Recently the first monophasic contraceptive pill containing estradiol has been developed which is thought to be a milestone in contraception. Nomegestrol acetate (NOM) is the progestogenic component. Progesterone receptor membrane component 1 (PGRMC1) is highly expressed in the tissue of breast cancer patients, and can predict a progestogen dependent risk of breast cancer.

Methods: MCF-7 cells were transfected with PGRMC1 expression plasmid, and were stimulated with estradiol (E2, 10(-12) and 10(-10) M). NOM, progesterone (P), medroxyprogesterone acetate (MPA) and norethisterone (NET) (each 10(-7) M) were added sequentially or continuously. Results: E2 at 10(-10) M elicited a significant increase of cell proliferation from 150 to 200%. No effect was seen at 10(-12) M. Addition of the progestogens to E2 at 10(-10) M had no significant effect. However, at an E2 10(-12) M, NET significantly stimulated cell proliferation more pronounced in the continuous combined model. No effect was seen for NOM, P and MPA. The E2/NET combined effect could be abrogated by the addition of an estrogen receptor (ER) antagonist. Conclusion: Since NOM did not increase proliferation it may be concluded that it will be neutral in terms of breast cancer risk when combined with E2 at least in women over expressing PGRMC1.
Nomegestrol acetate sequentially or continuously combined to estradiol did not negatively affect membrane-receptor associated progestogenic effects in human breast cancer cells

Xiangyan Ruan¹, Helen Schneck²*, Hans Neubauer², Yang Yang², Silke Schultz², Tanja Fehm², Michael A Cahill³, Harald Seeger², Alfred O Mueck²

¹ Beijing Ob/Gyn Hospital, Capital Medical University, Beijing, China
² University Women's Hospital, Tübingen, Germany
³ School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia

*: contributed equally

Short running title:

Key words: Progesterone, synthetic progestogens, progesterone receptor membrane component 1, breast cancer cells, proliferation

Corresponding Address
Professor Alfred O. Mueck, MD, PharmD, PhD
University Women's Hospital
Dept. of Endocrinology and Menopause, Head
Centre of Women's Health BW, Head
Calwer Strasse 7
D-72076 Tuebingen, Germany
E-Mail: endo.meno@med.uni-tuebingen.de
Abstract

Objectives:
Very recently the first contraceptive combined pill containing the physiological estradiol instead of ethinyl estradiol has been developed in a monophasic 24/4 system which is thought to be a milestone in contraception. One of the most important questions arises about the action of nomegestrol acetate (NOM), the progestogenic component of this pill, in the breast, since evidence is accumulating that the choice of the progestogen is decisive for a possible increased risk of breast cancer during hormonal applications.

Progesterone receptor membrane component 1 (PGRMC1) is highly expressed in the tissue of breast cancer patients. Previous own work revealed a stimulation of cell proliferation in PGRMC1 overexpressing cells by certain synthetic progestogens. The aim was to investigate the influence of NOM on human breast cancer cells overexpressing PGRMC1 when combined sequentially or continuously with estradiol in comparison to progesterone, medroxyprogesterone acetate and norethisterone.

Methods: MCF-7 cells were stably transfected with PGRMC1 expression plasmid (WT-12). To test the effects of NOM, progesterone (P), medroxyprogesterone acetate (MPA) and norethisterone (NET) (each 10^{-7} M) on cell proliferation, WT-12 cells were stimulated with different concentrations of estradiol (E2, 10^{-12} and 10^{-10} M). The hormones were combined in a sequential and in a continuous combined model.

Results: Estradiol at a concentration of 10^{-10} M elicited a significant increase of cell proliferation of about 150 to 200%. No effect was seen at 10^{-12} M. Addition of the progestogens to E2 at 10^{-9} M had no significant effect. However, at an E2 concentration of 10^{-12} M NET significantly stimulated cell proliferation in both regimen, the effect being more pronounced in the continuous combined model. No effect was seen for NOM, P and MPA. The E2- and NET-induced proliferation could be abrogated by the addition of an estrogen receptor antagonist.

