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Recent insight on the control of enzymes involved in estrogen formation and transformation in human breast cancer

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Abstract

The great majority of breast cancers are in their early stage hormone-dependent and it is well accepted that estradiol (E_2) plays an important role in the genesis and evolution of this tumor. Human breast cancer tissues contain all the enzymes: estrone sulfatase, 17β-hydroxysteroid dehydrogenase, aromatase involved in the last steps of E_2 bioformation. Sulfotransferases which convert estrogens into the biologically inactive estrogen sulfates are also present in this tissue. Quantitative data show that the 'sulfatase pathway', which transforms estrogen sulfates into the bioactive unconjugated E_2, is 100–500 times higher than the 'aromatase pathway', which converts androgens into estrogens.

The treatment of breast cancer patients with anti-aromatases is largely developed with very positive results. However, the formation of E_2 via the 'sulfatase pathway' is very important in the breast cancer tissue. In recent years it was found that antiestrogens (e.g. tamoxifen, 4-hydroxytamoxifen), various progestins (e.g. promegestone, nomegestrol acetate, medrogestone, dydrogesterone, norelgestromin), tibolone and its metabolites, as well as other steroidal (e.g. sulfamates) and non-steroidal compounds, are potent sulfatase inhibitors. In another series of studies, it was found that E_2 itself has a strong anti-sulfatase action. This paradoxical effect of E_2 adds a new biological response of this hormone and could be related to estrogen replacement therapy in which it was observed to have either no effect or to decrease breast cancer mortality in postmenopausal women. Interesting information is that high expression of steroid sulfatase mRNA predicts a poor prognosis in patients with +ER. These progestins, as well as tibolone, can also block the conversion of estrone to estradiol by the inhibition of the 17β-hydroxysteroid dehydrogenase type I (17β-HSD-1). High expressison of 17β-HSD-1 can be an indicator of adverse prognosis in ER-positive patients.

It was shown that nomegestrol acetate, medrogestone, promegestone or tibolone, could stimulate the sulfotransferase activity for the local production of estrogen sulfates. This is an important point in the physiopathology of this disease, as it is well known that estrogen sulfates are biologically inactive. A possible correlation between this stimulatory effect on sulfotransferase activity and breast cancer cell proliferation is presented. In agreement with all this information, we have proposed the concept of selective estrogen enzyme modulators (SEEM).

In conclusion, the blockage in the formation of estradiol via sulfatase, or the stimulatory effect on sulfotransferase activity in combination with anti-aromatases can open interesting and new possibilities in clinical applications in breast cancer.

Keywords: Breast cancer; Estradiol; Estrogens; 17β-HSD; Progestins; Sulfatase; Sulfotransferase

1. Introduction

Most breast cancers (about 95%), whether in pre- or postmenopausal women, are initially hormone-dependent, where the hormone estradiol plays a crucial role in their development and progression [1–4]. The hormone and estrogen receptor complex can mediate the activation of proto-oncogenes and oncogenes (e.g. c-fos, c-myc), nuclear proteins, as well as other target genes. Consequently, processes that modulate the intracellular concentrations of active estrogens can have the ability to affect the etiology of this disease.

After a period that may last several years, the tumor becomes hormone-independent by a mechanism, which though not yet fully elucidated is under scrutiny. One explanation for the progression towards hormone-independence may be the presence of estrogen receptor mutants [5,6]. In hormone-dependent cells, the interaction of the hormone with the receptor molecule is the basic step for eliciting a hormone re-

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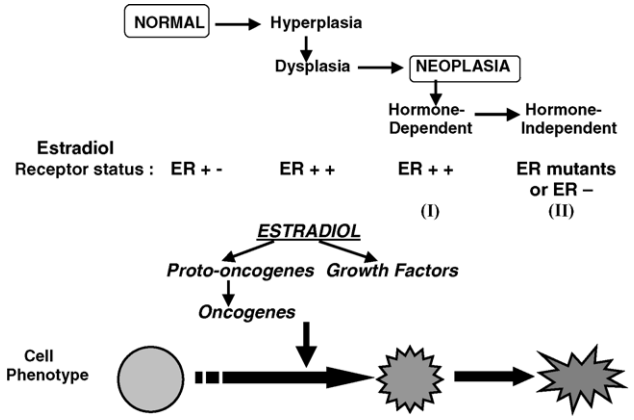

Fig. 1. Evolutive transformation of the breast cell from normal to carcinogen. Prognosis of the disease evolution is very good in the period when the breast cancer is hormone-dependent (I), but very poor when the cancer becomes hormone-independent (II). ER+, Estrogen receptor positive (detectable and functional); ER mutants, estrogen receptor detectable but non-functional; ER−, estrogen receptor negative (not detectable).

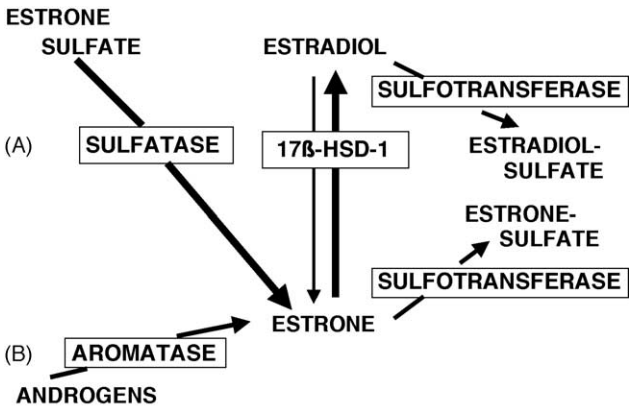

Fig. 2. Enzymatic mechanism involved in the formation and transformation of estrogens in human breast cancer. The sulfatase pathway (A) is quantitatively 100–500 times higher than that of the aromatase pathway (B). 17β-HSD-1 = 17β-hydroxysteroid dehydrogenase type 1.

sponse. As the cancer cell evolves, mutations, deletions and truncations appear in the receptor gene [7–9]; the estrogen receptor (ER) becomes 'non-functional' and, despite the estrogen binding, the cell fails to respond to the hormone. Fig. 1 describes the progression of normal mammary cells towards a hormone-independent carcinoma. A 'non-functional' estrogen receptor might explain why 35–40% of patients with ER-positive tumors fail to respond to antiestrogen therapy [10,11]. The remaining 5% of breast cancers, denoted BRCA-1, are considered hereditary. The gene was localized on Chromosome 17q21 [12,13] but its characterization and use as a marker are still a matter of great controversy (for a review see Ref. [14]).

The majority of breast cancers occur during the postmenopausal period when the ovaries have ceased to be functional. Despite the low levels of circulating estrogens, the tissular concentrations of estrone (E_1), estradiol (E_2) and their sulfates (E_1S, E_2S) are several times higher than those found in the plasma or in the area of the breast considered as normal tissue, suggesting a specific tumoral biosynthesis and accumulation of these hormones [15–19].

Several factors could be implicated in this process, including higher uptake of steroids from plasma and local formation of the potent E_2 by the breast cancer tissue itself. This infomation extends the concept of 'intracrinology' where a hormone can have its biological response in the same organ where it is produced.

