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Growth factor endocrine pathway crosstalk : can treatment with trastuzumab activate steroid signalling?

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I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree, Doctor of Medicine (MD), is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Abbreviations

ADCC	antibody dependent cellular cytotoxicity
AI	aromatase inhibitor
AIB1	amplified in breast 1
Akt	protein kinase B
AP-1	activator protein-1
APS	ammonium persulphate
ATCC	American type culture collection
ATM	ataxia telangiectasia mutated
BCA	bicinchonic acid
BRIP1	BRCA1 interacting protein 1
cAMP	cyclic adenosine monophosphate
CBP	cAMP response element binding protein
DAB	diaminobenzidine tetrahydrochloride
dH2O	distilled water
DLR	dual luciferase reporter
DNA	deoxyribonucleic acid
DNMT	deoxyribonucleic acid methyltransferases
dNTP	deoxyribonuleotide triphopshate
DTT	Dithiothreitol
E2	17β-estradiol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	oestrogen receptor
ERE	oestrogen responsive elements
ERK	extra-cellular signal-related kinase
Fab	fragment antigen binding
Fc	fragment crystallisable
FCS	foetal calf serum
FDA	food and drug administration
FKHR	forkhead homolog 1 rhabdomyosarcoma
FS	first strand
Grb2	growth factor receptor-bound protein 2
GSK-3	glycogen synthase kinase 3
H2O2	hydrogen peroxide
HER2	human epidermal growth factor receptor 2
HOX	homeobox genes in humans
HRP	horseradish peroxidase
IGF-1	insulin-like growth factor-1
IMS	industrial methylated spirits
kb	Kilobase
kDa	Kilodalton
LAR	luciferase assay reagent
MAPK	mitogen-activated protein kinase
MEM	minimum essential medium

MMLVRT	moloney murine leukemia virus reverse transcriptase
MMP	matrix metalloproteinase
MNAR	modulator of nongenomic action of oestroge receptor
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MUC4	membrane-associated glycoprotein mucin-4
NCoR	nuclear receptor co-repressor
P90RSK	p90 ribosomal S6 kinase
PAGE	polyacrylamide gel electrophoresis
PALB2	partner and localiser of BRCA2
PARP1	poly(ADP-ribose) polymerase 1
PAX2	paired box gene 2
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3-OH kinase
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
Raf	rapidly accelerated fibrosarcoma
Ras	RAt sarcoma
RIPA	radioimmunoprecipitation assay
SDS	sodium dodecyl sulphate
SERD	selective oestrogen receptor downregulator
SERM	selective oestrogen receptor modulator
SF-1	steroidogenic factor-1
SMRT	silencing mediator for retinoid and thyroid-hormone receptor
Sos	son of sevenless
SP-1	specificity protein-1
SRC-1	steroid receptor co-activator 1
STAR	study of tamoxifen and raloxifene
TAE	tris acetate ethylenediaminetetraacetic acid
Taq	thermus aquaticus
TBS	tris-buffered saline
TBST	tween in tris-buffered saline
TEMED	N,N,N,N-tetremethylethylenediamine
TFF1	trefoil factor 1
TGF	transforming growth factor
4-OHT	4-hydroxytamoxifen

Summary

Introduction

Breast cancer is the most commonly diagnosed malignancy in women in Ireland after non-melanoma skin cancer, with over 2,000 women diagnosed with the disease each year. It remains the leading cause of cancer-related mortalities in women, with an average annual rate of 644 deaths between 1994 and 2001. Although the mortality rate decreased between 1989 and 2006 by nearly 27%, it still remains one of the highest mortality rates in Europe.

Targeted therapy against the key receptors identified to date as being integral to the development and proliferation of breast cancers has significantly improved the overall and disease-free survival rates for patients. However, innate and acquired resistance to these therapeutic agents, including tamoxifen and trastuzumab, results in tumour recurrence which is not susceptible to further standard therapeutic interventions. Resistance to tamoxifen can occur in up to one quarter of patients after ten years, while the majority of patients with metastatic disease will develop resistance to trastuzumab within one year of treatment.

Resistance to tamoxifen is thought to occur through a number of proposed mechanisms. One such proposal is the concept of crosstalk between ER and the growth factor receptor pathways including the EGFR/HER2 family of receptors. This can result in resistance developing through a number of different mechanisms including Src kinase activation of the metalloproteinases with resultant activation of the MAPK pathway as well as Akt upregulation through PI3K and ras activation of ERK1 and ERK2.

While extensive *in vitro* research has been performed to determine how resistance to trastuzumab develops, the exact mechanisms remain inconclusive. Hypotheses include alteration in the interaction between the receptor and the antibody, alternative signalling through other HER or alternate pathways or failure to activate immune-mediated mechanisms to destroy tumour cells.

Understanding how resistance to trastuzumab develops would greatly improve the clinical prognosis for patients with HER2 expressing breast cancers, as it would enable clinicians to identify patients at risk of developing resistance and facilitate the tailoring of specific treatments to minimise the risk of such an outcome occurring.

Hypothesis

Research has highlighted the complex interactions that occur at a molecular level within breast cancer cells. Receptor crosstalk has been implicated in the development of resistance to endocrine therapies such as tamoxifen. We hypothesise that resistance to trastuzumab can be mediated by crosstalk between the growth factor signalling pathway and the steroid hormone pathway resulting in an uncontrolled upregulation of the steroid hormone pathway following trastuzumab treatment.

Aims

The overall aim of this work is to further elucidate the interactions between the steroid hormone and growth factor signalling pathways in breast cancer, in particular, to determine the effect of trastuzumab treatment on the steroid hormone pathway. The first objective will be to assess the effect of trastuzumab treatment on this pathway at a molecular level in breast cancer cells, looking at its effect at both a protein level and a transcriptional level. Secondly, we will verify our *in vitro* findings in breast cancer tissue samples utilising a large tissue microarray, assessing the clinical significance of our proposed crosstalk hypothesis.

Results

Protein expression levels of ER within the oestrogen sensitive MCF-7 cells was found to be increased following trastuzumab treatment while there was no effect observed in the oestrogen insensitive LCC-1 cells. Surprisingly, treatment of ER-negative SKBR-3 cells with trastuzumab resulted in the expression of ER.

Luciferase assay and RT-PCR was performed to assess whether trastuzumab had an enhancing effect on the functional activity of the steroid hormone pathway within the cell model. pS2, a well established ER target gene, was utilised to demonstrate activity. Increased levels of pS2 was observed following trastuzumab treatment in both the MCF-7 and SKBR-3 cell lines, with no difference in the LCC-1 cells.

Methylation studies were performed to determine whether they played a role in the expression of ER within the ER-negative SKBR-3 cells following trastuzumab treatment. Treatment was found to result in the demethylation of five CpG islands within the promoter region of the ER gene within the cells, thereby demonstrating that trastuzumab can result in epigenetic modifications in certain ER-negative cells resulting in the potential expression of a functional ER.

Immunohistochemistry staining for pS2 of a tissue microarray of almost five hundred breast cancer specimens was performed. Survival analysis of HER2 positive patients who expressed pS2 showed that patients had a significantly increased rate of recurrence if they received trastuzumab compared to those who did not. Similarly, a small cohort of patients who received trastuzumab prior to surgical resection were found to have significantly stronger staining for pS2 in their post-trastuzumab treated specimens when compared to their pre-treatment biopsies.

Conclusion

Resistance to trastuzumab remains a significant factor in the development of tumour recurrence in HER2-expressing breast cancers, resulting in reduced overall and disease-free survival times. To date, the exact mechanism by which resistance can develop remains elusive. In this work, molecular studies have demonstrated crosstalk between the steroid hormone and growth factor receptor pathways, suggesting that resistance can develop through upregulation of the endocrine signalling pathway. Furthermore, trastuzumab was also found to result in the expression of a functional ER in ER-negative cells, providing a previously unconsidered potential therapeutic target for these cancers. Translational studies

confirmed that the *in vitro* findings were replicable within a clinical cohort, affecting patient prognosis.

These findings constitute a minute fragment of the complex interactions between the steroid hormone and growth factor receptor pathways in breast cancer. The plasticity of breast cancer and its ability to overcome therapeutic interventions through receptor crosstalk as well as its capacity to alter receptor status demonstrates the complex molecular mechanisms involved in the disease process. Resistance to therapies is likely to present a significant challenge to both clinicians and academics for many years to come. However, translational research methods, as demonstrated within this body of work are essential to unravel our understanding of this complex disease.

Chapter 1

Introduction

1.1 Introduction to breast cancer:

Breast cancer is the most commonly diagnosed malignancy in women in Ireland after non-melanoma skin cancer, with over 2,000 women diagnosed with the disease each year (Ireland 2009). The rate of increase of new cases of breast cancer has fallen from 5.1% per year between 1999 and 2002 to 2.0% per year between 2002 and 2006. However, in 2006 the National Cancer Registry of Ireland predicted that the projected annual incidence would increase to almost 5,000 new cases by 2035 (figure 1.1) (Ireland 2006).

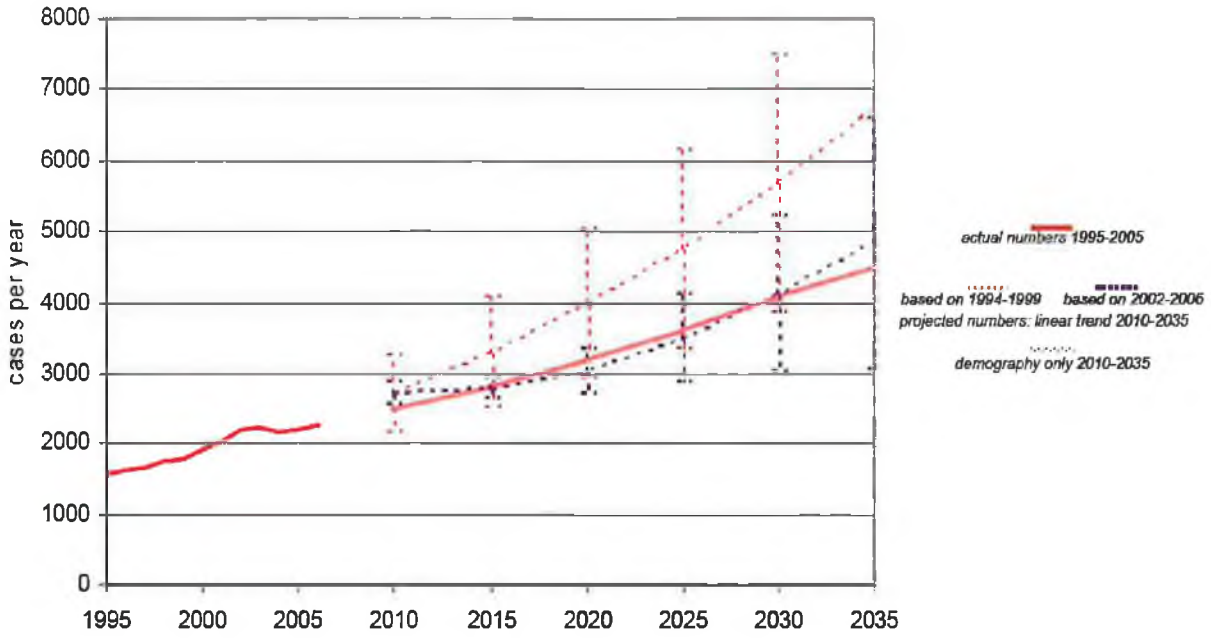


Figure 1.1: *Actual and projected number of breast cancer cases per year from 1995 to 2035 (National Cancer Registry Ireland, 2006).*

Breast cancer remains the leading cause of cancer-related mortalities in women, with an average annual rate of 644 deaths as a result of the disease between 1994 and 2001. While the overall number of deaths is expected to increase from 638 to 695 per year, the mortality rate is predicted to fall by 21% (O'Lorcain P 2006). Indeed, between 1989 and 2006 the mortality rate has decreased overall by nearly 27% (figure 1.2) with an almost 43% reduction in mortality rate in women under the age of 50 years. However, it still remains one of the highest mortality rates in Europe, third only to Denmark and England/Wales (Autier, Boniol et al. 2010).

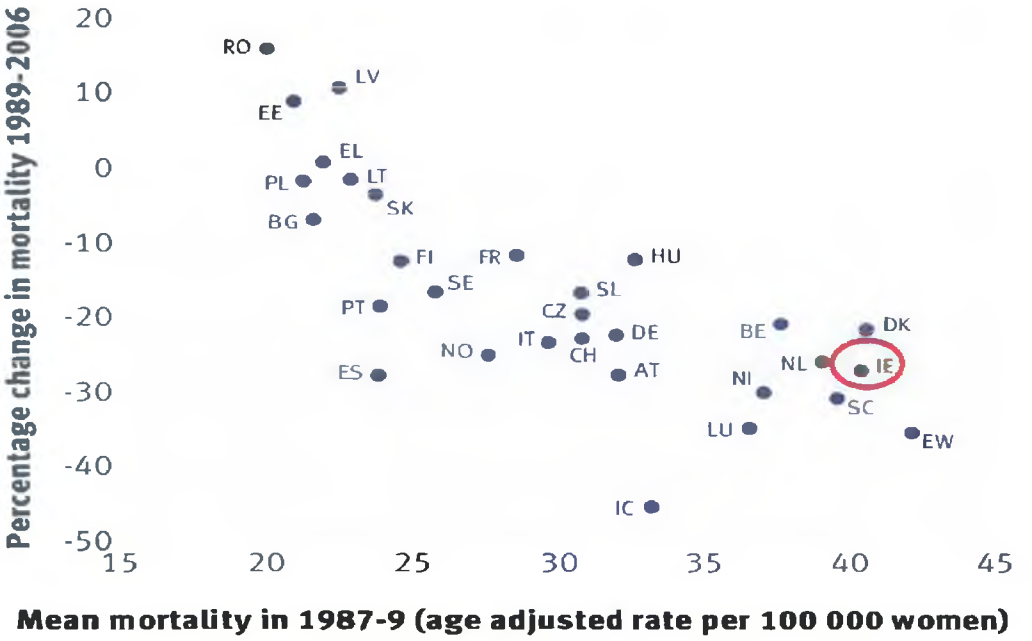


Figure 1.2: *Percentage changes in breast cancer mortality between 1989 and 2006 according to the mean breast cancer mortality in 1987 to 1989. Ireland is highlighted by the red circle (Autier, Boniol et al. 2010).*

Management of breast cancer has changed significantly over the last two decades. Improvements in detection techniques as well as the introduction of screening programmes has resulted in earlier diagnosis which, in conjunction with advances in surgical management and adjuvant therapies, has resulted in the improvements in overall survival rates. However, resistance to adjuvant therapies can develop as a result of the plasticity of tumour cells, with the challenge remaining to develop the ability to individualise treatment regimes according to tumour characteristics resulting in maximum patient benefit with minimal co-morbid effects and decreased risk of resistance developing.

1.2 Aetiology:

It is well established that breast cancer is a multi-factorial disease, with both genetic and environmental components identified which are associated with its development. Risk factors include family history, especially in first degree relatives, nulliparity, early menarche, late menopause as well as a previous history of breast cancer. Specific germline mutations are also known to significantly increase the risk of its

occurrence. In particular the high penetrance BRCA1 and BRCA2 mutations are associated with a 40% to 85% risk of developing breast cancer (Blackwood and Weber 1998; Antoniou, Pharoah et al. 2003). Other low penetrance genes have also been identified which confer a high risk for developing breast cancer including TP53, PTEN, ATM (Campeau, Foulkes et al. 2008) while CHEK2, PALB2 and BRIP1 double the risk compared to the normal population (Walsh and King 2007; Campeau, Foulkes et al. 2008). Although a number of genetic mutations have been identified which increase the predisposition to breast cancer, high penetrance BRCA1 and BRCA2 mutations account for only 25% of familial breast cancer susceptibility with the remaining 75% thought to be due to a large number of mutations affecting low penetrance genes (Pharoah, Antoniou et al. 2002). Specific treatments for BRCA1 and BRCA2 germline mutations have yet to developed but poly(ADP-ribose) polymerase 1 (PARP) has been shown to be effective *in vitro* (Bryant, Schultz et al. 2005; Farmer, McCabe et al. 2005) with clinical trials currently underway to further elucidate their benefits in these high penetrance mutations.

In breast cancer, there are a number of different factors which can determine treatment and influence outcome. These include the tumour tissue expression of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu). In particular the presence or absence of ER and/or HER2 can help predict the natural history and prognosis of the tumour and can also offer potential therapeutic targets. However, resistance to therapies that target these receptors can develop indicating a poor overall prognosis for the patient. An understanding of the plasticity of tumour cells and their ability to proliferate in the presence of current therapeutic agents is essential to overcome resistance and to improve patient survival.

1.3 The steroid hormone pathway

1.3.1 The oestrogen receptor

Steroid hormones including oestrogen influence a wide variety of cellular functions including proliferation and differentiation by influencing specific target gene expression (Tsai and O'Malley 1994; Mangelsdorf, Thummel et al. 1995). Prolonged exposure to oestrogen, both endogenously and exogenously, has been shown to result in the development of breast cancer (Colditz 1998; Hankinson, Colditz et al. 2004). The effects of oestrogen are mediated through ERs which are members of the nuclear receptor family (Kumar and Thompson 1999). ER induces transcriptional activity in the nucleus of cells in a number of ways including through a deoxyribonucleic acid (DNA)-binding domain which interacts with specific sites in the promoter region of target genes known as oestrogen-responsive elements (ERE) (Parker 1993; Osborne, Zhao et al. 2000). After ER was first identified over forty years ago, with subsequent isolation of encoding complementary deoxyribonucleic acids (cDNAs), it was thought that there was only one receptor isoform (Gorski, Toft et al. 1968; Jensen and DeSombre 1972; Green, Walter et al. 1986; Greene, Gilna et al. 1986). However, the subsequent discovery of a second subtype of ER, classified as ERβ with the initial receptor now classified as ERα, indicated a more complex nature to the oestrogen receptor than previously perceived (Kuiper, Enmark et al. 1996; Mosselman, Polman et al. 1996; Katzenellenbogen and Korach 1997). This stimulated intense interest in the role of ERβ and how it compared to that of ERα. *In vitro* research revealed a significantly lower transcriptional activity in both ovarian and breast cancer cell lines, while its tissue distribution was found to be similar but not identical to that of ERα (Kuiper, Enmark et al. 1996; Kuiper, Carlsson et al. 1997; McInerney, Weis et al. 1998). Indeed, they are now considered to play different roles in breast cancer through different DNA enhancers, with ERα appearing to be the predominant regulator of oestrogen-induced genes through its interaction with ERE, while ERβ appears to have minimal interaction with ERE (Bieche, Parfait et al. 2001; Hewitt, Harrell et al. 2005). From henceforth within this thesis, ER will refer only to ERα.

1.3.2 Regulation of ER expression

Approximately two thirds of breast cancers express ER at time of diagnosis (Osborne 1998). This has resulted in the development of therapies designed to interfere in the action of oestrogen on the ER to reduce cell proliferation and tumour growth. However, in the remaining one third of cancers, ER is not expressed and so is not a potential therapeutic target. The exact mechanism by which ER expression is lost remains unclear. In humans, chromosome 6 encodes for the ER protein gene which spans approximately 300kb. It contains eight coding regions which are transcribed from at least seven promoters (Kos, Reid et al. 2001). In keeping with the complex nature of the ER promoter, ER protein expression is known to be regulated by a number of molecular mechanisms including effectors of the chromatin structure. The ER negative phenotype could potentially result from homozygous deletion of the ER gene or loss of heterozygosity along with mutation of the remaining allele (Lapidus, Nass et al. 1998). However, homozygous deletion of the region surrounding the ER gene has not been identified in breast cancer. The loss of heterozygosity is not significantly different between ER positive and negative cancers and no significant gene alterations such as insertions, deletions or point mutations have been reported (Lapidus, Nass et al. 1998).

Epigenetic modifications are a mechanism by which loss of gene transcription can occur in the absence of gross mutations. There are a number of different modifications which can occur resulting in changes in gene expression including methylation, acetylation, phosphorylation and ubiquitination. Methylation of specific areas within DNA is a well described epigenetic modification by which gene expression is lost (Holliday and Pugh 1975). When cytosine-guanine rich areas, known as CpG islands, in the region of the 5' promoter of certain genes become methylated, the transcriptional activity of the associated gene becomes suppressed either through direct effects on the gene or as a result of a conformational change of the chromatin affecting transcription (Kass, Pruss et al. 1997). CpG island methylation is maintained by a number of DNA methyltransferases (DNMTs). Methylation has been shown to result in loss of ER expression in breast cancer cells, while ER positive cells do not demonstrate methylation of the ER gene (Ottaviano, Issa et al. 1994). Subsequent treatment with a DNMT inhibitor 5-aza-2'-cytidine

resulted in demethylation of the ER CpG island with re-expression of both ER mRNA and protein (Ferguson, Lapidus et al. 1995). In breast tumours, the degree of methylation of the ER gene has been shown to be inversely proportional to ER status, with ER positive tumours associated with low levels of methylation while ER negative tumours demonstrated the highest level of methylation (Lapidus, Nass et al. 1998).

1.3.3 Signalling through ER

In ER positive tumours, both oestrogens and growth factors can stimulate proliferation, invasion as well as the production and secretion of a number of proteins, while only growth factors drive these processes in ER negative tumours (Lippman, Dickson et al. 1986). ER belongs to a large superfamily of nuclear receptors which classically act as ligand-inducible transcription factors (Bjornstrom and Sjoberg 2005). When not bound to oestrogen, the receptor is sequestered in a multiprotein inhibitory complex as monomers bound to heat shock proteins either within the cytoplasm or nucleus of the cell (Jensen 1991). Lipid soluble oestrogens, most commonly 17β-estradiol (E_2), diffuse across the cell membrane, bind to the receptor resulting in dissociation of the receptor from the heat shock proteins followed by a series of sequential events. Initially, the binding of E_2 to the receptor induces phosphorylation of ER at several distinct serine/threonine residues (Le Goff, Montano et al. 1994). This results in alteration of its conformation which facilitates receptor dimerisation which then form complexes with a number of different co-regulatory molecules with subsequent binding to the promoter regions of target genes altering gene transcription (Kumar and Chambon 1988; Nilsson, Makela et al. 2001; Rosenfeld and Glass 2001; Osborne, Shou et al. 2005). The co-regulatory proteins function by modulating the transcriptional activity of ER target genes (McKenna, Lanz et al. 1999). Co-activators amplify ER-mediated gene transcription by altering chromatin structure via their histone acetylase activity (Osborne and Schiff 2003). Many of these co-activators, including AIB1 and CBP have been identified and studied (figure 1.3).