Conclusion: NOM seems to be neutral in terms of breast cancer risk when combined with estradiol at least in women overexpressing PGRMC1. The effect of progestogens on breast cancer tumorigenesis may clearly depend on the specific pharmacology of the various synthetic progestogens.

Introduction

Nomegestrol acetate (NOM) is a synthetic progestogen which is a norpregnane derivative, i.e. derived from 19-norprogesterone. In some countries NOMAC is used alone in the dosage of 2.5-10 mg/day particularly for menstrual cycle disorders or other bleeding problems or as subdermal silastic implant containing 38 mg NOMAC with a release rate of about 75 µg/day for one year for hormonal contraception [1-3]. In combination with estradiol (E2) NOM is used as hormone replacement therapy (HRT) in a dosage of 5 mg for 12-14 days for sequential and with 2.5-3.75-5 mg for continuous combined HRT, whereby larger prospective randomized trials until today still are lacking [4-7].

Very recently NOM also has been combined with E2 for the use in a new combined oral contraceptive pill (COC), the first hormonal contraception containing the physiological hormone estradiol instead of the synthetic ethinyl estradiol in a "monophasic" 24/28 system, i.e. with the same dosage of E2 (1.5 mg) combined with NOM (2.5 mg) given every day, applied for 24 days followed by 4 placebo tablets [8].
The development of the new pills based on the natural estrogen is thought as one of the most important milestones in 'gynecological endocrinology', because since 50 years only ethinyl estradiol has been used as component for combined hormonal contraception. However, up to date a monophasic pill with estradiol obviously could be achieved only with the unique progestogen NOM, new for oral contraception, and the first available progestogen derived from 19-norprogesterone, offering special properties like high endometrial activity, long elimination half-live and strong antigonatropic action, the preconditions to get cyclical stability comparable with combined hormonal contraception containing ethinyl estradiol [9].

However, since the progestogen component of hormonal applications is thought to be decisive for a possible increased risk of breast cancer, the question arises about the effect of this special progestogen in the breast. Particularly of interest should be the comparison with progestogens already shown to be able to increase the breast cancer risk, like medroxyprogesterone acetate (MPA) in the Women's Health Initiative (WHI) study [10], in contrast to estrogen-only therapy [11], or with norethisterone in several observational studies [12], in contrast to natural progesterone, which might be more neutral according to results like in the E3N-Study [13] correlating to our own previous experimental work [14]. Thus, our primary aim was to test the effect of NOM in human breast cancer cells comparing with these three progestogens when combined with estradiol, whereby we decided to test sequential versus continuous combination of the progestogens which should give us more information of the effect of the progestational component.

Thereby we decided to use an experimental model already validated by our recent previous work published elsewhere [15] accompanied by an editorial entitled "Can the increase in breast cancer observed in the estrogen plus progestin arm of the Women’s Health Initiative trial be explained by progesterone receptor membrane component 1 (PGRMC1)?" [16].

PGRMC1 has been found to be highly expressed in the tissue of breast cancer patients [17]. Previous own work revealed a stimulation of cell proliferation in PGRMC1 overexpressing cells by certain synthetic progestogens [15,18,19,20], underlining a good reproducibility of our experimental model.

PGRMC1 is associated with a high membrane-associated progesterone receptor activity in addition to the classical intracellular-located receptors, according to recent experimental data [21]. PGRMC1 was originally cloned from the endoplasmatic reticulum from porcine hepatocytes [22]. It contains several predicted motifs for protein interactions, and overlapping sites for phosphorylation, whose phosphorylation status might correlate with its localisation in the cell [21,23,24].

In our previous studies we could show that certain progestogens are able to stimulate particularly the proliferation of MCF-7 cells overexpressing PGRMC1, much more if compared to malignant cells not expressing this receptor [18,19,20]. In addition these cells were more sensitive against a stimulatory effect of E2 as compared to the wild-type MCF-7 cells [15].
Methods

Cell cultures
MCF-7, a human estrogen receptor (ER) positive primary breast cancer cell line, was purchased from American Type Culture Collection (ATCC). Cells were routinely cultured in RPMI-1640 medium containing 10% (v/v) heat inactivated fetal calf serum (FCS), 25mM HEPES and 1% penicillin /streptomycin at 37°C in a humid 5% CO₂ atmosphere.

