

**Collateral Resistance to Oestrogen and erbB Receptor Activated
Growth in Endocrine Resistant Breast Cancer**

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Declaration

I declare that the work present in this thesis is my own, except where otherwise stated.

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Abstract

Acquired resistance to endocrine therapy is a significant clinical problem in breast cancer. 'Cross-talk' between the ligand dependent and independent pathways, in particular the erbB receptor family, has been implicated in the development of resistance in ER α -positive breast cancer, with erbB2 overexpression occurring in approximately a quarter of cases. Overexpression of downstream signalling molecules Akt and ERK has also been implicated in carcinogenesis. The aim of this study was to evaluate the importance of 'cross-talk' in oestrogen insensitive and endocrine therapy resistant breast cancer.

A panel of breast cancer cells lines (MIII, LCC1, LCC2, LCC9, LY2) derived from the MCF-7 breast cancer cell line was used as a model to determine the signalling pathways which may contribute to the development of oestrogen independence and anti-oestrogen resistance. For ease of comparison to the parental oestrogen-dependent and anti-oestrogen-responsive MCF-7 cell line, the MIII, LCC1, LCC2, LCC9 and LY2 cell lines were termed 'resistant'. The MIII, LCC1 and LCC2 cell lines retained some degree of E₂ sensitivity.

Growth assays were performed and confirmed the phenotypes of the cell lines, where the resistant cell lines proliferated in the absence of stimuli. 1nM 17 β -oestradiol (E₂) significantly stimulated growth in MCF-7 and MIII cell lines on days 3, 5 and 7. For example, by day 7 1nM E₂ increased growth by ~63-fold and ~2-fold compared to day 7 control in the MCF-7 and MIII cell lines respectively (P<0.001). The growth of the LCC1 and LCC2 cell lines was only significantly enhanced after 5 days of treatment with 1nM E₂ (~1.4 and ~1.5-fold increases compared to day 5 control respectively, P<0.001). LCC9 cells were not growth stimulated by 1nM E₂ (days 3, 5 and 7, P>0.05). LY2 cells were not E₂ responsive at this concentration after 7 days. Resistant cell lines were insensitive to growth factors (TGF α and HRG β), while MCF-7 cells remained responsive (~11- and ~9-fold increases by day 7 compared to control respectively, P<0.001). MIII and LCC1 cells retained some tamoxifen sensitivity, while the remaining cell lines were unaffected. ER α expression was determined and 'cross-talk' was investigated by monitoring ER α activation via phosphorylation of serine residues 118 and 167 (P-S118/167) using western blotting.

MIII, LCC1 and LCC2 cell lines expressed more ER α than MCF-7 cells, which may account for elevated basal growth in these lines. The remaining cell lines expressed similar ER α levels to the MCF-7 cell line, hence another mechanism must account for elevated basal growth in these cells. ER α was subject to E₂ 'turnover' in all cells, indicating all cells contain functional ER α . ER α activation was then elucidated by observing P-S118 and P-S167. Of interest, E₂ significantly enhanced P-S118 in LCC1 cells to a greater extent than MCF-7 cells (~22-fold increase in the LCC1 cell line compared to ~5-fold increase, although both were considered to be significant compared to their relative controls and other treatments analysed simultaneously, P<0.001). Little or no P-S118 was observed in LCC9 cells irrespective of treatment. LCC1 and LCC9 cell lines were further investigated in comparison to MCF-7 cells as they displayed a progressive loss of E₂ and anti-E₂ sensitivity. No differences in P-S167 expression were observed between cell lines subject to control or E₂ treatment; HRG β enhanced P-S167 to an equal extent in all cells.

To investigate which upstream molecules may account for the changes in P-S118, the expression and activation of Akt, MEK and ERK were determined. Total levels of each of the three proteins were equivalent across the panel of cell lines. Akt was significantly constitutively phosphorylated in the LCC1 and LCC9 cell lines compared to the MCF-7 cell line (~2.2-fold increases for both cell lines compared to the MCF-7 cell line after 15 min control treatment, P<0.001), suggesting this pathway is important in the development of resistance. TGF α enhanced P-Akt to a similar final expression level in all three cell lines, with the constitutive activated Akt in the resistant cell lines producing a lower fold increase than that required in the MCF-7 cell line (~6-fold (P<0.001), ~1.5-fold (P>0.05) and ~1.8-fold (P<0.001) increases in the MCF-7, LCC1 and LCC9 cell lines respectively compared to control). HRG β also significantly enhanced P-Akt to a similar final level, although this was greater than that observed with TGF α , with increases of over ~30-fold (P<0.001) in the MCF-7 cell line, ~3.3-fold in LCC1 cells (P<0.001) and ~3.5-fold in the LCC9 cell line (P<0.01).

TGF α and HRG β increased P-MEK extremely significantly in MCF-7 cells (mean OD values of 1 and 5+/-0.2 arbitrary units compared to a control value of zero,

$P < 0.001$). $TGF\alpha$ significantly elevated the P-MEK in LCC1 cells by ~300-fold ($P < 0.001$, when analysed separately from HRG β data), and by ~43-fold in the LCC9 cell line ($P < 0.001$, when analysed separately separately from HRG β data). P-MEK expression was elevated by ~3000-fold and ~1500-fold with HRG β treatment in the LCC1 ($P < 0.001$) and LCC9 ($P < 0.01$) cell lines respectively when compared across several treatments. However, the relative HRG β -enhanced P-MEK expression was significantly reduced by ~1.8-fold between the MCF-7 and LCC1 cell lines ($P < 0.05$), and reduced further still between the LCC1 and LCC9 cell lines (~1.7-fold reduction, $P < 0.01$). The diminished signal was extremely statistically significant between the MCF-7 and LCC9 cell line (~3-fold reduction, $P < 0.001$).

However, expression of P-ERK, which is downstream of MEK, was equivalent across all three cell lines, indicating that P-ERK was not responsible for endocrine resistance in this model. Akt, ERKI/II and PLC γ were inhibited by PI3-Kinase inhibitor LY 294002, MEK inhibitor UO126 and U-71322 respectively in order to assess their role in the signalling and proliferation of these cells. LY 294002 and UO126 reduced cell proliferation via diminished P-Akt and P-ERKI/II respectively in a concentration-dependent manner in all cells. LY 294002 decreased cell proliferation to a greater extent than UO126 in all cells. HRG β had a 'protective' effect against either inhibitor, demonstrating some form of Akt dependence in all the cell lines. U-71322 was found to have oestrogenic-like action but this was disregarded as the metabolite of U-71322 is structurally similar to E_2 . Gene expression changes of a series of E_2 -responsive genes (ER α , PR, pS2, CTD) were investigated using qRT-PCR to monitor differences downstream of ER α . Generally, the resistant cell lines retained E_2 -sensitivity, but lost $TGF\alpha$ -sensitivity at the mRNA level, contrasting with MCF-7 cells.

Basal ER α mRNA levels were elevated in all three resistant cell lines compared to MCF-7 cells, with increases of ~4.3-fold ($P < 0.001$), ~2.2-fold ($P < 0.05$) and ~2.5-fold ($P < 0.01$) in LCC1, LCC9 and LY2 cell lines. Basal levels of pS2 mRNA were significantly elevated in all the resistant cell lines compared to MCF-7 cells (~19-fold ($P < 0.0002$), ~21-fold ($P < 0.0001$) and ~3.7-fold ($P < 0.0001$) higher in LCC1, LCC9 and LY2 cells respectively). Neither 10 μ M LY 294002 nor UO126 were able to

significantly affect basal or E₂ treated ER α mRNA expression in any of the four cell lines tested in this model. In contrast, UO126 significantly reversed pS2 expression when elevated by E₂ treatment in three of the four cell lines tested by ~2-fold (P<0.05) in MCF-7 cells, ~2.6-fold (P<0.001) in LCC1 cells, and ~1.7-fold (P<0.01) in the LCC9 cell lines. UO126 did not significantly affect pS2 expression in the LY2 cell line (P>0.05 comparison across all treatments).

The novel recombinant humanised anti-erbB2 monoclonal antibody 2C4 (2C4) was used to evaluate the importance of erbB2 and related signalling pathways in this model. 2C4 inhibited growth factor enhanced proliferation in MCF-7 cells via diminished P-Akt and P-ERKI/II activation as expected. 2C4 significantly reduced HRG β -enhanced P-Akt (~38- and ~33- fold reductions in LCC1 and LCC9 cells respectively, P<0.001 compared across treatments with and without 2C4 and control) whilst P-ERKI/II was abolished in the LCC1 cell line, and reduced by ~143-fold in the LCC9 cell line (P<0.001 across all 4 treatments conditions). This indicates these pathways may be partially responsible for some growth of these cell lines. However, 2C4 was unable to reduce resistant cell proliferation.

Initial results show tamoxifen appears to increase HRG β -enhanced P-Akt in MCF-7 and LCC1 cells by ~1.5-fold (P<0.05), but this increase was not observed in the LCC9 cell line. Tamoxifen significantly enhanced the proliferation of MCF-7 cells already increased by HRG β alone (P<0.01 compared to all other treatments). A ~0.9-fold (~92%, P<0.001) increase with the two agents was noted compared to a ~0.6-fold (~62%, P<0.001) increase when cells were treated with HRG β alone. This increase was significantly reduced when cells were pre-treated with 2C4 (P<0.001) to a level just above that produced by HRG β + 2C4. It should be noted that tamoxifen appears to be preventing the same extent of reversal as seen with 2C4 and growth factor alone (tamoxifen elevated the proliferation of this combination by ~16.5% compared to HRG β + 2C4 alone). This suggests tamoxifen interferes with the mechanism of action of 2C4. Apoptotic levels and cell cycle distribution were measured using FACS analysis. The resistant cells had a significantly increased proportion of cells in S-phase compared to MCF-7 cells (~30% and ~14% of LCC1 and LCC9 cells respectively are in the S-phase compared to only ~3% of MCF-7 cells (P<0.001 for

comparisons between all cell lines)). This may account for their elevated basal growth.

These data suggest that a combination of several factors including ER α and P-Akt overexpression, changes in P-MEK activation and P-S118 expression and an increased number of cells in S-phase contribute to the development and progression of resistance in this model.

Abbreviations

DCSS	=	Double charcoal stripped serum.
E ₂	=	oestrogen/ 17 β -oestradiol.
ER α	=	Oestrogen receptor α
HRG β	=	Heregulin β .
LCC1	=	MCF-7/LCC1 breast cancer cell line.
LCC2	=	MCF-7/LCC2 breast cancer cell line.
LCC9	=	MCF-7/LCC9 breast cancer cell line.
LY2	=	MCF-7/LY2 breast cancer cell line.
TGF α	=	Transforming growth factor- α .

- Figure 2. 5.** Schematic diagram depicting the flipping of phospholipids during apoptosis and the subsequent binding of annexin V molecules to the surface of the cell
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Chapter 1

Introduction

1. Introduction

Breast and ovarian cancer are the first and fourth most commonly diagnosed female cancers annually in the United Kingdom (U.K.) (<http://www.cancerhelp.org.uk/help/default.asp?page=143>). Oestrogen receptor (ER)-positive breast and ovarian cancer are hormone dependent. However, endocrine therapy resistance in ER-positive breast cancer remains a significant clinical problem for many patients, while ovarian cancers are often ER-positive and tend to be inherently resistant to endocrine therapy. One major theory as to why resistance to oestrogen and anti-oestrogens develops in these cancers is “cross-talk” between the oestrogen and epidermal growth factor (erbB) receptor signalling pathways. “Cross-talk” at the ER can be observed through specific serine residue phosphorylation. ER activation is associated with this phosphorylation. The purpose of this investigation was firstly to identify evidence of cross-talk *in vitro* using both breast and ovarian cancer model systems.

1. 1. Normal breast

The key role of a normal breast or mammary gland is the production of milk for the provision of nutrition to the young. The development of mammary glands begins during embryogenesis and is similar in both males and females until puberty. At puberty in females, mammary gland development is increased due to high levels of oestrogen produced by the ovaries, as well as elevated progesterone levels. The menstrual cycle, pregnancy and lactation all regulate the mammary gland through cycles of growth and involution (Mol *et al*, 2000). Further branching and end buds develop during pregnancy, which almost entirely regress post-partum through apoptotic cell death (Russo and Russo, 1987; Russo and Russo, 1994). The anatomy of a normal mammary gland is shown in figure 1. 1.

The shape of the breast is due to the fibrous septa system which attaches it to the skin which covers it, and the fatty deposits which are also enclosed (Marieb, 2006). A normal mammary gland contains between 15 and 20 lobes, and each lobe consists of a network of branched ducts which drain into the nipple (Marieb, 2006). The branched network of ducts lies on a fat pad which is attached to the chest wall by loose connective tissue and muscle. Lobules are distributed throughout the breast.

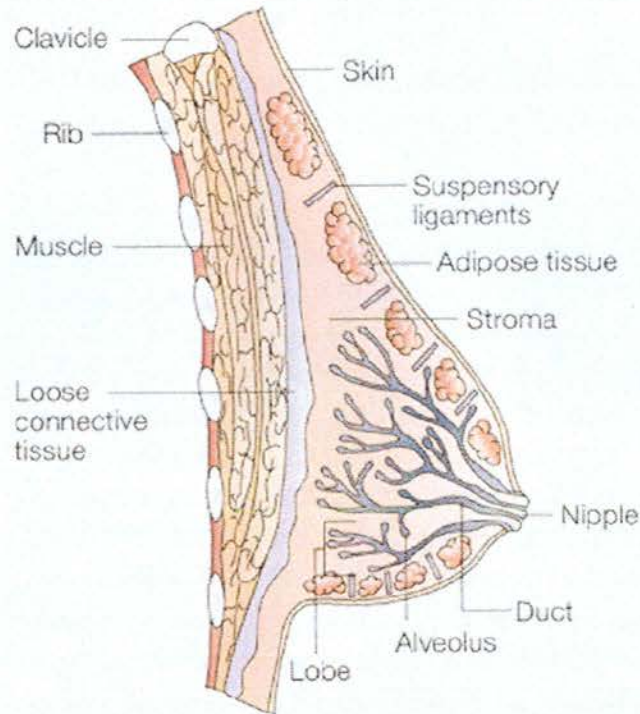


Figure 1. 1 The anatomy of a normal mammary gland. Each breast comprises of 15-20 lobes which are in turn made up of a network of branched ducts. The ducts drain into the nipple. The ducts and lobules rest in fibrotic stroma which lies on the chest wall above the ribs attached by loose connective tissue and muscle (reproduced from Ali and Coombes, 2002).

A continuous layer of epithelial cells line the surface of each duct, which are surrounded by an outer layer of myoepithelial cells. These cells possess contractile properties, but are not found in the lactiferous ducts close to the nipple. The glandular ducts are surrounded by fibroblast stroma. Stroma is the loose connective tissue which is separated from the epithelial compartment by the basement membrane. Together the epithelial cells, myoepithelial cells, and fibroblasts form the ducts, with the former being the site of oestrogenic action. The fibroblasts may also be involved in the oestrogen-induced breast development. During carcinogenesis, the fibroblast stromal tissue is drastically altered. Degradation and/or a reduction of synthesis of the basement membrane occurs. Direct contact between tumour cells and the surrounding stroma occurs simultaneously with inflammatory cell influx, neovascularisation and extensive remodelling of the extracellular matrix (Ronnov-Jessen *et al*, 1996).

1. 2. Normal Ovary

The ovaries form part of and play a significant role in the female reproductive system. They naturally produce oestrogen and progesterone and are responsible for breast development, body shape and egg production. The ovaries are dull white oval masses (3 x 2 x 1cm) of ~5-8g which lie in small depressions of the posterior wall of the broad ligament on either side of the peritoneal cavity just above the brim of the pelvis. They are connected to the fallopian tubes at the fimbriated ends and are enclosed in the tunica albuginea, a tough fibrous capsule. Each ovary consists of an outer cortex and an inner medulla. The outer cortex contains the follicles (including the remains of ruptured follicles) which are embedded in vascular fibrous tissue. The blood vessels, nerves and lymphatics enter the ovaries via the inner medulla (Marieb, 2006). Figure 1.2 depicts the structure of the female reproductive organs including the ovaries and an internal view of an ovary and the ovulatory cycle.

The ovaries control puberty and the menstrual cycle and stimulate the production of luteinising hormone (LH) and suppress the production of follicle stimulating hormone (FSH) in the pituitary gland. The ovaries require both LH and FSH to produce sex steroids. The thecal cells surrounding the follicle are stimulated into producing progesterone and androgens by LH. The androgens diffuse through to the granulosa cell layer via the basement membrane where they are converted under the action of FSH to oestrogens, mainly oestradiol. This enables the dominant developing follicle to fully mature (Marieb, 2006; Nussey and Whitehead, 2001).

Prior to puberty the ovaries are smooth, solid glands containing many primordial follicles. The surface of the glands becomes more corrugated between puberty and menopause as a result of the activity of the ovary during each ovarian cycle. The ovaries are shrunken and are scarred after the menopause due to these monthly follicular ruptures. Many cycles of ovulation are associated with repetitive disruption and repair of the ovarian surface epithelium. The stimulation of epithelial cells may increase the probability of spontaneous mutations in proto-oncogenes or tumour suppressor genes which may contribute to oncogenesis. It is also possible that inclusion cysts are formed when epithelial cells are trapped within the stroma, which subjects the epithelial cells to an alternative environment. Therefore, epidemiological studies have suggested that the number of ovulatory cycles is an important

contributory factor in the development of ovarian cancer (Nussey and Whitehead, 2001). The risk factors associated with ovarian cancer will be discussed further in section 1.4.5.

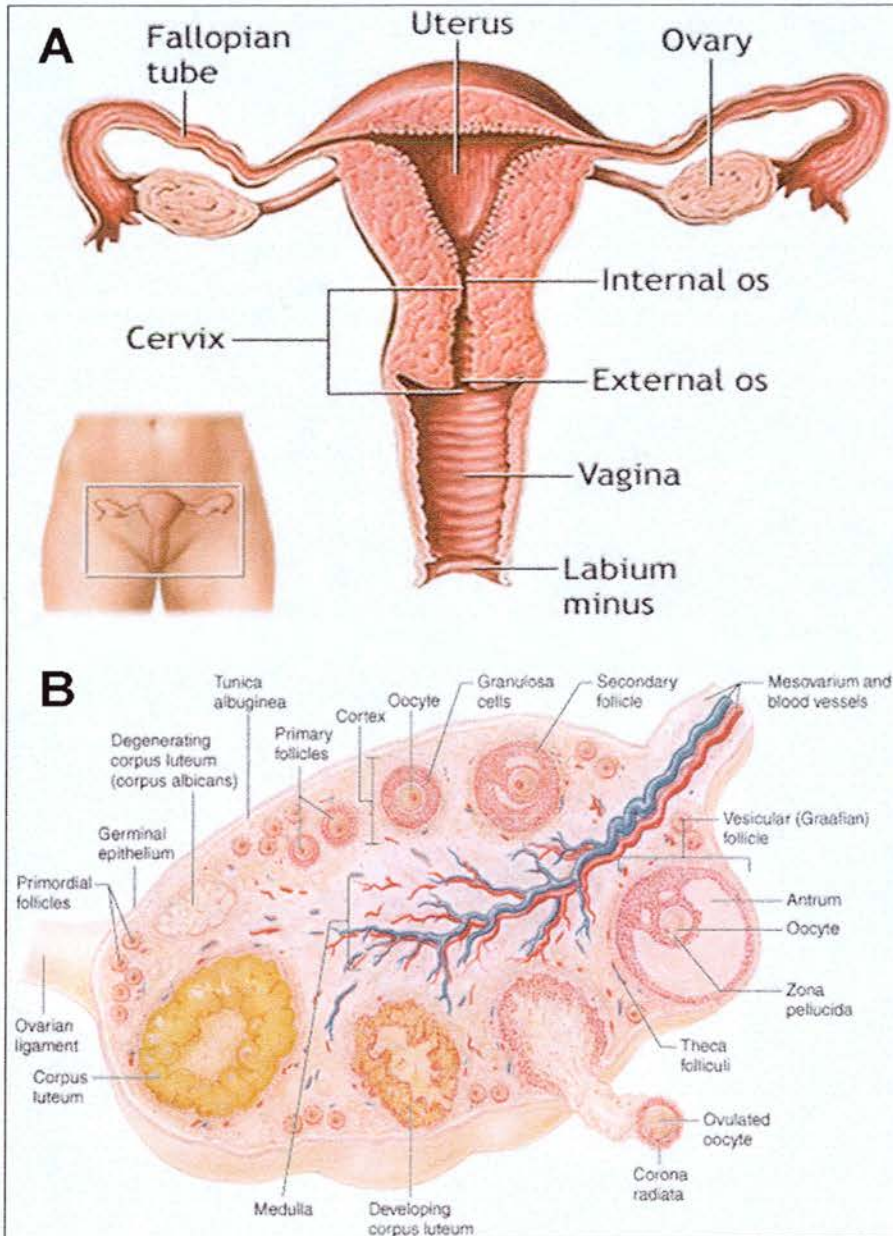


Figure 1.2 The structure of the female reproductive organs including the ovary and the stages of the ovulatory cycle. **A**, the structure of the female reproductive organs (reproduced from www.cdc.gov/cancer/nbccedp/bccgif/cc_basic.gif) **B**, the ovary and the ovulatory cycle. The cycle is shown from the development of primary follicles to ovulation and the degeneration of the corpus luteum (image reproduced from www.lakemichigancollege.edu/.../anat/reprod.html).

1. 3. Breast Cancer

1. 3. 1. Incidence and Survival Rates

Breast cancer accounts for 1 in 4 of all female cancers in the Western World, is the most frequent cancer in women and is the second leading cause of cancer death in women after lung cancer in the United States (U.S) (Stewart *et al*, 2004). It is the leading cause of death for women between the ages of 40-55 years in the U.S. A little under 42, 000 women and 300 men are diagnosed with breast cancer annually in the U.K. It is the most prevalent cancer in the U.K. as a whole, excluding non melanoma skin cancer. The majority of women who develop breast cancer are postmenopausal, however almost 8, 000 women under 50 years of age are diagnosed each year (U.K statistics reviewed at <http://www.cancerhelp.org.uk/help>).

There is a lifetime risk of developing breast cancer of 1 in 9 for women in the U.S.A and U.K (www.BreastCancerInfo.org/breast-cancer-riskfactors.html) and the incidence increases with every passing decade after 40 years of age (Lindley, 2002). The overall survival rates 5 years post-treatment are dependent on the tumour stage. The stage at which a tumour is diagnosed predicts the outcome and survival of the patient, with stage I having an overall 5 year survival of 84-93%, which falls dramatically to 18% in woman diagnosed with stage IV breast cancer (Taucher & Jakesz, 2004). Breast cancers diagnosed at stages I and II are considered curable, whilst those diagnosed at stage III show a decline in disease free survival, which falls to almost a zero value for those patients diagnosed with stage IV breast cancer (Lindley, 2002).

1. 3. 2. Epidemiology

There are several forms of breast cancer which can be categorised into stages and will be discussed in this section. **Lobular carcinoma *in situ***, (LCIS, figure 1. 3A) is confined to the lobules and is generally considered to be a **pre-cancerous** condition which arises in the skin or other tissues that cover the internal organs. Breast cancer arises through hyperproliferation of epithelial cells, progressing through **ductal carcinoma *in situ*** (DCIS, a preneoplastic phase, figure 1. 3B) to invasive breast cancer, where the basement membrane is breached (Fentiman and D'Arrigo, 2004).

The several forms of breast cancer include DCIS, invasive ductal carcinoma, invasive lobular carcinoma, inflammatory breast cancer, Paget’s disease and male breast cancer.

DCIS may cover a small or large area of the breast, but does not spread outside the ducts and is occasionally termed “pre-cancer” or “tumour in situ” (Tis) because of this. Approximately 20% of all breast cancer cases are classified as DCIS each year in the U.S (Adamovich and Simmons, 2003).

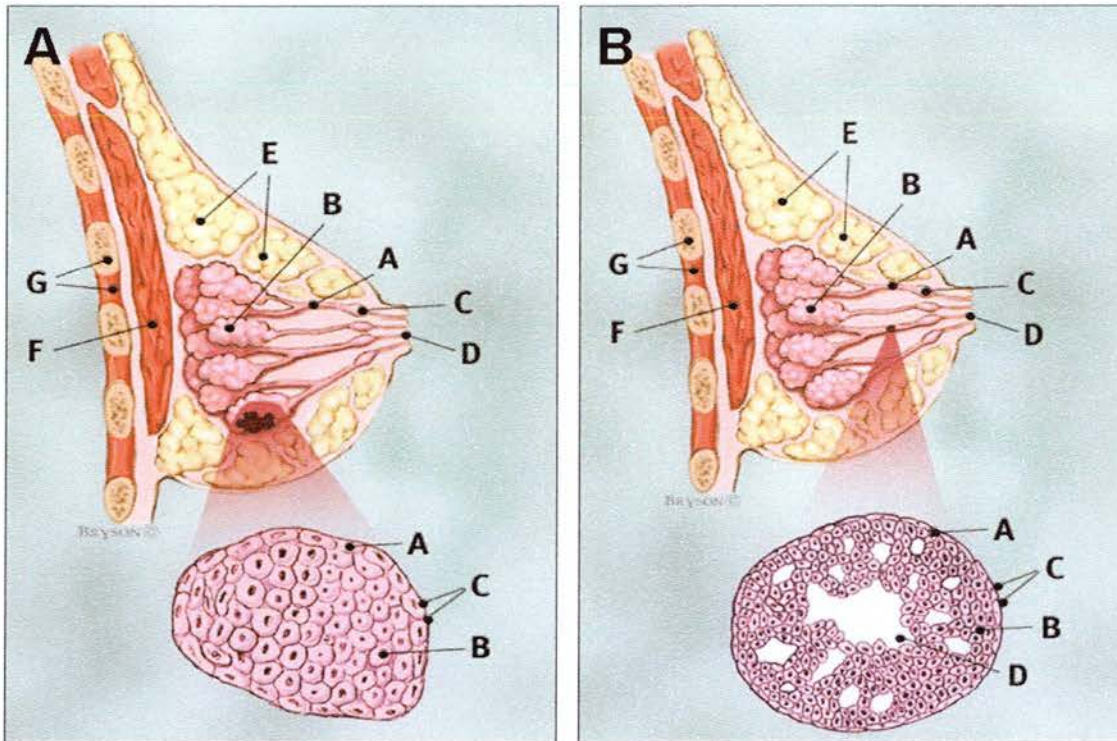


Figure 1.3 Normal breast with (A) lobular carcinoma in situ (LCIS) and (B) non-invasive ductal carcinoma in situ (DCIS) in an enlarged cross-section of the lobule; Breast profile: A Ducts, B Lobules, C Dilated section of duct to hold milk, D Nipple, E Fat, F Pectoralis major muscle, G Chest wall/rib cage Enlargements: A; A Normal lobular cells, B Lobular cancer cells, C Basement membrane; B; A normal duct cells, B ductal cancer cells, C basement membrane, D lumen (centre of duct) (reproduced from www.breastcancer.org).

Approximately 70-80% of breast cancers are diagnosed as **invasive ductal carcinoma (IDC)**, and can be further subcategorised into tubular, cribriform, mucoid (or mucinous) or papillary, although the majority of cases that present are diagnosed as of ‘no specific type’ (NST). Approximately 10% of breast cancers are classified as **invasive lobular carcinoma (ILC)**, and develop in women of any age, with the most

diagnoses in women between 45 and 55 years of age. ILC is difficult to diagnose by mammography as it does not always form a firm lump and may present instead as a thickened area of breast tissue. Therefore this may lead to ILC be of a larger size at diagnosis. **Paget's disease** constitutes ~1-2% of cases annually and is often mistaken for eczema as the symptoms are a scaly red rash of the skin of the areola and nipple. This can sometimes be itchy and if disturbed can bleed, ulcerate and scab over. Approximately 50% of patients diagnosed with Paget's disease, via a biopsy and mammogram, will have a cancerous lump or mass in the tissues behind the nipple, of which ~90% of cases are invasive. Four in ten patients diagnosed with Paget's disease have invasive breast cancer, but the majority of cases are DCIS.

1. 3. 3. Stages of breast cancer

Edlich *et al* (2005) report that a clinical examination and mammography are essential in detecting breast cancer. There is a universal staging system which is in operation for the classification of the extent of cancer once detected. The staging system enables physicians to understand and treat a variety of cancers worldwide, including that of the breast, and also allows the patient to have an understanding of their prognosis. The staging system when applied to breast cancer is discussed in depth at the www.breastcancer.org website.

Stage 0 applies to non-invasive breast cancer, thus DCIS is classified as Stage 0 breast cancer as it confined to the ducts and does not spread to the lymph nodes or other organs. **Stage I** cancer describes the disease when it has become invasive and the tumour measures up to 2cm but has not spread to the lymph nodes. **Stage II** breast cancer describes a tumour which is between 2 and 5cm in size, or the tumour has spread to the lymph node under the arm nearest the breast tumour which can be of any size.

The **Stage III** classification is further divided into Stages IIIA and B. **Stage IIIA** is applicable to cancers where the tumour is invasive and is larger than 5cm or the tumour has spread to the lymph nodes and the surrounding tissues and nodes are sticking to one another and clumping together. **Stage IIIB** breast cancer is invasive and has spread to the breast skin, internal mammary lymph nodes or chest wall. This

sub classification includes the uncommon but very aggressive **inflammatory breast cancer**, which manifests as redness of part or all of the breast and symptoms may include ridges and welts.

Stage IV, or advanced breast cancer is where the cancer is invasive and has spread beyond the breast, underarm, and internal mammary lymph nodes. The tumour may have metastasised to the supraclavicular lymph nodes (nodes located at the base of the neck), lung, brain, bone or liver.

1. 3. 4. Causes and risk factors of breast cancer

The majority of cases of breast cancer arise sporadically, however, there are a number of genetic and environmental factors which have been shown to affect the risk of occurrence. The epidemiological risk factors for breast cancer are reviewed by Okobia and Bunker (2005) and Dumitrescu and Cotarla (2005). This section details some of the risk factors associated with the development of breast cancer and table 1. 1. summarises the majority of these factors in order of magnitude of risk.

1. 3. 4. 1. Endogenous oestrogens

Oestrogens and other hormones play a crucial role in female sexual organ development and secondary sex characteristics, reproduction and regulation of the menstrual cycle (Greenstein, 1994). Oestrogen is a steroid hormone produced by the ovaries and the most abundant circulating form is 17β -oestradiol, or E_2 . It was first reported by Beatson (1896) that remission occurred in a proportion of women with metastatic breast cancer who had their ovaries removed. Subsequently, Jensen and Jacobson (1960; 1962) demonstrated using tritium labelled 17β -oestradiol that oestradiol was specifically retained by oestrogen target tissues, leading to the theory that an oestrogen-specific receptor must be present (the oestrogen receptor will be discussed in a later section). Oestrogen and other steroid hormones in the blood are bound largely with a high affinity to the sex hormone binding globulin, while the remainder are non-specifically bound to albumin with a low affinity. A small percentage of the hormone is in free circulation, which has been shown to diffuse readily into cells.

Abnormal oestrogenic signalling has been well documented over a long period of time as having a known association with breast cancer (reviewed by Russo and Russo, 1998). There is a strong hormonal aspect to breast cancer aetiology (Pike *et al*, 1993), where oestrogen exposure over a lifetime may be a critical factor in breast carcinogenesis (Pike *et al*, 1993 and reviewed by Okobia and Bunker, 2005). The risk of breast cancer rises less dramatically with age following the loss of ovarian function at menopause or due to a bilateral oophorectomy (Eerola *et al*, 2002), and this indicates that hormone production by the ovaries is a key breast cancer risk factor (Travis and Key, 2003). This is also supported by the fact that the disease does not occur before the onset of puberty.

The exact **mechanism** by which oestrogen influences the risk of developing breast cancer has not been fully established. Several mechanisms have been speculated. It has been hypothesised that oestrogens promote breast cancer via non-ER-mediated action of some of its metabolites. Cavalieri *et al* (1997) found evidence to indicate that catechol-oestrogens, hydroxylated products of oestrogens, can be undergo a conversion to quinones which cause DNA adduct formation, although this was only demonstrated in rodents. It is not entirely clear why the action of these and other carcinogens is restricted on the whole to the breast and uterus in humans.

It may be that these tissues have an increased level of oestrogen-metabolising enzymes (Clemons and Goss, 2001). Indeed, a four-fold increase in the risk of developing breast cancer was found to be associated with the genotype for polymorphism of catechol O-methyltransferase, an enzyme responsible for catechol oestrogen metabolism (Huang *et al*, 1999).

The increased risks of breast cancer due to oestrogen exposure may also simply be as a result of oestrogen's proliferative effects on the breast, as it is certain in a large number of individuals that oestrogen stimulates the progression of breast cancer by inducing malignant cell proliferation (Girdler and Brotherick, 2000). The fact that ER-positivity in breast tumours correlates with response to endocrine therapy sustains this argument.

Table 1. 1. Summary of Breast Cancer Risk Factors

Risk Factor	Risk	Reference
Early onset of menarche	~10-20% increase	Berkey <i>et al</i> , 1999
Late onset of menopause	Increased risk	Berkey <i>et al</i> , 1999
Menstrual irregularity	Increased risk	Okobia and Bunker, 2005
Female sex itself	99 in 100 cases are in women	Okobia and Bunker, 2005
Nullparous or 1 st full-term pregnancy after 30	Increased risk cf. pregnancy before 20 years	Berkey <i>et al</i> , 1999
Hormone Replacement Therapy (HRT)	5 extra cases per 1000 E ₂ alone 19 extra cases per 1000 with combined treatment	Million Women Study Collaborators, 2003
Oral contraceptives	Slightly elevated risk (although slight discrepancies as discussed by Travis and Kay, 2003)	Collaborative Group on Hormonal Factors in Breast Cancer, 1996
Increasing Age	2-fold increase with every decade	Lindley, 2002
<u>Lifestyle</u>		
High fat diet	Increased risk	Li <i>et al</i> 2005
Obesity	30% increased risk in obese postmenopausal women	International Agency for Research on Cancer, 2002;
Physical inactivity	Increased risk	International Agency for Research on Cancer, 2002
Smoking	~20% increase in risk	Baptista <i>et al</i> 2006
Excess alcohol intake	7.1% increase relative risk per extra alcoholic unit consumed on a daily basis	Silva, 2002
Race/ethnicity	Caucasians/blacks 2 x risk cf. Asians	Ziegler <i>et al</i> /1993
Having had breast cancer previously	5-fold increase risk cf. general population	www.cancerhelp.org.uk
<u>Genetic Factors</u>		
p53 mutation	~2-fold increased risk	Rohan <i>et al</i> 2006
BRCA1/2	50-85% increased risk	The Breast Cancer Linkage Consortium, 1999
Family history	~2-fold increase if close relation diagnosed with disease	www.cancerhelp.org.uk

In addition, ER has been suggested to be involved in the early processes of breast cancer as benign breast epithelium has a higher ER expression in breast cancer patients than those who do not have the disease (Khan *et al*, 1998). Therefore, although not fully understood, it may be possible that this is linked with the deregulated expression of ER in preneoplastic breast cancer cells.

Approximately one third of breast cancer cells are ER α -positive, and these proliferate in response to oestrogen (Cordera and Jordan, 2006). Stimulation of ER α -positive breast cancer with steroid hormones such as oestrogen results in an increase in cell proliferation and invasion (Osborne, 1998), with breast cancer cells themselves able to synthesise oestrogens (Bulun *et al*, 1993; Zhao *et al*, 1996). Therefore there is a link between the expression of ER and the development of breast cancer. The exact mechanism by which oestrogen signals via ER α will be reviewed in a later section (1.4).

There are several well-recognised risk factors which are linked with this hormonal risk including the **female sex** itself and reproductive characteristics including **age at menarche and menopause**, **menstrual irregularity**, and **age at first and last childbirth** (reviewed by Okobia and Bunker, 2005). **Breastfeeding** and **parity** have also been linked to the development of breast cancer. The early onset of menarche and a late menopause are linked with an elevated lifetime exposure to oestrogens. Specifically, an extended time period from Tanner stage 2 of breast development (breast budding with widening of the areola) to onset of ovulatory cycles, a long time frame of luteal inadequacy and anovulatory cycles which are characteristic of the years of perimenopause, harbour extended oestrogen time frames favoured for carcinogenesis. It has been hypothesised that oestrogens and progesterones may act in conjunction to stimulate the proliferation of cells (Raafat *et al*, 2001).

Berkey *et al* (1999) reported an increased risk of breast cancer of ~10-20% with the early onset of menarche, with the likely cause being a prolonged exposure of the breast epithelial cells to oestrogens and progesterone as a result of earlier ovulatory menstrual cycles (Bernstein, 2002). Adolescent women who had early menarche (less than 12 years of age) also had elevated levels of oestradiol during this time compared to those who had started their menstrual cycles at approximately 14 years

of age. Elevated levels of oestrogen may also be due to higher follicular, but not luteal, phase oestradiol levels and reduced sex-hormone-binding globulin (SHBG) in those individuals post adolescence. This results in oestradiol becoming more bioavailable allowing diffusion into the breast tissue. An increased number of menstrual cycles also results in an increased exposure to oestrogen, hence late menopause is associated with an increased risk of breast cancer.

The protective effect which has been observed with early age first full term pregnancy, parity and lactation may be due to the intense differentiation of the terminal duct lobule and the release of various hormones, paracrine and autocrine growth factors which occur during lactation. Xenoestrogens have thus been suggested to have a protective role. Miscarriage or induced abortion do not represent substantive risk factors for the future development of breast cancer (Brewster *et al*, 2005).

1. 3. 4. 2. Exogenous oestrogens

Exogenous oestrogens play a crucial role in the management of many conditions with the main sources of exposure arising from hormone replacement therapy (HRT) and the oral contraceptive pill. HRT is used in the management of menopausal symptoms. HRT is currently taken by ~33% of British women between 50 and 64 years of age either as oestrogen alone or in combination with progestins (Million Women Study Collaborations, 2002), while over 200 million women worldwide using the oral contraceptive pill (Beral *et al*, 1996).

1. 3. 4. 2. 1. Hormone replacement therapy (HRT)

Oestrogen exerts multisystemic effects, which include beneficial effects on blood lipids leading to a possible reduction in atherosclerosis with direct effects on the arterial wall endothelial tissue (Hodis *et al*, 2001). Hormone replacement therapy (HRT), usually in the form of synthetic 17 β -oestradiol derivatives (ethinyl estradiol or the 3-methyl ether form, mestranol), has long been prescribed in the management of menopausal women with deleterious affects of the loss of circulating oestrogens.

However, the use of combined oestrogen and progesterone HRT over a long duration of time (10 years) has shown to significantly increase the risk of developing breast cancer (www.cancerresearchuk.org). A study by the Million Women Study Collaborators (2003) reported that current users of all variations of HRT such as oestrogen only, oestrogen and progesterone combined, and tibolone, a synthetic hormone treatment, had an elevated risk of breast cancer than women who had never received HRT. The combined treatment was found to elevate the risk to a greater extent than oestrogen alone, with 19 cases for every 1000 post menopausal women taking the combined therapy as opposed to an extra 5 cases with oestrogen only HRT. The risk increased with longer term use of HRT, but declined within a few years of cessation of the treatment, and returned to normal after 5 years.

1. 3. 4. 2. 2. Oral contraceptives

Oral contraceptives have also been associated with an elevated risk of breast cancer. A report combining 54 studies of the use of the combined contraceptive pill (oestrogen and progesterone) in relation to breast cancer risk found a slightly elevated risk in women who were currently taking the pill, or those who had used them in the past decade (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). The linked cancers tended to be localised to the breast. The risk was deemed insignificant where the individual had ceased to take the pill 10 or more years prior to breast cancer diagnosis. Several later studies are generally in agreement with these conclusions, however some discrepancies still exist and alterations to the formulation of the combined contraceptive pill highlight the need for further research (Travis and Key, 2003).

1. 3. 4. 2. 3. Phyto-oestrogens

Phyto-oestrogens are naturally occurring plant compounds, or metabolites of these compounds, which exert weak oestrogenic action, or modulate the actions of a more potent endogenous oestrogen, more than likely via by binding to oestrogen receptors (UK Food Standards Agency Committee on Toxicity, 2002). The active element to the compound is believed to be isoflavone (www.cancerhelp.org.uk). It has been hypothesised that food rich in phyto-oestrogens, in particular soybeans, have a protective effect as breast cancer rates are lower in most Asian countries where this

diet is prevalent compared to Western Europe and the U.S (UK Food Standards Agency Committee on Toxicity, 2002). Thus this section also relates to **diet** and **geographical location** (country of origin) (see relevant sections for further details). It has also been suggested that phyto-oestrogens might decrease the risk by reducing the number of genotoxic metabolites via re-directing oestrogen metabolism or through non-oestrogenic pathways (Xu *et al*, 2000). Studies into the protective effect of phyto-oestrogens in humans remain inconclusive (Keinan-Boker *et al*, 2004) hence further investigation is necessary to make an accurate assessment of their role.

1. 3. 4. 3. Other hormones

Elevated circulating levels of the hormone **prolactin** have been found in nulliparous rather than parous women and this may be associated with higher breast cancer risk. In support of this, Perks *et al* (2004) reported that prolactin protected human breast cancer cell lines from apoptosis, which may have significant implications for cancer treatment. **Testosterone** may play a role in the aetiology of breast cancer (Lillie *et al*, 2003), where a pooled study of postmenopausal women estimated that the risk of breast cancer was more than double in women who were exposed to higher levels of testosterone (top quintile versus lowest quintile) (Endogenous Hormones and Breast Cancer Collaborative Group, 2002)

1. 3. 4. 4. Age

The incidence of breast cancer dramatically increases with age, doubling every 10 years until menopause, whereupon the rate slows. Approximately 25% of breast cancer develops in women under the age of 50 years, around 50% between 50 and 69 years and the remainder affect women over the age of 70 years (<http://www.netdoctor.co.uk/diseases/facts/breastcancer.htm>). The highest incidence rates and relative breast cancer risk among women carrying the BRCA1 occur before age 50. However, the risk of breast cancer as a whole actually diminishes dramatically with age following the loss of ovarian function at menopause or due to a bilateral oophorectomy (Eerola *et al*, 2002), but this is more than likely due to the loss of exposure to oestrogen as the ovaries are crucial to the production of the hormone as discussed in the previous section.

1. 3. 4. 5. Lifestyle

There are many factors which have been linked with the prevention or development of breast cancer. For example, most studies have reported a 20-40% reduction in the risk of breast cancer in those who are the most **physically active** (International Agency for Research on Cancer, 2002). This data is supported by Berstein *et al* (2005) who found an inverse association between physical activity and breast cancer among black and white women. **Excess alcohol intake** also increases the relative risk of developing breast cancer by 7.1% for each extra unit (alcoholic drink) consumed on a daily basis (10 g/day) (Silva, 2002). Li *et al* (2005) observed that the recency, length, and intensity of **smoking** were all associated with a modest increase in the risk of breast cancer, while the following of a **high-fat diet** can elevate the risk of developing breast cancer as excess fat can cause oestrogen-retention and the conversion of other hormones into a form of oestrogen.

Obesity has been indicated as a risk factor in breast cancer (International Agency for Research on Cancer, 2002; Tehard and Clavel-Chapelon, 2006), with the risk for an obese postmenopausal woman (body mass index (BMI) of more than 30kg/m²) being 30% higher than that of a normal woman with a BMI of less than 25kg/m². The elevated risk in post menopausal women is potentially due to the association between BMI and endogenous oestrogen concentrations as the latter is dependent on the extraglandular production of oestrogen in the adipose tissue (Travis and Key, 2003).

Contrastingly, premenopausal women do not share the same elevated risk with obesity and the development of breast cancer, in fact there is a link with a slight reduction in risk (International Agency for Research on Cancer, 2002). The majority of oestrogen production takes place in the ovaries and levels are homeostatically regulated by a negative feedback loop regulating by gonadotropins such as follicle-stimulating and lutenising hormones and are hence independent of adipose tissue. The reduction in breast cancer risk may be mediated by a decrease in exposure to ovarian progesterone via an increase in the number of anovulatory cycles. This decreased risk at an early age may also reduce the increased breast cancer risk observed in obese postmenopausal women (Hankinson and Hunter, 2002).

1. 3. 4. 6. Geographical location

Geographical location has a significant affect on the risk of breast cancer, with the most marked differences occurring in post menopausal women (risk factors reviewed by Dumitrescu and Cortarla, 2005). This is important as postmenopausal women in the westernised world tend to be more overweight than those in less industrialised countries (Pathak *et al*, 2000; Brinton *et al*, 1988). This factor was theorised to account for the majority of the difference in breast cancer rates occurring in Asian-American women after migration to America from China or Japan (Ziegler *et al*, 1996). Breast cancer studies have shown that migrants adopt the breast cancer risks of their destination country. For example, migrants who move from a country of low incidence, such as an Asian country, to a country of higher incidence such as the U.S have an increased risk of breast cancer as little as a decade after emigrating. These data highlight the importance of environmental and sociocultural elements in breast cancer risk (International Agency for Research, 2002; Li *et al*, 2005,; Silva *et al*, 2002).

1. 3. 4. 7. Genetic factors

Patients diagnosed with breast cancer are 20-30% more likely to have at least one relative with the disease (familial breast cancer). Only 5-10% of the cases are a direct result of germline mutations in highly penetrable genes, such as *BRCA1* and *BRCA2* as well as genes p53 and PTEN. Genetic testing for these mutations has been clinically available since 1996 (breast cancer genetics are reviewed by Edlich *et al*, 2005).

1. 3. 4. 7. 1. p53

The inactivated form of the tumour suppressor protein p53 plays a crucial role in tumorigenesis. It was the first tumour suppressor gene to be linked to hereditary breast cancer and is localised on chromosome 17p13 (Dumitrescu and Cortarla, 2005). Levine *et al* (1997) reported over 50% of human cancers to contain a mutated p53 and more specifically, mutations of the p53 gene (also known as TP53) are found in 20-40% of invasive breast cancers (Cremoux *et al*, 1999). A recent study showed that women with early-stage breast cancer with a p53 tumour suppressor mutation had a poorer prognosis than those who did not have the mutated version of the gene

(Linjawi *et al*, 2004). Stage I breast cancer patients with mutated p53 had a five-year survival rate of 74% compared to 83% for those without the mutation. The mean free survival was 70% compared to 98% in those with and without a mutation respectively. This study concluded that an individual with expression of a mutated p53 gene should receive chemotherapy. Testing for mutations is available for those considered high risk at specialised centres but results have been deemed inconclusive by the American Cancer Society (www.cancer.org) in determining the course of treatment to be followed.

p53 is regulated negatively by murine double minute-2 (Mdm2). Mdm2 is a proto-oncoprotein and functions as an ubiquitin E3 ligase for p53 to promote degradation (Haupt *et al*, 1997; Honda *et al*, 1997; Kubbutat *et al*, 1997; Fang *et al*, 2000). It physically interacts with and inhibits p53 and is up regulated in tumours in comparison to normal mammary glands (Murray *et al*, 2005).

Zheng *et al* (2004) observed that elevated levels of *c-erbB2* reduced wild-type (WT) p53 protein and stimulated Mdm2 nuclear translocation via the activation of the PI3-K/Akt pathway in the MCF-7 cell line. Subsequent inhibition of this pathway led to an increase in WT p53 protein, a reduction in cell proliferation and heightened sensitivity to chemotherapy agent adriamycin and radiotherapy. However, inhibition of this pathway did not affect these characteristics in the MDA-MB-453 cell line which expresses the mutated form of p53.

1. 3. 4. 7. 2. BRCA1 and BRCA2

Germline mutations of breast cancer-associated gene 1 (*BRCA1*) predispose women to breast and ovarian cancer and is reviewed by Narod and Foulkes (2004). *BRCA1* was first identified and cloned as a major breast cancer susceptibility gene over 10 years ago (Hall *et al*, 1990; Miki *et al*, 1994). However, families with a high incidence of male breast cancer did not express this mutation, hence further investigation lead to the discovery and cloning of the *BRCA2* gene (Wooster *et al*, 1994; Wooster *et al*, 1995). *BRCA1* mutations have been found to show a trend towards a worse survival for the ductal tumour type (Brekelmans *et al*, 2005).

There is an increased risk of 9- to 36-fold, and a 6- to 61-fold of developing breast and ovarian cancer respectively in women with detrimental mutations in the *BRCA1* or *BRCA2* genes compared to the general population (Antoniou *et al.*, 2003). Approximately 50% of people expressing a *BRCA1* or 2 gene mutation will develop breast cancer by the age of 50 years. The *BRCA1* gene is highly penetrant, which results in approximately 85% of people expressing the faulty gene developing breast cancer in their lifetime. A patient diagnosed at an early age with breast cancer and the number of first- and second-degree relatives with breast and ovarian cancer are significantly associated with an increased likelihood of carrying a *BRCA1* or *BRCA2* mutation (Narod and Foulkes, 2004).

A study by Nanda *et al.* (2005) compared the *BRCA1* and 2 mutations in white, Ashkenazi Jewish, African American, Hispanic and Asian families. African Americans were found to have a lower rate of deleterious *BRCA1* and *BRCA2* mutations but a higher rate of sequence variations compared to non-Hispanic and non-Jewish white families. The highest percentage of deleterious mutations in *BRCA1* and *BRCA2* were observed in Ashkenazi Jewish families (69%).

1. 3. 4. 7. 3. Other risk factors

To date, the data provided for the genetic determinants of endogenous hormones in relation to breast cancer risk has been inconclusive. For example, polymorphisms have been noted in genes involved in oestrogen synthesis such as *CYP17* and *CYP19*, and *HSD17B1* which codes for an enzyme responsible for the conversion of oestrone (Feigelson *et al.*, 2001; Dunning *et al.*, 1999). In addition, the oestrogen balance may be altered by polymorphisms in genes such as *COMT*, which is responsible for the methylation of oestrogen to harmless metabolites (Comings *et al.*, 2003; Wedren *et al.*, 2003). These have been classified as low-penetrance breast cancer susceptibility genes and may have a greater contribution to breast tumorigenesis when associated with exogenous factors, such as diet and smoking, and endogenous factors, such as hormones (reviewed by Dumitrescu and Cotarla, 2005).

Other mutations include germline mutations of **PTEN**, which occur in 80% of Cowden syndrome patients, which is a rare hereditary breast and thyroid

predisposition disorder which is linked with a 25-50% lifetime breast cancer risk (de Jong *et al*, 2002). PTEN (phosphatase and tensin homologue deleted on chromosome 10, also known as MMAC and TEP1) is tumour-suppressor gene which is found on chromosome 10q23.3. Mutations of other genes also increase the likelihood of developing the disease, including that of the **ATM** gene, which causes ataxia telangiectasia, and has been shown to elevate the risk of breast cancer by ~11% at 50 years, and 30% by the age of 70 (reviewed by Edlich *et al*, 2005).

Bcl-2 inhibits apoptosis and expression is a strong predictor of ER status of breast tumours. The gene predicts hormone receptivity and may indicate a favourable response to tamoxifen (Elledge *et al*, 1997). Regulation of the cell cycle and checkpoint machinery is crucial to the maintenance of normal cellular proliferation. Alterations to these components are central to the process of carcinogenesis and abnormal cell growth. Upregulated expression of **cyclin D1**, **murine double minute-2** (Mdm2), and Akt is found in tumours in comparison to normal mammary glands (Murray *et al*, 2005). In particular, cyclin D1 overexpression has been widely reported in the pathogenesis of breast carcinoma (Buckley *et al*, 1993; Bartkova *et al*, 1994) and a high percentage of primary breast cancers have elevated expression (Bartkova *et al*, 1994), including early stage cancers (Weinstat-Saslow *et al*, 1995).

Over expression of other **kinases** has also been implicated in breast carcinogenesis. A recent study by Lin *et al* (2005) showed that **PDK-1** is frequently elevated in breast cancer with subsequent increases in phosphorylation of downstream kinases, including Akt, mammalian target of rapamycin (mTOR), Stat3, p70S6K and S6. Lin *et al* (2005) suggested that PDK-1 may therefore promote oncogenesis to some degree via Akt and p70S6K activation. The group hypothesised that these molecules may be potential therapeutic targets for breast cancer treatment.

1. 3. 5. Current treatment of breast cancer

Treatment of breast cancer is dependent on the stage of the disease at diagnosis (Lindley, 2002), and usually involves some form of post-operative endocrine therapy, chemotherapy and/or radiotherapy in an attempt to eradicate the disease to the utmost extent. The current and future therapies will be discussed in the following sections.

1. 3. 5. 1. Surgery

Primary local therapy of breast cancer involves breast-conserving surgical removal of tumours. This has been the standard of care for the past decade (Lindley, 2002). The core aim of breast cancer surgery is to eradicate local disease in the breast and lymph nodes by complete resection with tumour-free margins. The extent to which surgery is performed depends on the stage of the tumour. DCIS tumours are usually removed via a lumpectomy, unless the tumour covers large area of the breast where a mastectomy may be more appropriate. Treatment involving surgical removal of the tumour is rarely effective where malignant cells have migrated from the primary tumour site, and only allows for local control of the disease (Taucher & Jakesz, 2004).

1. 3. 5. 2. Endocrine therapy

Endocrine therapy involves the targeting of hormone-responsive cellular proliferation through control of the endocrine system whether it is via inhibition of hormone receptors such as the oestrogen receptor (ER) or blockade of the production of oestrogen itself. Therefore, endocrine therapy is usually only used in the treatment of ER-positive patients.

1. 3. 5. 2. 1. Tamoxifen and other SERMs

Tamoxifen (Nolvadex®) was first documented as having anti-oestrogenic activity in late breast cancer by Cole *et al* (1971). It is the first choice of triphenylethylene compounds (figure 1. 4) over agents such as toremifene due to its lower toxicity and identical activity (Hayes *et al*, 1995; Holli *et al*, 2000). Tamoxifen is a selective ER α modulator (SERM) as it is an antagonist of the actions of oestrogen in relatively high

doses ($10\mu\text{M}$), but like oestrogen, tamoxifen can have an agonistic effect on $\text{ER}\alpha$ and cell proliferation at lower doses ($\sim 10\text{nM}$) (Howell *et al*, 2001). Tamoxifen has a complex mechanism of action and binds directly to the activation function-2 of $\text{ER}\alpha$ competing directly with the agonist action of oestrogen (Jordan, 1994). This mechanism is believed to be partly mediated through not only the repression of co-activator recruitment but also corepressor recruitment (Ali and Coombes, 2002) (see later section on the ER).

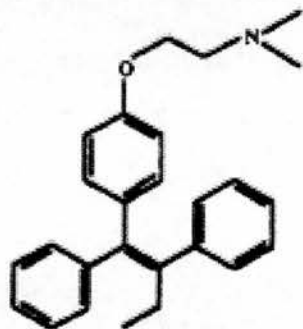


Figure 1. 4 The chemical structure of tamoxifen (reproduced from Cosman and Lindsay (1999)).

This is the main mechanism of action, which subsequently blocks the proliferative actions of oestrogen on mammary epithelium. A second proposed mechanism for this antiproliferative effect is the induction of the synthesis of the cytokine, transforming growth factor-beta ($\text{TGF-}\beta$) by tamoxifen. This acts as a negative autocrine regulatory molecule (Knabbe *et al*, 1987). Also, studies have shown that tamoxifen promotes the synthesis of $\text{TGF-}\beta$ in breast cancer stromal (mesenchymal) cells, which implicates a paracrine, as well as autocrine, mechanism of action, independent of an interaction with the $\text{ER}\alpha$ (Butta *et al*, 1992). There have been observations of a clinical effect of tamoxifen in ER-negative breast carcinoma which would support these mechanistic theories (Nolvadex Adjuvant Trial Organization, 1988). There are several other reports which corroborate of these findings, including the studies where tamoxifen lowered the levels of circulating insulin-like growth factor 1 (IGF-1) in breast cancer patients (Colletti *et al*, 1989; Pollack *et al*, 1990). **IGF-1** stimulates breast cancer proliferation acting via several possible routes such as paracrine, endocrine and autocrine mechanisms and will be discussed in further detail in section 1.6.

Tamoxifen was shown to be of the greatest benefit in ER-positive breast tumours (Osborne, 1998), with 5-year treatment reducing recurrence rates annually by 50%, and the death rate annually by 28% (Early Breast Cancer Trialists' Collaborative Group, 1998). Tamoxifen is beneficial in both pre- and post menopausal women of all ages, with a sequential strategy involving chemotherapy followed by tamoxifen desirable in post menopausal women with high risk breast cancer (Dellapasqua and Castiglione-Gertsch, 2005). It is now commonplace for pre- and post-menopausal women whose tumours are ER-positive to receive the first-line anti-oestrogen tamoxifen and is recommended for the prevention of tumour recurrence in dosages of 20mg/day for a period of 5 years (Lindley, 2002).

Metastatic breast cancer is also treated with tamoxifen, with an overall response rate of 30% for an average of 1 year, and disease progression is halted for 6 months in a further 20% of women. The ER-status is an important prognostic indicator, as is menopausal status, as post menopausal women have higher response rates than those of pre menopausal women (Osborne, 1998; Ravdin *et al*, 1992; Buchanan *et al*, 1986; Ingle *et al*, 1986; Muss *et al*, 1987). Complete or partial remissions were also reported in 48-63% of advanced breast cancers treated with toremifene (Valavaara *et al*, 1988; Gunderson, 1990; Modig *et al*, 1990).

The effectiveness of tamoxifen as an anti-cancer treatment led to speculation as to whether the drug could reduce the risk of breast cancer occurrence in high-risk women. Three studies were carried out, the largest being the National Cancer Institute (NCI) Breast Cancer Prevention Trial or National Surgical Adjuvant Breast and Bowel (NSABP) P-1 Study (Fisher *et al*, 1998). This study found there was an overall breast cancer incidence reduction of approximately 50%. Risk reduction for invasive breast cancer was 44% in women under 49 years of age and 55% for those over the age of 60 years. Non-invasive breast cancer risk reduction was ~49% in the tamoxifen-treated group. These reductions were only found in women with ER-positive tumours.

Preliminary results of the phase III randomized study of tamoxifen and raloxifene (STAR) for the prevention of breast cancer were released early in 2006

(www.cancer.gov/pressreleases/STARresultsApr172006/). The study was led by the National Surgical Adjuvant Breast and Bowel Project. Initial results suggested that the osteoporosis drug raloxifene was as effective as tamoxifen in preventing invasive breast cancer. Both tamoxifen and raloxifene were found to reduce the risk of developing invasive breast cancer by approximately 50%. Fewer cases of uterine cancer and blood clots (36% and 29% reductions respectively) were reported with raloxifene treatment compared to tamoxifen. Equivalent numbers of heart attacks, strokes and bone fractures occurred with each treatment. Raloxifene was found to be ineffective at reducing the incidence of non-invasive breast cancers in agreement with Continued Outcomes Relevant to Evista (CORE) Trial (2004) data. The final results of the STAR trial are yet to be published in a peer-reviewed journal. It is only at this point will a decision regarding whether tamoxifen or raloxifene is more efficacious at reducing the risk of developing breast cancer be possible.

1. 3. 5. 2. 2. 1. Adverse effects of tamoxifen

There is an increase in the risk of **benign uterine disease**, including **fibroid tumours**, **endometrial hyperplasia**, and **adenomyosis** as well as **uterine cancer** with tamoxifen treatment (Fisher *et al*, 1998; Shushan *et al*, 1996; Cohen *et al*, 1993; Fisher *et al*, 1994; Lahti *et al*, 1993). There is also an increased risk of **endometrial cancer** with five years of tamoxifen therapy (Nordenskjöld *et al*, 2005). Nordenskjöld *et al* (2005) found that there were few deaths caused by endometrial cancer. The group reported 41 cases of endometrial cancer and 7 deaths after 5 years of tamoxifen therapy, compared to 24 cases of endometrial cancer and 6 endometrial cancer deaths after 2 years of tamoxifen. Toremifene was shown to produce similar stimulatory effects on the uterus to tamoxifen (Tomas *et al*, 1995).

Many reports have documented the increased risk of **venous thromboembolic disease** in women with breast cancer treated with tamoxifen (Saphner *et al*, 1991; Fisher *et al*, 1994). Healthy women treated with tamoxifen in the Breast Cancer Prevention Trial also had an elevated risk of pulmonary emboli and deep vein thrombosis compared to the placebo group (Fisher *et al*, 1998). Lesser adverse effects of tamoxifen include increased “**hot flushes**” in both pre- and post menopausal women alike (Sawka *et al*, 1986; Love *et al*, 1991). The Breast Cancer

Prevention Trial (Fisher *et al*, 1998) found that “bothersome” hot flushes occurred in 46% of patients treated with tamoxifen compared to 29% of the control group. Tamoxifen has also been associated with an increase in vaginal discharge that was classed as moderately bothersome, and in some instances, worse (Fisher *et al*, 1998).

Tamoxifen has also been reported to have slightly adverse effects on bone metabolism (Wright *et al*, 1994). The STAR trial data will provide further data on the fracture inducing potential of tamoxifen therapy.

1. 3. 5. 2. 2. Advantageous effects of tamoxifen

Tamoxifen has been reported to **decrease the risk of coronary heart disease**, with the Scottish breast cancer trial observing fatal myocardial infarctions (MI) were 63% less common in those patients treated with tamoxifen than placebo (McDonald and Stewart, 1991). The follow-up study of this trial also documented a reduction in risk of MI and favoured of a reduction in other ischaemic heart diseases in those patients receiving tamoxifen (McDonald *et al*, 1995). These data are supported by the Stockholm Breast Cancer Study (Rutqvist and Mattsson, 1993). Updates to these studies are in agreement with a more recent study by Nordenskjöld *et al* (2005), which found that five years of tamoxifen reduced the risk of death from coronary heart disease by more than 30% compared to 2 years treatment. Five years treatment with tamoxifen also reduced the risk of death from breast cancer or from all causes combined as well as the reducing the risk of developing contralateral breast cancer. The effect was delayed however, only emerging after 7 or 8 years.

1. 3. 5. 2. 2. Second- and third-generation SERMs

Raloxifene, arzoxifene (LY35381) and EM-652, like tamoxifen, are also SERMs with anti-oestrogenic action (Gu *et al*, 2002). **Raloxifene** (figure 1. 5) has significant clinical activity, with improved toxicological profiles to that of tamoxifen (Clarke *et al*, 2001). Raloxifene was licensed for the prevention of non-traumatic vertebral fractures in postmenopausal women at increased risk of osteoporosis, in the UK in August 1998 (Layton *et al*, 2005). The benzothiophene is generally well tolerated with only minor gastrointestinal adverse symptoms and vaginal haemorrhage. A few

cases of visual disorders and venous thromboembolism were documented, but these require further investigation.

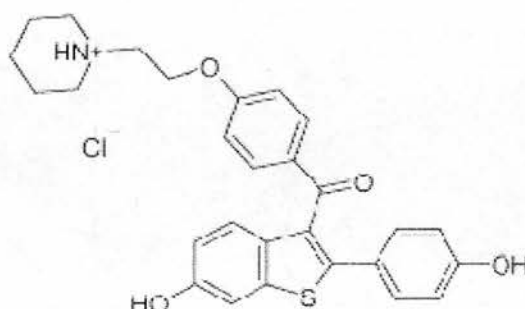


Figure 1. 5 Structure of raloxifene (reproduced from www.cancerquest.org).

Raloxifene was not originally developed as an anti-oestrogen for breast cancer, although it does possess this property, and has shown promising early results as a chemopreventative agent in the treatment of breast cancer in the MORE trial (Cummings *et al*, 1999). Raloxifene does not appear to affect the uterine or vaginal tissue (Delmas *et al*, 1995), and may even reduce the risk of uterine cancer (Cummings *et al*, 1998).

Arzoxifene is a benzothiophene which is more potent than raloxifene or tamoxifen in inhibiting breast cancer cells *in vitro* and *in vivo* (Fuchs-Young *et al*, 1997). A phase II study performed by Buzdar *et al* (1998) in women with tamoxifen-sensitive or resistant advanced or metastatic breast cancer was subsequently continued into a phase II trial. This trial was terminated early however as there was no significant difference between tamoxifen or arzoxifene.

EM-652 is non-steroidal SERM and a so-called pure anti- E_2 . The agent appeared to be a much more potent antagonist on a per-dose effect compared to raloxifene or tamoxifen. Subsequently, the pro-drug of EM-652, EM-800, was administered to postmenopausal women who had developed tamoxifen-resistance. This study found that there was an overall response rate of 14% (Labrie *et al*, 1999), however, a recent multicentre phase III trial with EM-800 was terminated early due to inferior responses with EM-800 compared to tamoxifen.

1. 3. 5. 2. 2. 1. Faslodex (ICI 182,780, Fulvestrant)

Faslodex® (ICI 182, 780, fulvestrant) is a selective ER downregulator which was developed by Wakeling *et al* (1991) and retains pure oestrogen antagonist activity. It was the first in a new class of novel, steroidal, 'pure' antioestrogen which down regulates the ER and stimulates the degradation of ER preventing receptor dimerisation, inhibiting estrogen-dependent gene transcription (Mckeage *et al*, 2004; Piccart *et al*, 2003). It has significant anti-tumour activity in patients with advanced breast cancer (Howell *et al*, 1996). The absence of partial agonist activity and the novel mechanism of action made the agent an ideal candidate for the treatment of advanced breast cancer in both pre- and postmenopausal women (Robertson, 2001) and in patients where tamoxifen resistance has developed (Martin *et al*, 2005; Mckeage *et al*, 2004).

Faslodex has a similar effectiveness in patients with tamoxifen-resistant disease to that of the aromatase inhibitor anastrozole. A number of clinical trials have reported that Faslodex may be a suitable alternative to nonsteroidal antiestrogens and aromatase inhibitors as a first-line endocrine treatment (Vergote and Robertson, 2004). Faslodex is not linked with an increased risk of endometrial cancer which has been associated with tamoxifen (Wakeling and Bowler, 1988).

However, it has been shown that MCF-7 cells were able to develop resistance and proliferate in the presence of ICI 182, 780 through enhanced erbB signaling after long-term culture in the presence of the pure antiestrogen ICI 182,780 (McClelland *et al*, 2001). Shaw *et al* (2006) also recently developed an ICI 182, 780-resistant MCF-7 cell line, and indicated this was due to a mechanism other than ER α alteration. These studies suggest that resistance may develop in clinical situations. The current and future roles of Faslodex in the management of breast cancer are discussed by Howell (2006).

1.3.5.2.3. Aromatase Inhibitors

Aromatase inhibitors (AIs) such as anastrozole are being considered as alternative first-line agents of choice in the treatment of postmenopausal women with ER-positive advanced breast cancer to tamoxifen (Cuzick, 2005). The previous AIs of choice such as aminoglutethimide and 4-OH androstenedione have now been superseded by the newer non-steroidal AIs anastrozole and letrozole and the steroidal inhibitor exemestane (figure 1. 6) due to their greater therapeutic ratio (Iveson *et al*, 1993; Plourde *et al*, 1995; Gershanovich *et al*, 1998; Zilembo *et al*, 1995). The newer AIs are more potent and have a lower profile of side effects than the older AIs.

Anastrozole (Arimidex®) has been reported to completely down-regulate the ER, which subsequently prevents the agonist activity of the drug (Howell *et al*, 2001). In clinical trials, anastrozole has been shown to be superior to tamoxifen in first-line therapy for advanced breast cancer in post-menopausal women (Nabholtz *et al*, 2000). The ATAC ('Arimidex', Tamoxifen, Alone or in Combination) clinical trial, where the AI was tested alone and in combination with tamoxifen versus tamoxifen alone found that there was a significant improvement in disease-free survival with anastrozole compared to tamoxifen alone (Smith *et al*, 2005).

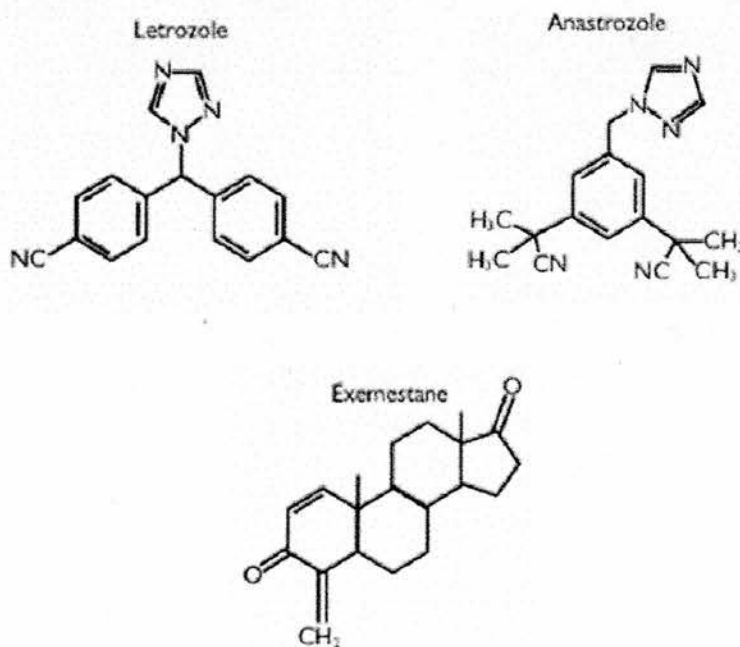


Figure 1. 6. The structures of three aromatase inhibitors, letrozole, anastrozole and exemestane (reproduced from Choueri *et al*, 2004).

It was hypothesised that the Immediate Preoperative Anastrozole, Tamoxifen, or Combined With Tamoxifen (IMPACT) trial would predict the long-term outcome in adjuvant therapy of the ATAC trial. The IMPACT trial predicted the negative effect of the combination treatment and suggested the benefit of anastrozole. However, the IMPACT Trial did not confirm this result, perhaps because the end-points and size of study were such that the study was underpowered to measure the modest additional benefit of anastrozole. It could only be concluded from this study that neoadjuvant anastrozole was as effective and well tolerated as tamoxifen in ER-positive operable breast cancer in postmenopausal women (Smith *et al*, 2005).

Letrozole (Femara®), the most potent of the new generation of AIs, inhibits oestrogen biosynthesis by inhibiting the conversion of androgens to oestrogens by the aromatase enzyme, the last stage in this biosynthetic sequence (Miller *et al*, 2002). A diagram of the conversion of androgens is shown in figure 1. 7 and is applicable to all AIs.

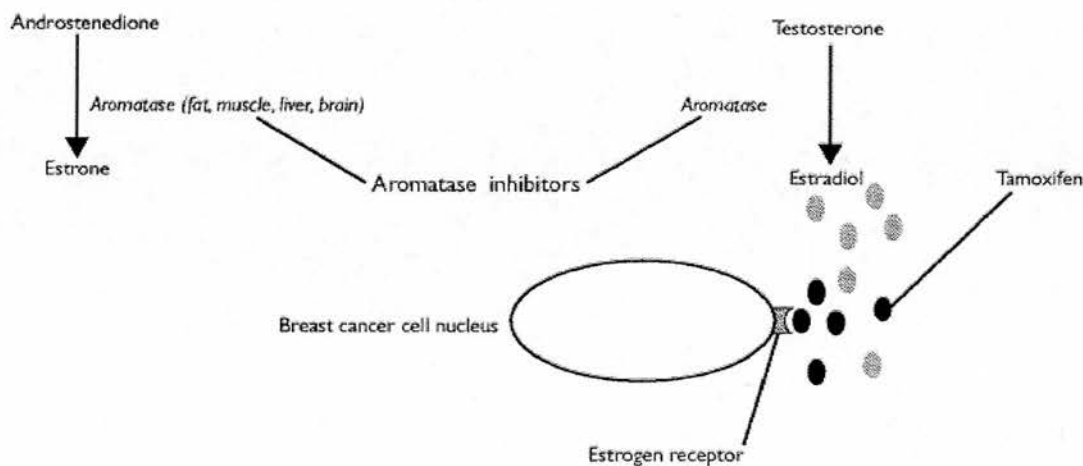


Figure 1. 7 The mechanism of action of aromatase inhibitors compared with tamoxifen in postmenopausal women (reproduced from Toni *et al*, 2004).

Letrozole has also proved to have a higher efficacy than tamoxifen as first-line treatment in post-menopausal women with advanced breast cancer (Smith *et al*, 2000).

The International Letrozole Breast Group carried out a clinical trial using adjuvant treatment with letrozole or tamoxifen in hormone-receptor positive postmenopausal women, and found that letrozole was significantly efficacious over tamoxifen in reducing the risk of recurrent disease in these patients (The Breast International Group (BIG) 1-98 Collaborative Group, 2005). The EORTC trial showed **exemestane** also has some promising activity and toxicity data as a first-line hormonal therapy in metastatic breast cancer patients (Paridaens *et al*, 2000).

Another class of endocrine therapy is the luteinising-hormone-releasing-hormone (LHRH) family, of which **goserelin** (Zoladex®) remains the first-line agonist of choice for the treatment of pre-menopausal patients with advanced breast cancer (Howell *et al*, 2001). Goserelin is rarely used as a single agent as it is more effective in combination with not only tamoxifen but also an AI as ovarian E₂ production is halted, improving the efficacy of the agents in pre-menopausal women. The pituitary gland produces luteinising hormone (LH), which stimulates E₂ production by the ovaries. Inhibition of LH production by goserelin, a gonadotropin-releasing hormone analogue, reduces plasma/serum E₂ levels (Cheer *et al*, 2005) which in turn slow tumour cell proliferation and may actually shrink the tumour. Improvements in response and survival and a reduction in time to progression were seen when goserelin was combined with tamoxifen compared to goserelin alone (reviewed by Jonat *et al*, 2006). Side effects of goserelin include the cessation of menstruation, hot flushes, weight gain, sore joints and perhaps initial tumour enlargement.

1. 3. 5. 3. Radiotherapy

Radiotherapy involves the direct irradiation of breast tumours and perhaps the surrounding tissues. It is generally used externally although the radioactive material may be temporarily placed internally (also known as 'brachytherapy' or 'high-dose intracavity radiation') in the site where the tumour was removed from at the end of is used in the treatment (www.breastcancer.org.uk). An important constituent of therapy for a large proportion of women with early stage breast cancer is adjuvant radiotherapy (Patt *et al*, 2005). The supplementary treatment of women post-surgery with radiotherapy allows the patient to retain their breast whilst improving their chances to those of women who have had a mastectomy initially (Fisher *et al*, 1995; Fisher *et al*, 2002). The application of radiotherapy after mastectomy also reduces

the risk of local recurrence of the disease and may also improve overall survival (Overgaard *et al*, 1997; Ragaz *et al*, 1997). Both these applications of radiotherapy post surgery reduce breast cancer mortality (Early Breast Cancer Trialists' Collaborative Group, 2000), although radiotherapy has been associated with an increased risk of cardiovascular mortality with long-term treatment, particularly of tumours of left breast (Patt *et al*, 2005).

1. 3. 5. 4. Chemotherapy

Chemotherapy is a systemic drug treatment and usually involves the combination of several agents. Chemotherapy is a treatment option for a large number of patients with metastatic breast cancer (O'Shaughnessy, 2005). It is always used where there is lymph node involvement and is recommended in pre-menopausal women with invasive tumours, as these tend to be more aggressive. It is used in the treatment of patients classified as ER-negative, or where endocrine therapy resistance has occurred. It is not recommended for the treatment of non-invasive breast cancer. Chemotherapy can also be used prior to surgery in order to shrink the tumour.

There are several agents which have been found to have single-agent activity, including the anthracyclines and taxanes, which are considered to have the most activity. Agents such as capecitabine (Xeloda®), gemcitabine (Gemzar®), and vinorelbine (Navelbine®) have also demonstrated substantial activity in the metastatic setting (Gralow, 2005). Single agent survival rates are generally improved when a second agent is administered as a follow-on agent, for example Paclitaxel median response rate of 34% was increased to 47% upon the addition of doxorubicin, which had a median response rate of 36% alone (Sledge *et al*, 2003).

Other combinatorial studies have confirmed this improvement. A recent 5-year study found that for women with early-stage, node-positive breast cancer, treatment with docetaxel (Taxotere®) was more effective than 5-fluorouracil in combination with adriamycin and cyclophosphamide at reducing the risk of recurrence. Other studies have shown that not all combinations are effective as current treatments in use. For example, in a study of the treatment of women with metastatic cancer with the GET combination, Gemzar (gemcitabine), epirubicin, and Taxol (paclitaxel), there was no significant improvement compared to the FEC combination of 5-

fluorouracil, epirubicin, and cyclophosphamide. This was important as the women who received the GET combination had more deleterious side effects (Musib *et al*, 2003; Zielinski *et al*, 2003).

Recent trials have also seen improvements in survival and other benefits of a combination of not only chemotherapeutic agents, but also in conjunction with targeted therapies such as Herceptin (see later section for further detail on herceptin) (Slamon *et al*, 2001; Marty *et al*, 2005).

1. 3. 5. 5. Monoclonal antibodies

The role of the cell membrane receptors in the balance between cell proliferation and programmed cell death (apoptosis) has widely been investigated. Angiogenesis and cell migration also play crucial roles in the spread of cancer and the investigation into these areas as a whole imparted the development of novel targeted cancer therapies (Mass, 2004). The epidermal growth factor family of receptors (erbB, EGFR, or HER family) is one such group of cell membrane receptors, which have been widely implicated in the development of many cancers, including breast cancer.

1. 3. 5. 5. 1. Anti-ErbB2 monoclonal antibodies

1. 3. 5. 5. 1. 1. Herceptin (trastuzumab)

Herceptin (trastuzumab) is a humanised monoclonal antibody against a membrane-proximal epitope in the extracellular region of erbB2 (Nagy *et al*, 2005). It binds competitively to the extracellular domain of the erbB2 receptor and blocks intracellular signalling (Bianco, 2004). Herceptin was originally developed by Axel Ullrich and co-workers at Genetech Inc. in 1990. Herceptin comprises of the antigen-binding loops from a murine monoclonal antibody against the extracellular domain of the erbB2R and the human variable region framework residues plus the IgG1 constant domains (Carter *et al*, 2002).

The **mechanism of action** of herceptin has yet to be entirely elucidated, with several possible theories speculated to date. A proposed mechanism is the enhancement of erbB2 degradation (Molina *et al*, 2001), while Artega *et al* (2001) found herceptin to act in part by inducing receptor endocytosis. Le *et al* (2003) and Jackson *et al* (2004)

speculated that the activity of herceptin arose as a result of the inhibition of cell cycle progression via inhibition of the MAPK pathway while Yakes *et al* (2002) and Mohsin *et al* (2005) suggested that the suppression of the anti-apoptotic PI3-K/Akt pathway was responsible.

There are also reports of the action of herceptin in mediating antibody-dependent cellular toxicity (ADCC) (Cooley *et al*, 1999; Clynes *et al*, 2000; Carson *et al*, 2001; Repka *et al*, 2003; Gennari *et al*, 2004). However, the mechanisms are not necessarily confirmed when comparing *in vitro* data from cells with erbB2 overexpression, to *in vivo* studies (Mohsin *et al*, 2005). The most recent study by Fujita *et al* (2006) has suggested that PTEN activity may play a significant and major role in the antitumour effect of herceptin via the erbB2/PI3-K/Akt pathway. Therefore, the group suggested that PTEN could be a useful biomarker for predicting the efficacy of herceptin in the treatment of breast cancer.

Herceptin was the first monoclonal antibody to be licensed for targeted therapy (Mass, 2004) and can effectively treat tumours with amplification of the *erbB2*-gene in 25% of patients as a monotherapy (Vogel *et al*, 2003). 50% of patients can effectively be treated with herceptin with a combination treatment such as taxane or other chemotherapy (Slamon *et al*, 2001), although tumour cells become resistant after 1-2 years of remission to any herceptin treatment (Ross and Gray, 2003; Slamon *et al*, 2001).

Herceptin is well tolerated in erbB2-positive patients with metastatic breast cancer when received as first line treatment (Vogel *et al*, 2002) and in combination with chemotherapy in patients with disease progression (Cobleigh *et al*, 1999). Herceptin treatment in combination with chemotherapy prolonged survival from a median of 20.3 to 25.1 months, and increased the objective response rate from 32% to 50%. The time to progression in women with erbB2-positive metastatic breast cancer was also extended from 4.6 to 7.4 months when used as a first line agent combined with an anthracycline plus cyclophosphamide or paclitaxel (Slamon *et al*, 2001). The survival duration (26.8 months) and response rate (56%) were greatest in patients who received herceptin in combination with an anthracycline and cyclophosphamide, the former of which being doxorubicin in the majority of cases. Herceptin has been

linked with the increased incidence of cardiac toxicity in the form of congestive heart failure (Slamon *et al*, 2001; Tan Chiu *et al*, 2005). The use of anthracyclines such as liposomal doxorubicin and epirubicin are hoped to reduce this higher than expected incidence of cardiotoxicity seen with the use of other anthracyclines (Gianni, 2002).

Other studies have shown similar responses, however, no tumour regression was observed in the remainder of patients who did not respond to single or combination treatment. Interestingly, the *erbB2* gene was found to be amplified and the protein overexpressed in the primary tumour and metastases of these patients, indicating *erbB2*-positive status is not the sole contributor when determining herceptin sensitivity (Tanner *et al*, 2002). This has led to the study of various other combination treatments. Herceptin in combination with tamoxifen has also provided positive inhibitory results, with strong synergistic growth inhibition, inhibition of *erbB2* activity, enhancement of G(0)-G(1) cell cycle accumulation, and a cytostatic effect without cell death (Wang *et al*, 2005). Ropero *et al* (2004) reported reduction in BT 474 cell proliferation of 44% compared to 24% and 31% with herceptin and tamoxifen alone respectively. However, these data are dependent on the order in which each agent was added, as an antagonism of the agents was seen with an inhibitory concentration of 30% (IC30) when cells were treated with herceptin first. Herceptin has also been tested in combination with Tarceva as mentioned previously (Totpal *et al*, 2002), and a recent study by Nakamura *et al* (2005) found the combination of herceptin plus Iressa to be additive or synergistic in their anti-tumour activity in NSCLC.

Currently, herceptin is licensed at a second-/third-line monotherapy and first line with paclitaxel in metastatic breast cancer with *erbB2* overexpression. There are also four Phase III trials in combination with chemotherapy and/or radiotherapy as adjuvant therapy taking place (Mass, 2004), and a Phase IV trial where patients with metastatic breast cancer are selected by FISH for treatment with docetaxel or paclitaxel post herceptin treatment.

1. 3. 5. 5. 1. 2. RhuMab2C4 (Pertuzumab, OmnitargTM, 2C4)

Humanised anti-erbB2 monoclonal antibody 2C4 (2C4) was first developed by Fendly *et al* (1990) and is a novel agent that has shown particular promise in *in vitro* studies against MCF-7 cells (Agus *et al*, 2002). 2C4 binds to and sterically hinders the signalling action of erbB2 (figure 1. 8), inhibiting ligand-stimulated signalling in tumour cells that express both low and high erbB2 levels (Agus *et al*, 2002; Albanell *et al*, 2003). This is significant as previously only tumours with high erbB2 levels were able to be effectively targeted with the anti-erbB2 antibody Herceptin (Albanell *et al*, 2003). 2C4 will be used as a tool to investigate the role, if any, of erbB2 in the development of endocrine resistance in this model of resistance.

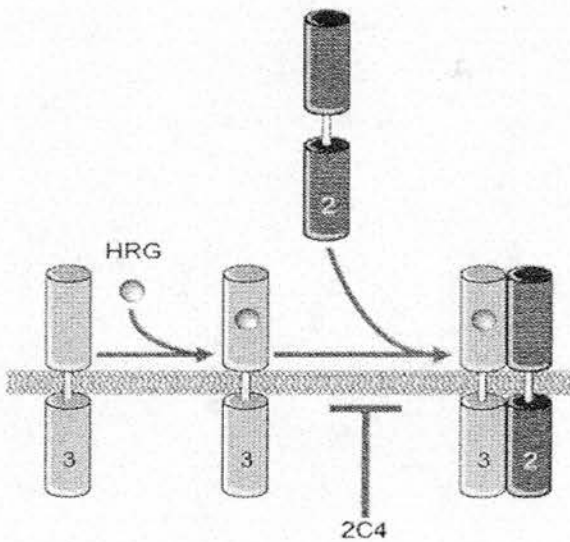


Figure 1. 8 A model for the proposed mechanism of 2C4 activity (reproduced from Agus *et al*, 2002). HRG binds to erbB3 which heterodimerises with erbB2. This dimerisation step is blocked by 2C4.

2C4 is produced in Chinese hamster ovary cells and is based on the human immunoglobulin (Ig) G1 (κ) framework sequences, consisting of two light chains (214 residues) and two heavy chains (449 residues) (Agus *et al*, 2005). The antibody binds to the dimerisation domain of erbB2 (Franklin *et al*, 2004), which sterically inhibits its ability to form dimers with other erbB receptors (Agus *et al*, 2002; Sliwkowski *et al*, 1994; Schaefer *et al*, 1997; Fitzpatrick *et al*, 1998; Liu *et al*, 2002;

Mendoza *et al*, 2002). 2C4 blocks both homo- and heterodimerisation of erbB2 and represents one of a new group of agents known as 'dimerisation inhibitors' (Agus *et al*, 2005). 2C4 has a distinct mechanism of action to Iressa and Tarceva, and represents a new class of targeted therapies known as erbB dimerisation inhibitors (Mass, 2004). 2C4 also has a separate binding site in domain II to that of herceptin which does not overlap with the epitope on erbB2 which herceptin recognises (Fendly *et al*, 1990; Cho *et al*, 2003).

A clinical study by Agus *et al* (2005) has found that 2C4 is well tolerated, has a pharmacokinetic profile which supports a 3-week dosing regime and is clinically active. 2C4 is currently in Phase II trials for second-/third-line monotherapy in advanced ovarian cancer, hormone-resistant prostate cancer and advanced NSCLC.

1. 3. 5. 5. 2. Inhibitors of the EGFR (erbB1)

1. 3. 5. 5. 2. 1. Iressa (gefitinib, ZD1839)

Iressa is a small molecule selective quinazoline derivative and is a reversible tyrosine kinase inhibitor which blocks the erbB1 receptor (Mass, 2004). Iressa (and Tarceva, see next section) binds competitively to the intracellular adenosine triphosphate binding site of erbB1 (Ciardiello and Tortora, 2001). The antitumour activity of Iressa is not dependent on the expression of erbB1, and conversely resistance to Iressa is not dependent on the expression levels either (Moasser *et al*, 2001). This indicates that receptor level expression is not an indicator of the sensitivity to Iressa. Interestingly, high levels of the erbB2 are also highly sensitive to Iressa, and erbB1 and erbB2 receptor phosphorylation occurs upon treatment with the inhibitor (Moasser *et al*, 2001). However, it has been reported that the erbB1 is required for this effect to take place and thus mediates the action of erbB2 phosphorylation induced by Iressa (Moulder *et al*, 2001). There is also evidence of activation of the PI3-K/Akt pathway via erbB3 interactions when cells are treated with Iressa (Moulder *et al*, 2001).

Iressa is orally active and has demonstrated cytostatic growth inhibitory properties in breast cancer cell lines expressing functional erbB1Rs. The agent also caused marked reductions in growth in a variety of tumours including breast and ovarian cancer

(reviewed by Ciardiello and De Vita, 2005 and Dowsett *et al*, 2005). Iressa is currently in the late phase of clinical development in several cancers (Mass, 2004) and has already been approved by the United States Food and Drug Administration (FDA) for pre-treated patients with advanced non-small cell lung carcinoma (NSCLC). This as a result of the phase III INTACT 1 and 2 and the IDEAL 1 and 2 trials with Iressa administered in combination with chemotherapy and as a monotherapy respectively (Mass, 2004). Phase II trials are currently taking place in various tumour types including glioma, where trials are ongoing as monotherapy, in combination with chemotherapy and/or radiotherapy, and other targeted agents.

1. 3. 5. 5. 2. 2. Tarceva (erlotinib, OSI-774)

Tarceva is also an inhibitor of erbB1. It is potent and highly selective and inhibits the activity of the isolated tyrosine kinase and erbB1 autophosphorylation at very low concentrations *in vitro* (Moyer *et al*, 1997). Tarceva also inhibits various downstream signalling molecules (Hidalgo *et al*, 2001; Akita *et al*, 2002), but a much higher concentration is required than for receptor phosphorylation. Tarceva operates via several mechanisms which include the inhibition of proliferation via cell arrest and induction of apoptosis (Moyer *et al*, 1997). The anti-tumour effects of Tarceva are likely to arise from the fact that inhibition of EGFR will subsequently inhibit angiogenesis, invasion, cell motility and the ability of the cell to recover from radiation and chemotherapy damage (Arteaga, 2001; Elesami *et al*, 2000; Woodburn, 1999). Tarceva has also been shown to inhibit the EGFRvIII (Iwata *et al*, 2002), which is expressed in what is believed to be the more aggressive phenotype, hence this may be of some clinical significance (Lal *et al*, 2002). Tarceva is well tolerated and responses have been observed in breast cancer, NSCLC and ovarian cancer (Mass, 2004), with dose-dependent inhibition of human head-and-neck carcinomas with EGFR overexpression (Pollack *et al*, 1999). The only side effects noted were diarrhoea and acneiform skin rash from the maximum tolerated dose of 150mg/day (uninterrupted schedule) (Hidalgo *et al*, 2001).

It has been hypothesised that the targeting of multiple pathways will produce an additive and perhaps synergistic effect due to the inhibition of 1, 2 or more pathways at once. This theory is supported by preclinical studies such as a study by Totpal *et al* (2002), who found the combination of Tarceva and the anti-erbB2 monoclonal

antibody 2C4 to have an additive effect on apoptosis. Further Phase I/II trials are being carried out in metastatic *erbB2*-positive breast cancer to investigate the potential benefits of combining Tarceva with Herceptin, which would target *erbB1* and *erbB2* respectively, thus inhibiting a significant proportion of the *erbB* receptor signalling network (Mass, 2004). A recent study by Friess *et al* (2005) has also reported promising additive and ‘more than additive’ anti-tumour effects of combining Tarceva with 2C4 in breast tumour xenografts.

1. 3. 5. 6. Tyrosine kinase inhibitors in development

There are currently a number of tyrosine kinase inhibitors in development, including Tarceva and Iressa discussed earlier. Other such inhibitors are the EGFR/*erbB2* inhibitor, **Lapatinib**, which is in Phase II trials in many tumour types including metastatic breast and colorectal cancers, the former under second-/third line studies in combination with Herceptin. Another agent targeting metastatic breast cancer is the pan *erbBR* irreversible inhibitor **CI-1033**. **PD153035** is in preclinical studies and targets *erbB1* and *erbB2*.

1. 3. 5. 7. Current opinion of treatment of breast cancer

The **International Consensus Guidelines** from the St. Gallens conference (2005) reviews the general opinion of European experts for the treatment of breast cancer (<http://www.breastcancersource.com>). The focus of the consensus shifted from initially considering risk factors such as tumour size in previous years to **endocrine responsiveness**. Breast cancer was categorised into endocrine responsive, responsive uncertain and non-responsive and then further classified according to low, intermediate or high **risk**, and whether the patient was **pre- or postmenopausal**. The risk factor was dependent on the tumour size, node status, age, whether the tumour was invasive and the *erbB2* gene status. For example, individuals with 1-3 nodes involved and amplified *erbB2* expression or 4 or more nodes involved were deemed to be ‘high risk’. The combination of factors presented at diagnosis identified which particular treatment would be the most efficacious for that individual postoperatively. For example, an endocrine responsive, intermediate risk postmenopausal breast cancer patient would be recommended tamoxifen, an AI, chemotherapy followed by

tamoxifen or chemotherapy with adjuvant AI therapy. There is also an indication for a switch to an AI (exemestane or anastrozole after 2-3 years, letrozole after 5 years) post tamoxifen therapy.

1. 4. Ovarian Cancer

1. 4. 1. Incidence and Survival Rates

Almost 7,000 ovarian cancer cases are diagnosed annually in the UK, making it the 4th most common female cancer after breast, bowel and lung cancer (<http://www.cancerhelp.org.uk/help/default.asp?page=143>). Ovarian cancer arises in 1 in 100 women within their lifetime in the UK, with survival rates of 54.8% & 26.3% for post-menopausal women 1 & 5 years post diagnosis respectively (CGS, Cancer Surveillance Group). Early diagnosis provides survival rates as high as 90%, although the vast majority of cases of ovarian carcinoma are not identified until late stage, where survival falls to 30-40% (Connolly *et al*, 2003). Women with no family of ovarian cancer have an average lifetime risk of developing the disease of approximately 1.5%. Thus, on average 1 in 70 women will develop the disease during their lifetime in the U.S. (www.ricancercouncil.org/facts/ovafacts.php).

1. 4. 2. Epidemiology

The epidemiology of ovarian cancer is reviewed at www.ricancercouncil.org/facts/ovafacts.php. Figure 1.9 shows the sites of origin of ovarian cancer. It arises from epithelial, germ or stromal cells which have been subjected to deregulation of normal cellular processes. There are three forms of ovarian cancer. **Epithelial carcinoma** is the most common form and arises in the epithelial cells lining the ovaries. **Germ cell cancer** comprises approximately 5% of the total number of ovarian cancers cases annually. It occurs in the cells that develop into the egg cells and is generally found in younger women. **Stromal cell cancer** also constitutes only about 5% of ovarian cancer cases and arises in the cells that form the tissues holding the ovary together.

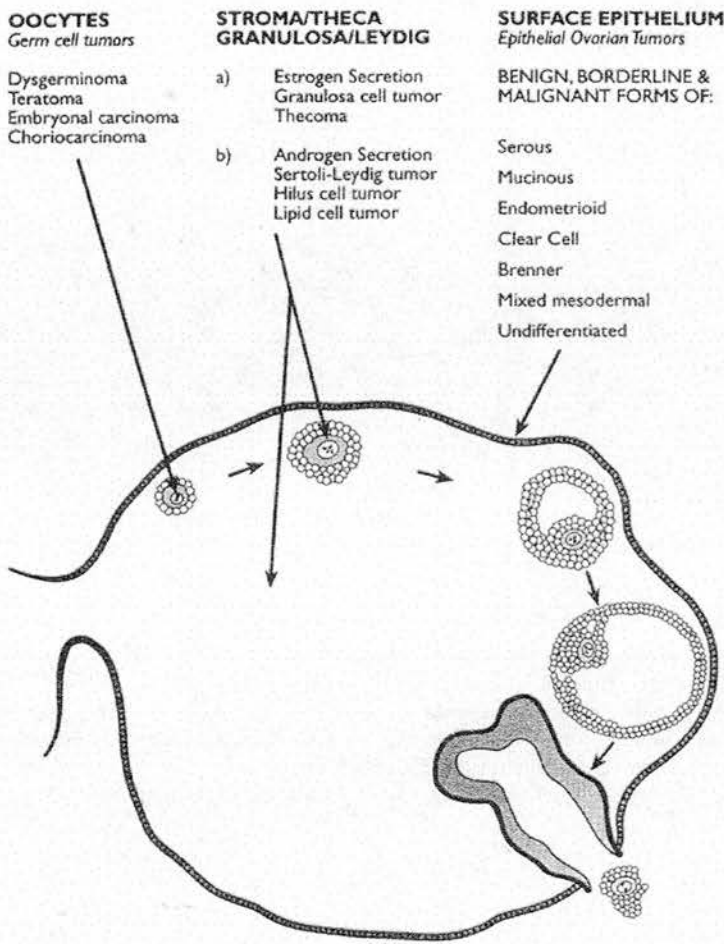


Figure 1.9 The points of origin of ovarian cancer (reproduced from Langdon *et al*, 1997).

1. 4. 3. Stages of ovarian cancer

Ovarian cancer can also be identified and diagnosed, as for breast cancer, by a series of stages. **Stage I** ovarian cancer may be located in one or both ovaries, but has not metastasised to any other organs. **Stage II** ovarian cancer is diagnosed where the tumour cells have metastasised to the other reproductive organs such as the uterus or fallopian tubes and/or has extended through the pelvis. Ovarian cancer which has spread to the abdomen above the pelvis, liver surface, lymph nodes and/or small bowel regions is classified as **Stage III**. **Stage IV** is advanced ovarian cancer which classifies carcinomas which have metastasised to organs outside the abdomen and the liver, for example the lungs.

1. 4. 4. *Diagnosis*

Ovarian cancer is diagnosed through a combination of methods such as a pelvic exam, a CA-125 blood test, transvaginal ultrasound, biopsy and perhaps via a CT scan (<http://www.cancerhelp.org.uk/help/default.asp?page=143>). Symptoms indicative of ovarian cancer include abdominal bloating and discomfort, fluid around the lungs/shortness of breath, indigestion and nausea, loss of appetite/sudden weight loss and vaginal bleeding not during menstruation.

1. 4. 5. *Causes and risk factors*

There are several known risk factors which may affect the relative risk of developing ovarian cancer. The most significant risk factor of developing the disease is a **family history** of the disease. The risk increases 3-fold if the woman has one or more primary relatives (mother/sister/daughter) who have had ovarian cancer (www.ricancercouncil.org/facts/ovafacts.php). The known mutations can be tested for and occur in the *BRCA1* and *BRCA2* genes, although the presence of a mutation in either gene does not necessarily lead to the development of ovarian cancer. Women in the 'high risk' group may choose to register on the UK Familial Ovarian Cancer Registry, and subsequently take part in the UK Familial Ovarian Cancer Screening Study (UKFOCSS) which is currently taking place.

Age is also a key risk factor, with ovarian cancer developing in women between the 40 and 70 years of age. The larger the number of **menstrual cycles** in a woman's lifetime also increases the risk. For example, a woman who has her first cycle before the age of 12 and/or begins the menopause after 50 years has an increased risk of ovarian cancer. A **high-fat diet** has been indicated to elevate the risk of ovarian cancer as excess fat can cause E_2 -retention and the conversion of other hormones into a form of E_2 . Ethnicity also plays a role in the risk of ovarian cancer, with studies in the U.S. reporting Caucasians and Hawaiians to have the highest risk and Native Americans to have the lowest risk (American Cancer Society Cancer Facts & Figures 2006).

Lifestyle changes can reduce the risk of developing ovarian cancer, including exercise, a low-fat diet, being a non-smoker and having a yearly gynaecological

and/or pelvic exam. Other factors which reduce the risk of ovarian cancer include breastfeeding, having more than one child and/or a child below the age of 30 years. Alternatively, a woman who has taken birth control medication for a period of more than 5 years may have a reduced risk of ovarian cancer, although studies have been inconclusive (<http://www.cancerhelp.org.uk/help>).

1. 4. 6. Current treatment of ovarian cancer

The current strategy for the treatment of ovarian cancer is dependent on the stage of the disease. It usually involves the surgical removal of the ovaries and womb followed by chemotherapy in the form of a platinum compound in combination with Taxol (The Advanced Ovarian Cancer Trialists Group, 1999). The Advanced Ovarian Cancer Trialists Group (1999) reviewed the advantages of platinum versus non-platinum agents in chemotherapy of advanced ovarian cancer. Surgery may not be necessary if the tumour grade is very low or borderline, but this is not generally the case. Post-surgery of stage I ovarian cancer, biopsies of the lymph nodes, diaphragm and tissue lining the abdomen and pelvis are taken to assess the extent of the spread of the disease. Adjuvant chemotherapy usually takes the form of cisplatin or carboplatin. The National Institute of Clinical Excellence (NICE) recommended a treatment choice of either Paclitaxel (Taxol) and a platinum agent, or a platinum drug alone in 2003.

Radiotherapy is rarely used in the treatment of ovarian cancer, but may be applied in the therapy of stage Ic or stage II cancers post-surgery where there is little or no sign of cancer in the abdomen or pelvis.

The majority of ovarian cancers are antioestrogen resistant, with a response rate of only approximately 15% (reviewed in Slotman and Rao, 1988). However, a distinct subset of patients do respond to endocrine therapy, while studies performed tended to be in small groups of patients in whom other therapies had proved unsuccessful (Clinton and Hua, 1997). Therefore, there is a rationale for hormonal therapy in this subset, particularly as abnormal oestrogenic signalling has a well known association with ovarian cancer (Pujol *et al*, 1998). In addition, as a result of the endocrine associations with ovarian cancer, the few deleterious side effects of hormonal therapy

and the established efficacy of these therapies in other endocrine organ-associated cancers, the role of endocrine therapy in ovarian cancer merits further investigation (Rao and Miller, 2006).

Endocrine therapy may be used in the treatment of ovarian cancer patients who have developed resistance to chemotherapy, while combination treatment with tamoxifen and goserelin has also been reported to be an active regimen in platinum-resistant ovarian cancer patients (Hasan *et al*, 2005). Trials currently underway involving endocrine therapy includes a Phase III trial performed by the Gynecologic Oncology Group of the efficacy of tamoxifen versus thalidomide in patients with recurrent ovarian epithelial cancer after first-line chemotherapy (www.clinicaltrials.gov).

1. 5. The oestrogen receptor (ER) and breast and ovarian cancer

As previously stated, the approximately a third of breast cancer cells are ER α -positive and proliferate in response to oestrogen, which has led to the conclusion that ER α plays a pivotal role in the development of breast cancer (Murphy *et al*, 1997; Schmitt *et al*, 1995). ER α are also expressed in the ovaries. ER α and oestrogen have been implicated in ovarian cancer and are perhaps related to its causation (Rao and Miller, 2006). Rao and Miller (2006) suggested that additional research was required into hormonal therapies as a treatment for ovarian cancer as discussed in the previous section. Therefore, ER α may play a crucial role not only in breast cancer, but also ovarian cancer.

ER mediate most biological effects of the hormone oestrogen including development & maintenance of female reproductive organs & lipid metabolism. 17 β -oestradiol is the most abundant circulating form of the ovarian hormone oestrogen (Gianni, 2002; Santen, 1986). ERs belong to a steroid/thyroid hormone superfamily of receptors, which act as transcription factors when specific ligands are bound to them (Joel *et al*, 1995). The ER was first identified by Toft and Gorski (1966), and isolated from several species including human (Gorski *et al*, 1968). Two groups eventually cloned the ER, now termed ER α (Walter *et al*, 1985; Green *et al*, 1986; Greene *et al*, 1986; Green *et al*, 1986a). The discovery of a second ER, ER β , was not made until the mid-

1990s (Kuiper *et al*, 1996). The cloning of the full-length 530 amino acid ER β molecule was performed by Ogawa *et al* (1998) and confirmed by Moore *et al* (1998), along with a collection of other isoforms.

ER signalling plays a role in sexual maturation (male and female), fertility, bone formation, cardiovascular and angiogenesis effects, and behaviour (McCauley *et al*, 2003; Windahl *et al*, 2002; Iafrati *et al*, 1997; Wersinger *et al*, 1997; Korach *et al*, 1996). ER α is the predominant receptor involved in mammary gland development (Herynk and Fuqua, 2004). It has been shown extensively that ER α overexpression is associated with breast and ovarian cancer, and ER β has also been linked with the development of these diseases (Rutherford *et al*, 2000; Speirs *et al*, 1999*a* and *b*; Pujol *et al*, 1998). ER β is expressed in breast cancer but the exact role it plays remains controversial (Platet *et al*, 2004; Gustafsson and Warner, 2000), hence this study will mainly focus on the role of ER α in breast and ovarian cancer.

1. 5. 1. ER α structure

Figure 1.10 shows the functional domains of ER α with ER β for comparison. ER α has six domains termed A-F (Green *et al*, 1986) including a variable N-terminal domain, which contains activation function-1 (AF-1), a moderately conserved C-terminal ligand binding domain, which contains activation function-2 and 2a (AF-2 and AF-2a) and a highly conserved DNA-binding domain. AF-1 and AF-2 mediate transcriptional activation. ER β has a very homologous DNA and ligand binding domain to that of ER α , and is also activated by oestrogen and inhibited by anti-oestrogens including tamoxifen (Ali and Coombes, 2002). Ogawa *et al* (1998) predicted a 96% homology in the DNA binding domain (C), and a 53% homology between the E/F domains, while the A, B, and hinge (D) domains are not well conserved between the two receptors.

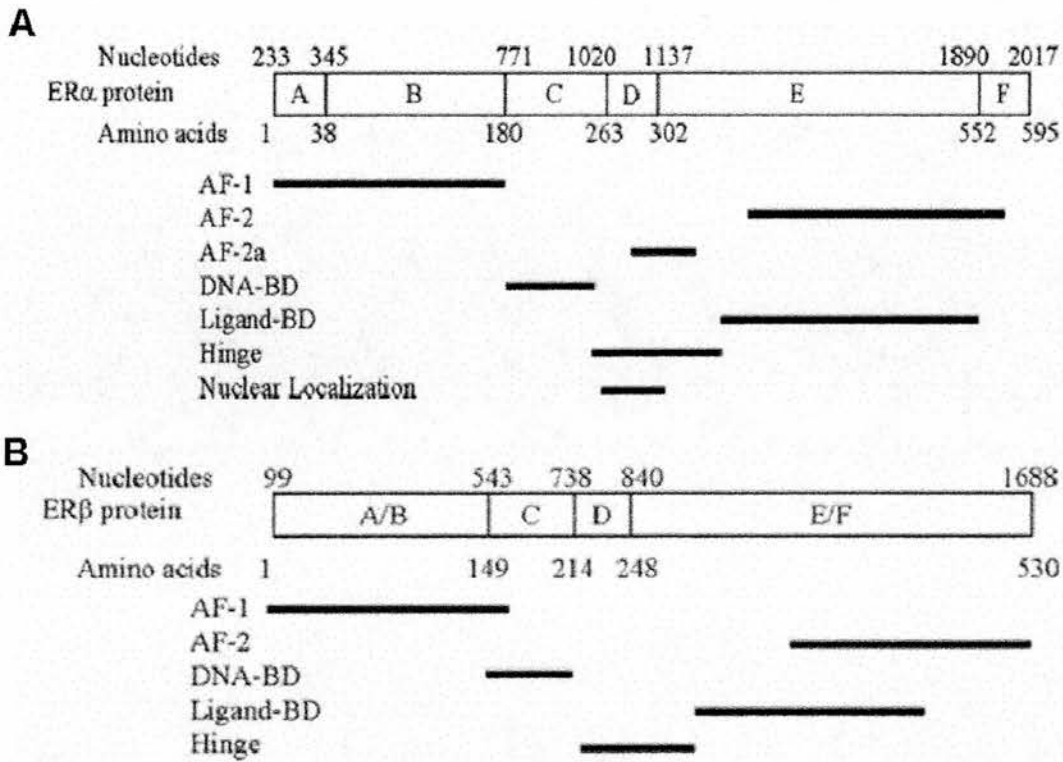


Figure 1.10. The ER α and ER β protein domains (A & B respectively). A-F, nucleotide numbers corresponding to the start of each domain are above, with amino acid numbers shown below. The solid bars beneath the ER schematic represent some of the known functional domains and their relative positions, with the binding domain indicated by BD (reproduced from Herynk and Fuqua, 2004)

ERs also contain functional domains alongside their structural domains (AF-1 and 2 mentioned previously). AF-1 is located within the amino-terminal A and B domains and contains ligand-independent activation function (Ribeiro *et al*, 1995; Metzger *et al*, 1995; Berry *et al*, 1990; Kumar *et al*, 1987). The A/B region contains a coregulatory domain, which binds a variety of ER coactivators and corepressors that modulate ER-mediated transcription (see later in section for further details). The C domain encodes the DNA binding domain and consists of two zinc finger motifs. This binds to the oestrogen responsive elements (EREs) within the promoters of oestrogen-responsive genes (Ribeiro *et al*, 1995). The dimerisation domain is divided between the C and E domains and is vital for the dimerisation of ERs, which allows them to bind to the entire ERE site (Ribeiro *et al*, 1995). ER α and ER β can both form homo- and heterodimers. The hinge region, a section of the ER nuclear localisation signal and a section of the ligand-dependent AF-2a domain are located in the structural D domain (Norris *et al*, 1990). The E and F regions at the carboxyl

terminal contain the ligand binding domain and the AF-2 domain as previously mentioned. The carboxy-terminal region is also involved in the formation of receptor dimers, the binding of chaperone proteins, including heat shock proteins 70 and 90 and the binding of coregulatory proteins (Scherrer *et al*, 1993; Chambraud *et al*, 1990).

1. 5. 2. ER transcription

Both ER α and ER β bind as dimers to short inverted palindromic repeat DNA motifs in the promoters of oestrogen regulated genes. Specifically, the ER dimer binds through the action of a pair of zinc fingers (Schwabe *et al*, 1993). The sequences surrounding these regions are also involved in the affinity for ER-DNA binding (Chen *et al*, 1999; Chen *et al*, 2000; Joel *et al*, 1995). ERs initiate gene transcription and expression by inducing the recruitment of the **general transcription machinery** to the start site of transcription via the activation domains (reviewed by Ali and Coombes, 2002 and summarised in figure 1.11).

