-HSD) Activity

GÉRARD SAMUEL CHETRITE[1], HUBERT H. THOLE[2], JEAN-CLAUDE PHILIPPE[1] and JORGE RAUL PASQUALINI[1]

[1]*Hormones and Cancer Research Unit, Institut de Puériculture, 26 Boulevard Brune, 75014 Paris, France;*
[2]*Solvay Pharmaceuticals GmbH, D-30173 Hannover, Germany*

Abstract. *Estradiol (E_2) is one of the main factors which control the growth and evolution of breast cancer. Consequently, to block the formation of E_2 inside cancer cells has been an important target in recent years. Breast cancer cells possess all the enzymatic systems (e.g. sulfatase, aromatase, 17β-hydroxysteroid dehydrogenase [17β-HSD]) involved in the conversion of estrogen precursors into E_2. Sulfotransferase, which converts estrogen to its sulfate, is also present in this tumoral tissue. Duphaston® is a synthetic progestogen with properties similar to the natural progesterone. In the present study we examined the effect of Duphaston® and its 20-dihydro-metabolite on the sulfatase and 17β-HSD activities in MCF-7 and T-47D breast cancer cells. The cells were incubated with estrone sulfate (E_1S) (5x10-9M) in the absence or presence of Duphaston® or its 20-dihydro-metabolite ($5x10^{-5}$ to $5x10^{-9}$M) for 24h at 37°C. In another series of experiments, estrone (E_1) ($5x10^{-9}$M) was incubated with T-47D cells in the absence or presence of the two progestogens ($5x10^{-5}$ to $5x10^{-9}$M) for 24h at 37°C. E_1S, E_1 and E_2 were characterized by thin layer chromatography and quantified using the corresponding standard. Duphaston® and its 20-dihydro-metabolite, at concentrations of $5x10^{-7}$ and $5x10^{-5}$M, inhibited the conversion of E_1S to E_2 by 14% and 63%, 65% and 74%, respectively, in MCF-7 cells; the values were 15% and 48% and 31% and 51%, respectively, in T-47D cells. In another series of experiments it was observed that, after 24-h incubation, E_1 ($5x10^{-9}$M) was converted in a great proportion to E_2 in the T-47D cells and that this transformation was significantly inhibited by Duphaston® and its 20-dihydro-metabolite. The IC_{50} value, corresponding to 50% of the inhibition in the conversion of E_1 to E_2, was $9x10^{-6}$M for 20-dihydro-metabolite in this cell line. It was concluded that the progestogen Duphaston® and its 20-dihydro-metabolite are potent inhibitory agents on sulfatase and 17β-HSD activities in breast cancer cells. Duphaston® is a progestogen with properties similar to the endogenous progesterone. The data open interesting perspectives to study the biological responses of these progestogens in clinical trials of patients with breast cancer.*

Correspondence to: Dr J.R. Pasqualini, Hormones and Cancer Research Unit, Institut de Puériculture, 26 Boulevard Brune, 75014 Paris, France. Tel: (33)-1 +45 42 41 21 or +45 39 91 09, Fax: (33)-1 +45 42 61 21, e-mail: Jorge.Pasqualini@wanadoo.fr

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There is substantial information that mammary cancer tissue contains all the enzymes responsible for the local biosynthesis of estradiol (E_2) from circulating precursors. Two principal pathways are implicated in the last steps of E_2 formation in breast cancer tissues: the 'aromatase pathway', which transforms androgens into estrogens (1,2) and the 'sulfatase pathway' which converts estrone sulfate (E_1S) into estrone (E_1) by estrone-sulfatase (3-5). The final step of steroidogenesis is the conversion of the weak E_1 to the potent biologically active E_2 by the action of a reductive 17β-hydroxysteroid dehydrogenase Type 1 activity (17β-HSD-1) (6-8).

Quantitative evaluation indicates that, in human breast tumour, E_1S '*via* sulfatase' is a much more likely precursor for E_2 than is androstenedione 'via aromatase' (9-11).

It is also well established that steroid sulfotransferases, which convert estrogens into their sulfates, are also present in breast cancer tissues (12-14). All this information extends the concept of 'intracrinology' where a hormone can have its biological response in the same organ as it is produced.