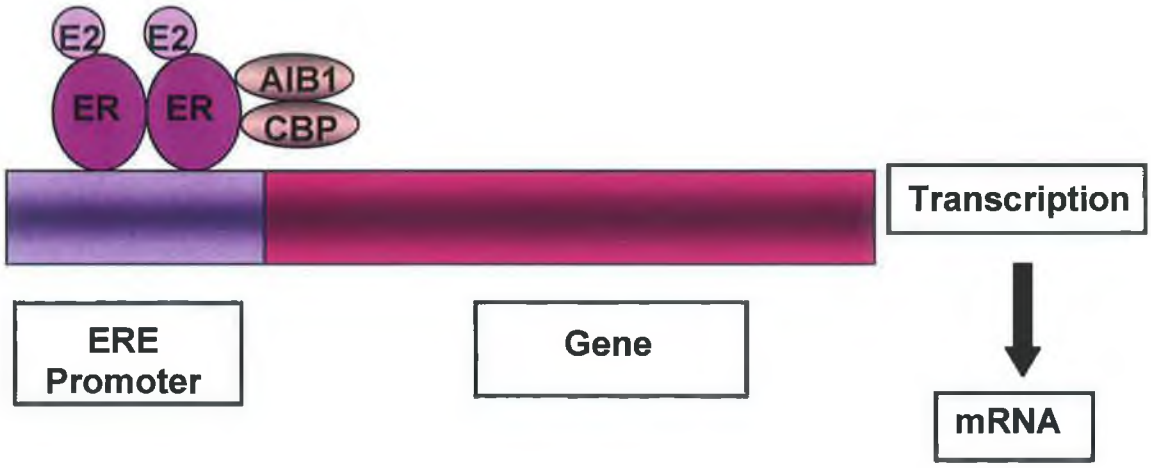


Figure 1.3: *Binding of E_2 to ER causes receptor dimerisation, binding of ER to ERE with recruitment of co-activators resulting in transcription of the target gene. Adapted from Osborne, Shou et al 2005.*
Abbreviations: E_2 - 17aβ-estrdiol; ER - oestrogen receptor; ERE - oestrogen response element; AIB1- amplified in breast 1; CBP - cAMP response element binding protein; mRNA - messenger ribonucleic acid

In contrast, when selective oestrogen receptor modulators such as tamoxifen bind to ER, a different conformational change occurs to the receptor dimer complex which facilitates the recruitment of co-repressors such as SMRT and NCoR along with their associated histone deacetylases (figure 1.4). These co-repressors inhibit transcriptional activity by preventing chromatin unwinding thereby decreasing the expression of target genes resulting in reduced cell proliferation (Smith, Nawaz et al. 1997; Osborne and Schiff 2003; Shou, Massarweh et al. 2004).

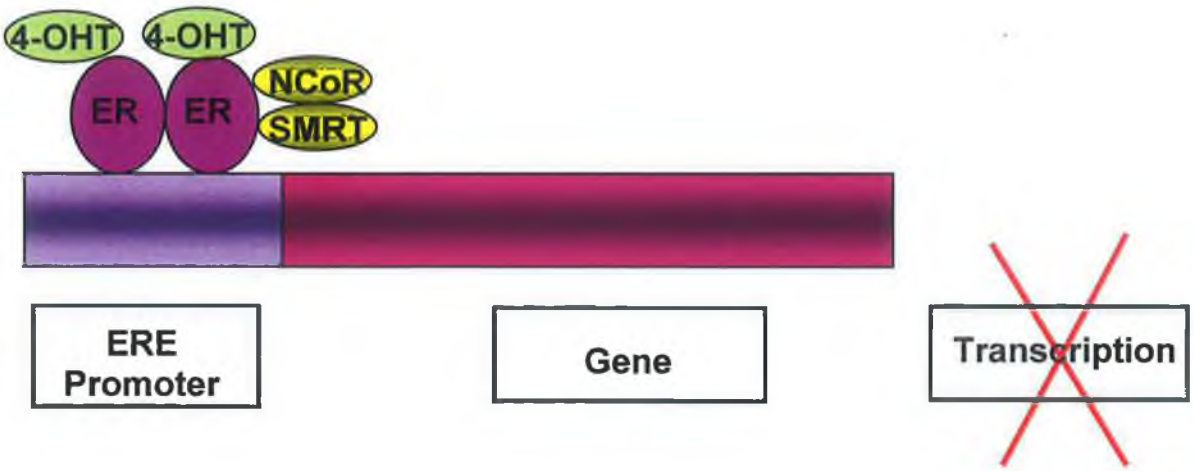


Figure 1.4: *Tamoxifen binding to ER results in recruitment of co-repressors to receptor complex which inhibits gene transcription. Adapted from Osborne, Shou et al 2005.*

Abbreviations: ER - oestrogen receptor; ERE - oestrogen response element; OHT - 4-hydroxytamoxifen; NCoR - nuclear receptor co-repressor; SMRT - silencing mediator for retinoid and thyroid-hormone receptor

ER dimers can also indirectly interact with DNA to promote transcriptional activity. This occurs through protein-protein interaction with other transcription factors such as Fos or Jun, resulting in the recruitment of acetyltransferases to complexes bound to activator protein-1 (AP-1) or specificity protein-1 (SP-1) sites on DNA, so-called ERE-independent genomic actions (figure 1.5) (Gottlicher, Heck et al. 1998; Kushner, Agard et al. 2000). There are also a number of oestrogen target genes which lack the ERE but instead contain ERE half-sites which are able to form interactions with the orphan nuclear hormone receptor steroidogenic factor-1 (SF-1). These are known as SF-1 response elements and serve as direct binding sites for ER (O'Lone, Frith et al. 2004).

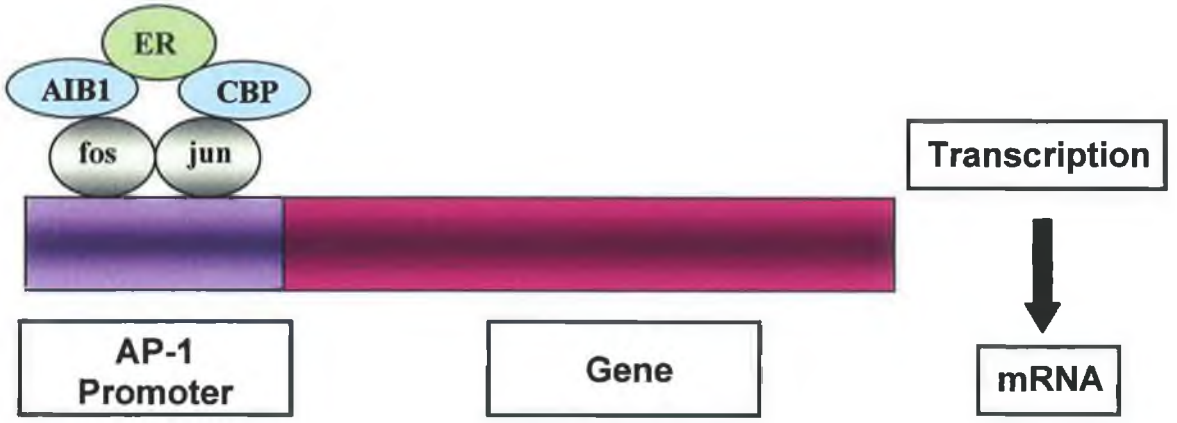


Figure 1.5: *ER can indirectly promote transcriptional activity through its interactions with transcription factors bound to AP-1 or SP-1 sites. Adapted from Osborne, Shou et al 2005.*
Abbreviations: ER - oestrogen receptor; AIB1- amplified in breast 1; CBP - cAMP response element binding protein; mRNA - messenger ribonucleic acid; AP-1 - activator protein-1; SP-1 - specificity protein-; mRNA - messenger ribonucleic acid

These three methods of ER induced transcriptional activity represent the genomic endocrine pathway, which can also be referred to as the nuclear-initiated steroid signalling pathway.

ER has also been shown to signal through another pathway known as the non-genomic or membrane-initiated steroid signalling pathway (figure 1.6).Signalling through this pathway allows ER functions to occur more rapidly within a cell when compared to protein synthesis from gene transcription (Beato 1989). *In vitro* studies have demonstrated the presence of ER located outside the nucleus, both within the cell cytoplasm or bound to the plasma membrane (Levin 1999; Razandi, Pedram et al. 2003; Shou, Massarweh et al. 2004). Non-genomic signalling can occur when the ligand, E_2 interacts with the plasma-membrane bound ER (Kousteni, Bellido et al. 2001). Other functional areas of the receptor can also participate in the signalling network through various protein-protein interactions.

ER that is bound to the plasma membrane has been shown to associate with a number of transcription factors and signalling molecules including the scaffold protein caveolin-1 (Chambliss, Yuhanna et al. 2000; Razandi, Oh et al. 2002), G

proteins (Razandi, Pedram et al. 1999; Wyckoff, Chambliss et al. 2001), the p85α regulatory subunit of phosphatidylinositol-3-OH kinase (PI3K) (Simoncini, Hafezi-Moghadam et al. 2000), Shc (Song, McPherson et al. 2002) and *ras* (Migliaccio, Piccolo et al. 1998). Membrane bound ER can also activate various protein-kinase cascades (Losel and Wehling 2003) including HER2/neu signalling (Chung, Sheu et al. 2002) as well as MAPK activation through direct interaction with the insulin growth factor-1 (IGF-1) receptor (Kahlert, Nuedling et al. 2000). E_2 activation of the membrane-bound ER can result in interaction with Src kinase through the scaffold protein MNAR (modulator of nongenomic action of oestroge receptor) thereby increasing Src kinase activity resulting in activation of the MAPK signalling pathway (Wong, McNally et al. 2002). ER activation of G proteins with resultant Src kinase and matrix metalloproteinase 2 activity cleaves heparin-binding epidermal growth factor (EGF) from the cell surface which can then interact with adjacent EGF receptors causing increased activity of the MAPK pathway (Razandi, Pedram et al. 2003). This non-genomic function can result in the mobilisation of intracellular calcium (Improta-Brears, Whorton et al. 1999) as well as the stimulation of cAMP production and adenylate cyclase activity (Aronica, Kraus et al. 1994; Improta-Brears, Whorton et al. 1999; Razandi, Pedram et al. 1999).

Clinically, this non-genomic signalling cascade may contribute to tumour resistance to endocrine therapies, in particular selective oestrogen receptor modulators (SERMs) in cells with abundant EGFR, HER2 and other cytoplasmic proteins that are able to sequester ER within the cytoplasm outside of the nucleus (Kumar, Wang et al. 2002; Wong, McNally et al. 2002; Shou, Massarweh et al. 2004).

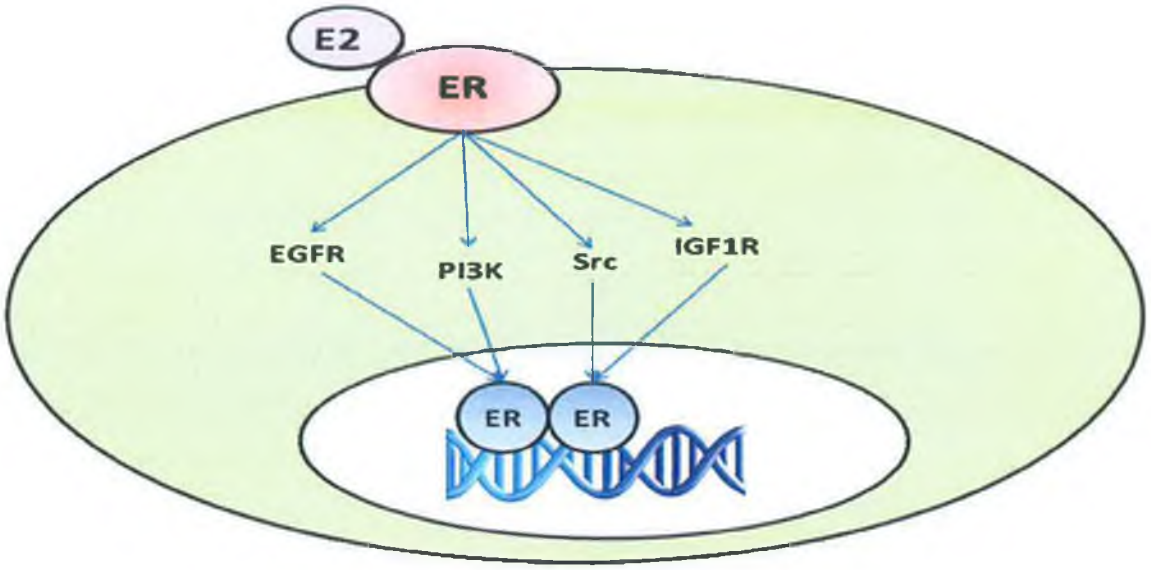


Figure 1.6: *Activation of membranous ER can increase signalling through different growth factor pathways in turn activating and augmenting nuclear ER functions. Adapted from Osborne, Shou et al 2005.*
Abbreviations: ER - oestrogen receptor; E_2 - 17aβ-estrdiol hydroxytamoxifen; EGFR - epidermal growth factor receptor; PI3K - phosphatidylinositol-3-OH kinase; IGF1R - insulin-like growth factor-1 receptor

1.3.4 pS2 – A classical ER target gene

pS2, also known as trefoil factor 1 (TFF1), is a small cysteine-rich protein of 84 amino acids (Jakowlew, Breathnach et al. 1984; Ather, Abbas et al. 2004) that is secreted by cells as a mature 60 amino acid protein which then undergoes dimerisation (Rio, Bellocq et al. 1988; Marchbank, Westley et al. 1998). It is expressed in a number of sites including the gastrointestinal tract (Rio, Bellocq et al. 1988; Piggott, Henry et al. 1991; Poulsom and Wright 1993), prostate (Ather, Abbas et al. 2004) and breast epithelium (Piggott, Henry et al. 1991). It is encoded by the TFF1 gene (Piggott, Henry et al. 1991) whose expression in breast cancer is stimulated in response to oestrogen (Westley, May et al. 1984) with pS2 mRNA concentrations increased up to 100-fold following oestrogen treatment (Masiakowski, Breathnach et al. 1982; May and Westley 1986; May and Westley 1988). Oestrogen stimulates gene expression through the binding of ER complex to a 13 base pair ERE which is located 400 bases upstream in the 5' region of the

transcription start site (Berry, Nunez et al. 1989; May and Westley 1997). It belongs to a family of small secretory proteins which are characterised by three intrachain disulphide bonds resulting in a trefoil motif (Reshkin, Tedone et al. 1999). pS2 is the most commonly identified target gene of ER which was first isolated from breast cancer cells in 1982 in an oestrogen dependent fashion (Masiakowski, Breathnach et al. 1982; Prud'homme, Fridlansky et al. 1985; May and Westley 1986; Nunez, Jakowlev et al. 1987). Its expression is found predominantly in ER positive breast cancer cell lines, with minimal expression found in ER negative cell lines (May and Westley 1986; May and Westley 1988; Henry, Nicholson et al. 1990; Carr, May et al. 1995). Rio et al also found that most pS2 positive breast tumours were also ER positive, suggesting a relationship between ER expression and pS2 production (Rio, Bellocq et al. 1987).

pS2 has been extensively studied in both clinical (Henry, Piggott et al. 1991; Foekens, van Putten et al. 1993; Gion, Mione et al. 1993) and cell culture studies (Nunez, Jakowlev et al. 1987; Nunez, Berry et al. 1989; Miyashita, Hirota et al. 1994; van Agthoven, van Agthoven et al. 1994). It has been shown to be expressed predominantly in ER positive tumours (Rio, Bellocq et al. 1987; Henry, Nicholson et al. 1990; Henry, Piggott et al. 1991; Soubeyran, Wafflart et al. 1995) with high protein levels predictive of a favourable prognosis (Foekens, van Putten et al. 1993; Soubeyran, Wafflart et al. 1995) as well as a favourable response to endocrine therapy in advanced breast cancer (Henry, Piggott et al. 1991). While pS2 has been well established as a target gene of ER and therefore a marker of ER activity, its biological function is as yet unknown, with the possibility that it might have a role to play as a chemoattractant for breast cancer cells facilitating cellular migration (Prest, May et al. 2002).

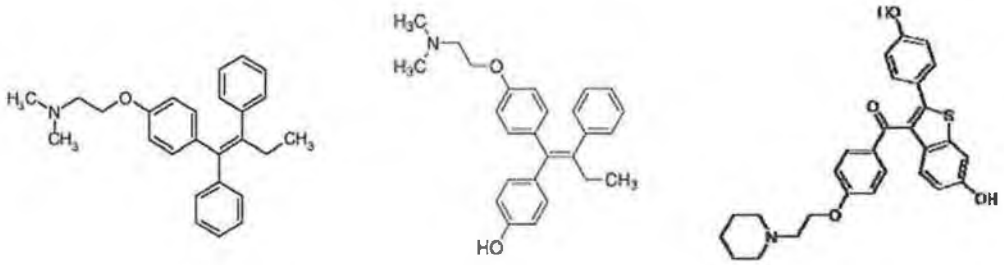
1.3.5 Anti-oestrogen therapy

At least 70% of breast cancers are classified as being ER positive (Harvey, Clark et al. 1999) which has resulted in the development of therapies designed to interfere in the action of oestrogen on the ER to reduce cell proliferation and tumour growth. The link between ovarian function through oestrogen production and secretion and the development of breast cancer has been known for over a century (Beatson 1896;

Boyd 1900). Today, pharmacological therapies have replaced surgical excision of the ovaries in the treatment of ER positive breast cancers. There are currently three different pharmacological methods of targeting the endocrine pathway: the selective oestrogen receptor modulators (SERMs), selective oestrogen receptor downregulators (SERDs) and aromatase inhibitors (AIs).

1.3.5.1 Selective oestrogen receptor modulators (SERMs)

SERMs are a class of compounds that structurally resemble oestrogens and act by competitively binding with ER. The most widely used and successful SERM to date is tamoxifen. Tamoxifen was initially synthesised in the early 1960s during an attempt to develop a new contraceptive agent (Harper and Walpole 1966; Harper and Walpole 1967). Its potential as an agent to treat and prevent breast cancer took many years to be recognised, however now it is the most widely used anti-oestrogen therapy. Tamoxifen is a prodrug that is converted to 4-hydroxytamoxifen, an anti-oestrogenic metabolite that has a high affinity for ER (figure 1.7) (Jordan, Collins et al. 1977; Allen, Clark et al. 1980).



Tamoxifen 4-Hydroxytamoxifen Raloxifene

Figure 1.7: *Molecular structures of tamoxifen, 4-hydroxytamoxifen and raloxifene*

The binding of the metabolically active form of tamoxifen to ER results in a conformational change to the receptor complex, resulting in its association with co-repressors and therefore no transcriptional activity (see figure 1.4) (Shang, Hu et al. 2000). SERMs, including tamoxifen, have both agonistic and antagonistic effects on ER depending on the tissue site of the receptor. It has an antagonistic effect in breast and mammary tissue (Jordan 1976), with agonistic effects in bone (Jordan, Phelps et

al. 1987). On uterine endometrial tissue it has both agonistic and antagonistic effects (Harper and Walpole 1967), with a recognised complication of tamoxifen therapy being a 2 to 4 fold increase in the development of endometrial cancer (Fornander, Rutqvist et al. 1989; Fisher, Costantino et al. 1994).

In the adjuvant setting, treatment with tamoxifen for five years has been shown to be effective in reducing the risk of both invasive breast cancer and ductal carcinoma *in situ* by up to 50% in both pre and post menopausal women (Fisher, Costantino et al. 1998; Cuzick, Forbes et al. 2002). It has also been shown to reduce the risk of contralateral breast cancer (Cuzick and Baum 1985) and can prevent the risk of breast cancer development in high risk women (Fisher, Costantino et al. 2005).

The effectiveness of tamoxifen stimulated an interest to develop other SERMs. One such drug is raloxifene (figure 1.7), which was developed for the treatment and prevention of osteoporosis (Ettinger, Black et al. 1999). When compared with tamoxifen in the STAR (Study of Tamoxifen And Raloxifene) trial, raloxifene had comparable overall patient survival rates however it was associated with lower rates of endometrial cancers and thromboembolic events (Vogel, Costantino et al. 2006).

1.3.5.2 Selective oestrogen receptor downregulators (SERDs)

SERDs such as fulvestrant, a 17α-alkylsulphinyl analogue of E_2, are another group of anti-oestrogens. They have a high affinity for ER but do not have the same agonistic effects as seen with tamoxifen (Addo, Yates et al. 2002), resulting in them being termed 'pure anti-oestrogens'. SERDs function by binding, blocking and degrading ER leading to an inhibition of signalling through the ER (Wakeling 1993; Wakeling 2000).

Fulvestrant has been shown to be at least as effective as the aromatase inhibitor anastrozole as a second line treatment (Robertson, Osborne et al. 2003), while high dose fulvestrant has been shown to be superior to anastrozole in improving time to disease progression and as effective as anastrozole in improving overall survival when used as a first line agent (Robertson, Llombart-Cussac et al. 2009). It has also been shown to be as effective as anastrozole in the treatment of ER positive patients

with metastatic disease refractory to tamoxifen treatment (Howell, Robertson et al. 2002; Osborne, Pippen et al. 2002).

