

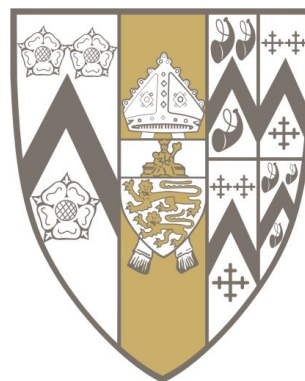
Response and resistance to lapatinib in HER2-overexpressing breast cancer

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Abstract

HER2 overexpression has been implicated in breast cancer. Lapatinib is a dual EGFR/HER2 tyrosine kinase inhibitor used in advanced HER2-positive breast cancer patients who have previously progressed on trastuzumab treatment. Recent meta-analysis of several clinical trials showed that lapatinib was inferior to trastuzumab as a neoadjuvant treatment combined with chemotherapy; however, the mechanism underlying this difference remains unclear.

This thesis aimed to assess the acute response and mechanism of resistance to lapatinib in HER2-overexpressing breast cancer cell lines SK-BR-3 and BT-474. Lapatinib inhibited the phosphorylation of EGFR, HER2 and HER3, but it recovered after eight hours of treatment. Lapatinib treatment induced NRG1 expression, correlating with this recovery. Lapatinib-resistant cell lines, SK250LR and BT250LR, were generated. These cells had an increased NRG1 mRNA expression, but the expression levels reduced within a week of lapatinib withdrawal.

Exogenous NRG1 rescued breast cancer cells from lapatinib inhibition, because lapatinib only incompletely inhibited NRG1-induced HER3 and HER4 signalling pathways. Combining lapatinib with pertuzumab (L+P) improved the inhibition on NRG1-induced signalling. This difference in inhibitory abilities correlated with the effect of treatment, as L+P induced growth inhibition and apoptosis more than either drug alone. *In vivo* experiments also showed that L+P triggered greater tumour regression than monotherapy in a BT-474 xenograft model. L+P induced more apoptosis in BT250LR cells but not in SK250LR cells, but it required further validation in a lapatinib-resistant xenograft model.

Analysis of RTKs activation suggested that SK250LR and BT250LR cell lines had different mechanisms of resistance. The SK250LR cell line underwent permanent changes in cell biology and became independent from EGFR/HER2 signalling. The BT250LR cell line developed a transient adaption by increasing HER signalling. Eight new RTK candidates were discovered, but their role in lapatinib resistance required further validation. This study suggested a novel strategy of drug combination between L+P in HER2-targeted therapy, which is promising for future investigation. It also uncovered new directions in RTK studies for their roles in lapatinib resistance.

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Abbreviations

ADAM	A disintegrin and metalloproteinase
AI	Aromatase inhibitor
Akt/PKB	Protein kinase B
ALK	Anaplastic lymphoma kinase
ALTTO	Adjuvant Lapatinib and/or Trastuzumab Treatment Optimisation
AML	Acute myeloid leukaemia
Ang	Angiopoietin
AR	Amphiregulin
ATP	Adenosine triphosphate
AV	Annexin V
BSA	Bovine serum albumin
BTC	Betacellulin
C-terminal	Carboxyl terminal
cAMP	Cyclic adenosine monophosphate
Cbl	Casitas B-lineage lymphoma protein
cDNA	Complementary DNA
CDK2	Cyclin-dependent kinase
Chk	C-terminal Src kinase-homologous kinase
CTF	Carboxyl-terminal fragment
DAB	Diaminobezidine
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ECD	Extracellular domain
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EML4-ALK	Echinoderm microtubule-associated protein-like 4-Anaplastic lymphoma kinase
Eph	Ephrin receptor

EPR	Epiregulin
EPG	Epigen
ER	Oestrogen receptor
FBS	Foetal bovine serum
FGFR	Fibroblast growth factor receptor
FOX	Forkhead box protein substrate
FRS	Fibroblast growth factor receptor
G protein	Guanosine nucleotide-binding protein
Gas6	Growth arrest-specific gene 6
GEF	Guanine nucleotide exchange factor
GI ₁₀	10% of growth inhibition
GI ₅₀	50% of growth inhibition
GDNF	Glial derived neurotrophic factor
Grb	Growth factor receptor-bound protein
HB-EGF	Heparin-binding epidermal growth factor
HRP	Horseradish peroxidase
HER	Human epidermal growth factor receptor
HGF	Hepatocyte growth factor
HGH	Human growth hormone
HRG/NRG	Heregulin/neuregulin
IC ₅₀	Half maximal inhibitory concentration
IDC-NOS	Invasive ductal carcinoma not otherwise specified
IDC-NST	Invasive ductal carcinoma of no special type
IHC	Immunohistochemistry
IHC-P	Immunohistochemistry – paraffin embedded
IP3	Inositol 1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
LDS	Lithium dodecyl sulphate
mAb	Monoclonal antibody
MAPK/Erk	Mitogen-activated protein kinase/Extracellular signal regulated kinase
MBC	Metastatic breast cancer
MOPS	3-Morpholinopropane-1-sulfonic acid
MK	Midkine
mRNA	Messenger RNA
MSPR	Macrophage-stimulating protein receptor

NeoALTTO	Neo-Adjuvant Lapatinib and/or Trastuzumab Treatment Optimisation
NMP-ALK	Nucleophosmin-Anaplastic lymphoma kinase
NSCLC	Non-small cell lung cancer
OD	Optical density
OS	Overall survival
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline (Dulbecco A)
PCR	Polymerase chain reaction
pCR	Pathological complete response
PFA	Paraformaldehyde
PFS	Progression-free survival
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PR	Progesterone receptor
PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homolog
PTN	Pleiotrophin
PVDF	Polyvinylidenedifluoride
qPCR	Quantitative real-time polymerase chain reaction
Raf	Rapidly accelerated fibrosarcoma family kinases
Ras	Rat sarcoma family kinases
RCC	Renal cell carcinoma
c-Ret	Rearranged during transfection receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RTK	Receptor tyrosine kinase
RYK	Receptor related to tyrosine kinases
SCC	Squamous cell carcinoma

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERM	Selective oestrogen receptor modulator
SH2	Src homolog 2
SH3	Src homolog 3
Shc	Src homolog 2 domain-containing protein
SOS	Son-of-sevenless
Src	Avian sarcoma viral oncogene homolog
STAT	Signal transducer and activator of transcription
TACE	Tumour necrosis factor- α converting enzyme
TAM	Tyro3/Axl/Mer receptors
TBS	Tris buffered saline
TGF- α	Transforming growth factor- α
Tie	Tyrosine kinase with immunoglobulin-like and EGF-like domains receptor
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TMA	Tumour microarray
TNBC	Triple negative breast cancer
WB	Western Blotting

1. Introduction

1.1 Breast cancer

1.1.1 Breast cancer in the UK

Breast cancer is the most commonly occurring form of cancer in women in the UK and it is the second most mortal type of cancer. In 2010, there were 49,564 women diagnosed with invasive breast cancer and 5,765 cases of breast cancer *in situ*. The number of deaths caused by breast cancer in women was 11,556 (CancerResearchUK, 2013). The incidence rate of breast cancer in Great Britain increased by 72% from the mid-1970s to 2010, but it has stayed relatively stable after the mid-2000s (Figure 1-1).

Although breast cancer is the second most common cause of cancer death, the mortality rate has been decreasing since 1990 (Figure 1-2). The five-year survival rate of women with breast cancer in England and Wales was 85.1% during 2005–2009. The improvement is likely to be caused by better detection of breast cancer through screening but mostly through an increased effectiveness in systemic treatments, such as the use of tamoxifen, chemotherapy, aromatase inhibitors and trastuzumab, and optimised radiotherapy (CancerResearchUK, 2013).

1.1.2 Classification of breast cancer

Traditionally, breast cancers are classified according to their histopathological characteristics in conjunction with several immunohistochemistry (IHC) markers. In the last decade, gene expression analysis provided a powerful tool for cancer molecular subtyping. This advancement offered important information with prognostic values and improved our knowledge in the complexity of the disease (Engstrom et al., 2013).

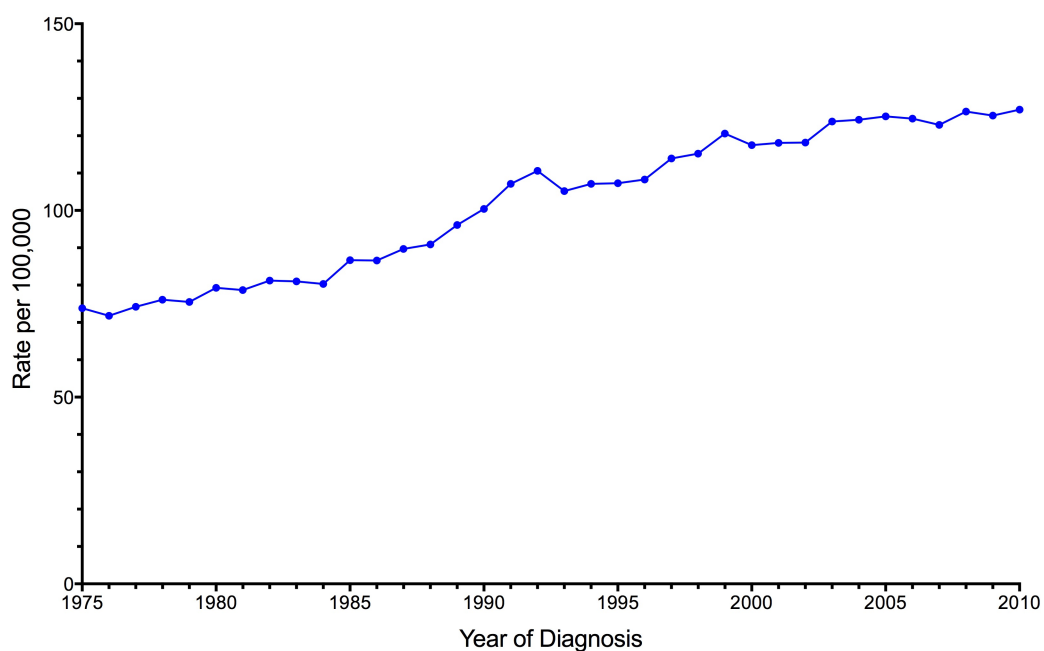


Figure 1-1 European age-standardised incidence rates of breast cancer in women of Great Britain, 1975-2010

Redrawn from Cancer Research UK, 2013.

