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IN VITRO EFFECT OF SYNTHETIC PROGESTOGENS ON ESTRONE SULFATASE ACTIVITY IN HUMAN BREAST CARCINOMA

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Summary—The effect of progesterone and nine synthetic progestogens on the activity rate of microsome estrone sulfatase obtained from human breast carcinoma tissues was studied. The progestogens were classified into three groups: group I with a strict inhibitor effect: demegestone and chlormadinone acetate; group II with a strict activator effect: medroxyprogesterone acetate, quingestanol acetate, lynestrenol and progesterone and group III with a nonsignificant effect: dydrogesterone, promegestone, norgestrel and danazol. Demegestone was the most potent inhibitor and medroxyprogesterone acetate and quingestanol acetate had the highest activator effect. The effect of Triton X-100, a nonionic detergent, was also tested. This detergent consistently increased the microsome estrone sulfatase activity. A comparison was made between the effects of demegestone, medroxyprogesterone acetate and danazol on estrone sulfatase activity measured with or without Triton X-100 in the incubation medium. The presence of the detergent modified the progestogen action. Our results suggest that synthetic progestogens can influence the estrone sulfatase activity measured in human breast carcinoma tissues. However, the effect of progestogens was dependent on experimental conditions. Progestogens such as demegestone and chlormadinone acetate which inhibited estrone sulfatase activity in intact preparations, can reduce the intracellular production of biological active estrogen *via* the sulfatase pathway.

INTRODUCTION

Animal studies and clinical trials of antiestrogens and inhibitors of estrogen biosynthesis, have suggested that estrogens are the major hormones involved in facilitating the growth of breast tumors [1, 2]. In postmenopausal women, with breast cancer, plasma concentrations of estrone-3-sulfate [3–5] are 10-fold higher than those of unconjugated estrone or 17β-estradiol [6–8] and can constitute sulfated estrogen precursors for an active estrogen production. In mammary cancer cells, the transformation of estrone-3-sulfate (E_1S) into 17β-estradiol (E_2) has been reported by Wilking *et al.* [9], Vignon *et al.* [10] and Pasqualini *et al.* [11].

The first step for the activation of E_1S into physiologically active E_2 and estrone [12–14] is its hydrolysis by sulfatases. Previous studies have demonstrated the existence of estrone sulfatase in human breast tumors [9]. This enzyme activity is always present in all breast carcinoma tissues regardless of their estrogen and progesterone receptor status [15, 16] and appears to be primarily responsible for intratissue estrone production in hormone-responsive breast carcinomas rather than the aromatase pathway [16]. The biological role of estrone-3-sulfate in breast tumors is supported by the fact that exogenous administration of this molecule can stimulate growth of carinogen-induced hormone-dependent mammary tumors in the rat [17].

Some treatments of breast carcinoma use the association of tamoxifen and synthetic progestogens to inhibit or decrease estrogen production or to reduce estrogen and progesterone receptors [18, 19]. In a solubilized microsomal preparation of pregnant guinea-pig uterus, Moutaouakkil [20] has demonstrated that several synthetic progestogens modify estrone sulfatase activity. In human hyperplastic endometrium associated with a high level of estrone sulfatase activity, progestogen treatment could simultaneously reduce hyperplasia and estrone sulfatase activity [21]. Carlström *et al.* [22, 23] have demonstrated that danazol inhibits steroid sulfatase activity in human liver

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cells as well as in breast carcinoma. However, the mechanism by which progestogens modify membrane-bound sulfatase activity, appears to be complex [20, 21, 23]. It seemed interesting to evaluate, *in vitro*, the inhibitory effect of synthetic progestogens on estrone sulfatase activity in human breast carcinoma. In postmenopausal women with low peripheral 17β-estradiol levels, estrogen production by the sulfatase pathway in mammary tumors might be reduced by the action of estrone sulfatase inhibitors.

The aim of this paper was to study the effect of some progestogens on estrone sulfatase activity in breast carcinoma and to identify those progestogens which displayed an inhibitor effect on this enzyme.

