In Vitro Regulation Of the Progesterone Receptor

A thesis submitted for the Degree Of Master of Science in the Faculty of Sciences.

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Abbreviations Used

AR	Androgen Receptor
BSA	Bovine Serum Albumin
°C	Degree Centigrade
cDNA	Complementary DNA
cpm	Counts Per Minute
DCC	Dextran Coated Charcoal
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra-Acetic Acid
ER	Oestrogen Receptor
ERE	Oestrogen Response Element
ETN	EDTA-Tris-NaCl buffer
FCS	Foetal Calf Serum
FSH	Follicle Stimulating Hormone
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
3H	Tritium Label
HED	HEPES-EDTA-Dithiothreitol
HEPES	4-(2-Hydroxyethyl-1-Piperazine)-Ethanesulphonic Acid
HIDCCFCS	Heat-Inactivated Dextran Coated Charcoal Stripped FCS
HRE	Hormone Reponse Elements
HRT	Hormone Replacement Therapy
LBA	Ligand Binding Assay
LH	Luteinizing Hormone
MR	Mineralocorticoid
mRNA	Messenger Ribonucleic Acid
OC	Oral Contraceptives
PBS-A	Dulbecco's Buffered Saline
PR	Progesterone Receptor
PRE	Progesterone Response Element
RNA	Ribonucleic Acid
Tam	Tamoxifen
VD3R	Vitamin D3 Receptor

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Summary.

The single commonest cause of cancer deaths among women is breast cancer, closely followed by lung cancer. As detection methods and treatments improve, the rate of breast cancer deaths will hopefully fall. Surgical removal of all clinically apparent disease is usually the treatment of primary breast cancer, often followed by adjuvant systemic treatments to treat subclinical metastases. There are several appropriate adjuvant systemic treatments, however, the one favoured for hormonally sensitive cancer is endocrine therapy. This treatment is based on the concept that certain tumour cells will retain their sensitivity to hormones (e.g. oestrogen and progesterone). The aim of this type of therapy must therefore be to negate or reverse the proliferative effects of such influential hormones.

While it is accepted by clinicians that antiestrogens and progestins provide effective treatment of advanced hormone-sensitive breast cancer, long term usage leads to drug resistance and relapse. A possible approach to overcoming the mechanism of resistance could be to use short bursts of alternative endocrine therapies. Leake *et al.*, (1992) demonstrated that a treatment with Tamoxifen and Megace could give significant advantages when compared to Tamoxifen alone. However, at the end of the progestin component of the cycle there was onset of vaginal bleeding in 13% of patients. One possible solution could be the maintenance of a low level of Norethisterone (10^{-9}M) through out the cycle. Studies contained within this thesis demonstrated that the Tamoxifen component of the cycle induced PR synthesis in ZR-75-1 cells. The ability of Tamoxifen to maintain PR synthesis was not impaired by the presence of Norethisterone ($1^{x}10^{-9}$M). However, higher doses of this progestin did significantly reduce PR levels. Investigations into the effect of Tamoxifen and

progestin either alone or together on the proliferation on ZR-75-1 or MCF-7 cells revealed that Tamoxifen induced cell growth. Norethisterone plus Tamoxifen had no effect on either cell line. However, on its own, Norethisterone induced some growth inhibition on MCF-7 cells.

1 BREAST CANCER

1.1 BREAST CANCER:- The Current Situation

Breast cancer is the commonest single cause of cancer deaths among women in the United Kingdom (closely followed by lung cancer). There are 26,000 women who are newly diagnosed as having breast cancer each year and, of those, 16,000 will eventually die of their disease. It has been estimated that 1 in 12 women will develop breast cancer at some time in their lives. World-wide over half a million women develop breast cancer each year but half of these cases occur in western developed countries which contain less than one fifth of the female world population.

The annual incident rate of breast cancer shows an eight fold variation world-wide. The annual death rate is highest in England and Wales with 28.4 deaths per 100,000 females. The United States has a lower rate of 22.1. The lowest incidence recorded is in Japan and is 5.8. Such international variation could suggest that there are genetic factors involved. However, the incidence of breast cancer in second generation Japanese living in the USA is the same as their Caucasian counterparts (Cancer Research Campaign, 1991). This suggests a role for social and/or environmental factors, including diet.

1.2 BREAST CANCER:- A Historical overview

Breast cancer has been a problem for treatment and prevention throughout the centuries. An Egyptian papyrus written in approximately 1600 BC is the earliest known reference to the disease (Moulin, 1983). It advocates no treatment other than cautery for ulcerated tumours. Surgery for breast tumours was later practised by Greek and Roman physicians. Records describe a prototype of radical mastectomy that was probably being performed in Rome around 100 BC. Greek and Roman medicine gave way to the barbarian societies of the so called Dark Ages when surgical treatments were no longer endorsed. Various doctrines adopted quasi-religious therapies that varied from mass prayer and the laying on of the hands, to topical

applications concocted from assorted natural elements often of an unspeakable nature. The mastectomy was reduced to a punitive rather than a therapeutic role. However, the Renaissance saw the reintroduction of the mastectomy as a therapeutic surgical practice refined from Greco-Roman protocols. Arrival of anaesthesia and antiseptics had still to occur before advances were seen in patient survival and morbidity.

It was not until 1775 that Perceval Pott (Pott, 1963) first suggested that the development of the human female breast was under the influence of the female sex secretions. While repairing a bilateral groin hernia, he removed the normal ovaries of a twenty-three year old woman; the woman subsequently ceased to menstruate and experienced significant loss in breast size, but remained otherwise healthy. Two years later, a French surgeon, Henry Francoise LeDran (Baum, 1981) significantly advanced the hope of a surgical cure. He proposed the theory that the cancer began as a local disease, spreading first to the lymphatics and then on to the general circulation.

Further important observations were made as early as 1836 by Cooper who observed a correlation between tumour growth and the menstrual cycle (Cooper, 1836). Sixty years later, at the end of the nineteenth century, Beatson reported that a relationship existed between the malignant growth of the breast and the ovary (Beatson, 1896). He described the regression of metastatic lesions of the breast of a thirty-three year old pre-menopausal woman after undergoing an oophorectomy. Boyd reiterated these views four years later (Boyd, 1900) The modern era of endocrine therapy for mammary cancer had begun.

Thirty years later oestrone and oestradiol-17β were characterised and proposed as oestrogen agents. However, it was not until after the second world war that oestrogens were proven to have a stimulatory affect on human breast cancer, reversing the benefits of ovarectomy (Pearson *et al.*, 1954). However, at the same time it was discovered that pharmacological doses of oestrogen could suppress the growth of some tumours. The next decade saw the utilisation of new biochemical techniques to identify and estimate steroids in blood and urine samples.

In 1952 Huggins and Bergensal reported that a bilateral adrenalectomy can induce remission of advanced breast cancer in post-menopausal women (Huggins *et al.*, 1952). Similar remissions were observed by Luft and by Pearson and Ray after hypophysectomy (Luft *et al.*, 1958; Pearson *et al.*, 1960). A molecular mechanism explaining the success of the above ablative endocrine therapies was slowly being pieced together. In 1959, oestrogens were shown to accumulate in the pituitary glands, mammary glands, uterus and vagina (Glascock and Hoekstra, 1959), which was consistent with the fact that glands responded to oestrogens (Folly, 1956). These observations were reiterated in 1960 by Jensen and Jacobson who found that tritiated oestradiol was specifically taken up by tissues that had a mitogenic response to the hormone. This suggested the existence of a protein that specifically bound oestrogen in target tissues and thus mediated its action. Therefore it was proposed that those tumours that responded to endocrine therapy would have oestrogen binding protein while those that failed did not (Jensen and Jacobson, 1960). Although not entirely accurate this hypothesis did provide impetus for a medical rather than a surgical endocrine treatment. The intracellular receptor for oestrogen predicted by Jensen *et al.*, was discovered in 1968 by two groups, Jensen and Gorski (Jensen *et al.*, 1968; Gorski *et al.*, 1968). They proposed the now classical two step model where the steroid enters the cell, binds to a cytostolic protein that translocates to the nucleus and binds to an oestrogen responsive gene. Thus the importance of receptor in breast cancer had been established. Largely due to McGuire and co-workers it was found that oestrogen receptors were present in about two thirds of human breast cancers (between 60-70% of cases). Furthermore their concentration was relative to the age and the menstrual status of a patient and to the degree of differentiation of the tumour (McGuire *et al.*, 1975). The progesterone receptor was commonly detected in those tumours that had oestrogen receptors in about 50% of oestrogen receptor positive patients (Horwitz *et al.*, 1985). The progesterone receptor was also detected in about 55% of all oestrogen negative patients.

As early as 1953 attention was brought to the inhibitory effects of anti-hormones. Kottmeir provided evidence for the inhibitory role of progestins on ovarian carcinomas. Exthamoxytriphetol (MER-25) synthesised at the Merrel Pharmaceuticals company was found to block the utrotrophic effect of oestradiol on mice (Kottmeier, 1953, Lerner and Jordan, 1990). Thus the precedent for the evaluation of a new form of endocrine therapy was established. During trials MER-25 was associated with unacceptable toxicities as an anti-tumour agent. However, the antiestrogen Tamoxifen developed in 1967 (Harper and Walpole, 1967) was found to have considerably fewer side-effects (Legha and Carter, 1976). In 1971 Tamoxifen first entered clinical trials in England (Cole *et al.*, 1971) and in 1978 the drug was approved by the FDA in the United States. From its initial introduction Tamoxifen (Nolvadex) proved to be an indispensable part of the clinicians arsenal in the treatment of preselected pre- and post-menopausal patients with any stage of breast cancer.

1.3 CAUSATION OF THE DISEASE

1.3.1 Epidemiology

Over the past two decades there have been many epidemiological studies of the aetiological basis of human breast cancer. However, useful conclusions are hard to postulate due to the interplay of various factors. A study by Anderson (1974) demonstrated that at least two types of breast cancer exist; a familial associated cancer, passed from one generation to another which could possibly be divided into further subclasses of genetic lesions, and a sporadic type of cancer not associated with family history. Investigations into possible risk factors associated with sporadic human breast cancer demonstrated that the younger the woman's age at menarche (MacMahon *et al.*, 1973) and the longer the duration elapsing between this event and the menopause, the higher the risk of breast cancer (Trichopoulos *et al.*, 1972). Analysis of this and other data led to the oestrogen window hypothesis (Sherman and Korenman, 1974) which proposed that the role played by anovulatory cycles was the cause of breast cancer. This has subsequently been shown to be incorrect.

Contraception has provided women with the means to control their own fertility. Hence the variation in time between menarche and first pregnancy amongst cultures has increased; woman from westernised or industrial societies tend to have fewer children and wait longer for them. MacMahon *et al.*, (1973) have managed to correlate early age at first pregnancy with a lower risk of breast cancer. This pattern could possibly be due to the short-term growth enhancement by elevated pregnancy oestrogens and progesterone on already generated clones, followed by a subsequent long-term protective effect induced by pregnancy terminal differentiation of susceptible mammary gland cells (Trichopoulos and Lippman, 1992). This hypothesis, therefore, predicts that pregnancy increases the short term risk of breast cancer in the mother before imparting a more substantial long-term reduction; this has been suggested by several reports (Pathak *et al.*, 1986; Bruzzi *et al.*, 1988; Williams *et al.*,

1990). The early excess risk should be more pronounced in mothers of multiple births since multiple birth pregnancies are characterised by higher levels of oestrogen and progesterone than for single birth pregnancies (Hseich *et al.*, 1993; Doherty, 1990). Subsequent births have little further influence on the chances of developing breast cancer (Tulinius *et al.*, 1978). Women with breast cancer that had fewer children overall, on average, had their first child at a later age. It is known that a first birth beyond a cross-over age limit between 30 and 35 years increases rather than reduces the overall risk of breast cancer (Trichopoulos *et al.*, 1983). Further factors may contribute to these observations, including diet, environment, cultural and genetic factors.

A further example can be observed when examining the low incidence of breast cancer in Asian women. It is widely believed that the relatively low fat diet of Asian women compared with the high fat of western women is responsible. However, studies attempting to link diet directly to breast cancer have largely been negative (Graham *et al.*, 1982; Willett *et al.*, 1987). No single dietary nutrient has been proven to contribute to the risk of breast cancer. Nevertheless, excessive calorific intake, combined with a sedatory lifestyle leading to excessive weight is thought to be a contributing factor (Albanes, 1987). Furthermore, a low protein diet experienced by the inhabitants of third world countries may delay the menarche; additionally the women may become pregnant on their first ovulation and may also become pregnant on several further ovulations. Women in the United States and Northern Europe tend to have diets in rich animal fats and proteins with the result that their first menstrual period is in their early teens (or before). Due to the dictation of their lifestyles, they may delay the first pregnancy, if at all, until twenty or more years. In conclusion, it is still not clear whether age of first menstruation, contrasting diet or environmental factors contribute the greater amount to the risk of breast cancer.

1.3.2 Oral Contraception and Hormone Replacement Therapy

Oral contraceptives have been widely used since the early 1960's by women to control ovulation and most oral contraceptives (OC) contain an oestrogen in combination with a progestin (Piper and Kennedy, 1987). There is concern whether exogenous hormones; either as contraception or hormone replacement (HRT) may increase the risk of breast cancer because most of the known risk factors are related to steroid hormones. The results of studies of oral contraceptives and breast cancer indicate that OC use generally has no effect on the aggregate life-time risk of developing breast cancer (Wingo *et al.,* 1993; WHO study 1990). However, under some circumstances there may be a slight increase in risk for young women taking the OC for a long time before their first pregnancy (Wingo *et al.,* 1993; WHO study 1990). This trend may be compensated by a possible decreased risk of breast cancer in older women (>45) using OC. The studies so far undertaken have involved women under the age of 60 years. In the future it will be possible to include older women who used OC permitting a more accurate assessment of the relationship between OC use and life-time risk.

Studies on the use of HRT have shown that a long term use may slightly increase the risk of breast cancer (Dupont and Page, 1991; Steinberg *et al.*, 1991). However, the beneficial effects of HRT in the prevention of osteoporosis, postponement of the onset of ischemic heart disease, improvement of the lipid profile and improved quality of life make it necessary for patients to be educated regarding HRT so that women can make their own decision regarding its therapeutic value.

1.3.3 Genetic and Familial Factors

A family history of breast cancer is now a well-established risk factor for the disease (Kelsey, 1979). Population based studies have shown that inherited factors maybe responsible for 4% to 10% of all breast cancer cases (Risch *et al.,* 1993; Hall *et al.,* 1990). The gene for early-onset familial breast cancer has been localised to the proximal portion of the long arm of the human chromosome 17 (Hall *et al.,* 1990; Narod *et al.,* 1991). A woman's risk of developing familial breast cancer is dependent upon how many relatives have had the disease and at the age that they developed their disease (Skolnick *et al.,* 1990). The increased risk becomes sufficiently significant for counciling when the woman has more than two relatives with the disease prior to forty-five years of age.

2 STEROIDS AND STEROID RECEPTORS

2.1 STEROID BIOSYNTHESIS

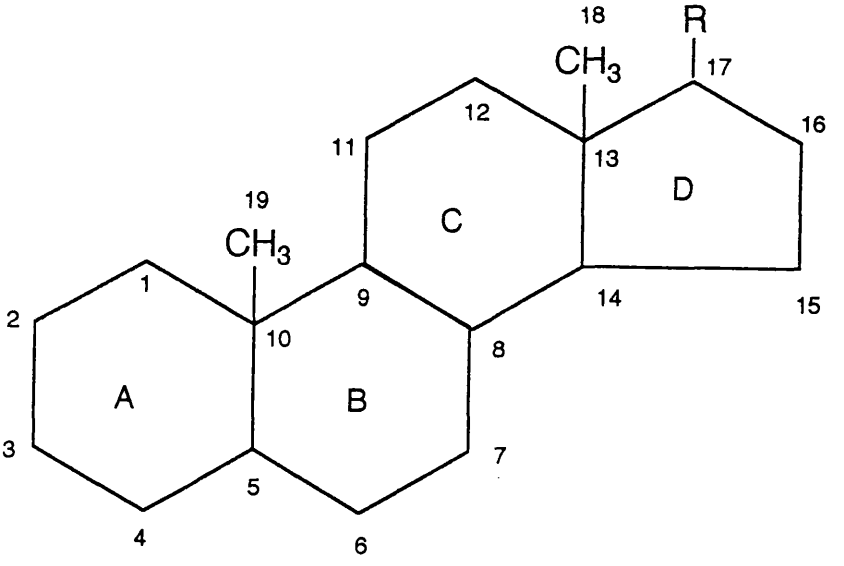
Steroids are widespread in both plant and animal kingdoms, and to date well over 225 naturally occurring steroids have been isolated and characterised. In addition, many other steroids and steroid analogues have been synthetically produced by pharmaceutical companies.

A steroid is an organic molecule whose structure is based on a tetracyclic ring system composed of four rings fused together with a specific stereochemistry (figure 1). The four rings are designated A, B, C, and D, beginning at the lower left, and the carbon atoms are numbered beginning in the A ring. The three six member rings (rings A, B, and C) can adopt strain-free chair conformations. However, the steroids are constrained to a rigid conformation and cannot undergo ring flips. The basic ring structure can be subject to a wide array of modifications by introduction of hydroxyl or carbonyl substituents and by introduction of double or triple bonds. In addition, halogens, sulphydryl or amino groups may replace steroid hydroxyl moieties and heteroatoms such as nitrogen or sulphur can replace the ring carbons

In mammalian systems cholesterol is the precursor of the five major classes of steroid hormones:- mineralocorticoids, glucocorticoids, oestrogens, progestins, and androgens. The steroid hormones are synthesised by sets of cells organised into glands which are members of the endocrine system. For example, cortisol and aldosterone, are produced by cells in the adrenal cortex; oestradiol and progesterone are made by interstital cells in the ovaries; and testosterone is made by leydig cells in the testes.

Steroidogenic tissues may synthesise cholesterol *de novo* from acetate, this occurs mainly in the testis (Hechter *et al.,* 1953). Other human steroidogenic cells derive most of their cholesterol from plasma low density lipoprotein (LDL) (Brown *et al* .,1979). The rate-limiting enzyme in cholesterol synthesis in adrenal cells is 3-hydroxy-3-methylgluteryl coenzyme A (HMGCoA) reductase which is suppressed by adequate

The Steroid Skeleton



(R= different side chains)

Fig. 1 A representative diagram of the basic steroid skeleton.

concentrations of LDL (Mason and Rainey, 1987). The human body takes its major source of cholesterol from its diet. Both HMGCoA reductase and LDL receptor and LDL cholesterol uptake are stimulated by trophic hormones that stimulate steroidogenisis (Golos *et al.*, 1985; Golos and Strauss, 1988). LDL cholesterol esters are taken up by receptor mediated endocytosis (Brown *et al.*, 1979). The esters may then be stored directly in the cell or converted to free cholesterol for steroid hormone synthesis. Free cholesterol is insoluble in the aqueous cytosol and maybe transported to the mitochondria by a Sterol Carrier Protein 2 (SCP-2) (Chanderbhan *et al.*, 1982; Chanderbhan *et al.*, 1983; Tanaka *et al.*, 1984). The flux of cholesterol across the mitochondrial membranes to the site of the first enzyme in the steroidogenic pathway, P450 side-chain cleavage (scc) enzyme, is stimulated by a 30 amino acid activator peptide, derived from the carboxy terminus of glucose regulatory protein (Pedersen and Brownie, 1983; Pedersen and Brownie, 1987; Ting and Lee, 1988). This peptide has a short half-life and appears to be very sensitive to cycloheximide (Privalle *et al.*, 1983).

Most steroidogenic enzymes are members of the cytochrome P450 group of oxidases (Miller and Levine, 1987; Nebert and Gonzalez, 1987). The generic term cytochrome P450 encompasses a large number of oxidative enzymes, all of which consist of about 500 amino acids and contain a single heme group. They all exhibit a characteristic shift in the Sorbet absorbance peak from 420 to 450nm upon reduction with carbon monoxide, hence the term P450 (pigment 450). Most P450 enzymes can metabolise multiple substrates catalysing a broad array of oxidations. This is also applicable to each steroidogenic P450 enzyme.

There are four distinctive P450 enzymes involved in adrenal steroidgenesis. As previously mentioned P450scc is found in the mitochondria and is the cholesterol side chain cleavage enzyme. P450c11, is also found in mitochondria and mediates 11-hydroxylase, 18-hydroxylase and 18-methyl oxidase activity. P450c17, found in the endoplasmic reticulum mediates both 17α-hydroxylase and 17, 20-lyase activities. P450c21, found in the endoplasmic reticulum mediates the 21-hydroxylation of both

glucocorticoids and mineralcorticoids. P450aro is found in the endoplasmic reticulum of cells in the gonads and in many other cell types mediating the aromatization of androgens and oestrogens.

The P450scc protein is a multimer of 16 subunits totalling over 850,00 daltons and is bound to the inner mitochondrial membrane (Shikita and Hall 1973(a); Shikita and Hall, 1973 (b)). It mediates the conversion of cholesterol to pregnenolone (Halkersten *et al.*, 1961), the first, rate limiting (Stone and Hechter, 1955), and hormonally regulated step in the synthesis of all steroid hormones (DiBlasio *et al.*, 1987; Golos *et al.*, 1987; Picado-Leonard *et al.*, 1988). This involves three distinctive chemical reactions, 20α-hydroxylation, 22-hydroxylation and scission of the cholesterol side chain at the bond between carbon atoms 20 and 22 to yield pregnenolone and isocaproic acid.

Pregnenolone may (see Figure 2) undergo one of two conversions either 17α-hydroxylation to 17-hydroxypregenolone mediated by P450c17 enzyme or converted to progesterone. The enzyme(s) catalysing this reaction is/are non-P450 enzyme(s) bound to the endoplasmic reticulum and mediate 3β-hydroxysteroid dehydrogenase and isomerase (isom) activities. After the synthesis of progesterone, this steroid is hydroxylated at the 21 position to yield deoxycorticosterone. Further hydroxylation mediated by P450c11 yields cortisol. P450c11 also mediates the three final steps in the synthesis of aldosterone from deoxycorticosterone (Yanagibashi *et al.*, 1986).

17-hydroxypregnenolone may undergo scission of the C-17,20 carbon bond to yield dehydroepiandrosterone (DHEA) or 17-hydroxlation to 17α–hydroxyprogesterone. Progesterone can also undergo 17α-hydroxylation to form 17α-hydroxprogesterone and further scission to yield androstenedione. After the synthesis of 17α–hydroxyprogesterone this steroid is hydroxylated at position 21 to yield 11-deoxycortisol, and further 11β-hydroxylation yields cortisol. Androstenedione can be synthesised from DHEA. Andostenedione is converted to testosterone, mediated by 17-ketosteroid reductase, principally in the testes (Kurosumi *et al.*, 1986). Androgens

Steroid Biosynthesis

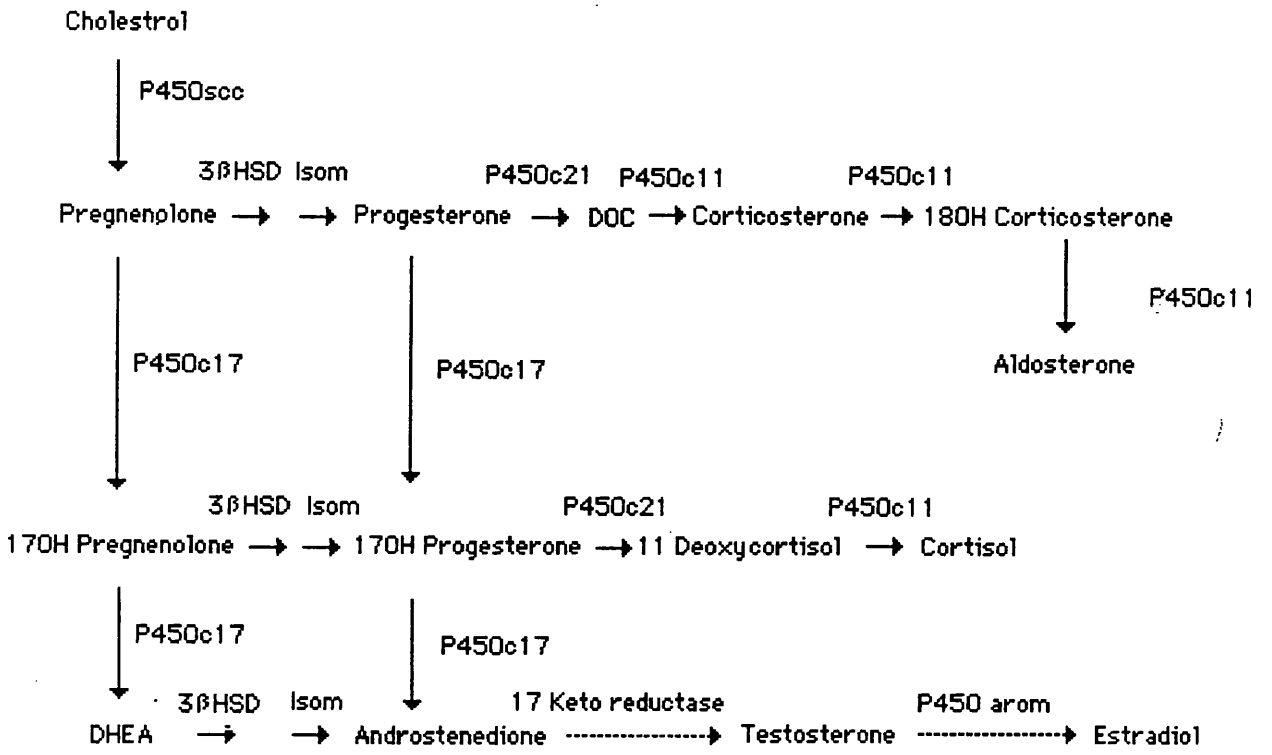


Fig. 2 Principal pathways of human steroid hormone synthesis. Other quantitatively and physiological minor steroids are also produced. Initially the mitochondrial cytochrome P450scc mediates the conversion of cholesterol to pregnenolone. Pregnenolone may undergo two conversions to either 17-Hydroxypregenolone or progesterone. Further hydroxylation of progesterone yields deoxycorticosterone. 17-OH Pregnenolone may undergo scission to yield either dehdroepiandrosterone (DHEA) or 17-hydroxylation to 17α hydroxyprogesterone. Progesterone may undergo further conversion by P450c17 to yield 17α-hydroxyprogesterone and androstenedione. 17α-hydroxyprogesterone may then be hydroxylated to yield 11-deoxycortisol and further hydroxylation yields cortisol. Androstenedione can also be synthesised from DHEA. Andostenedione is converted by a non-P450 enzyme, 17 Keto-reductase (also referred to as 17β steroid dehydrogenase) to testosterone which may then be converted to oestradiol by aromatase.

can also undergo aromatisation to form oestrogen steroids principally in the ovaries, but also in pheripheral adipose tissue and in breast tumour tissue.

2.1.1 Steroid Receptor Superfamily

Steroid hormone receptors mediate steroid action (Jensen *et al.*, 1968), and like many transcription factors they are single polypeptides that organised into discrete functional domains. Receptors have been characterised for each of the steroid hormone families: oestrogen (ER; Green *et al.*, 1986), progesterone (PR; Conneely *et al.*, 1986), glucocorticoid (GR; Hollenberg *et al.*, 1985), androgen (AR; Lubahn *et al.*, 1988), mineralocorticoid (MR; Arriza *et al.*, 1987), vitamin D3 (VD3R; McDonnell *et al.*, 1987) and also, more recently, the receptor superfamily has been shown to include receptors for nonsteroid, seemingly unrelated molecules, such as thyroid hormone (TRs; Weinberger *et al.*, 1986) and retinoic acid (RAR; Giguere *et al.*, 1987). All receptors in the superfamily:

(1) bind hydrophobic ligands that travel through cell membranes freely.

(2) are found intracellularly (usually in the nucleoplasm) and are not associated with cell membranes.

(3) are activated by binding ligand.

(4) achieve high affinity for specific response elements in the DNA, once activated.

(5) probably pass through several stages of phosphorylation.

(6) are associated with heat shock proteins when free of ligand.

(7) bind as dimers to hormone reponse elements which are palindromic in nature, causing gene activation.

These phenomena will be discussed in the following pages of this thesis.

2.1.2 Receptor Synthesis

Steroid receptors are believed to be synthesised on cytoplasmic microsomes from single mRNAs. Immunocytochemical evidence of perinuclear localisation of cytoplasmic receptors suggests that the synthesis of receptors *in vivo* may occur on the outer nuclear membrane, with the resultant protein either transversing the nuclear membranes, co-translationally or very shortly after synthesis (Tuohimaa *et al.*, 1988).

The progesterone receptor is unique among steroid receptors in that it exists as two isoforms, termed A and B. The molecular weights of the human PR isoforms, as deduced from complementary DNA (cDNA) sequence are 98k and 86k respectively. The two proteins are generated by initiation of translation at two in-frame ATGs. Two different oestrogen regulated promoters are responsible for the synthesis of isoform A and B specific human PR mRNA's (Kastner *et al.*, 1990). The two human PR isoforms have been demonstrated to activate transcription differently at target gene promoters (Tora *et al.*, 1988).

2.1.3 Subcellular Localisation of Steroid Receptors

The subcellular localisation of the receptors was initially thought to be cytoplasmic in the absence of hormone and nuclear in its presence (Gorski *et al.*, 1968; Jensen *et al.*, 1968). This hypothesis was based upon cell homogenisation and fractionation studies. However, the availability of specific monoclonal antibodies and immunocytochemical analysis, has shown that the oestrogen (King and Greene, 1984; Press *et al.*, 1989), progesterone (Press *et al.*, 1989; Gasc *et al.*, 1984; Perrot-Applant *et al.*, 1985), and androgen (Husmann *et al.*, 1990; Sar *et al.*, 1990) receptors to be intranuclear even in the absence of their ligands. By contrast the glucocorticoid (Fuxe *et al.*, 1985; Wikstrom *et al.*, 1987) and mineralocorticoid receptors (Farman et al., 1991; Lombes *et al.*, 1990) seem to be either cytoplamic or both cytoplasmic and nuclear in the absence of hormone, and accumulate in the nucleus in its presence (Fuxe *et al.*, 1985). *In vitro* mutagenesis studies of cloned receptors has led to the description of nuclear localisation signals in glucocorticoid (Picard and Yamamoto, 1987), and oestrogen receptors (Guiochon-Mantel *et al.*, 1989). In the case of the progesterone receptor two regions have been identified for nuclear localisation: one is located around position 638-642 and acts in the absence of hormone. The second is located in the steroid binding domain and has very little activity.

