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# The proliferative and apoptopic in vitro effects of progesterone and seven synthetic progestogens on normal and malignant human breast epithelial cells

**Inaugural-Dissertation**
**zur Erlangung des Doktorgrades**
**der Humanwissenschaften**

**der Medizinischen Fakultät**
**der Eberhard-Karls-Universität**
**zu Tübingen**

**vorgelegt von**

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**2007**

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*To Bernhard,*
*for making this possible.*

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## 1) INTRODUCTION

### *1.1 Hormone replacement therapy studies in women*

Hormone replacement therapy (HRT) in the form of estrogen replacement therapy gained widespread popularity in the 1960s and early 1970s, with 28 million prescriptions being issued in the USA for the non-contraceptive use of estrogens at the peak of 1974 (Ross *et al* 2000). This was followed by a decline in prescription numbers after the first definitive studies demonstrating a relationship between endometrial cancer and estrogen replacement therapy were published in 1975 (Smith *et al* 1975, Ziel *et al* 1975). Prescription numbers began to increase once again when a new strategy was introduced to protect the endometrium from the effects of exogenous unopposed estrogen. The new strategy was combined estrogen-progestogen replacement therapy.

There was, however, already suspicion that progestogens as well as estrogens were capable of stimulating the growth of dormant breast metastases. In 1967, Moore *et al* remarked on a case where the administration of medroxyprogesterone acetate caused marked stimulation of advanced breast cancer, revealed by acute hypercalcaemia (Moore *et al* 1967).

The first direct evidence that progestogens may increase the risk of breast cancer was published in 1989. A study of Swedish women found a 4.4-fold increase in the risk of breast cancer in those who had received an estrogen-progestogen combination treatment for more than 6 years (Bergkvist *et al* 1989). However, a subsequent report suggested that the study was not of statistical significance, as the risk was only based on 10 patients, and after using more precise risk estimates, a lesser 1.6-fold increase in risk was found (Persson *et al* 1992).

The Nurses' Health Study was established in 1976. A mailed questionnaire was completed every two years by 121,700 registered nurses aged 30 to 55 years. Baseline details including known or suspected risk factors for cancer or

cardiovascular disease were collected, and further questionnaires brought this information up to date and ascertained whether major medical events had occurred. Follow-up of participants was extended to 1992 to quantify the relationship between the use of hormones and the risk of breast cancer in post-menopausal women. Risk of breast cancer was found to be significantly increased among women who were currently using estrogen alone, or an estrogen-progestogen preparation, with relative risks of 1.32 and 1.41 respectively. The authors state that these relative risks did not differ significantly from each other. The increased risk was most pronounced among women over the age of 55, and was largely limited to those who had used HRT for five years or more. The study concluded that the addition of progestogens to estrogen therapy does not reduce the risk of breast cancer among postmenopausal women (Colditz *et al* 1995).

The Collaborative Group on Hormonal Factors in Breast Cancer (1997) brought together and reanalysed about 90% of the worldwide epidemiological evidence on the relationship between the risk of breast cancer and the use of HRT (Beral *et al* 1997). They found a relative risk of breast cancer of 1.35 for women who had used HRT for five years or longer, with no significant excess of breast cancer overall or in relation to duration of use five years or more after stopping HRT. Regarding hormonal constituents, they found no evidence of marked differences between preparations containing estrogen alone and preparations containing both estrogen and progestogen.

In contrast, a prospective study by Gambrell *et al* (1983) claimed that adding a progestogen to postmenopausal estrogen therapy significantly decreased the risk of breast malignancy. However, the study was limited by bias as the breast cancer risk factor profiles were not matched in the treated and untreated groups. A second study by Nachtigall (1992), a randomised placebo-controlled trial which reviewed the incidence of breast cancer in a 22 year study, also claimed a protective effect of combined therapy. However, the study was hampered by small patient numbers (Speroff 2000).

Two recent studies on hormone replacement therapy, the Women's Health Initiative (WHI) and the Million Women Study (MWS) have raised concern as to the relationship between progestogens and an increased risk of breast cancer in the climacteric and post-menopause (Writing Group for the Women's Health Initiative Investigators 2002, Million Women Study Collaborators 2003).

The WHI was the first randomised prospective study to investigate the possible primary prevention of coronary heart disease in postmenopausal women using combined HRT. The investigators recruited 16,608 postmenopausal women between the ages of 50 and 79 years with an intact uterus and aimed to assess the major health benefits and risks of the most commonly used combined hormone preparation in the United States (conjugated equine estrogens (CEE) 0.625mg/day plus medroxyprogesterone acetate 2.5mg/day). The combined HRT-arm of the study, with a planned duration of 8.5 years was famously terminated after a mean of 5.2 years of follow-up, because the overall health risks of therapy appeared to be exceeding the benefits, with an increased risk of breast cancer in the treatment group (Writing Group for the Women's Health Initiative Investigators 2002). There was evidence of a duration effect, with an increase in risk beginning to emerge three years after randomisation, but only in women with a history of HRT use prior to study entry (British Menopause Society Council Consensus Statement, 2004). The recently published results of the estrogen-only arm of the WHI, which used oral CEE (0.625mg/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, however this narrowly missed statistical significance (HR 0.77, CI 0.59-1.01) (Women's Health Initiative Steering Committee 2004).

The observational MWS recruited 1,084,110 women in the UK aged 50 to 64 years between 1996 and 2001 to study the effects of specific types of HRT on the incidence of breast cancer in postmenopausal women (Million Women Study Collaborators 2003). Information on lifestyle, socio-economic and

reproductive factors, past health and past and current use of HRT was collected. Incidence of breast cancer was found to be increased by a factor of 1.30 for current users of estrogen-only therapy, and by a factor of 2.00 for current users of estrogen-progestogen combination therapy. Use of HRT for 10 years was estimated to result in five additional cancers per 1000 users of estrogen only therapy, and 19 extra cases for users of combination therapy. The analysis also showed that the risk of breast cancer begins to decline when HRT is stopped, reaching the same level as women who have never taken HRT after five years.

A report published shortly after the MWS results were released stated, “the (Million Women) study demonstrates that alteration of a woman’s basic hormonal physiology over decades in the interest of long-term disease prevention is fraught with hazard” (Gann and Morrow 2003).

The MWS design, analysis and conclusions have, however, been questioned, as has the finding that breast cancer risk increases within one year of use, disappearing on cessation and irrespective of the duration of prior use. This is inconsistent with our understanding of tumour biology, and it has been stated that “the over-interpretation of marginal results has resulted in widespread loss of confidence in an important treatment option for millions of women” (Farmer 2004). The major HRT and breast cancer study results since 1997 are summarised in Table 1.

Whereas these studies all considered morbidity rates, in contrast, a meta-analysis published in July 2004, using pooled data from 30 randomised controlled trials of HRT from 1966 to 2003 showed that the odds ratio for total mortality associated with HRT was 0.98 (95% CI, 0.87-1.12) (Salpeter *et al* 2004). HRT was shown to reduce mortality in the younger age group (OR 0.61; CI 0.39-0.95) but not in the older age group (OR 1.03; CI 0.90-1.18). The authors concluded that HRT reduced mortality in trials with mean age of

participants under 60 years, and no change in mortality was seen in trials with a mean age of over 60 years.

***Table 1: Summary of the epidemiological studies of breast cancer risk under hormone replacement therapy since the Oxford Reanalysis (Beral et al Lancet 1997)***

| | | | **Relative risk of breast cancer (95% confidence interval if not stated otherwise)** | | | |
|---|---|---|---|---|---|---|
| **Study, year of publication & country** | **Duration of use** | **Aspect of use** | **Unopposed estrogen** | **Estrogen/progestogen combined** | **Sequential HRT** | **Continuous HRT** |
| Beral, Collaborative Group, 1997, Worldwide reanalysis | > 5 years | Current use or last use 1-4 years before diagnosis | 1.34 (SE 0.09) n=558 | 1.53 (SE 0.33) n=58 | | |
| Persson, 1997, Sweden | 1-6 years<br>6+ years<br><br>1-6 years<br>6+ years | Current or last use ≤ 1 year<br><br>Treatment ended ≥1 year ago | 1.0 (0.2-5.9) n=2<br>1.0 (0.3-3.4) n=18<br><br>1.0 (0.6-1.7) n=21<br>1.1 (0.6-2.0) n=17 | 2.8 (0.8-10.0) n=14<br>1.9 (0.6-6.1) n=35<br><br>0.9 (0.5-1.7) n=14<br>1.0 (0.5-2.1) n=9 | | |
| Magnuson, 1999, Sweden | Per year of use | | 1.03 (0.98-1.08) n=150<br>*p* for trend 0.001 | *Testosterone-derived progestogen:*<br>1.08 (1.03-1.13) n=324<br>*p* for trend 0.0007<br>*Progesterone-derived progestogen:*<br>0.95 (0.80-1.14) n=32<br>*p* for trend 0.38 | 1.03 (0.98-1.08) n=102<br>*p* for trend 0.27<br>*(Testosterone-derived progestogen)* | 1.19 (1.09-1.31) n=139<br>*p* for trend 0.0002<br>*(Testosterone-derived progestogen)* |
| Colditz (Nurses' Health Study) 2000, USA | 10 years | Taken between ages of 50 to 60 years | 1.23 (1-06-1.42) | 1.67 (1.18-2.36) | | |

| | | | Relative risk of breast cancer (95% confidence interval if not stated otherwise) | | | |
|---|---|---|---|---|---|---|
| **Study, year of publication & country** | **Duration of use** | **Aspect of use** | **Unopposed estrogen** | **Estrogen/progestogen combined** | **Sequential HRT** | **Continuous HRT** |
| Schairer, 2000, USA | Per year<br><br><4 years<br><br>>4 years | Recent users (defined as current use or use within the last 4 years) | 0.03 (0.01-0.06)<br>n=234<br>*p* for trend 0.001 | 0.12 (0.02-0-25)<br>n=52<br>*p* for trend 0.01<br>*Medroxyprogesterone acetate was majority progestogen* | *Medroxyprogesterone acetate was majority progestogen*<br><br>1.1(0.8-1.7) n=26<br>1.5(1.0-2.4) n=22 | n=12, therefore authors reported too few cases to derive stable estimates |
| Ross, 2000, USA | Per 5 years of use | | 1.06 (0.97-1.15)<br>n=742<br>*p* for trend 0.18 | *Medroxyprogesterone acetate was majority progestogen*<br>1.24 (1.07-1.45)<br>n=425<br>*p* for trend 0.0015 | *Medroxyprogesterone acetate was majority progestogen*<br>1.38 (1.13-1.68)<br>n=320<br>*p* for trend 0.0015 | *Medroxyprogesterone acetate was majority progestogen*<br>1.09 (0.88-1.35)<br>n=105<br>*p* for trend 0.44 |
| Newcomb, 2002, USA | >5 years | All users | 1.36 (1.17-1.58)<br>n=605 | *Medroxyprogesterone acetate was majority progestogen*<br>1.58 (1.16-2.15) n=117 | | |
| Kirsh & Kreiger, 2002, Canada | 5 to 9 years<br><br>>10 years | Ever use | 1.00 (0.44-2.24)<br>n=14<br>1.74 (0.93-3.24)<br>n=39 | 0.84 (0.31-12.11)<br>n=12<br>3.48 (1.00-12.11)<br>n=12 | | |
| Weiss, 2002, USA | >5 years | Current use | 0.81 (0.63-1.04)<br>n=292 | 1.37 (1.06-1-77) n=291 | 1.00 (0.69-1.46) n=84 | 1.54 (1.10-2.17)<br>n=132 |

| | | | Relative risk of breast cancer (95% confidence interval if not stated otherwise) | | | |
|---|---|---|---|---|---|---|
| **Study, year of publication & country** | **Duration of use** | **Aspect of use** | **Unopposed estrogen** | **Estrogen/progestogen combined** | **Sequential HRT** | **Continuous HRT** |
| Porch, 2002, USA | <5 years<br><br>>5 years | Current users | 0.96 (0.58-1-58) n=33<br>0.99 (0.65-1-53) n=68<br>*p* for trend 0.31 | 1.11 (0.81-1-52) n=85<br><br>1.76 (1.29-2.39) n=79<br><br>*p* for trend 0.0004 | <br><br>1.04 (0.74-1.16) n=61<br><br>*p* for trend 0.0003 | <br><br>1.82 (1.34-2.48) n=87<br><br>*p* for trend 0.0003 |
| Chen, 2002 | >5 years | | | 1.49 (1.29-1.74) n=402 | | |
| Daling, 2002, USA | >5 years Histological findings | Ever use | Ductal 0.7 (0.6-1.0) n=251<br>Lobular 1.3 (0.8.-2.2) n=57 | Ductal 1.0 (0.8-1.3) n=222<br>Lobular 2.0 (1.3-3.2) n=64 | Ductal 1.0 (0.7-1.3) n=112<br>Lobular 1.5 (0.8-2.6) n=28 | Ductal 1.2 (0.9-1.7) n=100<br>Lobular 2.5 (1.4-4.3) n=32 |
| HERS, 2002, USA | 6.8 years | | Not investigated | 1.27 (0.84-1.94) n=49 | | |
| Women's Health Initiative (WHI), 2002,USA | 5.2 years | Current use | Results from 2004 after 6.8 years.<br>0.77 (0.59-1.01) n=218 | *Medroxyprogesterone acetate used throughout*<br>1.26 (1.00-1.59) n=290 | | |
| De Lignieres, 2002, France | >5 years | Ever users | | | | Mainly transdermal gel formulation (83%)<br>0.98 (0.65-1.5) n=105<br>RR 1.005 (0.97-1.05) per year of use |
| Olsson, 2003, Sweden | >4 years | Ever use | 0.35 (0.07-1.86) *p*=0.219 | | 2.23 (0.90-5-56) *p*=0.084 | |

| | | | Relative risk of breast cancer (95% confidence interval if not stated otherwise) | | | |
|---|---|---|---|---|---|---|
| **Study, year of publication & country** | **Duration of use** | **Aspect of use** | **Unopposed estrogen** | **Estrogen/progestogen combined** | **Sequential HRT** | **Continuous HRT** |
| Women's Health Initiative (WHI), 2003, | >6 years | Current use | | *Medroxyprogesterone acetate used throughout*<br>1.35 (0.85-2.16)<br>n=45 | | |
| Li, 2003 | >25 years<br><br>>15 years | Ever use | 1.0 (0.7-1.4)<br>n=105 | <br><br>2.0 (1.3-3.3)<br>n=49 | <br><br>2.9 (1.3-6.6)<br>n=21 | <br><br>1.8 (1.0-3.3)<br>n=33 |
| Million Women Study (MWS), 2003, UK | Current use | Current use | 1.30 (1.21-1.40)<br>n=991<br>p<0.0001 | 2.0 (1.88-2.12)<br>n=1934<br>p<0.0001 | | |

## *1.2. Breast Cancer*

### 1.2.1 Epidemiology of breast cancer in women

With one million new cases in the world each year, carcinoma of the breast is the most common cancer in women, accounting for 19% of all new cases of female cancer (McPherson *et al* 2000, Hall *et al* 1998). In the year 2000, there were estimated to be 51,710 cases in Germany and 34,815 in the UK, with 19,149 and 14,415 deaths from the disease respectively (Globocan 2000). In contrast, it is a rare form of cancer in men. Most breast cancer cases (78%) occur in women over the age of 50, whereas only 6.5% of cases are diagnosed in women younger than 40 years. Breast cancer accounts for about a fifth of all deaths of women aged 40 to 50 years. Fig. 1 illustrates the age distribution of breast cancer mortality in women.

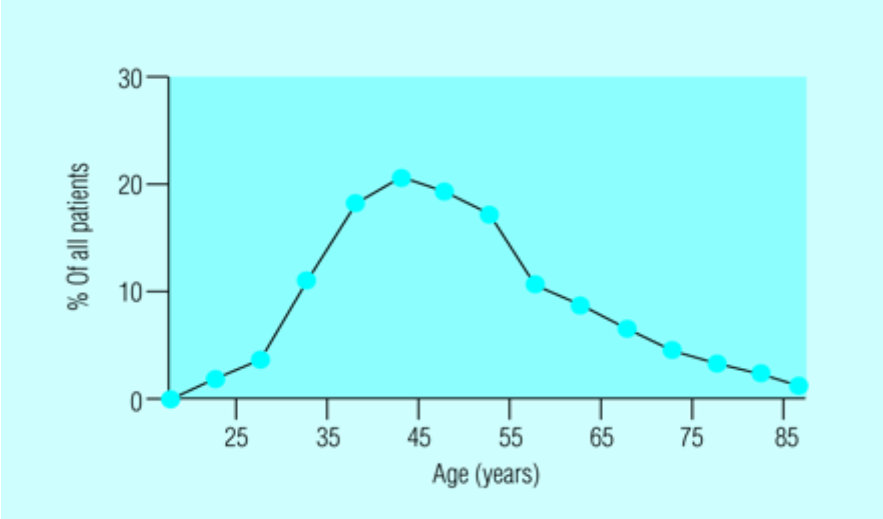



***Figure 1:Percentage of all deaths in women attributable to breast cancer (Taken from McPherson et al 2000)***

Breast cancer mortality rate varies worldwide, with Fig. 2 clearly illustrating geographical variation, with a higher incidence in the West.

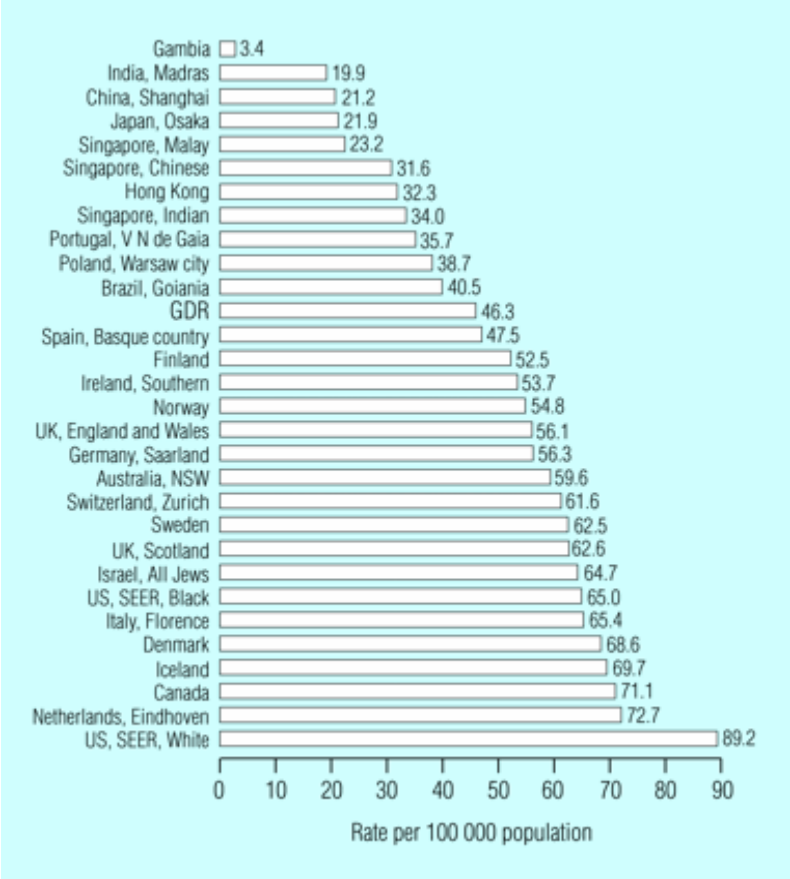



***Figure 2: Standardised mortality for breast cancer in different countries (Taken from McPherson et al 2000)***

In the USA, tumour registries noted a 1% increase in breast cancer each year until the 1980s, when the incidence rate increased dramatically. Some attribute this rise to life-style factors such as widespread oral contraceptive use, HRT, or earlier onset of the menarche in recent decades. Others attribute it to the introduction of widespread mammographic screening programmes in the 1980s (Gradishar and Morrow, 1996).

### 1.2.2 Risk factors for the development of breast cancer

The identification of risk factors for breast cancer enables high risk patients to be closely monitored and hence provide them with an earlier diagnosis and better prognosis, and allows prevention and treatment methods to be improved through better understanding of the relationship between risk factors and the disease. Two risk factors are without question: the female gender, and increasing age.

Table 2 shows other risk factors and their presumed relative risk.

***Table 2: Risk factors for the development of breast cancer.***

(Taken from McPherson *et al* 2000,Gradishar and Morrow 1996, Hall *et al*, 1998, Velicer *et al* 2004, Clemons and Goss 2001)

| Risk factor | Relative risk | High risk group | Comments |
|---|---|---|---|
| Female gender | 150 | | |
| Age | >10 | Elderly | Incidence increases about every 10 years until the menopause, when rate of increase slows dramatically |
| Age at menarche | 3 | Menarche before age 11 | |
| Age at natural menopause | 2 | Menopause after age 54 | Relative risk declines by 2.7% for each year after menopause |
| Parity | 1.4 | Nulliparity | |
| Age at first full pregnancy | 3 | First child in early 40s | |
| Geographical location | 5 | Developed country | |
| Benign breast disease | 4-5 | Atypical hyperplasia | |
| Cancer in the other breast | >4 | | |
| Family history of breast cancer | >2 | Especially if first degree relative with premenopausal diagnosis and/or bilateral disease | BRCA1 and BRCA2 genes account for substantial proportion of very high risk families |
| Serum estradiol concentration | 6.0 | Highest quartile | |
| Socioeconomic group | 2 | Groups I and II | |
| Oral contraceptive use | 1.24 | Current use | |
| HRT | 1.35 | Use for >10 years | |
| Body weight<br>Premenopausal<br>Postmenopausal | <br>0.7<br>2 | <br>Body mass index>35<br>Body mass index>35 | Increased production of estrone from androstenedione as fat cells are rich in aromatase, the enzyme necessary for this conversion |
| Alcohol consumption | 1.3 | Excessive intake | |
| Exposure to ionizing radiation | 3 | Abnormal exposure in young females after age 10 | |
| Antibiotic use | | | Odds ratio for breast cancer of 2.14 for 101-500 consecutive days of use for certain indications |

1.2.3 Types of breast cancer

Breast cancer can be divided into two main categories:

a) Non-invasive, or carcinoma *in situ*, and
b) Invasive

Non-invasive breast cancer does not metastasise, and remains in the area of the ducts or lobules of the breast. This type of carcinoma can be further divided into:

i. ductal carcinoma *in situ* (DCIS), which can appear in the pre- and post-menopause, typically presents as a mass with nipple discharge, and is commonly ipsilateral. It is the more common type of non-invasive cancer, making up about 3-4% of symptomatic cancers and 17% of screen detected cancers.
ii. lobular carcinoma *in situ* (LCIS), typically appears pre-menopausally, lacks clinical and mammographic signs and can be bilateral. It is a rare type of non-invasive cancer, making up about 0.5% of symptomatic cancers and 1% of screen detected cancers (Page *et al* 1995, Gradishar and Morrow 1996).

Invasive cancers are those in which cancer cells disseminate outside the basement membrane of the ducts and lobules into the surrounding adjacent normal issue. Of the invasive breast cancers, infiltrating ductal is the most common (70 to 80% of cases), followed by infiltrating lobular (5 to 10%) and then by other less common histological types.

Despite the apparent increased incidence of breast cancer in women who use HRT, most studies have shown either no effect on actual mortality, or a decrease (Nanda *et al* 2002, Salpeter *et al* 2004). It has been said that breast cancers which develop in users of HRT seem to be smaller and less clinically advanced, with a lower rate of node positivity, better differentiation and a more favourable histological type (Dixon 2002). Compared to tumours in never-users, those in ever-users are less likely to spread to axillary lymph nodes or to more

distant sites, and the excess risk of breast cancer is confined to localised disease (Beral *et al* 1997). However, in the estrogen plus progestogen group of the WHI, invasive breast cancers were similar in histology and grade to the placebo group, but were larger (p=0.04), more likely to be node positive (p=0.03) and were diagnosed at a significantly more advanced stage (Cheblowski *et al* 2003).

### 1.2.4 Relationship between cell proliferation and breast cancer

A general theory of carcinogenesis is that agents which increase the rate of cell proliferation increase the risk of development of new genetic mutations. Molecular genetic analysis of human cancers has shown that tumour cells contain multiple genetic defects including mutations, translocations and amplifications of oncogenes and they are reduced to homozygosity for putative tumour suppressor genes. These are all events which require cell division to occur. Increased cell division enhances the risk of such events happening. Propagation by cell replication leads to accumulation of a combination of such genetic errors and can result in a neoplastic phenotype. Hormones, drugs, infectious agents, chemicals, physical or mechanical trauma or chronic irritation are examples of promoters of increased cell division which can propel the accumulation of genetic errors (Preston-Martin *et al* 1990).

Estrogens are known to increase the rate of breast cell proliferation and could therefore potentially act in both the initiation and promotion of breast cancer.

## *1.3. Estrogens*

Estrogen deficiency symptoms in the peri- and post-menopause have successfully been treated with exogenous 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 five years after the menopause. However, a symptomatic patient, whether as a result of the natural or a surgically-induced

menopause, and with or without osteoporosis, may be given the option of replacement of her natural estrogens with HRT.

### 1.3.1 Pharmacology of estrogens

Estrogens are synthesised mainly by the ovaries, in large amounts by the placenta during pregnancy, in small amounts by the adrenal cortex in both sexes, and in the testes in males. Other extragonadal tissues such as liver, muscle, fat and hair follicles can convert steroid precursors into estrogens. The ovaries are the principal source of serum estrogen in premenopausal women, and little comes from the peripheral tissues; the case is opposite in postmenopausal women, with estrogen predominantly being produced from aromatisation of adrenal and ovarian androgens in the extragonadal tissues mentioned above.

The starting substance for endogenous estrogen synthesis is cholesterol, and immediate precursors to estrogens are androgenic substances (androstenedione and testosterone). The conversion to estrone ($E_1$) is made by aromatisation of the A-ring of the steroid molecule by aromatases (the 'aromatase pathway').

Estradiol ($E_2$) is the most potent estrogen, being the principle estrogen secreted by the ovaries. Its chemical structure is shown in Fig. 3. It can be locally synthesised by the reduction of estrone by 17$\beta$-hydroxysteroid dehydrogenase (17$\beta$-HSD). It is converted back to estrone by oxidation, and estrone may in turn be converted by hydroxylation to estriol ($E_3$), 16$\alpha$-hydroxyestrone (D-ring metabolites) or A-ring metabolites (2- and 4-hydroxyestrone, 2- and 4-hydroxyestradiol and 2-methoxyestradiol).

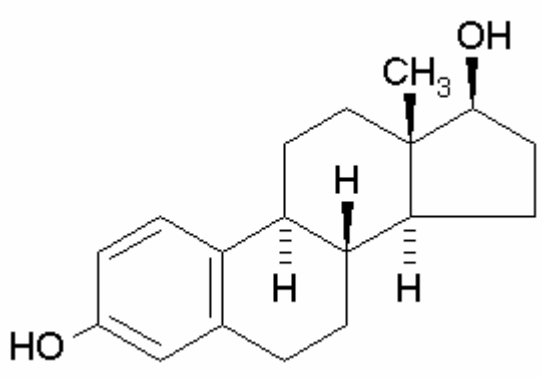



***Figure 3: Structure of Estradiol***

Of these metabolites, 4-hydroxyestrone and 16$\alpha$-hydroxyestrone are known to be carcinogenic. The 4- and 2-hydroxymetabolites are converted to anticarcinogenic methoxylated metabolites by catechol *O*-methyltransferase (COMT). The proportions of carcinogenic metabolites formed through the 2-hydroxylation and 16$\alpha$-hydroxylation pathways are important, as women who metabolise more endogenous estrogen through the 16$\alpha$-hydroxylation pathway may be at greater risk of breast cancer than those who metabolise more estrogen through the 2-hydroxylation pathway (Clemons and Goss 2001, Mueck *et al* 2002a).

Estradiol and estrone are the two main endogenous estrogens. They exist in equilibrium with each other in a ratio of 1:2 to 1:4. Estrone may be sulphated in the liver via estrone sulfotransferase to estrone sulphate, which can in turn be converted back to estrone by estrone sulfatase. Estrone may form a 'storage pool' of hormones. A summary of these conversion pathways is shown in Fig. 4

Estriol is short acting and has been regarded as of low efficacy. However, if plasma concentrations are constantly maintained, it can be as potent as estradiol (Rang and Dale 1991, Pasqualini 2003).

17$\beta$-HSD activity has been found to be higher in breast tumours than in normal breast tissue, and as this enzyme allows the conversion of estrone to more

active estradiol, the cancer cells are provided with an estrogenic environment, favourable for growth (Clemons and Goss 2001).

Estrogens control the early, proliferative phase of the menstrual cycle where endometrial regeneration occurs. Together with progestogens, they are responsible for the development of secondary sexual characteristics and the phase of accelerated growth at puberty.

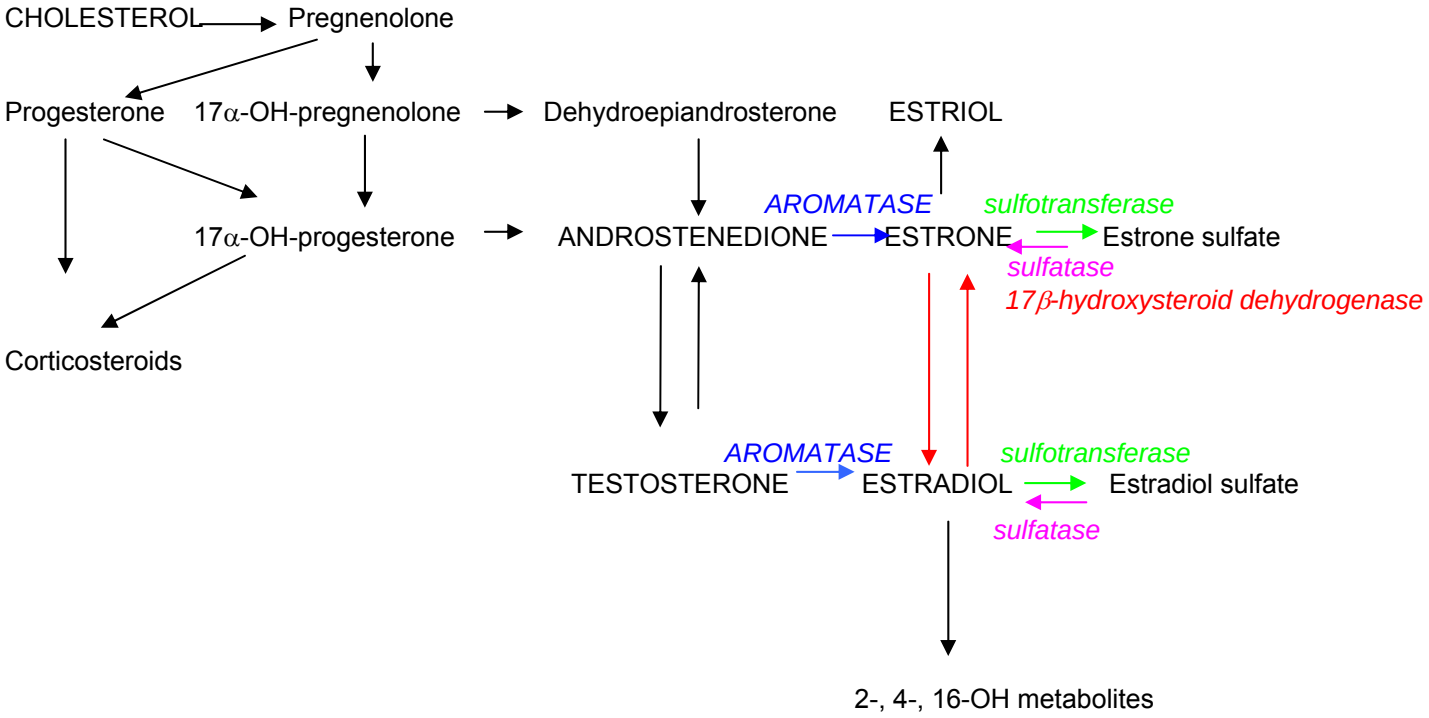



***Figure 4: Summary diagram of in vivo estrone, estradiol and estriol conversion pathways***

Symptoms of estrogen deficiency during the natural menopause and surgical menopause (which has proved which symptoms are due to true deficiency and not to increased age) have contributed significantly to knowledge of their function.

The connection between estrogens and breast cancer has been acknowledged for over 100 years since George Beatson demonstrated in 1896 that bilateral oophorectomy resulted in remission of breast cancer in premenopausal women (Beatson 1896), and ensuing evidence has implicated estrogen, both endogenous and exogenous, in the pathogenesis of breast cancer.

### *1.4. Progestogens*

Progestogens are now included in HRT for women with an intact uterus to inhibit endometrial proliferation under the action of unopposed exogenous estrogens, and hence endometrial hyperplasia and possible subsequent endometrial carcinoma.

#### 1.4.1 Pharmacology of progestogens

The natural hormone *progesterone* is normally produced and secreted in the human female by the corpus luteum (formed from old follicular cells in the ovary following ovulation), by the placenta during pregnancy, and in small quantities by the adrenal cortex (Schindler *et al* 2003). Its chemical structure is illustrated in Fig.5. *Progestogens* can be defined as substances that, like progesterone, can transform an endometrium primed by estrogens into a secretory status.

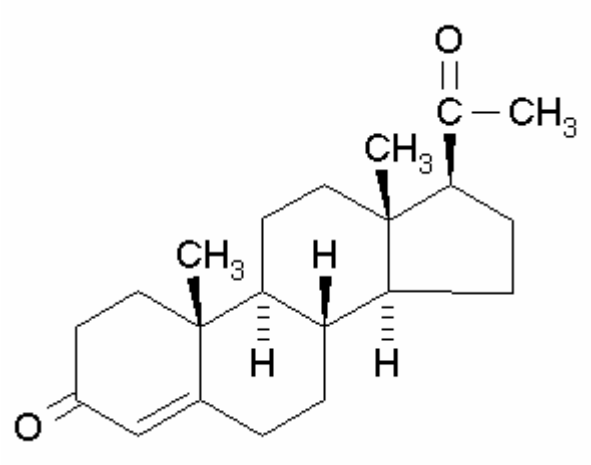



***Figure 5: Structure of Progesterone***

In general, progestogens down-regulate target tissue estrogen receptors and stimulate pathways of estrogen metabolism. They exert their activity by binding to the progesterone receptor, which exists in two forms (Form A, the most active, and Form B, the least active). Whether the progestogens available for use to date are able to bind specifically to PR-A or PR-B and its clinical relevance is unclear, and the biological importance of the different ratios of PR expression has not been explored (Druckmann 2003).

Progestogens may also interact with other steroid hormone receptors, including androgen, glucocorticoid, mineralocorticoid and estrogen. Besides having effects on the endometrium, progestogens exert important effects on the breasts, liver, bone, brain, lipids, carbohydrates, proteins, water and electrolyte regulation, haemostasis, fibrinolysis, the cardiovascular and immune systems (Pasqualini *et al* 1998).

Progesterone is the only natural progestogen with a clear biological function. The other progestogens which are used clinically are all synthetic, and are classified according to their chemical structure and distinct biological effects.

There are three main classes of progestogens: those derived from progesterone (C21-progestogens), those derived from androgenic testosterone (C19-

progestogens) and a more recent addition derived from spironolactone. Table 3 shows the classification and biological activities of progesterone and the synthetic progestogens. The examples highlighted in italics were used in the experiments in this study.

Desogestrel (a pro-drug, metabolised in the liver to 3-ketodesogestrel), gestodene and norgestimate have been referred to as ‘third-generation progestogens’. The ‘first generation’ includes norethynodrel, the first progestogen synthesised, and the ‘second generation’ includes norethisterone and levonorgestrel.