The ER may either interact directly with the general transcription components (Sadovskiy *et al*, 1995) or more recent studies have indicated trans-activation by nuclear receptors to occur via **co-activator** complexes. The co-activator complexes interact directly to recruit the transcription machinery, and more significantly the complexes mediate **chromatin remodelling** (reviewed by Glass and Rosenfeld, 2000 and McKenna and O'Malley, 2000). Such complexes include the TRAP/DRIP/SMCC complex, which is linked to polymerase II (Ito and Roeder, 2001), CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF). These are **histone acetyltransferase (HAT) complexes**, where hyperacetylation rather than hypoacetylation of histones appears to be associated with regions of the genome which are more actively transcribed. HAT recruitment may be vital in negating the repressive effects of chromatin on transcription (Strahl and Allis, 2000).

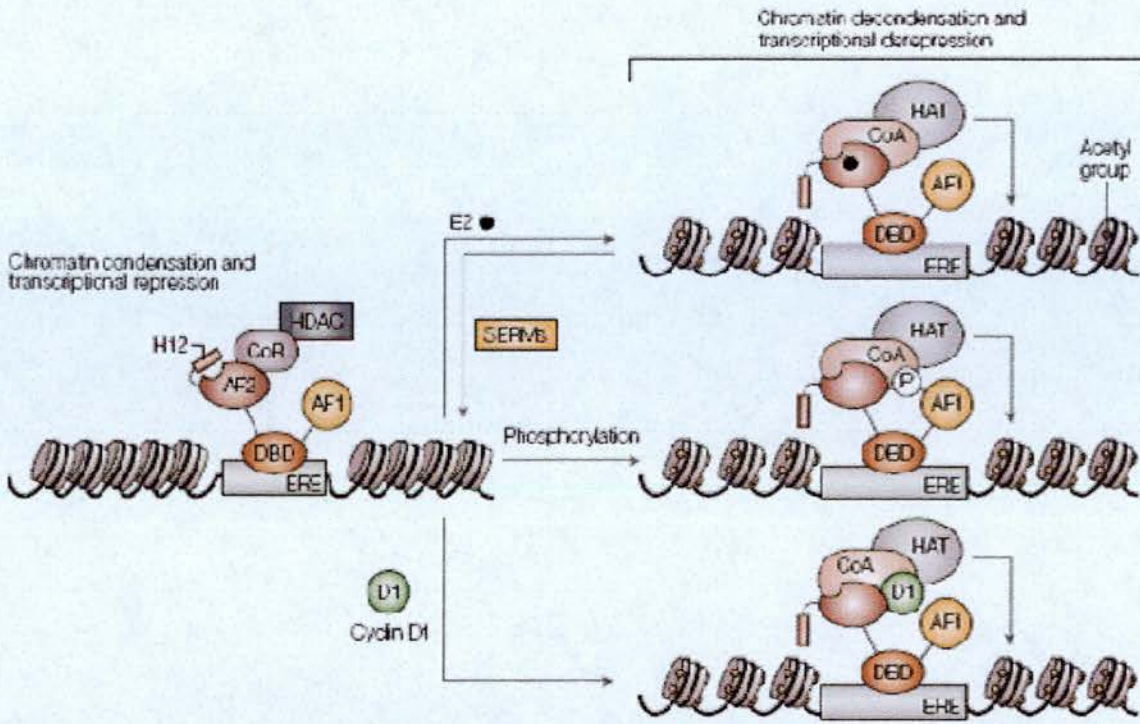


Figure 1.11 ER activation mechanisms (reproduced from Ali and Coombes, 2002). ER has three domains: AF1 and AF2 regulated by phosphorylation and the binding of E₂ respectively and a DNA-binding domain (DBD). In the **inactivated** state, ER is bound to corepressor (CoR) complexes, which recruit HDACs. HDACs maintain histones in a deacetylated state, favouring chromatin condensation. A conformational change occurs on E₂ binding in AF2 which facilitates interaction with co-activators (CoA), binding histone HATs. Histone acetylation by HATs results in chromatin decondensation, enabling transcriptional activation. SERMs modulate ER activity probably by balancing CoA and CoR complex recruitment to AF2, which is dependent on the SERM-induced conformation and other factors including tissue-specific differences in the availability of CoA/CoR. Cyclin D1 and growth factor-induced AF1 phosphorylation may also facilitate the recruitment of CoAs via ligand-independent mechanisms. ERE, oestrogen response element; H12, helix 12.

There is a group of three related co-factors which are known as the **p160 co-activators** that induce the activity of the ER after the binding of a ligand such as oestrogen via AF-2 (reviewed by McKenna *et al.*, 1999 and Leo and Chen, 2000). The three proteins are called **nuclear-receptor co-activator1 (NCOA1, SRC1)**, **NCOA2 (TIF2, GRIP1)** and **NCOA3 (P/CIP, ACTR, AIB-1 or TRAM1)** and facilitate histone acetylation by associating with the transcription factor CBP (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). NCOA1 and 3 also acetylate histones directly (Spencer *et al.*, 1997; Chen *et al.*, 1997). The ER α undergoes a conformational change in AF-2 to form a surface at which co-activators interact, and bind **histone acetyltransferases (HATs)**. The shape of this surface determines if a ligand acts as an agonist or antagonist (Russo and Russo, 1987). HAT histone

acetylation results in chromatin decondensation which facilitates transcriptional activation. Ligands such as IGF and TGF α activate the ligand-independent AF-1 domain (Webb *et al*, 1998; Arnold *et al*, 1995), and this is closely related to the phosphorylation status of the domain (Gayther *et al*, 2000; Smith *et al*, 1997). The ligand independent and non-genomic signalling pathways involving ER α phosphorylation will be discussed in a later section.

The inactive ER is bound to corepressor complexes, which recruit **histone deacetylases (HDACs)**. These molecules maintain the histone deacetylation state that is aligned with chromatin condensation. Repression of gene expression by a number of unliganded nuclear receptors can be mediated by the recruitment of HDACs via the interaction of the receptors with **nuclear-receptor corepressor 1 (NCOR1)** or **NCOR2 (SMRT)** (Nagy *et al*, 1997; Chen and Evans, 1995). AF-2 mediates NCOR recruitment without the need for a ligand, hence ligand binding leads to the dissociation of these complexes and the recruitment of co-activators. For the ER specifically, other histone-modifying proteins, including arginine methyltransferases, act as co-activators either through association with co-activators (Stallcup, 2001) or by directly interacting with the LBD (Koh *et al*, 2001). Ligand binding to the hydrophobic pocket of the carboxy-terminal of both the ERs induces the movement of helix 12 to position itself over the pocket (Steinmetz *et al*, 2001) thus stabilising the helix and allowing the recruitment of coactivators. These steps are required for full agonist action (Feng *et al*, 1998; Moras *et al*, 1998), while the bulky side chains of partial agonists or antagonists including tamoxifen, raloxifene or faslodex prevent the helix from aligning in the full agonist position.

This results in the blockade of co-activator binding to the ER (Pike *et al*, 2000). ER α -bound-tamoxifen represses gene expression by recruiting corepressors NCoR1 and 2 (Shang *et al*, 2000), which contrasts with ER β , which has been reported to recruit these corepressors not in the presence of antagonists but agonists (Webb *et al*, 2003). Decreased expression of NCoR and SMRT have both been correlated with tamoxifen resistance in breast tumours (Shang *et al*, 2000; Graham *et al*, 2000; Jackson *et al*, 1997).

ER β appears to have a greater affinity for phytoestrogens than ER (Kuiper *et al*, 1997), while ER β also displays reduced transactivation in the majority of cells in direct comparison with ER α . This is possibly due to the reduced ER β AF-1 activity (Kuiper *et al*, 1997). Contrastingly, the AF-2 activity for both ER α and ER β is similar, although this is dependent on the cell type (Cowley and Parker, 1999).

ER α and ER β differentially regulate transactivation of heterologous promoters (Kushner *et al*, 2000). ER α activating protein (AP)-1 activity is upregulated by oestrogens, in contrast to ER β AP-1 which is reduced. Tamoxifen and raloxifene both increase AP-1 activity of ER α and ER β . Herynk and Fuqua (2004) have reviewed the mutations of the ER which lead to alterations within the protein sequence, predominantly those affecting the function of AF-1 or AF-2. The review discusses the numerous ER mRNA splice variants which have been found and the subsequent variant forms of the ER proteins.

1. 5. 3. ER expression and breast cancer

Approximately 7-10% of normal human breast epithelial cells are thought to express ER α (Herynk and Fuqua, 2004), which fluctuates during the menstrual cycle (Battersby *et al*, 1992; Ricketts *et al*, 1991; Markopoulos *et al*, 1987). Normal cells which express ER α are not the same as those which are proliferating (Russo *et al*, 2000; Russo *et al*, 1999). Contrastingly, ER β expression is markedly higher at 80-85%, which is also unrelated to cellular proliferation (Palmieri *et al*, 2002) and does not alter during the menstrual cycle (Shaw *et al*, 2002). ER signalling is required for normal development of the mammary gland and it thought that deregulation of this signalling facilitates irregular cell proliferation possibly results in the breast cancer development and its progression (Herynk and Fuqua, 2004).

Low grade DCIS has been shown to express high levels of ER α in approximately 75% of its cells, which is similar to invasive breast cancer. However, high grade DCIS has a reduced expression of ER α in approximately 30% of its cells (Leal *et al*, 1995). DCIS have also been reported to have reduced expression of ER β in comparison to normal epithelial cells, while high grade DCIS displays the most dramatic decrease in ER β expression (Shaw *et al*, 2002). Herynk and Fuqua (2004) report that at present, there is an absence of large studies analysing ER β protein

expression in early lesions, invasive cancers and in normal breast tissue. The review concluded that ER α levels fluctuated while ER β expression levels rose during tumour progression.

1. 5. 4. ER α signalling & ‘cross-talk’

It was previously thought that ligand binding was required in order to activate ER α , however it has been shown that growth factors can stimulate ER α . AF-1 activity is regulated by phosphorylation while AF-2 requires E₂ to bind to the ligand-binding domain. It is possible that AF-1 and AF-2 may act independently and/or synergistically to activate transcription (Gianni, 2002) (figure 1.12). Phosphorylation is a common covalent modification of proteins that provides an important mechanism by which the activity of transcription factors is regulated (Chen *et al*, 1999).

Indirect evidence has shown that phosphorylation is involved in receptor function, for example, E₂ causes a rapid increase in phosphorylation by several fold, and TGF α is able to elicit ligand-independent ER α activation (Bunone *et al*, 1996; Joel *et al*, 1995).

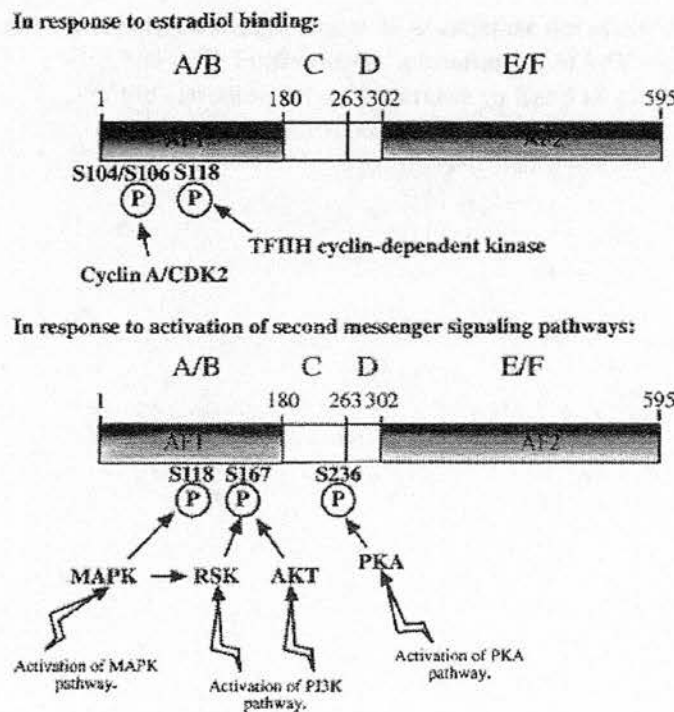


Figure 1.12 Schematic diagram of ER α domains. Phosphorylation of ER α is enhanced upon E₂ binding or activation of second messenger signalling pathways, via TGF α stimulation, for example. TGF α & other ligands of EGFR family are thought to stimulate the kinases shown (image reproduced from Lannigan, 2003).

Events subsequent to this initiate ERE-mediated gene expression. Oestrogens and anti-oestrogens, such as tamoxifen, and protein kinase activators also increase phosphorylation of the ER and/or proteins in the ER signalling cascade. Specifically, AF-1 of the ER α contains phosphorylation sites for a number of kinases including MAPK and cyclin A/ Cdk2 (Katzenellenbogen *et al.*, 1995).

There are two signalling pathways involved in normal cellular proliferation via ER α . The two pathways are believed to interact with one another leading to the development of endocrine therapy-resistance in ER α -positive cancers. The two pathways are termed the ligand-dependent and the ligand-independent pathways. Previous studies have shown that phosphorylation of serine residues on ER α at positions 104 and/or 106, serine 118 and serine 167 cause activation and that mutation of these residues reduces ER α activation, gene transcription and eventually cell proliferation. There is also evidence of the involvement of serine 236 and tyrosine 537 residues, however this is inconclusive (Lannigan, 2003). Figure 1.13 is a summary diagram of 'cross-talk' between the two pathways.

1. 5. 4. 1. The Ligand-Dependent Pathway

The ligand-dependent pathway requires the direct binding of hormones, such as oestrogen, to stimulate the growth of mainly epithelial cells via the AF-2 domain of ER α . Oestrogen binds to ER α inducing a conformational change (dimerisation), which triggers gene transcription & cell proliferation (Atanaskova *et al.*, 2002). In the ligand-dependent pathway the highly conserved serine residues 104, 106 and 118 are involved in ER α activation. Serine 104 and/or 106 are believed to be phosphorylated by the cyclin-A-cyclin dependent kinase 2 (Cdk2) complex (Rogatsky *et al.*, 1999). Chen *et al.* (1994) (Chen *et al.*, 2000) found that oestrogen treatment of the breast cancer cell line MCF-7 stimulated cell growth and transcription via serine 118 phosphorylation, located on AF-1 of ER α . Evidence has shown that phosphorylation of serine 118 is mediated through cdk7, which is a cyclin-dependent kinase associated with basal transcription factor TFIIH (Chen *et al.*, 2000). Chen *et al.* (1994) concluded that serine 118 was a major oestrogen-inducible phosphorylation site of the ER. It was observed that oestrogen enhanced serine 118 phosphorylation to a greater extent compared with serine 104 or 106 phosphorylation (Le Goff *et al.*, 1994).



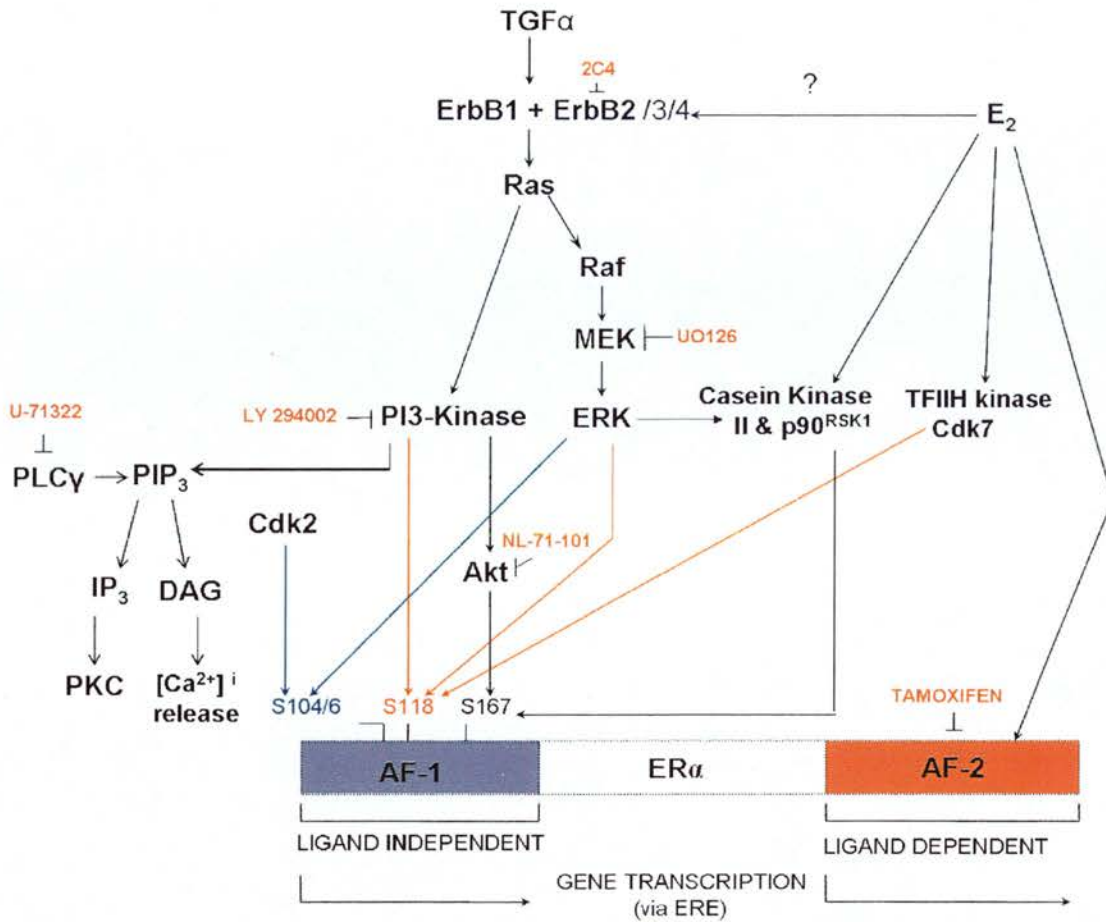


Figure 1.13 Schematic diagram of the ‘cross-talk’ between the ligand dependent and independent pathways of ERα. The ligand-dependent pathway: oestrogen (E₂) binds directly to and stimulates gene expression via activation function-2 (AF-2) of the ERα. E₂ also activates transcription via serine (S) 118 phosphorylation of activation function-1 (AF-1). The ligand-independent pathway: the erbBR pathway is shown as an example of ligand-independent signalling, where a ligand of the erbBR family such as TGFα (shown as an example here) binds to the erbBRs triggering receptor homo or heterodimerisation (erbB1/2 as one possible combination for TGFα), signal cascades and gene transcription via AF-1. These signals are mediated by at least two pathways including the PI3-K/Akt and MEK/ERK pathways, phosphorylating ERα residues S118 and S167, and S167 and S104/6 respectively. ERα activation via AF-1 and/or AF-2 results in ERα binding to the oestrogen response element (ERE) of the promoter region of genes stimulating gene transcription and ultimately a cellular response, dependent on ligand stimulation. The anti-oestrogen tamoxifen and a selection of inhibitors used in this study are shown at their various targets. Tamoxifen competes with E₂ at AF-2 of ERα.

1. 5. 4. 2. Non-genomic ER signalling; the Ligand-Independent Pathway and the Epidermal Growth Factor Receptor Family

The ligand-independent pathway refers to signalling which does not involve stimulation of ER by a hormone such as oestrogen. One such ligand-independent pathway operates via members of the Receptor Tyrosine Kinase (RTK) I family or erbB family of receptors (HER/ EGFR/ erbB family), which eventually enhance the phosphorylation of various ER α residues including serine 118 and 167. This family of receptors play an important role in the developmental processes and have been implicated in the development of breast cancer. These and other breast cancer markers are reviewed by Giancotti (2006).

The erbB family consists of four closely related receptors: EGFR (erbB1), erbB2, erbB3 and erbB4 (Sweeney and Carraway, 2000; Muthuswamy *et al*, 1999; Riese and Stern, 1998; Schlessinger and Ullrich, 1992) (figure 1.14). Ligands of the erbB family such as epidermal factor (EGF), transforming growth factor- α (TGF α) and heregulin- β (HRG β) bind to and activate erbB family members. This causes the receptors to undergo a conformational change, which leads to dimerisation and the formation of either a monomer or heterodimer (Sweeney and Carraway, 2000; Muthuswamy *et al*, 1999). This leads to phosphorylation of a specific set of tyrosine residues in the Src-homology 2 (SH2) domains of the receptors. These domains act as docking sites for adaptor proteins including Shc, Grb2, and Sos resulting in the activation of the Ras/Raf/MEK/ERK and PI3K/Akt pathways and ultimately transactivation, cell growth, proliferation and survival (Yarden *et al*, 2001; Tzahar *et al*, 1998).

ErbB2 is the preferred heterodimerisation partner for other members of the family (Vinolas *et al*, 1997). In non-cancerous cells erbB2 is involved in activating signalling pathways, which eventually result in cell growth and division (Nichols *et al*, 1998). ErbB2 adopts an open formation which is constitutively active and is similar to erbB1 when a ligand is bound (Humphreys and Hennighausen, 2000; Keski-Oja *et al*, 1988). This is an active conformation, and may be indicative of why other family members preferentially bind to erbB2 (Atanaskova *et al*, 2002). Experiments *in vitro* and *in vivo* have shown that erbB2 heterodimers induce a greater

mitogenic response than homodimers of the other receptors of the family. It was therefore hypothesised that ligand-induced activation of erbB1 or erbB3 receptors that formed heterodimers with the erbB2 receptor were important in tumour growth and resistance development (reviewed by Olayioye *et al*, 2000).

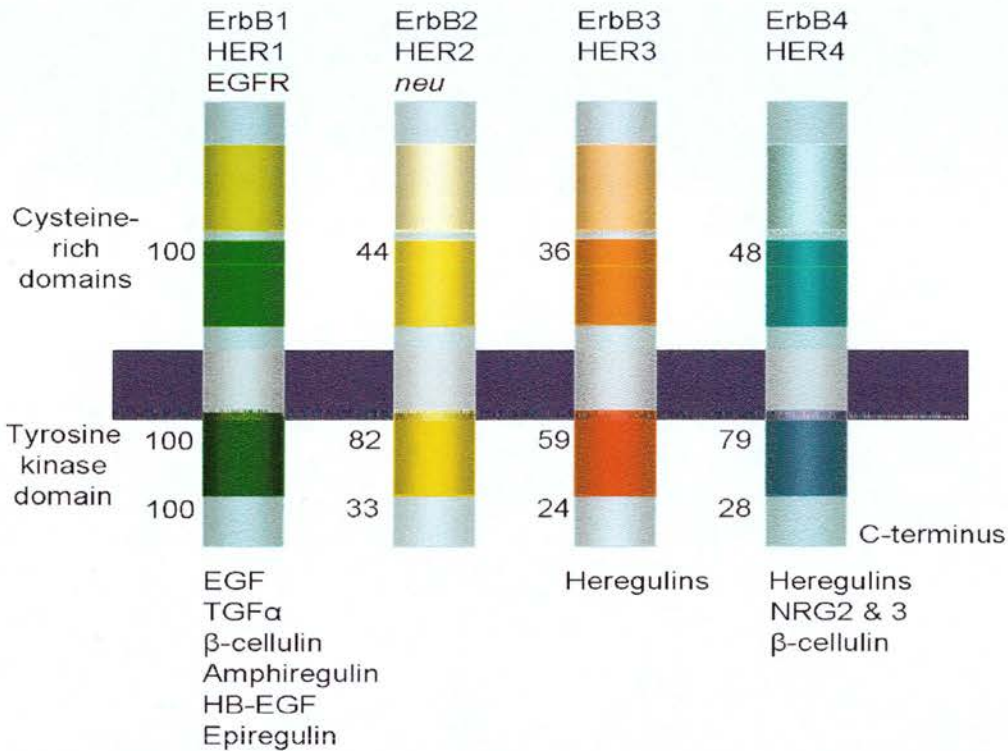


Figure 1.14 The erbB family of receptors and their associated ligands. ErbB receptors contain an extracellular ligand-binding domain, an intrinsic tyrosine kinase cytoplasmic domain and a single hydrophobic transmembrane domain (Sweeney and Carraway Sweeney, 2000; Riese and Stern, 1998; Schlessinger and Ullrich, 1992).

TGF α plays an important role in the normal and neoplastic development of the mammary gland (Humphreys and Hennighausen, 2000) and oncogenesis in a number of tissues (Humphreys and Hennighausen, 2000; Keski-Oja *et al*, 1988; Sporn and Roberts, 1985). It acts as a mitogen in most cells and initiates intracellular signalling pathways upon binding to erbBRs. TGF α stimulates erbB2 and the ERK/MAPK and the phosphatidylinositol 3-kinase (PI3-K)/ protein kinase B (PKB/Akt) pathways (Datta *et al*, 1999) which potentially leads to the phosphorylation of serine residues 118 and/or 167, followed by ER α activation and gene transcription (see figures 8 and 9). Serine 118 is phosphorylated via the E₂-independent pathway by mitogen-activated kinases (MAPK) ERK1 & II (Kato *et al*, 1995) and via PI3-K. Serine 167 is

also phosphorylated through this pathway via PI3-K and Akt and the 90kDa ribosomal S6 kinase RSK (Joel *et al*, 1998).

HRG β is a member of a polypeptide family of growth factors and is also known as a neu differentiation factor (Holmes *et al*, 1992) and regulates cell proliferation and differentiation (Plowman *et al*, 1993). HRG is often expressed in breast cancer tissues (Dunn *et al*, 2004) and is able to bind to erbB3 and erbB4, inducing heterodimerisation with erbB2. This in turn stimulates receptor tyrosine phosphorylation and induction of signal transduction pathways downstream (Fiddes *et al*, 1998; Sliwkowski *et al*, 1994; Plowman *et al*, 1993; King *et al*, 1988). HRG activates a number of signalling pathways in various systems, including activation of PI3-K, ERK and c-Jun N-terminal kinase (Bacus *et al*, 1996; Karunagaran *et al*, 1996; Ram *et al*, 2000).

HRG has mitogenic actions in a selection of various human mammary epithelial cells which co-express erbB2 and erbB3 receptors, and PI3-K is constitutively activated by erbB2 /erbB3 in breast carcinoma cells that overexpress the erbB2 receptor (Ram *et al*, 2000). Observations of breast cancer cell lines have shown that HRG has diverse effects which are cell-dependent (Marte *et al*, 1995; Peles *et al*, 1992). It is believed that the growth factor may be involved in regulating several biological effects of cancerous cells, for example adhesion, migration, invasion (Staebler *et al*, 1994; Bacus *et al*, 1993) and apoptosis (Daly *et al*, 1997). Further studies are needed to elucidate the integration of HRG-activated signals which lead to various biological effects as the overall picture remains unclear (Tan *et al*, 1999), although accumulating evidence suggests HRG increases breast cancer cell proliferation and promotes aggressive and invasive phenotypes as well as tumorigenesis (Atlas *et al*, 2003; Falls, 2003). This is supported by evidence where the inhibition of HRG expression suppressed breast cancer cell metastasis and tumorigenicity (Tsai *et al*, 2003).

1. 5. 4. 2. 1. 'Cross-talk' and Endocrine Therapy Resistance

Hua *et al* (1995) reported that ER α -positive ovarian cancer is often refractile to anti-oestrogen therapy, while approximately 30% of ER α -positive breast cancers are unresponsive to anti-oestrogen treatment and the majority of those which respond initially eventually become resistant (Ali and Coombes, 2002). Anti-oestrogen-

resistant growth of ER α -positive ovarian & breast tumours remains a significant clinical problem. It is believed that resistance to endocrine therapy arises through one of the following scenarios, or combination of scenarios; ER α activation in the absence of oestrogen, hypersensitivity to low levels of circulating oestrogen or activation as opposed to inhibition by oestrogen antagonists (Ali and Coombes, 2002).

Overexpression or alteration of a component of the ER α signalling pathways is believed to lead to the pathways interacting with one another increasing transcription and deregulating cellular proliferation.

In ovarian cancers erbB1 is present in 33% to 75% of cases (Owens *et al*, 1991; Battaglia *et al*, 1989) and is implicated in the progression and growth of the disease (Simpson *et al*, 1999). TGF α is often co-expressed with erbB1, is expressed in multiple breast cancer cell lines (Vyhlidal *et al*, 2000) and stimulates growth of ovarian cancer cells in culture (Crew *et al*, 1992; Morishige *et al*, 1991) supporting this theory. TGF α operating through overexpressed erbB1 and the ligand-independent pathway may account for the null effect of tamoxifen and the development of endocrine therapy resistance as TGF α operates via AF-1 as opposed to AF-2 which tamoxifen targets. TGF α has been shown to be induced by oestrogen treatment in ER-positive cell lines (Gong *et al*, 1992; Clarke *et al*, 1989).

The *c-erbB2* oncogene is overexpressed in 20-30% of many cancer types including breast and ovarian cancer (Gottesman *et al*, 2002; Yu and Hung, 2000; Hengstler *et al*, 1999; Revillion *et al*, 1998; Hynes and Stern, 1994) and is associated with malignant transformation, poor overall survival and oncogenesis (Yu and Hung, 2000; Hudziak *et al*, 1987). Pietras *et al* (1995) found that the erbB2 pathway targeted the ER α and promoted hormone-independent growth in human breast cancer cells. Overexpression of erbB3 or erbB4 is less frequent in breast cancer and does not appear to affect prognosis (Travis *et al*, 1996). However, more recent studies by Tovey *et al* (2004) and Witton *et al* (2003) have reported that overexpression of erbB4 may predict, surprisingly, for increased survival and lower proliferation indices in breast cancer.

Over expression of several elements downstream of the erbB family also leads to the development of cancer, for example, Ras, Raf, extracellular signal-regulated kinases I

and II (ERK1/II), mitogen-activated protein kinase (MAPK) and protein kinase C (PKC). PI3-K and Akt amplification is also seen in breast and ovarian cancer (Murray *et al.*, 2005; Kirkegaard *et al.*, 2005; deGraffenried *et al.*, 2004; Nakatani *et al.*, 1999; Shayesteh *et al.*, 1999; Bellacosa *et al.*, 1995). Suppression of ER α activation via ligand stimulation using protein kinase inhibitors has been used to show that ER bioactivity through the oestrogen-independent pathway involves protein kinases (Cho and Katzenellenbogen, 1993; Aronica and Katzenellenbogen, 1993). It is believed that the final proteins of the MEK/ERK and PI3-K/Akt signalling pathways phosphorylate various residues located on AF-1 of ER α . There is substantial evidence that ER α is activated via phosphorylation of only one or more of serine residues located on AF-1. Specifically, serine residues 104/6, 118 and 167 have been indicated as three potential sites. The exception to serine residue phosphorylation is the low level of phosphorylation found at tyrosine position 537 on the ER α .

[³²P] phosphopeptide tryptic map comparison suggested serine residues 104 & 6 (S104/6) as major sites of phosphorylation in MCF-7 mammary carcinoma cells which was confirmed using point mutation (Le Goff *et al.*, 1994). **Serine 118** (S118) was identified as a probable site of phosphorylation within AF-1 of ER α through point mutation (Le Goff *et al.*, 1994; Ali *et al.*, 1993). Kato *et al.* (1995) found that S118 was phosphorylated by MAPK *in vitro* and *in vivo* in a ligand independent manner, while Joel *et al.* (1995) also used point mutation to definitively identify S118 as a major site of phosphorylation in cells treated with oestrogen or phorbol ester. In a later paper Joel *et al.* (1998) stated that S118 phosphorylation of the human ER α (hER α) enhanced ER-mediated transcription and this was induced by oestrogen stimulation and activation of the MAPK (oestrogen-independent) pathway.

The latest research has shown that bidirectional cross talk between ER α and the erbB receptor family signalling pathways regulates **tamoxifen-resistant growth** where amphiregulin (AR) increased S118 phosphorylation (Britton *et al.*, 2005). Britton *et al.* (2005) indicated that the function of ER α in tamoxifen-resistant cells was maintained as a result of erbB/MAPK-mediated S118 phosphorylation, and that this subsequently generated a self-propagating autocrine growth-regulatory loop through the ER α -mediated production of AR.

Campbell *et al* (2001) and Martin *et al* (2000) both support the theory that ER α phosphorylation by Akt occurs at **serine 167** (S167), resulting in oestrogen-independent activation of ER α . However, serine residue phosphorylation is cell specific, with no S167 involvement in COS-1 cells, although phosphorylation of S104/6, S118 and S167 of hER α does occur in MCF-7 cells, which were used in this investigation. Nicholson *et al* (2004) and Shou *et al*, (2004) showed that increased nuclear ER α phosphorylation at S118 and 167 occurred as a result of increase erbB1R/erbB2R/IGF-1R-regulated MAPK and Akt activity in cells with acquired tamoxifen resistance. This promoted proliferation via stimulation of the transcriptional activity of the tamoxifen-ER α complex. Nicholson *et al* (2004) hypothesised that these kinases, among others, may be able to promote phosphorylation of the nuclear ER α , with subsequent ligand independent transcriptional activity, enabling ER α to act as a transcription factor in an oestrogen-depleted environment. Martin *et al* (2003) also implicated this genomic ER α mechanism in the development of oestrogen hypersensitivity.

The phosphorylation of serine residues on ER α is significant as it is a mechanism by which the activation of the ER α and ultimately cell growth and proliferation occurs. The ligand dependent mechanism provides a new aspect by which to study the activation of ER-positive endocrine therapy resistant cancer cell lines versus oestrogen-sensitive cancer cell lines. From this it may be possible to determine which pathways are involved in the progression from anti-oestrogen-responsive cancer to endocrine therapy resistant cancer.

1. 5. 4. 2. 1. 1. PI3-K/Akt pathway

The Akt signalling pathway is activated in cells subject to diverse stimuli such as growth factors, hormones and extracellular matrix components (Tsai *et al*, 2001; Gu *et al*, 2002). Activation of Akt has been reported by Sun *et al* (2001) to be present in ~40% of breast carcinomas and Jordan *et al* (2004) has suggested that the PI3-K pathway plays a role in the proliferation of their particular tamoxifen resistant cell lines. Akt activation has also been shown to predict the outcome of breast cancer patients treated with tamoxifen (Kirkegaard *et al*, 2005).

It has been inferred that increased expression of IGFR-I and increased levels of Akt and PI3-K, as well as IRS-1, may confer resistance to anti-oestrogens (Wiseman *et al*, 1993; Ahmad *et al*, 1999; Salerno *et al*, 1999; Campbell *et al*, 2001; Vivanco *et al*, 2002). Further to these studies, Frogne *et al* (2005) also found that elevated P-Akt levels were present in both tamoxifen and ICI 182, 780 resistant cell lines compared to the parental MCF-7 cell line from which they were derived. The cell lines with elevated levels of P-Akt were more sensitive than the parental cell line to wortmannin and SH-6, inhibitors of PI3-K and Akt respectively, therefore suggesting that Akt signalling was required for cell proliferation in anti-oestrogen resistant cell lines. Expression and activity of PTEN, a tumour suppressor protein, remained unaffected in the resistant cell lines with elevated P-Akt. PTEN dephosphorylates phosphatidylinositol 3, 4, 5-triphosphate (PIP-3), which in turn acts as a second messenger involved in Akt activation (Li *et al*, 1998). These data implicate Akt and signalling via Akt as a potential site and pathway for targeted therapy in resistant breast cancer.

A downstream component of the PI3-K/Akt pathway, mammalian target of rapamycin (mTOR), has been associated with tamoxifen resistance in breast cancer. mTOR is a serine-threonine kinase responsible for the function of transcriptional regulators p70s6 kinase and 4E-BP1 (reviewed by Hildalgo and Rowinsky, 2000) and is activated by Akt. Signalling via mTOR is therefore partly responsible for the regulation of cell cycle control, apoptosis and enhancement of growth factor production among other processes (reviewed by Nicholson *et al*, 2002). These functions are key to the oncogenic transformation of mammalian cells (deGraffenried *et al*, 2004). The PI3K/AKT/mTOR pathway has been correlated with the relapse and death of ER α -positive breast cancer patients post tamoxifen treatment. This concurs with *in vitro* evidence where Akt was demonstrated to mediate tamoxifen resistance (Kirkegaard *et al*, 2005; deGraffenried *et al*, 2004).

mTOR is actively being investigated as a potential therapeutic target with various inhibitors in clinical trials. Everolimus (RAD-001) is in a Phase III randomised clinical trial in the presence and absence of endocrine therapy for the treatment of metastatic breast cancer. Temsirolimus (CCI-779) is also in a Phase II trial in the presence and absence of endocrine therapy and is also in a Phase II clinical trial for

the treatment of metastatic breast cancer as a single agent. The progress of combining mTOR antagonists with endocrine therapy in the treatment of breast cancer is reviewed by Johnston (2006).

Akt activation has also been associated with NF κ B translocation and transactivation (Meng *et al*, 2002; Sizemore *et al*, 1999), inhibition of substrates linked positively with apoptosis and endothelial nitric oxide synthase activation. Bad and caspase 9 are substrates related to apoptosis which are inhibited by Akt (Ivanov *et al*, 2002).

1. 5. 4. 2. 1. 2. MEK/ERK pathway

Previous studies have suggested that P-ERK1/II is elevated in long term oestrogen deprived (LTED), tamoxifen-resistant and gefitinib-resistant MCF-7 cells under control conditions (Martin *et al*, 2005; Santen *et al*, 2005; Yue *et al*, 2002; Britton *et al*, 2005; Knowlden *et al*, 2003; Normanno *et al*, 2006). The elevated levels of P-ERK, and potentially PI3-K, were hypothesised to be due to residual oestrogens binding to ERs in the cytoplasm which then interacted and phosphorylated SHC via Src. This rapid 'non-genomic' mechanism of 'cross-talk' was put forward by Santen *et al* (2004) and was linked with cells adapting to long term oestrogen deprivation. This was associated with the conversion of their cell model to an oestrogen hypersensitive model. Santen *et al* (2004) and Song *et al* (2004) proposed that in breast cancer the activated insulin-like growth factor (IGFR-I) (discussed in section 1.6) recruited the SHC/ER complex to the plasma membrane, upstream of MEK activation. The results suggested this form of kinase signalling eventually converged on cell cycle components which promoted proliferation (Staka *et al*, 2005). Santen *et al* (2004) suggested this was independent of a direct ER transcriptional effect as even though oestrogen growth hypersensitive, this hypersensitivity was not mirrored at the ERE-regulated transcriptional level. Thus, an alternative genomic model was implicated for kinase/ER 'cross-talk' taking place at the nuclear ER level which must directly affect the transcriptional activity.

Cell proliferation after LTED was suggested to arise as a result of growth factor signalling, and various LTED models implicated not only erbB2, but also IGFR-I and downstream activation of ERK and PI3-K/Akt, which were implicated to interact with ER (Stephen *et al*, 2001; Martin *et al*, 2003; Santen *et al*, 2004).

1. 6. Vascular endothelial growth factor receptor (VEGFR)

The vascular endothelial growth factor (VEGF) (Brunner *et al*, 1993) gene is a major angiogenic factor which is stimulated by oestrogen and is involved in the progression of breast cancer (Hyder *et al*, 1999; Losordo *et al*, 2001). Breast cancer cells produce VEGF, which stimulates angiogenesis via a paracrine mechanism in the endothelial cells of tumours (Ferrara, 1999). VEGF also promotes cell growth via an autocrine pathway in tumour cells (Miralem *et al*, 2001; Bachelder *et al*, 2001). VEGF₁₂₁ and VEGF₁₆₅ are the predominant of the six alternatively spliced human isoforms of VEGF that exist (Ferrara, 1999). VEGF operates via VEGF receptors (VEGFRs), of which three are known; VEGFR-1, VEGFR-2 and the VEGF₁₅₆ receptor, NRP-1 (Guo *et al*, 2003). VEGFRs are located in breast cancer cells and research has shown that oestrogen and VEGF regulate a related group of genes in inducing breast cancer progression (Losordo *et al*, 2001). Guo *et al* (2003) showed that the overexpression of VEGF isoforms VEGF₁₂₁ and VEGF₁₆₅ in oestrogen-dependent MCF-7 cells stimulated breast tumour formation and that this occurred in an oestrogen-independent manner (no oestrogen treatment). VEGF also enhanced oestrogen-dependent breast cancer tumour formation in mice. The data provided by Guo *et al* (2003) suggest that VEGF up-regulation in oestrogen-dependent breast cancers contributes to the development of an oestrogen-independent phenotype by stimulating tumour progression and angiogenesis via both paracrine and autocrine mechanisms.

Interestingly, VEGF is increased in tumours and cancers with erbB2 overexpression, while VEGF overexpression itself is significantly correlated with higher levels of Akt and mTOR phosphorylation (Klos *et al*, 2006). Therefore, it appears that there are multi-faceted associations between many of the signalling proteins involved in breast cancer development and progression.

1. 7. Insulin-like Growth Factor receptor (IGFR) signalling

The type 1 IGF surface receptor (IGFR-I) was reported by Stewart *et al* (1990) to be an oestrogen-inducible protein, with several groups reporting the importance of the IGFR-I and ligands of the IGFR-I, insulin-like growth factor I and II (IGF-I/II), in the growth and survival of breast cancer cells (Arteaga and Osborne, 1989; Baserga *et al*, 1997; Jerome *et al*, 2003). It has long been established that IGF-I acts as a mitogen in human breast cancer cells including the MCF-7 cell line (Ullrich *et al*, 1986; Huff *et*

al, 1986). IGF-I binds to, activates and signals via the IGFR-I, which comprises of two extracellular α -subunits and β -subunits (Ullrich *et al*, 1986). IGFR-I acts as a tyrosine kinase upon IGF-I binding. IGFR-I activation leads to extensive phosphorylation of the 185kDa insulin receptor substrate 1 (IRS-1), an important substrate of the IGFR-I. The phosphorylated residues of IRS-1 bind to numerous SH2 domain-containing proteins, thus IRS-1 acts as a 'multisite docking protein' (Dufournay *et al*, 1997). Proteins which bind to IRS-1 include PI3-K, via its p85 regulatory subunit, and the guanine-nucleotide exchange factor Grb2/SOS (Backer *et al*, 1993; Skolnik *et al*, 1993). Potential downstream targets of PI3-K include the Rho family polypeptides Rac and Cdc42 (Chou and Blenis, 1996) and Akt (Burgering and Coffey, 1995). The former results in activation of the p21^{ras} and *raf* oncogenes and MEK1, which activates ERK. The latter kinases are primarily activated in response to proliferative stimuli (Marshall, 1995). Other kinases such as c-Jun N-terminal kinases (JNKs) and the p38 kinases respond to cell stresses (Kyriakis and Avruch, 1996). IGF-I (Monno *et al*, 2000) and growth factors such EGF have been shown to activate the JNKs (Logan *et al*, 1997).

The IGF-I growth stimulatory action occurs via the PI3-kinase/Akt pathway in cells adherent to the culture substratum (Dufournay *et al*, 1997). Dufournay *et al* (1997) suggested that PI3-K activity, but not that of MAPK is required for IGF-I signal transduction in the MCF-7 cell line, implying the MAPK signal transduction cascade does not play a role in IGF-I-enhanced growth induction. A further study by Suzuki and Takahashi (2000) reported that the IGF-I signal, which led to the stimulation of MCF-7 cell DNA synthesis, occurred via ERK through PI3-K only when the cells were anchorage-deficient. Upstream signalling kinases Raf-1, MEK and ERK were also activated by IGF-I in floating MCF-7 cells.

There is also considerable evidence of cross-talk between erbB1 and IGFR-I in breast cancer cells, with Knowlden *et al* (2005) suggesting a unidirectional IGFR-I/erbB1 cross-talk mechanism where the IGF-II ligand, operating via IGFR-I, controls ligand-activated and basal erbB1 signalling in conjunction with cell proliferation. This is believed to occur in a Src-dependent manner in cells which are tamoxifen resistant.

1. 8. Origin of cell lines

Breast and ovarian cancer cell models are being used in this investigation to study growth effects and serine phosphorylation of the ER α . The human breast cancer cell line MCF-7 was utilised in this study for this purpose in order to study breast cancer and was originally isolated from a malignant pleural effusion in a postmenopausal breast cancer patient (Soule *et al*, 1973). MCF-7 cells are widely used in studies as they are sensitive to the effects of oestrogen and thus susceptible to tamoxifen therapy. Several sublines of MCF-7 cells have been systematically isolated, including the MIII cell line, which was developed by prolonged withdrawal from potent oestrogenic stimuli *in vivo* in the nude mouse (Clarke *et al*, 1989). A schematic representation of the derivation of MCF-7 variant cell lines can be seen in Figure 1.15. The stepwise development of resistance in the MCF-7 derived cells is a clinically relevant model as it reflects what occurs in patients treated with tamoxifen followed by an AI, who may receive Faslodex on relapse. The current benefits and limitations of endocrine therapy are discussed by Nicholson and Johnston (2005). For example, some patients receive tamoxifen followed by an AI, and then may receive Faslodex on relapse (Nicholson and Johnston, 2005). Alternatively, patients may be treated with tamoxifen followed by an AI on relapse. Again, these cells are good models of stepwise induction of resistance which are a result of current treatment regimes.

MIII cells were originally isolated from tumours growing in ovariectomised mice and are E₂-independent. Their growth is inhibited by all major antioestrogen therapies *in vitro* (Brunner *et al*, 1993a). MCF7/LCC1 (LCC1) cells were obtained after a further *in vivo* selection of MIII cells, and display a shorter lag period from inoculation of tumour cells to the appearance of proliferating tumours (Yano *et al*, 1992). LCC1 cells are similar to MIII cells as they have acquired a hormone-independent phenotype and do not require E₂ for growth (Thompson *et al*, 1993). However, both cell lines respond mitogenically to E₂-supplementation *in vivo*, and have been found to retain sensitivity to anti-oestrogens and LH-RH antagonists (Clarke *et al*, 1989; Yano *et al*, 1992; Brunner *et al*, 1993a).

The MCF7/LCC2 (LCC2) cell line was derived from the LCC1 cell line. The cell line is a 4-hydroxytamoxifen resistant human breast cancer variant, which retains

sensitivity to the steroidal antioestrogen ICI 182, 780 (Brunner *et al*, 1993b), a characteristic often observed in patients with acquired tamoxifen resistance. LCC2 cells are E₂-independent when growing *in vitro* or *in vivo* (Brunner *et al*, 1993b). Brunner *et al* (1993a, b) demonstrated that by continuous sequential selection of MCF-7 cells in a stepwise manner a model could be produced to study endocrine resistance. A stable ICI 182, 780-resistant variant, MCF7/LCC9 (LCC9) was obtained using stepwise selections *in vitro* from 10pM to 1µM ICI 182, 780 against LCC1 cells (Brunner *et al*, 1997). The cells are also resistant to the nonsteroidal antioestrogen tamoxifen. Table 1. 2. shows a summary of characteristics of the cell lines used.

The human ovarian carcinoma cell line PEO1 was established and characterised by Langdon *et al* (1988) as E₂-insensitive. The SKOV-3 human ovarian carcinoma cell line is not responsive to E₂ and is not inhibited by antioestrogens OH-tamoxifen and ICI 164, 384 (Hua *et al*, 1995). They express ER at a level equivalent to the apparent K_d for E₂ binding. However, their E₂ resistance is believed to arise through a loss of E₂ regulation of selected growth regulatory gene products as opposed to defective transcriptional activation of ER by E₂. PEO1 cells were used as a model of E₂ and anti-oestrogen sensitive ovarian carcinoma and SKOV-3 cells were used as an *in vitro* model for E₂ and anti-oestrogen resistant ovarian cancer.

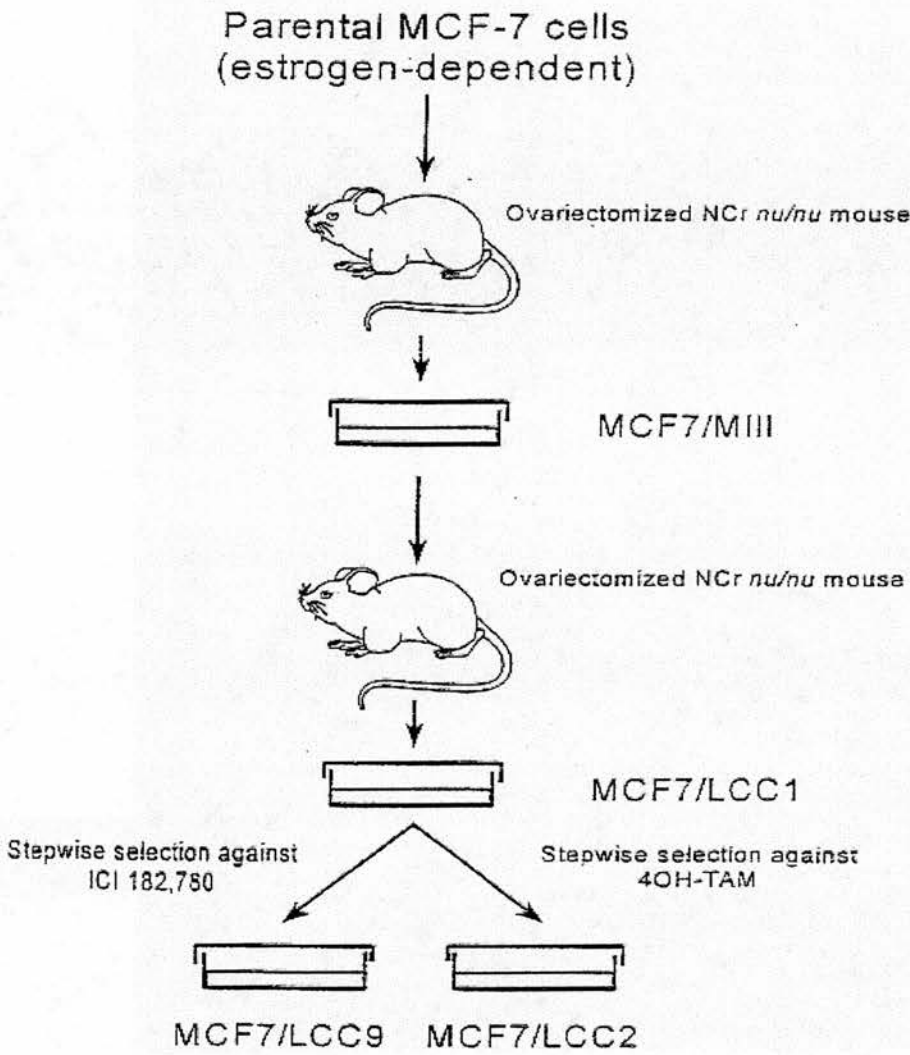


Figure 1.15 Schematic representation of the derivation of MCF-7 variant breast cancer cell lines and their relationship to the MCF-7 parental line (reproduced from Brunner *et al*, 1997).

Table 1. 2. E₂-sensitivity and ER α expression levels of ovarian and breast cancer cell lines.

Cell line	Phenotype	ER α Expression (sites/cell)	Reference
SKOV-3	E ₂ -sensitive	Less than MCF-7	Imai <i>et al</i> , 2005
PEO1	E ₂ -sensitive	More than or \approx MCF-7	Langdon <i>et al</i> , 1988,90
MCF-7	E ₂ -dependent Tam-, ICI 182, 780 & ICI 164, 384- sensitive	120,540 \pm 20,000	Soule <i>et al</i> , 1973
MIII	E ₂ -independent & responsive ICI 182, 780 & ICI 164, 384-sensitive	238, 660 \pm 94, 900	Clarke <i>et al</i> , 1989
LCC1	E ₂ -independent, & responsive anti-E ₂ -responsive	112,270 \pm 19, 440	Thompson <i>et al</i> , 1993
LCC2	E ₂ -independent, & responsive Tam-resistant ICI 182, 780 responsive	91,290 \pm 1,330	Brunner <i>et al</i> , 1993a
LCC9	E ₂ -independent, Tam- & ICI 182, 780 cross-resistant	133,200 \pm 21,140	Brunner <i>et al</i> , 1997
LY2	E ₂ -independent, Tam- & ICI 164, 384-cross resistant	54, 076 \pm 15, 066	Bronzert <i>et al</i> , 1985 Clarke <i>et al</i> , 1989

1. 9. Aims & Objectives

ER α activation via serine residue phosphorylation provides a new aspect by which to study the activation of ER-positive endocrine therapy resistant cancer cell lines versus E₂-sensitive cancer cell lines. The signalling pathways involved in the development of endocrine therapy resistance were investigated by comparing the E₂-sensitive cell line MCF-7 to lines with varying degrees of E₂-sensitivity (MIII, LCC1, LCC2, LCC9 & LY2). LY2 cells are resistant to tamoxifen and other antioestrogens. The LCC1 and LCC2 cell lines retain tamoxifen sensitivity and are fully sensitive to ICI 182 780 and yet their growth is hormone-independent, while remaining E₂-responsive. These cell lines represent an intermediate situation between hormone-sensitive and hormone-independent but hormone-responsive cells with increased metastatic potential. The cell lines provide a useful model to determine the molecular events associated with malignant progression to a hormone-independent and metastatic phenotype (Thompson *et al*, 1993) and changes associated with endocrine therapy resistance. The objectives were achieved by using two model systems, a breast cancer oestrogen-resistant model with MCF-7 variant lines and an ER-positive ovarian cancer model comparing an E₂-sensitive cell line, PEO1, with SKOV-3, an E₂-insensitive cell line.

The initial aim of this study was to confirm the **growth responses** elicited by E₂ and anti-E₂s in the breast cancer cell lines and also to characterise the effect of growth factors in the resistant cell lines, and to compare this characterisation with the characterisation of growth responses in MCF-7 variant cell lines. This characterisation also took place in the ovarian cell lines using E₂ and TGF α .

A secondary objective was to characterise **ER α levels and activation** through ER α phosphorylation of serine residues 118 and 167 (P-S118/167) using western blotting after oestrogen, antioestrogen and growth factor treatment of all breast cell lines. The novel aspect of the study was the characterisation of the same phosphorylation sites in the resistant cells and to compare these data with MCF-7 cell characteristics and observe any modifications which may have occurred between them. Studies into P-S118 and P-S167 expression were also performed in the ovarian cell lines.

The signalling pathways involved in the development of breast cancer resistance development were then investigated. This was achieved by using western blotting to investigate the total and activated levels of various signalling proteins in the **PI3-K/Akt** and **MEK/ERK** pathways. The effect of a panel of **tyrosine kinase inhibitors** on these pathways (via protein expression levels) and the cellular proliferation of the breast cancer cell lines in this model were then studied. The inhibitors were used to investigate the role of each signalling molecule.

A further aim of the study was to confirm the effect of E_2 and characterise the effect of TGF α on **mRNA expression levels** in a series of oestrogen-responsive genes (ER, progesterone receptor (PR), pS2, cathepsin D (CTD)) in the breast cancer model. The effect of tyrosine kinase inhibitors on these expression levels was also documented. The inhibitors were used to investigate the affect, if any, the blockade of these proteins has on mRNA expression.

The **cell cycle distribution and apoptosis** of the breast cancer cells was characterised after E_2 , growth factor and anti- E_2 treatment. The effect of novel anti-erbB2 agent **2C4** on the proliferation, cell cycle distribution and apoptosis in the breast cancer cell lines was also characterised to investigate the role of erbB2 in this model.

It is hypothesised that a combination of several factors are involved in the development of E_2 and anti- E_2 insensitivity ranging from elevated levels of signalling molecules such as Akt and ERK, mRNA expression to increased number of cells dividing. These avenues and their role in resistance will be explored in this investigation.

Chapter 2

Materials and Methods

2. 1. Materials

Chemicals and reagents were obtained from Sigma unless otherwise stated. Materials used are listed according to technique and antibody information is detailed in the Western blot section.

2. 1. 1. Cell culture

2. 1. 1. 1. Cell lines

MCF-7 cells were provided by Professor W. Miller (Edinburgh Breast Unit) and were routinely maintained in Dulbecco's minimal essential medium (DMEM) containing phenol red (PR) supplemented with 10% foetal calf serum (FCS), 0.1mg/ml penicillin/streptomycin (P/S). MCF-7 variant cell lines LCC1, LCC2, LCC9 and LY2 were kindly provided by Professor Robert Clarke (Cancer Centre, Georgetown University, Washington). The human ovarian carcinoma cell line PEO1 was provided by Dr. Simon Langdon and was derived from the ascites of a patient with a poorly differentiated serous adenocarcinoma at the Edinburgh Oncology Unit. The SKOV-3 ovarian cell line was obtained from the European Collection of Animal Cell Cultures, Porton Down, UK.

2. 1. 1. 2. Tissue culture reagents

<i>Product</i>	<i>Manufacturer/Supplier</i>
Cryovials	- Nuncleon, Invitrogen
Dulbecco's minimal essential medium + phenol red	- Invitrogen
Foetal calf serum	- Harlan Sera Lab
Penicillin/streptomycin	- Invitrogen
Phosphate buffered solution	- Invitrogen
RPMI 1640 growth media + phenol red	- Invitrogen
Trypsin/ EDTA	- Invitrogen

2. 1. 1. 3. *Inhibitors*

<i>Inhibitor</i>	<i>Manufacturer/Supplier</i>
LY 294002	- Calbiochem
NL-71-101	- Calbiochem
RhuMab2C4 (Pertuzumab)	- kindly donated by Roche pharmaceuticals
SB203580	- Calbiochem
4-hydroxy-tamoxifen	- Sigma
UO126	- Calbiochem
U-73122	- Calbiochem

Inhibitors were reconstituted in DMSO according to manufacturers' instructions to initial stock solutions of 10^{-2} M, from which dilutions were made. Inhibitors were aliquoted out in small volumes and stored at -20°C , with the exception of UO126 which was reconstituted just prior to use to avoid degradation and tamoxifen which was stored at 4°C . Light sensitive inhibitors were stored in the dark.

2. 1. 2. *Protein protocols*

<i>Product</i>	<i>Manufacturer/Supplier</i>
Chemiluminescence Western Blotting Kit	- Pierce
Gel apparatus	- BioRad
Protein concentration assay kit/SOLUTION	- BioRad
Tween-20	- BioRad

2. 1. 3. *Molecular protocols*

All molecular biology kits - Qiagen

Protein sequences were originally obtained from the SwissProt website (www.ebi.ac.uk/swissprot/). These sequences were then used to design primer pairs using the Primer3 database (http://frodo.wi.mit.edu/cgi-bin/primer3/primer_3www.cgi). The primer design allowed for differentiation between the amplified product of cDNA and any amplified product derived from contaminating genomic DNA. Primer pairs were manufactured by Sigma.

*Primer pairs***Actin:** fwd CTACGTCGCCCTGGACTTCGAGC/

rev GATGGAGCCGCCGATCCACACGG

CTD: fwd CCCGAGGTGCTCAAGAACTA/ rev TCACGTAGGTGCTGGACTTG**ER:** fwdCCACCAACCAGTGCACCATT/rev GTCTTTCCGTATCCCACCTTTC**PR:** fwd GTCAGTGGGCAGATGCTGTA/ rev AGCCCTTCCAAAGGA ATTGT**pS2:** fwd TTGTGGTTTTCTGGTGTCA/ rev CCGAGCTCTGGGACTAATCA**2. 2. Methods****2. 2. 1. Cell culture****2. 2. 1. 1. Routine cell line culture**

All MCF-7 variant cells were routinely cultured as monolayers in phenol red (PR)-free DMEM supplemented with 5% double charcoal stripped serum (DCSS), 0.1mg/ml penicillin/streptomycin and 2mM L-glutamine. Both ovarian cell lines were routinely cultured in RPMI 1640 media containing PR supplemented with 10% FCS, 0.1mg/ml P/S and 2mM L-glutamine. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂/ 95% O₂.

2. 2. 1. 2. Harvesting of cells

All cells were harvested at 70% confluence, with the exception of cells for RT-PCR, where ~50-60% confluence was required for initial seeding. In order to harvest cells for continued growth or for seeding for experiments, all cell lines were washed twice in PBS and trypsinised using trypsin/EDTA (1X) at 37°C for 5 min. The trypsin/EDTA was neutralised upon the addition of normal growth medium and the cell suspension was then centrifuged at 2,000 rpm for 5 min. Trypsin/EDTA containing media was discarded and the cell pellet resuspended in medium for growth or seeding at the appropriate cell density.

2. 2. 1. 3. Cell recovery from liquid nitrogen and cryopreservation

Cells were removed from liquid nitrogen storage and defrosted rapidly by the addition of warm medium. Cells were resuspended in a 10ml volume before centrifugation at 2,000 rpm for 5 min, whereupon the supernatant was replaced with fresh medium and cells seeded into 25cm² flasks. Cells were cryopreserved by harvesting and centrifuging as before, with the exception that cell pellets were resuspended in 1-2ml freezing media (10% DMSO in FCS) and transferred to cryopreservation vials for initial freezing at -70°C. Vials were transferred to liquid nitrogen storage tanks after 24h.

2. 2. 1. 4. Cell counting

Different experimental conditions required different concentrations of cells, thus cells were harvested as previously described, resuspended in 10ml of growth medium and cell number determined using a haemocytometer. The appropriate cell concentration was then achieved through dilution with media. Cells were grown in 24-well trays. Cells to be counted were washed twice with warm PBS and 300µl of trypsin/EDTA was added per well. The plates were then incubated at 37°C for 15-20 min, or until cells became detached from the wells. Once detached from the well surface, 200µl of 10% FCS DMEM was added to each well to stop any further trypsinisation. The plates were then kept on ice until cells were counted. The contents of each well were mixed using a 1ml BD Plastipak syringe with a gauge 3 BD Microlance needle. 200µl from each well was added to 1.8ml of 0.9% NaCl in a coulter pot. A Beckman Z2 Coulter particle counter and size analyser was used to count the number of cells present.

2. 2. 1. 5. Dextran-charcoal stripping of FCS

To study the functionality of the cell lines, it was necessary to grow the cells in medium containing charcoal-stripped FCS in order to remove endogenous stimuli that may have altered signalling. These endogenous steroids were stripped from FCS using dextran-coated charcoal and type IV sulphatase (EC 3.1.6.1). After thawing for 1h at room temperature, 1L of serum was heat inactivated at 56°C for 30 min, at

which point 2000U sulphatase was added and incubated for a further 2h at 37°C. The serum was pH adjusted to 4.2 using 2M HCl. A charcoal mix prepared prior to this stage was added and agitated overnight at 4°C. The charcoal mix consisted of 5g charcoal and 25mg dextran T70 in 50ml dH₂O, which was stirred overnight. The charcoal was removed from the serum via centrifugation at 10,000 rpm (30 min, 4°C), the pH was readjusted to 4.2 and then a second charcoal mix was added for further 24h agitation at 4°C. The serum was again centrifuged and a further spin required to remove any residual charcoal traces. 2M NaOH was used to return the serum to pH 7.2. The serum was then filter sterilised before aliquoting and storage at -20°C.

2. 2. 2 Functional assays

2. 2. 2. 1. Morphological study

Log-phase cells were seeded into 6-well tissue culture plates (optimised between $2-5 \times 10^4$ cells/ml). MCF-7 cells were seeded in 10% FCS DMEM (designated day - 2). After 24h cells were washed twice in PBS and media was replaced with 5% DCSS DMEM for 24h prior to treatment (day -1). As variant cell lines were routinely cultured in charcoal-stripped DMEM, these cell lines only required seeding 24h prior to treatment (day -1). On day 0, cells were treated with E₂ (1nM), TGF α (1nM), Tam (1 μ M) or E₂+Tam (1nM & 1 μ M respectively) for 48h before images of each cell line were taken using a Kodak MDS120 camera. These images were focused and processed using PhotoshopTM software.

2. 2. 2. 2. Growth assay

Log-phase cells were seeded into 24-well tissue culture plates (optimised between $2-5 \times 10^4$ cells/ml as before). Cells were seeded as previously described for the morphological study. Plates were treated for 0, 3, 5 and 7 days for each cell line. The day 0 plate only required cells seeding in the 2 outside columns to act as controls (no treatment). Plates for all other days were seeded in all 24 wells. A different treatment was added to each of the 4 middle columns to provide 4 wells and thus 4 cell counts for each treatment. After 48h charcoal stripping for MCF-7 and 24h post-

seeding for variant cells, day 0 plates were counted and plates for days 3, 5, and 7 were subjected to E₂ (1nM), TGF α (1nM), Tam (1 μ M) or E₂+Tam (1nM & 1 μ M). Ovarian lines were treated with E₂ and TGF α and a combination of the two ligands only. Cells were then counted and re-treated on days 3, 5 and 7.

2. 2. 2. 3. Inhibitor studies using the sulphorhodamine B assay

Log-phase cells were seeded into 96-well tissue culture plates (optimised between 2-5 x 10⁴ cells/ml) and treated with E₂, TGF α , HRG β 1 (all 1nM), Tam (1 μ M) or a combination of inhibitor and ligand for 72h. Control wells contained 0.05% DMSO as all inhibitors were reconstituted in DMSO. Specific inhibitor concentrations were determined through literature review and initial growth assays and were applied to western blotting for protein analysis (see Table 2.1 for inhibitor information). The treatment was halted by the addition of 50 μ l/well 25% trichloroacetic acid for 1h at 4°C. Plates were then washed 5 times in tap water and left to dry. Once dry, 50 μ l/well 0.4% SRB solution in 1% acetic acid was added and left for 30 min at room temperature prior to washing the plates 5 times in 1% acetic acid. Plates were again allowed to dry and then 150 μ l/well of 10mM Tris-Base (pH 10.5) was added for 1h at room temperature. The optical densities of each plate were ascertained at 540nm using a Biohit BP800 plate reader. Data were plotted as a percentage of control growth.

Table 2. 1 Inhibitor information

Inhibitor	Target	Chemical Name	Concentration range
LY 294002	PI3-Kinase	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one	0-70µM
NL-71-101	Akt	C ₂₆ H ₂₅ N ₃ O ₂ S (name not specified)	0-20µM
SB203580	p38 MAPK	4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole	0.1-70µM
UO126	MEK	1, 4-diamino-2, 3-dicyano-1, 4-bis (2-aminophenylthio) butadiene	0-60µM
U-73122	PLCγ	1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino) hexyl]-1H-pyrrole-2,5-dione	0-20µM
RhuMab2C4 (Pertuzumab)	ErbB2	Humanised anti-ErbB2 monoclonal antibody	100nM

2. 2. 2. 3. 1. SRB method development to determine correct seeding density

To determine the optimal seeding density for each cell line, cells were set up over a range of densities. Cells were trypsinised and counted on day 1. MCF-7 and resistant cell lines were seeded 72h and 24h prior to experimentation and MCF-7 cells exposed to charcoal-stripped FCS medium as previously described. S1 and S2 cell suspensions were prepared, S1 containing 250,000 cells/ml and S2 containing 25,000 cells/ml. Cell suspensions were counted after dilution to control the accuracy of dilution and altered with medium to reach the exact cell count. The difference from the theoretical number should be <20%. The different cell dilutions were prepared according to table 2. 2, with 150µl/well of each dilution from columns 2 to 11 in 7 96-well plates. The dilutions according to table 2. 3 were then prepared and 150µl/well of each plated from columns 2 to 11 in 1 plate. The calibration plate was precipitated the following day. After precipitation of the plates on days 1-7 the entire set of plates were stained according to the SRB protocol.

2. 2. 2. 3. 1. 1. Growth curves

The calibration plate was used to plot a graph with OD according to cell density. This is expected to be linear. The equation parameters were then calculated. The growth curves for each cell density were then plotted from the other plates (figures 2.1-3A). The calibration equation was used to calculate the number of cells/well on the day 2 plate. The latency, the time for the OD to increase by more than 20%, and the doubling times were also calculated (taken from data in figures 2.1-3C and D). Exponential growth implies that $Y = N_0 \cdot e^{kt}$, so the doubling time $t_{1/2} = \ln 2 / k$. This converted the optical densities (OD) to cell numbers, where upon a graph with cell number according to time using a logarithmic scale on the Y-axis was plotted (figure 2.1-3C). The optimal cell density for cytotoxicity studies was determined, which is the cell density that gives an OD>1.5 when cells are allowed to grow least 3 doubling times (figure 2.1-3D). It was observed that LCC9 and LY2 cell lines grew more slowly at lower densities than the other resistant cell lines, and proliferation only mirrored that of the other cell lines once cells reached $3-4 \times 10^5$ cells/ml. This may account for the non-linear aspect of the relationships between OD and cell number in these cell lines. LCC9 and LY2 cell lines were therefore seeded at $3-4 \times 10^5$ cells/ml.

Tables 2. 2 & 2. 3 Seeding for plates for SRB assay: stop one plate/day & calibration plate (lower table).

Cells/ well	cells/ml	vol S1	vol S2	medium	no. of wells	total vol cells	total vol medium	total vol
500	2500		0.02	0.18	56	1.12	10.08	11.2
1000	5000		0.04	0.16	56	2.24	8.96	11.2
1500	7500		0.06	0.14	56	3.36	7.84	11.2
2000	10000		0.08	0.12	56	4.48	6.72	11.2
2500	12500		0.1	0.1	56	5.6	5.6	11.2
3000	15000		0.12	0.08	56	6.72	4.48	11.2
3500	17500		0.14	0.06	56	7.84	3.36	11.2
4000	20000		0.16	0.04	56	8.96	2.24	11.2
4500	22500		0.18	0.02	56	10.08	1.12	11.2
5000	25000		0.2	0	56	11.2	0	11.2

S2 **61.6** (prepare 70ml)

500	2500		0.02	0.18	8	0.16	1.44	1.6
1000	5000		0.04	0.16	8	0.32	1.28	1.6
2000	10000		0.08	0.12	8	0.64	0.96	1.6
3000	15000		0.12	0.08	8	0.96	0.64	1.6
5000	25000	0.02	0.2	0	8	1.6	0	1.6
10000	50000	0.04		0.16	8	0.32	1.28	1.6
20000	100000	0.08		0.12	8	0.64	0.96	1.6
30000	150000	0.12		0.08	8	0.96	0.64	1.6
40000	200000	0.16		0.04	8	1.28	0.32	1.6
50000	250000	0.2		0	8	1.6	0	1.6

S1 **11.328** (prepare 15 ml)

S2 **3.68**

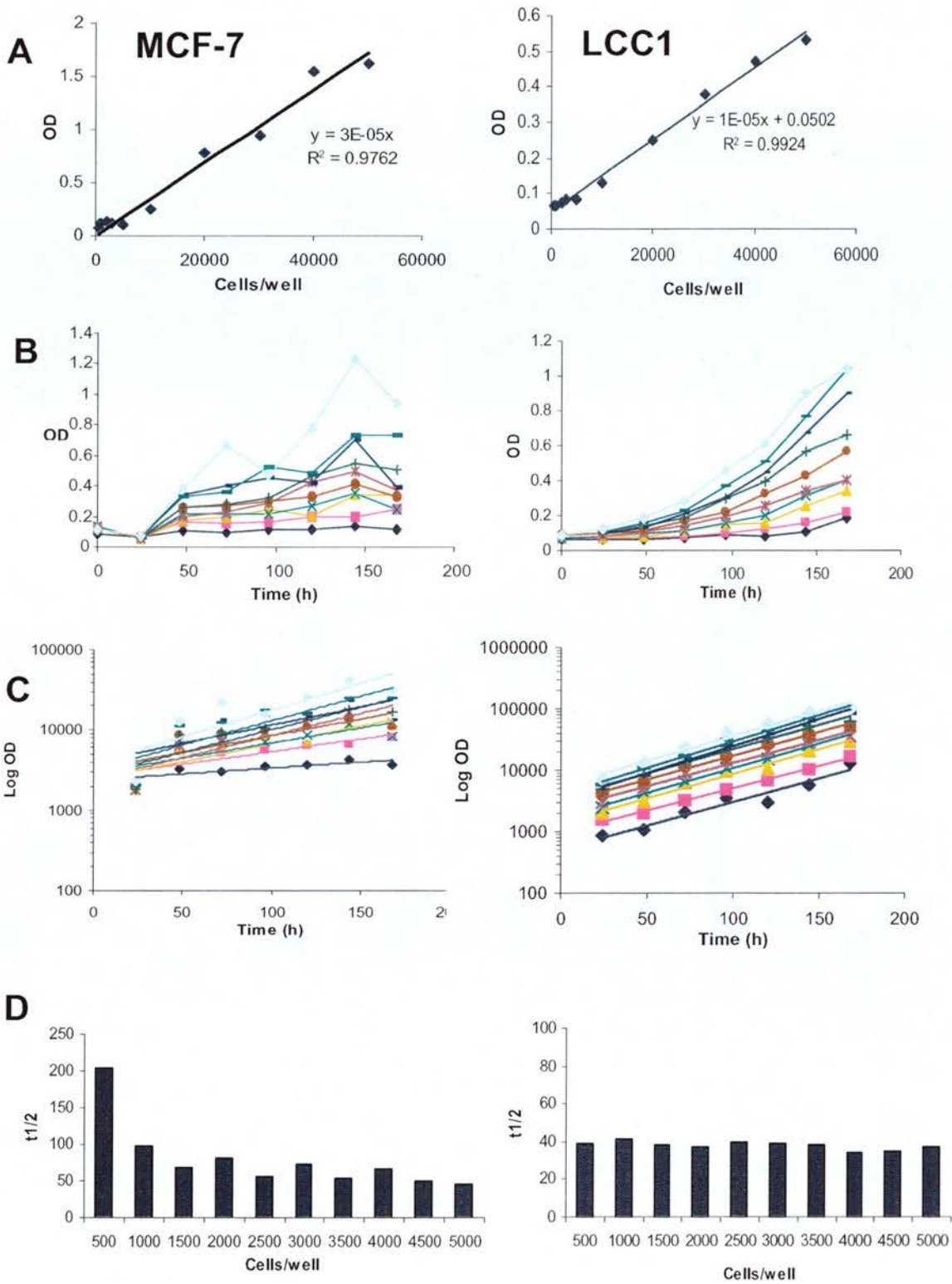


Figure 2. 1 Determination of the optimum seeding density of MCF-7 and LCC1 cells for the SRB assay in cytotoxicity and growth studies. Cells were seeded according to tables 2.2 and 2.3 and charcoal stripped 48h prior to the measurement of OD. ODs were obtained on days 1-7 and this could be directly related to cell number (A). B, proliferation, represented as OD values, according to seeding density over time (♦ =500, ■ =1000, ▲ =1500, × =2000, ✕ =2500, ● =3000, + =3500, - =4000, ▒ =4500, ◆ =5000 cells/ml). C, cell number according to time and (D) doubling times of each cell density. The final graph allows for the estimation of the optimum seeding density for subsequent SRB studies.

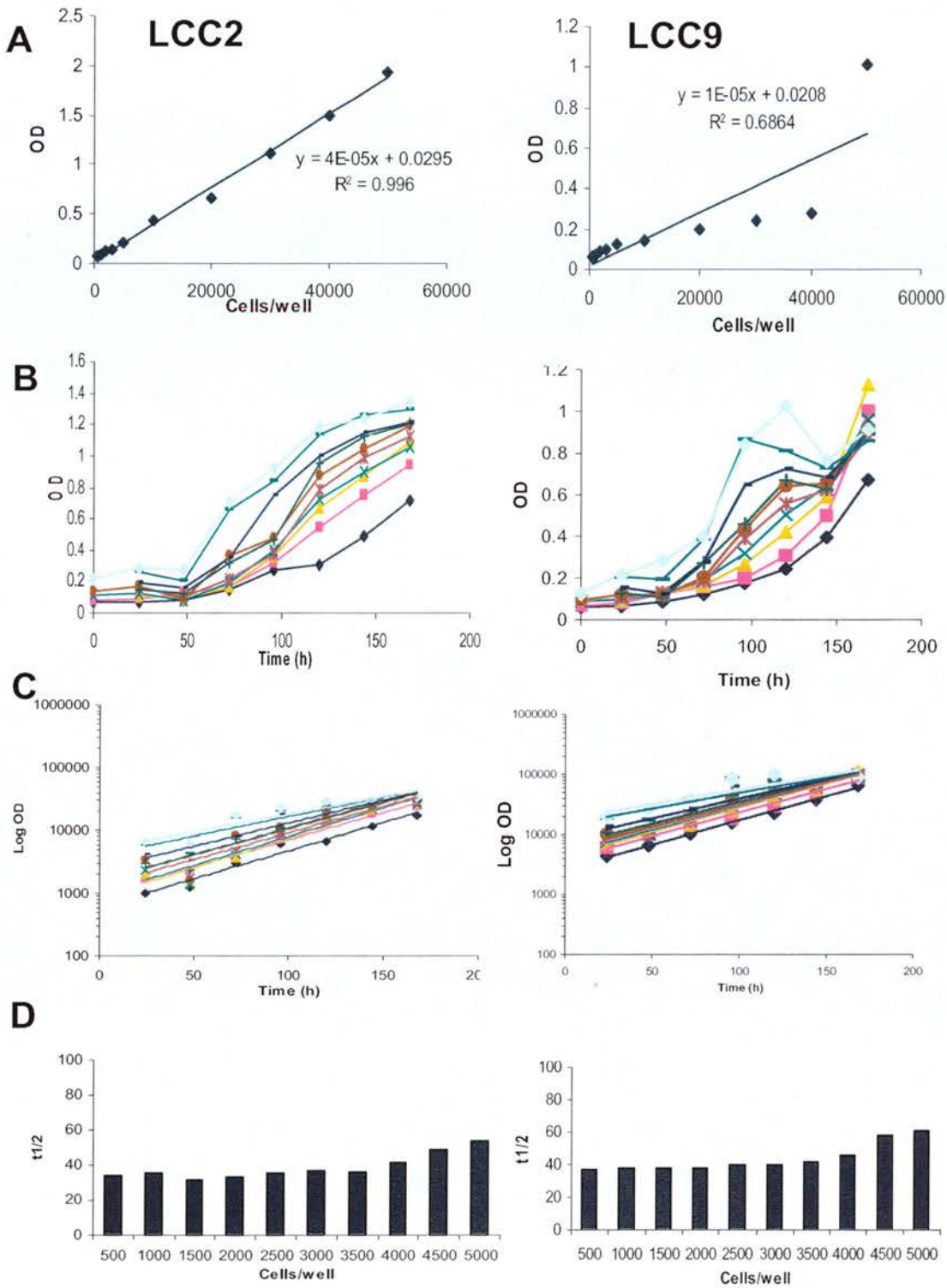


Figure 2. 2 Determination of the optimum seeding density of LCC2 and LCC9 cells for the SRB assay in cytotoxicity and growth studies. Cells were seeded according to tables 2.2 and 2.3 and charcoal stripped 48h prior to the measurement of OD. ODs were obtained on days 1-7 and this could be directly related to cell number (A). B, proliferation, represented as OD values, according to seeding density over time (♦ =500, ■ =1000, ▲ =1500, × =2000, × =2500, ● =3000, + =3500, - =4000, - =4500, ◊ =5000 cells/ml). C, cell number according to time and (D) doubling times of each cell density. The final graph allows for the estimation of the optimum seeding density for subsequent SRB studies.

LY2

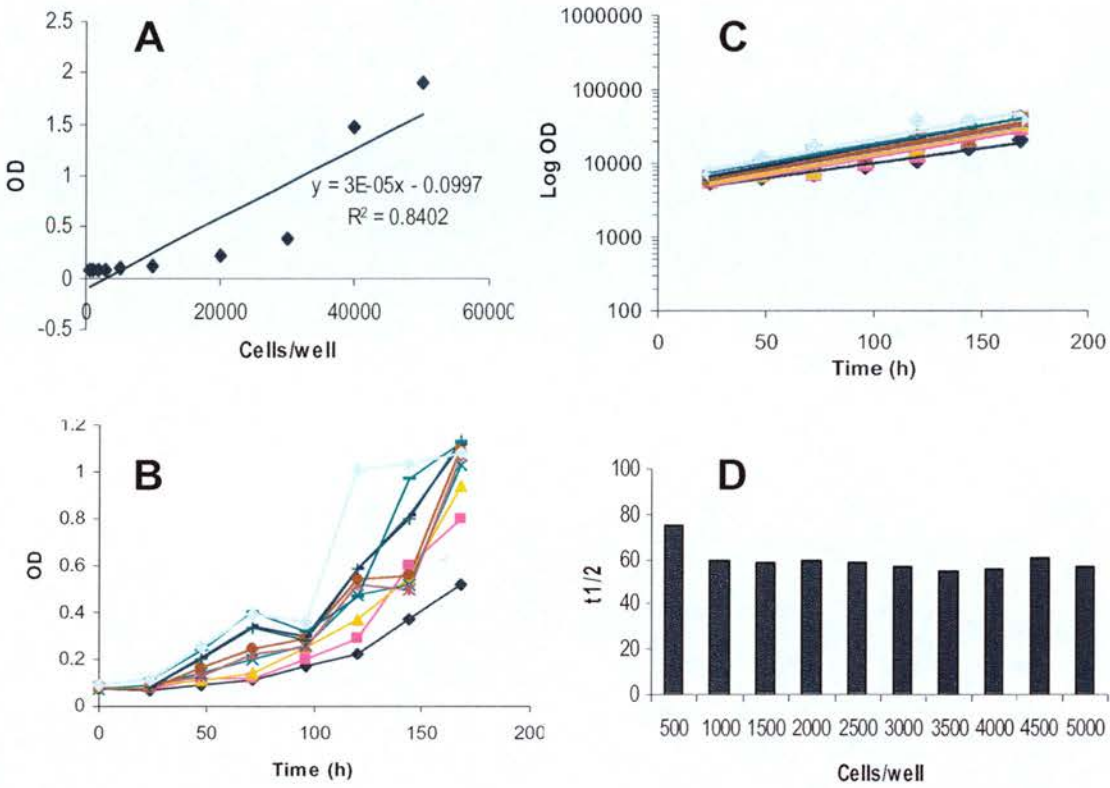


Figure 2. 3 Determination of the optimum seeding density of LY2 cells for the SRB assay in cytotoxicity and growth studies. Cells were seeded according to tables 2.2 and 2.3 and charcoal stripped 48h prior to the measurement of OD. ODs were obtained on days 1-7 and this could be directly related to cell number (A). B, proliferation, represented as OD values, according to seeding density over time (♦ =500, ■ =1000, ▲ =1500, × =2000, × =2500, ● =3000, + =3500, - =4000, - =4500, ◆ =5000 cells/ml). C, cell number according to time and (D) doubling times of each cell density. The final graph allows for the estimation of the optimum seeding density for subsequent SRB studies.

2. 3. Protein detection

2. 3. 1. Protein extraction

All cell lines were trypsinised at 70% confluence and seeded into 10cm diameter petri dishes (Nunc) at a density of 8×10^4 cells/ml, with the exception of LCC9 and LY2 cell lines, which were seeded at 1×10^5 cells/ml due to a slower growth rate. MCF-7 cells were grown for 24h in 10% FCS DMEM before being washed twice with PBS and then grown in 5% DCSS DMEM for a further 48h. Variant cell lines were seeded in 5% DCSS DMEM 24h before treatment. Cells were treated with E_2 (1nM), TGF α (1nM), Tam (1 μ M) or E_2 +Tam (1nM & 1 μ M) for either 15 or 30 min or at various time points spanning 0-24h. All agents used were diluted from stock to working solution using 5% DCSS DMEM. After the desired treatment time had elapsed the treatment solution was aspirated and cells washed twice with ice cold PBS. 1ml of ice cold PBS was added to each plate and the cells scraped into eppendorf tubes. Cell suspensions were centrifuged at 13,000 rpm at 4°C for 30s and PBS removed using a pipette. The remaining cell pellets were lysed in TNN lysis buffer for 30 min on ice. Following lysis, 10 μ l of the protein solution was used to perform a Bradford assay to obtain the protein concentration. Bradford protein assay dye reagent concentrate was obtained from Bio-Rad Laboratories GmbH (Munich, Germany). All samples were made up to either 0.5mg/ml or 1mg/ml depending on relative expression of ER α using lysis buffer. 5X loading buffer containing SDS and mercaptoethanol was added to each sample to give a final dilution of 1X SDS loading buffer. Aliquots were then stored at -20°C until required.

2. 3. 2. Western blotting

Stacking gels were poured onto 10% polyacrylamide running gels set up in Bio-Rad Mini Protean 3 cells. 10 μ g of each protein sample (including loading buffer) to be investigated was denatured at 95°C for 5 min, cooled on ice for 5min and then centrifuged for a further 5 min at 13,000 rpm at 4°C. Samples were loaded in individual wells of the stacking gel and run alongside a 1kb prestained protein marker broad range (premixed) (New England Biolabs inc.). Gels were run in 1X running buffer at 80V using a Power Pac 200 (Bio-Rad) for 15 min to allow all samples loaded to reach a similar level at the top of the running gel. The voltage was then increased to 200V for 1h, or until the loading buffer was run off the running gel.

Once run, gels were removed from the Mini Protean cells and placed in transfer tanks sandwiched next to nitrocellulose membrane at 4°C and subjected to 200mA/tank for 1.5h. After the wet transfer, nitrocellulose membranes were incubated in ponceau solution for 1 min to allow protein bands to become visible. Membranes were cut into the sections desired for separate antibody incubations, and then washed in water followed by 1X TBS for 5 min to remove the ponceau staining. Membranes were then incubated in 5% non-fat milk (Marvel) or 0.5% blocking solution (Roche) in 0.1% TBS-tween for 1h at room temperature to prevent non-specific binding. Following the 1h incubation, membranes were subjected to 3 x 5 min washes in 1X TBS-tween (0.1%). The membranes were then incubated in the appropriate primary antibody at 4°C overnight (optimum conditions for a phospho-antibody). The membranes were washed 3 times for 5 min each in 1X TBS-tween as before and then incubated in secondary antibody for 1h at room temperature. Post secondary antibody incubation, membranes were washed three times for 5 min each in 1X TBS-tween and then twice in 1X TBS for a further 10 min each wash to remove any detergent which may affect chemiluminescent detection.

Phospho-serine proteins were visualised by incubating the nitrocellulose membranes in a solution of equal parts of SuperSignal West Femto Maximum Sensitivity Substrate solutions (Pierce) at a 1:2 with H₂O. Stronger protein signals (ERK1/II etc) were detected using SuperSignal west pico stable solution and luminol/enhancer solution (Pierce) at a 1:2 with H₂O. Membranes were then sandwiched between 2 pieces of transparency in a photographic cassette where upon a piece of film was subjected to an initial exposure of 10s before being developed. Further film exposures for various lengths of time were then carried out on the basis of the strength of this initial signal.

2. 3. 3. Solutions

Running buffer (10X) consisted of 30g Trizma-Base, 144g glycine and 10g SDS (lauryl sulfate) made up in dH₂O, with transfer buffer (10X) containing Trizma-Base and glycine only. All solution components were supplied by Sigma as before. TBS (10X) comprised of 24.2g Trizma-Base and 80g NaCl (BDH Laboratory Supplies) and was reconstituted in dH₂O and titrated to pH 7.6. 10% Running gel comprised of 30% acrylamide/ bisacrylamide stock solution (Severn Biotech Ltd.), 1.5M tris (pH

8.8), 10% SDS, 25% ammonium persulfate and N, N, N', N'-tetramethylethylenediamine. These were all supplied by Sigma.

Human recombinant TGF α (0.1mg) was also provided by Sigma, with the initial stock solution being made up in 1ml PBS containing 0.5% BSA (1.67×10^{-5} M). E₂ and tamoxifen stock solutions (10^{-2} M) were made up in ethanol (BDH Laboratory Supplies). Lysis (TNN) buffer contained 150mM NaCl (BDH Laboratory Supplies), 0.5% NP-40 detergent (New England Biolabs inc.), a Mini complete protease inhibitor cocktail tablet (Roche), 1mM sodium fluoride, 1mM sodium orthovanadate, 50mM tris pH7.5 and 5mM EGTA.

2. 3. 4. Antibodies

ER α was detected using the primary ER α mouse monoclonal antibody clone and probed with secondary antibody goat anti-mouse IgG HRP conjugate. Phospho-oestrogen receptor α Ser 104/6, 118 and 167 were detected using the relevant specific primary antibodies made up in 1xTBS-tween with 0.5% blocking solution. All phospho-signals were probed for with the secondary goat anti-rabbit IgG HRP conjugate also made up in 1X TBS-tween with 0.5% blocking solution.

Protein loading was verified using actin ascites primary antibody and probed with the secondary antibody anti-mouse IgM, H & L Ch. (Goat) peroxidase conjugate. A summary of antibodies and antibody conditions is provided in tables 2.4 and 2.5.

Table 2. 4 Primary antibodies used in western blotting technique

Primary antibody	Dilution	Source	Type	Supplier details
Actin (α , β & γ) clone CP01	1: 50,000	Mouse	IgM	Calbiochem
Akt	1: 1000	Rabbit	IgG	NEB:# 9272
EGFR	1: 1000	Rabbit	IgG	NEB:# 2232
ER α F-10	1: 200	Mouse	IgG (mAb)	sc-8002
ErbB2	1:1000	Rabbit	IgG	NEB:# 2242
ERK1/II	1: 1000	Rabbit	IgG	NEB:# 9102
MEK	1: 1000	Rabbit	IgG	NEB:# 9122
P-Akt (Ser473)	1: 1000	Rabbit	IgG	NEB:# 9271
P-p44/42 MAPK (Thr202/ Tyr204)	1: 1000	Rabbit	IgG (polyclonal)	NEB:# 9101
P-MEK (Ser 217/221)	1: 1000	Rabbit	IgG	NEB:# 9121
P-ER α Ser 118 16J4	1: 1000	Mouse	IgG (mAb)	NEB:# 2511
P-ER α Ser 167	1: 500	Rabbit	IgG	NEB:# 2514
P-EGFR (Tyr845)	1: 1000	Rabbit	IgG	NEB:# 2231
P-ErbB2 (Tyr1248)	1: 1000	Rabbit	IgG	NEB:# 2247

New England Biotechnology (NEB) distributed by Cell Signaling technology; s-c- Santa Cruz Biotechnology Inc.

Table 2. 5 Secondary antibodies used in western blotting technique

Secondary antibody	Dilution	Supplier details
Anti-Mouse IgM, H & L Ch. (Goat) peroxidase conjugate	1: 500 (6ul in 30ml)	Calbiochem
Anti-rabbit IgG, HRP-linked	1: 1000	NEB:# 7074
Goat anti-mouse IgG HRP conjugate	1: 5000	Upstate, Cell Signaling Solutions

2.3.4.1. Antibody Optimisation

During this investigation, it was necessary to optimise the conditions when using certain antibodies as following manufacturer’s instructions did not necessarily produce western blots with clear bands and low background. Figure 2.4 illustrates the various conditions western blots were performed with using the phospho-ER α serine 118 antibody as an example. MCF-7 cells were treated with E₂ for 30 min as a positive control and harvested. Once transferred to nitrocellulose membrane, the membrane was cut into strips and different incubation conditions applied to each section. The optimum conditions found were a 1:1000 dilution of the antibody (as per manufacturer’s instructions) in 5% blocking solution (Roche) in 1X TBS. 5% 1X TBS containing 5% non-fat milk (NFM) also produced bands of a similar standard. Incubating the strips in 1X TBS without NFM or bovine serum albumin (BSA) or 1X TBS containing 5% BSA produced blots with a high background, making bands difficult to distinguish. Similar optimisation was performed for the phospho-ER α serine 167 antibody and any other antibodies where high background or multiple indistinguishable bands were observed.

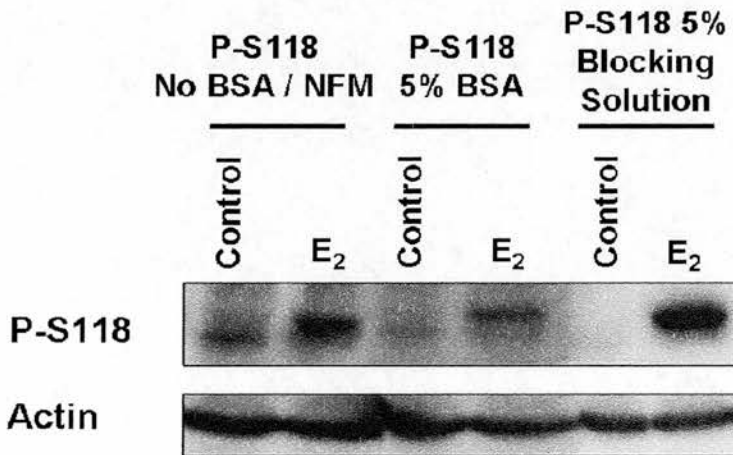


Figure 2. 4. Optimisation western blot of phospho-ER α serine 118 antibody (P-S118). MCF-7 cells were subjected to control media or media containing E₂ (1nM) for 30 min. Nitrocellulose membrane strips were incubated separately with P-S118 (1:1000) in 1X TBS without non-fat milk (NFM) or bovine serum albumin (BSA), 5% BSA or 5% blocking solution. Optimum conditions were found to be incubations performed in blocking solution.

2. 4. Flowcytometric analysis

For each culture condition, 5×10^5 cells were plated into 25mm³ petri dishes. Cells were seeded and charcoal stripped for 48h as previously described and then treated for 72h with control media, E₂ (1nM), HRG β (1nM), tamoxifen (1 μ M) and 2C4 (100nM) and various combinations of these treatments.

2. 4. 1. Cell cycle

After 72h the petri dishes were washed twice with 2ml PBS, 2ml of trypsin was then added per dish, taking care not to allow the cells to lyse. The trypsin was neutralised quickly with 2ml 10% FCS DMEM and the cells transferred to FACS tubes with individual syringes per treatment. The samples were centrifuged at 1600rpm for 5 min. Once centrifuged, the rims of the tubes were then blotted dry the pellets resuspended in 200ul vindelov citrate buffer at 4°C and vortexed gently. Samples were then stored at -20°C until time for analysis. Solutions A, B and C were thawed prior to use, with Solution C kept on ice after defrosting. Cell samples were thawed at room temperature and then 450 μ l of trypsin Solution A added per tube, briefly whirlmixed and incubated for 3 min. After 3 min 375 μ l of a trypsin inhibitor Solution B was added to neutralise the initial solution for 10 min. 250 μ l of Solution C containing propidium iodide was then added per tube and the tubes were then incubated on ice in the dark for a further 10 min. Stained cells were then immediately analyzed with a FACScalibur (Becton Dickinson) and data were obtained with the CellQuest 1.2.2 and the ModFit LT 1.01 software (Becton Dickinson).

2. 4. 2. Apoptosis

Annexin V can be utilized to detect apoptotic cells. Annexin V belongs to a family of calcium-dependent phospholipid-binding proteins which definitively belong to a family of ubiquitous cytoplasmic proteins involved in signal transduction. Annexin V binds preferentially with high affinity to phosphatidylserine (PS). PS is mainly located in membrane leaflets, facing the cytosol. During apoptosis, molecular machinery exposes PS at the cell surface, where it displays procoagulant and proinflammatory activity. Annexin V binds to the PS-exposing apoptotic cell, where its function is to inhibit the proinflammatory and procoagulant activities of a dying cell. Labelling with FITC permits direct detection by FACS analysis, and counterstaining with propidium iodide allows the discrimination of apoptotic cells.

Thus, the Annexin V-FITC Kit can be utilised to detect PS on the outer leaflet of the cell membrane using flow cytometry. Figure 2. 5 is a schematic diagram of the flipping of phospholipids during apoptosis and the subsequent binding of annexin V molecules to the surface of the cell.

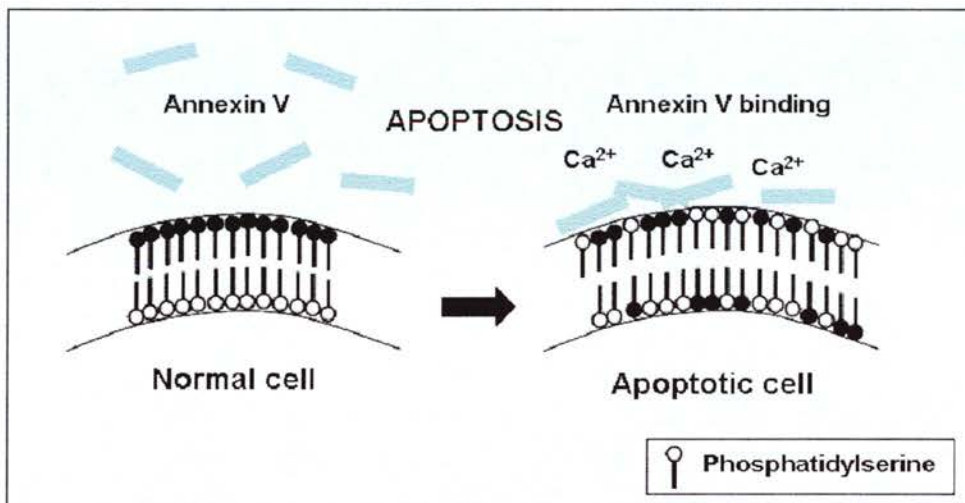


Figure 2. 5. Schematic diagram depicting the flipping of phospholipids during apoptosis and the subsequent binding of annexin V molecules to the surface of the cell.

Cells were treated with ligand or ligand in combination with inhibitor or vehicle for 48 hours as previously described for cell cycle analysis. Cells floating in the media were harvested into labeled FACS tubes and centrifuged at 1700rpm for 4 min. Supernatant was discarded and the pellets produced were pooled with cells trypsinised from the petri dishes (trypsinisation described in previous section 2. 4. 1., with the exception only 1.5ml 10% FCS DMEM was added to neutralize the trypsin). Samples were centrifuged at 1700rpm for 4 min. Supernatant was discarded as before and pellets resuspended in 1ml 10% FCS DMEM and incubated at 37°C for 5 min. Samples were further centrifuged at 1700rpm for 4 min. Cell pellets were resuspended in 1ml ice cold PBS and centrifuged as before. Cell pellets were then resuspended in 100µl ice cold annexin-v buffer (R&D Systems, 1:10 dilution in dH₂O). 10µl of propidium iodide (PI) and 1µl FITC were added per tube and incubated in the dark for 15 min. Three control tubes were also set up, one with only PI, another with FITC and the third containing neither agent. After 15 min 400µl annexin-v buffer was added per tube. Cytofluorometric analysis was performed (FACScalibur cytometer) within the hour and the background and experimental fluorescence intensities were evaluated by the CellQuest 1.2.2. software on 10,000 acquired events.

2. 4. 3. FACS Solutions

Citrate buffer contained 85.5g sucrose and 11.76g trisodium citrate (BDH Laboratory Supplies) in 800ml dH₂O. 50ml DMSO (BDH Laboratory Supplies) was then added and adjusted to pH 7.6. The solution was then made up to 1L with dH₂O. Stock Solution consisted of 200mg trisodium citrate (BDH Laboratory Supplies), 121mg Tris, 1044mg spermine tetrahydrochloride and 2ml Nonidet NP40 in 1.8L dH₂O. The solution was adjusted to pH 7.6 and made up to 2L with dH₂O. Solution A comprised of 15mg trypsin in 500ml Stock Solution (pH 7.6), aliquoted into 20ml volumes and stored at -20°C. Solution B contained 250mg trypsin inhibitor (Fluka Chemicals) and 50mg RNase A in 500ml Stock Solution (pH 7.6), aliquoted again into 20ml volumes and stored at -20°C. Solution C comprised of 208mg propidium iodide (Fluka Chemicals) and 500mg spermine tetrahydrochloride in 500ml Stock Solution (pH 7.6) aliquoted and stored as described for Solutions A and B. All chemicals were provided by Sigma unless otherwise stated.

2. 4. 4. Analysis of apoptosis results produced using the annexin-v assay and FACS analysis

A representative example of an apoptosis experiment produced using the annexin-v assay and FACS analysis with MCF-7 cells in the presence and absence of tamoxifen is shown in figure 2. 6. All cells were charcoal stripped for 48h as previously described and were then treated with control media (A) or tamoxifen (1µM) (B) for 72h. The graphs in A and B on the left hand side of the figure illustrate the area of scatter of cells selected for analysis (represented by forward and side scatter of cells). The graphs on the right hand side of the figure show the distribution of cells according to their apoptotic status dictated by the fluorescence produced. This example illustrates that the majority of MCF-7 cells in DCSS media are not undergoing apoptosis (Lower Left (LL) quadrant). Early apoptosis is represented in the Lower Right (LR) quadrant from which the apoptosis data were recorded. The Upper Right (UR) and Upper Left (UL) quadrants represent late stage apoptosis/necrosis and necrotic cells respectively. These processes were not under investigation here. The example in figure 2. 6 shows tamoxifen produced a small increase in the percentage of cells in the LR quadrant, and thus undergoing apoptosis.

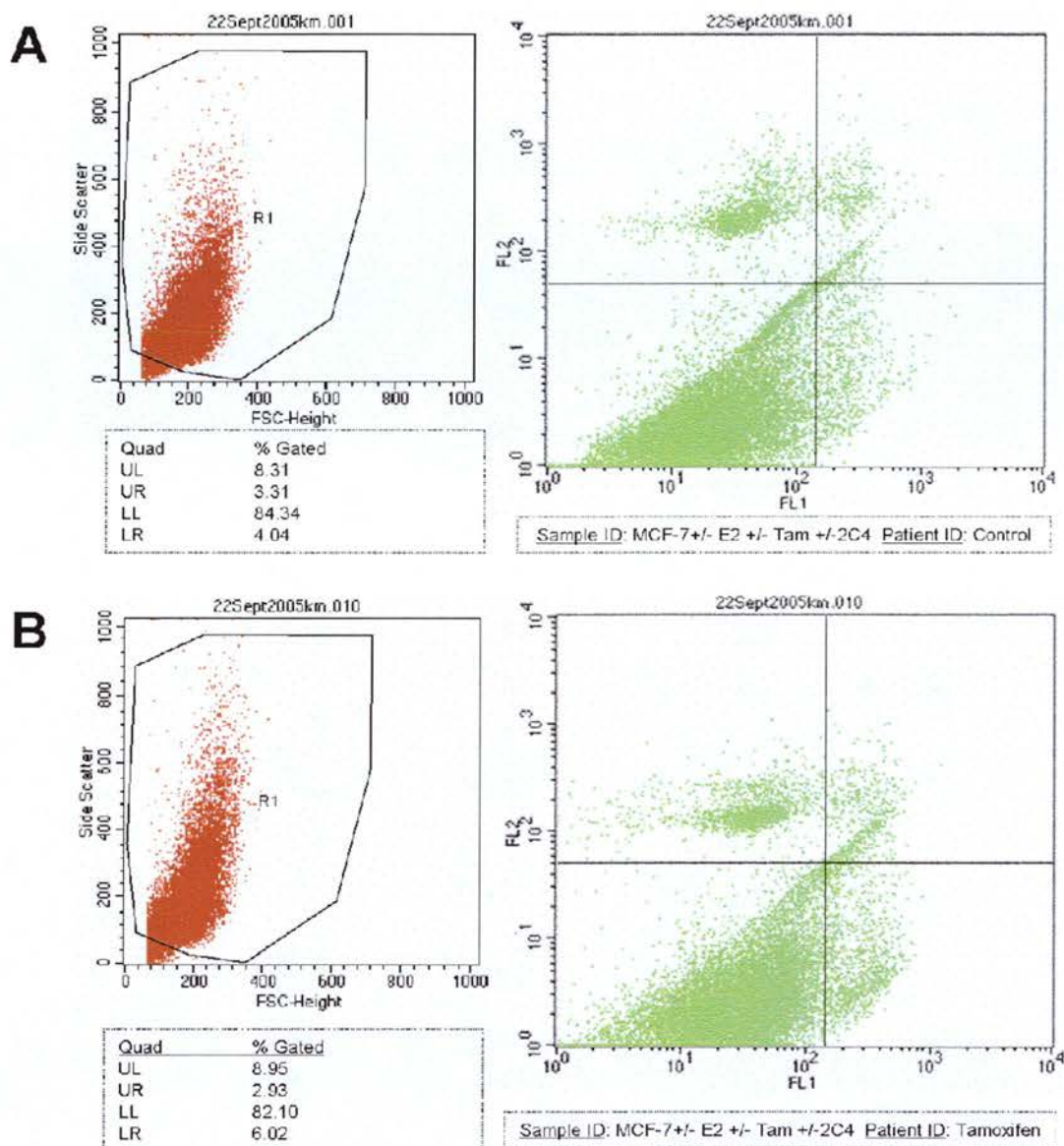


Figure 2. 5. Representative example of an apoptosis experiment produced using the annexin-v assay and FACS analysis with MCF-7 cells. Cells were charcoal stripped for 48h as previously described and were then treated with control media (A) or tamoxifen (1 μ M) (B) for 72h. The graphs in A and B on the left hand side of the figure illustrate the area of scatter of cells selected for analysis (represented by forward and side scatter of cells). The graphs on the right hand side of the figure show the distribution of cells according to their apoptotic status dictated by the fluorescence produced. This example illustrates that the majority of MCF-7 cells in DCSS media are not undergoing apoptosis (Lower Left (LL) quadrant). Early apoptosis is represented in the Lower Right (LR) quadrant from which the apoptosis data were recorded. The Upper Right (UR) and Upper Left (UL) quadrants represent late stage apoptosis/necrosis and necrotic cells respectively. These processes were not under investigation here. This example shows tamoxifen produced a small increase in the percentage of cells in the LR quadrant, and thus undergoing apoptosis.

2. 5. Inhibitor studies using quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

Log-phase MCF-7 cells were seeded into 75cm² flasks and charcoal-stripped 24h later and incubated for 48h prior to treatment. Resistant cell lines were seeded into 75cm² flasks 24h prior to treatment. Control times 0 and 24h flasks were set up alongside flasks treated with E₂ (1nM), TGF α (1nM), LY 294002 (10 μ M), UO126 (10 μ M), U71322 (5 μ M) and combinations of each ligand plus each inhibitor. Cells were pre-treated with U71322 for 1h while LY294002 and UO126 were added 30 min prior to ligand treatment. Control and treated flasks were harvested at 0 and 24h where appropriate.

2. 5. 1. Sample collection and RNA isolation

2. 5. 1. 1. RNA extraction

Cells were harvested into polypropylene tubes (Sarstedt) by the addition of 7.5ml TRI reagent per flask (1ml TRI reagent/10cm² of culture plate surface area). Once added, the cell lysate was passed through a pipette several times to form a homogenous lysate. Samples were left to stand for 5 min at room temperature to allow for complete dissociation of nucleoprotein complexes. 1.5ml chloroform was added per tube, the mixture shaken vigorously and the samples then left to stand for approximately 5 min at room temperature to allow for separation of the sample into layers. The samples were then centrifuged at 10K rpm for 15 min (SS34 rotor, Sorval RC5B centrifuge), 4°C, in order to separate the mixture into 3 phases (a red organic protein phase; a DNA interphase and a colourless RNA containing upper aqueous phase). The aqueous phase was transferred to a fresh tube and 3.75ml/sample of isopropanol was added and the tube inverted. This mixture was then centrifuged at 10K rpm for 10 min, 4°C. Supernatant was poured off and the pellet washed by adding 5ml 75% ethanol/tube, vortexing and centrifuging the sample at 13K rpm, 5min, 4°C. The sample was then briefly centrifuged again under the same conditions and the last few drops of ethanol removed. The pellets were briefly air-dried (in sterile hood) and 50 μ l dH₂O added and pipetted up and down before heating at 60°C for 10 min to aid dissolution.

2. 5. 1. 2. DNase treatment

2µl DNase 1 (RNase free) 10U/µl, 1µl RNasin 40U/µl and 5.8µl 10X buffer (all agents supplied by Boehringer Mannheim) were added to the 50µl volume of each sample and vortexed. Samples were then incubated at 37°C for 60 min. 60µl phenol:chloroform (50:50) was added to each sample, vortexed and centrifuged at 13K rpm, 15 min, 4°C. 55µl of the top layer was put in a separate tube to which 55µl of chloroform was added and vortexed for 5s. Samples were centrifuged for 15 min at 13K rpm, 4°C. Approximately 55µl of the top layer was transferred to a new tube on ice. Samples were then precipitated using 5.5µl 3M sodium acetate pH5.6 and 120µl ice cold 100% ethanol and stored overnight at -70°C. After a maximum of 24h samples were pelleted at 13K rpm, 15 min, supernatant removed and washed in 1ml 75% ethanol. Samples were centrifuged at 13,000 rpm (15 min, 4°C), supernatant removed and pellets centrifuged again to remove excess ethanol. RNA pellets were then resuspended in 50µl DEPC treated water and the concentration determined from the optical density (OD) at 260nm using a Unicam UV2 u.v. spectrophotometer.