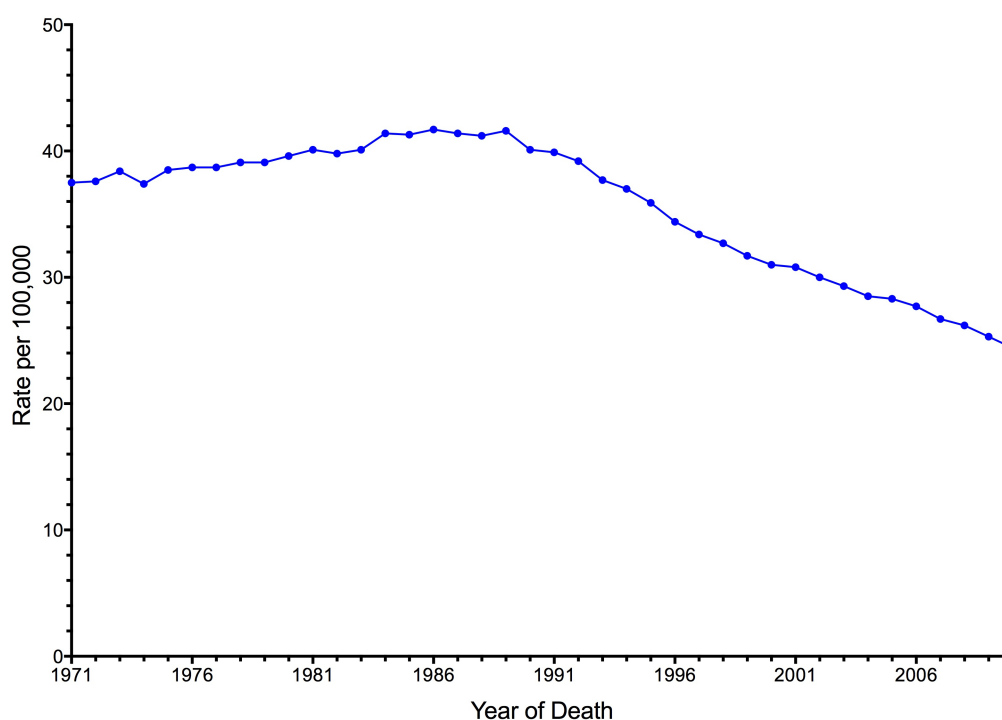


Figure 1-2 European age-standardised mortality rates of breast cancer in women of the UK, 1971-2010

Redrawn from Cancer Research UK, 2013.

1.1.2.1 Histopathological characterisation and tumour grade

Around 25% of breast cancers are classified based on their histological features into different subgroups such as lobular, tubular, medullary and inflammatory carcinoma. The rest of breast cancers fall into a group called invasive ductal carcinoma of no special type (IDC-NST) or not otherwise specified (IDC-NOS). NST or NOS is a heterogeneous group of breast cancers that cannot be classified into any histological group because of the lack of histological characteristics (Ellis et al., 2003). Most of the histological subtypes display homogeneity in transcriptome and molecular markers, meaning that tumours in the same histological subtype may share a similar mechanism of tumourigenesis (Weigelt et al., 2008). However, most cases of breast cancers are IDC-NST and hence the use of histological typing is limited.

In addition to histological typing, tumour histological grade provides useful prognostic information. Tumour grade assesses the degree of differentiation and growth rate of a tumour by evaluating tubule formation, nuclear pleomorphism and mitotic count (Rakha et al., 2010).

1.1.2.2 Immunohistochemistry markers

Immunohistochemistry staining of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) provides important molecular markers for breast cancer classification (Onitilo et al., 2009). These markers are important in the determination of treatment plans for the breast cancer patients, leading to their improved survival. Hormone therapies such as selective ER modulators (SERMs), for example, tamoxifen, and aromatase inhibitors (AIs), for example, anastrozole, are now commonly used in ER-positive breast cancer, which accounts for about 70% of patients. These therapies greatly improve the recurrence-free and overall survivals, and disease prevention in this subgroup (Morandi et al., 2011). Around 20% of patients are diagnosed

as HER2-positive and they are given HER2-targeted therapies such as trastuzumab (Arteaga et al., 2012). The remaining portion of breast cancers is classified as triple negative breast cancer (TNBC) because of the lack of ER, PR and HER2 expression. This group has limited treatment options because of the absence of therapeutic targets (Dawson et al., 2009).

1.1.2.3 Molecular subtypes based on genomic analysis

Using DNA microarray, breast cancers are classified into molecular subtypes based on the gene expression profile of around 500 “intrinsic” genes, which differs the least in the tumour of the same patient before and after adjuvant treatment, but varies the most among the samples of different patients. These intrinsic subtypes, namely basal, HER2+, luminal A, luminal B and normal breast-like, associate with clinical outcome such as time to develop distant metastasis and overall survival (OS) (Figure 1-3) (Sørlie et al., 2003). This classification has been later developed to an analysis of only 50 intrinsic genes (PAM50) (Nielsen et al., 2010). However, this kind of subtyping is debatable as the outcome of clustering is heavily based on the number and identities of genes used for the analysis. Pusztai *et al.* showed that when a different set of genes was used for analysis, normal breast-like subtype could be distributed into the other four groups, indicating that this subtype might not even exist (Pusztai et al., 2006). In addition, basal subtype is a heterogeneous group and can be subdivided, according to an immune response gene expression analysis of ER negative tumour, into four subgroups (Teschendorff et al., 2007).

With the advance in next-generation sequencing, it is now possible to incorporate the genomic differences, such as single nucleotide polymorphism and copy number variation, into the classification of breast cancer (Dawson et al., 2013). By integrating the genomic and transcriptomic variations, 10 novel subgroups, or integrative clusters, with distinct

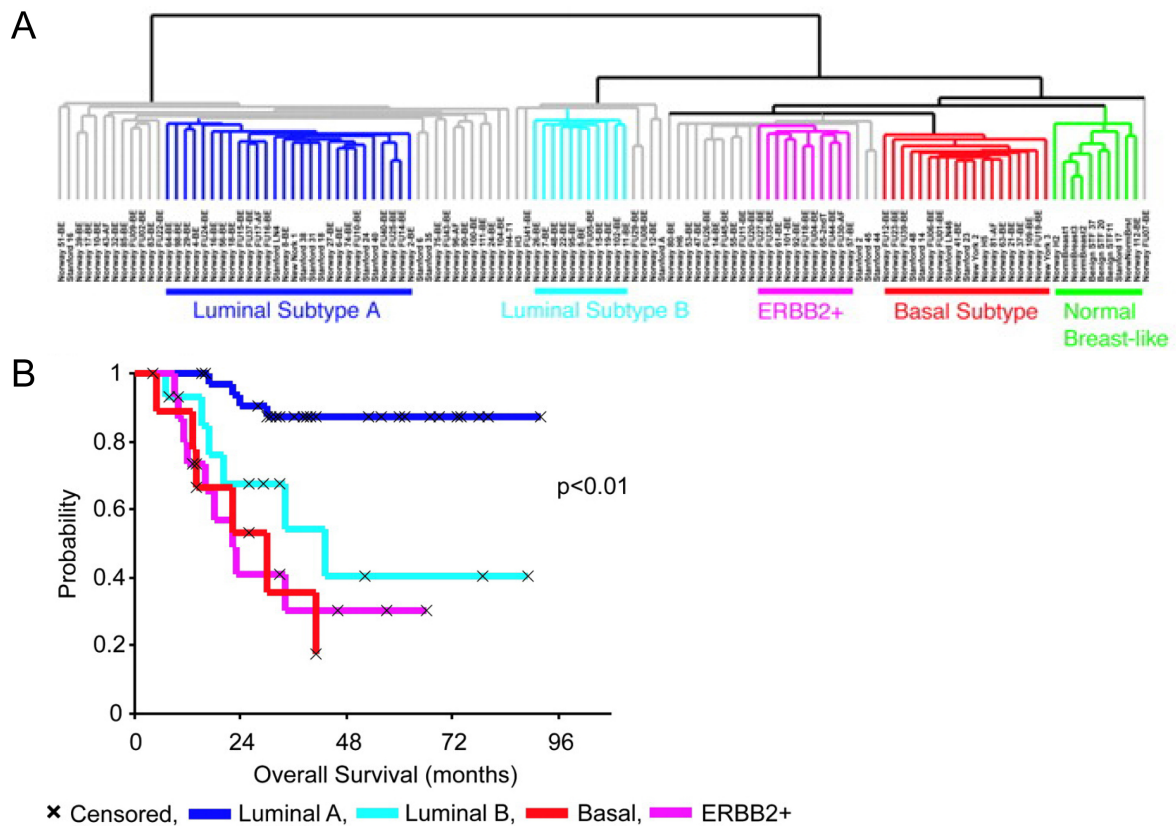


Figure 1-3 Molecular subtyping of breast cancer based on gene expression profile

From Sørlie *et al.*, 2003 (Reproduced with permission from PNAS, Copyright (2003) National Academy of Sciences, U.S.A.). (A) Dendrogram showing the clustering of tumour into subtypes. Grey branches denote tumours with low correlation with any subtype. (B) Overall survival of patients in different intrinsic subgroups (ERBB2+ = HER2+).

clinical outcome were found (Curtis et al., 2012). Interestingly, the intrinsic subtypes determined by PAM50 test, disperse among the integrative clusters, showing the heterogeneity of the intrinsic subtypes (Figure 1-4).

The study on molecular subtyping provides better understanding of breast cancer heterogeneity. Investigation on mutations, gene expression and genome integrity of these subtypes provides important information on the potential molecular drivers underlying each subgroup of cancer. This information will suggest crucial hints for the development of targeted therapies (Dawson et al., 2013).

