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Norelgestromin as selective estrogen enzyme modulator in human breast cancer cell lines Effect on sulfatase activity in comparison to medroxyprogesterone acetate☆

Jorge R. Pasqualini[a,*], Patrick Caubel[b], Andrew J. Friedman[c], Jean-Claude Philippe[a], Gérard Samuel Chetrite[a]

[a] *Hormones and Cancer Research Unit, Institut de Puériculture, 26 Boulevard Brune, 75014 Paris, France*
[b] *Johnson & Johnson Pharmaceutical Research and Development, Inc., Reproductive Medicine and Menopause, 920 Route 202, P.O. Box 300, Raritan, New Jersey, NJ 08869, USA*
[c] *Ortho-McNeil Pharmaceutical, Inc., 1000 Route 202, P.O. Box 300, Raritan, New Jersey, NJ 08869, USA*

Abstract

Human breast cancer tissue contains enzymes (estrone sulfatase, 17β-hydroxysteroid dehydrogenase, aromatase) involved in the last steps of estradiol (E_2) formation. In this tissue, E_2 can be synthesized by two main pathways: (1) sulfatase—transforms estrogen sulfates into bioactive E_2, and the (2) aromatase—converts androgens into estrogens. Quantitative assessment of E_2 formation in human breast tumors indicates that metabolism of estrone sulfate (E_1S) via the sulfatase pathway produces 100–500 times more E_2 than androgen aromatization.

In the present study, we demonstrated in T-47D and MCF-7 human breast cancer cells that norelgestromin (NGMN) (a metabolite of norgestimate) is a potent inhibitory agent of the estrone sulfatase activity. After 24 h incubation of physiological concentrations of E_1S (5×10^{-9} mol/l) the inhibitory effect of NGMN at concentrations of 5×10^{-9}, 5×10^{-7} and 5×10^{-5} mol/l was 43 ± 7, 74 ± 4 and 97 ± 2%, respectively, in T-47D cells; 25 ± 4, 57 ± 5 and 96 ± 2% respectively, in MCF-7 cells. Comparative studies using medroxyprogesterone acetate (MPA) showed that this progestin also has an inhibitory effect on sulfatase activity, but significantly less intense than that of NGMN. The inhibition for MPA at concentrations of 5×10^{-9}, 5×10^{-7} and 5×10^{-5} mol/l was 31 ± 5, 47 ± 3 and 61 ± 3%, respectively, for T-47D cells; 6 ± 3, 20 ± 3 and 63 ± 4%, respectively, for MCF-7 cells.

In conclusion, the present data show that NGMN is a very potent inhibitory agent for sulfatase activity in the hormone-dependent breast cancer cells, resulting in decreased tissue concentration of E_2. The clinical significance of this finding remains to be elucidated.

Keywords: Medroxyprogesterone acetate; Norelgestromin; Estradiol

1. Introduction

Breast cancer is one of the major causes of cancer-related deaths among women and recent statistical information indicates that in the United States one woman in eight will develop this disease during her lifetime; the values are one in twelve for countries of the European Community and one in eighty for Japan. It is now well-established that estradiol (E_2) is an important risk factor for the genesis and evolution of breast tumors and that most (about 95%) early stage tumors are estrogen-sensitive [1–3].

About two-thirds of breast cancers occur during the post-menopausal period when estrogen produced by the ovaries has essentially ceased. Despite the low levels of circulating estrogens, the tissue concentrations of estrone (E_1), 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 to be normal tissue, suggesting a specific tumoral biosynthesis and accumulation of these hormones [4–8].

There is substantial information that mammary cancer tissue contains 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

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* Corresponding author. Tel.: +33-1-4539-4121/9109; fax: +33-1-4542-6121.
E-mail address: jorge.pasqualini@wanadoo.fr (J.R. Pasqualini).

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in breast cancer tissues: the "aromatase pathway" which transforms androgens, mainly androstenedione, into estrogens [9,10] and the "sulfatase pathway" which converts E_1S into E_1 by the estrone-sulfatase (EC: 3.1.6.2) [11–15]. 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) [16,17]. Quantitative evaluation indicates that the majority of E_2 formation in human breast tumors is via the sulfatase pathway rather than via the aromatase pathway [5,15,18].