In this review is summarized the recent information on the control of the enzymes involved in the formation and transformation of estrogens in breast cancer.

2. Steroid enzymes and breast cancer

There is substantial information that mammary cancer tissue contains all the enzymes responsible for the local biosynthesis of 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 [20–22] and the 'sulfatase pathway' which converts E_1S into E_1 by the estrone sulfatase (EC: 3.1.6.2) [23–27]. 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; EC: 1.1.1.62) [28–30]. Quantitative evaluation indicates that in human breast tumor E_1S 'via sulfatase' is a much more likely precursor for E_2 than is androgens 'via aromatase' [17,31].

It is also well established that steroid sulfotransferases (ST), which convert estrogens into their sulfates, arte also present in breast cancer tissues [32,33]. Fig. 2 gives a general view of estrogen formation and transformation in human breast cancer.

3. Hydroxylated metabolites of estrogens and breast cancer

There is extensive information that the metabolic product of natural or synthetic steroids can have a biological response or more than other compounds. Concerning estrogens, three hydroxylated metabolites in positions C2, C4 and C16 are of biological importance. Fig. 3 gives a general view of this transformation.

It is interesting to note that 2-methoxy estradiol can inhibit the proliferation of breast cancer cells [34,35]. As this anti-proliferative effect can be obtained in negative estrogen receptor estrogen receptor cell lines, it is suggested that the biological response of 2-methoxy estradiol is mediated by another pathway, that of the classical estrogen receptor. This assumption is confirmed by the fact that the binding affinity to ER is only 0.1% compared with estradiol [36]. Zhu and Conney [37] suggest that 2-methoxy estradiol can have an-

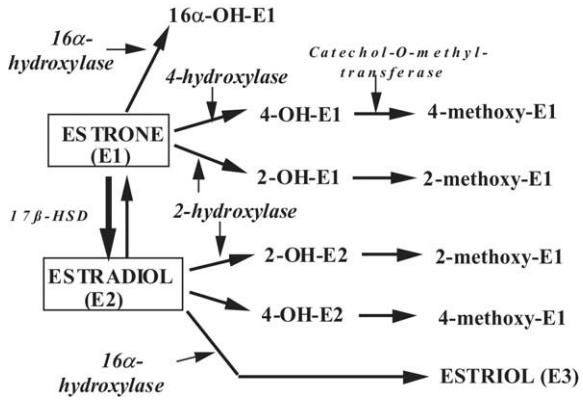

Fig. 3. Hydroxyl pathways of estrogens in human breast cancer. 2-OH-E1, 2-hydroxy-estrone; 2-OH-E2, 2-hydroxy-estradiol; 4-OH-E1, 4-hydroxy-estrone; 4-OH-E2, 4-hydroxy-estradiol; 16α-OH-E1, 16α-hydroxy-estrone.

titumorigenic and antiangiogenic effects, which can protect estrogen-induced cancer in target organs. It was demonstrated that the activity of 2-methoxy estradiol is mediated independently of estrogen receptor α or β [38].

In opposition to 2-hydroxy estrogens, 4-hydroxy estrone and 4-hydroxy estradiol possess estrogenic properties and exert a stimulatory effect on the growth of MCF-7 breast cancer cells [39,40]. Elevated 4-hydroxy enzyme activity was found in human breast cancer speciments [41]. Whether 4-hydroxy estrogens are involved in human carcinogenesis is still unclear, but it is interesting to note that high concentrations of 4-hydroxy estrogens are pressent in human breast tumors [42].

Also, 16α-hydroxy estrone has estrogenic activity which, based on the increased uterine weight of ovariectomized rats, is more potent than that of estradiol itself [43]. It was suggested that 16α-hydroxy estrone could be implicated in carcinogenesis, for instance a comparison of the E_2 metabolism of murine mammary epithelial cells revealed that 16α-hydroxylation was significantly elevated in high-risk animals [44,45]. In relation to the proliferative effect of 16α-hydroxy estrone, Lewis et al. [46] showed in MCF-7 breast cancer cells that this estrogen is capable of accelerating cell cycle kinetics and stimulating the expression of cell cycle regulatory proteins.

4. Estrone sulfatase activity

Estrone (or steroid) sulfatase has been implicated in the regulation of various physiological and pathophysiological processes in normal and malignant tissues, including during pregnancy, prodrug processing, cell-signaling pathways, neurotransmission and memorization, immune response, inherited skin disorder (e.g. X-linked ichthyosis) and hormone-dependent cancers (e.g. breast, endometrial and prostate cancers).

Human estrone (steroid) sulfatase is a member of an evolutionarily conserved protein family in a group of hydrolytic enzymes (human arylsulfatases family; at least six members: class A, B, C, D, E and F) and is ubiquitously expressed in mammalian tissues and target organs (e.g. liver, endometrium, ovaries, bone, brain, prostate, white blood cells, adipocytes, . . .), but is particularly prevalent in the placenta and in breast carcinoma tissue. Estrone (steroid) sulfatase corresponds to arylsulfatase C.

4.1. Structure of estrone (steroid) sulfatase

The steroid sulfatase gene has been mapped to the distal part of the short arm of the X chromosome (Xq22.3-qter), a region that escapes X inactivation, containing 10 exons over 146 kb pairs [47]. The encoded protein has a MW of 62 kDa [48]. Estrone (steroid) sulfatase is a single membrane-bound enzyme with a range of substrate specificities, made up of 587 amino acids and located in the endoplasmic reticulum.

The N- and C-terminal domains of the enzyme are located on the luminal side of the membrane and each contains a glycosylation site. The two domains are connected by a membrane spanning the hydrophobe region. It appears that the N-terminal domain, which shows the greatest degree of constant amino acid in the sulfatase family, contains the active site, while the less conserved C-terminal region contains the substrate-binding site [49]. The structure of steroid sulfatase, purified from the microsomal fraction of human placenta, has been determined at 2.60-A resolution by X-ray crystallography [50]. It appears that the enzyme possesses a «mushroom-like» shape, and a possible role of the lipid bilayer in catalysis is suggested. The catalytic active site for sulfate hydrolysis contains a conserved cystein residue that undergoes post-translational modification to *N*-formylglycine [51,52].

Recently, Zhu et al. [53] have shown the presence in adult female rat liver of a nuclear estrone (steroid) sulfatase isozyme with higher affinity for E_1S than microsomal isozyme. The data indicate the presence of two different isozymes.