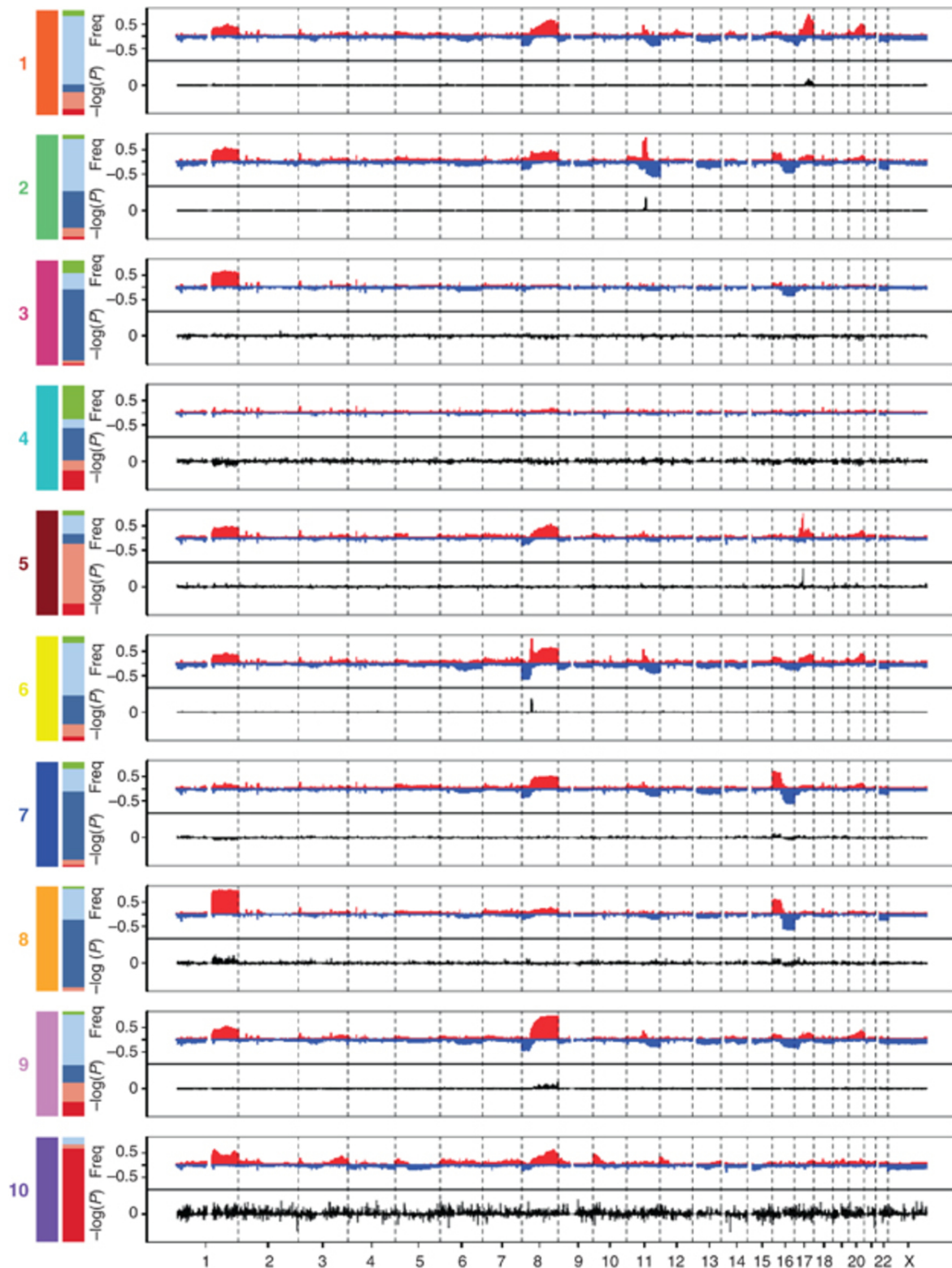


Figure 1-4 Genomic and gene expression landscape of integrative clusters

From Dawson *et al.*, 2013. (Reproduced with permission from John Wiley and Sons) Upper plot shows the genome-wide frequency of somatic copy number aberration (red: gain, blue: loss) and lower plot shows the subtype-specific association of gene expression (positive: overexpression, negative: underexpression). Intrinsic subtypes are indicated as red: basal, pink: HER2+, dark blue: luminal A, light blue: luminal B and green: normal-like.

1.2 Cell signalling cascade

A cell responds to external environment by reacting to specific chemicals and changing its enzymatic activity, gene expression and/or ion-channel activity. This process is called a cell signalling cascade. There are two main types of signalling molecules: nonpolar and polar. Nonpolar molecules such as oestrogen are able to penetrate cell membrane and bind to intracellular or nuclear receptors. Polar molecules such as hormone and molecules in food require membrane receptor proteins to transduce the signals into cytoplasm, activating secondary messengers such as cyclic adenosine monophosphate (cAMP) and inositol 1,4,5-trisphosphate (IP3) to mediate activation of effector proteins by phosphorylation. Then the signal is amplified when one effector protein activates multiple downstream molecules (Berg et al., 2002; Nelson et al., 2005).

There are several classes of membrane receptors. Seven-transmembrane-helix receptors change their conformation upon binding of ligand and then couple with guanine nucleotide-binding (G) proteins. G proteins activate adenylate cyclase and phospholipase C β to produce secondary messengers cAMP and IP3 respectively. Some receptors like human growth hormone (HGH) receptor dimerise upon ligand binding and activate their intracellular domains. Activated HGH receptors recruit protein kinases such as Janus kinase 2 to phosphorylate downstream effectors. A special class of growth hormone receptors has intrinsic kinase activity, possessing tyrosine kinase domain in their cytoplasmic domain, and hence they are named receptor tyrosine kinases (RTKs) (Berg et al., 2002; Nelson et al., 2005). RTKs, for example, the human epidermal growth factor receptor (HER) family, are discussed in the following section.

1.2.1 Receptor tyrosine kinases

RTKs are a large family with 58 members, which can be further divided into 20 subfamilies according to their ligand-binding domain sequence (Robinson et al., 2000). RTKs have a common structure that comprises an extracellular ligand-binding domain, a transmembrane domain, a cytosolic tyrosine kinase domain (TKD) and a tyrosine-rich carboxyl (C-) terminal (van der Geer et al., 1994). Most of the RTKs exist as a single polypeptide chain and monomer in the absence of ligand, except Met family receptors that consist of an extracellular α chain linked to a transmembrane β chain, and insulin receptor family members that consist of two extracellular α chains linked to two transmembrane β chains (Hubbard and Till, 2000).

When ligands bind to RTKs, two receptors dimerise and bring the cytoplasmic domains together, resulting in transphosphorylation of tyrosine residues within the kinase domain and the C-terminal regulatory sequence (Robertson et al., 2000). Activated RTKs transduce signals either by direct phosphorylation of downstream protein, for example, insulin receptor phosphorylates insulin receptor substrate 1 (Nelson et al., 2005), or the phosphorylated tyrosine residues serve as docking sites for downstream signalling molecules (Robertson et al., 2000).

1.2.2 Downstream pathways of RTKs

Activated RTKs phosphorylate the tyrosine residues on their C-terminal or their substrate proteins, creating docking sites for cytoplasmic proteins that contain Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (Reedijk and McGlade, 2005). These proteins form a signalling complex to mediate downstream signalling or catalyse the production of secondary messengers (Figure 1-5).

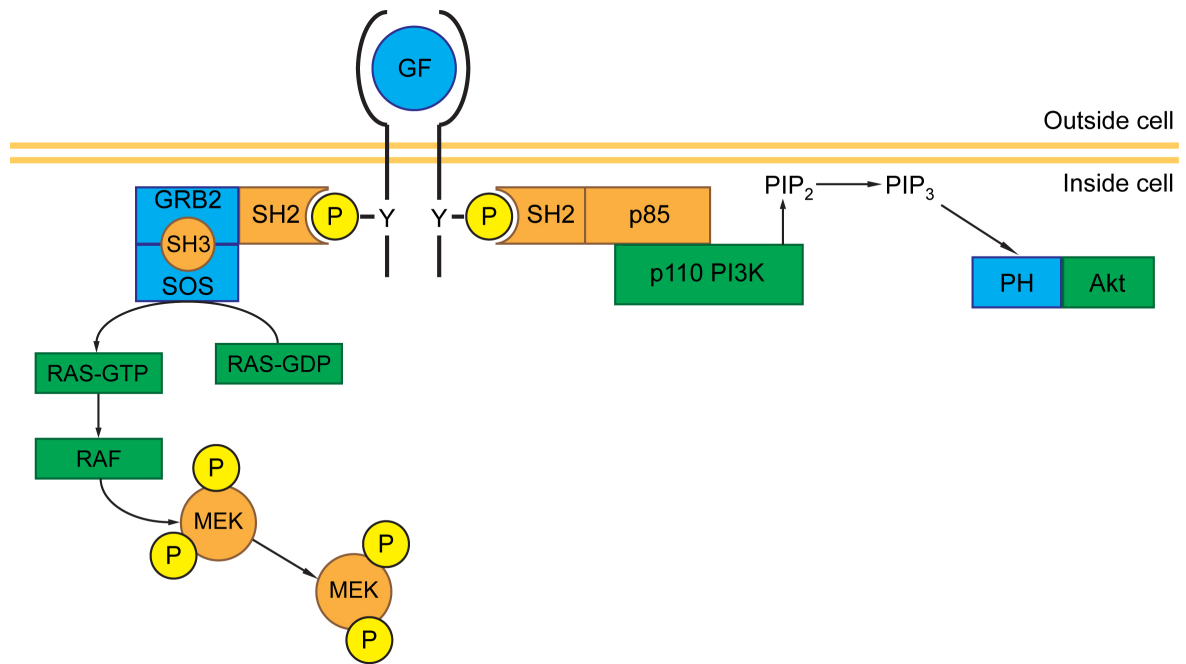


Figure 1-5 Receptor protein tyrosine kinases recruit cytoplasmic signalling molecules

Redrawn from Reedijk *et al.*, 2005. Upon ligand binding, RTKs autophosphorylate and recruit SH2 domain-containing protein, such as Grb2 and PI3K, to mediate downstream pathways.

Growth factor receptor-bound protein (Grb) 2, an adaptor protein without catalytic activity, contains SH2 and SH3 (Src homolog 3) domains. Grb2 binds to the phosphorylated C-terminal of RTKs via the SH2 domain, and recruits Son of Sevenless (SOS) and Grb2-associated-binding protein 1 via its SH3 domains. SOS is a guanine nucleotide exchange factor (GEF) that activates rat sarcoma (Ras) protein (Reedijk and McGlade, 2005). Ras is a small G protein that, when coupled with GEF, cycles between GDP-bound inactive state and GTP-bound active state, and has a central role to connect growth factor receptor and downstream pathways (Downward, 2006). Activated Ras stimulates rapidly accelerated fibrosarcoma (Raf) kinase, leading to the activation of the mitogen-activated protein kinase (MAPK) signalling pathway (Figure 1-5).

Phosphatidylinositol 3-kinase (PI3K) binds to the phosphotyrosine residue of RTKs through the SH2 domain on its p85 regulatory subunit. Once bound, the p110 catalytic subunit is activated and catalyses the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) from phosphatidylinositol (4,5)-bisphosphate (PIP2). PIP3 is a secondary messenger that binds to and activates protein kinase B (Akt/PKB) pathway (Figure 1-5).

1.2.3 Signalling pathway and cancer

Under normal circumstances, cell signalling and proliferation are carefully controlled by the regulated production and release of growth signals as well as negative feedback mechanisms. These regulation mechanisms maintain the homeostasis of cell number, tissue architecture and function. In cancer cells, cell signalling is dysregulated in one or more of the following ways: overproduction of growth factor ligands, increased level of signal receptor proteins, constitutive activation of downstream signalling pathway, and increased signal exchange with tumour-associated stroma (Hanahan and Weinberg, 2011).