2.1.4 Steroid Receptor Comparative Anatomy

Early protein chemical studies of the steroid hormone receptor suggested that they are structurally organised into different domains (Wrangler and Gustaffson 1978; Carlstedt-Duke *et al.*, 1982; Wrange *et al.*, 1984; Carlstedt-Duke *et al.*, 1987). In recent years the results of cDNA cloning of all the major steroid hormone receptors and comparing the deduced amino acid sequence has confirmed this prediction. All receptors thus far analysed are structurally organised into six regions of varying homology denoted A to F (see Figure 3) (Krust *et al.*, 1986). The two most highly conserved regions are C and E which correspond to DNA and ligand binding domains (Evans, 1988). The N-terminal region is variable, and is less well characterised but may have a modulatory effect on transactivation.

The high degree of conserved regions within the cDNA steroid receptor sequences led to the isolation of additional receptor genes. Screening genomic libraries with cDNA under low stringency hybridisation has identified sequences encoding receptors including oncogenes such as v-erb A. It is striking that the hormone family should evolve into a group of receptors that are capable of binding such a wide variety of biologically unrelated ligands.

A comparison of receptor structure and function indicates that the steroid receptor family can be divided into two subfamilies: one including the glucocorticoid, progesterone, androgen and mineralocorticoid receptors and the other including oestrogen, thyroid hormone, retinoic acid and vitamin D3 receptors. Within each subgroup the primary amino acid sequence of the DNA binding domain is much more closely conserved. However, the existence of two subgroups would make it conceivable that a further divergence occurred early in the evolution of the steroid receptor family.

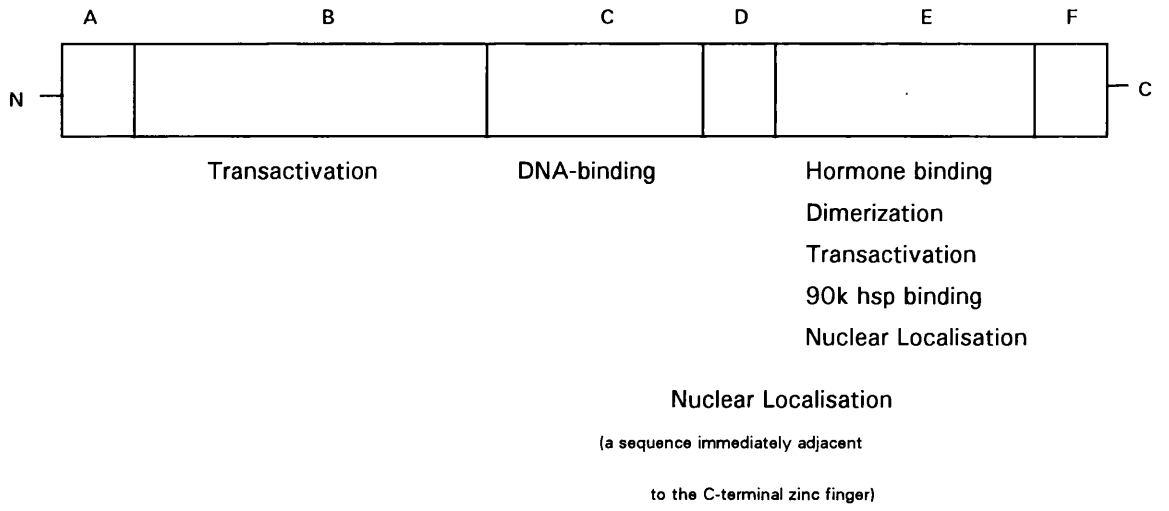


Figure 3. General structural and functional organisation of hormone receptors proteins.

2.1.5 Steroid Receptor Activation

Historically, steroid receptors were found in at least two distinctive forms. The first is a large heteromeric complex consisting of steroid-binding and non-steroid binding subunits with a sedimentation coefficient of 8-10S and was originally extracted in low ionic strength buffers. The second is a smaller unit consisting only of the steroid receptor and bound hormone which has a sedimentation coefficient of 4-5S, (Baulieu, 1987) and was originally extracted from the nuclear pellet using high (0.6M) salt. The 8-10S complex is unable to bind to DNA and has correspondingly been termed "nonactivated". Via the mechanisms referred to as "activation" and "transformation" the 8-10S complex can be converted into the 4-5S unit which can bind DNA and accordingly is termed "activated" (Grody *et al.*, 1982; Sakai and Gorski, 1984). There is some bimodal usage of the terms activation and transformation, however, in the case of the oestrogen receptor and perhaps others, these represent separate processes (Muller *et al.*, 1983). Receptor-activation can be defined as a dissociation of the non-steroid binding subunits from the 8-10S complexes producing a DNA binding receptor (Puri and Toft 1984). Whereas transformation of the oestrogen receptor is seen as a further increase from 4S to 5S to produce an oestrogen receptor species demonstrating slower oestrogen dissociation kinetics (Muller *et al.*, 1983). Further cross linking studies (Muller *et al.*, 1983) suggest that this 5S form of oestrogen receptor consists of a dimer of two steroid-binding subunits.

The inactive 8-10S complex is maintained by an association of the receptor with other proteins. There are several fairly clear examples of receptor associated proteins, one of which is the 90K heat shock protein. Clarification of a receptor: hsp90 complex was provided by several approaches: metabolic labelling studies (Howard and Distelhorst, 1988), yeast mutants (Picard *et al.*, 1990) and chemical cross linking experiments (Rexin *et al.*, 1992). However, while a role of hsp90 receptor state is strongly supported its function remains unclear. With the glucocorticoid receptor, hsp90 binding seems to be required for strong ligand activity, but once hormone

binding has occurred, it can be sustained without hsp90 (Bresnick *et al.*, 1989; Nemoto *et al.*, 1990). This dependence, however, is not a universal relationship. The androgen, oestrogen and progesterone receptors do not require hsp90 for ligand binding activity. Though hsp90 dimers are part of the 8-10S "inactive" complex and may be involved in masking the DNA binding domain until such times as the receptor is activated through ligand binding.

None of the receptor associated proteins so far known are unique to a single receptor or cell type; they all appear to be common cellular proteins that are much more abundant than steroid receptors. For example the ER-related protein hsp-27, appears to be a component of the oestrogen response machinery (King and Coffer, 1988) and is possibly a component of other steroid receptors. Therefore, the association of these proteins with steroid receptors may reflect their general function within a cell. One role often proposed is the efficient maintenance of inactive receptor unable to bind DNA without ligand. They might also serve to inhibit receptor degradation. Other possible functions include, aiding polypeptide chain folding, nuclear translocation, and molecular interactions with other cell signalling systems.

2.1.6 The DNA-Binding Domains Of Steroid Receptors

All steroid receptors analysed contain a cysteine and basic amino acid rich, highly conserved core of 66-68 amino acids (Evans, 1988). Green and Chambon conclusively demonstrated that this region was the DNA binding domain by creating a chimaeric receptor (Green and Chambon, 1987). By replacing this 66-amino acid region of the oestrogen receptor with that from the glucocorticoid receptor, they demonstrated the oestrogen induction of a glucocorticod inducible gene. The amino acid sequence of this region indicated that there are present two zinc-stabilised peptide loop structures. They are commonly referred to as "zinc fingers" and bind to the major groove of the DNA (see Figure 4) (Weinberger *et al.*, 1985; Danielson *et al.*, 1986, Evans and Hollenberg, 1988). This type of region has been shown to exist in a

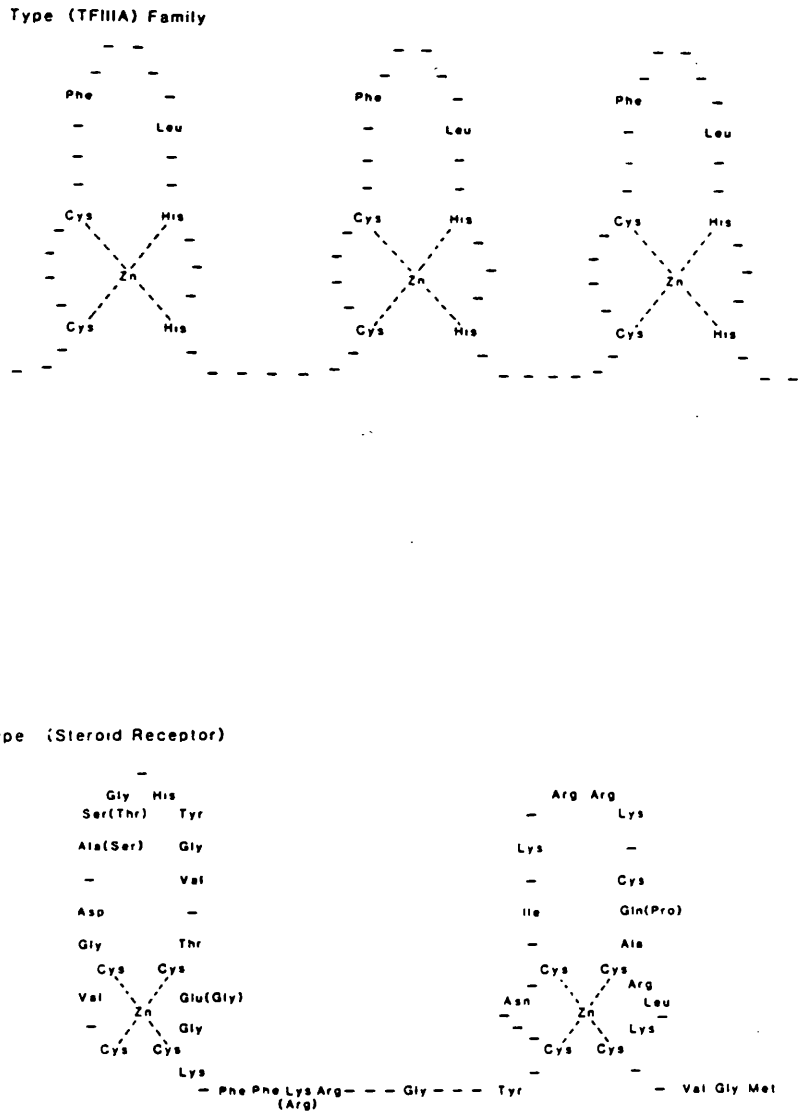


Fig. 4. Comparisons between TFIIIA related transcription factor zinc finger and steroid receptor zinc finger. The 'zinc finger' motif of TFIIA related transcription factors are defined by four metal ligands arranged cys x. -cys x. -His x. -His and three conserved hydrophobic residues. The steroid hormone receptors metal ligands are exclusively cysteine residues, but the spacing between these is similar to that in the TFIIIA related transcription factors.

number of DNA binding proteins and is similar to the folding scheme for the series of nine tandemly repeated sequences found in transcription factor IIIA (TFIIIA). However, TFIIIA forms its zinc fingers by binding a zinc atom with two cysteine and two histidine residues while the hormone receptors bind a zinc atom through 4 cysteine residues. Positive identification of the finger structures co-ordinated by cysteines was reported by Freedman and co-workers for the glucocorticoid receptor using EXAFS spectroscopy(Freedman *et al* .,1988). Two complexes of the glucocorticoid receptor DNA binding domains with two oligonucleotides, differing only in their half-site spacing were characterized using x-ray crystallography (Luisi *et al.,* 1991) The loop of the zinc finger consists of between 12 to 13 amino acids and the two fingers are linked by a region of 15-17 amino acids (Umesono *et al.,* 1989). Each finger is believed to have different function; the first has been demonstrated to be primarily responsible for determining target specificity (Green *et al.*, 1988). The second finger is important for nuclear localisation and probably stabilises the DNA receptor interaction (Picard *et al.*, 1987).

2.1.7 Hormone Response Elements

Steroid hormone receptors transduce extracellular hormonal signals to target genes by their interaction with specific enhancer gene sequences that are referred to as hormone response elements (HRE) (Evans, 1988; Green *et al.*, 1988). Each receptor recognises its own HRE, ensuring that a distinctive response is triggered by different hormones. The HRE's are cis-acting and they function in a relative orientation and position independent manner. Structurally they are related, but functionally they are not. Several reponse elements have been characterised. These include glucocorticoid response elements (GRE), oestrogen response elements (ERE) and thyroid response element (TRE) (see Figure 5).

HRE's are organised into palindromic pairs of hexameric "half-sites". The half sites can differ in sequence, for example two nucleotides per half site differ between GRE

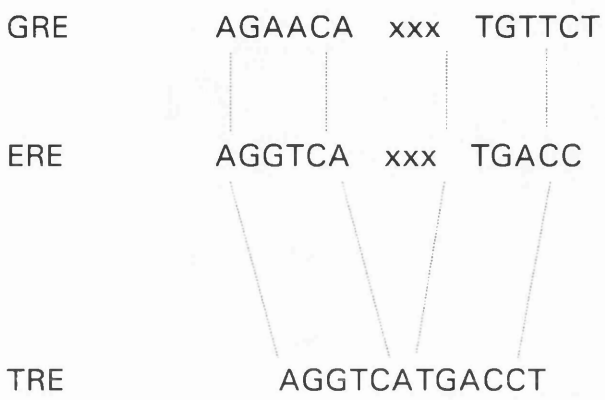


Fig. 5 Comparison of symmetrical DNA response elements. The DNA-binding sites of the glucocorticoid (GRE), oestrogen (ERE), and thyroid (TRE) hormone receptors are shown. The GRE and ERE differ only in two base-pairs in each half site. In contrast, the TRE differs from the ERE in the spacing of the two half sites.

and ERE (Klock *et al.*, 1987) or by the spacing between these sites. TRE and ERE have identical half-sites but differ in their spacing. Therefore, HRE's can diverge by at least two means.

Interestingly, GRE's are typically used not only as glucocorticoid response elements but also as progesterone, androgen and mineralocorticoid response elements, as shown by transcriptional regulatory studies involving these respective receptors (Stahle *et al.*, 1987; Darbre *et al.*, 1986; Cato *et al.*, 1987; Ham and Parker, 1988; Cato and Weihmann, 1988). The oestrogen response element is distinct but closely related to the GRE. In fact only 2bp mutations in both arms of the palindrome is sufficient to convert it to a GRE (Schutz, 1988; Martinez *et al.*, 1987).

2.1.8 DNA Binding Receptor Dimers

Because of the palindromic nature of HRE sequences it has been suggested that hormone receptors can bind to DNA as asymmetrical dimers. Activated GR can form either a homodimer or can be detected as a monomer in solution, absent of DNA (Eriksson *et al.*, 1990). The formation of oestrogen dimers has also been reported (Kumar and Chambon, 1988). The GR binds as a dimer to it's HRE (Perlman *et al.*, 1990). This clearly suggests that an equilibrium exists between GR monomer and dimer and that the GR could bind to the GRE by one of 2 possible mechanisms. Either in a stepwise manner, where a monomer binds to one half-site and strong positive cooperativity results in the binding of the second monomer or as a preformed dimer binding to the HRE.

Kumar and Chambon first showed that the dimerisation of the oestrogen receptor is principally linked to a region within the ligand binding domain (Kumar and Chambon, 1988). Using deletion and site-directed mutagenesis techniques, the dimerisation domain has been sublocalised to the ER residues between 500-540 (Farwell *et al.*, 1990). Comparisons with other members of the receptor superfamily, has detected a heptad repeat of hydrophobic amino acids that could possibly form a

dimerisation interface. Coiled-coil interactions between monomers could conceivably take place within this region allowing for the possibility of homo and heterodimers forming. Receptors containing the hormone binding domain form a more stable complex with the DNA.

Recent studies indicate that the PR exists preferentially in the dimer state and direct analysis of cPR dimerisation has revealed that this process is hormone independent (Rodriguez *et al.*, 1990). However, as yet no dimerisation motif has been found within the PR structure. Due to the PR's somewhat unusual ability, among the steroid hormone class of receptors, to synthesise two different sized polypeptides, analysis has revealed that the PR can bind to the PRE as one of three possible dimeric species, as two homodimer states and as a heterodimer state (Rodriguez *et al.*, 1990; DeMarzo *et al.*, 1991). The ability of the PR to form three dimeric species may provide a mechanism by which receptor molecules with different functional activities could be created. Also the preferential formation of dimers by the PR raises the possibility that the heat shock protein/inactivated steroid receptor complex may inhibit receptor DNA binding indirectly by blocking dimerisation.

2.1.9 The Hinge Region

The DNA binding domain and hormone binding domains are linked by a group of hydrophilic amino acids which are conserved neither in sequence nor in length among the different steroid receptors. There is, however, poor conservation among the ER from different species (Krust *et al.*, 1986; Koike *et al.*, 1987; Weiler *et al.*, 1987). This sequence of amino acids is referred to as a region D within the functional domains of steroid receptors. Region D is believed to act as a "molecular hinge" between the DNA binding domain and the hormone-binding domains during steroid receptor conformational changes (Kumar *et al.*, 1986). Mutagenesis analysis of this region demonstrates that a functional receptor does not require this region to adopt a precise structure (Kumar *et al.*, 1987).

2.2 Transcription Activation

Steroid receptors bind to specific DNA sequences ultimately eliciting specific effects at the transcription level. Transactivation occurs when defined regions of the steroid receptor molecule combine with the DNA binding activity to produce an increase in transcriptional activity. Using deletion mutants and chimeric receptor constructs it has been demonstrated, in PR, GR and ER, that there exist two domains responsible for the transcriptional activation of target genes (Tora *et al.*, 1989; Webster *et al.*, 1988; Meyer *et al.*, 1990). The two autonomous transcription activation functions (TAFs) are located, TAF-1 at the N-terminal region of the receptor, and TAF-2 within the hormone binding domain. The nature of the two TAFs in either ER and PR is unclear.

Initial investigations into the location and the number of TAFs within steroid receptors produced apparently contradictory results (Gronemeyer *et al.*, 1987; Kumar *et al.*, 1986; Godowski *et al.*, 1986). However, systematic studies of the discrepancies observed between different groups are explained by differing parameters in the various experimental protocols (Bocquel *et al.*, 1989). Thus it was demonstrated that the two TAFs could indeed be observed in both the PR and GR when transfection experiments were performed in Hela cells. The subsequent experiments revealed that the hormone binding domains of both the glucocorticoid and progesterone receptor has a repressor type function in the absence of the ligand. Also the relative function of each TAF was shown to be possibly related to cell specificity. Further studies demonstrated that the transcriptional action by TAF-2 in the ER is strongly dependent upon the promoter content. While the activity of TAF-1 is in general less affected by such variations (Berry *et al.*, 1990; Tora *et al.*, 1989).

Cell specific transcriptional activation by steroid receptor is thought not to be limited to specific domains within the steroid receptor but that the presence of

intermediary factors may be necessary. Such protein-protein contacts could occur with either the tata box or even the RNA polymerase II (Ham *et al.*, 1988). Additionally, the introduction of further steps to the nuclear receptor signal transduction pathway could add further combinational possibilities for regulation of transcription of the target gene.

2.2.1 Receptor Phosphorylation

Both enzymatic and non-enzymatic proteins can be regulated by the activities of reversible covalent modifications. Protein phosphorylation-dephosphorylation is a major regulatory process of cellular activity that is controlled by protein kinase and by phosphoprotein phosphatase (Cohen, 1982). All the steroid receptors that have been tested have been found to be phosphoproteins, yet the functional role for steroid receptor phosphorylation/dephosphorylation remains poorly defined. Attempts have been made to link phosphorylation to hormone (Mendal *et al.*, 1987) and receptor turnover and processing (Horwitz and McGuire, 1978). Hormone binding of crude androgen receptor has been shown to be increased by ATP (Goueli *et al.*, 1984). Purified calf intestinal alkaline phosphatase has been shown to inactivate glucocorticoid binding to the glucocorticoid receptor (Nrelson *et al.*, 1977).

Phosphopeptide-mapping studies with different progesterone receptor sources has indicated the presence of multiple phosphorylation sites. Predominately on serine residues (Moudgil, 1990; Sheridan *et al.*, 1989; Sullivan *et al.*, 1988) and this has been confirmed by the identification of some of those sites by amino acid sequencing (Bodwell *et al.*, 1991). The time at which progesterone receptor phosphorylation occurs is thought to be via a multi-step process that raises the possibility that different enzymes may phosphorylate different sites, potentially serving different functions. Initial phosphorylation is thought to occur at the basal level, as a post-translational modification occurring as the receptor matures (Sheridan et al., 1989). This nonactivated form of progesterone receptor becomes complexed with at least two heat

shock proteins, hsp90 and hsp70 (DeMarzo *et al.*, 1991; Kost *et al.*, 1989; Pratt *et al.*, 1990). Once progesterone binds to the progesterone receptor a further round of phosphorylation occurs, possibly decreasing the receptor's affinity for the multiprotein complex. Addition of progestins has been shown to generate hyperphosphorylated forms of the receptor within 5 mins of binding (Sullivan *et al.*, 1988). A third stage of phosphorylation occurs after the progesterone receptor has tightly bound to the DNA. This further act of hyperphosphorylation has been postulated to be complete receptor transformation and facilitate appropriate protein-protein interactions promoting transcription (Takimoto *et al.*, 1992). The steps cited above maybe incomplete. Further stages may occur during a steady progression of phosphorylation rather than set intervals once progestins are bound.

At present the format by which oestrogen receptors are phosphorylated is undetermined and is of need of resolution. A great deal of evidence has been provided over the preceding decade by Auricchio, Migliaccio and co-workers to suggest that the oestrogen receptor is regulated via a tyrosine phosphorylation systems (Auricchio *et al.*, 1990). Experiments using cell free systems suggest that a nuclear phosphatase inactivates the steroid binding capacity of oestrogen receptors and that a cytosolic kinase under the influence of Ca^{+2}-calmodulin (Migiliaccio *et al.*, 1986) restores this activity by phosphorylating tyrosines. Antibodies directed against phosphotyrosine were used to demonstrate that tyrosine phosphorylation also occurs *in vivo,* a reaction that was abolished by treatment with nuclear phosphatase (Migilaccio *et al.*, 1984). Tyrosine phosphorylation has been used to constitute newly synthesised oestrogen receptor to acquire ligand binding activity.

In sharp contrast with these observations but in agreement with other steroid hormones, it has been reported that oestrogen-stimulated phosphorylation of the human or calf oestrogen receptor occurred only on serine residues (Denton *et al.*, 1992; Washburn *et al.*, 1991). In addition studies of phosphorylation of oestrogen receptors in chicken oviduct and mouse uterine cells indicate phosphorylation/dephosphorylation of serine residues (Washburn *et al.*, 1991).

Although it is established that the oestrogen receptor is a phosphoprotein it is still necessary to determine which residues are phosphorylated in vivo, whether tyrosine/serine or both, which proteins mediate site specific phosphorylation and dephosphorylation, and the role it plays in receptor regulation.

General aspects of receptor phosphorylation are beginning to emerge. The receptors are basally phosphorylated in the unliganded form. They become hyperphosphorylated after association with hormone (Sheridan *et al.*, 1989; Sullivan *et al.*, 1988; Sheridan *et al.*, 1988). Residues that are phosphorylated have been identified on the chick progesterone (Bodwell *et al.*, 1991) and mouse glucocorticoid receptors (Denner *et al.*, 1990), most lie in the N-terminal domain. Almost all studies so far conducted point to serine as the main phosphorylated amino acid with minor amounts of threonine (Moudgil, 1990; Sheridan *et al.*, 1989; Hoeck and Groner, 1990; Sullivan *et al.*, 1988). The notable exception is the oestrogen receptor, where physiological phosphorylation of tyrosine residues takes place (Migliaccio *et al.*, 1986).

2.2.2 Regulation of Receptor Synthesis

Regulation of functional protein synthesis can occur at every stage of its production. From the regulation of initiation of gene expression to post-translational modifications. Both oestrogen and progesterone receptor levels in target tissues appear to be under dual hormone control (see Figure 6). Oestrogens have long been recognised as an important regulator of the intracellular content of the progesterone receptor (Clark *et al.*, 1971). It is almost certain that an oestrogen hormone response element exists 5' to the progesterone receptor gene (Ree *et al.*, 1989). Breast epithelial cells grown without oestradiol generally have only low basal levels of progesterone receptor. The notable exception being the cell-line T47D which has high PR protein and RNA levels independent of oestradiol (Berkenstam *et al.*, 1989). The T47D cells probably contain a duplication of chromosome 11 increasing the PR gene copy

number (Horwitz *et al.*, 1985; Law *et al.*, 1987). This, however, does not explain the oestrogen independence. In most PR positive cell lines, exposure of cells to oestrogens causes PR levels to rise and are maintained at new, elevated steady state levels (Horwitz *et al.*, 1978). Withdrawal of oestrogens in the absence of progestins results in a primary rapid decay with a half-life of approximately 2 days, followed by a secondary decay of 21 days. The half-life of empty PR in MCF-7 cells has been shown using dense amino acid containing medium to be approximately 28 hrs (Nardulli *et al.*, 1988). This value is identical in cells in which maximal or half-maximal levels of PR are maintained. Therefore, the increase in cellular PR during oestrogen exposure is not due to a slowing of receptor degradation.

In contrast to oestrogen exposure progestins decrease progesterone receptor levels in target tissues. Using density shift techniques it has been determined that unoccupied PR in the human breast cell-line T47D has a half life of 21 hrs (Nardulli *et al.*, 1988). However, in the presence of the synthetic progestin, R5020, receptor half life has been shown to be reduced to only 6 hrs. Besides an increase in receptor degradation, receptor synthesis was also shown to be markedly decreased. After 30 hrs of maintained exposure of cells to 20nM R5020, no newly synthesised receptor was subsequently detectable on sucrose gradients (Nardulli *et al.*, 1988). Clearly progestin exposure causes dramatic reductions in the level of the receptor present, the extent and the time course of receptor decline being dependent upon the concentration of the progestin. Low levels of progestins reduce receptor levels by a smaller percentage over a longer duration than would high concentrations. Replenishment rates are dependent upon the rate at which each progestin is metabolised by cells. Progesterone is rapidly metabolised by cells (half-life approximately 4 hrs) while synthetic progestins e.g. R5020 can take much longer to be metabolised. Evidence also exists that progestins down regulate PR mRNA through transcriptional inhibition (Wei *et al.*, 1988).

The effects of ligand on receptor turnover has been noted in other systems as well. The presence of ligand has been shown to be involved in the homologous down

regulation of thyroid and glucocorticoid receptor by increasing their rates of degradation (Raaka and Samuals, 1987; Rosewicz *et al.*, 1988). In contrast the presence of ligand upon the androgen receptor elicits a decrease in degradation rate resulting in increased receptor accumulation (Syms *et al.*, 1985).

Progestins have long been known to act as an antiestrogens in terms of growth regulation of both normal and cancer cells *in vitro* (Katzenellenbogen, 1980). It is possible that this inhibitory effect is caused, in part, by suppression of E2- elicited up regulation. It has been reported that progestins used on MCF-7 and T47D cell-lines down regulate the oestrogen receptor (Berkenstam *et al.*, 1989). In T47D cells the addition of the progestin R5020 causes ER message levels to be reduced to approximately 20% of control levels within 2 days of exposure. A reduction in ER protein by progesterone has also been documented in rat uterine (Bhakoo and Katzenellenbogen, 1977) and in hamster uterine cells (MacDonald *et al.*, 1982). Amino acid incorporation density shift experiments have revealed that decidual cells treated with oestrogen plus progesterone elicit a shortened oestrogen receptor half-life and have indicated that progesterone blocks E2- induced ER protein synthesis in these cells (Bahkoo and Katzenellen, 1977; Takeda *et al.*, 1986).

In MCF-7 and T47D cells, it has been shown that oestrogen binding to receptor results in the down regulation of oestrogen binding sites (Berkenstam *et al.*, 1989; Horwitz *et al.*, 1982). The decline in receptor protein to a new steady state level is accompanied by a parallel decreased in the level receptor mRNA (Saceda *et al.*, 1988). In sharp contrast to PR, the turnover of the ER with a half-life of 2.5-4.5 hr is the same in the presence or absence of oestrogen (Eckert *et al.*, 1984; Scholl *et al.*, 1984). Evidence would suggest that the level of mRNA is regulated almost exclusively by a post-transcriptional event (Brock and Shapiro, 1983; Saceda *et al.*, 1989). Both nuclear and cytoplasmic mRNA levels are suppressed when cells are exposed to oestrogens suggesting that the post-transcription suppression of the receptor is a nuclear event (Seceda *et al.*, 1989). Furthermore, it has been demonstrated that certain antiestrogen compounds have no effect on the level of ER mRNA and

effectively block the effect of oestradiol on ER mRNA levels. This would strongly suggest that the classical ER plays a key role in the post transcription regulation of ER mRNA. Inhibition of protein synthesis using cycloheximide has no effect on the suppression of ER mRNA when exposed to oestrogens in MCF-7 cells, at least in a 6 hrs-inhibition (Seceda *et al.*, 1989). This would suggest that the oestrogen-induced, ER mediated effect on the receptor mRNA level, is independent of protein biosynthesis. Steroid hormone receptors have been reported to form complexes with RNA (Chang *et al.*, 1982). Although data suggest that the effect of oestradiol on the level of receptor mRNA is a primary effect of the ER, other possibilities cannot be ruled out such as: the oestrogen receptor mediated effect is through the induction of an RNA species.