2. 5. 1. 3. qRT-PCR

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) allows for the quantitative detection and analysis of specific genes from small amounts of RNA using double-stranded DNA fluorescent dyes such as SYBR Green. RT-PCR amplification of a particular mRNA sequence requires a pair of primers which are specific for that mRNA sequence. The reverse transcriptase transcribes the RNA to cDNA, by extension from a primer complementary to the RNA sequence, where upon the PCR process immediately takes place. PCR amplification can be compartmentalised into three sections; an early background phase (little product accumulation), an exponential phase (or log linear phase, rapid product accumulation) and a plateau phase (no further product is amplified). The principle of RT-PCR is based on measuring fluorescence signals at the end of the extension phase of each PCR cycle that are generated following SYBR Green I (a double stranded (ds) DNA-binding dye) binding to PCR products. During the exponential phase of PCR theoretically the amount of DNA doubles at every cycle, and therefore during this phase the fluorescent signal increase should be directly proportional to the PCR product amount. The fluorescence signal increases as the product increases, resulting

in a sigmoidal-shaped curve. SYBR Green I dye is not specific therefore the reaction is made specific using “hot-start” PCR and by empirically determining annealing and signal acquisition temperatures for each gene-specific primer pair. Relative expression levels are quantified by constructing a standard curve using serial dilutions of a cDNA template to monitor the expression of a highly expressed gene. Any given piece of dsDNA will melt at a specific temperature which is mainly determined by the length and base content of the dsDNA. As temperature increases during PCR, the PCR product will denature and hence release any associated SYBR Green, causing a sharp decline in fluorescence. A good indication of the purity of the PCR product can be ascertained from construction of a melt curve, which is obtained by plotting the negative first derivatives values of the fluorescence intensity as a function of the temperature, resulting a peak. A single narrow peak is the optimal result.

β -Actin was used as the housekeeping gene in order to confirm initial equal mRNA concentration, i.e. the housekeeping gene acts as a loading control, where all test cDNA products were divided through by the amount of β -actin cDNA produced from the same sample. This gene was used to monitor loading as levels are equal and constant irrespective of treatment, unlike other genes whose expression may be up or downregulated.

qRT-PCR was carried out according to manufacturers instructions (QuantiTect™ SYBR® Green RT-PCR handbook), with the exception that the reaction mix per sample comprised of 7.5 μ l 2x Quantitect SYBR green, 0.375 μ l 20 μ M mix of primers, 0.15 μ l Quantitect RT Mix, 2.975 μ l RNase-free water and 4 μ l template RNA to give a final reaction volume of 15 μ l/sample. The Real Time cycler (Rotorgene RG-3000, Corbett Research) conditions were RT: 50°C for 30min (for primer annealing); PCR: initial activation 95°C for 15 min; followed by 40 cycles of denaturation 94°C for 15 sec, annealing 57°C for 30 sec, extension 72°C for 30 sec; and a final extension of 72°C for 60 sec. Details of primers used are detailed in the Materials section.

2. 5. 1. 3. 1. Confirmation of PCR products

The primers were confirmed to detect the correct cDNA (genes) via entering the forward and reverse primer sequences into the Blast database (<http://www.ncbi.nlm.nih.gov/Blast>). The database provides any sequences to which the primers could bind to and subsequently amplify. All primer pairs were found to only bind to and amplify the gene of interest for which they were designed. The specificity of the PCR products was determined by running all the products on an agarose gel (as well as referring to the melt curve produced during qRT-PCR). Positive control treated (E_2 (1nM), 24h) MCF-7 cell PCR product samples were run against a 100bp ladder (Invitrogen) on a 2% agarose gel (2g agarose heat dissolved in 100ml 1X TAE buffer (1L 50X TAE buffer consisting of 242g Tris-base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA, titrated to pH 8), plus 1 μ l ethidium bromide post-heating). 9 μ l of each qRT-PCR sample plus 1 μ l 10X DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in dH₂O) were loaded and run at 60V for approximately 1h, or until the bands of the ladder had spread sufficiently to distinguish between molecular weights. Bands were visualised using a dual intensity ultraviolet transilluminator (Ultra-Violet Products) and a picture produced using GDS Imagestore 7500 Version 7.12 software. Single bands were produced from each sample indicating only cDNA product of that size was produced per PCR reaction, and these band sizes each corresponded to their relevant sequence designed to be amplified by the primer pairs (gel shown in Figure 2. 7). This indicates the primers were gene-specific and the absence of multiple bands indicates the PCR products were not contaminated with products of different sizes. The correct molecular weights of each PCR product are listed below;

- ER α 107bp
- pS2 209bp
- PR 193bp
- CTD 196bp

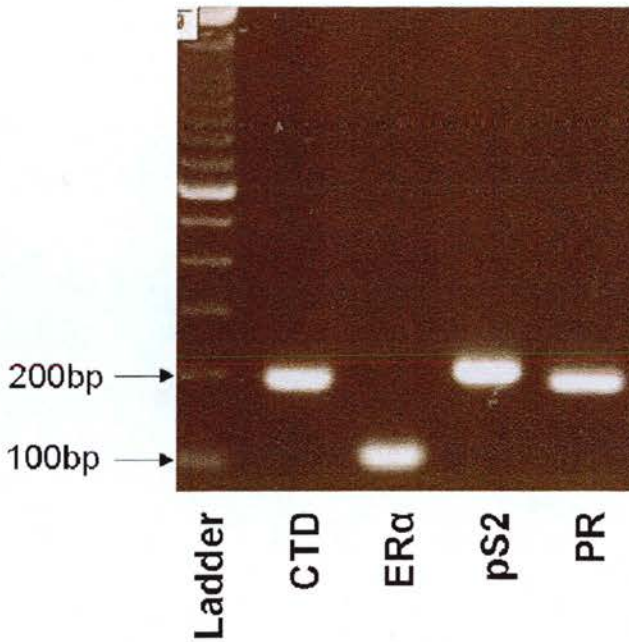


Figure 2.7 Confirmation of PCR products. The PCR products of MCF-7 cells treated with E_2 (1nM) for 24h after RT-PCR with CTD, ER α , pS2 and PR primers were run on a 2% agarose gel in order to confirm their molecular weights corresponded with the relevant sequence designed to be amplified by the primer pairs. All PCR products were of the correct molecular weight (ER α 107bp; pS2 209bp; PR 193bp; CTD 196bp).

2. 6. Statistical Analysis

One-way ANOVA with Tukey-Kramer post test was performed using GraphPad In-Stat version 3.06, for Windows 95 (GraphPad Software, San Diego California, USA, www.graphpad.com), where any changes which were considered statistically significant were allocated P values of $P < 0.05$ (*, significant), $P < 0.01$ (**, highly significant) and $P < 0.001$ (***, extremely significant).

Analysis of variance (ANOVA) permits comparison of population means, and if the null hypothesis is rejected, the Tukey-Kramer procedure can be utilised to determine any statistically significant differences between pairs of means, which is not possible with the ANOVA procedure. This provides an advantage over ANOVA alone as it specifies which treatment means have a statistically significant difference. Both simultaneously test for equality of all the mean values. The Tukey-Kramer test involves numerous statistics already computed in ANOVA, thus the former test is often used as a supplement to ANOVA rather

than as a replacement. It should be noted that ANOVA and the Tukey-Kramer tests both rely on data with a normal distribution, departure from which may cause errors. The robustness of the ANOVA and Tukey-Kramer statistical tests is discussed by Driscoll (1996).

The tests were utilised in conjunction to ascertain if cells treated with various agents such as TGF α , induced a significant change in growth, mRNA expression and phospho-signal expression for example, compared to not only untreated cells, but also to other treatments. Therefore it was possible to evaluate the effect of a treatment versus several others, including combination treatments. For example, HRG β and TGF α both enhanced phospho-Akt (P-Akt) expression compared to basal levels and statistical analysis of 3 replicate independent experiments provided information that not only were both growth factors able to elicit extremely significant increases in P-Akt compared to control untreated cells, but that HRG β enhanced P-Akt to a significantly greater extent than TGF α .

2. 7. Consistency of Data

In order to confirm the reproducibility and reliability of the data shown in representative individual experiments shown in each results chapter a summary table of some key data is shown in table 2. 3. The table is a summary of the consistency of some of the key results including percentage change in proliferation (reached by day 7, compared to day 7 control, with the exception of day 7 control, which was compared to the day 0 control), signalling characteristics (P-S118 and P-Akt shown as examples) and gene expression (ER α and pS2 shown as examples).

The data shown are final values from three independent replicate experiments. Each value was analysed using the ANOVA Tukey-Kramer test in relation to the other treatments performed in that experiment (for example, control compared to E₂, TGF α and HRG β treatments and also each treatment with one another). ANOVA & Tukey-Kramer were used to compare across treatments in each cell line, while control values were compared between cell lines to ascertain any significance between the parental & resistant phenotype. The mean of the three

values from the three independent experiments were then analysed in comparison with the means of all the other treatments using the ANOVA procedure to give a final significance value. This value is given in each box of the table. For example, although a modest increase in percentage cell proliferation was observed in the MCF-7 cell line under control conditions (as shown by the numbers being red in colour), the final value of $31.7 \pm 26.5\%$ ($P > 0.05$) was not a significant increase compared to the $2992.3 \pm 1252.8\%$, $1339.8 \pm 606.1\%$ or $1614.4 \pm 771.7\%$ increases observed with E_2 , $TGF\alpha$ and $HRG\beta$ treatment respectively ($P < 0.001$). Values shown in blue represent a decrease in percentage proliferation or gene expression for example. Numbers in black indicate either no change, or that the change is not significant.

Table 2. 3 Summary of the consistency of some of the study key results; Percentage Change in Proliferation, Relative P-S118 and P-Akt Expression and Relative ER α and pS2 Expression. Values shown in red and blue represent an increase or decrease respectively in proliferation for example. Values in black indicate either no change, or that the change was not found to be statistically significant using the ANOVA and Tukey-Kramer tests where * = P<0.05 and is significant, ** = P<0.01 and is deemed highly significant and *** = P<0.001 and is considered to be extremely significant. ANOVA & Tukey-Kramer were used to compare across treatments in each cell line, while control values were compared between cell lines to ascertain any significance between the parental & resistant phenotype. (Tx = treatment)

Assay/Cell	MCF-7	LCC1	LCC9	
% Change in Proliferation-day 7 Tx cf. day 7 Cont/ cell line conts	Cont	60.4 + 26.7 + 8.1 = 31.7 ± 26.5% (P>0.05)	2120 + 2813 + 4629.4 =3187.5 ± 1295.9% (P<0.001)	3138.4 + 2992 + 5450.5 = 3860.3 ± 1379.1% (P<0.001)
	E ₂	2360+2182.8+4434.2 = 2992.3 ± 1252.8% (P<0.001)	152.6 + 20.4 + 91.2 =88.1 ± 66.2% (P<0.01)	0.7 + 33.8 + 20.2 = 3.5 ± 13.9% (P>0.05)
	TGF α	982.6+2039.6+ 997.3 = 1339.8 ± 606.1% (P<0.001)	17.2 + 0.94 + 9.5 = 2.9 ± 13.5% (P>0.05)	12.5 + 11 + 12.1 = 4.3 ± 27.3% (P>0.05)
	HRG β	813 + 2352.5 + 1614.4 = 1614.4 ± 771.7% (P<0.001)	6.7 + 12.2 + 41.1 = 11.9 ± 66.2% (P>0.05)	6.9 + 17 + 13.8 = 8 ± 13% (P>0.05)
	Tam	0 + 0 + 50.2 = 16.7 ± 29% (P>0.05)	2.7 + 8.6 + 13.9 = 2.7 ± 11.25% (P>0.05)	11.32 + 13 + 5.9 = 10.1 ± 3.7% (P>0.05)
	E ₂ + Tam	90.1 + 70.3 + 63.5 = 74.6 ± 13.8% (P<0.001)	12.6 + 39+ 27.9 = 26.5 ± 13.3% (P>0.05)	4.2 + 0.2 + x = x ± y% (P>0.05)
Relative P-S118 Expression cf. Cont/ E₂ cf. E₂+Tam-30min	Cont	0.28 +0.26 + 0.18 = 0.24 ± 0.05 (P>0.05)	0.31 + 0.13 + 0.51 = 0.32 ± 0.19 (P>0.05)	0.06 + 0.11 + 0.08 = 0.08 ± 0.03 (P>0.05)
	E ₂	1.08 +0.88 + 1.03 = 1 ± 0.1 (P<0.001)	1.42 + 1.28 + 1.55 = 1.42 ± 0.14 (P<0.001)	0.02 + 0.06 + 0.07 = 0.05 ± 0.03 (P>0.05)
	TGF α	0.29 + 0.45 + 0.56 = 0.43 ± 0.14 (P>0.05)	0.3 + 0.71 + 0.5 = x ± y (P<0.001)	0.04 + 0.1 + 0.06 = 0.07 ± 0.03 (P>0.05)
	Tam	0.48 +0.52 + 0.33 = 0.44 ± 0.1 (P>0.05)	0.87 + 0.6 + 0.59 = 0.69 ± 0.16 (P>0.05)	0.03 + 0.05 + 0.1 = 0.06 ± 0.04 (P>0.05)
	E ₂ + Tam	0.48 +0.67 + 0.55 = 0.57 ± 0.1 (P<0.001)	1.14 +1.08 + 0.86 = 1.03 ± 0.15 (P<0.001)	0.07 +0.07 + 0.07 = 0.07 ± 0 (P>0.05)

<i>Relative P-Akt Expression cf. +ve control</i>	Cont	0.15 + 0.19 + 0.15 = 0.16 ± 0.02 (P>0.05)	1.31 + 1.82 + 2.7 = 1.94 ± 0.7 (P<0.001)	1.18 + 1.36 + 1.37 = 1.3 ± 0.11 (P<0.001)
	E ₂	0.12 + 0.18 + 0.39 = 0.23 ± 0.14 (P>0.05)	2.41 + 1.57 + 1.59 = 1.86 ± 0.48 (P<0.001)	0.98 + 1.34 + 1.2 = 1.17 ± 0.18 (P<0.001)
	TGFα	1 + 1 + 1 (+ve control) = 1 ± 0 (P<0.001)	2.79 + 3.2 + 2.07 = 2.69 ± 0.8 (P<0.001)	2.2 + 2.9 + 1.92 = 2.34 ± 0.5 (P<0.001)
	HRGβ	5.09 + 7.78 + 4.42 = 5.76 ± 0.02 (P<0.001)	6.5 + 5.24 + 5.74 = 5.83 ± 0.63 (P<0.001)	3.34 + 6.17 + 4.2 = 4.57 ± 1.45 (P<0.001)
<i>ERα Expression cf. Cont</i>	Cont	2.26 + 1.96 + 1.48 = 1.9 ± 0.4 (P>0.05)	3.86 + 3.66 + 4.31 = 3.94 ± 0.3 (P<0.001)	2.03 + 3.82 + 2.93 = 2.93 ± 0.9 (P<0.05)
	E ₂	1.49 + 1.24 + 1.21 = 1.31 ± 0.15 (P<0.05)	2.32 + 3.79 + 2 = 2.7 ± 0.95 (P<0.01)	1.82 + 1.72 + 3 = 2.18 ± 0.7 (P>0.05)
<i>pS2 Expression cf. Cont</i>	Cont	0.1 + 0.28 + 0.06 = 0.15 ± 0.12% (P>0.05)	1.01 + 1.2 + 1.85 = 1.35 ± 0.44% (P<0.001)	0.66 + 0.58 + 0.92 = 0.72 ± 0.18% (P<0.001)
	E ₂	1.04 + 1.53 + 1.27 = 1.28 ± 0.25% (P<0.001)	3.4 + 5.2 + 3.18 = 3.93 ± 1.11 % (P<0.01)	2.15 + 2.72 + 3.96 = 2.94 ± 0.93% (P<0.01)

Chapter 3

Characterisation of oestrogen and growth factor regulated responses in resistant breast cancer cell lines.

3. 1. Growth characterisation

A selection of ovarian and breast cancer cell lines were chosen as models of oestrogen sensitivity and resistance. The cell lines were first characterised in response to the SERMs 17β -oestradiol (E_2) and tamoxifen (Tamox, Tam) and the growth factors $TGF\alpha$ and $HRG\beta$. The breast cancer cell lines were characterised further in order to determine whether any differences in the mRNA expression of oestrogen-responsive genes, cell cycle distribution or apoptosis were present and if there were links to the development of endocrine-therapy resistance.

3. 1 .1. Breast cancer cell lines

3. 1. 1. 1. Growth responses

Breast cancer cell lines MIII, LCC1, LCC2, LCC9 and LY2 were characterised in conjunction with their parental breast cancer cell line MCF-7 in response to treatment with a concentration range of E_2 (figure 3. 1). The endocrine sensitivity of each cell line was then characterised by treating the cells with E_2 in the presence and absence of tamoxifen (figure 3. 2). The panel of cell lines was narrowed for growth characterisation with $TGF\alpha$ and $HRG\beta$ (figure 3. 3). MIII and LCC2 cell lines were excluded from more detailed studies as their growth phenotypes were similar to those of other lines in the panel and thus deemed superfluous. The morphology of the cells was observed after treatment with E_2 , $TGF\alpha$, Tamox, $E_2 + TGF\alpha$ and $E_2 + Tamox$ (figure 3. 4A-C).

In order to ascertain if any concentration of E_2 would stimulate the resistant cell lines and to determine the optimum E_2 concentration for further studies, cells were treated with 0.1-100nM E_2 . Control media used was phenol red-free DMEM containing 5% charcoal stripped FCS (DCSS). The relative E_2 sensitivity of the cell lines using a 1nM concentration after 7 days, was $MCF-7 > MIII > LCC1 = / > LCC2 > LCC9 = LY2$ (figure 3. 1). 1nM E_2 was used as this concentration induced the optimum increase in cell number in the majority of oestrogen-responsive cells lines and was used for further studies. The relative E_2 -sensitivity of the cell lines was determined by comparing the fold changes induced in proliferation of each cell line by a set concentration of E_2 on a set day.

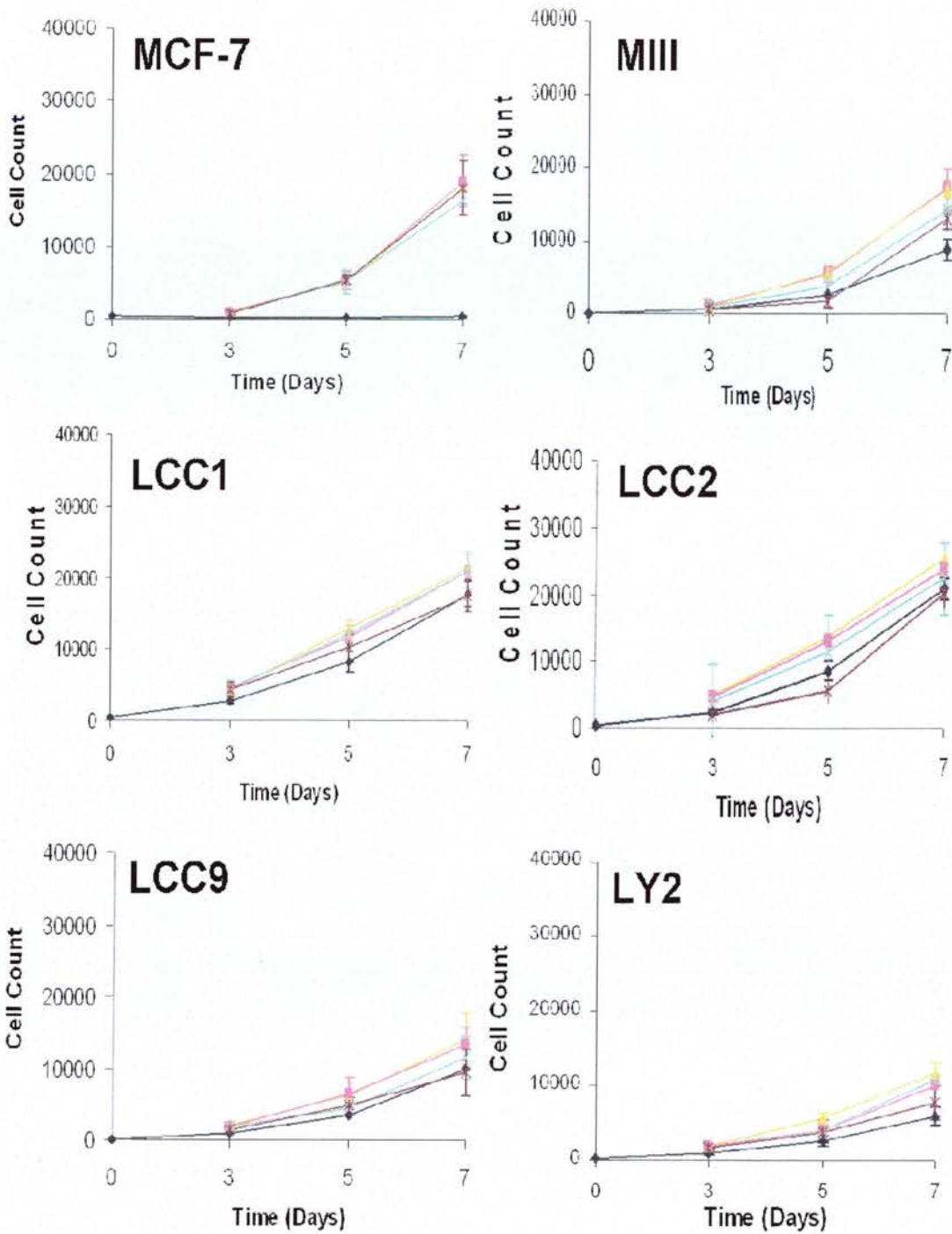


Figure 3. 1 The effect of various E₂ concentrations on the growth of resistant breast cancer cell lines versus the sensitive parental MCF-7 breast cell line. All cell lines were charcoal stripped 24h prior to treatment and were then treated with control media (◆) or 0.1nM (▲), 1nM (■), 10nM (x) or 100nM (■) E₂. Cells were treated and counted on days 0, 3 & 5 and a final count took place on day 7. Data were plotted as a mean of quadruplicate cell counts +/- SD for treated values and as a mean of 8 cell counts +/- SD for untreated cells. Results are representative of 1 of 3 independent experiments.

For example, 1nM E₂ produced a significant increase in MCF-7 cell proliferation of ~63-fold (day 7, P<0.001 compared with day 7 control). The fold increase fell to ~2-fold for the MIII cell line (P<0.001), and ~1.3-fold in both the LCC1 and LCC2 cell lines by day 7. The fold increase in the MIII cell line was still considered significant as the cell counts were very similar. In contrast, the cell counts for the LCC1, LCC2, LCC9 and LY2 cell lines varied somewhat, producing larger standard deviations and therefore even the increases of ~1.3 and ~1.6-fold for LCC9 and LY2 cell lines respectively were not found to be statistically significant (1nM E₂, day 7 compared to day 7 control, P>0.05). The LCC1 and LCC2 cell lines displayed some E₂-sensitivity at 1nM, but this was only significant at day 5 (~1.4 and ~1.5-fold increases respectively, P<0.001 compared to day 5 control). In contrast to observations here, Bronzert *et al* (1985) previously reported the LY2 cell line to be E₂-sensitive. However, the statistical increases noted by this author were observed after a minimum of 8 days in the presence of 10nM E₂, or 10 days in the presence of a range of concentrations compared to a maximum of 7 days in this experiment. The shorter incubation period in these studies may account for the absence of a significant increase in response to E₂ treatment. Also, DMEM media was used in this study in comparison to IMEM media utilised by Bronzert *et al* (1985), although this should not produce dissimilar results. As the growth of the LY2 cell line was not found to be significantly increased by 1nM E₂ after 0-7days incubation in these studies, and 1nM was used in further experiments, the cell line was considered to be E₂-insensitive under these conditions for the remainder of the study.

A similar E₂-responsive pattern to that seen with a 1nM concentration was also seen with 0.1nM E₂ on day 7, where the MCF-7 cell line was increased by ~62-fold (P<0.001), the MIII cell line by ~2-fold (P<0.001) and LCC1 and LCC2 cells by ~1.2-fold, values similar to those produced with 1nM E₂. Fold increases in the LCC1 and LCC2 cells were found to be significant on day 5 (only LCC1 cells were stimulated on day 3, and insignificant at this concentration on day 7). Any increase in growth in the LCC9 and LY2 cell lines was statistically insignificant (P>0.05, days 3, 5 and 7). Interestingly, the maximum concentration of E₂ used did not cause the

greatest increase in growth. 100nM increased growth by ~60-fold in MCF-7 cells compared to a ~63-fold increase with the lesser concentration of 1nM. This suggests the effect of E₂ stimulation plateaus between 1nM and 100nM in the MCF-7 cell line. This plateau effect may also occur in the MIIII cell line, or possibly a loss of growth stimulation is occurring, although all E₂-induced fold increases in growth remain significant compared to control conditions. For example, 10nM and 100nM concentrations produced smaller fold growth increases compared to lower concentrations, with ~1.6-fold and ~1.5-fold increases respectively (P<0.01)

LCC1 cells were significantly stimulated by all concentrations by day 3, with the greatest increase produced with 1nM E₂ (~1.8-fold, P<0.01). By day 5, E₂ concentrations of 1nM and 10nM significantly increased LCC1 cell proliferation by ~1.4 and ~1.5-fold respectively (P<0.01-0.001). By day 7 only 10nM significantly enhanced proliferation beyond the control level of proliferation, and only by ~1.2-fold (P<0.05). No significant growth increase was observed in LCC2 cells by day 7. Therefore, a higher E₂ concentration did not necessarily produce the greatest growth response in this panel of cell lines. 1nM E₂ was selected for use as firstly it produced a similar growth response to that of higher concentrations tested and secondly, it is a physiologically relevant dose at the upper end of the normal range for a premenopausal woman.

MCF-7 cells did not grow significantly in charcoal-stripped media in the absence of E₂ treatment, which contrasted with the resistant cell lines, which continued to proliferate in the absence of E₂ (a minimum 10-fold increase in proliferation from day 0 to day 7 control, P<0.001). The MCF-7 cell line was the most sensitive to all treatments (figures 3. 2 & 3. 3), with a cell growth increases of ~25-fold & ~11-fold (both P<0.001) with E₂ & TGF α treatments respectively (day 7). The slight discrepancy in size of the E₂-fold induction in the MCF-7 cells in the separate experiments is likely to be due to a difference in the initial seeding density.

Figure 3.2 shows that **tamoxifen** almost completely abrogated E₂-induced cell proliferation in MCF-7 cells, with a reduction in growth of ~10-fold (P<0.001) by

day 7 in the presence of E_2 + **tamoxifen** compared to E_2 alone. The resistant cells proliferated in the absence of any ligand stimulation (control) and this was observed in all growth experiments (figures 3. 1, 3. 2, 3. 3). MIII cells were sensitive to E_2 (1nM) and there was between a ~3 and ~2-fold ($P<0.05$ and $P<0.001$) induction in growth compared to control (figures 3. 1 and 3. 2 respectively).

LCC1 and LCC2 cells proliferated in response to E_2 (~2.5-fold increase by day 7, $P<0.001$) and tamoxifen significantly reduced this E_2 -induced growth but not to control level ($P<0.001$, day 7). **Tamoxifen** alone had no effect on growth in either the LCC1 or LCC2 cell line ($P>0.05$). LCC9 and LY2 cells were also insensitive to all treatments and grew in a similar manner irrespective of treatment ($P>0.05$).

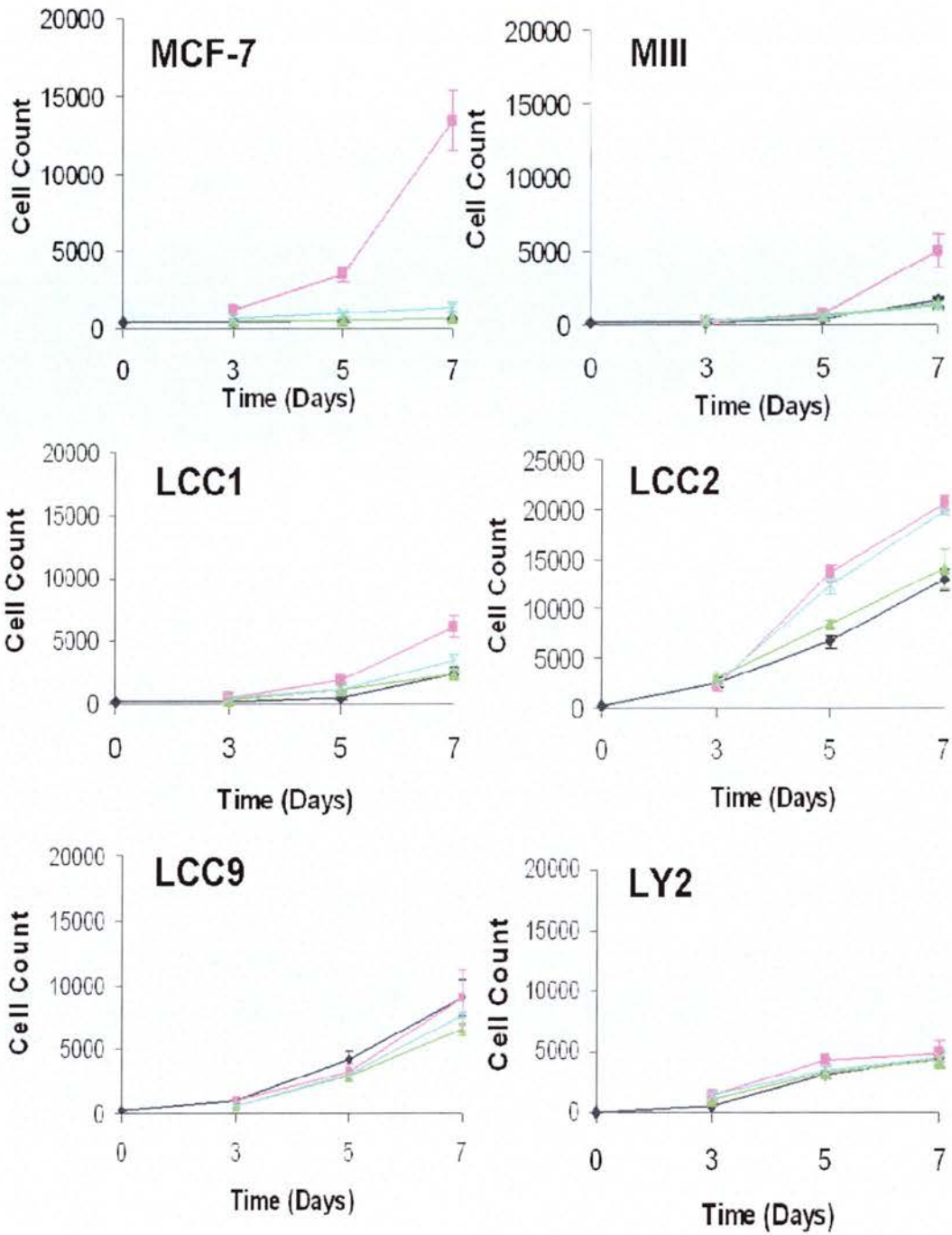


Figure 3. 2 The effect of endocrine agents on the growth of resistant breast cancer cell lines versus the sensitive parental MCF-7 breast cell line. All cell lines were charcoal stripped 24h prior to treatment and were then treated with control media (◆), E₂ (1nM) (■), tamoxifen (1µM) (▲) or E₂ + tamoxifen (1nM + 1µM) (×). Cells were treated and counted on days 0, 3 & 5 and counted on day 7. Data were plotted as a mean of quadruplicate cell counts +/- SD for treated values and as a mean of 8 cell counts +/- SD for untreated cells. Results are representative of 1 of 3 independent experiments.

Figure 3.3 shows the effect of growth factor stimulation on the growth of the parental MCF-7 cell line in comparison to the resistant LCC1, LCC9 and LY2 cell lines. As previously observed, control growth is minimal in MCF-7, compared to all resistant cell lines, which increased in growth by ~59, ~70 and ~80-fold for LCC1, LCC9 and LY2 cells respectively (day 7 compared to day 0, $P < 0.001$). **TGF α** and **HRG β** elicited an increase in cell proliferation of approximately the same size in MCF-7 cells (~11 and ~9-fold increases respectively, day 7), but to a lesser extent than E_2 (~23-fold increase by day 7, $P > 0.001$). The growth factors had no stimulatory effect in the resistant cell lines. However, initial observations indicated that HRG β treatment caused 'clumping' of the cells, perhaps more so in the resistant lines, which made them more adherent to each other and less adherent to the dish, hence the stimulatory effect may be slightly greater than recorded in the MCF-7 cell line. Thus, although no increase in resistant proliferation was observed with growth factor stimulation, there may be alterations in the extent of cell aggregation and cell-cell adhesion in the resistant cell lines.

These data suggest the resistant cell lines are not only increasingly insensitive to E_2 as previously documented, but also that the cells are insensitive to stimulation by other growth factors. It has been previously postulated that in some instances, endocrine resistance arises through initial stimulation by other growth factors, therefore further studies were carried out to determine the point at which the growth factors become ineffective in this model system.

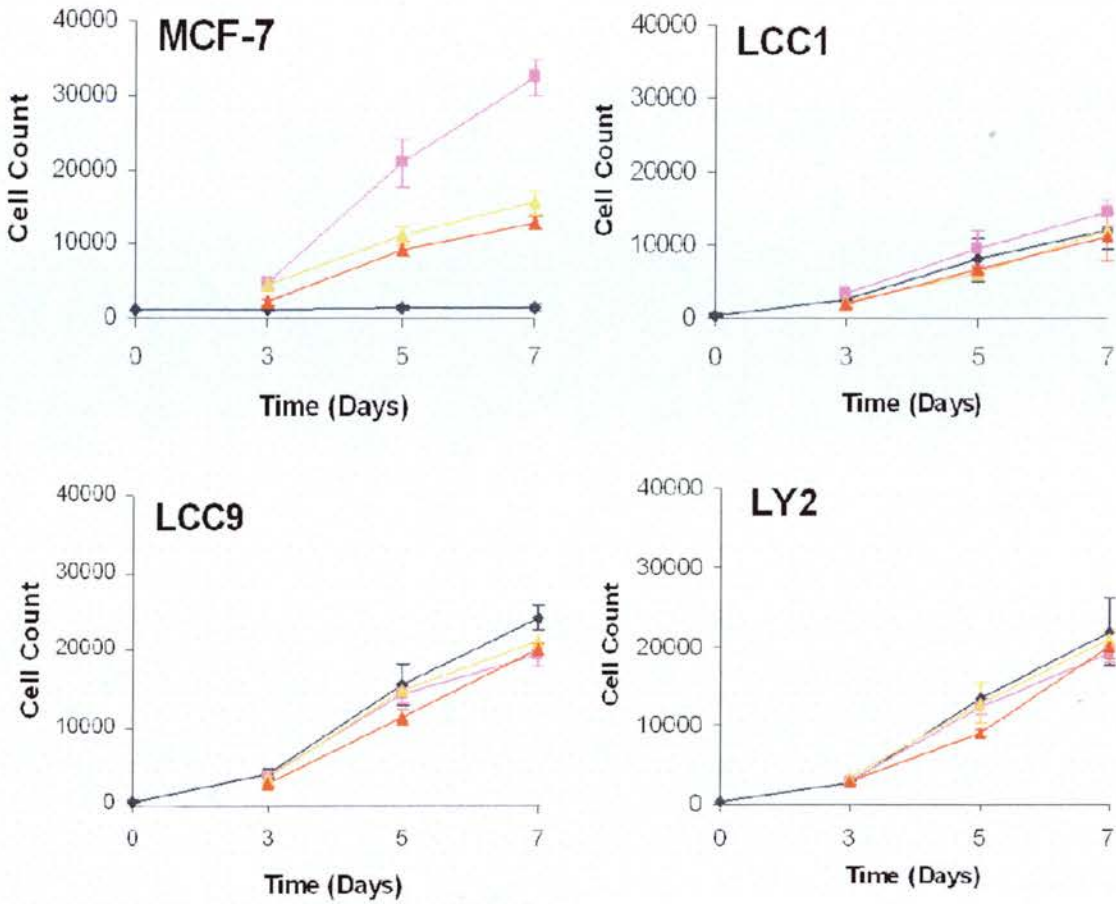


Figure 3. 3 The effects of growth factors on the growth of resistant breast cancer cell lines versus the sensitive parental MCF-7 breast cell line. All cells were charcoal stripped 24h prior to treatment and were then treated with control media (◆), E₂ (1nM) (■), TGF α (1nM) (▲) or HRG β (1nM) (▲). Cells were treated and counted on days 0, 3 & 5 and a final count took place on day 7. Data were plotted as a mean of quadruplicate cell counts +/- SD for treated values and as a mean of 8 cell counts +/- SD for untreated cells. Results are representative of 1 of 3 independent experiments.

3. 1. 1. 2. Cell morphology

The morphology of the panel of breast cell lines (excluding MIII at this stage) was observed after 3 days treatment with control medium, E₂ (1nM), tamoxifen (1µM), TGFα (1nM), E₂ + TGFα (both 1nM) and E₂ + tamoxifen (1nM + 1µM).

MCF-7 cells settled into a monolayer under control conditions with cell-matrix adhesions creating a 'cobblestone' effect. Treatment with E₂ caused the cells to form cell-cell adhesions as opposed to the cell-matrix adhesions, producing clumps of cells growing together with larger spaces in between. **TGFα** did not appear to alter the monolayer 'cobblestone' growth of the cells under control conditions. When grown in the presence of both ligands, the cells took upon an appearance somewhere between that of E₂ and control conditions. **Tamoxifen** treatment did not change the appearance of the cells when administered alone. The combination of E₂ **plus tamoxifen** also appeared to partially reverse the morphology change caused by E₂ alone. All resistant cell lines grew in monolayers under control conditions. There were a greater number of resistant cells under **control** conditions compared to the MCF-7 cells.

The morphology of the resistant cell lines was similar to that of MCF-7 cells treated with E₂, where cell-cell adhesions occurred. E₂-treatment caused clumping of the cells creating larger spaces than that observed in MCF-7 cells under the same conditions. **TGFα** and **tamoxifen** had no effect on the resistant cell morphology in any combination here.

A MCF-7

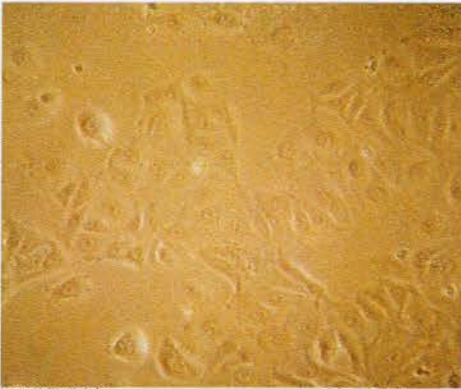
Control



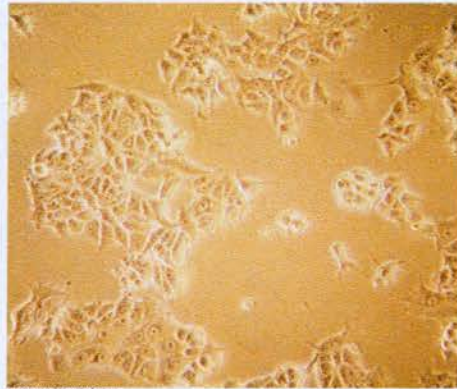
E₂



TGF α



E₂+TGF α



Tamoxifen



E₂+Tamoxifen

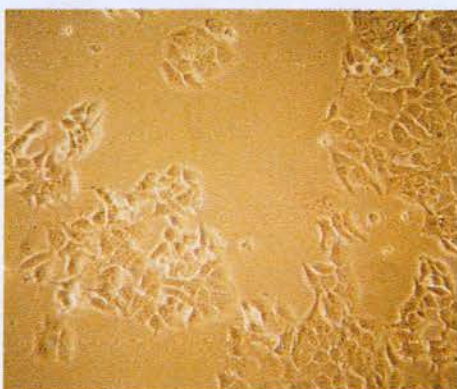
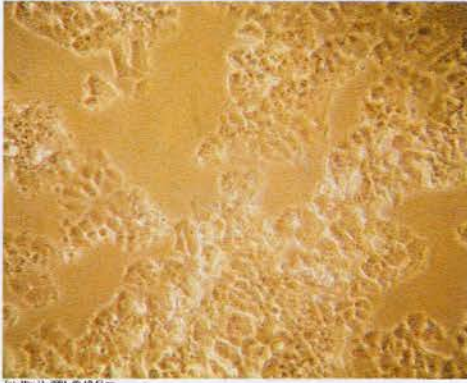


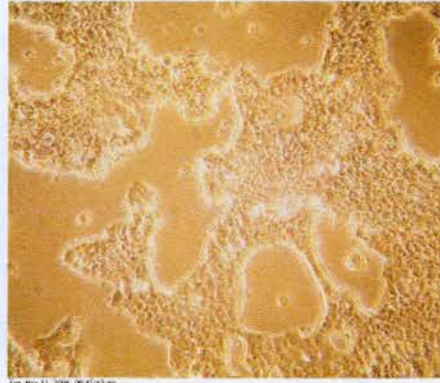
Figure 3. 4 The effect of various treatments on the cellular morphology of resistant breast cancer cell lines versus the sensitive parental MCF-7 breast cell line. (A) MCF-7 cells were charcoal stripped 24h prior to treatment and all cell lines were then treated with control media, E₂ (1nM), tamoxifen (1 μ M), E₂ + TGF α (both 1nM) or E₂ + tamoxifen (1nM + 1 μ M) (figure continued A-E). Magnification x 100.

B LCC1

Control



E₂



TGF α



E₂+TGF α



Tamoxifen



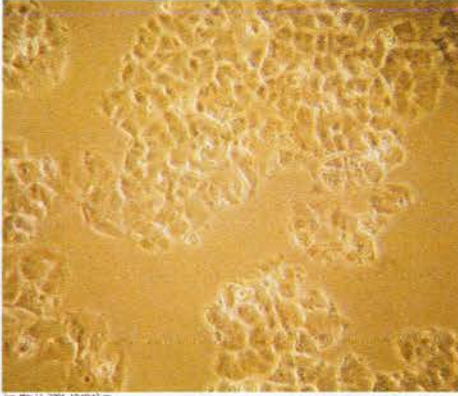
E₂+Tamoxifen



Figure 3. 4 The effect of various treatments on the cellular morphology of resistant breast cancer cell lines versus the sensitive parental MCF-7 breast cell line. (B) LCC1 cells were charcoal stripped 24h prior to treatment and all cell lines were then treated with control media, E₂ (1nM), tamoxifen (1 μ M), E₂ + TGF α (both 1nM) or E₂ + tamoxifen (1nM + 1 μ M) (figure continued A-E). Magnification x 100

C LCC2

Control



LCC2 Control

E₂



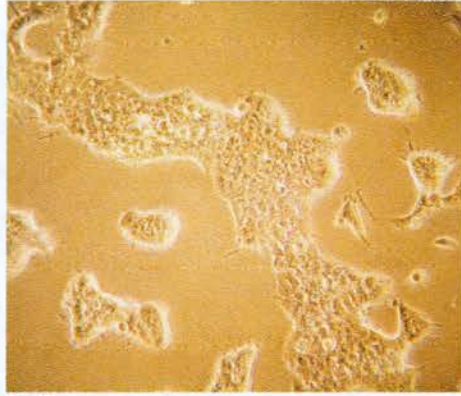
LCC2 E2

TGF α



LCC2 TGFα

E₂+TGF α



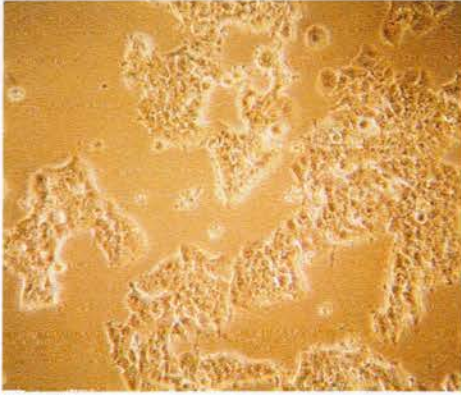
LCC2 E2+TGFα

Tamoxifen



LCC2 Tamoxifen

E₂+Tamoxifen



LCC2 E2+Tamoxifen

Figure 3. 4 The effect of various treatments on the cellular morphology of resistant breast cancer cell lines versus the sensitive parental MCF-7 breast cell line. (C) LCC2 cells were charcoal stripped 24h prior to treatment and all cell lines were then treated with control media, E₂ (1nM), tamoxifen (1μM), E₂ + TGF α (both 1nM) or E₂ + tamoxifen (1nM + 1μM) (figure continued A-E). Magnification x 100

D LCC9

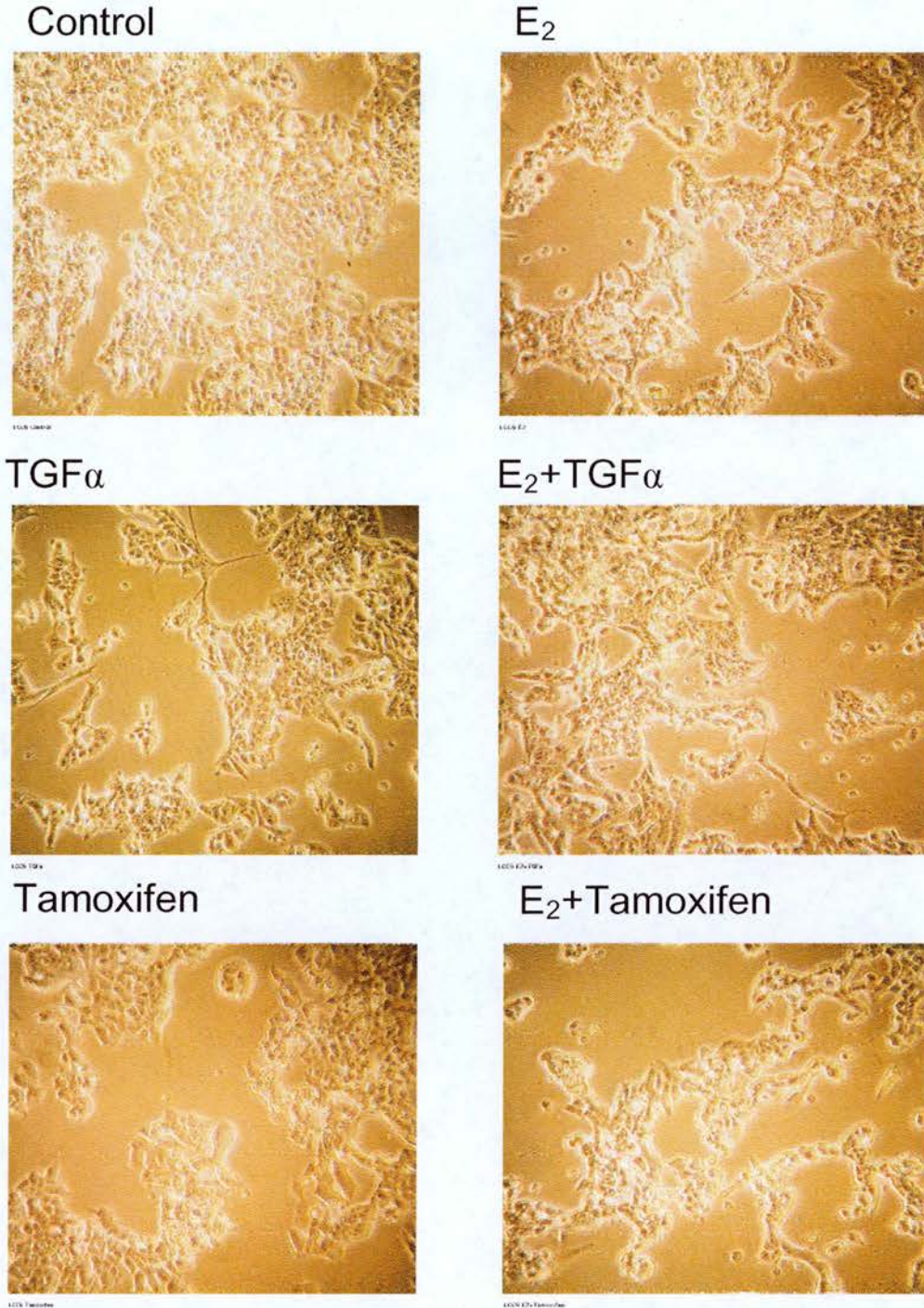


Figure 3. 4 The effect of various treatments on the cellular morphology of resistant breast cancer cell lines versus the sensitive parental MCF-7 breast cell line. (D) LCC9 cells were charcoal stripped 24h prior to treatment and all cell lines were then treated with control media, E₂ (1nM), tamoxifen (1 μ M), E₂ + TGF α (both 1nM) or E₂ + tamoxifen (1nM + 1 μ M) (figure continued A-E). Magnification x 100

E LY2

Control



E₂



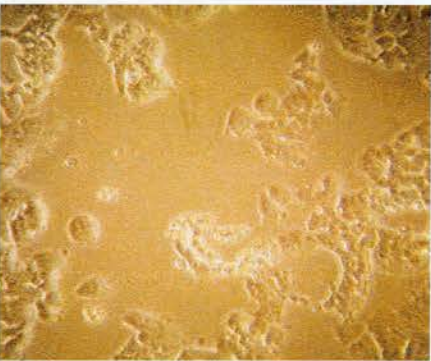
TGF α



E₂+TGF α



Tamoxifen



E₂+Tamoxifen

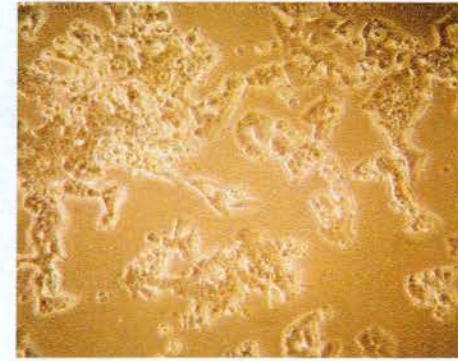


Figure 3. 4 The effect of various treatments on the cellular morphology of resistant breast cancer cell lines versus the sensitive parental MCF-7 breast cell line. (E) LY2 cells were charcoal stripped 24h prior to treatment and all cell lines were then treated with control media, E₂ (1nM), tamoxifen (1 μ M), E₂ + TGF α (both 1nM) or E₂ + tamoxifen (1nM + 1 μ M) (figure continued A-E). Magnification x 100.

3. 1. 2. Ovarian cancer cell lines

ER α and oestrogen have been implicated in ovarian cancer and are perhaps related to its causation (Rao and Miller, 2006). Rao and Miller (2006) suggested that additional research was required into hormonal therapies as a treatment for ovarian cancer as discussed in the introduction. Therefore, ER α may play a crucial role not only in breast cancer, but also ovarian cancer. Hence, ovarian cancer cell lines PEO1 and SKOV-3 were characterised in conjunction with the breast cancer cell line MCF-7 in response to treatment with E $_2$ and TGF α . The cell lines were subjected to increasing concentrations of E $_2$ and TGF α varying from 0.1 to 100nM for E $_2$ and 0.01nM to 10nM for TGF α (figures 3.4 and 3.5 respectively).

Figure 3.5 shows the effect of increasing E $_2$ concentrations on PEO1 and SKOV-3 cells. MCF-7 data are as previously described, where a higher E $_2$ concentration did not necessarily produce the greatest growth response in the cell line (figure 3.5A). E $_2$ did not effect the cell counts in SKOV-3 cells at any concentration (no fold change, P>0.05) and only a small increase in cell number was observed in PEO1 cells treated with the 0.1nM E $_2$ treatment (~1.4-fold increase, P<0.05, day 7) (figure 3.5C). Figure 3.6 shows that TGF α produced an increase in cell number in both SKOV-3 and PEO1 lines as with MCF-7 cells. 1nM TGF α increased MCF-7 cell numbers to the greatest extent, with a ~22.5-fold increase by day 7 (P<0.001). The higher concentration of growth factor did not increase growth to the same degree (~16-fold increase) although this was still considered extremely significant (P<0.001). This is in agreement with earlier E $_2$ data (figures 3.2 and 3.4).

PEO1 cells were more responsive to TGF α treatment than the SKOV-3 cell line. 1nM and 10nM TGF α produced similar fold increases of ~1.7-fold (P<0.001) in SKOV-3 cells, while both 1nM and 10nM TGF α produced a ~6-fold increase in PEO1 cell number (P<0.001) by day 7. 0.01nM and 0.1nM TGF α concentrations stimulated cell proliferation by ~3-fold and ~5-fold in PEO1 cells (day 7 data), which were considered extremely significant inductions (figure 3.5C). In contrast, 0.01nM TGF α did not significantly increase SKOV-3 cell proliferation by day 7 (P>0.05).

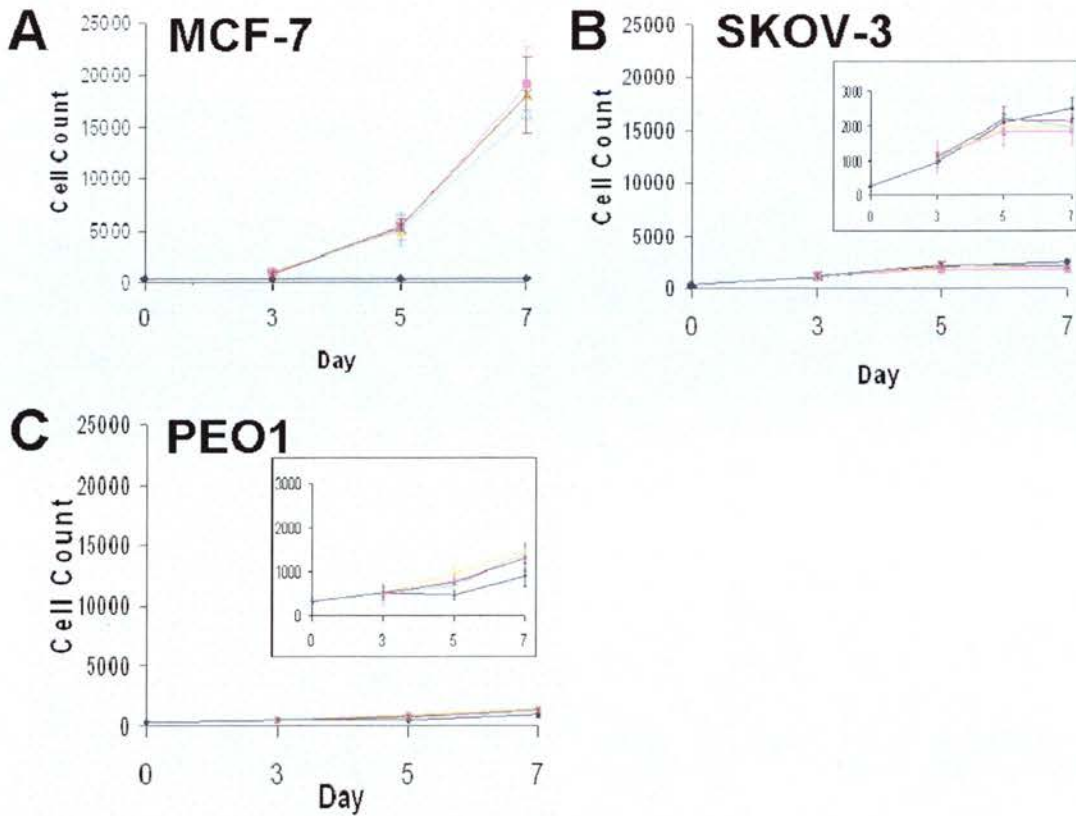


Figure 3.5 The effect of various E_2 concentrations on the growth of resistant ovarian cancer cell lines versus the sensitive parental MCF-7 breast cell line. MCF-7 cells were charcoal stripped 24h prior to treatment and all cell lines were then treated with control media (◆) or 0.1nM (▲), 1nM (■), 10nM (x) or 100nM (■) E_2 . Cells were treated and counted on days 0, 3 & 5 and a final count took place on day 7. Data were plotted as a mean of quadruplicate cell counts \pm SD for treated values and as a mean of 8 cell counts \pm SD for untreated cells. Results are representative of 1 of 3 independent experiments.

However, in contrast to MCF-7 and PEO1 cell lines, SKOV-3 cells were able to proliferate profusely in charcoal stripped media (between a \sim 5.5 and a \sim 11-fold increase, $P < 0.001$, figures 3.5B and 3.6B respectively), compared to a nominal increase in MCF-7 cell number between a zero value and a \sim 1.3-fold increase ($P > 0.05$ - < 0.05 , figures 3.5A and 3.6A). PEO1 cells proliferated to small degree under control conditions, but only by between \sim 1.3 and \sim 3-fold ($P < 0.05$ - 0.001) between days 0 and 7.

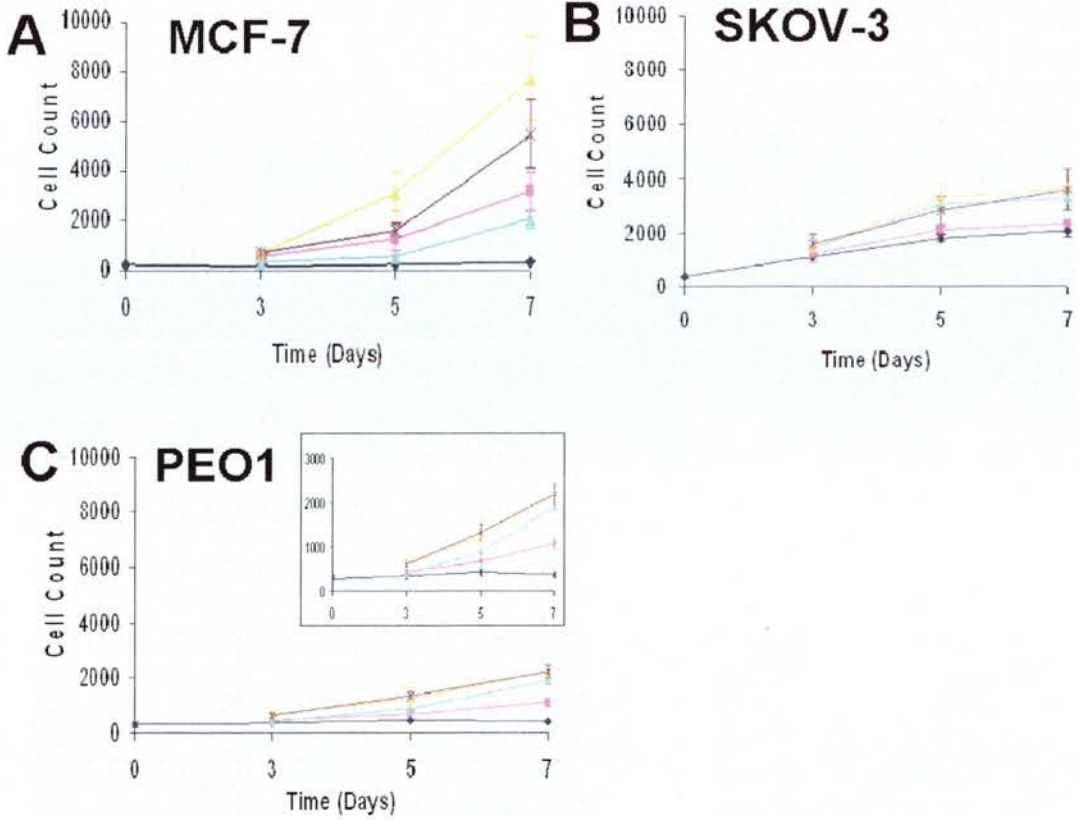


Figure 3. 6 The effect of various TGF α concentrations on the growth of resistant ovarian cancer cell lines versus the sensitive parental MCF-7 breast cell line. MCF-7 (A), SKOV-3 (B) and PEO1 (C) cell lines were charcoal stripped 24h prior to treatment and were then treated with control media (◆) or 0.01nM (■), 0.1nM (▲), 1nM (▲) or 10nM (■) TGF α . Cells were treated and counted on days 0, 3 & 5 and a final count took place on day 7. Data were plotted as a mean of quadruplicate cell counts +/- SD for treated values and as a mean of 8 cell counts +/- SD for untreated cells. Results are representative of 1 of 3 independent experiments.

3. 2. Expression profiling of a panel of breast cancer cell lines

The expression profiles of a panel of known oestrogen-responsive genes were investigated in the MCF-7, LCC1, LCC9 and LY2 breast cancer cell lines. mRNA expression levels of the pS2, ER α , PR and CTD genes were monitored in the presence and absence of E₂ and TGF α stimulation after 24h incubation.

Basal ER α mRNA levels were elevated in all three resistant cell lines compared to MCF-7 cells, with increases of ~4.3-fold (P<0.001), ~2.2-fold (P<0.05) and ~2.5-fold (P<0.01) in LCC1, LCC9 and LY2 cell lines (figure 3.7A). E₂ treatment reduced ER α mRNA expression by approximately a third (~31.6%, P=0.0105) in MCF-7 cells, ~two-thirds (~63.3%, P=0.0015) in LCC1 cells and an extremely significant reduction was noted in the LY2 cell line (74.2%, P<0.0001) (figure 3. 7B). In contrast, no significant change in ER α mRNA levels was observed in the LCC9 cell line (~10.7% reduction, P=0.4883). The reductions in the resistant cell lines compared to the MCF-7 cell line (as indicated by red asterices) were deemed very significant in the LCC1 cell line (P<0.01) and extremely significant in the LY2 cell line (P<0.001). The increase in ER α mRNA due to E₂ treatment was considered to be significant in the LCC9 cells compared to the MCF-7 cell line (P<0.05).

TGF α also reduced ER α mRNA expression (~37.3%, P=0.021) in MCF-7 cells. The ER α mRNA levels of LCC1 and LY2 cell lines remained insignificantly affected by TGF α (P>0.05, ~6.3% reduction in LCC1 and ~18.5% increase in LY2 cell line respectively), although ER α mRNA levels were increased by ~1.5-fold in LCC9 cells (P=0.005). The various reductions and increases in the ER α mRNA levels post TGF α treatment in the three resistant cell lines led to significant differences between these lines and the MCF-7 cell line. Values of P<0.05, P<0.001 and P<0.01 were recorded for the differences between the MCF-7 cell line and LCC1, LCC9 and LY2 cell lines respectively.

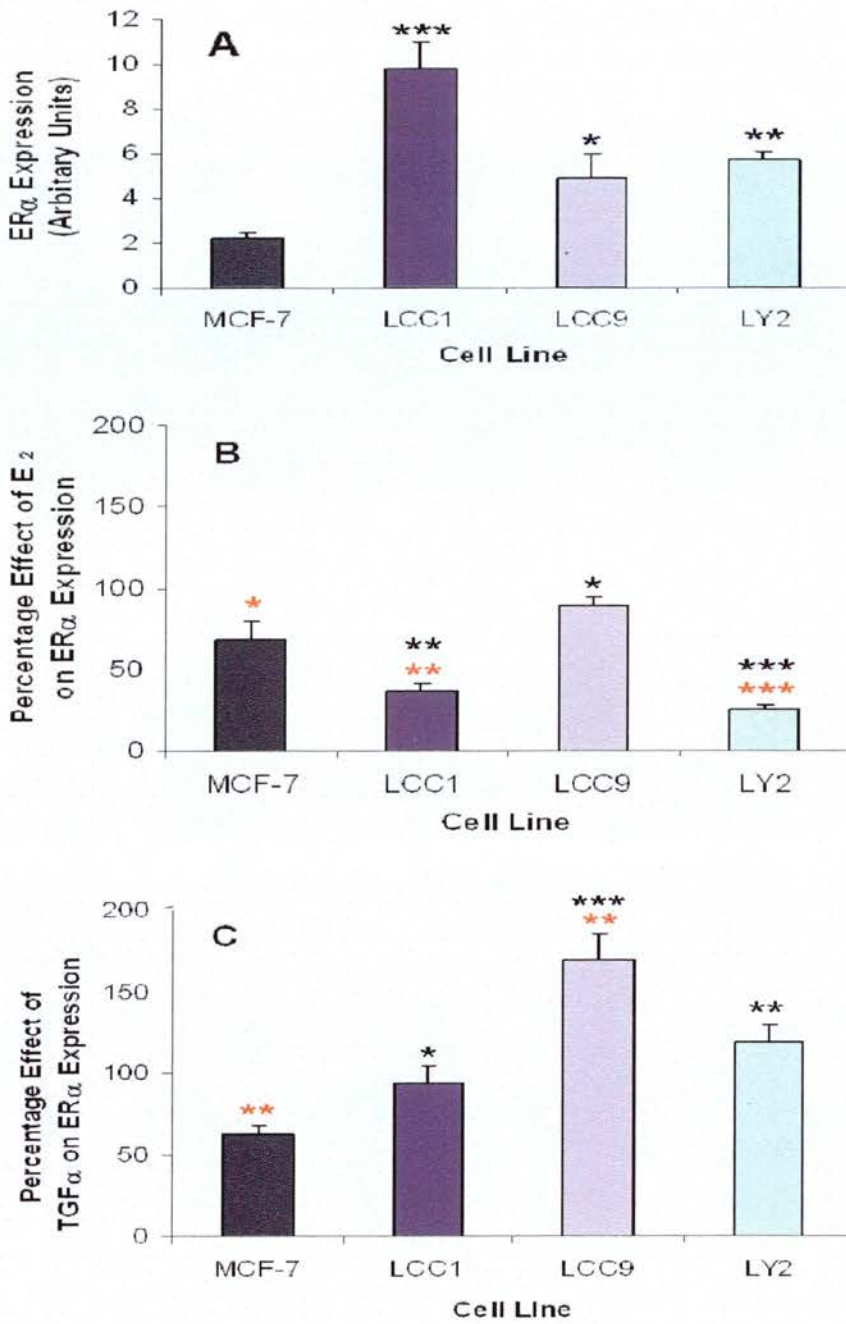


Figure 3. 7 The effect of E₂ and TGF α stimulation on ER α expression in resistant breast cancer cell lines versus MCF-7 cell line. MCF-7 (■), LCC1 (■), LCC9 (■) and LY2 (■) cell lines were treated with (A) control media to determine basal levels, (B) E₂ (1nM) or (C) TGF α (1nM) for 72h. Data were plotted as the mean of triplicates +/- SD relative to MCF-7 E₂ positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between MCF-7 and the resistant cell lines and those in red represent any statistically significant changes post-treatment with E₂ or TGF α compared to control. Results are representative of 1 of 3 independent experiments.

Figure 3.8A shows that **basal levels** of **pS2** mRNA were significantly elevated in all the resistant cell lines compared to MCF-7 cells (~19-fold ($P<0.0002$), ~21-fold ($P<0.0001$) and ~3.7-fold ($P<0.0001$) higher in LCC1, LCC9 and LY2 cells respectively). LY2 cell pS2 mRNA was not elevated as dramatically in control conditions compared to the LCC1 and LCC9 cell lines. E_2 significantly increased pS2 mRNA to a similar extent in MCF-7 and LY2 cell lines, with ~16-fold ($P=0.0003$) and ~14-fold ($P=0.0017$) increases in these lines respectively. E_2 also significantly increased pS2 mRNA levels in LCC1 and LCC9 cell lines by ~2.8-fold ($P=0.0015$) and ~2.3-fold ($P=0.0071$), but the increases were not as pronounced compared to the MCF-7 or LY2 cells (~5.5- and ~6.7-fold higher in the parental cell line compared to LCC1 and LCC9 respectively, both $P<0.01$). **TGF α** significantly enhanced pS2 in MCF-7 cells, but to a much lesser extent than E_2 (~1.7-fold, $P=0.0096$). No significant changes in pS2 levels were observed with TGF α treatment of any of the resistant cells ($P>0.05$). MCF-7 cells were significantly different in their percentage response to E_2 compared to LCC1 and LCC9 cells ($P<0.001$), and to all the resistant cell lines in response to TGF α treatment ($P<0.01$).

Basal PR mRNA levels were similar in the MCF-7 and LCC1 cell lines, while LCC9 PR mRNA were vastly elevated compared to MCF-7 cells (~5-fold increase, $P<0.001$) (figure 3.9A). Contrastingly, PR mRNA levels in the LY2 cell line were reduced by ~6.2-fold ($P<0.01$) compared to MCF-7 cells. PR expression was induced by 24h treatment with E_2 by ~12-fold ($P=0.0013$) in MCF-7 cells (figure 3.9B). PR mRNA levels were also significantly elevated in LCC1 and LCC9 cells (~5-fold, $P=0.0005$) and ~1.7-fold increases, but this was not found to be significant for the latter cell line. Interestingly, PR mRNA expression levels were increased significantly by ~19-fold in the LY2 cells with E_2 treatment compared to control cells ($P=0.0005$).

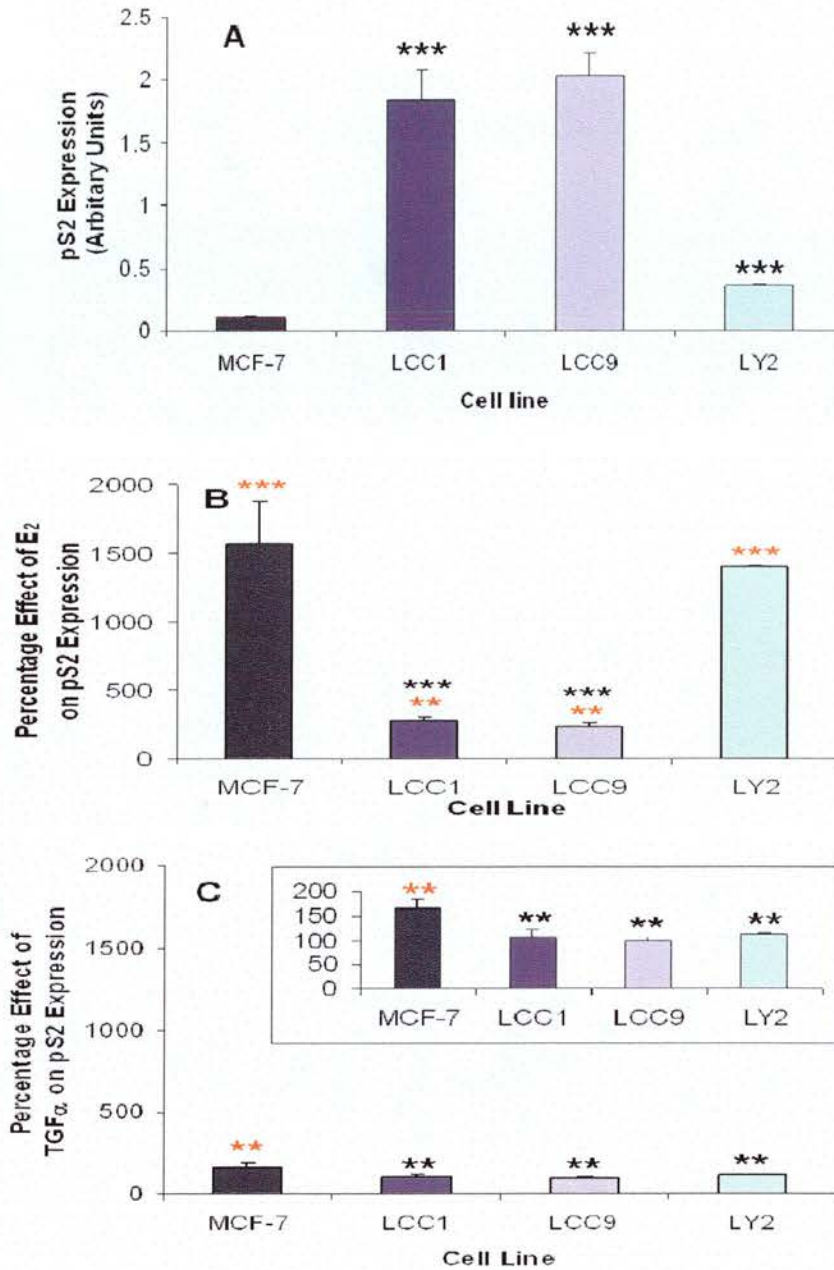


Figure 3. 8 The effect of E₂ and TGF α stimulation on pS2 expression in resistant breast cancer cell lines versus sensitive parental MCF-7 breast cell. MCF-7 (■), LCC1 (■), LCC9 (■) and LY2 (■) cell lines were treated with (A) control media to determine basal levels, (B) E₂ (1nM) or (C) TGF α (1nM) for 72h. Data were plotted as the mean of triplicate samples +/- SD relative to MCF-7 E₂ positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between MCF-7 and the resistant cell lines and those in red represent any statistically significant changes post-treatment with E₂ or TGF α compared to control. Results are representative of 1 of 3 independent experiments.

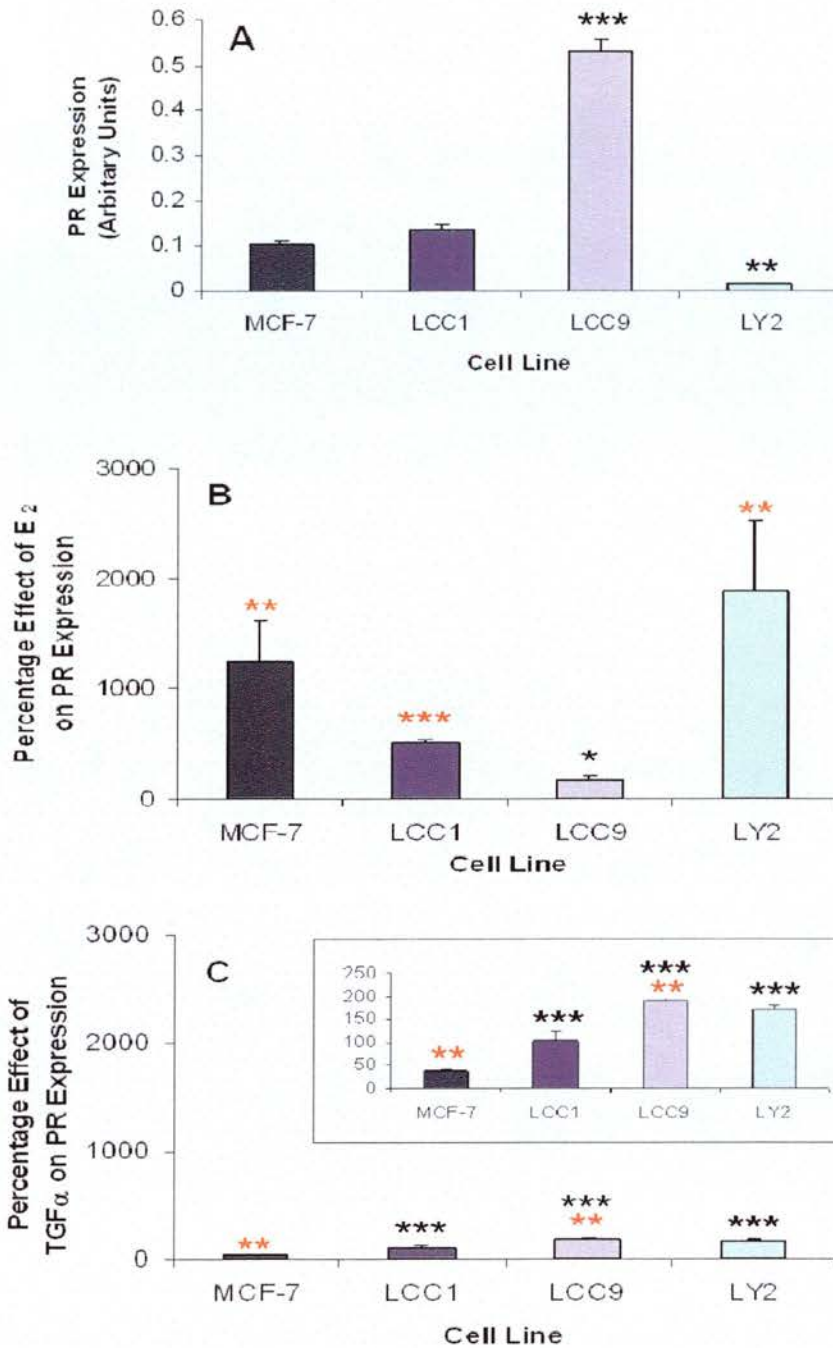


Figure 3. 9 The effect of ligand stimulation on PR expression in resistant breast cancer cell lines versus sensitive parental MCF-7 breast cell. MCF-7 (■), LCC1 (■), LCC9 (■) and LY2 (■) cell lines were treated with (A) control media to determine basal levels, (B) E₂ (1nM) or (C) TGF α (1nM) for 72h. Data were plotted as the mean of triplicate samples +/- SD. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between MCF-7 and the resistant cell lines and those in red represent any statistically significant changes post-treatment with E₂ or TGF α compared to control. Results are representative of 1 of 3 independent experiments.

In contrast to E_2 , figure 3.9C shows that **TGF α** reduced the PR mRNA expression levels in MCF-7 cells by ~ 2.6 -fold to $\sim 38\%$ ($P=0.0034$). TGF α elevated PR mRNA in both the LCC9 and LY2 cell lines, with increases of $\sim 88\%$ ($P=0.0063$) and $\sim 69\%$ respectively ($P=0.084$, considered not quite significant). There was no alteration in the level of PR expression upon treatment with TGF α in LCC1 cells ($P=0.805$, not significant). This is consistent with growth data where proliferation remained unchanged compared to that of untreated cells. MCF-7 cells were significantly different in their PR mRNA response to E_2 compared to LCC9 cells ($P<0.05$), and to all the resistant cell lines in response to TGF α treatment ($P<0.001$).

Figure 3.10A depicts a histogram of **CTD** mRNA **basal** levels in the panel of breast cancer cell lines. LCC1 cells have ~ 1.5 -fold less mRNA expressed than MCF-7 cells ($P<0.05$, significant), while the most notable reduction was observed in the LY2 cell line where a ~ 2.8 -fold reduction in mRNA was reported compared to MCF-7 cells ($P<0.001$, extremely significant). LCC9 cells expressed a similar level of CTD mRNA relative to positive control compared to the MCF-7 cell line ($P>0.05$). Figure 3.10B shows E_2 induced a $\sim 75\%$ increase in MCF-7 CTD expression ($P=0.017$). CTD expression was not increased significantly in the resistant LCC1 cell line by E_2 (~ 1.2 -fold). Basal CTD was inconsistently elevated in LCC9 cells to the level of MCF-7 treated with E_2 (24h), but the gene was shown to remain as E_2 -regulated and active as E_2 elevated the higher basal level by 1.4-fold ($P<0.05$, figure 3.9B). Basal LCC9 CTD expression was however elevated compared to that of LCC1 basal CTD expression ($P=0.0024$). The most notable CTD mRNA changes were again observed in the LY2 cell line. LY2 CTD mRNA levels were extremely significantly elevated by E_2 treatment, with an increase of ~ 3.4 -fold ($P=0.0002$) compared to basal LY2 CTD levels and relative to the positive control (figure 3.9B). Figure 3.10C illustrates that TGF α only induced minor changes to CTD expression, with varying degrees of significance. CTD mRNA levels were elevated marginally in the LCC1 cell line by ~ 1.3 -fold ($P=0.034$), while MCF-7 and LCC9 cell lines remained unaltered after **TGF α** treatment ($P>0.05$). The LY2 cell line was also increased by ~ 1.3 -fold with TGF α treatment, but this was insignificant.

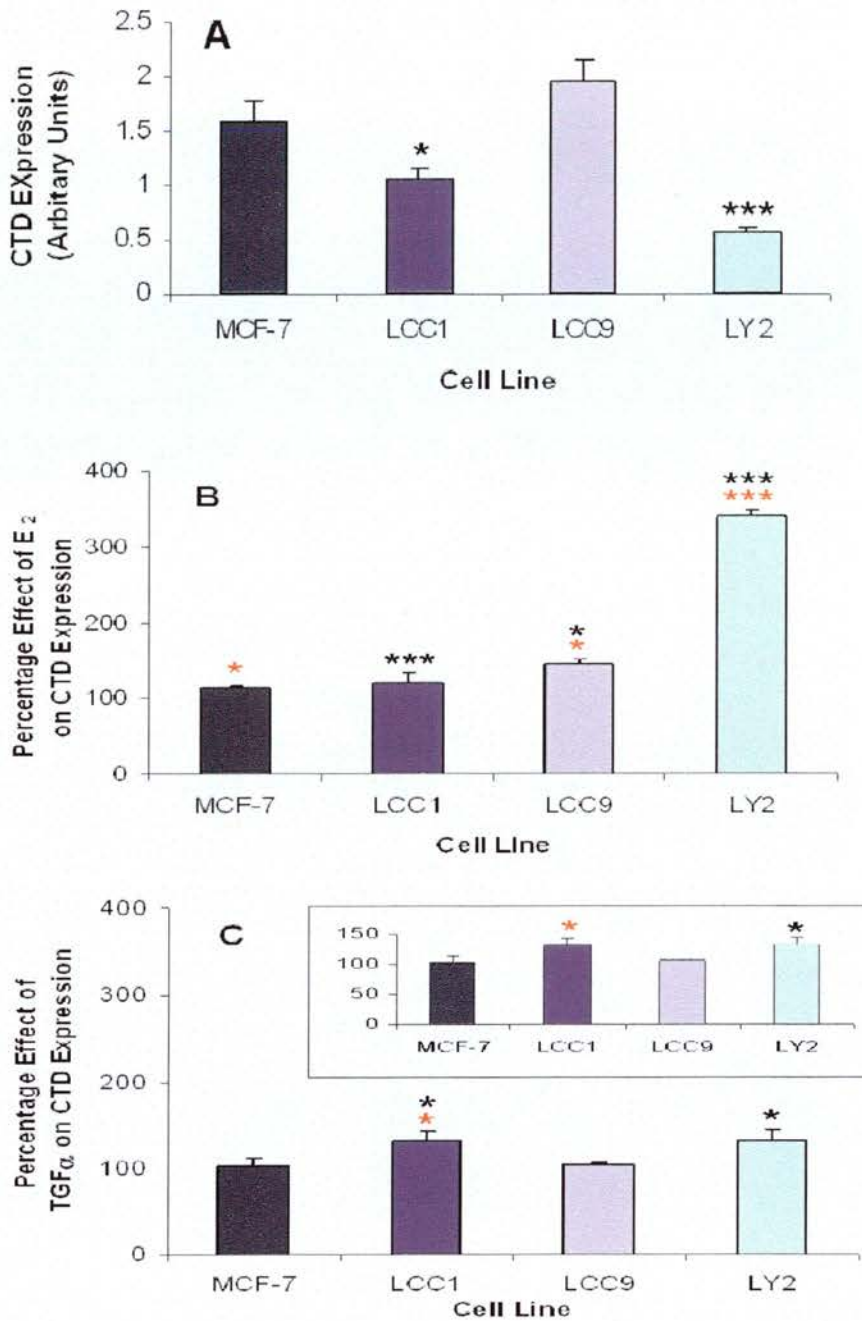


Figure 3. 10 The effect of ligand stimulation on cathepsin D (CTD) expression in resistant breast cancer cell lines versus sensitive parental MCF-7 breast cell. MCF-7 (■), LCC1 (■), LCC9 (■) and LY2 (■) cell lines were treated with (A) control media to determine basal levels, (B) E₂ (1nM) or (C) TGF α (1nM) for 72h. Data were plotted as the mean of triplicates +/- SD. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between MCF-7 and the resistant cell lines and those in red represent any statistically significant changes post-treatment with E₂ or TGF α compared to control. Results are representative of 1 of 3 independent experiments

3. 3. Cell cycle characterisation

In order to identify any cell cycle changes associated with the stepwise development of endocrine resistance seen in this system, the cell cycle distribution of MCF-7 cells was studied in comparison to the LCC1 and LCC9 cell lines. The three cell lines were treated with E_2 , $TGF\alpha$ and $HRG\beta$ to specifically activate the $ER\alpha$ ligand-dependent and independent pathways respectively. In addition, the effect of tamoxifen anti-oestrogen treatment on the cell cycle was assessed in order to identify changes in association with the development of resistance.

Figure 3.11 shows the effect of E_2 and growth factors on the cell cycle distribution of the three cell lines. All three ligands initiated cell cycle entry of the MCF-7 cell line (figure 3.11A). E_2 produced an increase in S-phase from ~3% to ~33% ($P<0.001$). $TGF\alpha$ and $HRG\beta$ produced similar increases from this ~3% to ~30% and ~34% in the S-phase respectively (both $P<0.001$). It is interesting to note that a higher percentage of cells of both of the resistant cell lines are in the S-phase of cell cycle under control conditions than the MCF-7 cell line (~30% and ~14% of LCC1 and LCC9 cells respectively are in the S-phase compared to only ~3% of MCF-7 cells ($P<0.001$ for comparisons between all cell lines)). In discord with the MCF-7 cell line, E_2 , $TGF\alpha$ and $HRG\beta$ all appear to reduce the proportion of LCC1 and LCC9 cells in the S-phase of the cell cycle (all $P<0.001$, with the exception of LCC9 $TGF\alpha$ treated cells, $P<0.01$) (figures 3.11B and C).

Figure 3.12 shows the effect of E_2 in combination with **anti-hormonal therapy** on the cell cycle distribution of MCF-7, LCC1 and LCC9 cell lines. In MCF-7 cells E_2 produced a dramatic phase distribution change as before, with a reduction from ~85% of cells in G_1/G_0 to ~44% ($P<0.001$), leading to corresponding increases from ~7% to ~37% in S-phase and ~7% to ~18% in G_2/M phases respectively (both $P<0.001$) (figure 3.12A). Tamoxifen alone did not significantly increase the number of cells in G_1/G_0 -phase ($P>0.05$). This may be explained by the fact that a large percentage of MCF-7 cells are already in G_1/G_0 arrest as the cells are subject to charcoal stripped conditions.

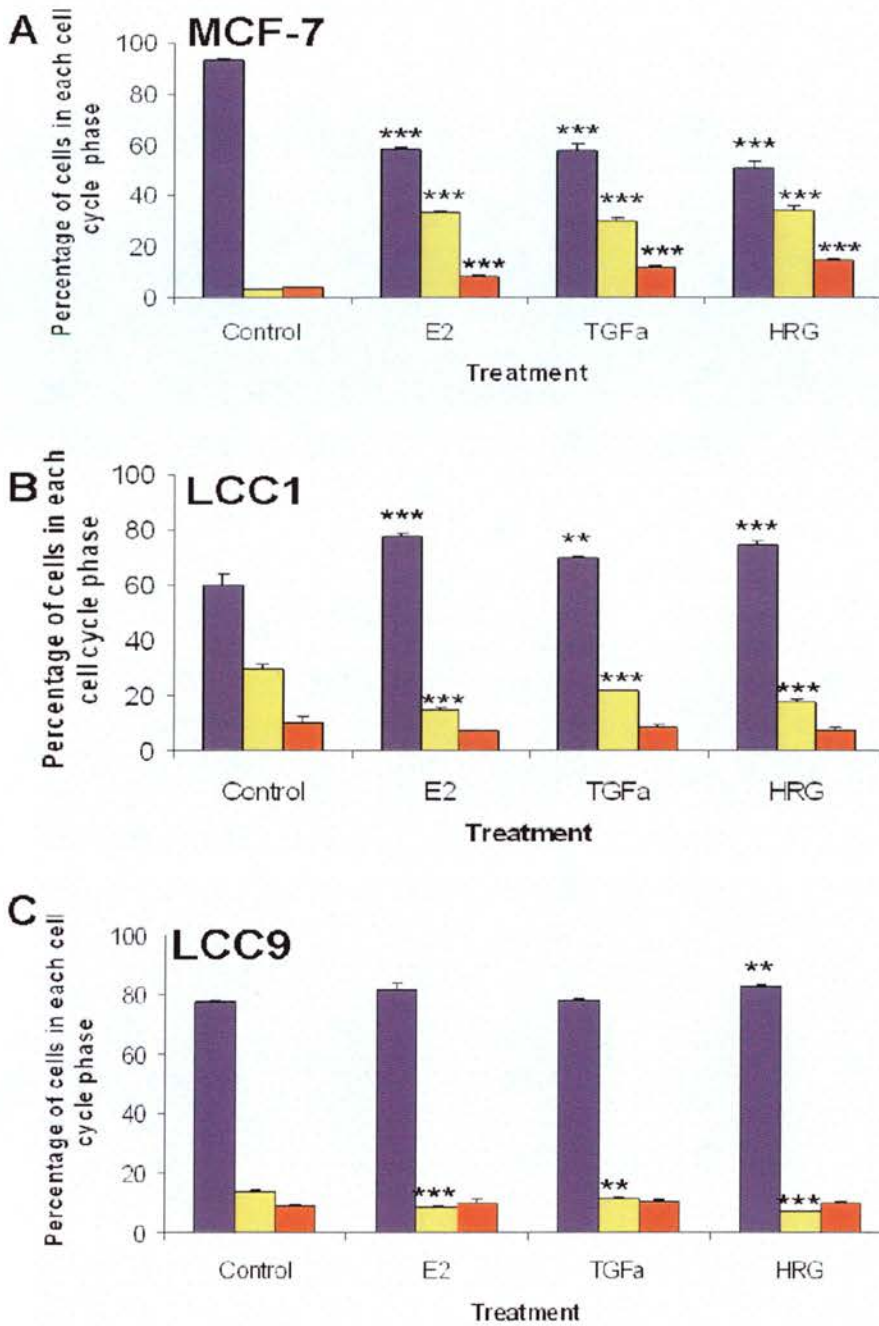


Figure 3. 11 The effect of ligand stimulation on the cell cycle of resistant breast cancer cell lines versus sensitive parental MCF-7 breast cell. All 3 cell lines were charcoal stripped 48h prior to treatment with control media, E₂ (1nM), TGFα (1nM) or HRGβ (1nM) for 72h. The percentage of cells in G0/G1 (■), S-(■) and G2/M (■) phases of the cell cycle were plotted as the mean of triplicate samples +/- SD as a percentage of the cell cycle. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks represent statistically significant changes between control and a treatment. Results are representative of 1 of 3 independent experiments.

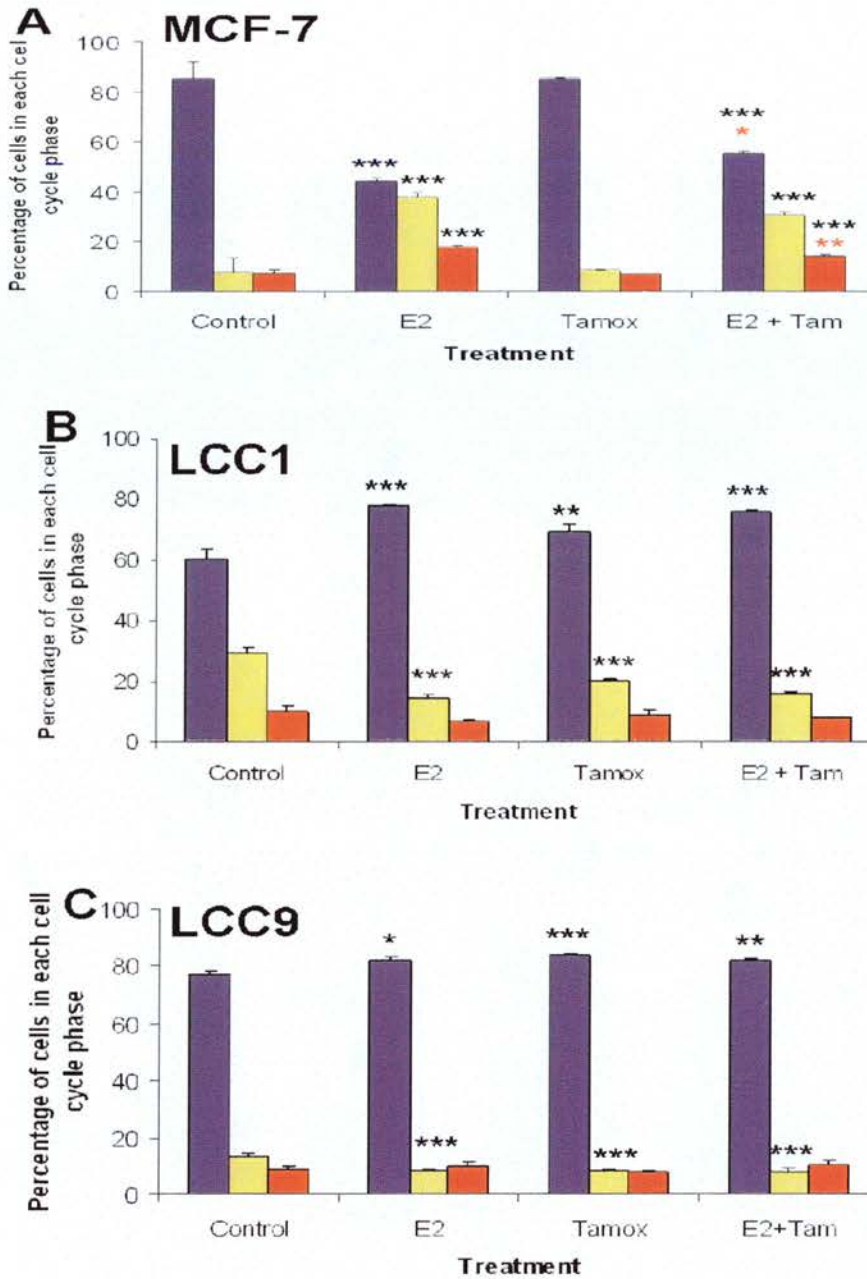


Figure 3. 12 The effect of endocrine therapy on the cell cycle of resistant breast cancer cell lines versus sensitive parental MCF-7 breast cell. All 3 cell lines were charcoal stripped 48h prior to treatment with control media, E₂ (1nM), tamoxifen (Tamox) (1µM) or E2+Tamoxifen (Tam) (1nM+1µM) (■) for 72h. The percentage of cells in G_{0/1} (■), S (■) and G_{2/M} (■)-phases of the cell cycle were plotted as the mean of triplicate samples +/-SD as a percentage of the cell cycle. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Black asterisks represent statistical significance between the same cell cycle phase with each treatment, while red asterisks represent statistical significance between the same phase post-treatment with tamoxifen compared to E₂ alone. Results are representative of 1 of 3 independent experiments.

Tamoxifen in combination with E_2 reduced the extent of the phase progression caused by E_2 treatment alone, increasing the percentage of cells in $G_{0/1}$ phase from ~44% to ~56% ($P<0.05$) and reducing the proportion of MCF-7 cells in the S-phase from ~38% to ~31% and from ~18% to ~14% in G_2/M phase ($P<0.01$).

In figures 3.12B and C, the percentage of cells in S-phase in the resistant lines was reduced by approximately half with E_2 treatment as before. In both cell lines this corresponded with an increase in cells in the G_1/G_0 phase. Figure 3.12B is the cell cycle distribution for the LCC1 cell line. Tamoxifen also reduced this value in LCC1 cells, but to a lesser extent than E_2 (from ~29% to ~20% ($P<0.001$) with tamoxifen treatment versus a reduction to ~15% with E_2 treatment). The combination of E_2 plus tamoxifen did in fact increase the number of cells in G_1/G_0 arrest to a value in between the two treatments (~16%, $P<0.001$ compared to control treated cells).

Figure 3.12C is a histogram of the LCC9 cell cycle distribution which display a very similar trend to that of the LCC1 cell line, with the exception that the percentage of LCC9 cells in S and G_2/M phases are approximately half those in the LCC1 cell line.

3. 4. Characterisation of the apoptotic nature of the panel of breast cancer cell lines

The effects of various ligands and the anti-oestrogen tamoxifen on apoptosis were monitored via annexin-v staining in the MCF-7, LCC1 and LCC9 cell lines. Figures 3.13 and 3.14 show the effects of E_2 and HRG β in the presence and absence of tamoxifen respectively. The data in figure 3.13A shows a non-significant reduction of ~1.4-fold occurs with E_2 treatment in MCF-7 cells. Tamoxifen increased the percentage of cells with positive annexin-v staining and thus apoptosis in MCF-7 cells, but not to a significant degree (~1.2-fold increase), however in combination with E_2 there was a significant increase in staining and hence this implicated an increase in early apoptosis of ~4-fold compared to E_2 alone ($P<0.001$).

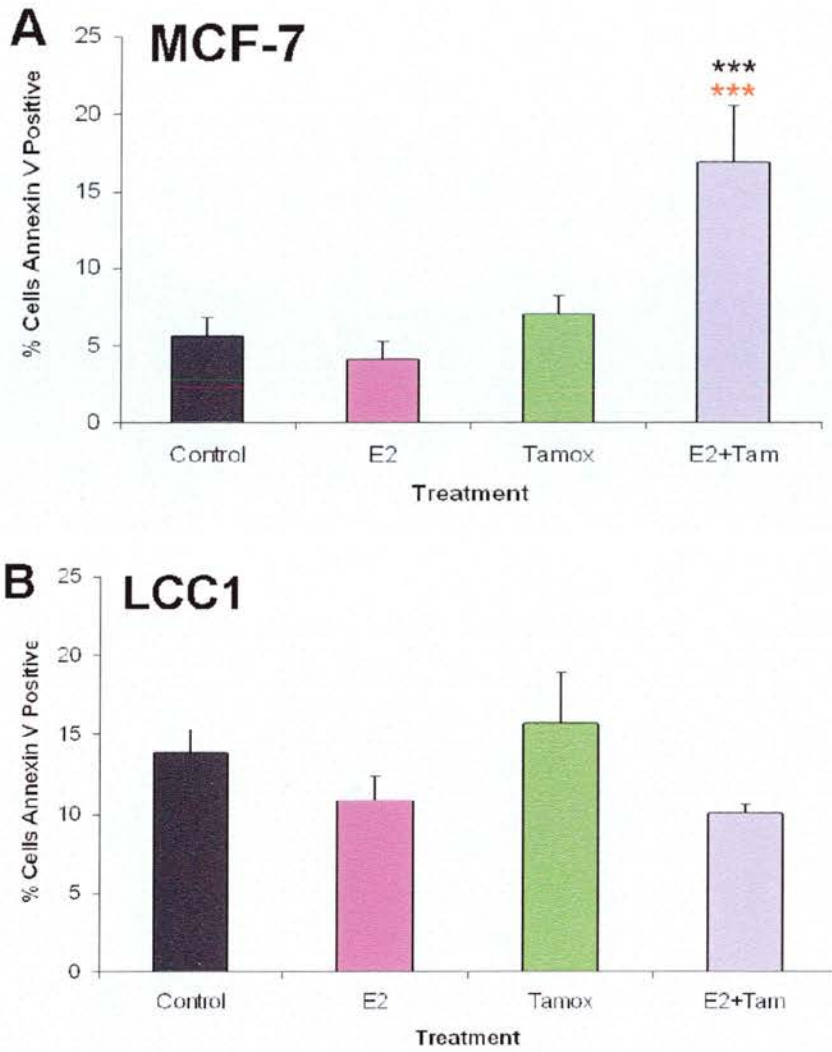


Figure 3. 13 The effect of endocrine agents on apoptosis of resistant breast cancer cell line LCC1 versus sensitive parental MCF-7 breast cell. Both cell lines were charcoal stripped 48h prior to treatment with control media (■), E₂ (1nM) (■), tamoxifen (Tamox) (1μM) (■), or E₂ + tamoxifen (Tam) (1nM + 1μM) (■) for 72h. Early apoptosis was monitored and data were plotted as the mean of triplicate samples +/- SD as a percentage of cells stained with annexin-v. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment and those in red represent any statistically significant changes post-treatment with tamoxifen compared to E₂ alone. Results are representative of 1 of 3 independent experiments.

The apoptotic levels of the LCC1 cell line, as for the MCF-7 cell line, remained significantly unaffected by E_2 , although a ~ 1.3 -fold reduction was observed in positive staining, which supports the growth phenotype of the LCC1 cell line. Tamoxifen did not significantly induce cell death via apoptosis (only a $\sim 1.8\%$ increase with tamoxifen treatment compared to control) and was unable to prevent any anti-apoptotic effect caused by E_2 (no change to the ~ 1.3 -fold reduction with E_2 alone).

Figure 3.14 illustrates the effect of HRG β in the presence and absence of tamoxifen on the apoptosis of MCF-7, LCC1 and LCC9 cells via observation of the annexin-v stained cells. HRG β treatment had an anti-apoptotic effect on MCF-7 cells, with a reduction in early stage apoptosis of $\sim 57\%$ ($P < 0.001$). Tamoxifen did not significantly affect annexin-v staining and thus apoptosis, while the addition of tamoxifen to HRG β significantly reduced the annexin-v staining and thus the number of cells identified as in the early stages of apoptosis by ~ 1.8 -fold ($P < 0.05$). HRG β and tamoxifen had no effect on apoptosis in LCC1 or LCC9 cells (figures 3.14B and C). This contrasts with the HRG β plus tamoxifen treatment, where a reduction in the annexin-v staining was observed in the LCC1 cell line ($P < 0.05$). The LCC9 cell actually saw an increase in the annexin-v staining of ~ 1.3 -fold ($P < 0.05$).

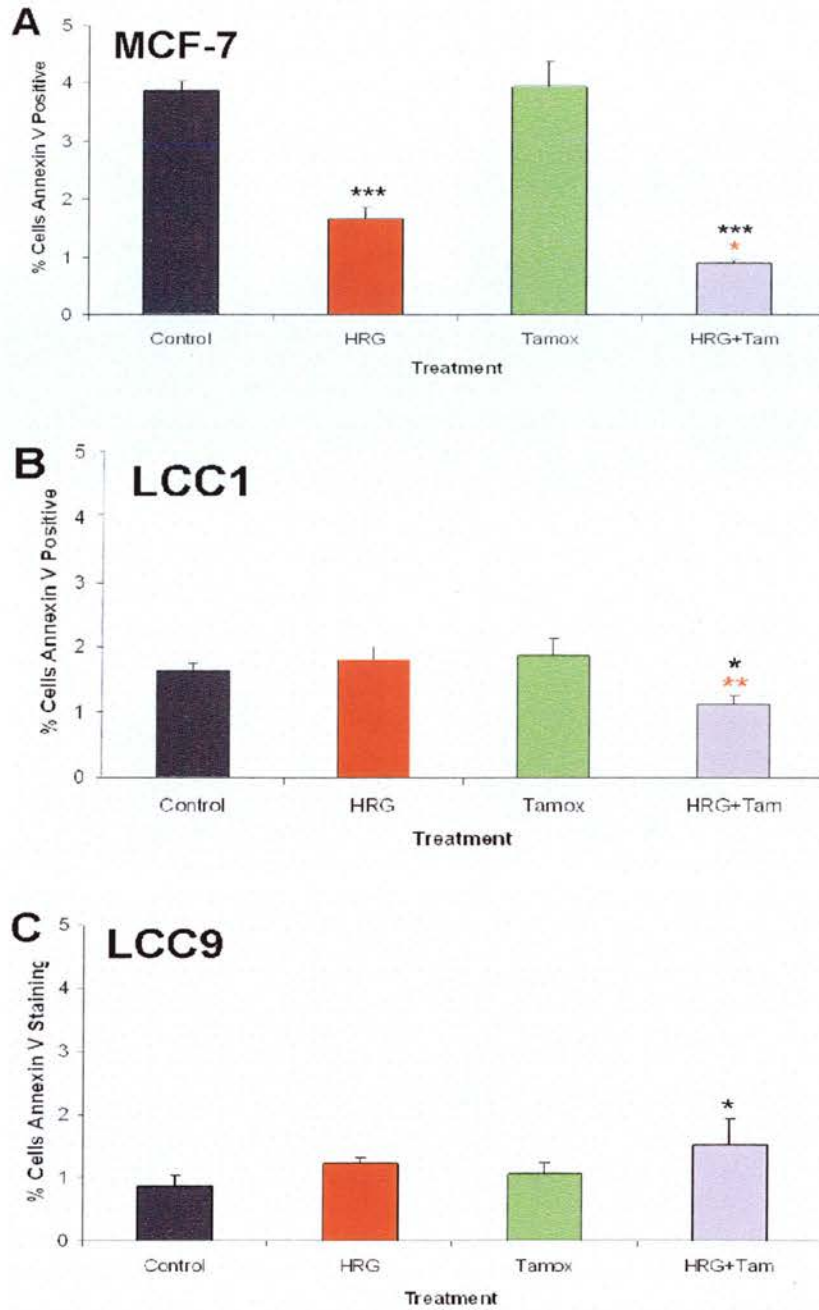


Figure 3. 14 The effect of HRG β and tamoxifen treatment on apoptosis of resistant breast cancer cell lines versus sensitive parental MCF-7 breast cell. MCF-7 cells were charcoal stripped 48h prior to treatment. All 3 cell lines were treated with control media, HRG β (1nM), tamoxifen (1 μ M), or HRG β + tamoxifen (1nM + 1 μ M) for 72h. Early apoptosis was monitored and data were plotted as the mean of triplicate samples +/- SD as a percentage of cells stained with annexin-v. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment and those in red represent any statistically significant changes post-treatment with tamoxifen compared to HRG β alone. Results are representative of 1 of 3 independent experiments

3. 5. Discussion

This section will discuss the characteristics defined by this chapter in order to ascertain the relevance of this panel of cell lines and whether the panel may be useful as a model of oestrogen and endocrine therapy insensitivity.

3. 5. 1. Growth characteristics

3. 5. 1. 1. Breast cancer cell lines

The breast cell line growth responses were characterised and the results were consistent with published data (Soule *et al*, 1973; Thompson *et al*, 1993; Brunner *et al*, 1993; Brunner *et al*, 1997). The following section will detail the findings for each cell line.

The parental **MCF-7** cell line was sensitive to growth stimulatory actions of E_2 , $TGF\alpha$ and $HRG\beta$. E_2 has been well documented as having a proliferative effect on breast cancer cells including MCF-7 cells (Thomas *et al*, 2005; Power and Thompson, 2003). Growth factors, including $TGF\alpha$ and $HRG\beta$ have also been reported to enhance the proliferation of MCF-7 cells (Normanno and Ciardiello, 1997; Keshamouni *et al*, 2002; Larsen *et al*, 1999). Tamoxifen alone did not elicit a positive or inhibitory growth response in this cell line, however the cells were found to be sensitive to the anti-oestrogenic actions of the drug when combined with E_2 , which is agreement with many published articles (Lippman *et al*, 1979; Kallio *et al*, 2005; Seeger *et al*, 2003).

Morphology studies showed MCF-7 cells had a flat ‘cobblestone’ appearance when untreated and that cell appearance changed only if treated with E_2 , or E_2 in combination with $TGF\alpha$ or tamoxifen. These observations are in agreement with previous findings where MCF-7 cultured cells were observed to be flat adherent epithelial-like cells (Thomas *et al*, 2005; Srivastava *et al*, 2004) which are sensitive to the proliferative and antiproliferative actions of E_2 and tamoxifen respectively (Palmary *et al*, 2000). MCF-7 cells have been reported to have protrusions of the plasma membrane (Thomas *et al*, 2005) and these were visible in all treatments. E_2

treatment increased the number of MCF-7 cells and altered their appearance from cell-matrix adhesions to cell-cell adhesions in agreement with a study by Srivastava *et al* (2004), who found E_2 to up regulate the cells morphologically and numerically. Tamoxifen did not alter the appearance of MCF-7 cells which is consistent with observations by Srivastava *et al* (2004) who noted no cytotoxic effect on the cell line post tamoxifen treatment. The morphology of treated MCF-7 cells agreed with the growth studies, where tamoxifen reversed E_2 -induced growth. TGF α did not appear to alter the morphology from that of control treated cells, although it did increase cell number in agreement with proliferation studies here and published data (Stewart *et al*, 1992).

The **MIII** and **LCC1** cell lines were characterised as having acquired a 'hormone-independent but hormone-responsive phenotype' by Thompson *et al* (1993). This statement is in agreement with the growth curves produced in this chapter. The **MIII** breast cancer cell line was therefore less sensitive to the proliferative effects of E_2 than the MCF-7 cell line as it proliferated in charcoal stripped media. **LCC1** cells displayed a limited sensitivity to the proliferative effects of E_2 . The cell lines retain sensitivity to antioestrogens and LH-RH antagonists (Artega *et al*, 1999; Clarke *et al*, 1989; Brunner *et al*, 1993a; Yano *et al*, 1992), which explains the reversal of the effect of E_2 in the presence of tamoxifen. Neither of the **growth factors** were able to stimulate proliferation in any of the resistant cell lines tested. These are novel results.

The **LCC2** cell line also proliferated in a hormone-independent manner. LCC2 cells displayed limited sensitivity to E_2 . Unlike the closely related LCC1 cell line, the LCC2 cell line was insensitive to the anti-proliferative effects of tamoxifen. These results are in agreement with a report on this cell line by Brünner *et al* (1993b). The LCC2 cell line was found to be insensitive to TGF α and HRG β as previously mentioned.

The **LCC9** and **LY2** cell lines were found to be completely insensitive to all ligands and also to the anti-oestrogenic properties of tamoxifen when combined with E_2 . The data on E_2 and tamoxifen are corroborated by Brünner *et al* (1997) and Clarke *et*

al (1987), and although as previously discussed in this chapter, Bronzert *et al* (1985) reported LY2 cells to be E₂-sensitive, this was after a longer incubation period compared to this study. The panel of resistant breast cancer cell lines had a less structured **morphology** than the MCF-7 cell line, perhaps reflecting their more chaotic, uncontrolled and profuse proliferation.