Amplification, overexpression or somatic mutations of RTKs are linked to a number of human developmental diseases, such as dwarfism, craniosynostosis, and cancer (Robertson et al., 2000). Amplification and overexpression of RTKs, for example, HER2, increase the concentration of active receptors, producing constitutive and uncontrolled cell signalling that leads to carcinogenesis (Slamon et al., 1987).

Sustaining proliferative signalling is one of the six traditional hallmark capabilities of cancer (Figure 1-6), which are generalised and validated abilities involved in cancer pathogenesis (Hanahan and Weinberg, 2000, 2011). Moreover, proliferative signalling is able to activate other hallmark capabilities. For example, HER signalling pathway activates PI3K/Akt pathway. Akt phosphorylates and inhibits Bcl-2-associated death promoter protein, a promoter of apoptosis (Datta et al., 1997). Activation of Ras by HER signalling contributes to multiple hallmark abilities, such as invasion and survival, because of its extensively branched downstream pathways (Hanahan and Weinberg, 2011). The above examples illustrate that dysregulated cell signalling participates in multiple aspects of cancer pathogenesis.

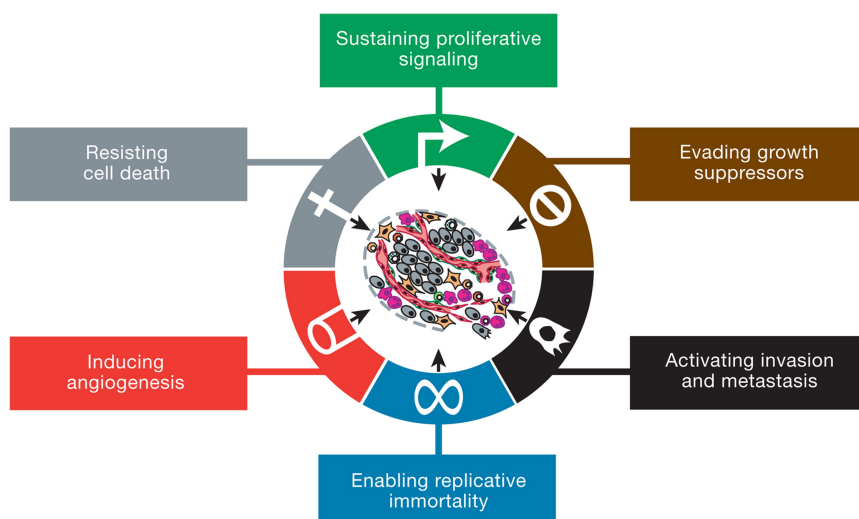


Figure 1-6 The six traditional hallmark capabilities of cancer

From Hanahan and Weinberg, 2011. (Reproduced with permission from Elsevier)

1.3 HER signalling pathway

The HER signalling pathway is a complex network involving 11 ligands and 4 RTKs. When the ligands bind and activate the RTKs, they stimulate downstream signalling and mediate apoptosis, migration, growth, adhesion and differentiation (Yarden and Sliwkowski, 2001).

1.3.1 HER receptors

The human epidermal growth factor receptor (HER, also known as ErbB) family consists of four transmembrane receptor tyrosine kinases, namely EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (Bazley and Gullick, 2005). The receptors were originally named as ErbB receptors because of the protein sequence similarity between EGFR and the v-erbB oncogenic protein of avian erythroblastosis virus (Downward et al., 1984). HER receptors are type I transmembrane RTKs that, upon ligand binding, activate their cytoplasmic tyrosine kinase domain to pass along cellular signals (Linggi and Carpenter, 2006).

The structure of a HER receptor comprises a glycosylated and disulphide-bonded extracellular ligand-binding domain, a single α -helical transmembrane domain and a large cytoplasmic domain made up of a TKD and a C-terminal tail characterised by multiple autophosphorylation sites (Baselga and Swain, 2009; Linggi and Carpenter, 2006). Figure 1-7 shows a schematic drawing of the structure of the four HER receptors. The characteristics of each HER receptor are discussed in the following sections.

1.3.1.1 EGFR

EGFR was the first RTK discovered as the membrane-bound receptor of epidermal growth factor (EGF) (Carpenter et al., 1978). It is also known as ErbB1 and HER1. Upon ligand

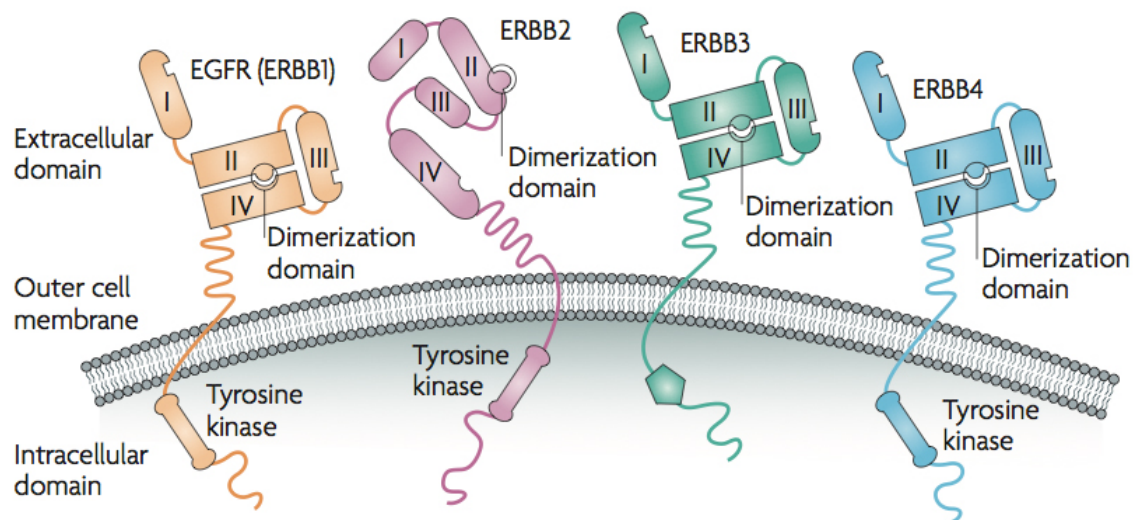


Figure 1-7 Schematic drawing of the structure of the HER receptors

From Baselga and Swain, 2009 (Reproduced with permission from Nature Publishing Group). HER family consists of four members: EGFR, HER2, HER3 and HER4 (or ErbB1–4). Each composes of a ligand-binding and dimerisation ectodomain, a transmembrane domain, a tyrosine kinase domain and a tyrosine-rich C-terminal. HER2 has constitutively “opened” ectodomain and HER3 has a defective tyrosine kinase domain.

binding, EGFR dimerises with another EGFR or other HER receptors and autophosphorylates, signalling downstream pathways. Among all members of the HER family, EGFR recruits the largest variety of signalling molecules and hence activates the widest range of downstream pathways (Schulze et al., 2005). EGFR has a few binding sites for adaptor proteins Grb2 and Shc, which recruit Ras and activate the Ras/Raf/MAPK signalling cascade (Baselga and Swain, 2009). Although EGFR has no binding sites for PI3K, the recruitment of Ras allows EGFR to trigger Ras/PI3K/Akt pathway indirectly (Citri and Yarden, 2006).

Phosphorylation of the Tyr845 residue of EGFR by avian sarcoma viral oncogene homolog (Src) kinase stabilises the activation loop of the kinase domain, maintaining the activity of the enzyme (Sato, 2013). Phospho-Tyr1045 is the docking site for ubiquitin ligase Cbl (named after Casitas B-lineage lymphoma), which mediates the subsequent ubiquitination and degradation after EGFR activation (Ravid et al., 2004). Interestingly, EGFR is the only HER family receptor that has a binding site for Cbl, making it the sole target of ubiquitination.

EGFR rapidly internalises via endocytosis upon ligand binding, but other HER receptors show impaired ligand-induced internalisation (Baulida et al., 1996). Furthermore, heterodimerisation of EGFR with other HER receptors slows down internalisation and affects the fate of EGFR, in which EGFR homodimers are degraded but heterodimers are recycled (Lenferink et al., 1998). The reason of the above phenomenon is still unclear. It is unlikely to be ubiquitin-dependent, as the Y1045F mutation of Cbl binding site in EGFR only impairs degradation but not internalisation (Ravid et al., 2004). A more recent study showed that mutations of three lysine residues, which are acetylated, to arginine defect the internalisation of EGFR, suggesting that the endocytosis process of EGFR may be acetylation-dependent (Goh et al., 2010).

1.3.1.2 HER2

HER2 is a special member in the HER family as no corresponding ligand has been discovered. However, it has been described as the preferred dimerisation partner for the rest of the HER family receptors (Graus-Porta et al., 1997). Downregulation of cell surface levels of either EGFR or HER2, using intracellular expression of endoplasmic reticulum (ER)-targeted single chain antibody (scFV) specific to the receptors, shows that HER3 and HER4 preferentially bind to HER2 upon ligand stimulation, and they only dimerise with EGFR when HER2 is not available (Graus-Porta et al., 1997). The lack of surface HER2 impairs EGF-induced HER3 and HER4 phosphorylation, suggesting the role in mediating the lateral transmission of signals between HER receptors.

Structural crystallography of the HER2 ectodomain revealed a conformational similarity between the ectodomains of HER2 and ligand-bound EGFR, proposing a model in which the ectodomain of HER2 is constitutively in the activated conformation and the dimerisation subdomain is always exposed (Garrett et al., 2003). The fact that HER2 has a constitutively “open” ectodomain may explain why it is the preferred dimerisation partner of other HER family members, and that HER2 requires no ligand for activation.

When ligand-bound HER receptors dimerise with HER2, the dissociation rate of ligand-receptor complex reduces, leading to a prolonged activation of downstream signalling pathways including MAPK and c-Jun N-terminal kinase (JNK) pathways (Karunagaran et al., 1996). Also, HER2-containing receptor dimers have a reduced rate of endocytosis and the internalised dimers are recycled to cell surface (Lenferink et al., 1998; Worthylake et al., 1999). This process maintains HER2 heterodimers for increased signalling.

HER2 mediates downstream signalling through adaptor proteins Grb2 and Shc (Olayioye et al., 2000; Schulze et al., 2005), hence activating Ras-mediated pathways like Ras/Raf/MAPK and Ras/PI3K/Akt pathways. It also has a docking site at Tyr1248 for Chk

(C-terminal Src kinase-homologous kinase), which phosphorylates and inhibits Src, inhibiting tumour growth (Zrihan-Licht et al., 1998). Coupling of Chk to HER2 serves as a negative regulator for HER2-driven proliferation.