4.2. Estrone sulfatase in breast cancer

At present two major endocrine therapies are available for the treatment of estrogen-dependent breast cancer. One using antiestrogens as selective estrogen receptor modulators (SERM) such as tamoxifen (Nolvadex®: tamoxifen citrate), droloxifene, raloxifene or fulvestrant, a new type of ER antagonist which down-regulates the ER. More recently, another approach used anti-enzyme agents, as selective estrogen enzyme modulators (SEEM), for reducing local biosynthesis and production of estrogens, such as aromatase inhibitors (anastrozole, letrozole or exemestane). Although these well-defined second-line and even first-line therapeutic treatments provide significant clinical benefits in advanced or early breast cancers, some hormone-dependent breast tu-

mors became resistant or/and fail to respond to SERM or anti-aromatase compounds. However, these anti-aromatases do not discriminate selective inhibition between peripheral and intratumoral aromatase activity. Moreover, various reports demonstrate that the reduction of the plasmatic estrogen concentration is relatively low (e.g. 50% for estrone) whereas the concentration of E_1S remains relatively high (400–1000 pg/ml) [54,55]. The fact that a third new generation of more potent and specific aromatase inhibitors does not significantly improve the clinical response suggests that the growth of breast cancer is influenced by locally synthesized estrogens. This information led to the exploration of the other source of estradiol formation in breast cancer tissue: the «sulfatase pathway».

Desulfation of inactive steroid-3-*O*-sulfates (e.g. estrone sulfate and dehydroepiandrosterone sulfate) by estrone (steroid) sulfatase plays a key role in the regulation of receptor-active estrogenic steroid levels (estradiol and androstenediol) in breast tumor cells. The importance of steroid sulfatase activity in breast cancer has emerged from the fact that circulating estrone sulfate can act as a reservoir for the local formation of free biologically active estrogens by an intracrine mechanism, particularly after the menopause when the incidence of breast cancer is higher (more than two-thirds of breast cancers). Steroid sulfatase associated with estrone sulfotransferase assumes the dynamic steady-state equilibrium of E_1S in breast cancer tissues.

Recent studies indicate that estrogen sulfates and estrone (steroid) sulfatase are important in breast cancer because of the following considerations:

(1) Estrone sulfate, the main substrate for estrone sulfatase is the most abundant circulating estrogen in the plasma of postmenopausal women [17,56].
(2) As estrogen sulfates do not bind to estrogen receptor (ER), only estrogen-3-sulfate is hydrolyzed by estrone (steroid) sulfatase to elicit a biological response, whereas estrogen-17-sulfates, which are not hydrolyzed by sulfatase, have no biological response [25,57].
(3) The half-life of E_1S (10–12 h) is considerably longer than that of estrone (20–30 min).
(4) The levels of estradiol and estrone sulfate are 7–11 times higher in breast tumoral tissue than in plasma of postmenopausal women [17].
(5) The concentration of estrone sulfate in the tumor is higher in postmenopausal than in premenopausal breast cancer patients [17].
(6) The tissular concentration of estrone sulfate is higher in tumoral than in normal breast tissue [18].
(7) The estrone sulfatase activity is very intense in malignant breast tumors compared to the normal breast tissue [18,58,59].
(8) Quantitative determinations indicate that estrone sulfatase activity is 40–500 times higher than that of aromatase in breast tumor [17,31].
(9) Estrone (steroid) sulfatase mRNA expression is well correlated with disease-free survival and is a significant, independent prognostic factor in human breast cancer [60–62].
(10) The addition of estradiol sulfate supports the growth of estrogen-dependent tumors in an in vivo model using ovariectomized nude mice bearing steroid sulfatase-transduced human breast cancer cells [63].

These studies show that estrone (steroid) sulfatase could be implicated in the growth of hormone-dependent breast cancer, and could be an important target for hormonal therapy in addition to aromatase inhibitors, thus increasing therapeutic interest in discovering how to control and block this enzyme.

5. Control of estrone (steroid) sulfatase activity

In recent years, the possibility was investigated of an inhibitory effect of a great number of compounds including: antiestrogens, progestins, tibolone and its metabolites, as well as steroidal and non-steroidal compounds. Attempts to discover structurally important features in sulfatase enzyme-inhibitor interaction have enabled the identification of the more potent, selective, metabolically stable and less estrogenic agents.

5.1. Inhibition by antiestrogens

The antiestrogens tamoxifen and its more active metabolite, 4-OH tamoxifen as well as ICI 164,384 have been reported to be inhibitors of sulfatase activity, probably by a non-competitve mechanism in hormone-dependent breast cancer cells and homogenates of breast tumor tissues [64–66]. Chu et al. [67] found that in the rat liver (*E*)- and (*Z*)-4-hydroxytamoxifen sulfamates are also sulfatase inhibitors with a K_i of 35.9 μM and >500 μM, respectively.

5.2. Inhibition by progestins

Various marketed progestins used as oral contraceptives, in estrogen-related disorders or in palliative therapy of breast and endometrial carcinoma have interesting inhibitory effects on the sulfatase activity.

Progesterone derivatives (e.g. medrogestone), retroprogesterone derivatives (e.g. dydrogesterone), 19-nortestosterone derivatives (e.g. norethisterone, norelgestromin), 17α-hydroxy-progesterone derivatives (e.g. medroxyprogesterone acetate), 17α-hydroxy-nor-progesterone derivatives (e.g. nomegestrol acetate), 19-nor-progesterone derivatives (e.g. promegestone) provoke a significant decrease of estradiol formation when physiological concentrations of estrone sulfate are incubated with breast cancer cells (MCF-7 and T-47D) [68–74]. Fig. 4 provides a comparative study of the inhibitory effect of different

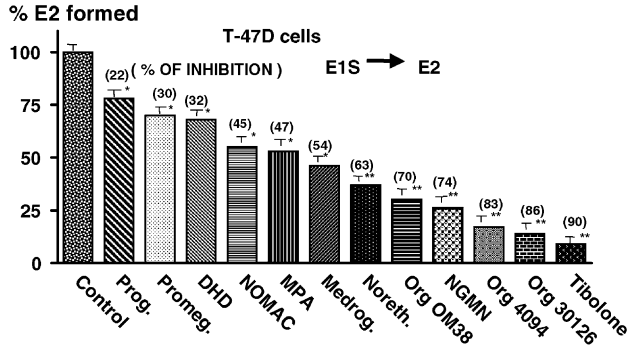

Fig. 4. Comparative effects of various progestins on the inhibition of the estrone sulfate (E_1S) conversion to estradiol (E_2) in the hormone-dependent T-47D human breast cancer cell line. Preconfluent cells were incubated 24 h at 37 °C with a physiological concentration (5×10^{-9} mol/l) of [^{3}H]-estrone sulfate (E_1S) alone or in the presence of progestins at the concentration of 5×10^{-7} mol/l. Results (pmol of E_2 formed/mg DNA from E_1S) are expressed in percent (%) of control value considered as 100%. The data represent the mean ± S.E.M. of duplicate determinations of 3–7 independent experiments. Prog., progesterone; Promeg., promegestone; DHD, 20-dihydro-dydrogesterone; NOMAC, nomegestrol acetate; MPA, medroxyprogesterone acetate; Medrog., medrogestone; Noreth., norethisterone; Org OM38, 4-en isomer of Tibolone; NGMN, norelgestromin; Org 4094, 3α-OH metabolite of tibolone; Org 30126, 3β-OH metabolite of tibolone; Tibolone, Org OD14; *$P \leq 0.05$ vs. control value; **$P \leq 0.01$ vs. control value.

progestins on the conversion of E_1S to E_2 in the T-47D hormone-dependent breast cancer cells.