Several new progestogens have been synthesised in the last decade. Dienogest is a hybrid progestogen, with a unique pharmacological and pharmacodynamic profile; derived from 19-nortestosterone, it combines these properties with those of progesterone derivatives. Drospirenone, an anti-mineralocorticoid progestogen, is derived from the aldosterone antagonist spironolactone and has pharmacological properties similar to natural progesterone.

Several risks are attributed to progestogens as a class-effect, however different progestogens have diverse pharmacological properties and do not induce the same side-effects. Natural progesterone, and some of its derivatives (such as the 19-norprogesterones nomegestrol acetate, promegestone, trimegestone and nesterone) do not bind to the androgen receptor and therefore do not the androgenic side-effects of some of the 19-nortestosterone derivatives, such as acne, greasy skin and hair. Chlormadinone acetate, cyproterone acetate and the newer drospirenone and dienogest have an anti-androgenic effect. Some progestogens, e.g., medroxyprogesterone acetate, norethisterone, lynestrenol (a pro-drug of norethisterone) and levonorgestrel can cause a reduction in glucose tolerance, and should therefore be used in caution in women with diabetes.

Conversely, micronized progesterone and some non-androgenic progestogens may improve the possible deleterious effect of estrogens on insulin sensitivity and should be preferred in women with diabetes or insulin resistance. Some of the 19-nortestosterone derivatives can cause a decrease in HDL-cholesterol and an increase in LDL-cholesterol in comparison to estrogen mono-therapy, and non-androgenic progestogens should therefore be chosen for women with hypercholesterolaemia (Schindler *et al* 2003, Sitruk-Ware 2002, Druckmann 2003, Rosano *et al* 2003).

### 1.4.2 Progestogens and breast cancer

The volume and morphology of the breast is a result of the fluctuations of gonadal steroids, mainly estrogens and progesterone. During the menstrual cycle, normal breast epithelium undergoes cyclical waves of proliferation, differentiation and apoptosis and after lactation arrest it undergoes remodelling by apoptosis. The maximal size of the breast occurs in the late luteal phase, when fluid secretion, mitotic activity and DNA production of non-glandular tissue and glandular epithelium peak. Factors including growth factors, glucocorticoids, insulin, aldosterone, hydrocortisone, prolactin, estradiol and progesterone, together with contact with the extra-cellular matrix co-ordinate these events, finally leading to mammary epithelium renewal. After a peak of increased mitotic activity corresponding with maximum levels of estrogen and progesterone, apoptosis occurs as the plasma levels of both hormones fall (Ory *et al* 2001).

A key issue to be addressed is whether progestogens exert proliferative, antiproliferative or neutral effects on the human breast. Epithelial cells in the breasts respond differently to those of the endometrium, where addition of a progestogen counteracts the proliferative effects of estrogen. In the human breast, maximal epithelial mitosis occurs during the luteal phase of the cycle between days 22 and 26 of the menstrual cycle, corresponding to the high serum levels of estradiol and progesterone (Pasqualini *et al* 1998), and this fact

has been used to support the role of progestogens in breast carcinogenesis. However, in a study by Gambrell *et al* (1983), incidence of breast cancer was significantly lower in estrogen-progestogen users compared to estrogen-only users, and both groups had a lower incidence than non-users. The resulting controversy had inspired a wide range of *in vitro* and *in vivo* studies to be undertaken in order to try and define the effects of the various progestogens on breast tissue.

In the USA, women have traditionally been given hormone replacement therapy in the form of conjugated equine estrogens (CEE) in combination with medroxyprogesterone acetate (MPA) as the progestogen of choice, i.e., a derivative of natural progesterone, and the results of the WHI were based solely on this hormone combination. However, in Europe the predominant estrogen used is 17β-estradiol in combination with a testosterone-derived progestogen if needed, i.e. norethisterone acetate (NET) or levonorgestrel (LNG), with MPA being used to a lesser extent. This raises the question of whether progestogens with differing androgenicity can influence the risk of breast cancer to a varying degree and therefore whether the results of studies in women using a particular combination of hormones can be extrapolated to cover all types of HRT.

As described in section 1.4.1, all progestogens are not alike in their structure and function, and depending on this and the tissue in which they are studied, they can exert either androgenic, synandrogenic, antiandrogenic, estrogenic, glucocorticoid-like or progestational effects (Santen *et al* 2001).

A study by Hofseth *et al* (1999) using breast biopsy samples found that the mitotic activity in the terminal ductal lobular unit of the breast was greater in postmenopausal women who were taking HRT as estrogen-progestogen (progestogen as MPA) in comparison to those taking estrogen alone, again proposing a link between progestogens and breast cancer. Mastodynia and oedema are often side-effects of treatment with certain progestogens, for example, norethisterone acetate, and increased mammographic density has

been clearly seen in such cases (Druckmann 2003). Lundstrom *et al* (1999) showed that an increase in mammographic density was much more common among women receiving continuous combination hormone therapy than among those receiving cyclic or estrogen-only treatment. Progestogens have also been shown to exhibit varying degrees of proapoptopic effects in human breast epithelial and in breast cancer cells (Seeger *et al* 2003, Kandouz *et al* 1999, Chang *et al* 1995, Gompel *et al* 2000, Franke and Vermes 2003)

Incidentally, Jasienka and Thune (2001) proposed that women from populations with a high risk of breast cancer are expected to have comparatively high concentrations of ovarian hormones, and using saliva samples from women in Bolivia, Congo, Nepal, Poland and the United States, showed that higher concentrations of ovarian progesterone in the mid-luteal phase were strongly associated with an increasing risk of breast cancer, the lowest rate being in the Congo (10.7 cases per 100,000 women), and the highest rate in the United States (80.7 cases per 100,000 women). They suggested that mean daily energy intake, lowest for the Congo and highest in the United States, is positively correlated with concentrations of serum progesterone, and thus poor energy status is associated with impaired ovarian function, anovulatory cycles or total amenorrhoea and therefore curtailed lifetime production of ovarian steroids and a reduced risk of breast cancer.

***Table 3: Classification of and biological activities of progesterone and synthetic progestogens*.**
***Key: (+) effective; (±) weakly effective; (-) not effective***
Taken from Schindler *et al* 2003, Pasqualini *et al* 1998

| Structure | Progestogen | Progestogenic | Anti-gonadotrophic | Anti-estrogenic | Estrogenic | Androgenic | Anti-androgenic | Glucocorticoid | Anti-mineralo-corticoid |
|---|---|---|---|---|---|---|---|---|---|
| **Progesterone and derivatives** | *Progesterone* | + | + | + | - | - | ± | + | + |
| | Medrogestone | + | + | + | - | - | ± | - | ± |
| **Retroprogster-ones** | Dydrogesterone | + | - | + | - | - | ± | - | - |
| **17α-Hydroxy-derivatives** | *Medroxyprogesterone acetate* | + | + | + | - | ± | - | + | - |
| | *Chlormadinone acetate* | + | + | + | - | - | + | + | - |
| | Megestrol acetate | + | + | + | - | ± | + | + | - |
| | Cyproterone acetate | + | + | + | - | - | ++ | + | - |
| **19-nor-progesterone derivatives** | Nomegestrol acetate | + | + | + | - | - | ± | - | - |
| | Promegestone | + | + | + | - | - | - | - | - |
| | Trimegestone | + | + | + | - | - | ± | - | ± |
| **19-nortestosteron e derivatives** | *Norethisterone* (=Norethindrone) | + | + | + | + | + | - | - | - |
| | Lynestrenol (pro-drug, converted to norethisterone) | + | + | + | + | + | - | - | - |
| | Norethindrol | ± | + | ± | + | ± | - | - | - |
| | *Levonorgestrel* | + | + | + | - | + | - | - | - |
| | Norgestimate | + | + | + | - | + | - | - | - |
| | *3-Ketodesogestrel* (metabolite of desogestrel) | + | + | + | - | + | - | - | - |
| | *Gestodene* | + | + | + | - | + | - | + | + |
| | *Dienogest* | + | + | ± | ± | - | + | - | - |
| **Spironolactone derivative** | Drospirenone | + | + | + | - | - | + | - | + |

### *1.5. Growth Factors*

Sex steroid hormones play a pivotal role in the development of breast cancer, however evidence also indicates that other regulatory molecules are involved and may work in conjunction with these hormones in facilitating carcinogenesis in breast tissue. 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. A diverse group of growth factors and other molecules are thought to have a paracrine role in breast cancer. These interactions involve infiltration of immune cells, the tumour vasculature, and the stromal fibroblasts, fibrocytes and adipocytes. Epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and mammary-derived growth factor 1 (MDGF-1) are among the growth factors involved in this paracrine role. Some of these growth factors (including EGF, FGF, and MDGF-1) are also released by the tumour cells themselves and have therefore been proposed to also exhibit an autocrine role *in vivo*, based on their *in vitro* effects (Dickson and Lippman 1995).

Growth factor expression *in vivo* is under the control of hormonal stimulation and it is believed that growth of estrogen and progesterone receptor-negative cells in normal breast epithelium is in response to growth factors derived from receptor-positive cells. EGF and TGF-α stimulate epithelial cell growth and TGF-β inhibits it.

*In vitro* experiments have suggested that that mitogenic events proceed most efficiently in an appropriate growth factor environment where there is substantial signal transduction cross-talk between mitogenic growth factors and sex steroid hormones. While many of the relevant growth factors and their receptors are expressed by the breast cancer epithelial cells themselves, additional paracrine factors (factors which are released and affect the function of other cells) may be

released from the surrounding stromal tissue of the breast (Nicholson and Gee 2000).

By stimulating the production of survival factors such as growth factors and cytokines, estradiol 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 (Rahman and Sarkar 2002).

### *1.6. Apoptosis*

The balance between cell proliferation and programmed cell death determines the growth rate of a tumour. Any variation in this balance may be a significant factor in the uncontrolled growth of malignant tumours (Franke and Vermes 2003). Too much growth and too little death of cells can lead to a severe disturbance, which may, ultimately, result in cancer. Cells have an intrinsic mechanism of self destruction called programmed cell death, or apoptosis. In multicellular organisms, many mechanisms controlling tissue homeostasis are linked to apoptosis, and defects in these pathways can lead to the expansion of a neoplastic cell population. Resistance to apoptosis can allow the cancerous cells to escape immune surveillance. In contrast to apoptosis, necrosis is not part of programmed cell death. It is accidental, and results in breakdown of organised cell structure and function after irreversible damage from an extrinsic stimulus.

Depending on the experimental model system, the cell context and the duration of treatment, progesterone has been shown to elicit either proliferative or anti-proliferative effects on breast epithelial cell growth (Lange *et al* 1999). When

used in high doses, the progestogens medroxyprogesterone acetate (MPA), megestrol acetate and norethisterone acetate (NETA) are licensed for the treatment of breast cancer. This shows a paradox in the effects of certain progestogens, e.g., in the case of MPA, the use of a progestogen which has been shown to increase the risk of breast cancer in healthy breast tissue, but is used in high dosages to treat breast cancer. This example illustrates the varying effects of progestogens on different cell types; i.e., the ability of one progestogen to induce proliferation in one cell line and apoptosis in another.

### *1.7 Aims of the following work*

One of the most important questions regarding HRT in the postmenopause is to what extent progestogens can increase the risk of breast cancer compared to estrogen monotherapy. Until recently, the general opinion was that progestogen supplementation could reduce the risk of breast cancer. This view stemmed mostly from experimental data, e.g. the reduction of proliferation of breast cancer cells by continuous progesterone therapy. However, *in vitro* studies have since been published, showing that the different progestogens could have varying effects on breast cancer cell proliferation.

The aim of this dissertation is to investigate and compare the effects of eight different progestogens, which are currently already being used in or are being considered for use in HRT preparations, on proliferation and apoptosis in different breast cell cultures. A normal human breast epithelial cell line as well as a human primary breast cancer cell line will be used for *in vitro* experiments.

The proliferative and apoptotic effects of the eight different progestogens on healthy and cancerous human breast cells in the presence of estradiol and /or growth factors will be studied, and the release of various markers of apoptosis will be determined.

# 2. MATERIALS AND METHODS

## *2.1 Materials*

### 2.1.1 Reagents

Progesterone (P), chlormadinone acetate (CMA), medroxyprogesterone acetate (MPA), norethisterone (NET), and estradiol (E2) 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 to give a concentration of $10^{-2}$ M and were stored as concentrated stock solutions at -20°C.

The growth factors Epidermal Growth Factor (EGF), Fibroblast Growth Factor-Basic (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.

4-Hydroxytamoxifen (4-OHTam), an active metabolite of tamoxifen, was purchased from Sigma Chemicals. Letrozole was kindly provided by Novartis, Germany. Androstenedione was purchased from Steraloids Inc., USA.

PD98059 and LY294002 were purchased from Calbiochem, Germany. The compounds were dissolved in dimethyl sulphoxide (DMSO) according to the manufacturer's instructions stated on the package insert, and were stored at -20°C.

### 2.1.2 Cells and cell culture

***MCF10A***

MCF10A, a human, non-tumorigenic, estrogen and progesterone receptor negative breast epithelial cell line was purchased from American Type Culture

Collection (ATCC), USA. The cell line stems from a 36 year old Caucasian female with fibrocystic breast disease. This cell line was chosen as only a small proportion (15-25%) of epithelial cells are ER+ in the normal breast, and these cells are largely non-dividing, in contrast to estrogen-regulated proliferation of ER+ breast tumours (Ali and Coombes 2002). This cell line can therefore be used to mimic the *in vivo* situation.

The cells were maintained in serum-free Mammary Epithelial Cell Medium purchased from PromoCell, Germany, supplemented with 100ng/ml cholera toxin purchased from List Biological Laboratories Inc., California, USA and 100U/ml penicillin plus 100μg/ml streptomycin.

Cells were incubated at 37°C in a 5% $CO_2$-in-air atmosphere in a 75$cm^3$ culture flask, and were fed every three to four days. Cultures were split weekly at a ratio of 1:3 to 1:4 after treatment with trypsin (0.04%)-EDTA (0.03%) for 15 minutes followed by trypsin neutralisation with Trypsin Neutralising Solution (TNS), both purchased from PromoCell, and centrifugation at 1200rpm for 5 minutes. The cell pellet was resuspended in the appropriate growth medium for subculture or assay work.

***HCC1500***

HCC1500, a human estrogen and progesterone-receptor positive primary breast cancer cell line was purchased from ATCC, USA. It was derived from a Stage IIB, invasive ductal carcinoma with 4/24 lymph node metastases in a 32 year old black female. This cell line was chosen because it is a primary cell line and therefore more closely represents the clinical *in vivo* situation compared to a cell line such as MCF-7 which was obtained from metastases.

The cells were maintained in RPMI-1640 medium (without phenol red) purchased from Sigma, which was modified to contain 1mM sodium pyruvate, 2mM L-glutamine, 4.5g/L glucose, 10%(v/v) heat inactivated foetal bovine serum and 100U/ml penicillin plus 100μg/ml streptomycin.

Cells were incubated at 37°C in a 5% $CO_2$-in-air atmosphere in a 75$cm^3$ culture flask and were fed every three to four days. Cultures were split weekly at a ratio of 1:2 to 1:3 after treatment with trypsin (0.05%)-EDTA (0.53mM), purchased from Gibco, Germany, for 5 to 10 minutes followed by trypsin neutralisation with complete growth medium, and centrifugation at 1200rpm for 5 minutes. The cell pellet was resuspended in the appropriate growth medium and gently passed through a fine-bore (subcutaneous) sterile needle to generate a single cell suspension for subculture or assay work.

2.1.3. Media used during working experiments

All assays were conducted using:

a) AIM-V serum-free medium for MCF10A cells
b) DMEM/FBS-stripped for HCC1500 cells

Stock concentrations of progestagens and growth factors were further diluted with these media during working experiments to give a final ethanol concentration of less than 0.01% per well.

AIM-V medium is a ready-made serum-free medium, purchased from Gibco, Germany, containing L-glutamine, streptomycin sulphate 50µg/ml and gentamicin sulphate 10µg/ml. No further additions were made to this medium.

DMEM/FBS-stripped was prepared in-house using Dulbecco's Modified Eagle Medium without phenol red (containing 1000mg/L glucose and sodium pyruvate, without L-glutamine), purchased from Gibco, supplemented with 10% heat inactivated (30 mins at 56°C) foetal bovine serum purchased from Gibco (which had been charcoal/dextran treated in-house to remove any steroid hormones present), 4mM/ml L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 250ng/ml amphotericin B as Fungizone®.

## *2.2 Methods*

### 2.2.1 Proliferation assays

Cells were counted using a Neubauer Cell Counter (Hemocytometer). 90μL of the marker 'tryphan blue' and 10μL of cell suspension were mixed thoroughly in a well in a 96-well plate, kept specifically for this purpose. 10μL of the marker/cell suspension mixture was placed on either side of a 0.1mm-deep chamber in the Hemocytometer and observed under a microscope. The number of cells in a defined area were counted and the cell concentration derived from the count.

Ninety-six well, sterile, transparent plates were seeded with approximately 1000 cells per well in:

a) 100μL AIM-V serum-free medium for MCF10A cells
b) 100μL DMEM/FBS-stripped medium for HCC1500 cells.

The cells were incubated for three days at 37°C. Progestagens alone or in combination with other test substances were then added at various concentrations and combinations three times over the next 7 days (see Table 4).

After 7 days, cell proliferation was measured by the ATP chemosensitivity test (Andreotti *et al* 1991), where proliferation is quantified by measuring light emitted during the bioluminescence reaction of luciferene in the presence of ATP and luciferase. The necessary reagents (Tumour Cell Extraction Reagent and Luciferene-Luciferase {Lu-Lu}), were purchased from DCS Innovative Diagnostik Systeme, Hamburg.

After removal of the old medium by tapping the plate on an absorbent pad, 50μl of Tumour Cell Extraction Reagent and 50μl of FBS-stripped medium were added to the wells. The plate was incubated at room temperature for 30 minutes on a plate shaker. 50μl of the solution in the wells was transferred with a multi-

pipette to a new sterile, opaque 96-well plate, and 50µl of Lu-Lu was added. Exactly two minutes after addition of Lu-Lu, the bioluminescence reaction was measured using a microplate luminometer. Measurements were recorded in 'relative light units' (RLUs), which are proportional to the cell count.

***Table 4: Diary plan of proliferation assays***

| **Procedure** | **Day of the week: Plan 1** | **Day of the week: Plan 2** |
|---|---|---|
| Seed 96 well plate, approx. 1000 cells/well in appropriate growth medium for cell line:<br>(MCF10A: AIM-V medium,<br>HCC1500: DMEM/FBS-stripped medium) | Friday | Tuesday |
| Remove medium, add reagents 1 | Monday | Friday |
| Reagent change 2 | Wednesday | Monday |
| Reagent change 3 | Friday | Wednesday |
| Measure using ATP chemosensitivity test | Monday | Friday |

The following proliferation assays were performed:

**MCF10A**

- E2 $10^{-7}$M to $10^{-10}$M alone and in combination with EGF, FGF and IGF $10^{-12}$M
- P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M to$10^{-10}$M alone and in combination with EGF, FGF and IGF $10^{-12}$M

**HCC1500**

- a) EGF, FGF and IGF combination $10^{-10}$M to $10^{-12}$M,
  b) E2 $10^{-9}$M and $10^{-10}$M,
  c) EGF, FGF and IGF combination $10^{-10}$M to $10^{-12}$M + E2 $10^{-10}$M
- P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M to$10^{-10}$M alone and in combination with E2 $10^{-10}$M
- P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M and $10^{-7}$M alone and in combination with EGF, FGF and IGF $10^{-12}$M

- P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M and $10^{-7}$M alone and in combination with EGF, FGF and IGF $10^{-12}$M + E2 $10^{-10}$M
- NET, LNG, DNG, GSD, KDG $10^{-6}$M and $10^{-7}$M in combination with
  a) 4-OHTam $10^{-6}$M + EGF, FGF and IGF $10^{-12}$M
  b) Letrozole $10^{-6}$M + EGF, FGF and IGF $10^{-12}$M

### 2.2.2 Cell death detection (CDD):proliferation assays

Cells were counted under a microscope using a Neubauer Cell Counter, as described in 2.2.1.

Ninety-six well sterile, transparent plates were seeded with approximately 1000 cells per well in:
a) 100µL AIM-V serum-free medium for MCF10A cells
b) 100µL DMEM/FBS-stripped medium for HCC1500 cells.

The cells were incubated for three days at 37°C. Substrates were then added at various concentrations and combinations three times over the next 7 days (see Table 4), in parallel to an identical proliferation assay, to enable the ratio of cell death detection:proliferation to be calculated.

Cell death was measured 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 (Roche 2003).

After completion of the incubation period with the test substances, cell death was induced by adding incubation buffer, provided in the kit, to the wells containing the cells followed by incubation for 30 minutes at 15-25°C. The

resulting solution containing cell lysates was used for the assay. Proliferation assays were measured as described in 2.2.1

The following CDD:proliferation assays and parallel proliferation assays were performed:

**MCF10A**

- P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M and $10^{-7}$M in combination with EGF, FGF and IGF $10^{-12}$M

**HCC1500**

- P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M and$10^{-7}$M + E2 $10^{-10}$M
- P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M and $10^{-7}$M + EGF, FGF and IGF $10^{-12}$M
- P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M and$10^{-7}$M + EGF, FGF and IGF $10^{-12}$M + E2 $10^{-10}$M

### 2.2.3 Proliferation assays in the presence of proliferation inhibitors

Cells were counted, seeded and incubated using the same method as for proliferation assays, described in 2.2.1.

On the days of the reagent changes, the old reagent medium was sucked away. 100µl of appropriate medium containing 1µM of the proliferation inhibitor PD98059 or LY294002 only was added to the wells containing cells to be incubated in the presence of the inhibitors. Cells were incubated at 37°C for one hour, after which the medium was sucked away and replaced with 100µl per well of fresh medium containing the reagent under test in the presence of PD98059 or LY294002 at a concentration of 1µM. The plates were then incubated as shown in Table 4. Reagents were changed only twice instead of three times as in the proliferation assay method.

The following proliferation assays in the presence of proliferation inhibitors were performed:

**MCF10A**

- PD98059 $10^{-6}$M or LY294002 $10^{-6}$M + EGF, FGF and IGF combination $10^{-12}$M
- MPA $10^{-6}$M and $10^{-7}$M + EGF, FGF and IGF combination $10^{-12}$M alone and in combination with PD98059 $10^{-6}$M or LY294002 $10^{-6}$M
- CMA $10^{-6}$M and $10^{-7}$M + EGF, FGF and IGF combination $10^{-12}$M alone and in combination with PD98059 $10^{-6}$M or LY294002 $10^{-6}$M
- PD98059 $10^{-5}$M or LY294002 $10^{-5}$M + EGF, FGF and IGF combination $10^{-12}$M

**HCC1500**

- PD98059 $10^{-6}$M or LY294002 $10^{-6}$M alone and in combination with EGF, FGF and IGF combination $10^{-12}$M
- PD98059 $10^{-6}$M or LY294002 $10^{-6}$M + E2 $10^{-10}$M
- PD98059 $10^{-6}$M or LY294002 $10^{-6}$M + EGF, FGF and IGF combination $10^{-12}$M + E2 $10^{-10}$M
- PD98059 $10^{-5}$M or LY294002 $10^{-5}$M + EGF, FGF and IGF combination $10^{-12}$M, E2 $10^{-10}$M and EGF, FGF and IGF combination $10^{-12}$M + E2 $10^{-10}$M
- LNG, GSD, NET, DNG, KDG $10^{-6}$M + EGF, FGF and IGF combination $10^{-12}$M alone and in combination with PD98059 $10^{-6}$M or LY294002 $10^{-6}$M

### 2.2.4 Apoptosis markers: cytochrome C, sFasL and p53

Cells were counted, seeded and incubated using the same method as for proliferation assays, described in 2.2.1.

For apoptosis marker measurement, sterile, transparent 24 well plates were seeded with approximately 60,000 cells per well in:

a) 500µL AIM-V serum-free medium for MCF10A cells

b) 500µL DMEM/FBS-stripped medium for HCC1500 cells.

A parallel proliferation assay was conducted on a sterile, transparent 96-well plate with 6,000 cells per well in 100µL medium. Reagents were changed only twice instead of three times as in the proliferation assay method. Previous in-house work showed that a minimum of two reagent changes (i.e. 96 hours of incubation in the presence of the progestogen and growth factor and/or E2 combination) was needed for a significant proliferative or inhibitory response to be seen with the HCC1500 cells.
The apoptosis marker proliferation reaction was stopped by removal of the medium in the wells by tapping the plate onto an absorbent pad, washing each well with 500µL of cold phosphate buffered saline (PBS) followed by addition of 250µL reconstituted lysis buffer from the cytochrome C measurement kit described below, followed by incubation for 1 hour at room temperature. The 24-well plates were then centrifuged at 1000g for 15 minutes, and 250µL supernatant was pipetted into labelled glass tubes and frozen at -20°C, ready for measurement.

Cytochrome C, sFasL and p53 were measured using ELISA kits purchased from BenderMed Systems, Austria.
Parallel proliferation assays were measured as described in 2.2.1.

The following apoptosis marker assays and parallel proliferation assays were performed:

**MCF10A**

- CMA, MPA $10^{-6}$M and $10^{-7}$M + EGF, FGF and IGF combination $10^{-12}$M

**HCC1500**

- MPA, NET, P $10^{-6}$M and $10^{-7}$M + EGF, FGF and IGF combination $10^{-12}$M
- MPA, NET, P $10^{-6}$M and $10^{-7}$M + E2 $10^{-10}$M
- MPA, NET, P $10^{-6}$M and $10^{-7}$M + EGF, FGF and IGF combination $10^{-12}$M + E2 $10^{-10}$M

### *2.3 Statistics*

Proliferation assays were measured versus controls and performed in quadruplicate. From these values, means were calculated alone with standard deviation (S.D.). Statistical analysis was done by ANOVA with the logarithmated values, followed by Dunnett's procedure. The overall alpha level was set at 0.05.

Proliferation and cell death in the cell death detection:proliferation assays were measured versus controls and performed in triplicate. From these values, the ratios of cell death to proliferation were calculated along with the standard deviation (S.D.). Ratios under the control value (assay medium only) favoured a proliferative response whereas ratios over control favoured 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.

Apoptosis marker assays were measured versus controls and performed in triplicate on the 24-well plates with 60,000 cells per well and in quadruplicate on the 96-well plates with 6,000 cell per well. 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

### *3.1 Suitability of chosen assay media*

Initial proliferation assays were conducted with both cell lines to check the suitability of the chosen assay media. This assay also included the addition of the growth factors EGF, FGF and IGF-I, and estradiol (E2) to the media to confirm the proliferative response expected for each cell line (i.e. a significant increase in proliferation for both cell lines in the presence of the growth factors; increase in proliferation of ER+ HCC1500 cells but not ER- MCF10A cells in the presence of estradiol) and to determine the concentrations of each to use to promote significant proliferative responses.

AIM-V medium, as described in 2.1.3, was initially chosen for both cell lines, as it is a serum-free medium.

MCF10A cells proliferated well in this medium, exhibiting a significant proliferative response to the growth factors at $10^{-12}$M but not to E2, as shown in Figure 6. Cell proliferation in the presence of both growth factors $10^{-12}$M and E2 over a concentration range of $10^{-7}$M to $10^{-10}$M also increased significantly compared to control, but not more than growth factors alone.

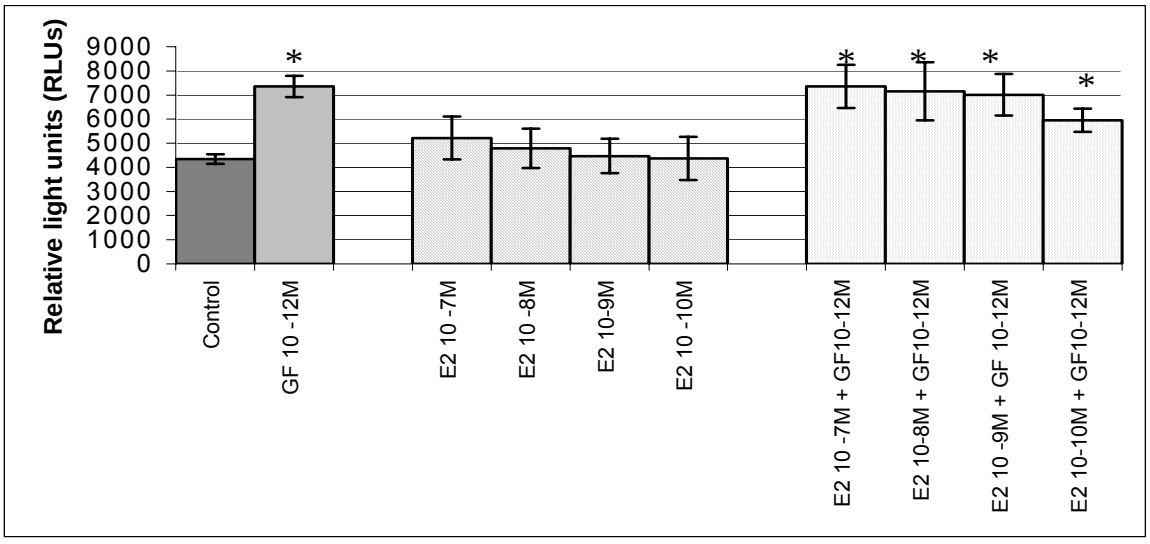



***Figure 6: Proliferative responses of MCF10A to growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M), estradiol (E2) $10^{-7}$M to $10^{-10}$M, and a combination of growth factors and estradiol in AIM-V medium. (Mean ± SD, n=4). * = p<0.05***

HCC1500 cells, however, did not respond significantly to AIM-V medium, with large variation errors in the results and poor proliferation, as shown in Fig.7. The assay was repeated using DMEM/FBS-stripped, as described in 2.1.3, and the cells proliferated significantly, with positive responses to the addition of both growth factors and E2 (Fig.8). Significant proliferative responses were seen with growth factors alone $10^{-12}$M, E2 alone $10^{-9}$M and $10^{-10}$M, and growth factors $10^{-12}$M in combination with E2 $10^{-10}$M. Growth factors alone at a concentration of $10^{-10}$M had a significant anti-proliferative effect.

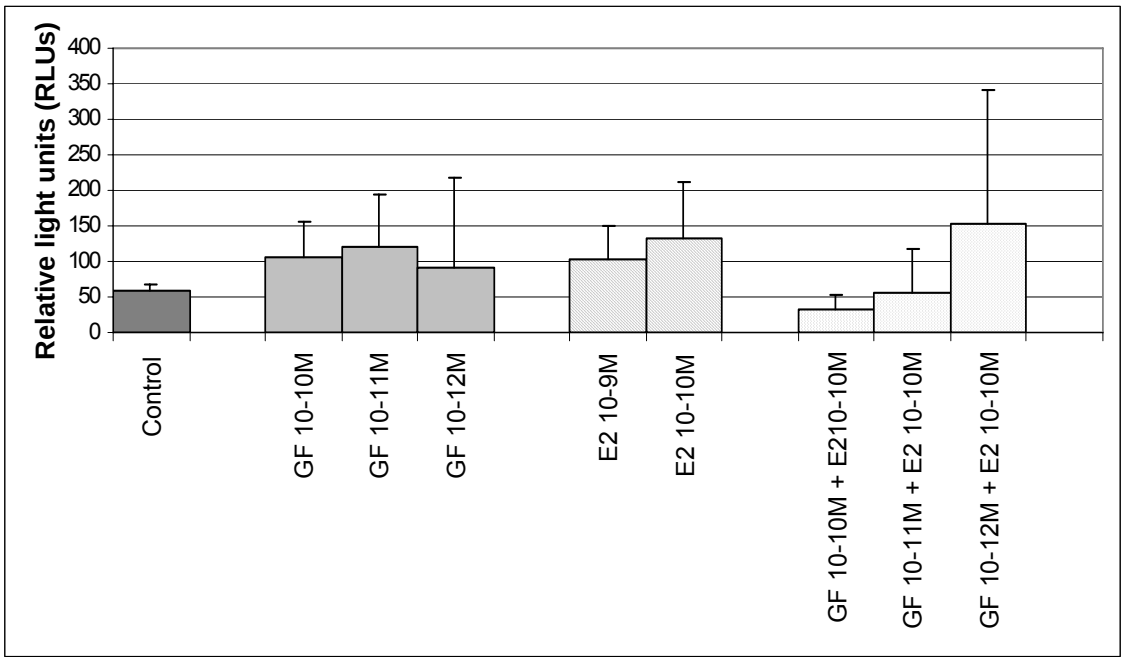



***Figure 7: Proliferative responses of HCC1500 to growth factors EGF, FGF and IGF-I (GF) $10^{-10}$M to $10^{-12}$M, estradiol (E2) $10^{-9}$M to $10^{-10}$M, and a combination of growth factors and estradiol in AIM-V medium. (Mean $\pm$ SD, n=4). * = p<0.05***

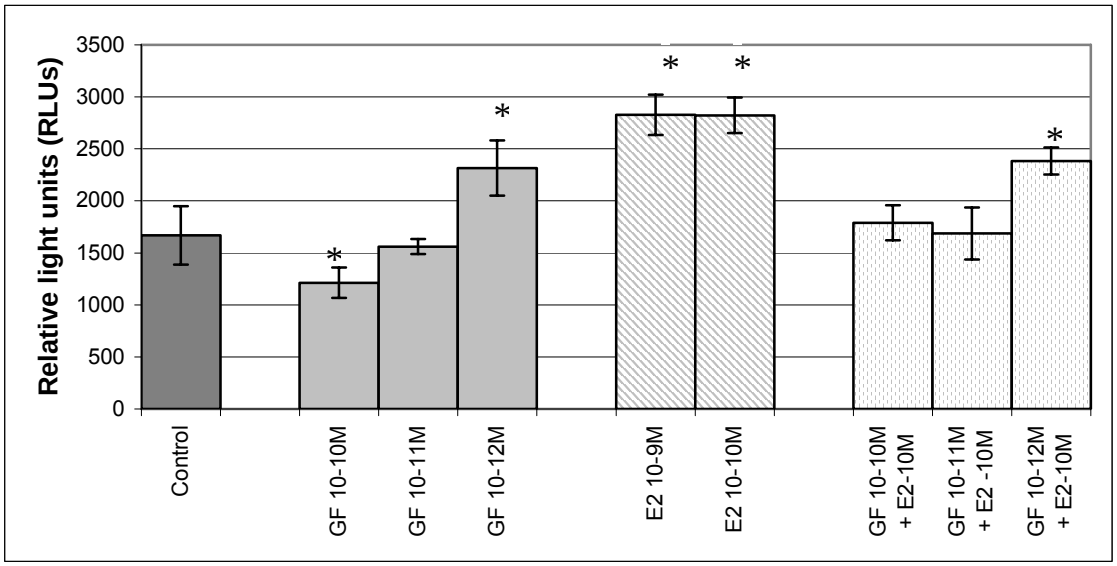



***Figure 8: Proliferative responses of HCC1500 to growth factors EGF, FGF and IGF-I (GF) $10^{-10}$M to $10^{-12}$M, estradiol (E2) $10^{-9}$M to $10^{-10}$M, and a combination of growth factors and estradiol in DMEM/FBS-stripped medium. (Mean ± SD, n=4). * = p<0.05***

Further assays were therefore conducted using AIM-V medium for MCF10A cells and DMEM/FBS-stripped medium for HCC1500 cells. The concentrations of growth factors (i.e., a combination of EGF, FGF and IGF-I) and estradiol used were $10^{-12}$M (1pM) and $10^{-10}$M (100pM) respectively.