There are a few splice variants of HER2 that have been previously described. Alternative splicing produces two secreted HER2 fragments, the p100 HER2 extracellular domain (ECD) and herstatin, which inhibit tumour growth probably by preventing receptor dimerisation (Wang et al., 2013b). A third splice variant, Δ 16HER2, lacks the exon 16, without which the Δ 16HER2-ECD has a conformational change. This change results in the constitutive activation of Δ 16HER2 homodimers (Marchini et al., 2011) and a lowered trastuzumab sensitivity (Sasso et al., 2011). Δ 16HER2 has also been reported to mediate *de novo* tamoxifen resistance via microRNA regulations (Sasso et al., 2011). Proteolytic cleavage or alternative transcription initiation generates a series of HER2 carboxyl-terminal fragments (CTFs) that is collectively called p95HER2. They are further classified, according their start codon number relative to full-length HER2, into 611-CTF, 648-CTF and 687-CTF (Sasso et al., 2011; Wang et al., 2013b). The 687-CTF is inactive, but the other two (611- and 648-CTF) are capable of activating downstream signalling (Wang et al., 2013b). Expression of p95HER2 is associated with HER2 mAb therapy resistance as these HER2 fragments lack the ECD for antibody-binding (Scaltriti et al., 2007).

1.3.1.3 HER3

HER3 is generally considered as kinase defective and its activation requires its dimerisation with another HER family receptor, particularly HER2, the preferred partner for other members of the family (Baselga and Swain, 2009; Sierke et al., 1997). However, recent studies uncover the competency of HER3 to bind ATP and autophosphorylate in an *in vitro* system, although its kinase activity is approximately 1000-fold less than EGFR (Shi et al., 2010). Moreover, single particle tracking using quantum dots shows that HER3

is actually capable of homodimerisation (Steinkamp et al., 2013), despite a previous chimeric receptor study showing the opposite (Berger et al., 2004). Steinkamp *et al.* confirms the activity of HER3-TKD in the SK-BR-3 cell line (Steinkamp et al., 2013), suggesting a need to review the paradigm of HER3-TKD inactivity.

The growing importance of HER3 in cancer research is implied by that HER3 activation might mediate drug resistance mechanism to EGFR- and HER2-targeted therapies such as gefitinib (Sergina et al., 2007) and trastuzumab (Gijsen et al., 2010). HER3 is particularly important in HER2-overexpressing breast cancer as HER3 knockdown reduces proliferation of cancer cells, while EGFR is expendable (Lee-Hoeflich et al., 2008).

HER3 is the main regulator of PI3K/Akt pathway in the HER signalling network as it has six binding sites for the regulatory p85 subunit of PI3K; for example, phospho-Tyr1222 and Tyr1289. HER3 is also capable of binding to Shc and Grb7 adaptor protein (Olayioye et al., 2000).

1.3.1.4 HER4

HER4 is the most recently discovered member of the HER receptor family. It has a very similar extracellular domain to HER3, and its cytoplasmic domain shares 77–79% sequence identity with that of EGFR and HER2 (Gilmour et al., 2001). Alternative splicing produces juxtamembrane (JM-a and JM-b) and cytoplasmic (CYT-1 and CYT-2) isoforms of HER4. JM-a isoform has a 23 amino acids sequence that is recognised by a disintegrin and metalloprotease (ADAM) 17, which facilitates the release of soluble intracellular domain of JM-a isoform. JM-b isoform has an alternative 13 amino acids sequence that cannot be recognised for cleavage (Elenius et al., 1997). Expression of JM-a isoform promotes cell proliferation; however, expression of JM-b isoform causes cell death (Sundvall et al., 2010).

CYT-1 isoform contains a unique 16 amino acids sequence with a tyrosine residue which, when phosphorylated, serves as a docking site for the p85 subunit of PI3K (Elenius et al., 1999). Soluble CYT-1 intracellular domain decreases proliferation and stimulates lumen formation in mouse mammary epithelium, while soluble CYT-2 domain promotes growth without lumen formation (Muraoka-Cook et al., 2009). Expression of different combinations of juxtamembrane and cytoplasmic isoforms produces HER4 receptor isoforms with opposing effects in tumour growth. However, the expression of HER4 is often found a good prognostic marker in breast cancer, and the loss of HER4 may contribute to tamoxifen resistance in breast cancer patients (Koutras et al., 2010).

When the ectodomain of HER4 JM-a isoform is cleaved by ADAM17, it forms a membrane-anchored fragment of 80 kDa (m80) that is still catalytic active (Carpenter, 2003; Vecchi and Carpenter, 1997) and capable of interacting with intact HER2 (Cheng et al., 2003). Subsequently, the m80 fraction of HER4 is cleaved by γ -secretase and becomes a soluble fraction (s80) (Ni et al., 2001). The s80 fraction associates with transcription factors such as STAT5 and ER, translocates into nucleus and regulates transcriptional activity (Jones, 2008; Williams et al., 2004).

1.3.2 HER ligands

There are 11 HER ligands that serve as specific agonists for HER receptors. HER ligands include EGF, transforming growth factor- α (TGF- α), heparin-binding EGF (HB-EGF), amphiregulin (AR), betacellulin (BTC), epigen (EPG), epiregulin (EPR) and neuregulin 1–4 (NRG1–4, also referred as heregulin [HRG] or neu differentiation factor [NDF]) (Linggi and Carpenter, 2006). They are small soluble proteins that have an EGF-like motif generated by three disulphide bonds (Linggi and Carpenter, 2006; Yarden and Sliwkowski, 2001). Each ligand is specific to one or two receptors as shown in Table 1-1 (Hynes and MacDonald, 2009; Linggi and Carpenter, 2006). Each of them has a different ability to

Ligand	Receptors			
	EGFR	HER2	HER3	HER4
EGF	+	–	–	–
TGF- α	+	–	–	–
HB-EGF	+	–	–	+
AR	+	–	–	–
BTC	+	–	–	+
EPG	+	–	–	–
EPR	+	–	–	+
NRG1	–	–	+	+
NRG2	–	–	+	+
NRG3	–	–	–	+
NRG4	–	–	–	+

Table 1-1 Specificity of HER receptors and ligands

Redrawn from Linggi and Carpenter, 2006.

activate different combinations of HER receptors (Table 1-2) and thus various signalling pathways (Beerli and Hynes, 1996; Harari and Yarden, 2000). However, there is no known ligand for HER2.

HER ligands are encoded as membrane-bound precursors (Linggi and Carpenter, 2006) and often require sheddases (membrane-bound enzymes that cleave extracellular domains of transmembrane proteins), such as ADAM family members, to release them for auto- or paracrine stimulation (Blobel, 2005). Some HER ligands like HB-EGF, however, are also competent for juxtacrine signalling. They can stimulate adjacent cells without being cleaved by ADAMs (Zahnow, 2006).

1.3.3 Mechanisms of action

Under the circumstances without any ligand, the dimerisation subdomain (subdomain II of ectodomain) of HER receptors, except HER2, is covered by the intramolecular interaction with subdomain IV of the ectodomain (Baselga and Swain, 2009; Linggi and Carpenter, 2006). Upon ligand binding to the subdomains I and III of the receptor ectodomain, this intramolecular interaction is resolved and the dimerisation subdomain II is exposed (Baselga and Swain, 2009; Linggi and Carpenter, 2006). This process is called a change from a “closed” to an “open” conformation (Figure 1-8A). HER receptors form homodimers with the same receptor or heterodimers with different receptors and bring their TKDs into close proximity (Baselga and Swain, 2009; Linggi and Carpenter, 2006). The two TKDs activate each other asymmetrically as the C-lobe of one TKD binds to the N-lobe of another TKD and acts as an allosteric activator (Figure 1-8B) (Baselga and Swain, 2009; Jura et al., 2009), then the activated TKD phosphorylates specific tyrosine residues.

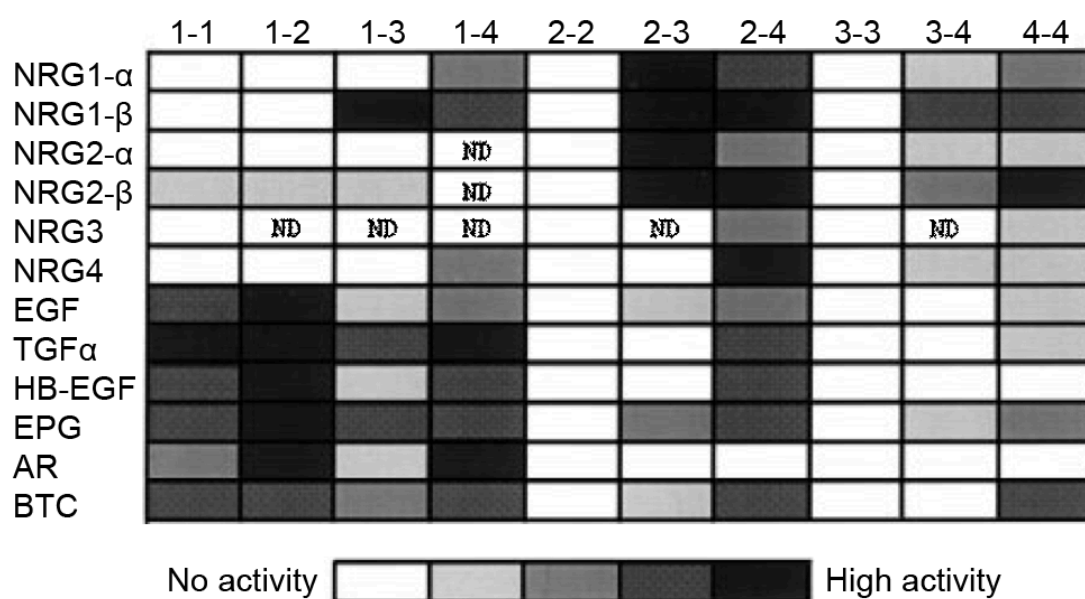


Table 1-2 Differential activation profile of HER ligands for different HER receptor combinations

Modified from Harari and Yarden, 2000. Activity was determined based on physiological mitogenic readout. Epigen was not included in this table because it was discovered in 2001 (Strachan et al., 2001). (ND = Not determined)