1.3.5.3 Aromatase inhibitors (AIs)

Aromatase is an enzyme of the cytochrome P-450 superfamily and is a product of the CYP19 gene (Evans, Ledesma et al. 1986). It is responsible for the synthesis of oestrogens from androgenic substrates, in particular oestrone (E1) from androstenedione and estradiol (E2) from testosterone. Therefore, aromatase inhibitors act to suppress the action of aromatase thereby suppressing plasma oestrogen levels. While E2 is the main oestrogen source in premenopausal women, E1 is the main source of plasma oestrogen in postmenopausal women (Lonning, Haynes et al. 2011). Although E1 is biologically inactive, the enzymes that are required to convert it into active E2 (i.e. sulfatases and reductases) are expressed by many tissues (Suzuki, Ishida et al. 2009). Anastrozole and letrozole are third generation AIs which function through reversible binding to the heme group of the enzyme while exemestane, another third generation AI, irreversibly binds to the same site on the aromatase molecule as androstenedione (Smith and Dowsett 2003).

Five year treatment with anastrozole has been shown to improve disease free survival and time to recurrence as well as reducing the incidence of contralateral breast cancer development in post-menopausal women when compared to tamoxifen (Forbes, Cuzick et al. 2008). In a similar patient cohort, letrozole has also resulted in more tumour regressions and was associated with a longer time to disease progression than tamoxifen (9.4 vs 6.0 months; p=0.0001) (Mouridsen, Gershanovich et al. 2001). Treatment with tamoxifen for two years with subsequent switching to anastrozole for the remaining three years of treatment also improves overall survival and reduces the risk of disease recurrence when compared to tamoxifen monotherapy for five years (Kaufmann, Jonat et al. 2007).

Trials are currently ongoing to evaluate the efficacy of third generation AIs in the neoadjuvant setting as well as in the prevention of breast cancer development in high risk women.

1.4 Human epidermal growth factor signalling pathways

1.4.1 Epidermal growth factor receptors

Human epidermal growth factor receptors (EGFR), also known as epidermal growth factor receptors (ErbB) are transmembrane tyrosine kinase receptors that are involved in the regulation of cell growth and differentiation as well as adhesion, migration and other responses (Yarden 2001). To date, four separate receptors have been identified within the growth factor receptor family: EGFR (HER1/ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (Yarden and Sliwkowski 2001). They comprise an extracellular binding domain, a transmembrane lipophilic segment and, except for HER3, a functional intracellular tyrosine kinase domain with a regulatory carboxyl terminal segment (Mendelsohn and Baselga 2003). Ligand binding to the extracellular domain activates the cytoplasmic catalytic function through receptor homo or heterodimerisation and self-phosphorylation of the intracellular tyrosine residues (Yarden and Sliwkowski 2001). Receptor activation results in a signal-transduction cascade promoting responses such as cellular proliferation and survival (figure 1.8). While each receptor differs slightly from each other, they have structural homology of 40-45% (Plowman, Culouscou et al. 1993). HER2 is over-expressed in up to 30% of breast cancers (Slamon, Clark et al. 1987). It has been implicated in cancer progression (Yarden 2001) and also can be used to predict breast cancer outcome (Winston, Ramanaryanan et al. 2004).

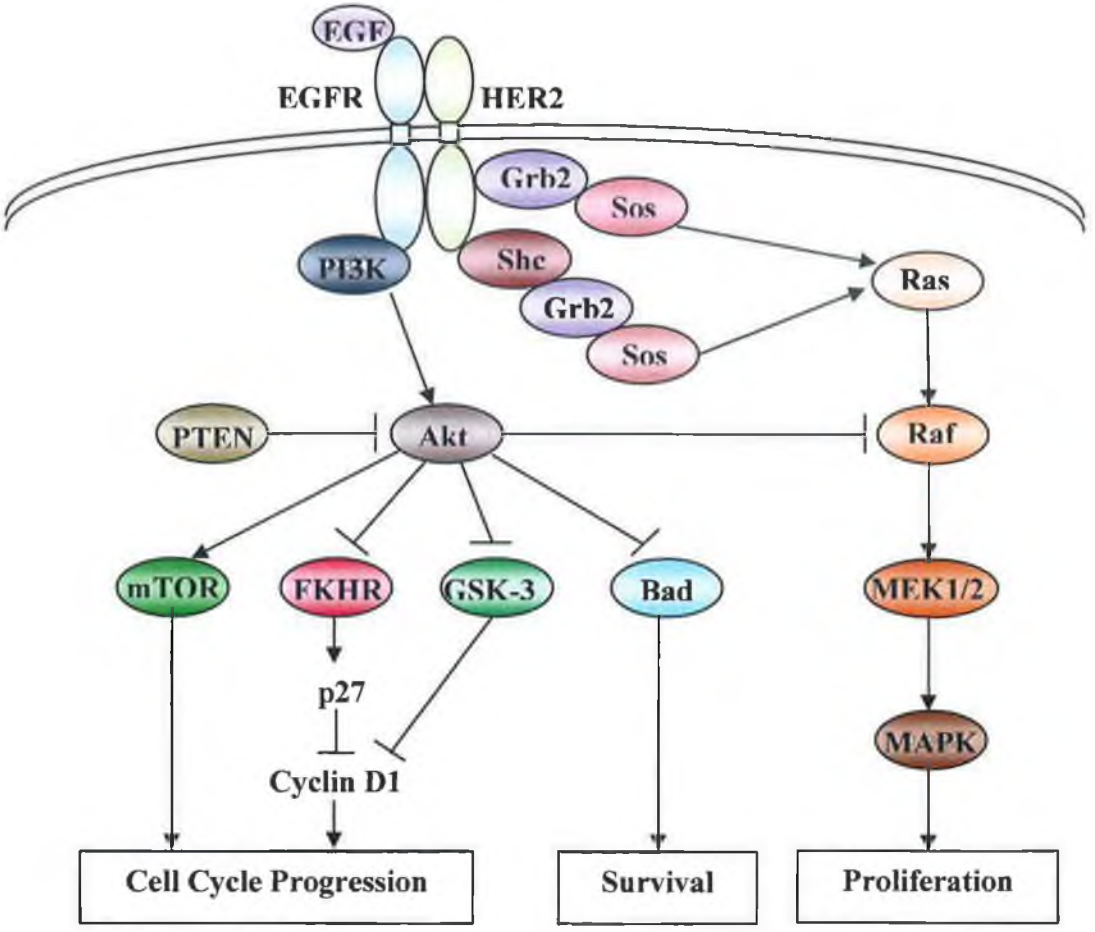


Figure 1.8: *Signalling through the epidermal growth factor receptors results in activation of a number of intracellular pathways that regulate key cellular responses. Adapted from Hudis 2007.*

Abbreviations: Akt - protein kinase B; EGF - epidermal growth factor; EGFR - epidermal growth factor receptor; FKHR - forkhead homolog 1 rhabdomyosarcoma; Grb2 - growth factor receptor-bound protein 2; GSK3 - glycogen synthase kinase 3; HER2 - human epidermal growth factor receptor 2; MAPK - mitogen-activated protein kinase; mTOR - mammalian target of rapamycin; PI3K - phosphatidylinositol-3-OH kinase; PTEN - phosphatase and tensin homolog; Raf - rapidly accelerated fibrosarcoma; Ras - RAt sarcoma; Sos - son of sevenless

1.4.2 HER2

HER2 is a 185kDa transmembrane glycoprotein receptor, encoded on chromosome 17q21, that was first described over two decades ago (Schechter, Stern et al. 1984; Coussens, Yang-Feng et al. 1985). Signalling via HER2 occurs through the Ras-MAPK pathway to promote cell proliferation and through the phosphatidylinositol 3'-kinase-AKT-mammalian target of rapamycin (mTOR) pathway to inhibit cell death (Cho, Mason et al. 2003). While the other HER receptors interact with ligands prior to dimerisation, no natural ligand has yet been identified for HER2 (Chang 2007), instead it can adopt a conformation which resembles a ligand-activated state which allows it to dimerise in the absence of a ligand (Cho, Mason et al. 2003). The other HER receptors preferentially form heterodimers with HER2, which produce more prolonged and stronger signals when compared to non-HER2 containing dimers (Chang 2007). Therefore, over-expression of HER2 in certain cell types can increase its sensitivity to growth factors resulting in uncontrolled cell proliferation leading to tumour growth (Rubin and Yarden 2001).

Over-expression of HER2 has been described in a number of different malignancies, however amplification of the HER2 gene is rare except for in breast cancer (Slamon, Godolphin et al. 1989; Onda, Matsuda et al. 1996; Latif, Watters et al. 2002). HER2 over-expression occurs in 20-30% of breast cancers (Slamon, Clark et al. 1987; Rubin and Yarden 2001; Hudis 2007). Over-expression in breast cancer is associated with decreased overall and disease free survival as well as varying responses to different chemotherapeutic and hormonal therapies (Albanell, Bellmunt et al. 1996; Ellis, Coop et al. 2001; Menard, Valagussa et al. 2001). In particular, HER2 amplification is associated with the development of resistance to tamoxifen (Osborne, Bardou et al. 2003; Shou, Massarweh et al. 2004).

1.4.3 Trastuzumab

In view of the important prognostic implications of HER2 over-expression in breast cancer, research has focused on developing new therapies which successfully inhibit the HER2 receptor and its signalling pathway. This has resulted in the development of biological agents targeted against the erbB family of tyrosine kinase receptors.

Trastuzumab (Herceptin®, Genentech/Roche) is a monoclonal antibody that was engineered by inserting the antigen-binding residues from a murine monoclonal antibody against the receptor into a cloned human immunoglobulin G framework (Carter, Presta et al. 1992). Its complementary-determining region amino acids bind to amino acids present on domain IV of the HER2 ectoderm (Cho, Mason et al. 2003). Its exact mechanism of action in vivo is unclear, but is thought to be divided into those mediated by Fab (fragment, antigen binding) or Fc (fragment, crystallisable) resulting in disruption of cell cycle progression and downstream proliferative signalling pathways (Albanell, Codony et al. 2003) as well as antibody-dependent cellular cytotoxicity (Cooley, Burns et al. 1999) and antiangiogenic effects (Izumi, Xu et al. 2002).

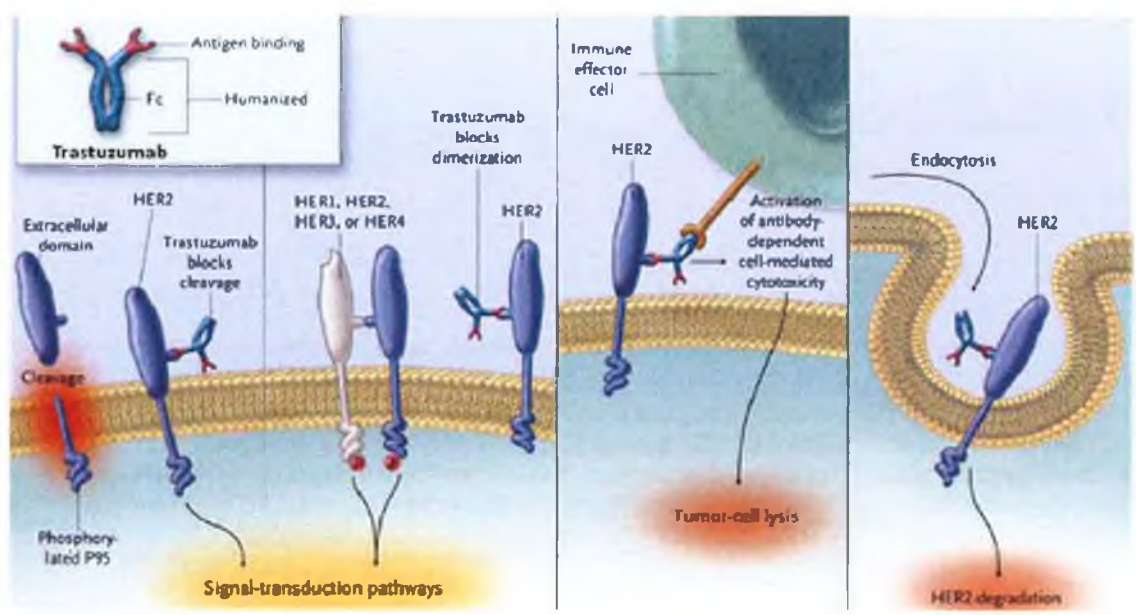


Figure 1.9: *Proposed mechanism of action of trastuzumab. Adapted from Hudis 2007.*

Following preclinical testing as well as a number of phase II clinical trials, in 1998 the US Food and Drug Administration (FDA) approved the use of trastuzumab for the treatment of HER2 positive patients with metastatic breast cancer. In 2001, a landmark phase III clinical trial revealed the benefits of combining trastuzumab with chemotherapy in patients with metastatic disease, improving both time to disease progression as well as overall survival rates (Slamon, Leyland-Jones et al. 2001).

Subsequent trials have revealed its benefits in both the adjuvant (Piccart-Gebhart, Procter et al. 2005; Romond, Perez et al. 2005) and neoadjuvant setting (Gianni, Eiermann et al. 2010).

While myelosuppression, nausea and alopecia is rare with trastuzumab monotherapy, combination therapy with an anthracycline chemotherapeutic agent when compared to anthracycline chemotherapy alone was found to increase the rate of cardiac dysfunction from 8% to 27% (Slamon, Leyland-Jones et al. 2001). While the underlying mechanism of cardiotoxicity is poorly understood, HER2 has been linked with embryonic cardiac development as well as the prevention of dilated cardiomyopathy (Negro, Brar et al. 2004). Accordingly, concurrent use of trastuzumab with anthracycline-based chemotherapy regimes is not advised with interval monitoring of cardiac function for patients receiving trastuzumab treatment.

1.5 Resistance to treatments

1.5.1 Tamoxifen resistance

Without question tamoxifen has revolutionised the treatment of ER positive breast cancers. Adjuvant tamoxifen therapy prolongs disease-free and overall survival in primary breast cancers which express ER, as well as reducing the annual risk of recurrence by up to 50% (Group 1992). It has also been shown to induce a clinical response in more than half of patients with ER positive cancers who present with metastatic disease (Osborne 1998). However, the Early Breast Cancer Trialists' Collaborative Group meta-analysis looking at tamoxifen treatment for five years in ER positive cancers found a recurrence rate of 15.1% at five years which increased to 24.7% at ten years post diagnosis (Group 1992). Furthermore, metastatic disease which initially responds to tamoxifen therapy will always develop resistance to the drug (Kurebayashi 2005). This would suggest that up to half of ER expressing cancers have a de novo resistance to tamoxifen with a significant proportion of responsive tumours acquiring resistance during treatment. De novo resistance occurs mainly through two mechanisms: lack of ER expression (i.e. ER negative tumours) or variant expression of the cytochrome P450 isoenzyme CYP2D6 resulting in the

inability to metabolise tamoxifen to its metabolically active form, 4 hydroxytamoxifen (4-OHT). There are many genetic variants of CYP2D6 which are broadly categorised into four distinct phenotypes: ultra rapid (high activity), extensive (normal activity), intermediate (reduced activity) and poor (no activity). Patients with two functional CYP2D6 allelles (i.e. extensive phenotype) have been shown to have better outcomes, while patients with non-functional (poor phenotype) or reduced activity (intermediate phenotype) have worse outcomes (Schroth, Goetz et al. 2009).

In acquired resistance, tumours initially respond to tamoxifen however, after a period of time, they re-occur and grow despite continued tamoxifen treatment, thereby demonstrating resistance to the medication. Extensive research has been undertaken to determine the mechanisms by which cancers develop resistance to tamoxifen, with a number of possibilities proposed. In general, acquired resistance is not a result of loss of or alteration to ER, with less than 20% of recurrences found to lose expression of ER (Kuukasjarvi, Kononen et al. 1996; Broom, Tang et al. 2009). Cancers which are resistant to tamoxifen frequently respond to other forms of anti-oestrogen therapies such as fulvestrant or aromatase inhibitors confirming that tumour development and progression is still influenced by signalling through the endocrine pathway (Howell, DeFriend et al. 1996).

One method by which tumours are thought to develop resistance to tamoxifen is through crosstalk between ER and the growth factor receptor pathways including the EGFR/HER2 family of receptors. This can result in the development of resistance through a number of differed mechanisms. Cell-membrane associated ER is a G protein coupled receptor, whose subunits have been shown to activate EGFR. The exact mechanism by which ER within the cell membrane activates this growth factor receptor pathway is unclear, however it is thought to involve Src kinase activation of the metalloproteinases, in particular MMP-2 and MMP-9, which subsequently induce the secretion of heparin bound epidermal growth factor thereby transactivating EGFR (Razandi, Pedram et al. 2003). This results in activation of the MAPK pathway followed by phosphorylation of extra-cellular signal-related kinase 1 (ERK1) and ERK2 (Razandi, Pedram et al. 2003; Shou, Massarweh et al. 2004).

Signalling through the growth factor receptor pathway consists of a protein kinase cascade as demonstrated in figure 1.8. Activation of the phosphatidylinositol 3-kinases (PI3K) pathway results in upregulation of Akt, which subsequently phosphorylates ER at serine 167 thereby enhancing its transcriptional activity (Campbell, Bhat-Nakshatri et al. 2001). Upregulation of ras results in the activation of various signalling intermediates which in turn phosphorylate and activate MAPK and subsequently ERK1 and ERK2. ERKs can then activate ER through a number of different mechanisms including phosphorylation of serine 118 (Kato, Endoh et al. 1995), phosphorylation of p90 ribosomal S6 kinase (P90RSK) (Joel, Smith et al. 1998) as well as phosphorylation of ER co-activators such as AIB1 (Font de Mora and Brown 2000). P38 MAPK can phosphorylate both ER and its co-activators thereby augmenting ER-mediated gene transcriptional activity (Lee and Bai 2002). Indeed, preclinical studies have confirmed that tamoxifen resistance is associated with increased levels of different kinases (Schiff, Reddy et al. 2000).

The nuclear receptor co-activators play an integral role in the mediation of ER transcription, therefore alterations in the expression of these co-regulator proteins may result in tamoxifen resistance. Over-expression of nuclear co-activator 3 (also known as AIB1) has been associated with reduced disease free survival in patients who are receiving adjuvant tamoxifen therapy (Osborne, Bardou et al. 2003). The paired domain transcription factor PAX2 has been shown to be a direct competitor with AIB1 for binding and regulation of ERB2 transcription (Hurtado, Holmes et al. 2008). *In vitro* studies revealed recruitment of PAX2 to an ER binding site within the ERB2 gene following treatment with both oestrogen and tamoxifen with evidence suggesting a role for PAX2 as a transcriptional repressor of ER. Subsequent translational studies comparing the relevance of PAX2 within the clinical setting found that out of a cohort of 109 patients with ER positive breast cancers, those patients who expressed PAX2 had a significantly improved recurrence-free survival. *In vitro* studies have also shown that overexpression of SRC1, a p160 nuclear receptor co-activator can result in the enhanced agonist activity of 4-hydroxy-tamoxifen (Smith, Nawaz et al. 1997). SRC-1 has also been shown to interact with the developmental transcription factor HOXC11 leading to the development of tamoxifen resistance through their regulation of the calcium-binding protein S100beta (McIlroy, McCartan et al. 2010).

1.5.2 Trastuzumab resistance

Development of resistance to trastuzumab is increasingly becoming an important clinical issue. While quantification of HER2 expression can identify patients who are likely to respond to trastuzumab treatment, a proportion of patients who receive adjuvant trastuzumab-containing therapy will relapse. Moreover, less than one third of patients with metastatic breast cancer who over-express HER2 will have an objective response to trastuzumab monotherapy and the majority of patients who do have an initial response develop resistance within one year. The exact mechanisms by which resistance develops remain inconclusive, with hypothesised mechanisms described in preclinical models but not yet validated in clinical samples. The proposed main mechanisms include alteration in the interaction between the receptor and the antibody, alternative signalling through other HER pathways or alternate pathways or failure to activate immune-mediated mechanisms to destroy tumour cells (figure 1.10) (Fiszman and Jasnis 2011). Determining which of these mechanisms is clinically relevant is an important goal of research, with it likely that, as with tamoxifen, clinical resistance occurring as the result of a multifactorial process.

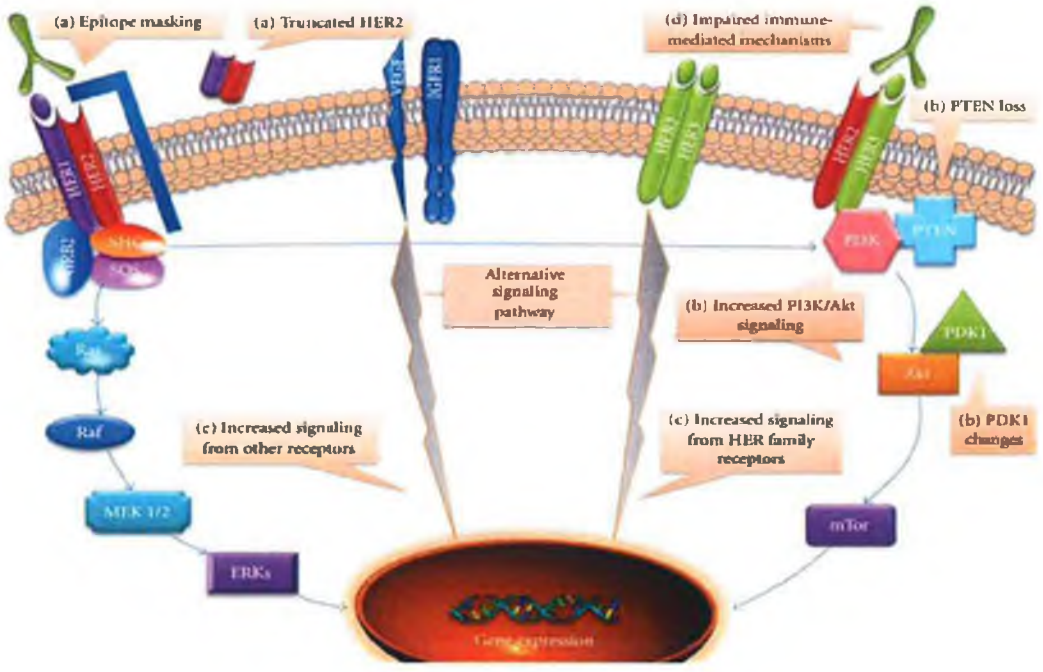


Figure 1.10: *Proposed mechanisms for trastuzumab resistance. Adapted from Fiszman and Jasnis 2011.*

The membrane-associated glycoprotein mucin-4 (MUC4) has been shown to interact directly with HER2, which is postulated to mask the receptor thereby preventing trastuzumab from effectively interacting with it (Price-Schiavi, Jepson et al. 2002). Preclinical studies have shown that the MUC4 protein level correlates with resistance to trastuzumab as well as being inversely proportional to the binding capacity of HER2 for the antibody (Nagy, Friedlander et al. 2005). Alteration in the interaction between the receptor and the antibody can also occur following cleavage of the extracellular domain of HER2 by proteases resulting in a truncated form of the receptor, known as p95HER2, to which trastuzumab is unable to bind (Molina, Saez et al. 2002). This truncated form of HER2 is constitutively active and therefore can mediate trastuzumab resistance (Scaltriti, Rojo et al. 2007).