EXPERIMENTAL

Chemicals

Estrone-3-sulfate was purchased from Sigma Chimie (L'Arbresle, France). [6,7-^{3}H]E_1S (ammonium salt; sp. act.: 53.0 Ci/mmol) and [4-^{14}C] estrone (sp. act.: 57.0 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). Before use, unconjugated steroids were eliminated by solvent partition [24]. Demegestone and promegestone were kindly provided by Roussel-Uclaf Laboratories (Paris, France), dydrogesterone by Duphar Laboratories (Villeurbanne, France), medroxyprogesterone acetate by Carlo Erba Laboratories (Paris, France), quingestanol acetate by Substantia Laboratories (Orléans-la-Source, France), chlormadinone acetate by Cassenne Laboratories (Osny, France), lynestrenol by Organon SARL (Saint Denis, France), norgestrel by Wyeth-Byla Laboratories (Paris, France) and danazol by Winthrop Laboratories (Longwic, France). Progestogens were dissolved in absolute ethanol at high concentrations so that the quantity of ethanol in the incubation medium did not represent more than 5% (v/v). All solvents and reagents were of analytical grade (Merck, Chelles, France).

Tumor tissue preparation

Human primary tumor specimens removed during surgery were promptly trimmed of fat and connective tissues and frozen in liquid nitrogen. In all cases, histological diagnosis of carcinoma was confirmed from block sections. Tissues were prepared for the assay of estrone sulfatase according to the method described previously [15].

The resulting microsome fraction (controlled by electron microscopy) from a pool of breast cancer tissues was used as enzyme preparation. No attempt was made to correlate estrone sulfatase activity and steroid receptor content since estrone sulfatase activity is always present in breast tumors regardless of their estrogen and progesterone receptor status [15, 16]. The protein content was determined by Lowry's method [25] with bovine serum albumin as standard.

Estrone sulfatase activity

The standard method has been described elsewhere [15, 24]. Briefly, the estrone sulfatase activity was measured in 0.05 M Tris–HCl buffer pH 7.6 with [^{3}H]E_1S (6.5 μmol/l; sp. act., 126 Ci/mol) in a final volume of 0.5 ml, including enzyme preparation (0.30 mg of protein by assay). The reaction was started by adding the enzyme preparation preincubated for 10 min with or without progestogen, and stopped after 30 min at 37°C by adding 1 ml of 0.1 M Na_2CO_3. [^{14}C]estrone (2000 dpm) was added to correct the methodological losses and unconjugated estrone was estracted with 5 ml of diethyl ether (peroxide-free). Controls without enzyme preparation were processed simultaneously. Each assay was done in triplicate. For kinetic studies increasing concentrations of E_1S were used as stated below and tracer amounts of [^{3}H]E_1S were added to obtain a constant specific activity (sp. act., 45 Ci/mol). One unit of estrone sulfatase activity was defined as the quantity of the enzyme preparation producing 1 pmol of estrone per min mg porotein^{-1}.

Statistical analysis of results

The statistical analysis of differences between assay and control was calculated using the Mann–Whitney U-test.

RESULTS

Effect of progestogens on microsome estrone sulfatase activity

The estrone sulfatase activity of breast cancer microsomes was measured according to the standard method in the presence of progestogens. Three concentrations were tested: 10, 40 and 80 μM (10 assays per concentration). Results represent variation rates of estrone sulfatase activity expressed in percentages with, as 100% the activity of control ($n = 6$) measured in the absence of progestogen. The between-run

Table 1. Progestogen effect on microsome estrone sulfatase activity in human breast carcinoma tissues

Group	Effect	Progestogen	Relative sulfatase activity[a] 10 μM[b] (%)	40 μM[b] (%)	80 μM[b] (%)
I	Inhibitor	Demegestone	−24[c]	−34[c]	−40[c]
		Chlormadinone acetate	−26[c]	−11	+3
II	Activator	Medroxyprogesterone acetate	+23[c]	+61[c]	+70[c]
		Quingestanol acetate	+14[c]	+29[c]	+36[c]
		Progesterone	+7	+13	+23[c]
		Lynestrenol	−12	+2	+24[c]
III	NSE[d]	Dydrogesterone	−8	−12	−15
		Promegestone	0	+8	+8
		Norgestrel	+13	+13	−9
		Danazol	−7	+4	−9

[a]Estrone sulfatase activity is expressed as a percentage of controls P − C/C × 100 (P: activity in the presence of progestogen ($n = 10$) and C: activity of control ($n = 6$) without progestogen).
[b]Concentration of progestogens in the test tube.
[c]Statistically significant difference ($P < 0.05$) vs control.
[d]NSE, no significant effect.