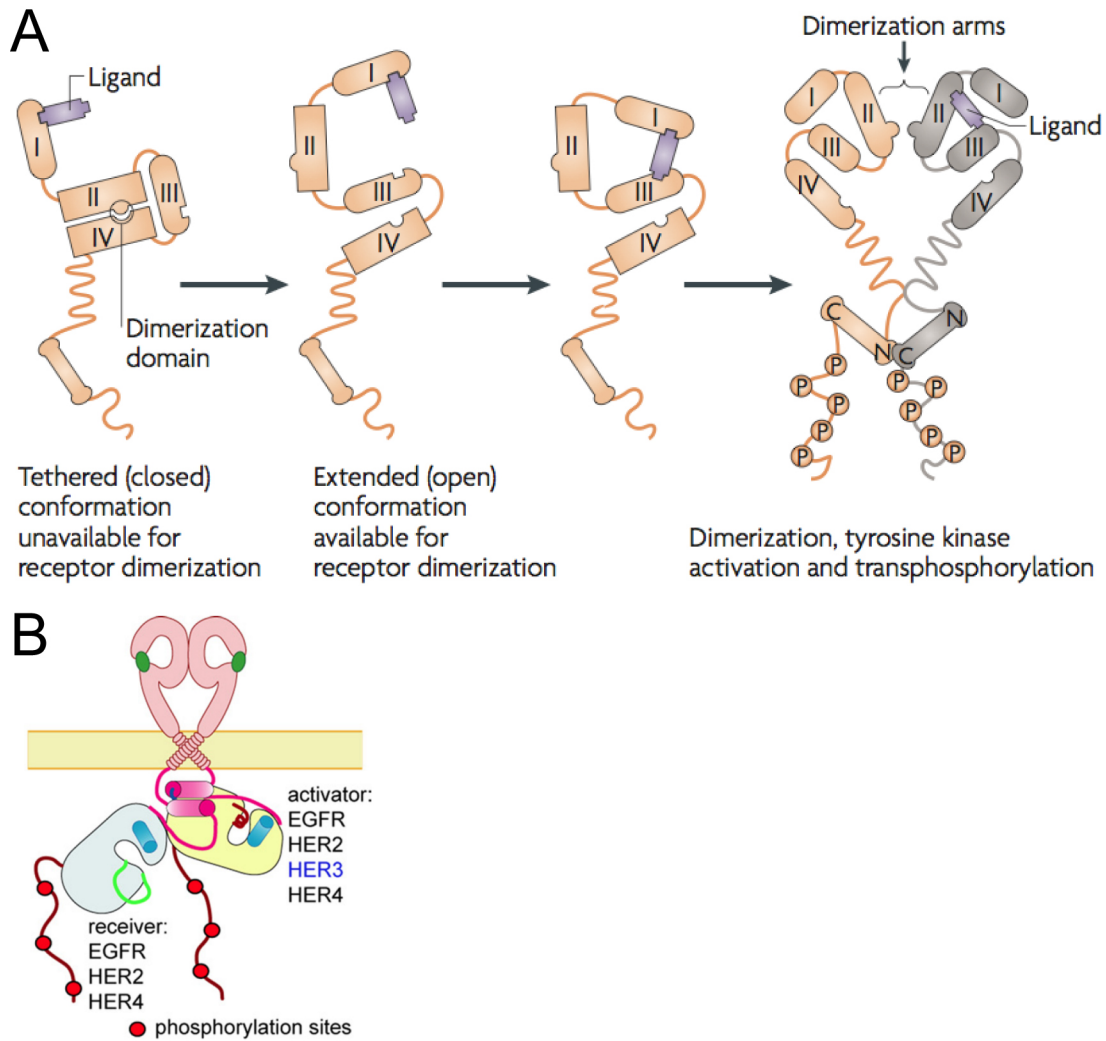


Figure 1-8 The process of HER receptor activation

(A) From Baselga and Swain, 2009 (Reproduced with permission from Nature Publishing Group). Without presence of ligands, the dimerisation subdomain II of HER receptor monomer is impounded by the intramolecular interaction with subdomain IV. Upon ligand binding, domain II is then exposed and allowed to dimerise with another “open” monomer. (B) From Jura *et al.*, 2009 (Reproduced with permission from PNAS). When the two tyrosine kinase domains of a HER dimer are brought together, one of them acts as an allosteric activator of the other and autophosphorylation occurs.

The phosphorylated tyrosine residues of the C-terminal serve as docking sites for adaptor proteins as well as other enzymes that activate downstream signalling (Figure 1-9) (Linggi and Carpenter, 2006; Olayioye et al., 2000).

1.3.4 Downstream targets of HER receptors

The phosphotyrosine residues at the C-terminal of HER receptors serve as docking sites for SH2- and PTB-containing proteins (Figure 1-9) (Olayioye et al., 2000). The downstream proteins that interact with HER receptors include adaptor proteins (such as Shc, Grb2 and Crk), kinases (such as Src, Chk and PI3K), and protein tyrosine phosphatases (such as PTP1B, PTP2C, Shp1 and Shp2).

These proteins produce secondary messengers and trigger downstream signalling pathways. The downstream pathways of HER receptors are Ras/Raf/MAPK pathway, PI3K/Akt pathway, JNK pathway and protein kinase C pathway. These downstream effectors in turn regulate apoptosis, adhesion, growth, migration and differentiation (Yarden and Slivkowski, 2001).

Different HER ligands bind to one specific or more HER receptors and they have different potencies in recruiting partner receptors (Table 1-2). In addition to the combination of dimerising receptors, the HER pathway is thus an extensive network of cell signalling cascades.

1.3.5 HER signalling pathway and cancers

Dysregulation of the HER signalling pathway is related to various forms of epithelial cancer; for instance, head and neck, lung and breast cancers. The mechanisms of HER family-related tumourigenesis include overexpression of ligands and receptors, and activating mutations (Mendelsohn and Baselga, 2006), which are discussed below.

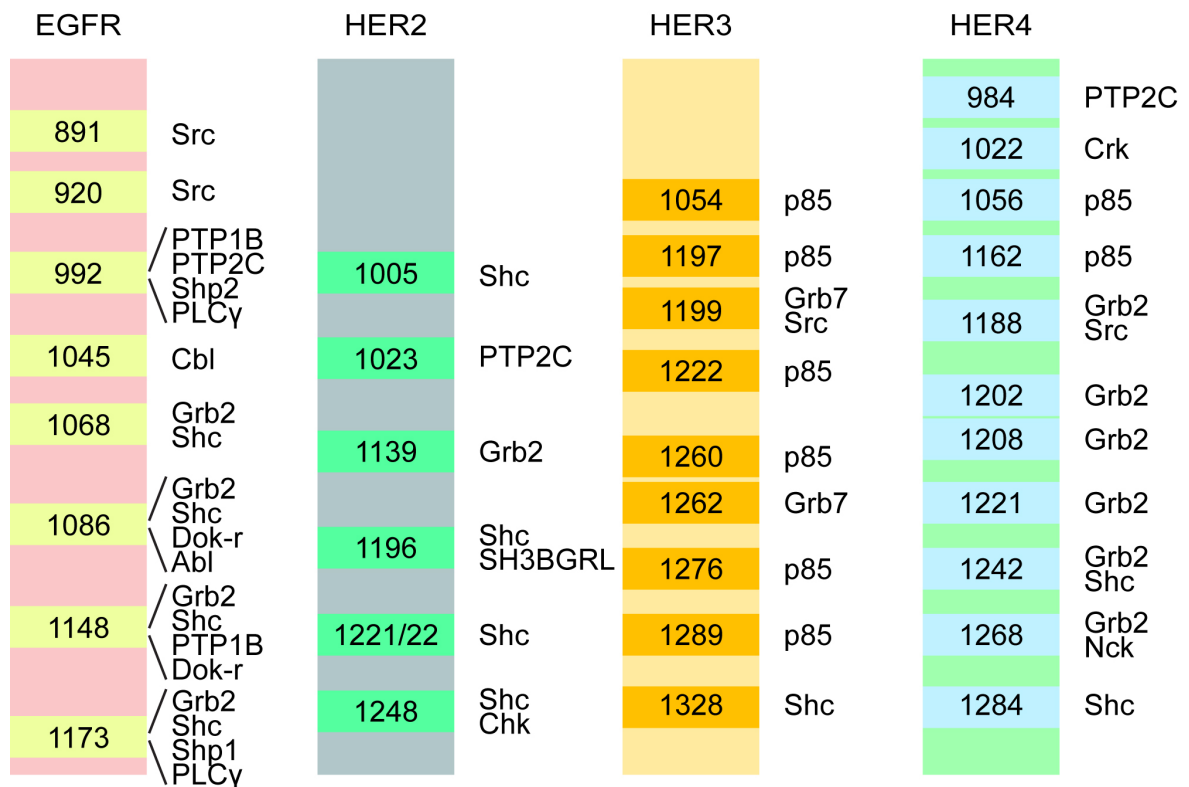


Figure 1-9 Proteins recruited to the specific phosphotyrosine residues of HER receptors

Data taken from references (Jorissen et al., 2003; Linggi and Carpenter, 2006; Olayioye et al., 2000; Schulze et al., 2005). Only the C-terminal of the four HER receptors are shown. Receptors are not drawn to scale.

1.3.5.1 Overexpression of ligands

Overexpression of ligands results in elevated activation of signalling pathway. TGF- α is a classic example of oncogenic ligand, which is implicated in brain, breast, colon, liver, lung, ovarian, skin, pancreatic and prostate cancers (Booth and Smith, 2007; Salomon et al., 1995; Scher et al., 1995; Yamanaka et al., 1993). EGF and AR expressions are also associated with various cancers (Busser et al., 2011; Salomon et al., 1995). In addition, some ligands such as BTC and NRG1 mediate targeted therapy resistance (Gijssen et al., 2010).

1.3.5.2 Overexpression of receptors

Overexpression of receptors can be caused by gene amplification or impaired expression regulation (Mendelsohn and Baselga, 2006). For instance, HER2 expression is maintained in low level by Forkhead box (FOX) protein P3, an X-linked tumour suppressor gene. Absence or deactivating mutation of FOXP3 causes increased HER2 expression (Zuo et al., 2007). Overexpression of EGFR is responsible for several carcinomas such as bladder, brain, breast, head and neck, kidney, lung and prostate cancers (Yarden and Sliwkowski, 2001). Upregulation of HER2 has been associated with breast, cervix, gastrointestinal, lung, ovarian and pancreatic cancers (Baselga and Swain, 2009; Yarden and Sliwkowski, 2001).

1.3.5.3 Activating mutations

In-frame deletion of extracellular domain is one of the most common activating mutations of EGFR, forming a mutant called EGFRvIII, which can be found in glioblastoma, breast, lung and ovarian cancers (Moscattello et al., 1995). In non-small cell lung cancer, in-frame deletions and amino acid substitutions are found, in a small group of patients, clustered around the ATP-binding pocket of the TKD, causing increased kinase activity but also increased response to EGFR TKI gefitinib (Lynch et al., 2004). However, such mutation is

rarely found in other types of cancer except in a small portion of patients with small cell lung cancer, oesophageal, ovarian and pancreatic cancers (Kwak et al., 2006; Okamoto et al., 2006; Schilder et al., 2005). There is a small subset of breast cancer having this activating mutation of TKD; however, the mutation rate is poorly defined (Generali et al., 2007).

