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Control of Sulfatase Activity by Nomegestrol Acetate in Normal and Cancerous Human Breast Tissues

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Abstract. *Nomegestrol acetate (NOMAC), a 17α-hydroxy-nor-progesterone derivative (17α-acetoxy-6-methyl-19-nor-4,6-pregnadiene-3,20-dione, the active substance in Lutenyl®), is a potent and useful clinical synthetic progestin for the treatment of menopausal complaints and is under current development for oral contraception. Previous studies in this laboratory demonstrated that NOMAC can block sulfatase and 17β-hydroxysteroid dehydrogenase, the enzymes involved in the biosynthesis and transformation of estradiol (E_2) in hormone-dependent MCF-7 and T-47D breast cancer cells. In the present study, the effect of NOMAC on sulfatase activity using total breast cancer tissue, compared to the effect in normal breast tissue, was explored. Slices of tumoral or normal breast tissues (45-65 mg) were incubated in buffer (20 mM Tris-HCl, pH 7.2) with physiological concentrations of [^{3}H]-estrone sulfate ($5x10^{-9}$ M), alone or in the presence of nomegestrol acetate ($5x10^{-5}$ - $5x10^{-7}$ - $5x10^{-9}$ M), for 4 h at 37°C. Estrone sulfate (E_1S), estrone (E_1) and E_2 were characterized by thin layer chromatography and quantified using the corresponding standard. It was observed that [^{3}H]- E_1S was only converted to [^{3}H]- E_1 and not to [^{3}H]- E_2, in normal or cancerous breast tissues, which suggests a low or no 17β-HSD activity under these experimental conditions. The sulfatase activity was more intense with breast cancer tissue than normal tissue, since the concentrations of E_1 were 42.5±3.4 and 27.2±2.5 pg/mg tissue, respectively. NOMAC, at the concentration of $5x10^{-5}$ M, inhibited this conversion by 49.2% and 40.8% in cancerous and normal breast tissues, respectively. The sulfatase inhibition at low concentration ($5x10^{-7}$ M) was 32.5% and 22.8%, respectively. It is concluded that sulfatase activity is almost twice as potent in cancerous breast tissues than in normal tissues. Nomegestrol acetate is a strong anti-sulfatase agent, in particular with cancerous breast tissues. The inhibition of estrone sulfatase activity by NOMAC in total normal or cancerous breast tissues can open attractive perspectives for future clinical trials.*

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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 in which it is produced.

In previous studies, it was observed that in the isolated MCF-7 and T-47D breast cancer cell lines, Nomegestrol acetate (NOMAC) can block estrone sulfatase activity (15).

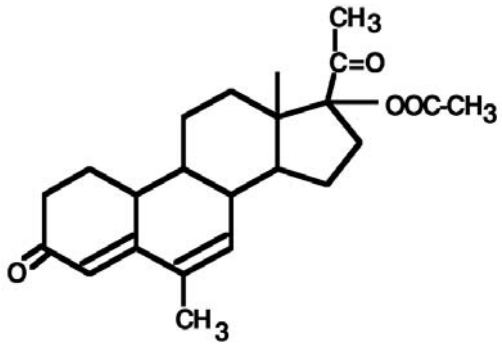

Figure 1. *Structure of the progestin nomegestrol acetate (NOMAC).*

In the present study, the effect of NOMAC was explored using total normal and cancerous human breast tissues.

Materials and Methods

Chemicals. [6,7-^{3}H(N)]-estrone-3-sulfate (^{3}H-E_1S), ammonium salt (53 Ci/mmol) and [4-^{14}C]-estrone (4-^{14}C-E_1) (51 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. Unlabelled E_1S, E_1 and E_2 were obtained from Sigma-Aldrich Chimie, (St. Quentin Fallavier, France). Nomegestrol acetate (NOMAC; 17α-acetoxy-6-methyl-19-norpregna-4,6-diene-3,20-dione) was a gift from Merck-Theramex Laboratories (Monaco). The structure of NOMAC is given in Figure 1. All chemicals were of the highest purity available.