It was hypothesised that the resistant cell lines would behave in a manner similar to that of long term oestrogen deprived cells (LTED) as the cells proliferate in the absence of stimuli. Several studies have implicated that hypersensitivity occurs in LTED breast cancer cells (Santen *et al*, 2004), where extremely low residual concentrations of hormones are able to stimulate cell proliferation. In a study by Yue *et al* (2002), LTED MCF-7 cells responded in a 'nearly identical' manner to E₂. These studies both contrast with data here, where E₂ response was progressively lost. In another study, increasing concentrations of E₂ reduced the proliferation of another model of LTED (Song *et al*, 2001), which also contrasts with data obtained here. There are some similarities, however, to a study by Staka *et al* (2005). This group found that their LTED MCF-7 cells, MCF-7X, were not oestrogen hypersensitive, and retained some degree of E₂ sensitivity. This is consistent with MIII and LCC1 data, which were both elevated by E₂ and proliferated in charcoal stripped media. However, the remainder of the resistant cell lines were unresponsive to E₂. The divergence in pathways implicated in the published studies will be investigated in the following chapters, including, the PI3-K/Akt and MEK/ERK pathways, with ER α serine phosphorylation also being considered.

3. 5. 1. 2. Ovarian cancer cell lines

Growth data for the PEO1 and SKOV-3 ovarian cancer cell lines was also consistent with published studies, where both cell lines were sensitive to the proliferative effects of TGF α , but only the PEO1 cell line was found to be sensitive to E₂. (Langdon *et al*, 1988; Langdon *et al*, 1990; Hua *et al*, 1995; Doraiswamy *et al*, 2000).

3. 5. 2. *Expression profiles*

The expression profiles of this model of resistance were monitored to assess whether acquisition of hormone-independence and endocrine therapy resistance were related to alterations in oestrogen-responsive genes.

LCC1 cells were reported to retain **ER α** levels equivalent to the parental MCF-7 cells (Brunner *et al.*, 1993), however, this is in discord with data reported here where LCC1, LCC9 and LY2 cells lines had elevated ER α mRNA expression compared to the MCF-7 cell line. Later data published by de Cremoux *et al.* (2003) contradicts this earlier study and finds in favour of the LCC1 data here, where LCC1 basal levels are higher than the parental line. This study did not confirm the LCC9 data reported here, and instead stated there was a 19-fold reduction in ER α levels compared to the LCC1 cell line, which was not observed here. It may be that differences in the time point at which the mRNA changes were measured (48h as opposed to 24h time point in this study), the primers used and/or the standard curve may account for these changes. Also, differences in confluency of the cell lines may affect the mRNA detected. All cell line ER α levels, with the exception of LCC9 cells, were reduced with E₂ treatment, with the greatest reduction observed in the LY2 cells. The reduction was previously been reported by de Cremoux *et al.* (2003) in the MCF-7 cell line.

The effects of growth factors on all the mRNA levels measured of the resistant cell lines have yet to be reported, hence data reported here are novel. The elevated expression levels of ER α in LCC1 and LCC9 cell lines may account for the elevation in expression with TGF α treatment. This theory may also apply to the basal PR level in the LCC9 cell line which is elevated even further with TGF α treatment. LCC1 PR levels are similar to MCF-7 cell levels, but there is no reduction with growth factor addition. This suggests the insensitivity of these cells to the proliferative effects of TGF α may arise as a result of the insensitivity at the mRNA level. LY2 cells generally reacted in a similar manner to MCF-7 cell lines, with perhaps only

increased levels rather than reductions, again suggesting some form of resistance is visible at the mRNA level in this cell line.

Basal levels of **pS2** expression were significantly elevated in LCC1 and LCC9 cells compared to MCF-7 cells, which is consistent with data published by Br nner *et al* (1993) in these cell lines specifically, and also by Cho *et al* (1991) in two E₂-insensitive sublines, also derived from the MCF-7 cell line. LY2 cell pS2 mRNA was not elevated as dramatically in control conditions compared to the LCC1 and LCC9 cell lines, suggesting a different mechanism of resistance to these cells. E₂ induced an extremely significant increase in pS2 mRNA in MCF-7 cells, which is consistent with published data (Sun *et al*, 2005; Cho *et al*, 1991). Cho *et al* (1991) showed that while ER α and PR mRNA expression remained the same in all lines, as shown in this study, pS2 expression was higher in the E₂-insensitive sublines. In agreement with these earlier findings, these data suggest that the alteration of the pS2 gene in the resistant cell lines is probably not due to a defective ER regulation or ER itself.

TGF α also enhanced pS2 in MCF-7 cells, in agreement with a study by El-Tanani and Green (1997), but to a lesser extent than E₂. pS2 expression remained unaltered by TGF α in all the resistant cell lines. Again, the effects of growth factors on all the mRNA levels of the resistant cell lines have yet to be reported, hence data here is novel. This null effect may contribute to the growth factor insensitive phenotype of the resistant cell lines.

Basal expression levels of **PR** mRNA were low in both MCF-7 and LCC1 cells, which is supported by studies by de Cremoux *et al* (2003) and Br nner *et al* (1993), while a study by Br nner *et al* (1997) found the LCC9 cell line to have an elevated PR expression, in agreement with levels reported in this study. LY2 cells had a reduced PR expression compared to MCF-7 cells. The LY2 cell line responded in a similar manner to the MCF-7 with E₂ treatment. PR expression was induced by E₂ in MCF-7 cells (Fritah *et al*, 2005; Cho *et al*, 1994), which was observed in these findings. Studies by de Cremoux *et al* (2003) and Br nner *et al* (1997) also reported

MCF-7 PR mRNA, unlike that of the LCC9 cell line, was still **oestrogen-inducible**. This statement again confirms findings here. PR expression was also elevated in LCC1 cells, but to a lesser degree. Interestingly, PR mRNA expression levels were increased significantly in the LY2 cells with E₂ treatment compared to control cells. This indicates that PR is still responsive to hormonal stimulation in all resistant cell lines at the mRNA level, but to a much lesser extent in the LCC1 and LCC9 cells than the completely E₂-sensitive MCF-7 cell line. PR mRNA also appears to be highly E₂-responsive in the LY2 cell line, although this did not translate to the proliferation data.

However, the resistant cell line mRNA levels behaved in a dissimilar manner to the parental line when treated with TGF α . MCF-7 cell PR mRNA levels were reduced upon TGF α treatment, while the levels remained unchanged in the LCC1 and LY2 cells (perhaps a slight, although insignificant, increase in the latter). The MCF-7 data contrasted with the LCC9 cell line mRNA levels which were increased with TGF α treatment. The unchanged response to TGF α in LCC1 and LY2 cells and the growth factor up regulation of PR levels in the LCC9 resistant cell line may be a possible contributing factor to the development of resistance in these cell lines.

Expression profiling here showed that ER α , PR and pS2 genes all responded to E₂ as expected in MCF-7 cells, and that the resistant cell lines had elevated levels of pS2, suggesting that the alteration of the pS2 gene in the resistant cell lines is probably not due to a defective ER α regulation or ER α itself (Cho *et al*, 1991). TGF α , in general, did not affect any of the resistant gene expression profiles, with the exception of the LCC9 cell ER α and PR mRNA levels, where increases were observed, and an increase in CTD expression in the LCC1 cells. This implies that there is a disjunction between growth stimulation and the effects on E₂-regulated gene expression in the resistant cell lines. The majority of the stimulatory actions of growth factors are ineffective at gene transcription level in E₂-regulated genes in the resistant cell lines and yet the genes themselves remain responsive to E₂.

MCF-7 basal **CTD** expression was not reduced in comparison to the resistant cell lines contrasts with reports of increased levels of the cathepsin D enzyme in the LCC1 cell line (Thompson *et al*, 1993). CTD has been shown to be elevated ~5-fold between LCC1 and LCC9 cell lines (Gu *et al*, 2002*b*), which reflects the data here where the LCC9 cell line had the highest relative CTD expression of any of the cell lines. CTD has also been documented as an oestrogen-responsive gene (Chalbos *et al*, 1993; Sun *et al*, 2004), which supports data reported here, where E₂ induced a significant increase in MCF-7 CTD expression. Basal CTD was inconsistently (and insignificantly) elevated in LCC9 cells to the level of MCF-7 treated with E₂ (24h), but the gene was shown to remain as E₂-responsive and active as E₂ still elevated the basal level significantly. These data are in agreement with studies mentioned earlier in other oestrogen-regulated genes where the mRNA of the resistant cell lines remains oestrogen-inducible.

CTD expression was unaltered in all cell lines treated with **TGF α** , with the exception of slight increases in the LCC1 and perhaps the LY2 cell line. Previous studies by Wang *et al* (2000) and Chalbos *et al* (1993) have shown an increase in the CTD gene in the MCF-7 cell line with EGF and IGF-1 growth factors. This disagrees with data here, where no significant change was observed. The absence of CTD induction may be due to the lower concentration used (5 to 10-fold) while the study by Chalbos *et al* (1993) used EGF, which although possesses very similar properties to TGF α , is not identical to TGF α and therefore may not be directly comparable.

In conclusion, the development of a hormone-independent phenotype with varying degrees of endocrine therapy resistance in human breast cancer may be associated with disruption in the expression of specific oestrogen-regulated genes. In particular, elevated expression of ER, pS2 and PR, but not CTD, mRNA occurs in the resistant cell lines.

3. 5. 3. Cell cycle characteristics

The cell cycle machinery is known to be under the direct control of oestrogen in breast cancer expressing ER α (Prall *et al*, 1997; Planas-Silva and Weinberg, 1997). Thus, to explore the causes of hormone independent growth, resistance to the antiproliferative effects of tamoxifen and the proliferative effects of growth factors cells were stained with propidium iodide and cell cycle analysis was performed using flow cytometry.

MCF-7 cells arrest in the G₀/G₁ phase of the cell cycle when subjected to reduced serum conditions (Dufournay *et al*, 1997), which supports the data obtained here. All three ligands stimulated MCF-7 cells in the cell cycle, observed as significant increases in the percentage of cells in S-phase of the cell cycle, which is consistent with earlier research (Thomas & Thomas, 1994; Foster & Wimalasena, 1996; Lewis *et al*, 1996; Prall *et al*, 1997; Abdelrahim *et al*, 2002). Tamoxifen alone did not stimulate MCF-7 cells to enter the cell cycle. This is consistent with published studies by Hodges *et al* (2003), Planas-Silva and Weinberg (1997) and Osborne *et al* (1983). Tamoxifen significantly reduced the amount of E₂ treated MCF-7 cells progressing through the cell cycle compared to those treated with E₂ alone which is in agreement with published studies (Watts *et al*, 1994).

In contrast to the parental cell line, LCC1 and LCC9 resistant cell lines both displayed a constitutive increase in the number of cells in the S-phase of cell cycle. This is indicative of an increased number of cells proliferating in the absence of ligand and supports cell proliferation studies. Wilcken *et al* (1997) demonstrated that overexpression and induction of cyclin D1 stimulated cells which were previously arrested in G1 to enter S-phase which supports results in this chapter. Wilcken *et al* (1997) postulated that this occurred by the formation of active cyclin D1/Cdk4 complexes and hyperphosphorylation of the retinoblastoma protein.

In contrast to the MCF-7 cell line, the resistant cell lines did not show an increased progression of cells into the S-phase of cell cycle post treatment with E₂ or either

growth factor. These cells had in fact a reduced number of cells in the S-phase post-treatment. Oestrogen has been shown to induce G₁-progression via the activation of Cdk4 and Cdk2 mediated through increased cyclin D1 expression, its regulatory subunit (Prall *et al*, 1997). It may be that the resistant cell lines do not possess or express these molecules to the same extent as MCF-7 cells, or more likely, the molecules are overexpressed and automatically drive the cell cycle in the absence of ligand stimulation. This is supported by Murray *et al* (2005), who reported that upregulated expression of cyclin D1 was found in tumours in comparison to normal mammary glands. Importantly, over expression of cyclin D1 was also reported by Hodges *et al* (2003) to be present in cell lines with tamoxifen resistance.

Recent studies by Riggins *et al* (2005) and Gu *et al* (2002) have provided evidence of the overexpression of an alternative molecule to support the cell cycle oestrogen-insensitivity of the LCC9 cell line. The LCC9 cell line was found to have elevated expression of the **p65 NFκB subunit** and **IκB kinase essential modulator**, the upstream regulator of NFκB. NFκB and other members of this family regulate inhibitors of apoptosis and the pro-proliferative affects of *c-myc* among many factors (Chen and Greene, 2004). The constitutive activation of NFκB has been implicated in breast cancer, and has been shown to be linked with resistance to agents which promote apoptosis (Baldwin, 2001). However, overexpression of the p65 NFκB subunit and IκB kinase was only found in the LCC9 cell line and not in the LCC1 cell line, suggesting a different mode of oestrogen-independent signalling in the LCC1 cell line. Perhaps the overexpression of a different molecule may play a role in the LCC1 cell line. The slight downward trend in the number of resistant cells in S-phase post ligand treatment may be as a result of superfluous ligand interfering with the over expressed molecules or other parts of the cell cycle machinery.

Tamoxifen and E₂ + tamoxifen had the same effect as E₂ alone on cell cycle regression in both the resistant cell lines. This reversal of the resistant cell lines into arrest may act as a protective mechanism, reducing the number of cells that can be targeted by a pro-apoptotic caspase or Bcl-2 family member.

3. 5. 4. *Apoptotic profile*

The regulation of apoptosis and proliferation are together thought to determine the response to radiotherapy, chemotherapy, and endocrine therapy response (Dowsett *et al*, 1999) the latter being of particular interest in this investigation. Deregulation or an imbalance of these factors has been implicated in the development of cancer (Wyllie, 1997). The annexin staining of each cell line pre and post treatment enabled the assessment of the extent of apoptosis of the resistant cell lines in comparison to the sensitive MCF-7 cells. Any differences noted at this downstream point may reflect changes in an upstream signalling molecule which led to the development of insensitivity.

In charcoal stripped serum under control conditions the **MCF-7** cell line was found to display a certain amount of apoptosis and this is supported by data provided by Rodrick *et al* (2005) who found that in the absence of growth factors, the MCF-7 cell line underwent apoptosis. This study and others also report that E_2 has an anti-apoptotic action in MCF-7 cells deprived of serum (Rodrick *et al*, 2005; Somai *et al*, 2003). This supports the results recorded here where a marginal reduction in apoptosis was observed with E_2 treatment, although this was not found to be significant. Rodrick *et al* (2005) suggested that the action of E_2 may occur in breast cancer cells via the promotion of survival signals through an mTOR-dependent elevation of Myc expression. The group also hypothesised that an increase in phospholipase D (PLD) expression, which is prevalent in breast cancer, may result in oestrogen independence.

Tamoxifen has been shown to inhibit the proliferation of breast cancer cells and induce apoptosis of these cells by $ER\alpha$ -dependent modulation of gene expression (Kallio *et al*, 2005). Autophagic cell death has also been implicated in MCF-7 cells when treated with tamoxifen (Bursch *et al*, 1996). The established apoptotic model consists of initiator caspases 8 and 9 and executioner caspases 3, 6 and 7, although MCF-7 cells do not contain caspase-3 (Jänicke *et al*, 1998). Different caspases are probably still involved in tamoxifen-induced MCF-7 cell death however, as the cells have been shown to undergo caspase-dependent cell death when subject to etoposide

or doxorubicin treatment (Jänicke *et al*, 1998). The absence of caspase-3 activation in MCF-7 cells has led to speculation that tamoxifen operates via a caspase-independent mechanism to induce cell death. Apoptosis and alternative mechanisms of cell death are reviewed by Guimares and Linden (2004), and may involve the downregulation of Bcl-2, as reported in breast cancer cell lines.

A study by Kallio *et al* (2005) suggested that there are several mechanisms by which tamoxifen causes breast cancer cell death. A possible mode of action is the rapid induction of cell death by mitochondrial mechanisms, which can be modulated by ERs, but are not solely dependent on these receptors. The group also demonstrated that the susceptibility of breast cancer cells to cell death induced by tamoxifen may be elevated by oestrogen withdrawal, or by pre-treating the cells with another anti-oestrogen. This theory may be consistent with the LCC1 and LCC9 cell lines which were developed in a similar manner.

1 μ M Tamoxifen has been documented as being able to inhibit proliferation and induce apoptosis of MCF-7 cells (Kallio *et al*, 2005), but under these charcoal stripped specific conditions in this study tamoxifen was unable to reduce cell proliferation further than control conditions thus it is unsurprising apoptosis was not increased significantly further than control levels. This is consistent with the cell and proliferation data provided here. The absence of tamoxifen-induced apoptosis under charcoal stripped conditions may be explained by a theory proposed by Evan and Littlewood (1998). These authors theorised that apoptosis could not occur unless cells could also proliferate. This suggests that cell proliferation is a requirement in order for apoptosis to occur, and that perhaps the withdrawal of a growth signal such as E₂ and/or the addition of an inhibitor of that signal (tamoxifen) disrupts cells trying to grow down an apoptotic pathway. Tamoxifen has also been shown to induce apoptosis in a time- and dose-dependent manner by modulating bcl-2 levels in breast cancer cells. The tamoxifen-induced downregulation of bcl-2 was not accompanied by alterations in p53 levels (Zhang *et al*, 1999).

HRG β treatment had an anti-apoptotic effect on MCF-7 cells, with a significant

reduction in early stage apoptosis, which is supported by earlier studies with growth factors (Guerra-Vladusic *et al*, 1999).

E_2 appeared to reduce the apoptosis taking place in the LCC1 cell line, which is consistent with the growth phenotype. Both resistant cell lines were insensitive to the anti-apoptotic effect of HRG β , again consistent with the growth data. A possible mechanism for the reduced rate of apoptosis of the LCC9 cell line was documented by Bouker *et al* (2004). The group indicated that the development of anti-oestrogen resistance present in the LCC9 cell line was mediated by a reduction in ER-mediated apoptosis by a loss of IRF-1 and IRF-1 regulation. Bouker *et al* (2004) also indicated that this reduction in apoptosis was responsible for resistance rather than a variation in cell cycle distribution. However, the reduction in IRF-1 was not observed in LCC1 cells, indicating again the progression of resistance in this model is also occurring at the molecular level.

Trecek *et al* (2004) observed that MCF-7 cells subjected to long term tamoxifen treatment displayed a significantly reduced apoptotic response to tamoxifen. This supports the data acquired in this study, where tamoxifen was unable to increase apoptosis in either the LCC1 or LCC9 cell lines. A summary of the characteristics of the cell lines are listed in Table 1. 2.

In conclusion the absence of cell cycle progression in response to ligand stimulation and a general reduction in the number of apoptotic resistant cells suggest insensitivity and resistance occurs in a pathway upstream of these processes. The next chapter will investigate the activation of these upstream pathways, in the form of the PI3-K/Akt and MEK/ERK, in order to ascertain whether the loss of growth factor activity and E_2 -insensitivity developed here.

This panel of cell lines is a useful model of not only oestrogen and endocrine therapy resistance but also growth factor insensitivity at several levels. The cell lines were originally derived from one source and show a gradual development of insensitivity, which is extremely relevant as this mirrors the clinical progression of breast cancer and how resistance develops.

Table 3. 1. Summary table of the characteristics of the panel of breast and ovarian cell lines.

Cell line/ treatment		MCF-7	MIII	LCC1	LCC2	LCC9	LY2	PEO1	SKOV-3	
Growth response	Control	-	+	+	+	+	+	-	+	
	E ₂	+++	++	++	-	-	-	++	-	
	TGF α	++	-	-	-	-	-	+	+	
	HRG β	++	-	-	-	-	-			
	Tamox	-	-	-	-	-	-			
	E ₂ +Tam	↓↓	↓↓	↓	-	-	-			
Morphology	Control	CS		C-M	C-M	C-M	C-M			
	E ₂	C-C		C-C	C-C	C-C	C-C			
	TGF α	CS		C-M	C-M	C-M	C-M			
	Tamox	CS		C-M	C-M	C-M	C-M			
	E ₂ +Tam	C-C/S		C-M	C-M	C-M	C-M			
mRNA Expression	Relative	ER α	+		+++		++	++		
		pS2	+		+++		+++	++		
		PR	+		+		+++	↓		
		CTD	+		↓		+	↓↓		
	+E₂	ER α	↓		↓↓		-	↓↓↓		
		pS2	+++		+		+	+++		
		PR	+++		++/+++		-	+++		
		CTD	++		-		+	+++		
	+TGFα	ER α	↓↓		-		++	-		
		pS2	+		-		-	-		
		PR	↓↓		-		++	-		
		CTD	-		+		-	-		
Cell cycle progression	Control	-		++		+				
	E ₂	+++		↓		↓				
	TGF α	+++		↓		↓				
	HRG β	+++		↓		↓				
	Tamox	-		↓		↓				
	E ₂ +Tam	↓		↓		↓				
Apoptosis	Control	+		+/\downarrow		↓				
	E ₂	↓		↓						
	HRG β (H)	↓↓		-		-				
	Tamox	+		-		-				
	E ₂ +Tam	+		↓						
	H+Tam	+		↓		-/+				

CS=cobblestone; C-M=cell-matrix adhesions; C-C=cell-cell adhesions; C-C/S=partial cell-cell
 -/NC= no change/effect; +=some expression/increase; ++ and +++=increase ↓=reduction.

Chapter 4

Differential expression and activation of ER α and upstream signalling pathways in resistant breast cancer cell lines

4. 1. Functionality and activation of ER α

Chapter 3 showed the resistant cell lines to be insensitive to not only oestrogen but also growth factor stimulation. In order to ascertain where this insensitivity arose from, the signalling pathways upstream of ER α were investigated. ER α itself was also probed at the protein level to assess whether it could be activated, or was functional at this stage in the cell proliferation pathway.

Previous studies have shown that phosphorylation of serine residues on ER α at positions 104 and/or 106, serine 118 and serine 167 cause activation of ER α and that mutation of these residues reduces ER α activation, gene transcription and eventually cell proliferation (reviewed by Lannigan, 2003). Indirect evidence has shown that phosphorylation is involved in receptor function, for example, oestrogen causes a rapid increase in phosphorylation by several fold, and TGF α is able to elicit ligand-independent ER α activation (Joel *et al*, 1995; Bunone *et al*, 1996). Events subsequent to this initiate ERE-mediated gene expression. Oestrogens and antioestrogens, such as tamoxifen, and protein kinase activators also increase phosphorylation of the ER and/or proteins in the ER signalling cascade (Katzenellenbogen *et al*, 1995), hence the effect of growth factors and oestrogen and tamoxifen were used to investigate the effect on the ligand independent and dependent pathways respectively.

4. 1. 1. Is the ER α still present and functional in resistant breast cancer?

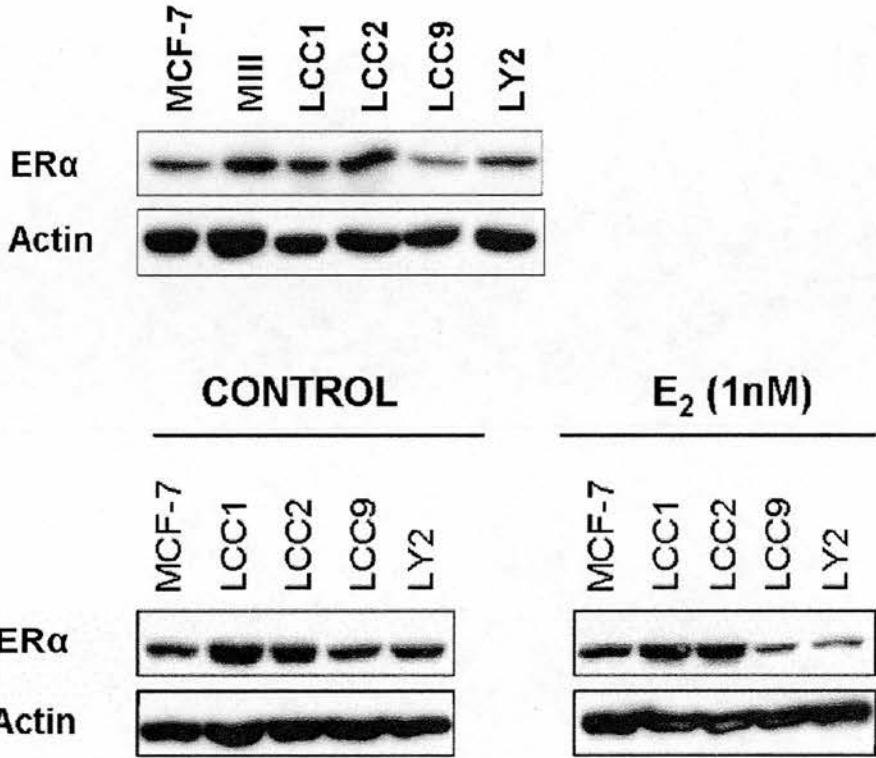
The ER α protein expression levels in the resistant and parental breast cancer cell lines were investigated. The cells were treated with oestrogen in the form of 17 β -oestradiol (E $_2$) (1nM) to observe if the receptors were 'turned over' and thus protein levels reduced by degradation. Turnover of ER α occurs when E $_2$ binds activating transcription and removing ER α from further E $_2$ activation and reducing the amount of protein detectable by western blotting (El Khissin and Leclercq, 1999; Nawaz *et al*, 1999; Lonard *et al*, 2000). The relative ER α expression was determined in the panel of cell lines. Initial studies of ER α and ER β were performed in all the breast

cancer cell lines, but the MIII cell line was excluded later as it was considered to have a very similar phenotype to the LCC1 cell line. The more significantly altered phenotypes of the LCC1 and LCC9 cell lines were deemed more suitable for further investigation. ER α protein levels were higher in the MIII, LCC1 and LCC2 resistant cell lines in comparison to the MCF-7, LCC9 and LY2 cell lines (figure 4. 1A).

The lower panel of ER α expression shows LCC1 and LCC2 ER α protein levels were \sim 1.8 and \sim 1.6-fold higher than the parental cell line. ER α levels were reduced by \sim 0.5-fold in MCF-7 cells upon E₂ treatment and LCC9 and LY2 cell lines were reduced by \sim 0.7-fold. The ER α protein levels were also reduced in LCC1 and LCC2 cell lines, but to a lesser degree (\sim 0.3 and \sim 0.2-fold reductions). These reductions were all seen at only 30 min, which is an early time point. Time course studies were also performed to investigate the effects of E₂ over a longer time period (see figure 4. 2). The reduction in protein expression suggests the ER α of the resistant cell lines are all able to bind E₂, however, this may not necessarily translate to the expression of functionally active ER α . ER β expression in the panel of cell lines was also investigated. There was no clear difference between the resistant and MCF-7 cell lines (figure 4.1B), although these studies do not show whether the ER α :ER β ratio is equivalent across the panel of cell lines.

Figure 4.2 shows the effects of E₂ (1nM), tamoxifen (1 μ M) and E₂ + tamoxifen (1nM + 1 μ M) over a 24h time course (0-1440 min). These studies, and subsequent more in-depth profiles, were performed in MCF-7, LCC1 and LCC9 cell lines as these lines displayed a clear progression in antioestrogen resistance and oestrogen independence. The western blots of the ER α protein expression levels are shown in the left hand panel with their corresponding β -actins beneath each blot as loading controls. The histograms in the right hand panel represent the optical density (OD) values taken from the western blots relative to each other using MCF-7 cells treated with E₂ for 30 min as a positive control (+). The maximum reduction was observed after 1440 min (24h) in all cells, with reductions of \sim 7, \sim 47 and \sim 63-fold for MCF-7, LCC1 and LCC9 cell lines respectively after E₂ treatment. Tamoxifen did not affect the levels of MCF-7 ER α as the values wavered around those of control treated cells. E₂ + tamoxifen did not induce or reduce ER α expression in this cell line.

A



B

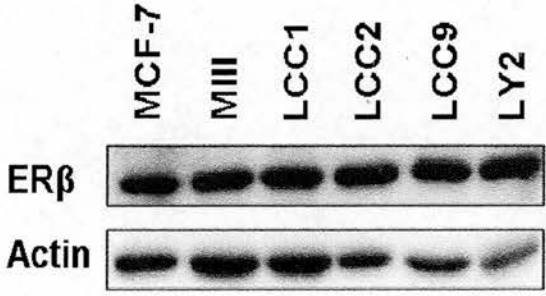
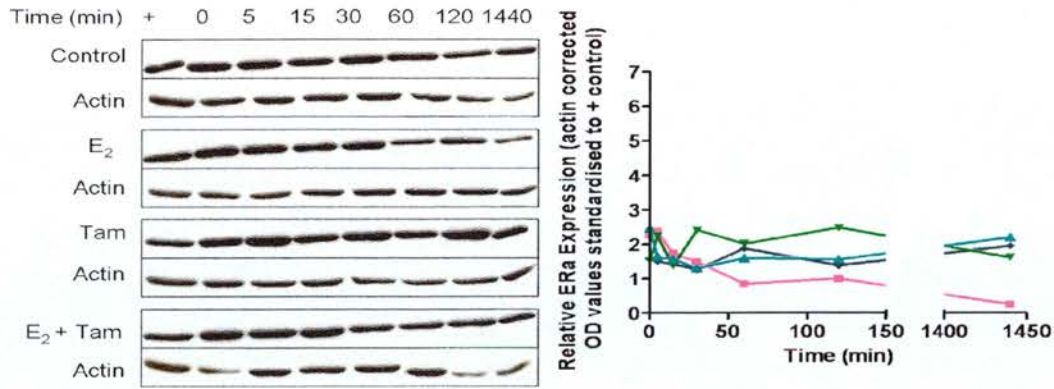
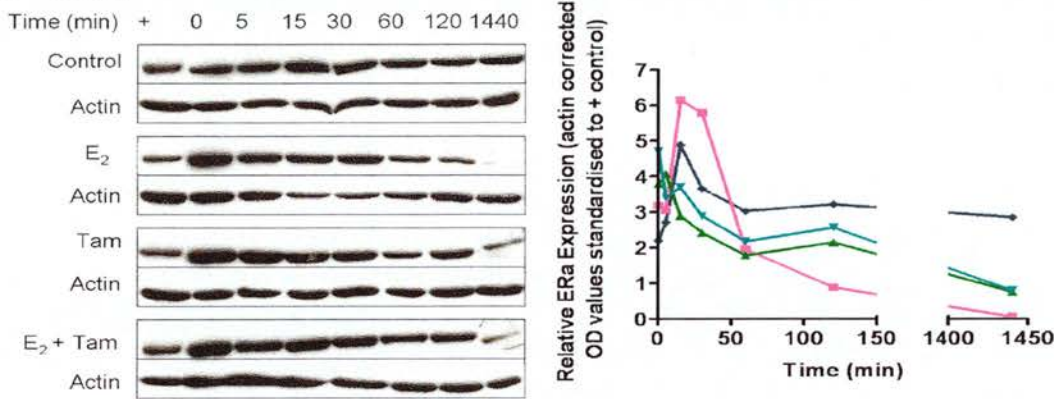


Figure 4. 1 ERα and ERβ protein expression levels. **A**, ERα expression levels in the panel of breast cancer cell lines. The effect of E₂ was assessed in a reduced panel of cells, where ERα levels decreased in the presence of E₂ in parental and resistant breast cancer cell lines alike. MCF-7 cells were charcoal-stripped for 24h and then the cell lines were treated with E₂ (1nM) for 30 min.; **B**, ERβ protein expression levels, where levels were similar across the panel of cell lines. Protein lysates were run on a 10% SDS-gel and membranes probed with ERα (F10, 1:200) or ERβ (H-150, 1:200) primary antibody. Results are representative of 1 of 3 independent experiments.

A MCF-7



B LCC1



C LCC9

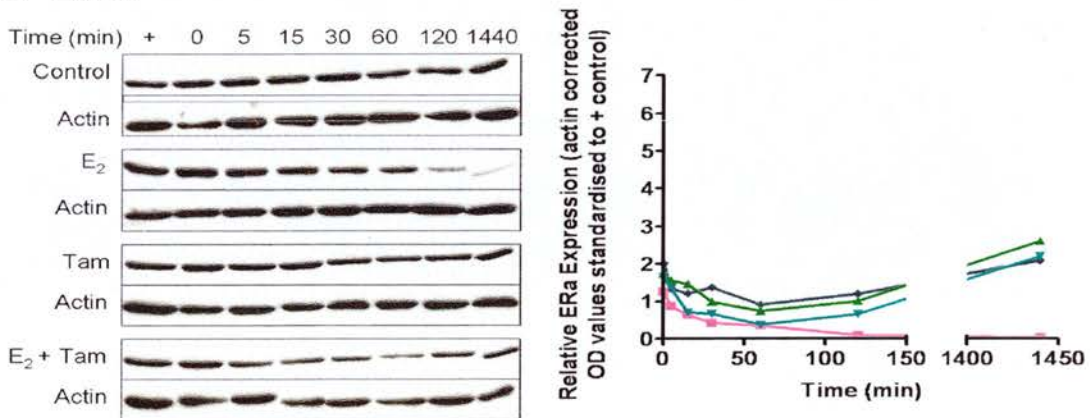


Figure 4. 2 ERα protein expression levels decreased in the presence of E₂ over time in parental and resistant breast cancer cell lines. MCF-7 cells were charcoal-stripped for 24h and then all cell lines were treated with control media (◆), E₂ (1nM) (■), tamoxifen (1μM) (▲) or E₂ + tamoxifen (1nM + 1μM) (▼), for 0-1440 min. Protein lysates were run on a 10% SDS-gel and membranes probed with ERα primary antibody (F10, 1:200). Histograms represent the western blots in the left hand panel. The positive control (+) was MCF-7 E₂ 30 min treatment, which was used to calculate relative ERα expression (OD units standardised to actins & + control) in the histograms of each western blot. Results are representative of 1 of 3 independent experiments.

In the LCC1 cell line the ER α levels were reduced over time with all treatments except control. Interestingly, tamoxifen appeared to reduce the level of protein by ~4-fold, which could be explained by the mild oestrogenic activity of tamoxifen. The trend of ER α expression after E₂ + tamoxifen treatment is similar to that of tamoxifen alone, where a ~4-fold reduction is observed. This is supported by previous growth data and published research, where the LCC1 cells were shown to be tamoxifen-resistant (Brunner *et al.*, 1993a). The ER α protein levels in the LCC9 cell line were only reduced by E₂. Tamoxifen alone and E₂ + tamoxifen treatment produced ER α trends similar to that of the untreated cells.

4. 1. 2. Is the ER α still activated in resistant breast cancer?

4. 1. 2. 1. P-S118 30 min single time point

As the ER α was shown to be turned over, phosphorylation of the ER α was then studied to determine any differences in the activation of resistant breast cancers compared to the parental MCF-7 cell line. The activation of the ER α via phosphorylation of serine residue 118 was investigated by treating the panel of breast cancer cell lines with E₂ (1nM), TGF α (1nM), tamoxifen (1 μ M) or E₂ + tamoxifen (1nM + 1 μ M), for 30 min. Lysates were run on 10% SDS gels and once transferred, membranes were probed with a P-S118 antibody. Figure 4.3A is a representative western blot and shows the antibody binds to the ER α P-S118 (at ~66kDa) and produced a number of bands at and around that position, depending on treatment. Control treatment produced relatively little or no P-S118 in all cell lines, while E₂ produced a significant increase in P-S118 in all cell lines (seen as a doublet, or a single band where the two bands merge together), with the exception of LCC9 cells. Figure 4.3B shows a histogram of western blot OD values of the MCF-7 and two chosen resistant lines (LCC1 and LCC9). Statistical analyses were performed on these data only. The small differences in control levels between the cell lines were statistically insignificant. There were, however, increases in P-S118 of ~5-fold for MCF-7 (P<0.001) and MIII cells with 30 min E₂ treatment compared to control levels, and of ~22-fold (P<0.001) compared to control in LCC1 cells.

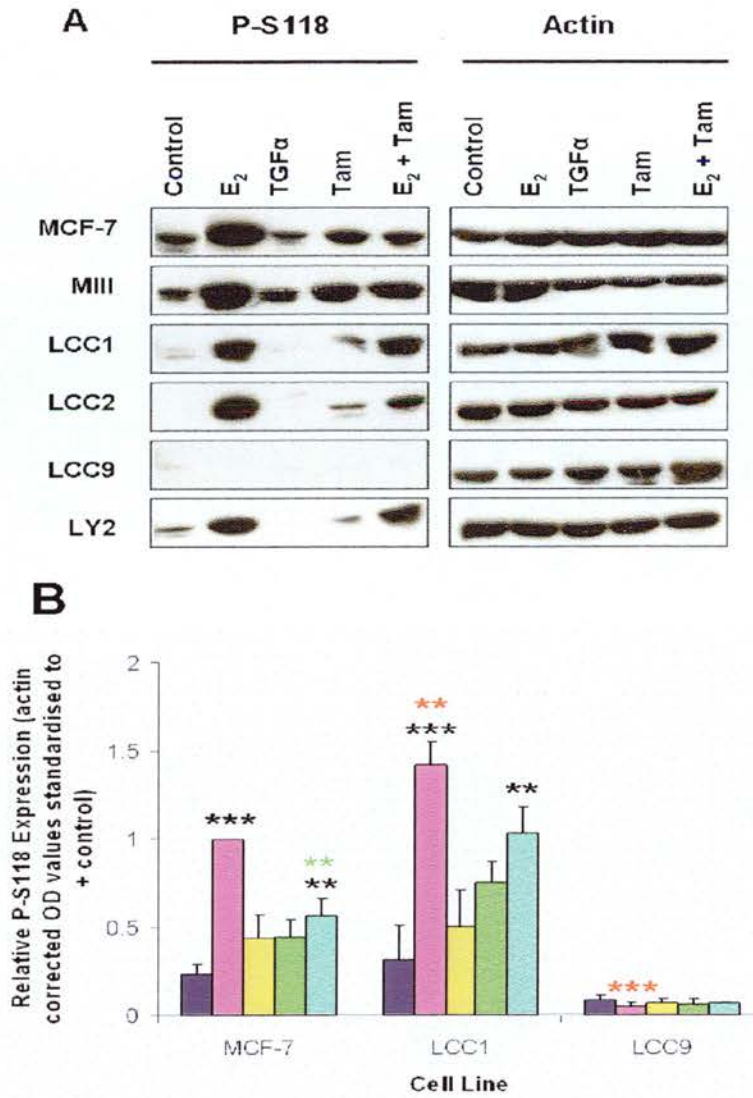


Figure 4.3 Effect of various treatment on the level of ERα P-S118 in resistant versus parental breast cancer cell lines. A, Western blot showing the panel of breast cancer cell lines were charcoal stripped for 24h then treated with control media, E₂ (1nM), TGFα (1nM), tamoxifen (1μM) and E₂ + tamoxifen (1nM + 1μM) for 30 min. Lysates were run on a 10% SDS gel and membranes probed with ERα P-S118 antibody (1:1000). Actins are shown as loading controls. B, Histogram representing optical densities (OD) from triplicate western blots, where cells were treated with control media (■), E₂ (■), TGFα (■), tamoxifen (■) and E₂ + tamoxifen (■), where the positive control used was MCF-7 E₂. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Black asterisks represent statistical significance between control and each treatment in each cell line, those in green represent statistical significance between cells treated with E₂ and E₂+Tamox in each cell line, while those in red represent statistical significance between MCF-7 and resistant cell lines treated with E₂. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

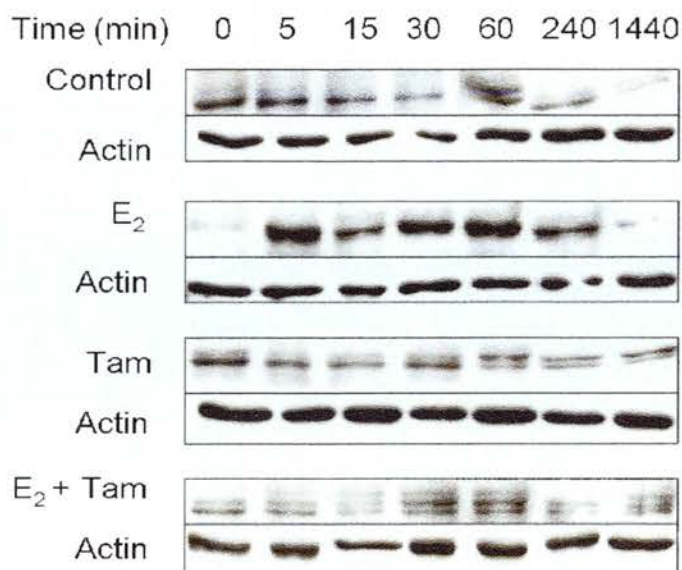
There was a similarly large induction of P-S118 in LCC2 cells, although the fold increase could not be determined due to the zero optical density reading of the control value (~1.3 OD units post E₂ treatment compared to zero units with control media alone). E₂ enhanced P-S118 by ~6.5-fold in the LY2 cell line. TGF α enhanced P-S118 after 30 min in the MIII cell line only (~3-fold compared to control). Tamoxifen enhanced P-S118 in MCF-7, MIII and LCC1 by approximately 2, 6 and 2.5-fold (P>0.05, not statistically significant) respectively and an increase of 0.2 OD units was noted in the LCC2 cell line. Tamoxifen reversed E₂-induced P-S118 in MCF-7 cells (~4-fold reduction, P<0.01, as indicated by the green asterices on figure 4.3B), but to a lesser degree in LCC1 (~0.1-fold reduction, P>0.05) and LCC2 cells (~0.25-fold reduction).

No reversal of E₂ enhanced P-S118 was observed in MIII cells with tamoxifen treatment (~0.1-fold increase). Tamoxifen reversed E₂-enhanced P-S118 by ~3-fold in the LY2 cell line. LCC9 P-S118 levels were low compared to all other cell lines and irrespective of treatment (P>0.05).

4. 1. 2. 2. P-S118 24h time course

The effect of E₂ (1nM), tamoxifen (1 μ M) and E₂ + tamoxifen (1nM + 1 μ M) on ER α P-S118 over a 24h time period (0-1440 min) in MCF-7, LCC1 and LCC9 cell lines was investigated (Figure 4.4). ER α P-S118 was detected using western blotting with a phospho-specific antibody as before. A representative western blot and histogram for MCF-7 cells are shown in figure 4.4A and B respectively. The only marked induction in ER α P-S118 was seen with E₂ treatment. E₂ treatment enhanced P-S118 after 5 min and this continued up to and including the 240 min time point. The maximum increase in P-S118 was observed between the 30 and 60 min treatments (~48-fold increase compared to control).

A MCF-7



B

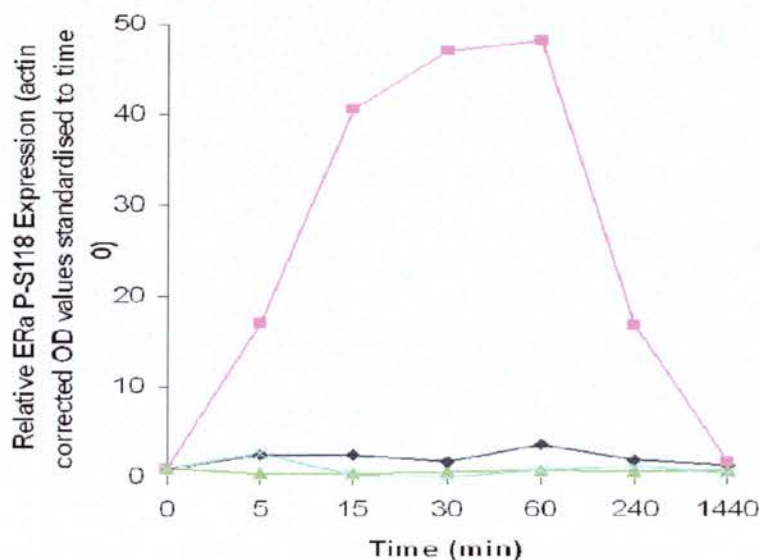


Figure 4.4 Effect of E₂ and tamoxifen on the level of ERα P-S118 in MCF-7 parental breast cancer cell line over a 24h time course. A, western blot showing the MCF-7 cell line was charcoal stripped for 24h then treated with control media, E₂ (1nM), tamoxifen (1μM) and E₂ + tamoxifen (1nM + 1μM) over a 24h time period (0-1440 min). Lysates were run on a 10% SDS gel and membranes probed with ERα P-S118 antibody (1:1000). Actins are shown as loading controls. B, Graphical representation of optical densities (OD) from the western blot shown in A, where cells were treated with control media (■), E₂ (■), tamoxifen (■) and E₂ + tamoxifen (■) and ODs were actin corrected and standardised to time 0. Results are representative of 1 of 3 independent experiments

Figures 4.5A and B show the western blot and histogram for the time course treatments of the LCC1 cell line. There is a massive induction in P-S118 with E₂ treatment after 5 min, which remained above control level up to and including the 60 min time point. P-S118 levels return to basal level after 240 min E₂ treatment. Tamoxifen enhanced P-S118 markedly after 5 min, and this remained the case for the duration of the time course, with the exception of the 30 min time point where the increase was only minor.

After 240 min exposure to E₂, the level of P-S118 returned to a level similar to that of control. Tamoxifen was able to almost completely abrogate E₂-induced P-S118 (95% reversal after 5 min), reducing P-S118 to just above that of control treated cells, and interestingly below that of tamoxifen treatment alone for the duration of the experiment.

The LCC9 cell line was treated as described for MCF-7 and LCC1 cell lines and is shown in figure 4.6A and B (representative western blot and histogram respectively). There was no obvious change in P-S118 levels irrespective of treatment at any time point, although a small increase was observed with tamoxifen alone and E₂ plus tamoxifen treatments after 24h (1440 min).

Chapter 3 reported the resistant cell lines to be growth insensitive to treatment with TGF α hence this ligand was further investigated for the potential to enhance P-S118 over time to see if the ligand had lost its ability to activate ER α . Figures 4.7A and B are the data for the MCF-7 cell line. Control levels were low as before. E₂ treatment, as previously described, induced a dramatic increase in P-S118 after 5 min to 240 min treatment, whilst TGF α also enhanced P-S118, but to a lesser degree. The greater area under the curve shows that E₂ enhanced P-S118 for a more prolonged time period, with the greatest increase around 30 min as before (~9-fold increase), compared to a smaller peak also seen after 30 min TGF α treatment (~6-fold increase).

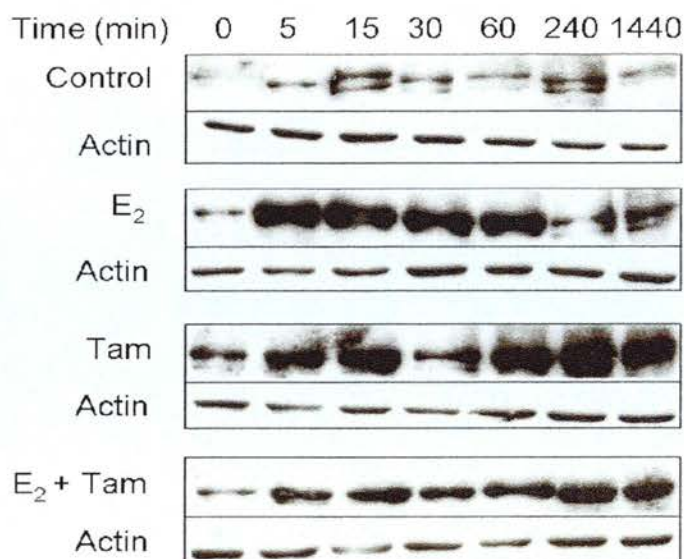
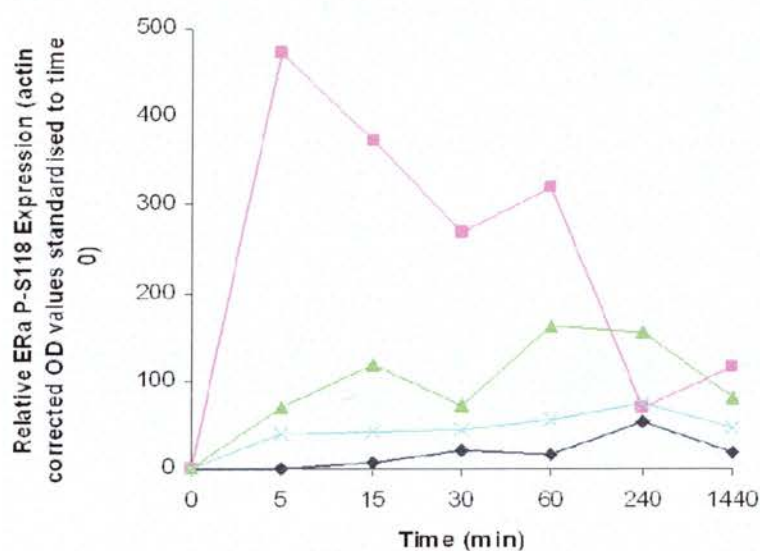
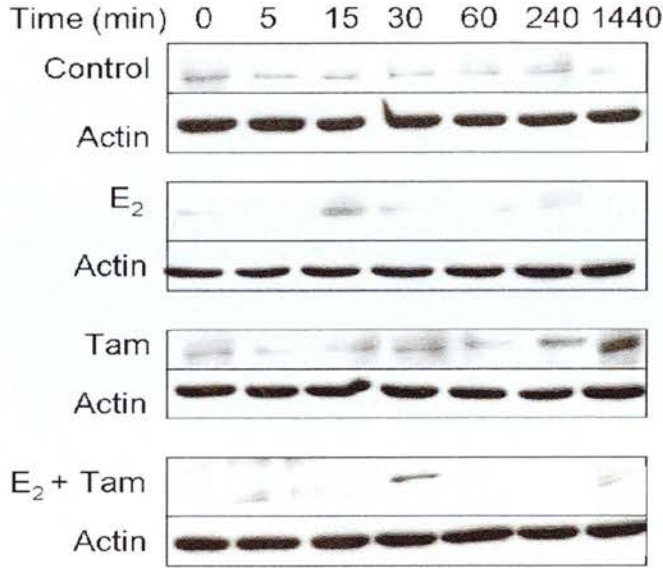
A LCC1**B**

Figure 4.5 Effect of E₂ and tamoxifen on the level of ERα P-S118 in the LCC1 breast cancer cell line over a 24h time course. A, western blot showing the LCC1 cell line was charcoal stripped for 24h then treated with control media, E₂ (1nM), tamoxifen (1μM) and E₂ + tamoxifen (1nM + 1μM) over a 24h time period (0-1440 min). Lysates were run on a 10% SDS gel and membranes probed with ERα P-S118 antibody (1:1000). Actins are shown as loading controls. B, Graphical representation of optical densities (OD) from the western blot shown in A, where cells were treated with control media (■), E₂ (■), tamoxifen (■) and E₂ + tamoxifen (■) and ODs were actin corrected and standardised to time 0. Results are representative of 1 of 3 independent experiments.

A LCC9



B

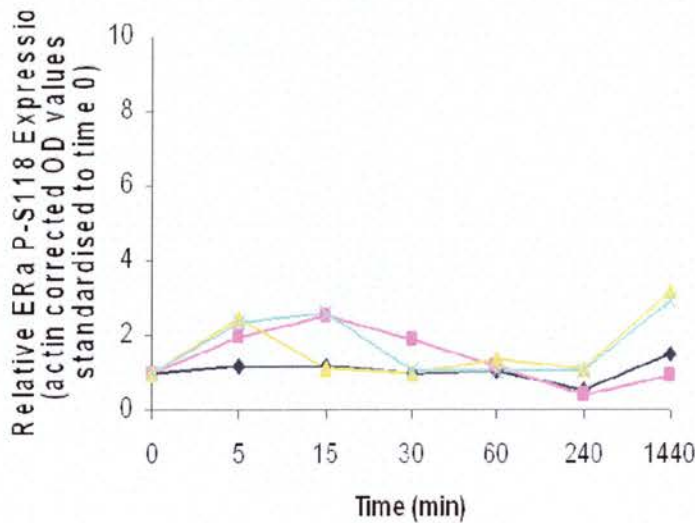
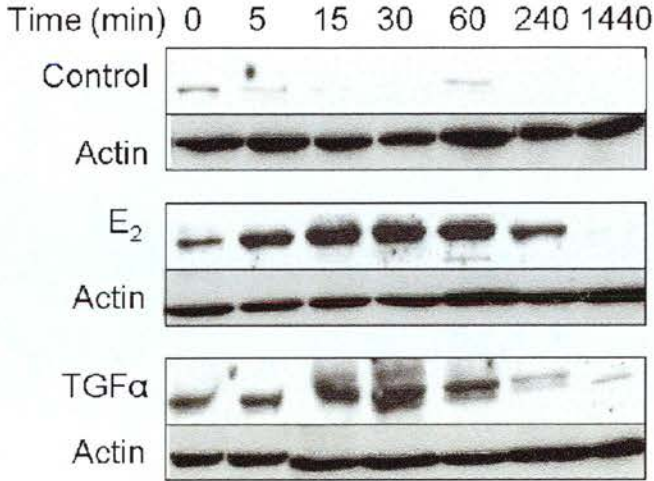


Figure 4.6 Effect of E₂ and tamoxifen on the level of ERα P-S118 in the LCC9 breast cancer cell line over a 24h time course. A, western blot showing the LCC9 cell line was charcoal stripped for 24h then treated with control media, E₂ (1nM), tamoxifen (1μM) and E₂ + tamoxifen (1nM + 1μM) over a 24h time period (0-1440 min). Lysates were run on a 10% SDS gel and membranes probed with ERα P-S118 antibody (1:1000). Actins are shown as loading controls. B, Graphical representation of optical densities (OD) from the western blot shown in A, where cells were treated with control media (■), E₂ (■), tamoxifen (■) and E₂ + tamoxifen (■) and ODs were actin corrected and standardised to time 0. Results are representative of 1 of 3 independent experiments.

A MCF-7



B

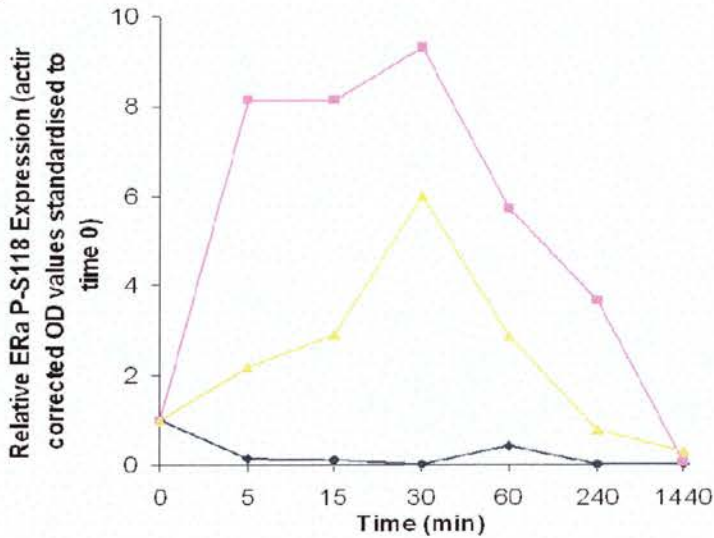
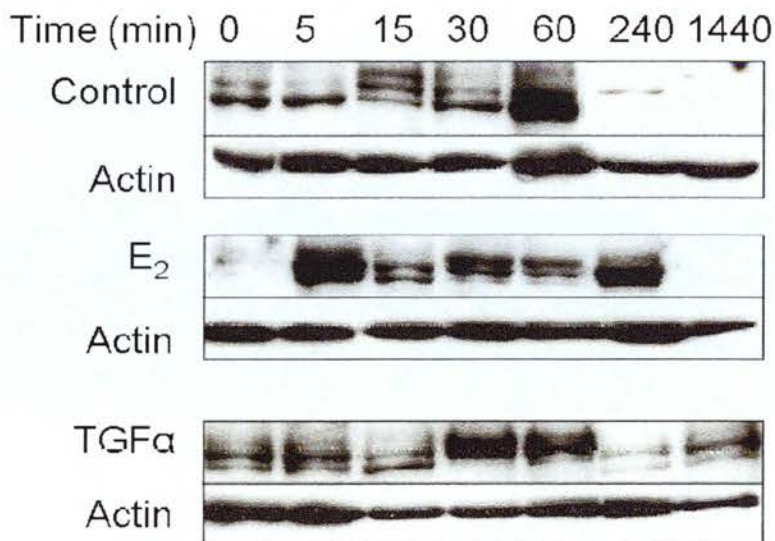


Figure 4.7 Effect of E₂ versus TGFα on the level of ERα P-S118 in the parental MCF-7 breast cancer cell line over a 24h time course. A, western blot showing the MCF-7 cell line was charcoal stripped for 24h then treated with control media, E₂ (1nM), and TGFα (1nM) over a 24h time period (0-1440 min). Lysates were run on a 10% SDS gel and membranes probed with ERα P-S118 antibody (1:1000). Actins are shown as loading controls. B, Graphical representation of optical densities (OD) from the western blot shown in A, where cells were treated with control media (■), E₂ (■), and TGF α (■) and ODs were actin corrected and standardised to time 0. Results are representative of 1 of 3 independent experiments.

A LCC1



B

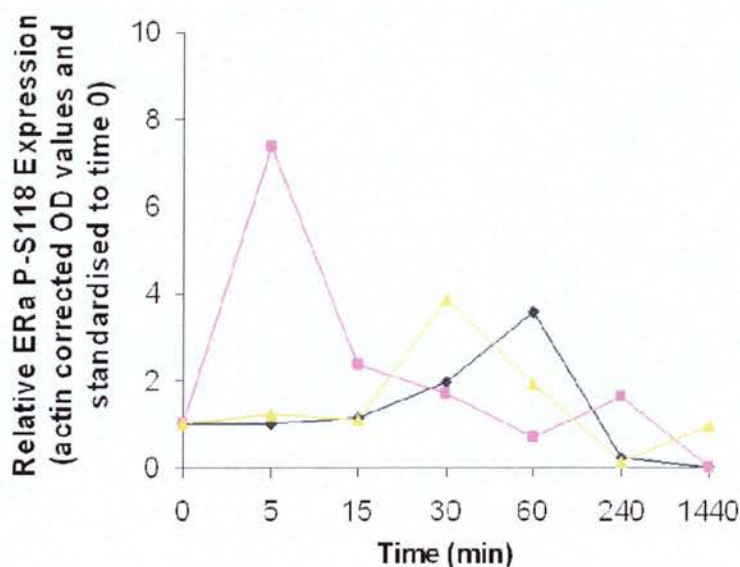


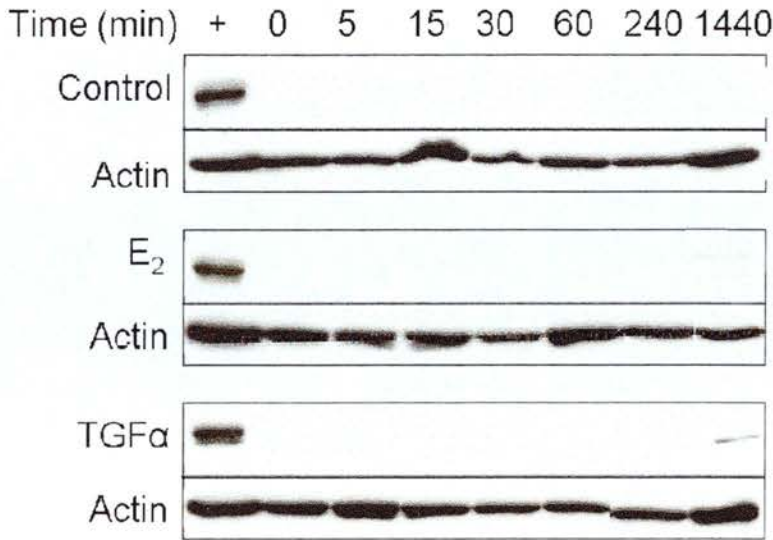
Figure 4.8 Effect of E₂ versus TGFα on the level of ERα P-S118 in the LCC1 breast cancer cell line over a 24h time course. A, western blot showing the LCC1 cell line was charcoal stripped for 24h then treated with control media, E₂ (1nM), and TGFα (1nM) over a 24h time period (0-1440 min). Lysates were run on a 10% SDS gel and membranes probed with ERα P-S118 antibody (1:1000). Actins are shown as loading controls. B, Graphical representation of optical densities (OD) from the western blot shown in A, where cells were treated with control media (■), E₂ (■), and TGF α (■) and ODs were actin corrected and standardised to time 0. Results are representative of 1 of 3 independent experiments.

TGF α treatment of LCC1 cells increased P-S118 levels after approximately 30 min compared to that of control treated cells (figure 4.8A and B) with levels returning to near basal level after 120 min. E₂ treatment enhanced P-S118 to greater extent than TGF α (~2-fold difference between peak values).

Exposure to E₂ or TGF α did not alter P-S118 expression in LCC9 cells (figures 4.9A and B). The LCC9 data was compared to a positive control (MCF-7 cells treated with E₂ for 30 min) to highlight the extremely low levels of expression in comparison to the MCF-7 cell line and also to confirm the activity of the antibody. As with figure 4.6, there was a minor increase in P-S118 after 1440 min (24h) with E₂ treatment. This also occurred with TGF α treatment.

E₂ enhanced P-S118 in resistant LCC1 and LCC2 cell lines to a greater extent than MCF-7 cells and MIII cells. These data are consistent with the elevated levels of ER α in these cell lines, which may account for this elevated P-S118, and are also consistent with the growth data. Exploratory analysis into the ratio of T-ER to P-S118 supports the elevated T-ER in resistant cell line hypothesis, with the LCC1 cell line having a larger proportion of T-ER than the parental cell line. The LCC1 cell line had a ratio of ~5.7:1 (T-ER: P-S118) compared to ~0.15:1 in the MCF-7 cell line after 5 min E₂ treatment, where maximum P-S118 enhancement was reported in the LCC1 cell line. The ratio remained in favour of a greater T-ER content in the LCC1 cell line even at the maximum P-S118 enhancement in the MCF-7 cell line after 30 min E₂ treatment, with ratios of 1.7:1 and 0.7:1 in the LCC1 and MCF-7 cell lines respectively. Initial exploratory analysis of this ratio in the LCC9 cell line produced a ratio of ~0.4:1 after 5 and 30 min of E₂ treatment. The ratio may not be as great as that between the LCC1 and MCF-7 cell lines, but the LCC9 cell line still has a greater T-ER: P-S118 than the parental cell line after 5 min treatment.

A LCC9



B

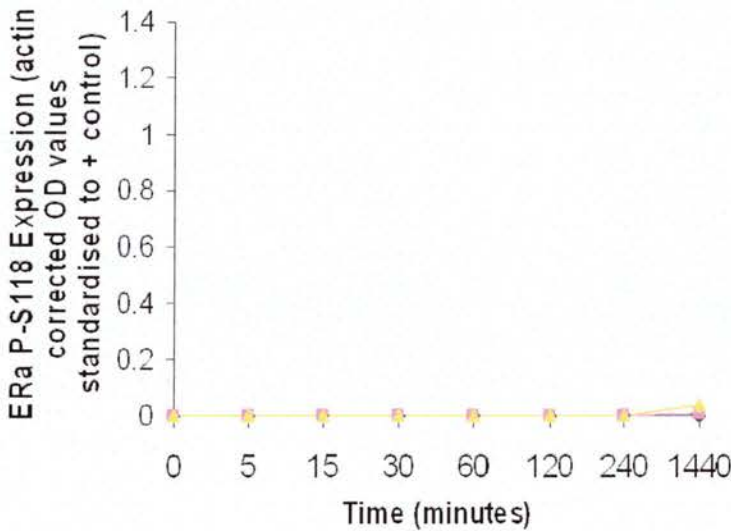


Figure 4.9 Effect of E₂ versus TGFα on the level of ERα P-S118 in the LCC9 breast cancer cell line over a 24h time course. A, western blot showing the LCC9 cell line was charcoal stripped for 24h then treated with control media, E₂ (1nM), and TGFα (1nM) over a 24h time period (0-1440 min). Lysates were run on a 10% SDS gel and membranes probed with ERα P-S118 antibody (1:1000). Actins are shown as loading controls. B, Graphical representation of optical densities (OD) from the western blot shown in A, where cells were treated with control media (■), E₂ (■), and TGF α (■) and ODs were actin corrected and standardised to time 0. Results are representative of 1 of 3 independent experiments.

4. 1. 2. 3. ER α P-S167

ER α activation was also monitored at serine residue 167 (S167) as this residue has been shown to be phosphorylated in MCF-7 cells as a result of stimulation via the PI3-K/Akt pathway and has been implicated in tamoxifen-resistance (Campbell *et al*, 2001). It was therefore hypothesised, as for P-S118 expression, that the resistant cell lines would be constitutively activated at S167; however this was not found to be the case.

The relative level of P-S167 in MCF-7 cells in comparison to LCC1 and LCC9 cell lines is shown in figure 4.10A. MCF-7 cells treated with HRG β for 15 min were used as a positive control. A clear band was observed in this lane, but no other, showing the MCF-7 and the resistant cells alike were not constitutively activated at this position. Figure 4.10B illustrates the activation of ER α at S167 in all three cell lines as bands are visible with HRG β treatment in the second column of all cell lines.

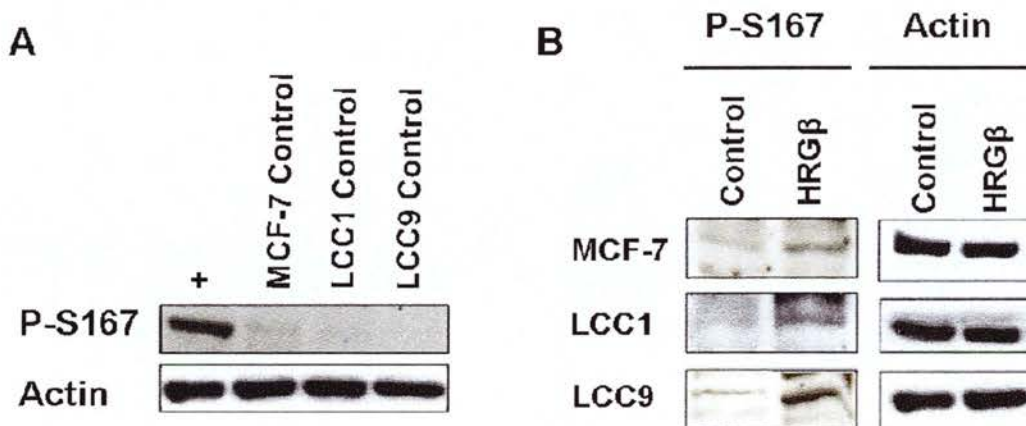


Figure 4.10. Basal levels and HRG β activation of ER α P-S167 in MCF-7 versus resistant breast cancer cell lines. A, western blot showing MCF-7, LCC1 and LCC9 breast cancer cell lines were charcoal stripped for 48h then treated with control media for 15 min. MCF-7 cells were also treated separately with HRG β (1nM) for 15 min to act as a positive control (+). B, Cells were treated as in (A), with control media and HRG β (1nM) for 15 min. Lysates were run on a 10% SDS gel and membranes probed with ER α P-S167 antibody (1:1000). Actins are shown as loading controls. Results are representative of 1 of 3 independent experiments.

4. 2. ER α expression and activation in ovarian cancer

4. 2. 1. ER α expression in ovarian cancer

The relative expression of ER α was confirmed in the ovarian PEO1 and SKOV-3 cell lines in comparison to the MCF-7 breast cancer cell line (figure 4.11). The western blot showed that the greatest ER α expression was found in MCF-7 cells, with PEO1 and SKOV-3 cells expressing dramatically reduced levels of this protein. PEO1 cells expressed approximately a third of the amount of protein, while SKOV-3 expression was relatively low compared to the other cell lines (at least ~20-fold reduced expression compared to MCF-7 and PEO1 cell lines).

4. 2. 2. ER α activation in ovarian cancer

The reduced expression of ER α in the ovarian cell lines was hypothesised to reduce the activation observed through phosphorylation. Figure 4.12 shows that the effect of E₂ and TGF α on P-S118 in MCF-7 cells in comparison to the PEO1 and SKOV-3 cell lines. MCF-7 cells treated with E₂ and TGF α for 30 min were used as positive controls for P-S118 and P-S167 respectively. PEO1 cells displayed no detectable P-S118 while E₂ induced a low level of P-S118 in SKOV-3 cells. There appeared to be some background P-S118 in the SKOV-3 cell line. No P-S167 was detected in either ovarian cell line with either E₂ or TGF α treatment.

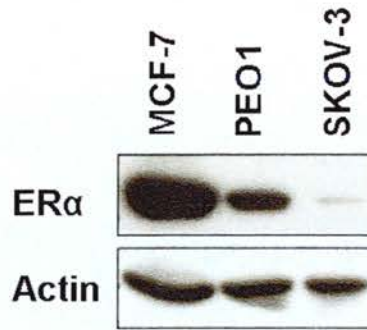


Figure 4.11. ERα expression levels in the MCF-7 breast cancer cell line compared to ovarian cancer cell lines. All cells were charcoal stripped for 24h and protein lysates were run on a 10% SDS-gel and membranes probed with ERα F10 antibody (1:200). Actins are shown as loading controls. Results are representative of 1 of 3 independent experiments.

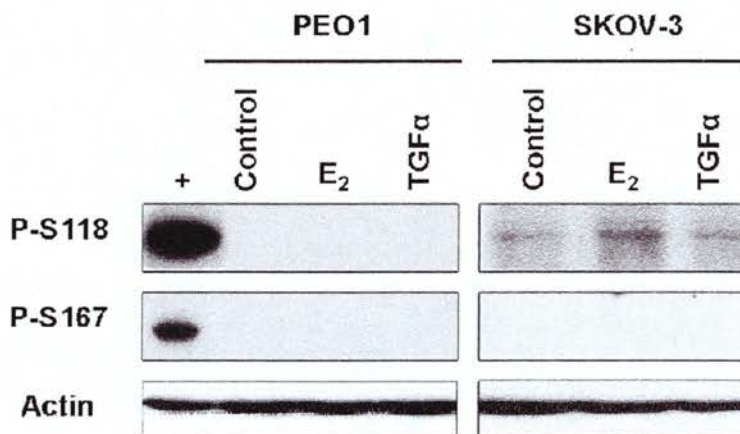


Figure 4.12. Effect of various treatments on the level of ERα P-S118 and P-S167 in MCF-7 breast cancer cell line in comparison to ovarian cancer cell lines. Western blots showing P-S118 and P-S167 expression in MCF-7 breast cancer in comparison with PEO1 and SKOV-3 ovarian cancer cell lines. All cells were charcoal stripped for 24h then treated with control media, E₂ and TGFα (both 1nM) for 30 min. MCF-7 cells treated with E₂ and TGFα for 30 min were used as positive controls for P-S118 and P-S167 respectively (+). Lysates were run on a 10% SDS gel and membranes probed with either ERα P-S118 or P-S167 antibodies (1:1000). Actins are shown as loading controls. Results are representative of 1 of 3 independent experiments.

4. 3. Are signalling molecules upstream of ER α involved in the development of resistant breast cancer?

Signalling molecules upstream of ER α were investigated in the presence and absence of hormonal and growth factor stimulation to determine their role and importance, if any, in the development of E₂ and endocrine-therapy-insensitivity. In particular, the activation status, through phosphorylation, of signalling molecules Akt, MEK and ERK1/II were characterised. These molecules were chosen as they have all previously been linked with the development of breast cancer.

4. 3. 1. P-Akt

Figure 4.13A shows a representational western blot of the protein expression of P-Akt in MCF-7, LCC1 and LCC9 cells treated with control media, E₂ (1nM), TGF α (1nM) or HRG β (1nM) for 15 min. Total-Akt levels were used as loading controls. Figure 4.13B shows a histogram representing triplicates of independent western blots performed, where P-Akt was significantly constitutively activated in both the LCC1 and LCC9 cells compared to MCF-7 cells (~2.2- (P<0.01) and ~2.1-fold (P<0.05) increases respectively compared to control treated MCF-7 cells). P-Akt was not elevated in any cell line with E₂ treatment (P>0.05) when compared to the control or other treatments. TGF α activated Akt through phosphorylation in all cell lines. TGF α elevated P-Akt ~6-fold in MCF-7 cells (P<0.001, when considered separately from HRG β data). Resistant cell lines were activated further than their elevated basal levels upon TGF α treatment, with inductions of ~1.5-fold for LCC1 cells (P>0.05) and ~1.8-fold (P<0.05) for LCC9 cells. HRG β significantly increased P-Akt in MCF-7 by over ~30-fold (P<0.001), ~3.3-fold in LCC1 cells (P<0.001) and ~3.5-fold in LCC9 cell lines (P<0.01). The final P-Akt expression levels were similar with HRG β -induction in all three cell lines.

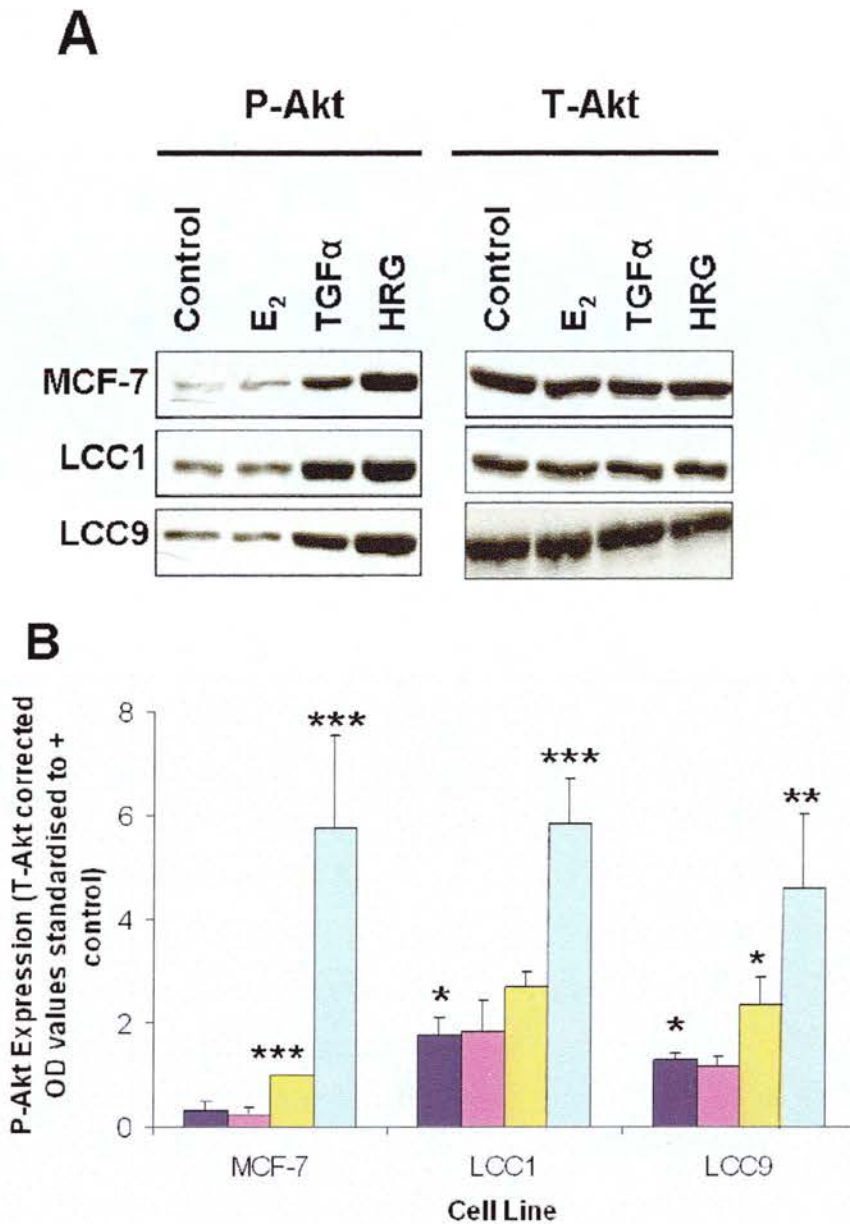


Figure 4. 13. Comparison of the levels of P-Akt and the effect of ligand stimulation on levels of P-Akt in resistant versus parental breast cancer cell lines. A, Western blot of P-Akt levels, where MCF-7 cells were charcoal stripped for 48h before all cell lines were treated with control media, E₂ (1nM), TGFα (1nM) or HRG (1nM) for 15 min. Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls. B, histogram of P-Akt levels of MCF-7, LCC1 and LCC9 cell lines treated with control media (■), E₂ (■), TGFα (■) or HRG (■) as before, where data are in triplicate +/-SD. MCF-7 cells treated with TGFα were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Black asterisks represent statistical significance between control and each treatment in each cell line. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

4.3.2. P-MEK

The levels of P-MEK in MCF-7, LCC1 and LCC9 cells were determined after 15 min treatment with control media, E₂ (1nM), TGF α (1nM) and HRG β (1nM) (Figure 4.14) as for previous signalling experiments. Figure 4.14A is a representative western blot of the phospho and total levels of MEK protein found in each cell line. T-MEK levels were used as loading controls. Figure 4.14B is a histogram of triplicate OD values taken from the western blots of separate experiments. The histogram shows statistical analysis of data including HRG β treatment. Very little P-MEK was observed, if at all, in any of the three cell lines treated with control media and there was no significant difference between the basal levels of any of the cell lines.

E₂ did not significantly increase P-MEK in any cell line as the SD values were large. TGF α and HRG β increased P-MEK extremely significantly in MCF-7 cells (mean OD values of 1 and 5+/-0.2 arbitrary units compared to a control value of zero, P<0.001). TGF α significantly elevated the P-MEK in LCC1 cells by ~300-fold (P<0.001, when considered separately from HRG β data), and P-MEK expression was elevated by ~3000-fold with HRG β treatment, which was also found to be extremely significant (P<0.001). LCC9 cells were also subject to an increase in P-MEK with TGF α (~43-fold) (P<0.001, when considered separately from HRG β data). HRG β increased P-MEK expression by ~1500-fold in the LCC9 cell line (P<0.01). Figure 4.14B shows the differences in P-MEK expression between the MCF-7 and the resistant cell lines when subjected to TGF α treatment to be extremely significant (P<0.001). P-MEK levels were also significantly higher in the parental line in comparison to the resistant lines when treated with HRG β (P<0.05 and P<0.001 for LCC1 and LCC9 cells respectively). LCC1 P-MEK expression was also elevated significantly when compared to the LCC9 cell line (P<0.01).

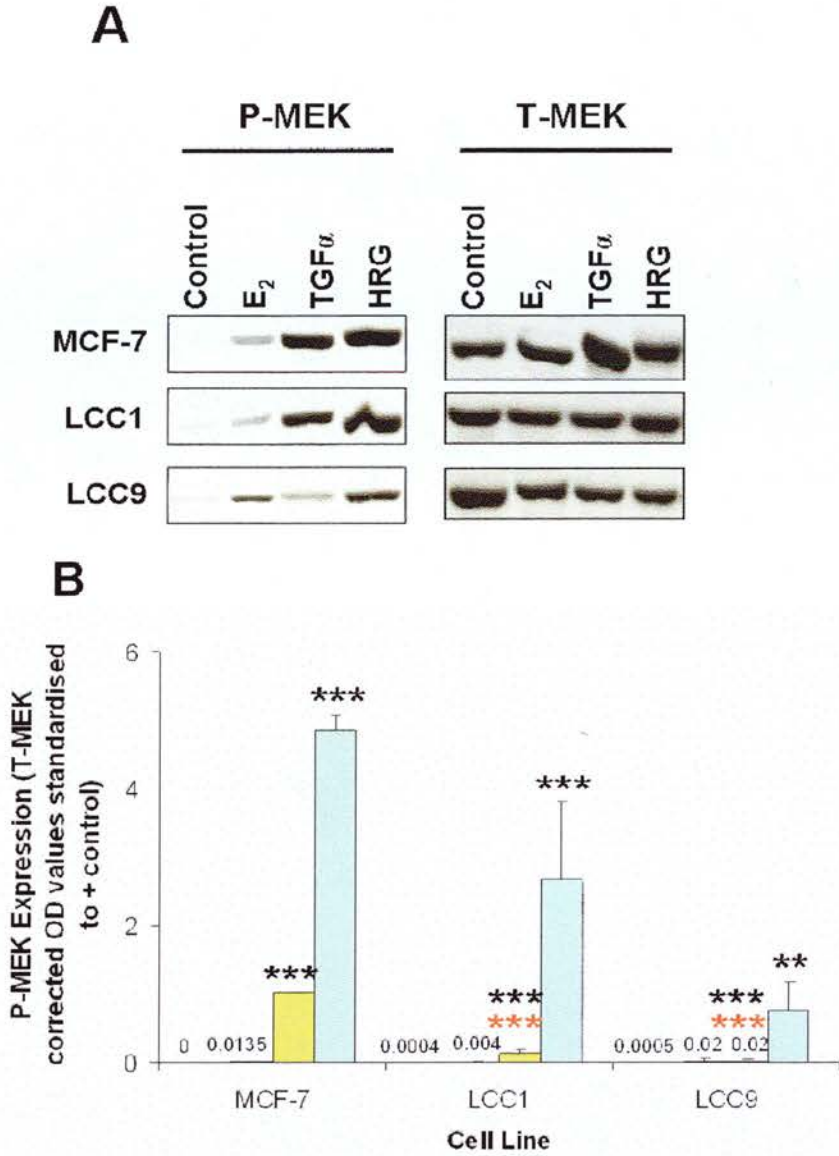


Figure 4. 14. Comparison of the levels of P-MEK and the effect of ligand stimulation on levels of P-MEK in resistant versus parental breast cancer cell lines. A, Western blot analysis of P-MEK levels, where MCF-7 cells were charcoal stripped for 48h before all cell lines were treated with control media, E₂ (1nM) or TGF α (1nM) for 15 min. Membranes were probed with P-MEK primary antibody (1:1000). T-MEK levels were used as loading controls. B, histogram of P-MEK levels of MCF-7, LCC1 and LCC9 cell lines treated with control media (■), E₂ (■), TGF α (■) or HRG (■) as before, where data are in triplicate +/-SD. MCF-7 cells treated with TGF α were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistical significance between control treated cells and another treatment and those in red represent statistical significance between MCF-7 and the resistant cell lines when treated with TGF α . Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

4. 3. 3. *P-ERKI/II*

Figure 4.15 shows the effects of 15 min E₂, TGF α and HRG β (all 1nM) treatment on the levels of P-ERKI/II in MCF-7, LCC1 and LCC9 cells. Total-ERK/II levels were used as loading controls. There were no significant differences between the basal levels of P-ERKI/II in any of the three cell lines ($P > 0.05$). E₂ did not increase P-ERKI/II in MCF-7 or LCC9 cell lines. There was a marginal increase in P-ERKI/II with E₂ in LCC1 cells (~1.4-fold), but this was not significant ($P > 0.05$).

TGF α activated P-ERKI and II significantly and equally in all three cell lines, with the production of bands at 44 and 42kDa respectively (figure 4.15A). Inductions of ~6, ~3.7 and ~6.5-fold were reported in MCF-7 ($P < 0.001$), LCC1 ($P < 0.05$) and LCC9 ($P < 0.001$) cell lines respectively with TGF α (figure 4.15B). HRG β also enhanced P-ERKI/II in all cells, with increases of ~6, ~4.5 and ~6.8-fold in MCF-7, LCC1 and LCC9 cells respectively (all $P < 0.001$). The histogram in figure 4.15B shows statistical analysis of data including HRG β treatment ($n = 3$).

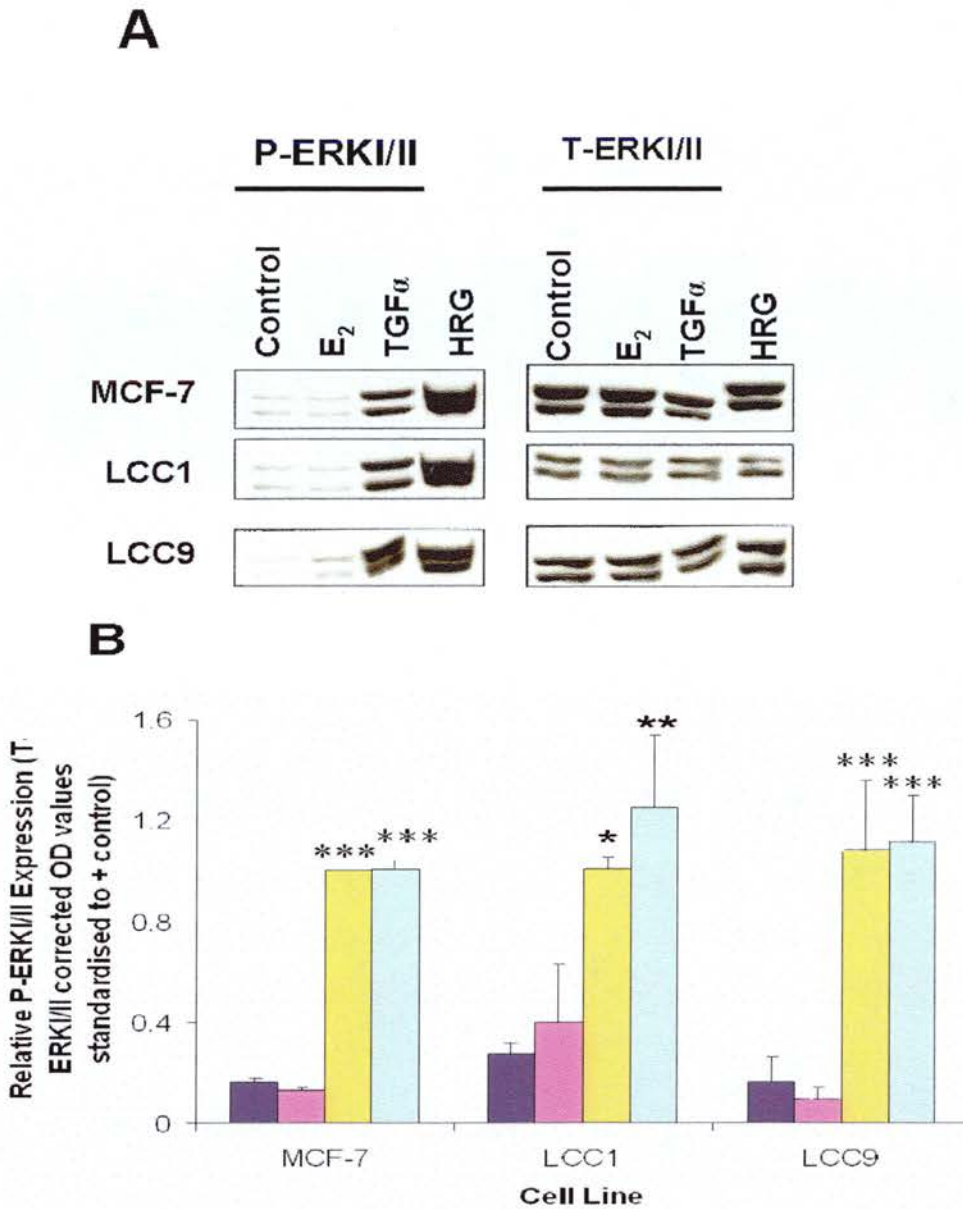


Figure 4. 15. Comparison of the levels of P-ERK1/II and the effect of ligand stimulation on levels of P-ERK1/II in resistant versus parental breast cancer cell lines. Western blot analysis of P-ERK1/II levels, where all cells were charcoal stripped for 48h before all cell lines were treated with control media, E₂ (1nM) or TGF α (1nM) for 15 min. Membranes were probed with P-ERK1/II primary antibody (1:1000). T-ERK1/II levels were used as loading controls. B, histogram of P-ERK1/II levels of MCF-7, LCC1 and LCC9 cell lines treated with control media (■), E₂ (■), TGF α (■) or HRG (■) as before, where data are in triplicate +/-SD. MCF-7 cells treated with TGF α were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistical significance between control treated cells and another treatment. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

4. 4. Does signalling differ in other MCF-7-derived resistant cells?

In addition to the LCC1 and LCC9 resistant cell lines, the expression and activation of Akt, MEK and ERK1/II were assessed in the LY2 cell line. LY2 cells are resistant to tamoxifen and LY 117018, but were derived separately to the LCC- cell lines. The LY2 cell line was investigated to ascertain if P-Akt was constitutively activated in this cell line also and perhaps indicate other changes which would account for differential growth responses between the LCC- and LY2 cell lines.

Figure 4.16 is a western blot of P-Akt expression in LY2 cells run in parallel with MCF-7, LCC1 and LCC9 cell lines. The cells were treated with TGF α and HRG β as these growth factors were previously shown to enhance MCF-7, LCC1 and LCC9 cells earlier in this chapter. Figure 4.16 shows that LY2 cells did indeed possess constitutively activated Akt under control conditions, which appeared to be to a similar level to that of LCC1 and LCC9 cell lines. TGF α marginally increased P-Akt expression in the LY2 cell line and was enhanced markedly by HRG β . These data are similar to the pattern already observed in the LCC- cell lines. The reduced level of T-Akt, and thus loading, in LCC9 cells treated with TGF α may account for a slight reduction in signal in comparison to data already recorded.

Figure 4.17 shows the effect of growth factors on P-MEK expression in the same panel of cell lines. The growth factors enhanced P-MEK in MCF-7 and LCC- cell lines as before, where HRG β produced a greater intensity of signal which diminished as resistance progressed. That is, P-MEK expression was less in the LCC1 cell line compared to the MCF-7 cell line, and reduced again in the LCC9 cell line compared to the LCC1 cell line when treated with either growth factor. HRG β enhanced P-Akt to a similar extent in the LY2 cell line as the LCC9 cell line. However, no discernable level of P-Akt was detected post TGF α treatment.

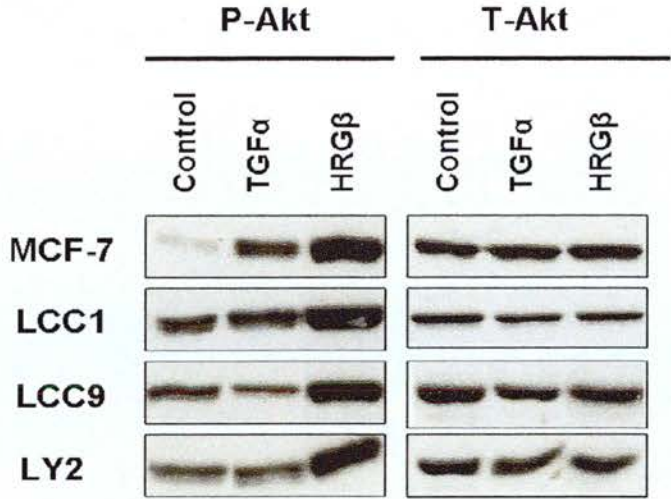


Figure 4.16. The effect of growth factors on P-Akt expression in the LY2 breast cancer cell line compared to the MCF-7 and LCC- cell lines. Western blot analysis of P-Akt levels, where all cells were charcoal stripped for 48h before all cell lines were treated with control media, TGFα or HRGβ (both 1nM) for 15 min. Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

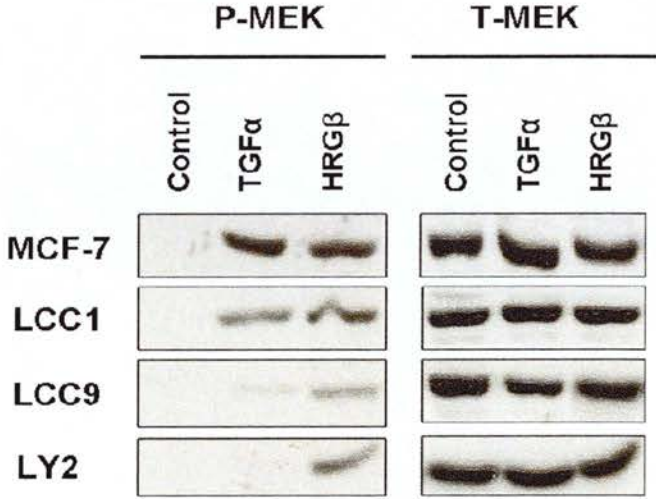


Figure 4.17. The effect of growth factors on P-MEK expression in the LY2 breast cancer cell line compared to the MCF-7 and LCC- cell lines. Western blot analysis of P-MEK levels, where all cells were charcoal stripped for 48h before all cell lines were treated with control media, TGFα or HRGβ (both 1nM) for 15 min. Membranes were probed with P-MEK primary antibody (1:1000). T-MEK levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

The effect of HRG β on P-ERKI/II expression in the LY2 cell line compared to the MCF-7 and LCC- cell lines is shown in figure 4.18. TGF α failed quality control tests performed by the manufacturer/supplier hence this ligand could not be used in this experiment. Figure 4.18 illustrates the expression of P-ERKI/II was elevated by HRG β in LY2 cells to a similar extent as in MCF-7, LCC1 and LCC9 cells, which is consistent with data produced earlier in this chapter.

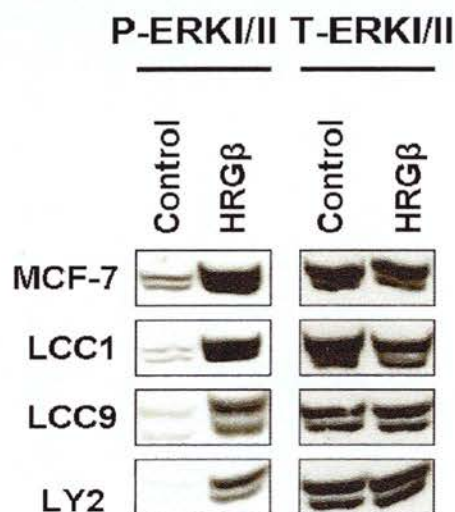


Figure 4.18. The effect of HRG β on P-ERKI/II expression in the LY2 breast cancer cell line compared to the MCF-7 and LCC- cell lines. Western blot analysis of P-ERKI/II levels, where all cells were charcoal stripped for 48h before all cell lines were treated with control media or HRG β (1nM) for 15 min. Membranes were probed with P-ERKI/II primary antibody (1:1000). T-ERKI/II levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

4. 5. The effect of serum-free conditions

The effects of E₂, TGF α and HRG β on P-Akt and P-ERKI/II expression were observed under serum-free conditions. This was to replicate the conditions of similar studies by Stoica *et al* (2003) to observe if the absence of E₂ effects on P-Akt and the absence of constitutively activated ERKI/II was due to experimental conditions or was a true differential response. Figure 4.19 shows that P-Akt expression under serum-free conditions did not differ to that of expression levels of cells treated in 5%

double charcoal stripped serum (DCSS). P-ERKI/II expression in the resistant cells under serum-free conditions was consistent with that produced under DCSS conditions. In contrast, MCF-7 P-ERKI/II expression did show some variation, but only when treated with E₂, where an elevated level of P-ERKI/II was observed compared to that of basal expression. The level of expression induced was not greater than that of either TGF α or HRG β .

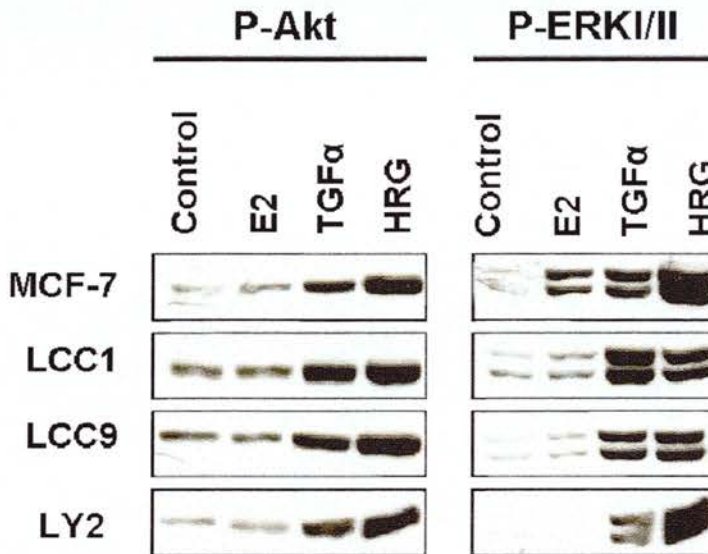


Figure 4. 19 Comparison of the levels of P-Akt and P-ERKI/II and the effect of various ligand stimulation on these levels in serum-free conditions in resistant versus parental breast cancer cell lines. Western blot analysis of P-Akt and P-ERKI/II levels, where all cells were charcoal stripped for 48h before all cell lines were treated with control media, E₂, TGF α or HRG β (all 1nM) for 15 min. Membranes were probed with either P-Akt or P-ERKI/II primary antibody (1:1000). Western blots are representative of 1 of 3 independent experiments.

4. 6. Discussion

This chapter investigated the role of ER α expression and activation in the development of resistance in breast and ovarian cancer. ER α has been well documented to play a critical role in the normal mammary gland development and also the development and progression of breast and ovarian cancer (Platet *et al.*, 2004; Rao and Miller, 2006). Overexpression and constitutive activation of ER has been implicated in tamoxifen-resistance (Britton *et al.*, 2005).

4. 6. 1. Breast cancer ER α expression and activation

4. 6. 1. 1. Loss of signalling via ER α is unlikely to confer tamoxifen resistance in endocrine resistant cell lines

The LCC1 and LCC9 cell lines were originally derived from MCF-7 cells through exposure to low levels of E₂ and anti-oestrogens (Brunner *et al.*, 1993). The MIII and LCC1 cell lines and the similar LCC2 cells expressed slightly elevated levels of ER α protein compared to the parental cell line. This is supported by published data where long-term oestrogen-deprived (LTED) cells were shown to have higher ER α levels than the wild type MCF-7 cells (Martin *et al.*, 2005; Staka *et al.*, 2005). The elevated ER α expression may explain the elevated basal rate of cellular proliferation in the MIII, LCC1 and LCC2 cell lines. However, the LCC9 cells were also developed in this manner but the level of ER α remained similar to that of the parental line. This may be explained by the further exposure of the LCC9 cell line to low levels of oestrogens and anti-oestrogens. The LY2 cell line also expressed a relatively unaltered level of ER α compared to MCF-7 cells, which may also be explained by the different manner in which this cell line was derived.

The down-regulation of ER α expression by E₂ has been previously reported by Lee *et al.* (2005), Hurd *et al.* (1995) and Saceda *et al.* (1988), and hence supports the reduction in ER α levels in MCF-7 cells with E₂ (1nM) treatment. Lee *et al.* (2005) reported that the reduction in ER α was also time dependent which is in agreement with the time course data provided here. The reduction in ER α levels in the resistant

lines after E₂ treatment is supported by these previous works which suggest E₂ is perhaps binding to and degrading ER α , and further suggests ER α are remaining functional in tamoxifen resistant cell lines. Tamoxifen-resistant tumours and breast cancer cell lines have repeatedly been reported to remain responsive to pure antioestrogens and hormone therapies (Lykkesfeldt *et al*, 1994; Brunner *et al*, 1993; Encarnacion *et al*, 1993) where it was concluded that it is unlikely that loss of signalling via ER α confers tamoxifen resistance (Nabha *et al*, 2005). However, this theory may only be applicable to resistant cell lines that retain sensitivity to pure antioestrogens, such as the LCC1 and LCC2 lines, and not the LCC9 cell line as LCC9 cells are insensitive to pure antioestrogens.

4. 6. 1. 2. 1. Changes in ER α P-S118 expression may contribute to the progression of resistance and insensitivity from the parental MCF-7 cell line to and between the resistant cell lines.

Basal levels of P-S118 MCF-7 were low as expected (Joel *et al*, 1995). P-S118 was hypothesised to be constitutively phosphorylated in the resistant cell lines of this model. This was found not to be the case and contrasts with other studies which have reported elevated basal expression of P-S118 in tamoxifen-resistant MCF-7 cell lines (Tam-R) (Britton *et al*, 2005). Britton *et al* (2005) routinely cultured the MCF-7 cell line in 5% DCSS medium rather than 5% FCS medium used here. 5% DCSS medium was utilised in the MCF-7 cell line only for experiments in this investigation. The reduction in serum may trigger the Tam-R cell line to use an alternative signalling pathway which is not utilised or available to the MCF-7 cells and may account for the elevation in ER α and subsequently P-S118 and P-ERK1/II levels.

E₂ enhanced P-S118 in MCF-7 cells, in agreement with many published studies (Joel *et al*, 1995) and reviewed by Lannigan (2003). The time course data showed rapid enhancement of P-S118 upon E₂ treatment after approximately 30 min in MCF-7 cells which is consistent with a study by Joel *et al* (1998) and time courses by Chen

et al (2002) and Martin *et al* (2005). Chen *et al* (2002) produced a time course which was consistent to that reported in this chapter, where P-S118 also peaked at 30 min and was reduced after 180 min. E_2 also enhanced P-S118 in MIII cells, indicating this cell line is activated by this ligand. This is consistent with growth data, where E_2 increased proliferation. However, P-S118 was enhanced to a similar extent in MIII compared to the parental cell line, indicating the overexpression of $ER\alpha$ did not correspond with elevated P-S118 expression in this cell line.

E_2 enhanced P-S118 in resistant LCC1 and LCC2 cell lines to a greater extent than MCF-7 cells and MIII cells. These data are consistent with the elevated levels of $ER\alpha$ in these cell lines, which may account for this elevated P-S118, and are also consistent with the growth data. This is also consistent with the study of LTED MCF-7 cells by Martin *et al* (2005), which reported their tamoxifen-resistance LTED cells overexpressed P-S118 compared to MCF-7 cells in line which also had elevated $ER\alpha$ expression. The ratio of T-ER to P-S118 supports this hypothesis, with the LCC1 cell line having a larger proportion of T-ER than the parental cell line. The hypothesis may also explain the reduction of P-S118 as T-ER levels also decline over time. The LCC9 cell line also had a higher ratio of T-ER: P-S118 than the MCF-7 cell line, although only after 5 min rather than 30 min, suggesting the level of T-ER changes over time.

The LY2 cell line differed to MIII, LCC1 and LCC2 cell lines as the $ER\alpha$ expression was not elevated and yet P-S118 was enhanced by E_2 and this was not reduced by tamoxifen. The level of P-S118 expression was similar to that of MCF-7 cells, in keeping with the similar $ER\alpha$ expression observed in the two cell lines. However, LY2 cells are generally E_2 -growth insensitive, hence, as for the LCC2 cell line, activation of $ER\alpha$ at S118 may be redundant downstream.

Growth factors have been shown to enhance P-S118 in MCF-7 cells (Kato *et al*, 1995, 1998) which reflected the $TGF\alpha$ -enhanced P-S118 observed in MCF-7 cells. The time course showing a sharp peak with $TGF\alpha$ at 30 min closely reflected that produced by Chen *et al* (2002). Growth factor stimulation was not observed at S118 in the resistant cell lines after 30 min, but was seen at a later time point in the LCC1

cells, but this was diminished compared to E_2 -enhanced P-S118. The absence of growth factor stimulation in the resistant cells is in discord with the expected elevated P-S118 levels which occur in other tamoxifen-resistant cells (Britton *et al*, 2005). The absence or reduced levels of P-S118 suggest the cells in this model are behaving in a manner consistent with a study by Fowler *et al* (2004). Fowler *et al* (2004) found $ER\alpha$ levels were elevated and this was thought to result in the activation of receptor transcriptional function via non-classical pathways which may not require ligand binding or growth factor phosphorylation. This supports the general absence of growth factor stimulated cellular proliferation reported in the resistant cell lines in this investigation. Fowler *et al* (2004) demonstrated this mechanism using a tetracycline-inducible $ER\alpha$ expression model of the MCF-7 cell line. Fowler *et al* (2004) reported that increased $ER\alpha$ activity was via AF-1 and was independent of P-S118. The elevated levels of $ER\alpha$ observed in the MIII, LCC1 and LCC2 cell lines (and perhaps a slight increase in the LCC9 cell line) are consistent with this study and also with the heightened levels reported by Staka *et al* (2005). However, Fowler *et al* (2004) also suggested this mechanism was independent of P-S118 which may partially explain the absence of P-S118 observed in the LCC9 cell line, but does not account for the elevated levels observed in the other three resistant cell lines upon ligand stimulation. In addition, Fowler *et al* (2004) demonstrated that P-ERK1/II levels were growth factor activated and not enhanced by oestrogen but were not elevated with elevated $ER\alpha$ expression. The group therefore concluded that the elevated transcriptional activity of $ER\alpha$ in the absence of ligand did not involve the MEK/ERK pathway. This is in agreement with results obtained in the LCC- and LY2 cell lines.

Tamoxifen slightly enhanced P-S118 in MCF-7 which is in agreement with the partial agonist action of the SERM. Tamoxifen also enhanced P-S118 in MIII, LCC1 and LCC2 cell lines. The increases in P-S118 expression were expected in the tamoxifen-sensitive MCF-7 cell line and perhaps even in the MIII and LCC1 cell lines which do retain some tamoxifen sensitivity. These results are consistent with a studies performed by Chen *et al* (2002) and Ali *et al* (1993) where tamoxifen was shown to increase P-S118. However, the increase in P-S118 in LCC2 cells was

surprising as LCC2 cells are growth insensitive to both oestrogen and tamoxifen. Tamoxifen significantly reduced E₂-enhanced P-S118 in MCF-7 and MIII cell lines, in accordance with the anti-oestrogenic properties of this agent (Bunone *et al*, 1996). E₂-enhanced P-S118 was only reduced marginally in LCC1 and LCC2 cells lines. The LCC1 data are consistent with the growth data. These data are consistent with the theory that tamoxifen is acting via both AF-1 to enhance P-S118 and via AF-2 to inhibit P-S118 and proliferation (Chen *et al*, 2002). Also, the reduction in the E₂-enhanced reversal by tamoxifen in the LCC1 and LCC2 cell lines is consistent with a recent study by Rayala *et al* (2006), although this study assessed the affect of tamoxifen resistance on ERE Luc activity rather than directly by P-S118 western blotting. Data from the Rayala *et al* (2006) study showed tamoxifen was unable to significantly reverse the E₂-enhanced ERE Luc activity in Tam-R cells, in contrast to MCF-7 cells where tamoxifen significantly reduced this activity.

The most interesting P-S118 expression pattern was produced in the LCC9 cell line. The cells expressed comparatively low levels of P-S118 which remained relatively unchanged irrespective of treatment. The absence of ligand stimulation shows the LCC9 cells differ to the remaining resistant cell lines and that ER α P-S118 may not be significant in the proliferation of this cell line.

It appears that ER α P-S118 is involved in the progression of resistance in MIII, LCC1, LCC2 and LY2 cell lines, but may be of less importance in the LCC9 cell line as indicated by a reduction or absence of P-S118 with ligand stimulation.

4. 6. 1. 2. 2. Changes in ER α P-S167 expression do not appear to contribute to the development of resistance

ER α S167 expression, as for S118, was also hypothesised to be constitutively phosphorylated in the resistant cell lines as activation of S167 has been linked with tamoxifen-resistance in breast cancer (Glaros *et al*, 2006; Campbell *et al*, 2001). Again, this was not found to occur, with basal levels remaining low in MCF-7, LCC1 and LCC9 cell lines alike. P-S167 was elevated with growth factor treatment in all cell lines consistent with published data (phosphorylation reviewed by Lannigan,

2003; Joel *et al*, 1998). This suggests phosphorylation of S167 may play a role in the activation of ER α in resistant cell lines, which has already been documented in MCF-7 cells, but this may not be directly associated with resistant cellular proliferation.

4. 6. 2. Ovarian cancer ER expression and activation

ER α expression was low in SKOV-3 cells compared to MCF-7 cells which is consistent with published data (Imai *et al*, 2005; Lau *et al*, 1999; Hau *et al*, 1995). Neither PEO1 nor SKOV-3 cells were constitutively phosphorylated at S167, suggesting these residues are not responsible for the elevated basal proliferation of the SKOV-3 cell line. P-S118 was not elevated in the PEO1 cell line either. However, there did appear to be some constitutive P-S118 under control conditions in the SKOV-3 cell line, suggesting this may account for the elevated basal rate of proliferation in this cell line. However, studies have shown that the SKOV-3 cell line expresses non-functional and E₂-unresponsive ER α (Imai *et al*, 2005); therefore the E₂-activation of ER α by phosphorylation of S118 appears to be irrelevant to the mechanism which drives proliferation of this cell line.

In contrast, the PEO1 ovarian cell line expressed a moderate level of ER α , consistent with published studies (Langdon *et al*, 1990), and expressed more ER α than SKOV-3. It was expected the increase in ER α in the PEO1 cell line would elevate P-S118 in response to E₂, especially as the cells proliferate upon E₂ addition, but surprisingly no activation at S118 or S167 was seen. These results imply ER α activation via S118 and S167 phosphorylation may not be crucial to PEO1 cellular proliferation. Due to the absence of any detectable P-S118 and P-S167 in the PEO1 cell line it was decided to limit further studies into the breast cancer cell model of resistance.

4. 6. 3. Changes in signalling molecule activation may contribute to the progression of resistance and insensitivity in the MCF-7 model

The signalling molecules Akt, ERK and MEK have all been previously implicated in breast cancer. Activation of Akt was reported by Sun *et al* (2001) to be present in ~40% of breast carcinomas and Navolanic *et al* (2003) reported MEK and ERK to be implicated in the development and progression of human breast cancer. A study by Campbell *et al* (2001) also reported Akt to play a role in the development of anti-estrogen resistance. The contribution of the MEK/ERK signalling pathway was investigated as this has a documented bifunctional role in proliferation and apoptosis (Cobb, 1999) and has also been implicated in the development and progression of breast cancer (Martin *et al*, 2005).