precision of the method was 10% (variation coefficient). This corresponded to the value usually reported for estrone sulfatase assay in the relevant literature [24]. The effect of progestogens on estrone sulfatase activity was analyzed statistically using a nonparametric test. Only significant variations ($P < 0.05$) were retained. Results are reported in Table 1. Progestogens were classified into 3 groups according to their effect on estrone sulfatase activity. Group I consisted of two progestogens with a significant inhibitor effect. Demegestone was the most potent inhibitor. Its inhibitory effect increased in terms of concentration. Chlormadinone acetate was a potent inhibitor at 10 μM. No significant action was found at the other two concentrations tested. Medroxyprogesterone acetate and quingestanol acetate were consistent activators (group II) with a greater significant effect as the concentration increased. Only progesterone, the natural progestogen, and lynestrenol had a significant activator effect at the highest concentration tested (80 μM). Group III included 4 compounds which did not have any significant effect on estrone sulfatase activity. The inhibitory effect of demegestone and chlormadinone acetate was evaluated in terms of kinetic parameters. A microsome fraction prepared from a pool of 4 breast tumors was used. The estrone sulfatase activity of this microsome fraction was measured with increasing concentrations of estrone sulfate [15]. Lineweaver–Burk analysis of the data showed for estrone sulfatase activity an apparent K_m of 4.8 μM and the maximum velocity value observed was 23.0 pmol $E_1 \cdot min^{-1} \cdot mg\ protein^{-1}$. The inhibitory effect of demegestone and chlormadinone acetate was tested by adding increasing concentrations (10, 20 and 40 μM) of each progestogen. The Hanes–Woolf plot was used to determine the type of inhibition (Fig. 1). Increasing concentrations of demegestone decreased the apparent velocity without affecting K_m. Demegestone acted as a noncompetitive inhibitor with a dissociation constant of the inhibitor from

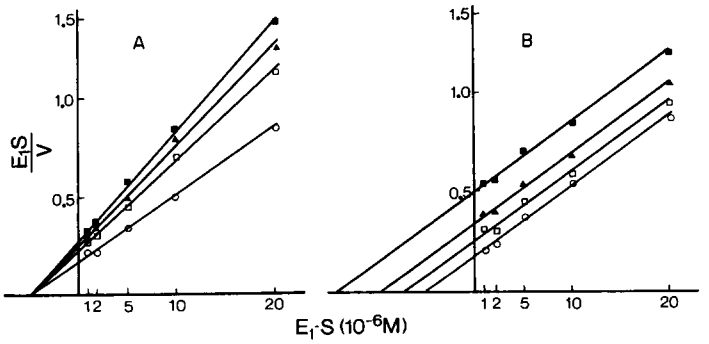

Fig. 1. Inhibition of estrone sulfatase by demegestone (A) and chlormadinone acetate (B). The standard method was used with increasing amounts of estrone sulfate [E_1-S] from 1 to 20 μM. The Hanes–Woolf Plot was drawn with [E_1-S] (○) alone or in the presence of 10 (□) 20 (▲) and 40 (■) μM progestogens. The lines were calculated by linear square regression. Each point represents the mean of four determinations. (V pmol estrone·min^{-1}·mg protein^{-1}).

the enzyme–substrate complex of 87.5 μM. Conversely, increasing concentrations of chlormadinone acetate increased the apparent K_m without affecting the maximum velocity. Chlormadinone acetate was a competitive inhibitor with a dissociation constant of the enzyme–inhibitor complex of 28.2 μM.

Effect of Triton X-100 on estrone sulfatase activity

Estrone sulfatase is a membrane-bound enzyme [15, 24, 26]. It is known that the specific activity of a membrane-bound enzyme varies according to the presence or the absence of detergent in the enzyme preparation [27, 28, 14]. Estrone sulfatase activity was measured using the standard method on the microsome fraction prepared from a pool of 4 breast tumors in the absence (control) or in the presence of an efficient concentration [28, 29] of Triton X-100 (0.2%; v/v). The microsome fraction was preincubated with Triton X-100 for 15 min at 37°C. Estrone sulfatase activity was expressed as a percentage of control (Fig. 2). Triton X-100 consistently increased estrone sulfatase activity. The activity was maximal with the lowest concentrations of estrone sulfate tested (1 and 2 μM) and declined nonlinearly at higher substrate concentrations. The kinetic properties of estrone sulfatase were affected by Triton X-100 treatment. This usual behavior of membrane-bound enzymes could have influenced the progestogen effects [20, 28, 29]. The effect of progestogens was also reinvestigated using microsome fractions preincubated with Triton X-100.