In the present study, we explored the effects of dydrogesterone (Duphaston®) and its 20-dihydro-derivative on estrone sulfatase in the MCF-7 and T-47D breast cancer cell lines and on reductive 17β-hydroxysteroid dehydrogenase Type 1 in T-47D cells.

Materials and Methods

Chemicals. [6,7-^{3}H(*N*)]-estrone sulfate (^{3}H-E_1S), ammonium salt (sp. act. 53 Ci/mmol), [6,7-^{3}H(*N*)]-Estrone (^{3}H-E_1) (sp. act. 49 Ci/mmol) and [4-^{14}C]-estradiol (^{14}C-E_2) (sp. act. 57 mCi/mmol) were purchased from New England Nuclear Division (PerkinElmer Life Sciences, Courtabœuf, France). The purity of the radioisotopes was assessed by thin-layer chromatography (TLC) in the appropriate system before use. E_1S, ammonium salt, unlabelled E_1 and E_2, (analytical grade) were obtained from Sigma-Aldrich Chimie (St Quentin Fallavier, France). Dydrogesterone (Duphaston®: (9β,10α)-pregna-4,6-diene-3,20-dione) and its metabolite 20-dihydro-dydrogesterone were provided by Solvay Pharmaceuticals GmbH (Hannover, Germany). All other chemicals were of the highest grade commercially available.

Cell culture. The hormone-dependent MCF-7 and T-47D human mammary cancer cell lines were kindly provided by Dr R. C. Clarke (Georgetown University, Washington, USA). The cells were grown in Eagle's Minimal Essential Medium (MEM) buffered with 10 mmol/l HEPES (pH 7.6), supplemented with 2mmol/l L-glutamine, 100 U/ml penicillin-streptomycin and 5% fetal calf serum (FCS) (A.T.G.C., Marne-la-Vallée, France) for T-47D, or 10% FCS for MCF-7 cells, and incubated at 37˚C in a humidified atmosphere of 5% CO_2. The media were changed twice a week. The cells were passed every 10-12 days and replated in 75-cm^2 flasks (A.T.G.C.) at 3x10^6 cells/flask. Four days before the experiments, the cells were transferred to MEM containing 5% steroid-depleted treated FCS. The FCS had been treated overnight at 4˚C with dextran-coated charcoal (DCC) (0.1-1% w/v, DCC-FCS) to remove endogenous steroids.

Isolation and quantification of [^{3}H]-estradiol from human mammary cancer cells incubated with [^{3}H]-estrone sulfate. Preconfluent cells were cultivated in MEM-DCC-FCS with the addition of 5 x 10^{-9} mol/l of [^{3}H]-E_1S, alone (control cells) or in combination with the different compounds: dydrogesterone (Duphaston®) or its metabolite, 20-dihydro-dydrogesterone, dissolved in ethanol (final concentration < 0.3%), at a range of concentrations of 5x10^{-5} - 5x10^{-7} - 5x10^{-9} mol/l. Control cells received ethanol vehicle only. Twenty-four hours later, the medium was removed, the cells washed twice with ice-cold Hank's Buffered Saline Solution (HBSS, calcium- magnesium-free) (A.T.G.C.) and harvested by scraping. After centrifugation, the pellet was treated with 80% ethanol and the radioactivity extracted for at least 24h at -20˚C. The cellular radioactivity uptake was determined in the ethanolic supernatant and the DNA content in the remaining pellet was evaluated according to Burton (15). [^{14}C]-E_2 (5,000 dpm) was added to monitor analytical losses and unlabelled E_1 and E_2 (50 μg) were used as carriers and reference indicators. In the total ethanolic extracts, E_2 was isolated by thin layer chromatography (TLC) on silica gel 60F254 (Merck, Darmstadt, Germany), developed with the chloroform-ethylacetate (4:1, v/v) system. After visualization of the estrogens under U.V. at 254 nm, the appropriate areas were cut off into small pieces, placed in liquid scintillation vials with ethanol (0.5 ml) and allowed to extract for 30 min. Three ml of Opti-fluor (Packard, PerkinElmer Life Sciences) were added and the vials were analyzed for ^{3}H and ^{14}C contents with quench correction by external standardization. The quantitative evaluation of E_2 was calculated as a percentage of the total radioactivity associated with the cells and then expressed as fmol of E_2 formed / mg DNA.