4. 6. 3. 1. Constitutive P-Akt expression in the resistant cell lines

Overexpression of the Akt signalling molecule has been implicated in breast cancer (Murray *et al*, 2005) and has been demonstrated to mediate tamoxifen resistance (Kirkegaard *et al*, 2005; deGraffenried *et al*, 2004). Activated Akt has a variety of biological effects, which include the suppression of apoptosis by phosphorylation and the inactivation of some members of the pro-apoptotic pathway, for instance caspase-9 (Cardone *et al*, 1998) or Bad, a member of the Bcl-2 family (Datta *et al*, 1997; del Peso *et al*, 1997). Therefore it was hypothesised that this upstream signalling molecule may be involved in tamoxifen-resistance observed in this model.

P-Akt was constitutively activated in LCC1, LCC9 and LY2 resistant cell lines. These data are consistent with published findings, where a tamoxifen-resistant MCF-7 cell line and other tamoxifen-resistant cells were found to have elevated basal levels of P-Akt compared to those of the parental MCF-7 cell line (Jordan *et al*, 2004; Frogne *et al*, 2004; Lin *et al*, 2005). A study by Campbell *et al* (2001) also supports this and showed that MCF-7 cells transfected with Akt were less sensitive to

tamoxifen. Jordan *et al* (2004) suggested that the PI3-kinase pathway played a role in the proliferation of their particular tamoxifen resistant cell lines, which will further be investigated in this model with the use of the inhibitor LY 294002 in the following chapter. Baccus *et al* (2002) also reported overexpression of the c-erbB2 oncogene correlated with overexpression of Akt2 and P-Akt. This may be a potential mechanism by which the cells in this model develop resistance.

Opinion is divided as to the effect of E₂ on Akt activation. A recent study by Rodrick *et al* (2005) found that E₂ did not enhance Akt in MCF-7 cells, which is in agreement with data reported in this chapter. Contrastingly, a previous study by Stoica *et al* (2003) described the rapid activation of Akt by E₂. This occurred after 10 min of treatment and used a similar design to that of this report, although their study was carried out in MCF-7 cells stably transfected with Akt rather than the LCC- model. Their experimental design also involved a 2-day incubation period in medium containing charcoal-stripped serum before the medium was changed into serum-free medium. The absence of this additional step may account for the corresponding absence of E₂ stimulation of P-Akt in this instance. A further study by Lee *et al* (2005) reported that E₂ only enhanced P-Akt after 24h, 48h and 72h, which are later time points indicating that the effects of E₂ on P-Akt are not as rapid as those suggested by Stoica *et al* (2003) which is consistent with data accrued.

In contrast to studies reporting elevated P-Akt expression and the data found here, Santen *et al* (2005) did not report an elevated level of P-Akt in their LTED cell line. The discrepancy between this finding and this published data may be due to the variations in the cell lines due to the manner in which they were derived, and the experimental conditions used.

Growth factors enhanced P-Akt in MCF-7 cells in this investigation, supported by Rodrick *et al* (2005), Jordan *et al* (2004) and Martin *et al* (2000). Jordan *et al* (2004) found P-Akt to be constitutively activated in Tam-R MCF-7 cells as previously mentioned, and the group also reported growth factor activation of this cell line. This concurs with P-Akt enhancement documented in the LCC- and LY2 cell lines.

However, Jordan *et al* (2004) noted that the Tam-R cells were enhanced to a greater extent than the parental MCF-7 cell line, which differs to the enhancement between the MCF-7 and LCC- and LY2 cell lines. The discrepancy is more than likely due to the differences between the cell lines being characterised. It is interesting to note that HRG β enhanced P-Akt to a greater extent than TGF α . This indicates the cell lines may preferentially express erbB heterodimers which have a higher HRG β binding affinity such as erbB2/3 or erbB2/4 rather than those with affinity for TGF α (erbB1/1 or erbB1/2 heterodimers). This may be in agreement with a theory proposed by Takai *et al* (2005) who hypothesised that ovarian cells may preferentially form certain heterodimers.

4. 6. 3. 2. P-MEK expression is altered with progression in resistance

P-MEK basal levels were relatively low and equal in MCF-7, LCC1 and LCC9 cell lines. This was also the case for E₂ treated cells. Basal levels of MEK activation were low in MCF-7 cells in agreement with studies by Martin *et al* (2003), although in contrast to this investigation, Martin *et al* (2003) reported elevated basal levels of P-MEK in their LTED cell line. This group also reported increased expression of ER α and P-ERK, the latter increase being inconsistent with data reported here. The discrepancies are more than likely due to the different models being tested as well as slight variations in experimental conditions where the addition of IGF-I appeared to 'super-sensitise' the cells to residual oestrogen and perhaps promote the tamoxifen-resistance (Staka *et al*, 2005).

Differences in P-MEK expression appeared upon treatment with growth factors, where expression fell as resistance occurred from MCF-7 to LCC1 cells and then progressed from the LCC1 to LCC9 cell line. This is consistent with the study by Glaros *et al* (2006), who reported that transfection of MCF-7 with MEK led to the maintenance of tamoxifen sensitivity. The study by Glaros *et al* (2006) also reported this increased P-S118, which would be consistent with data produced in the LCC9

cell line. The overexpression of Akt and oestrogen ER α activation via a non-genomic pathway may account for elevated P-S118 in the LCC1 cell line.

Moelling *et al* (2002) documented that Akt suppressed Raf kinase activity when activated by high concentrations of growth factor IGF-I, while lower doses still stimulated proliferation but do not repress Raf1 activity in the MCF-7 cell line. PMA, a ligand known to induce differentiation in this cell line, markedly activated the Raf/MEK/ERK pathway but only weakly stimulated the PI3-K/Akt pathway and did not induce crosstalk. This report would support the theory that the elevated levels of Akt in the LCC1, LCC9 and LY2 resistant cell lines suppressed MEK activation, via Akt-Raf crosstalk, to a greater extent than in the MCF-7 cell line. It also suggests that MEK activity is suppressed further in the LCC9 cell line than LCC1 cells.

4. 6. 3. 3. *ERK1/II activation is unaltered in this model of tamoxifen resistance*

Previous studies have suggested that P-ERK1/II is elevated in LTED and tamoxifen-resistant MCF-7 cells under control conditions (Martin *et al*, 2005; Santen *et al*, 2005; Yue *et al*, 2002; Britton *et al*, 2005; Knowlden *et al*, 2003). This is in disagreement with data here where basal P-ERK1/II levels were found to be low in parental and resistant cell lines alike. Martin *et al* (2005) reported the rapid P-S118 stimulated by E₂ was not associated with an elevation in P-ERK1/II expression. This supports the expression profile seen in all the breast cancer cells. Joel *et al* (1998) and Keshamouni *et al* (2002) both saw an increase in P-ERK expression with growth factors, but not with E₂ after 15 min which supports data here. Gaben *et al* (2004) and Keshamouni *et al* (2002) reported an increase in P-ERK1/II with E₂ only after a prolonged time period (approximately 6h and 2h treatment respectively), which is again consistent with the absence of signal observed at the 15 min time point in this investigation. TGF α and HRG β activated P-ERK1/II in MCF-7 cells in agreement with published data (Fowler *et al*, 2004; Thottassery *et al*, 2004). The growth factors

also enhanced P-ERK1/2 in all the resistant cell lines to a similar extent. A study by Britton *et al* (2005) supports growth factor activation of P-ERK1/2 in Tam-R cells.

Moreover, TGF α activated P-ERK1/2 significantly and equally in all three cell lines, suggesting the point of divergence did not occur at this signalling molecule and that P-ERK1/2 is not the point of origin for the development of resistance in this model. Any differences that were observed upstream of P-ERK1/2 at P-MEK were not observed here, indicating P-MEK differences are insignificant alone, 'cancelled out' or the ligand signals via another pathway such as the PI3-K/Akt or IGFR-I pathways. A study by Zimmerman and Moelling (1999) showed that growth factor Akt activation was adequate to surmount the growth inhibitory action of prolonged ERK activation in breast cancer cells. Campbell *et al* (2001) hypothesised that growth factors may activate ER α via the PI3-K/Akt pathway, thus conferring hormone-independent growth. This may be the rationale behind the elevated basal proliferation in the resistant cell lines.

This chapter shows that ER α , P-S118, P-Akt and P-MEK expression varies between MCF-7 cells and cell lines possessing a resistant phenotype. The impact of the PI3-K/Akt and MEK/ERK signalling pathways will be assessed further in the following chapter using specific tyrosine kinase inhibitors, while a novel anti-erbB2 inhibitor will be utilised to ascertain the importance of erbB2 in this model in Chapter 6.

Chapter 5

The roles of Akt and ERK signalling pathways in resistant breast cancer cell lines

5. 1. Do tyrosine kinase inhibitors block non-genomic signalling pathways, inhibit cell line proliferation and produce changes in expression profiles differentially in this model of resistance?

Breast cancer cells have been reported to proliferate in response to ligand stimuli via several pathways including the ligand dependent pathway, the IGFR-I pathway and this ligand independent pathway. Elements of 'cross-talk' between the former two pathways have widely been published (reviewed by Lannigan, 2003), whilst more recently, evidence has come to light of interactions between the latter two pathways (Dudek *et al*, 1997; Kauffmann-Evan *et al*, 1997; Gee *et al*, 2005). These pathways and interactions are fully discussed in chapter 1. This chapter will mainly focus on the ligand dependent and ligand independent erbB pathways.

The ligand dependent pathway requires the direct binding of E₂ to ER α , and the ligand-independent pathway requires the stimuli, for example by growth factors TGF α and HRG β , upstream of ER α . The ligand-independent pathway can signal via the erbB receptor family of tyrosine kinases. ErbB receptors play a pivotal role in the growth and differentiation of cells and irregular erbB activity and overexpression has been implicated in several human cancers including breast cancer (Slamon *et al*, 1987; Slamon *et al*, 1989; Blume-Jensen and Hunter, 2001). TGF α acts as a mitogen in most cells and initiates intracellular signalling pathways upon binding to erbB receptors (Derynck, 1988). It can stimulate erbB2 and the MEK/ERK and the PI3-K/AKT pathways (Datta *et al*, 1999) which potentially lead to the phosphorylation of serine residues 118 and/or 167 of ER α , followed by ER α activation and gene transcription. Growth factor signalling via the erbB receptors has been implicated in the development of acquired antioestrogen resistance (Nicholson *et al*, 2004).

Previous studies have reported that the involvement of components of the PI3-K/Akt and MEK/ERK signalling pathways can be determined by the use of specific inhibitors such as LY 294002 and UO126 (Marks *et al*, 2000; Stoica *et al*, 2003; Staka *et al*, 2005). The specificity of these agents for their targets provides a

powerful tool for the investigation of the physiological role of these signalling pathways. Several inhibitors were initially used to detect any differences in a range of targets, with further studies being continued with LY 294002 and UO126 (and U-73122 in expression profiling) only as Akt and MEK had previously been implicated in the development of antioestrogen resistance in tamoxifen resistant cell lines (Britton *et al*, 2005; Campbell *et al*, 2001).

To assess the potential role of Akt, MEK and ERK1/II in the development of resistance to anti-hormonal therapies these tyrosine kinases in the erbB receptor pathway, and other relevant pathways which may 'cross-talk' with this pathway, were targeted with a series of specific tyrosine kinase inhibitors (TKI) and these will be discussed individually. The inhibitors used were LY 294002, NL-71-101, U-71322 and SB 203580. Proliferation assays were performed to determine the effect of these tyrosine kinase inhibitors on cell proliferation. Protein expression levels of the molecules within the relevant signalling pathways were then examined to verify the specificity of the inhibitors. Any changes between the MCF-7 and resistant cell lines LCC1, LCC9 and LY2 were monitored. All four cell lines were subjected to a suitable concentration range of inhibitor determined from previous publications in the presence and absence of E₂, TGF α and HRG β (all 1nM). LY2 cell line was excluded from protein expression studies as this cell line possessed features similar to those of the other resistant lines. Protein expression studies were performed using three appropriate concentrations selected from the proliferation assay studies to explore any reduction produced in a dose-dependent manner. The selection of inhibitors was narrowed in order to monitor their downstream effects via the mRNA expression levels of several E₂-responsive genes in the presence and absence of E₂ and TGF α . This was to discover if the inhibition of a second messenger could be directly linked to specific gene expression as well as cell proliferation. The reasons for the selection of LY 294002, UO126 and U-73122 in this study will be discussed further later in the chapter.

5. 1. 1. The effect of LY294002 and NL-71-101 on elevated P-Akt in this model of resistance

5. 1. 1. 1. Proliferation Studies

5. 1. 1. 1. 1. LY 294002

LY 294002 is a competitive inhibitor for the ATP binding site of PI3-kinase and is specific for PI3-kinase even at a dose of 50 μ M (Vlahos *et al*, 1994). NL-71-101 is a specific Akt inhibitor (Reuveni *et al*, 2002) which operates just downstream of LY 294002. LY 294002, and NL-71-101 initially, were used to assess the importance of the elevated basal levels of Akt in the development of resistance in the LCC1, LCC9 and LY2 resistant cell lines compared to the parental MCF-7 cell line. Akt1, 2 and 3 were detected as the investigation used a pan-Akt antibody.

Under normal conditions, such as in the MCF-7 cell line, day 0 and day 3 control values are both approximately equal (100%). Any differences in inhibitor concentration required to inhibit cellular proliferation occurring between cell lines were noted as this may be indicative of altered signalling pathways in the development of resistance in this model. Figure 5.1 shows the effect of a concentration range of LY 294002 on cell number in the presence and absence of E₂, TGF α and HRG β (all 1nM) in resistant cell lines compared to the parental MCF-7 cell line. LY 294002 alone inhibited proliferation in all four cell lines, but was unable to reduce the cell number of the resistant cell lines to that of the MCF-7 cells. LY 294002 was able to significantly inhibit MCF-7 cell proliferation with the lowest concentration of 5 μ M (P<0.001). LY 294002 concentrations between 20 μ M and 70 μ M did not significantly reduce MCF-7 cell proliferation beyond that achieved with 20 μ M. LY 294002 significantly reversed the growth stimulation induced by E₂ and TGF α in MCF-7 cells in a similar manner. However, the ligand stimulated cell proliferation was not fully reversed to that of control level with 5 μ M LY 294002, and proliferation was only reduced to that of basal growth with concentrations of ~7 μ M, ~8 μ M and ~23 μ M for E₂, TGF α and HRG β treatments respectively. The elevated LY 294002 concentration required to inhibit HRG β -induced growth to the same

extent as the other treatments suggests HRG β treatment appears to prevent LY 294002 induced growth reversal in MCF-7 cells.

LY 294002 also reversed the growth proliferation induced by E₂ in LCC1 cells, with significant reductions occurring with every concentration used (5-70 μ M, P<0.001). LCC1 cells treated with 5 μ M LY 294002 alone were reduced from ~316% day 3 growth to ~183%. E₂, TGF α and HRG β elevated the concentration required to reduce proliferation to this level from 5 μ M to ~6 μ M, ~8 μ M and ~11 μ M respectively. LY 294002 reduced cell proliferation when cells were treated with TGF α and HRG β , but the growth factors appeared protective to the effect of LY 294002, as for the MCF-7 cell line, with HRG β preventing LY 294002 growth inhibition to the greatest extent. The fact that the growth factors still prevent LY 294002 induced growth inhibition in LCC1 cells compared to LY 294002 treatment alone is surprising as the resistant cell lines did not proliferate in response to the growth factors. The shape of the curves for all the cell lines were very similar, implying the cells are inhibited by LY 294002 in a similar manner.

LY 294002 in combination with E₂ required concentrations similar to those required to inhibit control growth to a similar extent, with values of ~9.5 μ M, ~43 μ M, ~69 μ M and ~55.5 μ M for MCF-7, LCC1, LCC9 and LY2 cell lines respectively to reduce growth to approximately 50% of day 0. Slightly elevated concentrations of ~18 μ M, ~70 μ M and ~68 μ M were recorded to inhibit proliferation to the same extent in MCF-7, LCC1 and LY2 cells respectively treated with LY 294002 and TGF α . A corresponding concentration was not obtained for LCC9 cells treated with LY 294002 and TGF α .

HRG β was reported to 'protect' all cell lines to a greater extent at 10 μ M, a therapeutically suitable concentration, than cells treated with E₂, TGF α or control conditions. There are statistically significant differences of ~60% with control and E₂ treatments, and ~52% with TGF α treatment compared to HRG β treatment in MCF-7 cells. This effect is mirrored in the LCC1 and LCC9 cell lines, with HRG β 'protecting' cells by ~17%, ~9% and ~6.5% with control, (P<0.001), E₂ (P<0.01) and

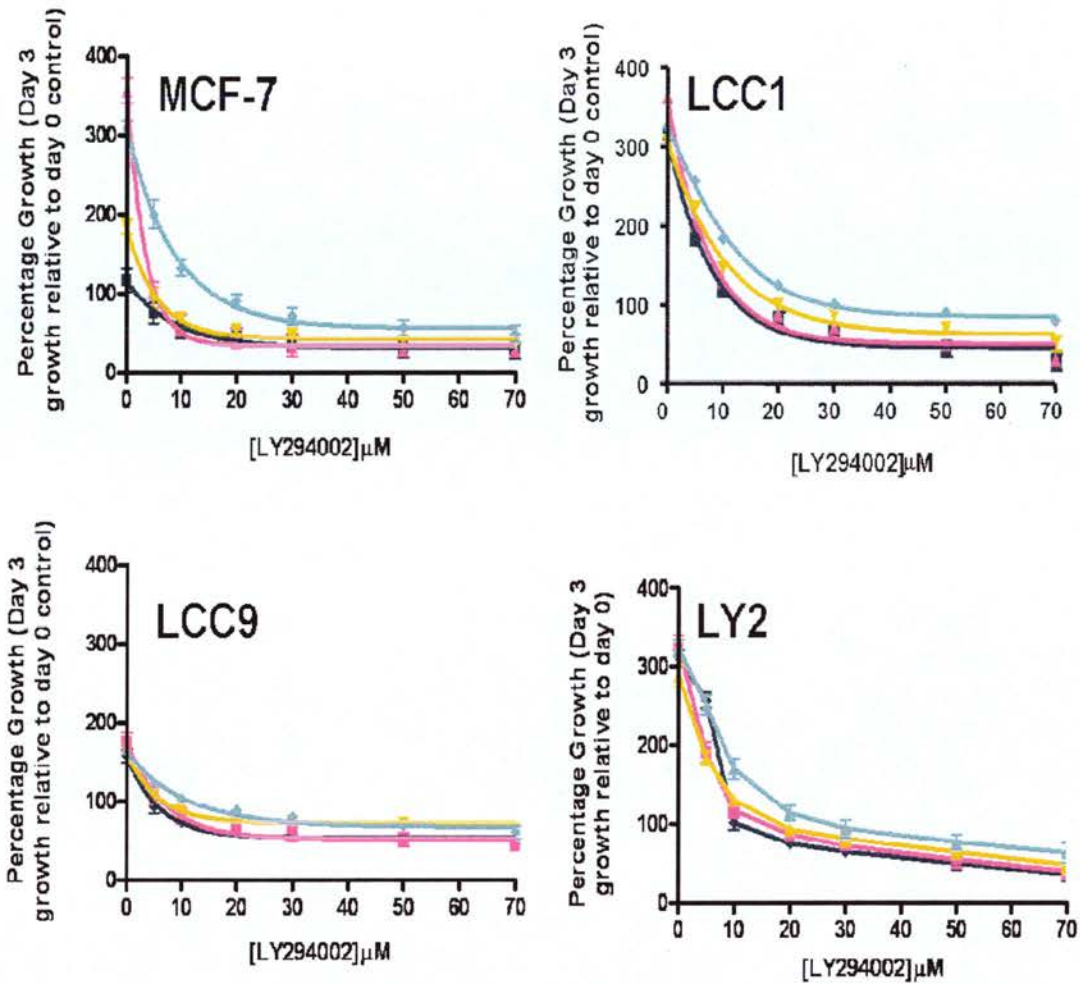


Figure 5.1 Growth inhibitory effect of LY 294002 in MCF-7 cells versus resistant breast cancer cell lines.. All cells were charcoal stripped 24h prior to treatment and were then treated with a concentration range of LY 294002 spanning 0-70 μ M plus control media (\blacklozenge), E_2 (1nM) (\blacksquare), TGF α (1nM) (\blacktriangle) or HRG β (1nM) (\blacktriangle). Cells were treated on day 0 and treatment halted on day 3. Data were plotted as a mean of 6 OD values \pm SD for treated values and as a mean of 12 OD values \pm SD for untreated cells. Graphs are representative of 1 of 3 replicate experiments.

TGF α treatments respectively in the LCC1 cell line, although this difference was not statistically significant in the latter treatment. In the LCC9 cell line HRG β ‘protected’ cells by ~18% ($P < 0.001$), ~14.5% ($P < 0.001$) and ~9% ($P < 0.01$) compared to cells subject to control, E_2 , and TGF α treatment. 70 μ M LY 294002 reduced day 3 growth in the resistant cell lines treated with HRG β to ~79%, ~60% and ~63% in LCC1, LCC9 and LY2 cells respectively, in keeping with the growth factor having a ‘protective’ effect in the resistant cell lines as well as in the MCF-7 cell line.

All ligands increased proliferation in MCF-7 cells as previously described (chapter 3), E₂, TGF α and HRG β treatment increasing proliferation by ~240%, ~68% and ~192% respectively (all P<0.001). HRG β increased MCF-7 cell number to a greater extent in this chapter compared to the increase noted in chapter 3. The discrepancy is probably due to the differences in experimental protocol between growth curves produced via 24-well plates with washes (chapter 3) versus the SRB technique where cells were grown in 96-well plates without media changes or certain wash steps. Cells grown in 24-well plates were observed to be less adherent once treated with HRG β , in agreement with published studies indicating HRG β to be involved in the invasion and metastases of breast cancer (Tsai *et al*, 2003). This effect was abolished in the SRB technique as cells were fixed to the plate surface post-treatment. This increase A higher concentration of LY 294002 was required to inhibit day 3 growth back to 50% when cells were treated with HRG β (69 μ M) in comparison to E₂ which produced approximately the same increase in proliferation but required only ~9.5 μ M to produce the same proliferation inhibition. TGF α and control treatments only required concentrations of ~18 μ M and ~11 μ M respectively to reduce proliferation to the same degree.

TGF α did not increase cell number in LCC1 cells, while E₂ and HRG β increased cell number by ~47% (P<0.001) and ~9% (P>0.05, not significant) respectively. 5 μ M LY 294002 significantly reduced LCC1 cell proliferation in the presence of E₂, TGF α and HRG β by ~161%, ~83% and ~109% respectively and by ~133% in untreated cells. The maximum concentration of 70 μ M reduced growth to ~51% and ~79% day 3 growth compared to day 0 with TGF α and HRG β treatment respectively in the LCC1 cell line.

Figure 5.1 shows LY 294002 also inhibited growth in LY2 cells irrespective of ligand treatment, with the exception of HRG β as before, while both growth factors increased the concentration of LY 294002 required to achieve a similar level of inhibition as control and E₂ treatments in LCC9 cells. 70 μ M LY 294002 reduced growth to only ~70% when LCC9 cells were treated with either growth factor, compared to ~43% in cells treated with control media or E₂ with the same LY 294002 concentration. Neither LCC9 nor LY2 cells were significantly growth stimulated by any ligand in agreement with previous data (chapter 3). LY 294002 concentrations of ~47 μ M,

~55.5 μ M and ~68 μ M were recorded for inhibition to 50% of day 0 proliferation in LY2 cells when untreated and when treated with E₂ and TGF α respectively. In contrast, LY 294002 was unable to produce 50% inhibition of LY2 cells relative to day 0 incubated with HRG β .

5. 1. 1. 1. 2. NL-71-101

The inhibitory action of NL-71-101 on the Akt signalling pathway was investigated using the SRB assay to study the effect of increasing NL-71-101 concentration on cellular proliferation. NL-71-101 studies were performed in the MCF-7, LCC1 and LCC9 cell lines only. All cell lines used were charcoal stripped 48h prior to treatment and were then treated with a concentration range of NL-71-101 spanning 0-20µM plus control media, E₂ (1nM), TGFα (1nM) or HRGβ (1nM). Cells were treated on day 0 and treatment halted on day 3. Data were plotted as a mean of 6 OD values +/- SD for treated values and as a mean of 12 OD values +/- SD for untreated cells. All three cell lines responded to E₂, TGFα and HRGβ treatment as previously described (chapter 3), where MCF-7 proliferated in response to all three ligands, while LCC1 cells only increased in cell number with E₂ treatment. LCC9 cell proliferation was not significantly altered compared to that of control treated cells irrespective of ligand treatment.

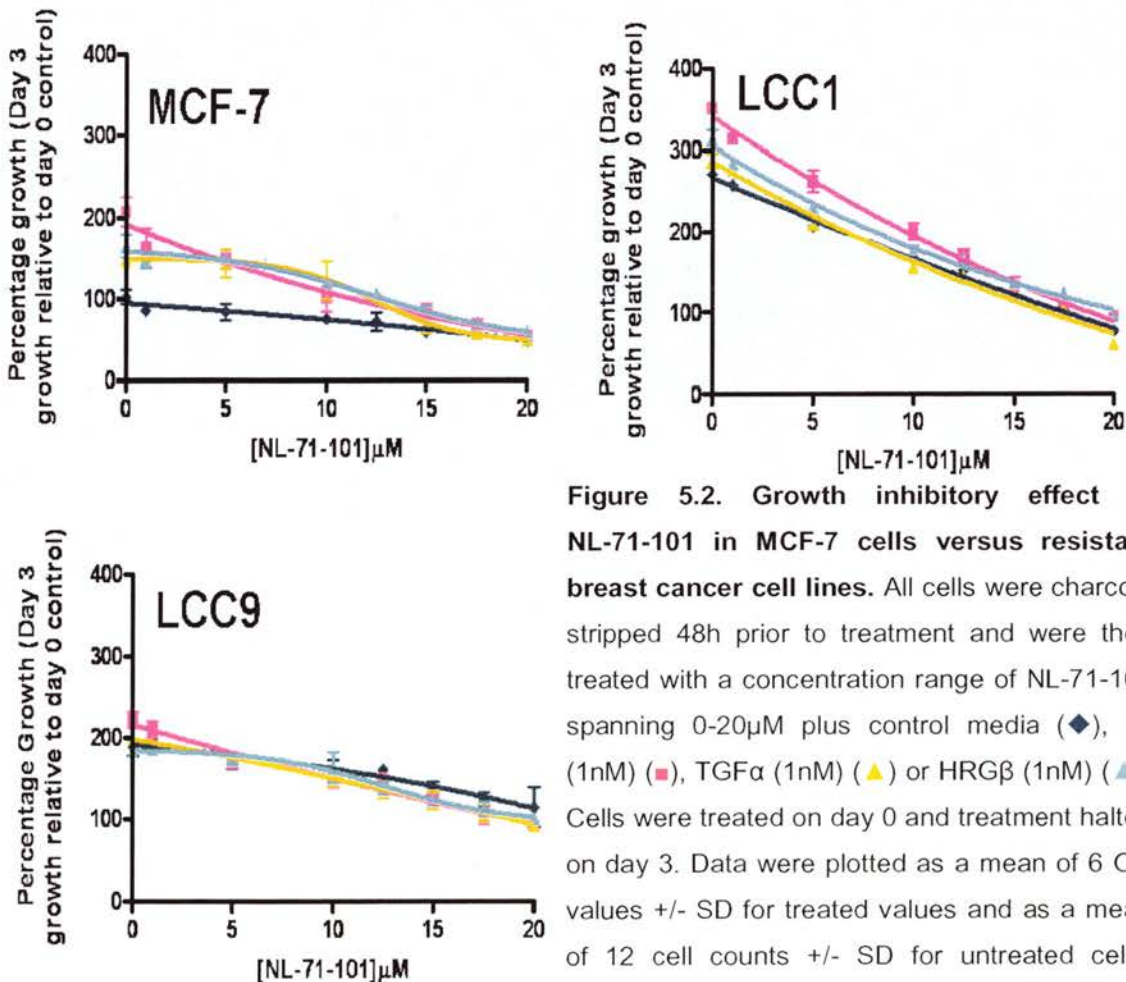


Figure 5.2. Growth inhibitory effect of NL-71-101 in MCF-7 cells versus resistant breast cancer cell lines. All cells were charcoal stripped 48h prior to treatment and were then treated with a concentration range of NL-71-101 spanning 0-20µM plus control media (◆), E₂ (1nM) (■), TGFα (1nM) (▲) or HRGβ (1nM) (△). Cells were treated on day 0 and treatment halted on day 3. Data were plotted as a mean of 6 OD values +/- SD for treated values and as a mean of 12 cell counts +/- SD for untreated cells. Graphs are representative of 1 of 3 replicate experiments.

NL-71-101 inhibited day 3 proliferation relative to day 0 control in MCF-7, LCC1 and LCC9 cell lines (figure 5.2). The maximum concentration of $\sim 20\mu\text{M}$ NL-71-101 reduced proliferation to approximately 50% of day 0 in the parental cell line only under control conditions and TGF α treatment. The maximum concentration of $20\mu\text{M}$ reduced day 3 growth relative to day 0 control growth of MCF-7 cells in the presence of E₂ and HRG β to $\sim 53\%$ and $\sim 59\%$ respectively. NL-71-101 reduced proliferation to $\sim 76\%$, $\sim 94\%$, $\sim 61\%$ and $\sim 96\%$ in LCC1 cells treated with NL-71-101 alone and in combination with E₂, TGF α and HRG β respectively. LCC9 cell proliferation was also decreased with NL-71-101 alone and in combination with E₂, TGF α and HRG β to $\sim 113.5\%$, $\sim 90\%$, $\sim 92.5\%$ and $\sim 101\%$ respectively.

In general, NL-71-101 inhibited proliferation according to the ligand stimulation, with E₂, TGF α and HRG β all elevating the concentration required to inhibit growth by the same extent as NL-71-101 alone in MCF-7 cells. Only E₂ elevated this concentration in the LCC1 cells, although this was not statistically significant, while the concentrations were approximately equal irrespective of treatment in the LCC9 cell line in accordance with the resistant cell line growth responses.

Relatively few studies have been performed with NL-71-101, hence due to the novel nature of NL-71-101 and the incomplete pharmacokinetic profile of this inhibitor it was decided to continue further studies with LY 294002 only. The novel aspect of NL-71-101, and the lack of evidence that there was a profound difference in the sensitivity of the inhibitor between the sensitive and resistant cell lines would demand substantial additional work to see if the data were informative.

5. 1. 1. 2. The effect of LY 294002 on P-Akt protein levels enhanced by TGF α

Figure 5.3 shows the inhibitory effect of LY 294002 in the presence and absence of TGF α on the Akt signalling pathway in MCF-7 cells versus resistant breast cancer cell lines. Figure 5.3A shows a representational western blot of P-Akt levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1 μ M, 5 μ M or 30 μ M LY 294002 for 30 min then the cells were treated with control media or TGF α (1nM) for 15 min.

Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls. Figure 5.3B is a histogram of P-Akt OD values of all cell lines treated with control media, 5 μ M LY 294002, TGF α (1nM) or TGF α + 5 μ M LY 294002 (1nM + 5 μ M), where data are in triplicate +/-SD. MCF-7 cells treated with TGF α were used as the positive control. LCC1 and LCC9 cell lines were significantly constitutively phosphorylated at the Akt position ($P < 0.05$) compared to the parental MCF-7 cell line as previously described in chapter 4. TGF α significantly enhanced P-Akt in all three cell lines ($P < 0.001$). The basal levels of P-Akt in MCF-7 cells treated with increasing concentrations of LY 294002 alone remained constant, while these levels were reduced with increasing concentrations in both resistant cell lines ($P < 0.05$ with 5 μ M concentration, figure 5.3B).

The western blot in figure 5.3A shows that TGF α -enhanced P-Akt levels were reduced with increasing LY 294002 concentrations in all three cell lines, with the greatest reduction taking place with 30 μ M LY 294002. 1 μ M LY 294002 noticeably reduced P-Akt levels increased by TGF α treatment in MCF-7 and LCC1 cell lines, but less so in LCC9 cells. The clearest reductions in bands were visible at 5 μ M and reductions were further investigated at this concentration (figure 5.3B).

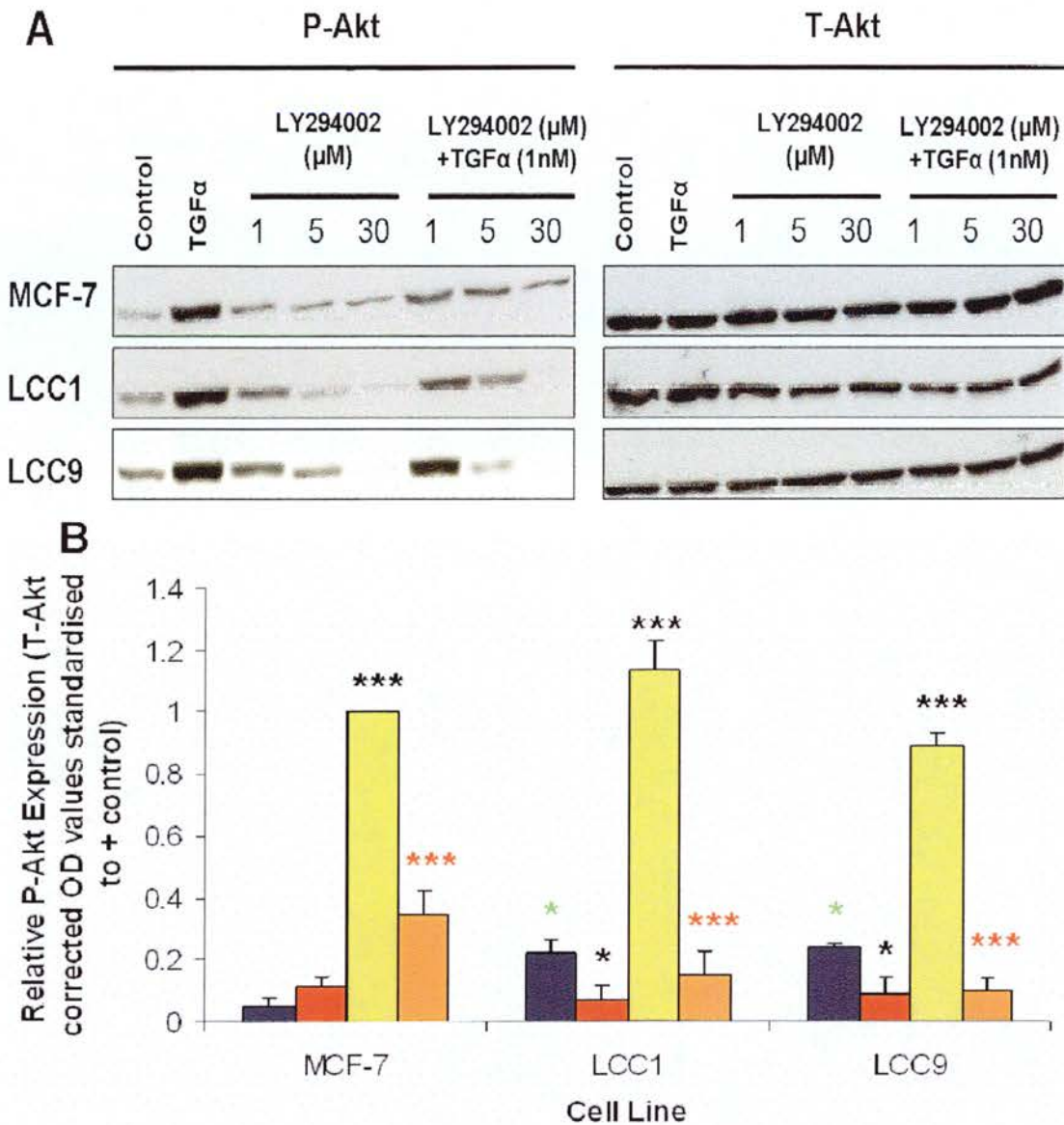


Figure 5.3 Inhibitory effect of LY 294002 in the presence and absence of TGFα on the Akt signalling pathway in MCF-7 cells versus resistant breast cancer cell lines. A, Western blot of P-Akt levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1μM, 5μM or 30μM LY 294002 for 30 min then the cells were treated with control media or TGFα (1nM) for 15 min. Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls. B, histogram of P-Akt OD values of all cell lines treated with control media (■), 5μM LY 294002 (■), TGFα (■) or TGFα+ 5μM LY 294002 (■), where data are in triplicate +/-SD. MCF-7 cells treated with TGFα were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment, those in green represent statistical significance between control treated MCF-7 and resistant cell lines and those in red represent any statistical significant changes between TGFα pre and post LY 294002. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

Figure 5.3B shows 5 μ M LY 294002 reduced constitutively phosphorylated Akt in the resistant cell lines as described above ($P<0.05$), with MCF-7 low basal levels remaining unaffected by any concentration of LY 294002 alone. 5 μ M LY 294002 significantly reduced P-Akt enhanced by TGF α in all three cell lines ($P<0.001$), with the greater reductions occurring in the resistant cell lines (~7.5 and ~8.7-fold reductions in LCC1 and LCC9 cell lines compared to ~3-fold reduction in MCF-7 cells).

5. 1. 1. 3. The effect of LY 294002 on the MEK/ERK/II pathway

5. 1. 1. 3. 1. The effect of LY 294002 on P-MEK protein levels enhanced by TGF α

The effect of LY 294002 on the MEK/ERK/II was subsequently investigated in order to ascertain consequences of PI3-kinase inhibition on downstream pathways. Figure 5.4 shows the effects of LY 294002 on the MEK signalling molecule. Figure 5.4A shows a representative western blot of P-MEK protein levels subjected to LY 294002 treatment, where cells were treated as previously described for figure 5.3, with the exception that membranes were probed with P-MEK and T-MEK antibodies for phospho- and total levels. TGF α significantly enhanced P-MEK in MCF-7 ($P<0.001$) and LCC1 ($P<0.05$) cell lines. Any increase in P-MEK in the LCC9 cell line was small in agreement with previously described data (chapter 4).

P-MEK levels were not significantly inhibited in the MCF-7 cell line at the two lower concentrations of 1 μ M and 5 μ M LY 294002 (figure 5.4A and B). P-MEK levels were inhibited by ~13.6-fold in the parental cell line with 30 μ M LY 294002. The western blot in figure 5.4A depicts a decline in TGF α -enhanced P-MEK signal in LCC1 and LCC9 cell lines when treated with 1 μ M and 5 μ M LY 294002. This reduction was found to be significant at 5 μ M in the LCC1 cell line ($P<0.05$). The increase in P-MEK signal was smaller in the LCC9 cells, and any reduction was also small.

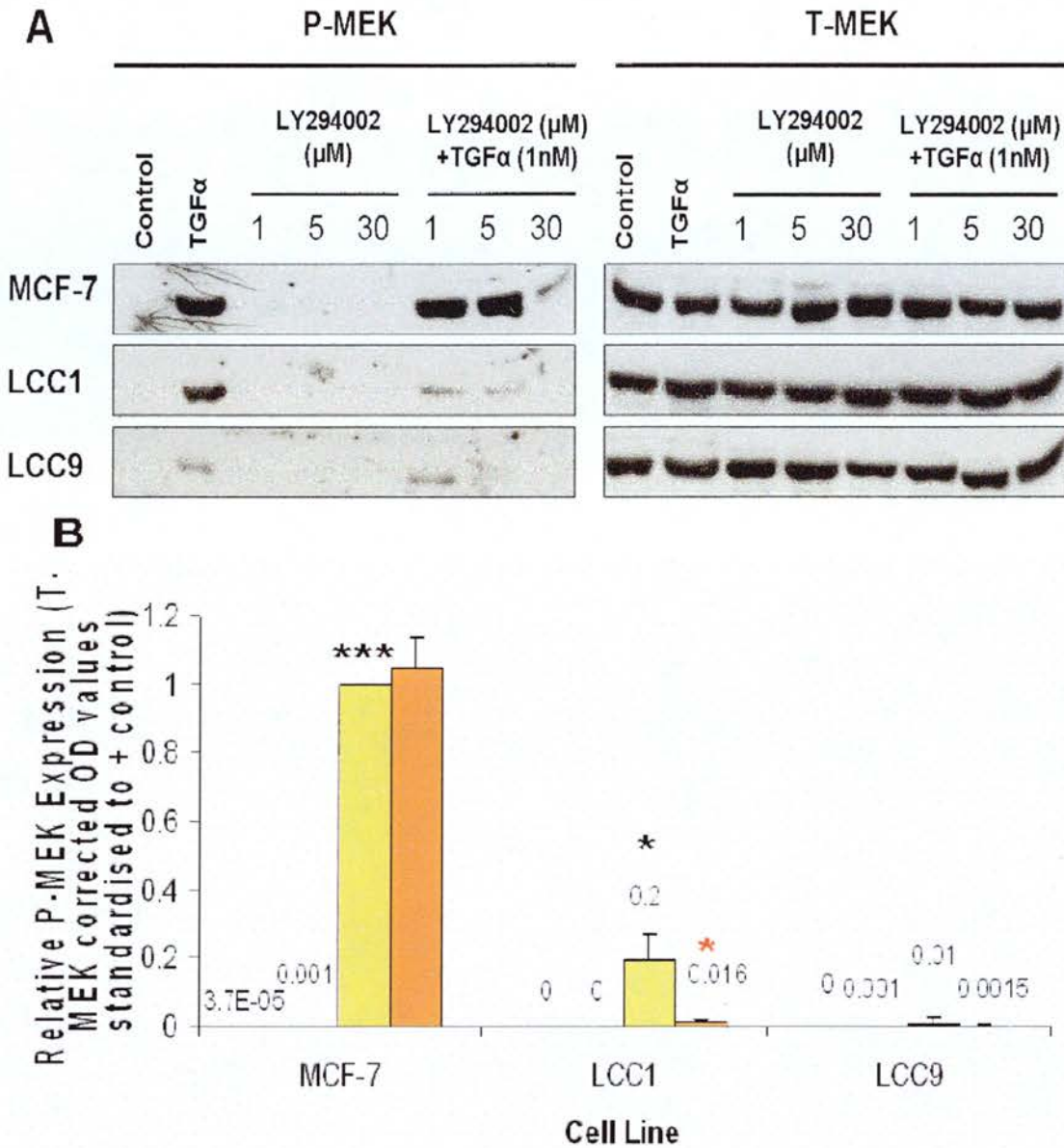


Figure 5.4 Inhibitory effect of LY 294002 in the presence and absence of TGFα on the MEK signalling pathway in MCF-7 cells versus resistant breast cancer cell lines. A, Western blot of P-MEK levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1μM, 5μM or 30μM LY 294002 for 30 min then the cells were treated with control media or TGFα (1nM) for 15 min. Membranes were probed with P-MEK primary antibody (1:1000). T-MEK levels were used as loading controls. B, histogram of P-MEK OD values of all cell lines treated with control media (■), 5μM LY 294002 (■), TGFα (■) or TGFα + 5μM LY 294002 (■) as before, where data are in triplicate +/-SD. MCF-7 cells treated with TGFα were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment and those in red represent any statistical significant changes between TGFα pre and post-treatment with LY 294002. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

5. 1. 1. 3. 2. The effect of LY 294002 on P-ERK1/II protein levels enhanced by TGF α

The effects of LY 294002 on TGF α -enhanced P-ERK/II levels are shown in figure 5.5. Figure 5.5A is a representational western blot of this experiment, where basal P-ERK1/II levels were comparably low in MCF-7, LCC1 and LCC9 cell lines. TGF α (1nM) significantly elevated P-ERK1/II in all three cell lines ($P < 0.001$) compared to basal levels treated with control media. LY 294002 did not significantly affect P-ERK1/II levels in the presence or absence of TGF α stimulation at any concentration used in any of the cell lines tested.

5. 1. 1. 4. The effect of LY 294002 on signalling in cells treated with E₂

The role of E₂ in the Akt and MEK/ERK1/II signalling pathways in the possible development of resistance in this model was investigated using the PI3-kinase inhibitor LY 294002 in the presence and absence of E₂ (figure 5.6). Figure 5.6A and B are western blots of P-Akt and P-ERK1/II levels respectively, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1 μ M, 5 μ M or 30 μ M LY 294002 for 30 min then the cells were treated with control media or E₂ (1nM) for 15 min. Membranes were probed with P-Akt and P-ERK1/II primary antibodies (1:1000). T-Akt and T-ERK1/II levels were used as loading controls. MCF-7 cells treated with TGF α were used as the positive control.

Figure 5.6A depicts the P-Akt levels of MCF-7, LCC1 and LCC9 cell lines. LCC1 and LCC9 cell lines were significantly constitutively phosphorylated at the Akt position ($P < 0.05$) compared to the parental MCF-7 cell line as previously described in chapter 4 and in figure 5.3. P-Akt levels were not significantly altered by E₂ treatment of any cell line. In agreement with figure 5.3B, the basal levels of P-Akt in MCF-7 cells treated with increasing concentrations of LY 294002 alone remained constant, while these levels were reduced with increasing concentrations in both resistant cell lines ($P < 0.05$ with 5 μ M concentration for LCC1 and LCC9 cells, figure 5.3B).

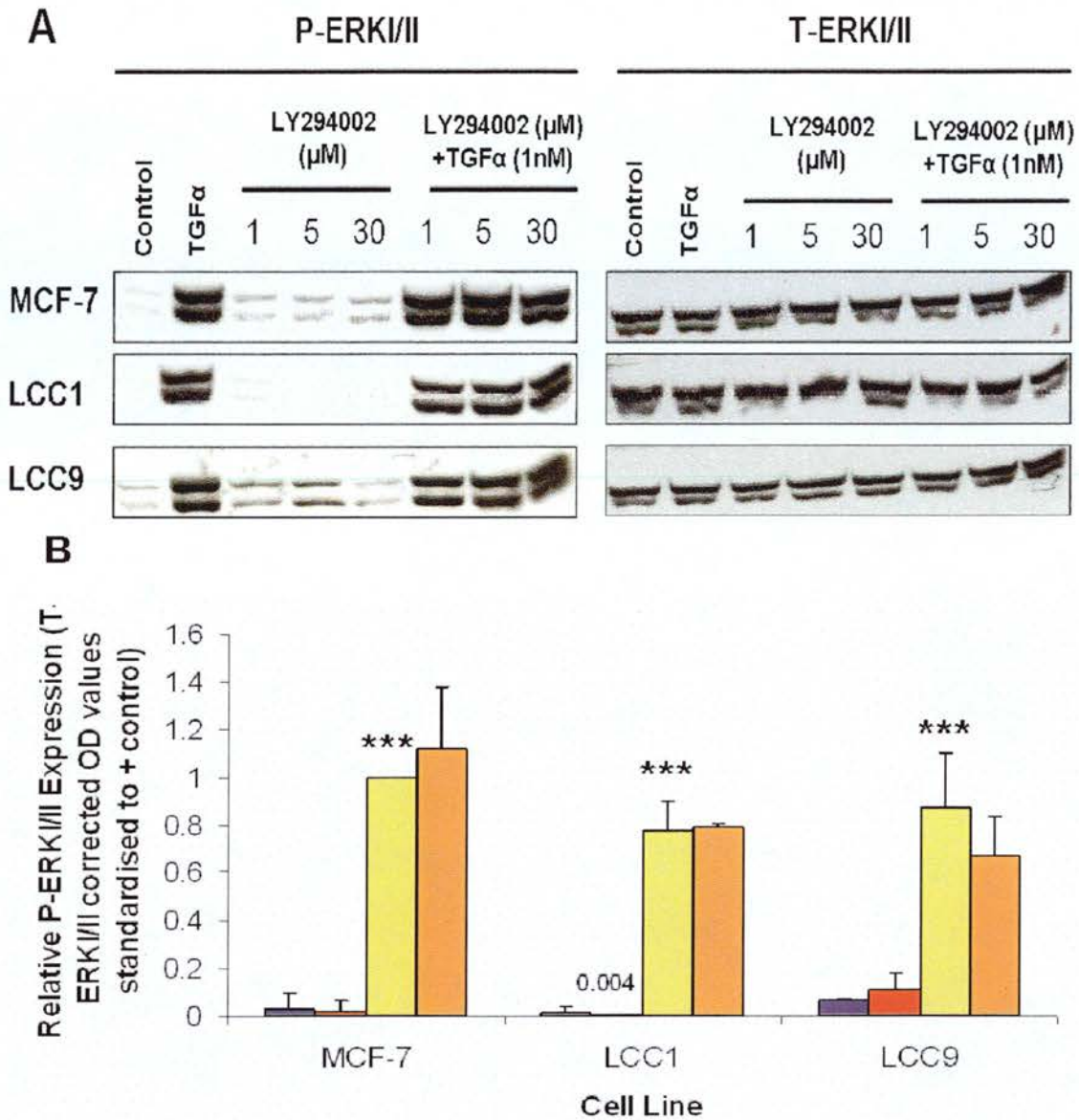


Figure 5.5 The effect of LY 294002 in the presence and absence of TGFα on the ERK1/II signalling pathway in MCF-7 cells versus resistant breast cancer cell lines. **A**, Western blot of P-ERK1/II levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1μM, 5μM or 30μM LY 294002 for 30 min then the cells were treated with control media or TGFα (1nM) for 15 min. Membranes were probed with P-ERK1/II primary antibody (1:1000). T-ERK1/II levels were used as loading controls. **B**, histogram of P-ERK1/II OD values of all cell lines treated with control media (■), 5μM LY 294002 (■), TGFα (■) or TGFα + 5μM LY 294002 (■) as before, where data are in triplicate +/-SD. MCF-7 cells treated with TGFα were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment and those in red represent any statistically significant changes between TGFα pre and post-treatment with LY 294002. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

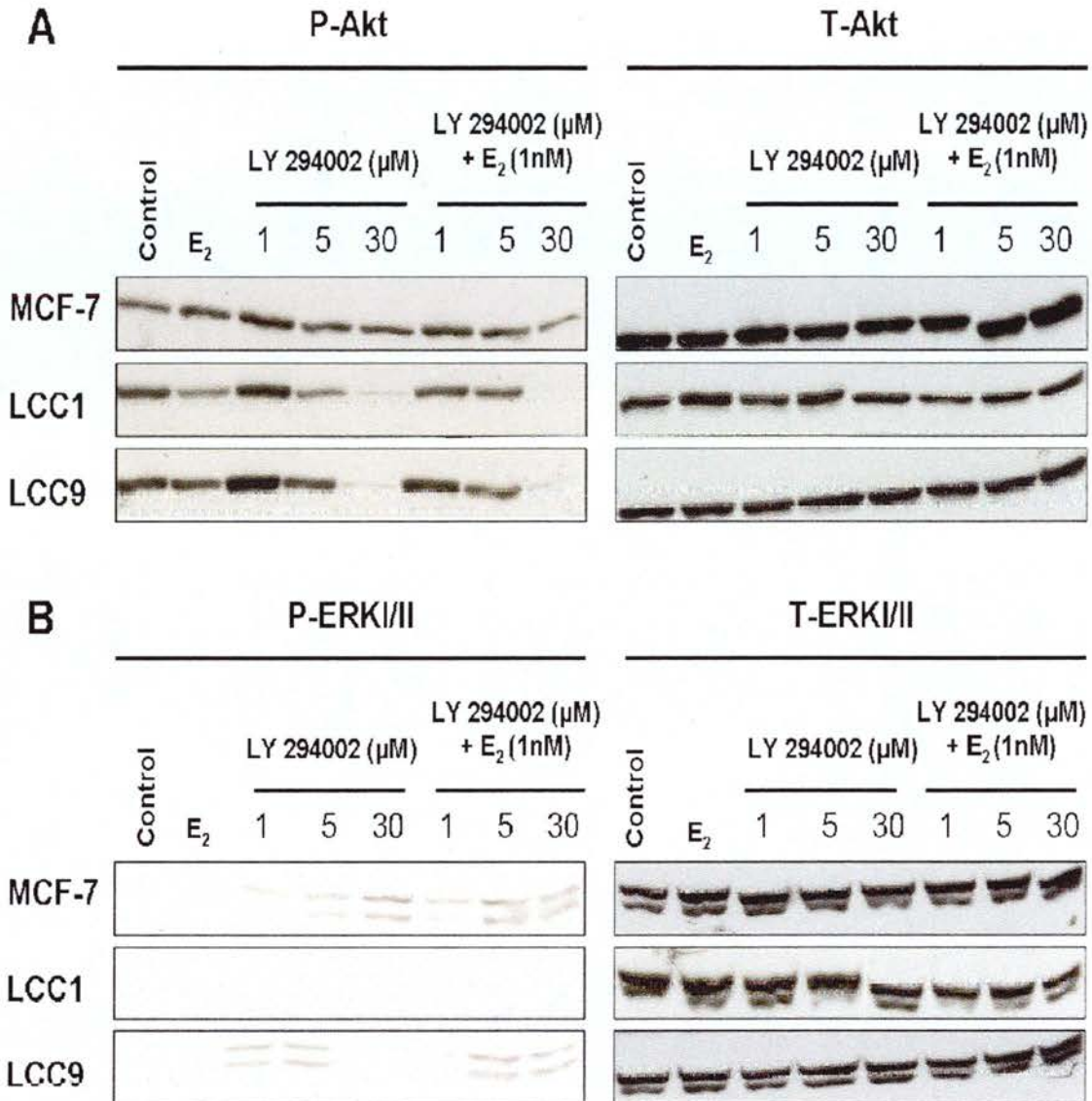


Figure 5.6 The effect of LY 294002 in the presence and absence of E₂ on the Akt and ERK1/2 signalling pathways in MCF-7 cells versus resistant breast cancer cell lines. A and B, Western blots of P-Akt and P-ERK1/2 levels respectively, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1μM, 5μM or 30μM LY 294002 for 30 min then the cells were treated with control media or E₂ (1nM) for 15 min. Membranes were probed with P-Akt and P-ERK1/2 primary antibodies (1:1000). T-Akt and T-ERK1/2 levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

E₂ was unable to increase or significantly alter the levels of P-ERK1/2 protein expression in MCF-7, LCC1 or LCC9 cell lines, which remained comparatively low irrespective of treatment or cell line (figure 5.6B).

5. 1. 1. 5. The effect of LY 294002 on mRNA expression levels of E₂-responsive genes in this model of resistance

The 'protective' nature of the growth factors in the resistant cell lines in the presence of the inhibitor LY 294002 was an interesting observation, given their inability to stimulate proliferation in the same cell lines. Gu *et al* (2002) reported that one of the mechanisms by which breast cancer cells become resistant is via alterations to gene networks controlling cell proliferation and apoptosis. Therefore, the effect of the growth factors, run in conjunction with E₂, was assessed downstream of ER α at the mRNA level.

5. 1. 1. 5. 1. E₂ response

The effects of LY 294002 in the presence and absence of E₂ on mRNA expression levels of E₂-responsive genes are illustrated in figure 5.7. Histograms representing the effect of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines were obtained. All cells were charcoal stripped 48h prior to treatment and were then pre-treated with 10 μ M LY 294002 for 30 min and then treated with control media or E₂ (1nM) or E₂ + 5 μ M LY 294002 (1nM + 10 μ M) for 24h. Data are in triplicate +/- SD and MCF-7 cells treated with E₂ were used as the positive control.

The ER mRNA basal expression levels of MCF-7, LCC9 and LY2 cell lines were found to be similar (~1.5, ~2 and ~2.4 relative expression units for MCF-7, LCC9 and LY2 cell lines), while the control expression of LCC1 cells was significantly elevated to a value of ~6.3 relative expression units (P<0.001, figure 5.7A, supported by data in chapter 3) (figure 5.7A). This is in agreement with the ER α protein expression levels observed in chapter 4. E₂ significantly decreased ER mRNA expression compared to basal levels in MCF-7 cells (P<0.05), LCC1 cells (P<0.01) and LY2 cells (P<0.01), but not in the LCC9 cell line (in agreement with data in chapter 3). 10 μ M LY 294002 alone did not significantly alter the ER mRNA expression levels of any cell line. 10 μ M LY 294002 was unable to significantly reverse or alter the affect of E₂ on ER mRNA expression in any of the four cell lines tested in this model.

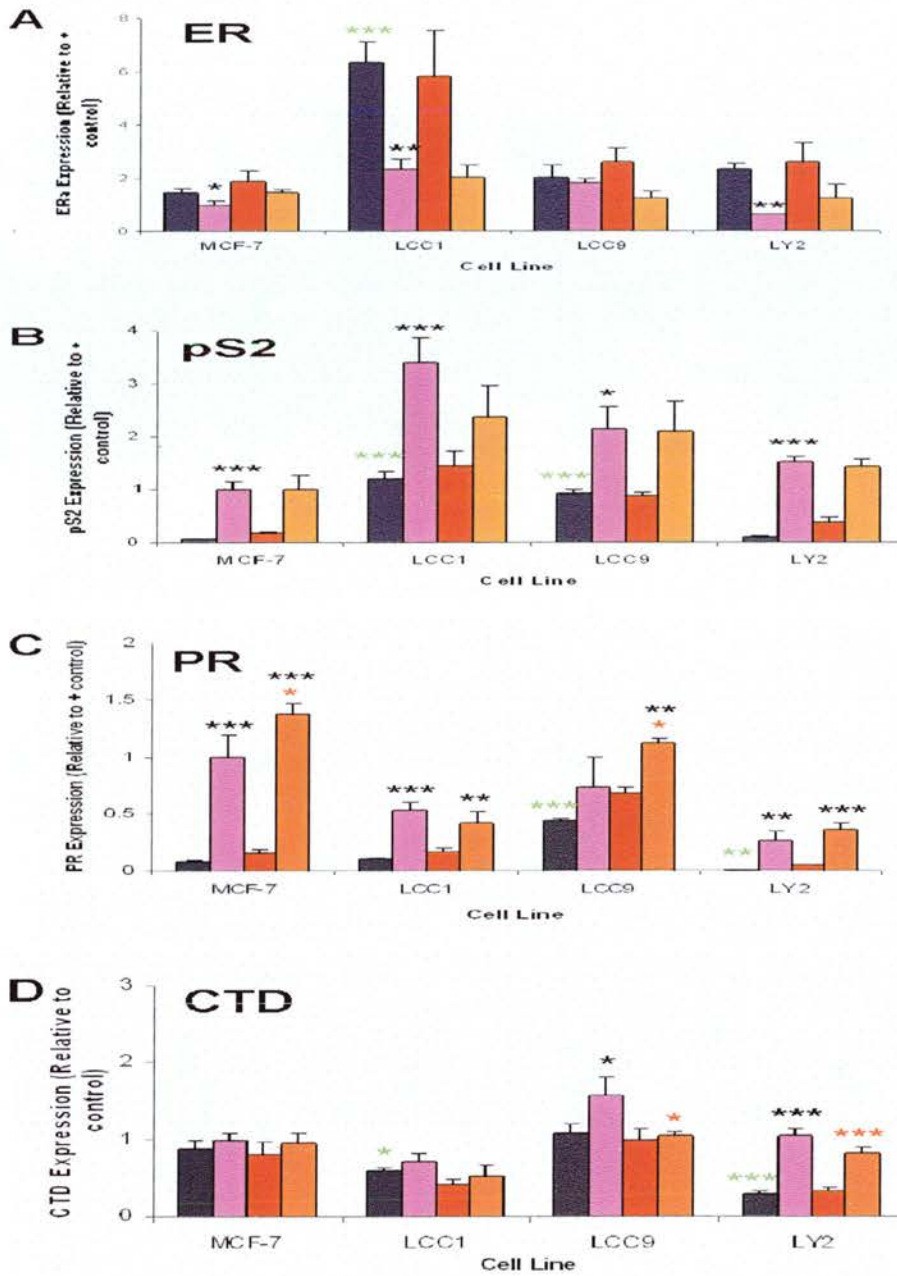


Figure 5.7 The effect of LY 294002 in the presence and absence of E₂ on mRNA expression levels of E₂-responsive genes. Histograms of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 10µM LY 294002 for 30 min and then treated with control media or E₂ (1nM). Cells were treated with control media(■), 5µM LY 294002(■), E₂(■) or E₂ + 10µM LY 294002(■) for 24h, where data are in triplicate +/-SD. MCF-7 cells treated with E₂ were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment, those in green represent statistical significance between MCF-7 cells and the resistant cell lines and those in red represent any statistical significant changes between E₂ pre and post-treatment with LY 294002. Histograms are representative of 1 of 3 independent experiments.

The mRNA expression levels of the E₂-responsive **pS2** gene are depicted in figure 5.7B relative to the positive control (MCF-7 treated with E₂). Basal pS2 mRNA levels were elevated significantly in LCC1 and LCC9 resistant cell lines compared to the MCF-7 parental cell line (P<0.001), with values of ~1.2 and ~0.9 relative expression units versus ~0.06 relative expression units. The basal pS2 mRNA level in the LY2 cell line was insignificantly different to that of MCF-7 cells (P>0.05). pS2 mRNA levels were elevated significantly with E₂ treatment in all four cell lines. The most significant increases were observed in the MCF-7, LCC1 and LY2 cell lines (P<0.001) compared to P<0.05 significance value for the LCC9 cell line. These data are supported by results presented in chapter 3. 5µM LY 294002 treatment alone did not statistically significantly alter basal pS2 levels and 5µM LY 294002 was unable to significantly reverse the increase in pS2 expression produced with 1nM E₂ in any cell line.

As previously discussed in chapter 3 basal **PR** mRNA expression levels were elevated in LCC9 cells by ~5.4-fold (P<0.001) and reduced in LY2 cells by ~5.8-fold (P<0.01) in comparison to MCF-7 cells (figure 5.7C). LCC1 basal PR expression was not significantly different to that of MCF-7 cells. PR mRNA expression was increased by E₂ treatment in MCF-7 (P<0.001), LCC1 (P<0.001) and LY2 cells (P<0.01) but not in LCC9 cells. LY 294002 did not alter the PR expression of any cell line when administered as a single agent. LY 294002 significantly increased PR expression over and above that achieved by E₂ alone in the MCF-7 and LCC9 cell lines (P<0.05). PR expression remained relatively unaltered in LCC1 and LY2 cell lines when treated with LY 294002 and E₂ in combination (P>0.05).

Figure 5.7D shows the basal **CTD** mRNA expression levels were reduced in LCC1 and LY2 cells (P<0.05 and P<0.001 respectively) compared to MCF-7 cells as previously discussed. Basal LCC9 CTD expression was not significantly changed to that of control treated MCF-7 cells. LCC1 CTD expression levels were significantly reduced in general to those of MCF-7 cells (P<0.05). The CTD mRNA levels of MCF-7 and LCC1 cells were unaltered by E₂, 10µM LY 294002 alone or 10µM LY 294002 + 1nM E₂ (P>0.05) treatments. E₂ increased CTD expression by ~1.4-fold (P<0.05) in LCC9 cells, which was subject to some reversal by pre-treatment with 10µM LY 294002 (P<0.05). LY 294002 did not alter the basal LCC9

CTD expression levels. The mRNA expression levels of the LY2 cell line responded in a similar manner to LCC9 cells. The level was significantly increased by E₂ by ~3.4-fold (P<0.001), which was again reduced by LY 294002 by ~0.5-fold (P<0.001). The reversals observed in the LCC9 and LY2 cells lines require confirmation in the form of dose-response data as these may be false positive results if one considers that measuring changes between three cells lines multiplied by four markers incurs a >50% chance that one result will be 5% significant despite no real changes occurring.

5. 1. 1. 5. 2 TGF α response

The effects of LY 294002 in the presence and absence of TGF α on mRNA expression levels of E₂-responsive genes are illustrated in figure 5.8. Histograms representing the effect of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines were obtained. All cells were charcoal stripped 48h prior to treatment and were then pre-treated with 10 μ M LY 294002 for 30 min and then treated with control media or TGF α (1nM) or TGF α + LY 294002 (1nM + 10 μ M) for 24h. Data are in triplicate +/- SD and MCF-7 cells treated with E₂ were used as the positive control.

Basal **ER** mRNA expression levels were as per described for E₂ treated cells in section 5. 1. 1. 5. 1. Figure 5.8A shows TGF α treatment reduced MCF-7 ER expression by ~1.6-fold (P>0.05). LY 294002 did not affect MCF-7 cells when administered alone. LY 294002 in combination with TGF α significantly reduced ER mRNA levels relative to control (P<0.01), but this reduction was insignificant compared to TGF α treatment alone. TGF α did not alter ER expression in LCC1 or LY2 cells, however the ligand did increase ER expression in LCC9 cells by ~1.7-fold (P<0.01). LY 294002 did not significantly alter ER expression in LCC1 cells, but interestingly reduced ER mRNA levels in combination with TGF α compared to control and TGF α alone (P<0.01). LY 294002 alone increased ER expression in LCC9 and LY2 cells significantly (P<0.01 and P<0.05 respectively).

Figure 5.8B shows the basal **pS2** mRNA expression levels were as per described for E₂ treated cells in section 5. 1. 1. 5. 1. Generally the pS2 mRNA levels were elevated irrespective of treatment in LCC1 and LCC9 cell lines compared to MCF-7 and LY2 cell lines. TGF α increased pS2 mRNA levels by ~0.7-fold (P<0.05) in MCF-7 cells, which was not reduced by LY 294002. Treatment of this cell line with LY 294002 alone did not change pS2 mRNA expression from that of control treated cells.

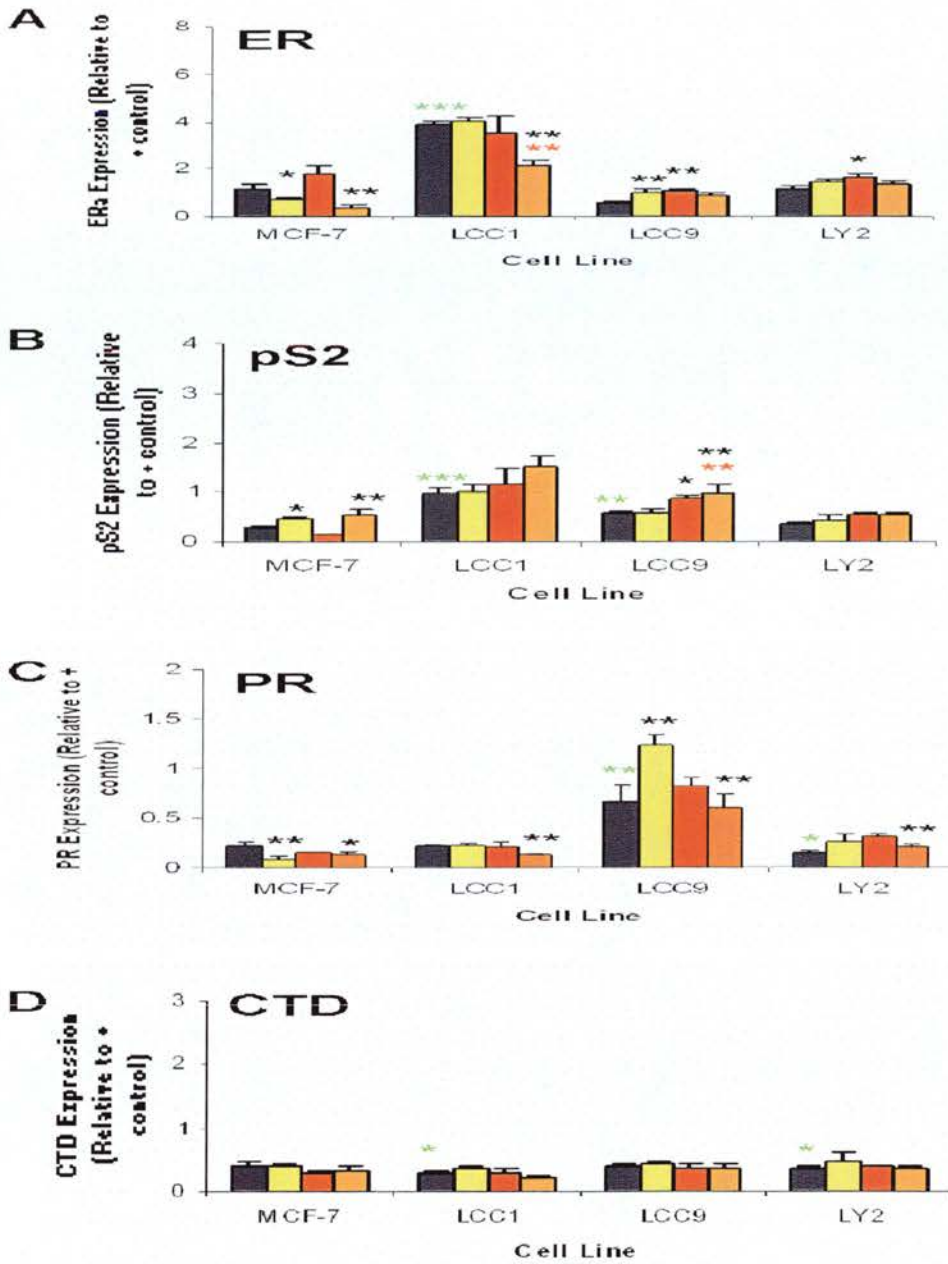


Figure 5.8 The effect of LY 294002 in the presence and absence of TGFα on mRNA expression levels of E₂-responsive genes. Histograms of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 10μM LY 294002 for 30 min and then treated with control media or TGFα (1nM). Cells were treated with control media(■), 10μM LY 294002(■), TGFα (■) or TGFα+ 10μM LY 294002(■) for 24h, where data are in triplicate +/- SD. MCF-7 cells treated with E₂ were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment, those in green represent statistical significance between MCF-7 cells and the resistant cell lines and those in red represent any statistically significant changes post-treatment with LY 294002. Histograms are representative of 1 of 3 independent experiments.

TGF α did not affect pS2 expression in any of the resistant cell lines compared to basal levels. pS2 mRNA levels in LCC1 cells were also unaffected by LY 294002 alone and in combination with TGF α ($P>0.05$). The pS2 expression levels of the LCC9 cell line were, however increased by ~ 1.5 -fold with LY 294002 alone compared to control and by ~ 1.6 -fold when LY 294002 was combined with TGF α compared to control. pS2 levels were also elevated in the combined treatment in LCC9 cells when compared to cells treated with TGF α alone (~ 1.1 -fold increase, $P<0.01$). The pS2 mRNA levels of LY2 cells remained unaffected irrespective of conditions.

Figure 5.8C illustrates the effect of LY 294002 and TGF α on the **PR** mRNA expression levels in the panel of cell lines. TGF α produced a significant decrease in PR expression in the MCF-7 cell line ($P<0.01$), while an increase was seen in the LCC9 cell line ($P<0.01$) compared to levels recorded in untreated control cells. TGF α did not significantly change PR expression in LCC1 or LY2 cells. LY 294002 did not alter PR expression in the MCF-7 cells alone, but the combination of inhibitor plus growth factor raised the reduced mRNA value, although not significantly compared to the combination treatment. No changes were observed in the resistant cell lines treated with LY 294002 alone. There were no significant differences in PR mRNA post-treatment with LY 294002 compared to TGF α alone in MCF-7 and LCC9 cells, however, the combination treatment appeared to be less significantly elevated in these cell lines compared to control than when the inhibitor was administered alone ($P<0.01$ reduced to $P<0.05$ and $P<0.001$ lessened in significance to $P<0.01$ in MCF-7 and LCC9 cells respectively). Also the combination treatment did not produce significant changes in LCC1 and LY2 cell lines compared to TGF α alone, but the mRNA value produced was considered significant compared to control treated cells.

CTD mRNA levels fluctuated around basal level irrespective of treatment in all four cell lines (figure 5.8D). LCC1 and LY2 basal levels were significantly lower than that of MCF-7 and LCC9 cell lines ($P<0.05$). The CTD mRNA levels of LCC9 control treated cells were slightly elevated compared to MCF-7 cell control expression levels but this was not found to be significant (~ 1.04 -fold increase).

TGF α did not affect pS2 expression in any of the resistant cell lines compared to basal levels. pS2 mRNA levels in LCC1 cells were also unaffected by LY 294002 alone and in combination with TGF α ($P>0.05$). The pS2 expression levels of the LCC9 cell line were, however increased by ~ 1.5 -fold with LY 294002 alone compared to control and by ~ 1.6 -fold when LY 294002 was combined with TGF α compared to control. pS2 levels were also elevated in the combined treatment in LCC9 cells when compared to cells treated with TGF α alone (~ 1.1 -fold increase, $P<0.01$). The pS2 mRNA levels of LY2 cells remained unaffected irrespective of conditions.

Figure 5.8C illustrates the effect of LY 294002 and TGF α on the **PR** mRNA expression levels in the panel of cell lines. TGF α produced a significant decrease in PR expression in the MCF-7 cell line ($P<0.01$), while an increase was seen in the LCC9 cell line ($P<0.01$). TGF α did not significantly change PR expression in LCC1 or LY2 cells. LY 294002 did not alter PR expression in the MCF-7 cells alone, but the combination of inhibitor plus growth factor raised the reduced mRNA value, although not significantly compared to the combination treatment. No changes were observed in the resistant cell lines treated with LY 294002 alone. There were no significant differences in PR mRNA post-treatment with LY 294002 compared to TGF α alone in MCF-7 and LCC9 cells, however, the combination treatment appeared to be less significantly elevated in these cell lines compared to control than when the inhibitor was administered alone ($P<0.01$ reduced to $P<0.05$ and $P<0.001$ lessened in significance to $P<0.01$ in MCF-7 and LCC9 cells respectively). Also the combination treatment did not produce significant changes in LCC1 and LY2 cell lines compared to TGF α alone, but the mRNA value produced was considered significant compared to control treated cells.

CTD mRNA levels fluctuated around basal level irrespective of treatment in all four cell lines (figure 5.8D). LCC1 and LY2 basal levels were significantly lower than that of MCF-7 and LCC9 cell lines ($P<0.05$). The CTD mRNA levels of LCC9 control treated cells were slightly elevated compared to MCF-7 cell control expression levels but this was not found to be significant (~ 0.04 -fold increase).

5. 1. 2. The effect of MEK inhibitor UO126 on reduced growth factor stimulated P-MEK in LCC1, LCC9 and LY2 cell lines

UO126 was identified as an AP-1 transactivation inhibitor, with specific action against MEK-1 and MEK-2 superior to that obtained against protein kinase C, ERK and several other kinases (Favata *et al*, 1998). This inhibitor was used to investigate the role of the MEK/ERK1/II pathway in the resistant cell lines as MEK was previously shown to be differentially phosphorylated in the resistant cell lines compared to MCF-7 cells upon growth factor stimulation (see chapter 4). These data showed that growth factor-enhanced P-MEK expression significantly decreased from the MCF-7 cell line to the LCC1 cell line ($P < 0.001$), and this further diminished between the LCC1 and LCC9 cell line (Figure 4.14). In contrast E_2 did not significantly alter P-MEK due to large standard deviation between experiments ($P > 0.05$). These alterations in expression may or may not be relevant in the development of resistance in this model, therefore, if inhibition was altered in the resistant cell lines showing reduced levels, this would support the theory that the MEK/ERK pathway may be a contributing factor to resistance.

5. 1. 2. 1 Growth inhibitory response

Figure 5.9 illustrates the growth effect of 0-60 μ M UO126 in MCF-7, LCC1, LCC9 and LY2 cell lines in the presence and absence E_2 , TGF α and HRG β . Proliferation assays were performed as detailed in earlier inhibitor studies, where cells were treated on day 0 and treatment halted on day 3. Data were plotted as a mean of 6 OD values \pm SD for treated values and as a mean of 12 OD values \pm SD for untreated cells.

All cell lines proliferated in response to ligand treatment as reported earlier (chapter 3, figures 5.1 and 5.2). 60 μ M UO126 reduced control growth by \sim 2.4-fold in MCF-7 cells from 125% to 53% day 3 growth relative to day 0 control with UO126 ($P < 0.001$). 60 μ M UO126 inhibited proliferation by \sim 3.6, \sim 3 and \sim 4-fold with E_2 , TGF α and HRG β treatment respectively in the parental cell line ($P < 0.001$). The growth stimulation was inhibited by 50% of day 0 growth with UO126 concentrations of \sim 27.5 μ M for E_2 , \sim 45 μ M for TGF α and \sim 36 μ M for HRG β treatment in MCF-7

cells. Interestingly, HRG β proliferation was reduced significantly even with 1 μ M UO126 ($P < 0.001$) while the increase in proliferation due to E₂ was not significantly reduced with 1 μ M UO126. This also contrasts with control treated MCF-7 cells which were significantly reduced with this concentration ($P < 0.01$). However, this did not occur at higher concentrations and in general the shape of the curves indicate that E₂ stimulation was more easily reversed than HRG β . Also, TGF α increased growth was not significantly reduced until MCF-7 cells were treated with 10 μ M UO126.

60 μ M UO126 inhibited proliferation by ~2.9, ~2.6, ~2.7 and ~2.5-fold in LCC1 cells subject to control, E₂, TGF α and HRG β conditions ($P < 0.001$). However, as these cells proliferated in the absence of any stimulation (control conditions), the final percentage proliferation of cells on day 3 was elevated compared to the MCF-7 cell line, with values of ~150%, ~155, ~ 101.5% and ~113% for LCC1 cells treated with control media, E₂, TGF α and HRG β respectively.

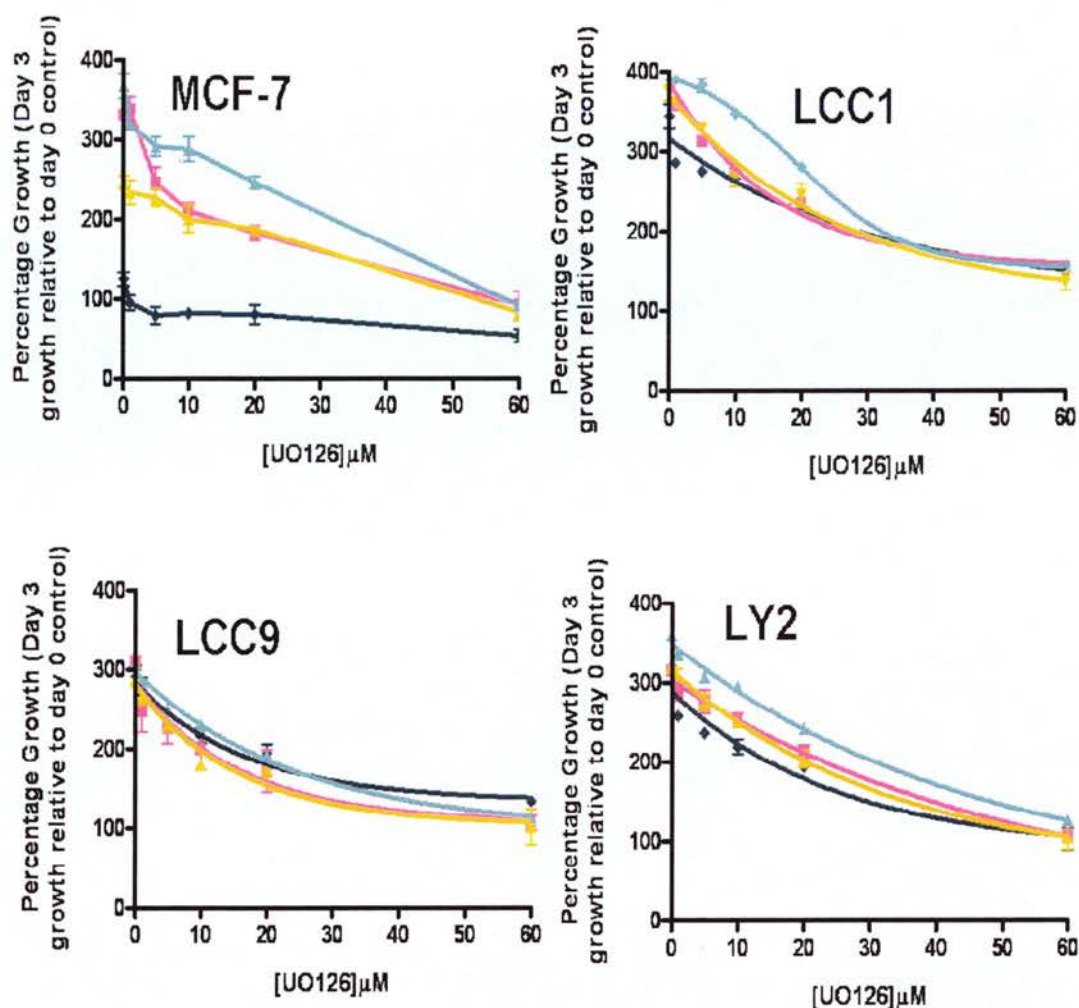


Figure 5.9 Growth inhibitory effect of UO126 in MCF-7 cells versus resistant breast cancer cell lines. All cells were charcoal stripped 24h prior to treatment and were then treated with a concentration range of UO126 spanning 0-60 μ M plus control media (\blacklozenge), E₂ (1nM) (\blacksquare), TGF α (1nM) (\blacktriangle) or HRG β (1nM) (\blacktriangle). Cells were treated on day 0 and treatment halted on day 3. Data were plotted as a mean of 6 OD values \pm SD for treated values and as a mean of 12 OD values \pm SD for untreated cells. Graphs are representative of 1 of 3 independent replicate experiments.

The lowest concentration of 1 μ M UO126 used significantly inhibited control and E₂ treated LCC1 cells ($P < 0.001$), but higher concentrations of 5 μ M and 10 μ M UO126 were required to significantly inhibit cells treated with TGF α and HRG β respectively. LCC9 cells were inhibited by \sim 2.2, \sim 2.9, \sim 2.9 and \sim 2.7-fold when treated with 60 μ M UO126 alone, E₂, TGF α and HRG β respectively.

The LY2 cell line was inhibited by \sim 3-fold with 60 μ M UO126 irrespective of treatment. 1 μ M UO126 significantly inhibited proliferation treated with control

media, E₂ and HRGβ. TGFα treatment required 5μM UO126 to be inhibited significantly. 60μM UO126 inhibited proliferation to ~103% in control conditions, ~105% with E₂, ~104% with TGFα and ~125% with HRGβ treatment.

It is interesting to note that at lower UO126 concentrations, in particular 10-20μM, HRGβ is again 'protecting' MCF-7, LCC1 and LY2 cells in comparison to all other treatments. Moreover, in contrast to LY 294002 data, E₂ and TGFα are both 'protective' against UO126 in not only MCF-7 cells as for LY 294002, but in the LCC1 cell line also. The LCC9 cell line behaved differently to the other cell lines, where proliferation was inhibited by UO126 to a similar extent irrespective of treatments.

5. 1. 2. 2 The effect of MEK inhibitor UO126 on the MEK/ERK pathway in LCC1 and LCC9 cells versus MCF-7 cells

The role of MEK/ERK signalling was investigated in resistance by using the MEK inhibitor UO126 to abrogate signalling via this pathway.

5. 1. 2. 2. 1 The effect of UO126 on P-ERK/II in cells treated with TGFα

Figure 5.10 shows the inhibitory effect of UO126 in the presence and absence of TGFα on the ERK1/II signalling pathway in MCF-7, LCC1 and LCC9 cell lines. Figure 5.10A is a representational western blot of P-ERK1/II levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1μM, 20μM or 60μM UO126 for 30 min; the cells were then treated with control media or TGFα (1nM) for 15 min. Membranes were probed with P-ERK1/II primary antibody (1:1000). T-ERK1/II levels were used as loading controls. The western blot illustrates the low and similar levels of P-ERK1/II in MCF-7, LCC1 and LCC9 cells. There were significant increases in P-ERK1/II with TGFα (1nM) in all cell lines, with the production of doublets at 44 and 42kDa respectively, as previously documented in chapter 4 and in figure 5.5 (P<0.001, comparison between control and TGFα treatment between all the cell lines), which were decreased with increasing UO126 concentration.

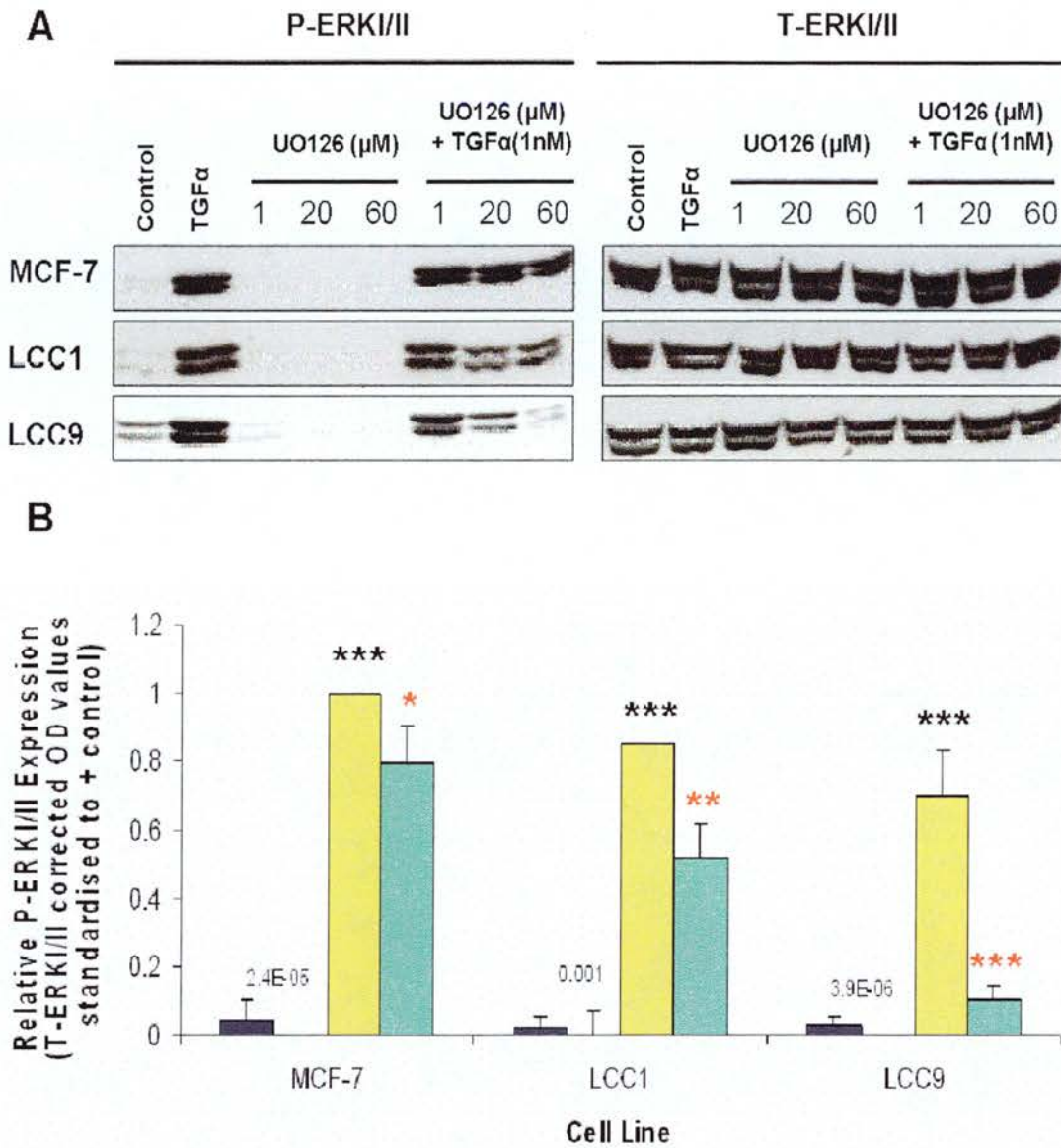


Figure 5.10 Inhibitory effect of UO126 in the presence and absence of TGFα on the ERK1/II signalling pathway in MCF-7 cells versus resistant breast cancer cell lines. A, Western blot of P-ERK1/II levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1μM, 20μM or 60μM UO126 for 30 min then the cells were treated with control media or TGFα (1nM) for 15 min. Membranes were probed with P-ERK1/II primary antibody (1:1000). T-ERK1/II levels were used as loading controls. B, histogram of P-ERK1/II OD values of all cell lines treated with control media (■), 20μM UO126 (□), TGFα (■) or TGFα (1nM) + 20μM UO126 (■) as before, where data are in triplicate +/-SD. MCF-7 cells treated with TGFα were used as the positive control. ANOVA test: * (P<0.05), ** (P<0.01) and *** (P<0.001) represent significant, highly significant and extremely significant changes. Asterisks in black represent significant changes between control and a treatment and those in red represent significance between TGFα pre and post-treatment with UO126. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

Analysis of multiple western blots using OD values of cells treated with a single concentration of 20 μ M (figure 5.10B) showed the reduction in P-ERK1/II signal to be most significant in LCC9 cell line (~6.4-fold reduction, $P < 0.001$) rather than MCF-7 cells (~0.3-fold reduction, $P < 0.05$) or LCC1 cell line (~0.7-fold reduction, $P < 0.01$).

5. 1. 2. 2 The effect of UO126 on P-MEK in cells treated with TGF α

Figure 5.11 illustrates the effect of UO126 in the presence and absence of TGF α on the MEK signalling pathway in MCF-7 cells versus resistant breast cancer cell lines. A representational western blot of P-MEK levels is shown in figure 5.11A, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1 μ M, 20 μ M or 60 μ M UO126 for 30 min. The cells were then treated with control media or TGF α (1nM) for 15 min. Membranes were probed with P-MEK primary antibody (1:1000). T-MEK levels were used as loading controls.

TGF α significantly enhanced P-MEK in MCF-7 ($P < 0.001$) and LCC1 ($P < 0.01$) cell lines. Any increase in P-MEK in the LCC9 cell line was small in agreement with previously described data (chapter 4). The western blot shows that P-MEK increased in signal strength with increasing UO126 concentration from 1 μ M to 20 μ M to 60 μ M in all cell lines. The histogram in figure 5.11B allows statistical analysis of the 20 μ M combinations as data was in triplicate.

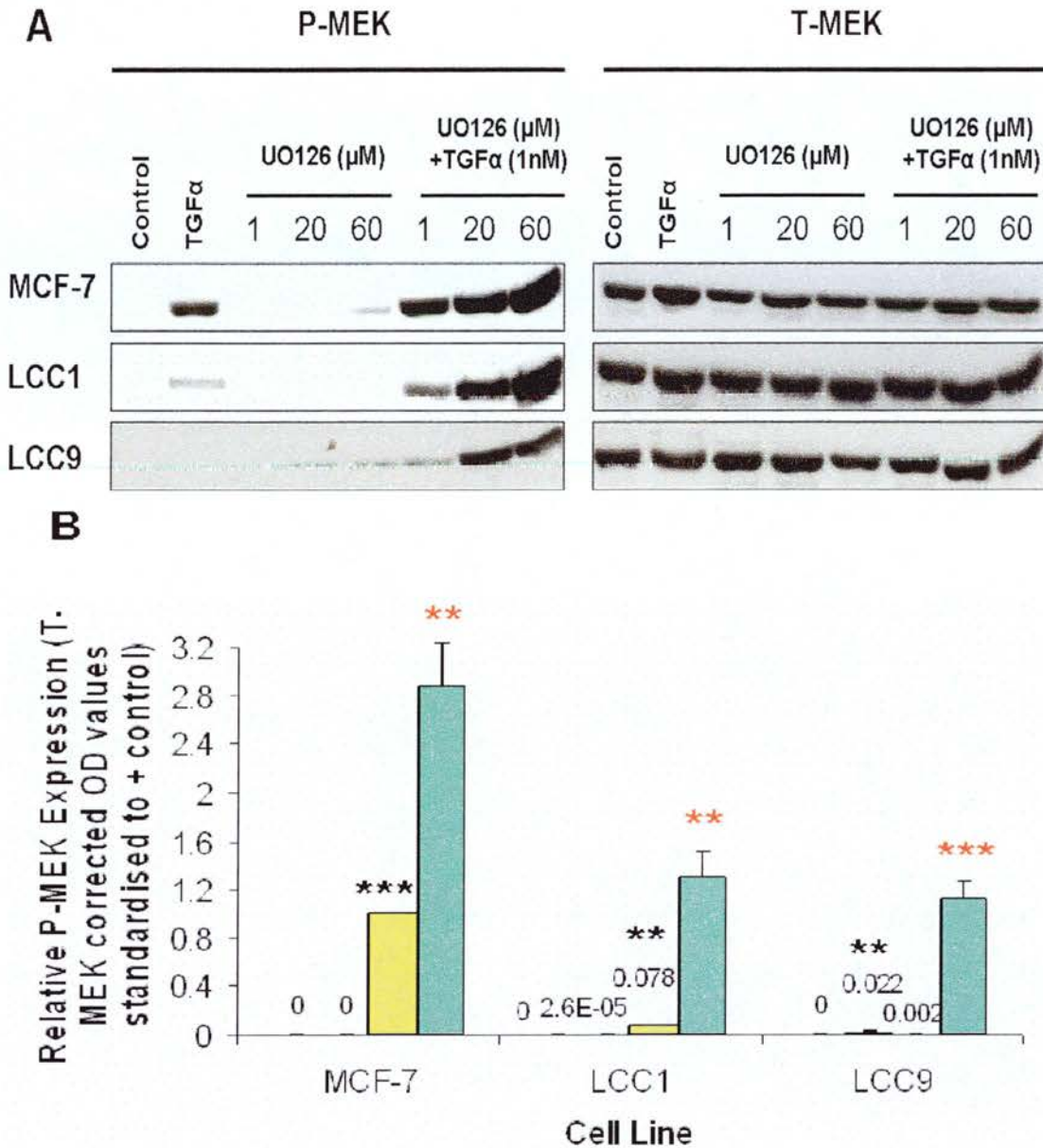


Figure 5.11 The effect of UO126 in the presence and absence of TGFα on the MEK signalling pathway in MCF-7 cells versus resistant breast cancer cell lines. A, Western blot of P-MEK levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1μM, 20μM or 60μM UO126 for 30 min then the cells were treated with control media or TGFα (1nM) for 15 min. Membranes were probed with P-MEK primary antibody (1:1000). T-MEK levels were used as loading controls. B, histogram of P-MEK OD values of all cell lines treated with control media (■), 20μM UO126 (□), TGFα (■) or TGFα+ 20μM UO126 (■) (columns 1-4 respectively) as before, where data are in triplicate +/-SD. MCF-7 cells treated with TGFα were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment and those in red represent statistical significance between TGFα pre and post-treatment with UO126 in each cell line. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

The combination of 20 μ M UO126 and TGF α (1nM) enhanced P-MEK significantly in all three cell lines with increases from \sim 1 to \sim 2.9 arbitrary units ($P < 0.01$) in MCF-7 cells, \sim 0.08 to \sim 1.3 units ($P < 0.01$) in LCC1 cells and \sim 0.002 to \sim 1.12 units ($P < 0.001$) in the LCC9 cell line. The increase in P-MEK produced from the combination of 20 μ M UO126 and TGF α was significantly greater in the parental MCF-7 cell line compared to both LCC1 and LCC9 resistant cell lines ($P < 0.01$). There was no significant difference between the LCC1 and LCC9 cell lines themselves when subjected to this combination ($P > 0.05$).

5. 1. 2. 2. 3. The effect of UO126 on P-Akt in TGF α -treated cells

The effect of UO126 in the presence and absence of TGF α on the Akt signalling pathway in MCF-7 cells versus resistant breast cancer cell lines is shown in figure 5.12. All cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1 μ M, 20 μ M or 60 μ M UO126 for 30 min then the cells were treated with control media or TGF α (1nM) for 15 min. Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls.

The western blot in figure 5.12A shows TGF α enhanced P-Akt levels were significantly increased further with increasing UO126 concentrations in all cell lines, with what appears to be the greatest enhancement occurring with 60 μ M ($P < 0.001$ with 20 μ M concentration in all cells compared to TGF α treatment alone, figure 5.12B). The basal levels of P-Akt in MCF-7 cells treated with increasing concentrations of UO126 were increased, but not significantly compared to control treated cells. In contrast, UO126 increased LCC1 and LCC9 cell line elevated constitutive P-Akt levels ($P < 0.01$ and $P < 0.001$ respectively).

Figure 5.12B is a histogram of P-Akt OD values of all cell lines treated with control media, 20 μ M UO126, TGF α (1nM) or TGF α + UO126 (1nM + 20 μ M), where data are in triplicate \pm SD. MCF-7 cells treated with TGF α were used as the positive control. LCC1 and LCC9 cell lines were significantly constitutively phosphorylated at the Akt position ($P < 0.05$) compared to the parental MCF-7 cell line as previously described in chapter 4 and in figure 5.3.

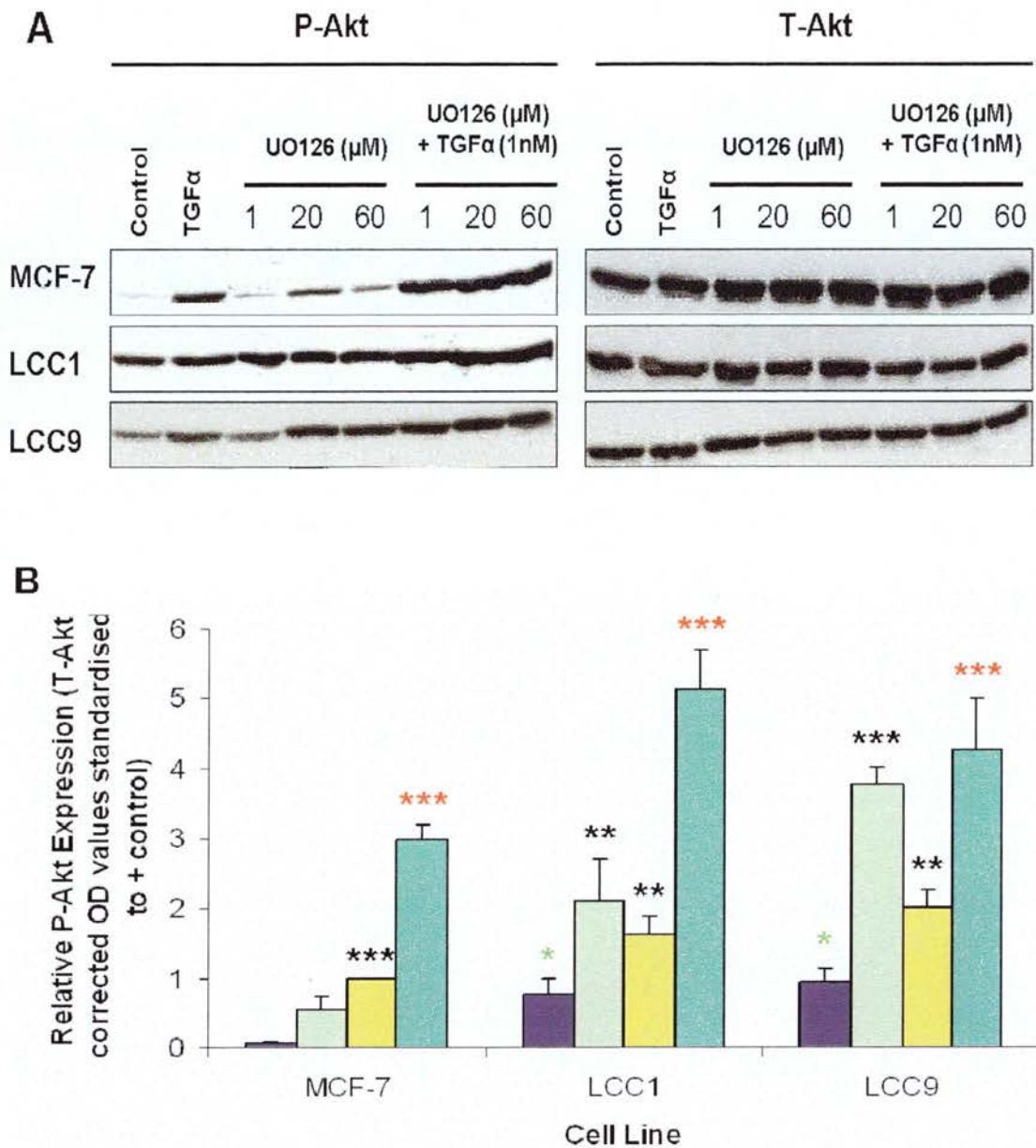


Figure 5.12 The effect of UO126 in the presence and absence of TGFα on the Akt signalling pathway in MCF-7 cells versus resistant breast cancer cell lines. A, Western blot of P-Akt levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1μM, 20μM or 60μM UO126 for 30 min then the cells were treated with control media or TGFα (1nM) for 15 min. Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls. B, histogram of P-Akt OD values of all cell lines treated with control media (■), 20μM UO126 (□), TGFα (■) or TGFα (1nM) + 20μM UO126 (■) as before, where data are in triplicate +/-SD. MCF-7 cells treated with TGFα were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment and those in red represent statistical significance between TGFα pre and post-treatment with UO126 in each cell line. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

TGF α significantly enhanced P-Akt in all three cell lines ($P < 0.001$ in MCF-7 and $P < 0.01$ for the resistant cell lines) also as previously described. UO126 potentiated TGF α -enhanced P-Akt from ~ 1 to ~ 3 relative expression units in MCF-7 cells, from ~ 1.6 to ~ 5 relative expression units in LCC1 cells and from ~ 2 to ~ 4.3 relative expression units in LCC9 cells (all $P < 0.001$). As for UO126 alone, the resistant cell line P-Akt levels were elevated further than the MCF-7 cell line. However, there was no difference between LCC1 and LCC9 cells lines, indicating P-Akt stimulation has peaked at the same level in both cell lines.

5. 1. 2. 3. The effect of MEK inhibitor UO126 on signalling pathways in cells treated with E₂

5. 1. 2. 3. 1. P-ERK/II

The effect of UO126 on P-ERK/II was observed in the cell model when cells were treated with E₂ (figure 5.13A). Basal P-ERK/II levels were low and relatively equal in all three cell lines and were not enhanced by treatment with E₂ (1nM). UO126 appeared to reduce the low basal levels even further with reductions of ~ 3 , ~ 5 and ~ 12 -fold in MCF-7, LCC1 and LCC9 cell lines with a concentration of 1 μ M. E₂ appears to prevent this reduction in the basal level of P-ERK/II in the MCF-7 cell line, but only at the lowest concentration of 1 μ M of UO126. 20 μ M and 60 μ M UO126 abolished any basal signal remaining.

5. 1. 2. 3. 2. P-Akt

The effect of UO126 on P-Akt was observed in the cell model when cells were treated with E₂ (figure 5.13B). Basal and TGF α -treated P-Akt levels were as previously described in chapter 4 and in figures 5.3 and 5.12. The pattern of P-Akt treated with UO126 alone reflects that of figure 5.12, where UO126 increased P-Akt in all cell lines with increasing concentration. Interestingly, the addition of E₂ further increased UO126 (1 μ M) enhanced P-Akt from ~ 2 -4-fold in all cell lines. The increase in P-Akt appears to plateau at 20 μ M UO126 + E₂ and does not increase any further with 60 μ M UO126 in combination with E₂. This also occurs in the absence of E₂.

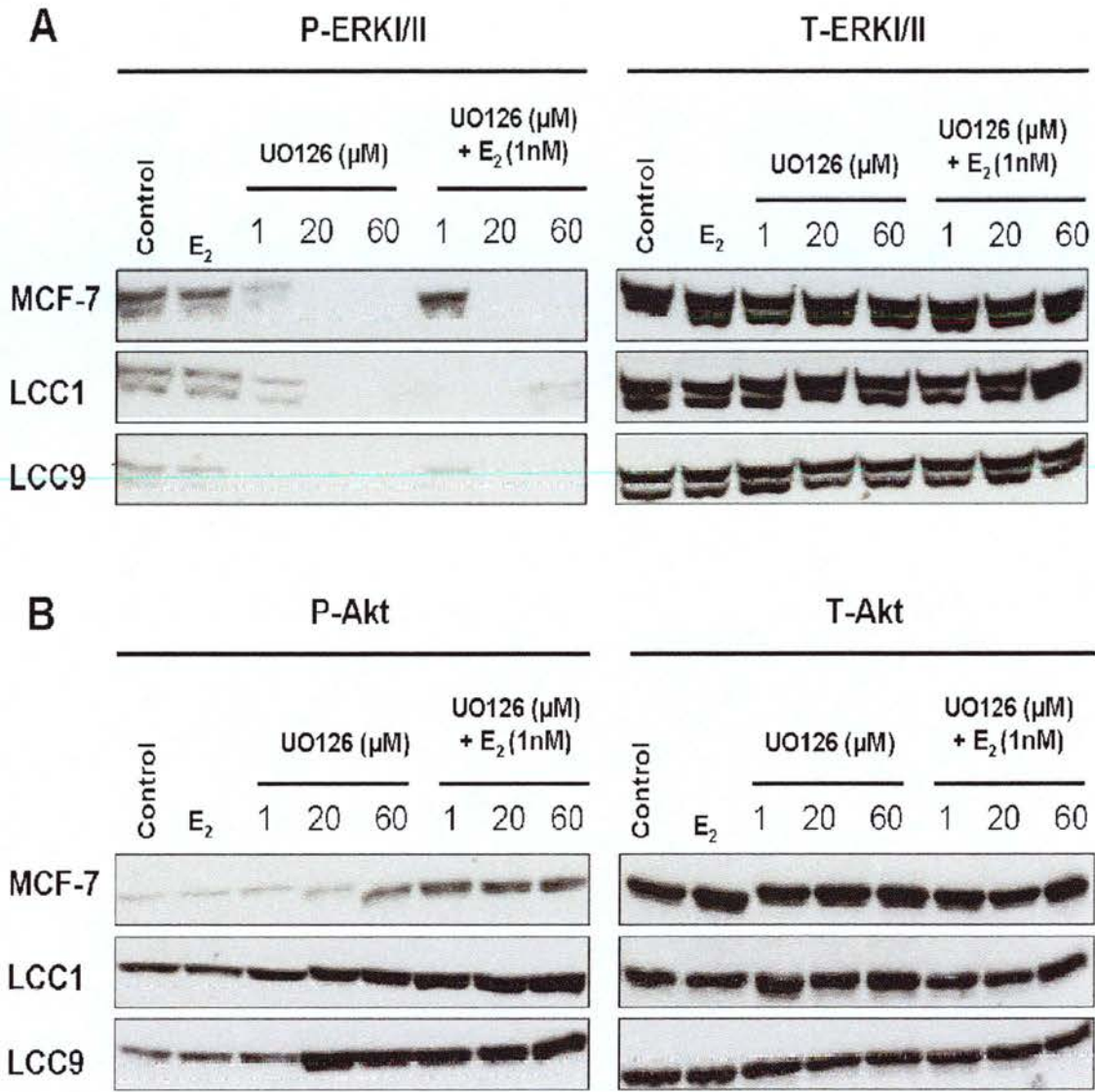


Figure 5.13 The effect of UO126 in the presence and absence of E₂ on the Akt and ERK1/II signalling pathways in MCF-7 cells versus resistant breast cancer cell lines. A and B, Western blots of P-Akt and P-ERK1/II levels respectively, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 1μM, 20μM or 600μM UO126 for 30 min then the cells were treated with control media or E₂ (1nM) for 15 min. Membranes were probed with P-Akt and P-ERK1/II primary antibodies (1:1000). T-Akt and T-ERK1/II levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

5. 1. 2. 4 The effect of MEK inhibitor UO126 on mRNA expression levels of E₂-responsive genes in this model of resistance

As previously reported, one of the proposed mechanisms by which breast cancer cells become resistant is via alterations to gene networks controlling cell proliferation and apoptosis (Gu *et al.*, 2002). Therefore, the effect of the growth factors, run in conjunction with E₂, was assessed downstream on E₂-regulated gene expression in the presence and absence of MEK inhibitor UO126.

5. 1. 2. 4. 1 E₂ response

The effects of UO126 in the presence and absence of E₂ on mRNA expression levels of E₂-responsive genes are illustrated in figure 5.14. Histograms representing the effect of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines were obtained. All cells were charcoal stripped 48h prior to treatment and were then pre-treated with 10µM UO126 for 30 min and then treated with control media or E₂ (1nM) or E₂ + UO126 (1nM + 10µM) for 24h. Data are in triplicate +/-SD and MCF-7 cells treated with E₂ were used as the positive control.

The basal and E₂-treated mRNA levels in all four genes tested are described in section 5. 1. 1. 5. **ER** mRNA expression levels were reduced significantly in MCF-7 cells treated with 10µM UO126 alone ($P < 0.01$) (figure 5.14A). UO126 was unable to significantly reverse the reduction in mRNA levels caused by E₂, although combined treatment produced less of a reduction in ER mRNA than treatment with E₂ alone ($P > 0.05$). 10µM UO126 did not significantly alter LCC1 cells treated with control media or E₂, although a slight reduction of ~0.5-fold was recorded ($P > 0.05$). UO126 significantly reduced LCC9 ER mRNA expression when combined with E₂ from ~1.7 to ~1 arbitrary expression units ($P < 0.05$). LY2 cell ER mRNA levels were again significantly reduced with E₂ treatment from ~2.2 to ~0.6 arbitrary expression units ($P < 0.001$), which was significantly reduced to ~1 unit by pre-treatment with 10µM UO126 ($P < 0.01$). UO126 did not significantly change LY2 ER mRNA expression when administered alone. Figure 5.14B depicts the effect of UO126 on pS2 mRNA expression levels in this cell model.