Patients and biological materials. This study was carried out with three post-menopausal patients (aged 54-73 years) with breast cancer. None of the patients had a history of endocrine, metabolic or hepatic diseases or had received treatment in the previous 2 months. Each patient received local anaesthesia (lidocaine 1%) and two regions of the mammary tissue were selected for the various analyses: A) the tumoral tissue, B) a distant zone (glandular tissue) which was considered as normal. The different tissue specimens removed at the time of surgery were promptly trimmed of fat and connecting tissues. A sample of each tissue was used for histology studies and classified according to the epithelial density. Samples were placed in liquid nitrogen and stored at –80˚C until enzyme activity analysis. Breast cancer histotypes were ductal and postmenopausal stages were T2. The tissular status of both estrogen receptor (ER) and progesterone receptor (PR) was positive.

Isolation and quantification of [^{3}H]-estrone from normal or cancerous human breast tissues incubated with [^{3}H]-estrone sulfate. After thawing, breast tissues were washed twice with cold 0.02 M Tris-HCl buffer (pH:7.2) and minced with scissors. Slices of tumor or normal breast tissues (45-65 mg) were incubated in buffer (final vol. 0.6 ml) with physiological concentrations of [^{3}H]-E_1S ($5x10^{-9}$ mol/l) alone or in the presence of nomegestrol acetate, prepared in ethanol (final concentration <0.3%, v/v), at a range of concentrations of $5x10^{-5}$, $5x10^{-7}$ and $5x10^{-9}$ mol/l, for 4 hours at 37˚C in a shaking bath. Control breast tissues received ethanol vehicle only. After centrifugation, the supernatant and pellet were separated and each fraction treated by 80% ethanol (2 ml) . The pellet fraction was homogenized using an ultraturrax apparatus (Ika-Werk, Janke & Kunkel, Staufen, Germany) and sonicated for 10 seconds. The radioactivity was extracted for at least 24 hours at –20˚C and [^{14}C]-E_1 was added to monitor analytical losses. The cellular radioactivity uptake was determined in the ethanolic supernatant. After evaporation of the organic phase, the extracts were redissolved in 50 µl of ethanol and the qualitative analysis and quantitative evaluation of E_1-E_2 were carried out after isolation by TLC on silica gel $60F_{254}$ plates (Merck, Darmstadt, Germany) developed with chloroform-ethylacetate (4:1, v/v). Unlabelled E_1S, 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 minutes. Three ml of Opti-fluor (Packard Division - PerkinElmer Life Sciences) 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 [^{3}H]-E_1S to [^{3}H]-E_1 or [^{3}H]-E_2, corresponding to the sulfatase activity at 4 hours, was calculated as a percentage of the total radioactivity associated with the tissue slices and then expressed as pg E_1 or E_2 formed/mg tissue.

Statistical analysis. The data are expressed as the mean±standard error of the mean (SEM) values. The 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 nomegestrol acetate on the conversion of estrone sulfate to estrone in human breast tissues.

a) Normal breast tissues. Many studies of the sulfatase pathway, the main route for the intracellular biosynthesis of E_2, have been done previously using breast cancer cells in culture. However, breast tissue slices are an interesting new experimental *in vitro* model, since the integrity of the organ is in part preserved and this represents a first approach to evaluate more physio- and pathological conditions before *in vivo* analyses. Normal breast tissues correspond to tissue distanced from the tumor in the same patient, allowing a more comparative study to evaluate differences between normal and tumor breast tissues.