## *3.2 MCF10A Results*

### 3.2.1 MCF10A proliferation assays with progestogens in combination with growth factors

- *P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M to $10^{-10}$M alone and in combination with EGF, FGF and IGF-I $10^{-12}$M*

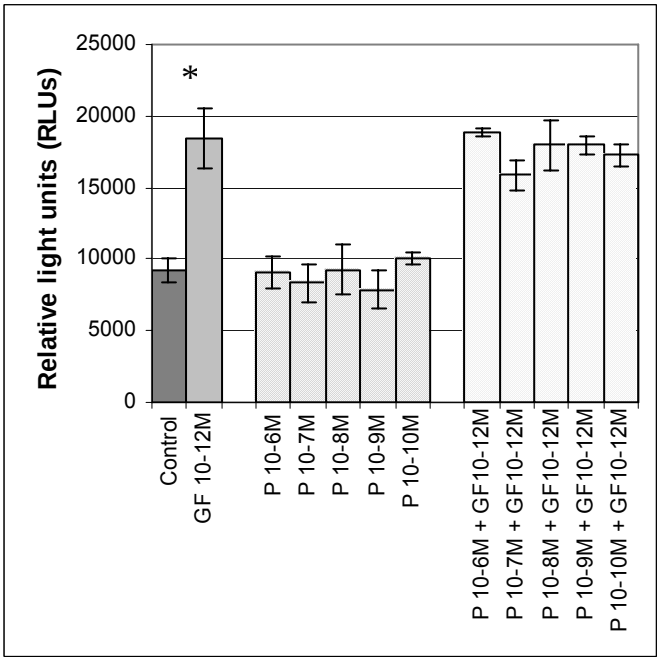
The results of these assays are illustrated in Fig. 9(a-h)

In comparison to the medium-only control, alone, none of the progestogens was able to significantly affect the proliferation of the MCF10A cells.

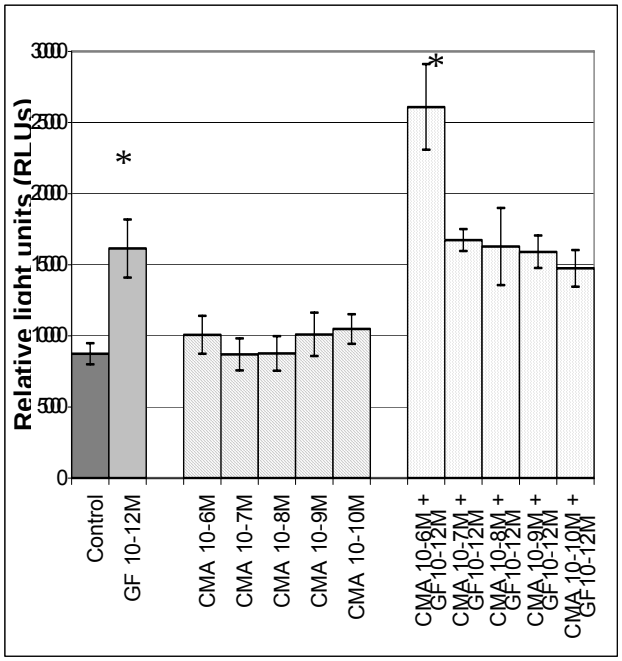
In combination with growth factors, P, NET, LNG, DNG, GSD and KDG had no significant effect on growth factor-induced proliferation.

MPA had the greatest proliferative effect, significantly enhancing the response to growth factors at the two highest concentrations, with 138% and 125% increases in cell proliferation respectively compared to the growth factor control. CMA followed, with a significant 62% increase in proliferation at $10^{-6}$M only.

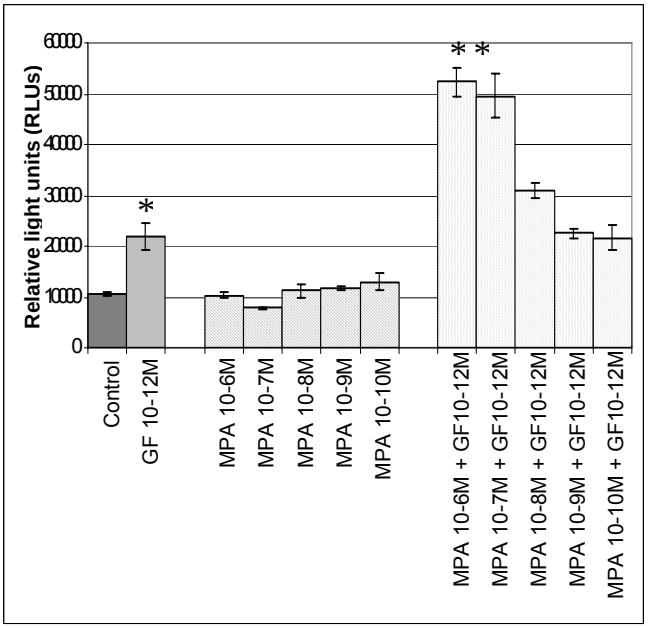
***Figure 9 (a-h): Proliferative responses of MCF10A to the various progestogens in concentrations of $10^{-6}$M to $10^{-10}$M, alone and in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) in AIM-V medium. (Mean ± SD, n=4). * = p<0.05***



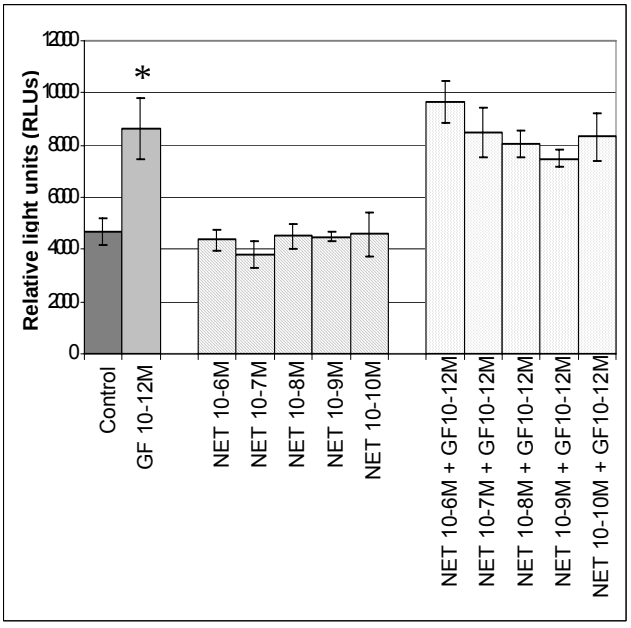

*a) Progesterone (P)*



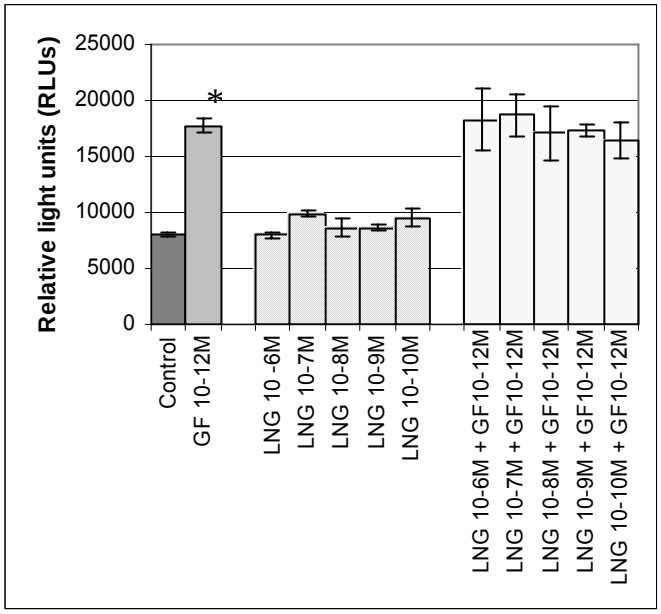

*b) Chlormadinone acetate (CMA)*



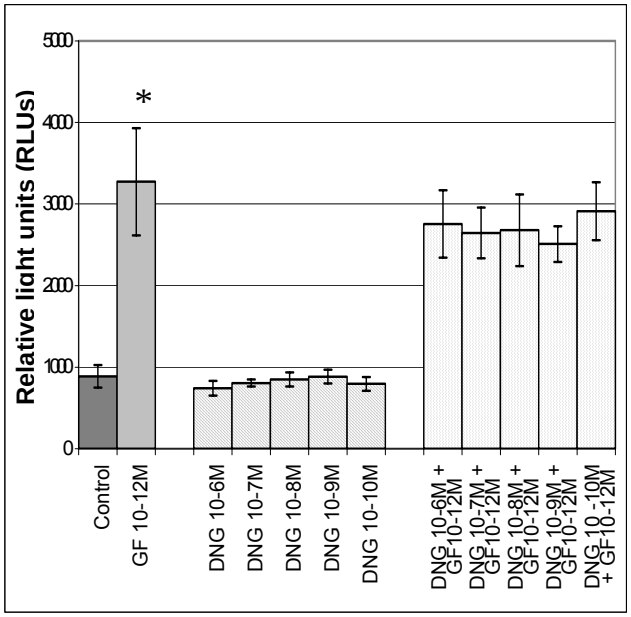

*c) Medroxyprogesterone acetate (MPA)*



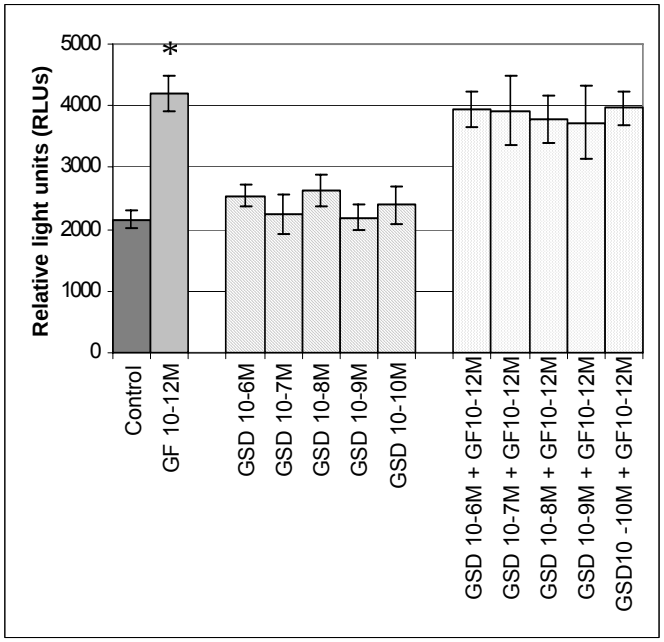

*d) Norethisterone (NET)*



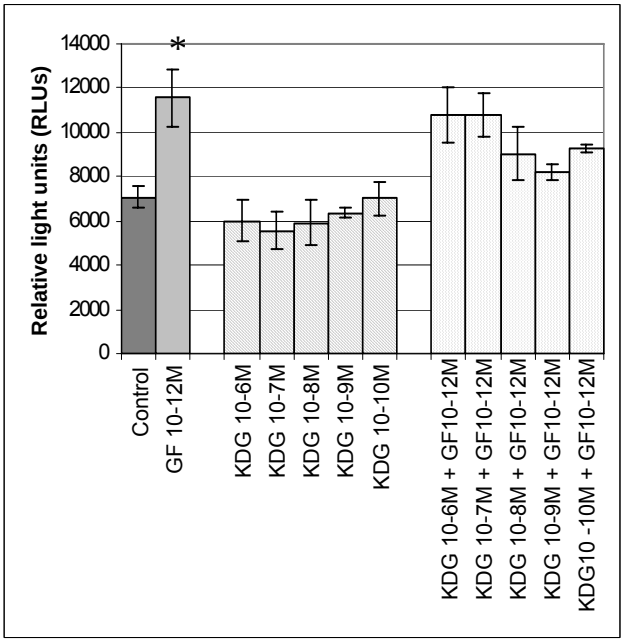

*e) Levonorgestrel (LNG)*




*f) Dienogest (DNG)*




*g) Gestodene (GSD)*




*h)3-Ketodesogestrel(KDG)*

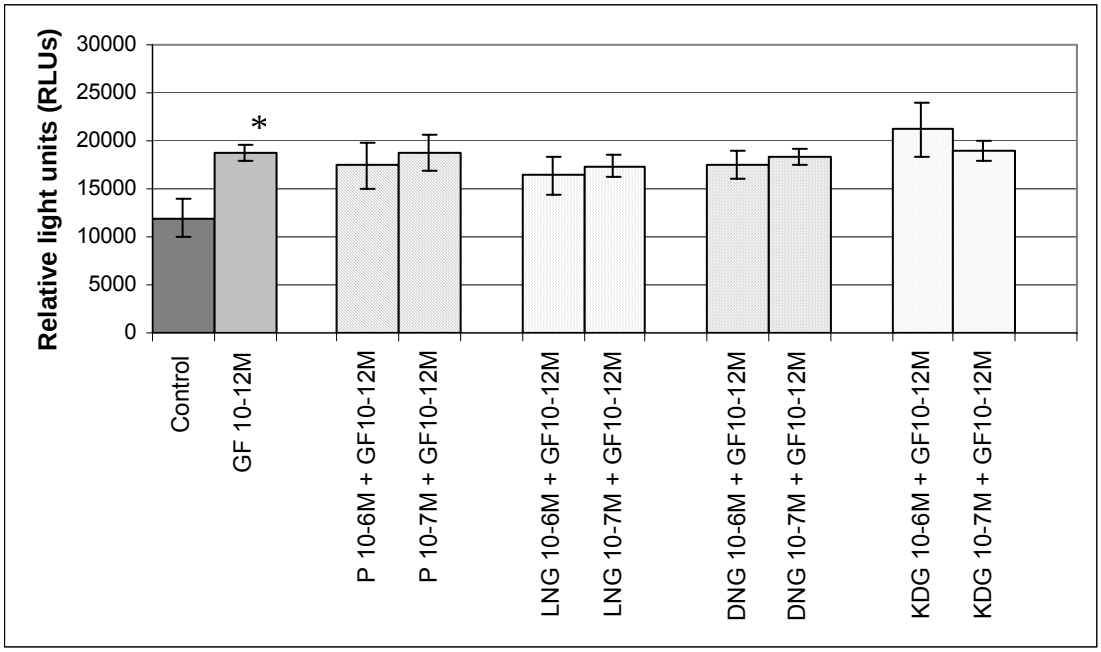
3.2.2 MCF10A Cell death detection:proliferation assays with progestogens in combination with growth factors

MCF10A cells were incubated with each of the progestogens in turn at concentrations of $10^{-6}$M and $10^{-7}$M alone and in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M, as described in 2.2.1. Progestogens in concentrations on $10^{-6}$M and $10^{-7}$M were used here as significant effects were seen at these concentrations in the proliferation assays in 3.2.1. Cell death detection (CDD) assays, as described in 2.2.2, were carried out in parallel to the proliferation assays, using the same progestogens and combinations of growth factors.

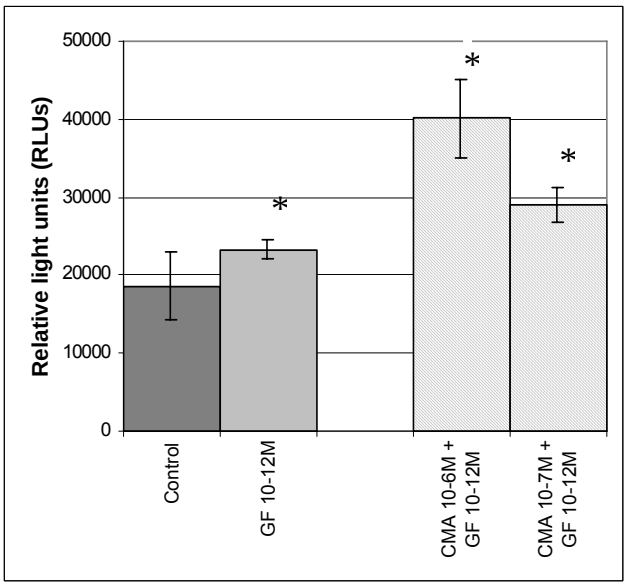
Ratios of cell death detection:proliferation were calculated as described in 2.3.

The results of the proliferation assays are illustrated in Fig.10 (a-e), cell death detection assays in Fig. 11 (a-d) and final results of cell death detection:proliferation ratios in Fig.12.

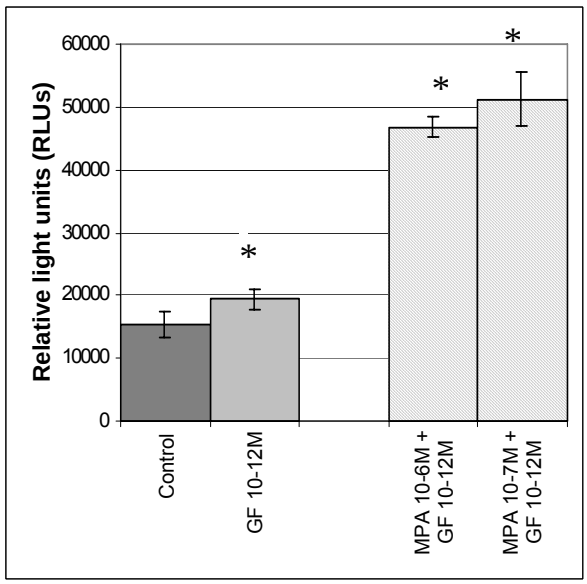
***Figure 10 (a-e): Proliferative responses of MCF10A to the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) in AIM-V medium. (Mean ± SD, n=3). * = p<0.05***



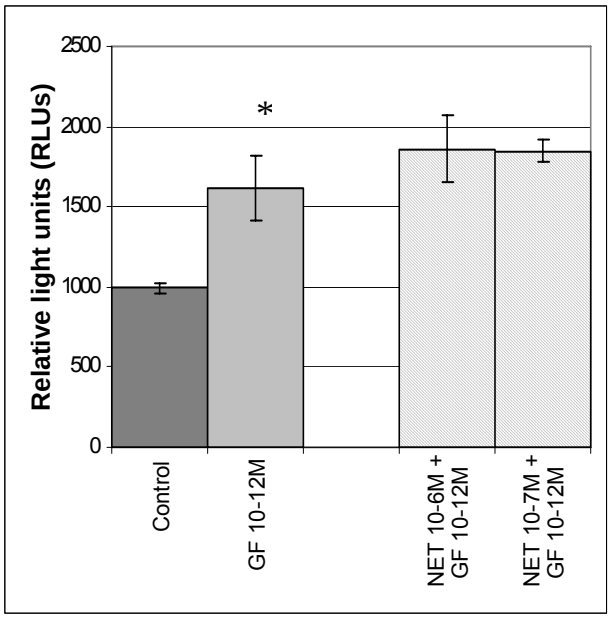

*a)Progesterone (P), Levonorgestrel (LNG), Dienogest (DNG) and 3-Ketodesogestrel (KDG)*



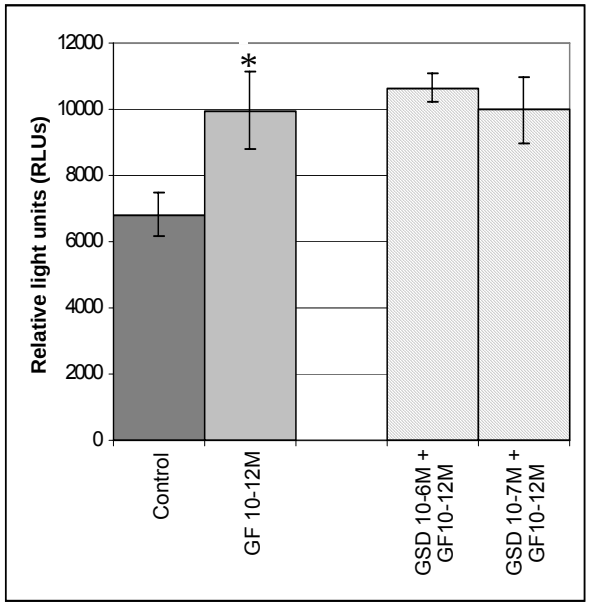

*b) Chlormadinone acetate (CMA)*




*c)Medroxyprogesterone acetate(MPA)*



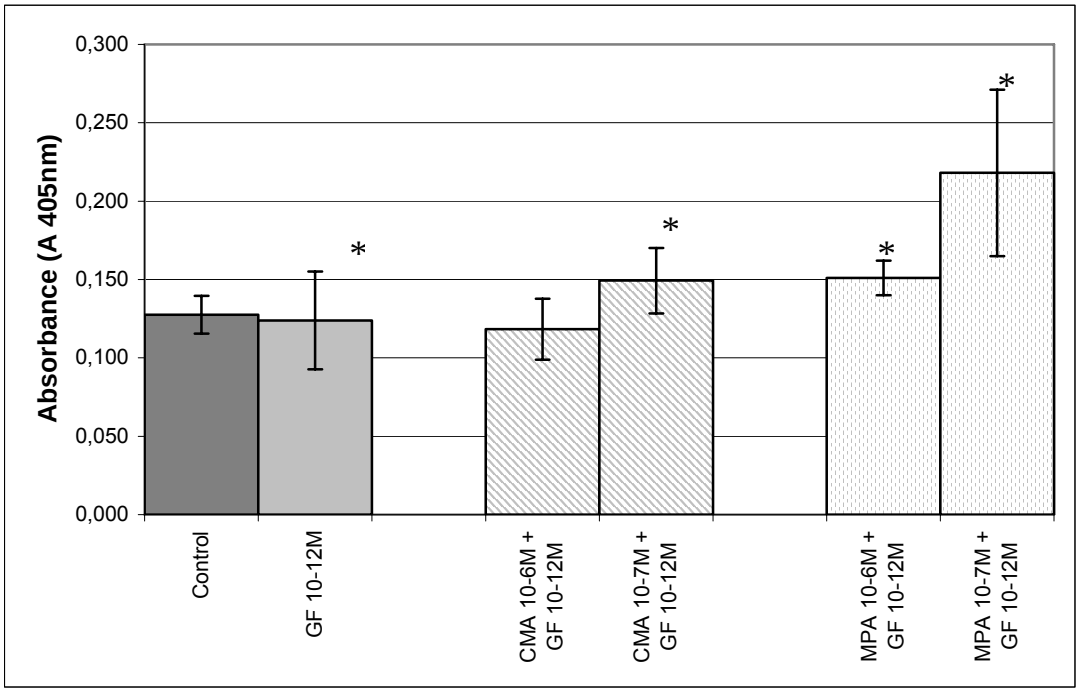

*d) Norethisterone (NET)*



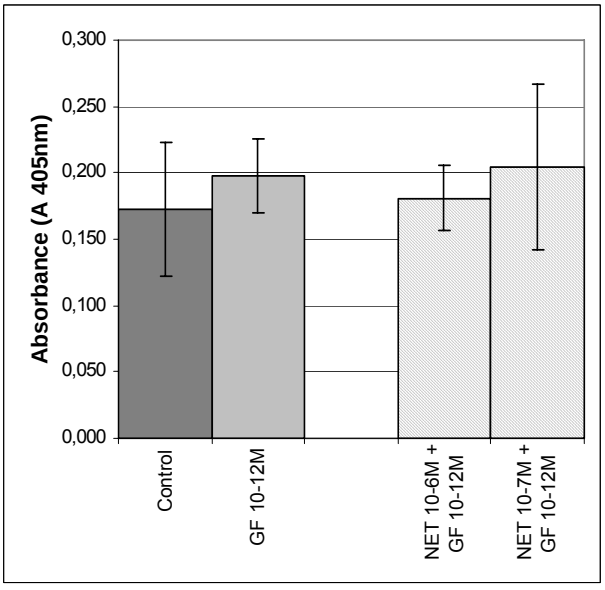

*e) Gestodene (GSD)*

Proliferation assay results in Fig. 10 (a-e) agreed with the initial proliferation assay results shown in Fig. 9 (a-h), i.e., only CMA and MPA were able to significantly further enhance the proliferative response of MCF10A cells to growth factors. No progestogen at a concentration of $10^{-6}$M or $10^{-7}$M significantly reduced the proliferative response. Cell death detection results are shown in Fig. 11 (a-d). They can only be discussed in context with proliferation assays as a ratio of cell death detection:proliferation (Fig. 12).

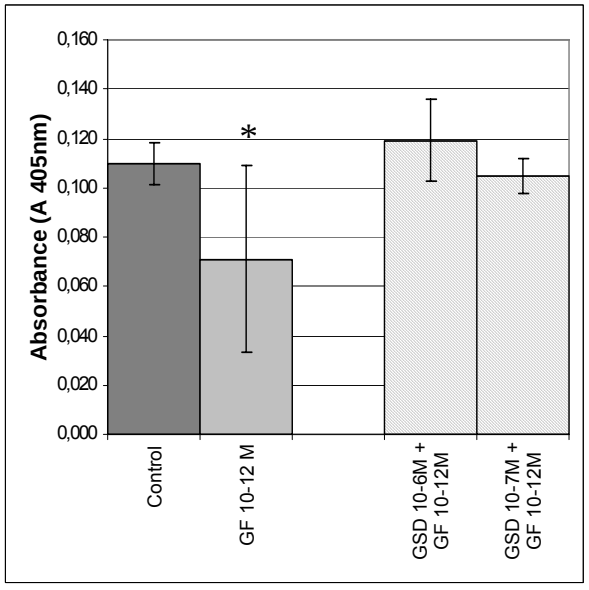
***Figure 11 (a-d): Cell death detection measurements of MCF10A after incubation with the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) in AIM-V medium. (Mean ± SD, n=3). * = p<0.05***



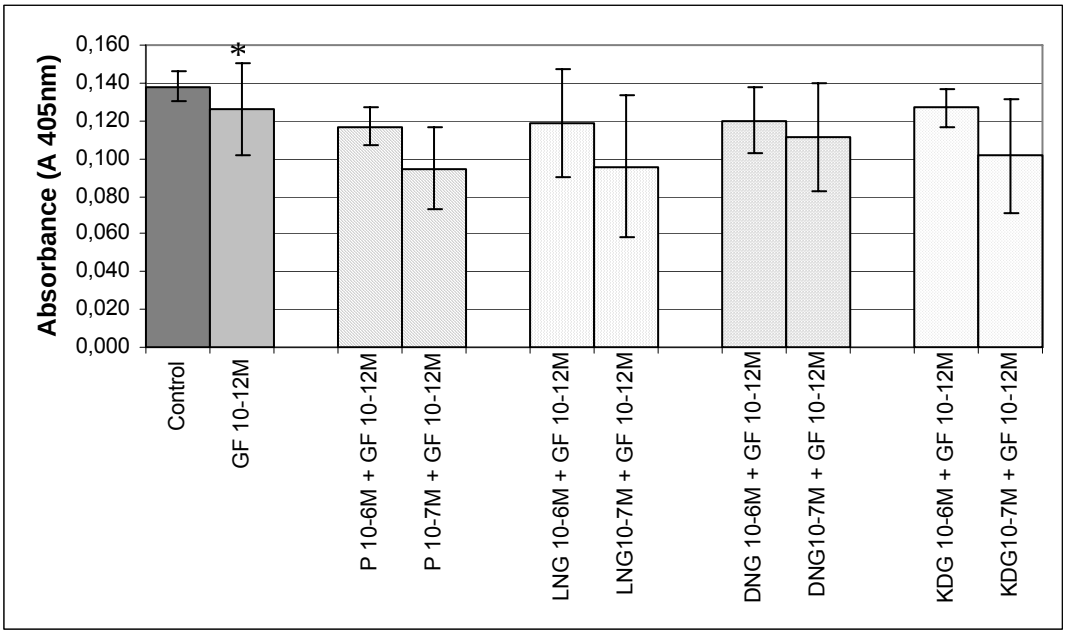

*a) Chlormadinone acetate (CMA), Medroxyprogesterone acetate (MPA)*




*b) Norethisterone (NET)*




*c) Gestodene (GSD)*



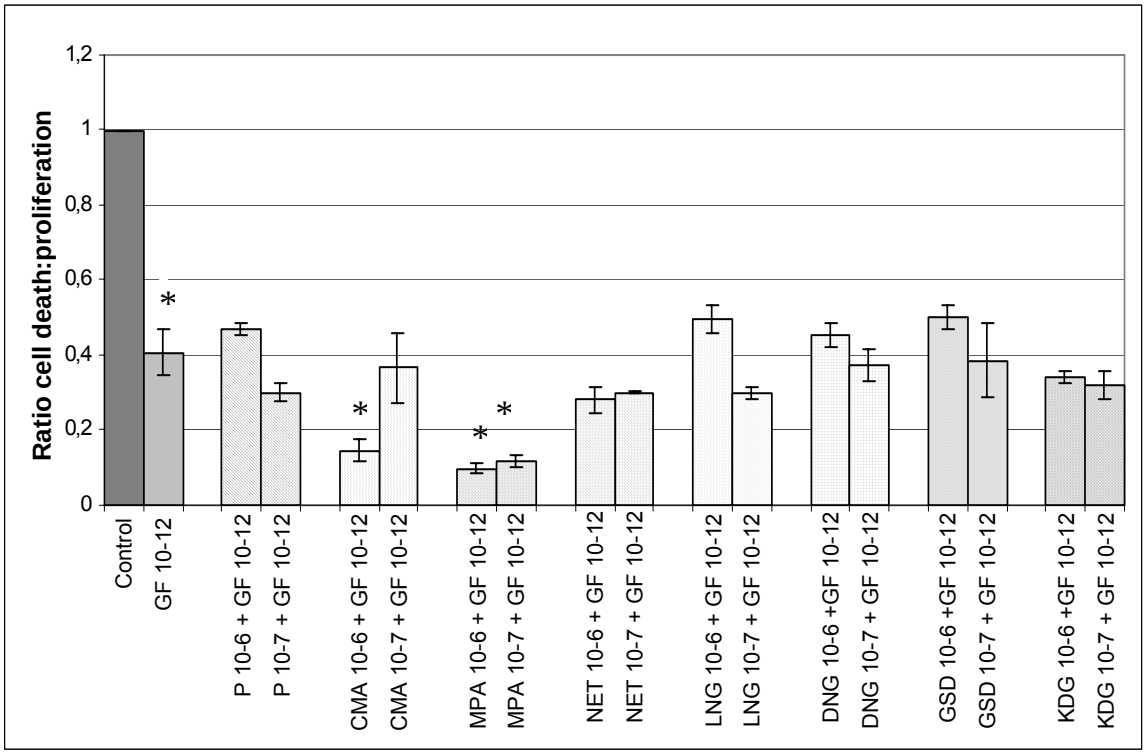

*d) Progesterone (P), Levonorgestrel (LNG), Dienogest (DNG) and 3-Ketodesogestrel (KDG)*

The results of the cell death detection:proliferation effects of the progestogens in combination with growth factors on the MCF10A cell line are illustrated in Fig. 12. The combination of the growth factors EGF, FGF and IGF-I alone confirmed a proliferative response compared to control.

In combination with growth factors, the ratio was reduced significantly compared to the growth factor control by MPA and CMA (i.e. favouring an additional proliferative effect as ratios below that of growth factor control favour a proliferative response, ratios above control favour an inhibitory response).

MPA induced 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 $10^{-6}$M only, reducing the ratio 3-fold. P, NET, LNG, DNG, GSD and KDG had no significant effect on the cell death detection:proliferation ratio for growth factor-induced stimulation of MCF10A.

***Figure 12: Cell death detection:proliferation measurements of MCF10A after incubation with the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) in AIM-V medium. (Mean ± SD, n=3). * = p<0.05***



### 3.2.3 MCF10A proliferation inhibitor assays with progestogens alone and in combination with growth factors

In the previous proliferation and cell death detection: proliferation assays, MPA and CMA were the only two progestogens capable of further stimulating the proliferative response of MCF10A cells to growth factors. To further investigate the pathway by which this proliferative effect occurs, the cells were incubated with the proliferation inhibitors PD98059 and LY294002 in the presence of the growth factors alone, MPA or CMA alone, and MPA or CMA together with growth factors.

The results for the effect of the progestogens in combination with growth factors on the MCF10A cell line are illustrated in Figs. 14 and 15. The combination of the growth factors EGF, FGF and IGF-I alone confirmed a significant proliferative response compared to control. Assays were conducted with MPA and CMA at a concentration of $10^{-6}$M in combination with growth factors at $10^{-12}$M, as significant proliferative effects were previously seen with both progestogens at these concentrations.

MPA and CMA alone had no significant effect on cell proliferation. However, MPA in combination with growth factors induced a significant 83% increase in cell proliferation at $10^{-6}$M in comparison to growth factors alone ($p$= <0.05). CMA in combination with growth factors also induced a proliferative response, but less than that of MPA, with a significant effect at $10^{-6}$M, (61% increase in cell proliferation in comparison to growth factors alone).

Proliferation inhibitors were dissolved in dimethyl sulfoxide (DMSO), and a control of DMSO 0.01% in AIM-V medium indicated that this concentration had no significant effect on cell proliferation, as shown in Fig. 14.

The results of the effects of the two proliferation inhibitors on the growth factors alone are shown in Fig. 13. Neither of the inhibitors at a concentration of $10^{-6}$M had a significant effect on growth factor-induced cell proliferation. The assay was therefore repeated with the inhibitors at $10^{-5}$M, and at this concentration both inhibitors significantly reduced growth factor-induced cell proliferation, with LY294002 having the greatest effect, as shown in Fig. 16. Neither inhibitor

significantly affected cell proliferation in the presence of growth medium alone. The concentration of DMSO was 0.1%, and a control of DMSO alone at this concentration had no significant effect on normal cell growth.

The results of combining the two progestogens and growth factors in the presence of the proliferation inhibitors are shown in Figs. 14 and 15. PD98059 and LY294002 significantly inhibited cell growth induced by MPA in combination with growth factors, with PD98059 exerting the greatest blockade effect. Neither of the inhibitors was able to significantly reduce CMA and growth factor-induced cell proliferation.