Transfection of MCF-7 cells (WT-12 cells)
MCF-7 cells were stably transfected with expression vector pcDNA3.1 containing hemeagglutinin-tagged (3HA) PGRMC1 described earlier [18]. Stable transfection was verified by PCR using chromosomal DNA and primers spanning intron 1 to distinguish integrated PGRMC1 cDNA from the chromosomal sequence. For experiments we used MCF-7/PGRMC1 clone WT-12.

Dissolution of estradiol, progesterone, synthetic progestogens and receptor antagonists
Estradiol (E2), progesterone (P) and the synthetic progestogens medroxyprogesterone acetate (MPA) and norethisterone (NET) were purchased from Sigma (Munich, Germany). Nomegestrol (NOM) was purchased from LGM Pharma (Boca Raton, USA). AG-205, an antagonist selective for PGRMC1, was purchased from Timtec, Inc. (Newark, USA). Fulvestrant, an antagonist of estrogen receptor-alpha, and RU 486, an antagonist of progesterone receptor A/B were purchased from Sigma (Munich, Germany). All compounds were dissolved in ethanol, stored as 10⁻² M at -20°C and further diluted during experiments to a final ethanol concentration of less than 0.01%.

Cell proliferation
Proliferation of treated cells was determined measuring the enzymatic cleavage of the tetrazolium ring of the yellow tetrazolium salt 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) by mitochondrial dehydrogenase resulting in a blue, water insoluble formazane salt. The MTT salt was dissolved in RPMI 1640 medium without phenol red to a concentration of 1 mg/ml, filtered sterile and further diluted 1:4 with RPMI 1640 medium without phenol red.

For the assay, medium was decanted from the 96 well plate used to incubate the cells. 100 μl MTT solution was added to each well and incubated for 3 h at 37°C. Then the crystallized formazane salt was centrifuged for 10 min at 400 g and the supernatant was discarded. The resulting salt was solubilized in 100 μl sterile DMSO added into each well and the plate was shaken for 7 min at room temperature. Analysis was performed using an ELISA-Reader at a wavelength of 550 nm. The resulting extinction is proportional to the number of cells present in the well. In internal experiments we have validated the MTT-assay against the bromodeoxyuridine (BrdU)-assay. In these head-to-head experiments the correlation coefficient was 0.96.

Hormone combination models
Sequential hormone combination was imitated by using estradiol at a concentration of 10⁻¹² and 10⁻¹⁰ M for 3 days and then addition of progestogens at a concentration
of $10^{-7}$ M for further 3 days. Continuous hormone combination was imitated by using estradiol at concentrations of $10^{-12}$ and $10^{-10}$ M in combination with the progestogens at a concentration of $10^{-7}$ M for 6 days incubation.

Expression of estrogen receptor alpha
Cells were seeded in a small flask (75mm$^3$) and cultured at 37°C and 5% CO2 in RPMI medium without phenol red and either 10% normal FCS or FCS deprived of hormones ('stripped FCS'). After reaching about 80% confluency cells were lysed with ice cold M-PER Mammalian Protein Extraction Reagent containing protease inhibitors. Protein concentration was determined using the BCA Protein Assay Kit (both from PIERCE, Rockford, USA). 20µg protein was loaded per lane on a 10% polyacrylamide gel and separated by electrophoresis.

The gel was blotted on Hybond ECL nitrocellulose membrane (Amersham, Piscataway, USA) and blocked overnight at 4°C using 5% milk in TBST buffer. Western blot for ERalpha was performed using rabbit anti ERalpha (H-184, 1:1000, Santa Cruz biotechnology, Santa Cruz, USA) over night at 4°C. As secondary antibodies goat anti-rabbit IgG-horse raddish peroxidase (1:2500, Santa Cruz) followed by streptavidin/HRP (1:1000, DakoCytomation, Hamburg, Germany) was applied. Loading control was performed with rabbit anti-actin (I-19; 1:500, Santa Cruz) for 2 h at room temperature followed by goat anti-rabbit IgG-horse raddish peroxidase (1:2500, Santa Cruz) for 1 h. Chemoluminescence was generated using ECL Western Blotting Analysis System (Amersham). The signals were measured with a Lumi-Imager (Boehringer, Mannheim, Germany).