Dual Regulation of Oestrogen and Progesterone Receptor Levels

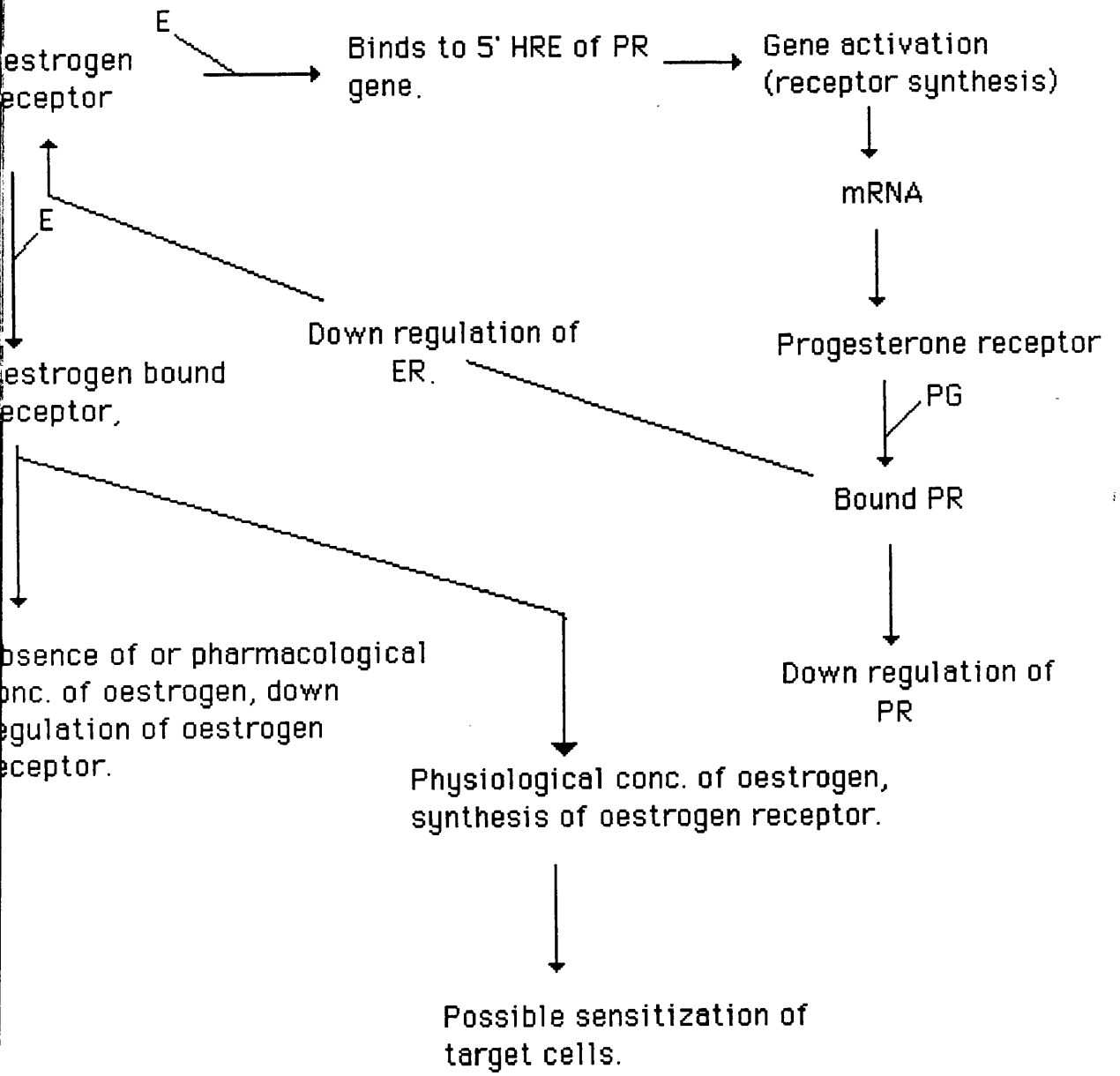


Fig. 6 A model for the dual regulation of oestrogen and progesterone receptor levels. Activated oestrogen receptor binds to the oestrogen hormone response element 5' to the progesterone receptor gene initiating progesterone receptor synthesis. Newly synthesised progesterone receptor binds progesterone causing dramatic reductions in the level of the progesterone receptor. Active progesterone receptor down regulates the oestrogen receptor. In the absence, or at pharmacological concentrations of oestrogens, the oestrogen receptor is down regulated.

3 STEROID RECEPTORS IN ENDOCRINE MANAGEMENT

3.1 Endocrine Management Of Breast

Treatment of primary breast cancer usually involves the surgical removal of all clinically apparent disease. However, it is well established that subclinical metastases are often present before clinical detection of the primary cancer. Therefore, following surgery, appropriate adjuvant systemic treatments such as radiotherapy and chemotherapy are considered. A comprehensive description of these treatments is not within the scope of this thesis, but improved endocrine therapy is directly concerned with this research.

Endocrine therapy is based on the idea that certain tumour cells retain their sensitivity to hormones that regulate function and growth of their normal progenitor cells. Oestrogen is a highly potent mitogenic agent in the normal mammary gland. Endocrine therapy must therefore, aim to negate or even reverse the proliferative effects of oestrogens or other influential hormones. This goal can be achieved in a significant number of patients using either ablative or additive hormone therapy.

Approximately two thirds of all breast cancer patients will not respond to endocrine therapy. However, it has been observed that tumours containing ER and/or PR are more likely to respond to this treatment (Souter and Leake, 1987) : 60% of patients with oestrogen receptor positive tumours and up to 75% of patients with both ER and PR will respond. PR being taken as a measure of active ER. The measurement of steroid receptors can therefore assist physicians in the selection of a treatment for a patient which will provide the best response.

3.2 Ablative Hormone Therapy.

Ablative hormonal therapy is the surgical removal of endocrine organs which are the major sources of oestrogens or their precursors, as adrenalectomy and hyophysectomy in post-menopausal or oophorectomy in pre-menopausal patients. Adrenalectomy and hypophysectomy are seldom practised today, since they carry large surgical risks and major secondary side-effects. However, for premenopausal women oophorectomy is still commonly performed either by surgical or radiation methods, resulting in reponse rates of 31% in non-selected patients (Kardinal 1988). This response rate can be increased to 55% by selecting ER positive patients and to 78% by selecting ER and PR positive patients.

3.3 Adjuvant Therapy

On the assumption that many patients with primary disease may already have micro-metastatic deposits, many studies have been set up to establish whether systemic therapy, given after first surgery, can result in prolonged survival. All eligible studies have recently been the subject of an overview (Peto, 1992). The general conclusion is that adjuvant chemotherapy is a significant advantage for premenopausal patients as is adjuvant endocrine therapy for post-menopausal patients.

3.4 Antiestrogens

Antiestrogen therapy is the hormonal therapy most commonly used to treat breast cancer. Clinically used antiestrogens such as Tamoxifen are referred to as steroid antagonists. Antiestrogens bind to the oestrogen receptor and if present at sufficient concentrations they competitively inhibit the binding of oestrogens, inhibiting their biological activity (Berry *et al.,* 1990). Several studies have found that these steroid antagonists induce changes in the oestrogen receptor that can influence its

physiochemical responses (Rochefort and Borgnal, 1981; Sasson, and Notides, 1982; Pavlik *et al.,* 1985; Pavik *et al.*, 1986; Faye *et al.*, 1986; Hanson, and Gorski, 1986). Further studies have shown that different ligands bound to the oestrogen receptor elicit different conformational changes. Polyclonal antibodies raised against the oestrogen receptor have been shown to inhibit the binding of [^{3}H] oestradiol but not [^{3}H] 4-hydroxytamoxifen (Tate *et al.,* 1984 (a); Tate *et al.,* 1984(b)). Furthermore, it has been discovered that the binding of antiestrogens to the oestrogen receptor exposes an antigenic determinant not recognised when oestrogens are bound (Martin *et al.*, 1988). This site can be exposed even in the presence of oestradiol indicating that antiestrogens may interact with regions separate from the oestrogen binding site. In related studies, the sedimentation characteristics of ligand bound oestrogen receptor complexes has been shown to vary depending on the type of ligand bound, oestrogen or antiestrogen (Eckert and Katzenellenborg, 1982). However, Elliston and Katzenellenbogen found that both types of ligand bound to a similar sequence in the steroid receptor (Elliston and Katzenellenbogen, 1988). Therefore, the distinctive biological activities of oestrogens and antiestrogens cannot be attributed to different ligand binding sites but more than likely to subtle changes in the receptor's conformation.

Current research has focused upon structure-relationships of different ligand oestrogen receptor complexes. Antiestrogens fall into two categories each inducing a response by a different method. The triphenylethylene type form antiestrogen/oestrogen receptor complexes displaying a lesser affinity than oestradiol/ER complexes for target DNA (Evans, *et al.,* 1982), and differ in their interactions with nuclear chromatin (Singh *et al.,* 1986). Recently it has been found that 4-hydroxytamoxifen/receptor complexes bind to oestrogen response elements (Metzer *et al.,* 1988). However, interestingly, the antiestrogen/receptor complexes do not stimulate all transcriptional events (Webster *et al.,* 1988). Therefore, triphenylethylene antiestrogens may exert their response by allowing oestrogen

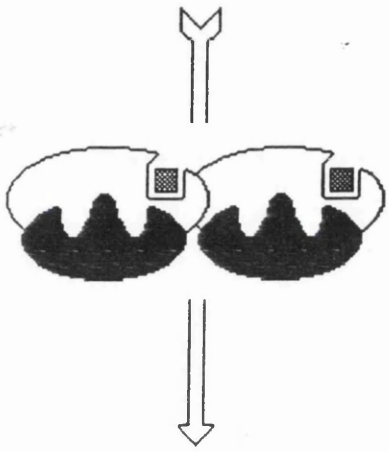
Fig. 7 A Schematic Model of Antiestrogen Action in Breast Cancer Cells. This diagram attempts to summarise the current understanding of antiestrogen action upon the oestrogen receptor in terms of ligand binding, dimerisation and DNA binding.

In this simplified scheme antiestrogens bind to the oestrogen receptor. 'Pure' antiestrogens exert their biological activities by preventing the oestrogen receptor from dimerising and binding to DNA. Triphenylethylene antiestrogens allow the receptor to dimerise and to bind to the DNA. However, these antiestrogen/oestrogen receptor complexes display a lower affinity with hormone response elements and do not stimulate all transcriptional events.

Schematic Model of Antiestrogen Action in Breast Cancer Cells

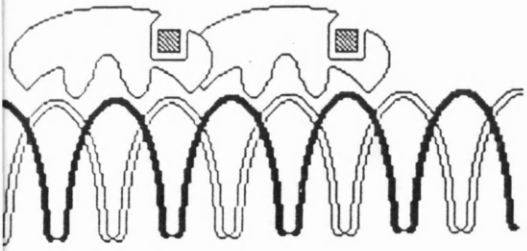
Pure, and triphenylethylene type antiestrogens binds to Steroid Receptors.

Oestrogen inactive 8S Steroid Receptors. Subunits (white) and hsp 90 (black).



Antiestrogen binds to receptor. Clinically used antiestrogens such as tamoxifen allow receptor activation and dimerisation. In contrast pure antiestrogens exert their biological activities by preventing oestrogen receptor dimerisation and DNA binding.

Dissociation of the non-steroid binding subunits results in steroid activation.



Triphenylethylene type antiestrogens (eg. tamoxifen) form antiestrogen /oestrogen receptor complexes. These display a lower affinity for hormone response elements and differ in their interactions with nuclear chromatin. The antiestrogen tamoxifen does no stimulate all transcriptional events.

Receptor dimerisation and DNA binding but fail to stimulate transcription at either of the two transactivation domains (Leese *et al.*, 1989). In contrast, pure antiestrogens are believed to exert their biological activities by preventing oestrogen receptor dimerisation and DNA binding (Fawell *et al.*, 1990) (see Figure 7).

Oestrogens modulate the growth of breast cancer cells by inducing growth factors or their receptors which in turn invoke a stimulatory or mitogenic effect (Leake *et al.*, 1990). Alternatively antiestrogens interfere with the growth factor stimulated proliferation possibly by increasing the production of inhibitory growth factors and decreasing the production of stimulatory growth factors.

The effect of steroids upon the induction of growth factors in breast cancer is the subject of numerous reports. The transforming growth factors α and β have readily been shown to be influenced by the presence or absence of oestrogens. Work by Lippman's group has (Bates *et al.*, 1988) demonstrated that oestrogen stimulation of breast cancer cells results in the elevated secretion of TGF-α, known to stimulate colony formation, while decreasing the levels of growth inhibitor, TGF-β (Knabbe *et al.*, 1987). Antiestrogen treatment reduces TGF-α secretion (Dickson *et al.*, 1986) and induces the release and activation of TGF-β. Another potent mitogen known to be inhibited by antiestrogens is the insulin like growth factor-I (IGF-I). Some proto-oncogenes have also been shown to be down regulated by antiestrogens e.g. c-myc (Le Roy *et al.*, 1991). It is probable that the known number of growth factors affected by antiestrogens will increase as the role of growth factors in response to hormonal treatments is further defined. Oestrogens and antiestrogens elicit their respective responses probably through their interactions with the oestrogen receptor.

It has long been recognised that the effects of antiestrogens are sometimes mediated through mechanisms independent of the oestrogen receptor. Work by Sutherland and co-workers demonstrated that radioladelled Tamoxifen will bind to an "antiestrogen-binding site" (AEBS), later shown to be located in the microsomal fraction of all tissues investigated (Sutherland *et al.*, 1980(a); Sutherland *et al.*, 1980(b); Kon, 1983; Watts and Sutherland, 1986). Interest in AEBS lead to broad

ranging investigations of antiestrogen interactions with other receptors e.g. dopamine D2 receptors (Hiemke *et al.*, 1984) and histamine receptor (Kroeger *et al.*, 1985). However, these receptor systems do not appear to represent the AEBS (Watts and Sutherland, 1987). Development of compounds that bind solely to AEBS will help in defining the role of the AEBS in cellular biochemistry.

Antiestrogen action is not limited to the direct site of the tumour itself. Studies indicate that Tamoxifen might act as a potent antiestrogen at the hypothalamo-pituitary axis, causing a decrease in the mid-cycle peak of prolactin but little or no increase in the concentrations of circulating LH and FSH in premenopausal patients (Groom and Griffiths, 1976; Manni *et al.*, 1979). However, there is an increase in circulation oestrogen (Sherman and Korenman, 1974). In postmenopausal patients Tamoxifen causes a decrease in both LH and FSH, demonstrating mild oestrogenic properties (Golder *et al.*, 1975, Golder *et al.*, 1976). Tamoxifen does not increase circulating oestradiol in postmenopausal patients.

The most commonly used clinical antiestrogen administered to postmenopausal breast cancer patients is Tamoxifen. In general patient populations, Tamoxifen can yield response rates of greater than 30% and in ER positive selective populations this response can go up to 60% to 75%. In randomised trials with postmenopausal patients, Tamoxifen has been compared with other hormonal treatments, oestrogens, androgens, progestins, adrenalectomy and hypophysectomy (Henderson *et al.*, 1987). The response rates for all of these therapies are not significant different, except for androgens that appear inferior. However, Tamoxifen is significantly less toxic. The most common side effects suffered by patients receiving Tamoxifen include nausea and worsening of hot flashes which occur in approximately in 10% of patients. Other less frequently occurring side effects include bone pain and hypercalcemia. Tamoxifen has been shown to induce ovarian steroidogenesis in premenopausal women, elevating oestradiol levels (Manni and Pearson, 1980; Sherman *et al.*, 1979). Despite this, circulating levels of FSH and LH are unchanged from, or only slightly elevated over

the norm. Tamoxifen has been associated with an increased incidence of fibroid ovaries and ovarian cysts (Powles et al., 1989).

3.5 Progestins and Antiprogestins

Progestin antagonists are relatively new compounds that have proven activity in the treatment of breast cancer. The precise mechanism by which both antiprogestins and high dose progestin agonists inhibit growth of breast cancers remains unclear. However, in human breast cancer cell lines high dose progestins exert a direct antiproliferative effect and abolish the stimulatory effect of oestradiol on growth (Allegra and Kiefer, 1985). In contrast antiprogestins are antiproliferative even at low dose and are believed to act directly through the progesterone receptor. Investigators postulate that there are at least two types of antiprogestins: type 1 that block PR binding to PREs; and type 2 that promote PR binding to PREs but impede transcription, echoing a general mechanism of antagonist action proposed by Guiochon-matel and co-workers (Guiochon-mantel *et al.*, 1988). Antiprogestins can also form filled receptor complexes which only down regulate partially, if at all, allowing nuclear receptor levels to remain high for hours if not for days. This is in sharp contrast to PR bound agonists which initiate receptor down regulation approximately an hour after DNA binding.

The actions of a progestin are not limited to the site of the tumour. They may cause oestrogen deprivation indirectly through the suppression of pituitary ACTH secretion resulting in reduced production of adrenal androgen precursors (Mathews *et al.*, 1970). This was demonstrated by the fall in the serum levels of 11-deoxycortisol before and after metyrapone administration (Alexieva-Figusch *et al.*, 1984). Additional endocrinological effects observed with megestrol acetate administration include, suppression of circulating levels of oestradiol, sex hormone globulin and gonadotropins and an increase in basal as well as TRH stimulated plasma progesterone levels.

The overall success of progestin therapy is dependent on the drug entering the target tissue in sufficient quantities to interact with and activate the appropriate numbers of receptors in each cell. Therefore, both plasma concentration of the drug and the blood supply to the tumour influence availability of the drug to the cancer tissue. Additionally, the functional progesterone receptor content of the tumour needs to be sufficiently high to pull in relatively small amounts of the progestin against a concentration gradient. A higher reponse rate to progestin therapy has been observed in women who are more than 5-10 years beyond the menopause (Alexieva-Figusch, *et al.*, 1980; Ansfield *et al.*, 1982). Presumably because the proportion of women with hormone-sensitive disease continues to increase with age.

The two most commonly used progestins that have been used regularly in clinical studies are medroxy-progesterone acetate (MPA) and megestrol acetate (MA). Chemically they differ only slightly in that megestrol has an unsaturated bond between carbons 6 and 7. Megestrol acetate has been found to be effective as a first line therapy and in several randomised trials has shown itself to have similar antitumour actions as Tamoxifen in postmenopausal women with advanced breast cancer. However, when used at conventional doses (< 500mg daily) MPA has been found to be less effective. This difference appears to be due to the increased solubility of MA, relative to MPA in terms of transport across the human intestinal mucosa. This is most readily demonstrated by measuring the plasma concentration of the respective drugs in otherwise equivalent patients.

Clinical studies of megestrol acetate indicate that high dose therapy is significantly superior with regard to response rate than conventional (160mg/day) dosage (Muss *et al.*, 1989; Aisner *et al.*, 1987). However, there are serious side-effects of the higher dosage including weight gain and increased appetite in virtually all patients. These symptoms are of course certainly helpful in treating women with severe weight lose and anorexia, secondary to the advanced stage their disease.

3.6 Other Modalities of therapy

As more biological information is gathered further light is shed upon the effectiveness of antihormones and their mode of action upon regulatory pathways. This has or will provide endocrine treatments directed at pathways not directly related to antiestrogen and progestin therapy.

The oestrogen that drives the growth of a hormone sensitive breast tumours can be the product of adrenal or ovarian synthesis, or it can be synthesised locally. Hence, therapies involved in blocking oestrogens may induce inhibition of HP function, inhibit aromatases and inhibit oestrogen function.

3.7 Systemic Therapy

Progestins and antiestrogens are known to be effective in treating advanced hormone-sensitive breast cancer. However, when used individually over prolonged periods beyond 2 to 3 years they provide little or no benefit (Stoll, 1991) and continuous therapy, no matter how good the initial response, leads to drug resistance and relapse. Long term treatment with antiestrogens usually Tamoxifen, causes the levels of oestrogen receptor and progesterone receptor to fall (Crawford *et al.*, 1987) and in the same way long term action of progestin results in loss of progesterone receptor.

There are two possible approaches to overcoming these mechanisms of resistance. Firstly, regimens can be designed which maintain high receptor levels. Secondly, short bursts of alternative endocrine therapies may delay the onset of resistance to either drug. An approach to the first idea is to use the effects of Tamoxifen to give a short-term increase in PR then follow Tamoxifen with Megestrol Acetate to take advantage of the increased PR levels. Tamoxifen is reintroduced after a period in order to replenish the Megace induced fall of PR. Of course, the potential success of this

cyclical approach also be due to the second idea, as it may delay onset of Tamoxifen resistance.

So far alternating treatment with Tamoxifen and Megestrol acetate (Megace) or medroxyprogesterone acetate have been analysed in clinical trials. A recently published study (Leake *et al.*, 1992) has shown that alternating the treatment with Tamoxifen and Megace gives a significant advantage when compared with Tamoxifen alone. This observation was made in postmenopausal women with locally advanced breast cancer.

4 AIMS

4. Aims

This thesis was based upon a recently published study (Leake *et al.*, 1992) which demonstrated that a cycle of 2 weks of Tamoxifen, followed by three weeks of megestrol acetate (MA), followed by one week of nothing (wash-out) gave a significant survival advantage when compared with the standard treatment of continuous Tamoxifen. This observation was made in post-menopausal women with locally advanced breast cancer. The initial theory behind this study was based on the concept that, when a patient recieves Tamoxifen over a long time peroid, the levels of oestrogen receptor (ER) in her breast tumour cells become reduced. Nevertheless, Tamoxifen, in the short-term, induce synthesis of the progsterone receptor (through its agonist activities). Progestins, on the other hand suppress synthesis of the progesterone receptor (PR). Thus, the cycle might maintain biologically effective levels of both ER and PR and, therefore, prolonging the time peroid over which endocrine therapy is effective. Because of the success of this initial study in prolonging overall survival of advanced breast cancer patients, it would seem reasonable to extend the use of the Tamoxifen-MA cycle to a larger group of breat cancer patients. The only serious side-effect observed in the initial study was the appearance of vaginal bleeding in 14% of patients in the cycle arm (less than 1% in the Tamoxifen alone arm). Norethisterone (NET) is able to abolish postmenopausal vaginal bleeding in many cases. Thus, use of the Tamoxifen-MA cycle would become realisitic in most-menopausal women, if the NET can be shown to abolish the bleeding without influencing the Tamoxifen-induced synthesis of PR.. Thus the aims of this thesis are to establish the full charateristics of Tamoxifen induction of PR, in relation to cell growth rates, and to test whether the sensitivity of PR induction to different levels of NET.

METHODOLOGY

Materials

REAGENT	SOURCE
Anti-PPLO Agent	Gibco BRL Ltd
Bradford Reagent	Bio Rad Ltd
BSA Standard II	Bio Rad Ltd
Charcoal (Norit A Activated)	Sigma
Diethylstiboestrol	Sigma
Dextran	Sigma
Dethiothreitol	Boehring Mannhein GmbH
DMSO	Fisons
DNA (Calf Thymus)	Sigma
EDTA	Bio Rad Ltd
FCS	Gibco BRL Ltd
HEPES	Fisons
Hoeschst 33258	Sigma
ICI 182,172	Zeneca
Megestrol Acetate	Bristol Myers
Norethisterone	Ortho Division of Cilag Ltd
[^{3}H] Oestradiol-17β	Amersham
Oestradiol-17β	Amersham
ORG 2058	Amersham
[^{3}H] ORG 2058	Amersham
RPMI-1640 Medium	Gibco BRL Ltd
Tamoxifen Base/Nolvadex	Zeneca
Trypsin	Gibco BRL Ltd

All other basic chemicals were purchased from BDH.

All plasticware for use in cell culture was supplied by NUNC.

1. Buffers and Solutions

1.1 Buffers.

1.1.1 ETN Buffer

10mM EDTA; 10mM Tris-HCl; 100mM Sodium Chloride; pH 7.0

1.1.2 Hank's Modified Buffer

1.3mM Calcium Chloride; 5.4mM Potassium Chloride; 0.5 mM Magnesium Chloride, 0.5mM Magnesium Sulphate; 137mM Sodium Chloride; 4mM Sodium Hydrogen Carbonate; 0.4mM Sodium Dihydrogen Orthophosphate; pH 7.2-7.4

1.1.3 Dextran Coated Charcoal For Dialysis

100mls sterile PBS-A; 0.25% w/v Norit A charcoal; 0.0025% w/v Dextran.

1.2 Cell Culture Solutions

1.2.1 PBS-A

170mM NaCl; 1mM Na2HPO4; 2mM KH2PO4; pH 7.2

1.2.2 Versene Solution

125mM NaCl; 2.7mM KCl; 6.3mM Na2HPO4; 3.2mM KH2PO4 and 0.5mM EDTA adjusted to pH 7.4 using HCl.

1.2.3 Dialysed Heat Inactivated Dextran Coated Charcoal Stripped Serum

100mls of FCS was dialysed against 4x 1litre changes of Hank's modified buffer over 48hrs at 4°C. Following dialysis the serum was transferred to a glass container and heat inactivated for 45mins at 56°C. The serum was then cooled to 4°C before being added to a pellet of dextran coated charcoal. This solution was allowed to stir at 4°C for 30mins before centrifuging at 10000g for 30mins at 4°C. The supernatant was filtered through 0.2 micron filters.

1.3 Receptor Assay Solutions

1.3.1 HE Buffer

20mM Hepes; 1.5mM EDTA; pH 7.5

1.3.2 HED Buffer

20mM HEPES; 1.5mM EDTA; 0.25mM dithiothreitol (DTT); made in a 15% glycerol solution; pH 7.5

1.3.3 Dextran Coated Charcoal

0.15% w/v Norit A charcoal, 0.0015% w/v dextran T70 in 0.25M sucrose; 1.5mM EDTA; 20mM HEPES; pH 7.4

2. Cell Culture Methods

2.1 Cell Lines

Two established human breast cell lines were used: ZR-75-1 and MCF-7. The ZR-75-1 cells are derived from the malignant ascitic effusion of a metastic breast tumour (Engel *et al* .,1978) and have a doubling time of approximately 14 hours. MCF-7 is a stable epithelioid cell line originally obtained from the pleural effusion of a female patient with breast cancer whose disease responded to endocrine therapy (Horwitz *et al* .,1975) and has a doubling time of approximately 22 hours. Both cell lines are oestrogen receptor and progesterone receptor positive and were maintained in RPMI media, 10% foetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere.

2.2 Routine Cell Culture

2.2.1 Culture Conditions

Both cell lines were routinely subcultured using RPMI 1640 media with L-Glutamine supplemented with 10% foetal calf serum (FCS), penicillin (100 iu/ml)/streptomycin (100 μg/ml) and Anti-pseudo pneumonia-like organism agent (Anti-PPLO agent) (30 μg/ml). Cell medium was changed every two to three days. The majority of routine subculture was carried out using phenol red free RPMI -1640 supplied as a powder, reconstituted as per manufacturers instructions. Supplementation was as above except for FCS which was heat inactivated, dextran coated charcoal-stripped, dialysed FCS (HIDCCFCS) and used as 5% during hormone experiments. Stocks of cells were grown routinely in 175cm^2 plastic tissue culture flasks seeded at a concentration of $4^{x}10^{6}$ cells per flask in 25 mls RPMI 1640 and 10% FCS. Experiments used 6 well plates seeded at a cell density of $4^{x}10^{4}$ cells per

well or 24 well plates seeded at a cell density of 1.7-2.5 $\times 10^4$ cells per well or $175cm^3$ plates (as indicated in the text). Cells were subcultured when the substrate surface area was estimated to be 80-90% confluent.

2.2.2 Drugs and Hormone Supplements Used

Drugs and hormones were dissolved in absolute alcohol to form stock solutions. These solutions could then be added aseptically to phenol red free RPMI 1640 media providing working solutions of minimal alcohol content (never > 1%). (See individual protocols for details of the ligands used and concentrations).

2.3 Management Of Cell Lines

2.3.1 Cryopreservation of Cell lines

Whenever possible frozen stocks of MCF-7 and ZR-75-1 cells were prepared as followed: cells were grown in $175cm^2$ flasks in RPMI 1640 media + 10% FCS until 80-90% confluent. The cells were washed and harvested as described in routine cell culture. The cell suspension was transferred to a sterile universal, centrifuged at 1000g for 2 mins at 4^oC and resuspended in 1ml 90% FCS + 10% DMSO per flask. The cell suspension was transferred to sterile "Biofreeze" vials and frozen overnight at -70^oC before ultimate long term storage under liquid nitrogen.

2.3.2 Recovery of Frozen Cells

Cells were recovered from the -170^oC freezer, thawed rapidly and transferred to a 75 cm^2 flask containing 100mls of prewarmed medium (RPMI 1640 + 10% FCS) and incubated at 37^oC. After 24hrs the cells were washed and fed with fresh medium.

3. DNA and Protein Assays

3.1 Hoechst DNA Assay

3.1.1 Assay Principle

The method used was modified from the original Leake and Habib (1987) protocol. This assay is dependent upon the intercalation of Hoechst dye with solubilised DNA; the resulting complex is fluorescent with a maximum emission at 450nm.

3.1.2 Assay Protocol

Samples were solubilised by incubating for 30mins at 37°C in 0.1% SDS in ETN buffer. 100µl aliquots of each solubilised specimen were transferred to RT-30 test-tubes to which 3 mls of ETN containing Hoechst 33258 (100ng/ml) and RNase (5.0 µg/ml) were added. The tubes were vortexed and incubated at room temperature for 30mins. The fluorescence enhancement at 450 nm was then measured using a Hitachi Perkin-Elmer MPF-2A fluorescent spectrophotometer with an excitation wavelength of 360nm and both slit widths at 5mm. The results obtained were compared against a standard curve range of calf thymus DNA of 0, 5, 10, 15, 20, 25, 30, 40, 50 µg/ml.

3.2 Bradford Protein Assay

3.2.1 Assay Principle

This assay is based on the principle that the absorbance maximum for an acidic solution of Coomassie Brilliant blue G-250 shifts from 465nm to 595nm when binding to protein occurs (Bradford, 1976).

3.2.2 Assay Protocol

The Bradford assay reagent was diluted 1:5 with distilled water and filtered. 100µl of each sample to be assayed was placed in a 10ml glass test-tube to which 5mls of dilute Bradford reagent was added. The tubes were incubated on ice for 5 mins and

the absorbance of each solution measured at 595nm. Where necessary the sample was diluted to within the range of the assay. Protein concentration was read against a standard curve made using bovine serum albumin (BSA) at 20, 40, 60, 80, 100, 120, 140, 200 μg/ml of protein.

4. Steroid Receptor Assays

4.1 Ligand Binding Assays

4.1.1 Assay Principle

These assays can determine the total specific steroid receptors in a variety of tissues and cell monolayers (Leake, 1981). The calculations of Scatchard (Scatchard, 1949) are the basis of this principle: cytosol and nuclear samples are incubated over a concentration range of radioactive hormone with or without a 100-fold excess of unlabelled hormone to determine specific receptor binding. The soluble hormone-receptor complex can be separated from any free hormone in the cytosol fraction using dextran coated charcoal (DCC) to which free steroid is absorbed whereas receptor-steroid complexes are not. Hormone receptor complexes tightly bound to the nuclear fractions are separated from any free hormone by passing the suspension through a filter to which free steroid passes through freely whereas DNA receptor-steroid complexes cannot.

4.1.2 Ligand Binding Assay (LBA) Protocol

Cell monolayers were grown in 175cm^2 flasks until 80% confluent. The cells were washed twice with PBS-A, harvested with a rubber policeman after the addition of 2mls of HED buffer to the flask. The cell suspension was transferred to a sterile universal and cells lysis occurred by fast freezing for 5mins in -70^{o}C freezer, followed by fast thawing at 37^{o}C for 10mins. The sample was checked by microscope for >95% lysis before centrifuging at 1000g for 5mins. The nuclear pellet was resuspended in 2mls of HED buffer while the cytostolic supernatant was spun at 18,000 rpm at 4^{o}C for 30 mins, removing the membrane fraction. 150µl aliquots of each suspension was added to 50µl of each steroid radioactive standard. Each suspension was incubated for 18hrs at 4^{o}C (overnight) to allow steroid binding to take place.