Crosstalk is known to occur among the different receptors of the HER family, therefore, while trastuzumab can reduce signalling through HER2 it might not affect signalling through the other receptors. Thus, even in the presence of trastuzumab, HER1/HER3 heterodimers and HER1/HER1 homodimers might initiate signalling through the MAPK and PI3K pathways (Motoyama, Hynes et al. 2002). In vitro models examining the effect of increased levels of the HER family ligands heregulin and epidermal growth factor (EGF) showed inhibition of the trastuzumab-mediated growth reduction in HER2 overexpressing breast cancer cell lines (Robinson, Turbin et al. 2006). Likewise, transforming growth factor-β (TGF-β), through its action on HER3 ligand shedding, has been shown to have a role in the development of resistance to anti-HER2 therapies (Wang, Xiang et al. 2008). Increased levels of insulin-like growth factor-1 receptor (IGF-1R) in HER2 overexpressing breast cancer cell lines appear to affect the efficacy of trastuzumab resulting in the development of resistance, with inhibition of IGF-1R signalling restoring the sensitivity of cells to the agent (Casa, Dearth et al. 2008). It is thought that IGF-1R induced trastuzumab resistance involves the PI3K signalling pathway resulting in enhanced degradation of p27 (Lu, Zi et al. 2004). Co-expression of IGF-1R in operable HER2 overexpressing breast tumours treated with neoadjuvant trastuzumab in conjunction with vinorelbine was associated with significantly lower response rates (50% versus 97%, p=0.001) when compared to tumours which did not co-express IGF-1R (Harris, You et al. 2007). Antibody-dependent cellular cytotoxicity (ADCC) is thought to be significantly involved in the functional activity of

trastuzumab, with interactions between the antibody and the Fc receptor critical in the activation of natural killer lymphocytes and the ADCC response. Preclinical studies suggest that germline polymorphisms as well as post-translational modifications such as glycosylation of the Fc receptor result in a reduction of the ADCC response to trastuzumab, thereby impairing the immune-mediated ability to destroy tumour cells.

1.6 Hypothesis

Recent research has highlighted the complex nature of the signalling pathways within breast cancer cells. Crosstalk between the main two pathways, the steroid hormone and growth factor pathways, has already been implicated in the development of resistance to anti-oestrogen therapies such as tamoxifen. We hypothesise that resistance to trastuzumab, as well as other tyrosine kinase inhibitors, can, in part, be mediated by crosstalk between the growth factor signalling pathway and the steroid hormone pathway that results in an uncontrolled upregulation of the steroid hormone pathway following trastuzumab treatment.

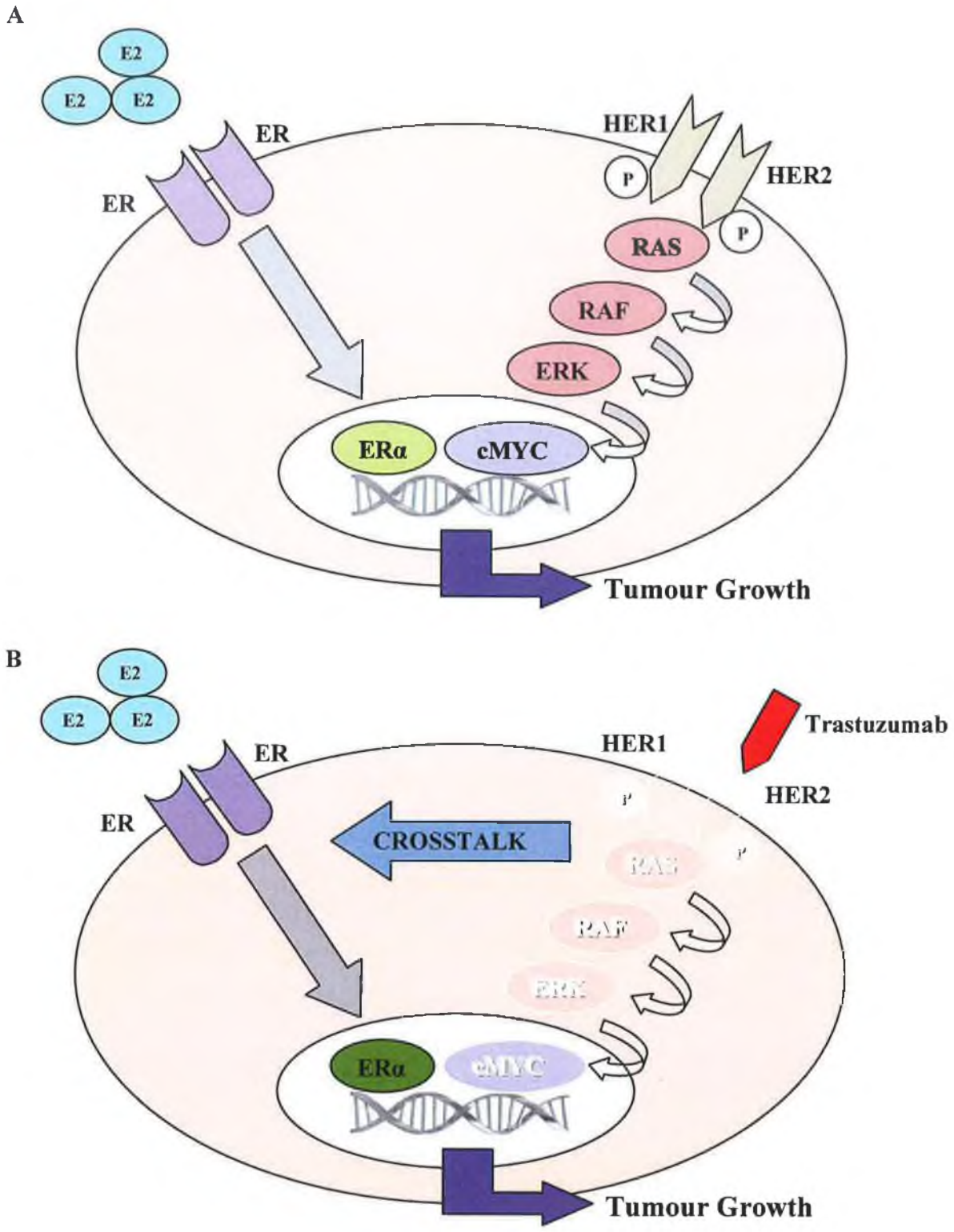


Figure 1.11: *Schematic representation of overall hypothesis.*
(a): Steroid hormone and growth factor pathways play an integral role in tumour growth (b): We hypothesise that growth factor pathway inhibition by trastuzumab results in upregulation of the steroid hormone pathway

1.7 Aims

The overall aim of this work is to further elucidate the interactions between the steroid hormone and growth factor signalling pathways in breast cancer, in particular, to determine the effect of trastuzumab treatment on the steroid hormone pathway. The first objective will be to assess the effect of trastuzumab treatment on this pathway at a molecular level in breast cancer cells, looking at its effect at both a protein level and a transcriptional level. Secondly, we will verify our *in vitro* findings in breast cancer tissue samples utilising a large tissue microarray, assessing the clinical significance of our proposed crosstalk hypothesis.

Chapter 2

Materials and Methods

2.1 Breast cancer cell culture techniques

2.1.1 MCF-7 breast cancer cell line

MCF-7 cells, a well established breast cancer cell line which overexpress ER and PR but not HER2 were obtained from the American Type Culture Collection (ATCC, Virginia, USA). They were cultured in minimum essential medium (MEM) (Sigma Aldrich, Stenheim, Germany) supplemented with 10% foetal calf serum (FCS) (Sigma Aldrich), 10,000 units of penicillin (Sigma Aldrich), 10mg of streptomycin (Sigma Aldrich) and 1ml of 200mM L-glutamine (Sigma Aldrich) per 100mls of medium.

2.1.2 LCC-1 breast cancer cell line

LCC-1 cells are an isogenic derivative of MCF-7 cells which overexpress both ER and HER2. Although they express ER, they are independent of oestrogen however they retain sensitivity to the effects of anti-oestrogen therapies such as tamoxifen (Brunner, Boulay et al. 1993). They were a kind gift from Dr. Robert Clarke, Department of Oncology, Georgetown University, USA. Phenol red is a weak oestrogen mimic, therefore to maintain their oestrogen independence, they were cultured in phenol red free MEM supplemented with 10% charcoal dextran stripped foetal calf serum (Sigma Aldrich), 10,000 units of penicillin (Sigma Aldrich), 10mg of streptomycin (Sigma Aldrich) and 1ml of 200mM L-glutamine (Sigma Aldrich) per 100mls of medium. Charcoal dextran stripping reduces the serum concentration of a number of different hormones as well as certain growth factors including cortisol and prostaglandins.

2.1.3 SKBR-3 breast cancer cell line

SKBR-3 cells carry multiple copies of the c-erbB2 gene resulting in overexpression of HER2; however they do not express ER. Cells were obtained from the ATCC and cultured in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% foetal calf

serum (FCS), 10,000 units of penicillin and 10mg of streptomycin per 100mls of medium.

2.1.4 Culturing of cells from cryo-storage

Cryovials containing cells were removed from storage in liquid nitrogen and thawed quickly. The contents of the cryovial were transferred to a sterile 15mls conical tube (Greiner Bio-One, Frickenhausen, Germany) containing 5mls of the appropriate culture medium. The cell suspension was centrifuged at 1,250 rpm for 4 minutes, the supernatant was discarded and the cell pellet resuspended in 2mls of medium. Cells were then transferred to a $75cm^2$ tissue culture flask (Sarstedt, Nümbrecht, Germany) to which a further 8mls of medium was added. The flasks were then incubated at 37°C.

2.1.5 Cell culture

All cell culture techniques were performed under sterile conditions using a laminar airflow cabinet. Cells were maintained in $75cm^2$ tissue culture flasks in a Heraeus Hera Cell incubator (Kendro Laboratory Products, Hanau, Germany) at 37°C with a humid 5% (v/v) CO_2 atmosphere. Cells were passaged when confluent. Following washing in PBS (PBS, Oxoid Ltd, Hampshire, England) cells were incubated in 2mls of 1x trypsin/EDTA solution (Sigma Aldrich) containing 0.05% trypsin and 0.02% EDTA for 5 minutes at 37°C. Trypsin was inactivated by the addition of full growth medium and transferred to a 15mls conical tube which was then centrifuged at 1,250rpm for 4 minutes. The supernatant was discarded and the cell pellet was resuspended in the required amount of medium which was then divided into fresh tissue culture flasks. Cells were routinely tested for mycoplasma every 4 to 6 months (MycoAlert mycoplasma detection kit, Lonza, Basel, Switzerland) to ensure that contamination of the cell lines did not occur.

2.1.6 Cell counting

Prior to seeding cells in cell culture well-plates, cells must be counted to ensure that they attain adequate confluency within their wells, without becoming over or under

confluent. This is achieved using a haemocytometer to count cells in suspension. After trypsinisation and formation of a cell pellet as described in 2.1.5, the cell pellet was resuspended in 5mls of medium and mixed by vortex to ensure an even distribution of cells within the suspension. A 50µl sample of this solution was mixed with 50µl of trypan blue (Sigma Aldrich). 10µl of this mixture was placed onto the haemocytometer with the number of healthy cells present within the central $1mm^2$ grid counted. Apoptotic cells were recognisable by weakened cell membranes that allowed the trypan blue to penetrate into the cell and were not counted. This process was repeated three times with the average value multiplied by both the dilution factor and 10,000 to calculate the number of cells per millilitre. This was then used to determine the required volume of cell suspension which was then seeded into the relevant cell culture well-plate.

2.1.7 Cell treatments

Prior to treatment, culture medium was removed from the flasks and cells were washed twice in sterile PBS. MCF-7 and SKBR-3 cells were then cultured for 48 hours in phenol red free MEM containing 10% charcoal dextran stripped foetal calf serum, followed by an additional 24 hours in serum free MEM. LCC-1 cells were washed twice in sterile PBS and cultured for 24 hours in serum free MEM. All cells were then treated with 17β-estradiol (E_2) (Sigma Aldrich) at 10^{-8}M concentration, 4-hydroxytamoxifen (4-OHT) (Sigma Aldrich) at 10^{-8}M concentration and trastuzumab (kind gift from pharmacy department, Beaumont Hospital) at 100µM/ml concentration. Sterile water was used as a vehicle control.

2.2 Western blot analysis

2.2.1 Protein lysate extraction

Western blotting is a technique employed to detect proteins in cell lysates. Protein lysate extractions were performed on ice. 10µl of protease inhibitor (P8340, Sigma Aldrich) was added to 1ml of radioimmunoprecipitation assay (RIPA) lysis buffer. Cell pellets were harvested by centrifugation at 1850 rpm for 7 minutes, the medium was removed and the cell pellets were resuspended in 80µl of the protease inhibitor/lysis buffer mixture. The samples were placed on ice for 10 minutes followed by a 30 second vortex, which was repeated a further two times. The samples were then centrifuged at 4°C at 13,000rpm for 20 minutes after which the supernatant was transferred to a chilled, labelled eppendorf and stored at -20°C for further use.

2.2.2 Protein quantification

Protein quantification was performed using a bicinchonic acid (BCA) assay (P23225, Pierce, Il, USA), as per manufacturer's instructions. A standard curve was created by serially diluting bovine serum albumin in dH_20 (Appendix I).

A 1:20 dilution of lysate was made in dH_20, ensuring the solution was well mixed. 25µl of each standard was pipetted into a 96 well plate in duplicate. 25µl of each sample was also pipetted into the 96 well plate in duplicate. The BCA working reagent was made up by mixing 1 part of BCA Reagent B (4% w/v $CuSO_4$) with 49 parts of BCA Reagent A (containing Na_2CO_3, $NaHCO_3$, BCA detection reagent and sodium tartrate in 0.5M NaOH). 200µl of the working reagent was then added to each well containing either a standard or sample. The plate was incubated at 37°C for 30 minutes after which the absorbance was read at 560nm using a plate reader (KC4, BioTek, VT, USA). The average absorbance value for each sample was calculated. Linear regression analysis was performed to determine the equation of the standard curve which was then used to calculate the protein concentrations of each sample.

2.2.3 Sodium dodecyl sulphate polyacrylamide gel electorphoresis (SDS-PAGE)

Electrophoresis is one of the methods used to separate proteins according to molecular weight or charge. Sodium dodecyl sulphate (SDS) is a detergent which gives the protein samples a negative charge by wrapping around the polypeptide backbone, thereby conferring a negative charge in proportion to the length of the polypeptide. This enables the relevant antibody to subsequently gain access to the epiptope of interest and also ensures that in SDS-PAGE, the protein separation is determined by molecular weight.

Polyacrylamide gels are formed by the polymerisation of acrylamide (Sigma Aldrich) and N,N-methylene-bis-acrylamide. The addition of ammonium persulphate (APS) (Sigma Aldrich) as well as N,N,N,N-tetramethylethylenediamine (TEMED) (Sigma Aldrich) initiates the polymerisation. APS determines the size of the pores within the gel, the higher the concentration of APS the smaller the pore size. Pore size is varied depending on the size of the protein of interest to ensure adequate separation of proteins occurs.

Gels were cast using the ATTO gel system (ATTO Corporation, Tokyo, Japan) and allowed to polymerise for 35 minutes at room temperature, with a small layer of isopropanol (Sigma Aldrich) on top to ensure the gel set evenly. Once set, the isopropanol was removed and a 5% stacking gel was poured on top of the gel. A 10-well, 1.5mm comb was inserted into the stacking gel which was then left for 35 minutes at room temperature to polymerise. The function of the stacking gel is to ensure that all the samples begin travelling down the polyacrylamide gel at the same time. The composition of the various gels is outlined in table 2.2. A 10% resolving gel was used when blotting for ER. Gels were then placed in the ATTO tank which was filled with 1x running buffer (Appendix I).

Table 2.1 Composition of various percentage gels for SDS-PAGE

	6% Resolving	8% Resolving	10% Resolving	12% Resolving	5% Stacking
H20	5.3	4.6	4.0	3.3	1.4
30% acrylamide mix	2.0	2.7	3.3	4.0	0.33
1.5M Tris (pH 8.80)	2.5	2.5	2.5	2.5	0.25
10% SDS	0.1	0.1	0.1	0.1	0.02
10% APS	0.1	0.1	0.1	0.1	0.02
TEMED	0.008	0.006	0.004	0.002	0.002

Each volume is in millilitres and used in the preparation of 10mls of resolving gel and 2mls of stacking gel.

2.2.4 Loading, running and transferring samples

For all western blot experiments, 80µg of protein was added to 6x laemelli SDS buffer (Sigma Aldrich) as well as varying amounts of protease inhibitor/lysis buffer mixture (as described in 2.2.1) so that each sample volume was equal to 25µl. This was then vortexed briefly and boiled at 95°C for 5 minutes to denature the protein samples. 20µl of each sample was then loaded carefully in to the relevant well. A molecular weight marker, Precision plus protein standard (Bio-Rad, CA, USA), was run alongside the samples to allow for determination of molecular weight. Gels were run at a constant voltage of 110V for 3.5 hours.

After SDS-PAGE electrophoresis, gels were transferred on to a nitrocellulose membrane using an ATTO semi-dry transfer system (ATTO Corporation). A constant current of 250mA was used with a transfer time of 1 hour. Protein transfer was confirmed by rinsing the nitrocellulose membrane with Ponceau S solution (Sigma Aldrich).

2.2.5 Western blotting

Non-specific protein binding sites were blocked by placing the nitrocellulose membrane in 5% non-fat dry milk (Chivers, Dublin, Ireland) in tris-buffered saline (TBS) containing 0.1% Tween (TBST) (Sigma Aldrich) overnight at 4°C. The primary antibody was diluted in 2% non-fat dry milk in TBST to a volume suitable for the membrane size to the required concentration as documented in table 2.3. Membranes were incubated in primary antibody with gentle rocking for one hour at room temperature when blotting for ER and overnight at 4°C when blotting for β-actin. The membrane was then washed three times for 10 minutes each in TBST. It was then incubated for 1 hour at room temperature with the relevant Horseradish Peroxidase (HRP) conjugated secondary antibody (Sigma Aldrich) at concentrations outlined in table 2.3. The membrane was again washed three times for 10 minutes each in TBST before being developed with the Enhanced Chemiluminescence (ECL) (Pierce) substrate solution. Light emitted during the enzyme-catalysed decomposition reaction was captured by exposure to Fuji X-ray film (FujiFilm, Tokyo, Japan) for 30 seconds to 40 minutes. The film was developed by immersion in developing solution followed by fixer solution (Kodak, USA) and left to air dry. The size of the visualised bands was determined using the molecular weight markers.

Table 2.2 Western blot antibody concentrations

Protein	***Primary antibody***	***Secondary antibody***	***Chemiluminescent reagent***
ER 66kDa	Santa Cruz Anti-ER (SC-543) 1:200 in 2% milk	Anti-rabbit 1:2000 in 5% milk	ECL x 1 minute
β-actin 42kDa	Anti-β-actin 1:7500 in 5% milk	Anti-mouse 1:7500 in 5% milk	ECL x 1 minute

2.3 Promoter activity assay

2.3.1 Luciferase assay

Eukaryotic gene expression and cellular physiology can be studied using genetic reporter systems. In the luciferase reaction, when the luciferase acts upon the appropriate substrate, light is emitted which can be detected and measured by a luminometer thereby allowing for the evaluation of biological processes. Luciferase assay is commonly used to assay the transcriptional activity of DNA sequences which have been transfected with a genetic construct containing the luciferase gene that is regulated by the promoter of interest. The simultaneous expression and measurement of two individual reporter enzymes within a single system is known as a dual reporter system. A dual reporter system allows for the experimental reporter to assay the effect of an experimental condition while the co-transfected control reporter provides an internal control that can measure the baseline cellular response. The activity of the experimental reporter than then be normalised against the internal control thereby minimising the experimental variability that can occur within cells.

The Dual Luciferase Reporter (DLR) Assay System (Promega, WN, USA) utilises the activity of the firefly (*Photinum pyralis)* and *Renilla* (*Renilla reniformis*) luciferases to determine cellular transcriptional activity. Luciferase Assay Reagent II (LAR II) (Promega) is added initially to measure the firefly luciferase reporter. After this is recorded, the reaction is quenched by adding Stop & Glo Reagent (Promega) to the sample which simultaneously initiates the *Renilla* luciferase reaction. Renilla activity is measured and used as the internal control to normalise the experimental reporter measurement.

