



The effect of progesterone, testosterone and synthetic progestogens on growth factor- and estradiol-treated human cancerous and benign breast cells

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Abstract

Objective: The effects of progesterone (P), testosterone (T), chlormadinone acetate (CMA), medroxyprogesterone acetate (MPA), norethisterone (NET), levonorgestrel (LNG), dienogest (DNG), gestodene (GSD) and 3-ketodesogestrel (KDG) were investigated in normal human breast epithelial MCF10A cells. In addition, the effects of these steroids were tested in estrogen and progesterone receptor positive HCC1500 human primary breast cancer cells.

Study design: MCF10A cells were incubated with each progestogen at 1 μ M and 100 nM for 7 days with growth factors epidermal growth factor (EGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF-I) (GFs, each 1 pM), and HCC1500 cells with GFs and/or estradiol (E2) 100 pM. Cell proliferation rate was measured by ATP-assay and cell death by photometric enzyme immunoassay. Ratios of cell death:proliferation were calculated.

Results: MPA and CMA with GFs induced proliferation of MCF10A cells. P, T, NET, LNG, DNG, GSD and KDG had no significant effect. In HCC1500 cells, MPA and CMA with GFs had an inhibitory effect compared to GFs alone. NET, LNG, DNG, GSD, KDG and T enhanced the proliferative effect of GFs. P had no significant effect. No progestogen could further enhance the stimulatory effect of E2 on HCC1500 cells; all but KDG inhibited it. MPA, GSD, T, CMA and NET had an anti-proliferative effect on the mitotic GF and E2 combination. P, LNG, DNG and KDG had no significant effect.

Conclusions: Estrogens and mitogenic growth factors from stromal breast tissue are significant in growth-regulation of breast cells and may alter responses to progestogens. Certain progestogens are able to induce proliferation of or inhibit growth of benign or malignant human breast epithelial cells independently of the effects of growth factors and E2; therefore, choice of progestogen for hormone therapy may be important in terms of influencing possible breast cancer risk.

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1. Introduction

Estrogen deficiency symptoms in the peri- and post-menopause have successfully been treated with estrogens since the 1940s. Around 25% of women go through the menopause without suffering debilitating vasomotor, psychological and physiological symptoms, and most symptomatic women become symptom free about 5 years after the

menopause. However, a symptomatic patient, whether as a result of a natural or surgically induced menopause, and with or without osteoporosis, may be given the option of replacement of her natural estrogens with hormone replacement therapy (HRT). The connection between estrogens and breast cancer has been acknowledged for over 100 years since Beatson demonstrated that bilateral oophorectomy resulted in remission of breast cancer in post-menopausal women [1], and ensuing evidence has implicated estrogen, both endogenous and exogenous, in the pathogenesis of breast cancer [2]. The addition of

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progestogens to estrogen replacement therapy to protect the endometrium from the proliferative effects of unopposed estrogen in non-hysterectomised patients has added a new dimension to the HRT controversy.

Two recent studies, the Women's Health Initiative (WHI) and the Million Women Study (MWS), have raised concern over the relationship between progestogens and increased risk of breast cancer in the climacteric and post-menopause [3,4]. The WHI, with a planned duration of 8.5 years, was terminated early after a mean of 5.2 years due to an increased incidence of breast cancer in the treatment group (i.e. those patients taking conjugated equine estrogens (CEE) 0.625 mg/day plus medroxyprogesterone acetate 2.5 mg/day) compared to those in the placebo (no treatment) group. This was the first randomised prospective study to investigate the possible primary prevention of coronary heart disease in post-menopausal women using this HRT combination. In contrast, the results of the estrogen-only arm of the WHI, which used oral CEE (0.625 mg/day) alone, showed a possible reduction in breast cancer risk, with a 23% lower rate of breast cancer in the CEE group than in the placebo group [5]. The MWS concluded that breast cancer risk was increased two-fold in current users of combined HRT compared to a factor of 1.3 for estrogen-only therapy; however, the accuracy of the MWS design, analysis and conclusions have been much questioned.