Mutations at the TKD of HER2 are reported in lung cancer (Shigematsu et al., 2005; Stephens et al., 2004; Wang et al., 2006). These mutations are in-frame insertion at exon 20 of HER2, resulting in constitutive activation of EGFR/HER2 signalling (Wang et al., 2006). However, equivalent insertion mutation is only found in one breast cancer patient so far (Bose et al., 2013). Missense and deletion mutations are found in a small percentage of patients with breast, colorectal and gastric cancers, but the consequences of these mutations are still unclear and may be different from the insertion mutation in non-small cell lung cancer (Lee et al., 2006). In a recent study, 25 patients with HER2 somatic mutation were identified from eight cancer-genome sequencing projects. These mutations are functionally characterised into several phenotypes. Substitutions at the TKD and the dimerisation domain as well as insertion at the TKD activate HER2 phosphorylation. Deletion of amino acids 755-759 from the TKD, which is equivalent to EGFR exon 19 deletions, increases phosphorylation of the dimerisation partners of HER2 but not HER2 itself. Substitution mutation L755S at the TKD results in lapatinib resistance but not oncogenic transformation (Bose et al., 2013).

1.4 HER2-overexpressing breast cancer

1.4.1 HER2 in breast cancer

Overexpression of HER2 protein and/or amplification of the HER2 gene occur in around 20% of breast cancer patients (Arteaga et al., 2012; Baselga and Swain, 2009). In breast cancer, the amplification of HER2 gene and overexpression of HER2 protein associate closely, while the incidence of overexpression of single HER2 gene via transcriptional and translational dysregulation is rare (Ross et al., 2009). Overexpression of HER2 increases cancer cell proliferation and it enhances motility, tumour invasiveness and reduces apoptosis. It also promotes tumour metastasis and angiogenesis (Moasser, 2007).

Overexpression of HER2 results in ligand-independent EGFR and HER2 activation (Worthylake et al., 1999) and HER2 homodimerisation (Penuel et al., 2002). As aforementioned, the ectodomain of HER2 is constantly in the “open” conformation and it exposes the dimerisation subdomain. This event leads to constitutive activation of downstream signalling, in particular Ras/Raf/MAPK and PI3K/Akt pathways that regulate cell growth, survival and differentiation (Yarden and Sliwkowski, 2001).

HER2 overexpression correlates with adverse prognosis in breast cancer patients and poor clinical outcome. Before the discovery of HER2-targeted therapies, HER2-overexpressing breast cancer patients used to have high recurrence rates and increased mortality (Arteaga et al., 2012; Ross et al., 2009). HER2 overexpression correlates with tumour size, lymph node positivity, high tumour grade and aneuploidy (Yarden and Sliwkowski, 2001). In addition, HER2-overexpressing breast cancers were found less sensitive to anti-oestrogen therapy, but clinical studies showed that the combination of anti-oestrogen therapy and HER2-targeted therapy improves progression-free survival (PFS) and objective response rate (Johnston, 2010; Montemurro et al., 2013; Yarden and Sliwkowski, 2001).

1.4.2 HER2-targeted therapy

Since HER2 overexpression is implicated in poor prognosis of breast cancer, various HER2-targeted therapies have been developed. There are two main classes of HER2-targeted therapy: monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs) (Arteaga et al., 2012).

mAbs bind to a specific epitope of the extracellular domain of HER2 receptor to inhibit its function. As discussed in Section 1.3.3, the extracellular domain of HER receptors is responsible for ligand-binding and dimerisation, targeting the ectodomain of a RTK effectively blocks these two processes. HER2 has no known ligands and always stays in the “open” conformation; therefore, blocking ligand-binding has not been a strategy for HER2. However, ligand-blocking antibodies are available for other HER receptors such as cetuximab for EGFR (Goldstein et al., 1995) and SPG1 for HER3 (Rajkumar and Gullick, 1994). In addition, antibodies activate Fc receptors of natural killer (NK) cells and trigger antibody-dependent cell-mediated cytotoxicity (ADCC), which enhances the anti-tumour activity of these antibodies (Clynes et al., 2000). However, antibodies cannot penetrate the cell membrane and can only work on membrane proteins with an extracellular domain.

TKIs are small chemical molecules that inhibit the tyrosine kinase activity. TKIs can function as competitive inhibitor with respect to ATP or substrate, allosteric inhibitor or inhibitor to conformational changes (Posner et al., 1994). They can penetrate the cell membrane to target the cytoplasmic domain of the HER receptors. TKIs are generally less specific than mAbs because different receptors may share a similar structure of ATP-binding pocket or substrate-binding site; however, the reduced specificity allows the development of broad-spectrum TKIs that targets pan-HER receptors.

Table 1-3 lists the therapeutic agents that target HER2 for cancer treatment. The marketed HER2-targeted therapies: trastuzumab, pertuzumab and lapatinib, are discussed in detail below.

1.4.2.1 Trastuzumab

Trastuzumab, a humanised antibody against the subdomain IV of the HER2 ectodomain (Figure 1-10) (Cho et al., 2003), was developed as the first-line targeted therapy for HER2-overexpressing breast cancer. Clinical studies revealed benefits from combining trastuzumab and chemotherapy in patients with HER2 positive breast tumours. Trastuzumab plus chemotherapy was first used in metastatic breast cancer pre-treated with chemotherapy, with objective response rate of 11.6–15% (Baselga et al., 1996; Cobleigh et al., 1999). A subsequent randomised trial showed that the addition of trastuzumab to chemotherapy increases the rate of objective response, the duration of response, disease-free and overall survivals, and decreases the risk of patient death (Slamon et al., 2001). In the adjuvant setting, trastuzumab combined with chemotherapy improves disease-free and overall survivals (Slamon et al., 2011). The U.S. Food and Drug Administration (FDA) approved the use of trastuzumab with paclitaxel in metastatic breast cancer patients whose tumour overexpresses HER2 (Siegel, 1998).

The precise mechanisms of action of trastuzumab are still unclear, but there are several working models to describe these mechanisms. Trastuzumab downregulates HER2 receptor and thereby attenuates abnormal HER2 signalling (Cuello et al., 2001; Gijzen et al., 2010; Sliwkowski et al., 1999). Upon binding of trastuzumab to HER2, the Fc region of trastuzumab interacts with immune effector cells. This immune response triggers NK cells action and activates ADCC (Clynes et al., 2000; Cooley et al., 1999). It was also

Agent	Company	Stage of development	Mechanism of action
Afatinib (BIBW-2992)	Boehringer Ingelheim	Phase III	Irreversible TKI to EGFR and HER2
ARRY-334543	Array BioPharma	Phase II	Reversible TKI to EGFR, HER2 and HER4
Ertumaxomab	Fresenius Biotech	Phase II (Terminated)	Bispecific to HER2 and CD3, allowing recruitment of T cells
Lapatinib	GlaxoSmithKline	Marketed	Reversible TKI to EGFR and HER2
Neratinib (HKI-272)	Puma	Phase III	Irreversible TKI to EGFR and HER2
Pertuzumab (2C4)	Roche	Marketed	HER2 dimerisation blocker
Trastuzumab	Roche	Marketed	Multiple (see Section 1.4.2.1)
Trastuzumab emtansine	Roche	Marketed	Targeted delivery of maytansine derivative, an anti-microtubule cytotoxic agent

Table 1-3 List of HER2-targeted therapies

Data from Arteaga *et al.*, 2012 and Baselga and Swain, 2009.

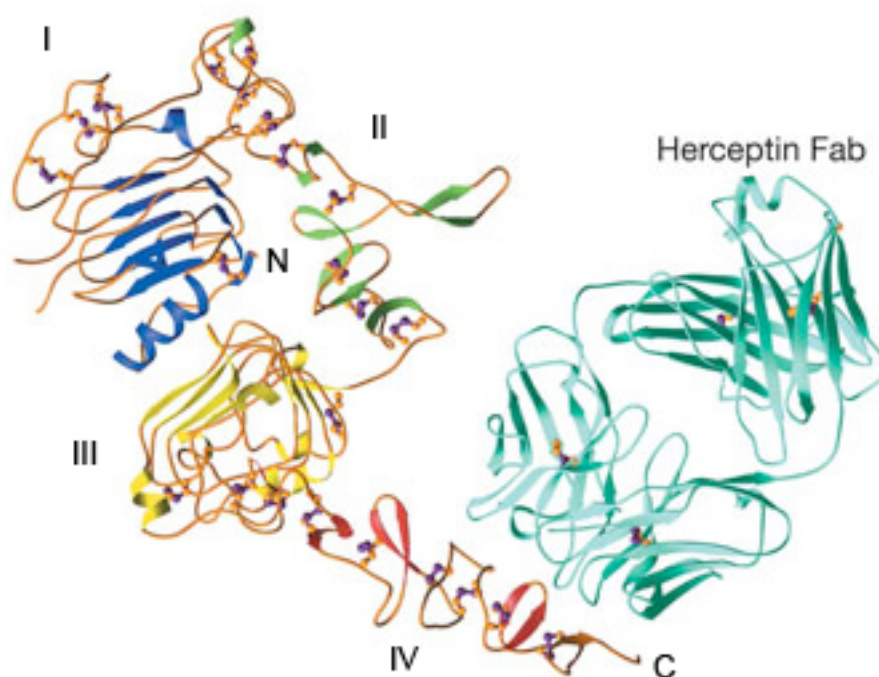


Figure 1-10 Binding of trastuzumab to ectodomain subdomain IV of HER2

From Cho *et al.*, 2003 (Reproduced with permission from Nature Publishing Group). Ribbon diagram of HER2 ectodomain. Subdomains I – IV, and the N- and C-termini are indicated.

reported that trastuzumab increases phosphatase and tensin homolog (PTEN) membrane localisation and phosphatase activity, which is mediated by Src inhibition and dephosphorylation of PTEN (Nagata et al., 2004). PTEN dephosphorylates PIP3, the product of PI3K, antagonising PI3K function and negatively regulating Akt activity. The decrease in PI3K/Akt pathway activities also increases p27Kip1 levels and reduces cyclin-dependent kinase (CDK2) levels, inhibiting a number of proteins required for DNA synthesis and hence resulting in cell cycle G1 arrest (Lane et al., 2001; Le et al., 2006). Finally, trastuzumab prevents proteolytic cleavage of HER2 extracellular domain and the production of the truncated HER2 fragment, which is constitutively active (Molina et al., 2001).