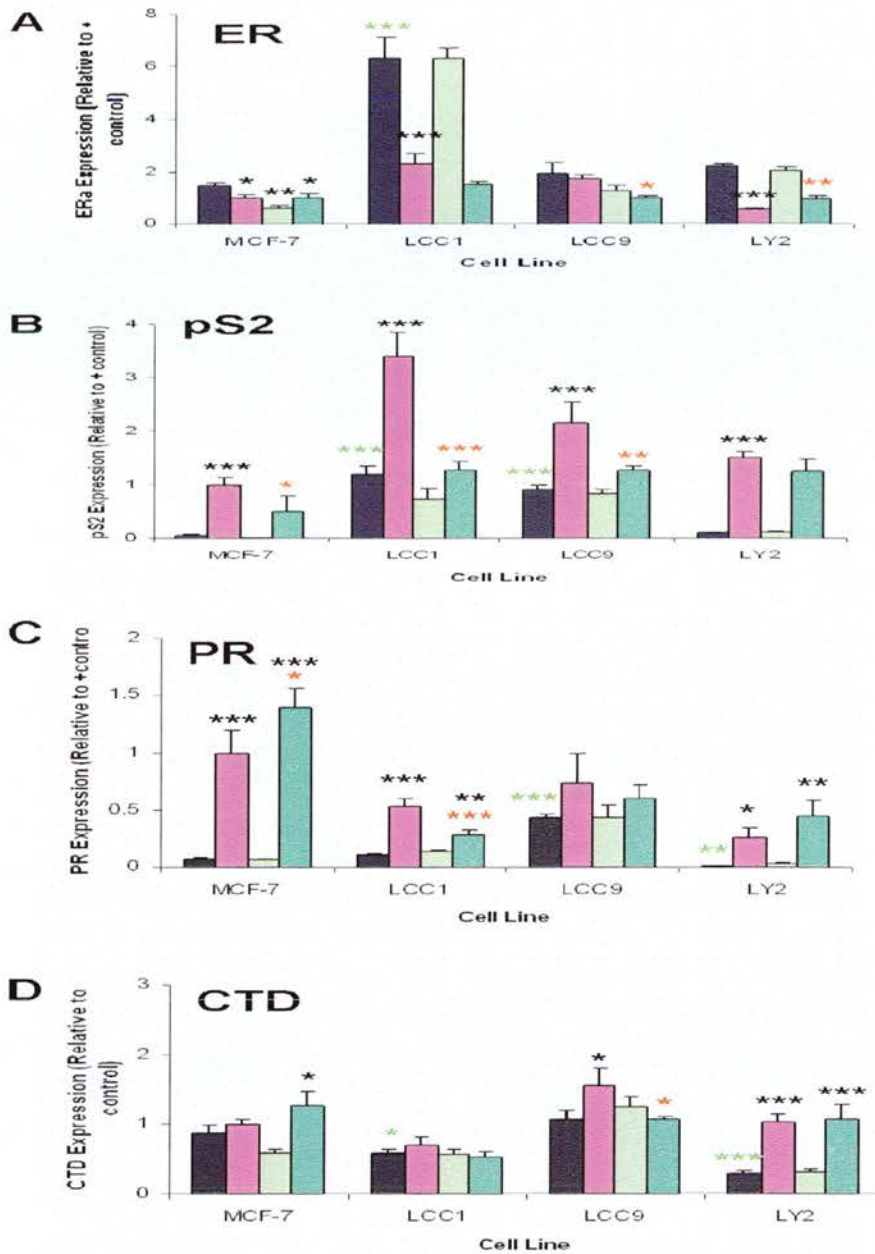


Figure 5.14 The effect of UO126 in the presence and absence of E₂ on mRNA expression levels of E₂-responsive genes. Histograms of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 10µM UO126 for 30 min and then treated with control media or E₂ (1nM). Cells were treated with control media (■), 5µM UO126 (□), E₂(■) or E₂+ 5µM UO126 (■) for 24h, where data are in triplicate +/-SD. MCF-7 cells treated with E₂ were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in that cell line, those in green represent statistical significance between MCF-7 cells and the resistant cell lines and those in red represent any statistical significant changes post-treatment with UO126 in combination with E₂ compared to E₂ alone in each cell line. Histograms are representative of 1 of 3 independent replicate experiments.

UO126 significantly reversed the elevated pS2 expression induced by E₂ by ~2-fold (P<0.05), but was unable to fully abolish the induction in the MCF-7 cell line. UO126 did not affect pS2 expression relative to control levels in the parental cell line. UO126 reversed control pS2 expression by ~0.6-fold (P<0.05) in LCC1 cells and almost completely abolished E₂-induced pS2 mRNA expression, with an extremely significant reversal of ~2.6-fold (P<0.001). UO126 also reversed the E₂-elevated pS2 levels in the LCC9 cell line by ~1.7-fold (P<0.01). UO126 reduced LY2 cell mRNA expression elevated by E₂, although this was not found to be statistically significant. UO126 did not significantly affect the pS2 mRNA levels of LY2 control treated cells (~0.2-fold reduction, P>0.05).

Figure 5.14C shows the PR mRNA expression levels of this model when subjected to 10µM UO126 in the presence and absence of E₂ (1nM). PR mRNA expression remained unchanged by pre-treatment with UO126 alone in all cell lines compared to that of control, whilst UO126 combined with E₂ elevated PR mRNA levels significantly in MCF-7 cells (~1.4-fold increase, P<0.05). Interestingly, E₂-enhanced PR expression was reduced significantly in the LCC1 cell line by ~0.9-fold (P<0.001), and to a minor extent in LCC9 cells (~1.2-fold reduction, P>0.05). UO126 plus E₂ PR mRNA expression was elevated compared to E₂ alone in LY2 cell lines (~1.7-fold), but this change was not found to be significant.

The CTD mRNA expression of all four cell lines treated with 10µM UO126 in the presence and absence of E₂ (1nM) are illustrated in figure 5.14D. UO126 reduced basal CTD expression in MCF-7 cells by ~0.5-fold, but this was not considered significant (P>0.05). CTD expression was elevated in MCF-7 cells when treated with UO126 and E₂ (P<0.05). LCC1 cell line PR mRNA expression did not significantly differ irrespective of treatment. E₂-enhanced CTD expression in the LCC9 cell line was significantly reduced by pre-treatment with 10µM UO126 from ~1.6 to ~1.1 relative arbitrary expression units (P<0.05). UO126 did not significantly modify CTD mRNA of LY2 cells compared to basal levels or E₂-enhanced CTD levels. CTD mRNA values remained significantly elevated in the presence of UO126 (P<0.001 compared to control).

5. 1. 2. 4. 2 *TGF α* response

The effects of UO126 in the presence and absence of TGF α on mRNA expression levels of E₂-responsive genes are illustrated in figure 5.15. Histograms representing the effect of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines were obtained. All cells were charcoal stripped 48h prior to treatment and were then pre-treated with 10 μ M UO126 for 30 min and then treated with control media or TGF α (1nM) or TGF α + UO126 (1nM + 10 μ M) for 24h. Data are in triplicate +/-SD and MCF-7 cells treated with E₂ were used as the positive control.

Basal and TGF α treated **ER** mRNA expression levels were as described in sections 5. 1. 1. 5. 1 and 5. 1. 1. 5. 2 respectively, although TGF α produced what was considered an even more significant reduction in ER mRNA expression in MCF-7 cells in figure 5.15A (P<0.001 compared to P<0.05 in figure 5.8A). Figure 5.15A shows UO126 reduced the ER mRNA value relative to control by ~1.3-fold (P<0.001), but the combined UO126 plus TGF α treatment did not affect the reduction in ER expression induced by TGF α treatment alone. UO126 also reduced LCC1 cell line values of ER mRNA expression by ~0.4-fold (P<0.05), which was reduced even further in the presence of TGF α (~2-fold compared to control, P<0.01). UO126 reduced ER mRNA expression when combined with TGF α from ~1.2 versus ~0.8 relative arbitrary units (P<0.05) in LCC9 cells and from ~0.26 to ~0.16 units (P<0.01) in LY2 cells compared to TGF α alone. UO126 did not decrease ER mRNA expression when administered alone in either LCC9 or LY2 cell lines.

pS2 expression was significantly decreased by UO126 alone in MCF-7 (P<0.01), LCC1 (P<0.05) and LCC9 cell lines (P<0.001) (figure 5.15B). UO126 alone did not significantly change the pS2 expression in LY2 cells. TGF α -enhanced pS2 expression was reduced with UO126 in MCF-7 (~2.2-fold reduction), LCC1 (~0.7-fold reduction) and LCC9 (~1.5-fold reduction) cell lines (P<0.001, P<0.05 and P<0.001 respectively).

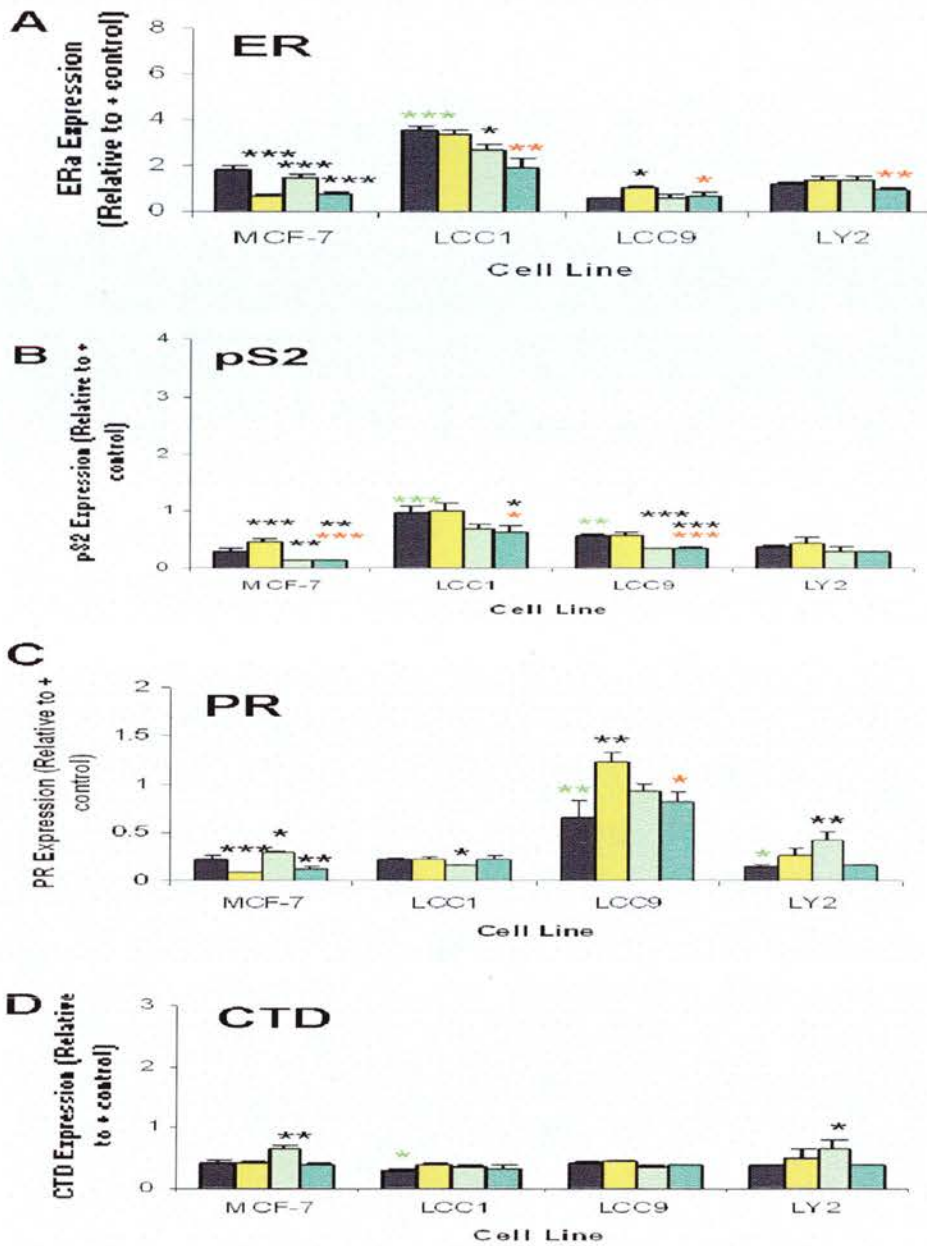


Figure 5.15 The effect of UO126 in the presence and absence of TGFα on mRNA expression levels of E₂-responsive genes. Histograms of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 10μM UO126 for 30 min and then treated with control media or TGFα (1nM). Cells were treated with control media (■), 10μM UO126 (□), TGFα (■) or TGFα+ 10μM UO216 (■) for 24h, where data are in triplicate +/-SD. MCF-7 cells treated with E₂ were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in that cell line, those in green represent statistical significance between MCF-7 cells and the resistant cell lines and those in red represent any statistical significant changes post-treatment with UO126 in each cell line. Histograms are representative of 1 of 3 independent experiments.

Figure 5.15C shows the relative **PR** mRNA expression of MCF-7, LCC1, LCC9 and LY2 cell lines post treatment with UO126 in the presence and absence of TGF α . UO126 alone elevated PR mRNA values in the MCF-7 and LY2 cell lines, with significance values of $P < 0.05$ and $P < 0.01$ respectively. The reduction in PR mRNA expression was elevated from 0.08 to 0.1 arbitrary expression units when MCF-7 cells were treated with UO126 combined with TGF α compared to UO126 alone (~1.5-fold increase, $P < 0.05$). UO126 reduced PR expression treated with TGF α in LCC1 cells by ~1.3-fold ($P < 0.05$). TGF α combined with UO126 did not differ from TGF α treatment alone in LCC1 or LY2 cells. As previously observed in chapter 3, PR expression was significantly elevated in control LCC9 cells compared to MCF-7 cells ($P < 0.01$). TGF α increased LCC9 PR expression by ~1.9-fold ($P < 0.01$), which was significantly reduced by UO126 pre-treatment by ~1.5-fold ($P < 0.05$). This reversal was not complete, and remained above the elevated control PR expression.

Figure 5.15D shows the effect of UO126 in the presence and absence of TGF α on **CTD** mRNA expression in this model of resistance. All cell lines were unaffected by UO126 or TGF α alone or combination of UO126 plus TGF α , with the exception of the MCF-7 and LY2 cell lines which had elevated CTD expression of ~1.6-fold ($P < 0.01$) and ~1.8-fold ($P < 0.05$) respectively in the presence of UO126 alone.

5. 1. 3. The effect of PLC γ inhibitor U-73122 in resistant cell lines versus parental MCF-7 cell lines

U-73122 is an amino steroid PLC γ inhibitor which in turn inhibits PLC-dependent processes (Thompson *et al*, 1991; Bleasdale *et al*, 1990; Smith *et al*, 1990). It has been used to link the PLC γ -mediated motility pathway with tumour invasion in several cancers, including breast (Kassis *et al*, 1999) and to elucidate other roles of PLC γ in tumour development and progression. U-73122 was used in cytotoxicity studies here as PLC γ has previously been linked with the erbB receptor pathway (Anderson *et al*, 1990) and development of resistance and hence may play a part in the development of oestrogen and antioestrogen resistance in this particular model

5. 1. 3. 1. Growth inhibitory response

Figure 5.16 illustrates the growth inhibitory effect of 0-20 μ M U-73122 in MCF-7, LCC1, LCC9 and LY2 cell lines in the presence and absence of E₂, TGF α and HRG β . Proliferation assays were performed as detailed in earlier inhibitor studies, where cells were treated on day 0 and treatment halted on day 3. Data were plotted as a mean of 6 OD values \pm SD for treated values and as a mean of 12 OD values \pm SD for untreated cells.

All cell lines proliferated in response to ligand treatment as reported earlier (chapter 3, figures 5.1, 5.2 and 5.9). U-73122 appeared to increase cell proliferation in a manner similar to that of E₂ at lower concentrations, with MCF-7 control (basal) growth elevated to ~203% day 3 growth (relative to day 0 control) from ~130% with 1 μ M (P<0.001). Elevated proliferation was only decreased lower than the initial untreated control level (~114%) with 7.5 μ M U-73122. This reduction was significant compared to the increase produced with 1 μ M U-73122 (P<0.001). Only 15 μ M and 20 μ M U-73122 concentrations produced significant reductions of the initial control growth of MCF-7 cells (P<0.001). The IC₅₀ values produced by U-73122 were ~16 μ M and ~18 μ M for MCF-7 cells treated with control media and E₂ in combination with the inhibitor.

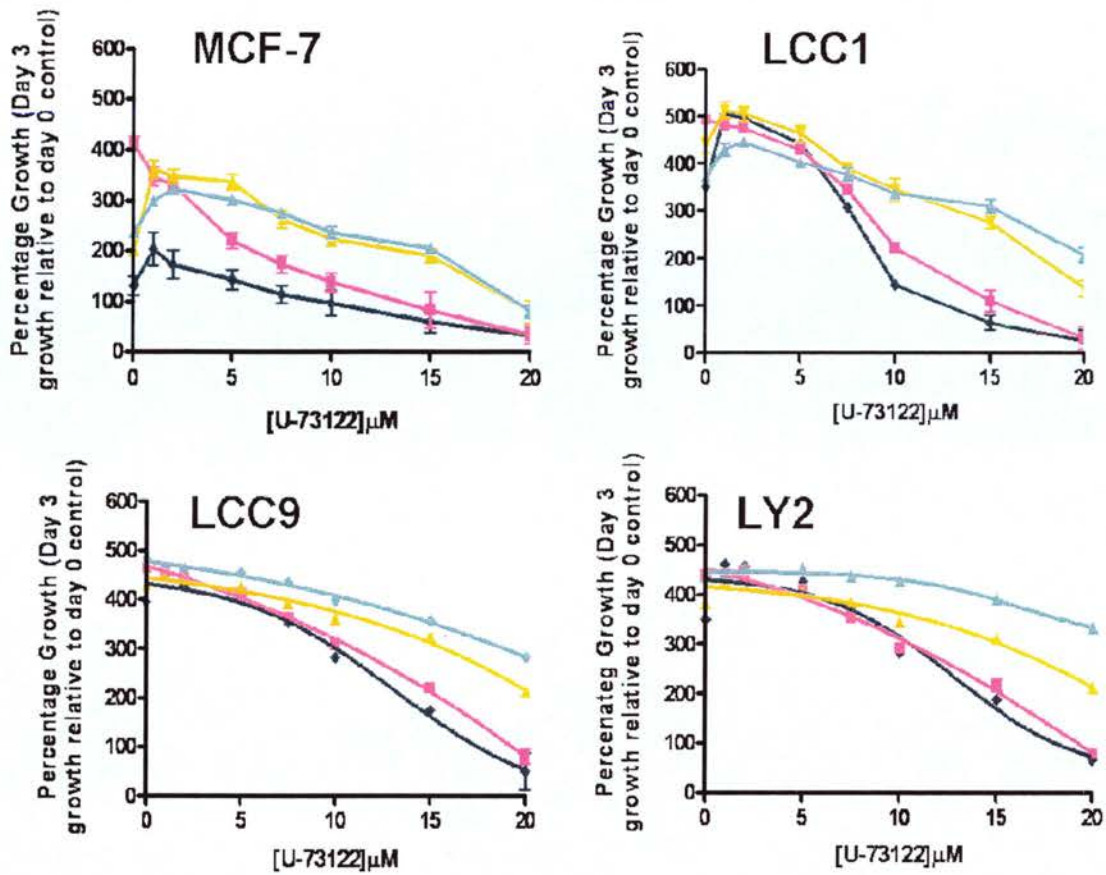


Figure 5.16 Growth inhibitory effect of U-73122 in MCF-7 cells versus resistant breast cancer cell lines. All cells were charcoal stripped 24h prior to treatment and were then treated with a concentration range of U-73122 spanning 0-20 μ M plus control media (\blacklozenge), E₂ (1nM) (\blacksquare), TGF α (1nM) (\blacktriangle) or HRG β (1nM) (\blacktriangle). Cells were treated on day 0 and treatment halted on day 3. Data were plotted as a mean of 6 OD values \pm SD for treated values and as a mean of 12 OD values \pm SD for untreated cells. Graphs are representative of 1 of 3 independent experiments.

The maximum concentration of 20 μ M U-73122 reduced MCF-7 proliferation to \sim 80% (day 3 growth relative to day 0 control) for both TGF α and HRG β treatments in contrast to \sim 31% and \sim 33% for control and E₂ treated cells. Similar trends were observed in all three resistant cell lines, with the two growth factors increasing the concentration of U-73122 required to reduce day 3 proliferation (relative to day 0 control). HRG β was more effective at increasing the concentration required for equivalent inhibition with U-73122 alone. The concentration required to inhibit proliferation back to 50% of day 0 growth in LCC1 cells treated with control media was very similar to that required in MCF-7 cells, with values of \sim 16 μ M and \sim 19 μ M respectively. An initial peak was observed with all combinations of treatment

involving U-73122 in E₂-sensitive MCF-7 and LCC1 cell lines suggesting some stimulatory effect of this inhibitor, which will be discussed in section 5. 2. 3. Treatment of the LCC1 cell line with TGF α and HRG β , in agreement with data obtained for the parental cell line, increased the concentration of U-73122 required to reduce cell proliferation to the same extent as control. The reductions in proliferation were to values of ~137% and ~207% for TGF α and HRG β treated LCC1 cells respectively, which are elevated values compared to those produced for MCF-7 cells.

LCC9 cells were not growth stimulated by any ligand or U-73122. ~20 μ M U-73122 alone reduced proliferation in LCC9 cells to 50% of day 0 control growth. LCC9 cells treated with E₂, TGF α and HRG β were growth inhibited but 20 μ M U-73122 was only able to reduce day 3 proliferation (relative to day 0 control) to ~79%, ~212% and ~281% for cells treated with E₂, TGF α and HRG β respectively. Moreover, these reductions were still significant compared to untreated cells (all P<0.001). 20 μ M U-73122 reduced day 3 growth (relative today 0 control) of LY2 cells treated with control media to ~66%, ~79% with E₂, ~212% with TGF α and ~332% with HRG β . All reductions were significant compared to cells that were not treated with U-73122 (P<0.001).

The combination of U-73122 with either growth factor prevented the fall in cell proliferation as observed with the same concentration of U-73122 in any cell line treated with E₂ or U-73122 alone. Thus, growth factors 'protected' the cells from the growth inhibitory action of U-73122, while U-73122 alone acted as an agonist at low concentrations in E₂-sensitive MCF-7 and LCC1 cell lines. The agonist effect of U-73122 alone was not observed in LCC9 or LY2 cell lines at any concentration.

5. 1. 3. 2. The effect of U-73122 on mRNA expression levels of E₂-responsive genes in this model of resistance

The proliferation assay data implicated U-73122 increased cell proliferation and thus acted as an agonist in the E₂-sensitive MCF-7 and LCC1 cell lines. MCF-7, LCC1, LCC9 and LY2 cell lines were treated with U-73122 to investigate any differential effects on E₂-responsive genes.

5. 1. 3. 2. 1. E₂ response

Figure 5.17 shows the effect of 5µM U-73122 in the presence and absence of E₂ on mRNA expression of E₂-responsive genes. Histograms representing the effect of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines were obtained. All cells were charcoal stripped 48h prior to treatment and were then pre-treated with 5µM U-73122 for 1h and then treated with control media or E₂ (1nM) or E₂ + U-73122 (1nM + 5µM) for 24h. Data are in triplicate +/-SD and MCF-7 cells treated with E₂ were used as the positive control.

The basal and E₂ treated mRNA levels in all four genes of all four cell lines tested are described in section 5. 1. 1. 5. MCF-7 cell line **ER** mRNA expression level remained unchanged with U-73122 alone compared to control. The reduction in ER expression was decreased marginally by ~1.1-fold by pre-treatment of MCF-7 cells with U-73122, but the final value was still deemed significant compared to control treated cells (P<0.05) (figure 5.17A). U-73122 alone also significantly reduced ER mRNA expression in LCC1 cells (P<0.001). LCC1 ER mRNA levels behaved in a similar manner to the parental line when treated with U-73122 plus E₂, with a minor increase in the ER expression (from ~2.3 to ~2.6 arbitrary relative expression units) with the combined treatment, but this remained an extremely significant reduction in ER mRNA (P<0.001).

Treatment of LCC9 cells with either E₂ or U-73122 alone reduced ER mRNA expression by ~0.5 and ~0.1-fold respectively, but these reductions were not found to be significant. However, the expression was further reduced and became significant when treated with the combination treatment of U-73122 and E₂ compared to control

treated cells ($P < 0.001$) or LCC9 cells treated with E_2 alone ($P < 0.01$). U-73122 significantly reduced ER mRNA levels in LY2 cells by ~ 0.5 -fold ($P < 0.001$). This reduction was not as great as that produced by E_2 (~ 0.75 -fold, $P < 0.001$). However, when combined, U-73122 appeared to significantly reverse some of the reduction induced by E_2 back to the level ER mRNA expression achieved with U-73122 alone ($P < 0.05$).

pS2 mRNA expression profiles are shown in figure 5.17B. E_2 , U-73122 alone and the combined treatment significantly elevated pS2 mRNA expression compared to the basal levels in MCF-7 cells ($P < 0.001$). The increases in mRNA induced by U-73122 and U-73122 plus E_2 were greater than that produced by E_2 alone with fold inductions of ~ 20 -fold in both lines compared to ~ 16 -fold for E_2 alone, but not significantly so. pS2 expression was also elevated in LCC1 cells by E_2 , U-73122 alone and $E_2 + U-73122$, with fold inductions of ~ 2.5 -fold ($P < 0.001$) with all treatments. The combined $E_2 + U-73122$ treatment increased mRNA levels to a greater extent than either agent alone, but the value was not significantly different. The pS2 mRNA profile for the LCC9 and LY2 cell lines showed that again the combined treatment increased pS2 expression further than either agent alone. E_2 increased LCC9 cell basal pS2 expression by ~ 0.9 -fold, U-73122 increased expression by ~ 1.4 -fold and $E_2 + U-73122$ by ~ 1.4 -fold. Only the latter treatment was found to be a significant increase ($P < 0.01$). The pS2 mRNA values of LY2 cells were also increased with E_2 and U-73122 alone (both $P < 0.01$).

PR mRNA expression is shown in figure 5.17C and was elevated with E_2 , U-73122 alone and $E_2 + U-73122$ in MCF-7 and LCC1 cells ($P < 0.001$). The combined treatment appeared to increase PR mRNA values above and beyond that of either E_2 or U-73122 alone in MCF-7 cells, but the increase was not significantly above that of either agent alone ($P > 0.05$). U-73122 alone was the only treatment which increased PR mRNA expression in LCC9 cells significantly ($P < 0.05$). LY2 cell PR mRNA expression was elevated by E_2 , U-73122 (both $P < 0.05$) and more so with $E_2 + U-73122$ treatment ($P < 0.01$) compared to control.

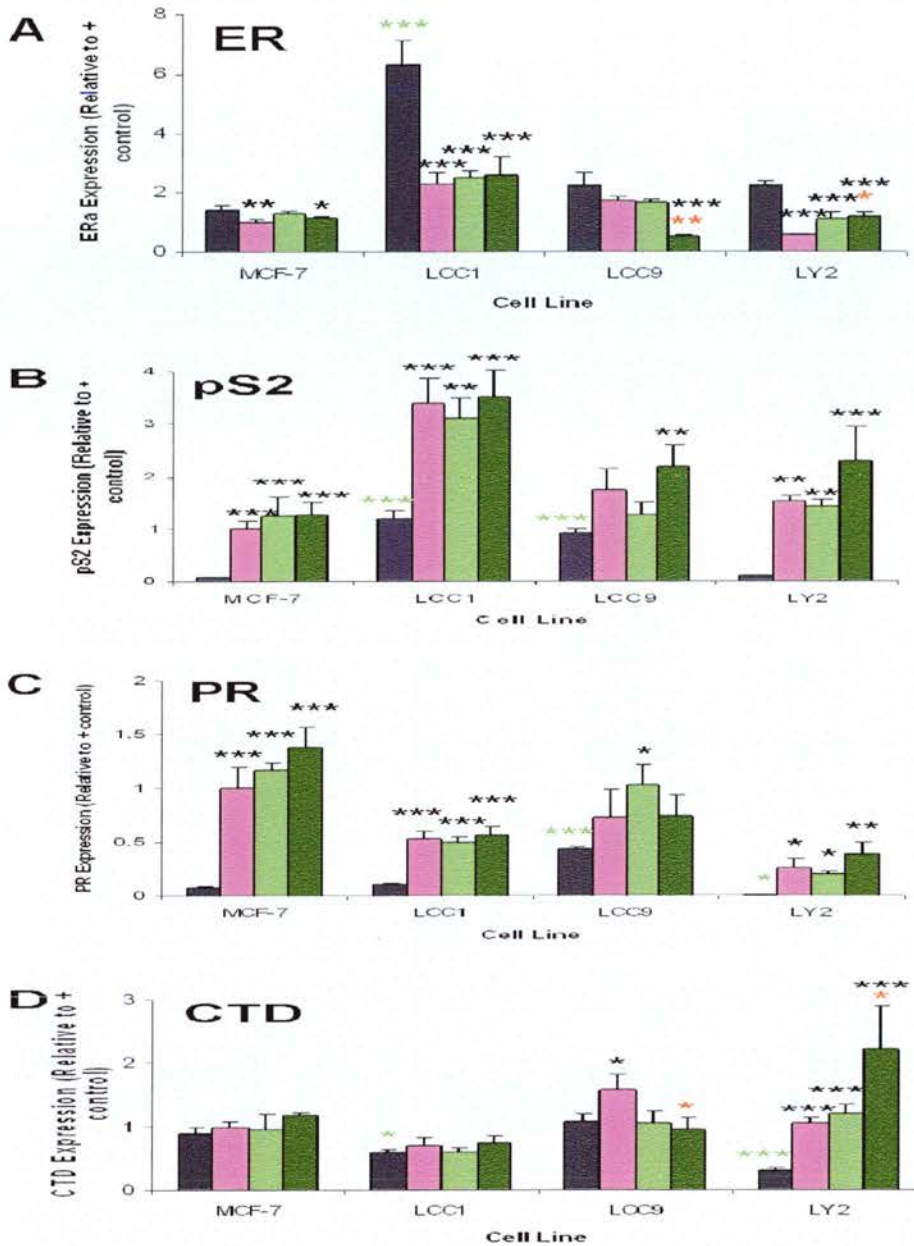


Figure 5.17 The effect of U-73122 in the presence and absence of E₂ on mRNA expression levels of E₂-responsive genes. Histograms of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines where all cells were charcoal stripped 48h prior to treatment and where applicable were then pre-treated with 5μM U-73122 for 1h and then treated with control media or E₂ (1nM). Cells were treated with control media (■), 5μM U-73122 (■), E₂ (■) or E₂ + 5μM U-73122 (■) for 24h, where data are in triplicate +/-SD. MCF-7 cells treated with E₂ were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in that cell line, those in green represent statistical significance between MCF-7 cells and the resistant cell lines and those in red represent any statistically significant changes post-treatment with U-73122 in each cell line. Histograms are representative of 1 of 3 independent experiments.

CTD mRNA expression was not significantly increased in MCF-7 cells irrespective of treatment (figure 5.17D). E₂ + U-73122 did increase CTD expression from ~0.9 to ~1.2 arbitrary relative expression units, but this was not a significant increase. LCC1 cells were also unaffected by any treatment with E₂ or U-73122. The CTD expression was elevated in LCC9 cells with E₂ treatment by ~0.4-fold (P<0.05), which was completely abolished by U-73122 pre-treatment (P<0.05). E₂ and U-73122 alone increased CTD expression in LY2 cells by ~1.4 and ~2-fold respectively, which increased to ~5-fold when the inhibitor and ligand were combined (P<0.001 compared to control, P<0.05 compared to E₂ or U-73122 alone).

5. 1. 3. 2. 2. TGF α response

U-73122 was investigated in combination with the growth factor TGF α on the mRNA expression levels of a panel of E₂-responsive genes (figure 5.18). Histograms representing the effect of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines were obtained. All cells were charcoal stripped 48h prior to treatment and were then pre-treated with 5 μ M U-73122 for 1h and then treated with control media or TGF α (1nM) or TGF α + U-73122 (1nM + 5 μ M) for 24h. Data are in triplicate +/-SD and MCF-7 cells treated with E₂ were used as the positive control.

The basal and TGF α treated mRNA levels of all four genes in all four cell lines tested are described in section 5. 1. 1. 5. **ER** mRNA was reduced further upon the addition of U-73122 prior to TGF α compared to TGF α alone in MCF-7 cells (~0.7 to ~0.4 arbitrary relative expression units). This reduction was not found to be any more significant than TGF α alone (P<0.05). LCC1 cell ER mRNA expression was not affected by TGF α , but the growth factor reduced ER mRNA expression beyond that of U-73122 alone (~1-fold reduction (P<0.001) versus ~0.5-fold reduction (P<0.01) respectively). In the LCC9 cell line, ER expression was increased by ~0.3-fold with U-73122 pre-treatment versus control expression (P<0.05). 5 μ M U-73122 almost completely abolished any increase observed with TGF α treatment from ~1 to ~0.64 arbitrary relative expression units.

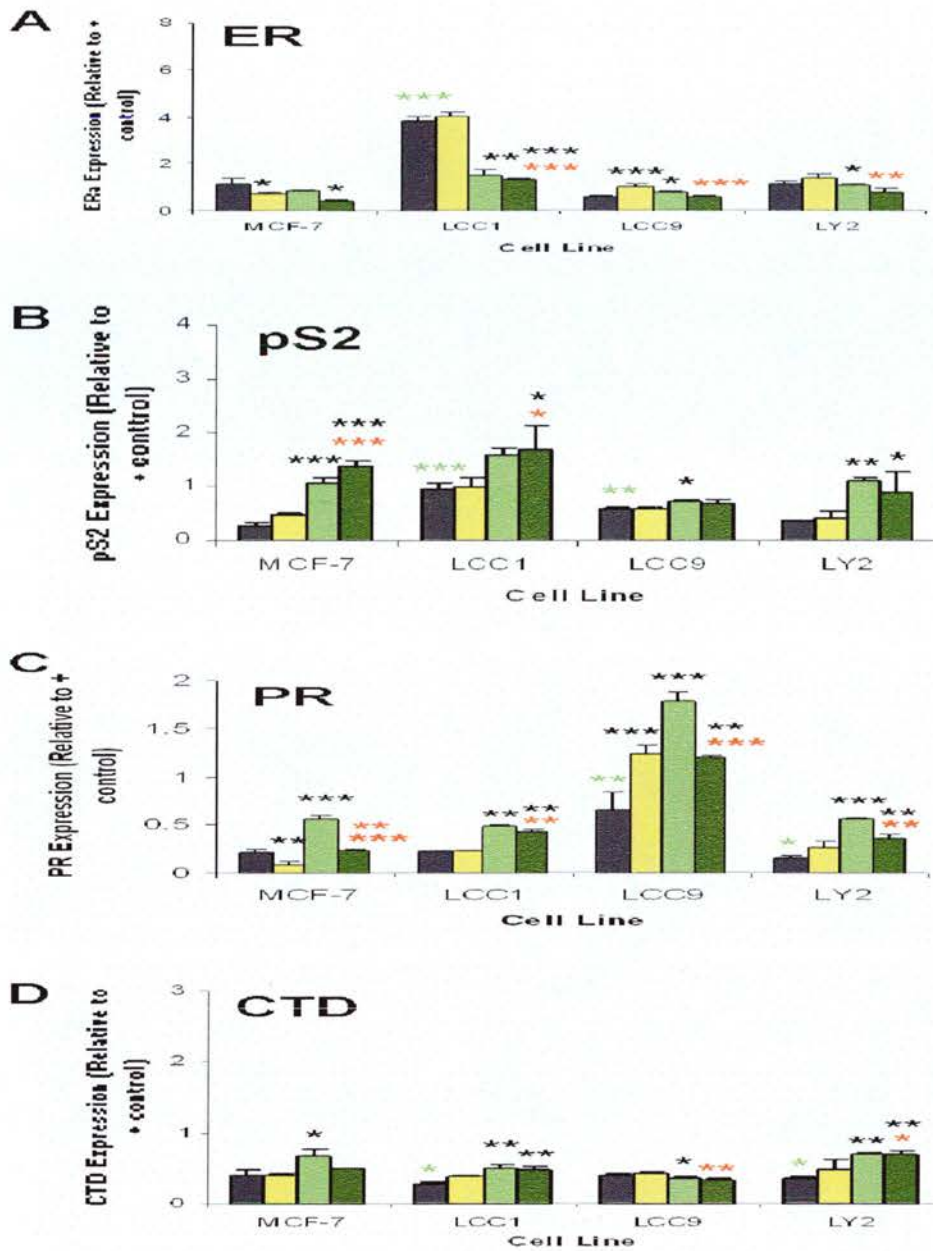


Figure 5.18 The effect of U-73122 in the presence and absence of TGFα on mRNA expression levels of E₂-responsive genes. Histograms of ER (A), pS2 (B), PR (C) and CTD (D) expression levels of all cell lines where all cells were charcoal stripped 48h prior to treatment and where applicable were then pre-treated with 5μM U-73122 for 1h and then treated with control media or TGFα (1nM). Cells were treated with control media (■), 5μM U-73122 (■), TGFα (■) or TGFα+ 5μM U-73122 (■) for 24h, where data are in triplicate +/-SD. MCF-7 cells treated with E₂ were used as the positive control. ANOVA test: * = P<0.05, ** = P<0.01 and *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in that cell line, those in green represent statistical significance between MCF-7 cells and the resistant cell lines and those in red represent any statistical significant changes post-treatment with U-73122 in each cell line. Red bold asterisks represent statistical significance between U-73122 and U-73122+TGFα in each cell line. Histograms are representative of 1 of 3 independent experiments.

U-73122 alone reduced LY2 cell line ER expression compared to basal levels ($P < 0.05$), while TGF α + U-73122 reduced ER expression further than U-73122 alone ($P < 0.01$).

Figure 5.18B illustrates the effect of U-73122 in the presence and absence of TGF α on mRNA expression levels of the **pS2** E₂-responsive gene. pS2 expression was elevated significantly in MCF-7 cells treated with U-73122 alone and even further in combination with TGF α relative to control and TGF α treated cells (both $P < 0.001$). U-73122 was unable to significantly increase pS2 expression alone in LCC1 cells (~0.6-fold increase), but when combined with TGF α this increase was ~0.7-fold which was found to be significant compared to basal and TGF α treated levels. TGF α , as for the E₂ treatment, did not interact with U-73122 in LCC9 cells, and the only change in pS2 expression was the increase with the inhibitor alone as described in the previous section. The combined effect of U-73122 + TGF α in the LY2 cell line was a minor reduction in pS2 mRNA expression compared to U-73122 alone (~1.1 to 0.9 arbitrary relative expression units). The effect of the growth factor and inhibitor alone are described in the previous sections.

Figure 5.18C shows **PR** mRNA expression was increased by ~1.6, ~1.2 and ~1.7-fold in MCF-7, LCC1 and LCC9 cell lines respectively with 5 μ M U-73122 pre-treatment ($P < 0.001$, $P < 0.01$ and $P < 0.001$ respectively). TGF α appeared to reverse some of the stimulation of PR expression induced by U-73122 alone, with a fold decrease of ~1.3-fold in the MCF-7 cell line ($P < 0.001$), as indicated by the three red asterisks in bold. Pre-treatment of MCF-7 cells with U-73122 prior to TGF α treatment produced a statistically significant increase ($P < 0.01$) compared to TGF α alone. LCC1 cell PR expression was also elevated by ~0.9-fold compared to TGF α alone when cells were pre-treated with U-73122 ($P < 0.01$). The combined treatment did not significantly differ to that of U-73122 alone. LCC9 cell PR expression was again elevated with TGF α (1nM) ($P < 0.01$). Pre-treatment with U-73122 significantly increased PR expression in LCC9 as mentioned earlier, and this was abolished with U-73122 treatment to levels similar to those obtained with TGF α alone. Red asterisks in bold indicate a significant reduction between U-73122 alone and U-73122 plus growth factor ($P < 0.01$) in LCC9 cells. The PR mRNA expression of the LY2 cell line was significantly raised by U-73122 pre-treatment in the presence and absence of TGF α

($P < 0.001$ and $P < 0.01$ respectively). TGF α treatment significantly reduced U-73122 elevated PR expression by ~ 0.6 -fold ($P < 0.01$).

U-73122 elevated **CTD** mRNA expression in MCF-7 cells by ~ 0.7 -fold ($P < 0.05$), ~ 0.8 -fold in LCC1 cells ($P < 0.01$) and ~ 0.9 -fold in LY2 cells ($P < 0.01$). In contrast, CTD expression was actually decreased in the LCC9 cell line by ~ 0.1 -fold with U-73122 compared to control treated cells ($P < 0.05$), which was marginally reduced further still by the addition of TGF α (total fold reduction ~ 0.2 -fold, $\sim P < 0.01$). LY2 cells pre-treated with U-73122 were subject to a raise in CTD mRNA of ~ 1.4 -fold ($P < 0.01$), which was further increased by TGF α , but not significantly compared to U-73122 alone (fold change remained ~ 0.9).

5. 1. 4. The effect of p38 MAPK inhibitor SB 203580 in resistant cell lines with elevated P-Akt expression

SB 203580 is a p38 MAPK inhibitor that specifically targets this molecule with a concentration 100-fold less than that required for inhibition of other MAP kinases (1 μ M cf. 100 μ M) (Cuenda *et al*, 1995). Gutierrez *et al* (2005) reported that tamoxifen resistant growth was associated with an increase in phospho-p38 MAPK. Therefore, SB 203580 was used to discover if the inhibition of p38 MAPK was directly related to resistance in this model.

5. 1. 4. 1 Growth inhibitory response

An SB 203580 concentration of just below ~60 μ M was required for MCF-7 cells to be inhibited to ~50% of day 0 growth under control and TGF α treatment conditions. E₂ increased cell proliferation as previously reported in this chapter and in chapter 3, and prevented this value being reached in MCF-7 cells. The maximum SB 203580 concentration of 60 μ M reduced E₂ stimulated proliferation (day 3 relative to day 0 control) by ~235% to ~69%. This reduction was deemed extremely significant (P<0.001). Reductions from ~108% to ~50% were observed with SB 203580 alone and from ~190% to ~52% with SB 203580 + TGF α (P<0.001).

MCF-7 cells treated with control media were only significantly reduced by SB 203580 concentrations higher than 50 μ M (P<0.001). Lower SB 203580 concentrations inhibited proliferation induced by E₂ and TGF α , although only to a similar final level as control treated cells in the case of TGF α treatment. 5 μ M SB 203580 reduced MCF-7 cell E₂ and TGF α -induced proliferation by ~40% (P<0.01) and ~30% (P<0.05) respectively. As previously stated, SB 203580 did not significantly affect MCF-7 control proliferation at this concentration, with a negligible reduction of only ~1%.

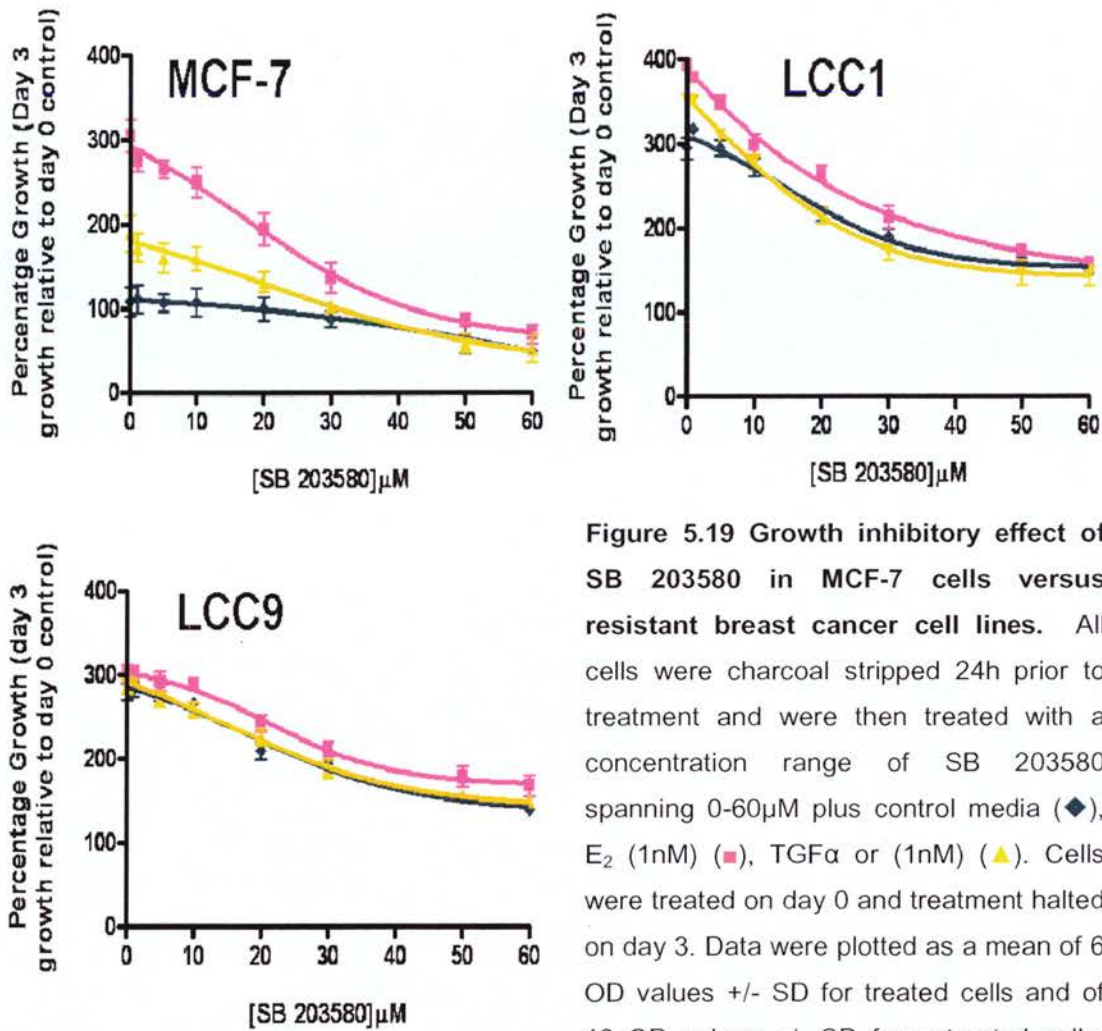


Figure 5.19 Growth inhibitory effect of SB 203580 in MCF-7 cells versus resistant breast cancer cell lines. All cells were charcoal stripped 24h prior to treatment and were then treated with a concentration range of SB 203580 spanning 0-60 μM plus control media (◆), E₂ (1nM) (■), TGF α or (1nM) (▲). Cells were treated on day 0 and treatment halted on day 3. Data were plotted as a mean of 6 OD values \pm SD for treated cells and of 12 OD values \pm SD for untreated cells. Graphs are representative of 1 of 3 independent experiments.

LCC1 cells proliferated in the absence of stimuli as previously reported in this and other chapters ($\sim 300\%$ day 3 growth relative to day 0 in LCC1 cells compared to $\sim 100\%$ observed in MCF-7 cells on both days 0 and 3). Day 3 growth was not found to be reduced with 5 μM SB 203580 alone, but was found to be reduced from $\sim 394\%$ to 349% in combination with E₂ and from $\sim 352\%$ and $\sim 310\%$ with TGF α . The LCC1 graph in 5.19 shows the SB 203580 inhibitory curves follow a similar trend, with E₂ having a slightly stimulatory and thus ‘protective’ effect which is consistent with the growth data for this cell line.

LCC9 cells proliferated in the absence of growth stimuli as before. E₂ treatment did not significantly alter the inhibitory action of SB 203580 compared to SB 203580 alone between 1 μM and 5 μM , the more therapeutic range of SB 203580. Some

protective effect was observed at 10 μ M and with higher concentrations, which is interesting to note as LCC9 cells are insensitive to the growth stimulatory action of E₂. TGF α did not significantly alter the inhibitory action of SB 203580 at any concentration of the inhibitor. A higher concentration of ~50 μ M was required to inhibit LCC9 proliferation by approximately half of day 3 proliferation compared to ~30 μ M in LCC1 cells. However, the level to which proliferation was reduced to was similar in both cell lines (~147% and ~140% in LCC1 and LCC9 cell lines). This suggests the final anti-proliferative effect is similar in both resistant cell lines.

In general, SB 203580 did not appear to be as efficacious as LY 294402 or UO126 at reducing the proliferation of any of the cell lines. MCF-7 cells treated with SB 203580 alone were not significantly inhibited at any concentration, while E₂ and TGF α -enhanced cell proliferation was reduced significantly. This suggests SB 203580 was only effective at reducing stimulated cells, rather than basal levels of proliferation. The trends of each curve are dissimilar to that of control, implying E₂ and TGF α are not 'protecting' MCF-7 cells as for LY 294002 or UO126 inhibitors. Exceptions did occur at the higher end of the concentrations administered, where E₂ did exert some preservative qualities. These were not considered to be relevant however as these concentrations (50 μ M and 60 μ M) are well outside the therapeutic window (Davies *et al*, 2000). Also, neither of the resistant cell line proliferation was reduced to the initial MCF-7 100% growth, with the growth remaining elevated compared to that of the resistant cell treated with any of the other inhibitors (~145% and ~140% in LCC1 and LCC9 cells respectively treated with 60 μ M SB 203580 only).

In contrast to the MCF-7 cell line, basal proliferation of both LCC1 and LCC9 cell lines was reduced in the presence of SB 203580 alone. This is consistent with the theory that SB 203580 is only targeting cells which are actively proliferating and not in a state of quiescence like the MCF-7 cell line. This inhibitor may be useful at reducing resistant cell proliferation to a certain extent, but the concentrations required to inhibit resistant cell growth by even ~100% are encroaching on the border of non-specific effects (60 μ M). Therefore, p38 MAPK may not be the only kinase inhibited and the antiproliferative effects of this drug may not be accredited to the blockade of this protein solely.

5. 2. Discussion

Oestrogen-independent proliferation has been shown to occur as a result of hyper- or supersensitivity to oestrogen through the overexpression and/or increased phosphorylation, and thus activation, of various signalling molecules including Akt and MEK and ERK (Santen *et al*, 2005; Yue *et al*, 2003; Britton *et al*, 2005; Campbell *et al*, 2001; Martin *et al*, 2005). In particular, P-Akt overexpression has been associated with oestrogen hypersensitivity (Yue *et al*, 2003) and resistance to aromatase inhibitors (and thus oestrogen deprivation) (Tokunaga *et al*, 2006), while Jelovac *et al* (2005) reported that MCF-7 cells with stable expression of aromatase, conferring resistance to the aromatase inhibitor letrozole, overexpressed P-ERK. This resistance was reversed with MEK inhibitors. Also of interest are MCF-7 cells selected for resistance to the pure anti-oestrogen ICI 182,780 which display an increase dependence on the erbB receptor/ERK signalling pathway (McClelland *et al*, 2001). This is of particular relevance in the LCC9 cell line, which is ICI 182, 780 resistant (Lykkesfeldt *et al*, 1997; Brunner *et al*, 1997).

Therefore, resistance to anti-hormonal therapies such as pure ER antagonists and aromatase inhibitors remains a significant clinical problem and the signalling pathways by which it develops are poorly understood. The following section will discuss the role of differential signalling and the effect inhibiting these proteins has on cellular proliferation. It was hypothesised that inhibition of molecules which are overexpressed such as Akt would produce a differential form of inhibition in the resistant cell lines compared to the parental MCF-7 cell line.

5. 2. 1. Akt and LY294002 inhibition of cell proliferation via the PI3-Kinase pathway

Elevated levels of P-Akt have been implicated in the development of resistance to both tamoxifen and ICI 182 780 in breast cancer *in vitro* (Lykkesfeldt *et al*, 2005) and to tamoxifen in clinical breast cancer (Kirkegaard *et al*, 2005). Chapter 4 showed that Akt was constitutively phosphorylated under basal conditions in the resistant cell lines which is consistent with studies by Clark *et al* (2002) and Campbell *et al* (2001). LY 294002 was utilised to block PI3-K and subsequently Akt, a downstream effector of PI3-K.

LY 294002 did not affect the basal P-Akt levels of the parental cell line, consistent with studies by Gaben *et al* (2004), but did reduce TGF α -enhanced P-Akt which is in agreement with studies by Monno *et al* (2000). LY 294002 reduced TGF α -enhanced P-Akt in a concentration-dependent manner, which is again consistent with published results using this inhibitor (Moelling *et al*, 2002). LY 294002 did not alter P-ERK expression in MCF-7 or the resistant cell lines, which is consistent with data reported by Martin *et al* (2003), showing LY 294002 is specifically inhibiting the PI3-K/Akt pathway rather than the MEK/ERK pathway.

LY 294002 inhibited P-Akt enhanced by TGF α and elevated P-Akt basal levels to a greater extent in the resistant cell lines than MCF-7 cells, in particular the LCC9 cell line, which is consistent with this theory and a published study by Yue *et al* (2003). Yue *et al* (2003) reported that their LTED MCF-7 cell line was more sensitive to the inhibitory effects of LY 294002 than the parental MCF-7 cell line. This study confirms the greater reduction in P-Akt expression brought about by LY 294002 treatment observed in the LCC- cells compared to the MCF-7 cell line. The LTED cell line developed by Yue *et al* (2003) is valid for comparison as this cell line also expressed constitutively phosphorylated Akt. A study using other Tam-R MCF-7 cells also supports these observations (Jordan *et al*, 2004).

The greater inhibition of P-Akt observed in the resistant cell lines led to speculation that the resistant cell lines would be more sensitive to the anti-proliferative nature of LY 294002 than in the MCF-7 cell line. This theory is supported by other studies. For example, Lykkesfeldt *et al* (2005) reported that inhibition of Akt phosphorylation by another PI3-K inhibitor wortmannin or the Akt inhibitor SH-6 resulted in a more pronounced growth inhibitory effect on the anti-oestrogen-resistant cells compared with the parental cells. This implicated Akt signalling was playing a role in their resistant cell line models. Subsequently, it was hypothesised that elevated levels of P-Akt would increase the sensitivity of the resistant cell line proliferation in the model used here to the PI3-kinase inhibitor LY 294002.

However, contrary to this, the resistant cell lines were not more sensitive to LY 294002 than the parental cell line. LY 294002 was unable to reduce resistant cell proliferation to the same extent as that of the parental MCF-7 cell line treated with the

same concentration of inhibitor. Similarly, the reduction in constitutively activated Akt in the resistant cell lines did not correspond with a reduction in proliferation beyond that observed in MCF-7 cells treated with the same concentration of LY 294002. It is interesting to note that both the LCC1 and LCC9 resistant cell lines were inhibited to a similar degree in the presence of LY 294002. The inhibition of the latter cell line with LY 294002 is consistent with a study in MCF-7 cells transfected with AND-34, which also conferred ICI 182, 780 resistance (Felekkis *et al.*, 2005). These cells also displayed constitutively phosphorylated Akt, which was seen in the LCC9 cell line. These data are similar to those reported by Zhang *et al.* (2004). This group reported that an MCF-7 cells overexpressing erbB2, which proliferated more rapidly than MCF-7 cells, were inhibited back to a similar level as the MCF-7 cell line in the presence of LY 294002. The slightly elevated concentration required to inhibit proliferation in this investigation is more than likely due to the differences between the cell lines and the protocols which were followed in their derivation.

LY 294002 is a specific competitive inhibitor of PI3-K (Vlahos *et al.*, 1994) and as such this may account for the 'protective' effect of HRG β . LY 294002 was also observed to be more efficacious than the MEK inhibitor UO126 at inhibiting cell proliferation which is also consistent with studies by Hu *et al.* (2001) and Lobenhofer *et al.* (2000) in MCF-7 cells. This suggests all the cells, including the resistant cell lines, are more dependent on the PI3-K/Akt than the MEK/ERK signalling pathway for proliferation.

The expression studies showed that LY 294002 alone did not alter ER, pS2 or PR mRNA levels in any cell line, which is consistent with a study in MCF-7 cells by Stoica *et al.* (2003) (expression studies are summarised in Table 5.1). This group found LY 294002 partly reversed the effects induced by E₂ in MCF-7 cells, which was not observed here. However, the data collected by Stoica *et al.* (2003) was after only 6h treatment rather than 24h. Therefore, this indicates any changes induced by LY 294002 treatment may only be observed at an earlier time point.

The specific Akt inhibitor **NL-71-101** also inhibited cellular proliferation in all cell lines in the presence and absence of growth factors, consistent with published data

(Qiang *et al*, 2004). However, the absence of a profound difference between the MCF-7 and resistant cell lines sensitivity to NL-71-101 indicate that substantial additional work is required to determine if these data are informative. Qiang *et al* (2004) utilised 20 μ M in their proliferation inhibition studies, a concentration equivalent to the maximum dose utilised in this investigation. Perhaps an increased concentration may prove to have a greater anti-proliferative effect in the MCF-7 and resistant cell lines. Interestingly, this group reported that this inhibitor was unable to abrogate or reduce the level of P-Akt, but did reduce the activation of the downstream target of Akt, FKHRL1, in a concentration-dependent manner.

5.2.1.1. The role of 'Raf-Akt' crosstalk in this model

As previously stated, LY 294002 did not alter P-ERK1/II expression in MCF-7 or the resistant cell lines, which is consistent with data reported by Gaben *et al* (2004) and Martin *et al* (2003). However, LY 294002 did reduce P-MEK in a concentration-dependent manner in all cell lines. Growth-factor enhanced P-MEK was reduced to a greater extent in the LCC1 cell line indicating that somehow as P-Akt is reduced so is P-MEK. Moreover, as LY 294002 specifically targets PI3-K and its downstream effectors this effect was not expected. Thus, it was hypothesised that some form of crosstalk may be occurring between the PI3-K/Akt and MEK/ERK pathways in all cell lines.

Moelling *et al* (2002) and Zimmerman and Moelling (1999) documented the existence of this crosstalk between the PI3-K/Akt and Ras/Raf/MEK/ERK pathways in the MCF-7 cell line. Inactivation of the crosstalk between the pathways in the MCF-7 cell line was stated to 'switch the biological response from proliferation to cell cycle arrest'. The 'Raf-Akt' crosstalk involves the inhibition of Raf via Akt when cells are stimulated with a high dose of growth factor. Low doses of growth factor did not induce this crosstalk. The presence of elevated levels of Akt in the resistant cell lines suggests that these cell lines may be able to inhibit cell cycle arrest activated by the MEK/ERK pathway via Raf-1 inhibition, even in the absence of high concentrations of growth factor. This would account for the elevated basal proliferation as the signalling via the MEK/ERK growth arrest pathway is dampened, allowing more cells to proliferate or survive (figure 5.20). Inhibition of P-Akt with LY 294002 has been shown to abrogate this crosstalk allowing growth arrest and

differentiation to resume. As LY 294002 abrogated P-Akt to a greater extent in the resistant cell lines, this implies any crosstalk occurring due to elevated levels would also be abolished, again recommencing growth arrest and differentiation.

No elevation in P-ERKI/II expression was seen in any of the cell lines upon LY 294002 treatment. Moelling *et al* (2002) did observe an increase in P-ERKI/II but their studies were performed using a higher concentration of inhibitor (20µM vs 5µM) and the increase was only observed at an earlier time point than the 15 min monitored here.

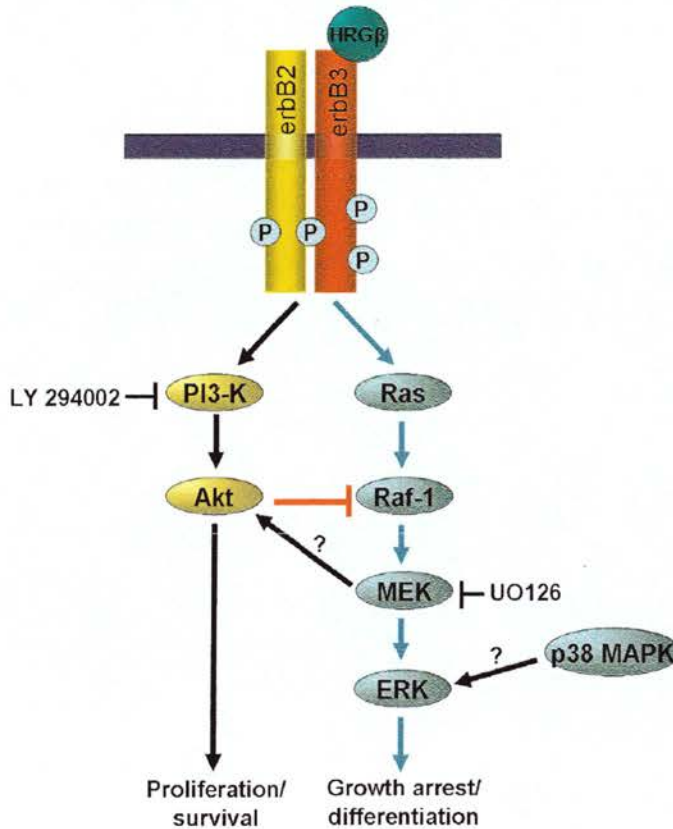


Figure 5.20 Proposed model for the Akt-Raf and MEK-Akt interaction in MCF-7 and resistant breast cancer cells. In the resistant cell lines constitutively phosphorylated Akt phosphorylates Raf which may in turn lead to ‘cross-talk’ and inhibition of the Ras/Raf/MEK/ERK cascade and proliferation (red bar between Akt and Raf-1). This may occur in the presence or absence of ligand stimulation (HRGβ shown as an example with relevant erbB heterodimer). LY294002 unblocks the ‘cross-talk’ and allows Raf to induce growth arrest (blue arrows). Moelling *et al* (2002) first documented this cross-talk and that low growth factor concentration did not induce ‘cross-talk’ in MCF-7 cells thus signalling occurred via both PI3-K/Akt and Raf/MEK/ERK pathways. Elevated levels of P-MEK occurred in conjunction with UO126 inhibition and an increase in P-Akt expression suggesting some form of ‘survival’ feedback on the PI3-K/Akt pathway (arrow between MEK and Akt). The similar levels of P-ERKI/II in the resistant cell lines compared to the parental MCF-7 cell line suggest input from another pathway, perhaps p38 MAPK, which has been implicated in the activated of other ERK family members (Zimmermann *et al*, 2001).

Moelling *et al* (2002) observed levels were not dissimilar to basal P-ERK1/II expression at later time points, which would be consistent with data reported in this chapter. According to the 'Raf-Akt' cross-talk theory, it should follow that P-MEK and P-ERK1/II expression would be diminished in the resistant cell lines due to the elevated levels of Akt inhibiting Raf more strongly than in the MCF-7 cell line. This theory is consistent with the reduced growth factor enhanced P-MEK seen in the resistant cell lines compared to MCF-7 cells. However, P-ERK1/II levels in the resistant cell lines were not diminished when cells were treated with growth factors. Resistant P-ERK1/II expression levels were in fact very similar to those of the parental cell line, suggesting perhaps another pathway is feeding into the activation of P-ERK1/II, such as p38 MAPK, which is known to activate ERK family members, including ERK3 (Zimmermann *et al*, 2001) which is very similar in structure to ERK1 (Cheng *et al*, 1996; Boulton *et al*, 1991)

Moelling *et al* (2002) did not document P-MEK expression. The majority of studies only assess the ability of UO126 to abrogate P-ERK1/II. In contrast to the reduced P-MEK signal observed in the resistant cell lines when treated with growth factors, UO126 was observed to increase P-MEK expression. An increase in P-MEK with UO126 treatment may be due to a blockade of downstream signalling which causes a build up of P-MEK as it is not being 'turned over' for downstream activation of ERK.

P-Akt was also elevated with UO126 treatment in a concentration-dependent manner. This implies that some form of feedback may occur at Akt as a result of elevated P-MEK signalling. This theory is consistent with a review by Chang *et al* (2003), who proposed that under a specific set of conditions, elevated Raf signalling resulted in the inactivation of transcription factors downstream of Raf, such as NF- κ B and c-Myc. This may explain Raf-induced antiproliferative responses which were observed in a number of studies. A study by Zhang *et al* (2004b) observed the opposite effect of high and low concentrations of IGF-I to those reported by Moelling *et al* (2002). However, Zhang *et al* (2004) used Lewis lung carcinoma subline H-59 cells rather than MCF-7 cells, suggesting Raf-Akt 'cross-talk' is cell line specific.

In summary of cross-talk, the mechanisms which are responsible for the stimulation of one pathway over another at varying ligand concentrations remain unclear. Navab *et al* (2001) proposed that any variation in the number of cell surface receptors and/or

downstream substrate expression may take part in these mechanisms. This is again in agreement with the theory that elevated P-Akt expression enhances 'Raf-Akt' cross-talk in the resistant cell lines. A propose model for the Raf-Akt interaction in MCF-7 and resistant breast cancer cells integrating the possible role of PLC γ in cell motility and survival (Figure 5. 21).

5. 2. 2. UO126 inhibited MEK/ ERK-driven cell proliferation

Chapter 4 showed that P-ERKI/II expression remained unaltered in the resistant cell lines, which contrasted with the reduction in P-MEK signal observed in the resistant cell lines. P-ERK overexpression has been implicated in the development of resistance against aromatase inhibitors which prevent the production of oestrogen, indicating P-ERK overexpression may be responsible for oestrogen-independent growth (Jelovac *et al*, 2005). As previously mentioned, Martin *et al* (2005) also implicated elevated P-ERK expression in the development of oestrogen-independent proliferation. In contrast to these studies, as reported in Chapter 4, P-ERKI/II overexpression did not occur in the LCC- or LY2 resistant cell lines. However, due to the growth factor-activation of the MEK/ERK pathway in the resistant cells it was hypothesised that this pathway still performed some role in their proliferation.

UO126 inhibited MCF-7 cells below the ~100% day 0 and 3 value in a concentration-dependent manner, in agreement with studies published by Yue *et al* (2003) and Lobenhofer *et al* (2000). As previously noted, UO126 was observed to be less efficacious than LY 294002 which is also consistent with the study by Lobenhofer *et al* (2000). The latter group showed that neither E₂ nor UO126 affected basal levels of P-ERKI/II, which reflected results observed at similar concentrations here.

In general, basal P-ERKI/II expression was reduced by UO126 in all cell lines in agreement with a published article in MCF-7 cells by Gaben *et al* (2004). The fact that P-ERKI/II expression of the resistant cell lines was also slightly reduced indicates these cells are behaving in the same manner as the parental cells, suggesting they still signal via the MEK/ERK pathway to some degree.

UO126 blocked the phosphorylation of P-ERKI and II in MCF-7 cells which is in agreement with previously reported data (Gaben *et al*, 2004; Martin *et al*, 2003) while

Keshamouni *et al* (2002) confirmed UO126 inhibited MCF-7 cell proliferation. Keshamouni *et al* (2002) reported that UO126 inhibited MCF-7 cells to a similar extent irrespective of treatment with E₂ or growth factor, but this study was only performed for 24h, as opposed to 72h. The additional incubation period in the current investigation may explain the elevated concentration of UO126 required to inhibit HRGβ-treated cells to the same extent as with the other treatments. Martin *et al* (2003) also noted that UO126 abrogated P-ERK1/II expression in their LTED MCF-7 cells, which proliferated in the absence of E₂, and are therefore similar in this respect to the resistant model here. However, this was a model of LTED which possessed elevated P-ERK1/II expression rather than P-Akt expression, which is the opposite of the expression pattern observed here. This could again be due to the differences between the cell models utilised. However, it is more likely that the data provided by Martin *et al* (2003) confirms that the cell lines utilised in this model are not LTED cells and the behavioural differences imply the mechanism of resistance is therefore different, which was confirmed by the signalling effects reported here. Thus, these are in fact two different models of resistance.

In contrast to LY 294002, UO126 significantly reduced basal levels of LCC9 ER mRNA expression and significantly reduced E₂-enhanced pS2 expression in all lines, except LY2 cells, where only a minor decrease was observed. The effect of UO126 on basal and E₂-inducible mRNA levels is partly consistent with published data by Martin *et al* (2003). This group found that UO126 significantly reduced basal transcriptional levels of LTED cell lines. However, no effect on E₂-mediated transcriptional activity was reported. This is probably due to the differential P-Akt and P-ERK expression profiles of the cell models utilised. The MCF-7 cell line was less sensitive to UO126 inhibition at a transcriptional level than the LTED cell lines, supporting their theory that elevated levels of P-ERK were important in the development of E₂-independent growth in the LTED model. This theory of overexpression was thought to be applicable in terms of P-Akt overexpression increasing LY 294002 sensitivity in the LCC- and LY2 cell lines at the mRNA level. This theory was refuted as LY 294002 was in general ineffective in the resistant cell lines. pS2 is often used, and was used in this investigation, as an indicator of E₂-sensitivity for growth. Human pS2 gene expression is specifically controlled at the

transcriptional level by oestrogens in the MCF-7 cell line (Masiakowski *et al*, 1982; Brown *et al*, 1984).

Rio *et al* (1987) also established pS2 as an E₂-dependent biochemical marker in breast cancer. This is interesting considering that the concentration of UO126 that inhibited pS2 did little to affect growth. This suggests that the anti-proliferative effect of UO126 via blockade of the MEK/ERK pathway and the subsequent reduction in pS2 expression is not as great as that achieved via the inhibition of Akt using LY 294002, where the reduction in LY 294002 may occur at an earlier time point (as observed by Stoica *et al*, 2003). This indicates the MEK/ERK pathway and perhaps even pS2 expression regulated by this pathway are not as important as Akt in terms of cellular proliferation in this model. Therefore, the use of the pS2 gene in this model may not be an accurate indicator of growth when observing cells treated with UO126. This supports the conclusions reached by Rio *et al* (1987), who reported that screening for pS2 gene expression may provided a basis for establishing subclasses of ER-containing tumours, but that it remained unclear as to whether the expression would provide additional information concerning the clinical unresponsiveness to hormone therapy seen in ~40% of ER-positive patients.

The UO126 data contrasts with LY 294002, which altered growth more dramatically at lower concentrations, but did not alter pS2 mRNA levels. In contrast to this investigation, Martin *et al* (2003) found LY 294002 to be similarly effective in reducing the transcriptional rates of these cell lines as UO126. The differential results are more than likely due to the increased concentration of LY 294002 utilised by Martin *et al* (2003) (50µM versus 5µM in this investigation). This may also account for the absence of the effect of LY 294002 on pS2 expression.

Observations here showed P-ERK1 and II to be low in the parental MCF-7 and resistant LCC1 and LCC9 cell lines alike, which disagrees with published data by Martin *et al* (2005), who reported P-ERK1/II to be elevated in LTED cell lines. However, the model used by Martin *et al* (2005) also displayed hypersensitivity to oestrogen and elevated basal P-S118 of ER α , neither of which were observed in the LCC- and LY2 cell lines. As previously proposed, these differential observations are more than likely due to the different cell models used.

Martin *et al* (2003) combined UO126 and LY 294002 and reported a further decrease than observed with either inhibitor alone. This suggests a combinatorial approach of targeting two or more pathways would be more efficacious than a single agent alone. Other studies, including Yue *et al* (2003) have implicated combinations of agents to be of greater benefit than the use of an individual agent alone.

5. 2. 3. Concentration dependent U-73122 oestrogen-like properties and inhibition of cell proliferation

PLC γ has been shown to play a role in motility signalling of several receptors such as IGFR-I (Bornfeldt *et al*, 1994) and is activated by many growth factors receptors, including erbB2 (DiFore *et al*, 1990; Margolis *et al*, 1990). PLC γ has been implicated in the development of breast cancer and inhibition of this protein with U-73122 has been shown to interfere with growth factor-mediated breast cancer migration and tumour invasion (Price *et al*, 1999; Xie *et al*, 1998; Chen *et al*, 1994). Therefore, U-73122 was utilised to elucidate if PLC γ played a role in the development of resistance in this model of breast cancer.

U-73122 initially stimulated cell proliferation before inhibiting the growth of all cell lines. This is consistent with the oestrogen-like actions of the metabolite of U-73122 which was documented by Cenni and Picard (1999). This action was also reflected in the mRNA expression profiles, with the inhibitor behaving in an extremely similar manner to oestrogen alone (summarised in Table 5.1). These data are again consistent with the elevated pS2 expression reported by Cenni and Picard (1999) upon U-73122 treatment. Any mRNA expression changes are therefore more likely to be due to the potential oestrogen-like action of U-73122 rather than the antagonistic properties of the inhibitor at this concentration.

Higher concentrations of U-73122 were able to inhibit proliferation (5-20 μ M), which indicates that inhibition of the PLC γ has the dominant effect on proliferation, but only at higher concentrations. All cells were 'protected' from U-73122 inhibition with growth factor treatment, in particular HRG β . This suggests that perhaps the elevated levels of P-Akt in the resistant cells are interacting with PLC γ , increasing expression or activation of this protein and thus elevating the concentration of U-73122 required to inhibit cells to the same extent as in the parental cell line.

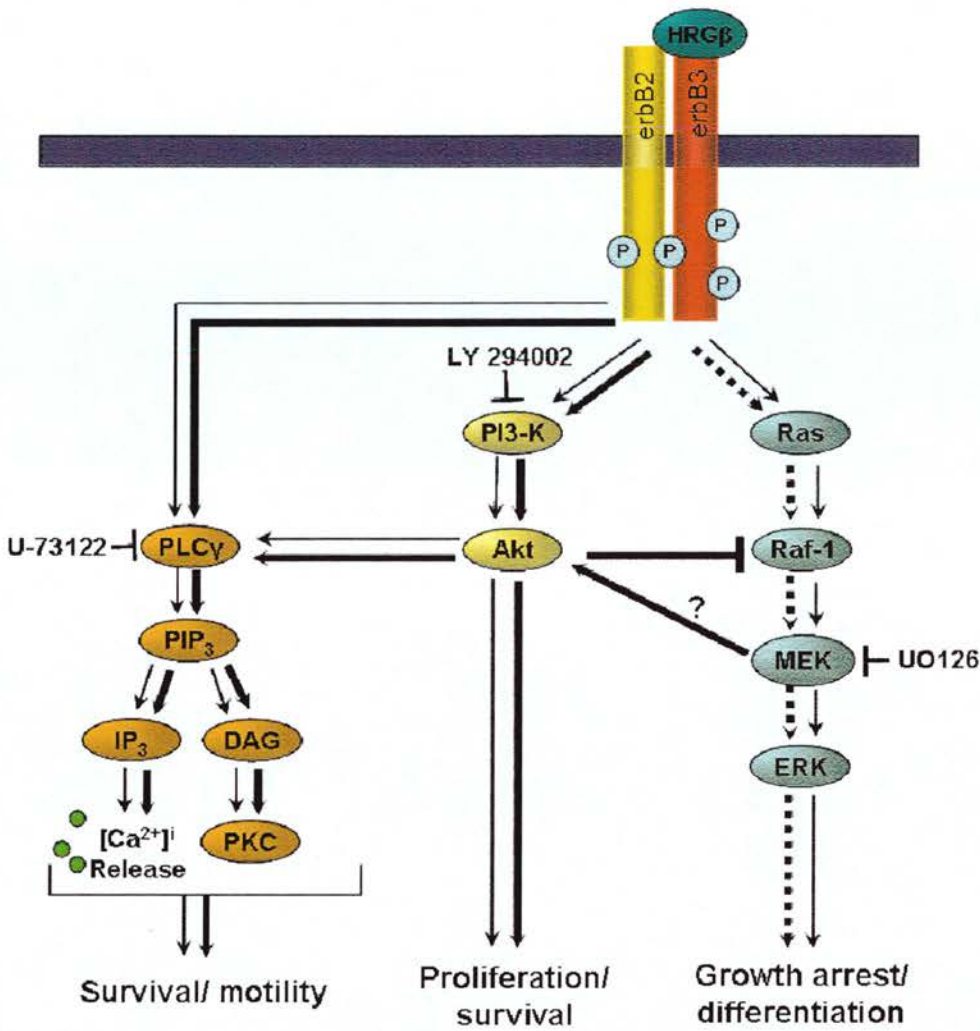


Figure 5.21 Proposed model for the Akt-Raf and MEK-Akt interaction in MCF-7 and resistant breast cancer cells integrating the possible role of PLC γ in cell motility and survival. Legend as for figure 5.20. The elevated P-Akt in the resistant cell lines may induce 'Raf-Akt' cross-talk (shown by horizontal bar between the aforementioned proteins) which may subsequently account for the reduction in P-MEK observed in the resistant cell lines (bold dashed lines). Increased signalling in the resistant cell lines is represented by bold lines. Fine lines indicate potentially weaker signalling occurring in the parental cell line. Studies by Wang *et al* (2006) support the proposed theory that overexpression of Akt in the resistant cell lines results in increased interaction with PLC γ . This increased interaction potentially increases the activation of PLC γ and thus elevates the concentration of U-73122 required to inhibit cells to the same extent as in the parental cell line. Wang *et al* (2006) also reported this interaction was growth factor-dependent, which is again consistent with the 'protective' effect of HRG β and TGF α reported in this model. The Akt-PLC γ interaction has been linked with cell survival (Deb *et al*, 2004) and motility via PIP $_3$, IP $_3$ and DAG subsequently intracellular calcium release ([Ca $^{2+}$] $_i$) and PKC respectively (pathway as described by Wang & Wang, 2003).

This theory is consistent with a study published by Wang *et al* (2006), who reported that the SH3 domain of PLC γ interacts with the proline rich motifs of Akt and that this process is dependent on growth factor stimulation. This interaction led to Akt phosphorylating PLC γ at S1248, which stimulated cell motility. The link between the Akt and PLC γ interaction and cell survival has also previously been suggested by Deb *et al* (2004).

5. 2. 4. SB 203580 did not differentially inhibit proliferation of resistant cell lines

p38 MAPK has been implicated in the development of tamoxifen-resistant proliferation (Gutierrez *et al*, 2005). p38 MAPK was also associated with the activation of AIB1 phosphorylation (Wu *et al*, 2004) which may relate to the activation of transcription factors such as AP-1 and NF κ β . Gutierrez *et al* (2005) proposed that the upregulation of growth factor signalling and subsequent activation of p38 MAPK may modulate ER α function and that of other transcription factors resulting in an increased resistance to tamoxifen. It was therefore hypothesised that this mechanism may also be responsible for the oestrogen-independence observed in the resistant cell lines. SB 203580 was utilised to investigate the effect of inhibition of p38 MAPK on the proliferation of MCF-7, LCC1 and LCC9 cell lines. In contrast to the parental cell line, basal proliferation of both LCC1 and LCC9 cell lines was reduced in the presence of SB 203580 alone. This is consistent with the theory that SB 203580 is only targeting cells which are actively proliferating and not in a state of quiescence like the MCF-7 cell line in DCSS media.

SB 203580 may be useful at reducing resistant cell proliferation to a certain extent, but the concentrations required even to inhibit resistant cell growth by half are encroaching on the border of non-specific effects (>30 μ M). Moreover, p38 MAPK activity is effectively and completely abrogated at 10 μ M in cell based assays (Davies *et al*, 2000), indicating p38 MAPK is probably not be the only kinase inhibited and the antiproliferative effects of this drug may not be accredited to the blockade of this protein solely. At higher concentrations LCK, GSK3 β , PKB α and Raf are also sensitive to the inhibitory properties of SB 203580 (Davies *et al*, 2000). Non-specific inhibition suggests any reductions in proliferation beyond 10 μ M SB 203580 may result from inhibition of downstream effectors of molecules such as Raf including the

MEK/ERK pathway. Davies *et al* (2000) concluded that caution must be taken when evaluating the data provided by inhibitors such as SB 203580 and that the use of two or more inhibitors may provide more reliable inhibition of a specific protein.

In summary, this chapter provides evidence that several pathways are involved in the mRNA expression regulation and proliferation of the MCF-7 and resistant cell lines. All cell lines appear to display Akt-dependent proliferation which is inhibited by LY 294002, which also suggests some form of 'Raf/Akt' crosstalk. UO126 inhibited E₂-regulated pS2 expression in all cell lines, indicating the involvement of the MEK/ERK pathway in mRNA expression in parental and resistant cell lines alike. In contrast, the general failure of LY 294002 to affect mRNA expression suggests the PI3-K/Akt pathway is not as crucial in the control of E₂-regulated genes as the MEK/ERK pathway.

Table 5. 1 Summary of the effects of various inhibitors on mRNA expression levels in a panel of breast cancer cell lines

Cell line/ treatment		MCF-7	LCC1	LCC9	LY2	
<i>mRNA Expression + LY 294002</i>	Control	ER α	-	-	-	-
		pS2	-	-	-	-
		PR	-	-	-	-
		CTD	-	-	-	-
	+E ₂	ER α	-	-	-	-
		pS2	-	-	-	-
		PR	minor +	-	minor +	-
		CTD	-	-	↓	↓
	+TGF α	ER α	-	-	-	-
		pS2	-	-	minor +	-
		PR	+	-	↓	minor ↓
		CTD	-	↓	-	-
<i>mRNA Expression + UO126</i>	Control	ER α	↓	-	-	-
		pS2	-	-	-	-
		PR	-	-	-	-
		CTD	-	-	-	minor +
	+E ₂	ER α	-	-	↓	minor +
		pS2	↓	↓↓	↓↓	minor ↓
		PR	minor +	minor ↓	-	-
		CTD	-	-	minor ↓	-
	+TGF α	ER α	-	↓↓	↓	↓
		pS2	↓↓	↓↓	↓↓	minor ↓
		PR	-	-	minor +	-
		CTD	-	-	-	-
<i>mRNA Expression + U-71322</i>	Control	ER α	↓	↓↓	↓	↓
		pS2	+	+	minor +	+
		PR	++	+	+	+
		CTD	-	-	-	+ / ++
	+E ₂	ER α	++	++	++	++
		pS2	↓	↓↓	↓↓↓	↓
		PR	++ / +++	+	+	+
		CTD	-	-	↓	+ / ++
	+TGF α	ER α	-	↓↓	↓↓	↓↓
		pS2	++	+	-	+
		PR	+	+	+	+
		CTD	-	+	↓	+

- = no change/effect; +=some expression/increase; ++ and +++=increase ↓=reduction

Chapter 6

Does inhibition of the erbB2 receptor overcome endocrine resistance in this model of breast cancer?

6. 1. Does the blockade of the erbB2 receptor inhibit signalling or growth responses in the resistant cell lines?

One route by which resistance to oestrogen and antioestrogens can develop in breast cancer is “cross-talk” between the oestrogen and erbB2 signalling pathways (Gee *et al*, 2005; Campbell *et al*, 2001). Overexpression of the erbB2 receptor occurs in 15-25% of human breast cancers (Revillion *et al*, 1998; Hynes & Stern, 1994) and is associated with malignant transformation and oncogenesis (Hudziak *et al*, 1987). Contrastingly, overexpression of erbB3 or erbB4 is less frequent in breast cancer and does not appear to affect prognosis (Travis *et al*, 1996). TGF α is often co-expressed with erbB1 and found in multiple breast cancer cell lines (Vyhlidal *et al*, 2000); it stimulates growth of ovarian cancer cells in culture (Crew *et al*, 1992; Morishige *et al*, 1991) supporting the resistance theory. Co-expression in ovarian cancer has also been associated with growth stimulation (Prenzel *et al*, 2001), again supporting this theory. TGF α and other growth factors operating through overexpressed erbB receptors and the ligand-independent pathway may account for the null effect of tamoxifen and the development of endocrine therapy resistance as erbB receptor ligands operate via AF-1 as opposed to AF-2, which tamoxifen targets. TGF α has also been shown to be induced by E₂ treatment in ER-positive cell lines (Clarke *et al*, 1989; Gong *et al*, 1992).

The discovery that monoclonal antibodies raised against the erbB2 receptor were able to inhibit growth of cancer cells with amplified levels of this receptor on their cell surface led to the development of herceptin (Hudziak *et al*, 1989). However, resistance to herceptin has been documented in the creation of the JIMT-1 cell line from a breast cancer patient, which overexpresses the HER-2 oncogene and should therefore have been a suitable candidate for herceptin (Tanner *et al*, 2004). This group reasoned that factors other than erbB2 receptor expression must play a role in determining the response to herceptin. Chapters 3, 4 and 5 showed that the LCC-model of resistance was insensitive to anti-oestrogens and to the growth effects of TGF α and HRG β , and also E₂ in the LCC9 and LY2 cell lines, and that this potentially occurs via changes in upstream signalling molecules such as the overexpression of Akt.

Investigation into the development of insensitivity was performed upstream of Akt by targeting the erbB2 receptor with the novel recombinant humanised anti-erbB2 monoclonal antibody 2C4. The monoclonal antibody, or dimerisation inhibitor 2C4 (Agus *et al.*, 2005), binds to the extracellular domain II of the erbB2 receptor, blocking dimerisation with other members of the erbB receptor family.

In contrast to herceptin, which is more effective in breast cancer with over expression of the erbB2 receptor, 2C4 is able to target cancers which express moderate to low levels of erbB2 receptor (Albanell *et al.*, 2003; Mass, 2004). This is of particular relevance in this model of resistance as these cell lines have been documented as having erbB2 levels relatively similar, and even somewhat reduced, to those of the parental MCF-7 cell line (Gu *et al.*, 2001). Totpal *et al.* (2003) and Agus *et al.* (2002) found that the blockade of dimerisation formation involving erbB2 receptors reduced ligand-activated signalling via this family of receptors, including diminished phosphorylation of the erbB2 receptor itself and activation of MAPK and Akt (Agus *et al.*, 2002; Totpal *et al.*, 2003). It was hypothesised that by targeting erbB2, an upstream regulator of Akt, and the dimerisation of erbB2 with 2C4, that this would be of particular significance in the resistant cell lines where Akt over expression occurred. It may be that the resistant cell lines are more sensitive to the anti-proliferative effects of 2C4 due to Akt over expression, but this is dependent on the role of Akt in the proliferation of these cell lines.

6. 2. Anti-proliferative Response

The anti-proliferative effect of 2C4 on the MCF-7 cell line has previously been documented (Agus *et al.*, 2002). This characterisation of 2C4 in the parental cell line allowed for subsequent direct comparison to the resistant cell lines when subjected to the same treatment conditions. Any differences between the sensitive and resistant cell lines due to signalling via the erbB2 receptor will hopefully become apparent. It was hypothesised that the elevated expression of Akt in the resistant cell lines was triggered upstream by the erbB pathway. Therefore, the blockade of the erbB pathway using a dimerisation inhibitor such as 2C4 would suppress proliferation in the resistant cell lines, perhaps to a greater extent than the MCF-7 cells due to the enhanced levels of constitutively active Akt.

All cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media 100nM 2C4 alone, TGF α , HRG β or E₂ (all at 1nM), or a combination of ligand plus 2C4 (100nM + 1nM) for 72h. TGF α and HRG β were again chosen as these ligands signal via combinations involving erbB1 and erbB3 receptors respectively, hence different heterodimer combinations could be tested. Phosphorylated erbB1/2 and 1/3 heterodimers have been linked with the development of tamoxifen resistance in MCF-7 cells (Knowlden *et al.*, 2003).

6. 2. 1. Does 2C4 inhibit proliferation when cells are treated with E₂?

Figure 6.1 shows the effects of 2C4 on E₂ treated breast cancer cells. All cell lines were treated with control media, E₂, 2C4 alone and a combination of the inhibitor plus hormone. Figure 6.1 shows that all the resistant cell lines proliferated under control conditions in the absence of any stimuli as previously reported while the MCF-7 and LCC1 cell lines were the only cell lines to proliferate significantly in response to E₂ as previously described (P<0.001). 2C4 reduced proliferation in MCF-7 cells (~9% reduction), but not significantly so, and did not significantly affect proliferation in any of the resistant cell lines. 2C4 was unable to reverse the E₂-enhanced proliferation in either MCF-7 or LCC1 cells to any degree, with day 3

proliferation remaining elevated with values of ~437% and ~450% in the absence and presence of 2C4 in MCF-7 cells and ~288% and ~278% in the absence and presence of 2C4 in the LCC1 cell line. However, the absence of proliferation inhibition does not necessarily correlate with an absence of stimulation of signalling pathways downstream of erbB2 via E₂. The combination treatment of E₂ + 2C4 did not produce any significant differential effects in the LCC9 or LY2 cell line to those of E₂ or 2C4 treatments alone.

6. 2. .2. Does 2C4 inhibit proliferation when cells are treated with growth factors?

6. 2. 2. 1 TGF α

Figure 6.2 shows the effects of 2C4 on TGF α treated breast cancer cells. All cell lines were treated with control media, TGF α , 2C4 alone and a combination of the inhibitor plus TGF α . All the resistant cell lines proliferated under control conditions in the absence of any stimuli as previously reported, while the parental MCF-7 cell line was the only cell line to proliferate significantly in response to TGF α as previously described (P<0.05). 2C4 alone reduced proliferation in MCF-7 cells, but not significantly so (~12.5% reduction), and did not significantly affect proliferation in any of the resistant cell lines.

2C4 reduced TGF α -enhanced proliferation in MCF-7 cells from ~133% to ~110%, which was considered significant (P<0.05). However, 2C4 was unable to completely abrogate TGF α -induced MCF-7 cell proliferation, with a combined treatment value ~10% above that of cells treated with control media.

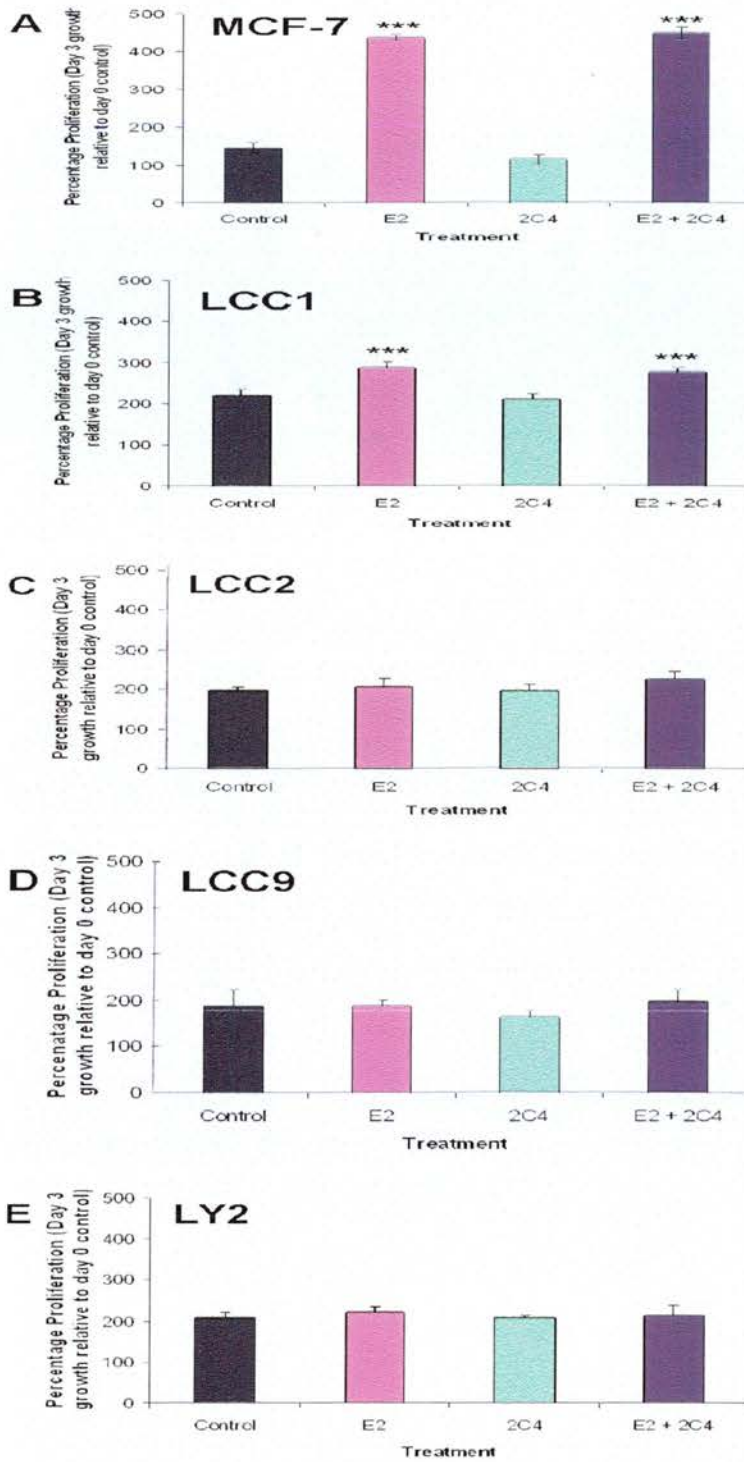


Figure 6.1. The effect of 2C4 in the presence and absence of E₂ on the proliferation of MCF-7 cells and a panel of resistant breast cancer cell lines. A-E, histograms of the proliferation of a panel of breast cancer cell lines, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media (■), E₂ (■) (1nM), 2C4 alone (■) or 2C4+E₂ (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in the MCF-7 cell line. Histograms are representative of 1 of 3 independent experiments.

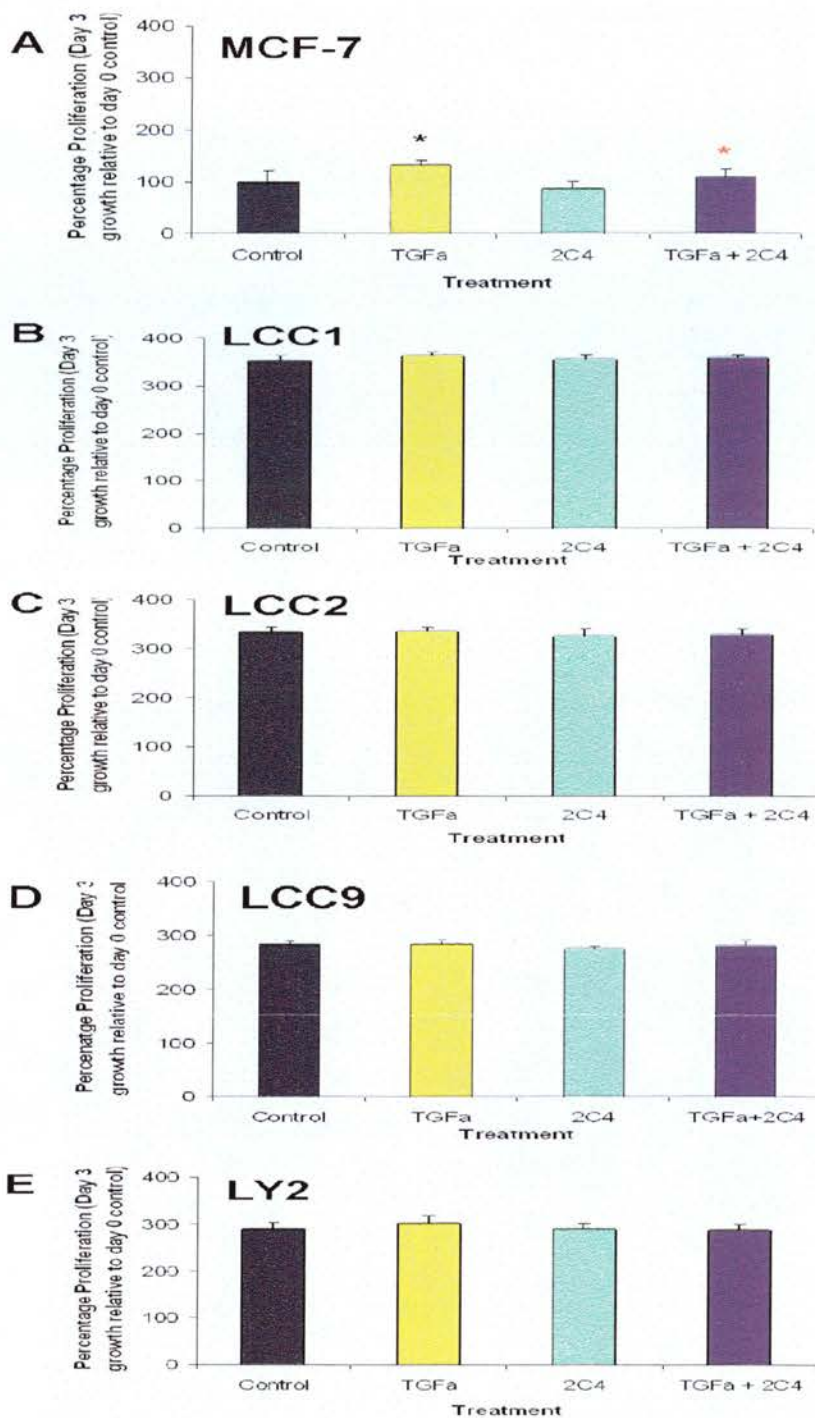


Figure 6.2 The effect of 2C4 in the presence and absence of TGFα on the proliferation of MCF-7 cells and a panel of resistant breast cancer cell lines. A-E, histograms of the proliferation of a panel of breast cancer cell lines, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media (■), TGFα (■) (1nM), 2C4 alone (■) or 2C4+TGFα (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in the MCF-7 cell line and those in red represent any statistically significant changes post-treatment with 2C4 in the MCF-7 cell line. Histograms are representative of 1 of 3 independent experiments.

6. 2. 2. 2 HRG β

Figure 6.3 shows the effects of 2C4 on HRG β treated breast cancer cells. All cell lines were treated with control media, HRG β , 2C4 alone and a combination of the inhibitor plus HRG β . All the resistant cell lines proliferated under control conditions in the absence of any stimuli as previously reported, while the parental MCF-7 cell line was the only cell line to proliferate significantly in response to HRG β as previously described ($P < 0.001$ when compared across all treatments in that cell line). 2C4 alone did not significantly affect proliferation in any of the cell lines. 2C4 almost completely abolished HRG β -enhanced proliferation in MCF-7 cells from ~161% to ~105%, which was considered extremely significant ($P < 0.001$). 2C4 was unable to completely abrogate HRG β -induced MCF-7 cell proliferation, with a combined treatment value ~5% above that of cells treated with control media.

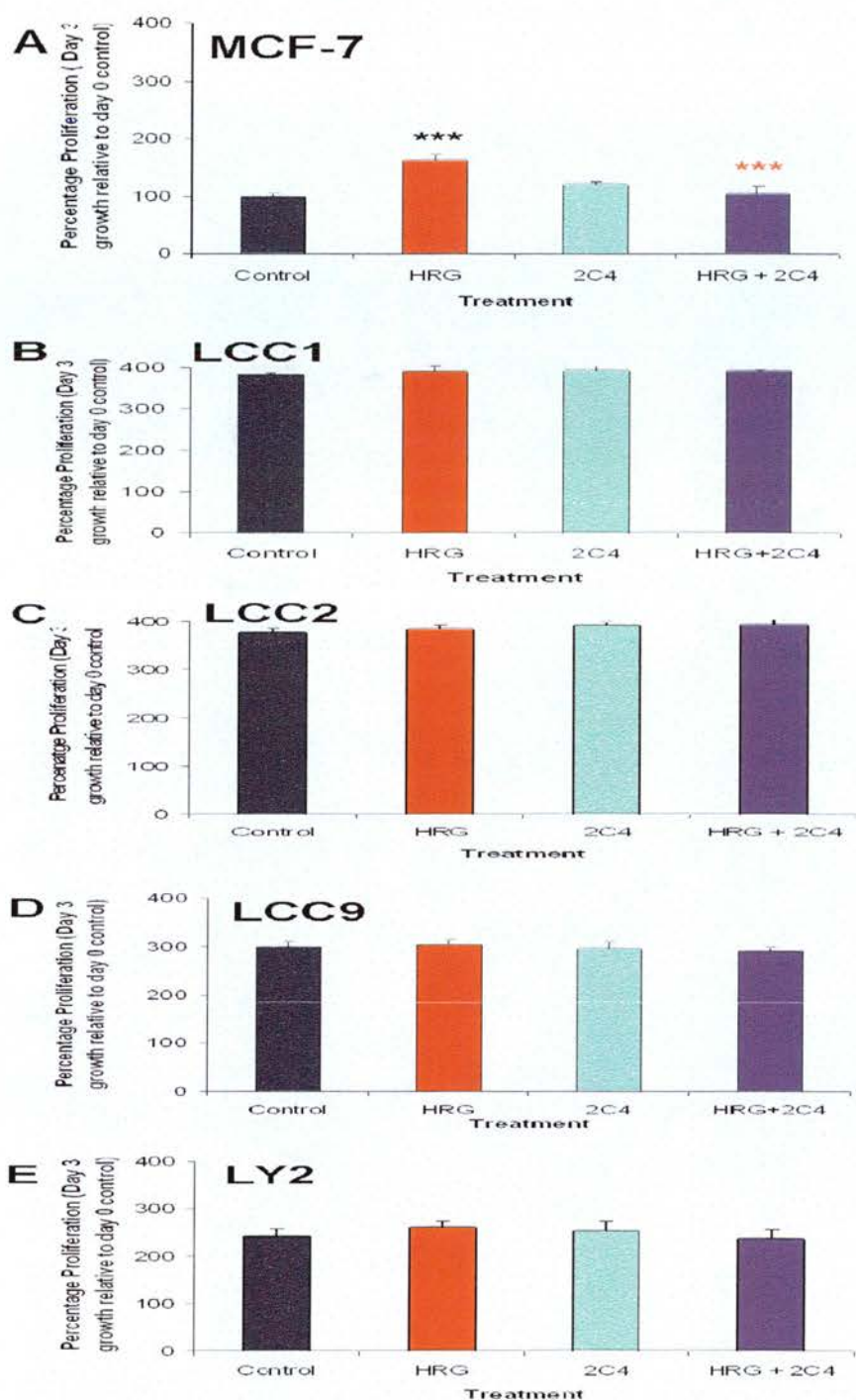


Figure 6.3 The effect of 2C4 in the presence and absence of HRG β on the proliferation of MCF-7 cells and a panel of resistant breast cancer cell lines. A-E, histograms of the proliferation of a panel of breast cancer cell lines, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media (■), HRG β (■) (1nM), 2C4 alone (■) or 2C4 + HRG β (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment and those in red represent any statistically significant changes post-treatment with 2C4 in the MCF-7 cell line. Histograms are representative of 1 of 3 independent experiments.

6. 3. The Effect of 2C4 on signalling pathways

Pre-treatment of the MCF-7 cell line with 2C4 produced inhibition of cellular proliferation as expected, however, in contrast to the initial proposed hypothesis, the resistant cell lines appeared to be completely insensitive to the effects of this anti-erbB2 agent. It was therefore hypothesised that signalling via Akt and ERK, molecules downstream of erbB2, may not be inhibited as is the case for the MCF-7 cell line. In particular, it was newly hypothesised that the constitutively phosphorylated Akt may in fact *de-sensitise* the resistant cell lines to the anti-proliferative nature of 2C4, in contrast to what was initially believed to occur. The following section will scrutinise the Akt and ERK signalling molecules known to be inhibited by 2C4 in the MCF-7 cell line. TGF α and HRG β growth factors were used as they operate via different erbB2 heterodimer conformations, which has been suggested to play a role in the 2C4 sensitivity of certain ovarian cell lines (Takai *et al*, 2005). Cells were also subjected to E₂ treatment to investigate the possibility of hormonal interaction with erbB2 via the Akt and ERK pathways. These studies will hopefully enable any alterations in the resistant cell lines to become apparent and enable elucidation of a potential mechanism of resistance.

6. 3. 1. The Effect of 2C4 on the Akt signalling pathway

6. 3. 1. 1. E₂

The effect of 2C4 pre-treatment in the presence and absence of E₂ on MCF-7, LCC1 and LCC9 cell lines is illustrated in figure 6.4. Figure 6.4A is a representative western blot which shows the absence of effect of either E₂ or 2C4 alone on P-Akt expression as previously described in chapter 4. 2C4 did not appear to significantly alter P-Akt levels of any treatment in any cell line as shown by bands of similar signal intensity across all treatments and cell lines. This is confirmed in the histogram in figure 6.4B, where no significant difference was observed between any treatment or cell line ($P > 0.05$).

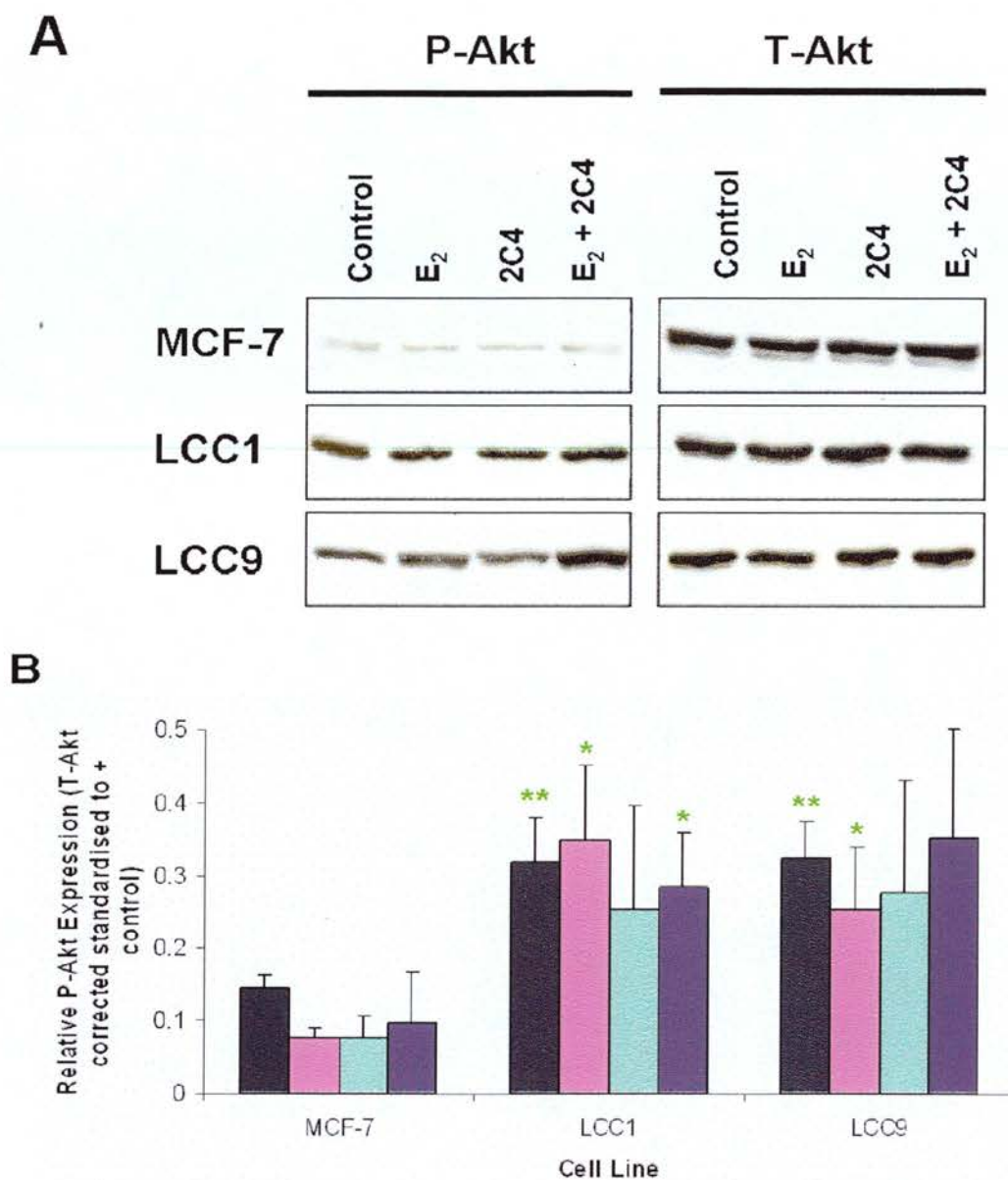


Figure 6.4 The effect of 2C4 in the presence and absence of E₂ on P-Akt signalling in MCF-7, LCC1 and LCC9 cell lines. A, western blot of the effect of 2C4 on P-Akt, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, E₂ (1nM), 2C4 alone or 2C4 + E₂ for 72h. Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls. B, histogram of P-Akt OD values of all cell lines treated with control media (■), E₂ (■) (1nM), 2C4 alone (■) or 2C4 + E₂ (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in that cell line, those in green represent statistically significant differences between the MCF-7 and resistant cell lines (between the same treatments). Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

6.3.1.2. *TGF α*

Figure 6.5 shows the effect of 2C4 in the presence and absence of *TGF α* on the Akt signalling pathway in MCF-7 cells versus resistant breast cancer cell lines. Figure 6.5A shows a representational western blot of P-Akt levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media or *TGF α* (1nM) for 15 min. Membranes were probed with P-Akt primary antibody (1:1000).