4.1.3 Analysis of the Cytostolic Fraction

After incubation, to each cytosol suspension, 200µl of DCC and HED was added, to remove unliganded steroid. This treatment continued on ice for 15min, vortexing twice during this period, before centrifuging for 5mins at 1000g to pellet the DCC absorbed steroid. 200µl aliquots of each suspension were transferred to scintillation vials to which 4mls of Ecoscint A was added. The vials were counted for tritium each for 1min and the cpm used to carry out computerised scatchard calculations.

4.1.4 Analysis of the Nuclear Fraction

100µl aliquots of each nuclear suspension was added to 5ml aliquots of 0.9% buffered NaCl immediately prior to pouring on a prewetted whatman Gf/c filter disc (2.5cm) held in a millipore filter apparatus. The test-tube and chimney of the apparatus were washed with 3x4ml aliquots of saline. The filters were transferred to scintillation vials to which 4mls of Ecosint A was added. The vials were counted for tritium each for 1min and the cpm used to carry out computerised scatchard calculations.

4.1.5 Steroid Standards

Ten radioactive steroid standards were prepared, seven radiolabelled standards within the stated range (0.2-3.0nM of [^{3}H] oestradiol-17β (ER assay) and 0.5-5.0 nM of [^{3}H]-Org 2058 (PR assay)) (standards 1-7) plus an additional three duplicate standards of the highest three concentrations with a 100-fold excess of non-labelled competitor (standards, 8,9 &10). First unlabelled steroid was added to tubes 8, 9 and 10 and then the solvent was evaporated using compressed air. To each vial, appropriate volumes of tritiated steroid, ethanol and HED buffer were added to a volume of 1ml. Aliquots of 50µl of each standard were counted to obtain a value of the "totals". The cpm were used to carry out computerised Scatchard calculations. Scatchard analysis (Scatchard, 1949) of ligand binding assay data was routinely carried out using a BBC microcomputer program as described by Leake *et al.*, (1987). The program calculates the mean non-specific binding correction factor from assay samples 8-10. This mean value is then used to calculate the component of the counts in samples 1-7 which represents specific binding. A saturation curve is plotted and then analysed by the methods of Scatchard.

The concentrations and ligands used for each receptor assay standard were as follows.

- For ER assay: 0.2, 0.3, 0.5, 0.75, 1.5, 2.0, & 3.0 nM final concentration [^{3}H]-oestradiol-17β was used as the radiolabel with a 100-fold excess of diethylstilboestrol (DES) as the competitor in the highest three concentrations. The [^{3}H]-oestradiol-17β had a specific activity of 55.4 Ci/mmol. (HE buffer replaces HED buffer in the ER assay).

- For PR assay: 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, & 5.0 nM final concentration [^{3}H]-Org 2058 was used as the radiolabel with a 100-fold excess of cold Org 2058 as the competitor in the highest three concentrations. The [^{3}H]-Org 2058 had a specific activity of 52 Ci/mmol.

4.1.6 Progesterone "Two-point competition" Steroid Receptor Assay

The ten point LBA of cell culture extracts were initially used to assay the progesterone receptor content of cells exposed to drug, and hormone supplements grown in 175cm^2 flasks. However, these experiments were very difficult to perform because the cells would not always continue to grow once the supplement had been added. Attempts were made to alter the initial seedings numbers of cells before adding the supplements in the hope that a large enough number of cells could be maintained for the duration of the experiments. This, however, proved fruitless. Over a peroid of four months the experiments were only completed with a limited number of flasks. Due to this lack of success, the abbreviated two-point assay was used. This relies upon straightforward competition between tritated ligand and excess unlabelled competitor (Leake and Habib 1987). The experiments were carried out in triplicate, per experiment, in 6 well tissue culture plates. The assay involves incubating the cells with 2mls of medium containing either tritiated Org 2058 ($1^{x}10^{-9}$M) (1) or tritiated Org 2058 ($1^{x}10^{-9}$M) plus excess cold Org 2058 ($1^{x}10^{-7}$M) (2) for 30mins at 37^{o}C. The cells were washed 3 times with PBS-A to remove all tritiated media and the cells were incubate for 15mins at 4^{o}C after the addition of 1ml of absolute alcohol. The alcohol was transferred to scintillation vials to which 4mls of Ecosint A was added. The vials were counted for tritium, each for 1min and the concentration of bound steroid was determined by subtracting (2) from (1). The counts per minute were then converted to the number of moles of bound steroid per litre. This was achieved by converting the cpm into cpm per litre and then into the disintegrations per minute. This gave the number of μCi and by knowing the number of Ci per mole of stock it was possible to calculate number of mole of bound steroid per litre. So that samples could be compared, the total number of moles of bound steroid was converted into the number of femtamoles per μg of DNA. Because of problems with DNA recovery of cells harvested (alcohol extracted) for receptor content, DNA content was determined

for cells in parallel wells. DNA content was determined using the Hoechst assay as descibed in Methodology section 3.1..

EXPERIMENTAL

1 Oestrogen and Progesterone Receptor Analysis of ZR-75 and MCF-7 Cells

1.1 INTRODUCTION

The long-term objective of this study was to assess the *in vitro* regulation of the progesterone receptor. In particular, it was of interest to establish whether the minimum gynaecologically-effective dose of Norethisterone (approx. 10^{-9}M) had any influence on the effect of Tamoxifen plus/minus Megace on the synthesis of the progesterone receptor. Initially it was necessary to quantitate the normal content of oestrogen and progesterone receptor in the human breast cell lines ZR-75 and MCF-7 grown under normal cell culture conditions in routine RPMI medium. Quality control samples were also assayed for PR and ER content to establish the accuracy and reproducibility of the assay.

1.2 METHOD

ZR-75 and MCF-7 cells were cultured routinely in 175cm^2 plastic tissue culture flasks as described in METHODOLOGY section 2.2. When the cells reached about an 80% confluent cell monolayer, cells were washed with PBS-A and 2mls of the appropriate buffer for either the oestrogen or progesterone receptor assay was added. The cells were harvested with a rubber policeman and transferred into sterile universals. The cell suspension was then analysed for either oestrogen or progesterone receptor content as described in METHODOLOGY section 4.1.2. Aliquots (100µl) of either cytosol or nuclear fractions were assayed for protein using the Bradford Protein assay or DNA using the Hoeschst DNA assay described in METHODOLOGY section 3.2 and 3.1 respectively. Lyophilised quality control samples were resuspended in a 10% glycerol deionised H_20 solution. These samples were then treated as cytostolic fractions for steroid receptor analysis. 100µl aliquots of the suspension were assayed for protein content.

1.3 RESULTS

The results described in TABLE 1 are for quality control material provided by the EORTC Receptor Group and represent the means of 3 separate experiments, each experiment consisting of triplicate ER or PR assay so that the values represent the mean of 9 assays. The mean results published by the EORTC Receptor study group are also shown. The results in TABLE 1 also describes the total content of PR and ER found in ZR-75 and MCF-7 cells. The result as shown in FIGURES 8 and 9 represent the mean of four experiments for each cell line, each experiment consisting of cells from one 175cm^2 plastic tissue flask. Receptor content is expressed as: cytosolic fraction, femtomoles per mg of protein and nuclear fraction, femtomoles per μg of DNA in FIGURE 8. In FIGURE 9 the nuclear and cytosol values are expressed as femtomoles per μg of DNA, allowing comparisons to be made between the relative distribution of ER and PR in the cell lines.

TABLE 1

Oestrogen And Progesterone Receptor Analysis of Quality Control Sample 1991-A

Steroid Receptor Assay	Receptor Content (fmol/mg protein)	Std Devn	Kd ($x10^{-10}M$)	Std Devn	No. Of Expts
ER	4.94	2.35	5.35	2.14	3
PR	86.63	6.61	7.74	1.03	3

EORTC RECEPTOR STUDY GROUP QUALITY CONTROL FIGURES

	Mean	St. Devn.	
ER	18.60	8.40	95
PR	106.70	54.70	95

The Total Concentration of ER and PR Determined by the Ligand Binding Assay in MCF -7 and ZR-75 Cells

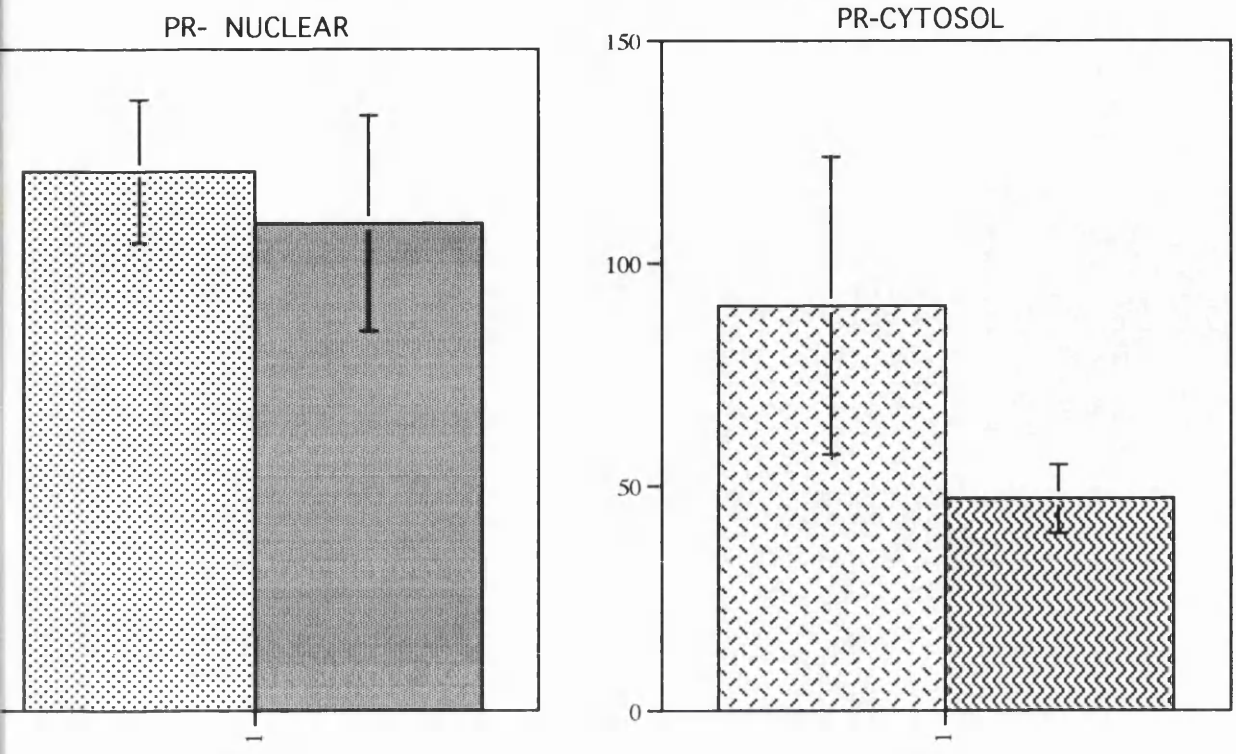
Steroid Receptor Assay	Receptor Content (fmol/µg of DNA)	St. Devn	No. Of Expts
MCF-7 Cells			
ER	2.41	0.63	4
PR	13.56	1.64	4
ZR-75 Cells			
ER	6.83	1.50	4
PR	9.92	1.96	4

Fig.8 Oestrogen and progesterone receptor analysis of ZR-75 and MCF-7 cells grown in routine medium. Both cell lines were grown in separate 175cm^2 plastic tissue culture flasks in routine RPMI medium until the cells reached about an 80% confluent cell monolayer formed. The cells were harvested and the ER and PR content was measured as described in METHODOLOGY section 4.1.2.

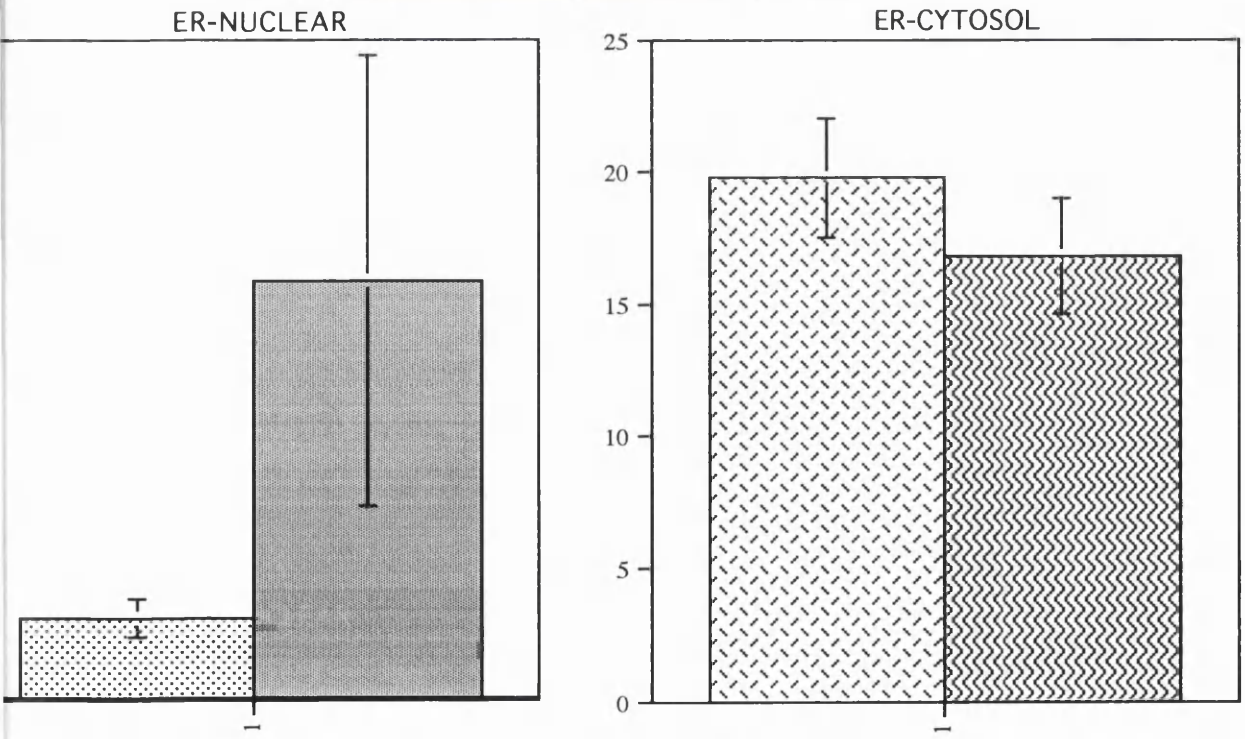
Error bars represent the Standard Deviation of the Data.

Oestrogen and Progesterone Receptor Analysis of ZR-75 and MCF-7 cells

Progesterone Receptor Ligand Binding Assay



Oestrogen Receptor Ligand Binding Assay



MCF-7 (NUCLEAR -fmol per ug of DNA)

ZR-75 (NUCLEAR -fmol per ug of DNA)

MCF-7 (CYTOSOL- fmol per mg of protein)

ZR-75 (CYTOSOL- fmol per mg of protein)

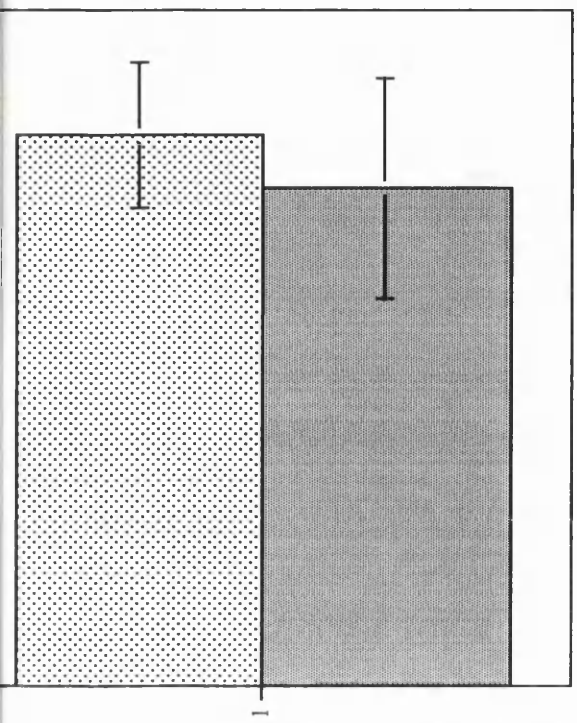
Fig. 9 Oestrogen and progesterone receptor analysis of ZR-75 and MCF-7 cells grown in routine medium. Both cell lines were grown in separate 175cm^2 plastic tissue culture flasks in routine RPMI medium until the cells reached about an 80% confluent cell monolayer formed. The cells were harvested and the ER and PR content was measured as described in METHODOLOGY section 4.1.2. The nuclear and cytosol values are expressed as femtomoles per μg of DNA.

Error bars represent the Standard Deviation of the Data.

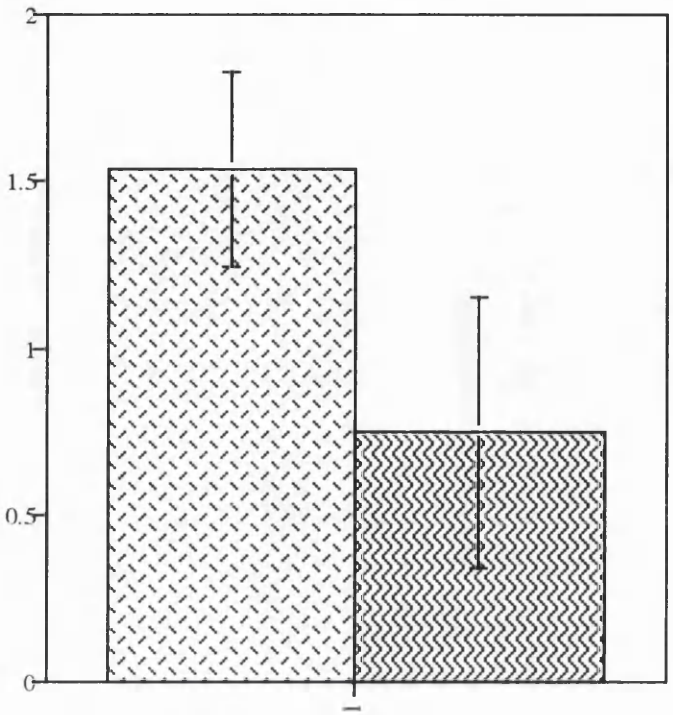
Oestrogen and Progesterone Receptor Analysis of ZR-75 And MCF-7 Cells

Progesterone Receptor Ligand Binding Assay

PR - NUCLEAR

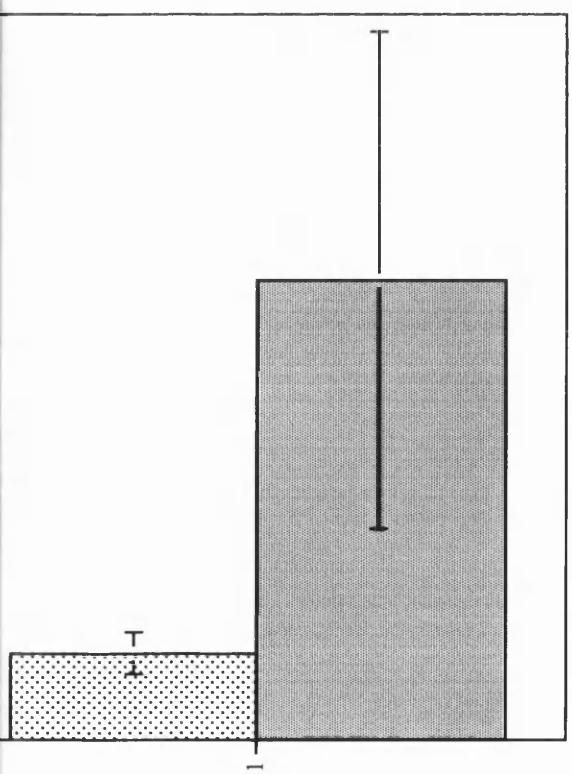


PR - CYTOSOL

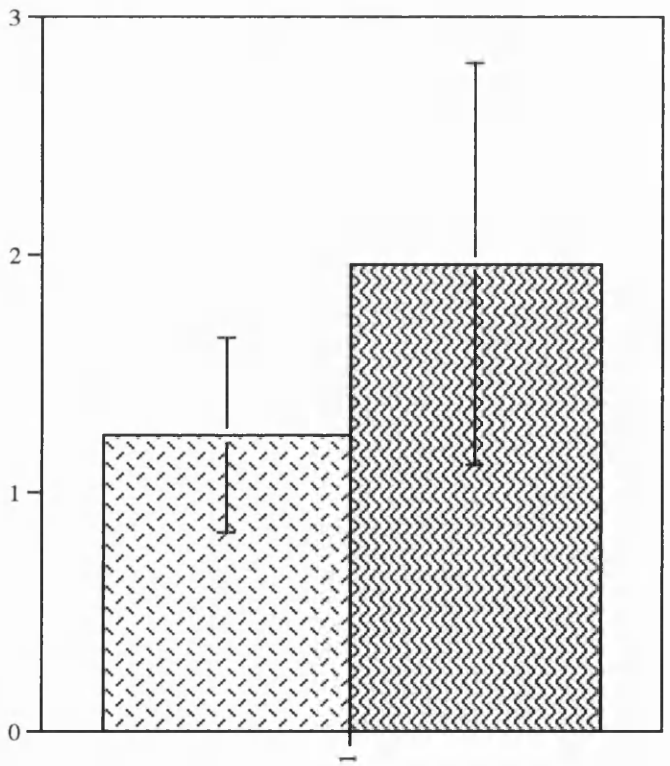


Oestrogen Receptor Ligand Binding Assay

ER - NUCLEAR



ER - CYTOSOL



MCF-7 (NUCLEAR -fmol per ug of DNA)

ZR-75 (NUCLEAR -fmol per ug of DNA)

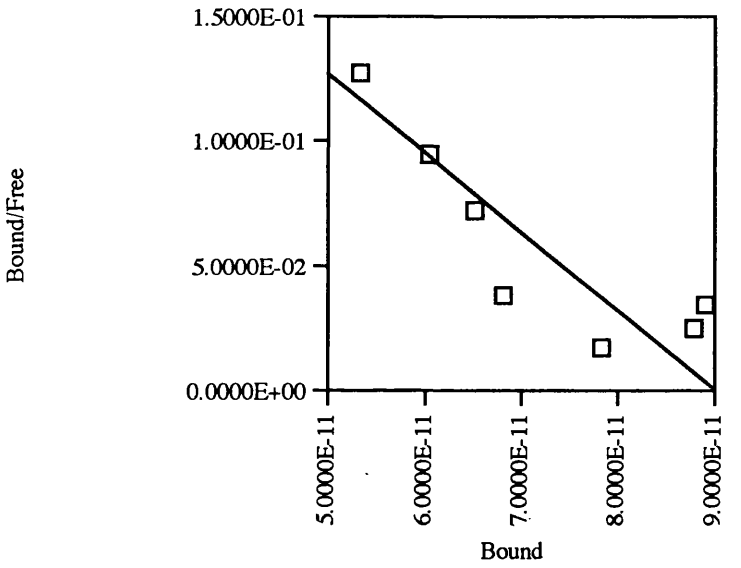
MCF-7 (CYTOSOL- fmol per ug of DNA)

ZR-75 (CYTOSOL- fmol per ug of DNA)

Fig. 10 Representative Scatchard Analysis of Ligand Binding Assay Data. The total concentration of receptor was determined from the total number of radioactive counts per minute in each fraction by Scatchard analysis (Scatchard, 1949). The Scatchard calculations were carried out using a BBC microcomputer program. A minimum of four points were selected to generate each plot (Leake *et al.*, 1987).

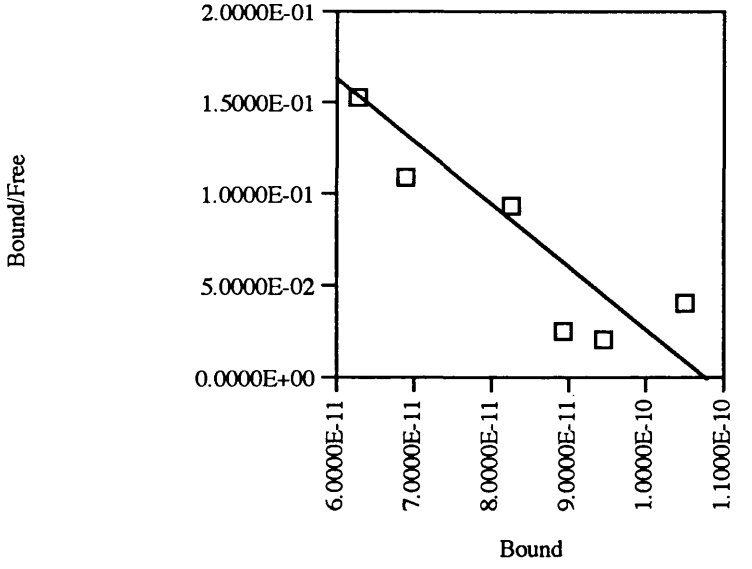
Scatchard Analysis Of Ligand Binding Data

A Representative Scatchard Plot of the Oestrgoen Ligand Binding Assay Of MCF-7 Cells



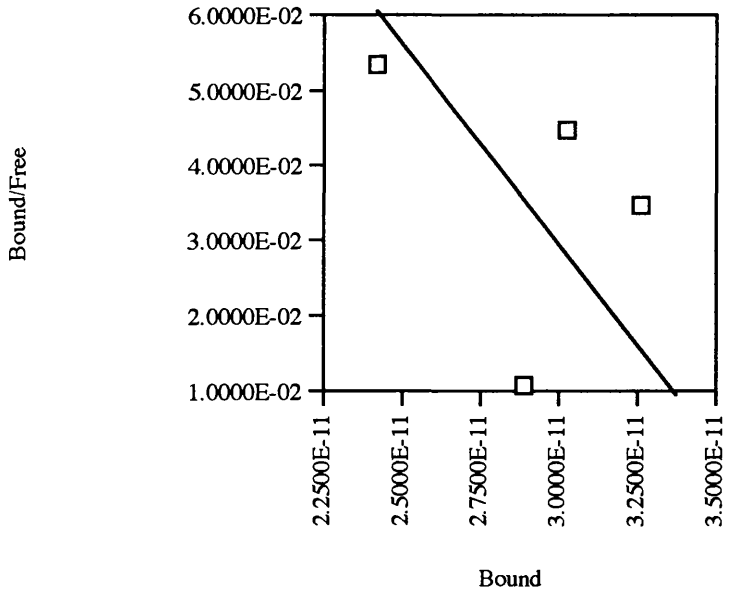
Kd = 3.850x10-10M

A Representative Scatchard Plot of the Progesterone Ligand Binding Assay Of ZR-75 Cells



Kd = 3.366x10-10M

A Representative Scatchard Plot of the Oestrogen Ligand Binding Assay of EORTC Samples



Kd = 5.048x10-10M

1.4 CONCLUSION

There is a large difference in the concentration of ER published by the EORTC Receptor Study Group and those found in this study. This difference is probably due to the age of the Quality Control sample used. Over duration between storage (approx. 2 years) and use, it is likely that some of the ER in the sample has degraded. The concentration of PR cited in this study for the qualtiy control sample is well within the range quoted by EORTC. Therefore, this demonstrates that the ligand binding assay for either ER or PR is reproducible, though absolute accuracy of ER was not confirmed.

As can be seen from FIGURE 8 both cell lines are oestrogen and progesterone receptor positive. MCF-7 and ZR-75 cells (TABLE 1 and FIGURE 9) in their total concentration of ER and PR but demonstrated equivalent distribution of each receptor. The MCF-7 cells produce a higher concentration of that PR than ZR-75 cells which contains higher levels of ER. The distribution of PR is favoured towards the nuclear region of each cell line while the ER receptor demonstrates an ability to be evenly distributed thought the cells.

2 Progesterone Receptor Induction By Tamoxifen In ZR-75 Cells

2.1 INTRODUCTION

Having established that PR content could be reliably measured it was necessary to determine the effects of Tamoxifen on the induction of PR in ZR-75 cells during 96 hours of exposure. The concentration of Tamoxifen used throughout this study was that which previous experiments in our laboratory had been shown to be effective in breast cancer cells *in vitro*.

2.2 METHOD

ZR-75 cells were cultured routinely in 175cm^2 plastic tissue culture flasks until the cell monolayer was between 80-90% confluent. The cells were harvested using trypsin/versene solution and reseeded into ten 6-well plastic tissue culture plates at a cell density of 7.5 to 8.5x10^4 cells per well. The cells were allowed to "plate down" for 24 hours. The cells were washed with PBS-A and five 6-well plastic tissue plates were fed with phenol red free medium with 5% HIDCCFS plus Tamoxifen (1x10^{-9}M) or ethanol at a volume equivalent to the supplement (as the control). Every 24 hours starting from time zero for 96hours, one 6 well plastic tissue plate was selected from each medium to determine the PR content using the progesterone two point competition steroid assay as described in METHODOLGY section 4.1. Four wells were used for the progesterone receptor analysis and from the other two wells the cells were harvested using trypsin/versene solution and the cell suspension was transferred to eppendorf. 200µl of ETN buffer containing 0.1% SDS was added to each eppendorf and incubated for 30 mins at 37°C. Aliquots (100µl) of solubilised cells were assayed for DNA using the Hoeschst DNA assay as described in METHODOLGY section 3.1.