2.3.2 ER expression vector and pS2 promoter

The ER-HEGO/pSG5 expression vector was a kind gift from Professor Pierre Chambon, University of Strasbourg, France. The control vector was created by restricton digest of the ER sequence from the HEGO vector and religating the ends.

The pGL3-pS2 promoter was a kind gift from Dr Kumar and colleagues at MD Anderson Cancer Centre, Texas, USA.

2.3.3 Cellular transfection

Cells were cultured in 24-well plates until they reached 90% confluency. Transfections were performed using Lipofectamine™ 2000 (Invitrogen) according to manufacturer's instructions. For each transfection sample, 12.5ng of *Renilla* (Invitrogen, CA, USA) and 500ng of each relevant plasmid and vector (table 2.4) was diluted in Opti-MEM reduced serum medium (Invitrogen) to a total volume of 50µl. 2µl of Lipofectamine™ 2000 was diluted in 48µl of Opti-MEM and incubated for 5 minutes at room temperature. The diluted Lipofectamine™ 2000 was combined with the diluted plasmid and vector sample and incubated at room temperature for 20 minutes. Each complex was added to wells containing cells along with 500µl of Opti-MEM and incubated for 6 hours at 37°C and 5% CO_2. The Opti-MEM was then removed and cells were incubated in fresh media for 16 hours. Cells were then treated with E_2 and trastuzumab as per section 2.1.7 for 6 hours.

Table 2.3 DNA transfection protocol

	DNA(ng)	DNA (ng)
Renilla	**12.5**	**12.5**
pS2/PGL3	**500**	**500**
ER/pSG5	**500**	**0**
Empty pSG5	**0**	**500**

2.3.4 Luciferase assay protocol

Following transfection, the media was removed from the wells and the cells were washed with PBS. Luciferase assay was performed according to manufacturer's instructions (Promega). Cells were lysed by adding 100µl of 1X passive lysis buffer (Promega) to the wells and placing the plates on a rocker for 15 minutes at room temperature. The cell lysates were transferred to eppendorfs and 10µl of each was

mixed with 50µl of LAR II. Firefly luciferase activity was recorded using a luminometer programmed to perform a two second pre-measurement delay followed by a ten second measurement period for each assay. Subsequently 50µl of Stop & Glo reagent was added to quench the firefly activity and initiate the *Renilla* luciferase activity which was similarly measured using the luminometer. Readings were entered onto a Microsoft Excel spreadsheet (Microsoft, WA, USA) with the firefly luciferase activity normalised to control *Renilla* luciferase activity. Results were graphed on bar charts. All luciferase experiments were performed in triplicate with the average reading used to obtain the final result. Each experiment was repeated a further two times to determine the standard error.

2.4 Nucleic acid biochemistry

2.4.1 RNA extraction

Cells were cultured in 6-well plates until they reached 90% confluency. They were then treated as described in section 2.1.7 for 6 hours after which time RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. 10µl β-mercaptoethanol was added to 1ml of Buffer RLT before use. To each cell monolayer 350µl of Buffer RLT was added, the cells were then scraped off the plate using a pipette tip and the lysate was transferred to an eppendorf. Each lysate was homogenised by passing it through a 20-guage syringe fitted to a 2.5ml RNase-free syringe. 350µl of 70% ethanol was then added to each lysate and pipette mixed, the sample was transferred to an RNeasy spin column and centrifuged for 15 seconds at 10,000rpm. The flow through was discarded and the membrane was then washed with 700µl of Buffer RW1 followed by two washes with 500µl of Buffer RPE, each time centrifuged at 10,000rpm for 15 seconds. A further 2 minute centrifuge at 15,000rpm was performed to dry the spin column membrane. Each spin column was then placed in a fresh 1.5ml eppendorf and 30µl of RNase free water (Qiagen) was applied to each membrane to elute the RNA. Each column was centrifuged at 10,000rpm for 1 minute after which the eluate from the eppendorf was re-applied to each membrane and centrifuged again to increase the RNA yield. RNA was quantified using the Nanodrop spectrophotometer (Thermo Scientific) and stored at -80°C until further use.

2.4.2 Reverse transcription

Reverse transcription is the process in which a single strand of RNA is transcribed in to complementary DNA (cDNA) using a reverse transcriptase enzyme. The reverse transcriptase enzyme used is the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) enzyme (Invitrogen). To a 0.2ml domed nuclease-free microcentrifuge tube (Starlab, Ahrensburg, Germany) 1µg of sample RNA was added, 0.5µl of 500µg/ml oligo dT (Invitrogen), 0.5µl of 10mM deoxyribonucleotide

triphosphates (dNTP) mix (10mM each of dATP, dTTP, cCTP and dGTP) (Invitrogen) and RNase-free water (Qiagen) to a total volume of 12µl. This is heated to 65°C for 5 minutes using a thermal cycler (DNA Engine Tetrad 2, Peltier Thermal Cycler, BioRad, Hercules, CA) and then placed on ice. To each sample 2µl of 5x first strand (FS) Buffer, 1µl of 0.1M dithiothreitol (DTT) and 0.5µl of RNasin inhibitor (Promega) was added. After gentle mixing, each sample was incubated at 37°C for 2 minutes after which 0.5µl (100 units) of M-MLV RT was added to each sample, apart from the negative control sample, followed by a further incubation for 50 minutes at 37°C in the thermal cycler. The samples were then heated to 70°C for 15 minutes to inactivate the reaction after which they were stored at -20°C until further use.

2.4.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique used to amplify segments of DNA using the DNA obtained in the reverse transcription step as templates from which a new DNA strand is produced. Specific DNA primers are used to initiate DNA synthesis and to ensure amplification of the region of interest within the DNA template. The primers are non-complementary and are present in excess in the reaction. Primers were designed using Oligoperfect software (Invitrogen) and were purchased from Invitrogen. Details of primers used are listed in table 2.5. As well as the DNA template and primers, *Thermus aquaticus* (Taq) DNA polymerase enzyme and the dNTPs are included to achieve amplification of the DNA fragment of interest. The concentration of the PCR reaction reagents is detailed in table 2.6 to a total volume of 50µl.

Table 2.4 Primers used to amplify DNA

PCR	Sequence (5' - 3')
β-Actin Forward	TCACCCACACTGTGCCCATCTA
β-Actin Reverse	CAGCGGAACCGCTCATTGCCA
pS2 Forward	GTCCCCTGGTGCTTCTATCC
pS2 Reverse	GAGTAGTCAAAGTCAGAGCAGTCAATCT

Table 2.5 PCR reaction reagents

Component	Volume (µl)
cDNA	3µl
10x PCR Buffer	5µl
50mM MgCl	1.5µl
0.1µM pS2 primers	1µl
10mM dNTP mix	1µl
Taq DNA polymerase	0.25µl
RNase free water	38.25µl

PCR reactions were performed using a thermal cycler. Following incubation at 94°C for 3 minutes to achieve DNA denaturation, each reaction comprised a number of different stages (table 2.7) as described below:

- DNA denaturation - sample is heated to 94°C for 45 seconds to ensure DNA denaturation.
- Primer annealing - temperature is reduced to allow the primers to anneal to the target DNA.
- Extension - DNA polymerase adds dNTPs in the 5' to 3' direction to synthesise a new cDNA strand.
- The denaturation-annealing-extension cycle is repeated with the sequence of interest between the primers exponentially increased with each cycle.
- Final elongation - following the last cycle, a final ten minute incubation at 72°C is performed to ensure that any remaining single-stranded cDNA is fully extended.

Table 2.6 Cycling conditions for PCR reactions

PCR Product	Denature		Anneal		Extend		No. of Cycles
	Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)	
B-Actin mRNA	94	45	60.0	30	72	60	35
pS2 mRNA	95	60	60.0	120	72	120	25

2.4.4 PCR analysis using DNA agarose gels

PCR products were analysed using agarose gel electrophoresis to separate products according to size. Agarose powder (Promega) was heated in 1x Tris acetate EDTA (TAE) Buffer (Appendix I) at a concentration of 1.5% (0.75mg agarose in 50mls TAE). After cooling, 7µl of Sybersafe (Invitrogen), (used as an intercalating agent) was added to the gel which was then poured into a casting rig containing a well-comb and allowed to solidify at room temperature. Once set, the gel was covered with 1x TAE Buffer and the samples and loading dye were added to each well. An electric current of 100V was then applied to the gel for 30 minutes allowing for adequate migration of the samples. Gels were visualised using ultraviolet light with the images recorded using the LAS3000 Image software (Fujifilm, Japan).

2.5 Epigenetic studies

As described in 1.3.2, epigenetic modifications are a mechanism by which loss of gene transcription can occur in the absence of gross mutations. Methylation of specific CpG regions within the promoter region of genes can result in the loss of gene expression.

2.5.1 DNA extraction

Cells were cultured in T_{25} flasks until they reached 90% confluency. They were then treated as described in section 2.1.7 for 24 hours. DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Cells were harvested as described in 2.1.5 after which they were resuspended in 200µl of PBS. 20µl of proteinase K was added to each solution followed by the addition of 200µl of Buffer AL and the solution was vortexed to ensure a homogenous mixture. They were incubated at 56°C for 10 minutes, 200µl of 100% ethanol was added to each sample which was then transferred to a DNeasy spin column and centrifuged for 1 minute at 8,000rpm. The flow through was discarded and the membrane washed with 500µl of Buffer AW1 after which it was again centrifuged for 1 minute at 8,000rpm. 500µl of Buffer AW2 was added to the membrane and the sample centrifuged for 3 minutes at 14,000rpm. The column was then placed in a 1.5ml eppendorf, 200µl of Buffer AE was added to the membrane and the sample was left to stand at room temperature for 1 minute. It was the centrifuged for 1 minute at 8,000rpm with the sample from the eppendorf re-applied to the membrane and centrifuged again to increase the DNA yield. DNA was quantified using the Nanodrop spectrophotometer (Thermo Scientific) and stored at -20°C until further use.

2.5.2 Bisulfite conversion and sequencing

The aim of bisulfite conversion is to identify the methylated and unmethylated CpG islands within the promoter region of interest. When unmethylated DNA is treated with bisulfite, the cytosine residues are converted to uracil residues. However, when

there is a methyl group attached to the cytosine residue, this prevents the conversion occurring and it remains a cytosine residue. Therefore, bisulfite conversion results in specific changes occurring to DNA sequences that are dependent on the methylation status of the cytosine residues. During PCR amplification, the uracil is converted to thymidine which can be identified using specific primers. This can therefore determine the methylation status of a segment of DNA, in the case of this research, the promoter region of ER.

Bisulfite conversion was performed using the EZ DNA-methylation Gold Kit (Zymo, CA, USA) following the manufacturer's instructions. 130µl of CT Conversion Reagent was added to 500ng of DNA with the total volume increased to 20µl using RNase free water (Qiagen). The sample was pipette mixed and then heated in a thermal cycler at 98°C for 10 minutes followed by 64°C for two and a half hours. The sample was then loaded in to a Zymo-Spin IC Column (Zymo) along with 600µl of M-Binding Buffer and was centrifuged at 13,000rpm for 30 seconds with the flow-through then discarded. 100µl of M-was Buffer was added with the sample centrifuged at 13,000rpm for 30 seconds. 200µl of M-Desulphonation Buffer was added and the column left to stand at room temperature for 15 minutes and then centrifuged at 13,000rpm for 30 seconds. It was washed with 200µl of M-Wash Buffer and centrifuged at 13,000rpm for 30 seconds which was repeated a second time. The column was transferred to a 1.5ml microcentrifuge tube, 10µl of M-Elution Buffer was added to the membrane and it was centrifuged at 13,000rpm for 30 seconds to elute the DNA which was stored at -20°C until further use.

Samples were then sent for sequencing to MWG Biotech (Ebersberg, Germany) using primers as detailed in table 2.8, which had been used previously in the identification of methylated and unmethylated ER (Lapidus, Nass et al. 1998; Pirouzpanah, Taleban et al. 2010).

Table 2.7 Primers used to amplify methylated and unmethylated ER

PCR	Sequence (5' - 3')
Methylated ER Forward	GATACGGTTTGTATTTTGTTCGC
Methylated ER Reverse	CGAACGATTCAAAAACTCCAACT
Unmethylated ER Forward	GGATATGGTTTGTATTTTGTTTGT
Unmethylated ER Reverse	ACAAACAATTCAAAAACTCCAACT

2.6 Translational Research

Ethical approval for all studies using patient tissue samples was obtained from the Medical Ethics Committee, Beaumont Hospital, Dublin 9 and Waterford Regional Hospital, Co. Waterford. Samples were only used from patients who provided informed consent for the use of their tissue in any subsequent translational research. All patients who presented with breast cancer between 1996 and 2008 were included. The only exclusion criterion was patients who were diagnosed with metastatic disease at presentation.

Table 2.8 Demographics of the patient cohort used to construct the tissue microarray

Patient Demographics	Number of Patients
Total number of patients	488
Median age	56.6 years (range 28.5 – 92.6)
Median follow-up	4.29 years (range 0.4 – 13.9)
Receptor status	
ER positive	342 (70.0%)
PR positive	262 (53.7%)
HER2 positive	147 (30.1%)
Tumour grade	
Grade I	67 (13.7%)
Grade II	220 (45.1%)
Grade III	201 (41.2%)
Tumour stage	
1	157 (32.2%)
2	263 (53.9%)
3	62 (12.7%)
4	7 (1.4%)
Positive lymph nodes	236 (48.4%)
Tumour recurrence	147 (30.1%)

2.6.1 Immunohistochemistry

Immunohistochemistry is a staining technique that is used to identify the presence and location of specific proteins within tissue specimens, in this case pS2. Immunohistochemistry was performed on previously constructed tissue micro-arrays that were constructed using formalin fixed paraffin embedded tissue. The tissue micro-arrays consisted of 0.8mm diameter cores of tumour specimens. Sections were taken at a thickness of 5μm using a microtome and mounted on Superfrost Plus slides (VWR International, Leuven, Germany).

All slides were baked for 4 hours at 65°C after which they were deparaffinised by soaking in xylene for a total of 6 minutes. Subsequent rehydration was performed by passage through industrial methylated spirits (100% for 6 minutes and 70% for 3 minutes). The slides were then washed twice in PBS for 5 minutes. The activity of endogenous peroxidase was depleted by incubating the slides twice in 3% hydrogen peroxide (H_2O_2, Sigma Aldrich) in the dark for 10 minutes. They were then washed in double-distilled H_2O for 5 minutes followed by a wash in PBS with 0.1% Tween (Sigma Aldrich) (PBST) for 5 minutes. Antigen retrieval was performed by heating the slides in 10mM sodium citrate buffer (pH 6.0) for 7 minutes in an enclosed plastic container using a domestic microwave. The slides were then cooled on the bench in the sodium citrate buffer for another 20 minutes followed by a further two washes in PBST for 5 minutes each.

Pre-incubating the slides at room temperature for 90 minutes in a 3% solution of goat serum (same species from which the secondary antibody was raised) reduces the binding of secondary antibody to non-specific endogenous immunoglobulins. Primary pS2 antibody (Novocastra, Leica Microsystems, Germany) concentration was determined according to the manufacturer's instructions and subsequent optimisation with a concentration of 1:300 found to result in optimal staining of slides. Slides were incubated in primary antibody at 4°C overnight. Following 3 washes in PBST for 5 minutes each, the slides were then incubated for 30 minutes at room temperature in the Vectastain Elite PK-1600 series biotinylated goat secondary antibody (Burlingame, CA) which was diluted by a factor of 1:200 in PBS containing 15μl of the goat blocking serum. Amplification of the signal was

achieved by incubating the slides for a further 30 minutes at room temperature in the Avidin-biotin complex from the Vectastain Elite kit. Diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich) was applied to the slides for 5 minutes to develop the product. The DAB reaction was stopped by washing the slides in double-distilled H_2O for 5 minutes. Counter-staining was achieved by incubating the slides in haematoxylin (Sigma Aldrich) for 3 minutes at room temperature after which the slides were washed in flowing water for 5 minutes. Dehydration of the slides was performed by passing them through 70% IMS for 3 minutes followed by two 3 minute passages through 100% IMS with two subsequent 3 minute passages in xylene. Coverslips were applied by mounting the slides with DPX mounting solution (Sigma Aldrich).

Stained slides were reviewed by two observers using light microscopy and scored according to the Allred scoring system (Allred, Clark et al. 1993). This system scores slides according to two variables: the area of tissue stained (none =0, <1%=1, >1% but <10%=2, >10% but <33%=3, >33% but <66%=4 and >66%=5) and the average intensity of staining of tumour cells (none=0, weak=1, intermediate=2, strong=3). This results in a combined score ranging between 0 and 8. A total score of three or greater was deemed positive.

2.7 Statistical Analysis

Anonymous databases were maintained on Microsoft Excel (Microsoft, WA, USA). Statistical analyses were carried out using StatsDirect software (Chesire, UK). P values <0.05 were considered significant. Each experiment was carried out three times (i.e. n=3) with the mean result and standard error presented. Densitometry was performed using ImageJ software, version 1.45 (National Institute of Mental Health, USA). For molecular studies the probability of difference between two paired samples was analysed using a two tailed paired Student's t-Test. Multivariate analysis was performed using STATA 10 data analysis software (Stata Corp. Texas, USA) and was carried out using Cox's proportional hazard model, using the Breslow method for ties. Survival times between groups were compared using the Wilcoxon Breslow test adjusted for censored values.

Chapter 3

Identifying the effects of trastuzumab treatment on the steroid hormone pathway *in vitro*

3.1 Introduction

Resistance to adjuvant therapies, whether de novo or acquired, adversely effects the overall prognosis of a person with breast cancer. While the benefits of tamoxifen therapy are well established (Group 1992; Osborne 1998), the phenomenon of acquired resistance is also widely documented (Group 1992; Kurebayashi 2005). As previously discussed in chapter 1.5, one proposed mechanism of tamoxifen resistance is through crosstalk between the steroid hormone and growth factor receptor pathways (Schiff, Reddy et al. 2000; Razandi, Pedram et al. 2003; Shou, Massarweh et al. 2004; Osborne, Shou et al. 2005). This has been demonstrated in a number of phase II clinical trials which have shown that the use of gefitinib, a tyrosine kinase inhibitor that targets HER1, in combination with anti-oestrogen therapy, can improve the progression free survival in ER positive, HER2 negative breast cancers (Cristofanilli, Valero et al. 2010; Osborne, Neven et al. 2011)

Similarly, crosstalk between the two signalling pathways has been implicated in the development of acquired resistance to lapatinib, a small-molecule tyrosine kinase inhibitor that inhibits signalling through the growth factor receptor pathway by reversibly binding to HER1 and HER2 (Xia, Bacus et al. 2006). Xia et al suggest that, as a result of this crosstalk, the efficacy of lapatinib might be enhanced if used in combination with anti-oestrogen therapies such as a selective oestrogen receptor downregulator e.g. fulvestrant, or an aromatase inhibitor. This combination therapy might even be relevant in breast cancers that are technically deemed to be ER negative based on diagnostic criteria but still express detectable ER.

In humans, chromosome 6 encodes for the ER protein gene which comprises eight exons separated by seven intronic regions, spanning more than 140 kilobases (Green, Walter et al. 1986; Greene, Gilna et al. 1986). It contains eight coding regions which are transcribed from at least seven promoters (Kos, Reid et al. 2001; Herynk and Fuqua 2004). Transcription of this gene results in the formation of a number of forms of ER including truncated versions whose sizes can vary and splice variants (Fuqua, Fitzgerald et al. 1992; Kos, Denger et al. 2002; Al-Bader, Al-Saji et al. 2010). Most commonly, ER is observed at 66kDa (Walter, Green et al. 1985).

Analysis of the functional activity of ER within our breast cancer cells required the use of a target gene whose expression is known to be dependent on the activity of ER. pS2, also known as trefoil factor 1, is a small cysteine-rich protein whose expression has been revealed to be dependent on an active ER. Therefore, in this study this classical ER target gene was used to demonstrate activity of ER within the cell lines.

The interactions between the steroid hormone and growth factor receptor pathways are undoubtedly extremely intricate and complex. Crosstalk invariably plays an integral role in the development of acquired resistance to tamoxifen and possibly other anti-oestrogen therapies. *In vitro* models suggest that it might also be a factor in the development of resistance to small-molecule tyrosine kinase inhibitors such as lapatinib. On this background, the effect of trastuzumab on the steroid hormone pathway was investigated in breast cancer cell lines to determine whether or not it had an effect on the steroid hormone pathway thereby indicating the presence of crosstalk between the two pathways.

3.2 Aims:

The aims of this chapter relate to the first objective of the study: to investigate the effect of trastuzumab treatment on the steroid hormone pathway in the breast cancer cell lines. The following specific objectives were defined:

a. characterise the effect of trastuzumab treatment on the expression of ER in the breast cancer cell lines.
b. determine the effect of trastuzumab treatment on the transcriptional activity of the steroid hormone pathway in the cell model.

3.3 Results:

3.3.1 Effect of trastuzumab treatment on ER protein expression in MCF-7, LCC-1 and SKBR-3 breast cancer cell lines

ER protein expression can play a role in the responsiveness of cells to endocrine therapies. To examine the effect of trastuzumab on ER protein expression in the breast cancer cell lines, cells were treated with 100μM/ml trastuzumab for 24 hours after which expression of ER protein was evaluated using western blotting techniques. All experiments were performed in triplicate (n=3) with the mean expression and standard error calculated. As shown in figure 3.1, MCF-7 cells had the highest expression of basal levels of ER, with LCC-1 cells only expressing a low basal level and SKBR-3 cells not expressing detectable levels of ER at all. Treatment with trastuzumab was found to significantly increase ER protein expression in the MCF-7 cells (mean increase = 2.09 +/- 0.247; p=0.048) while there was no significant change in protein expression in the LCC-1 cells (mean decrease = 0.77 +/- 0.0583; p=0.06). Trastuzumab treatment was noted to result in the expression of ER in the SKBR-3 cells (mean increase = 8.22 +/- 1.45; p=0.038).