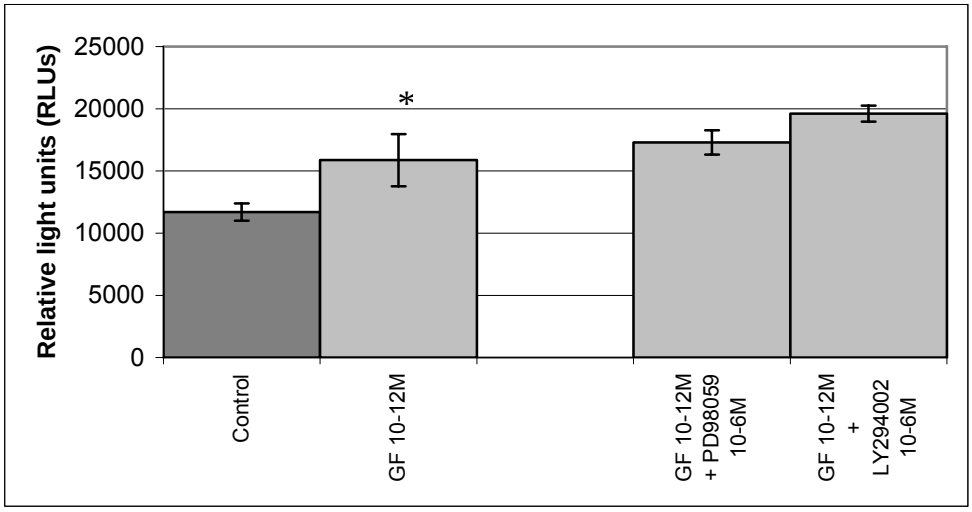



***Figure 13: Proliferative responses of MCF10A to growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) alone and in the presence of the proliferation inhibitors PD98059 $10^{-6}$M and LY294002 $10^{-6}$M in AIM-V medium. (Mean ± SD, n=4). * = p<0.05***

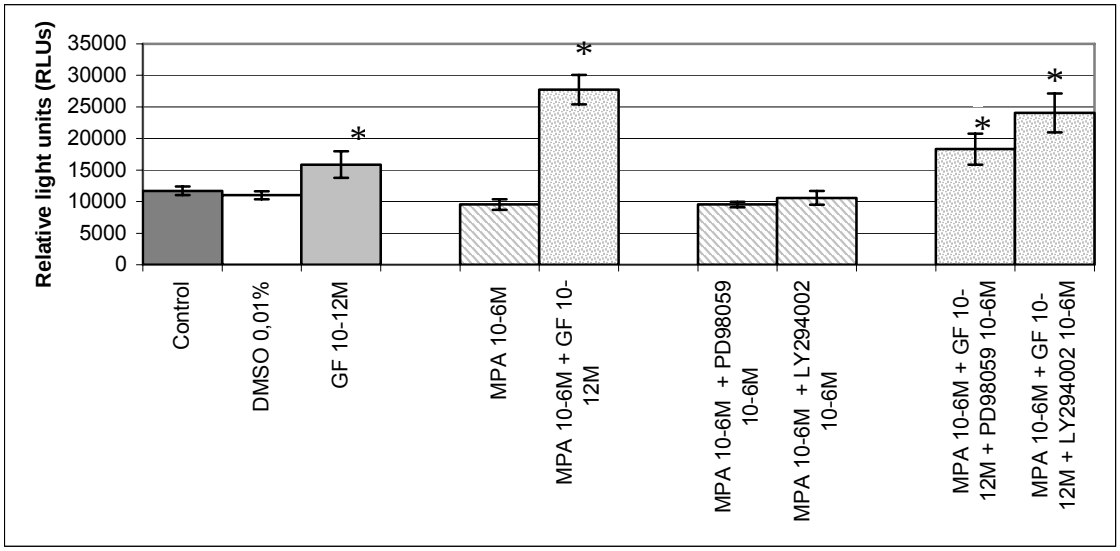



***Figure 14: Proliferative responses of MCF10A to growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and medroxyprogesterone acetate (MPA) $10^{-6}$M alone and in the presence of the proliferation inhibitors PD98059 $10^{-6}$M and LY294002 $10^{-6}$M in AIM-V medium. (Mean ± SD, n=4). * = p<0.05***

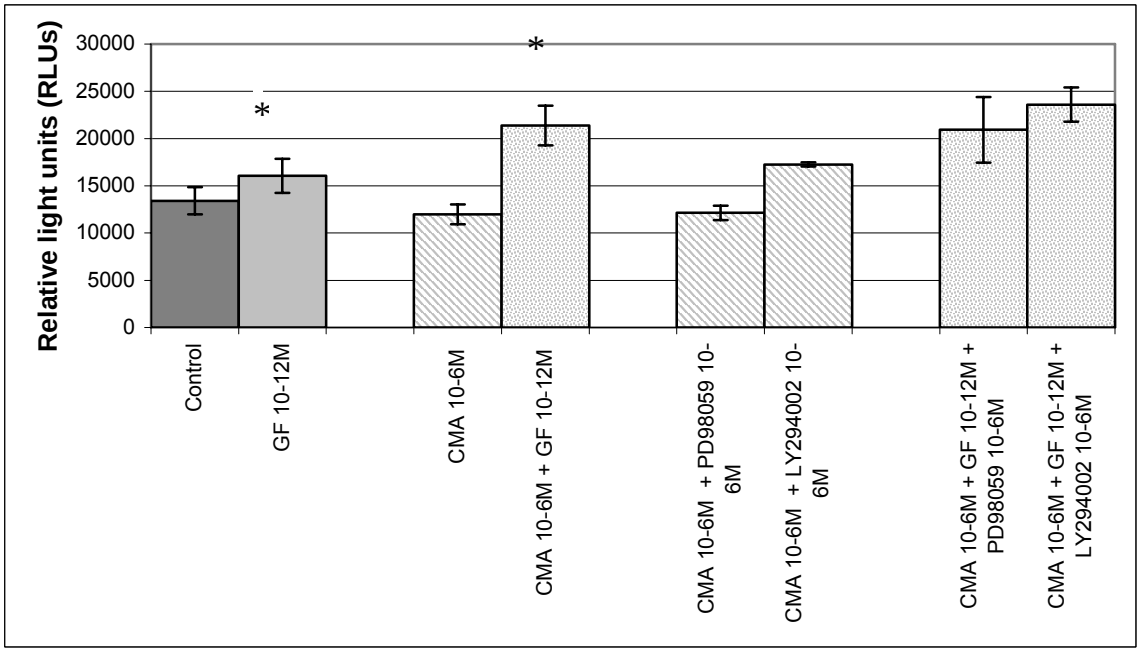



***Figure 15: Proliferative responses of MCF10A to growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and chlormadinone acetate (CMA) $10^{-6}$M alone and in the presence of the proliferation inhibitors PD98059 $10^{-6}$M and LY294002 $10^{-6}$M in AIM-V medium. (Mean ± SD, n=4). * = p<0.05***

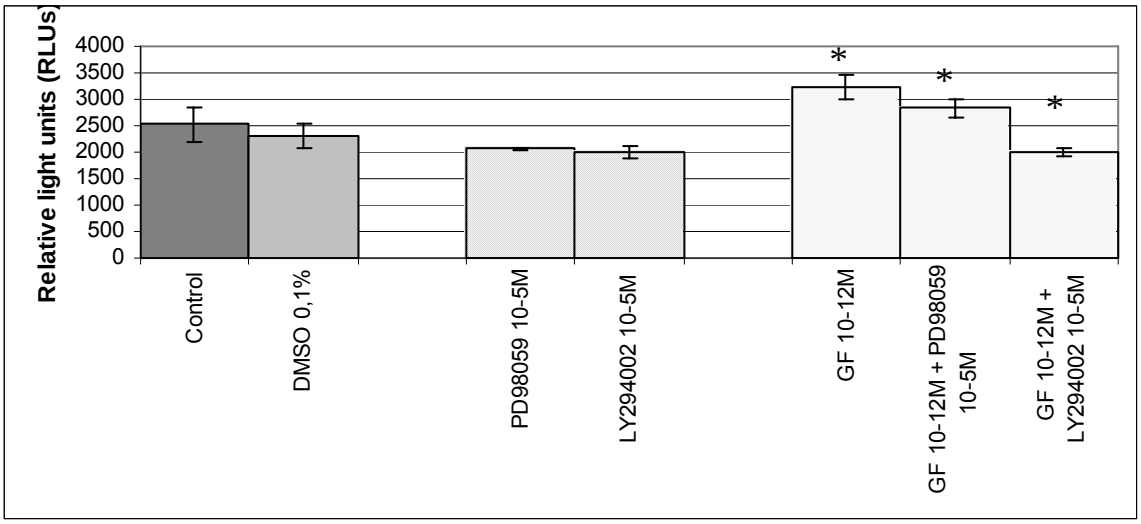



***Figure 16: Proliferative responses of MCF10A to growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) alone and in the presence of the proliferation inhibitors PD98059 $10^{-5}$M and LY294002 $10^{-5}$M in AIM-V medium. (Mean $\pm$ SD, n=4). * = p<0.05***

### 3.2.4 MCF10A apoptosis marker assays (cytochrome C, sFasL, p53) with progestogens in combination with growth factors

In the previous cell death detection:proliferation assays, only MPA and CMA were able to further stimulate the proliferative effects of growth factors on MCF10A cells. In order to investigate whether this proliferative pathway involves the inhibition of apoptosis, MCF10A cells were incubated with CMA and MPA in turn in the presence of growth factors. This incubation was followed by measurement of the apoptosis markers, cytochrome C, sFasL and p53 in cell lysates. Results are represented as percentage change in concentration of the markers compared to growth factor control.

Fig. 17 shows that growth factors alone reduced cytochrome C by 36%, and CMA $10^{-7}$M in combination with growth factors reduced cytochrome C release by a further 11%. CMA $10^{-6}$M reduced cytochrome C by a further 9% compared to growth factors alone, but this result was not significant due to a large variation in the results. MPA had no significant effect on cytochrome C release. Neither of the progestogens significantly affected sFasL inhibition by growth factors, as seen in Fig. 18. Both MPA and CMA at both concentrations were able to further reduce p53 release, which was already inhibited by growth factors. Growth factors reduced p53 release by 11% compared to control, CMA $10^{-6}$M and $10^{-7}$M by 11% and 14% respectively compared to growth factors alone and MPA $10^{-6}$M and $10^{-7}$M by 20% and 28% respectively, as illustrated in Fig. 19.

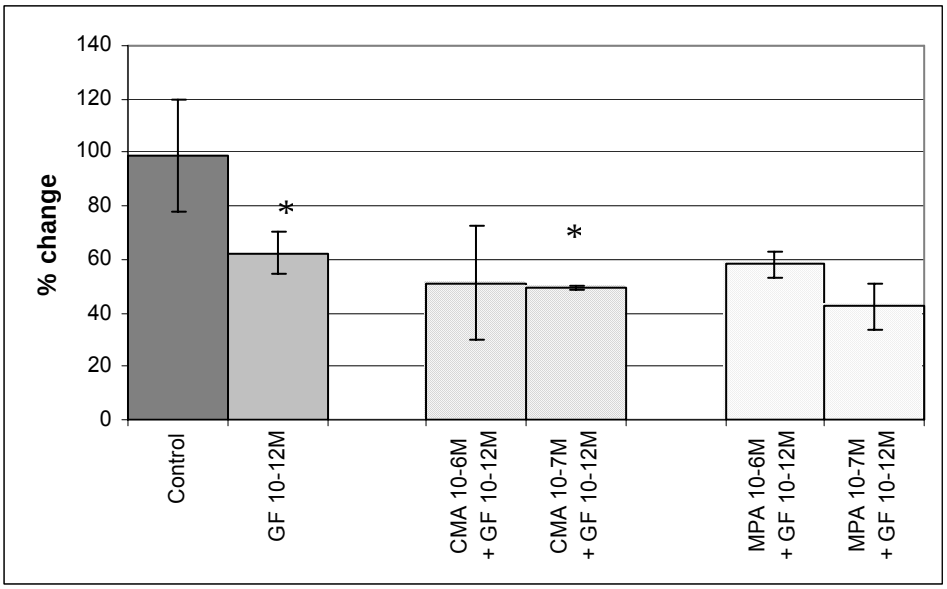



***Figure 17: Changes in cytochrome C release from MCF10A cells after addition of growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) alone and in combination with chlormadinone acetate (CMA) $10^{-6}$M and $10^{-7}$M and medroxyprogesterone acetate (MPA) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control. (Mean ± SD, n=4). * = p<0.05***

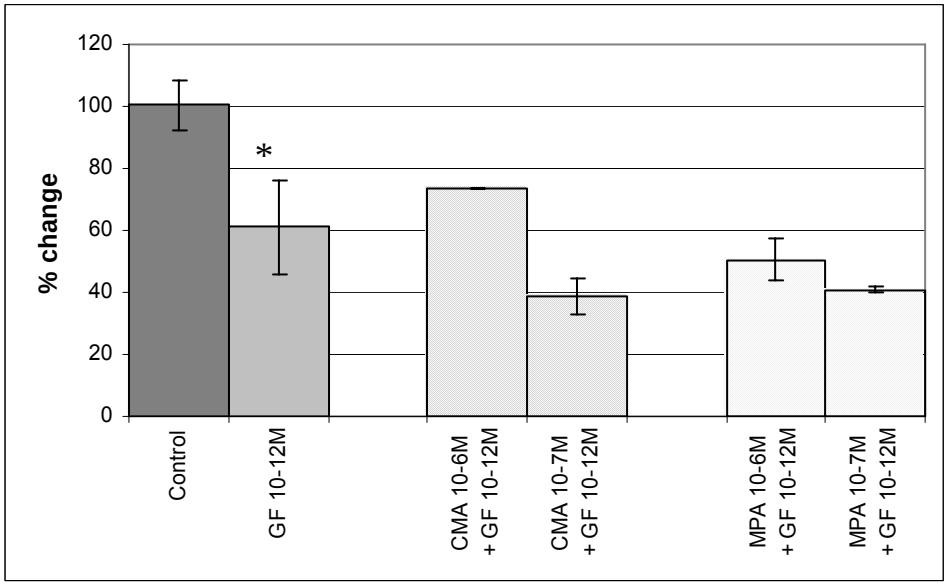



***Figure 18: Changes in sFasL release from MCF10A cells after addition of growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) alone and in combination with chlormadinone acetate (CMA) $10^{-6}$M and $10^{-7}$M, and medroxyprogesterone acetate (MPA) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control. (Mean ± SD, n=4). * = p<0.05***

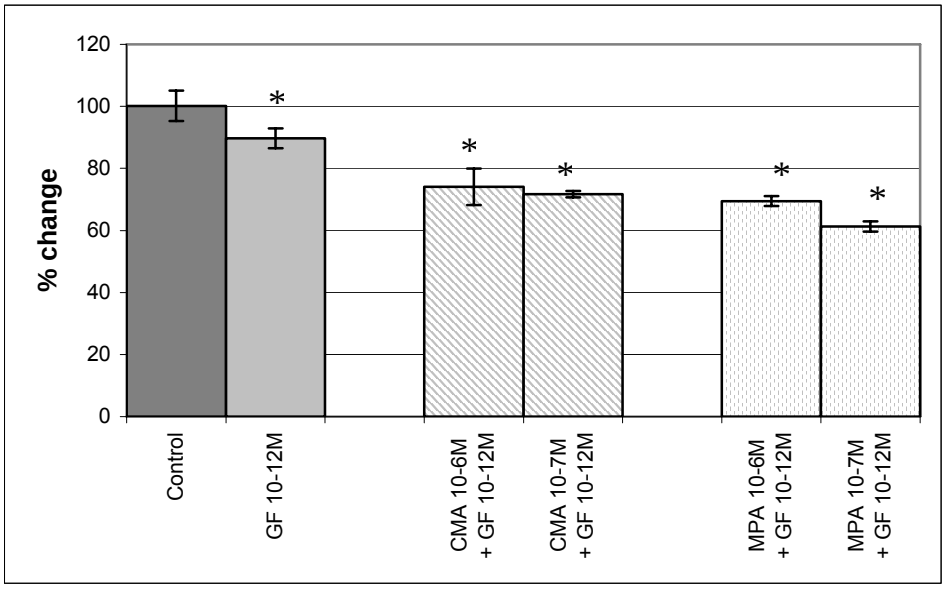



***Figure 19: Changes in p53 release from MCF10A cells after addition of growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) alone and in combination with chlormadinone acetate (CMA) $10^{-6}$M and $10^{-7}$M and medroxyprogesterone acetate (MPA) $10^{-6}$M and$10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control. (Mean ± SD, n=4). * = p<0.05***

### *3.3 HCC1500 results*

3.3.1 HCC1500 proliferation assays with progestogens in combination with estradiol

- *P, CMA, MPA, NET, LNG, DNG, GSD and KDG $10^{-6}$M to $10^{-10}$M alone and in combination with estradiol $10^{-10}$M*

The results of these assays are illustrated in Fig. 20 (a-h).

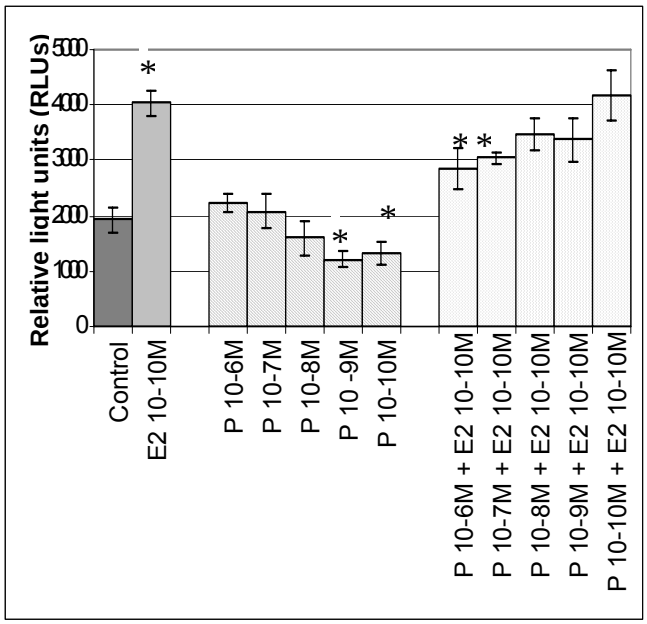
In comparison to control, alone, only GSD and KDG had no significant effect on cell proliferation over the whole concentration range. In contrast, NET was able to significantly increase cell proliferation over the whole concentration range by between 133% and 62% compared to control. CMA increased cell proliferation between 45% and 18% over the $10^{-6}$M to $10^{-8}$M concentration range. LNG also significantly increased cell proliferation at $10^{-6}$M and $10^{-7}$M by 58% and 30% respectively. DNG significantly increased proliferation over the $10^{-6}$M to $10^{-9}$M range by between 23% and 36%.

Proliferation was significantly inhibited by MPA $10^{-6}$M to $10^{-9}$M by up to 27%, by P $10^{-9}$M and $10^{-10}$M by 37% and 31% respectively.

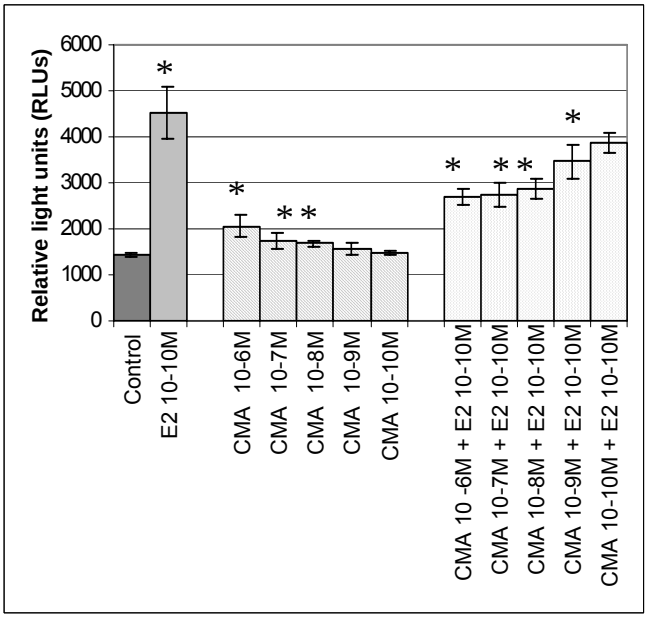
In combination with estradiol (E2), DNG and KDG had no significant effect on E2 induced proliferation. None of the remaining progestogens was able to further enhance the proliferative effects of E2, and in contrast to this, all inhibited the proliferative response.

MPA had the greatest anti-proliferative effect, significantly inhibiting the response to E2 over the $10^{-6}$M to $10^{-9}$M concentration range by up to 50%. CMA followed with significant inhibition over the same concentration range of up to 40%. NET, LNG, P and GSD $10^{-6}$M reduced E2-induced proliferation by 36%, 35%, 30% and 23% respectively.

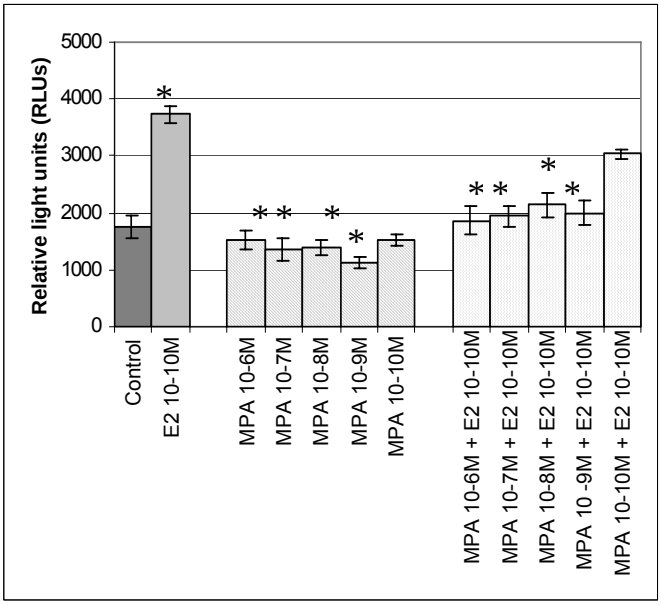
***Figure 20 (a to h): Proliferative responses of HCC1500 to the various progestogens in concentrations of $10^{-6}$M to $10^{-10}$M, alone and in combination estradiol (E2) $10^{-10}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=4). * = p<0.05***



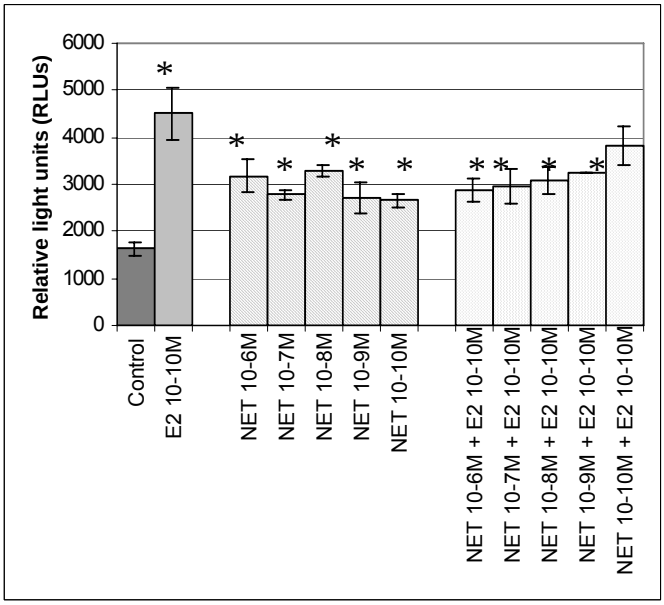

*a) Progesterone(P)*



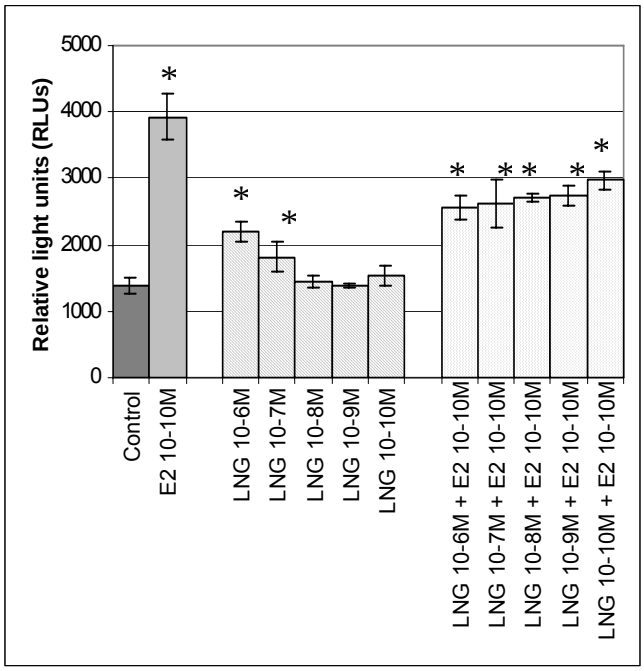

*b) Chlormadinone acetate (CMA)*



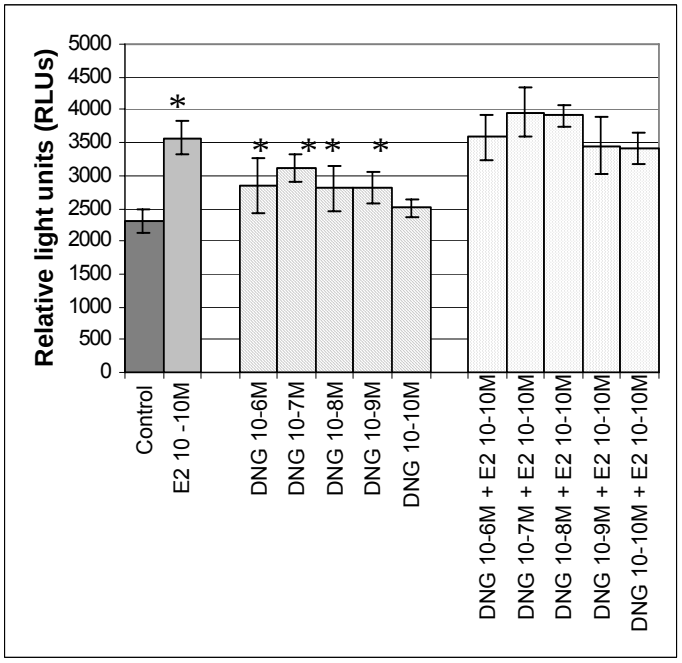

*c) Medroxyprogesterone acetate (MPA)*



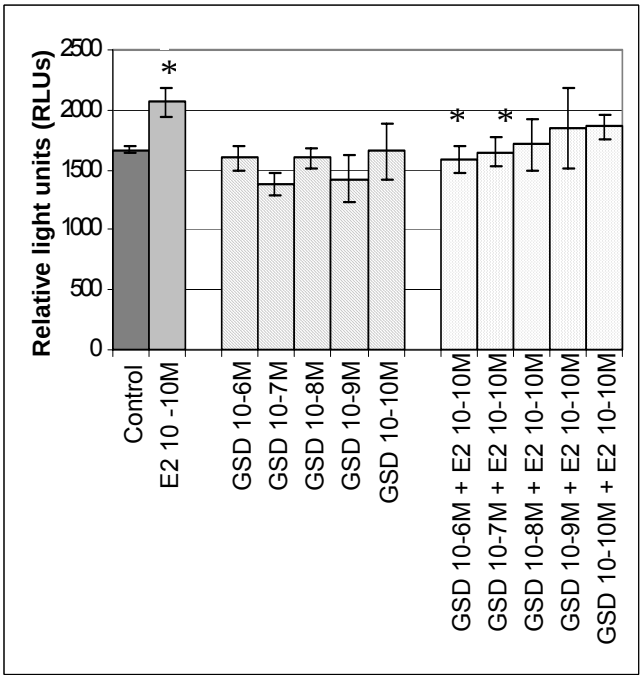

*d) Norethisterone (NET)*



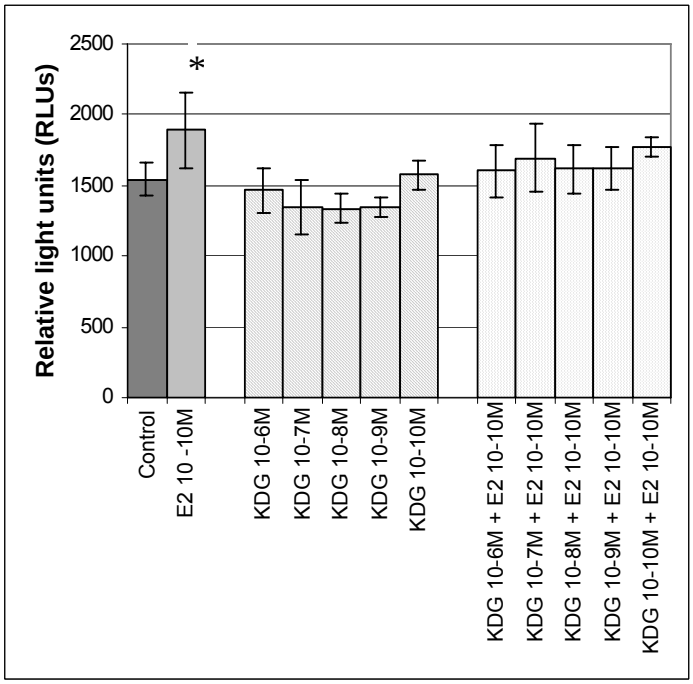

*e) Levonorgestrel (LNG)*




*f) Dienogest (DNG)*




*g) Gestodene (GSD)*




*h) 3-Ketodesogestrel (KDG)*

3.3.2 HCC1500 cell death detection:proliferation assays with progestogens in combination with growth factors and/or estradiol

HCC1500 cells were incubated with each of the eight of the progestogens in turn at concentrations of $10^{-6}$M and $10^{-7}$M (as significant effects had been seen at these concentrations in the proliferation assays in 3.3.1) according to the method in 2.2.1, as follows:

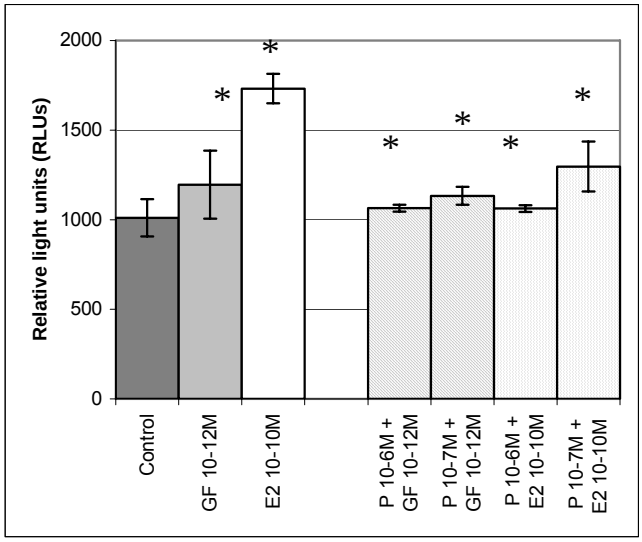
a) in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M,
b) in combination with E2 $10^{-10}$M,
c) in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M and E2 $10^{-10}$M

The results of these proliferation assays are shown in Figs. 21 (a-h) and 25 (a-b).

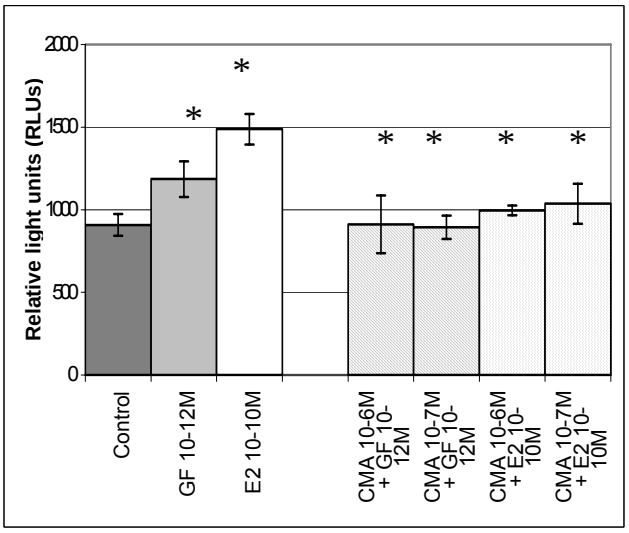
Cell death detection (CDD) assays, as described in 2.2.2, were carried out in parallel to the proliferation assays, using the same progestogens and combinations of growth factors and/or E2.

Ratios of cell death detection:proliferation were calculated as described in 2.3, and the final results are shown in Figs. 23, 24 and 27.

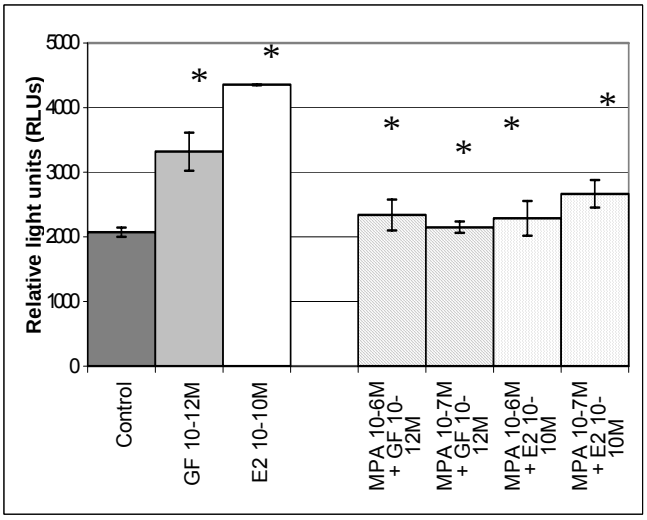
***Figure 21 (a to h): Proliferative responses of HCC1500 to the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) or estradiol (E2) $10^{-10}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=3). * = p<0.05***



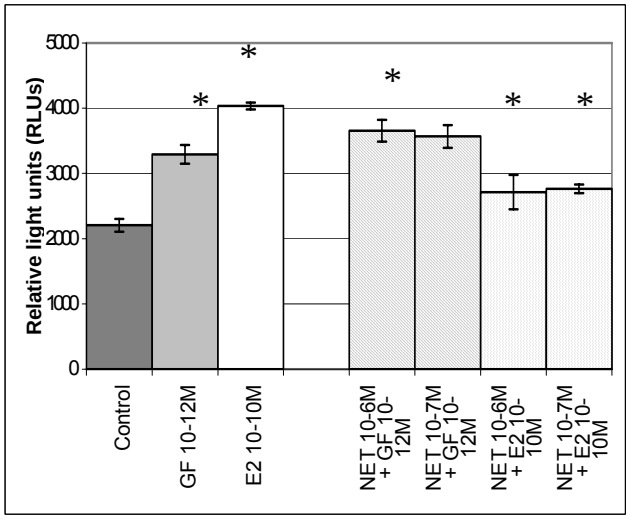

*a) Progesterone*



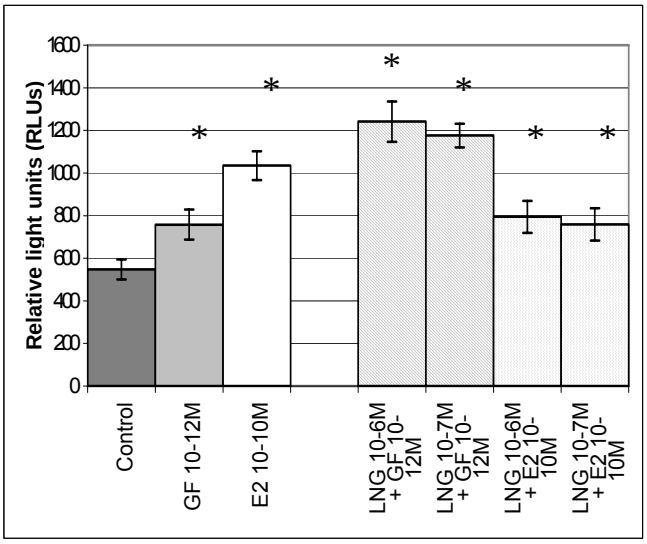

*b) Chlormadinone acetate*



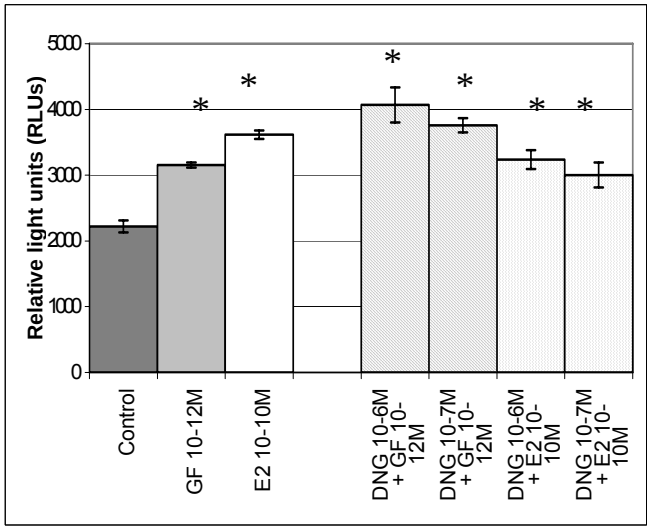

*c) Medroxyprogesterone acetate (MPA)*



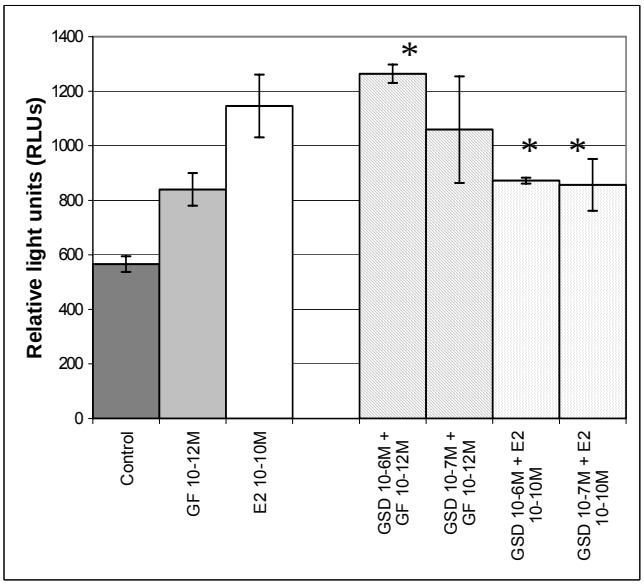

*d) Norethisterone (NET)*



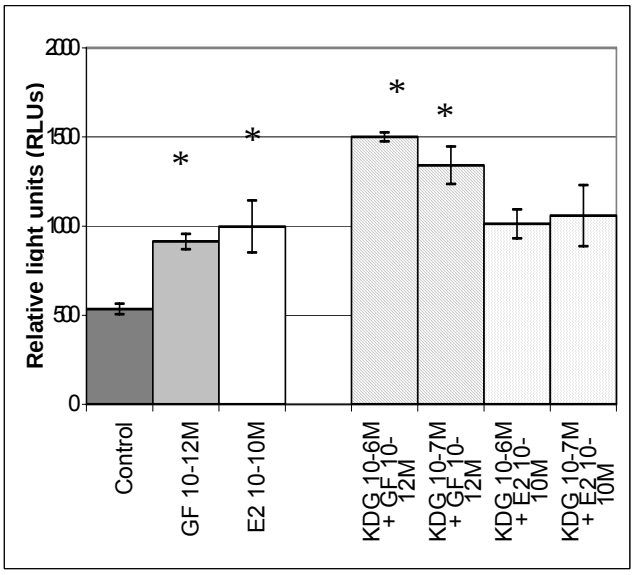

*e) Levonorgestrel (LNG)*




*f) Dienogest (DNG)*




*g) Gestodene (GSD)*



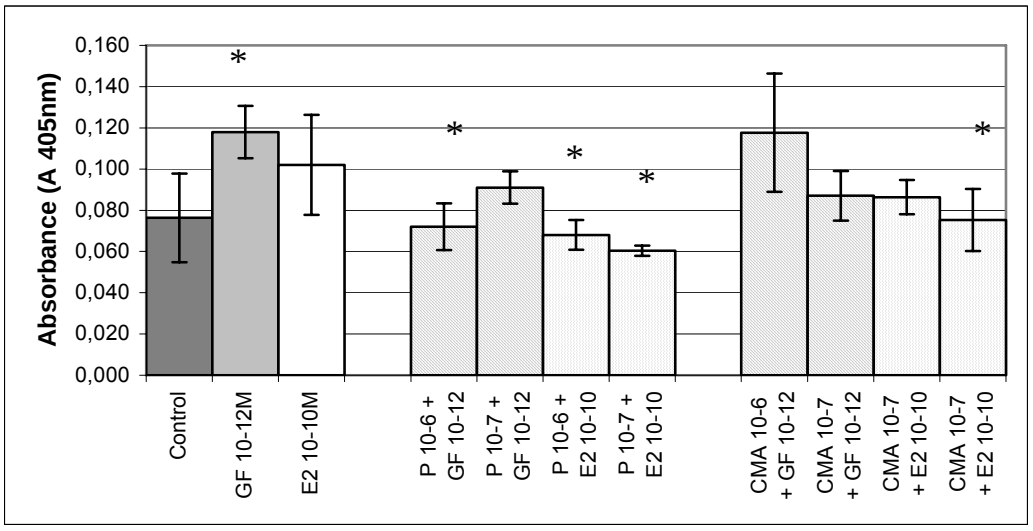

*h) 3-Ketodesogestrel (KDG)*

Proliferation assay results for the progestogens in the presence of E2 in Fig. 21 (a-h) agreed with the initial proliferation assay results shown in Fig. 20 (a-h) for all progestogens except DNG $10^{-7}$M + E2 10-$^{10}$M (significant anti-proliferative effect in these results, non-significant effect in the initial results).