5.3. Inhibition by tibolone and its metabolites

Tibolone (Org OD14, active substance of Livial®) is a 19-nortestosterone derivative with weak estrogenic, progestagenic or androgenic properties. This compound has a tissue-specific action and is used in hormone replacement therapy to treat menopausal complaints and prevention of osteoporosis, without stimulating the endometrium. Tibolone and its more active metabolites Org 4094, Org 30,126 (3α- and 3β-hydroxy derivatives) and its 4-en isomer (Org OM-38) are potent sulfatase inhibitors at low concentrations (5×10^{-7} M) in MCF-7 and T-47D hormone-dependent breast cancer cells [75].

5.4. Inhibition by steroidal compounds

Estrone (steroid) sulfatase is competitively inhibited by a number of natural steroids and steroid sulfates [26,76,77]. Carlstrom et al. [78] show a significant decrease in the concentration of circulating steroids as a consequence of the therapeutic application of danazol; an anabolic steroid with a blocking isoxazol group at position 2,3 of the steroidal skeleton. This compound is also active in breast cancer cells [79].

Estrone-3-*O*-sulfamate (EMATE) is a potent synthesizised sulfatase inhibitor, as at the concentration of 10^{-7} M the inhibition of estrone sulfatase is 99% in MCF-7 cells [80,81]. This inhibition is described as time- and concentration-dependent and is classified as an active-site directed irreversible inhibitor [82]. Unfortunately, the potent estrogenic activity of this compound precludes its use in clinical application [83]. However, a new 2-substituted analogue of EMATE: 2-difluoromethylestrone 3-*O*-sulfamate has an IC_{50} of 100 pM and is 90-times more potent sulfatase inhibitor than EMATE in placental microsomal preparations [84].

In other studies, Boivin et al. [85] and Poirier and Boivin [86] attempted to develop sulfatase inhibitors without residue estrogenic activity by synthesizing a series of E_2 derivatives bearing an alkyl, a phenyl, a benzyl substituted or not, or an alkyan amide side chain at position 17α. These authors showed that sulfatase inhibitors act by a reversible mechanism and that the hydrophobic group at the 17α position increased the inhibitory activity, while steric factors contributed to the opposite effect. The most potent inhibitor is a 17α-benzyl substituted E_2 derivative with an IC_{50} value of 22 nM. When these 17α-substituents were added to the 3-*O*-sulfamate estradiol structures, the combined inhibitory effect was more potent. The IC_{50} value is 0.15 nM [87]. Recently, a more potent derivative has been synthesized: 3β-sulfamoyloxy-17α-t-butylbenzyl-5-androsten-17β-ol, showing non-estrogenic and non-androgenic activity [88].

New irreversible inhibitor compounds described an estrone formate type [89] or steroidal 2′,3′-oxathiazine structures, able to inhibit the growth of MCF-7 cells induced by estrone sulfate [90].

5.5. Inhibition by non-steroidal compounds

Anderson et al. [91,92] showed that the basic structure for the binding of inhibitors does not include the steroid nucleus. These authors determine that the non-steroidal phosphate compound, *n*-lauroyl tyramine phosphate is a good inhibitor and suggested that sulfatase can differentiate the phosphoryl group from the sulfuryl group with respect to catalysis only and not to binding.

A new interesting family of compounds has been synthesized with a tricyclic coumarin sulfamate structure [93–96]. These non-steroidal sulfatase inhibitors are active in vitro and in vivo, are non-estrogenic and possess, in vitro, an IC_{50} value of approximately 1 nM. However, the most potent inhibitor in vivo does not correspond to the better compound in vitro. Recently, one such inhibitor, 667 COUMATE, has now entered a Phase I trial in postmenopausal women with breast cancer.

Compounds based upon cyclic esters of 4-[(aminosulfonyl)-oxy]-benzoate are also potent sulfatase inhibitors. The effecs are stronger than COUMATE (4-methylcoumarin-7-*O*-sulfamate), 667-COUMATE (its tricyclic derivative) and EMATE [97,98].

In another study, Billich et al. [99] propose a new class of non-steroidal irreversible inhibitors with substituted chromenone sulfamates. These compounds are exempt of estrogenic activity and can block E_1S and DHEAS-stimulated growth of MCF-7 cells. Recently, new potent reversible sulfatase inhibitors of this type of compound: 2-

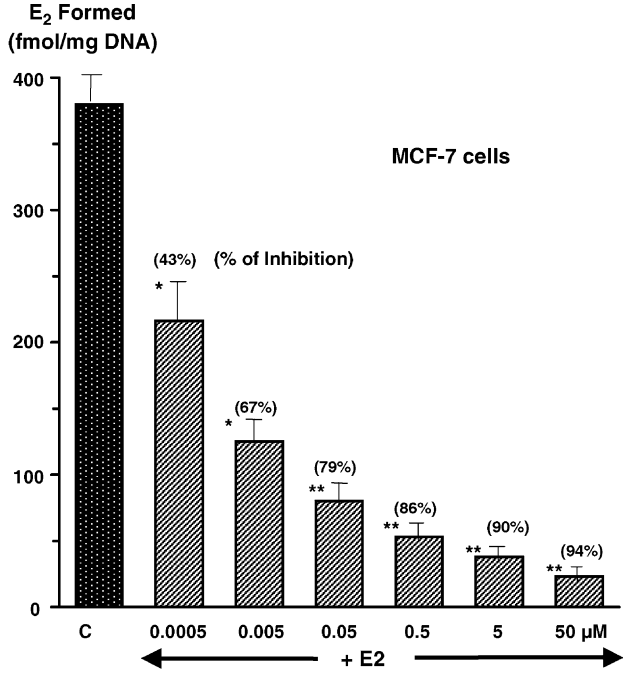

Fig. 5. Effect of estradiol (E_2) on the conversion of estrone sulfate (E_1S) to E_2 in the MCF-7 human breast cancer cells. The percentage of inhibition was obtained by calculating the ratio [(control − test)/control] × 100. The values are the mean ± S.E.M. of duplicate determinations of five independent experiments. *$P \leq 0.05$ vs. control value; **$P \leq 0.005$ vs. control value (quoted from Ref., [108]).

(1-adamantyl)-4-(thio)chromenone-6-carboxylic acids and 6-[2-(adamantylidene)-hydroxybenzoxazole]-*O*-sulfamate, were reported by these authors [100,101].

New non-steroidal compounds, corresponding to a 1-(*p*-sulfamoyloxyphenyl)-5-(*p*-*t*-butylbenzyl)-5 alkanols series have been proposed by Ciobanu et al. [102]. The best inhibitors are the undecanol derivatives in the sulfamates series (IC_{50} value, 0.4 nM).

Interesting new class of sulfatase inhibitors: sulfamoyloxy-substitued 2-phenylindoles and sulfamoyloxy-substitued stilbenes, show a dual mode of action as they block gene expression by inhibition of estrone sulfatase and by antiestrogenic action [103,104].