Norelgestromin (NGMN) is a synthetic progestin and the primary metabolic product of norgestimate, a progestin used in several marketed oral contraceptives and in a US marketed hormone replacement therapy.

In this study, we explored the effects of NGMN and medroxyprogesterone acetate (MPA) on the sulfatase activity in two hormone-dependent breast cancer cells: MCF-7 and T-47D.

2. Materials and methods

2.1. Chemicals

[6,7-^{3}H(*N*)]-estrone sulfate (^{3}H-E_1S), ammonium salt (specific activity 53 Ci/mmol) and [4-^{14}C-estradiol] (^{14}C-E_2) (specific activity 57 mCi/mmol) were purchased from New England Nuclear Division (DuPont de Nemours, Les Ulis, France). The purity of the radioisotopes was assessed by thin-layer chromatography (TLC) in the appropriate system before use. E_1S, ammonium salt, unlabeled E_1 and E_2 (analytical grade) were obtained from Sigma–Aldrich Chimie (St. Quentin Fallavier, France). NGMN (13-ethy17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one oxime) was a gift from the Johnson & Johnson Pharmaceutical Research and Development Inc., Medicinal Chemistry Department (Raritan, NJ, USA); medroxyprogesterone acetate (MPA; 17α-acetoxy-6α-methylprogesterone) was obtained from Sigma–Aldrich Chimie. All other chemicals were of the highest grade commercially available.

2.2. Cell culture

The hormone-dependent MCF-7 and T-47D human mammary cancer cell lines were kindly provided by Dr. R.B. Dickson (Georgetown University, Washington, DC, USA). The cells were grown in Eagle's Minimal Essential Medium (MEM) buffered with 10 mmol/l HEPES (pH 7.6) supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin–streptomycin and 5% fetal calf serum (FCS) (ATGC, 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. Media were changed twice a week. The cells were passed every 10–12 days and replated in 75 cm^2 flasks (ATGC) at 3×10^6 cells per 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).

2.3. 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 5×10^{-9} mol/l of [^{3}H]-E_1S alone (control cells) or in combination with the different compounds: NGMN or MPA, dissolved in ethanol (final concentration <0.2%) at a range of concentrations from 5×10^{-5} to 5×10^{-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) (ATGC) and harvested by scraping. After centrifugation, the pellet was treated with 80% ethanol and the radioactivity extracted for at least 24 h 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 [19]. [^{14}C]-E_2 (5000 dpm) was added to monitor analytical losses, and unlabeled E_1 and E_2 (50 μg) were used as carriers and reference indicators. In the total ethanolic extracts, E_2 was isolated by TLC on silica gel $60F_{254}$ (Merck, Darmstadt, Germany) developed with chloroform–ethylacetate (4:1, v/v) system. After visualization of the estrogens under UV 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 milliliter of Opti-fluor (Packard, Rungis, France) 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 radioactivitiy associated with the cells and then expressed as fmol of E_2 formed/mg DNA.

2.4. Statistical analysis

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

3. Results

3.1. Effect of norelgestromin (NGMN) on estrone sulfatase activity in hormone-dependent MCF-7 and T-47D human breast cancer cells

As E_1S is the most important precursor of E_2 in breast cancer tissues, the inhibition of estrone sulfatase activity can be an important way of preventing the liberation of unconjugated active estrogens from their inactive sulfo-conjugated

forms. Previous studies in this laboratory have clearly demonstrated that various progestins (e.g. medrogestone, nomegestrol acetate) or tibolone, can act as selective estrogen enzyme modulators by inhibiting estrone sulfatase and 17β-HSD or stimulating sulfotransferase activities in breast cancer cells. To this aim, we tested the capacity of the synthetic 19-norprogestin NGMN to block the sulfatase pathway in breast cancer cells.

When physiological concentrations (5×10^{-9} mol/l) of [^{3}H]-E_1S are incubated with the hormone-dependent T-47D and MCF-7 breast cancer cell lines for 24 h at 37 °C, the intracellular production of E_2 in both cells is elevated (1805 ± 152 and 2185 ± 101 fmol/mg DNA for T-47D and MCF-7 cells, respectively) (Figs. 1 and 2).