Statistical Analysis
All proliferation experiments were done in triplicate and were repeated at least three times, with each experiment yielding essentially identical results. Statistical analysis was done by ANOVA with the logarithmated values followed by Dunnett's procedure from triplicates of at least three independent experiments. The overall alpha level was set at 0.05.

Results
Incubation of estradiol alone for 6 days resulted in a significant 2-3 fold increased proliferation at the concentration of $10^{-10}$ M, no significant effect was seen at $10^{-12}$ M. Addition of progesterone or the synthetic progestogens in a sequentially or continuously combined manner did not significantly alter the estradiol-induced proliferation (Fig. 1a and 1b). However, NET showed a significant increased proliferation rate when combined with estradiol in a concentration of $10^{-12}$ M. The NET-induced increase was about 50 % in the sequential regimen and about 150% in the continuous regimen. The difference between these regimen was statistically significant (p<0.01).

In Figure 2 it is shown that the effect of NET is only prominent in MCF-7 cells transfected with PGRMC1 and not in MCF-7 transfected with empty vector.

In blocking experiments antagonists of the estrogen receptor alpha (fulvestrant), progesterone receptor (RU 486) or PGRMC1 (AG 205) were used alone and in the various combinations to reveal the potential involvement of these receptors in the
E2/NET-induced proliferative signalling in WT-12 cells. Figure 3 shows that only the presence of fulvestrant was able to totally block the EP effect combination, i.e. estradiol plus NET. Partial significant effects could be found in the presence of AG 205, no effect was observed for RU 486.

The expression of estrogen receptor alpha is enhanced in WT-12 cells as compared to MCF-7 wild-types cell (Figure 4). The results on PGRMC1 mutants, although showing a stronger expression of estrogen receptor alpha in this Figure, suggest the exclusion of a clonal selection.

Discussion

The results the Women’s Health Initiative (WHI) indicate a negative effect of progestogens with respect to breast cancer risk [10,11]. However, it remains unclear whether the combination of estrogens with synthetic progestogens and/or natural progesterone may elicit the same increased risk. Thus, many questions concerning the extrapolation of the WHI results to all synthetic progestogens and to natural progesterone remain unanswered. The new NOM/E2 pill recently has been approved in the European Union with respect to granted marketing authorization by the European Commission for the prevention of pregnancy [9]. This intended us to test this special progestogen regarding its effect in breast cancer cells, because until now studies are lacking investigating its effect on breast cancer development.

Experimental results indicate that progestogens are different in their ability to induce proliferation or inhibit the growth of benign or malignant human breast epithelial cells dependently or independently of the effects of stromal growth factors and estradiol [14,25-29]. In previous experiments we could show that in MCF-7 cells PGRMC1 is able to mediate an up to tenfold increase in proliferation when activated by certain testosterone-related progestogens e.g. norethisterone [20]. Apart from progesterone also certain progesterone-related progestogens such as medroxyprogesterone acetate did not cause an increase in proliferation in WT-12 cells. The mechanism(s) by which PGMRC1 mediates proliferation elicited by progestogens is currently under investigation. However, we already published that ERK2 expression is downregulated in MCF-7/PGRMC1 cells [18].

Additionally in a previous work we demonstrated that estradiol in a dosage that increased cell numbers of MCF-7 cells was able to induce an effect in WT-12 cells that is 3 to 4-fold higher than in MCF-7 cells. This publication has been positively commented by a special editorial [16]. This effect could be blocked by the addition of the potent estrogen receptor antagonist fulvestrant indicating that the intracellular estrogen receptor-alpha is involved. However, since the proliferation was twice as high as in MCF-7 cells, in the presence of PGRMC1 a mechanistic interaction between the estrogen receptor-alpha and PGRMC1 signaling systems seems to be highly possible. The mechanism(s) of interaction is currently unknown. Our investigations furthermore showed that the E2 effect on WT-12 cells is clearly dose-dependent. E2 elicited a significant increase in cell numbers only at a concentration of $10^{-10}$ M. Thus oral or transdermal administration may be of importance in the presence of PGRMC1. The clinical study of Opatrny et al. indicated that indeed transdermal hormone therapy may have a lower breast cancer risk than oral
administration [30].