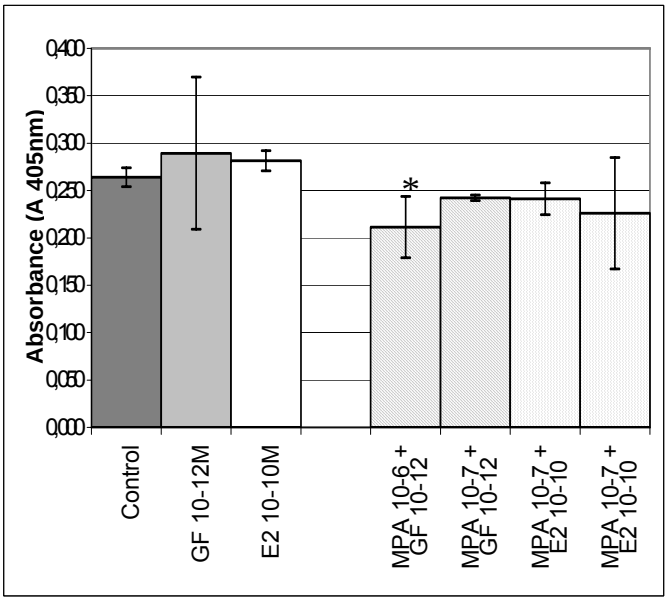
In combination with growth factors alone, (Fig. 21 (a-h)), only P, CMA and MPA at both concentrations were able to significantly inhibit the proliferative cell response to growth factors, with MPA $10^{-7}$M having the greatest effect, producing a 35% inhibition in proliferation. LNG, DNG and KDG at both concentrations and NET $10^{-6}$M and GSD $10^{-6}$M all significantly increased the proliferative response to growth factors. LNG increased proliferation by up to 64%, DNG up to 29%, KDG up to 47%, NET by 11% and GSD by 50%.

The cell death detection results (Fig. 22 (a-f)) can only be discussed in context with proliferation assays as a ratio of cell death detection:proliferation.

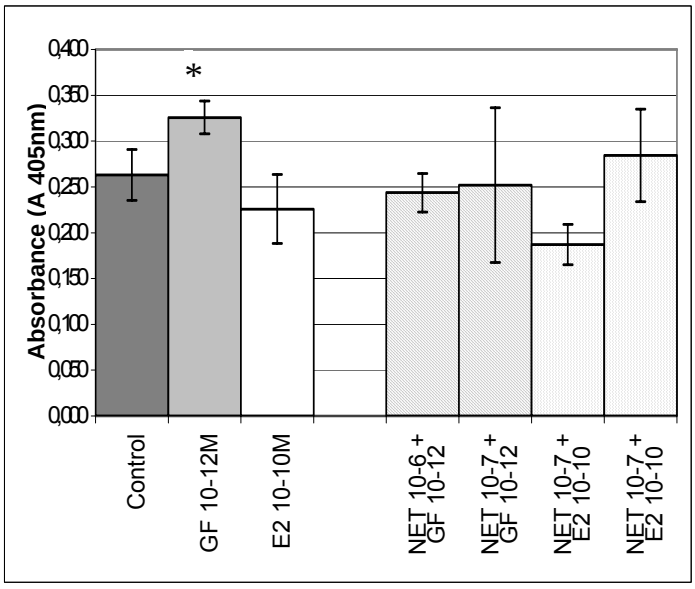
***Figure 22 (a to f): Cell death detection measurements of HCC1500 after incubation with the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) or estradiol (E2) $10^{-10}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=3). * = p<0.05***



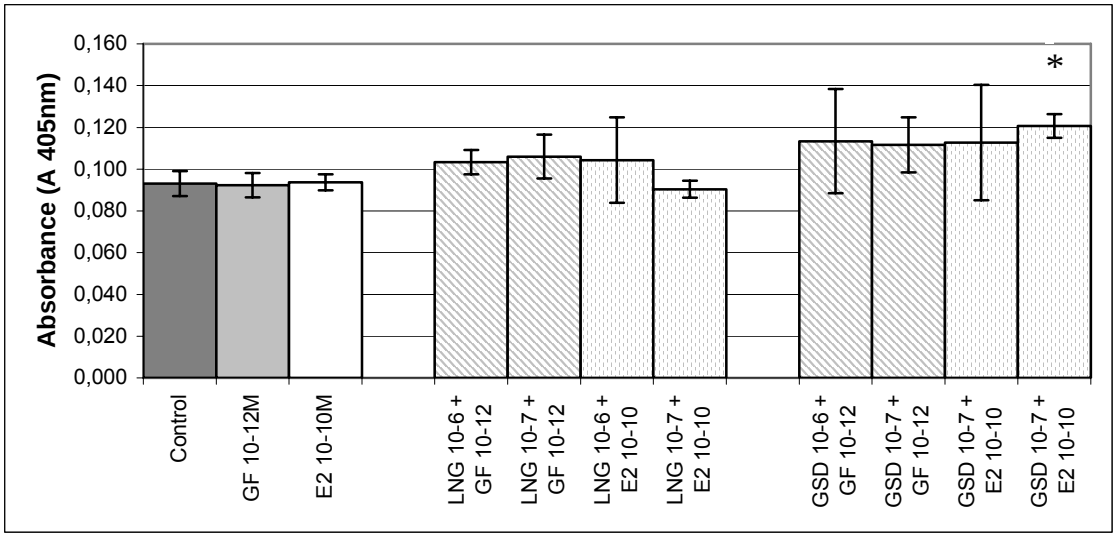

*a) Progesterone (P) and Chlormadinone acetate (CMA)*



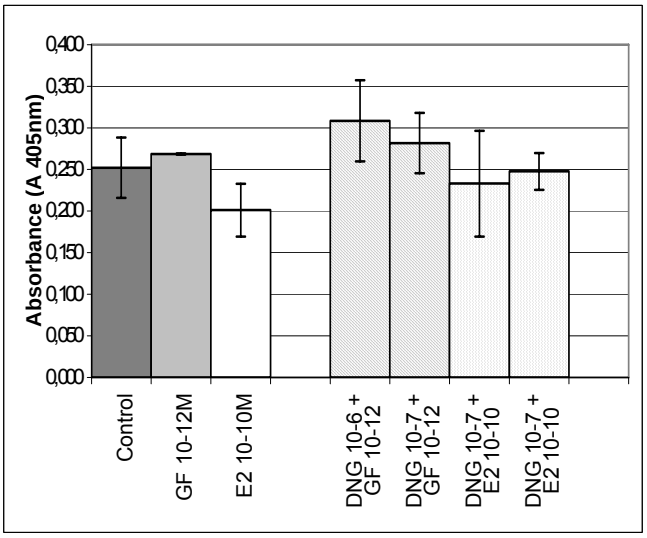

*b) Medroxyprogesterone acetate (MPA)*



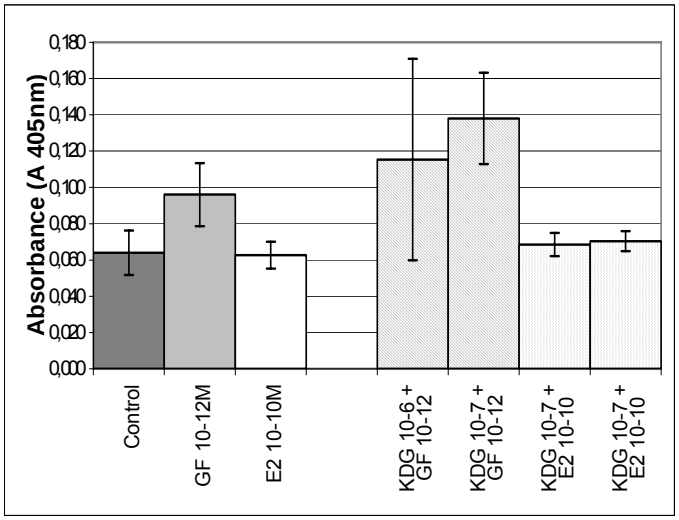

*c) Norethisterone (NET)*




*d) Levonorgestrel (LNG) and Gestodene (GSD )*




*e) Dienogest (DNG)*




*f) 3-Ketodesogestrel (KDG)*

The cell death detection:proliferation results for the effect of the progestogens in combination with growth factors on the HCC1500 cell line are illustrated in Fig. 23. The combination of the growth factors EGF, FGF and IGF-I alone confirmed a proliferative response compared to control. MPA in combination with growth factors caused a significant increase in the ratio at both concentrations ($p$=<0.05), the effect greatest at $10^{-7}$M, 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 $10^{-6}$M, yielding over a 2-fold ratio increase. Conversely, NET, LNG, and DNG at both concentrations and GSD, KDG at $10^{-6}$M led to a significant reduction in the ratio, favouring an enhancement of the initial proliferative effect induced by the growth factors. P had no significant effect at either concentration.

Fig. 24 shows the results of the combination of progestogens and estradiol (E2 $10^{-10}$M) on the estrogen-receptor positive (ER+) HCC1500 cells. As expected, E2 alone promoted a proliferative effect on cell growth (reduction in the ratio). In combination with E2, the progestogens CMA, MPA, NET, LNG, DNG and GSD at both concentrations, and P at $10^{-6}$M significantly increased the ratio towards an anti-proliferative effect to varying degrees, with MPA $10^{-6}$M having the greatest effect, followed by NET at $10^{-7}$M. 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.

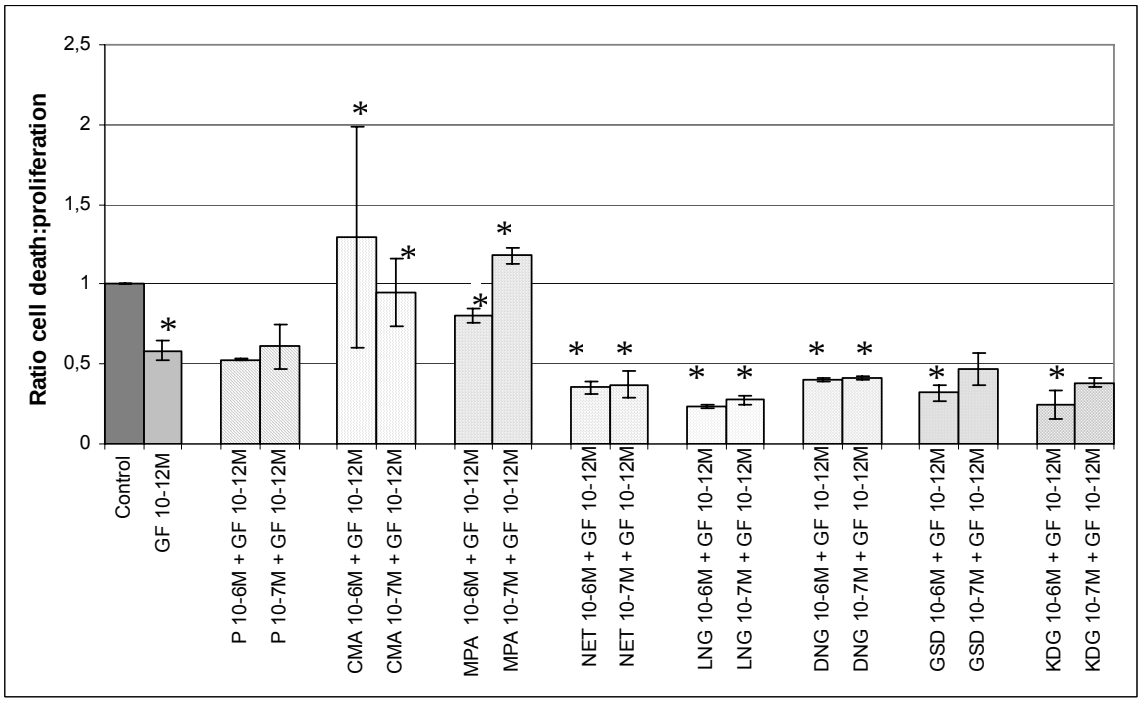



***Figure 23: Cell death detection:proliferation measurements of HCC1500 after incubation with the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) in DMEM/FBS-stripped medium. (Mean ± SD, n=3). * = p<0.05***

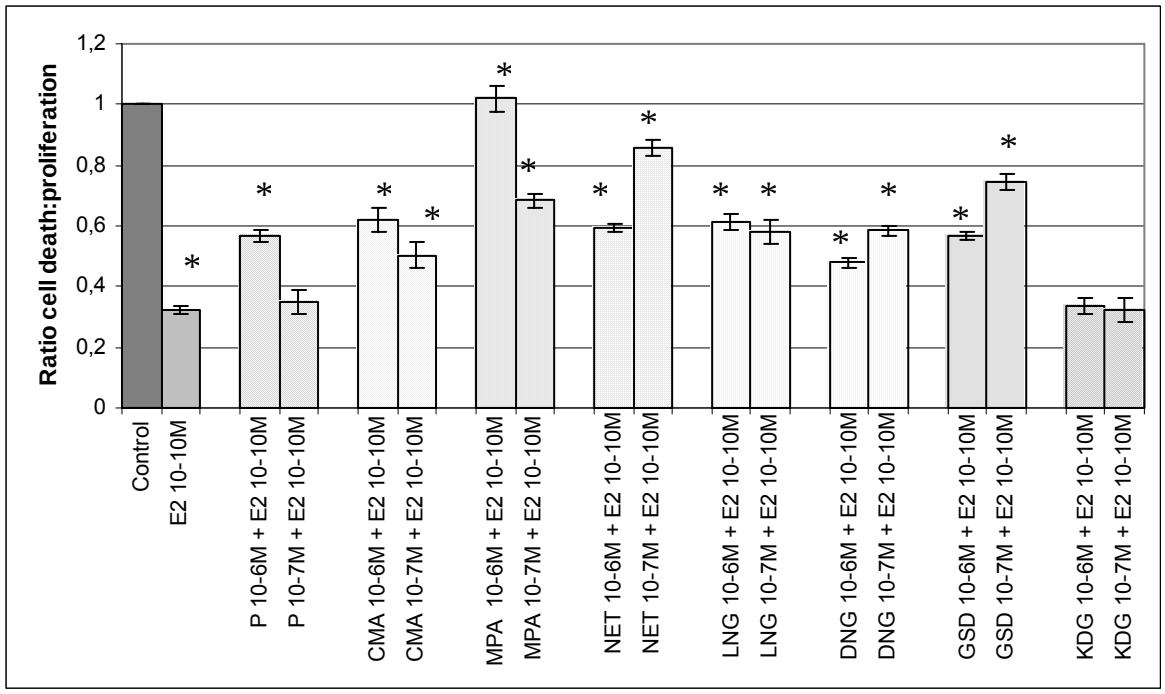
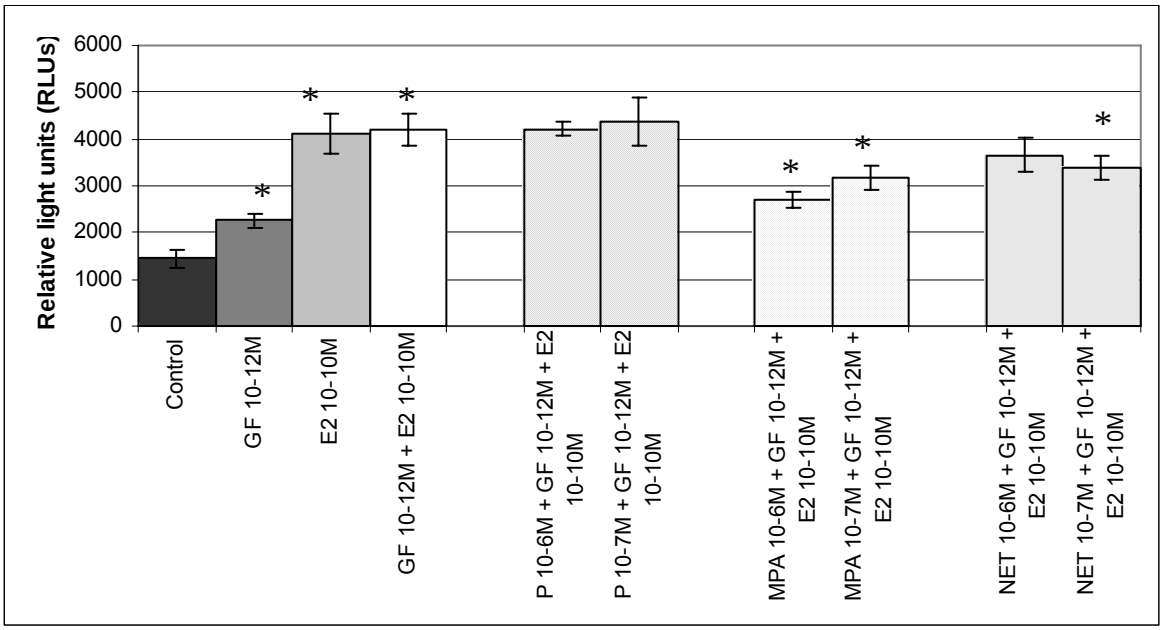


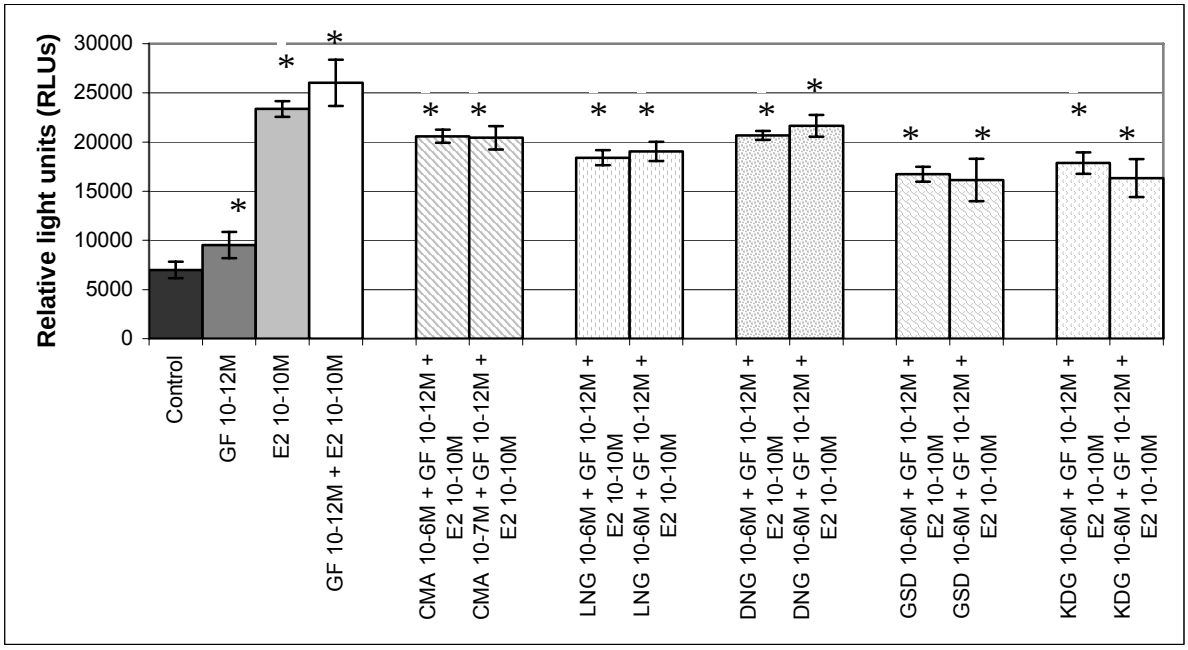

***Figure 24: Cell death detection:proliferation measurements of HCC1500 after incubation with the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with estradiol (E2)$10^{-10}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=3). * = p<0.05***

Proliferative responses of HCC1500 to the progestogens in the presence of both growth factors and estradiol in combination are shown in Fig. 25 (a-b). All progestogens but P inhibited the proliferative response. The maximal inhibitory responses were shown by GSD $10^{-7}$M and MPA $10^{-6}$M with 38% and 36% reductions in proliferation respectively. NET $10^{-6}$M had the least effect, reducing growth factor and E2 induced proliferation by only 13%.

***Figure 25 (a to b): Proliferative responses of HCC1500 to the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and estradiol (E2) $10^{-10}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=3). * = p<0.05***



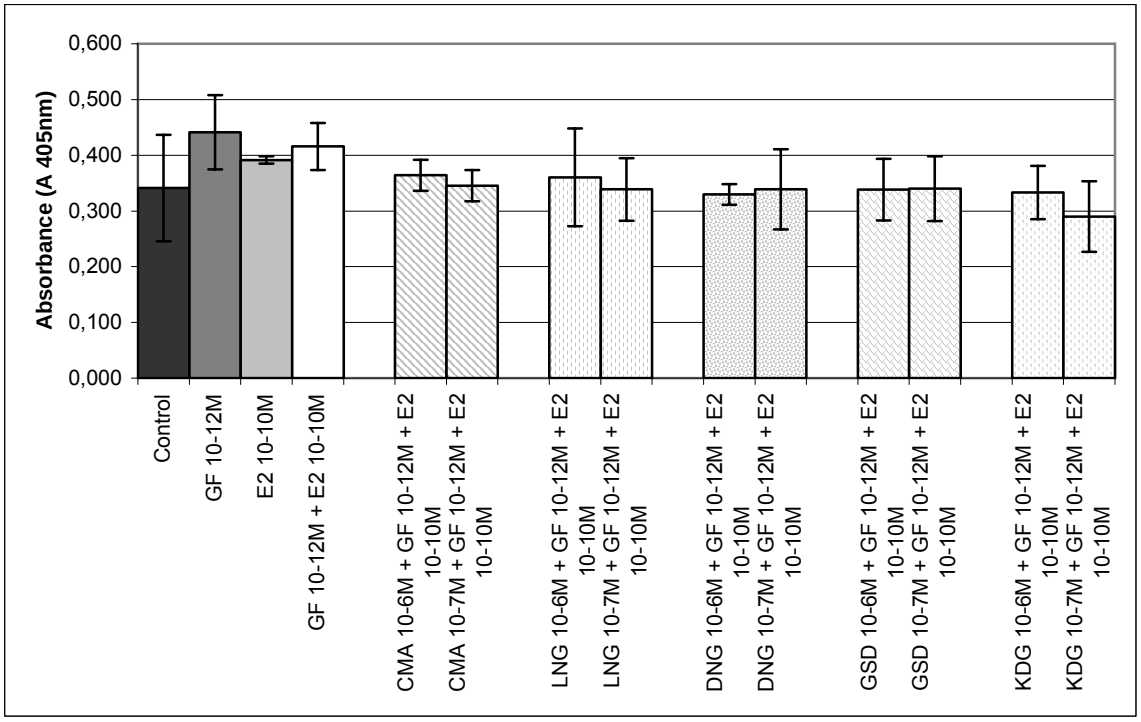

a) *Progesterone (P), Medroxyprogesterone acetate (MPA) and Norethisterone (NET)*



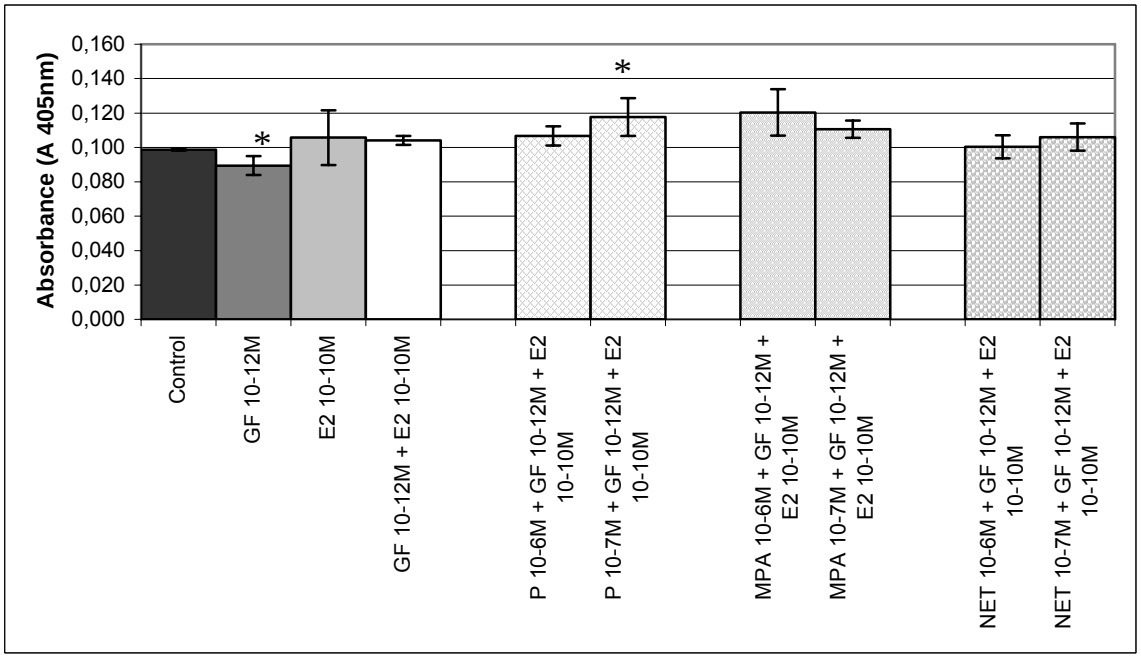

*b) Chlormadinone acetate (CMA), Levonorgestrel (LNG), Dienogest (DNG), Gestodene (GSD) and 3-Ketodesogestrel (KDG)*

The cell death detection:proliferation results of combining the progestogens with the combination of growth factors (EGF, FGF and IGF-I) and E2 on HCC1500 cells are illustrated in Fig. 27. MPA and GSD at both concentrations and CMA $10^{-6}$M and NET at $10^{-7}$M all 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.

***Figure 26 (a to b): Cell death detection measurements of HCC1500 after incubation with the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and estradiol (E2) $10^{-10}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=3). = p<0.05***

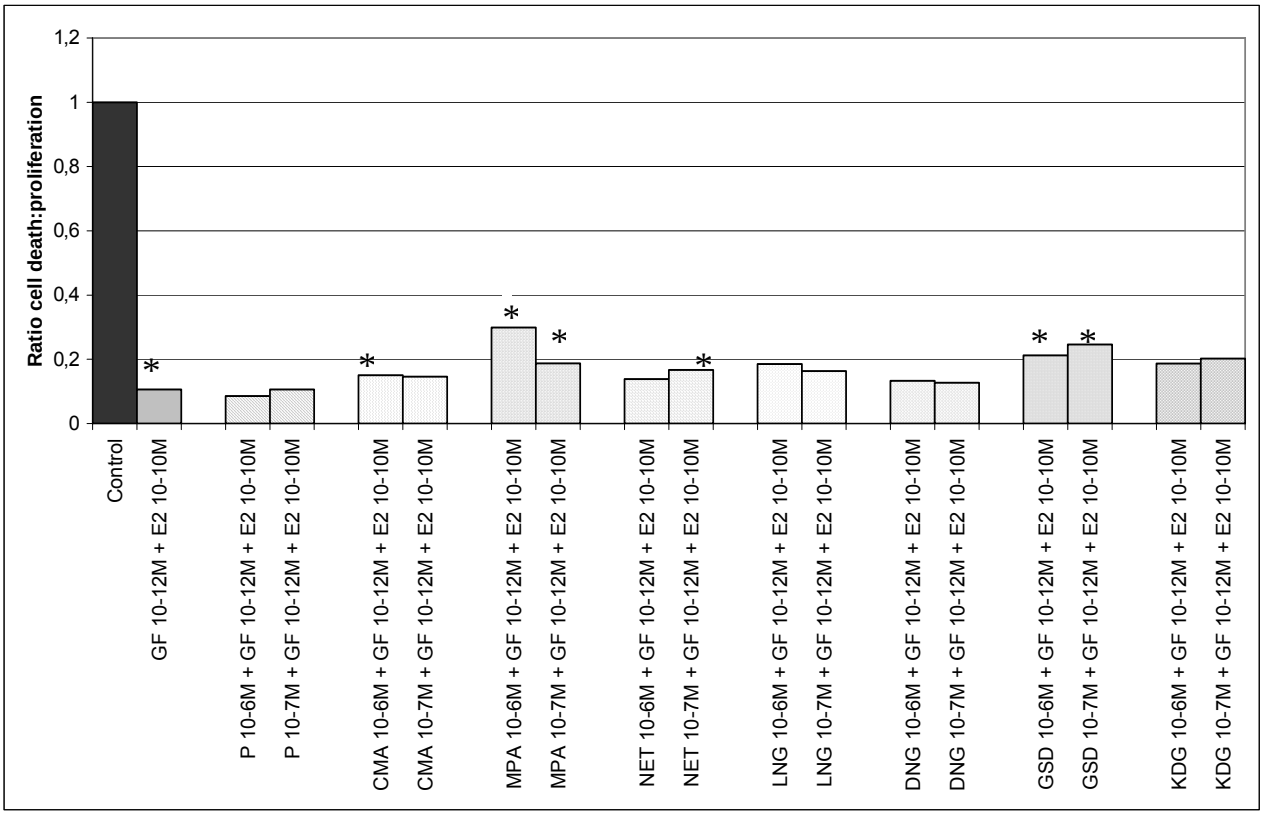



*a) Chlormadinone acetate (CMA), Levonorgestrel (LNG), Dienogest (DNG), Gestodene (GSD) and 3-Ketodesogestrel (KDG)*




*b) Progesterone (P), Medroxyprogesterone acetate (MPA) and Norethisterone (NET)*

***Figure 27: Cell death detection:proliferation measurements of HCC1500 after incubation with the various progestogens in concentrations of $10^{-6}$M to $10^{-7}$M in combination with growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and estradiol (E2)$10^{-10}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=3). * = p<0.05***



### 3.3.3 HCC1500 proliferation inhibitor assays with progestogens alone and in combination with growth factors and/or E2

In the previous proliferation and cell death detection:proliferation assays, growth factors and E2, alone and in combination, and the progestogens NET, LNG, GSD, DNG and KDG $10^{-6}$M in combination with growth factors all induced a mitotic response in HCC1500 cells, i.e., cell proliferation occurred. Therefore, these agents were chosen for further proliferation assays in the presence of proliferation inhibitors try and determine the pathway via which this proliferative response occurs. Both of the proliferation inhibitors (PD98059 and LY294002) at a concentration of $10^{-6}$M were able to significantly reduce cell proliferation of the HCC1500 cells alone and in the presence of growth factors, as shown in Fig. 28. In the case of E2 alone and in combination with growth factors, again, both inhibitors blocked the proliferative response of the cells as illustrated in Fig.29. Because the inhibitors at a concentration of $10^{-5}$M appeared to have a more marked effect on proliferation of MCF10A cells, this concentration was also tested on HCC1500 cells, as shown in Fig. 30. However, the results were the same as those using $10^{-6}$M, i.e., both inhibitors were able to significantly inhibit cell proliferation in the presence of growth factors, E2 and a combination of the two.

With regard to the progestogens, again, the proliferative response to the growth factor and progestogen combination was significantly inhibited by both agents in each case, as shown in Fig. 31.