Tissue regulation by estrogen and progesterone at local tissue level is modulated in a very complex arrangement by locally acting growth factors, by epithelial cellular differentiation, by epithelial cell–cell and cell–stromal adhesion, by various stromal cell types and by additional, poorly understood serum factors [6]. By stimulating the production of survival factors such as growth factors and cytokines, estradiol (E2) and other steroid hormones can increase cell proliferation. High affinity binding of these proteins to their relevant steroid hormones causes favoured retention of the steroid in the target tissue, and gives then a major role in the development and growth of the target tissue and in breast cancer development. Epithelial and stromal cell-derived growth factors are understood to be significant in the regulation of breast cancer cells directly via autocrine or intracrine, paracrine or juxtacrine and endocrine pathways. Further responses stimulated by growth factors may activate signalling pathways which support the growth of cancer cells [7].

To further explore these findings *in vitro*, we investigated the effect of progesterone, testosterone and seven synthetic progestogens, currently used in or under investigation for use in HRT preparations, on growth factor- and/or E2-treated normal breast epithelial MCF10A cells and estrogen-receptor positive HCC1500 primary breast cancer cells. Previous in-house experiments (unpublished) have shown that clinically significant effects of the progestogens were only seen in the presence of growth factors and/or estradiol, and that this environment takes steps to more realistically represent the *in vivo* milieu compared to a hormone-free

environment. Therefore, the results of the effects of the progestogens alone are not presented.

2. Materials and methods

Progesterone (P), chlormadinone acetate (CMA), norethisterone (NET), medroxyprogesterone acetate (MPA), testosterone (T) and estradiol were purchased from Sigma Chemicals. Gestodene (GSD) and 3-ketodesogestrel (KDG) were kindly provided by Wyeth Pharma, Münster, Germany, and dienogest (DNG) by Jenapharm, Jena, Germany. The compounds were dissolved in ethanol and were stored as concentrated stock solutions at -20°C .

Epidermal growth factor (EGF), basic-fibroblast growth factor (bFGF) and insulin-like growth factor (IGF-I) were purchased from Sigma Chemicals. The compounds were reconstituted according to the manufacturer's instructions stated on the package insert and were stored in aliquots at -20°C .

MCF10A, a human, non-tumorigenic, estrogen and progesterone receptor-negative breast epithelial cell line, was purchased from American Type Culture Collection (ATCC), USA. Cells were maintained in serum-free Mammary Epithelial Cell Medium purchased from Promo-Cell, Germany, supplemented with 100 ng/ml cholera toxin purchased from List Biological Laboratories Inc., CA, USA, and 100 U/ml penicillin plus 100 $\mu\text{g}/\text{ml}$ streptomycin.

HCC1500, a human estrogen and progesterone receptor-positive primary breast cancer cell line, was also purchased from ATCC. Cells were maintained in RPMI-1640 medium (without phenol red) purchased from Sigma, which was modified to contain 1 mM sodium pyruvate, 2 mM L-glutamine, 4.5 g/l glucose, 10% (v/v) heat inactivated foetal bovine serum and 100 U/ml penicillin plus 100 $\mu\text{g}/\text{ml}$ streptomycin.

In-house experiments using RT-PCR techniques have demonstrated the absence of estrogen (ER- α and ER- β), progesterone (PR-A and PR-B) and androgen receptors (AR-A and AR-B) in the MCF-10A cells and the presence of estrogen, progesterone and androgen receptors in the HCC1500 cell line.

All assays were conducted using serum-free medium for MCF10A cells and charcoal-treated serum-containing medium for HCC1500 cells. Stock concentrations of progestogens, growth factors and E2 were further diluted with these assay media during working experiments to give a final ethanol concentration of less than 0.01% per well.

Ninety-six well plates were seeded with approximately 1000 cells per well. The cells were incubated for 3 days at 37°C in the appropriate culture medium. Progestogens in the concentrations of 1 μM (10^{-6} M) and 100 nM (10^{-7} M) in combination with growth factors (EGF, FGF and IGF) 1 pM (10^{-12} M) and/or E2 100 pM (10^{-10} M) were then freshly added three times over the next 7 days, i.e. at days 1,

3 and 5. Due to a very low inter- and intra-assay variability, the experiments were only performed in triplicate.

Cell proliferation was measured on day 7 by the ATP chemosensitivity test, where proliferation is quantified by measuring light emitted during the bioluminescence reaction of luciferene in the presence of ATP and luciferase [8]. This assay has been validated in the routine laboratory of our hospital, where it has been in use for several years to evaluate the efficacy of chemotherapy agents for the treatment of breast cancer patients.