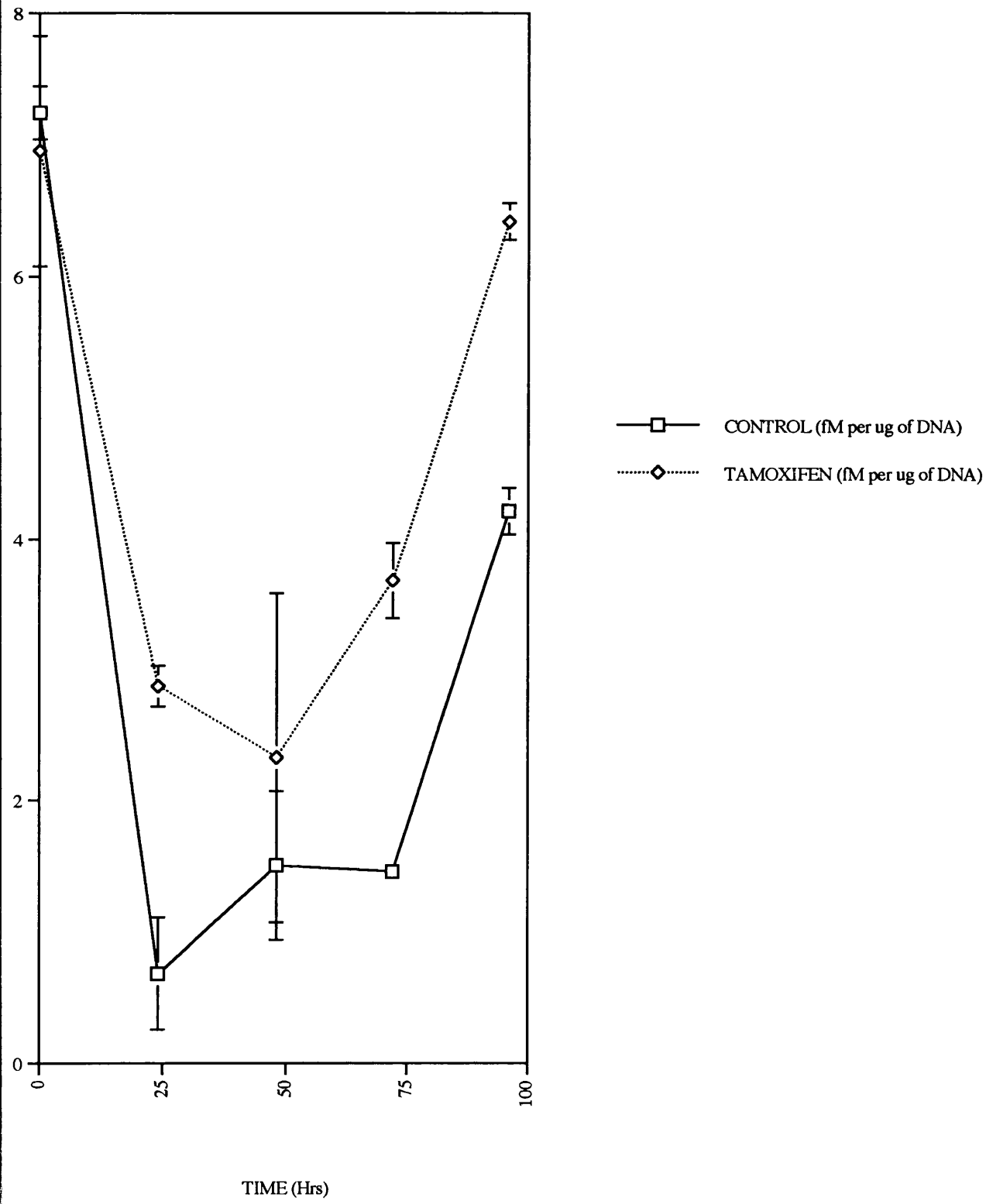
2.3 RESULTS

FIGURE 11 shows the time course of Tamoxifen regulation of PR synthesis. Each data point is the mean of 3 separate determinations. The parameter being measured is progesterone receptor content and so the results are expressed as femtomoles per μg of DNA.

Fig.11 Time course of Tamoxifen induction of PR in ZR-75 cells. ZR-75 cells grown in $175cm^2$ plates were reseeded into 6 well plates at a density of 7.5 to $8.5x10^4$ cells/well before the addition of Tamoxifen ($1x10^{-9}M$) or vehicle. The cells were then incubated for up to 96hrs at 37^oC and the media was changed after 2 days. Wells were selected every 24hours to measure PR content using a two point receptor assay as described in METHODOLOGY section 4.1. Each data point represents PR content expressed as femtomoles per μg of DNA.

Error bars represent the standard Deviation of the Data.

Progesterone Receptor Induction in ZR-75 cells



2.4 CONCLUSION

FIGURE 11 demonstrates the effects of Tamoxifen and the control on the induction of PR in ZR-75 cells. What is interesting is that the PR levels should fall so dramatically after the initial introduction of the new medium. Possibly this could be due to a trauma response induced by the changes from one type of media to another. After 48 hours exposure to the new media, Tamoxifen treated cells start to show induction of the PR. Future experiments would therefore need to have a duration longer than 48 hours, allowing the effects of the supplements on PR synthesis to be observed. Interestingly this phenomenon can also be observed in those cells treated as the control after 72 hours. The control media contained no supplement that should have been able to induce PR. Therefore, the most likely explanation is that the cells were starting to become over confluent. However, initial work demonstrated that the cells tended to become over confluent some time between 96 hours and 120 hours in 6 well plates when seeded at the cell density described in this method section. When the cells reached a confluent state it had been observed that they synthesised higher levels of PR than when they are growing normally (observations of R. E. Leake and co-workers). This would imply that the duration of further experiments studying the role of the PR needs to start 24 hours after the seeding of the wells, and needs to be longer than 48 hours after the initial introduction of the supplement, but no longer than 96 hours when cells become over confluent.

3 The Effects of Drug And Hormone Treatments on Progesterone Receptor Content In ZR-75 cells.

3.1 INTRODUCTION

Studies were developed to determine the influence of oestrogens and antiestrogens on PR content. On the basis of experiment 2.3 the experiments were designed so that the cells were exposed to a supplement for 96hours at 37°C. The cells progesterone receptor content was measured by using the progesterone two point competition steroid receptor assay.

3.2 METHOD

ZR-75 cells were cultured routinely in 175cm^2 plastic tissue culture flasks until the cell monolayer was between 80-90% confluent. The cells were harvested using trypsin/versene solution and reseeded into 6-well plastic tissue culture plates at a cell density of 7.5 to 8.5 x10^4 cells per well. The cells were allowed to plate down for 24hours. The cells were washed with PBS-A and each plastic tissue plate was fed with phenol red free medium with 5% HIDCCFS plus ethanol to an equal volume as the supplements (as the control) or a hormone supplement; Oestradiol (1x10^{-9}M and 3x10^{-9}M), Tamoxifen (1x10^{-9}M) or ICI 182,780 (1x10^{-9}M) was added. After 48hours each set of plates was washed with PBS-A and fed. After 96 hours the cells were washed with PBS-A and then assayed for PR using the progesterone two point competition steroid receptor assay described in METHODOLOGY section 4.1.

The concentrations of Oestradiol used throughout this study are equivalent to the range of Oestradiol found in the plasma of pre-menopausal women. The antiestrogen ICI 182,780 has not been administered to patients, but the level chosen for this supplement is equivalent to that for Oestradiol because the affinity of ICI 182,780 for ER is similar to that of E_2.

3.3 RESULTS

The results shown in FIGURE 12 represent the mean of 3 experiments, each experiment consisting of 3 samples for each medium used. The parameter being measured is the progesterone receptor content, so the results are expressed as the concentration of bound receptor as a percentage of the control. The results described in TABLE 2 represent the above data illustrated as the progesterone receptor content expressed as fmol/μg DNA of the original cell pellet. Because of problems with DNA recovery of cells harvested (alcohol extracted) for receptor content, DNA content was determined for cells grown in parallel wells. (See EXPERIMENTAL section 13.3 - TABLE 6). Variation in DNA content amongst wells (for identical growth conditions) had been previously demonstrated to be less than 10%.

TABLE 2

Progesterone Receptor Analysis Of ZR-75 Cells Exposed to Hormone and Drug Supplements.

Hormone or Drug Supplement	Receptor Content (fmol/mg of DNA)	ST Devn	No. Of Expts
Control	0.479	0.036	3
Oestradiol ($1x10^{-9}M$)	0.688	0.342	3
Oestradiol ($3x10^{-9}M$)	0.641	0.368	3
Tamoxifen ($1x10^{-9}M$)	0.568	0.079	3
ICI 182,780 (1x10-9M)	0.423	0.046	3

Fig.12 Effects of Oestradiol, Tamoxifen and ICI 182,780 on progesterone receptor concentration in ZR-75 cells. ZR-75 cells grown in 150 cm^2 plates were reseeded into 6 well plates at a density of 7.5 to $8.5^{x}10^{4}$ cells/well before the addition of compounds. Oestradiol ($1^{x}10^{-9}$M and $3^{x}10^{-9}$M), Tamoxifen ($1^{x}10^{-9}$M), ICI 182,780 ($1^{x}10^{-9}$M), or vehicle were incubated for up to 96hrs at 37^{o}C. Media was changed after 2 days. Progesterone receptor concentration was measured using a two point progesterone receptor assay as described in METHODOLGY section 4.1.

The 't' test was used to measure the difference between the PR content of cells exposed to a supplement and the control cells and values are given below for each condition.

(1) $P < 0.2$, comparing Oestradiol ($1x10^{-9}$M) to the control.

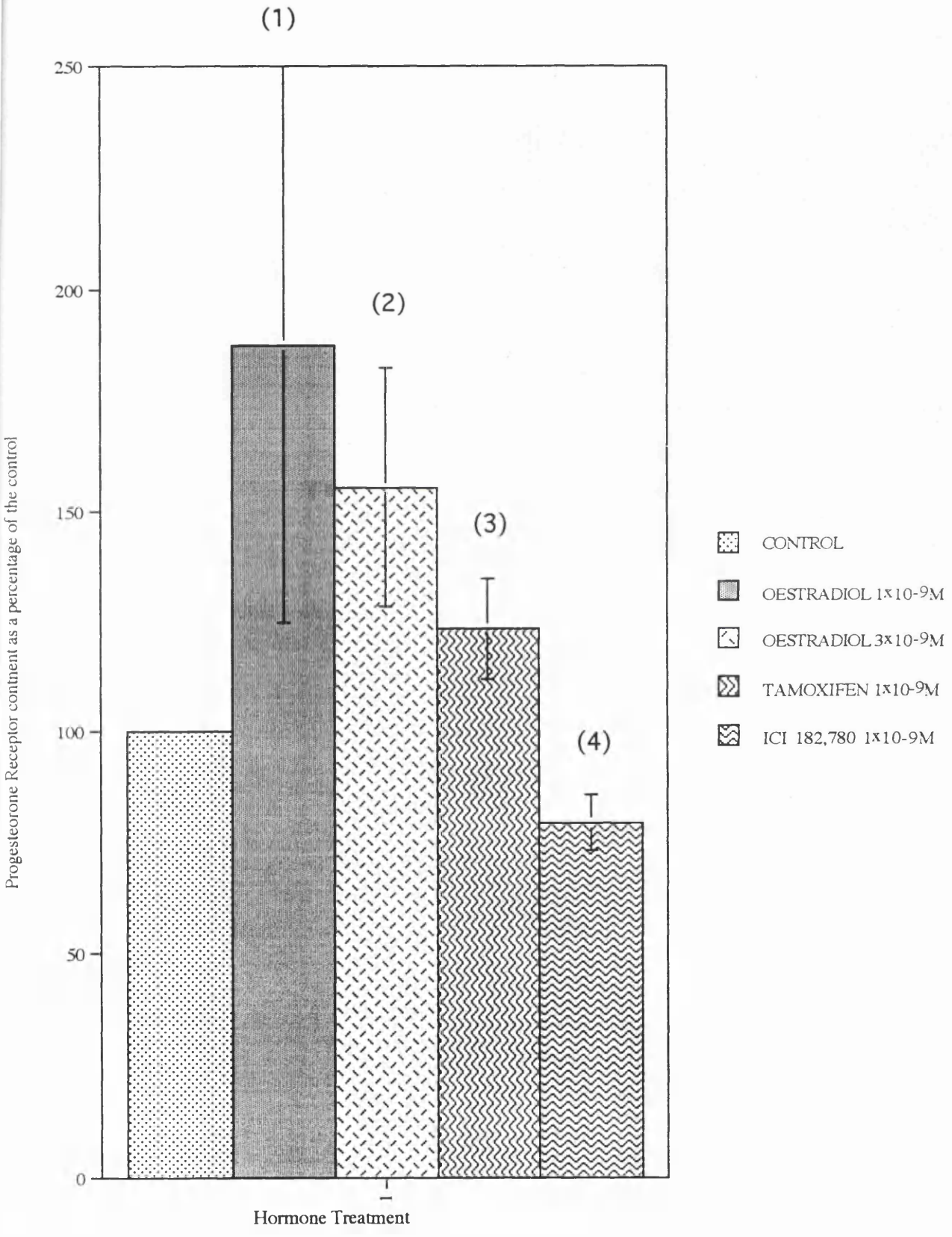
(2) $P < 0.1$, comparing Oestradiol ($3x10^{-9}$M) to the control.

(3) $P < 0.1$, comparing Tamoxifen ($1x10^{-9}$M) to the control.

(4) $P < 0.05$, comparing ICI 182,780 ($1x10^{-9}$M) to the control.

Error bars represent the Standard Deviation of the Data.

Summary of the Effects of Drug and Hormone Treatments on Progesterone Receptor Content in ZR-75 cells



3.4 CONCLUSION

Initial observations of FIGURE 12 reveal that the PR concentration in 1 and $3x10^{-9}$M ($P<0.2$ and $P<0.1$, respectively) Oestradiol and Tamoxifen ($P<0.1$) treated cells are higher than the control value (the 'P' values are not significant due to the small number of samples). This profile is due to the ability of oestrogens to stimulate the synthesis of the progesterone receptor. As previously stressed, activated ER has the ability to bind to ERE's 5' to and 3' to the PR gene, inducing synthesis. Tamoxifen demonstrates partial oestrogenic qualities with its ability to induce synthesis of PR.

It was interesting to note that the PR levels in ZR-75 cells after exposure to ICI 182,780 were significantly ($P<0.05$) lower than those in the control level. This implies that something in the control growth medium remains oestrogenic (RIA showed no detectable E_2 in stripped medium) or that the pre-experimental washing does not fully remove oestrogens.

4 The Effect Of Progestins On Progesterone Receptor Content In ZR-75 Cells

4.1 INTRODUCTION

Progestins, such as Megace, are known to be effective in treating hormone-sensitive breast cancer. Their action is assumed to be mediated through the PR. It was of interest to compare the effects of Norethisterone at the range used to reduce vaginal bleeding (~10^{-9}M), with a dose of Megace ($1x10^{-7}$M), a progestin used in the study by Leake *et al* (1993).

4.2 METHOD

The experiment was performed as described in EXPERIMENTAL section 3.2. except the following supplements were used instead of those described in EXPERIMENTAL section 3.2; Norethisterone (1, 3 and $5x10^{-9}$M) or Megace ($1x10^{-7}$M).

4.3 RESULTS

The results shown in FIGURE 13 represent the mean of 3 experiments, each experiment consisting of 3 samples for each medium used. The parameter being measured is progesterone receptor content, so the results are expressed as the concentration of bound receptor as a percentage of the control. The results described in TABLE 3 represent the above data illustrated as the amount of PR in femtomoles per μg of DNA. Consistent DNA samples could not be obtained from the experimental (alcohol-treated) wells and so the results in TABLE 3 were calculated from the DNA content of samples in identical growth conditions measured by the Hoescht assay. (See EXPERIMENTAL section 13.3 - TABLE 6).

TABLE 3

Progesterone Receptor Analysis Of ZR-75 Cells Exposed to Progestin Supplements

Progestin Supplement	Receptor Content (fmol/mg of DNA)	ST Devn	No. Of Expts
Control	0.786	0.317	3
Norethisterone (1x10-9M)	0.969	0.399	3
Norethisterone (3x10-9M)	0.668	0.585	3
Norethisterone (5x10-9M)	0.536	0.428	3
Megace ($1^{X}10$-7M)	0.552	0.391	3

Fig.13 Effects of Norethisterone and Megace on progesterone receptor concentration in ZR-75 cells. ZR-75 cells grown in $150cm^2$ plates were reseeded into 6 well plates at a density of 7.5 to $8.5x10^4$ cells per well before the addition of compounds. Norethisterone (1, 3, and $5x10^{-9}M$), Megace ($1x10^{-7}M$) or vehicle were incubated for up to 96hrs at 37^oC. Media containing compounds were changed after 2 days. Progesterone receptor concentration was measured using the progesterone "two-point competition" steroid receptor assay as described in METHODOLOGY section 4.1.

The 't' test was used to measure the difference between the PR content of cells exposed to a supplement and the control cells and values are given below for each condition.

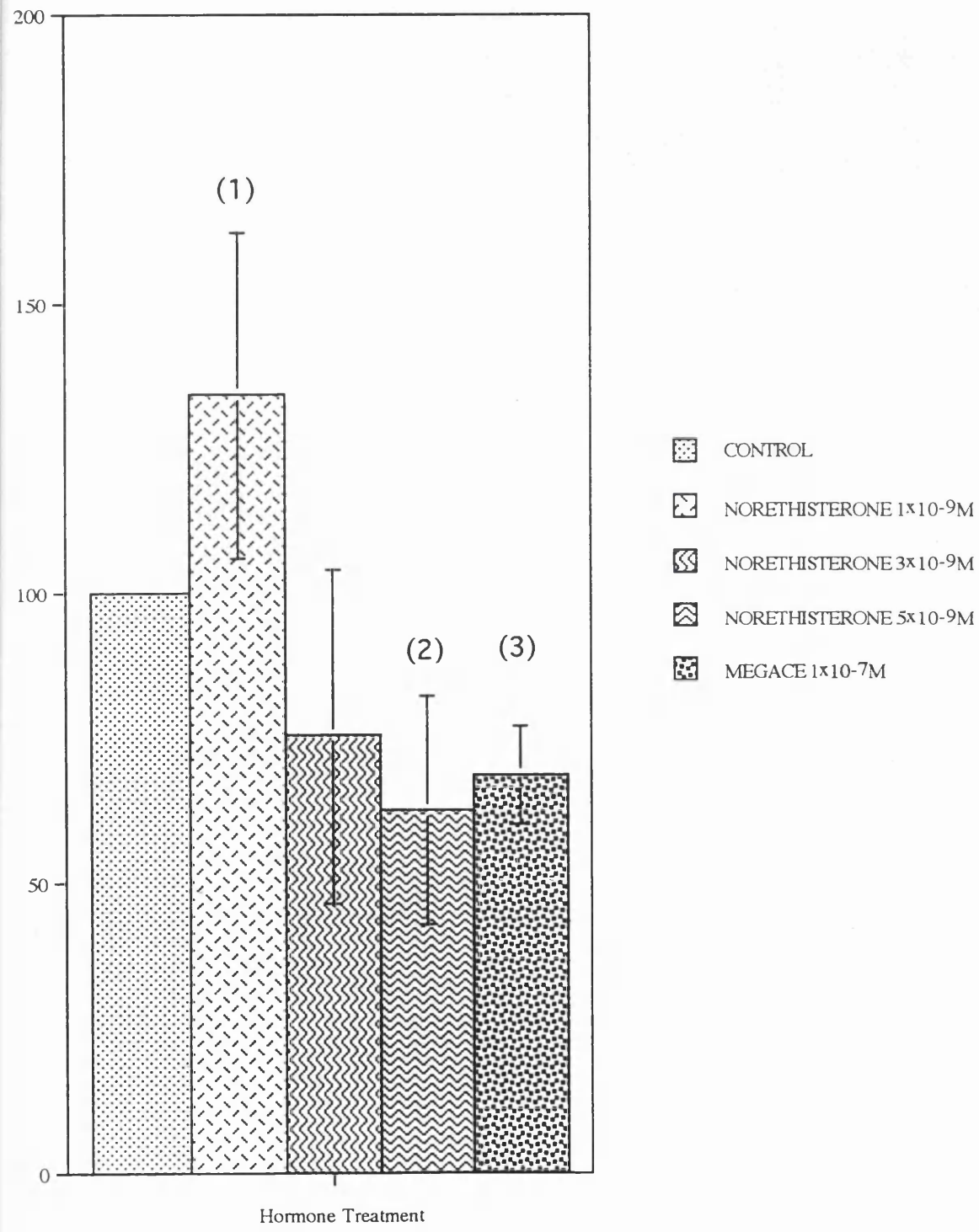
(1) $P < 0.2$, comparing Norethisterone ($1x10^{-9}M$) to the control.

(2) $P < 0.5$, comparing Norethisterone ($5x10^{-9}M$) to the control

(3) $P < 0.1$, comparing Megace ($1x10^{-7}M$) to the control.

Error bars represent the Standard Deviation of the Data.

Summary of the Effects of Progestins on Progesterone Receptor content in ZR-75 cells.



4.4 CONCLUSION

FIGURE 13 demonstrates the role of progestins in the regulation of the progesterone receptor content. Treatment with a low dose progestin, Norethisterone($1x10^{-9}M$) induces ($p<0.2$) a higher concentration of PR in comparison to the control. Alternatively the highest dose of Norethisterone and Megace have ($p<0.5$ and $p<0.1$ respectively) lower values than the control. Norethisterone appears to span the contrasting results. (The 'P' values may not be significant due to the small sample size).

In conclusion, it appears that the actions of progestins on the PR is dependant upon the dosage of the treatment. Low dose treatments of progestins maintain higher levels of PR either by inducing the synthesis of PR or by slowing down the degradation of the protein. Higher doses of treatments reduce the level of PR in the cells.

5 The Effects Of Tamoxifen plus Progestin On Progesterone Receptor Content In ZR-75 Cells

5.1 INTRODUCTION

It has been established that Tamoxifen due to its partial oestrogen properties can maintain high levels of PR over a 96 hour period. In view of these results it was decided to determine the effect of progestins on Tamoxifen induction of PR after 96 hours.

5.2 METHOD

The experiment was performed as described in EXPERIMENTAL section 3.2. except the following supplements were used instead of those described in EXPERIMENTAL section 3.2; Tamoxifen ($1x10^{-9}M$) or Tamoxifen plus Norethisterone (1, 3 and $5x10^{-9}M$) or Megace ($1x10^{-7}M$ and $1x10^{-8}M$).

5.3 RESULTS

The results shown in FIGURE 14 represent the mean of 3 experiments, each experiment consisting of 3 samples for each medium used. The parameter being measured is progesterone receptor content, so the results are expressed as the concentration of bound receptor as a percentage of the control. The results described in TABLE 4 represent the above data illustrated as the amount of PR in femtomoles per μg of DNA using DNA content of competition wells as described in EXPERIMENTAL section 13.3 - TABLE 6.

TABLE 4

Progesterone Receptor Analysis Of ZR-75 Cells Exposed to Tamoxifen Plus Progestin Supplements

Tamoxifen + Progestin Supplement	Receptor Content (fmol/mg of DNA)	ST Devn	No. Of Expts
Control	0.476	0.034	3
Tamoxifen ($1x10^{-9}M$)	0.519	0.051	3
Tamoxifen + Norethisterone ($1x10^{-9}M$)	0.569	0.038	3
Tamoxifen + Norethisterone ($3^{x}10^{-9}M$)	0.231	0.020	3
Tamoxifen + Norethisterone ($5^{x}10^{-9}M$)	0.334	0.179	3
Tamoxifen + Megace ($1^{x}10^{-7}$)	0.252	0.043	3
Tamoxifen + Megace ($1^{x}10^{-8}$)	0.357	0.126	3

Fig.14 Effects of Tamoxifen plus Norethisterone or Megace at varying molarities on progesterone receptor concentration in ZR-75 cells. ZR-75 cells grown in $150cm^3$ plates were reseeded into 6 well plates at a density of 7.5 to $8.5x10^4$ cells/well before the addition of compounds. Tamoxifen ($1x10^{-9}M$), Tamoxifen plus Norethisterone (1, 3, and $5x10^{-9}M$), Megace ($1x10^{-7}M$ and $1x10^{-8}M$) or vehicle were incubated for up to 96hrs at 37°C. Media containing compounds were changed after 2 days. Progesterone receptor concentrations were measured using a two point receptor assay as described in METHODOLOGY section 4.1.

The 't' test was used to measure the difference between the PR content of cells exposed to a supplement and control cells and values are given below for each condition.

(1) $P < 0.1$, comparing Tamoxifen ($1x10^{-9}M$) to the control.

(2) $P < 0.05$, comparing Tamoxifen plus Norethisterone ($1x10^{-9}M$) to the control.

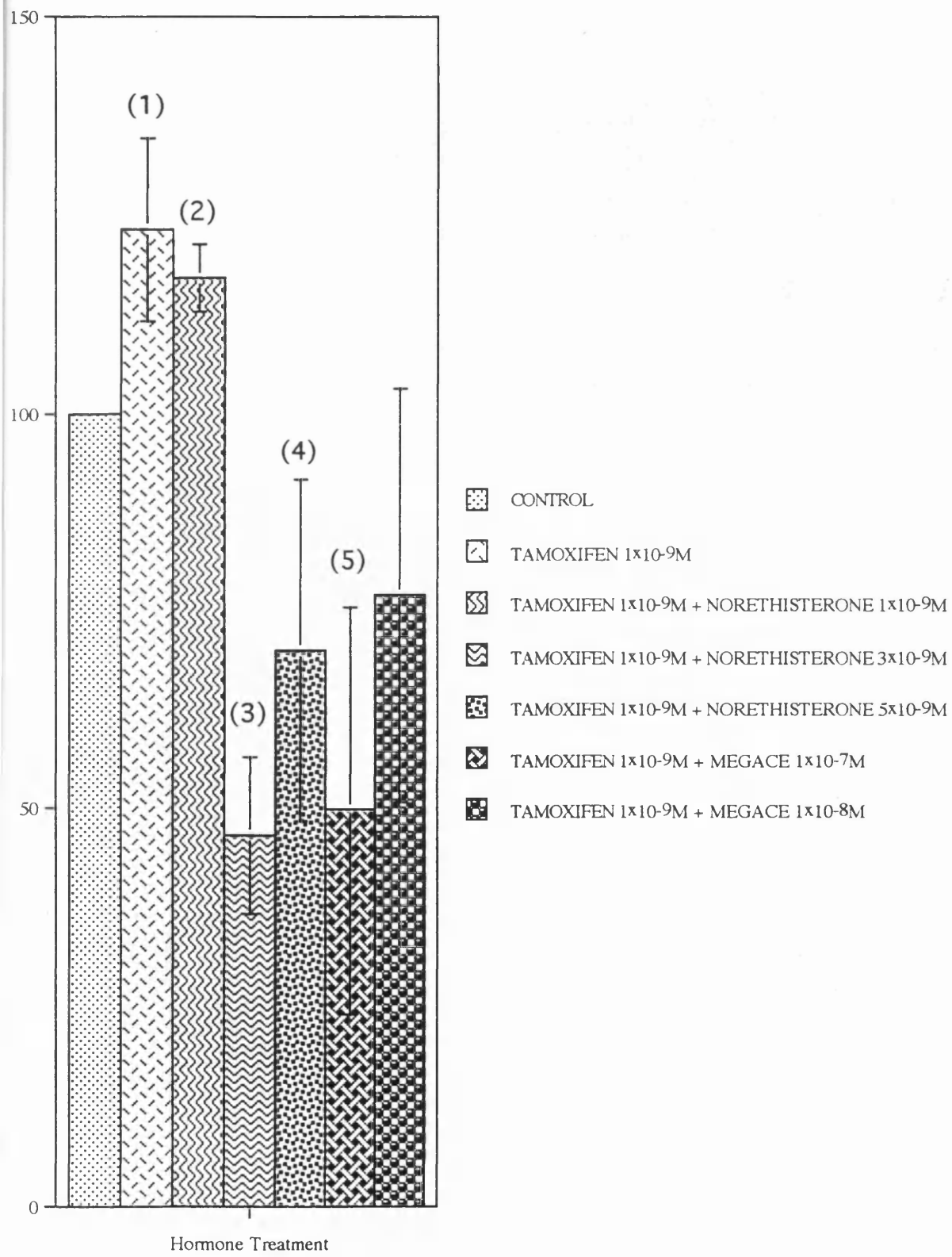
(3) $P < 0.01$, comparing Tamoxifen plus Norethisterone ($3x10^{-9}M$) to the control.

(4) $P < 0.1$, comparing Tamoxifen plus Norethisterone ($5x10^{-9}M$) to the control.

(5) $P < 0.05$, comparing Tamoxifen plus Megace ($1x10^{-7}M$) to the control.

Error bars represent the Standard Deviation of the Data.

Summary of the Effects of Tamoxifen plus Progestin on Progesterone Receptor Content in ZR-75 cells.



5.4 CONCLUSION

FIGURE 14 demonstrates that progestins can overcome the oestrogenic effects of Tamoxifen on PR . It has been demonstrated in the cell line T47D that the half-life of the PR is reduced in the presence of progestins (Nardulli *et al.*, 1988).

However, what is interesting is that the progestin, Norethisterone at a concentration of $1x10^{-9}M$ with ($P<0.05$) or without ($P<0.2$) Tamoxifen should induce higher concentrations of PR in comparison to the control. Upon comparing these treatments with Tamoxifen alone it can be observed that there is no significant difference between any of the treatments. Therefore in terms of the long term aim of this study Norethisterone ($1x10^{-9}M$) dose not affect the ability of Tamoxifen to induce the synthesis of PR. However, the results also propose that progestins can also regulate the level of PR within hormonally sensitive cells as well as oestrogenic compounds.

6 The Effects Of ICI 182,780 plus Progestin On Progesterone Receptor Content In ZR-75 Cells.

6.1 INTRODUCTION

It has been established that the pure antiestrogen, ICI 182,780 does not induce any synthesis of the PR. On the basis of experiments 3.3, 4.3 and 5.3, Norethisterone ($1x10^{-9}M$), in the presence or absence of Tamoxifen, demonstrates no significant difference to that induced by Oestrodial *in vitro.* In view of these results it was decided to determine whether low dose progestin could maintain high levels of PR in the presence of ICI 182,780.

6.2 METHOD

This experiment was performed as described in EXPERIMENTAL section 3.2, except the following supplements were used instead of those described in EXPERIMENTAL section 3.2; Tamoxifen ($1x10^{-9}M$) or Tamoxifen plus Norethisterone (1, 3 and $5x10^{-9}M$) or Megace ($1x10^{-7}M$ and $1x10^{-8}M$).

6.3 RESULTS

The results shown in FIGURE 15 represent the mean of 3 experiments, each experiment consisting of 3 samples for each medium used. The parameter being measured is progesterone receptor content, so the results are expressed as the concentration of bound receptor as a percentage of the control. The results described in TABLE 5 represent the above data illustrated as the amount of PR in femtomoles per μg of DNA. Consistent DNA samples could not be obtained from the experimental (alcohol-treated) wells so the results in TABLE 5 were calculated from the DNA content of samples in identical growth conditions measured by the Hoescht assay. (See EXPERIMENTAL section 13.3 - TABLE 6).