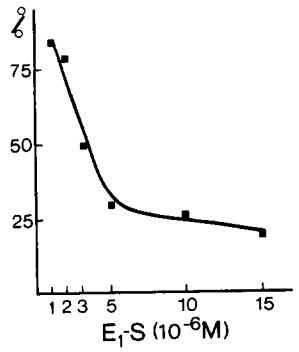

Fig. 2. Effect of Triton X-100 on microsome sulfatase activity. Microsomes were preincubated in the absence (control C) or in the presence (T) of Triton X-100 (0.2%; v/v) for 15 min at 37°C. Estrone sulfatase activity was measured by the standard method with increasing concentrations of estrone sulfate [E_1-S]. Estrone sulfatase activity is expressed as a percentage of controls: $T - C/C \times 100$. Each point is the mean of triplicate determinations.

The action of three compounds: demegestone, medroxyprogesterone acetate and danazol on the E_1S hydrolysis was tested in untreated or Triton X-100 treated microsome fractions. The results are reported in Fig. 3. Triton X-100 added to the incubation medium modified the effect of progestogens. In the presence of Triton X-100, the inhibitor effect of demegestone was reduced and even an activator effect appeared at a progestogen concentration of 60 μM. Medroxyprogesterone acetate, an estrone sulfatase activator, and danazol which had no significant effect, were converted into inhibitors when Triton X-100 was added to the microsome fraction.

DISCUSSION

The effect of synthetic progestogens on estrone sulfatase activity was measured in human breast cancers using 12,500 *g* supernatant resulting from resuspended 105,000 *g* pellet (obtained from tumor tissue preparation for receptor assays). In the absence of a detergent, this 12,500 *g* supernatant fraction corresponded to a nonsolubilized preparation of microsomes [15]. In the presence of increasing concentrations of progestogens, the modifications of estrone sulfatase activity were complex and varied according to the tested progestogen. In this study, 10 progestogens including progesterone and danazol were tested. Due to their action on estrone sulfatase activity they were grouped into activators (medroxyprogesterone acetate, quingestanol acetate, lynestrenol and progesterone); inhibitors (demegestone and chlormadinone acetate) or without effect (dydrogesterone, promegestone, norgestrel and danazol). The synthetic progestestogens can be classified according to the basic steroid molecule from which they are derived. Demegestone, chlormadinone acetate, medroxyprogesterone acetate, dydrogesterone and promegestone result from a modification of a progesterone molecule. Quingestanol acetate, lynestrenol and norgestrel result from a transformation of the testosterone molecule, such as danazol whose structure exhibits a greater modification of the basic steroid molecule. Danazol is not a progestogen, but some of the actions it has on the human endometrium, may be explained by a progestin-like action [30, 31]. No correlation could be established between the molecular structure of progestogen and a particular effect on microsome sulfatase activity. Moreover, demegestone