The data show that the progestin NGMN very intensively decreases the production of E_2 from E_1S in a dose-dependent manner. At low concentrations (5×10^{-9} and 5×10^{-7} mol/l) NGMN is a potent inhibitory agent of the sulfatase pathway in T-47D cells: the inhibition is 43 ± 7 and 74 ± 4%, respectively. These effects are lower in MCF-7 cells where the inhibition found was 25 ± 4 and 57 ± 5%, respectively.

At high concentrations (5×10^{-5} mol/l) the inhibition is very strong in both cells: 97 ± 2 and 96 ± 2% in T-47D and MCF-7 cells respectively. Using non-linear regression analyses, the IC_{50} values, corresponding to the concentration of NGMN which decreases the formation of E_2 by 50%, are 1.27×10^{-8} mol/l and 1.78×10^{-7} mol/l in T-47D and MCF-7 cells, respectively, indicating that NGMN is 14 times stronger in the progesterone receptor (PR)-rich T-47D cells than in MCF-7 cells (Table 1).

Table 1
Determination of the IC_{50} values for NGMN and MPA in the conversion of estrone sulfate to estradiol in MCF-7 and T-47D breast cancer cells

	[IC_{50} values (μmol/l)]	
	T-47D cells	MCF-7 cells
+ NGMN	0.0127	0.178
+ MPA	2.15	26.10

IC_{50} values correspond to 50% inhibition of the conversion of estrone sulfate (E_1S) to estradiol in MCF-7 or T-47D cells by NGMN or MPA. [^{3}H]-E_1S was incubated with cells at physiological concentrations (5×10^{-9} mol/l). The IC_{50} values were determined by using non-linear regression analyses.

3.2. Effect of MPA on estrone sulfatase activity in the hormone-dependent MCF-7 and T-47D human breast cancer cells

MPA is a synthetic progestin derived from 17-hydroxyprogesterone, used as injectable contraceptive, and at high doses as palliative treatment of breast and endometrial carcinoma. The anti-tumoral action of MPA may be due to an effect on the hypothalamo–hypophysis axis, on the estrogen receptor and/or on the metabolism of steroids in tissues.

Figs. 1 and 2 show that at low doses (5×10^{-9} and 5×10^{-7} mol/l) MPA significantly inhibits the E_2 produc-