Cell death was measured on day 7 using a Cell Death Detection ELISA kit, purchased from Roche Applied Science. The assay is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. Photometric enzyme immunoassay quantitatively determines cytoplasmic histone-associated DNA fragments after induced cell death [9]. This kit was validated according to the manufacturer's instructions using a cytostatic cisplatin test.

2.1. Statistical analysis

Proliferation and cell death were measured versus controls and performed in triplicate. From these values, the ratios of cell death:proliferation were calculated along with the standard deviation (S.D.). Ratios under the control value (assay medium only) favour a proliferative response, whereas ratios over control favour an anti-proliferative response. We have evaluated this ratio several times in previous experiments with good reproducibility.

Statistical analysis was done by ANOVA with the logarithmated values followed by Dunnett's procedure from triplicates of two independent experiments. The overall alpha level was set at 0.05.

3. Results

Preliminary experiments were performed to explore the time response curves and the effective dosages of the individual growth factors and estradiol. The results revealed that the maximum proliferative effect of the individual growth factors and estradiol were 1 and 100 pM, respectively. Therefore, these dosages were used for the following experiments. The maximum proliferative effect occurred between 3 and 7 incubation days.

The cell death:proliferation ratio results for the effect of the steroids in combination with growth factors on the MCF10A cell line are illustrated in Fig. 1. The combination of the growth factors EGF, FGF and IGF-I alone confirmed a proliferative response compared to the assay medium-only control.

In combination with growth factors, the ratio was reduced significantly compared to the growth factor alone control by MPA and CMA (i.e. favouring an additional proliferative effect). MPA produced a four-fold reduction in the ratio in comparison to growth factors alone at both concentrations ($p < 0.05$), CMA had a significant effect at 1 μM only, reducing the ratio three-fold. P, NET, levonorgestrel (LNG), DNG, GSD, KDG and T had no significant effect on the growth factor-induced stimulation of MCF10A.

The results for the effect of the steroids in combination with growth factors on the HCC1500 cell line are illustrated in Fig. 2. Again, the combination of the growth factors EGF, FGF and IGF-I alone confirmed a proliferative response compared to the assay medium-only control. MPA in combination with growth factors caused a significant increase in the ratio at both concentrations compared to growth factors alone ($p \leq 0.05$), the greatest effect being at 100 nM, with a doubling of the ratio, i.e. an inhibitory effect. CMA also caused a significant increase in the ratio, with the greatest effect seen at 1 μM , yielding over a two-fold ratio

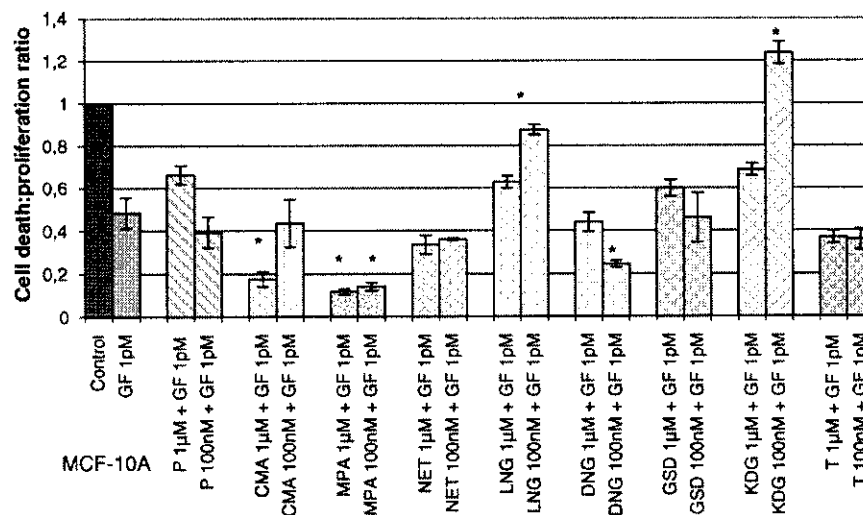


Fig. 1. Mean of the cell death:proliferation ratios in MCF10A cells by various steroids in combination with EGF, FGF and IGF-I (each 1 pM) (control, medium-only control; GF, EGF + FGF + IGF-I; P, progesterone; CMA, chlormadinone acetate; MPA, medroxyprogesterone acetate; NET, norethisterone; LNG, levonorgestrel; DNG, dienogest; GSD, gestodene; KDG, 3-ketodesogestrel; T, testosterone). $p < 0.05$.