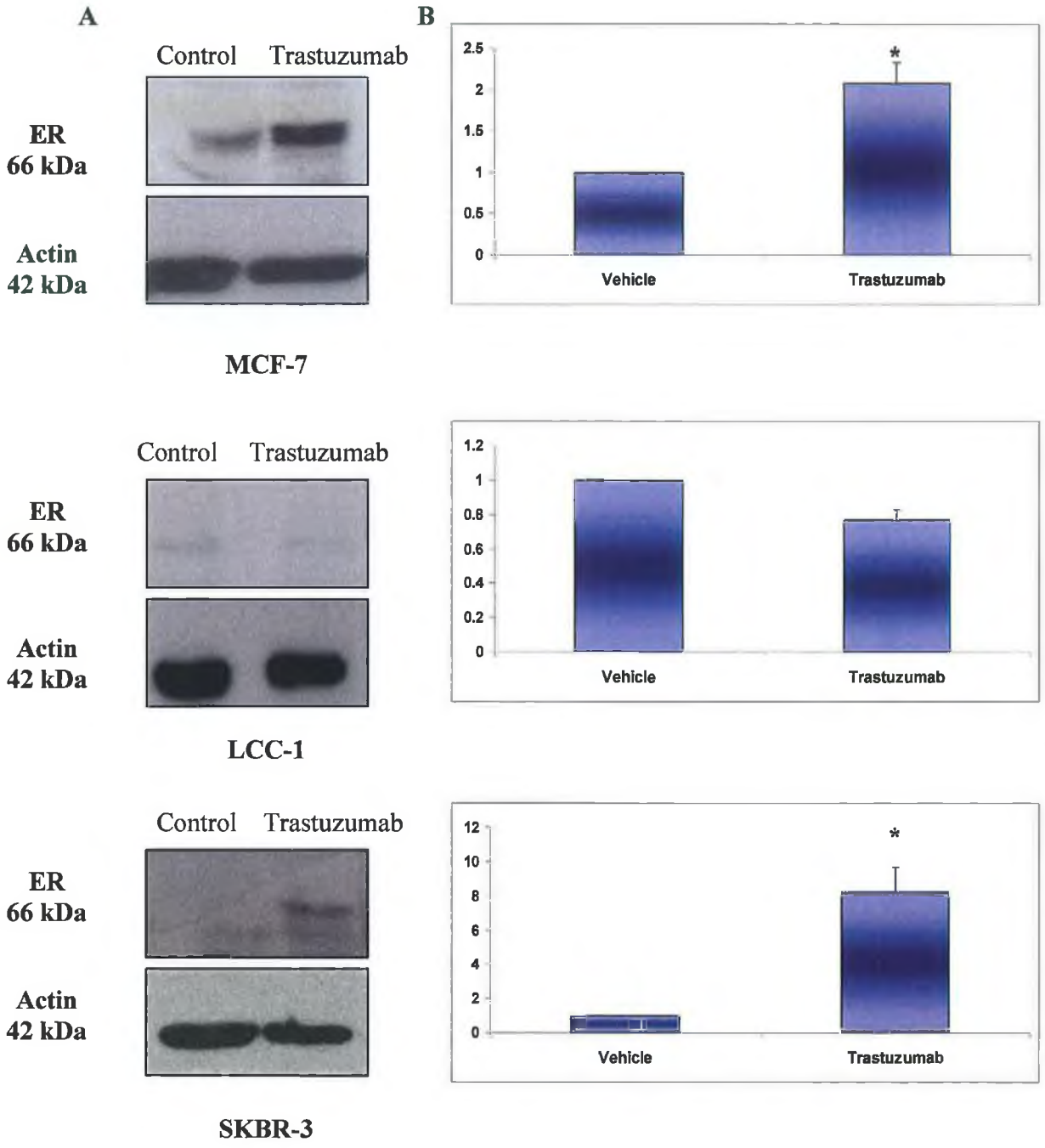


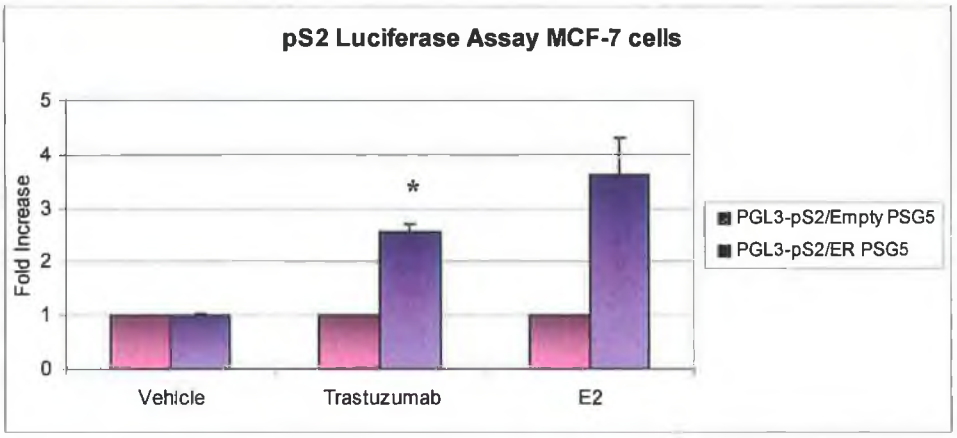
***Figure 3.1: A** - Western blot analysis of ER expression in protein lysates from MCF-7, LCC-1 and SKBR-3 cells. Cells were treated with 100μM/ml trastuzumab for 24 hours prior to performing western blot analysis. Treatment with trastuzumab resulted in a significant increase in ER protein expression in MCF-7 cells ($p=0.048$), no change in ER protein expression in LCC-1 cells and expression of ER in SKBR-3 cells ($p=0.039$).*

***B** – densitometry graph illustrating the changes in ER expression following treatment with trastuzumab. The asterisks indicate statistically significant changes.*

3.3.2 Effect of trastuzumab treatment on the transcriptional activity of the steroid hormone pathway in MCF-7 and LCC-1 breast cancer cell lines

The effect of trastuzumab and E_2 treatment on the transcriptional activity of the steroid hormone pathway in the MCF-7 and LCC-1 cell lines was assessed using a Dual Luciferase Reporter Assay System. Cells were treated with 100μM/ml trastuzumab and 10^{-8} E_2 for 6 hours prior to performing the luciferase assay. Each assay was performed in triplicate and repeated three times to determine the mean expression levels and standard error rates. Treatment of MCF-7 cells with both E_2 and trastuzumab resulted in upregulation of the transcriptional activity of the steroid hormone pathway as demonstrated by the increased activity of the PGL3-pS2 promoter (mean increase = 3.614 +/- 0.691; p=0.06 for E_2; mean increase = 2.546 +/- 0.159; p=0.0097 for trastuzumab) (figure 3.2). In the LCC-1 cells there was no noticeable difference in promoter activity after both treatments (mean increase = 1.446 +/- 0.084; p=0.09 for E_2; mean increase = 0.801 +/- 0.107; p=0.43 for trastuzumab).

A



B

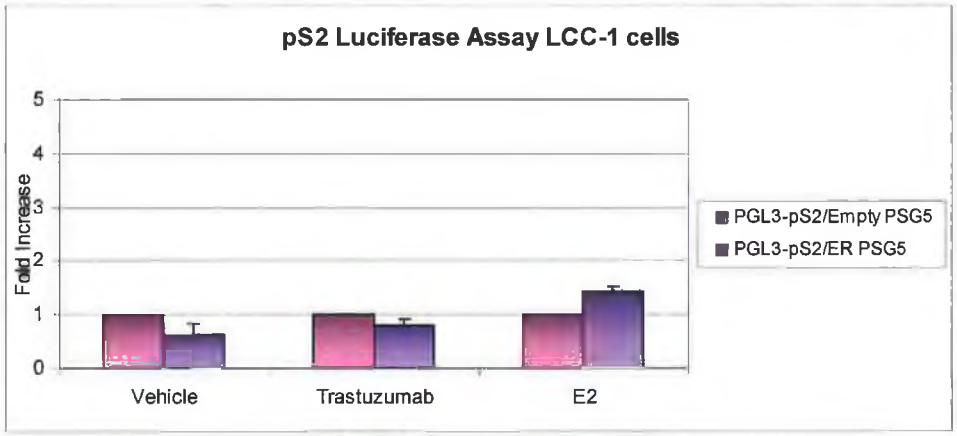
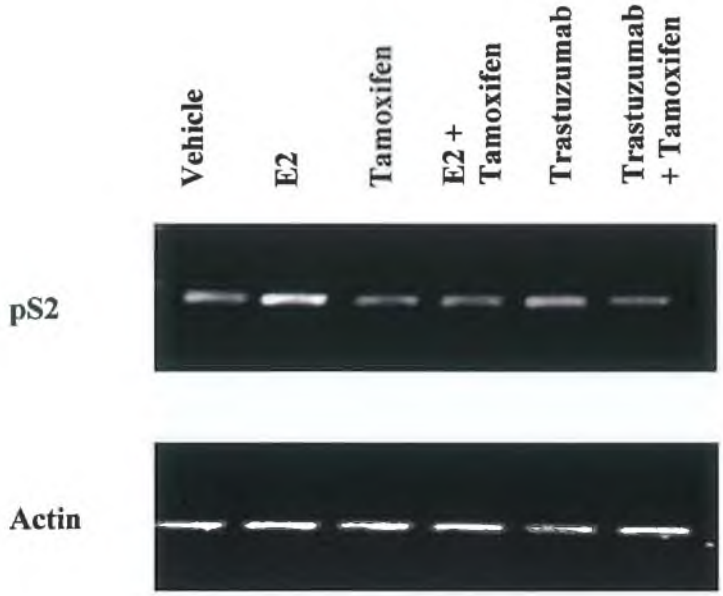


Figure 3.2: *Luciferase assay demonstrating the effect of E_2 and trastuzumab on the transcriptional activity of the steroid hormone pathway. Cells were treated with 10^{-8}M E_2 and 100μM/ml trastuzumab for 6 hours prior to performing luciferase assay. E_2 and trastuzumab increased transcriptional activity in MCF-7 cells (mean increase = 3.614 +/- 0.691; p=0.06 for E_2; mean increase = 2.546 +/- 0.159; p=0.0097 for trastuzumab) (A) while there was no significant change in activity in LCC-1 cells following treatment (mean increase = 1.446 +/- 0.084; p=0.09 for E_2; mean increase = 0.801 +/- 0.107; p=0.43 for trastuzumab) (B). The asterisk indicates a statistically significant change.*

Relative pS2 mRNA levels were also investigated using reverse transcription PCR (RT-PCR) in the three breast cancer cell lines treated with100μM/ml trastuzumab, 10^{-8} 4-OHT and10^{-8} E_2 for 6 hours both separately and in combination. All experiments were performed three times to determine the mean and standard error rates. In the MCF-7 cells, treatment with E_2 resulted in significantly increased levels of pS2 mRNA expression (mean increase = 2.281 +/- 0.131; p=0.01) whilst 4-OHT significantly reduced expression levels (mean decrease = 0.718 +/- 0.027; p=0.009). Treatment with trastuzumab was found to increase pS2 mRNA levels almost to a similar level as seen following treatment with E_2 (mean increase = 1.741 +/- 0/157; p=0.042) The addition of 4-OHT to both E_2 and was found to reduce pS2 levels to near baseline (mean increase = 1.235 +/- 0.08 for E_2 and 4-OHT, mean increase = 1.285 +/- 0.118 for trastuzumab and 4-OHT) (figure 3.3). There were no significant differences found in pS2 mRNA expression levels in LCC-1 cells following the various treatments (mean increase = 1.015 +/- 0.044; p=0.76 for E_2, mean increase = 1.067 +/- 0.014; p=0.359 for 4-OHT, mean decrease = 0.945 +/- 0.094; p=0.731 for E_2 and 4-OHT, mean increase = 1.362 +/- 0.05; p=0.305 for trastuzumab and mean increase = 1.063 +/- 0.015; p=0.807 for trastuzumab and 4-OHT) (figure 3.4). Treatment of SKBR-3 cells with E_2 had minimal effect on pS2 mRNA expression levels (mean increase = 1.015 +/- 0.044; p=0.246) as did 4-OHT treatment (mean increase = 1.067 +/- 0.014; p=0.431). However, following treatment with trastuzumab, there was a significant increase in pS2 mRNA levels noted (mean increase = 1.362 +/- 0.045; p=0.015) (figure 3.5).

A



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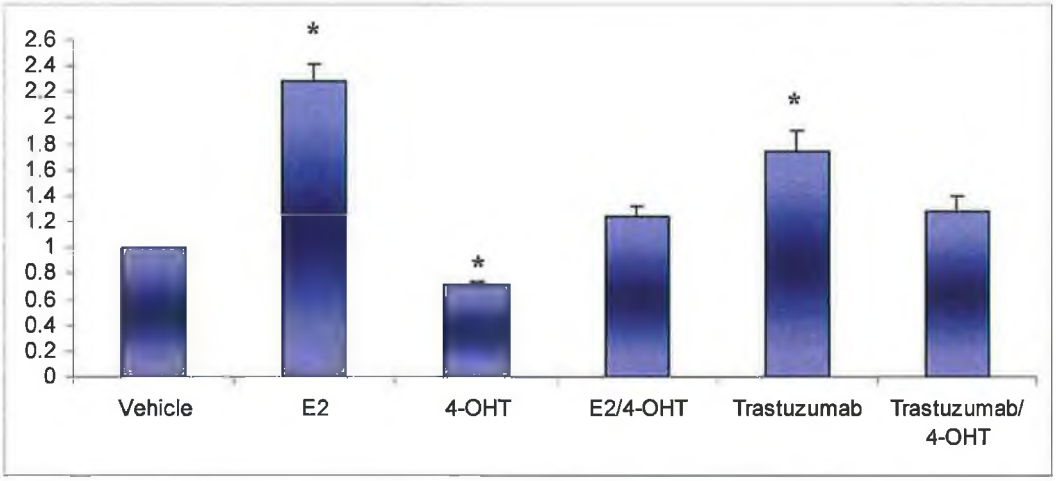
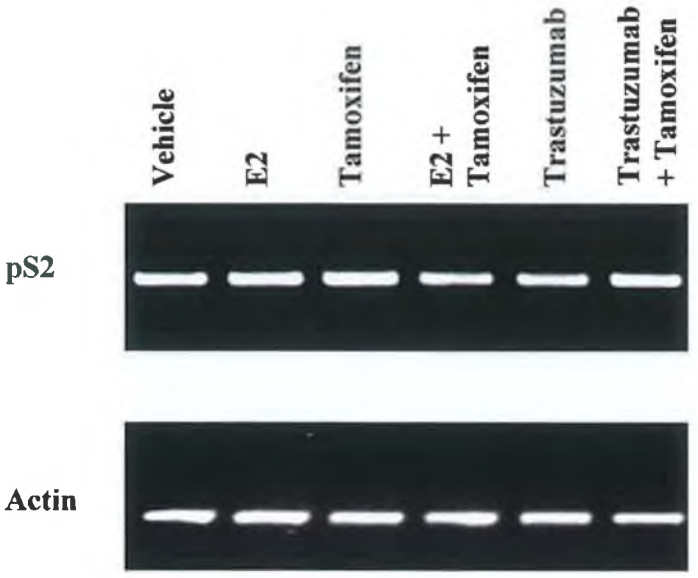


***Figure 3.3: A** - RT-PCR expression of pS2 mRNA in MCF-7 cells under the influence of 10^{-8}M E_2, 10^{-8}M tamoxifen and 100μM/ml trastuzumab, individually and in combination. mRNA levels were significantly increased following E_2 (mean increase = 2.281 +/- 0.131; p=0.01) and trastuzumab (mean increase = 1.741 +/- 0/157; p=0.042) treatment and reduced following tamoxifen treatment (mean decrease = 0.718 +/- 0.027; p=0.009).*

***B** – densitometry graph illustrating the increase in pS2 mRNA expression following treatment with E_2 and trastuzumab and the decrease following 4-OHT treatment. The asterisks indicate statistically significant changes in expression levels.*

A



B

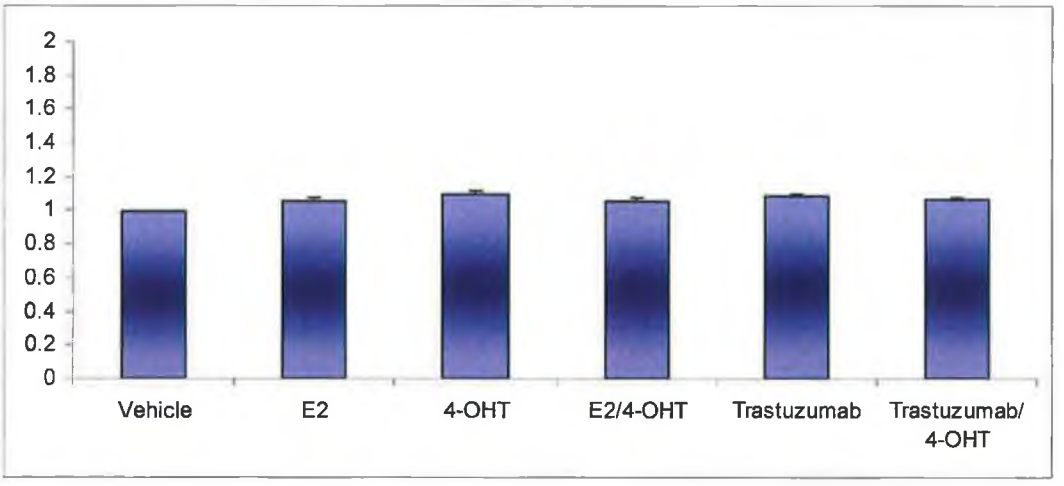
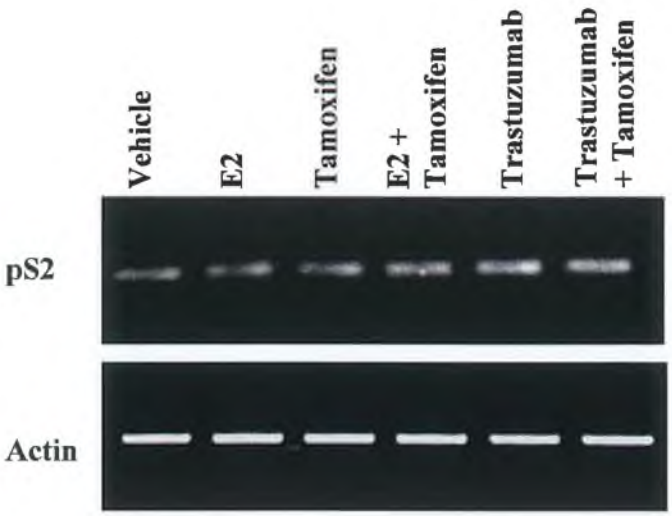


***Figure 3.4: A** - RT-PCR expression of pS2 mRNA in LCC-1 cells under the influence of 10^{-8}M E_2, 10^{-8}M tamoxifen and 100μM/ml trastuzumab, individually and in combination. mRNA levels were unchanged following all treatments (mean increase = 1.015 +/- 0.044; p=0.76 for E_2, mean increase = 1.067 +/- 0.014; p=0.359 for 4-OHT, mean decrease = 0.945 +/- 0.094; p=0.731 for E_2 and 4-OHT, mean increase = 1.362 +/- 0.05; p=0.305 for trastuzumab and mean increase = 1.063 +/- 0.015; p=0.807 for trastuzumab and 4-OHT).*

***B** – densitometry graph illustrating the similar expression of pS2 mRNA levels in LCC-1 cells following treatments.*

A



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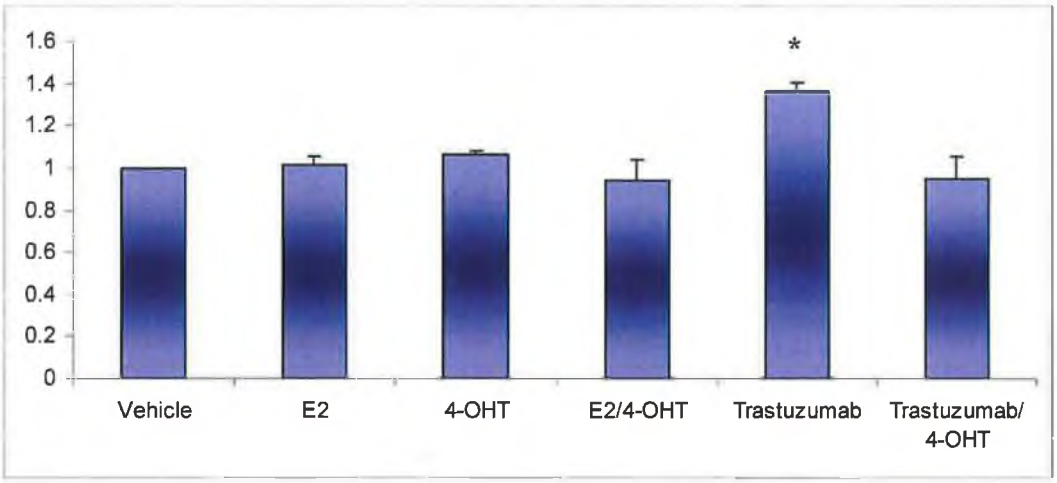


Figure 3.5: A - *RT-PCR expression of pS2 mRNA in SKBR-3 cells under the influence of 10^{-8}M E_2, 10^{-8}M tamoxifen and 100μM/ml trastuzumab, individually and in combination. Treatment of SKBR-3 cells with E_2 had minimal effect on pS2 mRNA expression levels (mean increase = 1.015 +/- 0.044; p=0.246) as did 4-OHT treatment (mean increase = 1.067 +/- 0.014; p=0.431). However, following treatment with trastuzumab, there was a significant increase in pS2 mRNA levels noted (mean increase = 1.362 +/- 0.045; p=0.015)*

B – *densitometry graph illustrating the increase in pS2 mRNA expression in SKBR-3 cells following treatment with trastuzumab but not with E_2. The asterisk indicates a statistically significant change.*

3.3.3 Trastuzumab induced expression of ER in SKBR-3 breast cancer cells through demethylation

As demonstrated above, treatment of ER negative SKBR-3 cells with trastuzumab results in the expression of the ER protein (figure 3.1). Ottaviano et al have previously demonstrated that methylation of CpG islands within the promoter region of the ER gene can result in the loss of expression of ER (Ottaviano, Issa et al. 1994). Treatment of ER negative breast cancer cells with a DNA methyltransferase inhibitor results in demethylation of the ER CpG islands and subsequent expression of ER protein (Ferguson, Lapidus et al. 1995). To determine whether trastuzumab treatment of SKBR-3 cells resulted in demethylation of CpG islands within the promoter region of the ER gene with subsequent expression of a functional ER, methylation studies were performed following treatment of cells with 100μM/ml trastuzumab for 24 hours. The results show that out of five detectable CpG islands within the ER promoter region (figure 3.6), all five were unmethylated in MCF-7 cells in both vehicle and trastuzumab treated samples (figure 3.7, table 3.1). In the SKBR-3 cells, all five islands were methylated prior to trastuzumab treatment, with all becoming unmethylated following treatment (figure 3.7, figure 3.8, table 3.2).