T-Akt levels were used as loading controls. Figure 6.5B is a histogram of P-Akt expression relative to positive control derived from triplicate OD values of all cell lines treated with control media, 100nM 2C4, *TGF α* (1nM) or *TGF α* + 2C4 (1nM + 100 μ M), where data are in triplicate \pm SD. MCF-7 cells treated with *TGF α* were used as the positive control. LCC1 and LCC9 cell lines were significantly constitutively phosphorylated at the Akt position ($P < 0.05$) compared to the parental MCF-7 cell line as previously described in chapter 4. *TGF α* significantly enhanced P-Akt in all three cell lines as shown by bands with a strong signal intensity in figure 6.5A and in the histogram in figure 6.5B ($P < 0.001$ compared to control and other treatments run simultaneously). 2C4 alone did not significantly affect any cell line ($P > 0.05$). 2C4 pre-treatment significantly reduced *TGF α* -enhanced P-Akt by ~ 6.4 -fold ($\sim 87\%$) in the MCF-7 cell line from an initial ~ 9 -fold induction with *TGF α* alone ($P < 0.001$). 2C4 did not significantly alter the *TGF α* -enhanced P-Akt expression levels of either of the resistant cell lines ($P > 0.05$ compared to MCF-7 reduction observed with the same treatments).

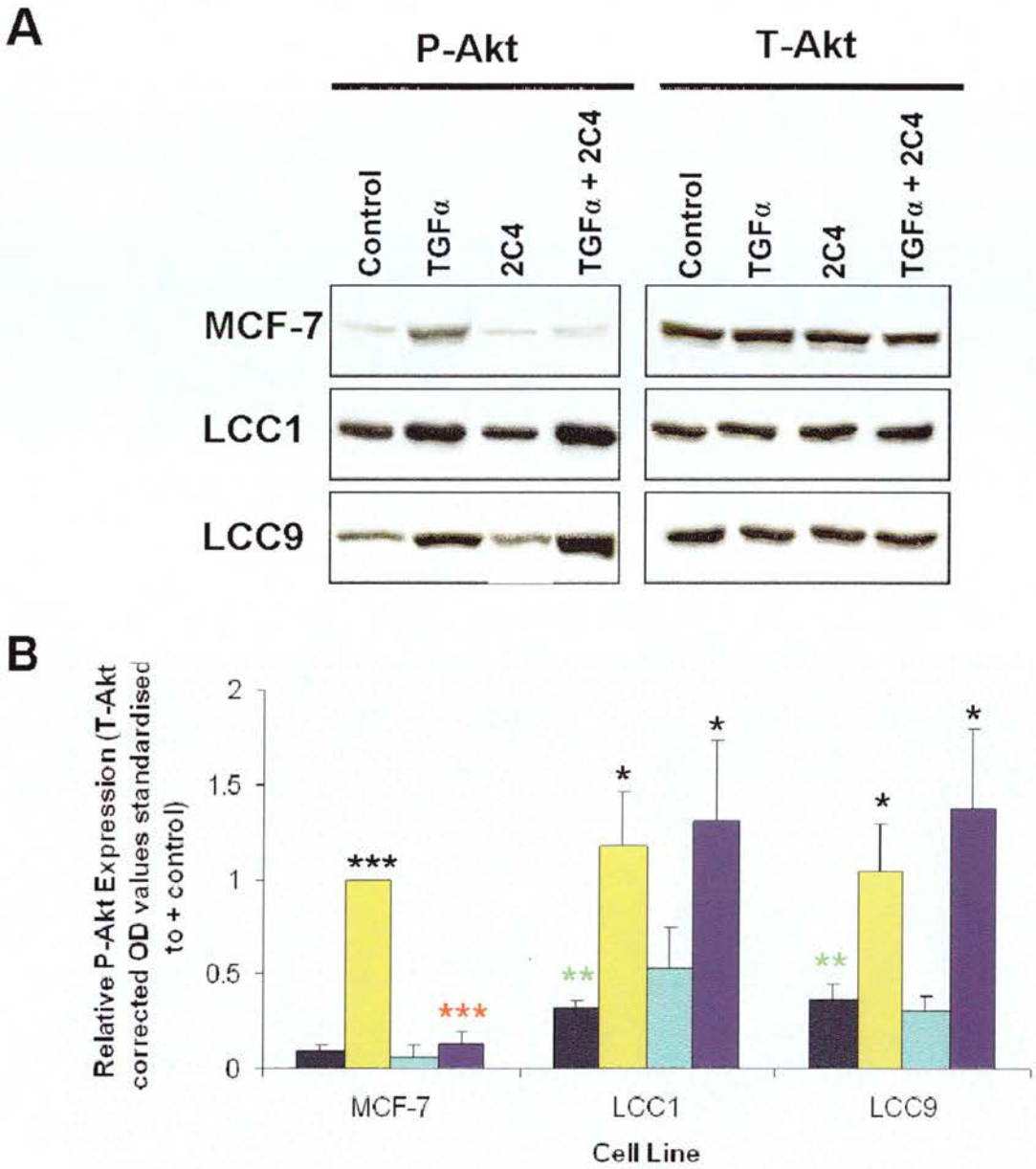


Figure 6.5 The effect of 2C4 in the presence and absence of TGF α on P-Akt signalling in MCF-7, LCC1 and LCC9 cell lines. A, western blot of the effect of 2C4 on P-Akt, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, TGF α (1nM), 2C4 alone or 2C4 + TGF α for 72h. Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls. B, histogram of P-Akt OD values of all cell lines treated with control media (■), TGF α (■) (1nM), 2C4 alone (■) or 2C4 + TGF α (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in that cell line, those in green represent statistically significance differences between the control treated MCF-7 and resistant cell lines, and those in red represent any statistically significant changes post-treatment with 2C4 in each cell line. Western blots are representative of 1 of 3 independent experiments amalgamated in the histogram (B).

6. 3. 1. 3. *HRG β*

Figure 6.6A is a representational western blot of the effect of 2C4 pre-treatment on HRG β -enhanced P-Akt in MCF-7, LCC1 and LCC9 cell lines. The blot shows basal levels were increased in the resistant cell lines in the first column and that TGF α enhanced P-Akt expression in all three cell lines which was observed as increased signal intensity in the second column. This is in agreement with previous data in chapter 4. 2C4 did not affect the basal signal of any cell line. The histogram in figure 6.6B showed that 2C4 reduced P-Akt significantly in all three cell lines ($P < 0.001$), with fold reductions of ~15-fold (~93%) in the MCF-7 cell line, ~37-fold (~97%) in the LCC1 cell line and ~31-fold (~97%) in LCC9 cells.

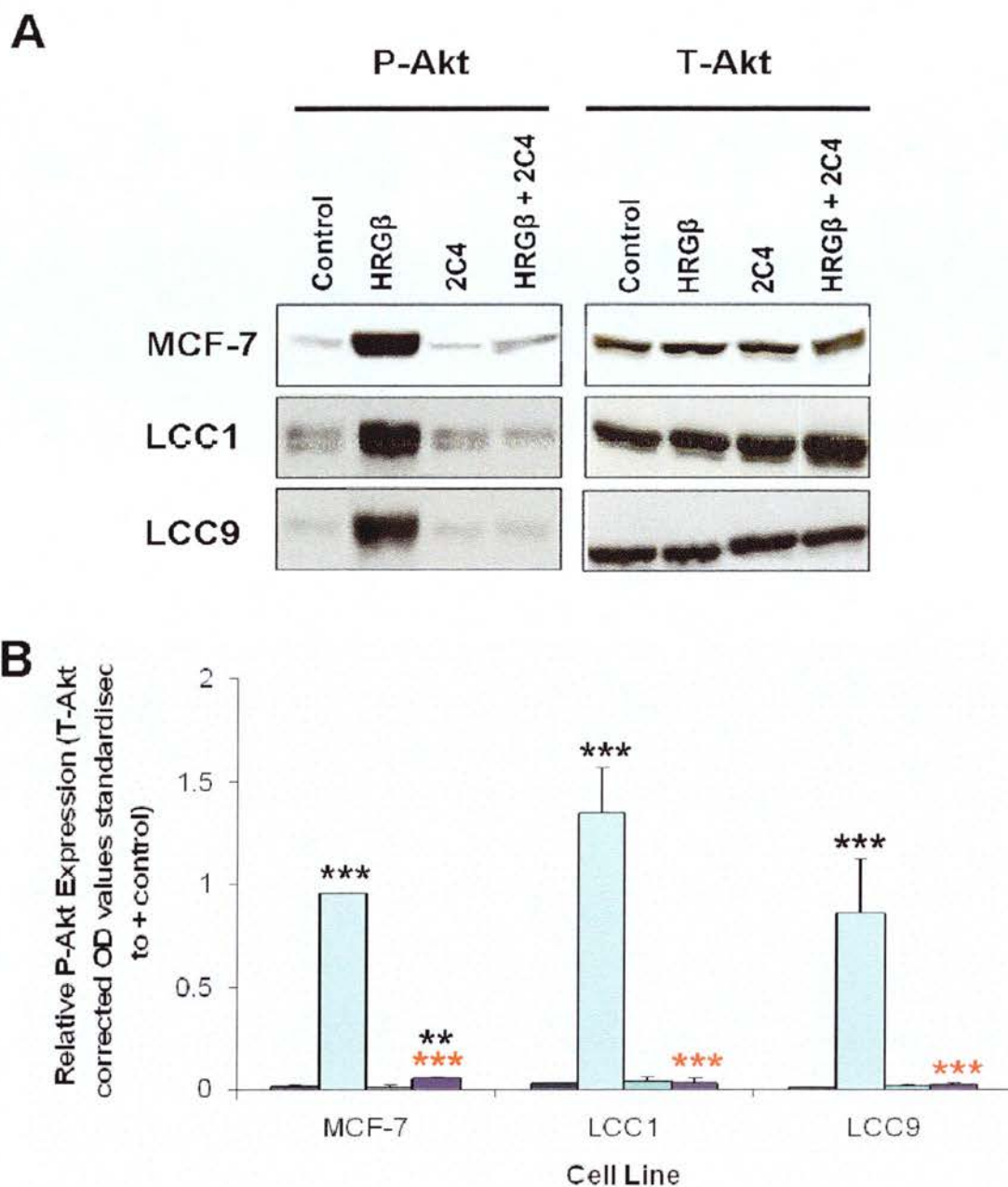


Figure 6.6 The effect of 2C4 in the presence and absence of HRGβ on P-Akt signalling in MCF-7, LCC1 and LCC9 cell lines. A, western blot of the effect of 2C4 on P-Akt, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, HRGβ (1nM), 2C4 alone or 2C4 + HRGβ for 72h. Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls. B, histogram of P-Akt OD values of all cell lines treated with control media (■), HRGβ (■) (1nM), 2C4 alone (■) or 2C4 + HRGβ (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in that cell line and those in red represent any statistically significant changes post-treatment with 2C4 in each cell line. Western blots are representative of 1 of 3 independent experiments amalgamated in the histogram (B).

6. 3. 2. The Effect of 2C4 on the ERK signalling pathway

6. 3. 2. 1. E₂

The effect of 2C4 pre-treatment in the presence and absence of E₂ on the three cell lines is shown in figure 6.7. As before, a western blot which was representative of a series of repeat experiments is depicted in figure 6.7A, while a composite of triplicate OD values is shown in figure 6.7B. No treatment alone or in combination had any significant differential effect on P-ERKI/II expression (P>0.05).

6. 3. 2. 2. TGF α

Figure 6.8 shows the effect of 2C4 on the P-ERKI/II signal enhanced by TGF α in the MCF-7, LCC1 and LCC9 cell lines. Figure 6.7A depicts a representational western blot, and figure 6.8B shows a histogram of relative P-Akt expression derived from triplicate OD values of all cell lines treated with control media, TGF α , 2C4 alone or TGF α + 2C4. Single agent treatments were as before, with no change in basal level expression, and significantly elevated P-ERKI/II levels upon treatment with TGF α . Pre-treatment with 2C4 alone had no significant effect on the levels of P-ERKI/II in any of the cell lines (P>0.05). 2C4 reduced TGF α -enhanced P-ERKI/II in the MCF-7 cell line only (~0.4-fold or by ~30%, P<0.01 compared to control in conjunction with treatments). No significant changes were observed in the P-ERKI/II bands of either of the resistant cell lines when the cells were pre-treated with 2C4 and then subjected to TGF α (P>0.05, comparison across all treatments in each cell line separately or simultaneously).

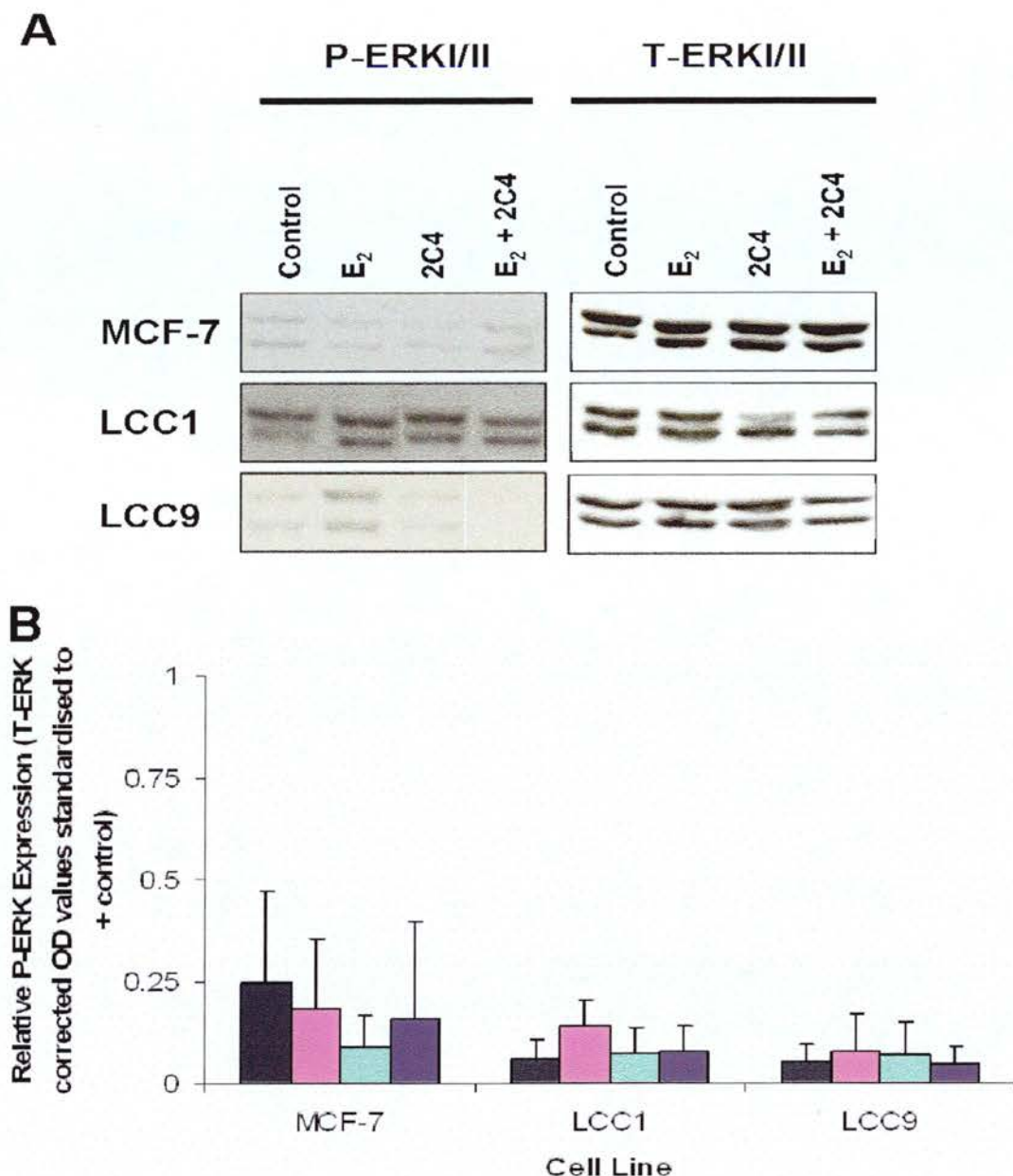
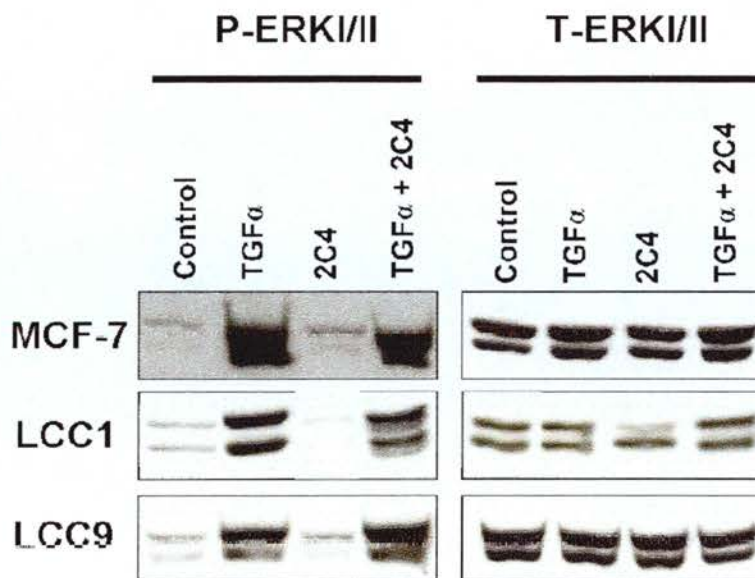


Figure 6.7 The effect of 2C4 in the presence and absence of E₂ on P-ERKI/II signalling in MCF-7, LCC1 and LCC9 cell lines. A, western blot of the effect of 2C4 on P-ERKI/II, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, E₂ (1nM), 2C4 alone or 2C4 + E₂ for 72h. Membranes were probed with P-ERKI/II primary antibody (1:1000). T-ERKI/II levels were used as loading controls. B, histogram of P-ERKI/II OD values of all cell lines treated with control media (■), E₂ (■) (1nM), 2C4 alone (■) or 2C4 + E₂ (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. The absence of any asterisks indicates no treatment was significant in any cell line individually or between the cell lines. Western blots are representative of 1 of 3 independent experiments which are amalgamated in the histogram (B).

6. 3. 2. 3. *HRG β*

The effect of 2C4 pre-treatment on P-ERK expression is illustrated in figure 6.9. Figure 6.9A and B are a representational western blot and histogram of triplicate OD values from western blots respectively. Cells were treated as in section 6. 3. 1. 2 and were probed with a P-ERKI/II antibody. As previously described, control treatment did not enhance P-ERKI/II ($P>0.05$), while HRG β significantly enhanced P-ERKI/II ($P<0.001$) (Chapter 4). Pre-treatment of the cell lines with 2C4 dramatically reduced HRG β -enhanced P-ERK in MCF-7 cells as demonstrated by a loss of signal intensity (figure 6.9A). There was a ~61% reduction which was extremely significant ($P<0.001$, figure 6.9B). 2C4 pre-treatment completely abolished HRG β -enhanced P-ERKI/II signal in the resistant cell lines with reductions of ~100% and ~99% in the LCC1 and LCC9 cell lines respectively (figure 6.9A). This was again found to be extremely significant in both cell lines ($P<0.001$, figure 6.9B). Thus, HRG β -enhanced P-ERKI/II signal appears to be at least as susceptible to 2C4 inhibition in the LCC1 and LCC9 cell lines.

A



B

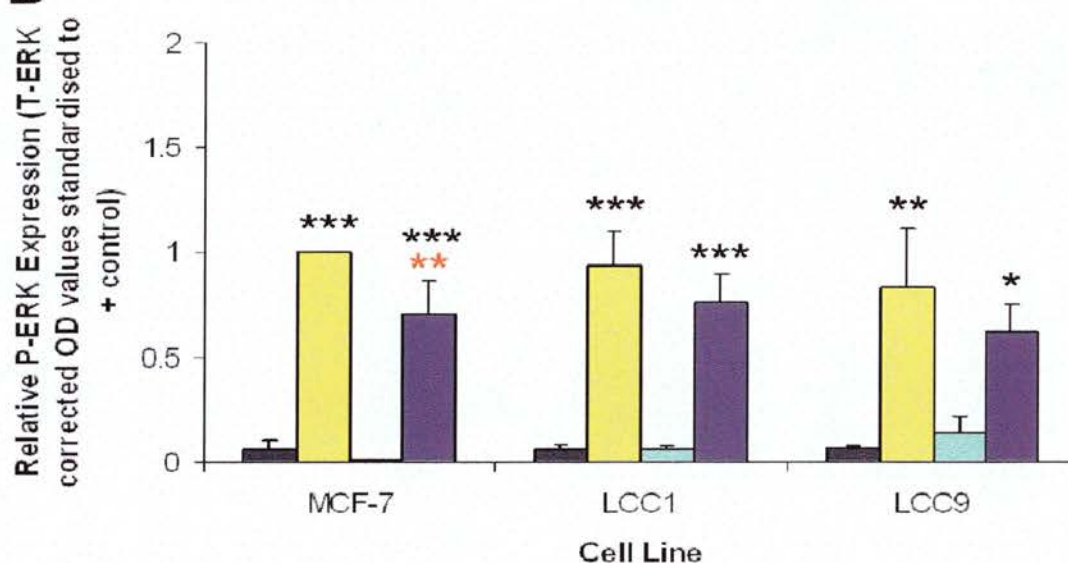


Figure 6.8. The effect of 2C4 in the presence and absence of TGF α on P-ERK1/II signalling in MCF-7, LCC1 and LCC9 cell lines. A, western blot of the effect of 2C4 on P-ERK1/II, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, TGF α (1nM), 2C4 alone or 2C4 + TGF α for 72h. Membranes were probed with P-ERK1/II primary antibody (1:1000). T-ERK1/II levels were used as loading controls. B, histogram of P-ERK1/II OD values of all cell lines treated with control media (■), TGF α (■) (1nM), 2C4 alone (■) or 2C4 + TGF α (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in that cell line and those in red represent any statistically significant changes post-treatment with 2C4 in the MCF-7 cell line. Western blots are representative of 1 of 3 independent experiments amalgamated in the histogram (B).

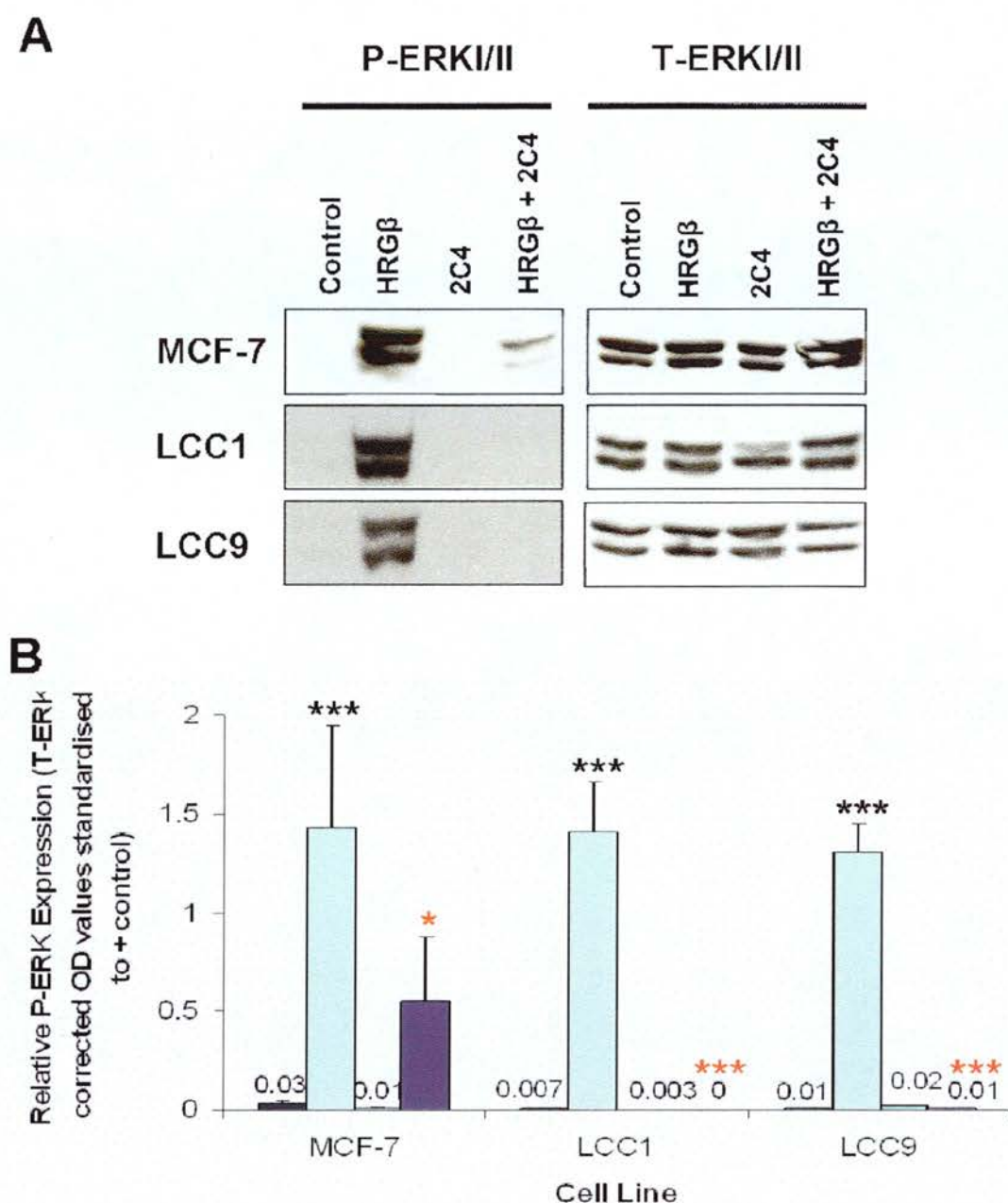


Figure 6.9. The effect of 2C4 in the presence and absence of HRGβ on P-ERKI/II signalling in MCF-7, LCC1 and LCC9 cell lines. A, western blot of the effect of 2C4 on P-ERKI/II, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, HRGβ (1nM), 2C4 alone or 2C4 + HRGβ for 72h. Membranes were probed with P-ERKI/II primary antibody (1:1000). T-ERKI/II levels were used as loading controls. B, histogram of P-ERKI/II OD values of all cell lines treated with control media (■), HRGβ (□) (1nM), 2C4 alone (■) or 2C4 + HRGβ (■) for 72h. ANOVA test: * = $P < 0.05$, ** = $P < 0.01$ & *** = $P < 0.001$. Asterisks in black represent statistically significant changes between control and a treatment, in that cell line and those in red represent any statistically significant changes post-treatment with 2C4 in each cell line. Western blots are representative of 1 of 3 independent experiments amalgamated in the histogram (B).

6. 4. The Effect of 2C4 in combination with anti-oestrogen treatment

It has been hypothesised by a number of studies that endocrine therapy resistance is a result of the ‘cross-talk’ between the erbB receptor pathway and the ligand dependent pathway and this resistance may be overcome by targeting the two pathways simultaneously (Mass, 2005; Martin *et al.*, 2005). The discovery that 2C4 alone was ineffective at reducing the proliferation of the resistant cell lines but remained able to inhibit HRG β -enhanced Akt and ERK signalling of these cells led to speculation that a combination strategy may be more successful. The JIMT-1 cell line is resistant to the anti-erbB2 agent herceptin despite overexpressing the *HER-2* oncogene (Tanner *et al.*, 2004). There is evidence from other studies that combining an anti-oestrogen such as tamoxifen with an erbB receptor blocker, such as herceptin leads to a reduction in cell proliferation and signalling via the erbB receptor pathway.

It was therefore hypothesised that in this model system, the inhibition of both the erbB2 receptor pathway with 2C4 and the ligand-dependent pathway with tamoxifen could prove to be more effective than either single agent alone in reducing cellular proliferation.

6. 4. 1. Does combined 2C4 and anti-oestrogen treatment inhibit proliferation in this model?

6. 4. 1. 1. E₂

The effect of 2C4 in various combinations with tamoxifen and/or E₂ on the proliferation of the panel of breast cancer cell lines is shown in figure 6.10. Figure 6.10A is a histogram of the effect of the various treatments on the MCF-7 cell line. The histogram shows that, as before, E₂ significantly increased proliferation by ~3-fold (P<0.001) and this enhanced proliferation was not significantly affected by 2C4 pre-treatment (P>0.05).

Tamoxifen did not alter cell proliferation when administered alone, and proliferation was also unchanged when tamoxifen was combined with 2C4 pre-treatment (P>0.05).

Tamoxifen significantly reduced MCF-7 proliferation enhanced by E₂ by ~0.7-fold (~175% reduction, P<0.001). This reduction was not significantly affected when cells were pre-treated with 2C4 (P>0.05), with proliferation remaining at ~250% on day 3 relative to day 0 control growth.

Figure 6.10B is a histogram of the effects of these treatments on LCC1 proliferation. As previously described in chapter 3, E₂ significantly increased the already elevated basal level of proliferation by ~0.3-fold (P<0.001) from ~220% to ~288% day 3 growth relative to day 0 control. 2C4 did not affect E₂-enhanced proliferation (P>0.05), while tamoxifen significantly reduced this increase by ~38% (P<0.001). 2C4 did not significantly alter the reduction in proliferation caused by tamoxifen.

Figures 6.10C to E are histograms of the LCC2, LCC9 and LY2 resistant cell lines subjected to the same treatments. As previously described earlier in this chapter, the cells all proliferated in the absence of stimulation (between ~200-300% by day 3 compared to day 0). No significant differences were observed with any treatment combination in any of these cell lines (P>0.05).

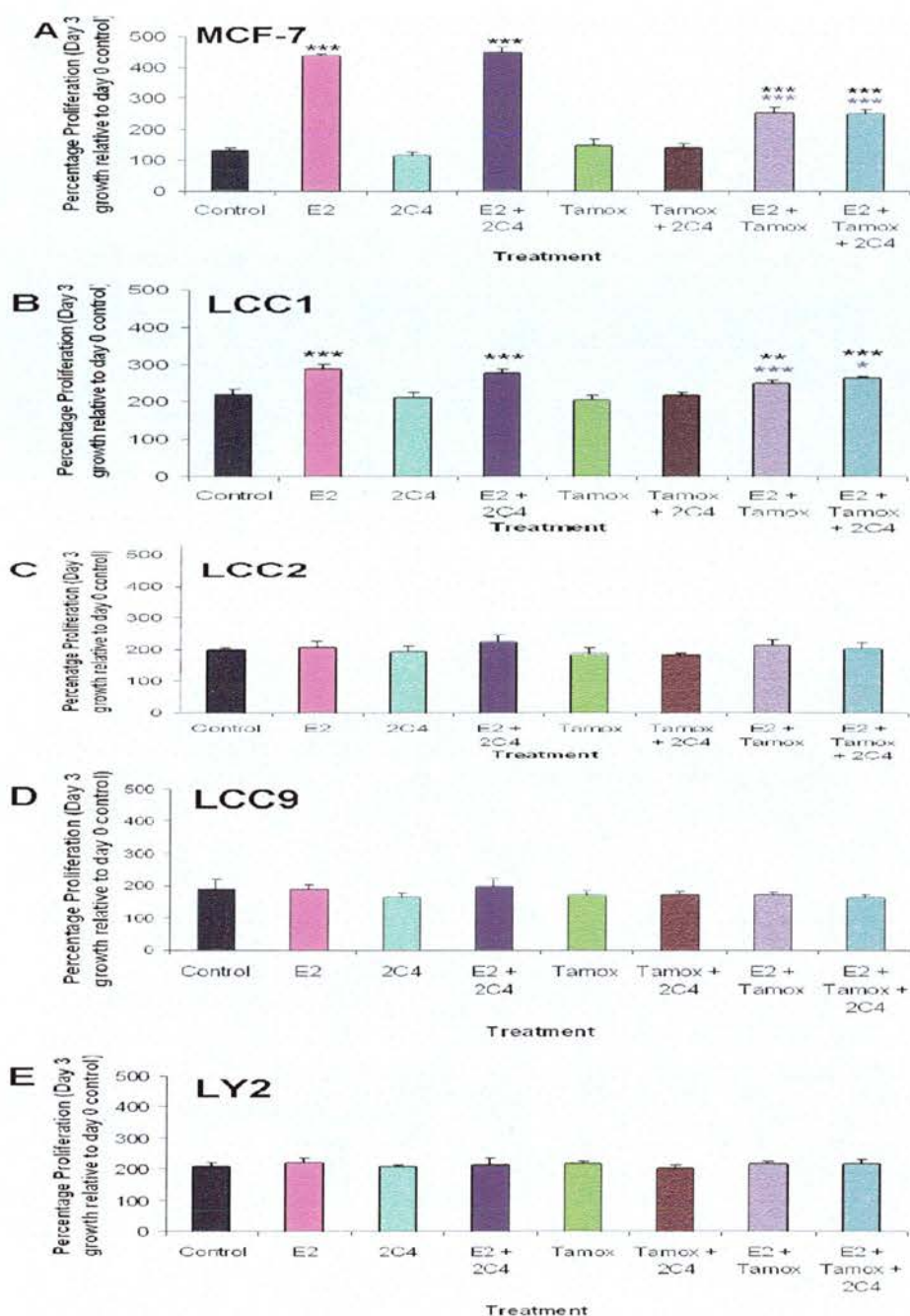


Figure 6.10. The effect of 2C4 combined with tamoxifen in the presence and absence of E₂ on the proliferation of MCF-7 cells and a panel of resistant breast cancer cell lines.

A-E, histograms of the proliferation of a panel of breast cancer cell lines, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media (■), E₂ (■) (1nM), 2C4 alone (■), 2C4 + E₂ (■), tamoxifen (1μM) (■), tamoxifen + 2C4 (■), E₂ + tamoxifen (■) or E₂ + tamoxifen + 2C4 (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in each cell line, those in blue represent statistical significance post treatment with tamoxifen in each cell line compared to the other treatment combinations performed without tamoxifen. Histograms are representative of 1 of 3 independent experiments.

6. 4. 1. 2. *TGF α*

The effect of 2C4 in various combinations with tamoxifen and/or TGF α on the proliferation of the panel of breast cancer cell lines is shown in figure 6.11. Figure 6.11A is a histogram of the effect of the various treatments on the MCF-7 cell line. The histogram shows that, as before, TGF α -enhanced proliferation was reduced by 2C4 pre-treatment ($P < 0.01$). 2C4 alone did not statistically significantly alter the proliferation of the MCF-7 cell line ($P > 0.05$).

Tamoxifen did not alter cell proliferation when administered alone, and proliferation was also unchanged when tamoxifen was combined with 2C4 pre-treatment ($P > 0.05$). Also of interest are the data showing tamoxifen to significantly enhance the proliferation of MCF-7 cells already increased by TGF α alone, although this further increase itself was not found to be significant. A ~59% ($P < 0.001$) increase with the two agents was noted compared to a ~33% ($P < 0.01$) increase when cells were treated with TGF α alone. This increase was significantly reduced when cells were pre-treated with 2C4 ($P < 0.01$) to a level just above that produced by TGF α + 2C4.

Figures 6.11B to E are histograms of the LCC1, LCC2, LCC9 and LY2 resistant cell lines subjected to the same treatments. As previously described earlier in this chapter, the cells all proliferated in the absence of stimulation (between ~200-300% by day 3 compared to day 0). No significant differences were observed with any treatment combination in any of the cell lines ($P > 0.05$).

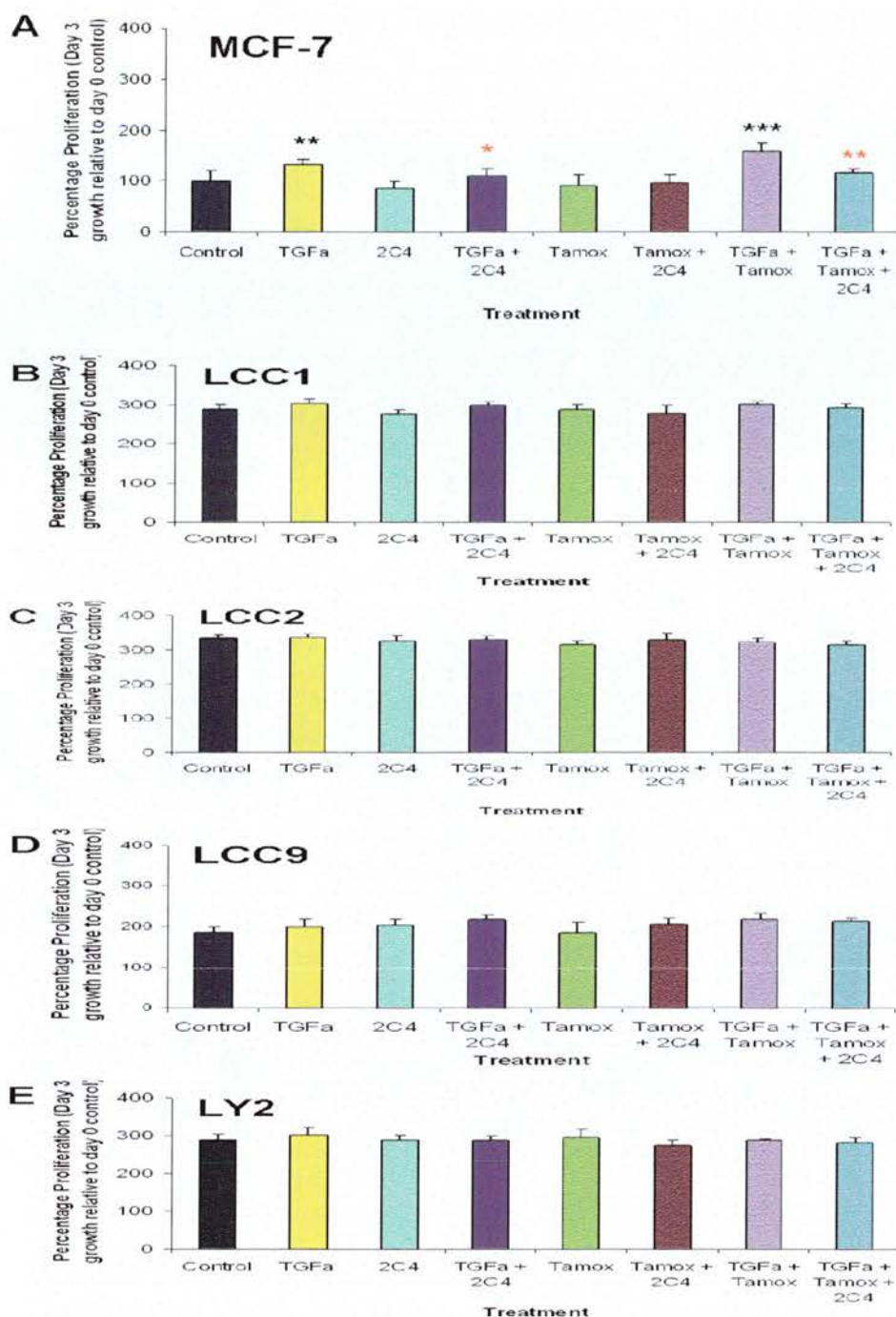


Figure 6.11. The effect of 2C4 combined with tamoxifen in the presence and absence of TGF α on the proliferation of MCF-7 cells and a panel of resistant breast cancer cell lines. A-E, histograms of the proliferation of a panel of breast cancer cell lines, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media (■), TGF α (■) (1nM), 2C4 alone (■), 2C4 + TGF α (■), tamoxifen (1 μ M) (■), tamoxifen + 2C4 (■), TGF α + tamoxifen (■) or TGF α + tamoxifen + 2C4 (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment in that cell line and those in red represent any statistically significant changes post-treatment with 2C4 in each cell line. Histograms are representative of 1 of 3 independent experiments.

6. 4. 1. 3. HRG β

The effect of 2C4 in various combinations with tamoxifen and/or HRG β on the proliferation of the panel of breast cancer cell lines is shown in figure 6.12. Figure 6.12A is a histogram of the effect of the various treatments on the MCF-7 cell line. The histogram shows that, as before, HRG β -enhanced proliferation was reduced by 2C4 pre-treatment ($P < 0.001$). Tamoxifen did not alter cell proliferation when administered alone, and proliferation was also unchanged when tamoxifen was combined with 2C4 pre-treatment ($P > 0.05$). Interestingly, as with TGF α treatment, data here showed tamoxifen significantly enhanced the proliferation of MCF-7 cells already increased by HRG β alone ($P < 0.01$). A ~ 0.9 -fold ($\sim 92\%$, $P < 0.001$) increase with the two agents was noted compared to a ~ 0.6 -fold ($\sim 62\%$, $P < 0.001$) increase when cells were treated with HRG β alone. This increase was significantly reduced when cells were pre-treated with 2C4 ($P < 0.001$) to a level just above that produced by HRG β + 2C4. It should be noted that tamoxifen appears to be preventing the same extent of reversal as seen with 2C4 and growth factor alone (tamoxifen elevated the proliferation of this combination by $\sim 16.5\%$ compared to HRG β + 2C4 alone).

Figures 6.12B to E are histograms of the LCC1, LCC2, LCC9 and LY2 resistant cell lines subjected to the same treatments. As previously described earlier in this chapter, the cells all proliferated in the absence of stimulation (between ~ 200 - 300% by day 3 compared to day 0). No significant differences were observed with any treatment combination in any of the cell lines ($P > 0.05$).

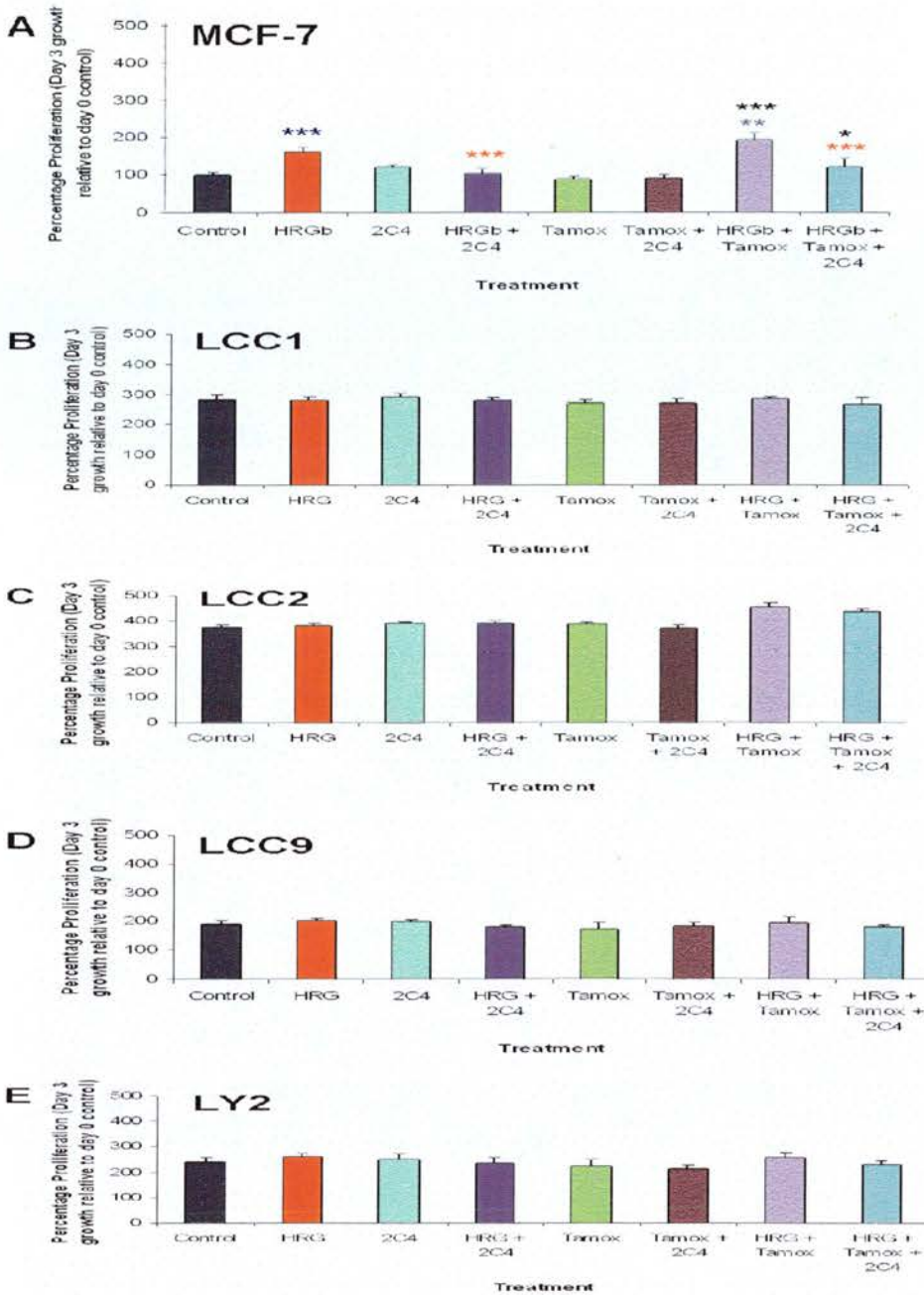


Figure 6.12. The effect of 2C4 combined with tamoxifen in the presence and absence of HRGβ on the proliferation of MCF-7 cells and a panel of resistant breast cancer cell lines. A-E, histograms of the proliferation of a panel of breast cancer cell lines, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media (■), HRGβ (■) (1nM), 2C4 alone (■), 2C4 + HRGβ (■), tamoxifen (1μM) (■), tamoxifen + 2C4 (■), HRGβ + tamoxifen (■) or HRGβ + tamoxifen + 2C4 (■) for 72h. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment, in the MCF-7 cell line, those in blue represent statistical significance post treatment with tamoxifen compared with the same treatment without in MCF-7 cells, and those in red represent any statistically significant changes post-treatment with 2C4 in MCF-7 cells. Histograms are representative of 1 of 3 independent experiments.

6. 5. The Effect of 2C4 in combination with tamoxifen on signalling pathways

The proliferation studies discussed in the previous section showed that tamoxifen appeared to be interfering with the anti-proliferative nature of 2C4 when MCF-7 cells were treated with growth factors. This is the opposite of what was expected to occur. The blockade of the two pathways was believed to act synergistically in the inhibition of proliferation, not interfere or compete with each other. Importantly, these data demonstrated that the combined treatment of 2C4 and tamoxifen was ineffective at inhibiting proliferation with any ligand in any combination in any of the resistant cell lines. The following section demonstrates the effect of 2C4 and tamoxifen on the Akt and MEK/ERK signalling pathways in this model. Akt, ERK and MEK were investigated in an attempt to observe any changes which may account for the differential response to 2C4 between TGF α and HRG β .

Unfortunately, due to a failure in the suppliers' batch of TGF α it was not possible to complete some aspects of the investigation in the LCC9 cell line in combination with tamoxifen, therefore P-Akt and P-ERK1/II expression data were not obtained. Also, studies into the levels of P-MEK in all cell lines treated with TGF α were not monitored due to the failure of TGF α to enhance either P-Akt or P-ERK1/II under positive control conditions.

6. 5. 1. The Effect of 2C4 and tamoxifen on the Akt signalling pathway

Figure 6.13 shows western blots performed to characterise the effects of 2C4 and tamoxifen with either TGF α , HRG β or E₂ in various combinations on P-Akt expression. Figure 6.13A shows combinations involving TGF α , where band intensities for control, TGF α , 2C4 and TGF α + 2C4 were as previously detailed in this chapter. Tamoxifen alone or in combination with 2C4 did not appear to alter P-Akt expression in either MCF-7 or LCC1 cell line from their original basal levels. In agreement with proliferation studies, the signal intensity for P-Akt treated with TGF α + tamoxifen did not appear too dissimilar to that of TGF α alone in MCF-7

cells. Tamoxifen did not appear to affect the reversal of TGF α -enhanced P-Akt by 2C4 in the MCF-7 cell line. In the LCC1 cell line, tamoxifen appeared to increase the signal intensity of the TGF α -enhanced P-Akt band, although 2C4 was able to reverse this back to the level of TGF α + 2C4 and TGF α alone. This effect was not observed in proliferation studies as all levels remained constant irrespective of treatment.

Figure 6.13B illustrates western blots of P-Akt expression of the MCF-7, LCC1 and LCC9 cell lines when subjected to 2C4 in the presence and absence of tamoxifen and HRG β . Only the tamoxifen combinations will be discussed as other treatments have been previously described. In agreement with the MCF-7 cell proliferation, tamoxifen treatment further increased HRG β -enhanced P-Akt signal intensity. 2C4 reduced the HRG β + tamoxifen enhancement of P-Akt signal, but was unable to reduce the signal to that of HRG β + 2C4. The same pattern occurred in both the LCC1 and LCC9 cell lines as in the parental cell line. This pattern was not reflected in the cell proliferation of the resistant cell lines.

Figure 6.13C depicts any changes in P-Akt expression when the three cell lines were treated with 2C4, E₂ and tamoxifen. Only the tamoxifen combinations will be discussed here as all other single and dual combinations are detailed previously in this chapter. Tamoxifen did not affect the original P-Akt basal expression or any other treatment combination in any cell line.

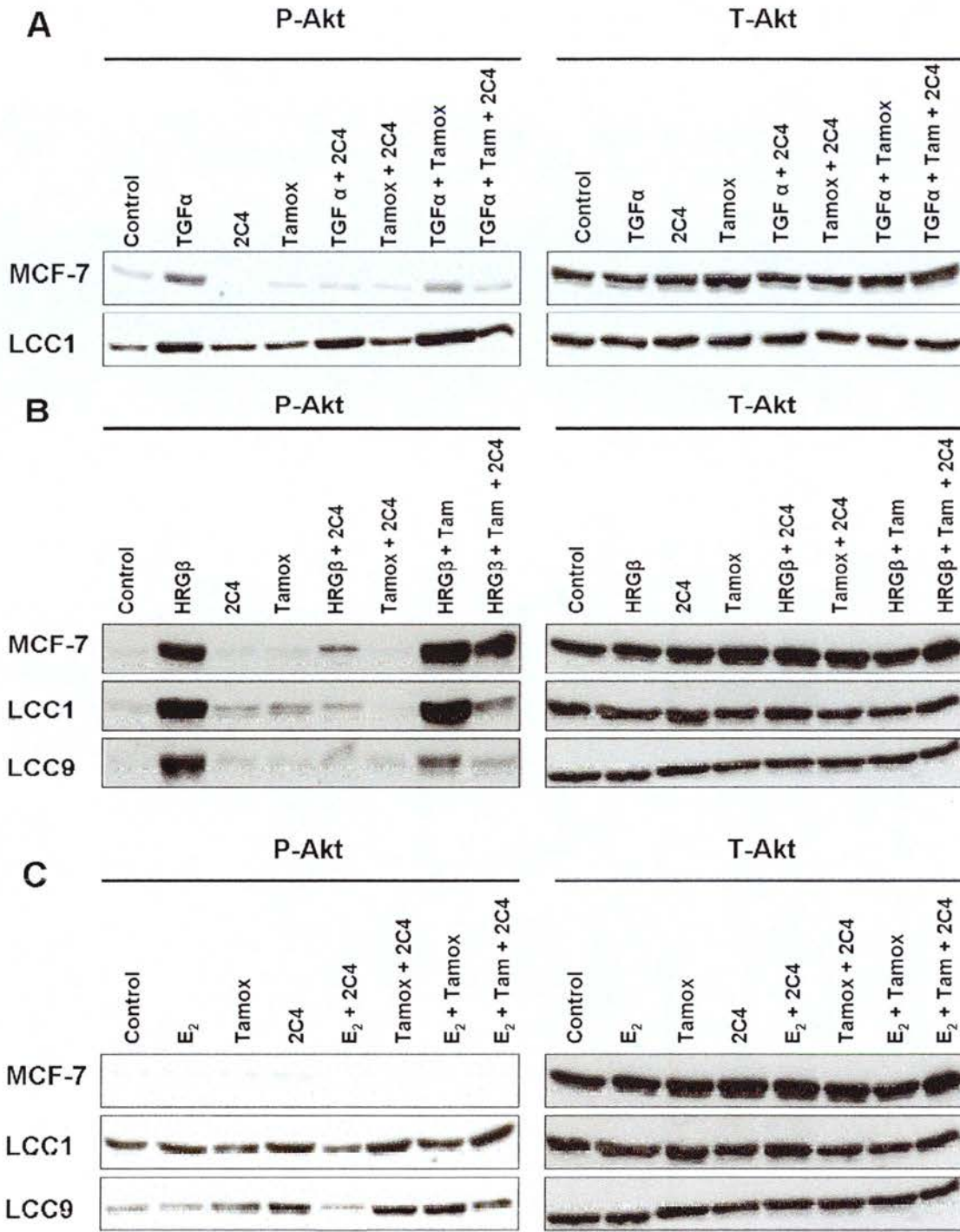


Figure 6.13 The effect of 2C4 in combination with tamoxifen on P-Akt expression levels in breast cancer cells treated with TGF α , HRG β and E₂. A-C, western blots of P-Akt levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, TGF α , HRG β or E₂ (all 1nM, A-C respectively), tamoxifen (1 μ M), or various combinations of these treatments for 15 min. Membranes were probed with P-Akt primary antibody (1:1000). T-Akt levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

6. 5. 2. *The Effect of 2C4 plus tamoxifen on the MEK/ERK signalling pathway*

6. 5. 2. 1. *ERKI/II*

Figure 6.14A shows the effects of 2C4, TGF α and tamoxifen on the pattern of P-ERKI/II expression were very similar to that of P-Akt in MCF-7 and LCC1 cell lines, where tamoxifen increased TGF α -enhanced P-ERKI/II signal intensity. Also, 2C4 reversed P-ERKI/II expression when both cell lines were treated with TGF α + tamoxifen to that of TGF α + 2C4.

The effect of 2C4, HRG β and tamoxifen on P-ERKI/II expression is shown in figure 6.14B. Again, tamoxifen increased the growth factor-enhanced P-ERKI/II band intensity in MCF-7, LCC1 and LCC9 cell lines. In the MCF-7 cell line, 2C4 reduced HRG β + tamoxifen enhanced P-ERKI/II signal, but not to the level of HRG β + 2C4. In the LCC1 and LCC9 cell lines 2C4 appeared to be more effective and the P-ERKI/II signal was almost completely abrogated back to the level of HRG β + 2C4. The western blots for P-ERKI/II levels post treatment with 2C4, E₂ and tamoxifen are presented in figure 6.14C. Levels tended to fluctuate around basal levels but no treatment clearly changed the expression.

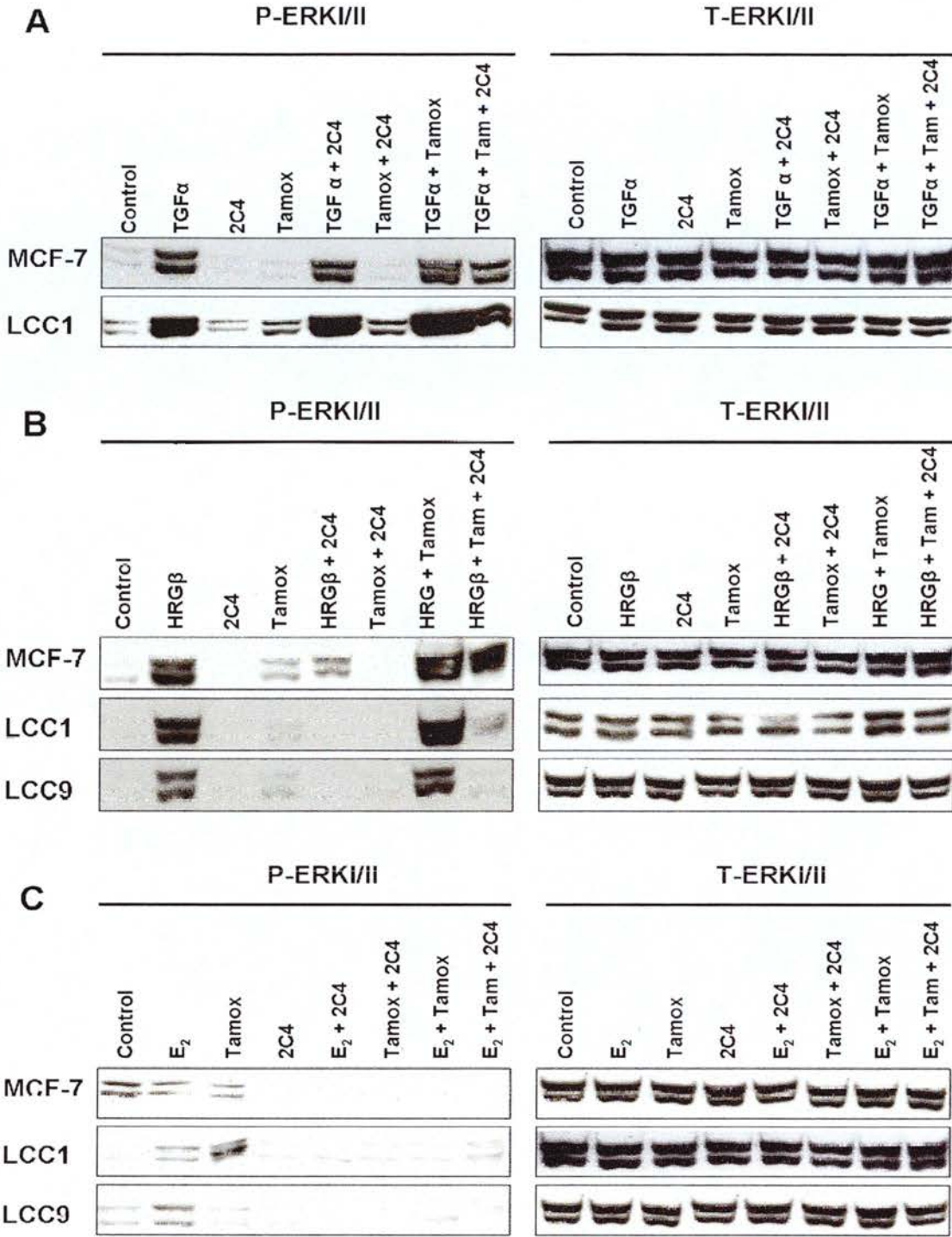


Figure 6.14 The effect of 2C4 in combination with tamoxifen on P-ERK1/II expression levels in breast cancer cells treated with TGF α , HRG β and E₂. A-C, western blots of P-ERK1/II levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, TGF α , HRG β or E₂ (all 1nM, A-C respectively), tamoxifen (1 μ M), or various combinations of these treatments for 15 min. Membranes were probed with P-ERK1/II primary antibody (1:1000). T-ERK1/II levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

6. 5. 2. 2. MEK

The effects of various treatment combinations involving a ligand, 2C4 and tamoxifen on the P-MEK expression pattern of the MCF-7, LCC1 and LCC9 cell lines are shown in figure 6.15. The effects of single agents have previously been discussed in this chapter, as have cells pre-treated with 2C4 and a ligand. Figure 6.15A shows the effect of tamoxifen on the HRG β and 2C4 study. The western blots show that tamoxifen may slightly reduce the P-MEK signal enhanced by HRG β in the MCF-7 and LCC1 cell lines, but clearly dramatically diminished the HRG β -enhanced P-MEK signal in the LCC9 cells. 2C4 reversed HRG β -enhanced P-MEK in the presence and absence of tamoxifen in all cell lines, although a signal was still faintly visible in the presence of tamoxifen in all the cell lines (visible on western blots themselves, signal less clearly seen in scanned images). Extremely faint bands were also visible in all cells treated with tamoxifen, although again these are less clear in the scanned images.

Figure 6.15B illustrates the effect of oestrogen and antioestrogen treatment in the presence and absence of the anti-erbB2 agent 2C4 on P-MEK expression. A longer film exposure showed that E₂ did in fact increase P-MEK in the MCF-7 cell line and that 2C4 was able to reverse this stimulation. 2C4 alone was also seen to reduce the control treatment level of P-MEK. Tamoxifen was observed to reduce E₂-enhanced P-MEK, but did not alter the basal level of P-MEK when administered alone. Tamoxifen did not alter the extremely low level of P-MEK signal intensity caused by 2C4 treatment. Tamoxifen did not drastically alter the P-MEK band intensity of E₂ + 2C4, although a slight increase was observed compared to E₂ + 2C4 and even E₂ + tamoxifen.

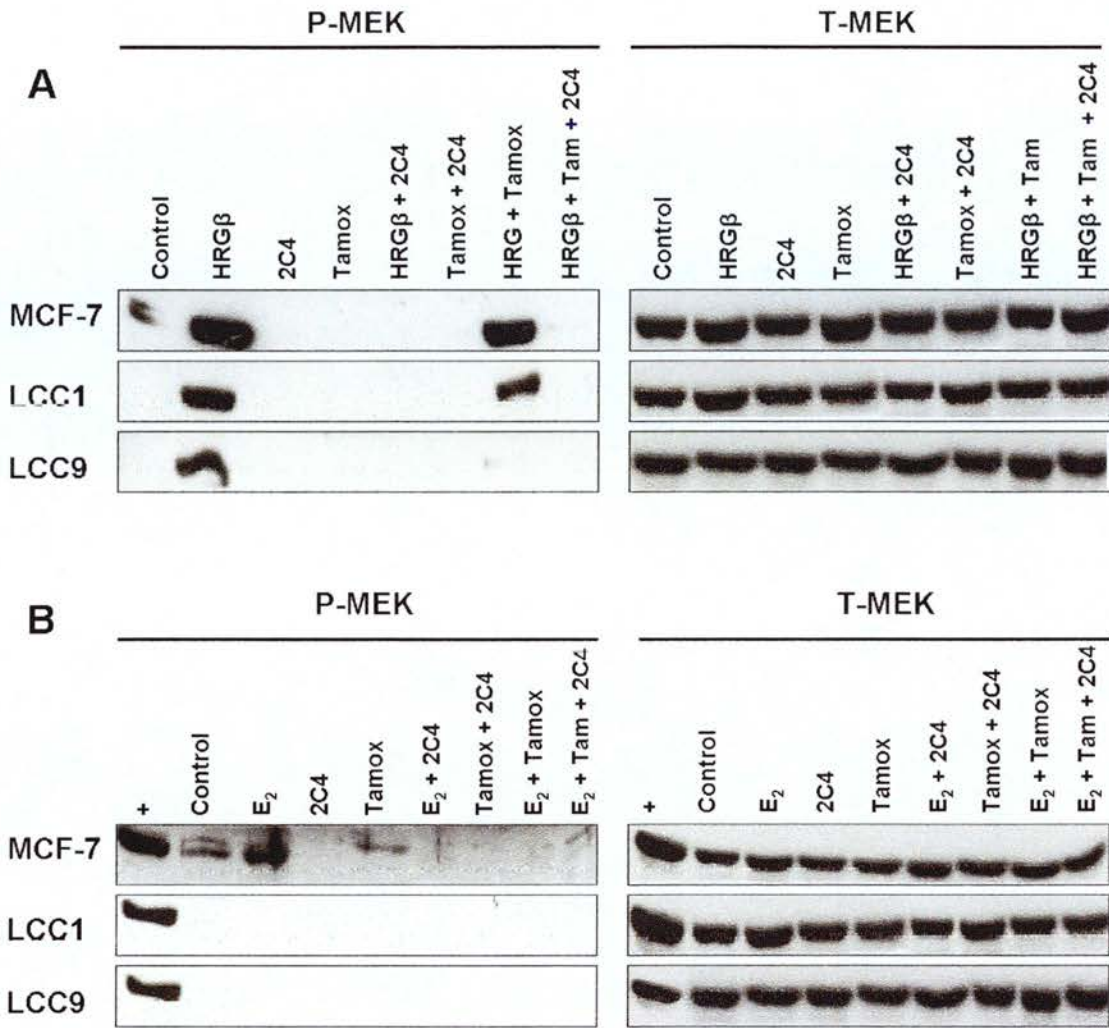


Figure 6.15 The effect of 2C4 in combination with tamoxifen on P-MEK expression levels in breast cancer cells treated with HRGβ and E₂. A-B, western blots of P-MEK levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, HRGβ or E₂ (both 1nM, A & B respectively), tamoxifen (1μM), or various combinations of these treatments for 15 min. Membranes were probed with P-MEK primary antibody (1:1000). T-MEK levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

6. 6. *ErbB* receptor expression

Figure 6.16 represents a western blot of total erbB1 and erbB2 (T-erbB1 and 2) expression in the MCF-7 cell line subjected to control, E₂, HRGβ, 2C4 and HRGβ + 2C4 treatments. The blot shows that this cell line expresses a low level of T-ErbB1 which remains relatively constant irrespective of treatment. T-ErbB2 levels were higher in the MCF-7 cell line than T-erbB1. The levels were unaffected by E₂ but appeared to be elevated by HRGβ. 2C4 alone appeared to slightly reduce T-erbB2 expression, and also appears to return HRGβ-enhanced T-ErbB2 expression back to control level.

Figure 6.17 shows T-erbB2 expression in MCF-7, LCC1 and LCC9 cell lines and the effect of E₂ and HRGβ treatment on P-ErbB2 activation at position 1248. MCF-7 cells treated with HRGβ (1nM, 15 min) were used as the positive control. Figure 6.17A shows the T-erbB2 expression levels of the MCF-7, LCC1 and LCC9 cell lines. Control (15 min) samples were used and showed that LCC1 cells had a marginally increase expression of T-erbB2 compared to the MCF-7 cell line. In contrast, LCC9 cells had a marginally reduced T-erbB2 expression level compared to the MCF-7 cell line. It was hypothesised that differential T-erbB2 expression would result in differential efficacy of anti-erbB2 agents such as 2C4. However, elevated *and* reduced T-erbB2 expression was reported in LCC1 and LCC9 cell lines respectively, and as neither cell line was affected by the anti-proliferative nature of 2C4, this suggests T-erbB2 expression is not the sole contributing factor to 2C4 resistance.

Figure 6.17B shows MCF-7, LCC1 and LCC9 cell lines did not constitutively express P-erbB2 (1248) at control level. E₂ did not affect P-erbB2 levels in any of the cell lines. HRGβ only enhanced P-erbB2 (1248) expression in the MCF-7 cell line. To explore the mechanism by which tamoxifen interacted with 2C4, cells were treated with HRGβ, a ligand found to enhance P-erbB2 (1248) in the MCF-7 cell line, 2C4 and tamoxifen (figure 6.18). Neither tamoxifen nor 2C4 alone or in combination altered P-erbB2 expression compared to control treated cells. 2C4 completely abrogated HRGβ-enhanced P-erbB2 expression in MCF-7 cells.

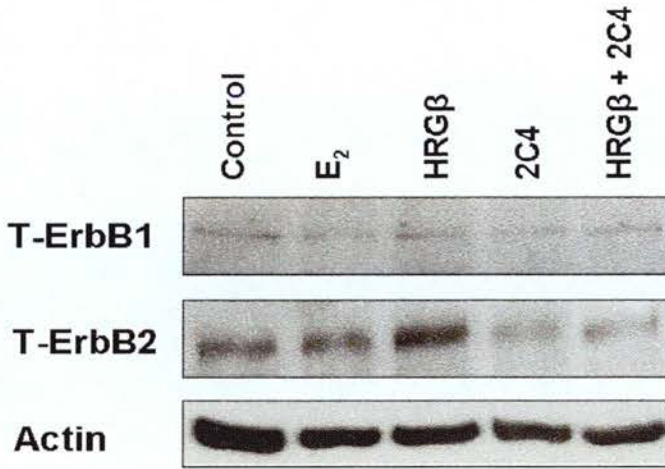


Figure 6.16 The effect of E₂, HRGβ and 2C4 on T-ErbB1 and T-ErbB2 expression levels in the MCF-7 breast cancer cell line. Western blots of T-ErbB1 and 2 levels, where all cells were charcoal stripped 48h prior to treatment and then cells were then pre-treated with 100nM 2C4 or control media for 30 min and then treated with control media or HRGβ (1nM) for 15 min. Membranes were probed with T-erbB1 and T-erbB2 primary antibodies (1:1000). Actin levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

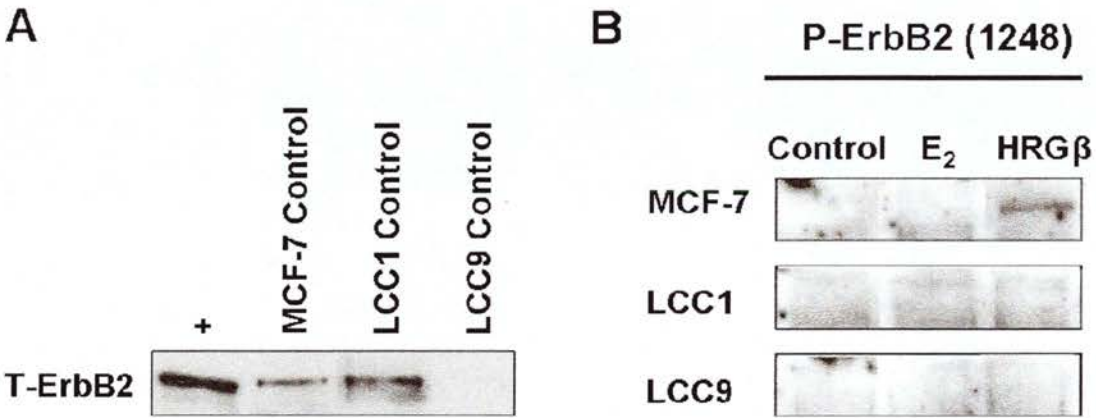


Figure 6.17 Comparison of T-ErbB2 expression and the effect of E₂ and HRGβ on P-ErbB2 (1248) expression levels in parental and resistant breast cancer cells. Western blots of T-rbB2 (A) and P-erbB2 (1248) (B) expression levels, where all cells were charcoal stripped 48h prior to treatment and were then cells were treated with control media, E₂ or HRGβ (both 1nM) for 15 min. Membranes were probed with T-erbB2 or P-erbB2 (1248) primary antibody (1:1000). Western blots are representative of 1 of 3 independent experiments.

The most apparent alteration to P-erbB2 expression was the dramatic increase in HRG β -enhanced P-erbB2 signal upon the addition of tamoxifen. 2C4 was able to return this induction back to basal levels, but not to the low levels reached with HRG β and 2C4 solely. No P-erbB2 was detected in either the LCC1 or LCC9 cell line irrespective of treatment.

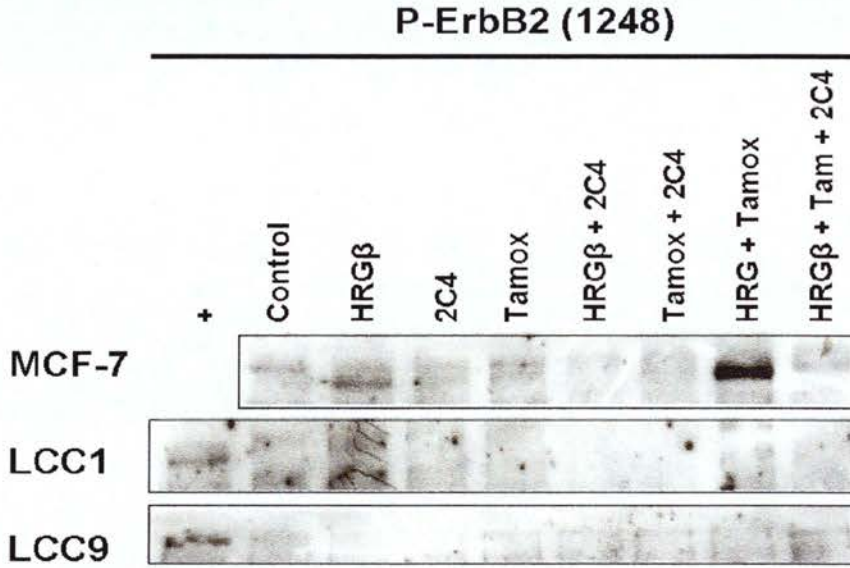


Figure 6.18 The effect of 2C4 alone and in combination with tamoxifen on P-ErbB2 (1248) expression levels in breast cancer cells treated with HRG β . Western blots of P-erbB2 levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, HRG β (1nM), tamoxifen (1 μ M), or various combinations of these treatments for 15 min. Membranes were probed with P-erbB2 (1248) primary antibody (1:1000). Western blots are representative of 1 of 3 independent experiments.

6. 7. The Effect of 2C4 on phosphorylation of ER α

The data provided in this chapter show that 2C4 inhibited signalling in the resistant cell lines, but this did not correlate with a reduction in cell number. Therefore, another component possibly downstream of the PI3-K/Akt and MEK/ERK pathways may play a role in proliferation in this model. The effect of 2C4 on the activation of ER α via phosphorylation of serine residues is discussed in the following sections. It was hypothesised that 2C4 would inhibit ER α activation at both S118 and S167 in the MCF-7 cell line as these cells responded to the antiproliferative nature of 2C4. Subsequently it was further hypothesised that 2C4 would be unable to inhibit P-S118 and P-S167 in the LCC1 cell line and P-S167 in the LCC9 cell line.

6. 7. 1. 2C4 and P-S118

Figure 6.19 represents western blots of the effect of E₂, TGF α , HRG β and 2C4 alone and in combination with tamoxifen on P-S118 expression levels in MCF-7, LCC1 and LCC9 cell lines. E₂, TGF α and HRG β all enhanced P-S118 in the MCF-7 cell line. 2C4 alone reduced P-S118 slightly, and reduced E₂-enhanced P-S118 and abrogated growth factor-enhanced P-S118 back to control level. E₂ induced a dramatic increase in P-S118 in the LCC1 cell line in agreement with previous findings. TGF α and HRG β both enhanced P-S118, but to a much lesser degree than E₂, while 2C4 alone did not alter P-S118 levels from that of control treated LCC1 cells. In contrast to MCF-7 cells, 2C4 was unable to reverse, or even reduce E₂-enhanced P-S118 to any extent. 2C4 abolished HRG β -enhanced P-S118, but was unable to reduce TGF α -enhanced P-S118. These findings are similar to the data obtained for P-Akt and P-ERK when subjected to the same treatment conditions. Very little if any P-S118 was observed in the LCC9 cell line, as previously detailed, irrespective of treatment.

The MCF-7 cell line was the only cell line in which tamoxifen actually reduced the antiproliferative nature of 2C4, hence the combination of tamoxifen plus 2C4 was investigated against P-S118 of MCF-7 cells. Figure 6.20 shows the effect of 2C4 alone and in combination with tamoxifen on ER α P-S118 expression levels in MCF-7 breast cancer cells treated with E₂. 2C4 and tamoxifen were both able to reduce E₂-enhanced P-S118 alone, but appeared to antagonise each other when combined against E₂ as the signal was not reduced to the same extent.

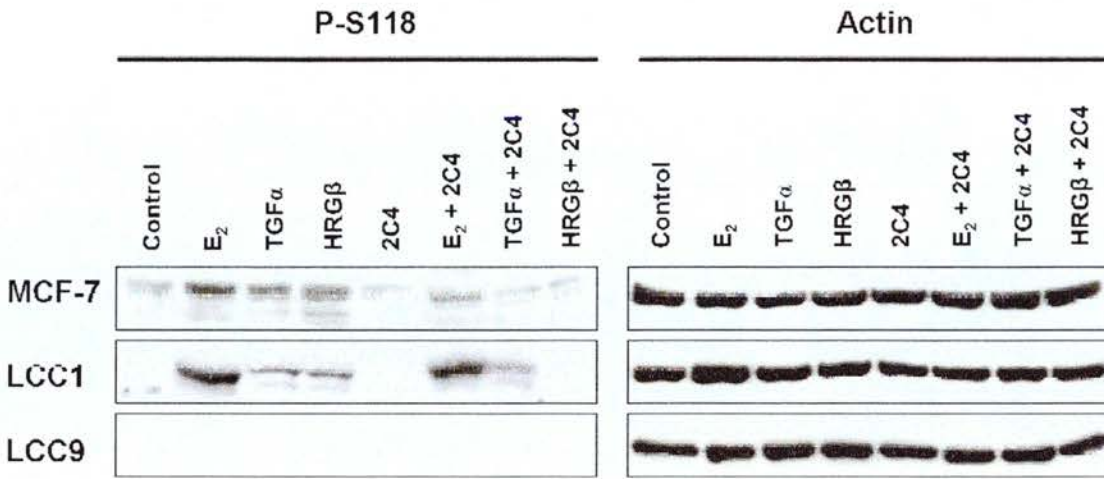


Figure 6.19 The effect of E₂, TGF α , HRG β and 2C4 alone and in combination with tamoxifen on ER α P-S118 expression levels in breast cancer cells. Western blots of P-S118 levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, E₂, TGF α , HRG β (all 1nM), tamoxifen (1 μ M), or various combinations of these treatments for 15 min. Membranes were probed with P-S118 primary antibody (1:1000). Actin levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

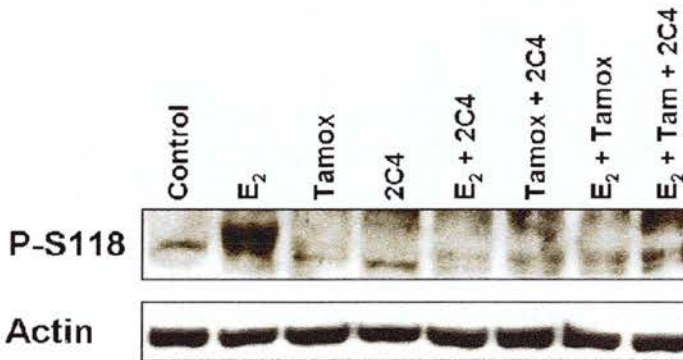


Figure 6.20 The effect of 2C4 alone and in combination with tamoxifen on ER α P-S118 expression levels in MCF-7 breast cancer cells treated with E₂. Western blots of ER α P-S118 levels, where MCF-7 cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, E₂ (1nM), tamoxifen (1 μ M), or various combinations of these treatments for 15 min. Membranes were probed with P-S167 primary antibody (1:1000). Actin levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

6. 7. 2. 2C4 and P-S167

HRG β is known to regulate ER α activity via the erbB2/PI3-K/Akt pathway (Stoica *et al*, 2003) and was found to enhance P-S167 in not only MCF-7 cells, but also both LCC1 and LCC9 cell lines. Figure 6.21 shows the effect of 2C4 alone and in combination with tamoxifen on ER α P-S167 expression levels in MCF-7, LCC1 and LCC9 cells treated with HRG β . Control levels were equivalent in all cell lines and HRG β enhanced P-S167 in all cell lines. 2C4 and tamoxifen alone did not affect P-S167 of any cell line. 2C4 reduced HRG β -enhanced P-S167 in all cells. Tamoxifen did not appear to affect HRG β -enhanced P-S167 in the MCF-7 and LCC1 cell lines. Tamoxifen appeared to decrease P-S167 in LCC9 cells treated with HRG β . Tamoxifen prevented 2C4 from fully reversing HRG β -enhanced P-S167 in MCF-7 cells, while it did not appear to affect the ability of 2C4 to abrogate P-S167 increased by HRG β in the resistant cells.

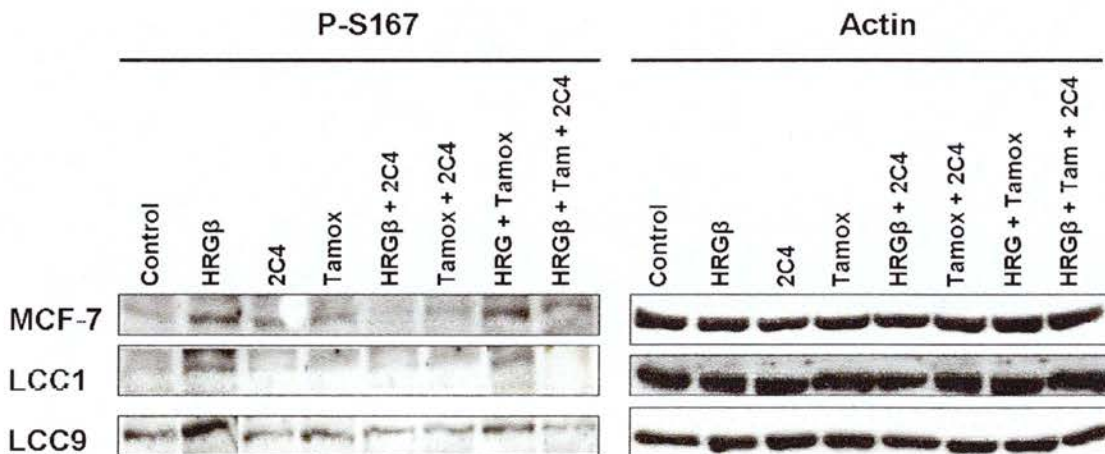


Figure 6.21 The effect of 2C4 alone and in combination with tamoxifen on ER α P-S167 expression levels in breast cancer cells treated with HRG β . Western blots of ER α P-S167 levels, where all cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, HRG β (1nM), tamoxifen (1 μ M), or various combinations of these treatments for 15 min. Membranes were probed with P-S167 primary antibody (1:1000). Actin levels were used as loading controls. Western blots are representative of 1 of 3 independent experiments.

6. 8. The effect of 2C4 on apoptosis in this model of resistance

The effect of 2C4 on Annexin V staining, and thus apoptosis, on MCF-7, LCC1 and LCC9 cell lines treated with HRG β is illustrated in figure 6.22. Cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min. The cells were then treated with control media, HRG β or HRG β +2C4 (1nM + 100nM) for 15 min. The histogram of MCF-7 cells shows that Annexin V staining is significantly diminished by the addition of HRG β as previously described in chapter 3. 2C4 did not significantly affect the percentage of MCF-7 cells with positive Annexin V staining and thus the number of cells undergoing apoptosis. 2C4 was able to significantly increase Annexin V staining originally reduced by HRG β by ~0.6-fold ($P<0.01$) implying that the proportion of apoptotic cells was increased.

No significant differences were observed in Annexin V staining in the LCC1 cell line, indicating HRG β and 2C4 do not affect the number of cells undergoing apoptosis in the LCC1 cell line. The only significant difference observed with this combination of treatments in the LCC9 cell line was with the combination of HRG β plus 2C4. However, this was only a small increase (~0.1-fold, $P<0.05$) in Annexin V staining and did not correlate with a reduction in proliferation. It is interesting to note that both the LCC1 and LCC9 control levels of Annexin V staining are less than that of the MCF-7 cell line (~1.7% and ~0.9% in the LCC1 and LCC9 cell lines respectively, compared with ~3.9% in MCF-7 cells). This implies the NETT growth rate balance has shifted, indicating a reduction in the number of LCC- cells undergoing apoptosis may coincide with an increase in the proliferation of the resistant cell lines.

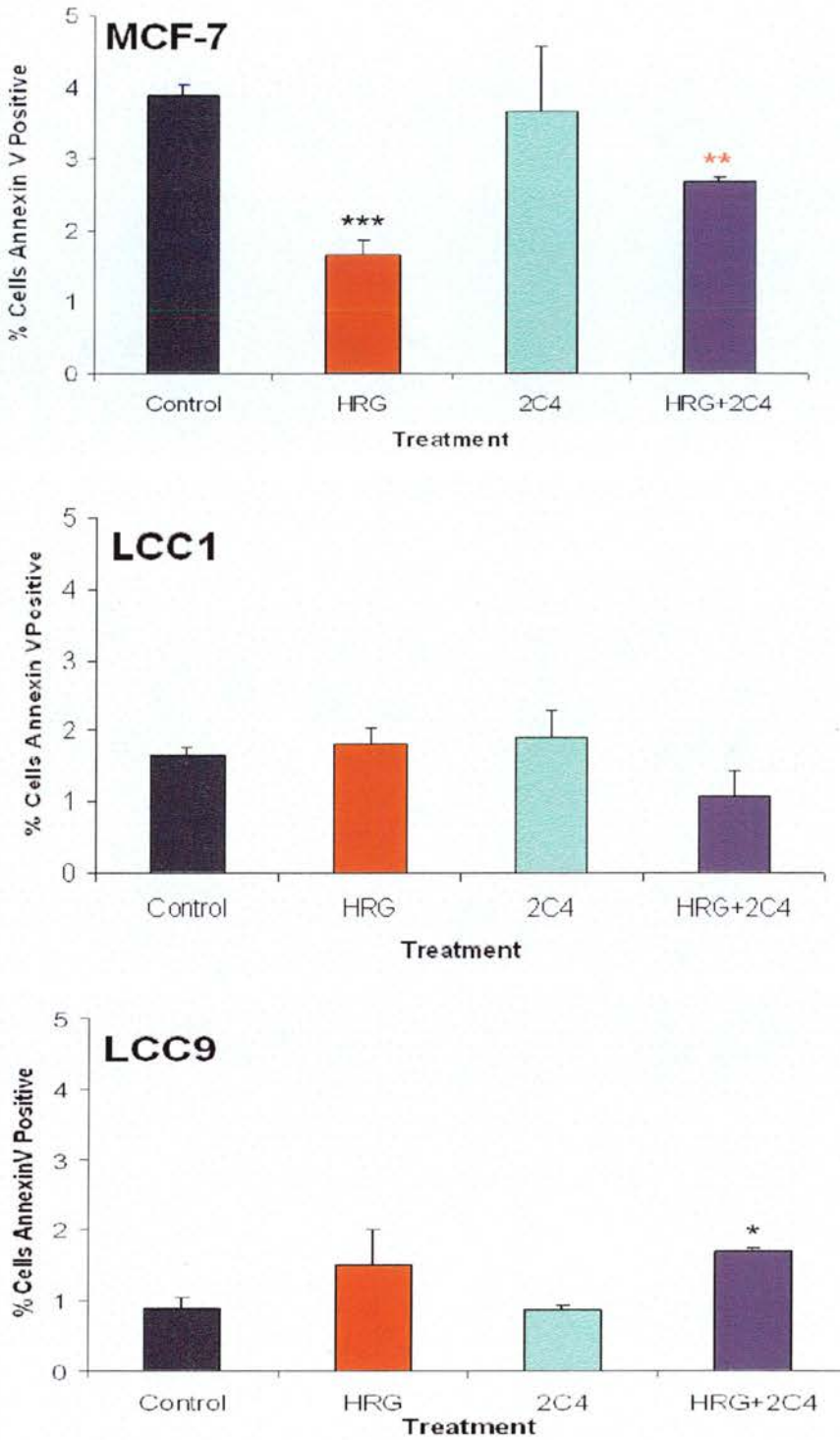


Figure 6.22 The effect of 2C4 on annexin-v staining in breast cancer cells treated with HRGβ and 2C4. Cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, HRGβ or HRGβ +2C4 (1nM + 100nM) for 15 min. ANOVA test: * = P<0.05, ** = P<0.01 & *** = P<0.001. Asterisks in black represent statistically significant changes between control and a treatment and those in red represent any statistically significant changes post-treatment with 2C4 in each cell line. Histograms are representative of 1 of 3 independent experiments.

6. 9. The effect of 2C4 on cell cycle progression in this model of resistance

6. 9. 1. E₂

Histograms of the effect of 2C4 and E₂ on cell cycle distribution of MCF-7, LCC1 and LCC9 cell lines are shown in figure 6.23. Control and E₂ treatments in all cell lines are as previously reported in chapter 4. Figure 6.23A is a histogram of the effect of these treatments on the percentage of MCF-7 cells in G_{0/1}, S and G₂/M phases of cell cycle.

2C4 pre-treatment did not alter E₂-induced progression in general, with the only significant increase of ~2% observed in the G_{0/1} phase (P<0.05) (as indicated by the red asterisk). The E₂+2C4 combined treated was significantly different to the control treatment (P<0.001), but was generally similar to E₂ alone.

The LCC1 cell cycle distribution pattern is shown in figure 6.23B. Interestingly, compared to E₂ treatment alone, the combined treatment significantly decreased the percentage of cells in G_{0/1} phase by ~30% and significantly increased cells in S- and G₂/M phases by ~23% and ~7.5% respectively (all P<0.001). This suggests 2C4 may be causing a block in cell cycle, or contrastingly, inducing progression. However, the effect of 2C4 on cell cycle did not translate to any significant affect on the proliferation of the LCC1 cell line in the presence or absence of E₂. Figure 6.23C is a histogram of the same experiment in the LCC9 cell line where 2C4 pre-treatment did not significantly alter the cell cycle distribution compared to E₂ alone

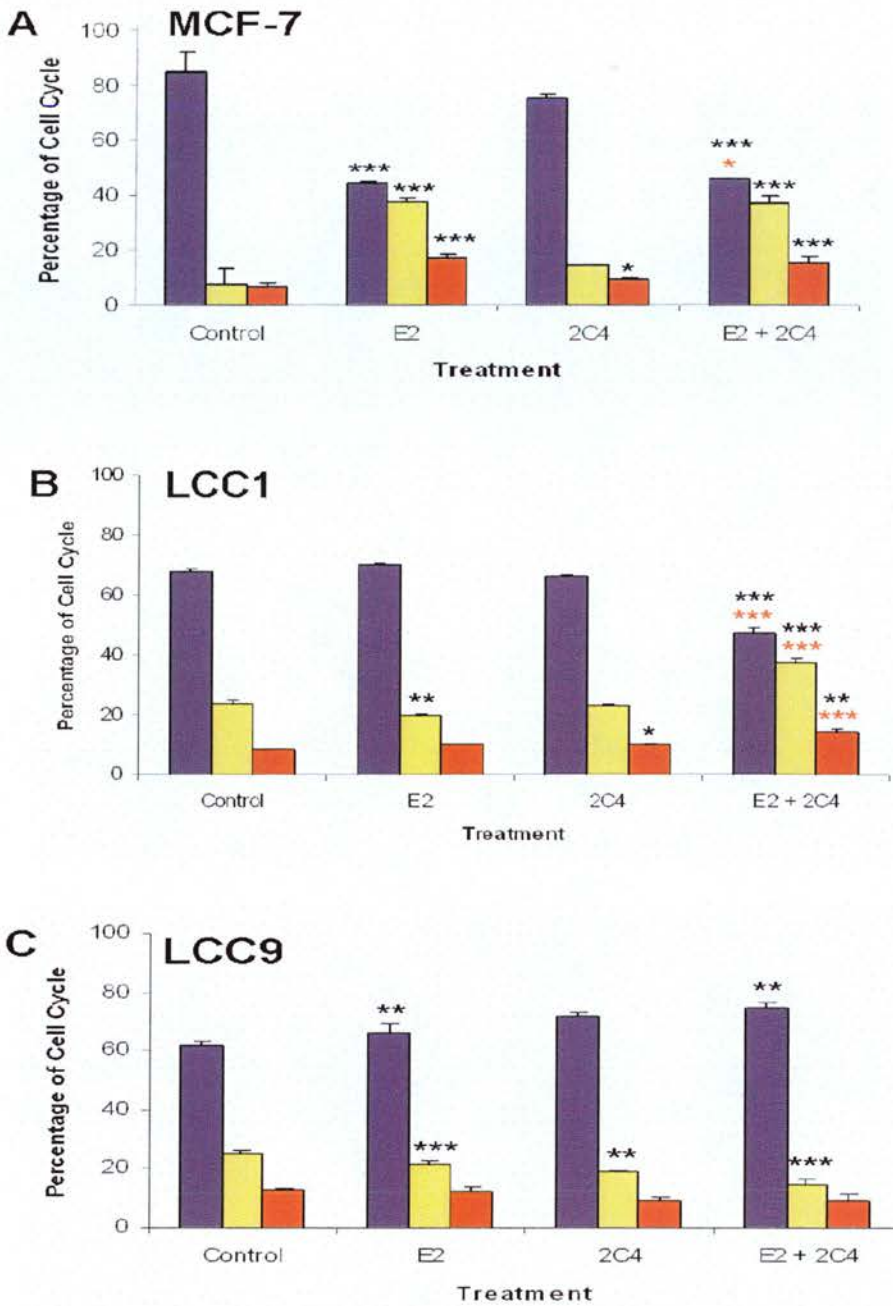


Figure 6.23. The effect of 2C4 pre-treatment on the cell cycle distribution of breast cancer cell lines treated with E₂. Cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, E₂ or E₂+2C4 (1nM+100nM) for 15 min. Changes in G_{0/1} (■), S (■) and G_{2/M} (■)-phases of the cell cycle were plotted as the mean of triplicate samples +/- SD as a percentage of the cell cycle. ANOVA test: * (P<0.05), ** (P<0.01) and *** (P<0.001) represent significant, highly significant and extremely significant changes. Black asterisks represent significance between the same cell cycle phase with each treatment, while red asterisks represent significance change between the same phase after E₂ treatment compared to E₂+2C4 in each cell line. Histograms are representative of 1 of 3 independent experiments.

6. 9. 2. *TGF α*

Histograms representing the effect of *TGF α* in the presence and absence of 2C4 on the cell cycle distribution of MCF-7, LCC1 and LCC9 cell lines are shown in figure 6.24. Figure 6.24A shows the effect of these treatments in the MCF-7 cell line. *TGF α* significantly increased the proportion of cells in S and G₂/M phase and reduced those in the G_{0/1} phase as previously described in chapter 3 ($P < 0.001$). 2C4 alone significantly elevated the percentage of cells in S and G₂/M phases ($P < 0.001$) and reduced the number of cells in G_{0/1} phase. However, 2C4 generally decreased MCF-7 proliferation slightly; therefore the increase in the proportion of cells in S- and G₂/M-phases could indicate a cell cycle block which would be consistent with a small degree of growth inhibition. 2C4 significantly reduced the increase in the percentage of cells in S-phase caused by *TGF α* by ~11% by decreasing the number of cells in S-phase to ~19% ($P < 0.001$), a value near to that of 2C4 treatment alone (~18%). There was a similar significant increase of cells in G_{0/1} phase of ~13% ($P < 0.001$).

A histogram of the same treatment combinations in the LCC1 cell line is presented in figure 6.24B. *TGF α* significantly increased the percentage of cells in G_{0/1}-phase by ~10% ($P < 0.01$), while a decrease of ~8% was observed in the S-phase of the cell cycle distribution ($P < 0.001$). 2C4 alone produced a similar pattern to that of *TGF α* treatment, with a reduction in G₂/M phase also documented. 2C4 pre-treatment did not affect cell cycle distribution when treated with *TGF α* compared to *TGF α* alone. *TGF α* only affected LCC9 cell cycle distribution between the S and G₂/M phases (figure 6.24C). 2C4 significantly increased the proportion of *TGF α* -treated cells in S-phase by ~1.8% ($P < 0.05$).

Generally speaking, the largest numerical percentage changes were observed in the MCF-7 cell line. The changes between treatments in the resistant cell lines may be classified as significant, but the actual percentages are not particularly large in comparison to the changes observed in the MCF-7 cell line. This indicates that changes between the cell lines are more important.

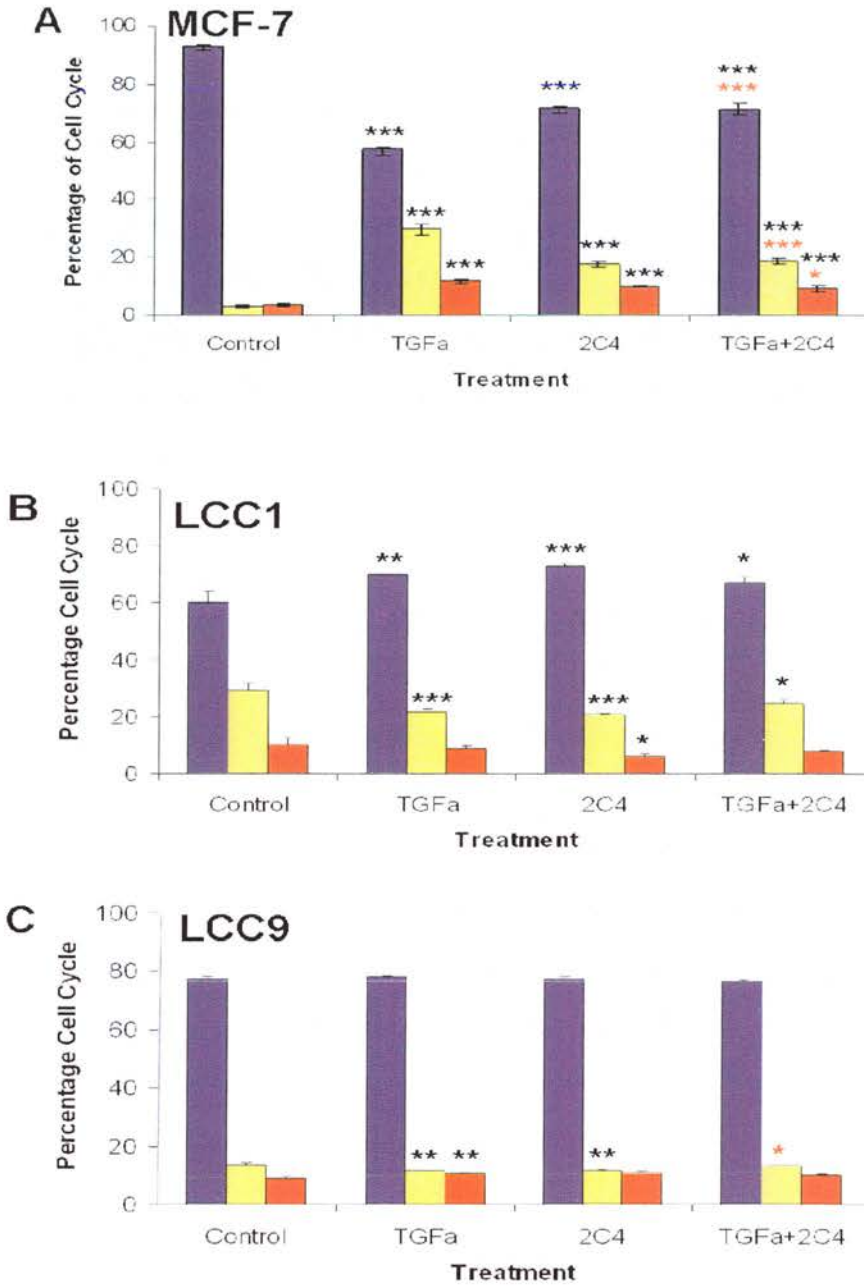


Figure 6.24 The effect of 2C4 pre-treatment on the cell cycle distribution of breast cancer cell lines treated with TGFα. Cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100μM 2C4 for 30 min then the cells were treated with control media, TGFα or TGFα+2C4 (1nM+100nM) for 15 min. Changes in G_{0/1} (■), S (■) and G_{2/M} (■)-phases of the cell cycle were plotted as the mean of triplicate samples +/- SD as a percentage of the cell cycle. * (P<0.05), ** (P<0.01) and *** (P<0.001) represent significant, highly significant and extremely significant changes. Black asterisks represent significance between the same cell cycle phase with each treatment in that cell line, while red asterisks represent significance change between the same phase after TGFα treatment compared to TGFα+2C4 in each cell line. Histograms are representative of 1 of 3 independent experiments.

6. 9. 3. HRG β

Figures 6.25A-C are histograms of cell cycle distribution in the three cell lines treated in the same manner as figure 6.24, but with HRG β substituted for TGF α . Control and HRG β treatments were as previously described in chapter 4 in all cell lines. In this experiment 2C4 did not significantly alter MCF-7 cell cycle distribution (figure 6.25A), which would support signalling and proliferation data where MCF-7 cell response to 2C4 was minimal. The cell cycle progression stimulated by HRG β was actually enhanced further by 2C4 pre-treatment, with a reduction in G_{0/1} phase of ~10.5% (P<0.05) and an increase of ~12% (P<0.01) in S-phase.

Figure 6.25B is a histogram representing the LCC1 cell line under the same conditions. The only significant change with 2C4 pre-treatment was an increase of less than 1% in G₂/M phase. 2C4 pre-treatment produced the opposite effect to that in the MCF-7 cell line. A significant reduction of ~5.5% (P<0.001) of cells in S-phase and an increase in cells in G_{0/1} phase (~5% increase, P<0.001) was observed in cells treated with the combination of HRG β and 2C4, indicating 2C4 may arrest HRG β -treated LCC1 cells, but this does not translate to a reduction in cell number.

Figure 6.25C is a histogram of the effect of HRG β in the presence and absence of 2C4 on LCC9 cell cycle distribution. Chapter 4 details the elevated percentage of resistant cells in S and G₂/M phases of cell cycle compared to the MCF-7 cell line which can again be observed here and in figures 6.23 and 6.24. 2C4 alone again had a minor affect only by reducing the number of LCC9 cells in S-phase (~4%, P<0.05). 2C4 pre-treatment did significantly change the cell cycle distribution compared to HRG β alone or control treatments.

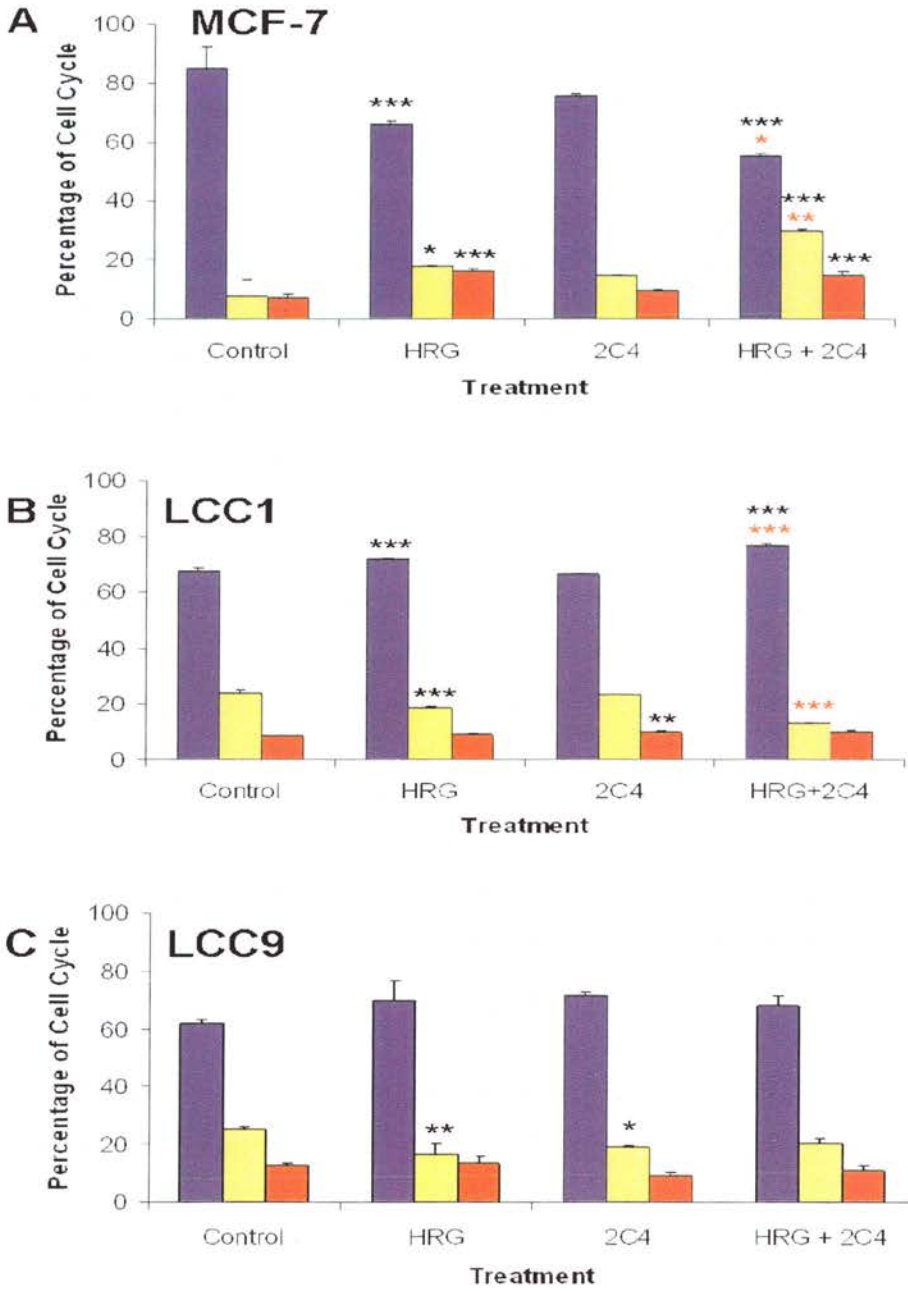


Figure 6.25 The effect of 2C4 pre-treatment on the cell cycle distribution of breast cancer cell lines treated with HRGβ. Cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, HRGβ or HRGβ+2C4 (1nM+100nM) for 15 min. Changes in G_{0/1} (■), S (●) and G_{2/M} (■)-phases of the cell cycle were plotted as the mean of triplicate samples +/- SD as a percentage of the cell cycle. ANOVA test: * (P<0.05), ** (P<0.01) and *** (P<0.001) represent significant, highly significant and extremely significant changes. Black asterisks represent significance between the same cell cycle phase with each treatment in that cell line, while red asterices represent significance change between the same phase after HRGβ treatment compared to HRGβ+2C4 in each cell line. Histograms are representative of 1 of 3 independent experiments.

6. 10. The effect of tamoxifen and 2C4 on cell cycle progression in this model of resistance

Figure 6.26 presents three histograms of the 2C4 combined with tamoxifen in the presence and absence of HRG β on the cell cycle distribution of MCF-7, LCC1 and LCC9 cell lines. Only the relevant combination treatments are shown (with control and tamoxifen alone for comparison) as all other treatments were as previously described. Tamoxifen significantly reduced the proportion of MCF-7 cells in G₂/M and dividing in S-phase due to HRG β -treatment, and significantly increased the percentage of cells undergoing arrest from ~51% with HRG β to ~71% (P<0.001) (figure 6.26A). Tamoxifen in combination with 2C4 also significantly increased the percentage of cells in G_{0/1} arrest from ~72% with 2C4 alone, to ~84% (P<0.001). The addition of tamoxifen to the HRG β +2C4 treatment reversed the effect of 2C4 on HRG β and increased MCF-7 cell arrest. Tamoxifen appeared to be reversing the effect of 2C4. This may have ramifications on the anti-proliferative effect of 2C4.

A similar pattern was observed in the LCC1 cell cycle distribution (figure 6.26B), where tamoxifen reduced the proportion of cells dividing in S-phase. Tamoxifen reversed the action of 2C4 on HRG β , increasing the number of cells in S-phase from ~13% with HRG β + 2C4 to ~16% in the presence of the triple treatment (P<0.001, as indicated by the green asterices).

Tamoxifen did not significantly affect the LCC9 cell cycle distribution when cells treated with HRG β (P>0.05). This contrasts with LCC1 and MCF-7 cell line data. The addition of tamoxifen to cells treated with HRG β + 2C4 pushed more cells into G_{0/1} arrest (~2% increase, P<0.05) and reduced S-phase dividing cells by ~4% (P<0.001).