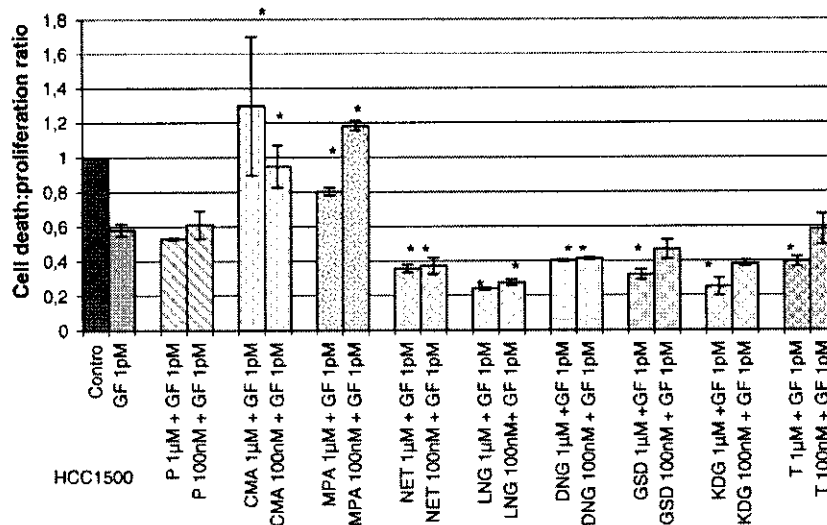


Fig. 2. Mean of the cell death:proliferation ratios in HCC1500 cells by various steroids in combination with EGF, FGF and IGF-I (each 1 pM) (control, medium-only control; GF, EGF + FGF + IGF-I; P, progesterone; CMA, chlormadinone acetate; MPA, medroxyprogesterone acetate; NET, norethisterone; LNG, levonorgestrel; DNG, dienogest; GSD, gestodene; KDG, 3-ketodesogestrel; T, testosterone). * $p < 0.05$.

increase. Conversely, NET, LNG and DNG at both concentrations and GSD, KDG and T at 1 μM led to a significant reduction in the ratio, enhancing the initial proliferative effect induced by the growth factors. P had no significant effect at either concentration.

Fig. 3 shows the results of the combination of the steroids and E2 100 pM on the estrogen-receptor positive (ER+) HCC1500 cells. As expected, E2 alone promoted a proliferative effect on cell growth. In combination with E2, the progestogens CMA, MPA, NET, LNG, DNG, GSD and T at both concentrations, and P at 1 μM significantly

increased the ratio towards an anti-proliferative effect to varying degrees compared to E2 alone, with MPA 1 μM having the greatest effect, followed by NET at 100 nM. KDG had no significant effect at either concentration. No progestogen used was able to further enhance the stimulatory effect of E2 on HCC1500 cells, and all but KDG actually inhibited this effect.

The results of combining the steroids with the combination of growth factors (EGF, FGF and IGF-I) and E2 on HCC1500 cells are illustrated in Fig. 4. MPA, GSD and T at both concentrations and CMA 1 μM and NET at 100 nM all