Recently, two new potent classes of non-steroidal steroid sulfatase inhibitors, without estrogenicity, have been reported: BENZOMATE (benzophenone-4,4′-*O*,*O*-bis-sulfamate) and related analogues [105], and some biphenyl-4-*O*-sulfamate derivatives (e.g. 2′,4′-dicyanobiphenyl-4-*O*-sulfamate) [106,107].

5.6. Effect of estradiol on the sulfatase activity in breast cancer cells

In a series of studies, a paradoxical effect of estradiol was demonstrated in MCF-7 and T-47D breast cancer cells in that it can block its own bioformation by inhibiting, in a dose-dependent manner, the conversion of E_1S to E_2 in the range of concentrations from 5×10^{-10} to 5×10^{-5} M (Fig. 5). Estradiol is a potent inhibitory agent of the estrone sulfatase activity as the IC_{50} values are 1.84×10^{-9} and 8.77×10^{-10} M in T-47D and MCF-7 cells, respectively [108]. Similarly, exposure to E_2 (10^{-8} M) was associated with 70% reduction in estrone sulfatase activity in the MCF-7 cells after 6 days [109]. Fig. 6 shows the hypothetical effect of estradiol as an anti-sulfatase agent during breast carcinogenesis.

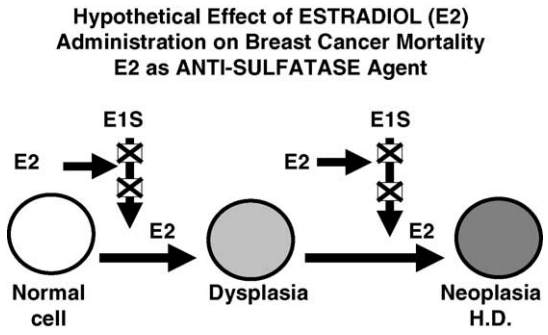

Fig. 6. Hypothetical effect of estradiol (E_2) as an anti-sulfatase agent during breast carcinogenesis.

6. Control of estrone sulfatase in the total breast cancer tissue

Using total breast cancer tissues from postmenopausal patients, it was demonstrated that nomegestrol acetate [110] or medrogestone could block the sulfatase activity. The effect was significantly more active in breast carcinoma than in the area of the breast considered as normal (Table 1).

7. Correlation of sulfatase activity and proliferation in breast cancer

Recent information has established that the growth and evolution of breast cancer in postmenopausal patients is due to the local formation of estrogens in the breast tumoral tissue in which the estrogen level is significantly higher than in the circulating estrogens in plasma. As estrogen sulfatase is one of the major routes in the biosynthesis of estradiol in the tissue itself, it was consequently of interest to explore a possible correlation between the effect of the tumor growth by anti-sulfatase agents. Nakata et al. [111] show that the anti-sulfatase compound 9: [(*p*-*O*-sulfamoyl)-*N*-tetradecanoyl tyramine] has no estrogenic activity and can block the tumor growth in female nude mice obtained by transplantation with MCS-2 cells, a human breast cancer cell over-expressed with sulfatase (Fig. 7).

8. Expression and control of estrogen sulfatase mRNA

In breast cancer cells, it was observed that the expression of mRNA sulfatase was correlated with the sulfatase activity

Table 1
Effect of nomegestrol acetate (NOMAC) on sulfatase activity

	Estrone sulfate (E_1S) (in pg/mg tissue)	Estrone (E_1) (in pg/mg tissue)	Estradiol (E_2)	R, (E_1/E_1S)	Percent of inhibition
(A) Normal breast tissues					
Control					
[^{3}H]-$E_1S = 5 \times 10^{-9}$ M	34.2 ± 3.1	27.2 ± 2.5	ND	0.80	
+ NOMAC					
5×10^{-9} M	35.6 ± 2.5	24.8 ± 2.4	ND	0.70	8.9
5×10^{-7} M	34.4 ± 2.1	21.0 ± 2.6*	ND	0.53	22.8
5×10^{-5} M	47.2 ± 2.8	16.1 ± 1.8*	ND	0.34	40.8
(B) Tumoral breast tissues					
Control					
[^{3}H]-$E_1S = 5 \times 10^{-9}$ M	13.4 ± 2.7	42.5 ± 3.4	ND	3.18	
+ NOMAC					
5×10^{-9} M	15.1 ± 2.4	36.8 ± 3.1	ND	2.44	13.4
5×10^{-7} M	28.4 ± 3.1	28.7 ± 2.4*	ND	1.01	32.5
5×10^{-5} M	37.2 ± 2.8	21.1 ± 3.8*	ND	0.58	50.3

^{3}[H]-E_1S, 5×10^{-9} mol/l, was incubated with slices of normal or tumoral breast tissues for 4 h at 37 °C in the absence (control) or presence of nomegestrol acetate (NOMAC) in the range of 5×10^{-9} to 5×10^{-5} mol/l. The data represent the average ± S.E.M. of three independent duplicate determinations. R, ratio concentration of E_1 to E_1S in normal or tumoral breast tissues; ND, not detectable.

* $p \leq 0.05$ vs. E_1 control value; quoted from Ref., [110].

[112], but little is known about the factors regulating steroid sulfatase gene expression in humans. However, it was demonstrated that the progestin promegestone (R-5020), at concentrations of 5 and 50 μmol/l, could inhibit the expression of estrone sulfatase mRNA levels in the MCF-7 and T-47D hormone-dependent breast cancer cell lines by 25 and 50%, respectively [112,113]. This inhibition is correlated with the reduction of the enzymatic activity [114].

Newman et al. [115] observed no effect of the cytokine tumor necrosis factor-α (TNF-α) or of interleukin-6 (IL-6) on mRNA sulfatase expression in MCF-7 cells. However, TNF-α and IL-6 increased steroid sulfatase activity in transfected MCF-7 cells with a sulfatase cDNA lacking promoter and enhancer elements. These results suggest that TNF-α and IL-6 may increase sulfatase activity via post-translational modification of the enzyme or by increasing substrate availability.

Miyoshi et al. [62] suggest that the sulfatase mRNA levels can serve as a significant, independent prognostic factor in ER-positive breast cancer. The authors considered that high levels of the mRNA sulfatase are associated with a poor prognosis.

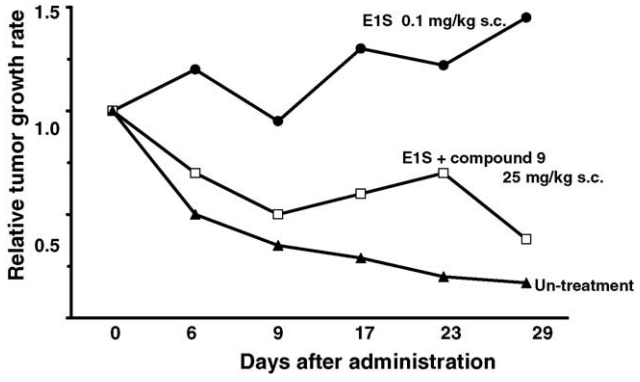

Fig. 7. Anti-tumor activity of sulfatase inhibitor (compound 9) against sulfatase overexpressed human breast cancer MCS-2 cells transplanted in female nude mice. Sulfatase inhibitor compound 9 correspond to [(*p*-*O*-sulfamoyl)-*N*-tetradecanoyl tyramine] structure (quoted from Ref., [111]).