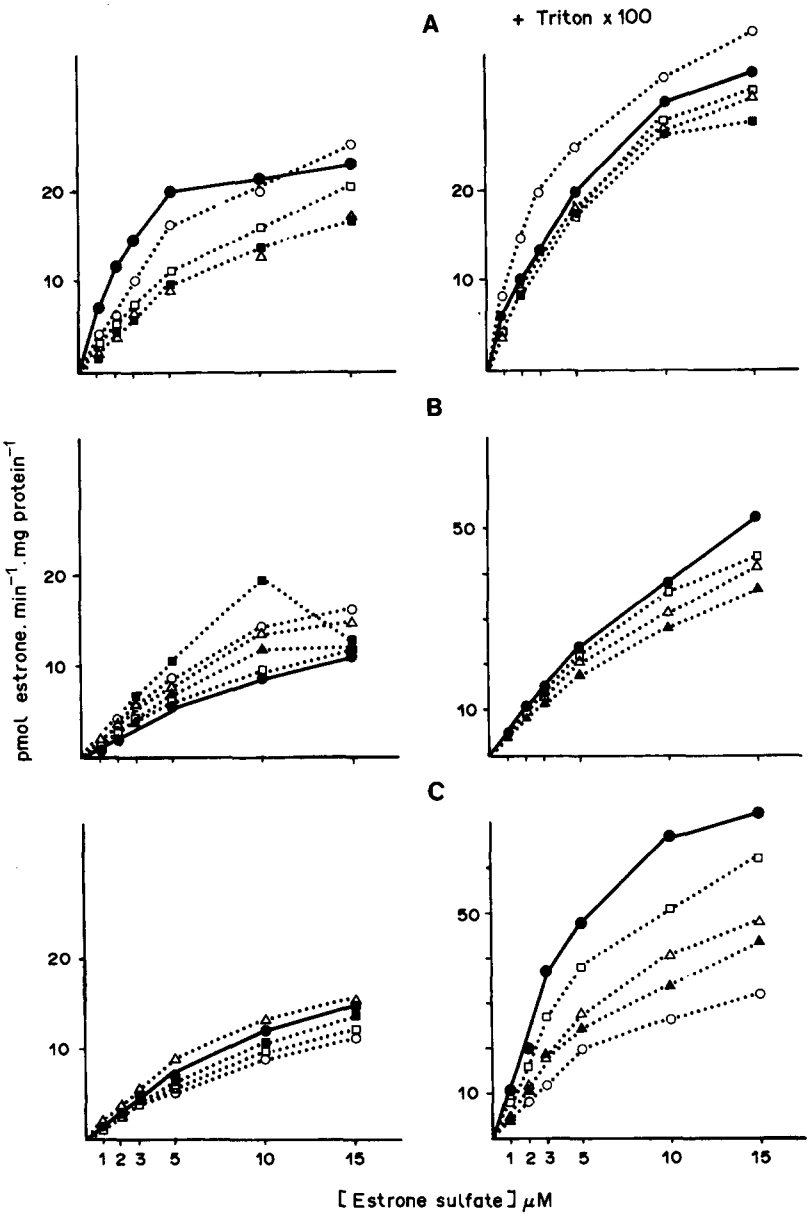

Fig. 3. Effect of demegestone (A), medroxyprogesterone acetate (B) and danazol (C) on estrone production (pmol estrone·min^{-1}·mg protein^{-1}] from different concentrations of estrone-sulfate by microsomes of human breast carcinomas in the absence (left diagrams) or in the presence (right diagrams) of Triton X-100 (0.2%, v/v) in the incubation medium. The microsome fraction was preincubated with Triton X-100 for 15 min at 37°C. The progestogen concentrations were 0 (●), 10 [□), 20 (△), 40 (■), 60 (○) and 80 μM (▲).

and chlormadinone acetate which have the same basic structure, act as a noncompetitive and a competitive inhibitor, respectively.

The activity of a membrane-bound enzyme is dependent on its local environment. Detergents used for solubilizing a membrane-bound enzyme could activate or inhibit its activity according to the nature and the concentration of the detergent used [28]. Chang *et al.* [27] demonstrated that the solubilization of membrane-bound arylsulfatase C considered to be identical to estrone sulfatase [29], depended on the concentration of Triton X-100 or Miranol H_2M, an amphoretic surface active agent. They noted that the specific activity of the enzyme increased after solubilization, but could also be inhibited with high concentrations of detergent. Several partial purifications of estrone sulfatase from different tissues using various detergents for solubilization and different purification

methods have been reported [29, 32–35]. These studies have resulted in different enzyme preparations, with variable specific activity. There seems to be no doubt that this variability was related to the fact that this enzyme is membrane-bound and difficult to dissolve in aqueous systems. Steroid sulfatase activity seems closely related to the phospholipid content of membranes [33, 36, 37]. This dependence can be modified by a detergent. In the same way, the action of synthetic progestogens on microsome estrone sulfatase activity can stimulate or inhibit the expression of the enzyme by modifying the membrane environment. Incubation of the microsome fraction with Triton X-100 resulted in an increase of the specific activity of estrone sulfatase. The action of synthetic progestogens was also affected by Triton X-100: medroxyprogesterone acetate, an estrone sulfatase activator, became an inhibitor. Thus, danazol although it does not have a significant effect in the absence of Triton X-100, became an inhibitor. This result is consistent with the inhibitor effect of danazol on estrone sulfatase activity in a solubilized microsomal preparation of guinea-pig pregnant uterus [20]. It would seem that the previous incubation of enzyme preparation with Triton X-100 solubilizes the membrane estrone sulfatase, and the modified action of synthetic progestogens on the enzyme expression would appear to result from the modification of the membrane environment of the enzyme. It is very important to keep in mind the fact that the effect of a progestogen is dependent on experimental conditions before any conclusion concerning a putative effect *in vivo* can be drawn. Thus, the progestogen action on estrone sulfatase activity measured in solubilized preparations cannot be compared with the progestogen action *in vivo*. In intact preparations, synthetic progestogens could modify the membrane environment of estrone sulfatase, and consequently the enzyme expression. In mammary cancer cells, the regulation of the estrone sulfatase activity rate could modulate the entry into the cell of sulfated estrone which has been demonstrated to have a biological activity in hormone-responsive tissues [10, 13, 17]. Some progestogens such as demegestone or chlormadinone acetate which inhibit estrone sulfatase activity in a non-solubilized preparation, could reduce the intracellular production of biological active estrogen *via* sulfatase pathway [16]. This possibility still remains to be proved *in vivo*.

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