Isolation and quantification of [^{3}H]-estradiol from T-47D human mammary cancer cells incubated with [^{3}H]-estrone. Preconfluent T-47D cells were incubated for 24h at 37˚C in MEM containing 5x10^{-9} mol/l of [^{3}H]-E_1 in the absence or presence of dydrogesterone (Duphaston®) or its metabolite 20-dihydro-dydrogesterone, (5x10^{-5} - 5x10^{-7}- 5x10^{-9} mol/l). At the end of the incubation, the medium was removed, the cells washed, collected, centrifuged and analyzed as previously described in the experimental section on the conversion of [^{3}H]-E_1S to [^{3}H]-E_2. The qualitative analysis and quantitative evaluation of E_1 and E_2 were carried out after isolation by TLC on silica gel 60F254 plates, which were developed with the chloroform-ethylacetate (4:1, v/v) system. [^{14}C]-E_2 (5,000 dpm) was added to monitor analytical losses and unlabeled E_1 and E_2 (50 μg) were used as carriers and reference indicators. After visualization of the estrogens under U.V. at 254 nm, the appropriate areas were cut off into small pieces, placed in liquid scintillation vials with ethanol (0.5 ml) and allowed to extract for 30 min. Three ml of Opti-fluor (Packard, Rungis, France) were added and the vials analyzed for ^{3}H and ^{14}C contents with quench correction by external standardization. The quantitative evaluation of the transformation of the [^{3}H]-E_1 to [^{3}H]-E_2, corresponding to the reductive 17β-HSD activity at 24h, was done by calculating the percentage of the total radioactivity associated with the cells or the medium and then expressed as fmol E_2 /mg DNA.

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

Effect of dydrogesterone (DYD) and of its metabolite 20-dihydro-dydrogesterone (DHD) on estrone sulfatase activity in the hormone-dependent MCF-7 and T-47D human breast cancer cell lines. As estrone sulfate is the most important precursor of estradiol in breast cancer tissues, the search for compounds which can act as selective estrogen enzyme modulators by inhibiting estrone sulfatase and 17β-hydroxysteroid dehydrogenase or stimulating sulfotransferase activities in breast cancer cells is particularly pertinent. To this aim, we tested the capacity of dydrogesterone (Duphaston®) and its 20-dihydro metabolite (see Figure 1) to block the sulfatase pathway in breast cancer cells.

When physiological concentrations (5x10^{-9}M) of [^{3}H]-E_1S were incubated with the hormone-dependent MCF-7 and T-47D breast cancer cell lines for 24h at 37˚C, the intracellular production of E_2 in both cells was elevated (1975 ± 211 and 1216±142 fmol/mg DNA, for MCF-7 and T-47D cells, respectively) (Tables I and II). The progestogen dydrogesterone interfered significantly with the estrone sulfatase activity only at the concentration of 5x10^{-5} M (-63% and -48% of inhibition in MCF-7 and T-47D cells, respectively). The 20-dihydro-metabolite strongly decreased the production of E_2 from E_1S in a dose-dependent manner in the two cell lines. This inhibitory effect was high at 5x10^{-5} M