9. Sulfotransferases in normal and carcinomous breast tissues

Human estrogen sulfotransferases (hEST) (*SULT 1E1* or *STE* gene) responsible for the conversion of estrogens to the biologically inactive estrogen sulfates involve two isoforms: the hEST-1, which is expressed in various breast cancer cells (e.g. MCF-7, ZR-75-1, T-47D) and is efficient in catalyzing the sulfation of 2-hydroxy-estrone and 2-hydroxy-estradiol, and the hEST-2, which selectively catalyzes sulfonation of estradiol, estrone, and ethinyl estradiol [33,116,117]. Sulfotransferases in normal and carcinomous breast is extensively demonstrated [118–120].

9.1. In the normal breast

Very high levels of estrone sulfotransferase (EST) were observed in a "normal" breast cell line produced by a Simian Virus (SV) 40, immortalization of breast epithelial cells obtained from reduction mammoplasty (Huma-7) [121]. The EST activity in this cell line far exceeded that in either MCF-7 or ZR-75-1 breast cancer cells. In the normal cell after 24 h culture, 50% of the substrate was sulfated compared with less than 10% in the malignant cells. This study was confirmed by Anderson and Howell [122] using two normal breast epithelial cells: the MTSV 1-7 and the MRSV 4-4 produced by SV 40 immortalization cells obtained from human milk [123].

Among the different human STs, only hEST has the affinity for estradiol sulfation in the namomolar concentration range. Consequently, hEST may be active in altering the lev-

els of unconjugated estrogens in the cell, and thus cellular responsiveness to estrogens, as estrogens in the nanomolar concentration range interact with the estrogen receptor.

When human mammary epithelial cells (HME) are established initially, they are estrogen-dependent [124]. Studies using immunohistochemical ER, a method more sensitive than the classical biochemical receptor assays, confirm the presence of ER in HME cells [125]. Estrogen-dependent cells with high EST levels grow more slowly than cells with lower levels of EST or no detectable EST. Metabolic evidence indicates that this is due to the ability of EST to render estrogens physiologically inactive via sulfate conjugation [33,126].

9.2. In breast cancer

There are discrepancies concerning sulfotransferase activities in breast cancer. Some authors found only PST (SULT 1A1) or HST (SULT 2A1) activity, but not EST, in the hormone-dependent breast cancer cells, as well as in the hormone-independent BT-20 cells and detected no ST activity in MDA-MB-231 or MDA-MB-468 cells, whereas other authors report EST and HST activity in MCF-7 and ZR-75 cells and in mammary tumours. These variations are probably caused by different factors, including cell origin, culture conditions, instability of human EST enzyme, and the condition of the enzyme assays.

Falany and Falany [33] considered that human estrogen sulfotransferase is not detectable in most breast cancer cell lines and suggested that the sulfoconjugated activity in the cells is mainly due to the human Phenol-PST, an enzyme that has a higher affinity with the estrogens at micromolar than at nanomolar concentrations. hP-PST has an affinity for estrogen sulfation about 300-fold lower than that of hEST [127,128].

To explore the difference in EST content between normal human mammary epithelial and breast cancer cells, and their correlation with cellular growth, Falany and Falany [33,129] transformed MCF-7 cells with an EST expression vector, and observed that after incubation of 20 nM of E_2, sulfation occurs more rapidly with MCF-7 cells transformed with EST than with the control cells, thereby rendering E_2 physiologically inactive. EST/MCF-7 cells require a higher concentration of E_2 to stimulate growth than do control MCF-7 cells, as EST inactivates E_2 via sulfatation, consequently rendering it incapable of binding to the estrogen receptor and inhibiting the process of cell growth.

10. Control of sulfotransferase activities in breast cancer

Comparative studies on the formation of estrogen sulfates after incubation of estrone with the hormone-dependent (MCF-7, T47D) and hormone-independent (MDA-MB-231) breast cancer cells show significantly higher sulfotransferases in the former [130,131].

10.1. Effect of various progestins

Medrogestone is a synthetic pregnane derivative used in the treatment of pathological deficiency of the natural progesterone. This compound produces secretory activity in the estrogen-primed uterus, is thermogenic and acts as an antiestrogen and antigonadotropin. Concerning the effect of medrogestone on sulfotransferase activity in MCF-7 and T47-D breast cancer cells it was observed that this progestin has a bi-phasic effect: at a low concentration (5×10^{-8} mol/l) it stimulates the formation of estrogen sulfates in both cells lines, whereas at a high concentration (5×10^{-5} mol/l) the sulfotransferase activity is not modified in MCF-7 cells or is inhibited in T-47D cells [132]. A similar dual effect on sulfotransferase was obtained with the progestins: nomegestrol acetate and promegestone (R-5020) [131,133] (Fig. 8).

10.2. Effect of tibolone and its metabolites

Tibolone is a 19-nortestosterone derivative with estrogenic, androgenic and progestagenic properties used to prevent climacteric symptoms and postmenopausal bone loss [134,135].

In a series of studies, the effects on sulfotransferase activity of tibolone and its metabolites: 3α-hydroxy (Org 4094), 3β-hydroxy (Org 30126) and the 4-ene isomer (Org OM-38) were explored in MCF-7 and T-47D breast cancer cells. These compounds also provoke a dual effect on sulfotransferase activity: stimulatory at low doses (5×10^{-8} mol/l) whereas an inhibition of this activity is observed at higher doses (5×10^{-5} mol/l). It is to be remarked that the 3β-hydroxy

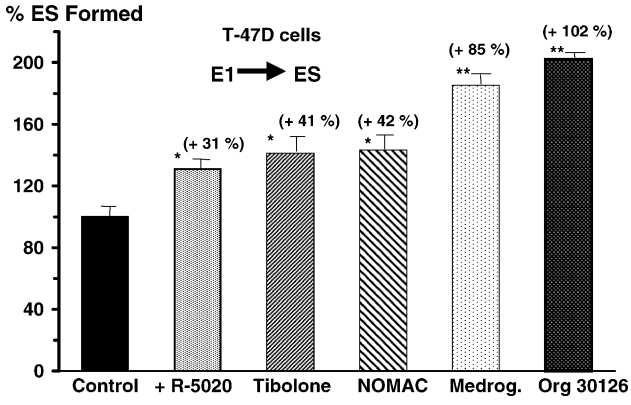

Fig. 8. Comparative effects of various compounds on the conversion of estrone (E_1) to estrogen sulfates (ES) in the hormone-dependent T-47D human breast cancer cell line. Preconfluent cells were incubated 24 h at 37 °C with 5×10^{-9} mol/l of [^{3}H]-E_1 alone (control; non-treated cells) or in the presence of various compounds at the concentration of 5×10^{-8} mol/l. Results (pmol of ES formed in culture medium per mg DNA) are expressed in percent (%) of control value considered as 100%. The data represent the mean ± S.E.M. of duplicate determinations of 3–6 independent experiments. R-5020: promegestone; Tibolone, Org OD14; NOMAC., nomegestrol acetate; Medrog., medrogestone; Org 30126, 3β-OH metabolite of tibolone; *$P \leq 0.05$ vs. control value; **$P \leq 0.01$ vs. control value.