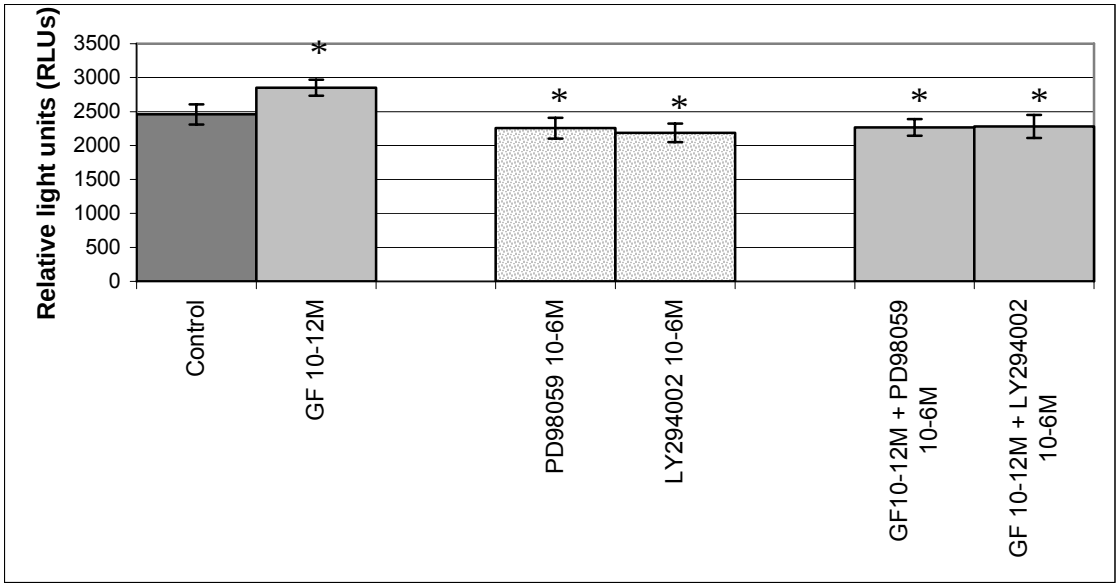



***Figure 28: Proliferative responses of HCC1500 to growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) alone and in the presence of the proliferation inhibitors PD98059 $10^{-6}$M and LY294002 $10^{-6}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=4). * = p<0.05***

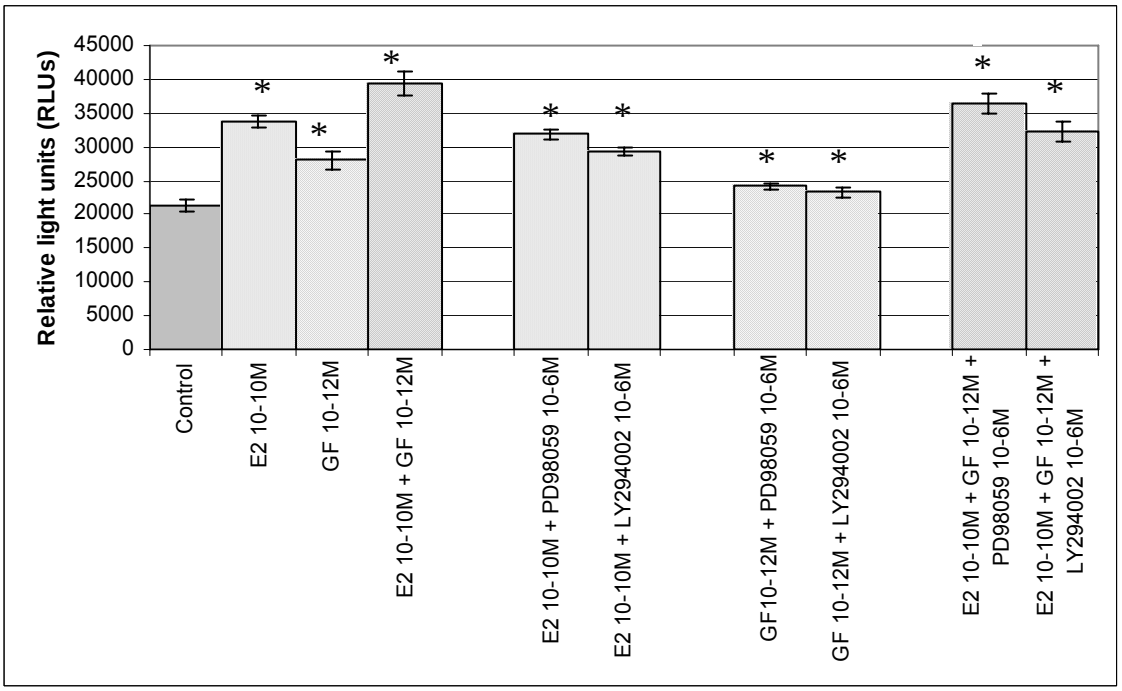



***Figure 29: Proliferative responses of HCC1500 to estradiol (E2) $10^{-10}$M, growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M), and a combination of growth factors and estradiol alone and in the presence of the proliferation inhibitors PD98059 $10^{-6}$M and LY294002 $10^{-6}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=4). * = p<0.05***

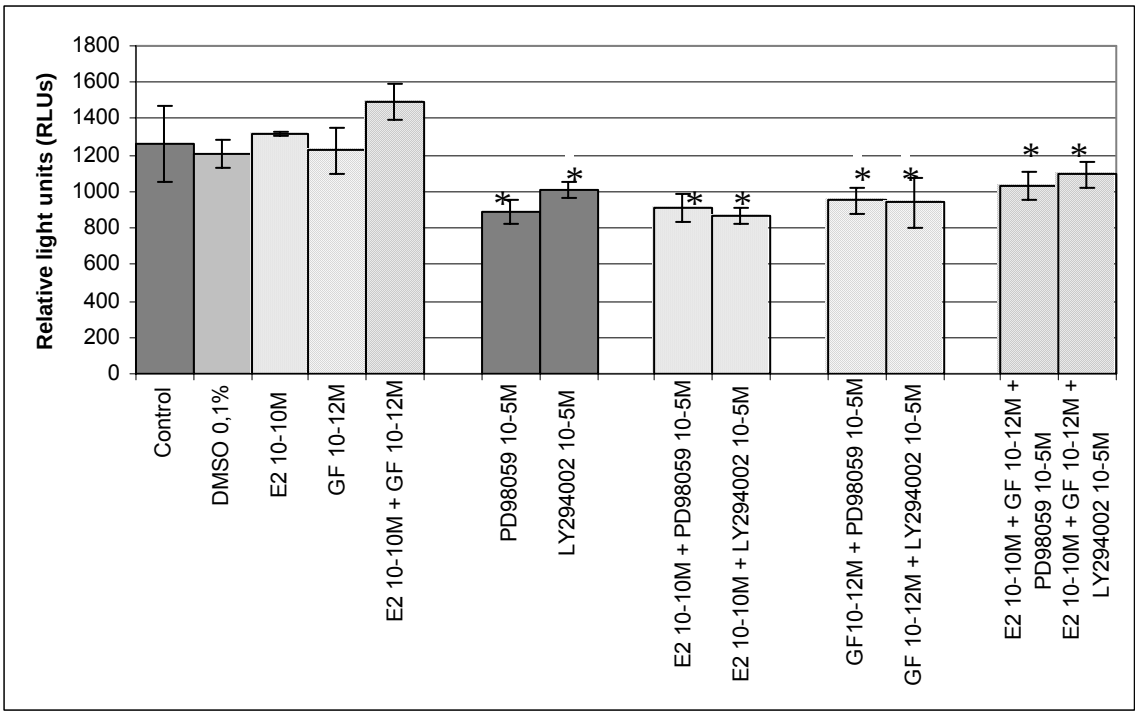



***Figure 30: Proliferative responses of HCC1500 to estradiol (E2) $10^{-10}$M, growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M), and a combination of growth factors and estradiol alone and in the presence of the proliferation inhibitors PD98059 $10^{-5}$M and LY294002 $10^{-5}$M in DMEM/FBS-stripped medium. (Mean $\pm$ SD, n=4). * = p<0.05***

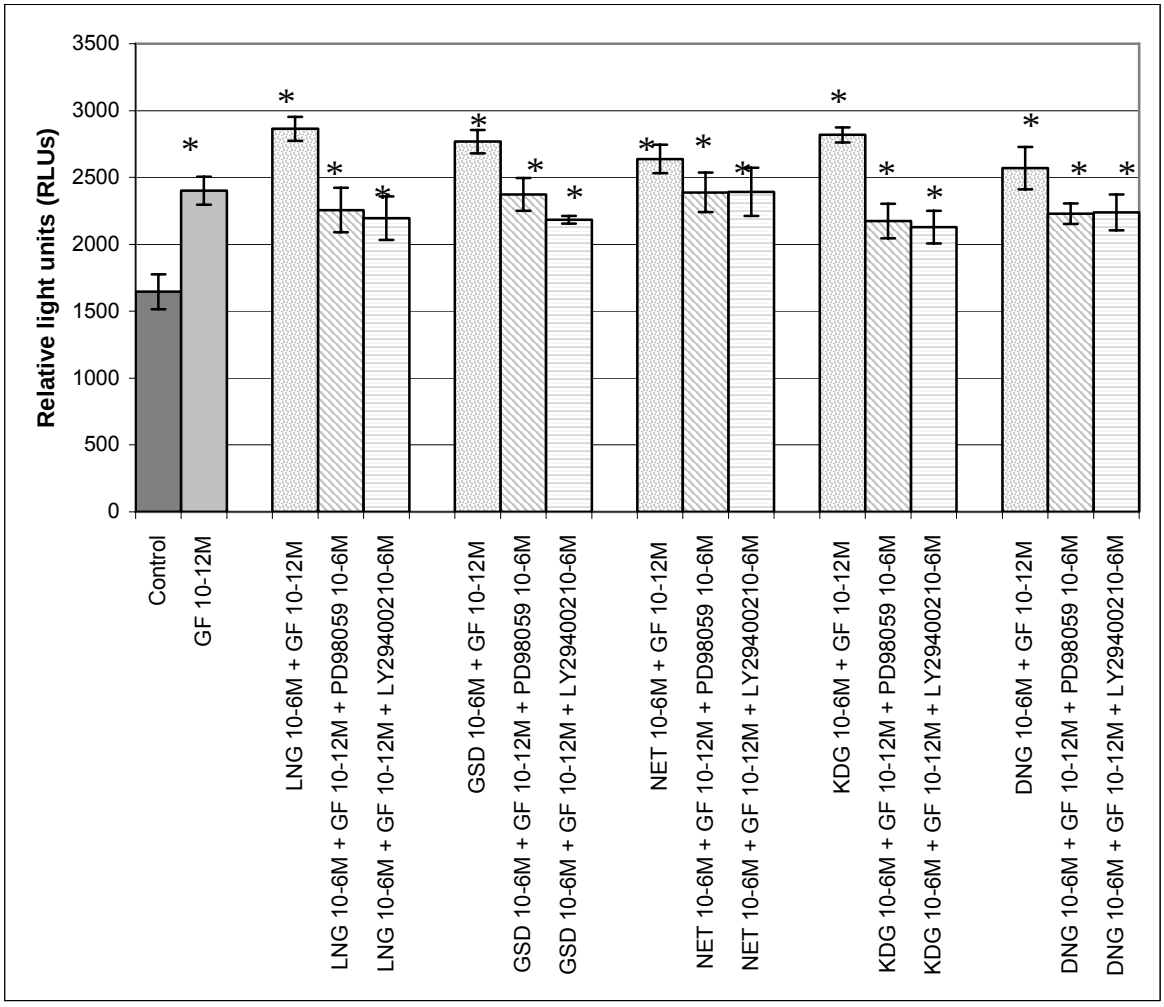



***Figure 31: Proliferative responses of HCC1500 to growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and levonorgestrel (LNG) $10^{-6}$M, gestodene (GSD) $10^{-6}$M, norethisterone (NET) $10^{-6}$M, 3-ketodesogestrel (KDG) $10^{-6}$M and dienogest (DNG) $10^{-6}$M alone and in the presence of the proliferation inhibitors PD98059 $10^{-6}$M and LY294002 $10^{-6}$M in DMEM/FBS-stripped medium. (Mean $\pm$ SD, n=4). * = p<0.05***

### 3.2.4 HCC1500 apoptosis marker assays (cytochrome C, sFasL, p53) with progestogens in combination with growth factors and/or estradiol

To further investigate the pathways by which the progestogens exert their various effects on growth factor- and/or estradiol-stimulated HCC1500 cells, the cells were incubated with MPA, NET, and P as representatives of a C-21 derivative, a C-19 derivative (assuming the same pathway is involved for the other C-21 and 19-nortestosterone derivatives respectively) and natural progesterone in turn in the presence of growth factors, estradiol or a combination of these two mitotic agents. Incubation was followed by measurement of the markers of apoptosis, cytochrome C, sFasL and p53 in cell lysates. Significant increases or decreases in these markers suggest induction or inhibition of apoptosis by the progestagen respectively. Results are represented as percentage change in concentration of the markers compared to growth factor and/or estradiol control.

Figs. 32 to 34 show the results of changes in cytochrome C. E2 and growth factors alone and in combination all significantly reduced cytochrome C release compared to the medium-only control.
In combination with E2, MPA $10^{-7}$M was the only progestogen to significantly alter cytochrome C release, increasing its release compared to E2 alone. NET and P tended to increase cytochrome C release, but not significantly.

In combination with growth factors, MPA $10^{-6}$M and $10^{-7}$M significantly increased cytochrome C release by 16% and 26% respectively compared to growth factor control and P $10^{-6}$M by 30% compared to growth factor control, as shown in Fig. 33. NET had no significant effect on its release.

Growth factors and E2 in combination significantly reduced cytochrome C release, as shown by the growth factor and E2 control in Fig. 34. MPA at both concentrations and NET at $10^{-6}$M significantly increased cytochrome C release compared to growth factor and E2 control, supporting their inhibitory effect on

HCC1500 cells. P had no significant effect on its release from growth factor- and E2-stimulated cells.

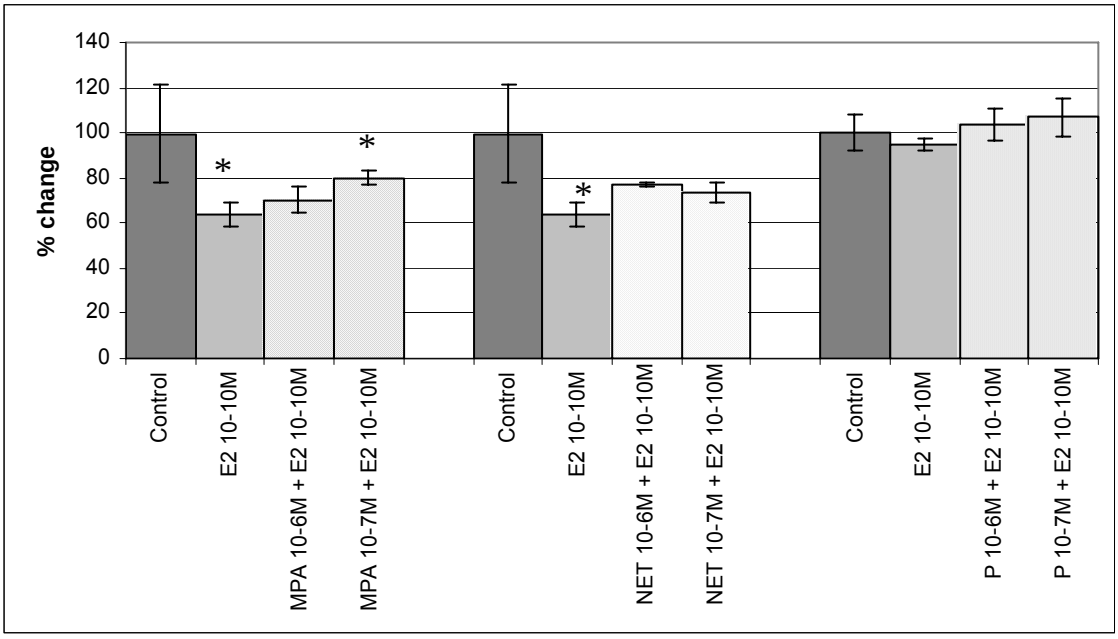



***Figure 32: Changes in cytochrome C release from HCC1500 cells after addition of estradiol (E2)$10^{-10}$M alone and in combination with medroxyprogesterone acetate (MPA) $10^{-6}$M and$10^{-7}$M, norethisterone acetate (NET) $10^{-6}$M and $10^{-7}$M, and progesterone (P) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control. (Mean ± SD, n=4). * = p<0.05***

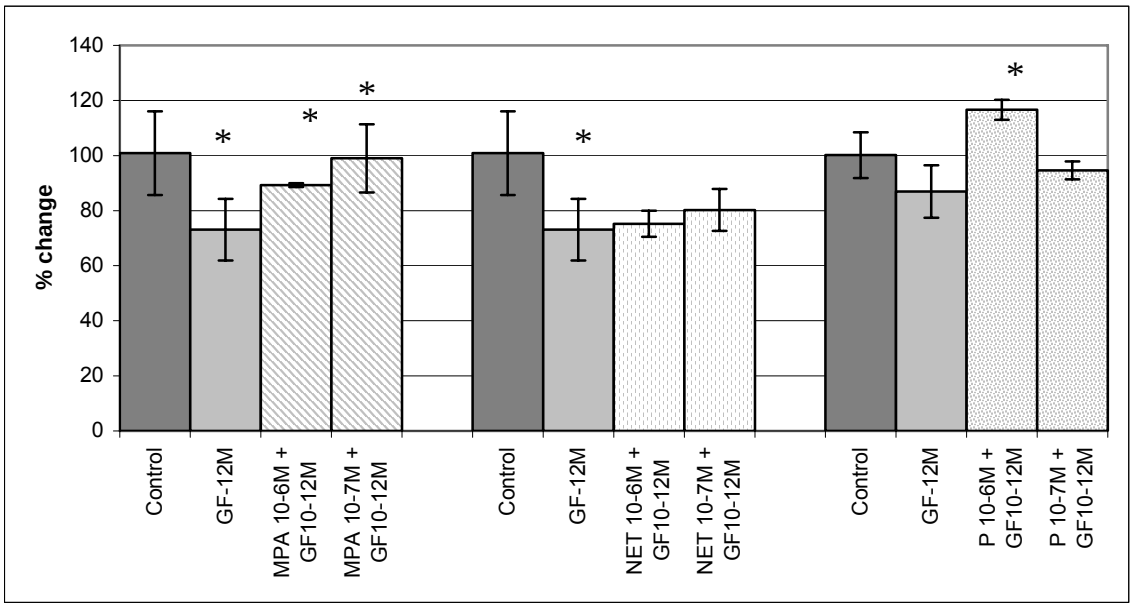



***Figure 33: Changes in cytochrome C release from HCC1500 cells after addition of growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) alone and in combination with medroxyprogesterone acetate (MPA) $10^{-6}$M and $10^{-7}$M, norethisterone acetate (NET) $10^{-6}$M and $10^{-7}$M, and progesterone (P) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control.***
***(Mean $\pm$ SD, n=4). * = $p<0.05$***

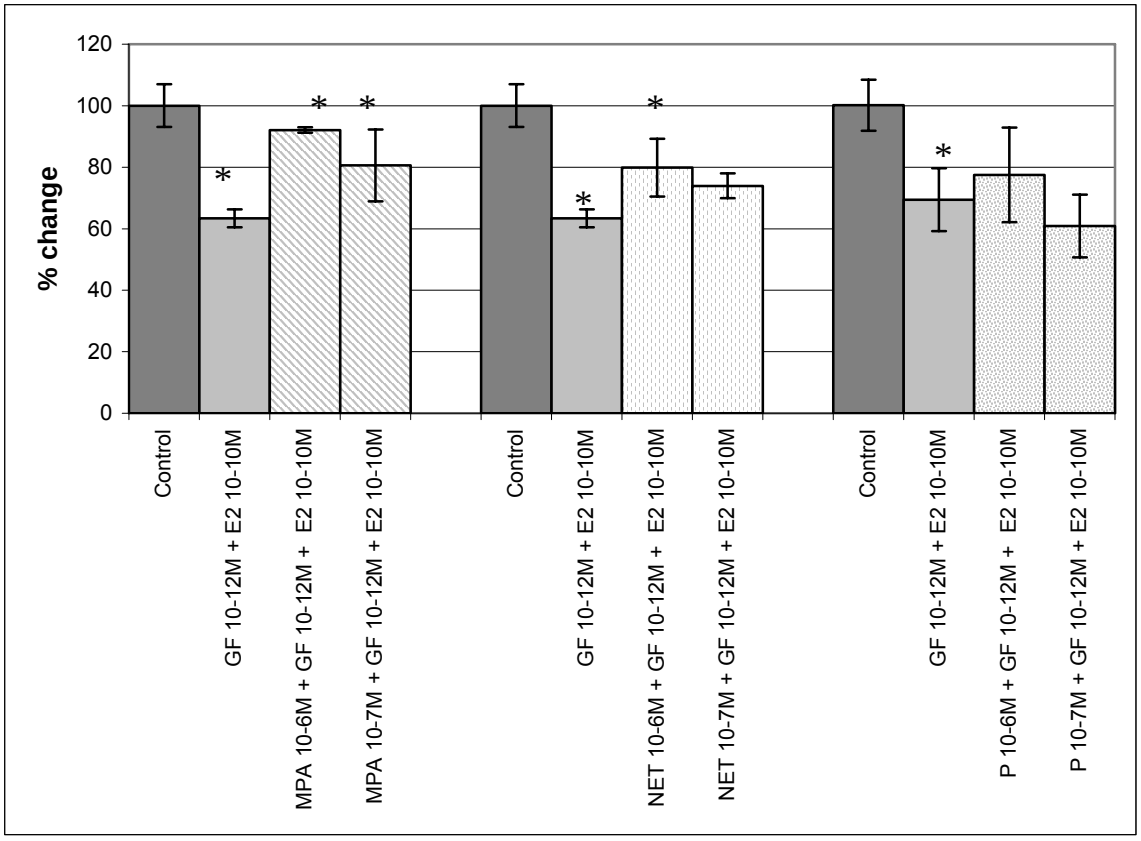



***Figure 34: Changes in cytochrome C release from HCC1500 cells after addition of growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and estradiol (E2) $10^{-10}$M alone and in combination with medroxyprogesterone acetate (MPA) $10^{-6}$M and$10^{-7}$M, norethisterone acetate (NET) $10^{-6}$M and $10^{-7}$M, and progesterone (P) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control. (Mean $\pm$ SD, n=4). * = p<0.05***

Figs. 35 to 37 show the effects of the progestogens on sFasL release in the presence of E2, growth factors or E2 plus growth factors respectively. E2 alone significantly reduced sFasL release by up to 43%, supporting its proliferative role. Compared to E2 alone, MPA, NET and P all both further increased sFasL release, by up to 26%, 14% and 36% respectively, in line with their anti-proliferative results in the cell death detection:proliferation assays.

Growth factors alone either significantly reduced sFasL release, again, supporting their proliferative role. NET reduced sFasL release compared to growth factor control by up to 25%. These results correspond with the proliferative effects of NET in the presence of growth factors. P increased sFasL release by 23% compared to growth factor control, but had no significant effect in the cell death detection:proliferation assay. The results for MPA were non-significant.

Growth factors and E2 in combination reduced sFasL release by up to 43%. This effect was significantly inhibited by both MPA and NET, where sFasL release was increased, supporting their anti-proliferative effect on the growth factor + E2 combination. P also significantly increased sFasL release, but had a non-significant effect in the cell death detetction:proliferation assay in the presence of growth factors and E2.

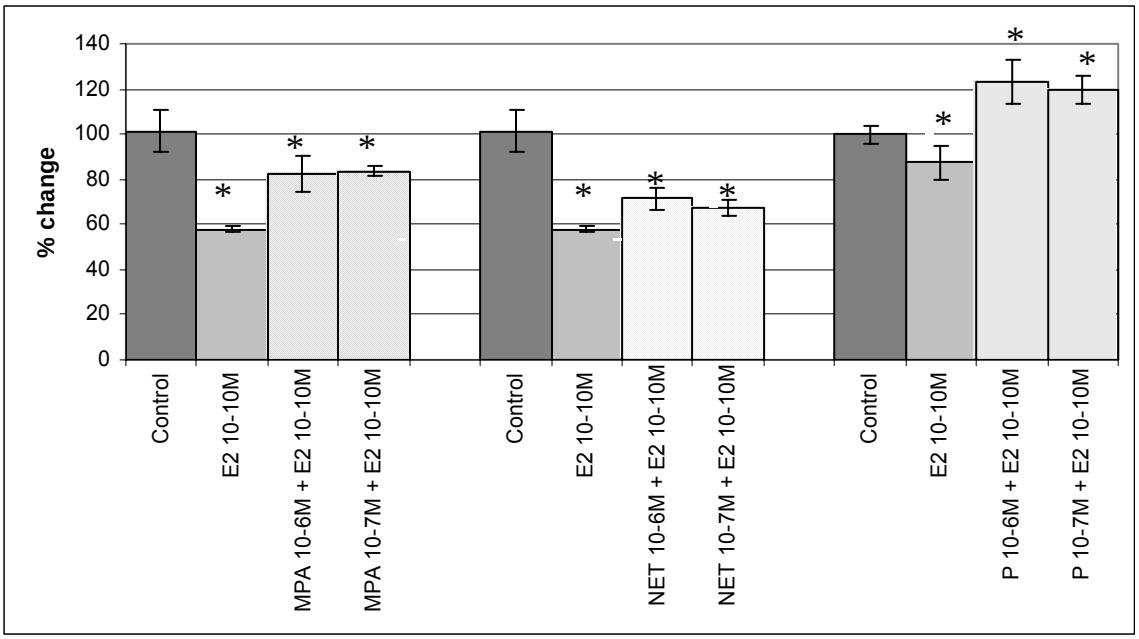



***Figure 35: Changes in sFasL release from HCC1500 cells after addition of estradiol (E2)$10^{-10}$M alone and in combination with medroxyprogesterone acetate (MPA) $10^{-6}$M and$10^{-7}$M, norethisterone acetate (NET) $10^{-6}$M and $10^{-7}$M, and progesterone (P) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control. (Mean ± SD, n=4). * = p<0.05***

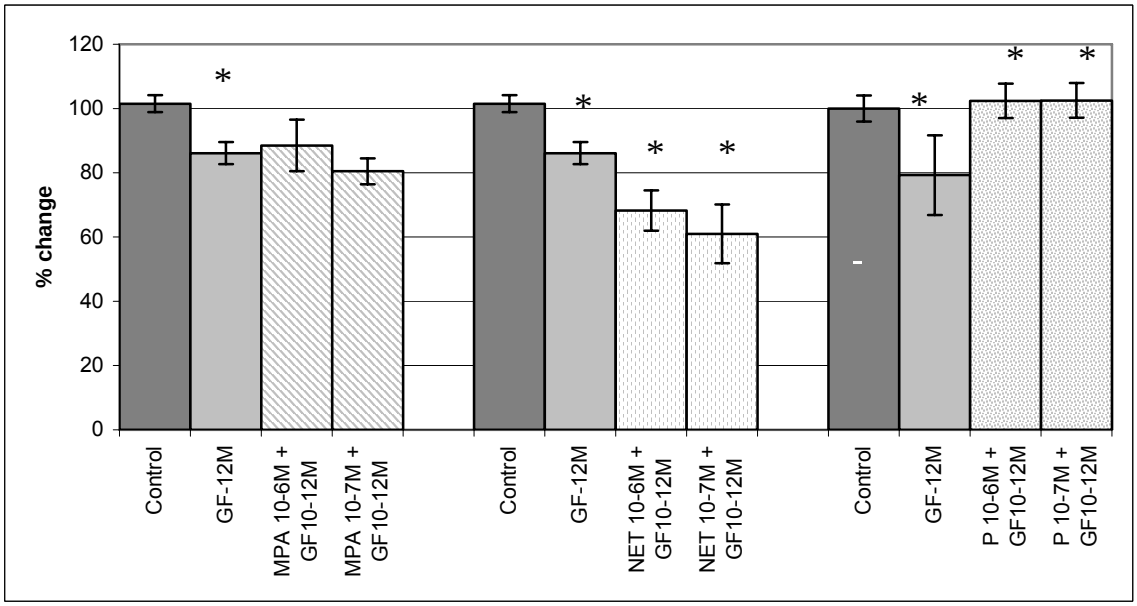



***Figure 36: Changes in sFasL release from HCC1500 cells after addition of growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) alone and in combination with medroxyprogesterone acetate (MPA) $10^{-6}$M and $10^{-7}$M, norethisterone acetate (NET) $10^{-6}$M and $10^{-7}$M, and progesterone (P) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control.***
***(Mean ± SD, n=4). * = p<0.05***

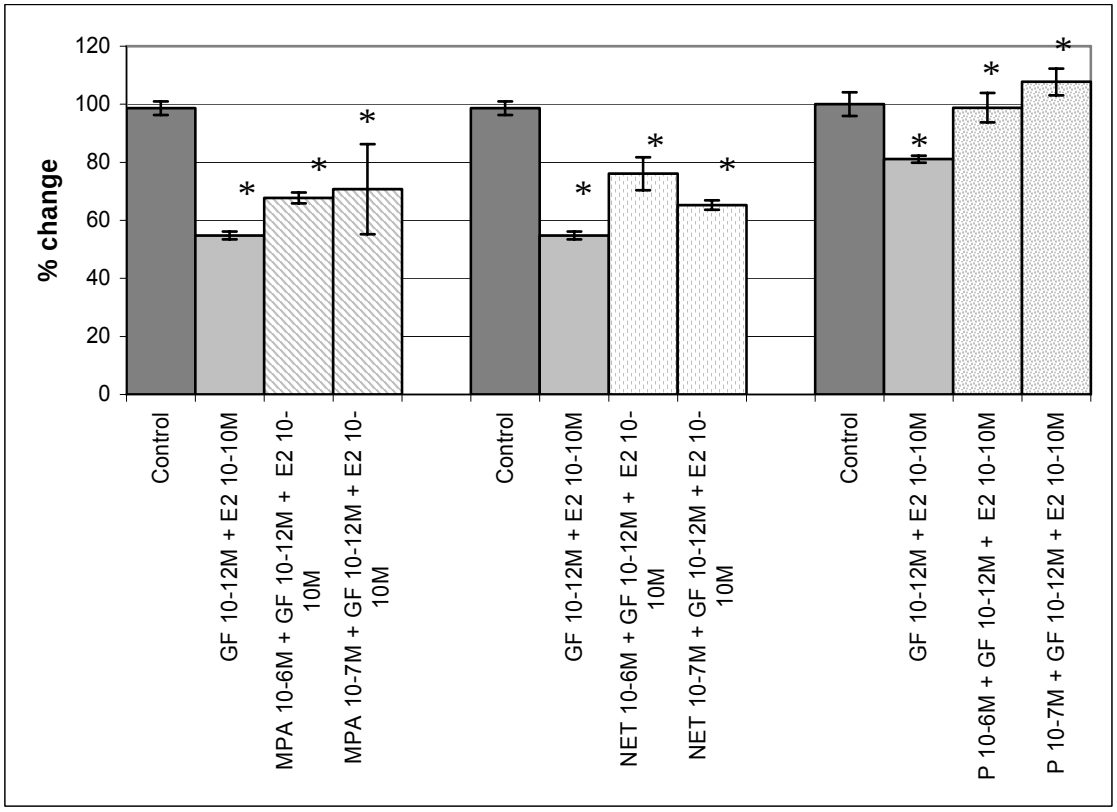



***Figure 37: Changes in sFasL release from HCC1500 cells after addition of growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and estradiol (E2) $10^{-10}$M alone and in combination with medroxyprogesterone acetate (MPA) $10^{-6}$M and$10^{-7}$M, norethisterone acetate (NET) $10^{-6}$M and $10^{-7}$M, and progesterone (P) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control. (Mean ± SD, n=4). * = p<0.05***

Figs. 38 to 40 show the effects of the progestogens on p53 release. E2 alone reduced its release by up to 43%. Both MPA and NET increased p53 release compared to the inhibitory effects of E2, in agreement with their apoptopic effects in the cell death detection:proliferation assays. P increased p53 release at $10^{-6}$M, however, the results were non significant, due to a large margin of error. The E2 control for the P assay also shows an increase in p53 release compared to medium-only control, a result which is probably due to experimental error.

Growth factors alone reduced p53 release by up to 27%. MPA and NET had no further significant effect. P $10^{-6}$M significantly increased p53 release.

Growth factors and E2 in combination reduced p53 release by up to 30%. All three progestogens tested significantly increased p53 release compared to the growth factors plus E2 control, in agreement with anti-proliferative effects of MPA and NET on the growth factor plus E2 combination in the cell death detection:proliferation assay.