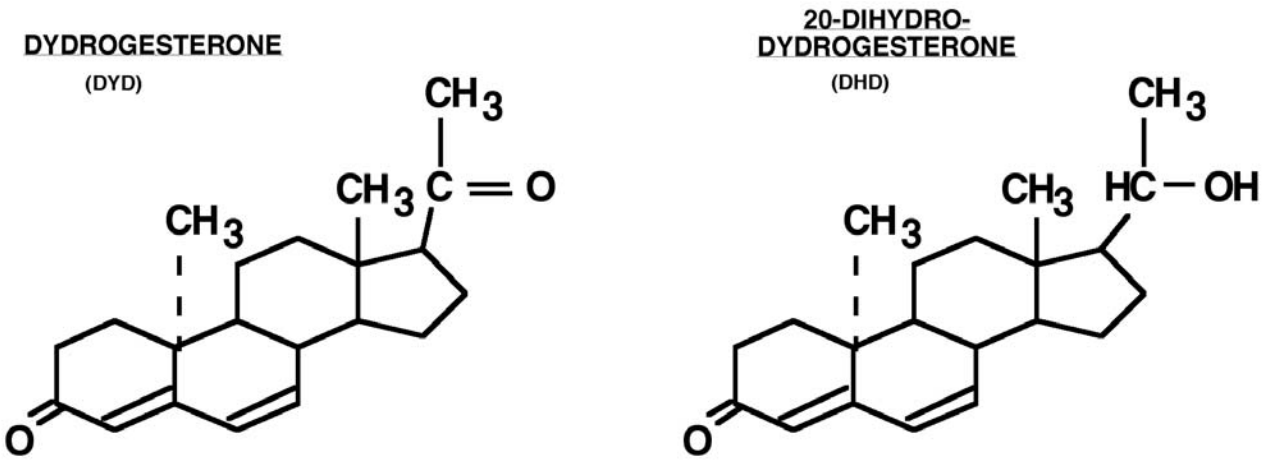

Figure 1. *Structure of the progestogens dydrogesterone and the metabolite 20-dihydro-dydrogesterone.*

(-74% and -51% in MCF-7 and T-47D cells, respectively). At lower concentrations the inhibitory effect on MCF-7 cells was -65% and -24% at $5x10^{-7}$ and $5x10^{-9}$ M, respectively. The values are -31% and -9%, respectively for T-47D cells (Figures 2 and 3). The IC_{50} values, corresponding to 50% of the inhibition in the conversion of E_1S to E_2, were respectively $9.8x10^{-6}$M and $7.1x10^{-8}$M for dydrogesterone and 20-dihydro-metabolite in the MCF-7 cell line.

Effect of dydrogesterone (DYD) and of its metabolite 20-dihydro-dydrogesterone (DHD) on 17β-hydroxysteroid dehydrogenase activity in the hormone-dependent T-47D human breast cancer cell line. The ultimate step of the 'sulfatase pathway' in the formation of the biologically active estrogen estradiol is the conversion of estrone (E_1) into E_2 by the reductive activity of the 17β-hydroxysteroid dehydrogenase. Consequently, it was attractive to explore the effect of dydrogesterone and its 20-dihydro-metabolite in this last metabolic pathway.

In recent studies, it has been shown that, in the hormone-dependent breast cancer cells this reductive direction (conversion of E_1 to E_2) is prevalent. When a physiological concentration ($5x10^{-9}$ M) of tritiated estrone ($[^3H]$-E_1) was incubated alone as precursor, without the addition of any co-factors, in T-47D cells for 24h at 37°C, the production of $[^3H]$-E_2 was 2110±134 fmol/mg DNA and the rate of conversion approx. 51%, as reported in Table III. The progestogen dydrogesterone very efficiently decreased the conversion of E_1 to E_2 in a dose-dependent manner. The inhibitory effect on the conversion of E_2 from E_1, *versus* control values, was -48%, -34% and -23%, at $5x10^{-5}$, $5x10^{-7}$ and $5x10^{-9}$ M, respectively. The values for the 20-dihydro-metabolite were -54%, -42% and -33%, respectively (Figure 4). The IC_{50} value, corresponding to 50% of the inhibition in the conversion of E_1 to E_2, was $9x10^{-6}$M for 20-dihydro-metabolite.

Table I. *Effect of dydrogesterone and of its metabolite 20-dihydro-dydrogesterone on the conversion of [3H]-estrone-sulfate to [3H]-estradiol by the hormone-dependent MCF-7 breast cancer cell line.*

	Estradiol (E_2) (fmol E_2/mg DNA)	
$[^3H]$-E_1S alone ($5 x10^{-9}$ mol/l)	1975 ± 211	
	+ Dydrogesterone (DYD)	+ 20-Dihydro-dydrogesterone (DHD)
$5x10^{-9}$	1791 ± 135	1520 ± 96*
$5x10^{-7}$	1701 ± 104	688 ±103*
$5x10^{-5}$	727 ± 90*	510 ± 86*

Preconfluent MCF-7 cells were incubated for 24h at 37°C with estrone-sulfate ($[^3H]E_1S$: $5x10^{-9}$ mol/l) alone or in the presence of dydrogesterone or its metabolite 20-dihydro-dydrogesterone in the range of concentrations $5x10^{-9}$ to $5x10^{-5}$ mol/l. Estradiol (in fmol/mg DNA) was analyzed and quantified as indicated in Materials and Methods. The values are expressed as the mean±S.D. of 2 independent duplicate determinations.
*$p<0.05$ *versus* control values ($[^3H]$-E_1S alone).