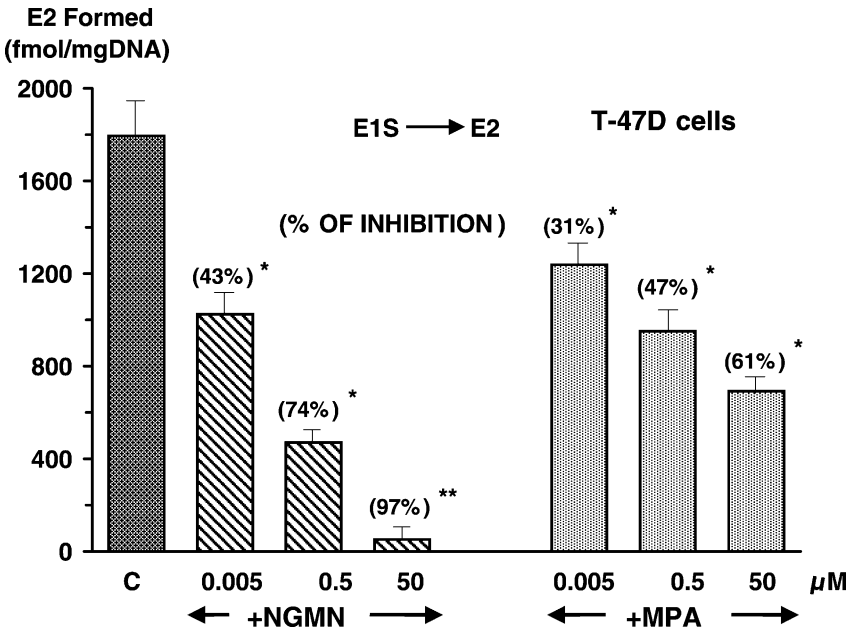

Fig. 1. Effects of NGMN and MPA on the conversion of estrone sulfate (E_1S) to estradiol (E_2) in the hormone-dependent T-47D human breast cancer cell line. Preconfluent cells were incubated for 24 h at 37 °C with a physiological concentration (5×10^{-9} mol/l) of [^{3}H]-E_1S alone (control: non-treated cells) or in the presence of NGMN or MPA at the range of concentrations from 5×10^{-9} to 5×10^{-5} mol/l. Qualitative and quantitative analyses of E_2 in the cell compartment were performed by thin-layer chromatography and liquid scintillation counting as indicated in Section 2. Results are expressed in fmol of E_2 formed/mg DNA from E_1S. The data are the mean ± S.E.M. of duplicate determinations of three independent experiments. * $P = 0.05$ vs. control values (non-treated cells). ** $P = 0.005$ vs. control values (non-treated cells).

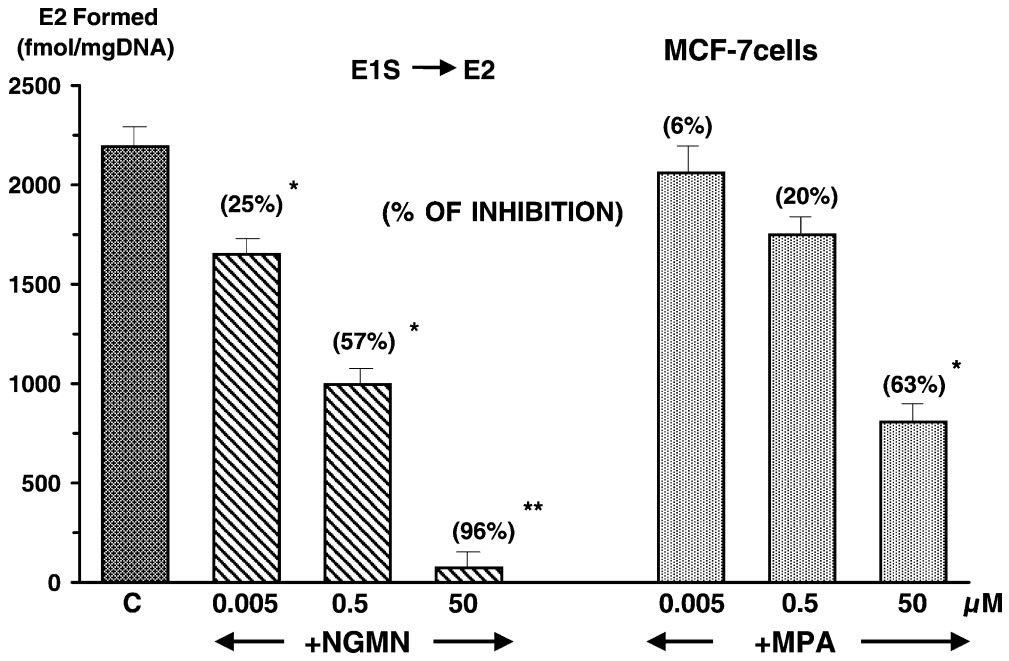

Fig. 2. Effects of NGMN and MPA on the conversion of estrone sulfate (E_1S) to estradiol (E_2) in the hormone-dependent MCF-7 human breast cancer cell line. Preconfluent cells were incubated for 24 h at 37 °C with a physiological concentration (5×10^{-9} mol/l) of [^{3}H]-E_1S alone (control: non-treated cells) or in the presence of NGMN or MPA at the range of concentrations from 5×10^{-9} to 5×10^{-5} mol/l. Qualitative and quantitative analyses of E_2 in the cell compartment were performed by thin-layer chromatography and liquid scintillation counting as indicated in Section 2. Results are expressed in fmol of E_2 formed/mg DNA from E_1S. The data are the mean ± S.E.M. of duplicate determinations of three independent experiments. $^*P = 0.05$ vs. control values (non-treated cells). $^{**}P = 0.005$ vs. control values (non-treated cells).