TABLE 5

Progesterone Receptor Analysis Of ZR-75 Cells Exposed To ICI 182,780 Plus Progestin Supplements.

ICI 182,780 + Progestin	Receptor Content (fmol/mg of DNA)	St Devn	No. Of Expts
Control	0.528	0.346	3
ICI 182,780 ($1x10^{-9}M$)	0.423	0.046	3
ICI 182,780 + Norethisterone ($1x10^{-9}M$)	0.358	0.068	3
ICI 182,780 + Megace ($1x10^{-8}$ M)	0.441	0.134	3
ICI 182,780 + Megace ($1x10^{-7}$ M)	0.379	0.088	3

Fig. 15 Effects of ICI 182,780 plus Norethisterone or Megace on progesterone receptor concentration in ZR-75 cells. ZR-75 cells grown in $150cm^2$ plates were reseeded into 6 well plates at a density of 40,000 cells/well before the addition of compounds. ICI 182,780 ($1x10^{-9}$), ICI 182,780 plus Norethisterone (1,3 and $5x10^{-9}M$) or Megace ($1x10^{-7}M$ and $1x10^{-8}M$) were incubated for up to 96hrs at $37^{o}C$. Media containing compounds were changed after 2 days. Progesterone receptor concentration was measured using a two point progesterone receptor assay as described in METHODOLOGY section 4.1.

The 't' test was used to measure the difference between the PR content of cells exposed to a supplement and control cells and values are given below for each condition.

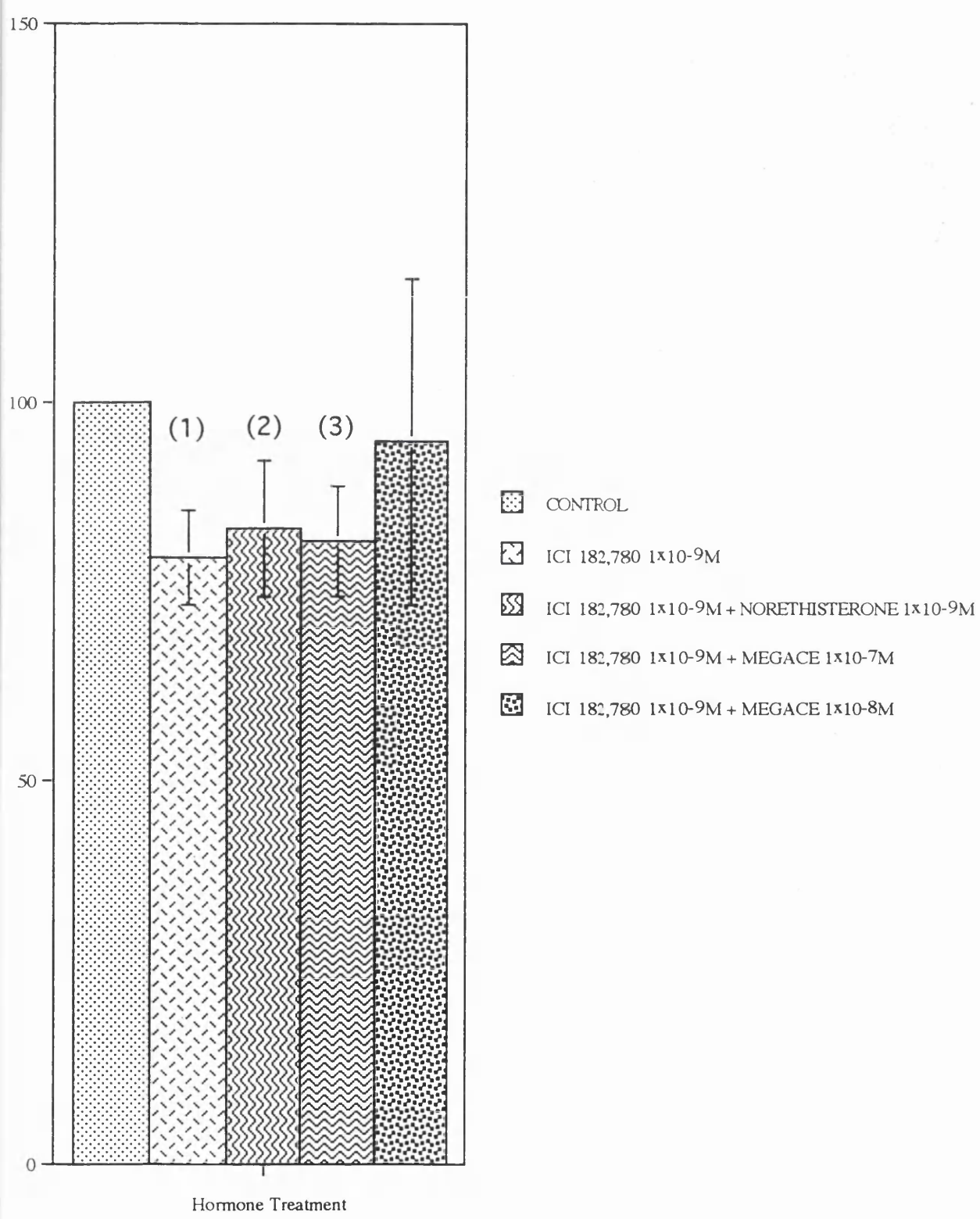
(1) $P < 0.05$, comparing ICI 182,780 ($1x10^{-9}M$) to the control

(2) $P < 0.2$, comparing ICI 182,780 plus Norethisterone ($1x10^{-9}M$) to the control

(3) $P < 0.1$, comparing ICI 182,780 plus Megace ($1x10^{-7}M$) to the control.

Error bars represent the Standard Deviation of the Data.

Summary of the Effects of ICI 182,780 plus Progestins on Progesterone Receptor Content in ZR-75 cells.



6.4 CONCLUSIONS

The low amount of progesterone receptor present in cells exposed to ICI 182,780 on its own or with a progestin in FIGURE 15 demonstrates the dependence of the PR on ER for synthesis. What is interesting is that the level of PR present in cells exposed to both antiestrogen and progestin should approximately range between 80%-90% of the control value. It has been demonstrated in previous experiments that progesterone on their own or in the presence of Tamoxifen have the ability to reduce PR levels to approximately 50% of that of the control. Data from the human breast cell line T47D demonstrates that the half-life of the progesterone receptor is reduced from 21 hours for unoccupied PR to 6 hours when progestins are present (Nardulli *et al.*, 1988). Therefore, if ICI 182,780 is preventing the synthesis of PR by ER and the progestins have increased the rate of PR degradation, the level of detectable PR in comparison to the control should be very low. It has also been established that a low dose of Norethisterone does not alter the level of PR in the absence of oestrogens. These results would suggest that ICI 182,780 has the ability to overcome some of the biological actions of progestins.

7 The Effect of Hormonal Treatments On the Growth of ZR-75 And MCF-7 Cells

7.1 INTRODUCTION

The synthesis of the progesterone receptor is regulated by an oestrogen hormone response elements 5' and 3' to the gene. In the presence of oestradiol the oestrogen receptor binds to the ERE stimulating the synthesis of the progesterone receptor. The oestrogen receptor also stimulates many other genes, some of which generate the mitogenic effects so readily seen in hormone sensitive cells. One of the objectives of this study was to investigate the way in which progestins modulate the agonist activity of Tamoxifen in inducing PR. As PR synthesis is partly cell cycle dependent it was necessary also to determine the effects of the various combinations of drugs on growth rates of MCF-7 and ZR-75 cells (Sutherland., 1993).

The cell line MCF-7 had proven difficult to work with while studying the levels of PR synthesis. However, this cell line in addition to ZR-75 cells proved to be an adequate model for studying the effects of hormonal supplements on cell proliferation.

7.2 METHOD

ZR-75 and MCF-7 cells were cultured routinely in 175cm^2 plastic tissue culture flasks until the cell monolayer was between 80-90% confluent. The cells were harvested using trypsin/versene solution and reseeded into 24 well plates at a cell of 1.7-2.5x10^{-4} cells per well. The cells were allowed to "plate down" for 24 hours. The cells were washed with PBS-A and each set of 10 wells were fed with phenol red-free medium with 5% HIDCCFCS plus alcohol at a volume equivalent to the supplements (as the control) or a hormone supplement; Oestradiol (1x10^{-9}M and 3x10^{-9}M), Tamoxifen (1x10^{-9}M) or ICI 182,780 (1x10^{-9}M) was added. The cells were washed and fed with fresh medium every 48hours. Samples were removed every 24hrs for 96hours from cells exposed to the various medium. At each time point two wells were

selected, washed and harvested using trypsin/versene solution for each hormonal or drug supplement being studied. The cells were transferred to eppendorfs, spun down and stored at -20°C. When all samples had been removed 200µl of ETN buffer containing 0.1% SDS was added to each eppendorf and incubated for 30mins at 37°C. Aliquots (100µl) of solubilised cells were assayed for DNA using the Hoechst DNA assay described in METHODOLOGY section3.1. To demonstrate that the concentration of DNA per sample was comparable with the number cells per ml, the two were plotted against each other (see figure 16). The cells were harvested from wells using trypsin/versene solution. The cells were transferred to eppendorfs, 100µl of the sample was then used to determine the cell number per ml using a haemocytometer. The rest of the sample was assayed for DNA as before.

7.3 RESULTS

The results in FIGURE 16 demonstrates a correlation between cell number and DNA content.

The results shown in FIGURES 17 and 18 each represent the mean of 3 experiments, each experiment consisting of 2 wells of cells for each time point. The parameter being measured is cell growth and so the results are expressed as the concentration of DNA (µg/ml) in relation to the duration of the experiment (hours).

Fig 16. A correlation between DNA content and cell number. During several growth experiments samples were taken and both cell number (counted on the haemocytometer) and DNA content were measured (using the Hoescht DNA assay)

A Correlation Between DNA Content And Cell Number

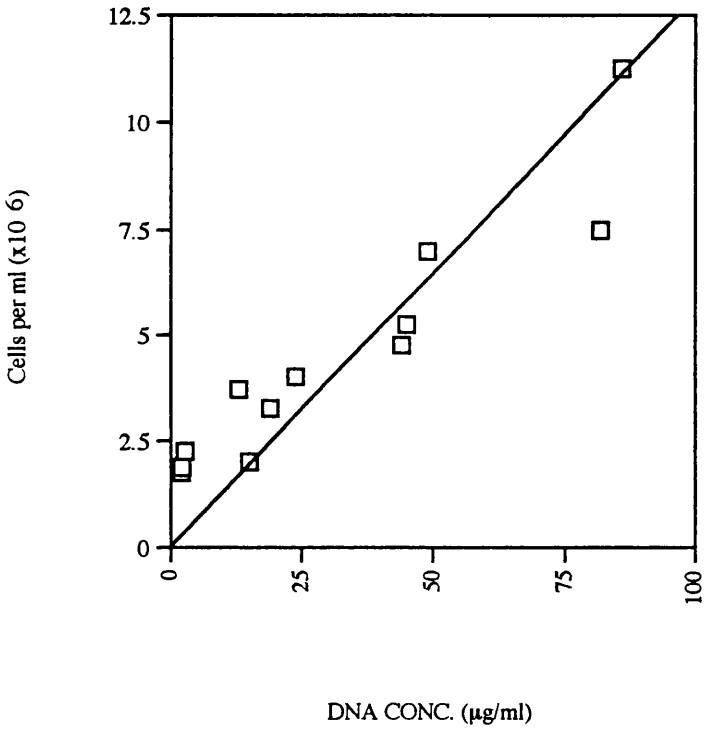


Fig.17 Time course of the effects of Tamoxifen, Oestradiol and ICI 182,780 on the proliferation of ZR-75 cells over a 96hr period. ZR-75 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, Tamoxifen ($1x10^{-9}$M), Oestradiol ($1x10^{-9}$M and $3x10^{-9}$M), ICI 182,780 ($1x10^{-9}$M) or vehicle. The cells were then incubated for 96hrs at 37°C. Media were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section 3.1. Each data point represents cell growth expressed as the concentration of DNA (μg/ml).

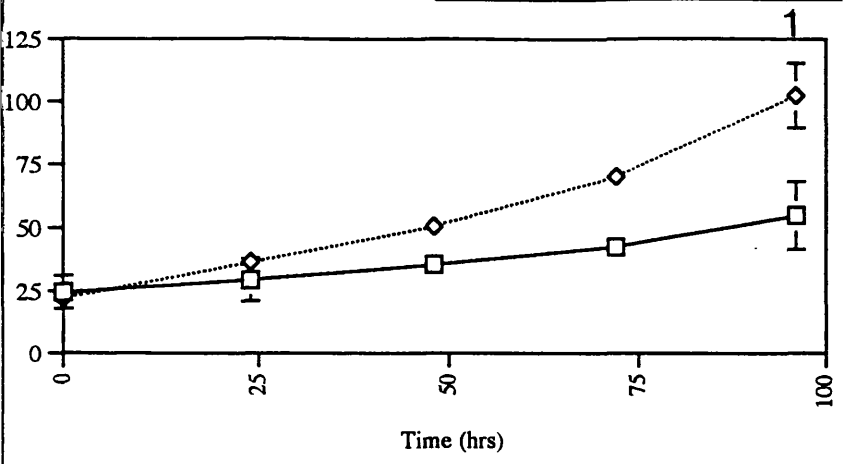
The 't' test was used to measure the difference in cell proliferation between cells exposed to a supplement and the control cells at 96hrs and values are given below for each condition.

(1) $P < 0.05$, comparing Oestradiol ($1x10^{-9}$M) to the control.

(2) $P < 0.05$, comparing Oestradiol ($3x10^{-9}$M) to the control.

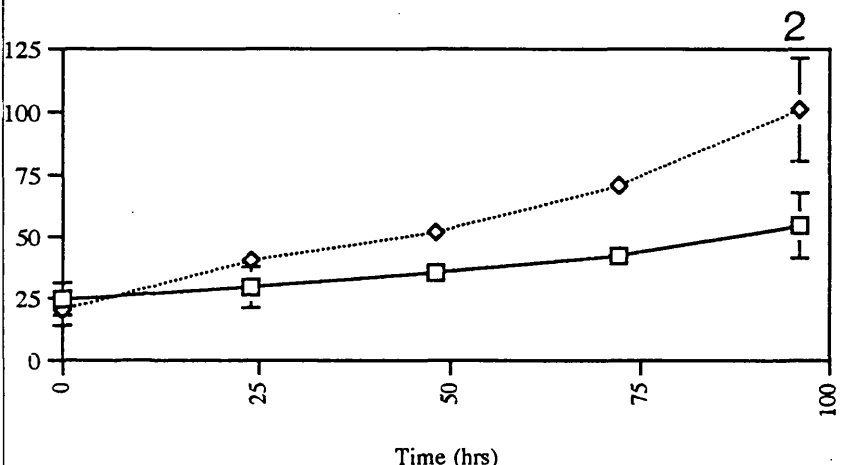
Error bars represent the Standard Deviation of the Data.

Summary of the Effects of Hormonal Treatments on the Growth of ZR-75 Cells



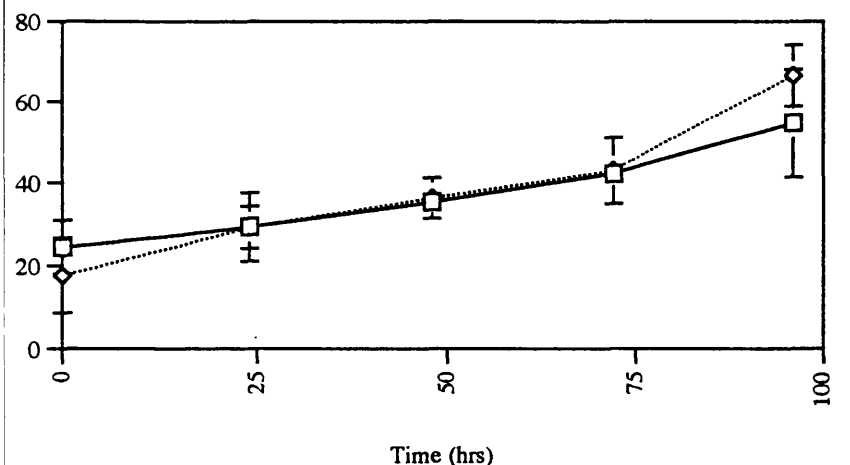
Control

Oestradiol (1x10-9M)



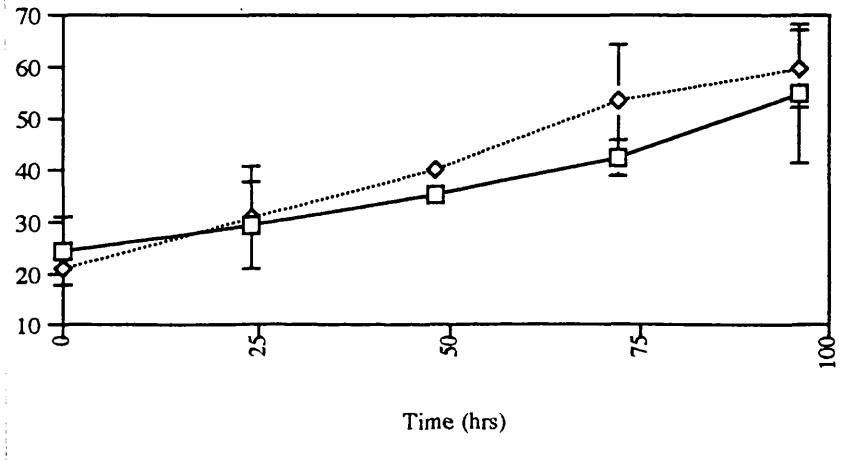
Control

Oestradiol (3x10-9M)



Control

Tamoxifen (1x10-9M)



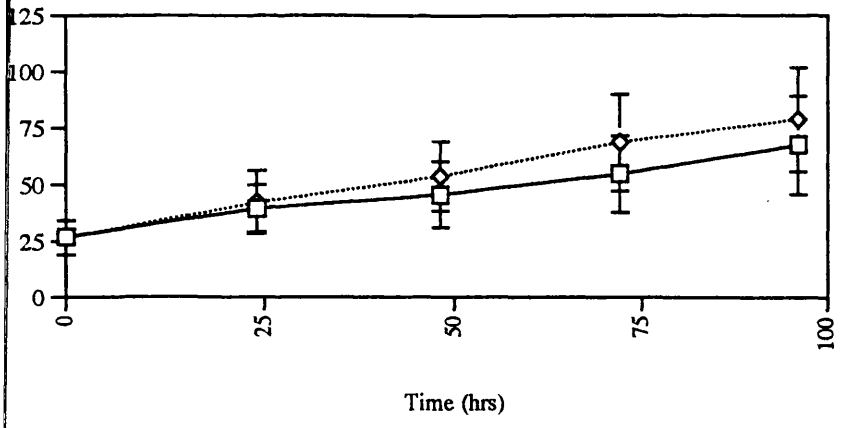
Control

ICI 182,780 (1x10-9M)

Fig.18 Effects of Tamoxifen, Oestradiol and ICI 182,780 on the growth of MCF-7 cells over 96hrs. MCF-7 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, ethanol (as the control), Tamoxifen ($1x10^{-9}$M), Oestradiol ($1x10^{-9}$M and $3x10^{-9}$M), ICI 182,780 ($1x10^{-9}$M) or vehicle. The cells were then incubated for 96hrs at 37°C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section 3.1. Each data point represents cell growth expressed as the concentration of DNA (μg/ml).

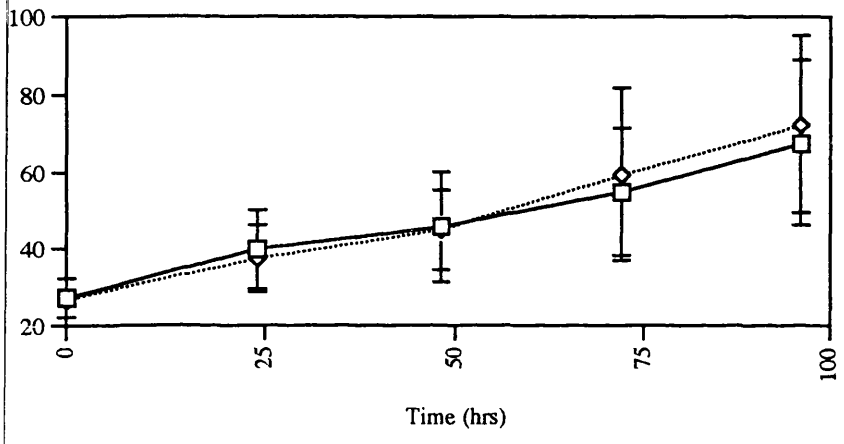
Error bars represent the Standard Deviation of the Data.

Summary of the Effects of Hormonal Treatments on the Growth of MCF-7 Cells



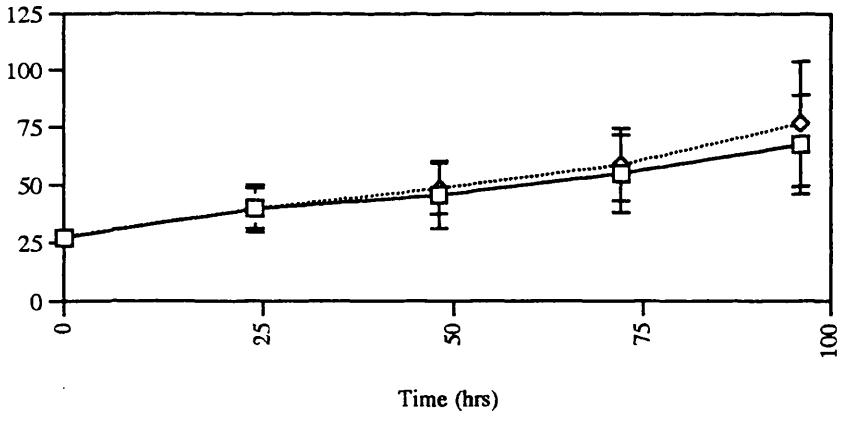
Control

Oestradiol (1x10-9M)



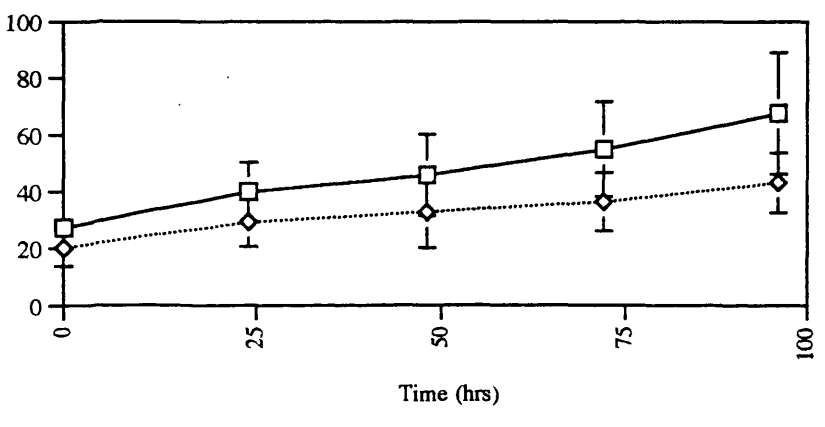
Control

Oestradiol (3x10-9M)



Control

Tamoxifen (1x10-9M)



Control

ICI 182,780 (1x10-9M)

7.4 CONCLUSION

FIGURE 17 demonstrates the mitogenic effect of oestradiol on the cell line ZR-75. The significant proliferative effect of oestradiol $1x10^{-9}$ M and $3x10^{-9}$ M ($P<0.05$ and $P<0.05$ repectively) during the time course experiment demonstrates that the oestrogen receptors measured in the initial experiment are functional and sensitive to hormonal treatment for ZR-75 cells. MCF-7 cells treated with oestradiol show no significant difference in growth rate when compared with the control (figure 18). This is also the case for the cells treated with Tamoxifen and ICI 182,780. The experiment in chapter 1 demonstrated that the MCF-7 cell-line is both ER and PR positive. Neither oestrogen or antiestrogen demonstrated an ability to induce the oestrogen receptor to influence the growth rate of the MCF-7 cells. This would suggest that the oestrogen receptors in the MCF-7 cell-line are not fully functional.

ICI 182,780 has been defined as a pure antiestrogen. The definition of a pure antiestrogen is a compound that prevents oestrogen from stimulating transcription.. ZR-75 cells incubated with ICI 182,780 showed no alteration in growth when compared with the control. Therefore, the ZR-75 cells can proliferate without the oestrogen receptor. Tamoxifen also demonstrated no significant difference in growth rate when compared with the control.

8 Dose Dependent Growth of ZR-75 and MCF-7 Cells to Norethisterone.

8.1 INTRODUCTION

Having established the influence of oestrogens and antiestrogens on the growth of ZR-75 and MCF-7 breast cancer cell lines, it was decided to assess the action of the progestin, Norethisterone, on cell proliferation of the human breast cell lines, over a dose range of $1x10^{-9}M$ to $1x10^{-7}M$. This would provide data comparing the effects of high and low dose Norethisterone on breast cancer cells *in vitro.*

8.2 METHOD

Both cell lines were subcultured as described in EXPERIMENTAL section 7.2. After 24 hours the cells were washed with PBS-A and each set of 10 wells were fed with phenol red free medium with 5% HIDCCFCS plus ethanol (as the control) or a synthetic progestin supplement; Norethisterone was used at $1x10^{-9}M$, $1x10^{-8}M$ and $1x10^{-7}M$. Samples were removed as described in EXPERIMENTAL section 7.2.

8.3 RESULTS

The results shown in FIGURES 19 and 120 represent the mean of 3 experiments, each experiment consisting of 2 wells of cells for each time point. The parameter being measured is cell growth and so the results are expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

Fig. 19 Effects of Norethisterone on the growth of ZR-75 cells over 96hrs. ZR-75 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, Norethisterone ($1x10^{-9}$M, $1x10^{-8}$M and $1x10^{-7}$M) or vehicle. The cells were then incubated for up to 96hrs at 37^{o}C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section 3.1. Each data point represents cell growth expressed as the percentage of DNA in relation to the control.

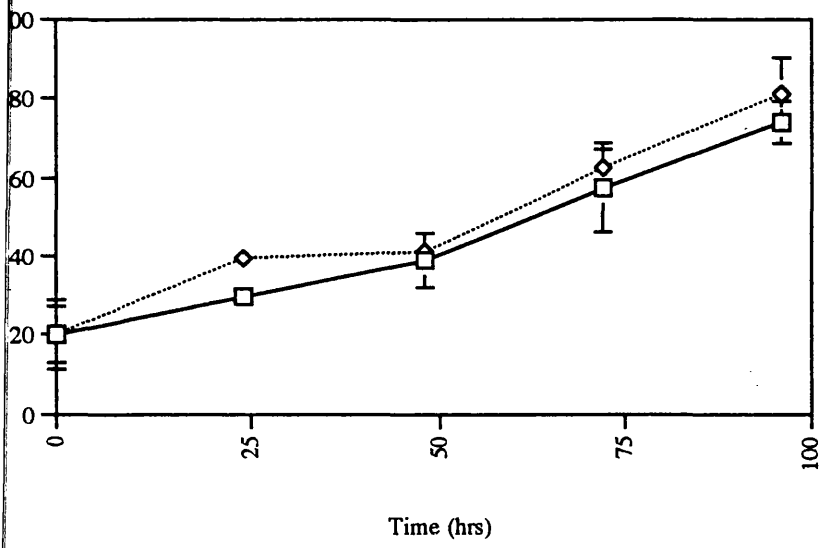
The 't' test was used to measure the difference in cell proliferation between cells exposed to a supplement and the control cells at 96hrs and values are given below for each condition.

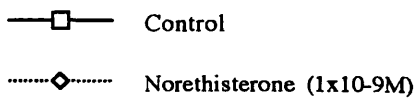
(1) $P < 0.1$, comparing Norethisterone ($1x10^{-8}$M) to the control.

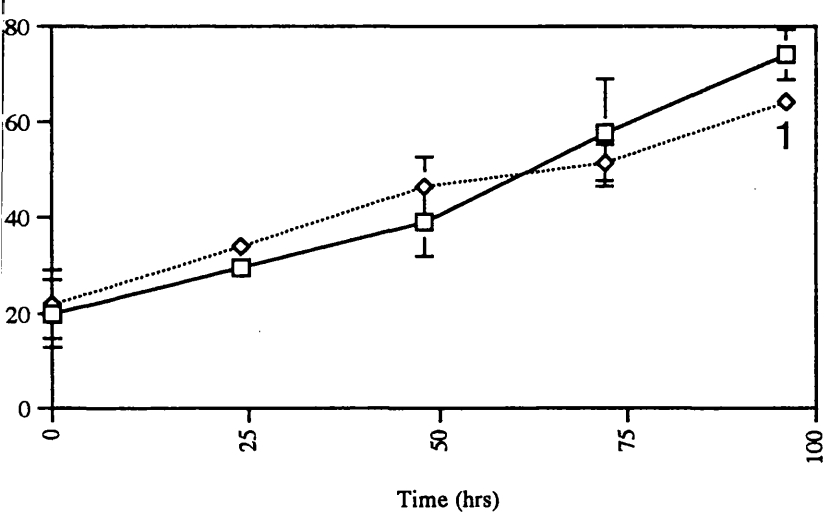
(2) $P < 0.05$, comparing Norethisterone ($1x10^{-7}$M) to the control.

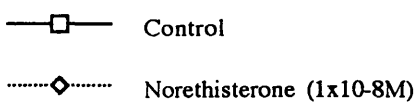
Error bars represent the Standard Deviation of the Data.