Discussion

For many years endocrine therapy in breast cancer has mainly consisted of antiestrogens, which block the estrogen receptor. Treatment with the antiestrogen tamoxifen (Nolvadex: tamoxifen citrate) of millions of women with breast cancer has shown a benefit of 30%-35% being free of symptoms of the disease and a 20-25% reduction in mortality.

Table II. *Effect of dydrogesterone and of its metabolite 20-dihydro-dydrogesterone on the conversion of [^{3}H]-estrone-sulfate to [^{3}H]-estradiol by the hormone-dependent T-47D breast cancer cell line.*

	Estradiol (E_2) (fmol E_2/mg DNA)	
[^{3}H]-E_1S alone (5 x10^{-9} mol/l)	1216 ± 142	
	+ Dydrogesterone (DYD)	+ 20-Dihydro-dydrogesterone (DHD)
5x10^{-9}	1279 ± 170	1114 ± 137
5x10^{-7}	1040 ± 139	846 ± 100*
5x10^{-5}	643 ± 84*	591 ± 58*

Preconfluent T-47D cells were incubated for 24h at 37˚C with estrone-sulfate ([^{3}H]E_1S: 5x10^{-9} mol/l) alone or in the presence of dydrogesterone or its metabolite 20-dihydro-dydrogesterone in the range of concentrations 5x10^{-9} to 5x10^{-5} mol/l. Estradiol (in fmol/mg DNA) was analyzed and quantified as indicated in Materials and Methods. The values are expressed as the mean±S.D. of 2 independent duplicate determinations.
*$p<0.05$ *versus* control values ([^{3}H]-E_1S alone).

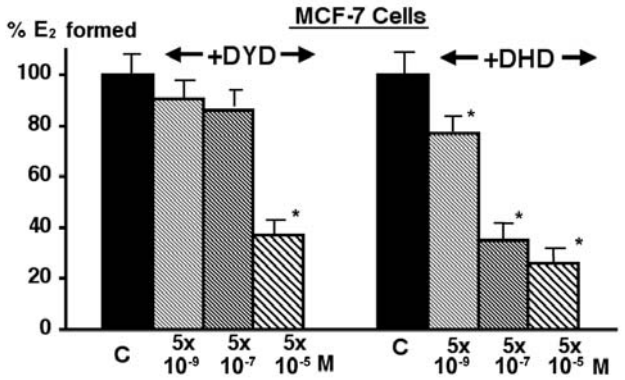

Figure 2. *Effects of dydrogesterone and its metabolite 20-dihydro-dydrogesterone on the conversion of estrone sulfate (E_1S) to estradiol (E_2) in the hormone-dependent MCF-7 human breast cancer cell lines.*
Preconfluent MCF-7 cells were incubated for 24h at 37˚C with a physiological concentration of estrone sulfate ([^{3}H]-E_1S: 5x10^{-9} mol/l) alone (control: non-treated cells) or in the presence of dydrogesterone (Duphaston®) or its 20-dihydro-metabolite at the range of concentrations: 5x10^{-9} mol/l to 5x10^{-5} mol/l. Estradiol was calculated after isolation of the hormones, as indicated in Materials and Methods. The percentage of the effect (in fmol E_2 formed/mg DNA from E_1S) was obtained by calculating the ratio: [(test - control) / control] x 100. The data are the mean ± SEM of duplicate determinations of 2 independent experiments.
**p≤0.05 vs control values*

Table III. *Effect of dydrogesterone and of its metabolite 20-Dihydro-dydrogesterone on the conversion of [^{3}H]-estrone to [^{3}H]-estradiol by the hormone-dependent T-47D breast cancer cell line.*