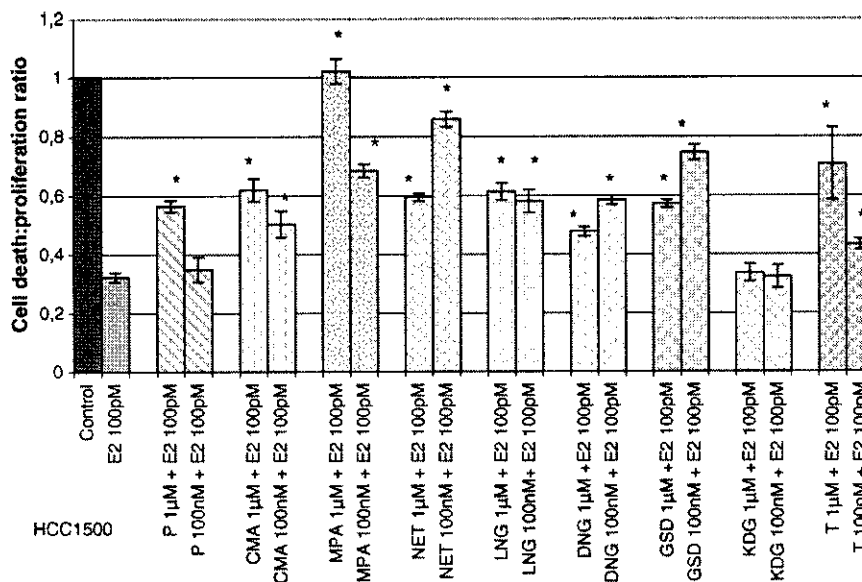


Fig. 3. Mean of the cell death:proliferation ratios in HCC1500 cells by various steroids in combination with estradiol (100 pM) (control, medium-only control; E2, estradiol; P, progesterone; CMA, chlormadinone acetate; MPA, medroxyprogesterone acetate; NET, norethisterone; LNG, levonorgestrel; DNG, dienogest; GSD, gestodene; KDG, 3-ketodesogestrel; T, testosterone). * $p < 0.05$.

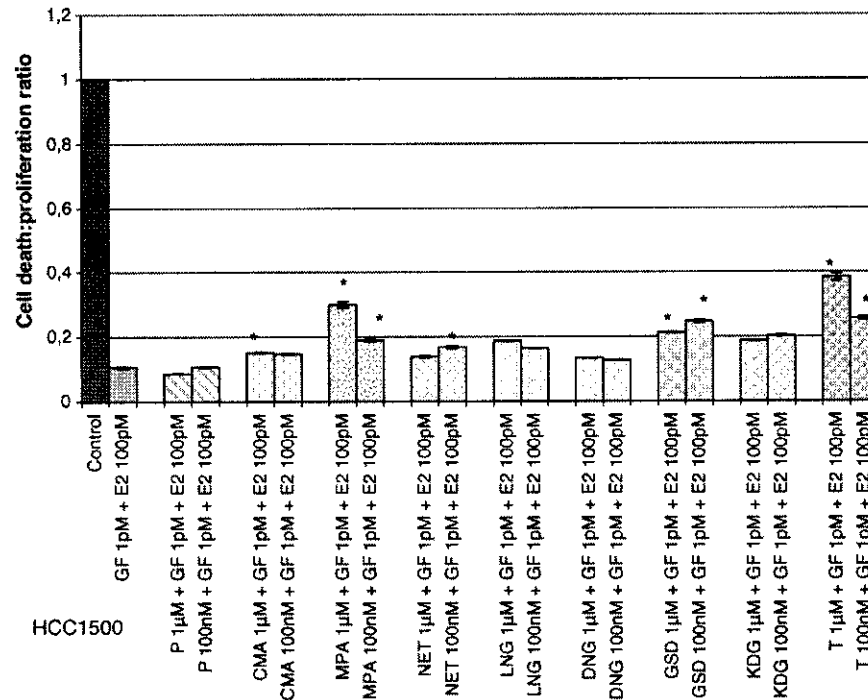


Fig. 4. Mean of the cell death:proliferation ratios in HCC1500 cells by various steroids in combination with EGF, FGF and IGF-I (each 1 pM) and estradiol (100 pM) (control, medium-only control; GF, EGF + FGF + IGF-I; E2, estradiol; P, progesterone; CMA, chlormadinone acetate; MPA, medroxyprogesterone acetate; NET, norethisterone; LNG, levonorgestrel; DNG, dienogest; GSD, gestodene; KDG, 3-ketodesogestrel; T, testosterone). * $p < 0.05$.

increased the ratio favouring an anti-proliferative effect compared to the proliferative effect of growth factors and E2 alone. P, LNG, DNG and KDG had no significant effect at either concentration.

4. Discussion

The proliferation of normal and malignant cells is under the control of both estrogen and growth factors. In normal epithelial cells, estrogen-receptor expressing cells represent only a minority of the total cells and do not proliferate [10]. Current opinion is that estrogens act proliferatively in a paracrine fashion by inducing the production of stromal-derived growth factors and cytokines or their receptors via the activation of epithelial or stromal estrogen receptors. Growth factors may play an important role in the promotion of receptor-positive breast cancer by cross-talk with the steroid-receptor and are mainly responsible for the progression of estrogen-receptor negative breast cancer. Among the growth factors which are important for cell growth are the epidermal growth factor family, insulin-like growth factors I and II (IGF-I and IGF-II), fibroblast growth factors (FGFs), transforming growth factor- α (TGF- α) and platelet-derived growth factors (PDGFs).