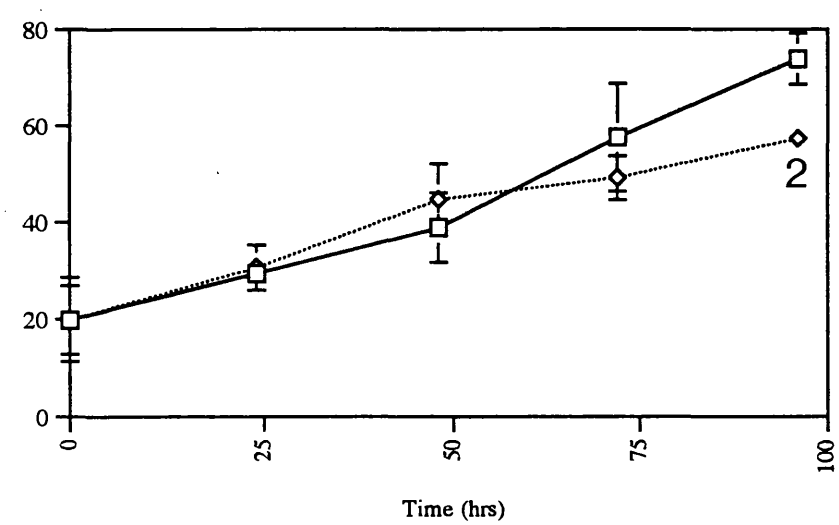
Summary of the Effects of Progestins on the Growth of ZR-75 Cells











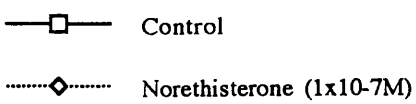
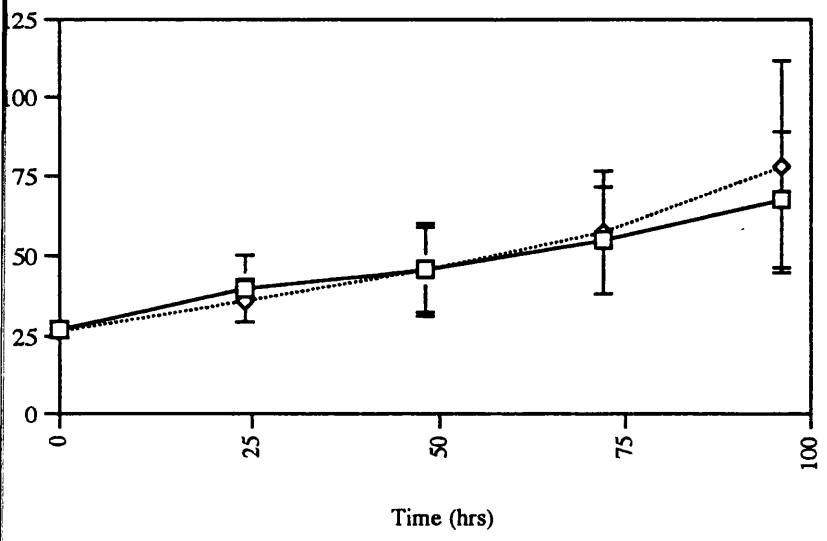
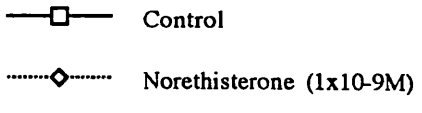


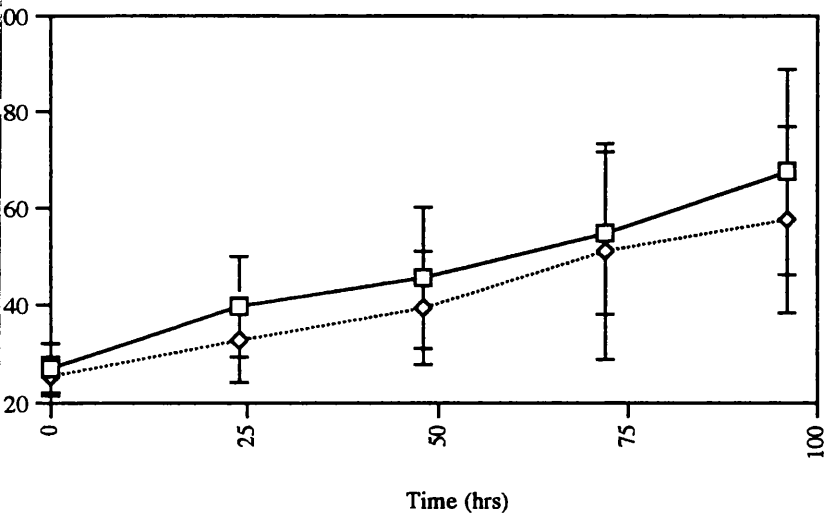
Fig. 20 Effects of Norethisterone on the growth of MCF-7 cells over 96hrs. MCF-7 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, ethanol (as the control), Norethisterone ($1x10^{-9}$M, $1x10^{-8}$M and $1x10^{-7}$M). The cells were then incubated for up to 96hrs at 37^{o}C. Media was changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section 3.1. .Each data point represents cell growth expressed as the percentage of DNA in relation to the control.

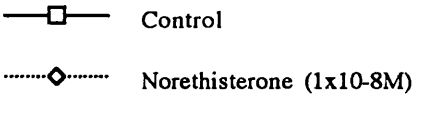
Error bars represent the Standard Deviation of the Data.

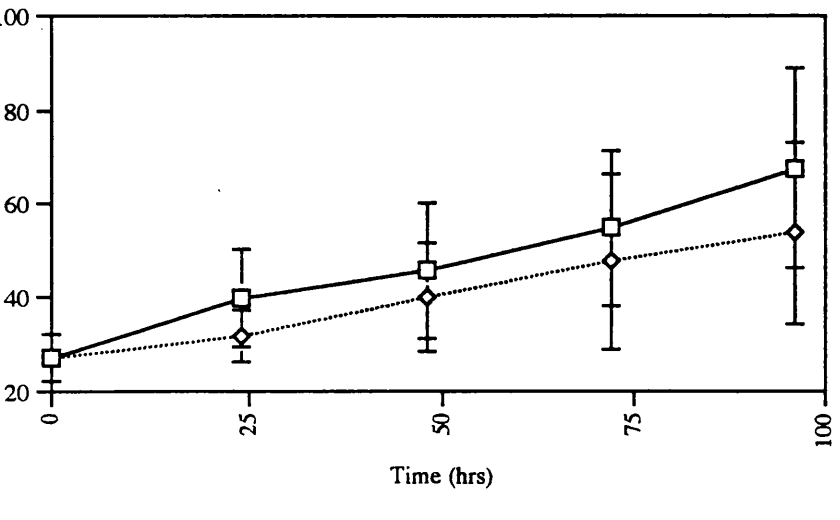
Summary of the Effects of Progestins on the Growth of MCF-7 Cells

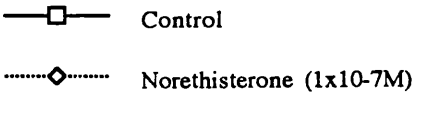












8.4 CONCLUSION

FIGURES 19 demonstrates the growth inhibition of the cell line ZR-75 mediated by the dose range of Norethisterone $1^{x}10^{-8}$ M and $1^{x}10^{-7}$ M ($P<0.1$ and $P<0.05$, respectively) over a period of 96 hours. As the concentration of progestin was increased so did the level of growth inhibition. What is interesting is that the progestin should have no effect on the ZR-75 cell line for at least the first 48 hours of exposure before growth inhibition is induced. Norethisterone ($1x10^{-9}$M) had no affect on the cell proliferation of ZR-75 when compared to the control.

The MCF-7 cells demonstrated no significant affects on cell proliferation when treated with Norethisterone (figure 20).

9 The Effect Of A Low Dose Range Of Progestins On The Growth Of ZR-75 And MCF-7 Cells

9.1 INTRODUCTION

It has been established that Norethisterone can inhibit cell proliferation of ZR-75 cells in the absence of oestrogens. The *in vivo* level of Norethisterone used to reduce vaginal bleeding is about 10^{-9}M. Given the differential effects of 1,3 and 5x10-9M Norethisterone on PR, it was decided to see if these doses had differential effects on growth rates. Additionally, the plasma level of Megace, when used as an anti-cancer agent, is about 10^{-7} M. Therefore, it was also decided to compare the effects of an anti-cancer dose of Megace with *in vivo* levels of Norethisterone.

9.2 METHOD

Both cell lines were subcultured as described in EXPERIMENTAL section 2.2. After 24hours the cells were washed with PBS-A and each set of 10 wells were fed with phenol red free medium with 5% HIDCCFCS plus ethanol (as the control) or a synthetic progestin supplement; Norethisterone (1, 3 and $5x10^{-9}$M) or Megace ($1x10^{-7}$M). Samples were removed as described in EXPERIMENTAL section 7.2.

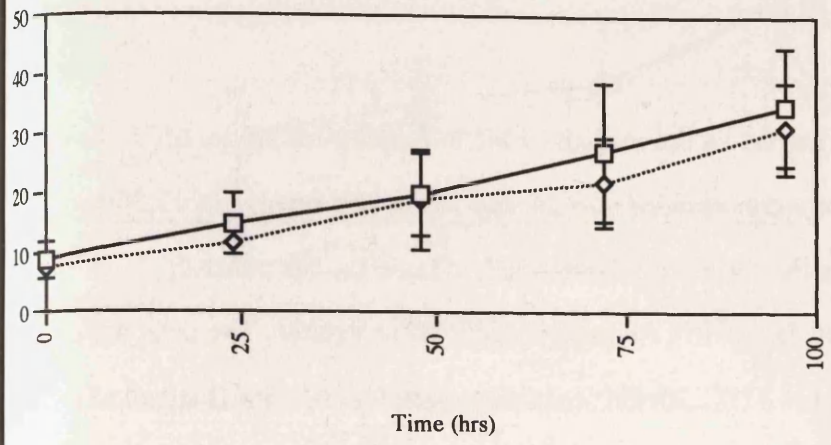
9.3 RESULTS

The results shown in FIGURES 21 and 22 represent the mean of 3 experiments, each experiment consisting of 2 wells of cells for each time point. The parameter being measured is cell growth and so the results are expressed as concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

Fig.21 Effects of progestins on the growth of ZR-75 cells over 96hrs. ZR-75 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, Norethisterone (1 3, and 5x10^{-9}M), Megace (1x10^{-7}M) or vehicle. The cells were then incubated for 96hrs at 37^{o}C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section 3.1. .Each data point represents cell growth as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

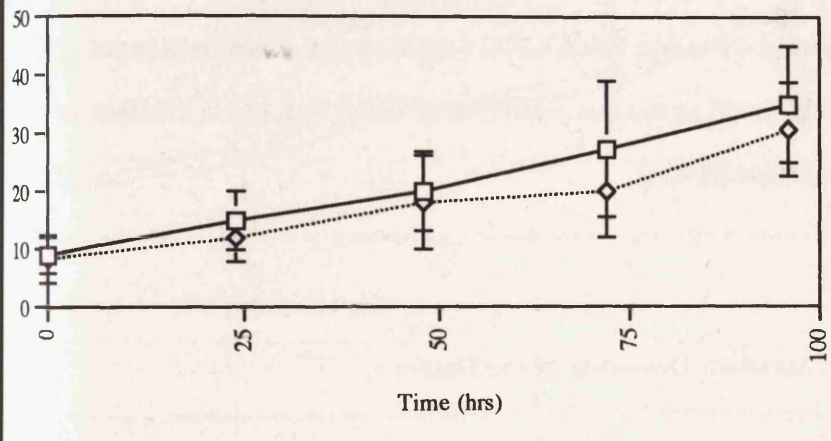
Error bars represent the Standard Deviation of the Data.

Summary of the Effect of Progestins on the Growth of ZR-75 Cells



Control

Norethisterone (1x10-9M)



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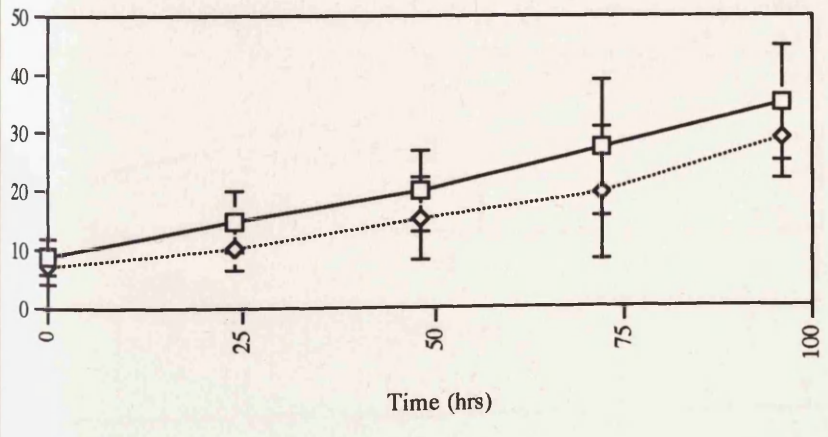
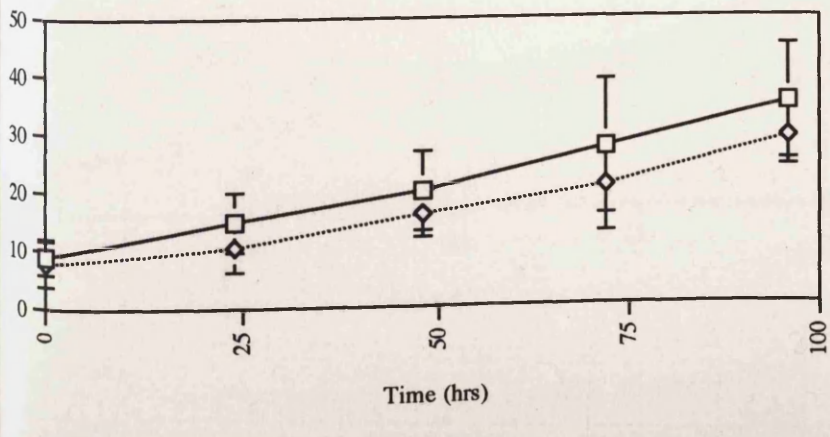
Norethisterone (3x10-9M)



Control

Norethisterone (5x10-9M)



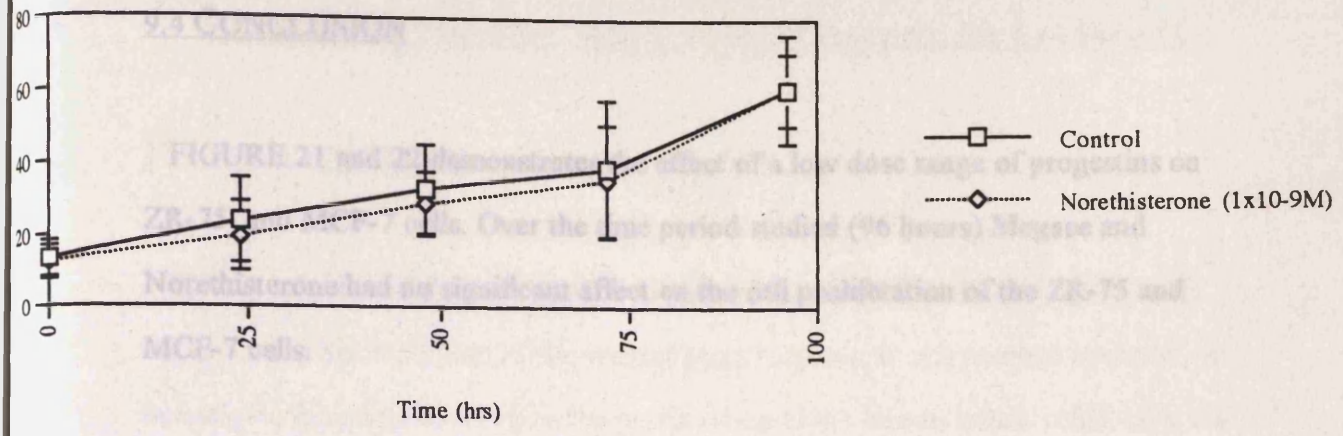
Control

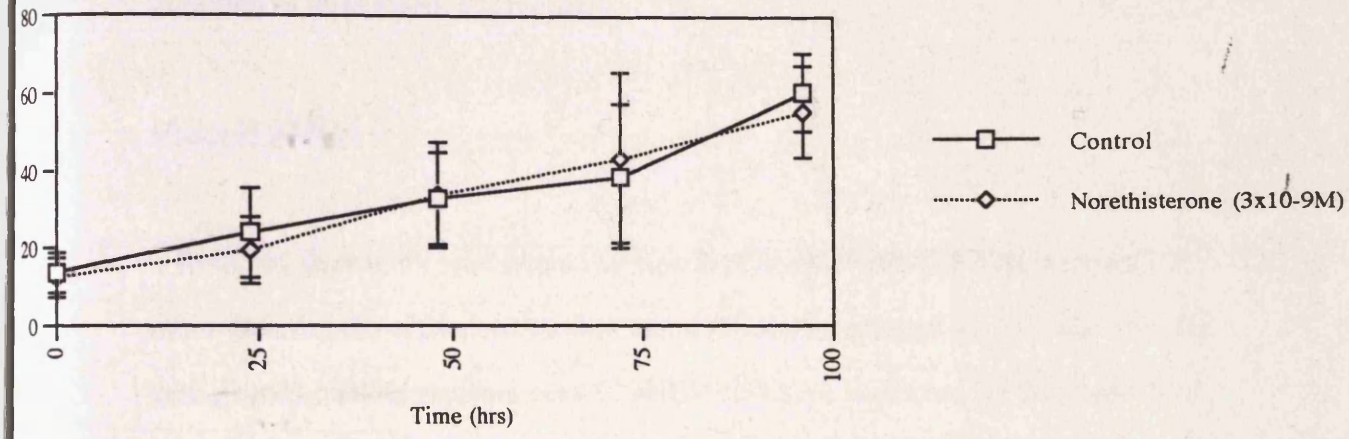
Megace (1x10-7M)

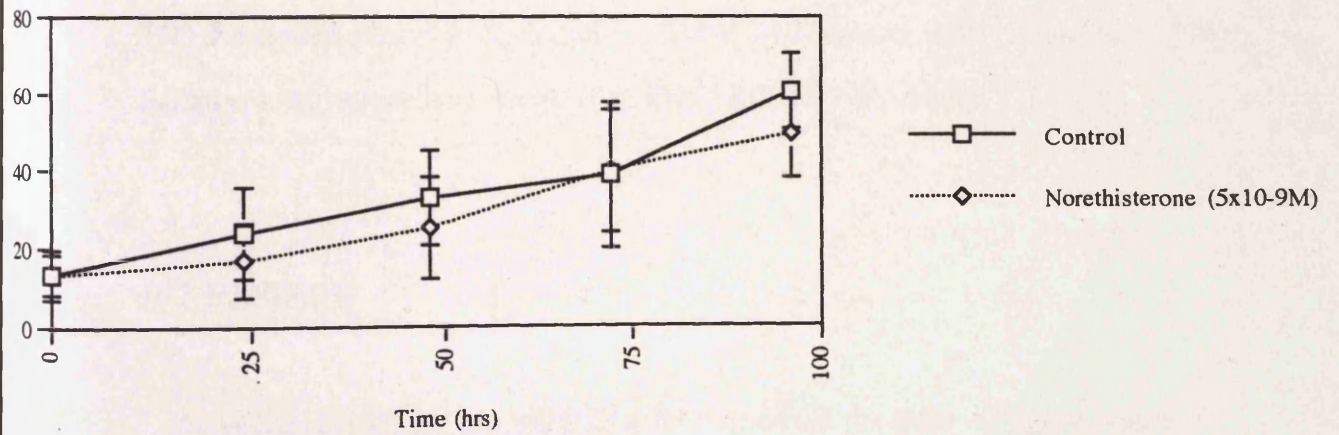
Fig.22 Effects of progestins on the growth of MCF-7 cells over 96hrs. MCF-7 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, ethanol (as the control), Norethisterone (1 3, and 5x10^{-9}M), Megace (1x10^{-7}M) or vehicle. The cells were then incubated for 96hrs at 37^{o}C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoeschst assay as described in METHODOLGY section 3.1. Each data point represents cell growth expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

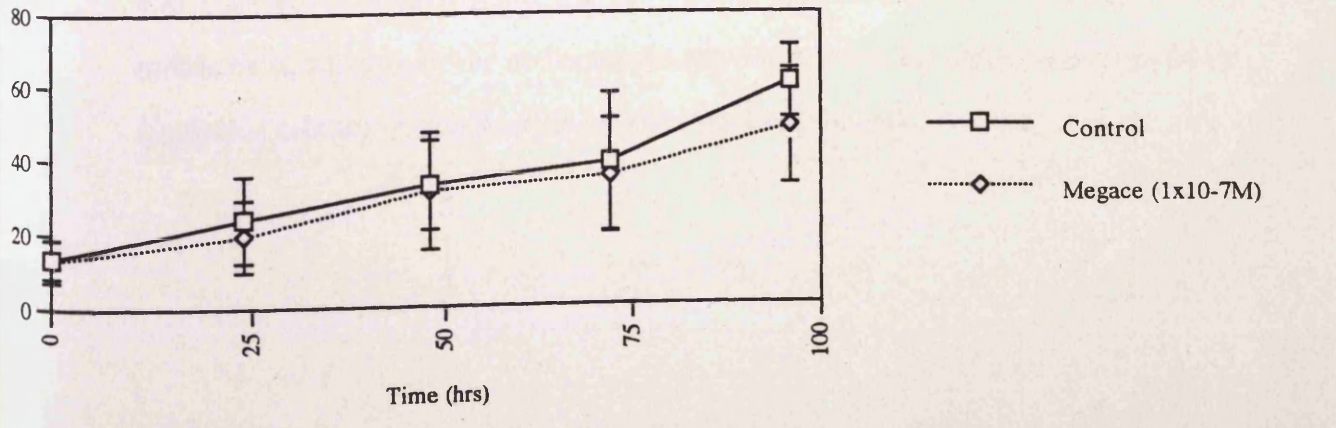
Error bars represent the Standard Deviation of the Data.

Summary of the Effects of Progestins on the Growth of MCF-7 Cells









9.4 CONCLUSION

FIGURE 21 and 22 demonstrates the effect of a low dose range of progestins on ZR-75 and MCF-7 cells. Over the time period studied (96 hours) Megace and Norethisterone had no significant affect on the cell proliferation of the ZR-75 and MCF-7 cells.

10 The Effects Tamoxifen, With or Without Progestin, On Cell Growth.

10.1 INTRODUCTION

Having established that the $1x10^{-9}M$ dose of Norethisterone did not significantly effect Tamoxifen induction of the progesterone receptor, it was deemed necessary to investigate the progestins role in the proliferation of the human breast cell lines in the presence or absence of Tamoxifen.

10.2 METHOD

Both cell lines were subcultured as described in EXPERIMENTAL section 2.2. After 24hours the cells were washed with PBS-A and each set of 10 wells were fed with phenol red free medium with 5% HIDCCFCS plus ethanol (as the control) or Tamoxifen or Tamoxifen plus a synthetic progestin supplement; Tamoxifen ($1x10^{-9}M$) Tamoxifen plus Norethisterone ($1x10^{-9}M$) or Megace ($1x10^{-7}M$ and $1x10^{-8}M$). Samples were removed as described in EXPERIMENTAL section 2.2.

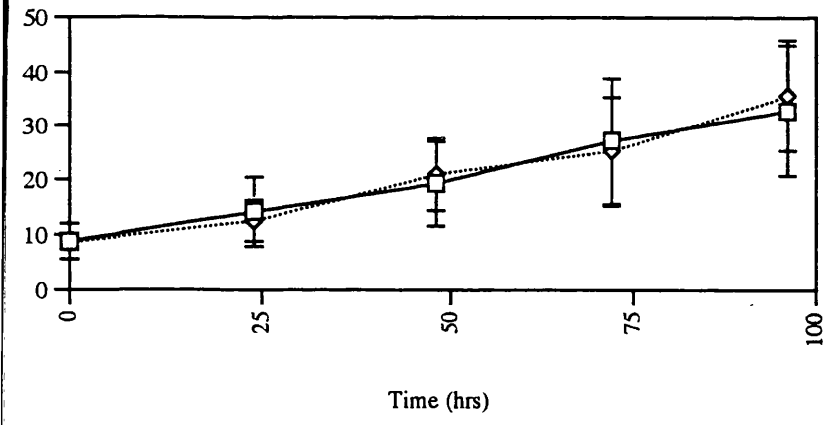
10.3 RESULTS

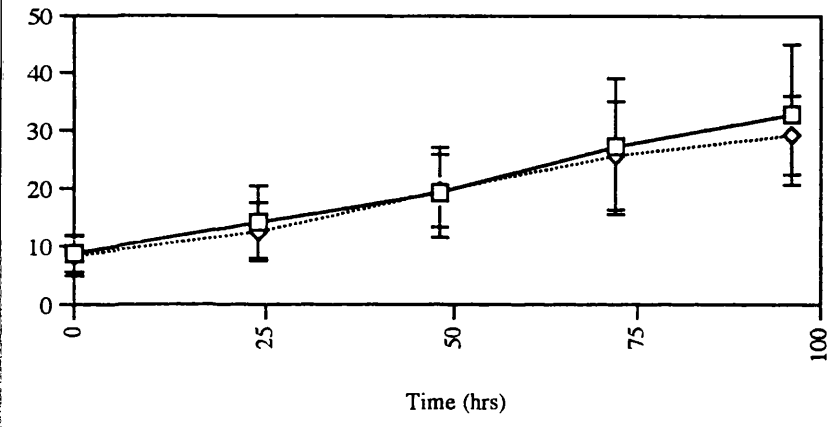
The results shown in FIGURES 23 and 24 represent the mean of 3 experiments, each experiment consisting of 2 wells of cells for each time point. The parameter being measured is cell growth and so the results are expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

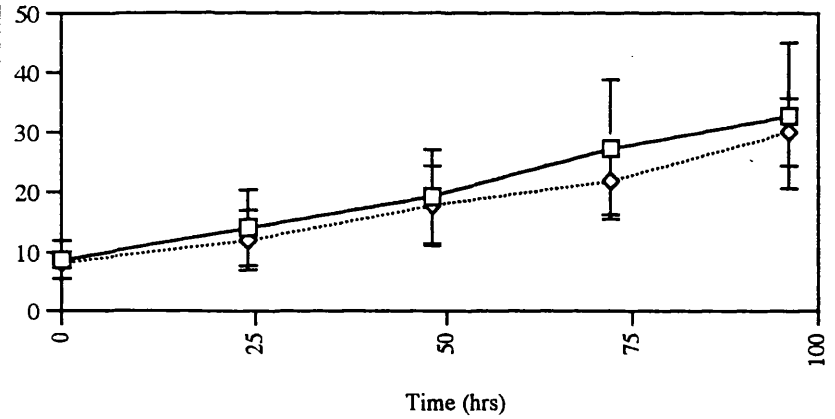
Fig.23 Effects of Tamoxifen plus Norethisterone or Megace on the growth of ZR-75 cells over 96hrs. ZR-75 cells grown in 175cm^2 plates were reseeded into 24well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, Tamoxifen ($1x10^{-9}$M), Tamoxifen plus Norethisterone ($1x10^{-9}$M) Megace ($1x10^{-7}$M and $1x10^{-8}$M) or vehicle. The cells were then incubated for 96hrs at 37°C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section 3.1. Each data point represents cell growth expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

Error bars represent the Standard Deviation of the Data.

Summary of the Effects of Tamoxifen Plus Progestin on the Growth of ZR-75 Cells







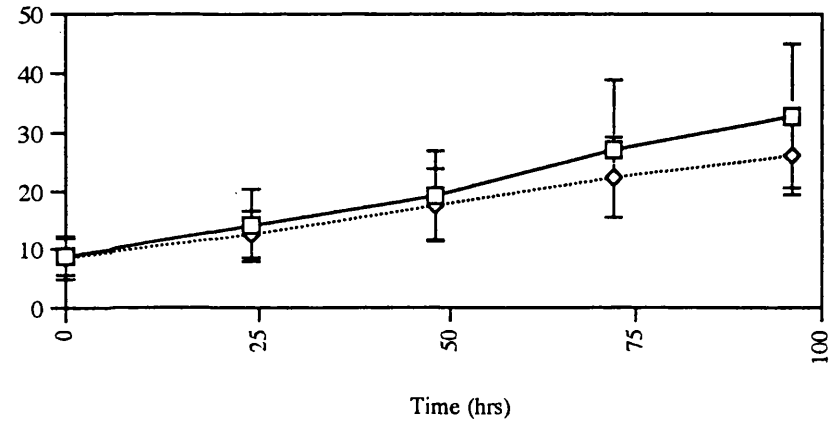
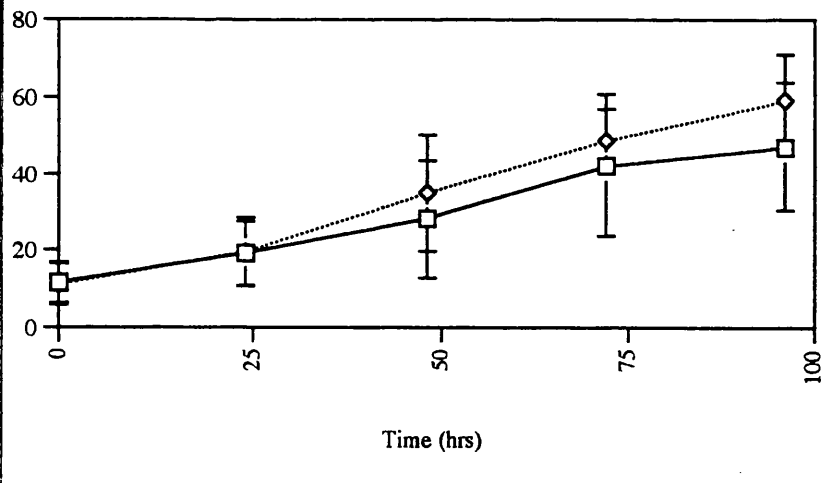
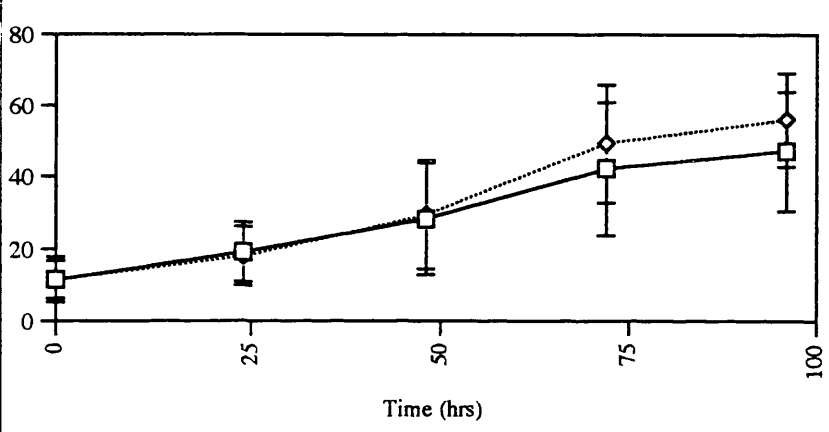


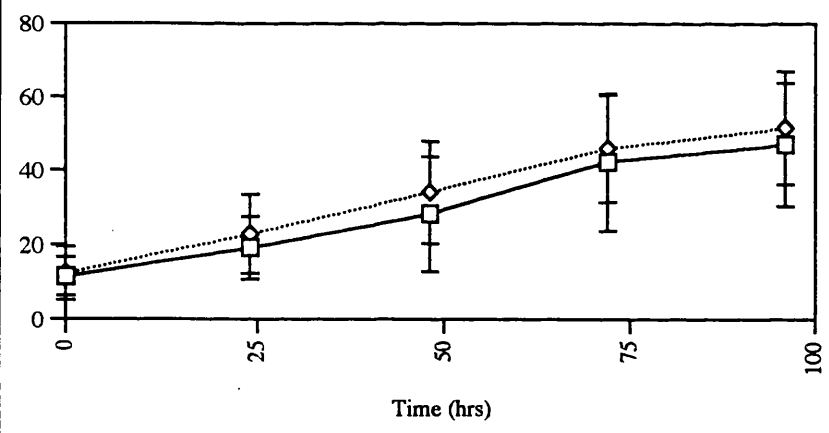
Fig.24 Effects of Tamoxifen plus Norethisterone or Megace on the growth of MCF-7 cells over 96hrs. MCF-7 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, ethanol (as the control) Tamoxifen ($1x10^{-9}$M), Tamoxifen plus Norethisterone ($1x10^{-9}$M), Megace ($1x10^{-7}$M and $1x10^{-8}$M) or vehicle. The cells were then incubated for 96hrs at 37^{o}C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section3.1. Each data point represents cell growth expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

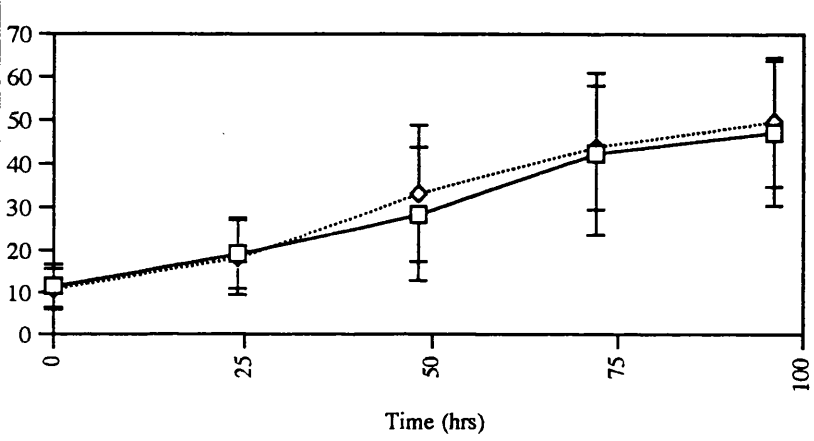
Error bars represent the Standard Deviation of the Data.