	Estrone (E_1)	Estradiol (E_2)		
	(fmol/mg DNA)			
[^{3}H]-E_1S alone (5 x10^{-9} mol/l)	2034 ± 151		2110 ± 134	
	+ Dydrogesterone (DYD)		+ 20-Dihydro-Dydrog. (DHD)	
	Estrone (E_1)	Estradiol (E_2)	Estrone (E_1)	Estradiol (E_2)
5x10^{-9}	2400 ± 128	1640 ± 105*	2110 ± 96	1428 ± 76*
5x10^{-7}	2860 ± 107	1400 ± 98*	2714 ± 125	1242 ± 104*
5x10^{-5}	2750 ± 146*	1102 ± 112*	2405 ± 82	975 ± 85*

Preconfluent T-47D cells were incubated for 24h at 37˚C with estrone ([^{3}H]E_1: 5x10^{-9} mol/l) alone or in the presence of dydrogesterone or its metabolite 20-dihydro-dydrogesterone, in the range of concentrations 5x10^{-9} to 5x10^{-5} mol/l. Estrone and Estradiol (in fmol/mg DNA) were analyzed and quantified as indicated in Materials and Methods. The values are expressed as the mean ± S.D. of 2 independent duplicate determinations.
*$p<0.05$ *versus* control values ([^{3}H]-E_1 alone).

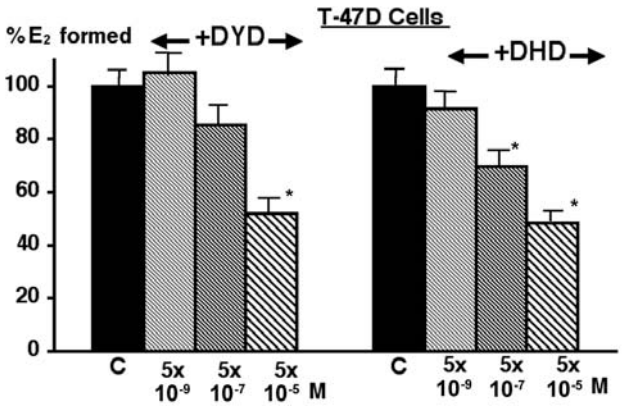

Figure 3. *Effects of dydrogesterone and its metabolite 20-dihydro-dydrogesterone on the conversion of estrone sulfate (E_1S) to estradiol (E_2) in the hormone-dependent T-47D human breast cancer cell lines.*
Preconfluent T-47D cells were incubated for 24h at 37˚C with a physiological concentration of estrone sulfate ([^{3}H]-E_1S: 5x10^{-9} mol/l) alone (control: non-treated cells) or in the presence of dydrogesterone (Duphaston®) or its 20-dihydro-metabolite at the range of concentrations: 5x10^{-9} mol/l to 5x10^{-5} mol/l. Estradiol was calculated after isolation of the hormones, as indicated in Materials and Methods. The percentage of the effect (in fmol E_2 formed/mg DNA from E_1S) was obtained by calculating the ratio: [(test - control) / control] x 100. The data are the mean ± SEM of duplicate determinations of 2 independent experiments.
**p≤0.05 vs control values*