In the present manuscript we compared the addition of progesterone and synthetic progestogens to estradiol in a sequentially or continuously combined regimen. When PGRMC1 is overexpressed, as in WT-12 cells, the E2-induced effect is dominating especially at a concentration of 10^{-10} M. However, the addition of NET triggered a strong proliferative signal in the presence of the low E2 concentration and the figures are not significantly different to those obtained for the combination with the high E2 concentration. No effect was found for progesterone, MPA and NOM. This indicates that also in WT-12 cells the presence of low E2 concentration sensitizes the cells against the addition of a synthetic progestin. However the NET effect was significantly lower in the sequentially combined regimen as compared to the continuous combined regimen suggesting that women expressing the PGRMC1 may benefit from a sequentially regimen when using NET as progestin for hormone therapy.

It appears that the presence of estrogen receptor-alpha is mandatory for the observed proliferative effect, since only fulvestrant was able to totally block the effect of estradiol/progestogen combination. Our Western blot experiments clearly showed that the expression of estrogen receptor alpha is significantly stronger in WT-12 cells than in wild-type MCF-7 cells. This might be one reason for the higher estrogen sensitivity of these cells and a cross-talk between estrogen receptor alpha and PGRMC1 seems to be highly possible.

Although so far clinical studies investigating the effect of NOM on breast cancer development are still lacking, experimental research is very encouraging that with NOM the hormone-dependent risk may be low or even not increased. NOM is able to alter estrogen metabolism via the sulfatase pathway in normal breast and also in breast cancer cells such that the amount of estradiol is reduced in these cells: NOM can block the sulfatase activity regarding the conversion of estrone sulfate (E1S) to estrone (E1), particularly in cancerous breast tissue, but also can exhibit a stimulatory effect on the sulfotransferase activity in different cell lines, resulting in the conversion of E1 to biologically inactive E1S [31,32].

In addition in human breast cancer cells NOM can decrease the activity of 17ß-hydroxysteroid-dehydrogenase Type 2 which blocks the reaction from E1 to E2 [32]. Moreover, in a stable aromatase expressing cell line, NOM was able to inhibit aromatase activity [33]. All those reactions lead to a decreased estrogenic activity in benign as well as in malignant breast cells. Since the presence of estrogen in the breast is suggested to be a precondition for additive proliferating progestogen effects, these effects even might lead to a decrease of risk [32,34].

Regarding proliferating effects, NOM did not stimulate cell proliferation in MCF-7 and T47-D human breast cancer cell lines, whereas norgestrel and gestodene had stimulatory effects, mediated via the estrogen receptor [27,35]. Our own experiments presented here demonstrated that NOM did not induce any proliferative action in MCF-7 cells overexpressing PGRMC1 in contrast to other progestogens used for hormonal contraception and hormone therapy.
Of special interest in the present manuscript is the investigation of an estradiol/nomegestrol acetate combination for the first time. NOM has been combined with E2 for the use in a new combined oral contraceptive pill, the first hormonal contraception containing estradiol in a "monophasic" 24/28 system [8]. This new contraceptive pill delivering the natural estrogen instead of the synthetic ethinyl estradiol (EE) is expected to elicit particularly good safety profiles regarding cardiovascular risks, since the risk of venous and arterial thromboembolism mainly has been associated with the use of EE. Large clinical studies on this issue are already running (9). In summary of our present results and all those other experimental data described above regarding effects in breast, it seems very promising to demonstrate the neutral effect of NOM also in a clinical endpoint study also in the breast.