Summary of the Effects of Tamoxifen plus Progestin on the Growth of MCF-7 Cells









10.4 CONCLUSION

The growth curves in FIGURES 23 and 24 demonstrate that Tamoxifen alone or in the presence of a progestin has no effect upon the ptoliferation of MCF-7 or ZR-75 cells..

11 The Effects Of Tamoxifen plus Norethisterone on the Growth of ZR-75 and MCF-7 cells

11.1 INTRODUCTION

It has been established that when low dose Norethisterone ($1x10^{-9}M$) is used in combination with Tamoxifen on MCF-7 and ZR-75 cells, the cells do not experience growth inhibition. This is in contrast to the growth inhibitory effects mediated by Norethisterone ($1x10^{-8}M$ and $1x10^{-7}M$) on ZR-75 cells. Because of the differential effects of 1, 3 and $5x10^{-9}M$ Norethisteorone on PR synthesis it was decided to investigate whether an increase in Norethisterone dosage in the presence of Tamoxifen could overcome the proliferative effects of the "antiestrogen".

11.2 METHOD

Both cell lines were subcultured as described in EXPERIMENTAL section 2.2. After 24hours the cells were washed with PBS-A and each set of 10 wells were fed with phenol red free medium with 5% HIDCCFCS plus ethanol (as the control) or Tamoxifen or Tamoxifen plus Norethisterone; Tamoxifen ($1x10^{-9}M$) or Tamoxifen plus Norethisterone (1, 3 and $5x10^{-9}M$) . Samples were removed as described in EXPERIMENTAL section 7.2.

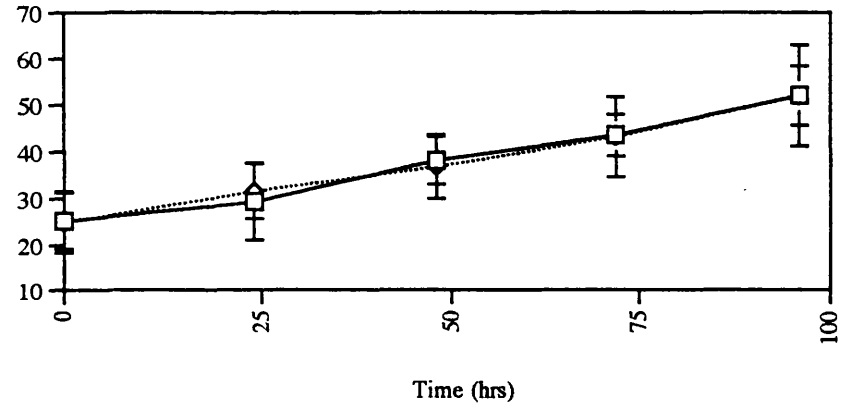
11.3 RESULTS

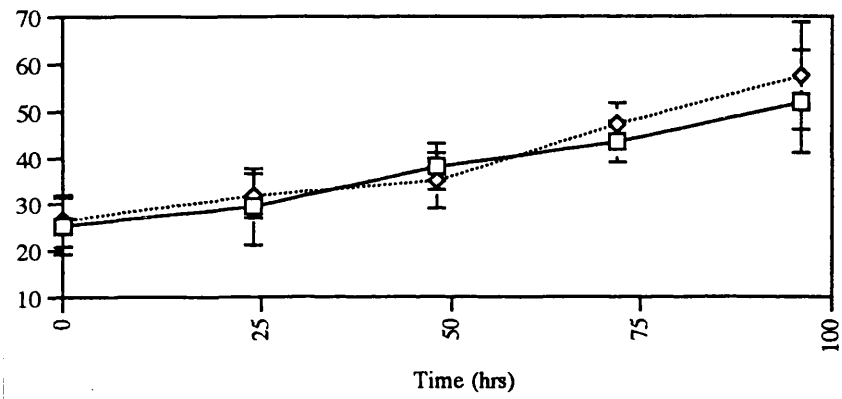
The results shown in FIGURES 25 and 26 represent the mean of 3 experiments, each experiment consisting of 2 wells of cells for each time point. The parameter being measured is cell growth and so the results are expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

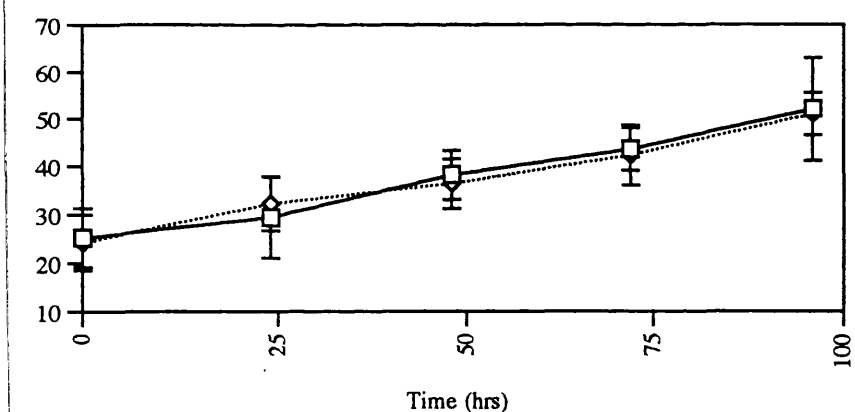
Fig.25 Effects of Tamoxifen plus Norethisterone on the growth of ZR-75 cells over 96hrs. ZR-75 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, Tamoxifen ($1x10^{-9}M$), Tamoxifen plus Norethisterone (1,3 and $5x10^{-9}M$) or vehicle. The cells were then incubated for 96hrs at 37°C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section 3.1. Each data point represents cell growth expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

Error bars represent the Standard Deviation of the Data.

Summary of the Effects of Tamoxifen Plus Norethisterone on the Growth of ZR-75 Cells







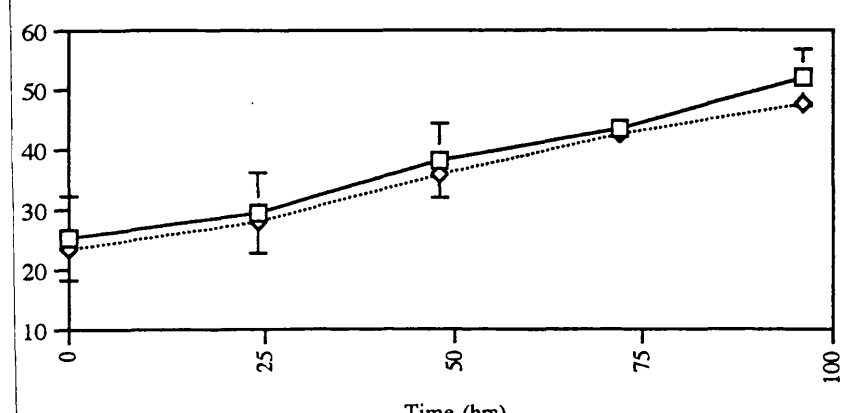
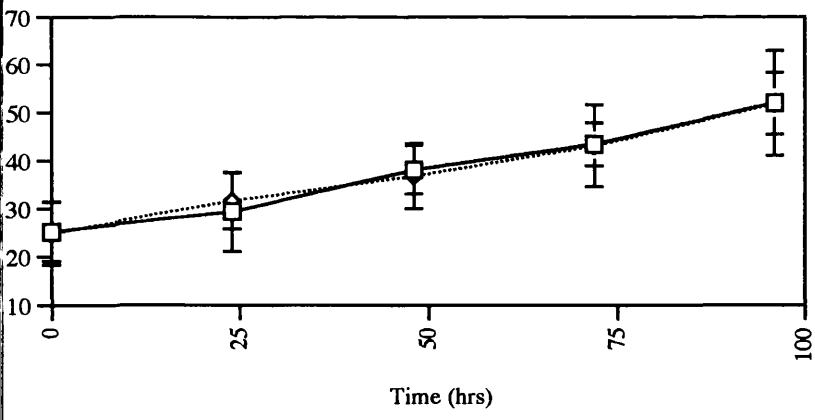
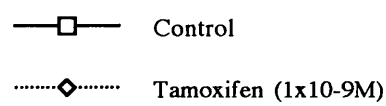


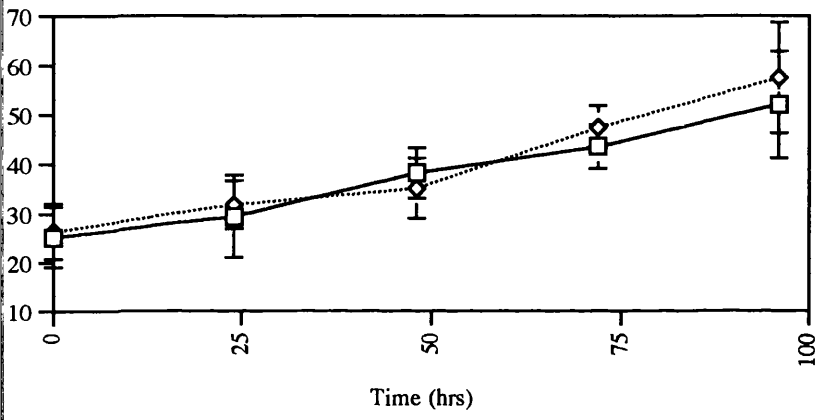
Fig.26 Effects of Tamoxifen plus Norethisterone on the growth of MCF-7 cells over 96hrs. MCF-7 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, ethanol (as the control), Tamoxifen ($1x10^{-9}$M), Tamoxifen plus Norethisterone (1,3 and $5x10^{-9}$M) or vehicle. The cells were then incubated for 96hrs at 37°C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section3.1. Each data point represents cell growth expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

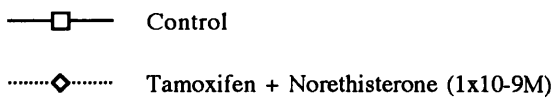
Error bars represent the Standard Deviation of the Data.

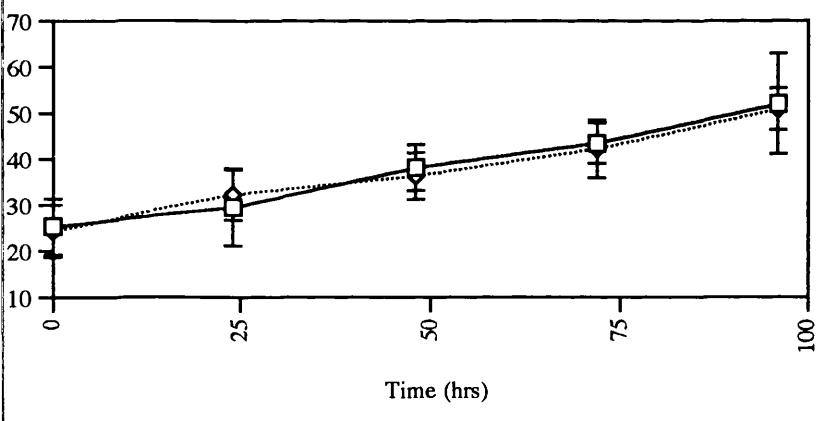
Summary of the Effects of Tamoxifen Plus Norethisterone on the Growth of MCF-7 Cells

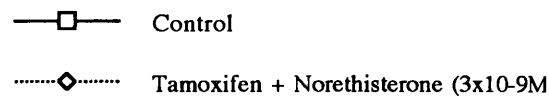


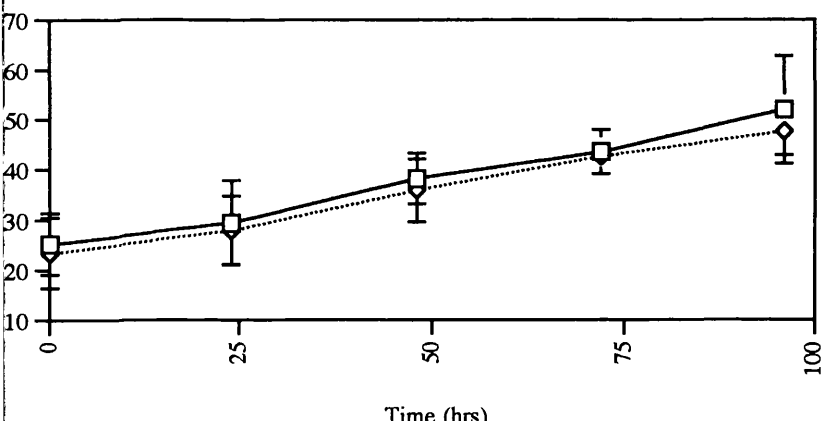


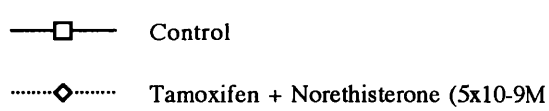












11.4 CONCLUSION

The data (figures 25 and 26) shows that exposure to Tamoxifen alone or in the presence of Norethisterone (1, 3 and 5x10-9M) has no effect on the proliferative rate of MCF-7 and ZR-75 cell lines

12 The Effect Of Inhibiting the Synthesis Of The Progesterone Receptor In The Presence Of A Progestin On Cell Growth

12.1 INTRODUCTION

Tamoxifen is a weak oestrogen agonist but the "pure" antiestrogen ICI 182,780 has no known agonist activity. ICI 182,780 exerts its biological activities by preventing the oestrogen receptor from acting as a transcription factor. Experiments were carried out to confirm that ICI 182,780 has no agonist growth activity in either cell line.

12.2 METHOD

Both cell lines were subcultured as described in EXPERIMENTAL section 2.2. After 24hours the cells were washed with PBS-A and each set of 10 wells were fed with phenol red free medium with 5% HIDCCFCS plus ethanol (as the control) or ICI 182,780 of ICI 182,780 plus Norethisterone; ICI 182,780 ($1x10^{-9}M$) or ICI 182,780 plus Norethisterone (1, 3 and $5x10^{-9}M$) . Samples were removed as described in EXPERIMENTAL section 2.2.

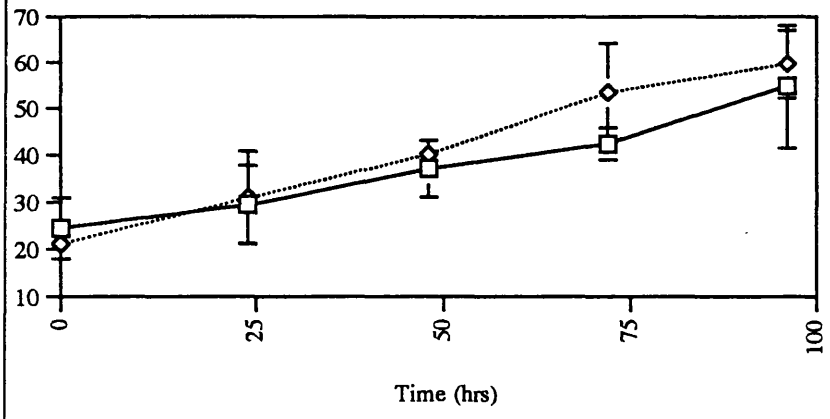
12.3 RESULTS

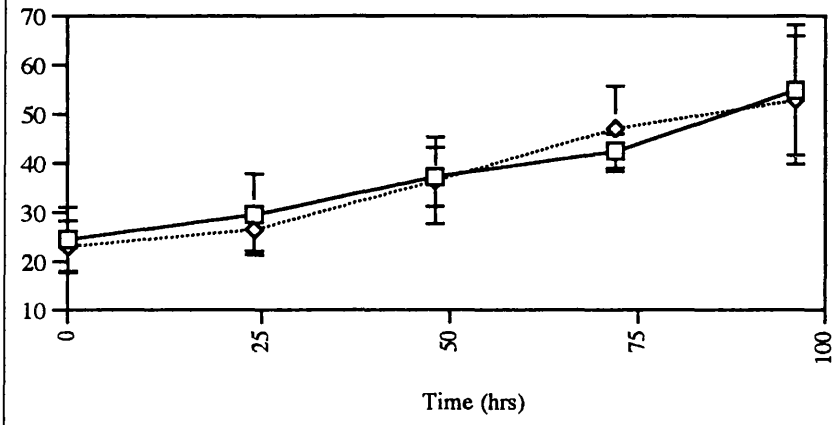
The results shown in FIGURES 27 and 28 represent the mean of 3 experiments, each experiment consisting of 2 wells of cells for each time point. The parameter being measured is cell growth and so the results are expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

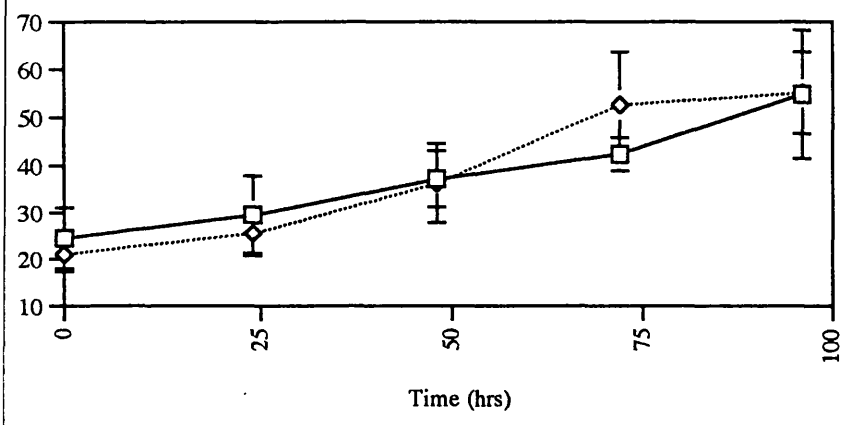
Fig.27 Effects of ICI 182,780 plus Norethisterone or Megace on the growth of ZR-75 cells over 96hrs. ZR-75 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, ICI 182,780 (1x10^{-9}M), ICI 182,780 plus Norethisterone (1x10^{-9}M), Megace (1x10^{-7}M and 1x10^{-8}M) or vehicle. The cells were then incubated for 96hrs at 37^{o}C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoechst assay as described in METHODOLOGY section 3.1. Each data point represents cell growth expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

Error bars represent the Standard Deviation of the Data.

Summary of the Effects of ICI 182,780 Plus Progestin on the Growth of ZR-75 Cells







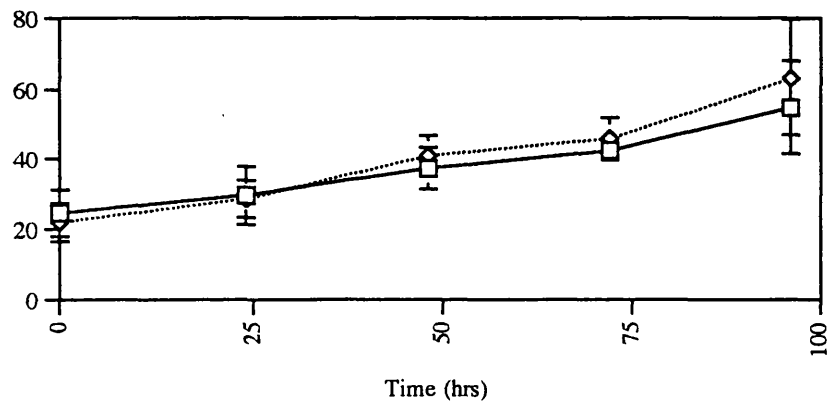
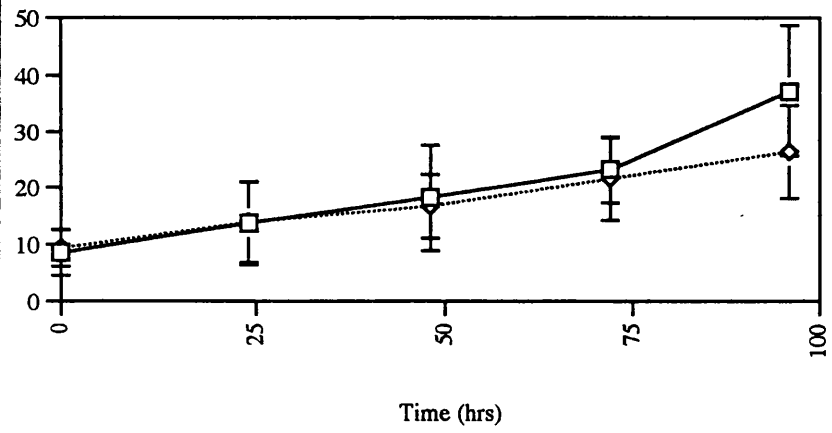
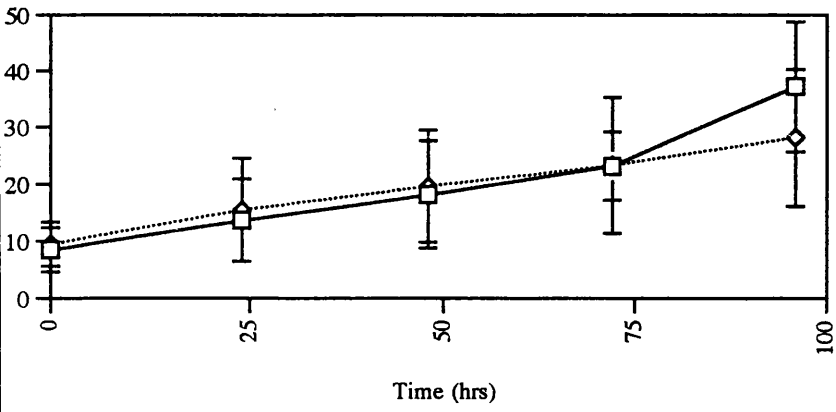


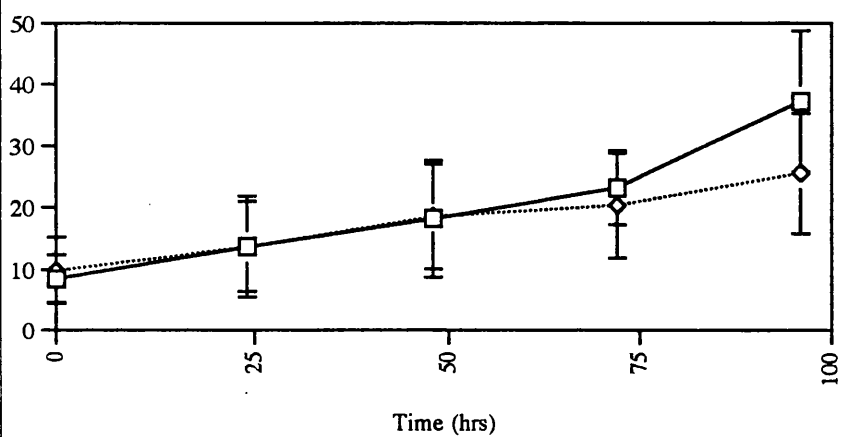
Fig.28 Effects of ICI 182,780 plus Norethisterone or Megace on the growth of MCF-7 cells over 96hrs. MCF-7 cells grown in 175cm^2 plates were reseeded into 24 well plates at a density of 17,500 to 22,500 cells/well before the addition of compound, ethanol (as the control), ICI 182,780 ($1x10^{-9}M$), ICI 182,780 plus Norethisterone ($1x10^{-9}M$), Megace ($1x10^{-7}M$ and $1x10^{-8}M$) or vehicle. The cells were then incubated for 96hrs at 37°C. Media containing compounds were changed after 2 days. Samples were removed every 24hrs and the DNA content was measured using the Hoeschst assay as described in METHODOLOGY section 3.1. Each data point represents cell growth expressed as the concentration of DNA (μg/ml) in relation to the duration of the experiment (hours).

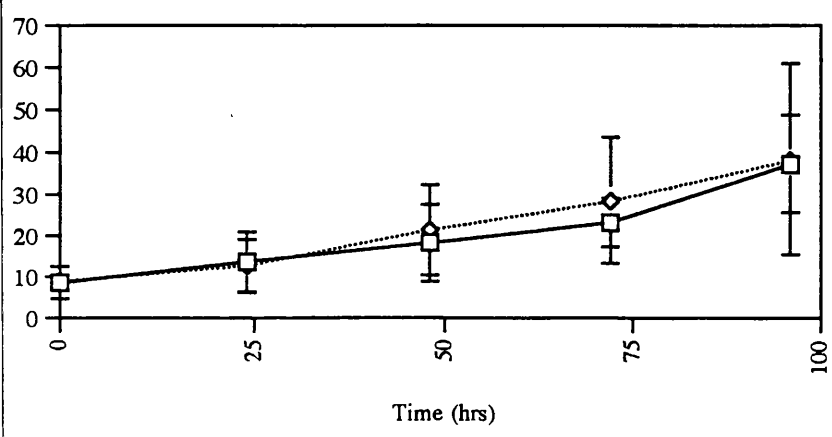
Error bars represent the Standard Deviation of the Data.

Summary of the Effects of ICI 182,780 plus Progestin on the Growth of MCF-7 Cells









Discussion

In this study, ' two point' competition progesterone receptor assays and growth time course experiments were used to investigate the effects of a gynaecological doses of Norethisterone (10^{-9}M) upon the *in vitro* regulation of the progesterone receptor. This dose of Norethisterone had no effect upon the proliferation of ZR-75 and MCF-7 cell lines. Norethisterone ($1x10^{-9}$M) did not alter the levels of PR in ZR-75 cells. Alternatively higher doses 5 x 10^{-9}M reduced the levels of PR. The effect of Norethisterone on PR messenger RNA levels was not investigated.

The results of Tamoxifen on PR in ZR-75 cells demonstrate that the induction of PR by Norethisterone $1x10^{-9}$M is comparable with that of Tamoxifen alone or with Norethisterone. Therefore these results demonstrate that low dose Norethisterone has the ability to be oestrogenic in nature, stimulating the synthesis of PR. However, higher concentrations of Norethisterone down regulate the synthesis of PR. The purpose of this dual control has not been established. Work by Wei *et al.*, (1988) suggests that the human PR autoregulates by binding to, and inhibiting transcription of the PR gene. If this proves correct then this explains why PR levels fall in the presence of Norethisterone ($5x10^{-9}$M). However, this does not provide an explanation why Norethisterone ($1x10^{-9}$M) manages to induce PR synthesis. It is possible that the shortening of receptor half-life only occurs at higher concentrations of progestin and so the low concentration appears to have a relatively positive effect on PR levels. To demonstrate that bound PR interfered with the transcription of its gene, it would be interesting to perform foot-printing assays on this region of DNA, in the presence of a range of doses of Norethisterone. This would provide data demonstrating which proteins are bound 5' to the PR gene during transcription and would define the role played by progestin bound PR. If there exists a type of "seesaw" control of the PR gene by progestins, then the concentration of the progestin needed for the induction of PR synthesis bound by PR may differ amongst cell lines depending on the basal level

of PR. Higher levels of basal PR may respond to lower levels of plasma progestin due to the ability of larger amounts of the unbound PR concentration to progestin within the cell.

Another possible explanation of the above results suggested by Jordan *et al* (1992) is that Norethisterone acts as a oestrogenic compound via the oestrogen receptor. This study used higher concentrations of progestin (10^{-7} M to 10^{-8} M), however, they demonstrated that the progestin Norethindrone could induce mitogenic effects. If this was a possible explanation for the induction of PR synthesis by Norethisterone ($1x10^{-9}M$) we would have expected the growth time course experiments of MCF-7 and ZR-75 cells to demonstrate cell proliferation greater than the control.

In the context of the study by Leake *et al.,* (1992) these results demonstrate that Norethisterone ($1x10^{-9}M$) does not affect the ability of Tamoxifen to induce the synthesis of PR. However, higher concentrations of Norethisterone significantly reduce this effect. Therefore, the dose of Norethisterone administered throughout the cyclical trial must be large enough to prevent vaginal bleeding in the post-menopausal patients but low enough so not to interfere with Tamoxifen's induction of PR synthesis.

Tamoxifen is administered to patients as an anticancer treatment which acts primarily via the oestrogen receptor. This antiestrogen demonstrates an ability to induce PR synthesis almost equivalent to Oestradiol. Tamoxifen's inability to interfere with the ER's induction of PR synthesis suggests that Tamoxifen's antiestrogenic properties lie outwith this phenomenon.

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