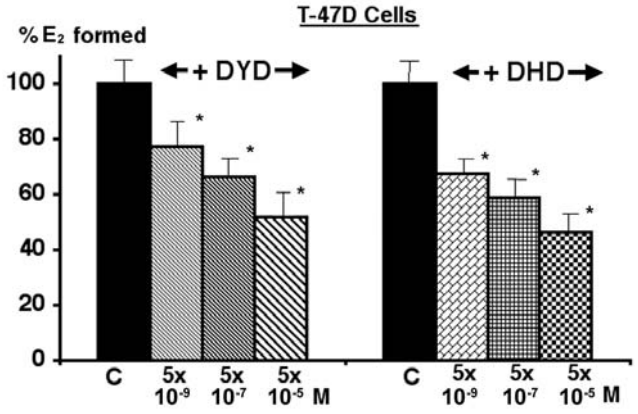

Figure 4. *Effects of dydrogesterone and its metabolite 20-dihydro-dydrogesterone on the conversion of estrone (E_1) to estradiol (E_2) in the hormone-dependent T-47D human breast cancer cell lines.*
Preconfluent T-47D cells were incubated for 24h at 37°C with a physiological concentration of estrone ([^{3}H]-E_1: 5x10^{-9} mol/l) alone (control: non-treated cells) or in the presence of dydrogesterone or its 20-dihydro-metabolite at the range of concentrations: 5x10^{-9} mol/l to 5x10^{-5} mol/l. Estrone and estradiol were calculated after isolation of the hormones, as indicated in Materials and Methods. The percentage of the effect (in fmol E_1 or E_2 formed/mg DNA) was obtained by calculating the ratio: [(test - control) / control] x 100. The data are the mean ± SEM of duplicate determinations of 2 independent experiments.
**$p \leq 0.05$ vs control values*

More recently, another endocrine therapy has been explored by inhibiting the tissular E_2 production using different anti-enzyme agents involved in the biosynthesis of this hormone. At present, the positive effect of anti-aromatase compounds in breast cancer patients is well documented (16, 17).

It is recognized that the 'sulfatase pathway' is essentially responsible for the intratissular E_2 production in hormone-responsive mammary carcinomas or in breast cancer models (9, 11). Estrone sulfatase activity is potentially relevant as it is the main factor controlling the production of unconjugated estrogens from the high concentrations of sulfoconjugated precursors which prevail in breast cancer tissues, particularly for postmenopausal patients (9).

The present data showed that the synthetic progestogen dydrogesterone and its 20-dihydro-metabolite can reduce the conversion of E_1S to E_2 in the hormone-dependent MCF-7 and T-47D breast cancer cells by blocking the 'sulfatase pathway'. In the MCF-7 cells, dihydro-dydrogesterone was significantly more active than its precursor, dydrogesterone. It is notable that the inhibition was observed with very low concentrations ($5x10^{-9}$M) of this metabolite. These data confirm that, in studies and treatment with different progestogens, it is more relevant to explore the biological activity of its metabolic products. Similarly, in the T-47D cells, the effect of the 20-dihydro-derivative was more active than dydrogesterone in blocking the 'sulfatase pathway'.

Concerning the 17β-HSD activity, the preferential conversion in breast tumor (*in vivo* and *in vitro* studies) is the reduction of E_1 to E_2. The 17β-HSD Type 1 is located in the cytoplasm of malignant epithelial cells of breast tumors (18). It was observed that the orientation of the enzymatic activity (oxidative or reductive) in breast cancer is also greatly dependent on the local, metabolic or experimental conditions, including: the nature and concentration of the cofactors (*e.g.* NADPH or NADP) and of the substrate, pH and subcellular localization of enzymes. *In vitro* studies using human tumor homogenates indicated that the predominant 17β-HSD activity was oxidative rather than reductive (7). However, *in vivo* studies, after isotopic infusion of estrogens to postmenopausal breast cancer patients, have shown that the reductive direction is greater than the oxidative (19). In hormone-dependent breast cancer cell lines (MCF-7, T-47D, R-27, ZR-75-1) 17β-HSD Type 1 was the predominant reductive isoform, but Type 2 and 4 isoforms with oxidative activities (formation of E_1) were also detected (18, 20-22).

In the present study, both dydrogesterone and its 20-dihydro-metabolite were able to block the reductive 17β-HSD activity in the T-47D breast cancer cells, where a significant effect was observed with low concentrations ($5x10^{-9}$M - $5x10^{-7}$M) of these progestogens.

On the basis of all this information, we can consider that these two progestogens; dydrogesterone and its 20-dihydro-metabolite, act as selective estrogen enzyme modulators (SEEMs).

The present data confirmed that in studies and treatment with different progestogens, it is more often relevant to explore the biological activity of the metabolic products. Similarly, in the T-47D cells, the effect of the 20-dihydro-derivative was more active than dydrogesterone in blocking the 'sulfatase pathway'.

In conclusion, dydrogesterone and its 20-dihydro-metabolite are potent inhibitors of sulfatase and 17β-HSD activity in breast cancer cells, resulting in decreased tissue concentrations of E_2. This data opens interesting perspectives to explore the action of these progestogens in clinical trials of patients with breast cancer.

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