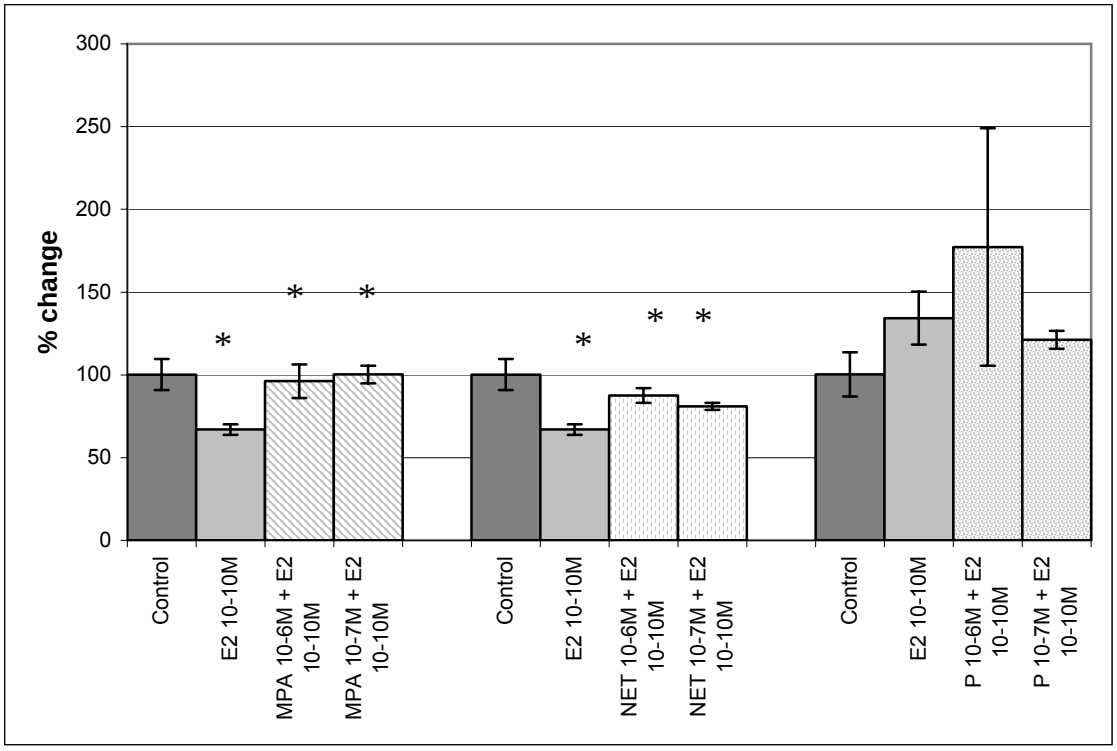



***Figure 38: Changes in p53 release from HCC1500 cells after addition of estradiol (E2)$10^{-10}$M alone and in combination with medroxyprogesterone acetate (MPA) $10^{-6}$M and$10^{-7}$M, norethisterone acetate (NET) $10^{-6}$M and $10^{-7}$M, and progesterone (P) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control. (Mean ± SD, n=4). * = p<0.05***

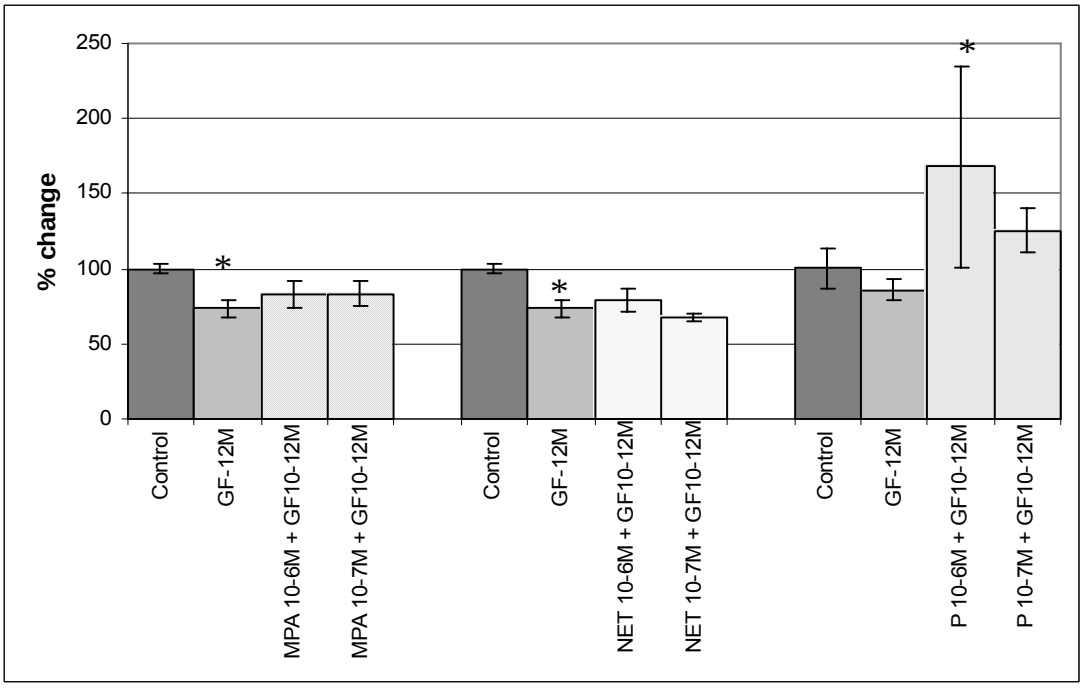



***Figure 39: Changes in p53 release from HCC1500 cells after addition of growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) alone and in combination with medroxyprogesterone acetate (MPA) $10^{-6}$M and $10^{-7}$M, norethisterone acetate (NET) $10^{-6}$M and $10^{-7}$M, and progesterone (P) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control.***
***(Mean ± SD, n=4). * = p<0.05***

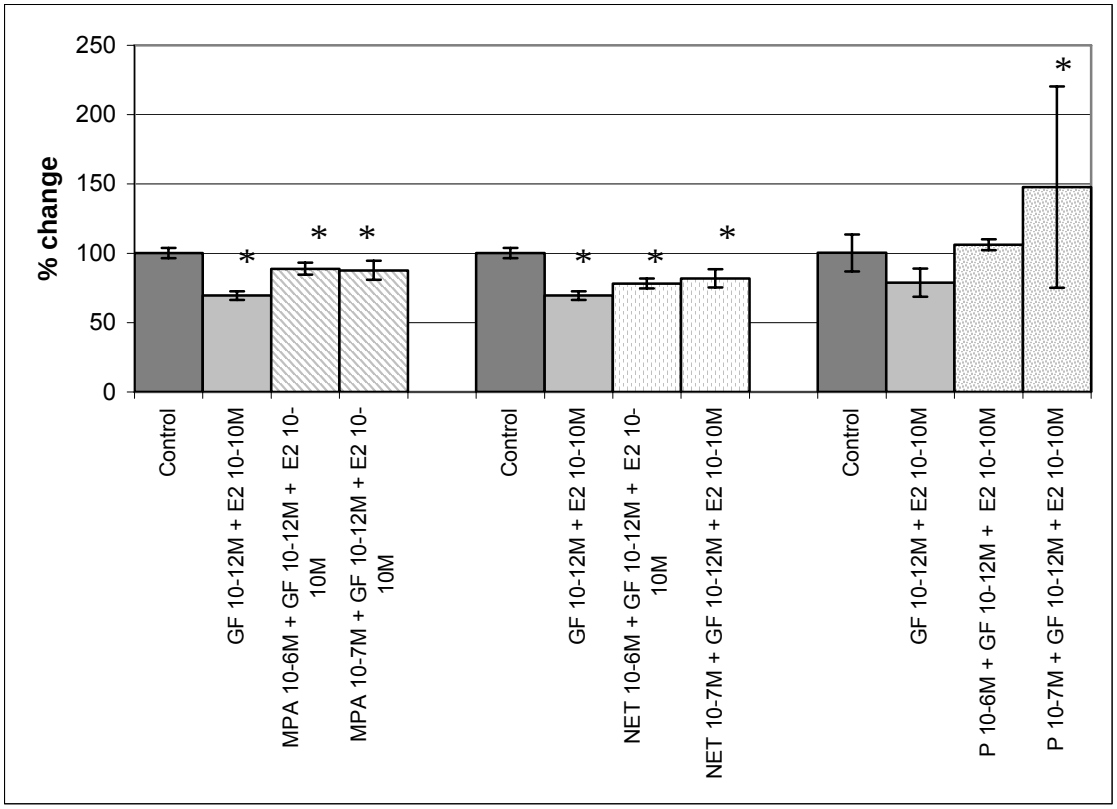



***Figure 40: Changes in p53 release from HCC1500 cells after addition of growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and estradiol (E2) $10^{-10}$M alone and in combination with medroxyprogesterone acetate (MPA) $10^{-6}$M and$10^{-7}$M, norethisterone acetate (NET) $10^{-6}$M and $10^{-7}$M, and progesterone (P) $10^{-6}$M and $10^{-7}$M. Values are given as percentage change in the markers compared to medium-only or growth factor control. (Mean ± SD, n=4). * = p<0.05***

### 3.3.5 HCC1500 proliferation assays with progestogens in combination with estradiol in the presence of tamoxifen or letrozole.

To determine whether the proliferative effects of NET, LNG, DNG, GSD and KDG on HCC1500 cells in the presence of growth factors were induced via stimulation of the estrogen receptor, or if aromatase was involved in the cell proliferation process, HCC1500 cells were incubated with each of the progestogens at $10^{-6}$M and $10^{-7}$M in the presence of growth factors $10^{-12}$M, along with 4-hydroxytamoxifen $10^{-6}$M (an active metabolite of tamoxifen) or letrozole $10^{-6}$M using the proliferation assay method. The results are shown in Fig. 41.

Tamoxifen, an antagonist for the estrogen receptor, inhibited the proliferative effect of all the progestogens tested, whilst letrozole (an aromatase inhibitor which blocks the aromatisation of precursors to estrogen) failed to inhibit any progestogen and growth factor-induced cell proliferation.

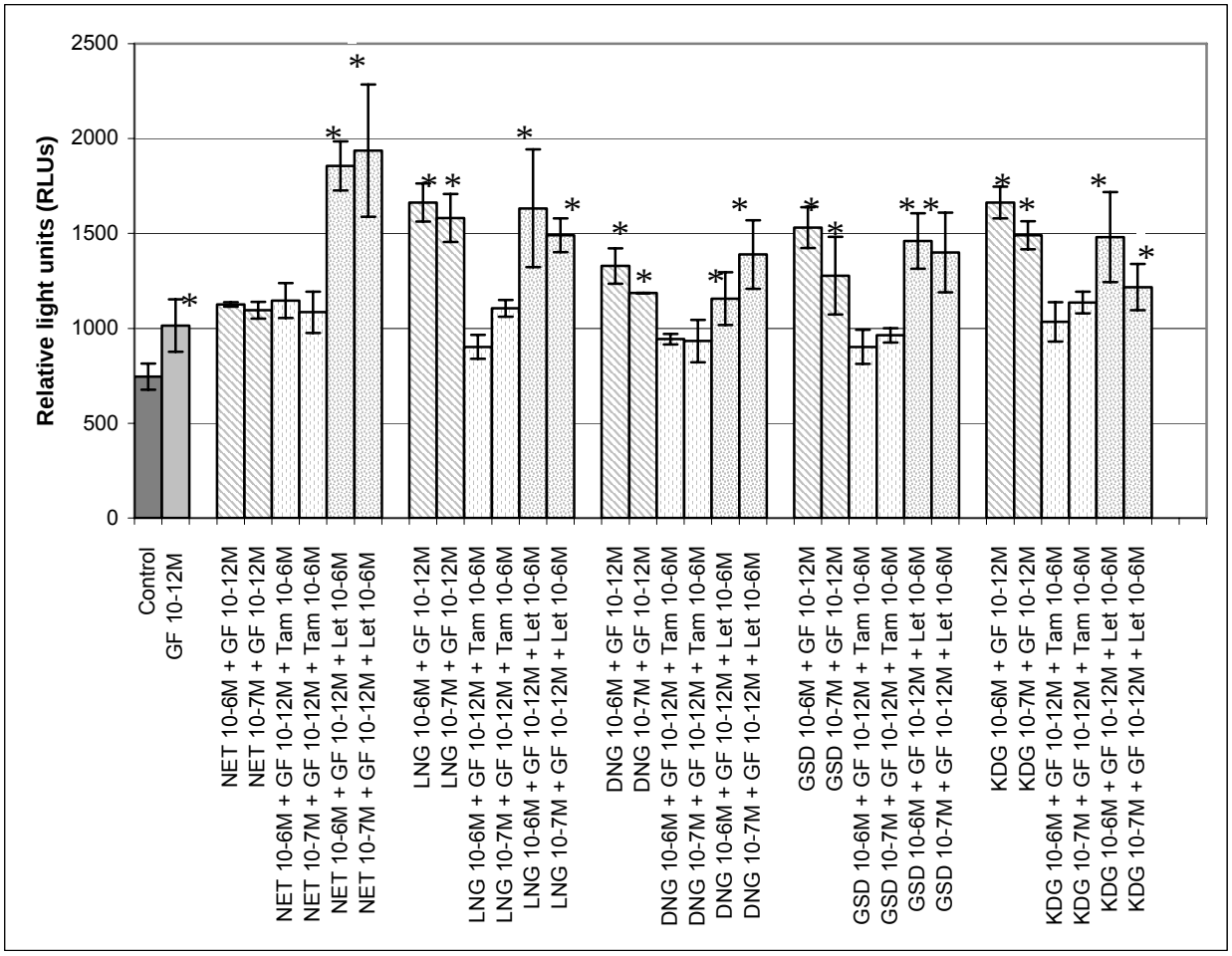



***Figure 41: Proliferative responses of HCC1500 to growth factors EGF, FGF and IGF-I $10^{-12}$M (GF $10^{-12}$M) and norethisterone (NET), levonorgestrel (LNG) $10^{-6}$-$10^{-7}$M, dienogest DNG $10^{-6}$-$10^{-7}$M, gestodene (GSD) $10^{-6}$-$10^{-7}$M, $10^{-6}$-$10^{-7}$M, 3-ketodesogestrel (KDG) $10^{-6}$-$10^{-7}$M alone and in the presence of tamoxifen $10^{-6}$M or letrozole $10^{-6}$M in DMEM/FBS-stripped medium. (Mean ± SD, n=4). * = p<0.05***

## 4. DISCUSSION

The traditional HRT in America is conjugated equine estrogens (CEE), i.e., estrone- and dihydroestrone sulphate, equilin sulphate and equilenin sulphate in combination with the progestogen medroxyprogesterone acetate (MPA), either on a cyclical (sequential) or continuous basis. The regimen commonly prescribed in Europe is 17β-estradiol with testosterone-based progestogens such as norethisterone (NET) or levonorgestrel (LNG), using the less androgenic MPA to a lesser extent (Stahlberg *et al* 2003). This difference in preferences can lead to potential problems when extrapolating results from published studies, many of which have originated in America. Studies may include one major preparation, however they conclude that progestogens as a drug class have the general effect found in the study, therefore not allowing for potential differences between them. Results do not necessarily apply to lower dosages of the same drug, to other formulations of oral estrogens and progestogens or to transdermal estrogen and progestogen preparations. Transdermal estradiol with progesterone closely mimics the normal physiology and metabolism of endogenous sex hormones and therefore has a different risk-benefit profile to oral preparations (Writing Group for the Women's Health Initiative Investigators 2002).

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 (ER+) represent only a minority of the total cells and do not proliferate (Ali and Coombes 2002). 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 estrogens and are mainly responsible for the progression of estrogen-receptor negative (ER-) breast cancer. Many studies examining growth factor synthesis, bioactivities, and employing growth factor transfection of cells or the generation

of transgenic mice have demonstrated the potential of growth factors to induce preneoplasia and neoplasia in the mouse mammary gland and promote breast cancer growth *in vivo* (Imagawa *et al* 2002).

It is important to differentiate between normal and malignant estrogen-receptor positive breast cells. Therefore, for the first time, this study investigated the effect of eight different progestogens on the proliferation of benign and malignant breast epithelial cells in the presence of growth factors and/or estradiol.

The results of the investigations demonstrate that with respect to their effects on normal and cancerous cells, there are distinct differences between the eight chosen progestogens, i.e., natural P, the synthetic C-21 progesterone derivatives CMA and MPA and synthetic C-19 testosterone-derived NET, LNG, DNG, GSD, and KDG (a metabolite of desogestrel). Differences between these progestogen types also appear to exist for cardiovascular surrogate markers, shown by our department in previous experiments (Lippert *et al* 1996, Seeger *et al* 2001, Mueck *et al* 2002b). Consequently, the choice of progestogen may be of significance regarding a possible antagonistic effect on E2's benefits in hormone replacement therapy. Progestogens also have effects on the enzymes in breast tissue, such as sulfatase, sulfotransferase and 17β-HSD, which are responsible for the local synthesis of estradiol. It has been shown that progestogens with no androgenic action are potent sulfatase inhibitors and exert a stimulatory effect on sulfotransferase activity (therefore converting estrone to estrone sulphate). The same effect has not been shown with androgenic progestogens, showing that different progestogens may induce different effects on breast cells (Druckmann 2003, Pasqualini *et al* 1998).

## *4.1 MCF10A*

### 4.1.1 MCF10A proliferation assays with growth factors

None of the progestogens alone was able to significantly affect cell normal proliferation, however, for the first time it was found that the highest concentrations of MPA and CMA were able to further enhance the mitotic effects of growth factors on estrogen receptor-negative normal breast epithelial cells. P, NET, LNG, DNG, GSD and KDG had no significant effect on the proliferative effects of growth factors.

Clinical and animal trials have already demonstrated a possible negative effect of MPA on the proliferation of normal breast epithelial cells. Hofseth *et al* (1999) showed that HRT with estrogen alone or estrogen plus MPA was associated with increased epithelial proliferation in the normal postmenopausal breast, which is consistent with our *in vitro* findings. Combination therapy caused more proliferation than estrogen alone. Furthermore, with estrogen and MPA, breast proliferation was localised to the terminal duct-lobular unit of the breast, which is the site of development of most breast cancers. Postmenopausal adult female cynomolgus macaques treated with conjugated equine estrogens (CEE) alone or with medroxyprogesterone acetate, exhibited a greater proliferative response of healthy mammary gland epithelium with combined therapy than with estrogens alone (Cline et al 1996). Suparto *et al* (2003) later confirmed these results by showing that CEE and MPA induced a diffuse epithelial proliferation in the mammary glands of surgically postmenopausal cynomolgus monkeys. Ross *et al* (2000) showed a odds ratio of 1.24 (95%CI 1.07-1.45) for breast cancer risk after five years of treatment with HRT in a study of postmenopausal women in America. The cohort included mostly women receiving CEE and MPA. Greendale *et al* (1999) assessed changes in breast radiographic density on mammography in patients treated with placebo, conjugated equine estrogens (CEE) alone, CEE plus cyclic MPA, CEE plus daily MPA, and CEE plus cyclic micronized P (MP). After 12 months, the odds of an increase in mammographic density were 13.1 (95% CI, 2.4-73.3) with CEE plus cyclic MPA,

9.0 (CI, 1.6-50.1) with CEE plus daily MPA and 7.2 (CI, 1.3-40.0) with CEE plus MP compared to CEE alone. The placebo group had no density grade increases.

These and our results indicate that MPA may enhance the mitotic rate of normal epithelial breast cells in the presence of growth factors and therefore long-term use may increase the probability of faults in DNA-replication. Furthermore, the results of the WHI indicated that patients who were not using hormones prior to the start of the study had no increased hazard ratio for breast cancer, however subjects with prior hormone use for up to five, five to ten and more than 10 years showed an increasing risk (Writing Group for the Women's Health Initiative Investigators 2002).

These data suggest that long-term use of MPA may increase breast cancer risk by enhancing the mitotic rate of normal epithelial cells.

CMA has been shown to have no influence on breast cancer risk in a case-control study involving combined oral contraceptives containing CMA. Risk was not elevated in ever-users, did not increase with duration of use and did not change with time since initial exposure or with time since most recent use (Ebeling *et al* 1991). In contrast to this, another study of the use of oral contraceptives containing CMA found an elevated relative risk for breast cancer of 1.3. The authors of this study state that although not statistically significant, the positive correlation observed for breast cancer may be real (Nischan and Ebeling 1984), which is consistent with our stimulatory results. Up to now, there is a paucity of data available regarding the effects of CMA on the proliferation of normal epithelial breast cells.

P in combination with estrogen has been shown to increase healthy breast epithelial cell proliferation when injected daily into ovariectomized mice for 56 days (Rafaat *et al* 2001). In contrast, in a small clinical trial involving 40 postmenopausal women, exposure to P for 14 days reduced the E2-induced proliferation of normal breast cells *in vivo* (Foidart *et al* 1998). In their study of women receiving exclusively or mostly a transdermal E2 gel and a progestogen other than MPA (oral micronized P was used in 58% of patients, MPA in less

than 3% of patients), de Lignières *et al* (2002) concluded that early interruption of such a type HRT is not justified, as they found no increase in breast cancer risk in combined HRT users, even after >5 years of use. Plu-Bureau *et al* (1999) showed at least an absence of deleterious effects caused by percutaneous progesterone use in women with benign breast disease in their study of 1150 premenopausal French women, 58% of whom were prescribed percutaneous progesterone treatment. P did not alter the proliferative effects of E2 on mammary epithelial proliferation in ovariectomized rhesus monkeys treated with E2 alone or in combination with P (Zhou *et al* 2000). Laidlaw *et al* (1995) found that P either alone or after E2 priming did not affect proliferation of normal human breast tissue implanted into athymic nude mice. Chang *et al* (1995) found that topical application of an E2 and P gel to the normal breast reduced E2-induced proliferation of the breast epithelial cells. Using a culture system of normal human breast epithelial cells, Malet *et al* (2000) observed an inhibitory effect on cell growth of 7 days of P treatment in the presence of E2. Wiebe *et al* (2000) compared the capacity of non-tumourous and tumourous breast tissue to convert P to its metabolites and then tested the effects of these metabolites on breast cell proliferation. Metabolites identified were 4-pregnenes, produced more by normal breast tissue, and 5α-pregnanes, which were produced in higher levels by tumourous breast tissue due to its elevated 5α-reductase activity. *In vitro* proliferation studies on MCF-7 and MCF10A cells showed that each of the 4-pregnenes isolated from breast tissue suppressed, whereas each respective 5α-reductase product stimulated cell proliferation. They concluded that a change in *in situ* progesterone metabolism, resulting in an increased 5α pregnane:4 pregnene ratio may promote breast cancer by promoting increased cell proliferation, whereas increases in 4-pregnenes may retard these tumourigenic processes. There is no data available from other research groups using a P and growth factor combination with which to compare our results.

LNG in a combination HRT estrogen-progestogen preparation was shown to increase the relative risk of breast cancer in a Swedish study, with a standard incidence rate of 1.4 (1.1-1.8) after 10 years of follow-up (Persson *et al* 1996).

In another study, relative risk of breast cancer increased significantly with increasing cumulative dose of LNG as part of a combined oral contraceptive pill (Dumeaux *et al* 2003). Using fine needle aspiration biopsies, Isaksson *et al* (2001) assessed epithelial proliferation in normal breast tissue of 106 healthy pre-menopausal women with or without oral contraceptives. There was a positive correlation between proliferation and progesterone levels in non-users and with serum LNG concentrations in women using oral contraceptives containing this progestogen. These results are in contrast to our null effect results of LNG on growth factor-stimulated MCF10A cells.

Regarding the null effects of NET, DNG, GSD and KDG on growth factor-stimulated MCF10A cells, there is a relative paucity of data available from other study groups on the effects of these progestogens on healthy breast cells. NET in combination with E2 was shown to increase the risk of breast cancer more than E2 alone in a Swedish study (OR 1.4 (0.9-2.2) for 1-10 years' combined treatment, 2.4 (0.7-8.6) for 11+ years (Persson *et al* 1997). Another study investigated the effects on mammographic breast density on two different continuous combined regimens for hormone therapy: either estradiol valerate/DNG or estradiol/norethisterone acetate (NETA). An increase in mammographic density was recorded in approximately 50% of the women, and there were no differences between the treatments. The investigators concluded that continuous combined hormone therapy with different progestogens has a marked impact on the breast (Conner *et al* 2004). Mueck *et al* (2001) studied the effects of estradiol valerate with or without the addition of DNG as hormone replacement therapy on the ratio of the main D-ring and A-ring metabolites of estradiol in postmenopausal women, and the effects of ethinylestradiol plus DNG or ethinyestradiol plus NETA as oral contraceptives in women of reproductive age. There were no significant differences in the ratios before and after 3 months of treatment in the HRT group and the ethinyestradiol plus NETA group, and a significantly lower ratio in the ethinylestradiol/DNG oral contraceptive group. The authors concluded that the estrogen-progestogen combinations tested did not impose any negative effects on estradiol

metabolism, i.e. they did not elicit a higher D-ring metabolism, which is considered to increase breast cancer risk. In contrast, in a study of the effect of oral and transdermal estradiol/NETA on the ratio of the main D-ring metabolite (16-OHE1) to the main A-ring metabolite (2-OHE1), there was a tendency for the ratio to rise after oral treatment, but no significant change in the transdermally treated group, showing a possible difference in metabolism depending on the route of administration. (Seeger *et al* 2000). Magnusson *et al* (1999) showed a positive association between increasing breast cancer risk and the use of estrogen combined with continuous addition of testosterone-derived progestogens (i.e. NETA or LNG) in women without previously diagnosed breast cancer. However, in a study of samples of normal post-menopausal breast epithelium from patients taking mainly NET or norgestrel-containing combined HRT, cell proliferation was not found to be significantly altered (Hargreaves *et al* 1998). In a study by Suparto *et al* (2003), treatment of surgically postmenopausal cynomolgus monkeys with ethinylestradiol plus NETA did not induce epithelial tissue proliferation in the mammary gland. In a cohort study of 1150 French women with benign breast disease who were treated with progestogens and followed-up for 10 years, 19-nortestosterone derivatives were found to be significantly associated with a lower risk of breast cancer (RR 0.48; 95% CI 0.25-0.90) (Plu-Bureau *et al* 1994). The authors suggest that high doses of 19-nortestosterone derivatives may have a beneficial effect on the risk of breast cancer in premenopausal women with benign breast disease. In a primary efficacy and safety study of an oral contraceptive containing 150mcg desogestrel (pro-drug of KDG) and 30mcg ethinylestradiol, with a total of 11,656 cycles of exposure in 1,221 women, pre-existing benign breast disease was generally found to improve (Walling 1992). However, the effects of progestogens on breast cancer risk when they are used at high dose in premenopausal women (leading to suppression of follicular maturation and reduced estradiol levels) may be different to the situation in post-menopausal women who are treated with both estrogen and progestogen at various doses and ratios, where the results are different and an increase in risk may be seen in long-term users. (Sitruk-Ware and Plu-Bureau 2004).

4.1.2 MCF10A cell death detection:proliferation assays

The multi-step, multi-pathway process of apoptosis, inherent in every cell in the body, is initiated either via by death receptors on the cell surface (the extrinsic pathway), or the intrinsic pathway by mitochondria inside the cell. Caspases that cleave cellular substrates leading to characteristic biochemical and morphological changes are activated in both pathways, although caspase independent-types of cell death can also occur. Apoptosis leads to fragmentation of aged or unwanted cells, blebbing and the formation of apoptopic bodies. The remains of the dying cell are engulfed by phagocytes (Igney and Krammer 2002).

The molecular regulation of apoptosis, which is still not fully understood, involves two main categories of proteins, the bcl-2 family members and a family of cysteinyl aspartase-specific proteases, the caspases. Proteins of the bcl-2 family may dimerize, and the implementation of apoptosis depends on the net balance of the expression of anti-apoptopic and pro-apoptopic members. Proteins of the caspase family are activated by binding to apoptosis-related death receptors and/or by sequential cleavages, a process which ultimately leads to the appearance of characteristic apoptopic morphology such as cell shrinkage, membrane blebbing, chromatine condensation and DNA fragmentation (Ory *et al* 2001).

The relationship between proliferation and cell death might reflect the fact that cells require ‘survival signals’. Lack of these signals triggers apoptosis (‘death by neglect’). Survival signals include growth factors, cytokines such as IL-2 and IL-3, and hormones such as insulin. In general, survival signals are mediated by means of the activated phosphatidylinositol 3-kinase (PI3K) / Akt pathway which results in interference with the apoptopic machinery and inhibition of pro-apoptopic members of the bcl-2 protein family (Igney and Krammer 2002).

Increasing evidence suggests that cell proliferation and apoptosis are intrinsically linked. Potent mitogenic stimuli result in proliferation when adequate survival factors are present, and programmed cell death if not (Santen *et al* 2002). To our knowledge, there is a paucity of other studies using the cell death:proliferation ratio method used in this work to quantify the differential effects of progestogens on breast cancer cell lines. Up to now, only one other paper has been published by Franke and Vermes (2003), who used the ER+ MCF-7 breast cancer cell line. No other groups have used a non-malignant cell line in this way. The cell death:proliferation ratio results using progestogens at concentrations of $10^{-6}$M and $10^{-7}$M in the presence of growth factors $10^{-12}$M confirmed the results of the initial proliferation assays. Of the eight progestogens tested, only MPA and CMA significantly affected the proliferative response of MCF10A cells to growth factors by increasing cell proliferation.

4.1.3 MCF10A and proliferation inhibitors

The most important families and pathways affected by growth factors are mitogen activated protein kinases (MAP kinases), phosphatidylinositol-3-kinase (PI3K) and the Akt-pathway. MAP kinases are a key family of serine/threonine protein kinases which are involved in many cellular programs such as cell proliferation, differentiation, movement and death. The PI3K pathway, activated by many survival factors, leads to the activation of Akt, an important player in survival signalling (Akt is also known as protein kinase B, PKB). Activated Akt inhibits the proapoptopic bcl-2 family member Bad, directly inhibits caspase-9 and inhibits additional anti-apoptopic pathways. Many growth factors and cytokines induce anti-apoptopic bcl-2 family members.

To investigate the mechanism of growth factor- and progestogen-induced proliferation, we used specific inhibitors of the different pathways, i.e., PD98059, a selective inhibitor of MAP kinases and LY294002, a specific phosphatidylinisotol 3-kinase inhibitor. According to our results, the stimulation of MCF10A cells by growth factors appears to be mainly mediated via both pathways, since both specific inhibitors were able to significantly block the

stimulation. In agreement with these results, Makarevich *et al* (2002) showed that addition of EGF to rabbit granulosa cells activated ERK-related MAP kinase.

Neither of the proliferation inhibitors was able to inhibit the proliferative effects of MPA or CMA alone. However PD98059 and LY294002 were both able to further inhibit the proliferation of growth factor stimulated cells in the presence of MPA. This indicated that MPA may elicit its proliferative effects via mixed pathways, including activation of MAP kinase and subsequent substrates and activation of PI3K. Cell proliferation induced by a combination of CMA and growth factors could not be significantly reduced by either of the two inhibitors, indicating that CMA may exert its proliferative effects via other pathways.

### 4.1.4 MCF10A and apoptosis markers (cytochrome C, sFasL and p53)

Cytochrome C has been identified as a component required for the crucial steps in apoptosis, caspase-3 activation and DNA fragmentation and has been shown to redistribute from mitochondria to the cytosol during apoptosis in intact cells. Release of cytochrome C into the cytosol leads to activation of an apoptopic program via activation of a caspase dependent pathway. Measurement of cytochrome C release from mitochondria is a tool to detect the first early steps for initiating apoptosis in cells.

Fas is a membrane protein that belongs to the TNF/nerve growth factor receptor family. Fas mediates apoptosis when it is cross-linked with specific binding partners. The natural binding partner of Fas is FasL. sFasL is a soluble form of FasL. The soluble form has been shown to induce apoptosis in susceptible cells.

p53 is the most commonly mutated gene in human cancer. It is considered a stress response gene, and the p53 protein acts to induce cell cycle arrest or apoptosis in response to DNA damage, thereby maintaining genetic stability in the organism. These functions are executed by a complex and incompletely

understood series of steps known as the p53 pathway. p53 therefore has a vital role in suppressing the development of cancer. There is very little data available from other research groups regarding these apoptosis markers and progestogens in relation to their effects on healthy breast cells.

Using surgically menopausal cynomolgus macaques treated continuously for 35 months with either conjugated equine estrogens (CEE), MPA or a combination of CEE and MPA, Isaksson *et al* (1999) found an increased expression of p53 in normal breast and endometrial tissue linked to this treatment. In the breast alveoli, there was an association between proliferation and p53 expression in all groups. The study showed increased expression of p53 by long-term hormonal treatment and a higher rate of breast alveolar proliferation in animals with p53 expression (this higher mitotic index having already been associated with p53 protein expression in women with breast cancer).

Our results showed that growth factors reduced the release of cytochrome C, sFasL and p53 in MCF10A cells. CMA 10-7M was able to further reduce cytochrome C release, and MPA and CMA $10^{-6}$M and $10^{-7}$M were both able to further reduce p53 release, but did not significantly affect sFasL inhibition by growth factors. These results suggest that CMA, MPA and growth factors may exert their proliferative responses via inhibition of sFasL and/or p53 and CMA also by inhibition of cytochrome C. Our results for MPA are in contrast to those of Isaksson *et al*, above. Our results only consider short-term treatment, and since p53 acts as a ‘guardian of the genome’, after long-term exposure to MPA, it is possible that increased proliferation and genomic instability increase its levels.

## *4.2 HCC1500*

### 4.2.1 HCC1500 proliferation assays with growth factors and/or estradiol

In contrast to their neutral effects on MCF10A cells, P, CMA, MPA, NET, LNG and DNG alone were all able to affect the proliferation of HCC1500 cells. Only GSD and KDG had no effect. However, the *in vivo* significance of these results can be questioned, as progestogens do not appear in the female body without the presence of growth factors or E2, and are therefore subject to the further influences of these mitogenic substances.

In the presence of E2, DNG and KDG had no effect, and the remaining progestogens all inhibited the proliferative effect of E2 on cell growth. In the presence of growth factors, P and the C21-progestogens CMA and MPA at $10^{-6}$M and $10^{-7}$M were able to significantly inhibit growth factor-induced cell proliferation. However, the mitotic effects of growth factors on HCC1500 cells were significantly enhanced by the 19-nortestosterone derivatives, NET at $10^{-6}$M, and LNG, DNG, GSD and KDG at both concentrations. In the presence of both E2 and growth factors, only P at both concentrations and NET $10^{-6}$M had no significant effect on the proliferative effects of E2 and growth factors. MPA, CMA, NET $10^{-7}$M, LNG, DNG, GSD and KDG were all able to significantly inhibit the proliferative response.

Varying results regarding the effects of MPA on cancerous breast cells have been published by other research groups in agreement or in contrast to our inhibitory results in the presence of growth factors and/or E2. MPA alone has been shown to induce a modest, but statistically significant cell growth at $10^{-6}$M in a specific subgroup of MCF-7 cells (Schoonen *et al* 1995), and inhibition of E2-stimulated proliferation at the higher MPA concentrations has been illustrated by others (Schoonen *et al* 1995, Cappelletti *et al* 1995, Seeger *et al* 2003). However, MPA combined with E2 has also been found to stimulate proliferation of MCF-7 cells (Franke and Vermes 2003). Cappelletti *et al* (1995)

showed that MPA was able to effectively counteract cell growth induced by the growth factor TGF-$\alpha$ in MCF-7 cells.

Regarding P, other groups have published supporting results, where E2-induced stimulation of MCF-7 cells has been shown to be inhibited by P (Schoonen *et al* 1995, Cappelletti *et al* 1995, Seeger *et al* 2003, Mueck *et al* 2004). It has been proposed that progesterone inhibits the mitogenic effects of IGFs in breast cancer cells (Speroff 2000). Breast cancer cells express IGF-I receptors but not IGF, which comes from nearby stromal cells.

In agreement with our results using ER+ HCC1500 cells, NET alone at $10^{-6}$M has been found to stimulate the growth of ER+ MCF7 and T47DA18 human breast cancer cells, but not ER- MDA-MB-231, BT-20 and T47DC4 human breast cancer cells (Jeng *et al* 1992). The authors of this study suggest that the 19-nortestosterone derivatives have estrogenic properties and suggest that activation of the ER but not PR is the growth-stimulatory mechanism for these synthetic progestogens. The effect of NET on E2-induced proliferation of breast cancer cells has been explored in only a few *in vitro* studies up to now. NET alone has been shown to stimulate proliferation of MCF7 cells at high pharmacological doses of $10^{-7}$M and $10^{-6}$M (Schoonen *et al* 1995). Catherino *et al* (1993) showed that NET had a proliferative effect on E2 stimulated MCF-7 cells and Schoonen *et al* (1995) found that NET had no significant effect on the E2-stimulated proliferation of a subclone of MCF-7 cells, in contrast to our inhibitory results of NET on cancerous cells in the presence of E2 and E2 with growth factors.

CMA has been shown to be able to reduce E2-stimulated proliferation in cancerous MCF-7 cells (Seeger *et al* 2003) and was able to inhibit cell proliferation in the breast cancer cell line ZR-75-1, which expresses both estrogen and progesterone receptors (Poulin *et al* 1991). This is in agreement with out inhibitory results on HCC1500 cells. Up to now, there is a paucity of

data available regarding the effects of CMA on the proliferation of malignant epithelial breast cells.

LNG alone has been found to stimulate the growth of ER+ MCF7 and T47DA18 human breast cancer cells, but not ER- MDA-MB-231, BT-20 and T47DC4 human breast cancer cells (Jeng *et al* 1992), supporting our results obtained using ER+ HCC1500 cells. LNG alone has been shown to stimulate proliferation of MCF-7 cells at high pharmacological doses of $10^{-7}$M and $10^{-6}$M, but reduce E2-stimulated growth of only a specific subgroup of malignant MCF-7 cells by approximately 60% over the concentration range of $10^{-6}$M to $10^{-9}$M (Schoonen *et al* 1995). Another research group showed no suppression of E2-induced stimulation of MCF-7 cells (Seeger *et al* 2003). Van der Burg *et al* (1992) demonstrated that LNG was able to inhibit the mitogenic effect of E2 on MCF-7 cells but was not able to inhibit the synergistic combination of E2 with insulin where, in combination with insulin, LNG stimulated proliferation at 1μM ($10^{-6}$M). Our results agree with other groups' findings where LNG has been found to have an inhibitory effect in the presence of E2 alone. However, they do not help to explain LNG's proliferative effect in the presence of growth factors, and inhibitory effect when combined with growth factors and E2.

DNG has been shown to elicit potent anti-tumour activity against hormone-dependent cancer types in an animal model (Katsuki *et al* 1997), and has exhibited slight concentration-dependent inhibitory effects in combination with E2 (Schoonen *et al* 1995), in agreement with our results. However, we found that DNG was able to promote the proliferative effects of growth factors in both cell lines. This effect was negated in the presence of growth factors and E2, when DNG significantly inhibited their mitotic effects.