Despite their widespread use, in vitro models have certain limitations: the choice of culture conditions can unintentionally affect the experimental outcome, and cultured cells are adapted to grow in vitro; the changes which have allowed this ability may not occur in vivo. A further limitation of our work is the short incubation period of the cells with the substrates under investigation, in comparison to the longer time period for which hormone therapy is usually prescribed. Concerning the concentrations used for the steroids we have tried to imitate the clinical situation. Serum concentrations of progesterone during HT are in the range of $10^{-9}$ M to $10^{-7}$ M, i.e. concentrations which do in fact have an effect in our experiments. Estradiol serum concentrations are in the range of $10^{-10}$ to $10^{-9}$ M when using 2 mg estradiol. However, experimental investigations can only point to mechanism(s) and clearly cannot replace clinical studies. Additionally we did not compare the combination of E2 with NOM to that of EE with NOM in the present study. These studies are ongoing. However, we already showed that EE tends to have a lower proliferative potency than E2 [36] at least in human breast cancer cells and according to WHI the progestogenic component seems to be more important in terms of breast cancer risk.

**Conclusion**

Our results suggest that women with breast cancer cells that overexpress PGRMC1 may be more susceptible in developing breast cancer and thus may have a much higher breast cancer risk when treated with estrogen combined with certain synthetic progestogens e.g. medroxyprogesterone acetate and norethisterone than those not expressing PGRMC1. However, certain progestogens such as progesterone and nomegestrol acetate, derived from progesterone, had a neutral effect. In addition our results indicate that sequential estradiol/progestogen combinations may present a lower breast cancer risk as compared to a continuous combined regimen at least in breast tissue overexpressing PGRMC1 suggesting the significance of the progestogenic component. The use of nomegestrol acetate may add no further breast cancer risk when added to estradiol in hormone therapy or hormonal contraceptive pill. To prove this also in a clinical endpoint study seems to be important in the light of the new combined contraceptive monophasic pill, which for the first time has been very recently available on the basis on a natural estrogen component.
References


2) Coutinho EM. One year contraception with a single subdermal implant containing nomegestrol acetate (Uniplant). Contraception 1993; 47: 97-105


8) Mueck AO, Sitruk-Ware R. Nomegestrol acetate, a novel progestogen for oral contraception. Steroids 2011; 76: 531-539

9) Ruan X, Seeger H, Mueck AO. The pharmacology of Nomegestrol acetate. Maturitas 2012, in press


16) Stanczyk FZ. Can the increase in breast cancer observed in the estrogen plus progesterone arm of the Women’s Health Initiative trial be explained by progesterone receptor membrane component 1? Menopause 2011 18:833-4.


Legends

Figure 1: WT-12 cells were incubated with estradiol (E2, 10-12 or 10-10 M) alone or in sequential (a) or continuous (b) combination with progesterone (P), medroxyprogesterone acetate (MPA), norethisterone (NET) and nomegestrel (NOM) (each 10-7M). Cell proliferation was measured after 5 days. Data were normalized to unstimulated control cells. (Means ±SD; * p<0.05; ** p< 0.01 vs. control)

Figure 2: Proliferative response of different concentrations of norethisterone (NET) in MCF-7 cells, MCF-7 cells overexpressing PGRMC1 and MCF-7 cells transfected with a vector control (MCF7-HATAG). Means ± SD; ** p< 0.01 vs. controls

Figure 3: Blocking experiments with fulvestrant (FUL, 10-6 M), RU 486 (RU, 10-6 M) and AG205 (AG, 10-6 M) when added to an estradiol (10-12M, 10-10 M) / norethisterone (10-7 M) combination (EP). Means ± SD; ** p< 0.01 vs. EP

Figure 4: Expression of ERalpha in MCF7 and MCF7/PGRMC1 cells. Depicted are western blot results for parental MCF7 cells and cell lines derived thereof transfected with plasmids overexpressing PGRMC1 wild type or PGRMC1 with mutated serines 56 and 180 to alanine. Cells were cultured in medium containing normal or stripped FCS. Upper row: signals for ER-alpha; Lower row: signals for beta-actin
Figure 1

Figure 2
Figure 3

![Graph showing percentage change in ERalpha expression with different treatments and conditions.]

Figure 4

![Diagram showing ERalpha and Actin expression under different conditions: Normal FCS and Stripped FCS.]

Normal FCS  
'Stripped' FCS

MCFT  | MCFT/PGRMC1 (WT)  | MCFT/PGRMC1 (S56A)  | MCFT/PGRMC1 (S56/S180A)

ERalpha  
Actin