Normal breast tissues have the capacity to transform physiological concentrations ($5x10^{-9}$ mol/l) of [^{3}H]-E_1S, incubated for 4 hours at 37˚C, to E_1. This conversion corresponds to 44% of the initial [^{3}H]-E_1S substrate with a concentration ratio E_1 / E_1S of 0.80. Under our conditions, no conversion to E_2 was observed; thus, the measure reflects the sulfatase activity in the isolated tissues. The progestin NOMAC has a significant dose-dependent inhibitory effect

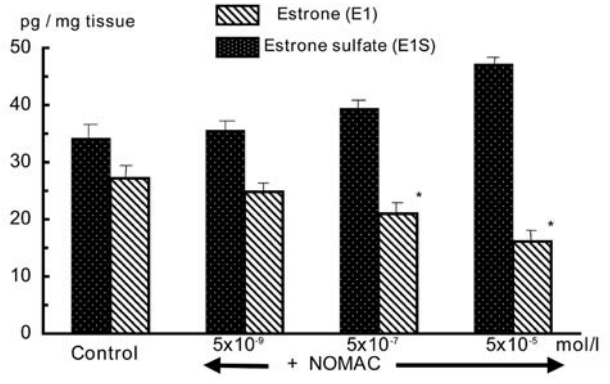

Figure 2. *Effects of nomegestrol acetate (NOMAC) on the conversion of estrone sulfates (E_1S) to estrone (E_1) in human normal breast tissues. Slices of normal breast tissues (45-60 mg) were incubated for 4 hours at 37°C with a physiological concentration of estrone sulfate ([^{3}H]-E_1S: $5x10^{-9}$ mol/l) alone (control: non-treated cells) or in the presence of NOMAC at the range of concentrations $5x10^{-9}$ mol/l to $5x10^{-5}$ mol/l. Estrogens were calculated after isolation of the hormones, as indicated in Materials and Methods. The data are the mean±SEM of duplicate determinations of 3 independent experiments.*
**$p \leq 0.05$ vs E_1 control values.*

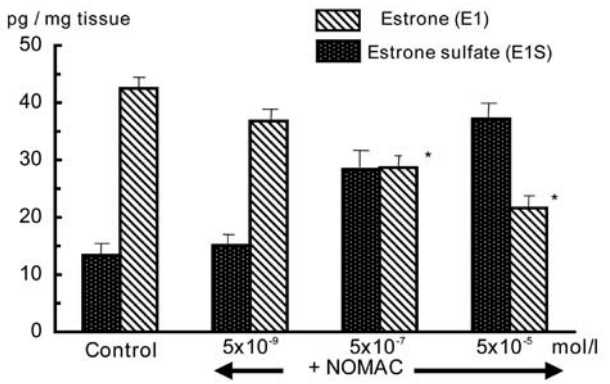

Figure 3. *Effects of nomegestrol acetate (NOMAC) on the conversion of estrone sulfates (E_1S) to estrone (E_1) in human cancer breast tissues. Slices of cancer breast tissues (50-65 mg) were incubated for 4 hours at 37°C with a physiological concentration of estrone sulfate ([^{3}H]-E_1S: $5x10^{-9}$ mol/l) alone (control: non-treated cells) or in the presence of NOMAC at the range of concentrations $5x10^{-9}$ mol/l to $5x10^{-5}$ mol/l. Estrogens were calculated after isolation of the hormones, as indicated in Materials and Methods. The data are the mean±SEM of duplicate determinations of 3 independent experiments.*
**$p \leq 0.05$ vs E_1 control values.*

Table I. *Effect of NOMAC on the sulfatase activity by normal breast tissues.*

	Estrone sulfate (E_1S) (in pg/mg tissue)	Estrone (E_1)	Estradiol (E_2)	R: E_1/ E_1S	% of inhibition
Control [^{3}H]-E_1S = 5 x 10^{-9}M	34.2±3.1	27.2±2.5	ND	0.80	
+ NOMAC					
5 x 10^{-9}M	35.6±2.5	24.8±2.4	ND	0.70	8.9%
5 x 10^{-7}M	34.4±2.1	21.0±2.6*	ND	0.53	22.8%
5 x 10^{-5}M	47.2±2.8	16.1±1.8*	ND	0.34	40.8%