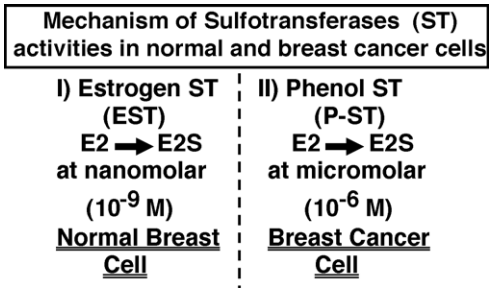

Fig. 9. Mechanism of sulfotransferase (ST) activity in normal and breast cancer cells. In normal breast cancer cells it is suggested that the action of hEST works at physiological (nanomolar) concentrations of estradiol to form estradiol sulfate, which is biologically inactive. This enzyme is absent from breast cancer cells where the phenol-ST activity acts only at micromolar (non-physiological) concentrations.

derivative is the most potent compound in the stimulatory effect of ST [130] (Fig. 8).

11. A possible correlation of the sulfotransferase activity and breast proliferation in the normal and carcinomous breast tissue

Maximal epithelial mitosis of the normal breast cell is found between 22 and 26 days of the cycle, which corresponds to the high levels of estradiol and progesterone [136]. During pregnancy, it is suggested that the elevated values of circulating progesterone are responsible for the induction of lobular–alveolar development, to prepare the breast for lactation [137,138]. The data on the effect of progesterone on breast epithelial proliferation are contradictory. It has been found that progesterone can increase DNA synthesis in normal breast epithelium in organ culture [139].

Using normal epithelial cells of human breast, it was demonstrated that the progestin promegestone could decrease cell proliferation [140,141]. These authors also found that progestins can inhibit the proliferative effect provoked by estradiol, whereas McManus and Welsch [142] and Longman and Buehring [143] demonstrated no effect.

The proliferative effect of progestins using various isolated breast cancer models: cell lines, organ culture, or transplantation of breast cancer cells in nude mice, is contradictory as it was reported that these compound can either inhibit [144–147], stimulate [148–150], or have no effect [151].

The data of Falany et al. [33,128] shows that hEST is present in the normal breast cells and is effective at nanomolar concentrations. The tissular estradiol is converted to estradiol sulfate and consequently proliferation can be blocked, as this conjugated is biologically inactive. However, in the breast cancer cells is present the phenol sulfotransferase (P-ST) which is active at micromolar concentrations of E_2 (Figs. 9 and 10) and the hEST is not present [33,125,128]. As the progestins nomegestrol acetate or medrogestone can stimulate hEST in breast cancer cells, and as these compounds can block the proliferation in breast cancer cells, it is suggested that the anti-proliferative effect of nomegestrol acetate or medrogestone is correlated with the stimulatory effect of human estrogen sulfotransferase in the hormone-dependent breast cancer cells (Fig. 11). More information on the correlation of the proliferative effect and hEST on breast cancer cells of various progestins or other substances are needed to verify this hypothesis.

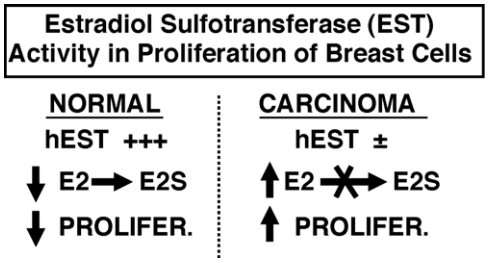

Fig. 10. Effects of estradiol sulfotransferase (EST) activity on the proliferation of breast cancer cells. In the normal breast cancer cells, as a consequence of the hEST activity, the proliferation is inhibited, as estradiol sulfate (E_2S) is biologically inactive. In opposition to breast cancer cells, hEST activity is very low or inexistent as E_2S is not formed and E_2 can stimulate proliferation.

12. 17β-Hydroxysteroid dehydrogenase and its control in breast cancer

17β-Hydroxysteroid dehydrogenase (17β-HSD) is a widely distributed enzyme in mammalian tissues, which is implicated in the interconversion of the inactive 17β-keto-<–> into active 17β-hydroxy in sex steroid hormones (estrogens and androgens). However, some types of 17β-HSD may metabolize further substrates such as bile acids, alcohols, fatty acids and retinols. 17β-HSD belongs to a superfamily of enzymes (to date up to 11 different isoforms are recognized).

The last step of biosynthesis of the potent biologically active estrogen, estradiol, in target tissues is the conversion of estrone to estradiol by the reductive 17β-HSD-1 activity.

In breast tumors, in vivo and in vitro studies indicate that the preferential conversion is the reduction of E_1 to E_2. The 17β-HSD type 1 is located in the cytoplasm of malignant ep-

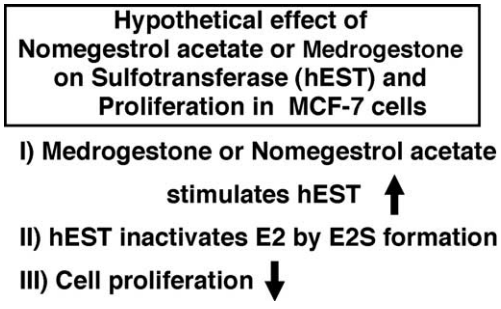

Fig. 11. Hypothetical effects of nomegestrol acetate or medrogestone on human sulfotransferase (hEST) and proliferation in T-47D and MCF-7 breast cancer cells. As nomegestrol acetate or medrogestone can stimulate hEST in the cancer cell, the effect of estradiol becomes inactive by the formation of estradiol sulfate and consequently cell proliferation is inhibited.

ithelial cells of breast tumors [152]. However, 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 substrate, pH, subcellular localization of enzymes. In vitro studies using human tumor homogenates indicated that the predominant 17β-HSD activity was oxidative rather than reductive [28]. 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 [29].

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 [30,152–154]. It was demonstrated that in intact cells, when the physiological conditions are more closely protected, the catalytic activity of each type of 17β-HSD is exclusively uni-directional, whereas in cell homogenates the bidirectional orientation prevails, but the physiological direction is favoured [155,156].

In contrast, when breast cancer cells evolve to a hormone-independent status (MDA-MB-231; MDA-MB-436; Hs-578S) they revert to the oxidative (E_2 to E_1) 17β-HSD activity as their preferential enzymatic orientation [30]. This observation suggests that there is a change in 17β-HSD phenotype in neoplastic cells and that the tumoral process of the breast is accompanied by a modification of estrogen metabolism [157].