Figure 3.6: *Demonstrates the clone sequence chromatogram of the DNA region for the ER gene. The CpG islands are highlighted by the stars. In the MCF-7 cells these were unmethylated both before and after treatment with 100 μM/ml trastuzumab. In the SKBR-3 cells these were methylated prior to treatment and unmethylated after treatment with 100 μM/ml trastuzumab.*

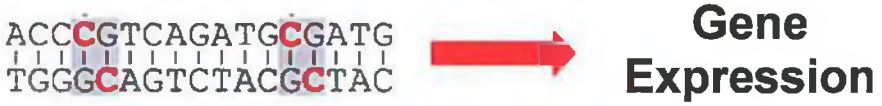


Figure 3.7: *Schematic representation of unmethylated DNA sequence in MCF-7 cells and in SKBR-3 cells following trastuzumab treatment resulting in ER gene expression.*

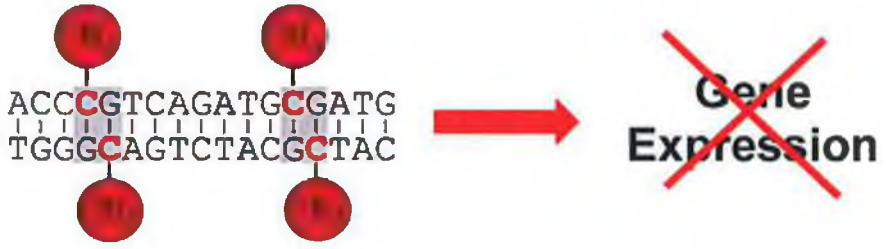


Figure 3.8: *Schematic representation of methylation of the DNA sequence in SKBR-3 cells inhibiting ER gene expression*

Table 3.1: Methylation studies assessing CpG islands within the promoter region of the ER gene in MCF-7 cells

All CpG islands were unmethylated both prior and subsequent to treatment with 100μM/ml trastuzumab.

CpG Site	1	2	3	4	5
Vehicle	Unmeth	Unmeth	Unmeth	Unmeth	Unmeth
Trastuzumab	Unmeth	Unmeth	Unmeth	Unmeth	Unmeth

Table 3.2: Methylation studies assessing CpG islands within the promoter region of the ER gene in SKBR-3 cells

All CpG islands were methylated prior to treatment with 100μM/ml trastuzumab, with all unmethylated subsequent to trastuzumab treatment.

CpG Site	1	2	3	4	5
Vehicle	Methylated	Methylated	Methylated	Methylated	Methylated
Trastuzumab	Unmeth	Unmeth	Unmeth	Unmeth	Unmeth

3.4 Discussion

Crosstalk between the steroid endocrine pathway and the growth factor receptor pathway has been previously implicated in the development of resistance to anti-oestrogen therapies (Schiff, Reddy et al. 2000; Razandi, Pedram et al. 2003; Shou, Massarweh et al. 2004; Osborne, Shou et al. 2005). Despite a large proportion of patients with amplification of the HER2 gene, in particular those with metastatic disease, developing resistance to anti-HER2 monotherapies such as trastuzumab, the exact mechanism by which this resistance develops is as yet unknown. In this study it was postulated that treatment of breast cancer cells with trastuzumab could result in upregulation of the steroid hormone pathway through crosstalk between the two pathways and therefore could result in the development of resistance to trastuzumab.

To investigate this hypothesis three separate breast cancer cell lines were chosen to study the effects of trastuzumab *in vitro*. Differential expression levels of ER was established in the three cell lines. The effect of trastuzumab on the steroid hormone pathway was then determined through a number of techniques examining the expression and functional activity of ER following treatment.

3.4.1 ER positive endocrine sensitive breast cancer cell model

MCF-7 breast cancer cells, a well established ER-expressing cell line sensitive to endocrine therapies, were used to study the effect of trastuzumab in ER positive cells. Following treatment with trastuzumab, these cells were noted to have increased levels of ER when compared to baseline levels. Further molecular studies confirmed that this increased expression enhances the transcriptional and functional activity of the steroid hormone pathway as demonstrated by the increased activity of the pS2 promoter as well as elevated levels of pS2 mRNA.

These findings corroborate the hypothesis of this study that crosstalk can occur between the steroid hormone pathway and the growth factor receptor pathway whereby treatment with trastuzumab can result in upregulation of the steroid hormone pathway.

Crosstalk between the two pathways has already been shown to result in resistance to endocrine therapies (Schiff, Reddy et al. 2000; Razandi, Pedram et al. 2003; Shou, Massarweh et al. 2004; Osborne, Shou et al. 2005) which has detrimental consequences in the form of tumour recurrence and proliferation that is non-responsive to currently available therapies. The molecular pathways behind the development of resistance to trastuzumab are not yet as well investigated as those in endocrine resistance, but their effect is equally devastating. As discussed in section 1.5.2, a number of mechanisms by which tumour cells develop resistance to trastuzumab have been investigated (Molina, Saez et al. 2002; Price-Schiavi, Jepson et al. 2002; Lu, Zi et al. 2004; Scaltriti, Rojo et al. 2007; Wang, Xiang et al. 2008), however our research highlights another possible mechanism – receptor crosstalk. This finding has potential consequences for the future clinical treatment of patients with breast cancer. It highlights the necessity to concurrently rather than sequentially target the steroid hormone pathway and the growth factor receptor pathway to minimise the uncontrolled stimulation of the steroid hormone pathway which could then lead to cell proliferation and tumour recurrence. Further molecular studies are required to identify the transcription factors involved in this crosstalk to allow for individualised patient therapy.

3.4.2 ER positive endocrine insensitive breast cancer cell model

LCC-1 cells, an isogenic derivative of MCF-7 cells, overexpress both ER and HER2. They are however insensitive to oestrogen therapy and so were used to demonstrate an endocrine insensitive breast cancer cell model. As expected, there was no response observed in cellular activity when treated with E_2. Interestingly, there was also no significant change in either the expression of ER or in the transcriptional or functional activity of these cells when treated with trastuzumab, unlike the endocrine sensitive MCF-7 cells.

This highlights the plasticity of tumour cells, demonstrating the varying effect that identical treatments can have on the molecular pathways involved in cellular growth and proliferation. These findings show that in endocrine insensitive breast cancer cells, the phenomenon of crosstalk between the two pathways does not appear to occur following trastuzumab treatment, unlike that observed in endocrine sensitive

cells. This suggests a possible link between the sensitivity of cells to oestrogen and the ability of cells to overcome the anti-proliferative effect of trastuzumab through interaction with the steroid hormone pathway. Further molecular studies are required to elucidate this hypothesis and determine why certain ER-expressing cells show upregulation of the steroid hormone pathway while others do not.

3.4.3 ER negative breast cancer cell model

SKBR-3 cells overexpress HER2 due to the amplification of the c-erbB2 gene however they do not express ER and so were used to demonstrate a HER2 positive, ER negative cell model. Baseline Western blots for ER expression levels demonstrated that, as expected, ER was not expressed. However, subsequent treatment with trastuzumab not only resulted in the expression of ER, it made it functionally active. Therefore, not only can trastuzumab enhance the function of ER, it can cause a complete switch in its expression in certain cells, which, as demonstrated is as a result of demethylation of the relevant CpG islands within the promoter region of ER.

Dual treatment with trastuzumab and an anti-oestrogen agent such as tamoxifen or an aromatase inhibitor has been shown to restore the sensitivity of resistant cells to anti-oestrogen therapies (Benz, Scott et al. 1992; Kunisue, Kurebayashi et al. 2000; Sabnis, Schayowitz et al. 2009). Recent data has also shown that treatment of letrozole-resistant cells with trastuzumab can restore cellular ER levels and thereby reverse the developed resistance to the aromatase inhibitor (Sabnis, Schayowitz et al. 2009). However, to date, there is no data published suggesting that trastuzumab has the ability to express a functionally active ER in certain ER negative breast cancer cells or indeed tumours. This has potentially significant clinical implications. Current therapeutic regimes do not include anti-oestrogen therapies if the breast cancer is deemed to be ER negative. However, if these same cancers are HER2 positive they would be considered for therapeutic interventions such as trastuzumab if clinically indicated. The *in vitro* findings in this study would suggest that a proportion of these tumours would switch from being ER negative to ER positive due to the demethylation effect of trastuzumab resulting in an untreated functioning steroid hormone pathway through which tumours can recur and proliferate.

Chapter 4

Translating the interaction between trastuzumab and the steroid hormone pathway into the clinical setting

4.1 Introduction

Translational studies enable researchers to recreate experimental hypotheses within clinical samples to determine whether *in vitro* findings correspond to similar outcomes within the clinical arena. This fundamental research tool allows for the quick and efficient transfer of pertinent research into medical therapies, with the potential to significantly alter clinical practice and patient outcomes.

In order to assess whether the *in vitro* findings correlated with clinical outcomes, translational studies were performed. Two hypotheses were investigated. Firstly, similar to the experimental model, did treatment with trastuzumab result in unregulated activation of the steroid hormone pathway? To demonstrate this hypothesis, three patients were identified with metastatic disease who received trastuzumab prior to resection of their breast cancer as well as a matched control patient who received neoadjuvant chemotherapy but did not receive trastuzumab as she did not over-express HER2. Tissue from the surgical resection specimens as well as from the pre-treatment core biopsy specimens underwent immunohistochemical analysis (as described in 2.6) to determine expression of pS2 and to assess for changes in the tissue expression levels of the ER target gene to identify trastuzumab-related increased activity of the steroid hormone pathway.

Secondly, it was endeavoured to establish whether the *in vitro* results had any significant correlation within a large patient cohort. After establishing the tissue microarray and gathering the relevant clinicopathologic details of all patients including receptor status, tumour grade and stage, disease recurrence as well as various treatment regimes such as trastuzumab therapy, immunohistochemical analysis was performed of these specimens to determine the tissue expression levels of pS2. The *in vitro* results show that trastuzumab increases the activity of the steroid hormone pathway through receptor crosstalk. Therefore the clinical data was analysed to assess for a correlation between pS2 expression and the clinicopathologic parameters, in particular disease recurrence and its relationship with the administration of trastuzumab with a view to establishing an activated

steroid hormone pathway as the link between trastuzumab treatment and disease recurrence.

4.2 Aims

The aim of the work presented in this chapter was to investigate whether the *in vitro* findings were translatable into the clinical arena. The following specific objectives were defined:

a. Identification and immunohistochemical staining of patients who received trastuzumab prior to surgery with a comparison of pS2 in tissue samples pre and post treatment
b. Immunohistochemical staining of a constructed tissue microarray to determine tissue expression levels of pS2
c. Correlation of pS2 expression levels and disease recurrence with various clinicopathological features of the relevant specimens

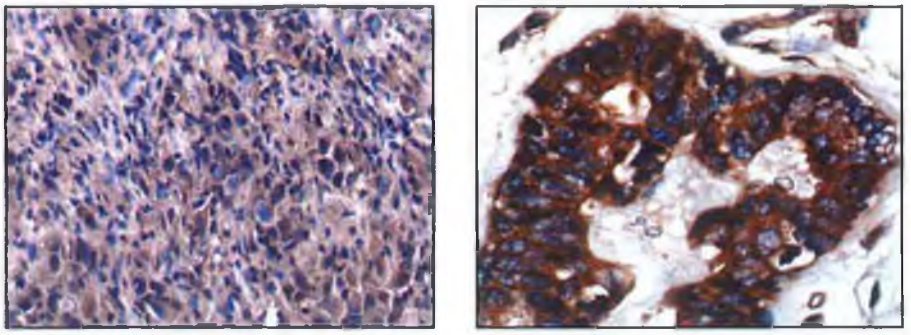
4.3 Results

4.3.1 Immunohistochemistry for pS2 following pre-operative trastuzumab

The use of trastuzumab in the neoadjuvant setting is not currently routine clinical practice therefore the number of patients who have, to date, received trastuzumab prior to surgical intervention in the two institutions involved in the study is relatively low. In this study, three patients with HER2 positive breast cancer were identified. They all presented with metastatic disease and so were initially treated with trastuzumab and chemotherapy, with subsequent resection of their breast tumours. The mean time to surgery was 9 months (range 2 - 15 months). A case-matched patient who received neoadjuvant chemotherapy but not trastuzumab as she did not over-express HER2 was used as a control. Immunohistochemistry staining of both the pre-treatment core biopsy and the post-treatment surgical resection specimen for pS2 was performed (figure 4.1). Unfortunately, tissue from the core biopsy of one of the patients who received trastuzumab prior to surgical resection was not available for inclusion.

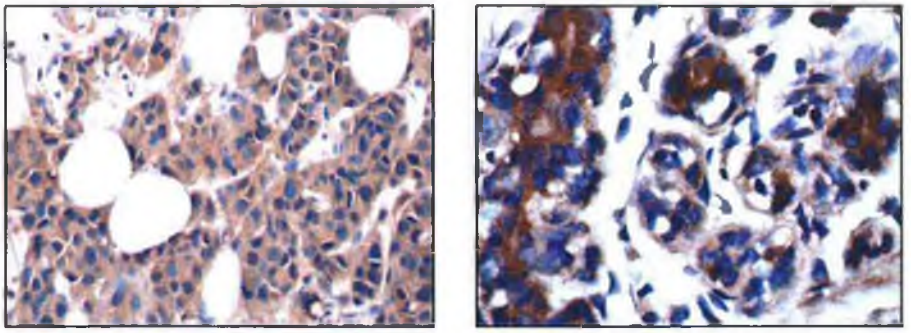
Staining of the pre-treatment core biopsy specimens showed low expression levels of pS2 with both of the post-treatment surgical resection specimens showed significantly stronger staining for pS2. Both the biopsy specimen and the surgical resection specimen from the control patient showed similar staining for pS2 indicating that the increased expression observed in the trastuzumab treated patients was not a result of the chemotherapy. Instead the increased expression could be attributed to the activity of the steroid hormone pathway being stimulated by trastuzumab as previously demonstrated *in vitro*.

A.



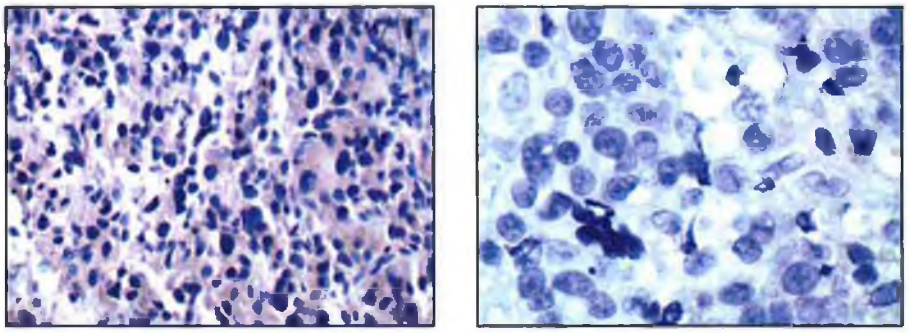
Core biopsy (x200) *Surgical specimen (x600)*

B.



Core biopsy (x200) *Surgical specimen (x600)*

C.



Core biopsy (x200) *Surgical specimen (x600)*

Figure 4.1: *Core biopsies (x200 magnification) and surgical resection specimens (x600 magnification) from patients who received trastuzumab prior to surgery as well as a case-matched control patient were stained for pS2 using immunohistochemistry. A & B: patients who received trastuzumab prior to surgery had greater expression of pS2 in the surgical resection specimens than core biopsy specimens. C: similar expression of pS2 was observed in a patient who received neoadjuvant chemotherapy but not trastuzumab as she did not over-express HER2.*

4.3.2 Immunohistochemistry of tissue microarray for pS2 expression

As discussed in 2.6, following ethical approval, a tissue microarray was constructed from paraffin-embedded tissue specimens of breast cancers from patients treated in both Beaumont Hospital and Waterford Regional Hospital. Tissue samples from a total of 488 patients with breast cancer were stained and graded by two individuals using the Allred scoring system (Allred, Clark et al. 1993) with samples graded according to the area of tissue stained and the average intensity of staining of tumour cells (see section 2.6.1) . In those tumour samples in which pS2 was expressed, it was demonstrated within the cytoplasm of cells (figure 4.2). pS2 was found to be expressed in just over 70% of tissue samples, which is in keeping with previously documented expression levels (Soubeyran, Wafflart et al. 1995). On univariate analysis, the only clinicopathological parameter that had a significant difference between pS2 positive and negative tumours was HER2 status, with HER2 positive tumours less likely to express pS2 than negative tumours ($p=0.048$). On multivariate analysis, there was no difference noted in tumours that expressed pS2 to those that did not with regard to any of the standard clinicopathological parameters, including receptor status, lymph node metastases, tumour stage or tumour grade.

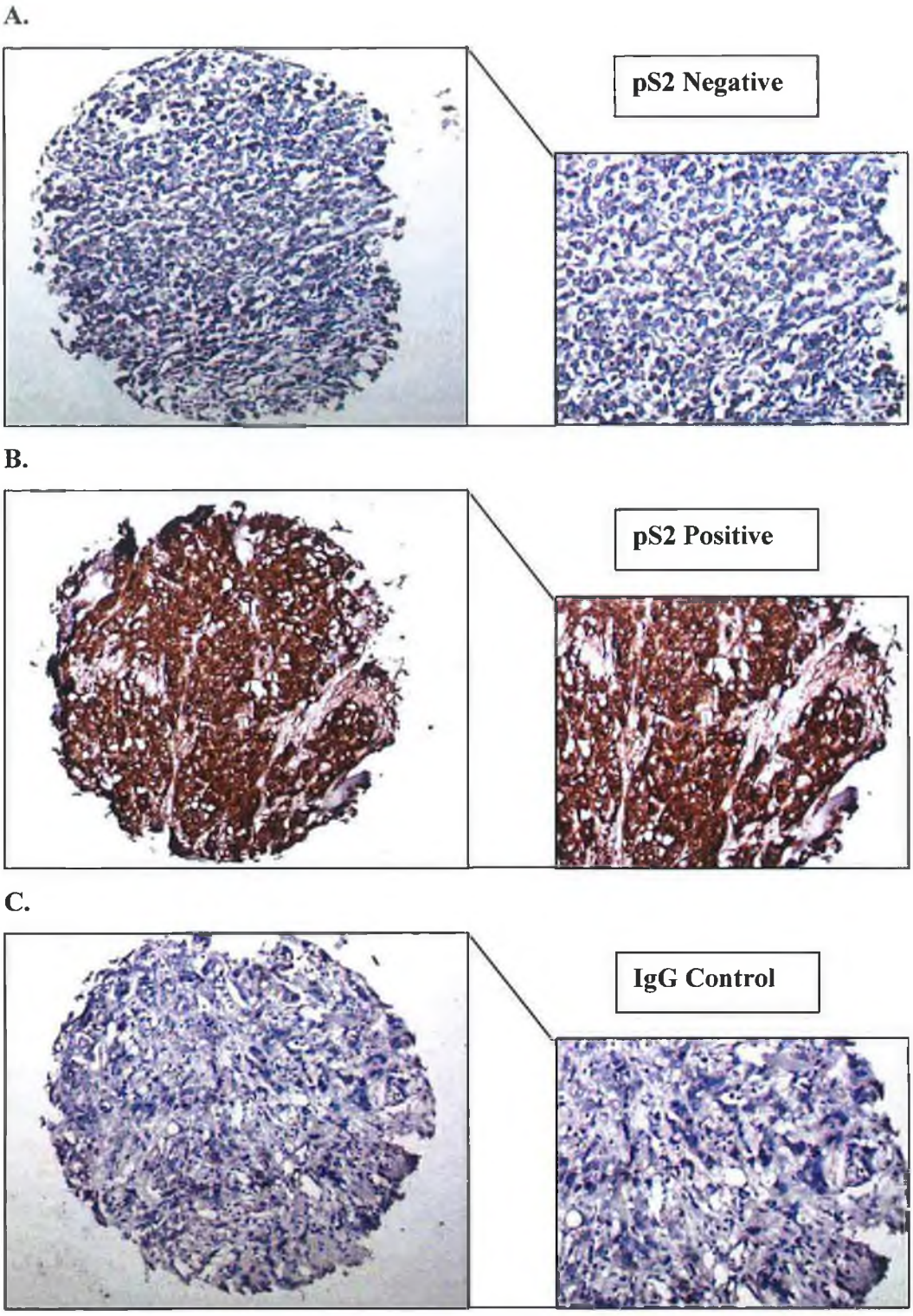


Figure 4.2: *A tissue microarray containing 488 cores of breast tumours was stained for pS2 using immunohistochemistry. Images are representative for a) tumour core negative for pS2; b) tumour core positive for pS2; c) tumour core stained with rabbit IgG control. Images are x100 magnification (left) and x200 magnification (right).*

4.4 Clinical ramifications of receptor crosstalk

To date, there is no significant data published examining the effect of trastuzumab on the steroid hormone pathway and the clinical ramifications of this crosstalk. Survival analysis of forty HER2 positive patients who expressed pS2 on immunohistochemistry (i.e. Allred score > 3) showed that patients had a significantly increased rate of recurrence if they received trastuzumab compared to those who did not (Wilcoxon Breslow test for equality of survivor function p=0.0315) (figure 4.3).