It is important to differentiate between normal and malignant estrogen-receptor positive breast cells. Therefore, for the first time, we have investigated the effect of eight different progestogens and of testosterone on the prolifera-

tion of benign and malignant breast epithelial cells in the presence of growth factors and/or estradiol.

Our results indicate that MPA may enhance the mitotic rate of normal epithelial breast cells in the presence of growth factors and thus may increase the probability of faults in DNA-replication when used in long-term. Indeed, the results of WHI indicate that patients who were not using hormones prior to the start of the study had no increased hazard ratio for breast cancer, whereas subjects with prior hormone use for up to 5, 5–10 and more than 10 years showed an increasing risk [1]. These data suggest that long-term use of MPA may increase breast cancer risk by enhancing the mitotic rate of normal epithelial cells.

We could further demonstrate that P had a neutral effect on growth factor stimulated healthy breast epithelial cells. In the case of cancerous breast cells, other groups have published supporting results, where E2-induced stimulation of MCF-7 cells has been shown to be inhibited by P [11–14]. Overall our results suggest that NET may have no negative effect on the mitotic rate of normal and cancerous breast cells. This is in agreement with the as yet available epidemiological studies [15]. Up to now, there is a paucity of data available regarding the effects of CMA and LNG on the proliferation of normal and malignant epithelial breast cells. There are also conflicting epidemiological data concerning these progestogens [16–18]. DNG has been shown to elicit potent anti-tumor activity against hormone-dependent cancer types in an animal model [19], and has exhibited slight concentration-dependent inhibitory effects in combination

with E2 [19], in agreement with our results. GSD and KDG have been shown to be able to inhibit cell proliferation of a specific sub-clone of MCF-7 in the presence of E2 [11]. Our results support the inhibitory effects of both GSD and KDG in combination with E2; however, we found both exhibited a proliferative effect on HCC1500 cells with growth factors alone. T was found to exhibit dose-dependent inhibition of cell growth in the four human breast carcinoma cell lines MCF-7, T47-D, MDA-MB 435S and BT-20 [20]. These inhibitory effects on E2 are consistent with our results.

By comparing the cell death:proliferation ratio results of growth factors alone, E2 alone and combination of growth factor and E2 on HCC1500 cells, we also found that the single proliferative effects of growth factors or E2 alone are magnified when in combination with each other, which, however, was not always statistically significant. The mechanism of the stimulatory effect of MPA (and of CMA) on MCF10A cells is currently unknown, as this cell line is both estrogen and progesterone receptor negative. The effects of the steroids on HCC1500 cells appear to be receptor-dependent, since the time course clearly shows a long-term effect rather than a rapid non-genomic action.

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*. Limitations of this *in vitro* study might be the high concentrations needed for an effective anti-proliferative effect. The clinically relevant blood concentrations for the progestogens most commonly used for HRT, MPA and NET are in the range of 4×10^{-9} to 10^{-8} M for MPA [21] and around 10^{-8} M for NET [22]. However, higher concentrations may be required *in vitro* in short-time tests in which the reaction threshold can only be achieved with supraphysiological dosages. Higher concentrations may also be reached *in vivo* in the vessel wall or organs compared to the concentrations usually measured in the blood.

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. That duration of therapy may indeed be an important factor for breast cancer risk is emphasized by the results of WHI, where breast cancer risk was significantly higher compared to placebo only in women given combined HRT for 10 years or more, but not in those treated only for the duration of the study period, i.e. 5.2 years [1]. *In vitro* experiments can support, but not replace, clinical trials, and therefore, further clinical studies are needed to determine which progestogens, if any, have the lowest breast cancer risk.

5. Conclusion

Despite the experimental limitations described, we suggest our results indicate that certain different progesto-

gens are able to induce proliferation of or inhibit the growth of benign or malignant human breast epithelial cells independently of the effects of growth factors and E2, and therefore the choice of progestogen for hormone therapy may be important in terms of influencing a possible breast cancer risk.

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