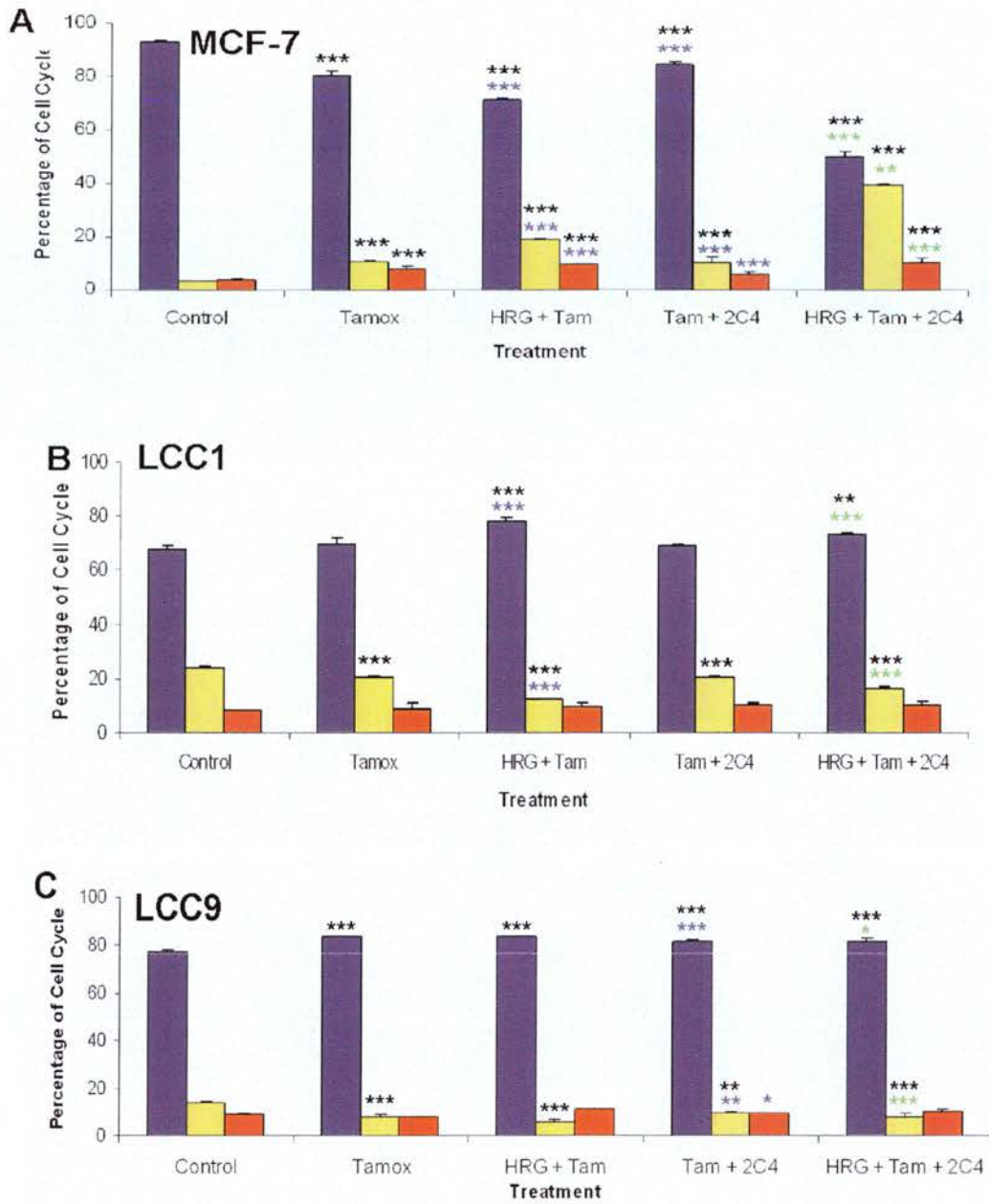


Figure 6.26 The effect of 2C4 combined with tamoxifen in the presence and absence of HRGβ on the cell cycle distribution of breast cancer cell lines. Cells were charcoal stripped 48h prior to treatment and were then pre-treated with 100nM 2C4 for 30 min then the cells were treated with control media, HRGβ (1nM), tamoxifen (1μM), and various combinations of these agents for 15 min. Changes in G_{0/1} (■), S (■) and G₂/M (■)-phases of the cell cycle were plotted as the mean of triplicate samples +/- SD as a percentage of the cell cycle. ANOVA test: * (P<0.05), ** (P<0.01) and *** (P<0.001) represent significant, highly significant and extremely significant changes. Black asterices represent significance between the same cell cycle phase with each treatment compared to control, blue asterices represent significance after tamoxifen treatment compared to 2C4 alone and green asterices represent significance between HRGβ + 2C4 vs. HRGβ + tamoxifen + 2C4 treatment. Histograms are representative of 1 of 3 independent experiments

6. 11. Discussion

Overexpression of erbB2 occurs in ~12-20% of ER α + human breast cancer (Witton *et al*, 2003), and is associated with malignant transformation, poor overall survival and oncogenesis (Yu and Hung, 2000). This receptor has therefore become a useful target for drug development for treatment of hyperproliferative diseases including breast cancer (Mass, 2004; Shawyer *et al*, 2002).

A reduction in the level of P-Akt (and P-ERK) had been suggested as a requirement for another anti-erbB2 agent herceptin to reduce cell proliferation, hence it has been hypothesised that this is also the mechanism of action of 2C4 (Tanner *et al*, 2004; Agus *et al*, 2003). The anti-erbB2 agent 2C4 abolished the stimulatory effects on both Akt and ERK and growth effects of TGF α and HRG β in the MCF-7 cell line in agreement with data produced by Agus *et al* (2003) in the same cell line and Totpal *et al* (2002) in ovarian cells. 2C4 was less effective at reducing TGF α than HRG β -stimulated proliferation suggesting that the nature of erbB2 heterodimer(s) formed in the MCF-7 cell line dictates the efficacy of this agent. This is supported by data found in ovarian cell lines by Takai *et al* (2005). This contrasts with T-erbB1 expression, which was found to be less than T-erbB2 expression in the MCF-7 cell line, suggesting less of a drive through TGF α heterodimers. Therefore, this indicates that perhaps even low T-erbB1 levels in this cell line are sufficient for some degree of TGF α signalling, perhaps via erbB1 homodimers.

The Akt and ERK1/II signalling pathways are intact in the parental MCF-7 and resistant LCC1 and LCC9 cell lines alike. Interestingly, 2C4 only abolished the elevated level of P-Akt and P-ERK (and P-S118 in the LCC1 cell line) enhanced by HRG β in the resistant cells, indicating these pathways may be partially responsible for some growth of the cell lines, and that this is dependent on the ligand involved. However, the abrogation of signalling did not translate to the growth data in the resistant cell lines, as neither growth factor increased proliferation by day 3, and 2C4 did not reduce cell proliferation in either resistant cell line. 2C4 was able to reverse the activation of certain pathways by certain ligands, in particular HRG β , but was unable to reverse P-Akt or P-ERK expression (or P-S118 in the LCC1 cell line) enhanced by TGF α in these cells. Again, this could be associated with the heterodimers formed. T-erbB1 levels in the MCF-7 cell line were found to be low,

which is consistent with published data (Hager *et al*, 2005), the T-erbB1 expression of the LCC1 and LCC9 resistant cell lines requires investigation in order to assess their involvement in 2C4 resistance.

The antiproliferative nature of 2C4 is dependent on the initial ligand stimulating growth. Takai *et al* (2005) suggested that in cells where 2C4 was able to inhibit MEK/ERK signalling such as the MCF-7 cell line, recruitment of erbB2 into erbB receptor complexes was necessary for maximal signalling response. Previous studies have shown that this occurs for erbB1, where the blockade of erbB2 recruitment into erbB1-ligand complexes does not abrogate signalling, but instead diminishes the signal intensity, duration and diversity (Anido *et al*, 2003). This information is summarised in figure 6.27.

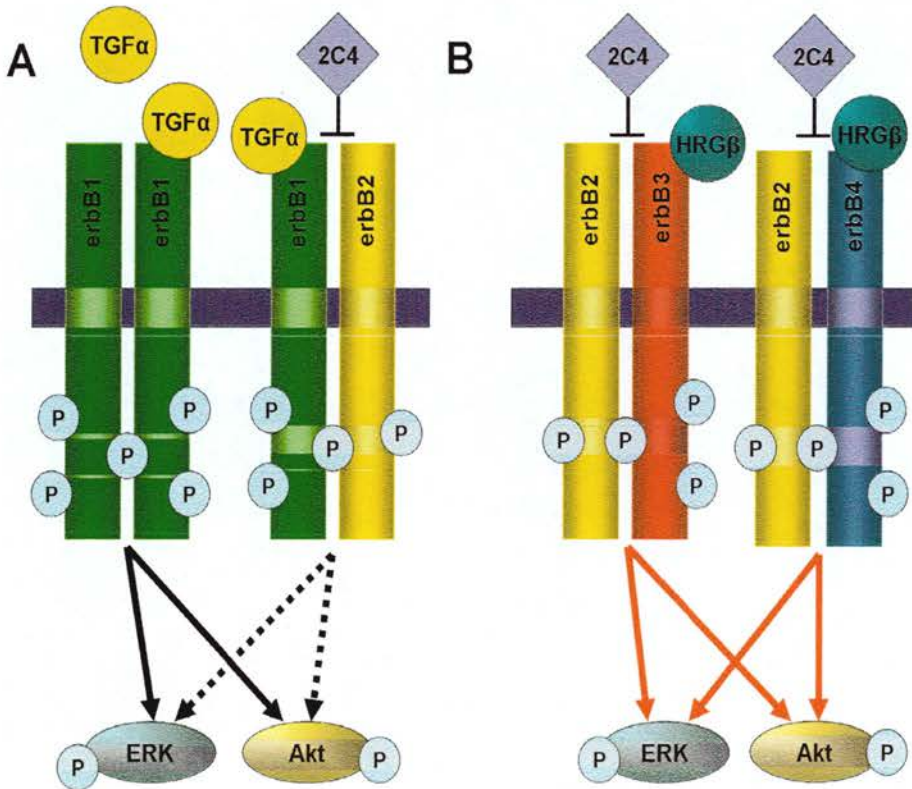


Figure 6.27 Schematic diagram showing 2C4 targeting erbB2-containing heterodimers. 2C4 acts as a 'dimerisation inhibitor' and blocks activation of ERK and Akt via any heterodimers erbB2 only. A, TGFα treatment facilitated phosphorylation of erbB1-based dimers, where signal intensity may not be completely abolished but instead reduced in intensity (Anido *et al*, 2003) (dashed black arrows). ErbB1 homodimers (shown as example) or erbB1/3 or 4 heterodimers induced by TGFα stimulation would not be inhibited by 2C4 (solid black arrows). B, 2C4 treatment abolished the formation of phosphorylated erbB2/3 heterodimers induced by HRGβ (red arrows) possibly by sequestration of the receptors into unphosphorylated inactive erbB1/2 and erbB1/3 heterodimers. The abrogation of HRGβ-induced P-Akt and P-ERK suggests only erbB2 containing heterodimers are formed in the presence of this ligand in MCF-7 and resistant cell lines alike.

This may account for the differential blockade of HRG β but not TGF α -enhanced signalling in the resistant cell lines as HRG β creates erbB2/3 heterodimers as opposed to erbB1/2 heterodimers formed due to TGF α stimulation.

Interestingly, a long western blot exposure showed that E₂ elevated P-MEK in MCF-7 cells, which is consistent with a study by Keshamouni *et al* (2002). This group reported that, in contrast to the rapid TGF α -enhanced ERK activation, E₂ only enhanced P-ERK after 2h. This is consistent with the weak P-MEK signal observed here after 15 min, which would, in time, activate ERK.

Cell cycle distribution data gathered here demonstrates that a smaller proportion of resistant cells than MCF-7 cells undergo cell cycle arrest. These data are in accordance with the fact that breast cancer is due to an imbalance between proliferation and apoptosis (Meteoglu *et al*, 2005; McCloskey *et al*, 1996). Thus, a heightened rate of cell division may account for the elevated basal proliferation of the resistant cell lines.

Published findings show that **tamoxifen** arrests breast cancer cells in G₁ phase and those cells which were not retained in the G₀/G₁ phase appear to progress into the remainder of the cell cycle at a similar rate to that of untreated control cells (Prall *et al*, 1997; Taylor *et al*, 1983). The slight oestrogenic action of tamoxifen may account for this, and while the majority of MCF-7 cells undergo this arrest, not all of the cells do, which may also contribute to induction of MCF-7 cells into the cell cycle. This is consistent with a study by Liu *et al* (2003), who reported that tamoxifen resistance involved changes preventing apoptosis and enhancing cell survival and proliferation.

The 2C4 reversal of TGF α -enhanced signalling and MCF-7 proliferation was linked with a reversal of cell cycle progression and an increased number of cells undergoing cell cycle arrest. This is supported by a study using another anti-erbB2 antibody Herceptin, which also was found to increase the proportion of cells arresting and subsequently decrease the percentage of cells in S-phase (Le *et al*, 2003). TGF α data contrasts with HRG β -enhanced signalling and proliferation which were completely abrogated by 2C4 and are linked with an *increase* in dividing cells. The variation in

responses to growth factors is in agreement with published data by Takai *et al* (2005) and Anido *et al* (2003). This indicates the erbB2/3 conformation is more sensitive to 2C4 than erbB1/2 heterodimer and supports the differential erbB heterodimer conformation theory proposed by these authors. Interestingly, cell cycle data indicates that 2C4 was more efficacious as an anti-proliferative agent when cells were pushed into S-phase as opposed to arrest.

The combination of HRG β and tamoxifen was greater than either agent alone on P-Akt, P-ERK and proliferation as previously discussed, and this was observed downstream as a reduction in MCF-7 cells in G_{0/1} phase and an increase in cells in S- and G₂/M phases compared to tamoxifen treatment alone.

Overall, **tamoxifen** enhanced growth factor signalling in all cell lines. This increase indicates that tamoxifen might act as a partial agonist via erbB2, which is in agreement with a theory proposed by Shou *et al* (2004). Shou *et al* (2004) proposed that increased bi-directional cross-talk between ER α and erbB2 converted tamoxifen into an agonist. Tamoxifen would then activate both the erbB1 and erbB2 signalling pathways including Akt and ERK, leading to the phosphorylation of the relevant ER α serine residues. This occurs in this scenario but only when tamoxifen was in the presence of a growth factor. Akt and ERK are both activated and this translated to an increase in P-S167 and P-S118.

Anido *et al* (2003) suggested that in cells overexpressing erbB2, the erbB1 inhibitor ZD1839 inhibited proliferation by sequestering the erbB2 and 3 into an inactive conformation with erbB1. It is possible that tamoxifen sterically hinders 2C4 by a similar mechanism or by binding to 2C4 itself or part of the extracellular domain II of the erbB2 receptor, where 2C4 binds, thus preventing the complete blockade of dimerisation with other members of the erbB receptor family

ErbB2 was phosphorylated by HRG β in MCF-7 cells, consistent with published data (Sadick *et al*, 1996) and further enhanced when this was combined with tamoxifen as previously discussed. However, unlike in the MCF-7 cell line, P-erbB2 was not enhanced in the resistant cell lines. This was unexpected and suggests that HRG β and tamoxifen may be acting on a component(s) downstream of erbB2 or via an entirely

different receptor. This component or receptor-ligand complex would subsequently have to activate both the PI3-K/Akt and MEK/ERK pathways. For example, IGFR may play a role in the phosphorylation of Akt and ER α S167 as it may activate PI3-K by direct binding to the p85 subunit (Jackson *et al.*, 1998). However, this activation was stimulated by IGF-I via IGFR, not HRG β . Therefore, an alternative and more likely theory may be that the phosphorylation of erbB2 is occurring at a different residue than that being detected by the P-erbB2 1248 antibody in the resistant cell lines. The phosphorylation of an alternative P-erbB2 residue may account for the activation of downstream targets Akt, ERK and ER α S167 in the resistant cell lines, while the phosphorylation of residue 1248 may account for the sensitivity of MCF-7 cells to 2C4. It has also been shown that growth factors activate JNKs (Logan *et al.*, 1997), thus it may be that HRG β is activating this pathway and subsequent cross-talk may allow for activation of Akt, ERK and ER α S167 via this route.

6. 11. 1. Resistance to 2C4

Resistance to anti-erbB2 agents has previously been reported (Mass, 2004; reviewed in Miller, 2004). Reports into resistance against agents which also target erbB2 may help explain why resistance to 2C4 develops. For example, Herceptin was found to be effective in ~40% of metastatic breast cancer patients, which can be increased to ~60-80% when combined with taxanes or vinorelbine (Cardoso *et al.*, 2002), however, no tumour regression was observed in the remainder of patients. This was despite the presence of amplified erbB2 gene and overexpression of the protein in the primary and metastatic tumour sites (Tanner *et al.*, 2002). Herceptin resistance was therefore due to another component other than erbB2 expression (Mass, 2004). This supports the data collected in this investigation, where the resistant cell lines were not activated at erbB2, suggesting another mechanism is responsible for resistance to 2C4.

LCC1 cells had a marginally increase expression of T-erbB2 compared to the MCF-7 cell line. In contrast, LCC9 cells had a marginally reduced T-erbB2 expression level compared to the MCF-7 cell line. This is consistent with data reported by Gu *et al.* (2002), where erbB1 gene expression was found to be 50% less in the LCC9 cell line than in LCC1 cells. The differential T-erbB2 expression and yet absence of effect in

any circumstance in the resistant cell lines suggests T-erbB2 expression may only be part of the mechanism of resistance to 2C4. As previously discussed, HRG β enhanced P-erbB2 in MCF-7 cells but did not appear to enhance erbB2 phosphorylation in the LCC- cell lines. Ovarian data suggest levels of P-erbB2 may predict sensitivity to 2C4 (Gordon *et al*, 2005). Gordon *et al* (2005) reported that the overall median time to progression was greater in P-erbB2-positive ovarian cancer patients (20.9 weeks) compared to P-erbB2-negative patients (5.7 weeks) when treated with 2C4. This theory is consistent with the absence of an anti-proliferative effect of 2C4 in the resistant cell lines, where no, or very low levels of P-erbB2 were detected.

The ineffectiveness of drugs targeting erbB1/erbB2 has been previously been suggested to be a result of constitutive activation of downstream signalling molecules such as ERK and Akt that support cell growth in the presence of antioestrogens. Studies have shown that the activation of **ERK** (Benz *et al*, 1993; Kurokawa *et al*, 2000) and/or **Akt** (Lin *et al*, 2005; Jordan *et al*, 2004; Campbell *et al*, 2001; Sun *et al*, 2001) are extremely relevant in the development of antioestrogen resistance. A study by Knowlden *et al* (2003), recorded elevated levels of **P-ERK1/II** in studies performed in similar resistant cell lines. This contrasts with the ERK levels reported in the resistant cell lines here, which were found to expressed at levels equivalent to the MCF-7 cell line, indicating that in this model ERK overexpression was not responsible for 2C4 resistance.

Contrastingly, **Akt** was observed to be constitutively overexpressed in the resistant cell lines (as previously discussed in chapter 4), suggesting Akt does indeed play a role in 2C4 resistance in LCC1 and LCC9 cell lines. However, as much as the resistant cell lines are dependent on Akt signalling, as shown via LY 294002 cytotoxicity studies (chapter 5), the basal rate of proliferation remained unchanged and still outweighed that of the MCF-7 cell line when HRG β -enhanced P-Akt was blocked with 2C4 in the resistant cells. These data suggest another mechanism of signalling is also responsible in the LCC1 and LCC9 cell lines other than erbB2, perhaps another receptor that is stimulated by TGF α , and may be overexpressed in the resistant cell lines. Alternatively, the autocrine secretion of TGF α by a tumour cell may lead to the extended stimulation of erbB1 and of the heterodimerised members

and may lead to resistance to anti-erbB2 agents (Valabrega *et al*, 2005). The group hypothesised that Herceptin resistance occurred not as a result of an intrinsic defect of the erbB2 degradation machinery, but was probably associated with impaired erbB2 downregulation due to Cbl uncoupling and ultimately altered endocytosis. Valabrega *et al* (2005) suggested that TGF α production was involved in the uncoupling mechanism and may therefore account for resistance to herceptin.

The presence of '**Akt-Raf** crosstalk' in MCF-7 cells has been documented by Moelling *et al* (2002) and is also discussed in chapter 5. Blockade of this negative feedback between Akt and Raf1 causes cell cycle arrest via elevation of MEK and ERK. It is possible that the elevated levels of P-Akt in the resistant cell lines compensate for any MEK/ERK pathway arrest and therefore the balance remains in favour of proliferation. 2C4 is unable to reverse basal P-Akt levels of the resistant cell lines and these alone may allow for the blockade of Raf1.

Other potential theories as to the growth factor receptor signalling redundancy and erbB2-neutralisation observed in the resistant cell lines include the possible activation of the insulin-like growth factor-I receptor as well as other members of the erbB family of receptors (Holboro and Hynes, 2004; Smith *et al*, 2004; Albanell and Baselga, 2001). These studies were carried out using Herceptin, resistance to which has also been linked with decreased levels of p27kip1 and a deficiency in PTEN (Nagata *et al*, 2004; Nahta *et al*, 2004).

It is more than likely the overexpression of ER α in the LCC1 cell line may be one direct mechanism of resistance. This would not apply to the LCC9 cell line. The possible mechanisms of resistance will be discussed in the following chapter, based on the findings of all chapters.

6. 11. 2. Overcoming resistance to 2C4

It remains uncertain as to whether the targeting of individual tyrosine kinases such as Akt and MEK will overcome anti-oestrogen resistance in general, or whether a combination strategy would be more effective. Several studies, reviewed by Miller (2004) and also by Mass (2004), have concluded that a combination of targeted

therapies may be required to bypass anti-erbB2 resistance when agents are used as a monotherapy. For example, as the binding sites for Herceptin and 2C4 do not overlap it may be more efficacious to administer the two agents together. Preliminary data supports this hypothesis (Nahta *et al*, 2004). Clinical studies looking into this combination are currently being planned.

It was thought that one of the reasons for the limited success of drugs targeted against erbB1/erbB2 to reverse or prevent the antioestrogen resistance was due to elevated levels of components of signalling pathways including Akt and ERK. However, the blockade of P-Akt did not abrogate elevated basal growth and the absence of ERK activation in this model suggests otherwise. It may be that a combination strategy would be more advantageous, with the blockade of not only elevated pathways, such as the PI3-K/Akt, but potential alternative pathways which operate as 'back up' when Akt is inhibited. A study by Nabha *et al* (2005) showed that rottlerin inhibition of Akt and ERK activity significantly reduced cell proliferation in a panel of four antioestrogen-resistant cell lines. The group suggested that over expression of PKC δ may play a role in the development of antioestrogen resistance in ER+ breast tumour cells and could therefore be a potential future drug target.

2C4 has been shown to inhibit EGF-induced P-ERK but not that induced by HRG β (Takai *et al*, 2005). This is in contrast to the effect observed in this model of resistance where HRG β -induced P-ERK was reversed upon 2C4 pre-treatment. Takai *et al* (2005) also observed that 2C4 was unable to reverse P-ERK levels of certain cell lines and suggested that the specificity of 2C4 in their panel of ovarian cell lines may be due to the expression of various combinations of homo and/or heterodimers of the erbBR family. The group suggested that 2C4 may be the most effective in tumours with where the ligand induced erbB2 heterodimers that stimulated proliferative signals.

Several other factors have been implicated in the development of resistance to tamoxifen. For example, Shou *et al* (2004) found that tamoxifen behaved as an oestrogen agonist in breast cancer cells which expressed high levels not only erbB2 but also the ER coactivator AIB1, with *de novo* resistance as a result. The group found that the anti-erbB1 agent Gefitinib eliminated cross-talk and re-established the

antitumour effects of tamoxifen. However, unlike the model used here, the cell lines used by Shou *et al* (2004) did not grow in the absence of oestrogen, hence AIB1 cannot be the only factor causing resistance in the model here.

6. 11. 3. Concluding remarks

In conclusion, 2C4 reversed P-Akt and P-ERK stimulated by HRG β in all three cell lines but this did not translate to an antiproliferative effect in the resistant cell lines. TGF α -enhanced P-Akt and P-ERK was not reduced by 2C4 in LCC1 and LCC9 cell lines, suggesting both pathways are partly responsible for the proliferation resistant cell lines. Therefore, the expression of particular homo and heterodimer combinations of the erbB receptor family in any given cell line may dictate the response to 2C4 and other anti-erbB agents.

Chapter 7

General Discussion

This investigation has implicated the involvement of several factors in the development of oestrogen independence and anti-oestrogen resistance. These factors and potential further studies which could be performed will be summarised in this final chapter.

7. 1. ER α dependence and overexpression

Elevated levels of ER α expression are associated with an increased rate of cellular proliferation (Jordan, 2006) and also with poor prognosis in patients with breast cancer who are not receiving adjuvant therapy (Jordan, 2006; Dowsett *et al*, 2005). Therefore, it has been hypothesised that these heightened levels may in turn be responsible for constitutive activation of ER α (Fowler *et al*, 2003). Fowler *et al* (2003) demonstrated this mechanism using a tetracycline-inducible ER α expression model of the MCF-7 cell line. The model had elevated ER α expression and subsequent gene activation via atypical promoter occupancy in the absence of oestrogen. They reported that increased ER α activity was via AF-1 and was independent of P-S118. The elevated levels of ER α observed in the MIII, LCC1 and LCC2 cell lines (and perhaps a slight increase in the LCC9 cell line) are similar to those found by Fowler *et al* (2003) and also with the heightened levels reported by Staka *et al* (2005). However, Fowler *et al* (2003) also suggested this mechanism was independent of P-S118 which may partially explain the absence of P-S118 observed in the LCC9 cell line, but does not account for the elevated levels observed in the other three lines upon ligand stimulation. A recent publication by our laboratory , (Kuske *et al*, 2006) in which data from this study was published, hypothesised the reduced P-S118 expression observed in the LCC9 cell line may more than likely affect coactivator binding. Initial results indicated that p160 binding, and in particular AIB1, was reduced in the LCC9 cell line, and suggested that this was again consistent with endocrine insensitivity as reported earlier by Kuske *et al* (2004).

Several studies using LTED cells have characterised the phenomena of oestrogen hypersensitivity and supersensitivity (Staka *et al*, 2005; Santen *et al*, 2005; Martin *et al*, 2005; Yue *et al*, 2003). Some of these studies indicated that oestrogen independence arose as a result of enhanced ER α expression, ERK activation and P-S118 via this hypersensitivity or supersensitivity to oestrogen. These factors are not in agreement with the results reported here, as no heightened ERK activation was

observed and in stark contrast, ER α did not display supersensitisation to growth factor activation (Martin *et al*, 2005; Martin *et al* 2003). Unlike LTED cell lines, which were hyper- or supersensitive (Martin *et al*, 2005; Santen *et al*, 2005; Yue *et al*, 2002), the resistant cells in this investigation displayed true oestrogen-independent growth (unpublished data produced by K. Macloud, Cell Biology Group). Moreover, studies using siRNA and ICI 182 780 performed by Kuske *et al* (2006) have shown that the growth of the LCC1 and LCC9 cell lines is ER α dependent. Our group reported that a reduction in ER α protein using siRNA was accompanied by a decline in PR protein, indicating gene expression in the MCF-7, LCC1 and LCC9 cell lines tested was ER α -dependent. The paper suggested that the elevated ER α expression alone may account for the increased binding of DNA. This was supported by the enhanced ER α binding to the pS2 promoter in the LCC1 and LCC9 cell lines in the absence of E₂, reported in this publication, and also by Fowler *et al* (2004, 2006).

The Fowler publications used a tetracycline-inducible system to increase ER α expression in the MCF-7 cell line and results suggested these elevated levels resulted in activation of the transcriptional function of the receptor, and that this occurred via a separate mechanism to those involving P-S118 or P-S104/6 activation via growth factors or ligand binding. Fowler *et al* (2004) hypothesised that elevated ER α in their unbound state stabilised any interactions with the transcriptional machinery, allowing transcription which may not be possible with normal potentially 'weaker' receptor levels. This theory could be applied to the resistant LCC1 and LCC9 cell lines utilised in this study and is consistent with the majority of reports of acquired anti-oestrogen resistance, where ER α expression is retained, indicating resistance is associated with altered ER α function.

Previous studies have shown that phosphorylation of serine residues on ER α at positions 118 and 167 cause activation of ER α and that mutation of these residues reduces ER α activation, gene transcription and eventually cell proliferation (reviewed in Lannigan, 2003). Indirect evidence has shown that phosphorylation is involved in receptor function, for example, oestrogen causes a rapid increase in phosphorylation by several fold, and TGF α is able to elicit ligand-independent ER α activation (Joel *et al*, 1995; Bunone *et al*, 1996). Staka *et al* (2005) demonstrated that levels of P-S118 were elevated in their MCF-7X cell line in keeping with the increase in ER α

expression. Heightened P-S118 was observed in the MIII, LCC1 and LCC2 cell lines, but only upon ligand stimulation, thus the results are only partially in agreement as ER α was not constitutively activated at S118. Kuske *et al* (2006) also directly features the P-S118 expression profiles reported in this study, where ER α overexpression, again reported in this study, may solely account for the elevated P-S118 levels observed upon E₂ treatment in the LCC1 cell line.

A study by Weitsman *et al* (2006) supports data reported here, as direct evidence is provided for a functional role of E₂-regulated P-S118 of ER α . The ER α and ER α P-S118 time courses shown in the MCF-7 cells, although only up to 1h, are very comparable and therefore support data shown in this study. Weitsman *et al* (2006) also used the chromatin immunoprecipitation assay (chIP) to 'pull down' any DNA associated with ER α P-S118, which was amplified using PCR and primers to specific genes. The group found that ER α P-S118 was present at the promoters of several E₂-regulated genes, including pS2 and PR which were used in this report, but was not altered due to *HER-2* overexpression when the receptor was overexpressed in the MCF-7 cell line. The group reported that overexpression of the erbB2 protein did not affect E₂-enhanced P-S118, or its presence at the promoters of E₂-regulated genes. This further supports conclusions reached in this study as to the redundant role of erbB2 (via the ineffectiveness of 2C4) in the development of endocrine resistance in these cell lines. Weitsman *et al* (2006) confirms this by stating the groups' data do not support the proposed theory of endocrine therapy resistance in breast tumours being attributed to constitutive ER α P-S118 via a constitutively activated MAPK pathway and ligand independent activation. This again supports the absence of any anti-proliferative activity of 2C4 in the LCC1 and LCC9 cell lines, and may account for the Akt inhibitor LY 294002 not having a greater efficacy in these 'resistant' cell lines. Weitsman *et al* (2006) did not use LY 294002, however, the group observed that the MEK/ERK inhibitor UO126 did not affect P-S118 in the presence or absence of E₂, which could be explained by the elevated Akt levels seen upon UO126 treatment. The increase may be a compensatory or bypass mechanism which operates in the absence of the MEK/ERK signalling pathway being operational. Weitsman *et al* (2006) concluded that another signalling molecule IKK- α , but not Cdk7, was partly involved in E₂-mediated P-S118 in the MCF-7 cell line. It may be that this molecule

is overexpressed in the LCC1 and/or LCC9 cell lines or other cell lines including LY2.

The study by Sarwar *et al* (2006) used immunohistochemical analysis of breast tumour biopsies to determine the relationship, if any, between ER α P-S118 expression and patient response to tamoxifen in breast cancer. The importance of such a publication (along with others such as Murphy *et al*, 2004) is in linking previously *in vitro* observations in cell line models such as the one here, with actual tumours and clinical responses. The group reported that ER α P-S118 expression was increased in tumour biopsies taken from patients who had relapsed after receiving tamoxifen therapy compared with biopsies taken from patients who had yet to receive treatment. This supports observations reported in this study, where the MIII, LCC1 and LCC2 cell lines with elevated P-S118 had diminished response to tamoxifen treatment compared to the fully tamoxifen-responsive MCF-7 cell line. Sarwar *et al* (2006) also produced P-S118 expression time courses and UO126 inhibitor studies in the MCF-7 cell line and a tamoxifen-resistant MCF-7 cell line which supported data reported in this study. Therefore, the data reported here and the publication by Sarwar *et al* (2006) support the theory that increased P-S118 may play a vital role in the development of endocrine resistance in breast cancer. However, the data produced by Sarwar *et al* (2006) does not account for tamoxifen resistance observed in the LCC9 cell line, which displayed reduced P-S118 expression. The tamoxifen resistant cell line used by Sarwar *et al* (2006) also had elevated MAPK activity, which shows their model of tamoxifen-resistance is not comparable to the one utilised here as different pathways are in operation. Sarwar *et al* (2006) also reported that P-S118 was elevated in more differentiated tumours, and suggested that P-S118 was therefore associated with a good prognosis in patients who had not previously received endocrine agents. The authors suggested that ER α P-S118 may therefore play a role in normal ER α function. This may also account for the endocrine agent response in the MCF-7 cell line which expressed lower levels of P-S118 than the LCC1 cell line, but higher than the LCC9 cell line. The growth of the LCC9 cell line must be operating via a distinct mechanism other than ER α P-S118.

The report by Staka *et al* (2005) used an oestrogen-independent MCF-7 cell line which was also insensitive to classical growth factor receptor stimulation. These MCF-7X cells possessed similar signalling alterations in relation to the LCC- model, including elevated P-Akt and similar P-ERK expression to the parental cell line. The data from the study by Staka *et al* (2005) evaluated the efficacy of ICI 182 780, LY 294002 and PD 98059 in combination and concluding that the only way to prevent resistance was to adopt a triple-combination strategy targeting multiple pathways and ER α simultaneously. The similarities in the profile of the MCF-7X model suggest this would be an appropriate strategy to adopt in the LCC- model.

Heightened ER and pS2 mRNA expression in the LCC1 and LCC9 resistant cell lines in the absence of oestrogen or growth factor stimulation was generally associated with elevated ER α expression. This is subsequently associated with increased transcription of the relevant genes and is consistent with published data (Staka *et al*, 2005; de Cremoux *et al*, 2003; Br nner *et al*, 1993). These results are consistent with ER α , pS2 and PR transcription being increased by ligand-independent mechanisms in the resistant cell lines, where oestrogen induced further ligand-dependent increases. This contrasts with CTD, which demonstrated only ligand-dependent activation (and no ligand independent activation) at this 24h time point.

Kuske *et al* (2006) also reported that growth responses to E₂ and tamoxifen were reflected in their response to transcriptional changes. The mRNA expression profiles produced in this study are supported by Kuske *et al* (2006), although it should be noted that the time points differ (48h versus 24h treatments used in this study).

7. 2. Changes in signalling in the resistant cell lines

7. 2. 1. P-Akt overexpression

Overexpression of P-Akt is frequently observed in breast cancer (Murray *et al*, 2005) and has been implicated in the development of tamoxifen resistance (Kirkegaard *et al*, 2005; deGraffenried *et al*, 2004). The LCC- and LY2 cell lines all expressed constitutively activated Akt consistent with this theory and with several reports using oestrogen-independent cell lines, including Tam-R MCF-7 cell line variants (Lin *et al*, 2005; Jordan *et al*, 2004; Frogne *et al*, 2004; Campbell *et al*, 2001). Soderlund *et al*

(2005) reported that MCF-7 cells transfected with constitutively active Akt increased resistance against apoptosis, which reinforces the reduction in apoptosis observed in LCC1 and LCC9 cell lines. Proliferation assays using LY 294002 confirmed the MCF-7 and resistant cell lines alike were Akt-dependent, although cell proliferation was reduced only to a slightly lesser extent using UO126 indicating the MEK/ERK pathway also remains actively involved in this model (data consistent with studies by several groups, including Moelling *et al* (2005)). In contrast to the total abrogation of P-Akt and P-ERK expression observed with LY 294002 and UO126 respectively, neither inhibitor reduced resistant cell proliferation to the same extent as the MCF-7 cell line. It may be that some resistant cells are Akt-dependent whilst others remain ERK-dependent, and therefore a combination of the two inhibitors would have been more effective. Alternatively, yet another pathway may be involved in oestrogen-independent growth.

Several reports have described the significance of the PI3-K/Akt pathway in erbB2 signalling (Dubska *et al*, 2005; Soderlund *et al*, 2005; Kim and Muller, 1999), thus the constitutive activation of Akt led to the hypothesis an anti-erbB2 agent, 2C4, would abrogate oestrogen-independent cellular proliferation. However, 2C4 did not affect the proliferation of the resistant cell lines. Moreover, P-Akt and P-ERK1/II expression were reduced, or even abrogated by 2C4, again indicating both pathways remain intact and are involved in proliferation, although they may not necessarily be solely responsible for the increased rate of basal proliferation. The inability of 2C4 to have any anti-proliferative action against the resistant cell lines may instead be due to the dominant erbB receptor conformation (Takai *et al*, 2005). It is possible that TGF α preferentially induces erbB1 homodimers as opposed to erbB1/2 heterodimers, and/or erbB1/2 heterodimers are formed, but are sequestered into an inactive conformation. Either possibility would render 2C4 ineffective, as demonstrated by the nil effect on TGF α -enhanced P-Akt and P-ERK signalling in the LCC1 and LCC9 cell lines.

HRG β signalling was still intact in the resistant cell lines. HRG β has been shown to be responsible for cell aggregation, which signals via the PI3-K/Akt pathway and can be abrogated with LY 294002 (Tan *et al*, 1999b). Therefore, although no increase in resistant proliferation was observed with growth factor stimulation, there may be alterations in the extent of cell aggregation and cell-cell adhesion in the resistant cell

lines. This theory is consistent with initial observations of all cell line growth assays in 24-well plates, where HRG β treatment caused ‘clumping’ of the cells, perhaps more so in the resistant lines, which made them more adherent to each other and less adherent to the dish. These observations suggest the involvement of cell-surface molecules such as cadherins, which are involved in signalling pathways controlling proliferation and apoptosis. Abnormal cadherin expression has been associated with breast carcinoma. For example, cadherin-E was found to be overexpressed in low-grade DCIS breast cancer (Tan *et al*, 1999a). Conversely, the same molecule was downregulated in high-grade breast cancer (Oka *et al*, 1993), suggesting cadherin expression is involved in the early stages of breast cancer development and progression. Tan *et al* (1999a) demonstrated that PI3-K was vital for cell aggregation induced by HRG β , in contrast with the MEK/ERK pathway, which was found not to play a role in cell aggregation. Tan *et al* (1999a) proposed that because aggregation can contribute to the invasion and metastasis of breast cancer cells, HRG β -activation of erbB receptors may affect these properties of MCF-7 cells. An increase in resistant cell aggregation may be attributed to the elevated P-Akt expression and diminished P-MEK signal in the LCC- and LY2 cell lines. Further studies are required in order to confirm these initial findings and whether cadherin expression is relevant in this particular model.

Another possible mechanism which may account for the redundancy of growth factor receptor signalling and resistance to 2C4 and neutralisation of erbB2, would be the activity of another ligand independent pathway, such as the IGFR-1 pathway (Smith *et al*, 2004; Holbro and Hynes, 2004; Albanell and Baselga, 2001). This may also explain the development of oestrogen-independence and anti-oestrogen resistance. Reduced expression of PTEN may also account for the absence of any anti-erbB2 anti-proliferative effect. PTEN negatively regulates Akt activity by antagonising PI3-K (Lu *et al*, 1999; Li and Sun, 1998). PTEN has been previously demonstrated to confer resistance to another anti-erbB2 agent herceptin in cells overexpressing erbB2 (Nagata *et al*, 2004). PTEN may also play a pivotal role in any anti-tumour effect mediated by erbB2/PI3-K/Akt and may prove to be a useful predictive marker for the efficacy of herceptin in erbB2 overexpressing breast cancer (Fujita *et al*, 2006). These data suggest PTEN may also be reduced in the LCC- and LY2 resistant cell

lines. It was therefore proposed that anti-erbB2 monotherapy resistance may only be overcome by using a combination of targeted therapies (Miller, 2004).

7. 2. 2. P-MEK and P-ERKI/II

P-MEK and P-ERKI/II have also both been implicated in the progression of breast cancer. The overexpression of both these tyrosine kinases has been documented to possess a strong association with breast cancer many times over (Martin *et al*, 2005; Navolanic *et al*, 2003). The reduction in P-MEK expression levels in the resistant cell lines is inconsistent with these studies. However, a study by Glaros *et al* (2006) reported that transfection of MCF-7 with MEK led to the maintenance of tamoxifen sensitivity, which is in agreement with the progressive loss of growth factor-enhanced P-MEK with a progression in resistance from MCF-7 to LCC1 to the LCC9 cell line. Moreover, the study by Fowler *et al* (2004) also reported that P-ERKI/II levels were not elevated in their MCF-7 Tet-On model overexpressing ER α . The group therefore concluded that the elevated transcriptional activity of ER α in the absence of ligand did not involve the MEK/ERK pathway, in agreement with results obtained in the LCC- and LY2 cell lines.

In addition, the elevated levels of Akt in the resistant cell lines may induce some form of 'Raf/Akt' crosstalk in these cells, which has previously only been observed in the presence of high ligand concentrations in the MCF-7 cell line (Moelling *et al*, 2002). This may account for the reduced growth factor enhanced P-MEK expression compared to the parental cell line. P-ERKI/II was not elevated in the resistant lines, with the expression pattern remaining similar to that of the parental MCF-7 cell line. This is again inconsistent with published data which report constitutively activated ERK in oestrogen-independent MCF-7 cells (Martin *et al*, 2005; Martin *et al*, 2003). However, the majority of these studies, as previously discussed, utilised LTED cell lines, which were either hyper- or supersensitive to oestrogen and did not have elevated P-Akt expression either, indicating different a signalling mechanism(s) was responsible for the regulation of proliferation.

7. 3. *Future Studies*

This investigation has proposed a number of areas of interest in the understanding of the development and progression of breast cancer. There are several further areas and studies which could be explored, a few examples of which have been detailed in the following section.

As previously stated, current studies in the Cell Biology group (performed by C. Naughton) are taking the form of ER α knockout using siRNA and ICI 182 780 and confirmed the involvement of ER α in the LCC1 and LCC9 resistant cell lines. Initial results showed a reduction in ER α reduced the proliferation of the resistant cell lines, consistent with data reported by Fowler *et al* (2003).

It is necessary to fully characterise the total and phosphorylated expression patterns of **erbB1**, **erbB2**, **erbB3** and **erbB4** receptors of all cell lines in the presence and absence of ligand stimulation (including E₂, HRG β and TGF α) and 2C4. This should be performed over a time course as initial studies have indicated that the effects of E₂ may be somewhat delayed to those of the growth factors. These expression patterns may indicate whether overexpression or reduced expression of a receptor, erbB2 for example, is partly responsible for the absence of any anti-proliferative effect of 2C4.

It would be useful to investigate the effect of **LY 294002** and **UO126** on **P-S118** and **P-S167** in this model of resistance. This has already been carried out by Staka *et al* (2005) in LTED MCF-7 cells. Staka *et al* (2005) found that along with ER α expression, S118 phosphorylation was elevated, in agreement with the data reported here. S167 phosphorylation was partly reduced with LY 294002 treatment in the study by Staka *et al* (2005). It is therefore hypothesised the elevated P-Akt expression in the resistant cell lines would be more sensitive to the inhibitory properties of LY 294002. Also of interest would be the effect of LY 294002 and UO126 on the **cell cycle** and **apoptosis** of MCF-7 cells compared to the resistant cell lines. LY 294002 appeared to reduce the number of cells below 100% irrespective of treatment, indicating some form of cell death is occurring. This may or may not occur in the resistant cell lines. It may prove valuable to investigate the effect of E₂ on **P-S167** expression to ascertain the involvement, if any, of non-genomic signalling in this model of breast cancer resistance. Initial western blots at a 15 min time point

showed E₂ did not enhance P-S167 in either the parental or resistant cell lines but this needs to be confirmed. A time course study would confirm whether there was any involvement at a later stage.

More confirmation is still required between proliferation and apoptosis and constitutively activated Akt and reduced P-MEK. It is proposed that observing the cell cycle distribution and the number of cells undergoing apoptosis when the cells are subject to **LY 294002** and **UO126** using **FACS analysis** may show the resistant cells to be more sensitive to the effects of LY 294002. An interesting observation reported in Chapter 6 was the effect of tamoxifen on growth factor-enhanced P-Akt, P-MEK and P-ERK in all cell lines. The exact mechanism by which this occurs needs to be confirmed. A potential first step in this process would be to confirm or rule out the involvement of the **erbB receptors** by further characterising the **effect of tamoxifen** in the presence and absence of growth factors and E₂ on these receptors. Also, as HRGβ has been implicated in tumour metastases and invasion (Tan *et al*, 1999), an **invasion assay** could be performed to assess the effect of repressing pathways stimulated by HRGβ (e.g. PI3-K/Akt and MEK/ERK in this instance).

p38 MAPK has been implicated in the development of breast cancer resistance via activation of **ERK3** (Zimmermann *et al*, 2001), hence it may prove useful to characterise the expression of ERK3 and p38 MAPK proteins in this model. Also of interest would be the characterisation of **PTEN** expression in relation to the development of resistance to anti-erbB2 agents such as 2C4 and Herceptin. PTEN antagonises PI3-K and subsequently negatively regulates Akt activation (Crowder *et al*, 2004; Nagata *et al*, 2004; Pandolfi, 2004).

The combined blockade of several signalling pathways simultaneously by combining inhibitors such as LY 294002, UO126 and an anti-oestrogen to target the PI3-K/Akt and MEK/ERK pathways and the ER directly may prove to be more efficacious than targeting a single pathway alone in this model. This strategy has previously been successfully implemented in other resistant breast cancer models (Martin *et al*, 2003; Yue *et al*, 2003).

7. 4. Proposed Mechanisms of Resistance

The development and progression of resistance from the parental oestrogen and anti-oestrogen sensitive MCF-7 cell line to the LCC- cell lines is summarised in figure 7.1. The development of oestrogen independent growth appears to occur alongside several alterations to signalling (in the form of constitutively activated Akt and a reduction in growth factor-enhanced P-MEK), cell cycle (in the form of an elevated proportion of cells undergoing division in S-phase) and apoptosis (observed as a reduction in the percentage of LCC1 and LCC9 cells undergoing apoptosis). The acquisition of resistance to tamoxifen and ICI 182 780 observed between the LCC1 the LCC9 cell lines was accompanied by a reduction in P-S118. The MIII cell line would logically fall between the MCF-7 and LCC1 cell lines, while LCC2 cells would lie somewhere between the LCC1 and LCC9 cell lines. The LY2 cell line was derived in a different manner to these cell lines, but some phenotypic aspects are similar. It is therefore not surprising that change such as elevated P-Akt expression also occurs in the LY2 cell line.

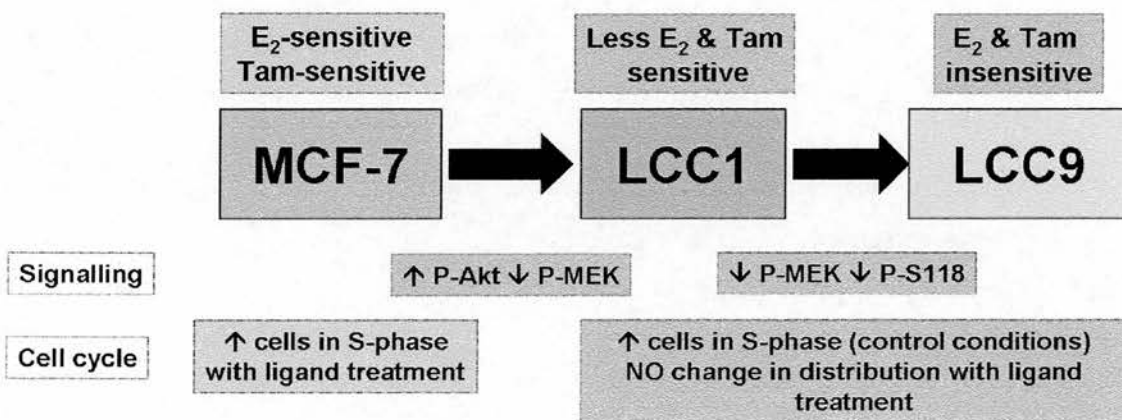


Figure 7.1. Development and progression of resistance in the LCC- breast cancer model. Schematic diagram showing the factors which may contribute to the development and progression of oestrogen insensitivity and anti-oestrogen resistance from the parental MCF-7 cell line to the LCC1 and LCC9 cell lines. Resistance progression is accompanied by alterations to signalling and cell cycle distribution.

Figure 7.2 shows the proposed mechanism of resistance for oestrogen independence and anti-oestrogen resistance. The proposed mechanism is based on the mechanisms suggested by Schiff *et al* (2004) and Moelling *et al* (2002). The mechanism takes into account the ability of LY 294002 to abrogate growth factor signalling (HRGβ is

shown as an example), crosstalk and proliferation in the resistant cell lines. The mechanism also factors in the ability of tamoxifen to further enhance growth factor-enhanced P-Akt, P-MEK and P-ERK1/2. This is consistent with data published by Hatakeyama *et al* (2002), who also observed LY 294002 abrogated HRG β signalling as reported here.

Figure 7.2 shows a growth factor binds to the appropriate erbB receptor, inducing erbB receptor homo or heterodimerisation (HRG β is shown as the example here, with a possible corresponding heterodimer conformation of erbB2/erbB3). Upon HRG β binding and heterodimer formation, the heterodimer undergoes a conformational change allowing activation of downstream PI3-K/Akt and Ras/Raf/MEK/ERK signalling cascades. The MCF-7 and resistant cell lines alike appear to signal via both these pathways, but to differing extents. The elevated P-Akt in the resistant cell lines may induce 'Raf-Akt' cross-talk which may subsequently account for the reduction in P-MEK observed in the resistant cell lines. This differential activation of Akt and MEK in the resistant cell lines is consistent with the reduced rate of apoptosis, the elevated percentage of cells in S-phase and therefore an increased rate of cellular proliferation.

Oestrogen and tamoxifen are shown to induce gene transcription by both directly binding to ER α (genomic action) and via the MEK/ERK signalling pathway (non-genomic action). Tamoxifen is also indicated to induce transcription via the PI3-K/Akt pathway. These genomic and non-genomic actions of oestrogen and tamoxifen are consistent with previously published theories (Schiff *et al*, 2004; Hatakeyama *et al*, 2002; Duh *et al*, 1997).

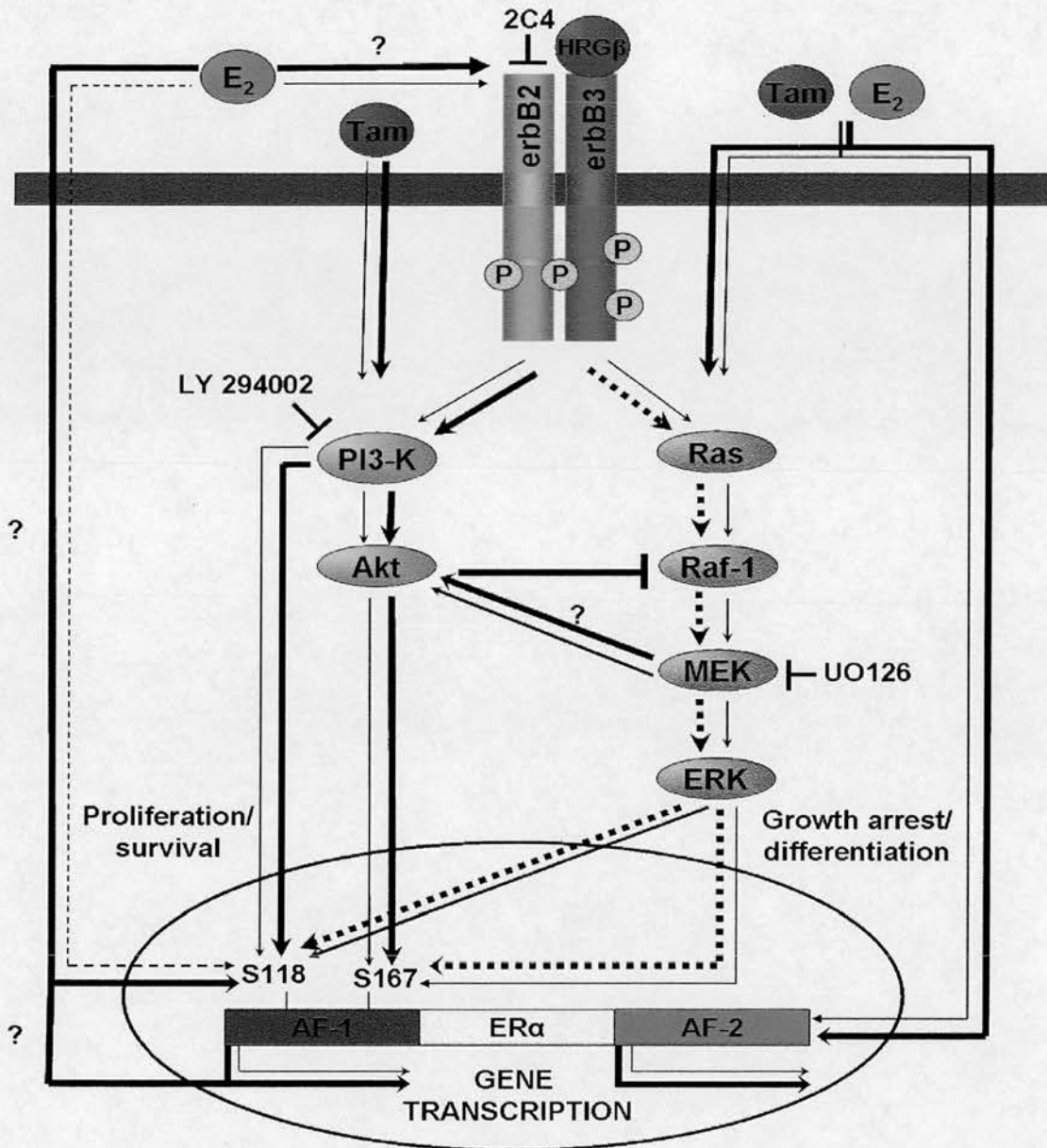


Figure 7.2. Proposed mechanism of resistance in the LCC- cell lines. The parental MCF-7 cell line signalling pathways are depicted by the lighter lines compared to the LCC-cell lines which are represented by the bold lines and arrows. A growth factor binds to the appropriate erbB receptor, inducing erbB receptor homo or heterodimerisation (heregulin β (HRG β) is shown as an example here, with one of the possible corresponding heterodimer conformation of erbB2/erbB3). Upon HRG β binding and heterodimer formation, the heterodimer undergoes a conformational change allowing activation of downstream PI3-K/Akt and Ras/Raf/MEK/ERK signalling cascades. The MCF-7 and resistant cell lines alike appear to signal via both these pathways, but to differing extents. The elevated P-Akt in the resistant cell lines may induce 'Raf-Akt' cross-talk (shown by horizontal bar between the aforementioned proteins) which may subsequently account for the reduction in P-MEK observed in the resistant cell lines (bold dashed lines). This differential activation of Akt and MEK in the resistant cell lines is consistent with the reduced rate of apoptosis, the elevated percentage of cells in S-phase and therefore an increased rate of cellular proliferation. Oestrogen (E_2) and tamoxifen (Tam) induce gene transcription by binding directly to ER α at AF-2, enhancing P-S118 at AF-1 or potentially via a non-genomic mechanism involving the Ras/Raf/MEK/ERK pathway. Tam may also act via the PI3-K/Akt pathway. Other pathways and proteins may also be linked with the overexpression of Akt, such as PLC γ activation as discussed previously in chapter 5.

7. 5. Potential contributing factors to resistance development

Gratton *et al* (2001) reported that VEGF-induced activation of the PI3-K/Akt pathway led to the inhibition of p38 MAPK and protected the cells from apoptosis. This and other studies (Park *et al*, 2002) suggest Akt may reduce stress kinase activation. Thus, constitutively activated Akt in the LCC- and LY2 cell lines may diminish apoptosis further still in conjunction with the effect of 'Raf/Akt' crosstalk. Zimmermann *et al* (2001) further explored this theory and reported that ERK3 expression arose as a consequence of p38 pathway activation and more than likely represented an intracellular defence or rescue mechanism against cell stress and damage induced by proteasome inhibition. In addition, ERK3 overexpression protected cells from the anti-proliferative effect induced by proteasome inhibition. Inversely, Zimmermann *et al* (2001) reported that inhibition of p38 specifically sensitised various cells to proteasome inhibitors. It may be that the resistant cells have elevated expression of ERK3, while any other activation of the p38 stress kinase pathway is reduced.

Ligand activated ER α classically interact with the ERE, and indirectly regulate gene expression by interacting directly with the AP-1 protein complex (Webb *et al*, 1999), the Sp1 protein (Safe, 2001) or the nerve factor- κ B (NF κ B) protein (Harnish *et al*, 2000). These are all genomic actions of oestrogen which occur in conjunction with or aside from non-genomic signalling (Kousteni *et al*, 2001). Alterations to any of these components may lead to the development or progression of resistance. Specifically, increased activity of the AP-1 transcription factor has been found to lead to tamoxifen-resistance in MCF-7 cells (Liu *et al*, 2002). Gu *et al* (2002) suggested that the LCC9 cell line may survive treatment with anti-oestrogens by 'bypassing specific growth inhibitory signals' induced by ER α occupied with an antagonist. This cell line was further studied by Riggins *et al* (2005), who reported that overexpression of the transcription factor NF κ B was associated with the development and progression of ICI 182 780 resistance in the LCC9 cell line, and inhibition of this factor restored ICI 182 780 sensitivity. NF κ B was not overexpressed in the LCC1 cell line and therefore anchorage-dependent proliferation was not affected by NF κ B inhibition.

Another factor which may contribute to tamoxifen resistance and oestrogen insensitivity is the inhibition of NK cell activity. Tamoxifen exerts some of its antitumour properties via elevated NK activity and resistance may arise as a result of elevated secretion of growth factors or cytokines which inhibit this activity. This would potentially account for tamoxifen resistance in the LCC2 cell line, as these cells secrete a significant quantity of cytokine TGF β 2 (Arteaga *et al.*, 1999). In addition, both IGFR and erbB1 expression was lower in the LY2 cell line than in the parent line (Boylan *et al.*, 1998), implicating the involvement of alternative signalling pathways.

*In summary, this study reports that multiple factors appear to be contributing to the development and progression of oestrogen independence and anti-oestrogen resistance in this model. These factors occur at different stages of oestrogen-independent growth and endocrine agent resistance development and progression and include elevated **ER α** and **P-S118** expression (in the MIII, LCC1 and LCC2 cell lines), which may be linked with elevated E₂-responsive **gene expression** in these cell lines (as shown in the LCC1 cell line), constitutive activation of **Akt** as shown in both the LCC1 and LCC9 cell lines, reduced expression of **MEK** as resistance to endocrine agents increases (decreasing in magnitude from the MCF-7 cell line to LCC1 cells and further still in the LCC9 cell line), a reduction in the number of 'resistant' cells undergoing **apoptosis** (LCC1 and LCC9 cell lines) and an increase in the number of cells undergoing **S-phase cell division** (LCC1 and LCC9 cells). The absence of elevated MEK/ERK activity (in the LCC1 and LCC9 cell lines) as observed in other tamoxifen-resistant models also supports the conclusion that several different pathways contribute to the resistance process. In addition, LCC9 cells have a comparable level of ER α expression to MCF-7 cells and yet possess reduced ER α P-S118 levels, which also indicates a distinct pathway to those utilised by the MIII, LCC1 and LCC2 cell lines which overexpress P-S118. Therefore ER α overexpression cannot solely account for endocrine resistance in this cell model, indicating resistance progression is likely to be a multifactorial process.*

Chapter 8

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Appendix

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Endocrine therapy resistance can be associated with high estrogen receptor α (ER α) expression and reduced ER α phosphorylation in breast cancer models

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Abstract

Hormone-dependent estrogen receptor (ER)-positive breast cancer cells may adapt to low estrogen environments such as produced by aromatase inhibitors. In many instances, cells become insensitive to the effects of estrogen but may still retain dependence on ER. We have investigated the expression, function, and activation of ER α in two endocrine-resistant MCF-7 models to identify mechanisms that could contribute to resistance. While MCF-7/LCC1 cells are partially estrogen dependent, MCF-7/LCC9 cells are fully estrogen insensitive and fulvestrant and tamoxifen resistant. In both MCF-7/LCC1 and MCF-7/LCC9 cell lines, high expression of ER α was associated with enhanced binding to the trefoil factor 1 (TFF1) promoter in the absence of estrogen and increased transcription of TFF1 and progesterone receptor. In contrast to the observations derived from hypersensitive and supersensitive models, these cells were truly estrogen independent; nevertheless, removal of ER α by siRNA, or fulvestrant, a specific ER downregulator, inhibited growth indicating dependence on ER α . In the absence of estrogen, neither ER α Ser¹¹⁸ nor Ser¹⁶⁷ were phosphorylated as frequently found in other ligand-independent cell line models. Addition of estrogen activated ER α Ser¹¹⁸ in MCF-7 and LCC1 cells but not in LCC9 cells. We suggest that the estrogen-independent growth within these cell lines is accounted for by high levels of ER α expression driving transcription and full estrogen independence explained by lack of ER α activation through Ser¹¹⁸.

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Introduction

Estrogen receptor α (ER α) is a major growth regulator for many breast cancers and has provided an exploitable target for therapy (Ali & Coombes 2002). Estrogen binding to ER α promotes conformational changes in the receptor leading to dimerization and attachment to DNA, generally at the site of conserved estrogen response elements in the promoter regions of target genes (Ali & Coombes 2002). Functional regulation of ER α is additionally mediated via phosphorylation of key residues in the activation function 1 (AF-1) domain of ER α including Ser¹¹⁸ and Ser¹⁶⁷ and these influence both DNA binding and

recruitment of cofactor molecules (reviewed in Lannigan 2003). The activation of ER involves crosstalk with other growth factor-signaling pathways. There is extensive evidence that activation of the mitogen-activated protein kinase (MAPK)-signaling cascade and the phosphoinositol 3 kinase (PI3-K) pathway phosphorylate ER α at Ser¹¹⁸ and Ser¹⁶⁷, via extracellular signal-regulated kinase (ERK)1/2 and Akt respectively (Bunone *et al.* 1996, Martin *et al.* 2000, Lannigan 2003). Transcriptional activation of ER α then involves a dynamic process where large transcription complexes incorporating co-activator proteins are assembled in an ordered and combinatorial manner

(Glass & Rosenfeld 2000, Metivier *et al.* 2003). Well-defined estrogen-regulated genes include trefoil factor 1 (TFF1)/pS2 (Masiakowski *et al.* 1982, Jakowlew *et al.* 1984) and progesterone receptor (PGR; Nardulli *et al.* 1988).

While tamoxifen has been the established form of treatment for ER-positive breast cancers for more than 20 years, other anti-estrogen strategies, notably aromatase inhibitors (Johnston & Dowsett 2003) and selective estrogen downregulators (SERDs), are increasingly being used (Robertson 2002). Despite initial responsiveness to these agents, most tumors eventually recur with acquired resistance (Clarke *et al.* 2001, 2003). Multiple mechanisms, dependent on the form of endocrine treatment, are involved in the development of resistance and, in many cases, these mechanisms remain unclear. During the acquisition of endocrine resistance, progressive changes are frequently observed, with ER-positive breast cancer cells progressing in a stepwise manner from a fully estrogen-sensitive phenotype to an estrogen-sensitive, but no longer dependent phenotype, to a fully resistant phenotype (Clarke *et al.* 2001, 2003).

With the increasing clinical use of aromatase inhibitors, such as letrozole, anastrozole, and exemestane which act by inhibiting estrogen synthesis (Johnston & Dowsett 2003), there has been great interest in how breast cancer cells can adapt to low estrogen environments and become resistant to the effects of these drugs. In most cases of acquired anti-estrogen resistance, expression of ER α is retained, suggesting that resistance involves either changed functionality or bypass of the receptor. Culturing breast cancer cells in estrogen-low conditions to produce long-term estrogen deprivation (LTED) has identified mechanisms of estrogen hypersensitivity and estrogen supersensitivity (Yue *et al.* 2002, Martin *et al.* 2003, 2005a,b, Santen *et al.* 2005). Estrogen hypersensitivity is characterized by the ability of cells to respond to levels of estrogen at concentrations 2–3 log lower than required to stimulate wild-type cells (Yue *et al.* 2002, Santen *et al.* 2005). This mechanism involves increased expression of ER α alongside enhanced phosphorylation of ER α Ser¹¹⁸ and is associated with activation of the ERK1/2 and PI3-K pathways. Estrogen supersensitivity, wherein cells are apparently estrogen independent, is a mechanism again associated with enhanced ER α expression, ERK activation, and activation of ER α Ser¹¹⁸ and involves ER α being supersensitized by growth factor activation (Martin *et al.* 2003, 2005a).

While higher levels of ER α expression are generally associated with enhanced estrogen response, in certain cases tumors expressing high levels of ER α can be insensitive to endocrine manipulation. High levels of ER α expression have been associated with increased proliferation rates (Black *et al.* 1983) and poor prognosis in breast cancer patients not receiving adjuvant therapy (Black *et al.* 1983, Thorpe *et al.* 1993). It has been suggested that a high level of ER α may lead to constitutive activation (Fowler *et al.* 2004). This mechanism has recently been demonstrated by Fowler *et al.* (2004, 2006) in a tetracycline-inducible ER α expression model of the MCF-7 cell line, wherein increased ER α expression resulted in aberrant promoter occupancy and gene activation in the absence of estrogen. The increased receptor activity required the amino-terminal domain and was not inhibited by tamoxifen, supporting the notion of AF-1 activation, yet was independent of Ser^{104/106} and Ser¹¹⁸ phosphorylation (Fowler *et al.* 2004).

In these models, the expression of ER α is still critical to the response and it has been suggested that use of a SERD such as fulvestrant (faslodex, ICI 182 780) would be a beneficial strategy once resistance to aromatase inhibitors has developed (Johnston *et al.* 2005, Martin *et al.* 2005b). A number of laboratories are developing models of resistance to this agent to identify strategies that might be tried at the onset of resistance (Dowsett *et al.* 2005, Howell 2005, Johnston *et al.* 2005, Martin *et al.* 2005b, Nicholson *et al.* 2005, Normanno *et al.* 2005).

We have investigated two MCF-7 cell lines (MCF-7/LCC1 and MCF-7/LCC9), which have acquired estrogen insensitivity and with variable sensitivity to tamoxifen and fulvestrant to identify novel mechanisms of endocrine resistance that might arise in clinical specimens. The wild-type ER-positive MCF-7 breast cancer cell line is both estrogen dependent and responsive to anti-estrogens, such as tamoxifen and fulvestrant. The MCF-7/LCC1 (LCC1) cell line was derived from an MCF-7 xenograft, which had grown in a low estrogen environment in an immuno-deprived mouse and which was known to be estrogen independent but with a degree of estrogen sensitivity (Brunner *et al.* 1993). Treatment of the cell line with fulvestrant produced the MCF-7/LCC9 (LCC9) cell line which is fully resistant to both estrogen and fulvestrant (Brunner *et al.* 1997). A number of novel features of these lines were identified within this study and are reported here.

Materials and methods

Cell proliferation

MCF-7 cells were routinely grown in phenol red containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 (g/ml). LCC1 and LCC9 cells (source: Dr Robert Clarke, V T Lombardi Cancer Research Center, Georgetown University Medical School, Washington, DC, USA) were routinely kept in phenol-free containing DMEM supplemented with 5% dextran-activated charcoal-stripped fetal calf serum (DCC), penicillin (100 units/ml), streptomycin (100 (g/ml), and 2 mM glutamine. All cells were grown at 37 °C in 5% CO₂. To determine the effects of 17β-estradiol (E₂) and tamoxifen on cell proliferation, MCF-7 cells were seeded in six-well plates in phenol red containing DMEM with 10% fetal bovine serum (FBS) for 24 h. The media were changed to phenol red-free DMEM with 5% DCC for 48 h. The cells were then supplemented with media containing either 1 nM E₂, 1 μM tamoxifen or both. LCC1 and LCC9 cells were seeded in six-well plates in phenol red-free containing DMEM with 5% DCC and after 24 h supplemented with E₂ and/or tamoxifen. Cell growth was evaluated using a Coulter counter. Fulvestrant was a kind gift from Dr Alan Wakeling (AstraZeneca, Macclesfield, Cheshire, UK). For studies exploring growth in DMEM without serum, the sulforhodamine-B (SRB) colorimetric assay was used.

Briefly, log phase cells were seeded into 96-well flatbottom tissue culture plates. The following day, cells were washed in PBS and media replaced with phenol red-free DMEM for 48 h. Cells were then treated with concentrations of E₂ varying from 10 fM to 1 μM in the absence or presence of 100 nM fulvestrant. After 72 h, plates were removed from the incubator and ice-cold 25% trichloroacetic acid (TCA) solution (50 μl) added to each well. All plates were placed on ice for 60 min after which the TCA solution was removed. The plates were washed under running water and dried prior to staining with SRB dye solution (30 min at room temperature) and the trays were washed with 1% glacial acetic acid (×4) at room temperature, air-dried, and resuspended in 10 mM Tris buffer (pH 10.5; 150 μl) before reading at 540 nm.

RNA extraction and RT-PCR

Extraction of total RNA from whole cells was performed using Tri-Reagent (Sigma) as per the manufacturers' instructions. RNA concentration was

measured using a spectrophotometer. QuantiTect SYBR Green system (Qiagen, cat no. 204243) was used according to the manufacturers instructions for one step RT-PCR in a total of 15 μl reaction volumes, including 0.5 μM each primer and 40 ng RNA. Real-time cyclers conditions were RT: 50 °C for 30 min; PCR: initial activation 95 °C for 15 min followed by 40 cycles of denaturation 94 °C for 15 s, annealing 57 °C for 30 s, extension 72 °C for 30 s, and a final extension of 72 °C for 60 s. The following primers were used:

TFF1: fwd TTGTGGTTTTCTGGGTGCA
rev CCGAGCTCTGGGACTAATCA
ERα: fwd CCACCAACCAGTGCACCATT
rev GTCTTTCCGTATCCCACCTTTC
PGR: fwd GTCAGTGGGCAGATGCTGTA
rev AGCCCTTCCAAAGGAATT
ACTIN: fwd CTACGTCGCCCTGGACTTTCGAGC
rev GATGGAGCCGCCGATCCACACGG

Western analysis

Cells were washed twice with PBS and lysed in ice-cold lysis buffer (50 mM Tris (pH 7.5), 5 mM EDTA (pH 8.5), 150 mM NaCl, 1% Triton X-100, aprotinin 10 μg/ml, and 1× protease cocktail inhibitor (Roche) for 10 min and the debris was cleared by centrifugation at 13 000 r.p.m. for 6 min at 4 °C). Protein lysates (100 μg) were resolved on 7.5–12% SDS-PAGE and electrophoretically transferred to Immobilon-P membranes. After transfer, membranes were blocked and probed with primary antibody overnight at 4 °C. Immunoreactive bands were detected using chemiluminescent reagents (ECL or SuperLuminol) and photographic paper (Hyperfilm, Amersham). The following antibodies were used: ERα (F-10; Santa Cruz Biotech, Santa Cruz, CA, USA sc-8002), PGR (ab-8; Neomarkers, Stratech Scientific Ltd, Newmarket, Suffolk, UK (MS-298)), P-ERK1/2 (1:1000, Cell Signaling, New England Biolabs, Hitchin, Herts, UK #9101), phospho-Ser¹¹⁸ ERα (1:500, Cell Signaling #2511), phospho-Ser¹⁶⁷ ERα (1:500, Cell Signaling #2514), and actin (1:120 000, CP01, Calbiochem, La Jolla, CA, USA). Integrated optical density absorbance values were obtained by densitometric analysis using a gel scanner and analyzed by 'Labworks' gel analysis software (UVP Life Sciences, Cambridge, UK).

Chromatin immunoprecipitation assays (ChIP)

Cells were grown to 85–90% confluence in phenol red-free DMEM with 5% DCC for at least 48 h. Cells were

cross-linked with 1% formaldehyde (37 °C for 10 min) at 10-min interval over a 90-min time course. Unreacted formaldehyde was quenched by gentle agitation at room temperature for 10 min with 0.125 M glycine. Cells were then washed twice with ice-cold PBS, collected into PBS containing protease inhibitors (Roche), and centrifuged for 4 min at 2000 r.p.m. at 4 °C. The pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), and 1× protease inhibitor cocktail), incubated on ice for 10 min, and sonicated (12×20 s at two amplitude microns, Soniprep 150, MSE) to fragment DNA to ~500 bp. Following centrifugation for 15 min at 13 000 r.p.m. and 4 °C, supernatants were collected and resuspended in dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, and 1× protease inhibitor cocktail). Chromatin were precleared with 1 µg anti-rabbit or anti-mouse IgG, 2 µg sheared salmon sperm DNA, and Protein-G-Agarose (50 µl of 50% slurry in dilution buffer) for 3 h at 4 °C. Immunoprecipitation using Protein-G-Agarose Beads (Roche) was performed overnight at 4 °C with anti-ER α HC-20 antibody (sc-543, Santa Cruz). Beads were washed sequentially for 5 min each at 4 °C with TSE I (20 mM Tris (pH 8.1), 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS), TSE II (20 mM Tris (pH 8.1), 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, and 0.1% SDS), and buffer III (10 mM Tris (pH 8.1), 0.25 M LiCl, 1 mM EDTA, 1% NP40, and 1% deoxycholate). Precipitates were then washed twice with TE buffer and the protein/DNA complexes were eluted twice with 0.1 M NaHCO₃ and 1% SDS. Heat treatment at 65 °C overnight reversed formaldehyde cross-links. DNA fragments were purified using QIAquick Spin Kit columns (Qiagen) and amplified using the QuantiTect SYBR Green system (Qiagen, cat no. 204242). TFF1 PCR conditions were: initial activation of 95 °C for 15 min followed by 45 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 5 min. TFF1 primer sequences: fwd GACGGAATGGGCTTCATGAGC and rev CTGAGACAATAATCTCCACTG. For the distal region, primers were: fwd GAGTTTGGCCTCC-CACATTA and rev CTTGCCTCTGCATTCTCTCC.

Short interfering (siRNA) transfections

MCF-7 cells were seeded at 0.5×10^6 cells per T75 flask in DMEM as mentioned previously. After 24 h, the media were changed to phenol red-free containing DMEM with 5% DCC for 48 h. LCC1 and LCC9 cells were seeded directly into phenol red-free containing

DMEM with 5% DCC for 24 h prior to transfection. Cells were transfected with siRNA for 4 h using Oligofectamine reagent (Invitrogen) after which time 1 nM E₂ was added for a further 48 h prior to RNA and protein extraction. For the 7-day time course, the media were left unchanged after the initial changes. For siRNA growth assays, cells were seeded as for growth characterization as mentioned previously. siRNA transfections were carried out as described earlier but scaled down for 24-well plates. Following siRNA treatment for 4 h, cells were treated with 1 nM E₂ or 100 nM fulvestrant or a combination and cell counts on days 0, 3, and 6 were estimated using a Coulter counter. The following siRNA sequences were used: ER RNAi 1; ESR1 SMARTpool (four pooled sequences; Upstate Biotechnology, Lake Placed, NY, USA; M-003401; 100 nmol), ER RNAi 2; 5'-AAACAGGAGGAA-GAGCTGCCA (Ambion; 40 nmol), ER RNAi 3; 5'-AACCTCGGGCTGTGCTCTTTT (Ambion, Huntingdon, Cambridgeshire, UK; 40 nmol), and negative RNAi: Upstate (D-001206; 100 nmol).

Results

Increased ER α expression in resistant cell lines

To explore the possibility that high ER α expression leads to estrogen-independent growth in endocrine-resistant cells, the expression levels of ER α in resistant lines (LCC1 and LCC9) were compared with levels in wild-type MCF-7 cells. Both resistant lines expressed between four- and elevenfold more ER α mRNA than wild-type cells (Fig. 1A). ER α protein levels were clearly elevated in LCC1 cells relative to MCF-7 cells (sevenfold) and less markedly in LCC9 cells (Fig. 1B). E₂ decreased ER α protein in MCF-7 cells at 48 h and this has been explained by proteosomal degradation, a process speculated to limit the action of estrogen signaling (Nawaz *et al.* 1999; Fig. 1C). Similarly, both resistant lines demonstrated ER α turnover, suggesting that ER α is binding to E₂ in all cases. In contrast, tamoxifen treatment results in maintenance of the receptor expression levels in all three cell lines (Fig. 1C).

Addition of 1 nM 17 β -estradiol (E₂) to MCF-7 cells produced a marked stimulation of growth to cells cultured in estrogen-depleted (double charcoal-stripped FCS) medium (Fig. 2A). In the absence of E₂, MCF-7 cells are essentially static (Fig. 2A). In contrast, LCC1 cells grow rapidly in estrogen-depleted conditions and show an approximately twofold stimulation of growth on addition of E₂ (Fig. 2B). LCC9 cells showed a lack of response to E₂, again

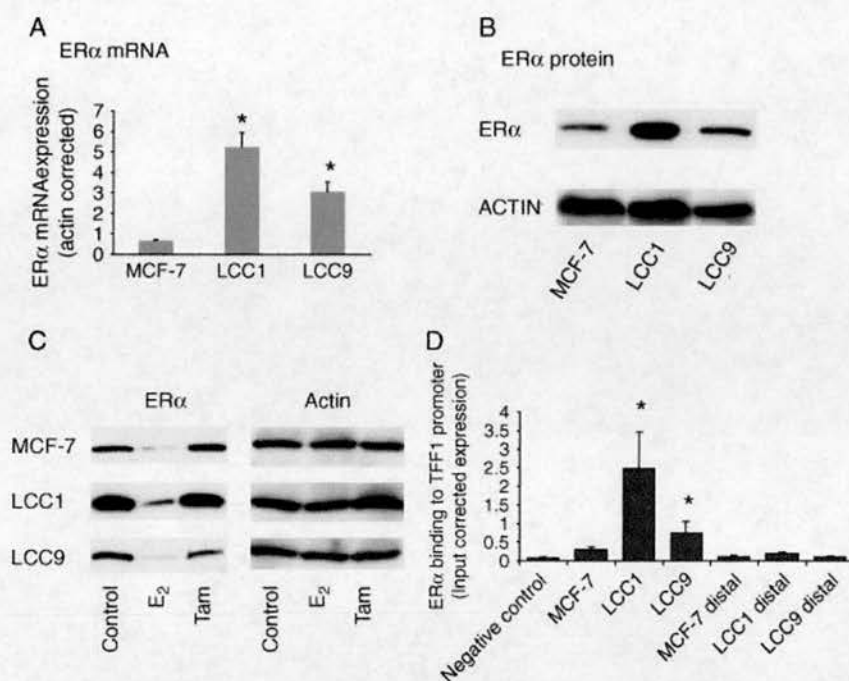


Figure 1 ER α expression in MCF-7, LCC1, and LCC9 cells. (A) ER α mRNA expression. Cells were grown in charcoal-stripped serum-containing medium for at least 48 h and RNA was collected. A representative experiment is shown of at least two experiments carried out. Each column presents mean of triplicate RT-PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars = s.d. Statistical significance noted for treatment groups versus matched control (one-way ANOVA and multiple comparison Tukey–Kramer test; * $P < 0.05$). (B) Western blot analysis of ER α (66 kDa) in breast cancer cell lines grown in charcoal-stripped serum-containing medium for 48 h prior to protein collection. One hundred micrograms of protein were loaded per lane and detected using either anti-ER α (Santa Cruz Biotech) or anti-actin (Calbiochem) antibodies as described in Materials and methods. (C) Western blot analysis of ER α (66 kDa) in breast cancer cell lines grown in charcoal-stripped serum-containing medium for at least 48 h prior to protein collection. One hundred micrograms of protein were loaded per lane and detected using either anti-ER α (Santa Cruz Biotech) or anti-actin (Calbiochem) antibodies as described in Materials and methods. (D) ER α binding to the TFF1 promoter. Basal recruitment of ER α to the TFF1 promoter was determined by ChIP analysis on untreated cells. The ChIP method used was as described in Materials and methods and immunoprecipitated TFF1 promoter was quantified by real-time PCR. The input-corrected expression values were determined by normalizing to the inputs. Data are presented as mean + s.e. Groups were compared with the Kruskal–Wallis test with Dunn's multiple comparison test (* $P < 0.05$). Binding to the promoter region is compared with binding to a region 3.5 kb distal to the promoter wherein only background binding was observed.

growing very rapidly in the absence of E₂ (Fig. 2C). Addition of 1 μ M tamoxifen to MCF-7 cells antagonized the E₂-stimulated growth in this cell line. Tamoxifen also inhibited the E₂-stimulated growth of LCC1 cells but had no effect on LCC9 cells (Fig. 2B and C). These results are consistent with wild-type cells being estrogen dependent, LCC1 cells demonstrating partial estrogen dependence and LCC9 cells being fully estrogen independent.

Reduced ER α Ser¹¹⁸ phosphorylation in LCC9 cells

Several frequently cited mechanisms of estrogen-independent activation of ER α involve phosphorylation of ER α at the Ser¹¹⁸ or Ser¹⁶⁷ residues mediated via ERK or Akt respectively (Bunone *et al.* 1996, Martin *et al.* 2000, Lannigan 2003). While the Ser¹¹⁸

residue is a major site of E₂-induced phosphorylation, Ser¹⁶⁷ is not (Lannigan 2003). The latter site is activated by growth factor signaling. In view of these previous observations, we first investigated whether ER α Ser¹¹⁸ or Ser¹⁶⁷ phosphorylation were increased in the absence of estrogen in the resistant cell lines. Neither was there evidence of increased Ser¹¹⁸ phosphorylation in the resistant lines relative to MCF-7 under basal conditions, nor was Ser¹⁶⁷ phosphorylation increased (Fig. 3A–C). Furthermore, phospho-ERK1/2 expression was unchanged in the lines (Fig. 3C). On E₂ addition, there was a marked increase in Ser¹¹⁸ phosphorylation in MCF-7 cells and this was also observed in the LCC1 cell line (Fig. 3A and B). However, minimal change was observed on E₂ addition to LCC9 cells (Fig. 3A and B). Ser¹¹⁸ phosphorylation has been proposed to affect cofactor recruitment and this might explain the reduced

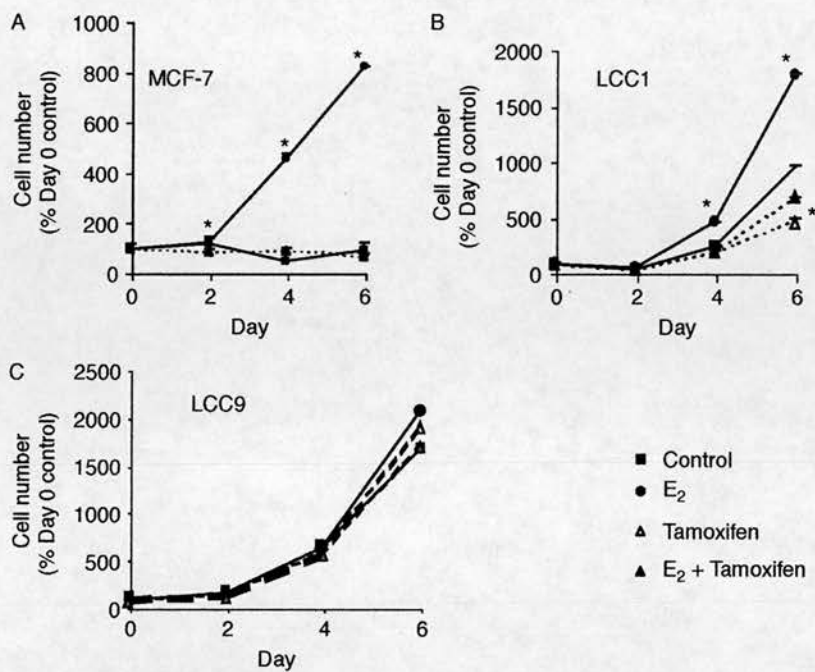


Figure 2 Growth characterization of MCF-7 and MCF-7 variant cells. (A) MCF-7 cells, (B) LCC1, and (C) LCC9 cells were plated for 24 h and maintained in reduced media for 48 h before treatment. Cells were then left untreated (control group), treated with 1 nM E₂, 1 μM tamoxifen or 1 nM E₂ and 1 μM tamoxifen. Cells were counted on day 0 (72 h after plating/day of treatment start) and days 2/4/6 using a Coulter counter. Mean cell counts of triplicate samples and duplicate counts for each time point in each treatment group are expressed. Error bars = s.d. A representative experiment is shown of at least four experiments carried out.

transcriptional (as mentioned below) and growth responses observed on E₂ addition to this cell line. Tamoxifen alone produced a small increase in Ser¹¹⁸ phosphorylation in MCF-7 and LCC1 cells but not in LCC9 cells (Fig. 3A and B). Tamoxifen also produced a reduction of estrogen's Ser¹¹⁸ phosphorylation in the MCF-7 and LCC1 cell lines (Fig. 3A and B).

Modified DNA binding of ERα in resistant cell lines

To explore whether high ERα expression was reflected in enhanced DNA binding in the absence of E₂, ChIP methodology was used to examine ERα binding to the promoter of the E₂-responsive gene TFF1 in the MCF-7, LCC1, and LCC9 cell lines. LCC9 cells had >2.5-fold greater ERα binding to the TFF1 promoter than MCF-7 cells (Fig. 1D). However, this binding was significantly higher in LCC1 cells with levels greater than eightfold above MCF-7 cells. This enhanced ERα binding in LCC1 cells was equivalent to the increased expression of ERα protein and is consistent with the suggestion by Fowler *et al.* (2004) that enhanced ERα protein expression can lead to increased DNA binding. As a control, binding to a region 3.5 kb distal to this region indicated only background levels as expected (Fig. 1D).

Growth responses to estrogen and tamoxifen in the wild-type and variant cell lines are reflected in transcriptional changes

To investigate the differences in estrogen and anti-estrogen activation processes, indicator genes that reflected the different growth responses were next investigated. Transcriptional changes in the estrogen-regulated genes TFF1 and PGR were measured and modulated expression was compared with the growth changes.

Expression of TFF1 mRNA in the absence of E₂ was higher in both resistant lines compared with MCF-7 cells (Fig. 4A). After 48-h E₂ (1 nM) treatment, TFF1 mRNA was increased by >20-fold in MCF-7 cells, but only one- to two-fold in the resistant lines although this increase was significant. Tamoxifen (1 μM) produced a small increase in TFF1 expression in MCF-7 and LCC1 cells but not in the LCC9 cell line (Fig. 4A). These levels broadly reflect the growth differences observed.

The expression of PGR mRNA in the absence of E₂ was greater in LCC1 and LCC9 lines compared with MCF-7 cells (Fig. 4B). As for TFF1, after 48-h E₂ treatment, PGR mRNA was increased by >20-fold in MCF-7 cells and 2–5-fold in LCC1 and LCC9 cell lines (Fig. 4B). Tamoxifen also increased the PGR mRNA

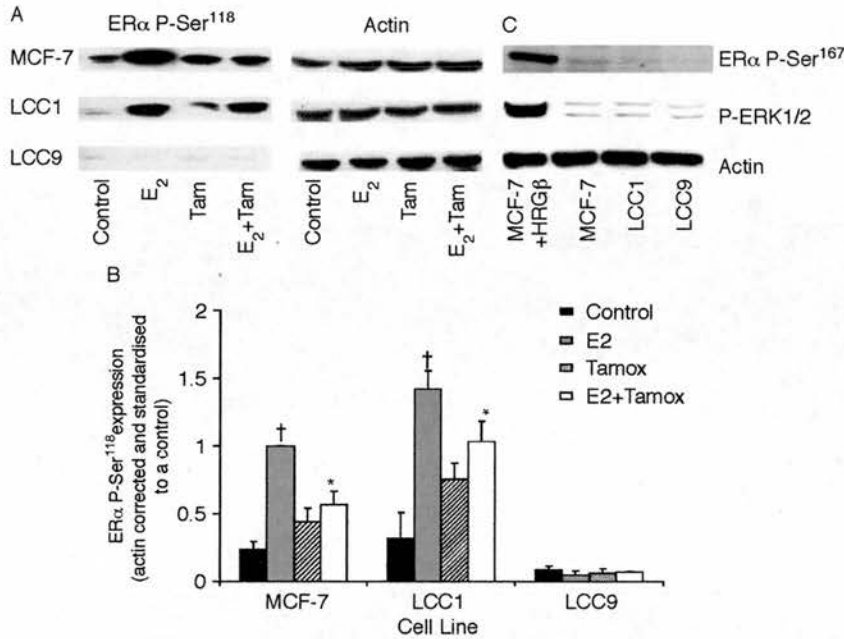


Figure 3 Effects of E₂ and tamoxifen on ER α phosphorylation in the resistant cell lines. (A) Western analysis of ER α phospho-Ser¹¹⁸ after 30-min treatment with control (no treatment), 1 nM E₂, 1 μ M tamoxifen, or 1 nM E₂ and 1 μ M tamoxifen. Lysates were run on a 10% SDS gel and membranes probed with anti-phospho ER α Ser¹¹⁸ antibody (1:1000). Lysates were also probed for actin expression to compare protein loading. (B) Histogram representing optical densities from triplicate western blots of ER α phospho-Ser¹¹⁸ after 30-min treatment with control (no treatment), 1 nM E₂, 1 μ M tamoxifen, or 1 nM E₂ and 1 μ M tamoxifen. Values were actin corrected and then standardized to a control sample. The control sample was a 30 min E₂-treated MCF-7 sample and was used on all gels as a standard to allow comparisons between runs. Statistical comparisons were made with each cell line's control level; * $P < 0.01$; [†] $P < 0.001$ (ANOVA). (C) Western analysis of ER α phospho-Ser¹⁶⁷ and phospho-ERK1/2 in cell lines. Untreated lysates were probed with antibodies specific for ER α phospho-Ser¹⁶⁷ and phospho-ERK1/2. A positive control lane of MCF-7 cells treated with 1 nM HRG β was used. Lysates were also probed for actin expression.

expression level not only in MCF-7 cells, but also in LCC1 cells producing effects equivalent to that of E₂ in the latter cell line. No change was observed in the LCC9 cell line.

These results are consistent with transcription of TFF1 and PGR being increased by ligand-independent mechanisms in LCC1 and LCC9 cell lines with estrogen and tamoxifen producing an additional ligand-dependent increase.

Effect of removal of ER α on the growth of the cell lines

To determine the relative importance of ER α on downstream gene expression and growth of MCF-7, LCC1, and LCC9 cells, we investigated the effects of removing ER α , either by specific siRNA inhibition of receptor synthesis or through inhibition and degradation of the receptor by fulvestrant.

A panel of interfering RNAs (siRNAs) were initially compared for their ability to transiently reduce ER α expression and were transfected into the MCF-7 cell

line. RNAi 1 is a pooled set of four targeted sequences (Imai *et al.* 2005) while RNAi 2 (5'-AAACAGGAG-GAAGAGCTGCCA) and RNAi 3 (5'-AACCT-CGGGCTGTGCTCTTTT) are individually targeted sequences (Leu *et al.* 2004). Of the three, RNAi 2 produced the best reduction of ER α mRNA and protein and was selected for further experiments (Fig. 5A and B). Quantitative RT-PCR analysis showed that, 48 h after transfection, ER α RNAi 2 treatment resulted in an 85% decrease in ER α mRNA expression and an 87% decrease in the presence of E₂ (Fig. 5C). LCC1 and LCC9 cells have significantly higher basal expression of ER α mRNA and siRNA removal caused an 82 and 73% decrease respectively with similar reductions in the presence of E₂ (Fig. 5C). Western analysis of the MCF-7 and LCC1 cell lines demonstrated that RNAi 2 produced ER α protein knockdown over a 7-day period (Fig. 5D) and it was effective in all three cell lines (Fig. 5E). This reduction in ER α protein was accompanied by a decrease in PGR protein (Fig. 5E). Thus, it appeared that gene expression in all three cell lines was ER α dependent.

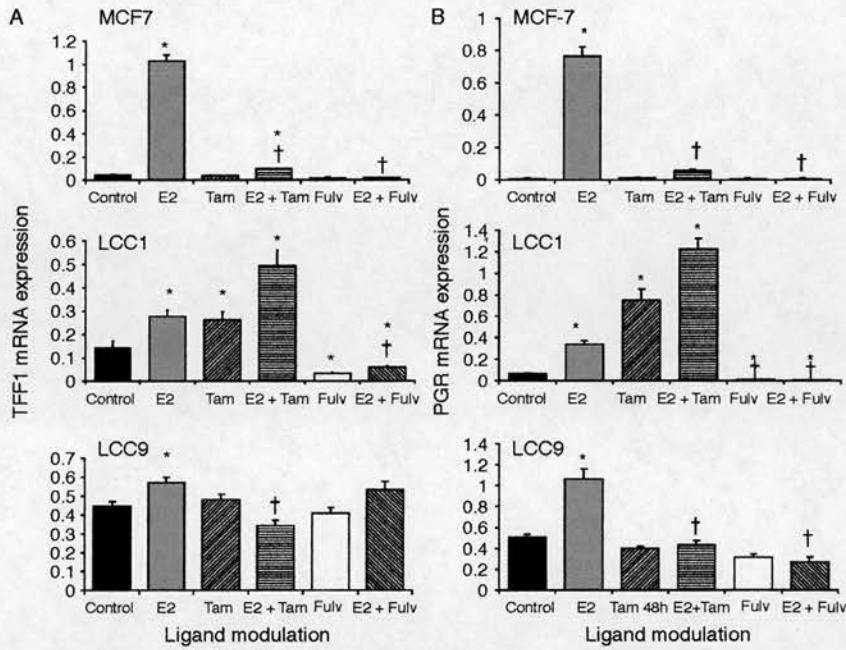


Figure 4 Effect of estrogen, tamoxifen, and fulvestrant on (A) TFF1 expression and (B) PGR mRNA expression in the cell lines. Relative mRNA expression values of TFF1 and PGR in the cell lines were measured by real-time RT-PCR using specific primer pairs. RNA was collected at 48 h and was extracted from either untreated (control) cells or cells treated with 1 nM E₂, 1 μM tamoxifen, 1 nM E₂+1 μM tamoxifen, 100 nM fulvestrant, or 1 nM E₂+100 nM fulvestrant. Each column represents mean of triplicate PCR analysis for each sample demonstrating mRNA expression relative to actin expression. Error bars = s.d. Statistical significance noted for treatment groups compared to matched control where **P* < 0.05, untreated control versus treatment group; †*P* < 0.05, E₂ control versus treatment group (ANOVA and multiple Tukey–Kramer comparison test).

This was investigated further using fulvestrant. Fulvestrant abrogates E₂-induced gene transcription by binding, blocking, and causing the degradation of ERα (Parker 1993). Fulvestrant treatment in MCF-7 cells blocked E₂-induced expression of TFF1 and PGR (Fig. 4A and B). In addition, ligand-independent and E₂-induced TFF1 and PGR expression in LCC1 cells were reduced on fulvestrant treatment. These data confirm that for LCC1 cells TFF1 and PGR induction are dependent on ERα expression. However, LCC9 cells are resistant to fulvestrant treatment and as such no change in TFF1 expression and only a minor change in PGR expression was observed. The effect of fulvestrant on the growth of all three cell lines was also investigated in the complete absence of serum (Fig. 6). Under these conditions, MCF-7 cells did not grow over a 72-h period. LCC1 cells, however, still proliferated and the addition of E₂ had little effect on growth confirming their independence of E₂. Under these conditions, fulvestrant was able to oppose the effect of low concentrations of E₂ again indicating dependence on ERα. In contrast, LCC9 cells were completely insensitive to both E₂ and

fulvestrant. Fulvestrant degraded ERα protein in all three lines which is shown in Fig. 7A.

To determine how critical levels of ERα expression were for the growth of MCF-7, LCC1, and LCC9 cell lines, we used RNAi removal with or without fulvestrant to inhibit the synthesis of ERα protein (Fig. 7B–D). E₂-induced MCF-7 cell growth was significantly decreased (33%) by ERα removal and abolished by all combinations of fulvestrant alone or with RNAi. LCC1 cells grew in the absence of E₂ and RNAi removal had only a minor effect on growth. E₂-induced LCC1 cell growth was reduced by approximately 40% when ERα was removed through RNAi, but, unlike MCF-7 cells, fulvestrant alone was not enough to abolish growth – this, however, could be accomplished though through combination with RNAi. LCC9 cell growth in the absence of E₂ was reduced by ERα RNAi. A similar decrease was observed in the presence of E₂. LCC9 cells are fulvestrant resistant and no effect on growth was observed with this agent. No combinations of fulvestrant or RNAi were able to totally abolish growth. These results indicate a varying degree of dependence on ERα for growth in the three cell lines.

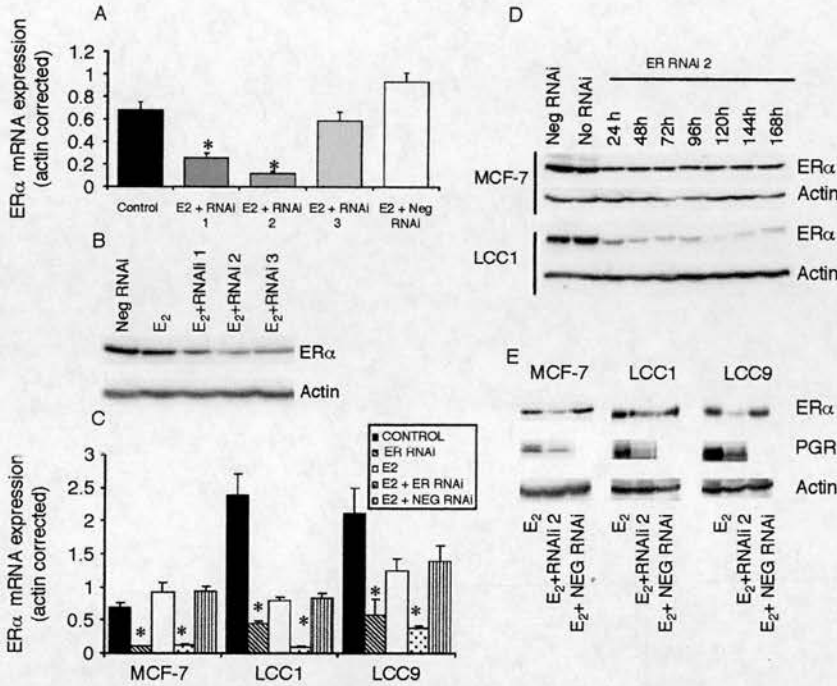


Figure 5 Effects of ER α RNAi on ER α and PGR expression in the cell lines. (A) Expression of ER α mRNA after treatment with a range of ER α mRNA-targeted RNAis. ER α mRNA expression was measured by quantitative RT-PCR of mRNA from MCF-7 cells 48 h after RNAi treatment in the presence of 1 nM E $_2$. Data are presented as mean \pm s.d. of actin-corrected values from triplicate samples. The RNAi transfection method and RNAi sequences used are described in Materials and methods. Statistical significance noted for treatment groups compared with matched control where * P < 0.05, untreated control versus treatment group (ANOVA and multiple Tukey–Kramer comparison test). (B) Western analysis of ER α protein expression in MCF-7 cells 48 h after siRNA treatment. ER α was probed with the F-10 antibody and actin is shown as a loading control. (C) Expression of ER α mRNA after treatment with RNAi 2. ER α mRNA expression was measured by quantitative RT-PCR of mRNA from cell lines 48 h after RNAi 2 treatment in the presence or absence of 1 nM E $_2$. Data are presented as mean \pm s.d. of actin-corrected values from triplicate samples. The RNAi transfection method and RNAi sequences used are described in Materials and Methods. Statistically significance differences are noted for treatment groups compared with matched control where * P < 0.05, untreated control versus treatment group (ANOVA and multiple Tukey–Kramer comparison test). (D) Western analysis time course of the effect of RNAi 2 treatment on ER α protein expression in the MCF-7 and LCC1 cell lines. ER α was probed with the F-10 antibody and actin is shown as a loading control. (E) Western analysis of ER α and PGR protein expression in the cell lines 48 h after siRNA treatment. ER α was probed with the F-10 antibody, PGR with Ab 8 and actin is shown as a loading control.

Discussion

Aromatase inhibitors are now used for the adjuvant treatment of most hormone receptor-positive early breast cancer. Despite the improvement they offer over tamoxifen alone, recurrences still occur, and thus models of resistance to both tamoxifen and estrogen deprivation are required. The series of MCF-7-derived cell lines provides an excellent model system for the exploration of mechanisms of stepwise acquisition of resistance to tamoxifen and estrogen deprivation. Most models to date have been derived *in vitro*, which makes LCC1 cells interesting as the initial estrogen deprivation was achieved *in vivo* and therefore might reflect features that could arise in a primary breast cancer (Brunner *et al.* 1993). In many of the *in vitro*-derived LTED models, acquired resistance is due to enhanced sensitization to low concentrations of estrogen, which

often involves crosstalk with growth factor-signaling pathways (Martin *et al.* 2003, 2005a,b). LCC1 cells have certain of the characteristics of the LTED phenotype (Yue *et al.* 2002, Martin *et al.* 2003, 2005a,b, Santen *et al.* 2005) such as a higher expression level of ER α , an ability to grow in low-estrogen conditions and elevated TFF1 expression. The continuous culturing of LCC1 cells in low estrogen conditions may well contribute to the increased expression of ER α in this cell line.

However, unlike most LTED-derived cells, which show little response to physiological levels of estrogen yet are sensitive to very low levels of estrogen, LCC1 cells appear truly insensitive to the addition of low levels of exogenous estrogen. Similarly, while most LTED cells show basal activation of ERK1/2 activation and ER α via Ser¹¹⁸ phosphorylation, LCC1 and

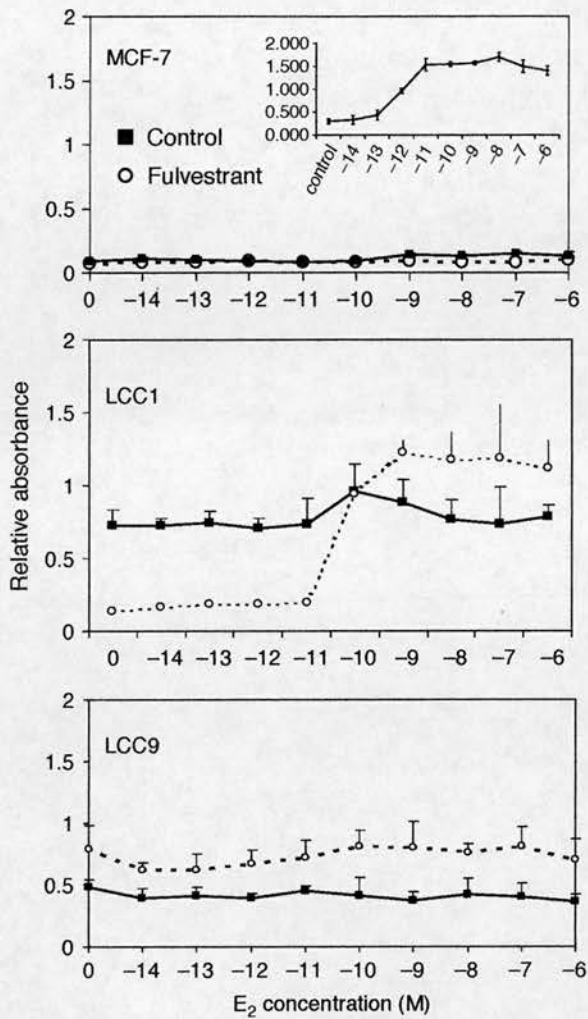


Figure 6 Effect of the growth of the cell lines in serum-free media and treated with varying concentrations of E₂ in the absence or presence of fulvestrant (1 nM). Cells were plated and after establishment placed in serum-free medium for 48 h. E₂ with or without fulvestrant was added and plates left for 72 h. Relative cell numbers were then assessed by SRB assay as described in Materials and methods. Inset in MCF-7 figure: effect of E₂ on MCF-7 cells grown in 5% double charcoal-stripped fetal serum.

LCC9 cells do not. The ER, however, is still clearly functional in LCC1 cells and linked to growth regulation as estrogen addition can produce an increase in growth which could be reversed by tamoxifen. ER α is also downregulated by the addition of estrogen and markedly phosphorylated at Ser¹¹⁸. Additionally, the ER α downregulator fulvestrant reduces expression of TFF1 and inhibits growth. These effects are more marked when cells are exposed to fulvestrant with siRNA removal of ER α .

While constitutive activation of ER α may be achieved in some instances by phosphorylation of

Ser¹¹⁸ mediated by growth factor-driven activation of ERK, an increased expression of ER α alone might account for increased DNA binding. In support of this, there was enhanced binding of ER α to the TFF1 promoter in the absence of added estrogen in both the LCC1 and LCC9 cell lines. In addition, TFF1 transcription was markedly increased in the resistant cell lines consistent with this enhanced ER α -binding driving transcription. Direct support for such a mechanism has recently been demonstrated in an MCF-7 cell line using a tetracycline-inducible ER α overexpression model (Fowler *et al.* 2004, 2006). As with the data mentioned earlier, the results suggested that elevated levels of ER α resulted in activation of receptor transcriptional function in a manner distinct from mechanisms that involve ligand binding or growth factor-induced phosphorylation of the Ser¹⁰⁴, Ser¹⁰⁶ or Ser¹¹⁸ sites. The mechanism required the amino-terminal A/B domain and was not inhibited by tamoxifen. It was also uncoupled from ERK activation. The hypothesis proposed was that overexpression of unliganded ER α stabilized interactions with the basal transcriptional machinery, which at normal receptor levels may be too weak to support effective transcription (Fowler *et al.* 2004).

These results together support a model wherein growth (and TFF1 transcriptional activation) in LCC1 cells is dependent on ER α . This dependency has some ligand (i.e., estrogen) responsiveness but is largely ligand independent. The ligand-dependent component may be reversed by tamoxifen. The ligand independence appears to involve neither growth factor activation via the Ser¹¹⁸ or Ser¹⁶⁷ phosphorylation routes nor hypersensitization (where low levels of estrogen produce apparent independence). Instead the ligand independence appears to be explained by the high level of ER expression leading to constitutive activation and promoting DNA binding and transcriptional activation.

We have shown that ER α is functionally active in the LCC1 model and since this has also been shown in models demonstrating LTED, a logical clinical strategy to attempt after development of resistance in a low estrogen environment (such as produced by aromatase inhibitor treatment) is to downregulate the receptor using fulvestrant (Johnston *et al.* 2005, Martin *et al.* 2005a,b). This strategy clearly is effective at inhibiting growth in LCC1 cells. However, the LCC9 variant was derived after exposure and development of resistance to fulvestrant (Brunner *et al.* 1997) and showed no growth response to either estrogen or tamoxifen. In this cell line, the negligible changes of

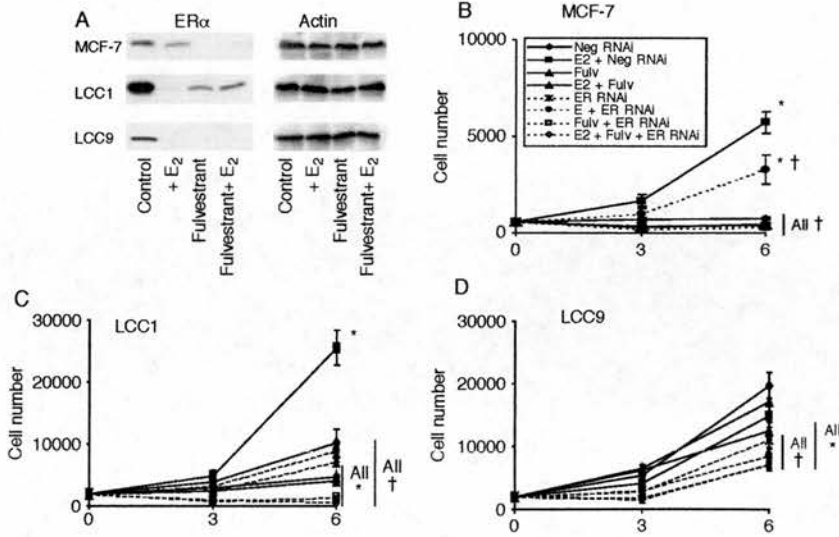


Figure 7 Effect of fulvestrant on ER α expression and combined with ER α siRNA on the growth of the cell lines. (A) Western blot analysis of ER α (66 kDa) in breast cancer cell lines in control, 1 nM E $_2$, 100 nM fulvestrant, or 1 nM E $_2$ and 100 nM fulvestrant-treated groups at 48 h. One hundred micrograms of protein were loaded per lane and detected using anti-ER α (Santa Cruz Biotech) antibody as described in Materials and Methods. Actin expression is also shown. (B–D) Effects of fulvestrant, ER α siRNA, or combinations of these on the growth of the cell lines. (B) MCF-7, (C) LCC1, and (D) LCC9 cells were treated with 1 nM E $_2$, 100 nM fulvestrant, 40 nM ER α siRNA, or combinations of these. siRNA treatment was for 4 h only, while E $_2$ and fulvestrant were present throughout the time course. Comparisons are made with the negative siRNA control which gave an equivalent growth effect to no treatment. Data are presented as mean \pm s.e. from quadruplicate samples. Statistical significance noted for treatment groups compared with matched control where * $P < 0.05$, negative RNAi control versus treatment group; † $P < 0.05$, negative RNAi + E $_2$ control versus treatment group (ANOVA and multiple Tukey–Kramer comparison test).

ER α Ser 118 phosphorylation obtained on estrogen or tamoxifen addition contrasted with observations in the other cell lines. Markedly reduced phosphorylation is likely to affect cofactor binding and our initial findings suggest that p160 binding (specifically AIB1) is reduced in this cell line, again consistent with endocrine insensitivity (Kuske *et al.* 2004). However, it is quite clear that fulvestrant can downregulate the receptor and even extremely high levels of fulvestrant (10 μ M) were unable to influence growth (data not shown). Despite this, siRNA removal of ER α produced some growth inhibition suggesting a reduced but still measurable dependency on ER α .

In conclusion, these results suggest that multiple changes contribute to endocrine resistance. While ER still demonstrates functionality in LCC1 cells, there is a major shift to ligand independence. This independence can be explained by the high level of ER expression found in these cells and could lead to constitutive activation of the receptor. These cells still show a degree of dependency on estrogen and this can be blocked by tamoxifen. Further changes were produced by exposure and development of resistance to fulvestrant including a loss of ER α Ser 118 activation, which could account for its loss of sensitivity to estrogen. These data support the view that in the early stages of resistance, SERDs may

provide a useful therapeutic option, but other approaches will be required when resistance has developed to these agents.

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