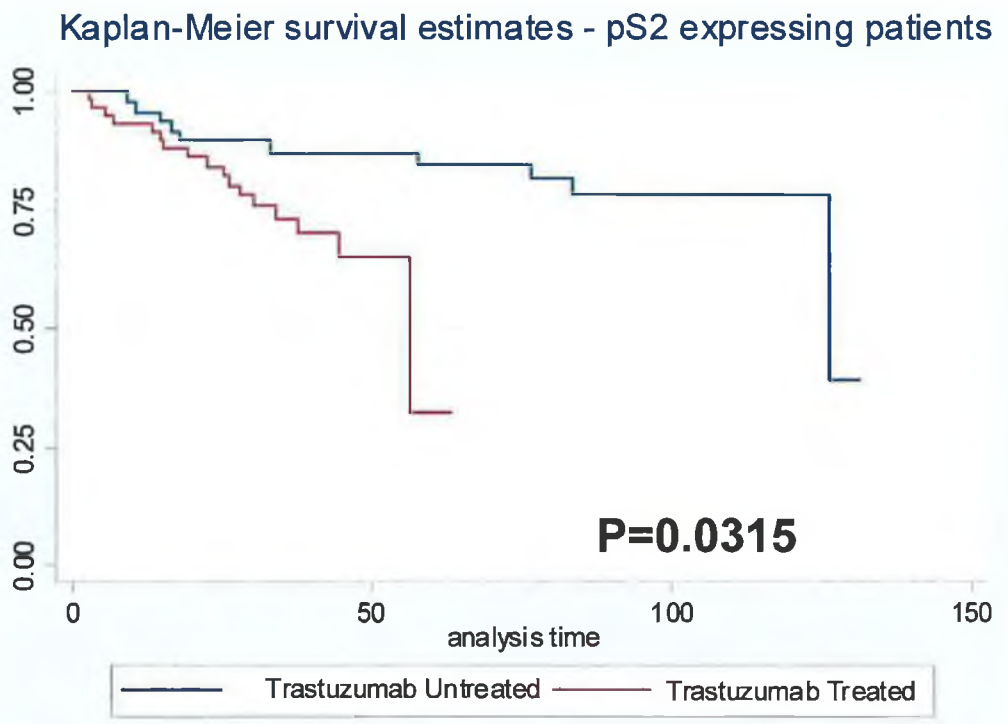


Figure 4.3: *Kaplan-Meier survival estimates were used to plot and compare disease recurrence in HER2 positive patients who expressed pS2 on immunohistochemistry. There were significantly more recurrence events noted during the follow up period in patients who received trastuzumab than patients who did not (Wilcoxon Breslow test for equality of survivor function p=0.0315).*

4.5 Discussion

In this chapter immunohistochemistry staining for pS2 of a tissue microarray containing samples from almost five hundred breast cancer patients was performed to correlate the *in vitro* findings with outcomes from the clinical arena. Tissue from a second smaller cohort of patients who received trastuzumab prior to surgical intervention was also stained. Statistical analysis was performed to compare staining intensity with tumour clinicopathological features and recurrence of disease.

The correlation between pS2 and standard clinicopathological parameters such as tumour grade and lymph node status has been widely debated with some studies finding an inverse correlation between the two (Stonelake, Baker et al. 1994; Racca, Conti et al. 1995; Soubeyran, Wafflart et al. 1995; Ioachim, Tsanou et al. 2003) and others finding no correlation (Foekens, van Putten et al. 1993; Gion, Mione et al. 1995; Nichols, Ibrahim et al. 1995). While the percentage of pS2-expressing samples was in keeping with previously published studies (Soubeyran, Wafflart et al. 1995), similarly there was no significant association found between pS2 and standard clinicopathological parameters. pS2 has, however, been well documented as an ER target gene (Masiakowski, Breathnach et al. 1982; Prud'homme, Fridlansky et al. 1985; May and Westley 1986; Foekens, van Putten et al. 1993). Therefore positive immunohistochemistry staining in this study highlights as active steroid hormone pathway. Looking specifically at HER2 positive patients who also had an active steroid hormone pathway indicated by their expression of pS2, there was a significantly increased rate of disease recurrence in the population of patients who received trastuzumab compared to those who did not. Similarly, in a smaller cohort of patients who received trastuzumab treatment prior to surgery, the expression of pS2 was markedly increased in the post-treatment specimens compared to the initial biopsy specimens. Samples from a case-matched control who received neoadjuvant chemotherapy did not show a change in expression levels confirming that the difference noted was not as a result of the chemotherapeutic agents.

Multiple randomised controlled trials have shown the benefit of trastuzumab therapy on disease free survival and overall survival in patients both with metastatic HER2

positive breast cancer (Slamon, Leyland-Jones et al. 2001; Marty, Cognetti et al. 2005) and in the adjuvant setting (Romond, Perez et al. 2005; Smith, Procter et al. 2007; Joensuu, Bono et al. 2009). Neoadjuvant chemotherapy in conjunction with neoadjuvant and adjuvant trastuzumab has also recently been shown to improve prognosis in locally advanced or inflammatory breast cancer when compared to neoadjuvant chemotherapy alone (3-year event-free survival, 71% [95% CI 61—78; n=36 events] with trastuzumab, *vs* 56% [46—65; n=51 events] without; hazard ratio 0·59 [95% CI 0·38—0·90]; p=0·013) (Gianni, Eiermann et al. 2010). While the benefit of trastuzumab in the treatment of HER2 positive metastatic or locally advanced breast cancer is indisputable, the timing of therapeutic regimes is not as clear cut. The timing of trastuzumab therapy in relation to chemotherapy has been investigated in a number of phase II and III clinical trials, however there has yet to date been a large randomised controlled trial assessing the impact of trastuzumab treatment with concurrent *vs* sequential hormonal therapy. Therefore, it remains unclear whether trastuzumab should precede, be added to or follow hormonal therapy in the subgroup of patients who have HER2 positive, ER positive breast cancer. One randomised controlled trial has looked at the addition of trastuzumab to an aromatase inhibitor in metastatic disease and found that this prolonged progression-free survival (Kaufman, Mackey et al. 2009). However, the trial did not compare concurrent therapy with sequential therapy (trastuzumab followed by an aromatase inhibitor), trastuzumab monotherapy or the use of chemotherapeutic agents in this patient population.

As previously discussed, the results in this chapter identify an increased rate of disease recurrence in HER2 positive, pS2 expressing patients who received trastuzumab compared to those who did not. We hypothesise that this increase in disease recurrence is, at least in part, due to the effect of trastuzumab on the steroid hormone pathway as previously demonstrated *in vitro* in chapter 3, with cellular plasticity allowing the tumour cells to overcome the effect of trastuzumab on growth and proliferation through the adaptive mechanisms of crosstalk between the signalling pathways. This effect is further demonstrated when tissue samples were compared from patients before and after they received trastuzumab with a definitive increase in the expression levels of pS2 following treatment noted, again

highlighting the effect of trastuzumab on the activity of the steroid hormone pathway.

These findings suggest a relationship between trastuzumab treatment and activity of the steroid hormone pathway that has not previously been investigated to a significant extent in pre-clinical or clinical trials. If, as we hypothesise, trastuzumab increases activity of ER through the steroid hormone pathway, then current clinical practice of treatment with sequential therapy of adjuvant trastuzumab and chemotherapy followed by hormonal therapy does not prevent the upregulation of the steroid hormone pathway by trastuzumab. In fact, it could potentiate the proliferation of residual cancer cells in an uncontrolled manner. While further research is required to determine the molecular pathway involved in the crosstalk between the signalling pathways, there is also the need to translate our results in to the clinical arena to further investigate our findings in a larger cohort of patients. Indeed, concurrent treatment of trastuzumab with an anti-ER agent such as tamoxifen might negate the effect of trastuzumab on the steroid hormone pathway, a change in the current therapeutic management of breast cancer which warrants further investigation.

Chapter 5

General Discussion

5.1 General Discussion

Despite recent phenomenal advances in both the detection and treatment of breast cancer, it still continues to affect one in ten women in the western world and remains the leading cause of cancer-related mortalities in women in Ireland today. This is due to a number of factors, not least the complex nature of the disease itself. Indeed, breast cancer is now not considered to be a single disease entity but rather a spectrum of disorders that vary immensely in disease pathology and progression which can affect response to treatments and overall patient prognosis. The ability to identify patients who would benefit from currently available adjuvant and neoadjuvant therapies as well as those for whom chemo and hormonal therapies would have no benefit still remains a challenge for clinicians today. To date, the use of clinicopathological parameters such as receptor status and lymph node positivity have been invaluable in informing the clinical management of breast cancer. The number and variety of therapies currently available has increased significantly over the last two decades. However, due to the complex nature of the disease and an, as yet, incomplete understanding of the intricate relationships among the various signalling pathways involved, targeted therapy that is selected based on a patient's individual cancer is not yet possible.

Indeed, a significant challenge that is faced by clinicians and patients is the development of resistance by cancers to therapeutic agents, with a significant number of patients with good prognostic tumour profile developing recurrence that does not respond to first-line therapies. The plasticity of the cancer cell allows it to adapt its signalling mechanisms to overcome treatments resulting in cell proliferation and tumour recurrence that does not respond to the original therapies. The challenge still remains to develop the ability to individualise treatment regimes according to tumour characteristics resulting in maximum patient benefit with minimal co-morbid effects and decreased risk of resistance developing. Elucidation of the mechanisms of how a tumour can adapt to therapy and consequently reoccur will provide mechanistically targeted therapies which will positively impact patient prognosis and survival.

One mechanism by which ER positive tumours are thought to develop resistance to hormonal therapies such as tamoxifen is through crosstalk between the steroid hormone pathway and the growth factor receptor pathway. Various protein kinases have been implicated in this crosstalk including Src (Razandi, Pedram et al. 2003), ERK1 and ERK2 (Kato, Endoh et al. 1995; Razandi, Pedram et al. 2003), PI3K (Campbell, Bhat-Nakshatri et al. 2001) and P90RSK (Joel, Smith et al. 1998) among others, with preclinical studies confirming the association of tamoxifen resistance with increased levels of various kinases (Schiff, Reddy et al. 2000).

With crosstalk between the two signalling pathways playing a significant role in resistance to endocrine therapy, we hypothesised that it must also play a part in the development of resistance to anti-HER2 therapies such as trastuzumab. Previous work by Xia *et al* has implicated ER signalling in the development of resistance by breast cancer cells to lapatinib, a therapeutic target of EGFR and HER2 (Xia, Bacus et al. 2006). We therefore determined to further elucidate the possibility of crosstalk playing a role in the development of resistance to trastuzumab.

Our first aim was to investigate our hypothesis *in vitro* using breast cancer cell models to replicate various tumour phenotypes (chapter 3). As suspected, treatment of endocrine sensitive MCF7 cells with trastuzumab resulted in the upregulation of the steroid hormone pathway. Similar upregulation was not observed in the endocrine insensitive LCC1 cells suggesting that trastuzumab might only influence the steroid hormone pathway in the presence of oestrogen sensitive ER. However, further molecular research is required to investigate this hypothesis. Subsequent immunohistochemical analysis of patient tumour samples looking specifically at the activity of the steroid hormone pathway found a significant increase in the rate of disease recurrence in HER2 positive patients who received trastuzumab compared to those who did not (chapter 4). We also observed increased levels of pS2 expression in a separate cohort of patient's tissue samples following treatment with trastuzumab. This, in conjunction with the previously described *in vitro* findings lends credence to our hypothesis that trastuzumab can drive activity through the steroid hormone pathway, increasing ER function leading to cell proliferation and tumour growth.

A second finding from our *in vitro* research was the expression of a functional ER in a well established ER negative breast cancer cell line following trastuzumab treatment (chapter 3). Epigenetic modifications have been shown to play a significant role in ER gene expression (Ottaviano, Issa et al. 1994; Yang, Ferguson et al. 2000). In both normal and the majority of ER positive breast cancer cell lines, the ER CpG island has been shown to be unmethylated, whereas it is methylated in most ER negative breast cancer cell lines (Lapidus, Nass et al. 1998). Reduced or absent ER expression is a result of methylation of these CpG islands and can be reversed through demethylation (Ferguson, Lapidus et al. 1995). We confirmed that the expression of ER following trastuzumab treatment was due to demethylation of the CpG islands within the ER promoter region.

The presence of crosstalk between the steroid hormone and growth factor receptor pathways reflects the plasticity of tumour cells and their ability to upregulate receptors to counteract the treatments used to combat them. Whether this mechanism has the potential to occur in all tumour cells or within a select few is not yet determined. By the latter process, treatment with certain therapeutic agents such as trastuzumab would result in the death of tumour cells that are sensitive to HER2 receptor inhibition. However, it would facilitate the differentiation of certain cells that possess the ability to proliferate through receptor crosstalk thereby allowing tumour growth in the presence of supposed appropriate therapies. Whether the phenomenon occurs through the former or latter mechanism is beyond the remit of this thesis however it does warrant further investigation in future work. Our results have identified the phenomenon of receptor crosstalk and so raise the question of how to tackle this problem within clinical practice.

The first issue that is raised is the timing of therapeutic interventions, particularly in HER2 positive, ER positive tumours. Currently, clinical practice is to treat suitable HER2 positive patients with trastuzumab concurrently with adjuvant chemotherapy with sequential tamoxifen or other anti-oestrogen therapy. However, our results would propose that this would result in uncontrolled stimulation of the steroid hormone pathway and therefore concurrent treatment of trastuzumab with an anti-oestrogen therapy would be preferential. The combination of trastuzumab with the aromatase inhibitor anastrozole has been shown to improve progression free survival

in patients with metastatic breast cancer (Kaufman, Mackey et al. 2009), however, as previously discussed in chapter 4, there were a number of limitations to this study. There are a number of clinical trials currently underway which are assessing the effect of concurrent trastuzumab and anti-oestrogen treatments (NCT00022672 ; NCT00238290 ; NCT00499681) which should help to determine the benefit of concurrent *vs* sequential treatment. Not all tumours are likely to progress and relapse during trastuzumab treatment therefore the use of concurrent treatment is unnecessary in a number of patients. Further research is required to identify characteristic tumour markers which would be able to distinguish cancers requiring concurrent therapy due to their predisposition to recurrence through crosstalk from those who are less likely to reoccur.

The second issue relates to our observation that trastuzumab treatment can result in the expression of a functionally active ER in ER negative breast cancer cells. This raises the question of the need for anti-oestrogen therapy in patients with ER negative tumours who are receiving trastuzumab therapy. Currently, if a tumour is found not to express ER, then the patient does not receive anti-oestrogen therapy, regardless of HER2 status. However, if trastuzumab treatment was to result in the re-expression of ER then these tumours would be able to proliferate due to the uncontrolled activity of the steroid hormone pathway. It is clear that not all patients with ER negative HER2 positive tumours develop re-expression of ER following trastuzumab treatment or else there would have been evidence of such a phenomenon within the multiple clinical trials assessing the efficacy of trastuzumab. Our findings suggest that a proportion of ER negative breast cancers will express a functional ER following trastuzumab therapy. Should all patients with ER negative tumours receive anti-oestrogen therapy despite their ER status if they are to receive trastuzumab? While this will inevitably be beneficial in a number of patients, it would result in the over-treatment of patients in whom ER expression does not occur following treatment. This has both financial implications in relation to healthcare expenditure and also could potentially result in unnecessary side effects of anti-oestrogen therapy. The next step therefore is to elucidate the mechanism by which trastuzumab can cause demethylation of the CpG islands within the promoter region of the ER gene to elicit a potential biomarker that would enable clinicians to identify patients in whom anti-oestrogen therapy would be beneficial.

In fact, receptor crosstalk resulting in re-expression of ER in ER negative tumours is a mechanism that can be used to a patient's advantage. Hormone receptor status is an important prognostic indicator in overall and disease free survival rates with ER positive tumours performing significantly better than ER negative tumours. The ability to re-activate ER and therefore convert an ER negative cancer into an ER positive one would offer clinicians a therapeutic target through which they can control cell growth and tumour recurrence. The ability to identify this sub-population of patients would greatly enhance therapeutic interventions and improve patient prognosis. In fact, it might widen the subset of patients in whom trastuzumab is prescribed from current practices of treating patients with metastatic or lymph node positive disease only, to include those with ER negative local disease in whom trastuzumab has been identified to result in a switch in ER status thereby facilitating broader therapeutic intervention.

Our findings of receptor crosstalk and in particular the expression of ER following trastuzumab therapy raises questions regarding the analysis of tumour specimens to determine receptor status. Currently receptor status is based upon the primary resection specimen with metastatic disease not routinely biopsied unless it is a locally recurrent or cutaneous lesion. However, if a proportion of ER negative tumours switch ER status following adjuvant trastuzumab treatment, this will result in a number of patients with metastatic disease failing to receive appropriate hormonal therapy. In fact, a prospective study comparing the receptor status of distant metastases with that of the primary breast cancer found that there was a substantial discordance resulting in altered management in 20% of cases (Simmons, Miller et al. 2009). Clinical management of patients with metastatic disease therefore needs to consider the possibility of receptor crosstalk resulting in a change in receptor status with a possible need for a more aggressive biopsy policy of accessible metastases until molecular studies can identify primary tumours in which receptor status is likely to switch.

5.2 Conclusion

The plasticity of breast cancer and its ability to overcome therapeutic interventions through receptor crosstalk as well as its capacity to alter receptor status demonstrates the complex molecular mechanisms involved in the disease process and highlights the need for further research to uncover the relevant mechanistics. The work contained within this thesis has uncovered the primary step in unravelling this complex pathway interaction. We have demonstrated the ability of trastuzumab to influence the steroid hormone pathway thereby facilitating tumour recurrence through receptor crosstalk which we have shown had a negative impact on disease recurrence within our patient population. We have also for the first time demonstrated the ability of a growth factor receptor inhibitor to alter the expression of ER through epigenetic modification of the gene promoter region. The findings presented in this work constitute a small fragment of a large and exciting puzzle. The understanding of the complex relationship between the steroid hormone and growth factor receptor pathways is likely to present a significant challenge to academics and clinicians for many years. However, every piece of the puzzle takes us one step closer to understanding the biology of this disease and therefore enables us to improve therapeutic options and ultimately benefits the patient.

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7 Appendix I

7.1 Cell Culture

Trypsin-EDTA

Trypsin 10 X liquid (25g/l GiBcoBRL®)

HBSS (GiBcoBRL®)

0.02% EDTA

20MM HEPES

1ml of trypsin and 1 ml of 0.02% EDTA was made up to 10ml HBSS filter prior to use through Acrodic® 32 filters (0.2μm pore size)

Treatment conditions

17β-estradiol (E_2)	10^{-8}M concentration
4-hydroxytamoxifen (4-OHT)	10^{-8}M concentration
Trastuzumab	100μM/ml concentration
Control	Sterile water

Phosphate buffered saline (PBS)

Dissolve one PBS tablet per 200mls dH_20

Each PBS tablet contains:

- 0.01M phosphate buffer
- 0.0027M potassium chloride
- 0.137M sodium chloride

Autoclave and filter prior to use

7.2 Western blot analysis

Protein standards used to create the standard curve for BCA assay

Standard	*Stock Albumin (µl)*	*dH_2O (µl)*	*Protein Concentration (µg/ml)*
A	0	100	0
B	10	90	200
C	20	80	400
D	30	70	600
E	40	60	800
F	50	50	1000
G	60	40	1200
H	70	30	1400

Radioimmunoprecipitation assay (RIPA) lysis buffer

1x PBS	495mls
1% Ipe	5mls
0.5% Deoxycholate	2.5g
0.1% SDS	0.5g

Tris buffered saline (TBS) (20X)

121.1 g Tris

175.5 g NaCl

Made up to 1 litre with dH2O

Use at 1X final concentration, pH 8.3

Wash buffer (TBS-T)

1x TBS

0.05% Triton X-100

1M Tris-HCl, pH 6.8

157.6g Tris-HCl

Made up to 1 litre with dH_20, pH 6.8

1.5 M Tris.HCl, pH 8.8

236.4g Tris- HCl

Made up to 1 litre with dH2O, pH 8.8

Blocking buffer

1X TBS

0.05% Triton® X-100

5% Molecular grade skimmed milk

Transfer buffer:

2.93 Glycine

5.8 g Tris Base

0.375 g SDS

200 mL Methanol

Made up to 1 litre with dH2O

Sample buffer (5X)

2 g SDS

5 ml 1M Tris.HCl (6.8)

3.0 ml dH2O

8 ML Glycerol

2 ml 0.1% Bromophenol Blue

Make up to 20ml with dH2O

5% ß-mercaptomethanol

Running buffer (10X)

288 gm Glycine

60.6 g Tris Base

20 g SDS

Made up to 2 litres with dH2O

Acrylamide/Bis-acrylamide 30%

Liquid easigel 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide was used directly

20% SDS

20 g SDS was dissolved in 100 ml of dH2O

10%Ammonium Persulphate

100 mg/ml was dissolved in dH2O

7.3 Nucleic acid biochemistry

10x Tris acetate EDTA buffer, pH 8.0

48.4g Tris

11.4ml glacial acetic acid

20ml 0.5M EDTA

Made up to 1 litre with dH_20, pH 8.0

7.4 Immunohistochemistry

Sodium citrate buffer, pH 6.0

2.94g 10mM sodium citrate

500µl 0.05% Tween

Made up to 1 litre with dH_20, pH 6.0