3[H]-E_1S, 5 x 10^{-9} mol/l, was incubted with slices of normal breast tissues for 4 hours at 37°C in the absence (control) or presence of NOMAC in the range of 5 x 10^{-9} to 5 x 10^{-5} mol/l. The values of ^{3}[H]-estrogens (E_1, E_2, E_1S) were determined after isolation of the hormones, as indicated in Materials and Methods. The data represent the average±SEM of three independent duplicate determinations.
R: ratio concentration of E_1 to E_1S in normal breast tissues.
ND: not detectable.
**$p \leq 0.05$ vs* E_1 control value

Table II. *Effect of NOMAC on the sulfatase activity by tumoral breast tissues.*

	Estrone sulfate (E_1S) (in pg/mg tissue)	Estrone (E_1)	Estradiol (E_2)	R: E_1/ E_1S	% of inhibition
Control [^{3}H]-E_1S = 5 x 10^{-9}M	13.4±2.7	42.5±3.4	ND	3.18	
+ NOMAC					
5 x 10^{-9}M	15.1±2.4	36.8±3.1	ND	2.44	13.4%
5 x 10^{-7}M	28.4±3.1	28.7±2.4*	ND	1.01	32.5%
5 x 10^{-5}M	37.2±2.8	21.1±3.8*	ND	0.58	50.3%

^{3}[H]-E_1S, 5 x 10^{-9} mol/l, was incubated with slices of tumoral breast tissues for 4 hours at 37°C in the absence (control) or presence of NOMAC in the range of 5 x 10^{-9} to 5 x 10^{-5} mol/l. The values of ^{3}[H]-estrogens (E_1, E_2, E_1S) were determined after isolation of the hormones, as indicated in Materials and Methods. The data represent the average ± SEM of three independent duplicate determinations.
R: ratio concentration of E_1 to E_1S in tumoral breast tissues.
ND: not detectable.
**$p \leq 0.05$ vs* E_1 control value

on the sulfatase activity in normal breast tissues (Figure 2). At the concentrations of $5x10^{-5}$ mol/l and $5x10^{-7}$ mol/l, NOMAC exerted an inhibition of 40.8% and 22.8%, respectively (Table I).

b) Cancerous breast tissues. In the tumoral area, the conversion of [^{3}H]-E_1S to E_1 was more intense than in normal breast tissues. This conversion corresponds to 76% of the initial [^{3}H]-E_1S substrate with a concentration ratio

E_1 / E_1S of 3.18. NOMAC had a strong anti-sulfatase activity (Figure 3) and exerted an inhibition on the sulfatase activity of 50.2% and 32.5% at concentrations of $5x10^{-5}$ mol/l and $5x10^{-7}$ mol/l, respectively (Table II).

Discussion

In previous studies, it was demonstrated that NOMAC is a potent inhibitor of estrone sulfatase in the isolated MCF-7 and T-47D cell lines (15). The present study confirms and extends this blockage of sulfatase activity, using total breast tissues. The data are of interest because the effect obtained using the total tissue is closer to the physiopathological conditions.

The sulfatase activity was significantly more intense in the breast cancer tissue than in the area considered as normal (for a review see Ref. 16). The fact that NOMAC was more active as an anti-sulfatase agent in the breast cancer tissues than in the normal breast provides interesting information on the specific effect of this progestin. It is notable that, in the present data, only E_1 was detected, indicating a very low 17β-hydroxysteroid dehydrogenase type I activity in the tissue used. This is of particular interest because, under the experimental conditions used, intact breast tissues can provide an interesting model to explore sulfatase activity, as only this enzyme is involved in the transformation of estrone sulfate to the unconjugated estrone.

In conclusion, NOMAC is an anti-sulfatase agent, not only in isolated breast cancer cells, but also in the intact tissue. The data can open attractive perspectives for future clinical trials with this progestin on patients with breast cancer.

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