GSD and KDG alone have been shown to stimulate proliferation of MCF-7 cells at high pharmacological doses of $10^{-7}$M and $10^{-6}$M (compared to our null-effect results on HCC1500 cells at these concentrations) and in contrast have been shown to be able to inhibit cell proliferation of a specific sub-clone of MCF-7 in

the presence of E2 (Schoonen). In the experimental work by Van der Burg *et al* (1992), they both exhibited similar effects to LNG, i.e. inhibition of proliferation of MCF-7 in the presence of E2, stimulation of proliferation with insulin and inability to inhibit the synergistic combination of E2 with insulin. Catherino *et al* (1993) have shown that GSD alone stimulates MCF-7 proliferation, and this effect occurs via an estrogen receptor-mediated mechanism. In contrast to these proliferative effects, it has been suggested that GSD inhibits the growth of neoplastic breast cells, possibly by displacing E2 from the cancer cell estrogen receptor and causing breast cancer cells to secrete the negative growth modulator, TGF-β (Baum *et al* 1991). Iqbal and Valyani (1988) found that the binding of GSD to the ER in malignant breast disease was refractory to competition by excess amounts of E2 or tamoxifen and suggested that in the already altered ER in the cancerous human breast, there is another binding site for GSD which closely approximates in number and affinity the binding site for E2, and that GSD itself is capable of bringing about a configurational change which greatly reduces the binding of E2 to the ER without completely abolishing it. Coletta *et al* (1989 and 1991) demonstrated a specific GSD binding protein in MCF-7 and T47D cells, which was suggested to be involved in the reduction of cell growth by GSD. The authors proposed that the growth-inhibitory action of gestodene may be mediated in part by an autocrine induction of TGF-β, a potent inhibitor of the growth of normal epithelial cells.

Our results support the inhibitory effect of GSD in combination with E2, however, we found both GSD and KDG exhibited a proliferative effect on HCC1500 cells with growth factors alone. This effect was negated with E2 and growth factors combined, to an anti-proliferative effect with both progestogens.

By comparing the proliferation 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, i.e., they exhibit a synergistic effect in combination. Considerable cross-talk has already been documented between the signalling pathways of estrogen and growth factors. Stewart *et al* (1992)

showed that E2 increased the response of MCF-7 cells to the proliferative effects of EGF, TGF-α, bFGF and IGF-I.

4.2.2 HCC1500 cell death detection:proliferation assays

The balance between programmed cell death and cell proliferation determines tumour growth rate, and any change in this balance may be a key element for the uncontrolled growth of malignant tumours. Franke and Vermes (2003) studied the effects of the progestogens MPA, norethisterone acetate (NETA), DNG and P, amongst others, all at a concentration of $10^{-6}$M, on the ratios of apoptosis: proliferation of MCF-7 cells in the presence of E2. The combinations of MPA + E2, NETA + E2 and DNG + E2 all stimulated proliferation, whereas P + E2 had no significant effect. These results are in contrast to our results, where MPA, NET, DNG and P all significantly inhibited the proliferative effects of E2 on HCC1500 cells. Of the 8 progestogens tested, only KDG had no significant effect; the remaining 7 all inhibited proliferation in the presence of E2. The cell death: proliferation results are in agreement with the initial proliferation assay results for the $10^{-6}$M and $10^{-7}$M concentrations tested.

Previous studies have shown that during the treatment of postmenopausal women with NET-containing preparations, a small proportion of NET is peripherally aromatised into ethinylestradiol, especially in fat and muscle tissue, (Klehr-Bathmann and Kuhl 1995) This effect has also been seen in two perimenopausal women, where a small but significant proportion of NET was converted to ethinlyestradiol *in vivo* (Reed *et al* 1990). The possible increased inhibitory effect of NET $10^{-6}$M in combination with E2 is compensated by a partial conversion of NET into ethinyestradiol. This may explain why NET $10^{-7}$M appears to have a significantly greater inhibitory effect than the $10^{-6}$M concentration in Fig. 24. The authors of this study also suggest that other testosterone derivatives may be subject to aromatization which may contribute to their estrogenic properties. This effect can also be seen on the same graph with the testosterone derivatives GSD and DNG. GSD has been confirmed to have estrogenic activity by another research group, again possibly via action of

its metabolites (Lemus *et al* 2001).This effect is not seen with LNG in our results, however, LNG is also thought to exhibit estrogenic activity via its metabolic conversion products, but with a 750-fold lower estrogenic potency than that of estradiol and a significantly lower relative binding affinity (Santillan *et al* 2001).

Unfortunately, no other research groups have conducted similar work on malignant breast cells using progestogens and growth factors +/- E2 for us to compare our results with. In the presence of growth factors, only MPA and CMA inhibited their proliferative effect. We are, however, unable to explain the greater inhibitory effect of MPA $10^{-7}$M in combination with growth factors compared to the lesser inhibitory effect of $10^{-6}$M.

The remaining progestogens (NET, LNG, DNG, GSD and KDG) enhanced the proliferative effects of the growth factors. These results are all in agreement with the proliferation assay results at $10^{-6}$M and $10^{-7}$M, except for P, which had an anti-proliferative effect on growth factor-treated HCC1500 cells in the proliferation assay, but a null effect in the cell death detection:proliferation assay.

In the presence of growth factors in combination with E2, MPA, CMA, NET and GSD all produced an anti-proliferative effect, in contrast to P, LNG, DNG and KDG, which had no significant effect. In fact, the stimulatory effects of NET, LNG, DNG, GSD and KDG on growth factor-treated HCC1500 cells was inhibited and reduced to either a neutral effect or anti-proliferative effect in the presence of E2. All results except for LNG, DNG and KDG agree with the proliferation assay results, where these three progestogens all had an anti-proliferative effect in the proliferation assays and no effect in the cell death detection:proliferation assay when in the presence of growth factors and E2.

The synergistic effects of growth factors and E2 in combination were once again confirmed in the cell death: proliferation assay results, where the additive

proliferative effects of growth factors and E2 were greater than their effects alone.

4.2.3 HCC1500 and proliferation inhibitors

In contrast to the results seen with MCF10A cells, the growth inhibitors PD98059 and LY294002 had significant effects on the proliferation of HCC1500 cells under various conditions. Both inhibitors were able to significantly inhibit the growth of HCC1500 cells alone (although this inhibitory effect could be attributed to the possible presence of growth factors in the DMEM/FCS-stripped assay medium), inhibit growth factor-induced proliferation and E2-induced proliferation, and growth factor plus E2-induced proliferation. This indicated that MAP kinases and PI3K are all involved in all these processes, perhaps with the PI3K pathway having the greatest influence. Other research groups have confirmed these results. Stoica *et al* (2003) demonstrated that EGF and IGF-I can activate Akt and that E2 can also rapidly activate PI3K/Akt in the MCF-7 cell line. Breast cancer cells can be stimulated to grow with growth factors in the absence of added estrogen or progesterone. MAP kinase activation increases in response to growth stimuli such as EGF, IGF-I, insulin, prolactin and TGF-α or -β (Santen *et al* 2002).

E2 mediates cell proliferation at least partially through the actions of MAP kinase. In ER+ breast tumours, MAP kinase pathways can exert “cross talk” effects at the cell cycle level and at the level of ER induced transcription. Using mechanisms which involve activation of MAP kinase, E2 stimulates cell proliferation either through rapid, non-transcriptional effects, or by increasing growth factor production and consequently MAP kinase. Progestogens and androgens also stimulate MAP kinase through both of these two mechanisms (Santen *et al* 2002). Using MCF-7 cells and measuring the fraction of cells entering DNA synthesis after E2 treatment in the presence of PI3K and MAP kinase inhibitors, Lobenhofer *et al* (2000) showed that estrogen initiates mitogenesis by inducing the transcription of immediate early genes, but cytoplasmic signalling pathways play an important role in the control of

subsequent events in the cell cycle. Another group proposed that the MAP kinase pathway is activated during the process of adaptation of breast cancer cells to a low estrogen environment, and that MAP kinase activation is responsible for E2 hypersensitivity in E2-deprived MCF-7 cells (Yue *et al* 2002). It has also been suggested that the induction of the cell cycle by E2 does not require a direct activation of MAPK/ERK or PI3K signalling protein kinase cascades, but that these kinases appear to have a permissive role in cell cycle progression (Gaben *et al* 2004).

It has been suggested that progesterone selectively increases the sensitivity of key kinase cascades to growth factors, thereby priming cells for stimulation by latent growth signals, supporting a model in which breast cancer cell growth switches from steroid hormone to growth factor dependence (Lange *et al* 1998).

In contrast to the above investigations considering blocking the mitotic pathway, Alkhalaf *et al (*2002) investigated the capacity of progesterone to induce apoptosis and alter the activity of a key regulator of cell growth and differentiation in MCF-7 cells. They concluded that (i) growth inhibition of breast cancer cells by progesterone is due to activation of cell differentiation and not to apoptosis, (ii) progesterone activates the PI3K/Akt pathway, (iii) inhibiting the PIK3/Akt pathway with LY294002 causes stimulation of apoptosis, and (iv) progesterone enhances LY294002-induced growth inhibition and apoptosis. Our proliferation assays support the inhibitory effects of progesterone when in the presence of E2 or growth factors.

Unfortunately, there is a paucity of data from other research groups considering these proliferation inhibitors in the presence of other specific progestogens.

### 4.2.4 HCC1500 and apoptosis markers (cytochrome c, sFasL and p53)

Apoptosis regulation is ensured by various genes, mostly pro-apoptopic and rarely antiapoptopic. Bcl-2 was the first antiapoptopic gene to be described. Some gene products derived from its family can hetero-dimerize, and the cell

survival or death depends on the relative ratio of bcl-2/bax. When the ratio is low, apoptosis occurs, mediated by the cytochrome C molecule located in the mitochondrial membrane. Cytochrome C is released from its membrane hold and activates the caspase cascade, essential to the apoptosis process (Gompel *et al* 2004).

Unfortunately, there is no data available from other study groups with which to compare our results. We found that cytochrome C release was significantly reduced by both E2 and growth factors, supporting their mitotic effects. MPA increased its release in the presence of E2, growth factors and a combination of the two, showing that MPA exerts its anti-proliferative effects on HCC1500 cells via a mechanism involving release of cytochrome C. NET was only able to significantly affect its release at $10^{-6}$M in the presence of E2 and growth factors. P increased its release in the presence of growth factors only. The results for NET and P show that other pathways must be also involved via which these two progestogens exert their effects on HCC1500 cells.

An emerging theme from studies defining the mechanisms of action of chemotherapeutic drugs is the involvement of death receptors, particularly Fas and its ligand, FasL, in drug-induced apoptosis. Fas activation by FasL activates a caspase cascade, leading to apoptosis. Treatment of different tumour cell types with DNA-damaging drugs has been shown to induce the expression of Fas and/or FasL. Blocking Fas activation in these cells inhibits drug-induced apoptosis of the tumour cells (Gibson *et al* 1999). In a study using T47D breast adenocarcinoma cells, MCF-7 malignant breast epithelial cells, and embryonic kidney epithelial (HEK293) cells, Gibson *et al* (1999) demonstrated that EGF stimulation of all three cell lines protected the cells from Fas-induced apoptosis. EGF stimulation of the MCF-7 cells also inhibited Fas-induced caspase activation and the proteolysis of Akt. Expression of activated Akt in MCF-7 cells was sufficient to block Fas-mediated apoptosis. The authors concluded that EGF stimulation of breast cancer cells has a significant survival function against Fas-induced apoptosis by a mechanism involving the activation

of Akt, and the activation of Akt appears to be both required and sufficient for the anti-apoptopic function of EGF. Our own results using HCC1500 in the presence of growth factors showed proliferation could be inhibited by LY294002, a PI3K inhibitor, and therefore subsequent inhibitor of Akt, again demonstrating that EGF, and possibly other mitotic growth factors may activate Akt.

Our results using HCC1500 cells support the results from other research departments; we found that growth factors (EGF, FGF and IFG-I combination) were able to significantly reduce sFasL release. We also found that sFasL release was significantly reduced by both E2 and a combination of E2 and growth factors, supporting their mitotic effects. Again, unfortunately no data is available from other research groups with which to compare our E2 results and progestogen results. MPA increased sFasL release in the presence of E2 and E2 plus growth factors, in line with its anti-proliferative effects. NET increased sFasL in combination with E2 and growth factors plus E2, and reduced it in the presence of growth factors alone, in agreement with the cell death detection:proliferation assay results. P increased sFasL release in all cases, which is surprising as P only had a significant anti-proliferative effect on E2-treated cells in the cell death detection:proliferation assays and had no significant effects on growth factor and growth factor plus E2-treated cells. However, in the initial proliferation assays, P was shown to also have an anti-proliferative effect on growth factor-treated HCC1500 cells.

The tumour suppressor protein, p53, can induce apoptosis through the bcl-2/bax pathway. Therefore, if the genome repair mechanisms activated by p53 after DNA damage were not effective, it induces apoptosis or cell cycle arrest, thereby protecting the tissue against the transmission of the DNA abnormalities and acting as a ‘guardian of the genome’. Mutations in the p53 gene have been demonstrated in a variety of malignant tumours including breast and endometrial cancer (Gompel *et al* 2004, Isakkson *et al* 1999) Using normal breast cells, it has been shown that progestogens and antiestrogens can

increase the level of p53, while E2 has an opposite effect on the level (Gompel *et al* 2004, Somaï *et al* 2003). Gompel *et al* (2004) studied p53 in T47D cells, a breast cancer cell line containing mutated p53, which does not have any pro-apoptopic function, and found in comparison to the wild-type form of p53 (normal type), that E2 induced p53 in parallel to its proliferative action, and progestogens and antiestrogens suppressed its expression. They concluded that progestogens have opposite effects on the normal and mutated form of p53, which is probably essential in their protective action on breast cancer, as p53 is mutated in 30-40% of breast cancers and some cases of breast hyperplasia. This increase in mutated p53 in E2-treated T47D cells has also been shown elsewhere, where in the same investigation, p53 was down-regulated by R5020 (promegestone), showing different responses in other cell lines (Hurd *et al* 1995). Our results showed that E2 reduces p53 release from HCC1500 breast cancer cells, as do growth factors and a combination of growth factors and E2. The HCC1500 cell line may contain the normal 'wild-type' form of p53, as our results are in agreement with those of Gompel *et al* (2004).

No data is available from other study groups on the effects of MPA and NET on p53. We found that all three progestogens increased p53 release in the presence of E2, in line with their anti-proliferative effects under these conditions. MPA and NET had no effects on the growth factor-induced reduction in p53 release, but were both able to increase its release in the presence of growth factors and E2.

Formby and Wiley (1998) found that progesterone at a concentration similar to that found in the third trimester of pregnancy exhibited a strong antiproliferative effect on T47D breast cancer cells, apoptosis had been induced and the expression of p53 had been up-regulated. However, there are conflicting reports about the regulation of p53 levels by progestogens in breast cancer cells, as another group showed that the synthetic progestogen R5020 (promegestone) lowered levels of p53 to 30% of the control level in T47D cells (Moudgil *et al* 2001). Using MCF7 cells, Alkhalaf and El-Mowafy (2003) showed that

progesterone inhibits cell growth and the inhibition is associated with down-regulation of p53 levels. However, in the same culture conditions using T47D and ZR75-1 cell lines, growth inhibition induced by progesterone was independent of p53 regulation. Progesterone has also been shown to induce apoptosis and up-regulation of p53 expression in two human ovarian carcinoma cell lines after exposure to P $10^{-5}$M (10μM) for 72 hours (Shi-Zhong *et al* 1997). Our results show that p53 release from HCC1500 cells is increased by P in the presence of E2, growth factors and a combination of the two. These contrasting results may be due to the properties of the different cell lines used and the presence of wild-type or mutated p53 gene.

4.2.5 HCC1500 proliferation assays with progestogens in combination with estradiol in the presence of tamoxifen or letrozole.

The role of estrogen, and the blocking of the estrogen receptor and of aromatase inhibition in the treatment of breast cancer have been supported by the success of the use of tamoxifen and anastrazole respectively (Early Breast Cancer Trialists' Collaborative Group 1998, The ATAC (Arimidex, Tamoxifen Alone or in Combination) Trialists' Group 2002).

The proliferative effects of the 19-nortestosterone derivatives, NET, LNG, DNG, GSD and KDG on growth factor-stimulated ER+ HCC1500 cells were completely inhibited by the estrogen receptor antagonist 4-hydroxytamoxifen (4OH-Tam), by not by letrozole, an aromatase inhibitor. This suggests that these progestogens exert their stimulatory effect via the estrogen receptor in this cell line, and not by their conversion to ethinylestradiol by aromatase. However, it is possible that these progestogens were converted into estrogenic metabolites, as suggested by other investigators (Larrea *et al* 1987 and Vilchis *et al* 1986).

Jeng *et al* (1992) investigated the estrogenic potential of progestogens in oral contraceptives to stimulate human breast cancer cell proliferation. They state

that the 19-nortestosterone derivatives NET, LNG and norethynodrel bind to the PR with high affinity, and investigated whether these progestogens could act through the ER. They showed that growth stimulation of ER+ MCF-7 cells by NET and LNG at $10^{-6}$M was blocked by the antiestrogens 4OH-Tam and ICI 164,384, but not the antiprogestogen RU486. They also ruled out the possibility that the progestogens were converted into aromatized estrogens, as the aromatase inhibitors 4-hydroxyandrostenedione and aminoglutethimide did not block progestogen-induced growth stimulation. Jordan *et al* (1992) also confirmed these results in their work. They found that NET, LNG, GSD and norethynodrel were able to stimulate the growth of ER+ MCF-7 cells but not ER- MDA-MB-231 breast cancer cells in culture. This effect was blocked by the 4OH-Tam, but not by mifepristone (RU486). They suggest that although the results could be interpreted as showing an estrogenic activity for the 19-nortestosterone derivatives through the ER, it is possible that the 4OH-Tam blocked the estrogen-induced production of the PR, therefore inhibiting the proliferative response through this receptor system. However, they dismiss this possibility as mifepristone was ineffective as an antiproliferative agent, and therefore propose that the PR does not appear to be involved in the proliferative response of 19-nortestosterone derivatives.

In a study of the effects of pregnane derivatives (including P and MPA) and 19-nortestosterone derivatives (NET, LNG, GSD, KDG and Org30659) on three subclones of MCF-7 cells, Schoonen *et al* (1995) again found that 4OH-Tam and ICI 164,384 but not the antiprogestogens mifepristone and Org 2058 could block the stimulatory effects of the 19-nortestosterone derivatives. In the presence of E2, the growth inhibitory effects of LNG, GSD and KDG could not be blocked by antiprogestogens.

Van der Burg *et al* (1992) showed that LNG, GSD, KDG and Org 30659 were able to stimulate proliferation of E2-treated MCF-7 cells, and that this effect could be blocked by 4OH-Tam, but not by the antiprogestogens RU486 or Org 31710. Catherino *et al* (1993) suggested that the 19-norprogestins norgestrel

and GSD stimulate MCF-7 cells by activating the estrogen receptor as this effect could be blocked by the antiestrogen ICI 164,384 but not by RU486.

### *4.3 Cell Culture*

Breast cancer cells which grow *in vitro* are one of the most widely used models of breast cancer, and for many studies, are the only means of analysis available. Many breast cancer cell lines can be maintained *in vitro*, and are generally stable with respect to their *in vitro* or *in vivo* responsiveness (Clarke *et al* 2000).

There are a number of advantages to using cell lines as *in vitro* models in cancer research; they are easy to handle, are a self-replicating source which can be cultured in almost infinite amounts, they can be re-cultured from frozen stocks if lost or contaminated for any reason and they show a relatively high degree of homogenicity. However, they can exhibit phenotypic and genotypic drift during repeated culture, this being more common in frequently used cell lines, which were established many years ago. This drift can lead to changes in cell growth rates, hormone receptor content and genetic content, even though the cells may appear morphologically identical. This drift away from the phenotype of the original cell is of relevance if cell lines are to be used as models for investigating the pathological processes of cancer or the effects of drugs, chemicals or physiological substances etc. on the cells as mirrors of what may occur *in vivo* (Burdall *et al* 2003).

In this study, the two cell lines used were a human, non-tumorigenic, estrogen and progesterone receptor negative breast epithelial cell line (MCF10A), isolated from the mastectomy tissue of 36-year old Caucasian woman with benign fibrocystic disease, and a human estrogen and progesterone-receptor positive primary breast cancer cell line (HCC1500), derived from the breast of a 32 year old black female with Stage II, Grade 2, invasive ductal carcinoma with

4/24 lymph node metastases, i.e., the cells used originated from healthy breast tissue or a primary breast tumour. A number of commonly used breast cancer cell lines, which are routinely used as 'breast cancer models' are, in fact, derived from tumour metastases such as aspirates or pleural effusions. Examples of these cell lines are MCF-7 and T47D (both from the pleural effusions from a patients with invasive ductal carcinoma of the breast), and MDA-MB-231 (pleural effusion from a patient with adenocarcinoma of the breast). These metastatic cells are not derived from the primary tumour itself, and, as they are metastatic cells, can be unrepresentative of the type, stage or grade of breast cancer, and are more representative of late stage, more aggressive or rapidly advancing disease than early stage disease. Using cells derived from a primary tumour where possible is more representative of an *in vivo* model, as most drug therapies or pathological agents act directly on these primary cells.

In general however, human breast tumours are highly diverse and contain many subpopulations of cells with different phenotypic characteristics, including ER+ and ER- cells. In contrast, breast cancer cell lines are relatively homogeneous, which can be an advantage or disadvantage. Cell lines can be representative of the response of a tumour subpopulation, even if they do not fully imitate the response of a multifaceted human tumour (Clarke *et al* 2000).

The way cells are handled, including duration of trypsinisation and time at room temperature during passage or treatment can affect outcomes of experimental studies. The culture conditions of a cell line in the laboratory can also influence outcomes. Many culture media include serum, which contains a wide range of minor components which may affect cell growth and therefore experimental results. These components include growth factors, hormones, minerals, lipids, nutrients (amino acids, nucleosides, sugars etc.), often in unknown quantities, with undefined actions and with batch-to-batch variance. For this reason, the first proliferation assays carried out were to test proliferation of MCF10A and HCC1500 in different culture media, ideally a serum-free media. MCF10A

proliferated successfully in serum-free AIM-V medium, however, HCC1500 did not, and therefore DMEM/FCS-stripped medium had to be used for this cell line. FCS-stripped is charcoal/dextran treated to remove steroid hormones, however other components which may affect cell growth, including growth factors, may remain in the medium. The inhibitory effect of the proliferation inhibitors PD98059 and LY294002 on the growth of HCC1500 cells alone was a possible result of using serum-containing media for the assay work with this cell line.

Using serum-free media for experimental work has the advantage of eliminating the serum content and therefore allowing the content of the media to be accurately defined. It does, however, have disadvantages; cell growth may be slower or inadequate, the media may be more expensive than conventional media and removal of serum also removes the protective, detoxifying action that some serum proteins may have, therefore a high degree of cleanliness and good aseptic technique is required (Freshney 2000).

Experimental work in this study was begun by plating both cell lines in AIM-V serum-free medium to test for an adequate proliferative growth response in this medium. MCF10A cells proliferated well, and further tests were therefore continued with AIM-V serum-free medium. However, proliferation assays initially carried out with HCC1500 cells and AIM-V serum-free medium yielded poor results, with low, extremely variable cell counts and high rate of cell death. The remainder of the assays with HCC1500 cells were therefore carried out using DMEM-FCS stripped medium, which the cells responded well to. Proliferative results of the cell lines to confirm the expected responses to estradiol and growth factors were as predicted, however growth factors at a concentration of $10^{-10}$M HCC1500 cells had a significant inhibitory effect on cell proliferation in contrast to the zero effect of $10^{-11}$M and proliferative effect of $10^{-12}$M. It has been shown that estradiol is capable of displaying a biphasic action on cell proliferation of MCF-7 cells, i.e. stimulatory at low concentrations ($10^{-8}$M) and inhibitory at a high concentration ($10^{-5}$M) (Lippert *et al* 2003), and it is possible that this same effect occurred here with growth factors and HCC1500 cells.

Phenol red is present in many commercially available media as a pH indicator. It has been shown that phenol red bears a structural resemblance to some non-steroidal estrogens and has significant estrogenic activity at the concentrations at which it is found in cell culture media. It can stimulate the proliferation of ER+ cells and reduce the degree to which exogenous estrogen is able to stimulate responses. It has been shown to have no effect on ER- breast cancer cells (Berthois *et al* 1986). Phenol red-free media were therefore used throughout for the culture of and experimental work with the HCC1500 cell line, which is ER+.

It has been reported that the culture of ER+ breast cancer cells *in vitro* in ethanol-containing medium was associated with an increased proliferation rate, with a 1.3-fold increase in cell proliferation after 6 days of culture of MCF-7 cells in the presence of 0.1% ethanol. This was, however, not exhibited in ER- cell lines (Etique *et al* 2004). This response has also been demonstrated with ethanol concentrations between 10mM and 100mM (1% to 10%) in MCF-7 and ZR75.1, both ER+ cell lines, but not in the ER- cell lines, BT-20 and MDA-MB-231 (Singletary *et al* 2001). Similar results were also obtained in our own work in-house work, with growth stimulation of ER+ MCF-7 cells at an ethanol concentration of 0.1% but not 0.01%, showing a dose-dependent effect of ethanol on this cell line. Stock solutions of progestogens and estradiol in a concentration $10^{-2}$ M in 100% ethanol were therefore diluted with the relevant medium for the cell type used to an ethanol concentration of ≤0.01% for all working experiments.

HCC1500 cells were found to grow in culture in clustered ‘domes’, rather than spreading into a cell layer across the flask. The cells occasionally remained in these clusters after trypsinisation and did not separate into a single cell suspension. All recommendations from ATCC to avoid clumping during trypsinisation were followed (i.e., refraining from tapping the flask base on a hard surface to encourage cells to dislodge, maintaining the flask and contents at 37°C during trypsin treatment, avoiding use of too much dissociation

solution). Clumping of the cells was noted when counting the cells under the microscope with the Neubauer cell counter (Hemocytometer). To overcome this problem, after trypsinisation, centrifugation and resuspension of the cell pellet in medium prior to counting cells, the cell suspension was gently passed through a fine bore (subcutaneous) sterile needle attached to a 1ml syringe to generate a single cell suspension for counting and more accurate plating.

### *4.4 Limitations*

Limitations of this *in vitro* study might be the high concentrations of progestogens needed for an effective antiproliferative effect. We present mainly only results of rather high progestogen concentrations of $10^{-7}$M and $10^{-6}$M, since lower *in vitro* concentrations did not show any relevant effect. The clinically relevant blood concentrations for MPA and NET are in the range of $4x10^{-9}$M to $10^{-8}$M for MPA (Svensson et al *1994*) and around $10^{-8}$M for NET (Stanzyk *et al* 1978). LNG, DSG and GSD may have similar concentrations to NET, but little data is available to support this. 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.

Other limitations include the reliability of the assay methods and kits used. The cell proliferation measurement assay using the ATP chemosensitivity test 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. However, large statistical variations present in some results may affect the interpretation of the findings. Many multiple statistical comparisons are made in this publication, and therefore some significant results could be due to chance.

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 emphasised 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 (Writing Group for the Women's Health Initiative Investigators 2002). *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.

### *4.5 Summary*

These results clearly indicate that different progestogens can induce proliferation of or inhibit the growth of benign or malignant human breast epithelial cells independently of the effects of growth factors and E2. A marked diversity was seen among the effects of the C-21 progesterone derived MPA and CMA (which further stimulated the proliferation of growth factor-treated normal MCF10A breast cells, and had an opposite inhibitory effect on growth factor- and/or E2-treated HCC1500 cells), and the C-19 testosterone derived NET, LNG, DNG, GSD and KDG (which had no effect on growth factor-treated MCF10A cells, proliferative effects on HCC1500 cells in the presence of growth factors, inhibitory or neutral effects in the presence of E2 and, most relevant to the *in vivo* situation, inhibitory or neutral effects in the presence of growth factors and E2). The proliferative effects of the C-19 testosterone derivatives NET, LNG, DNG, GSD and KDG on HCC1500 cells in the presence of growth factors were all inhibited by the estrogen receptor antagonist 4-hydroxytamoxifen, suggesting that the proliferative effects of these synthetic progestogens may involve stimulation of the estrogen receptor. Differences were also demonstrated and involvement of MAPK and PI3K in the proliferative pathways, and the release of the markers of apoptosis, cytochrome C, sFasL and p53.

## 5. CONCLUSIONS

There is little doubt that a reduced lifetime exposure to ovarian hormones will reduce breast cancer risk. However, for those women in need of post-menopausal or post-surgical replacement of their natural ovarian hormones with hormone replacement therapy (HRT), the choice of treatment must be modified to minimise any possible increase in breast cancer risk thereby produced, also considering the possible beneficial or adverse effects of treatment on coronary heart disease, osteoporosis, endometrial cancer, colon cancer and cognitive decline.

There is very little agreement among published studies addressing estrogen-progestogen therapy and breast cancer risk, and the results may often reflect either a very small effect or the impact of confounding bias. In addition to this, some major studies have been conducted using only one progestogen, leading to a general conclusion that all progestogens are detrimental to the health of women. However, the geographical variation in the choice of steroids used in HRT is well known, and this general conclusion cannot be applied to both testosterone-like and progesterone-like progestogens. Observational studies have added to the evidence of an increased risk of breast cancer following the administration of exogenous sex steroids, but the risk may be restricted to specific types of HRT only.

*In vitro* studies of the effects of progestogens on breast cancer risk have been very inconsistent, and one may question the ability of *in vitro* studies to fully replicate the *in vivo* environment. The normal function of breast cells requires growth factor modulation by surrounding stromal and fat tissue, an environment which is absent with the use of cultured cells. Extensive information has been provided by various laboratories about the proliferative effects of different progestogens on isolated models using breast cancer cells and, to a lesser extent, healthy breast cells, these substances having either an inhibitory or stimulatory action. However, in many publications, the effects of progestogens on breast cells have been tested alone, without the presence of estrogens or

growth factors, which does not attempt to mimic the *in vivo* situation. Therefore, in the presented work, most assays were conducted in the presence of estradiol and/or growth factors, as mitogenic growth factors from stromal breast tissue and estrogens are important in the growth-regulation of breast cells and may modify responses to progestogens.

In this study, the *in vitro* effects of progesterone and seven synthetic progestogens (C21-progesterone derivatives and C-19 testosterone derivatives) were investigated with respect to their effects on the proliferative and apoptopic responses of growth factor- and/or estradiol (E2)-treated normal and malignant breast epithelial cells.

The following results were obtained: in the proliferation assays, the synthetic C-21 progesterone derivatives medroxyprogesterone acetate (MPA) and chlormadinone acetate (CMA) had stimulatory effects on benign growth factor-stimulated MCF10A cells, but inhibitory effects both on growth factor and/or E2-treated malignant HCC1500 cells. Natural progesterone (P) was able to inhibit the proliferative effects of E2 and growth factors alone in the cancerous HCC1500 cells, but had no significant effect on MCF10A cells in the presence of growth factors and HCC1500 cells in the presence of E2 and growth factors in combination.

The synthetic C-19 testosterone-derived norethisterone acetate (NET), levonorgestrel (LNG), dienogest (DNG), gestodene (GSD), and 3-ketodesogestrel (KDG), a metabolite of desogestrel, had no effect on growth factor-stimulated MCF10A cells. NET, LNG and GSD significantly reduced HCC1500 proliferation in the presence of E2; DNG and KDG had no significant effect. In combination with growth factors, P, MPA and CMA reduced the proliferative response, however NET, LNG, DNG, GSD and KDG all further stimulated proliferation. In the presence of growth factors and E2, all progestogens tested inhibited the proliferative response, except P, which had no significant effect.

Assays using the proliferation inhibitors PD98059 and LY294002 showed that the proliferative effects of growth factors on MCF10A cells and of MPA in the presence of growth factors occur via mixed pathways, including activation of mitogen activated protein kinase (MAPK) and phosphatidylinisotol 3-kinase (PI3K). CMA, however, possibly exerts its proliferative effects via other pathways, as these effects could not be inhibited.

Using HCC1500 cells, the proliferative effects of growth factors were once again found to involve the MAP kinase and PI3K pathways, as were E2 and a combination of the two mitotic agents. The effects of the progestogens NET, LNG, GSD, DNG and KDG, which were all found to enhance the proliferative response to growth factors, were blocked by both PD98059 and LY294002, again, showing the involvement of MAPK and PI3K in these proliferative processes.

MPA and CMA were found to have anti-apoptopic effects on normal MCF10A cells and pro-apoptopic effects on malignant HCC1500 cells. Natural P exerted an apoptopic effect on E2-stimulated cancerous HCC1500 cells, but had no overall significant effect on MCF10A stimulated with growth factors and on HCC1500 cells in combination with growth factors alone or growth factors with E2.

NET, LNG, DNG, GSD, and KDG had varying anti-apoptopic, apoptopic or neutral effects on both cell lines, depending on the presence of growth factors, E2 or both. However DNG was found to have an overall apoptopic effect on E2-treated HCC1500 cells, compared to its neutral effects in the proliferation assays above. In combination with growth factors and E2, proliferation of HCC1500 was inhibited by MPA and CMA (apoptopic effect), as already described, as well as NET and GSD, with the remaining progestogens having no significant effect. In fact, the stimulatory effects of NET, LNG, DNG, GSD

and KDG on growth factor-treated HCC1500 cells were inhibited and reduced to either a neutral effect or apoptopic effect in the presence of E2.

Measurement of the apoptosis markers cytochrome C, sFasL and p53 in MCF10A cell lysates showed that growth factors alone reduce the release of all three markers. MPA reduced the release of p53 only and CMA of cytochrome C and p53, perhaps illustrating that part of the short-term proliferative process initiated by these two progestogens involved inhibition of the release of these important key players in the apoptotic process.

Measurement of the apoptosis markers cytochrome C, sFasL and p53 in growth factor and/or E2 treated-HCC1500 cell lysates showed that these proliferative agents reduce the release of these three markers, suggesting inhibition of the apoptopic process by these agents. MPA increased cytochrome C and p53 release from growth factor and/or E2-treated cells, and sFasL from E2- and growth factor plus E2-treated cells. NET's effects on the apoptosis markers were as varied as its effects on proliferation depending on the presence of growth factors, E2 or both. In the presence of growth factors and E2, it increased the release of all three markers, in line with its anti-proliferative effects under these conditions. In the presence of growth factors alone, it only affected sFasL by further reducing its release. P increased release of cytochrome C from growth factor-treated cells, and of sFasL and p53 from the cells under all three treatment conditions (although a large margin of error sometimes affected significance).

The proliferative effects of the C-19 testosterone derivatives NET, LNG, DNG, GSD and KDG on HCC1500 cells in the presence of growth factors were all inhibited by 4-hydroxytamoxifen, an active metabolite of tamoxifen and antagonist of the estrogen receptor, showing that the proliferative effects of these progestogens involve stimulation of the estrogen receptor. Proliferative effects were not blocked by the aromatase inhibitor letrozole, suggesting that

the stimulatory effects of these progestogens is not due to their conversion to ethinyestradiol by aromatase.

In general, from these results, it can be seen that the progestogens tested in this study have diverse effects on normal and cancerous human breast cells, distinct differences being seen between the effects of C-21 progesterone derived MPA and CMA (which further stimulated the proliferation of growth factor-treated normal MCF10A breast cells, and had an opposite inhibitory effect on growth factor- and/or E2-treated HCC1500 cells), and the C-19 testosterone derived NET, LNG, DNG, GSD and KDG (which had no effect on growth factor-treated MCF10A cells, proliferative effects on HCC1500 cells in the presence of growth factors, inhibitory or neutral effects in the presence of E2 and, most relevant to the *in vivo* situation, inhibitory or neutral effects in the presence of growth factors and E2). Differences were also demonstrated in the proliferative pathways and involvement of MAPK and PI3K, and the release of the markers of apoptosis, cytochrome C, sFasL and p53.

In conclusion, despite the experimental limitations described, these results indicate that certain different progestogens 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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## 7) ACKNOWLEDGEMENTS

I would like to thank PD Dr. Harald Seeger, Head of the Endocrine and Menopause Research Laboratory, and Frau Daniela Köndgen, MTA, both of the Universitäts Frauenklinik, Tübingen, and my husband, Dr. med. Bernhard Krämer, for their help, advice and support during the completion of this study.

Finally, I would like to thank my loving parents, Jackie and Alan Austwick, for their unfailing and continued support throughout the completion of all my studies.

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