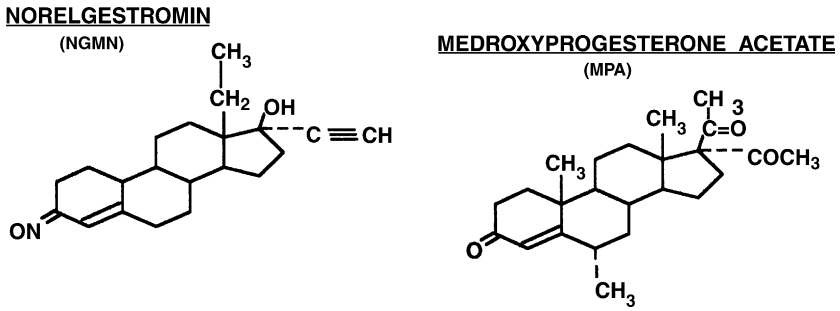

Fig. 3. Structures of the two progestins: norelgestromin (NGMN) and medroxyprogesterone acetate (MPA).

tion from E_1S in T-47D cells only, where the percentage of inhibition is 31 ± 5 and 47 ± 3%, respectively. For MCF-7 cells the inhibition is 6 ± 3 and 20 ± 3%, respectively. At a higher dose (5×10^{-5} mol/l) the inhibitory effect is significant in both cell lines: 61 ± 3 and 63 ± 4%, respectively, in T-47D and MCF-7 cells. The IC_{50} values are 2.15×10^{-6} and 2.61×10^{-5} mol/l in T-47D and MCF-7 cells respectively, indicating that MPA is 12 times stronger in the PR-rich T-47D cells than in the MCF-7 cells (Table 1).

Comparatively, NGMN is 170 and 147 times stronger for inhibiting the sulfatase pathway than MPA in T-47D and MCF-7 cells, respectively. The comparative structures of these two progestins are given in Fig. 3.

4. Discussion

It is recognized that the "sulfatase pathway" is essentially responsible for the intratissular estradiol (E_2) production in hormone-responsive mammary carcinomas or in breast cancer models. 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 from postmenopausal patients [5].

The present data establish that the synthetic progestin NGMN can reduce the conversion of E_1S to E_2 in T-47D

and MCF-7 human mammary cancer cells by blocking the "sulfatase pathway". Despite a similar sulfatase activity in non-treated T-47D and MCF-7 intact cells in culture, NGMN exerts a greater inhibitory effect on this enzyme in the former cell lines, particularly at low doses (5×10^{-9} and 5×10^{-7} mol/l). The concentration of NGMN needed to obtain 50% inhibition of this activity (IC_{50} value) was 1.27×10^{-8} mol/l for the T-47D cells and 1.78×10^{-7} mol/l for the MCF-7 cells. As T-47D cells express high functional levels of PR, a PR-mediated mechanism by which NGMN inhibits estrogen production could be an attractive path to explore.

Norgestimate belongs to the 19-norprogestin series with an oxime group on C-3 that, in combination with ethinyl estradiol, is contained in widely marketed oral contraceptives. It binds to the PR and causes conversion of a proliferative to a secretory endometrium in rabbits after direct injection in the uterine horn. It inhibits luteinizing hormone release from rat pituitary cells in culture, is not estrogenic and is relatively free of androgenic activity [20]. It is well established that norgestimate is rapidly converted into three major metabolites; the 17-deacetylated norgestimate, or NGMN, 3-keto norgestimate and levonorgestrel. NGMN is the primary active metabolite which carries the progestogenic properties of norgestimate. Significant concentrations (3500 pg/ml, 1.5 h after intake) of NGMN were found in the serum of women after administration of norgestimate and it is suggested that NGMN contributes to the pharmacologic response of norgestimate [21]. Studies using gastrointestinal mucosa show also that NGMN is the main metabolic product of norgestimate [22]. NGMN is the progestin contained in the new FDA-approved once-weekly contraceptive patch Ortho Evra/Evra (norelgestromin/ethinyl estradiol, transdermal system) [23]. The present study demonstrates previously unknown biological effects of NGMN.

MPA is extensively used in patients with advanced or recurrent breast cancer [24,25]. The fact that NGMN has more potent anti-sulfatase activity than MPA suggests possible new and interesting uses for NGMN in clinical trials of patients with breast cancer.

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