12.1. Control by progestins and by tibolone and its metabolites

Breast tumors from postmenopausal patients receiving lynestrenol display higher oxidative 17β-HSD activity than tumors from untreated patients. The activity depends on the ER or PR status of the tumor [158].

Progestins can induce 17β-HSD type 1 activity with an increase in both the 1.3 kb mRNA species and enzyme protein in hormone-dependent T-47D breast cancer cells [152,159,160]. Org 2058 increases the oxidative direction in T-47D cells only [152]. Coldham and James [161] showed that the progestin medroxyprogesterone acetate (MPA) stimulates the reductive (E_1 to E_2) activity of MCF-7 cells when phenol red was excluded from the tissue culture media. The authors suggested that this could be the way in which progestins increase cell proliferation in vivo. On the other hand, Couture et al. [153] observed that in the treatment of hormone-dependent ZR-75-1 breast cancer cells with MPA, the oxidative (E_2 to E_1) direction is predominant; this effect seems to implicate the androgen receptor. Other progestins, such as progesterone, levonorgestrel, and norethisterone, increase both the oxidative and reductive 17β-HSD activity in MCF-7 cells [162] whereas promegestone (R-5020) has no significant effect on the reductive activity of 17β-HSD [30] but can increase the oxidative (E_2 to E_1) activity in T-47D cells [163]. Nomegestrol acetate has an inhibitory effect on the 17β-HSD enzyme in T-47D cells (35 and 81% inhibition at 5×10^{-7} and 5×10^{-6} M, respectively) but no significant effect was found in MCF-7 cells, except at 5×10^{-5} M [70]. Medrogestone (Prothil®), a synthetic pregnane derivative of progesterone, significantly decreases the reductive 17β-HSD type 1 activity in MCF-7 and T-47D breast cancer cells. The inhibitory effect is dose-dependent and is more intense, even at low doses, in the T-47D cell line than in the MCF-7 cells; the IC_{50} values, which correspond to the 50% inhibition of the conversion of E_1 to E_2, are 0.45 and 17.36 μM, respectively [164].

Tibolone (Org OD14), a 19-nortestosterone derivative with tissue-specific estrogenic, androgenic or progestagenic properties, significantly decreases the reductive activity of 17β-HSD in hormone-dependent T-47D and MCF-7 breast cancer cells [165]. This inhibitory effect is dose-dependent and was significant at a concentration of 5×10^{-7} M. The 3α-OH and 3β-OH metabolites of tibolone (Org 4094 and Org 30126, respectively) also show a similar inhibitory effect. The 4-en isomer of tibolone (Org OM38) shows an inhibitory effect only at the concentration of 5×10^{-6} M; The IC_{50} values in T-47D cells are respectively: 1.44, 2.03, 4.83 and 35.25 μM for Org 30126, tibolone, Org 4094 and Org OM38.

12.2. Control by antiestrogens and other compounds

The anti-estrogen ICI 164,384 can inhibit by competition the enzyme 17β-HSD in human breast tumors (IC_{50} value, 890 μM) [66]. However, in our laboratory we found that ICI 164,384 at 5×10^{-6} M inhibits by 53% the conversion of E_1 to E_2 in T-47D cells [30].

Various potential irreversible or reversible inhibitors of 17β-HSD type 1 have been synthesized (e.g. bromoacetoxy or alkylamide derivatives of E_2 and of progesterone) [166–168]. Thus, for example, the compound 16α-(bromoalkylamide) derivative of E_2 inhibits the 17β-HSD type 1 in human placenta with an IC_{50} value of 10.6 μM [169]. Sawicki et al. [170] obtained 77% inhibition of 17β-HSD type 1 activity with equilin, a component used in estrogen replacement therapy, at the concentration of 1 μM.

In a recent interesting study, Gunnarsson et al. [171] observed that the expression of 17β-hydroxysteroid dehydrogenase type I or type II can correlate to recurrence-free survival (RFS) of patients with breast cancer; low levels of mRNA 17β-HSD type II was related to decreased RFS.

Oduwole et al. [172] suggest that 17β-HSD type I is an independent prognostic marker in breast cancer.

13. Aromatases and anti-aromatases in breast cancer

Aromatase inhibition by anti-aromatase agents is largely developed with very positive results in the treatment

of patients with breast cancer. These inhibitors include steroidal and non-steroidal compounds. The most useful are: aminoglutethimide, 4-hydroxy-androstenedione (Formestane; Lentaron®), Vorozole, Letrozole (Fermara®), Anastrozole (Arimidex®), Examestane (Aromasin®). A series of reviews has been published recently on the biological effects and the therapeutic applications of these anti-aromatases [22,173–175].

14. Conclusions

One of the possible ways of blocking the estradiol effect in breast cancer is the use of antiestrogens, which act by binding to the estrogen receptor. More than 15 years' experience has shown that breast cancer patients treated with the anti-estrogen tamoxifen (e.g. Nolvadex) have a significantly reduced risk of recurrence and an increased overall survival. Recently, tests using a series of new antiestrogens yielded very attractive clinical results. However, another way to block estradiol is by using anti-enzymes [anti-sulfatase, anti-aromatase, or anti-17β-hydroxysteroid dehydrogenase (17β-HSD)] which are involved in estradiol biosynthesis in breast cancer tissues. At present, anti-aromatases are extensively used in breast cancer treatment with positive benefits. However, estrone sulfatase is quantitatively the most important pathway in estradiol bioformation in breast cancer tissue. Very interesting data were obtained concerning the inhibitory activity of various progestins (promegestone, nomegestrol acetate, medrogestone, dydrogesterone, norelgestromin), as well as tibolone and its metabolites, on estrone sulfatase, as well as on 17β-hydroxysteroid dehydrogenase, enzymes involved in the other pathway of estradiol formation in breast cancer cells. Using another series of steroidal and non-steroidal anti-sulfatase agents, very stimulating results were obtained in pre-clinical studies.

The fact that estradiol (E_2) can block its own bioformation in the breast cancer cell provides another aspect of this very complex mechanism in breast cancerization which, in addition to growth factors, oncogenes, proto-oncogenes and other factors, needs extensive additional information to be clarified. The paradoxical effect of E_2 could be related to estrogen replacement therapy (ERT), a treatment that has been observed to have either no effect or to slightly increase breast cancer incidence [176] but significantly decrease mortality [177–181].

Recent data also show that some progestins (promegestone, nomegestrol acetate, medrogestone) as well as tibolone can stimulate sulfotransferase activity in hormone-dependent breast cancer cells. This is an important point in the physiopathology of this disease, as it is well known that estrogen sulfates are biologically inactive.

For these inhibitory or stimulatory effects on the control of the enzymes involved in the formation and transformation of estrogens in breast cancer, we have proposed the concept of selective estrogen enzyme modulators (SEEM).

In conclusion, it is suggested that sulfatase inhibitors in combination with anti-aromatases may have potential as a new therapeutic treatment in hormone-dependent cancer patients.

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