Nevertheless, some patients do not response to trastuzumab treatment or develop drug resistance after initial response. There are several models put forward to explain the mechanisms of resistance. Tumours expressing a truncated p95HER2 due to the alternative transcription start site do not response to trastuzumab because of the lack of HER2 ectodomain (Scaltriti et al., 2007). Since trastuzumab inhibits PI3K/Akt pathway via PTEN, loss of PTEN expression or function results in trastuzumab resistance (Nagata et al., 2004). Mutations in the PIK3CA gene, which causes constitutive activation of PI3K, also abolish the response to trastuzumab (Berns et al., 2007). Increased expression of HER family receptors and HER ligands such as TGF- α compensates for the loss of HER2 function and contributes to trastuzumab resistance (Arteaga et al., 2012). Trastuzumab treatment triggers alternative HER signalling pathways via autocrine HER ligands release mediated by ADAM17 (Gijssen et al., 2010), as well as alternative RTK pathways that involve insulin-like growth factor 1 receptor (Huang et al., 2010b) and Met (Shattuck et al., 2008).

The critical disadvantage of trastuzumab is that it does not normally penetrate blood-brain barrier due to its high molecular weight (Duchnowska and Szczylik, 2005; Pestalozzi and Brignoli, 2000) unless this barrier is disrupted by other treatments such as radiotherapy (Pienkowski and Zielinski, 2010). Clinical studies also suggested that there are no differences in the rate of central nervous system (CNS) metastasis in the patients who did or did not receive trastuzumab treatment (Duchnowska and Szczylik, 2005).

1.4.2.2 Pertuzumab

Pertuzumab (formerly known as 2C4) is a humanised monoclonal antibody against HER2. It binds to the dimerisation subdomain (subdomain II) of the extracellular region of the receptor, preventing it from dimerising with other HER receptors and hence inhibiting HER signalling pathway (Figure 1-11) (Capelan et al., 2013; Johnson and Jänne, 2006). In preclinical studies, it was found that pertuzumab, but not trastuzumab, inhibits NRG1-induced growth (Agus et al., 2002) and morphogenesis *in vitro*, and induced rapid tumour regression in NRG1-dependent xenograft model (Lee-Hoeflich et al., 2008). In NRG1-dependent xenograft, both pertuzumab and the combination of pertuzumab and trastuzumab produced comparable tumour regression, although the combination caused a stronger trend (statistically insignificant) of delayed tumour regrowth than pertuzumab alone after the treatment was stopped (Lee-Hoeflich et al., 2008). A later study showed that the combination of pertuzumab and trastuzumab induced synergistic tumour regression in HER2-overexpressing lung and breast xenograft tumour models (Scheuer et al., 2009).

Early clinical trials showed that pertuzumab has limited activity in breast (Cortes et al., 2005), lung (Herbst et al., 2007), ovarian (Gordon et al., 2006) and prostate (Agus et al., 2007; de Bono et al., 2007) cancers, but these studies did not select patients with HER2-overexpression, which might be the reason of their failure. The limited use of pertuzumab

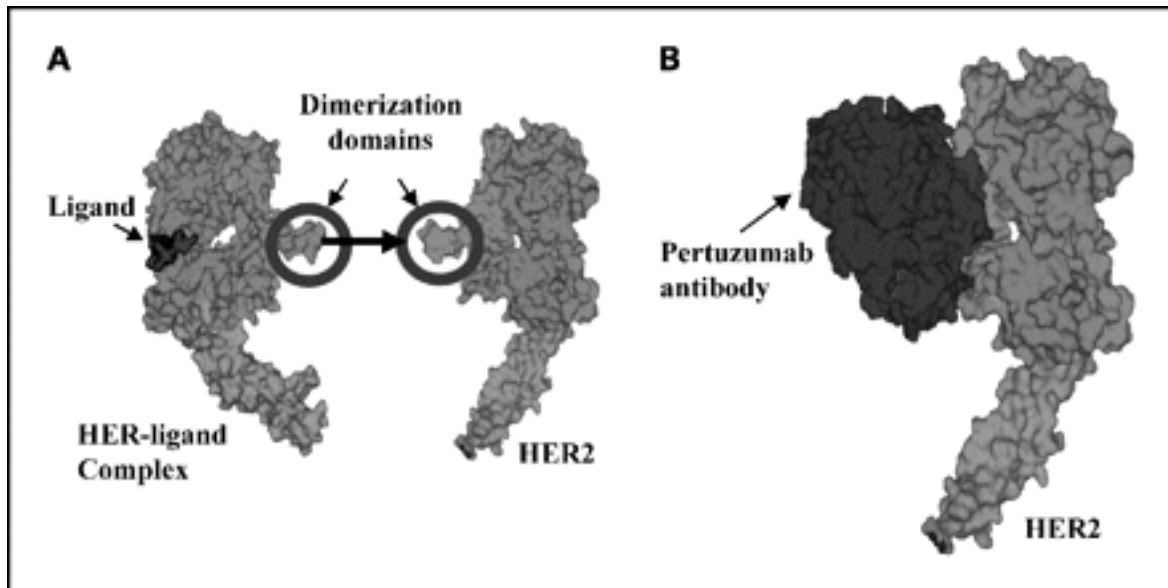


Figure 1-11 Binding of pertuzumab to HER2 dimerisation subdomain

Modified from Johnson and Jänne, 2006. (Reproduced with permission from The American Association for Cancer Research) (A) Exposure of dimerisation subdomain constitutively in HER2 and upon ligand binding to other HER receptors. (B) Pertuzumab binds to subdomain II of HER2 ectodomain, blocking its dimerisation subdomain to inhibit HER2 dimerisation.

in HER2-negative metastatic breast cancer (MBC) was confirmed by a later report (Gianni et al., 2010).

The combination of trastuzumab and pertuzumab was shown to be synergistic in inhibiting the survival of HER2-overexpressing breast cancer cell line BT-474 (Nahta et al., 2004); therefore, this combination was tested in the recent clinical trials. In 2012, CLEOPATRA (Clinical Evaluation of Pertuzumab and Trastuzumab), a phase III study on this combination in HER2-overexpressing MBC, showed that pertuzumab plus trastuzumab with chemotherapy prolonged PFS compared to trastuzumab with chemotherapy (Baselga et al., 2012b). This finding led to the FDA approval of the use of pertuzumab/trastuzumab combination with chemotherapy in HER2-overexpressing MBC in June (FDA, 2012). A neoadjuvant study of the combination of pertuzumab and trastuzumab, NeoSphere (Neoadjuvant Study of Pertuzumab and Herceptin in an Early Regimen Evaluation), suggested that this combination with chemotherapy improved the pathological complete response (pCR) of patients compared to trastuzumab alone with chemotherapy (Gianni et al., 2012). The FDA, after considering the significance of the combination in both MBC and neoadjuvant setting, granted accelerated approval to use the combination of pertuzumab and trastuzumab as neoadjuvant treatment for patients with early HER2-overexpressing breast cancer (FDA, 2013).

1.4.2.3 Lapatinib

Lapatinib (formerly known as GW2016, and later as GW572016) was developed by GlaxoSmithKline and first published in 2001 (Rusnak et al., 2001). It is a small molecule chemical inhibitor that targets the TKD of EGFR and HER2 (Konecny et al., 2006; Rusnak et al., 2001). Lapatinib acts as a competitive inhibitor that reversibly binds to the ATP-binding pocket of the TKD and prevents phosphorylation (Konecny et al., 2006).

In vitro enzyme inhibitory assay showed that lapatinib is highly specific to EGFR and HER2, with IC₅₀ of 10.8 nM and 9.2 nM respectively (Rusnak et al., 2001). The IC₅₀ values of lapatinib to inhibit HER4 and Src are 36-fold and 300-fold, respectively, higher than that for HER2 (Table 1-4). Fabian *et al.* developed an ATP-binding assay to study the interaction between small molecules and a panel of 119 protein kinases (Fabian et al., 2005). They found that lapatinib binds to serine/threonine-protein kinase 10 (STK10) and sterile 20-like serine/threonine-protein kinase (SLK) fairly weakly, with the dissociation constant (K_d) larger than 1 µM (Table 1-5). Lapatinib has been proven successful in inhibiting phosphorylation of the HER signalling pathway and its downstream targets such as Akt and MAPK (Konecny et al., 2006; Xia et al., 2002). Early research also showed that lapatinib reduced tumour growth *in vitro* and *in vivo* (Konecny et al., 2006; Rusnak et al., 2001). Lapatinib has an advantage over trastuzumab because it can inhibit a truncated form of HER2 receptor, p95HER2 (Xia et al., 2005), while trastuzumab can only bind and inhibit full-length HER2 with the extracellular domain (Scaltriti et al., 2007; Zagozdzon et al., 2011).

Clinical trials have shown that lapatinib plus chemotherapy (capecitabine) could significantly reduce disease progression and increase the time to progression compared to chemotherapy alone for advanced HER2-overexpressing breast cancer that progressed after receiving trastuzumab-based therapy (Cameron et al., 2008; Geyer et al., 2006). Hence, the FDA approved lapatinib in 2007 for treating advanced HER2-overexpressing breast cancer.

Following the success of lapatinib in advanced breast cancer, further trials were initiated to investigate the effect of lapatinib and/or trastuzumab in early breast cancer. The Adjuvant Lapatinib and/or Trastuzumab Treatment Optimisation (ALTTO) and NeoALTTO trials were designed to evaluate the effect of lapatinib, trastuzumab and their combination as

Enzyme	IC ₅₀ (nM)
EGFR	10.8 ± 0.53
HER2	9.2 ± 0.75
HER4	367 ± 4.2
Src	3,500 ± 650
Raf1	>10,000
MEK	>10,000
ERK	>10,000
Fms	>10,000
CDK1	>10,000
CDK2	>10,000
P38	>10,000
Tie-2	>10,000
VEGFR2	>10,000

Table 1-4 Enzyme inhibition activity of lapatinib

Redrawn from Rusnak *et al.*, 2001. (Error = SEM)

Enzyme	Dissociation constant (μM)
EGFR	0.0055
HER2	0.011
SLK	9.3
STK10	2.6

Table 1-5 Protein kinase binding affinity of lapatinib

Redrawn from Fabian *et al.*, 2005.

adjuvant treatment after surgery and neoadjuvant treatment before surgery respectively. To date, both trials have completed recruitment but no data has been published for the ALTTO trial. Data from NeoALTTO showed that the combination of lapatinib and trastuzumab with chemotherapy was superior to either drug alone in terms of the pCR rate, but there was no statistical difference in the pCR rate between lapatinib and trastuzumab when combined with chemotherapy (Baselga et al., 2012a). Two other neoadjuvant studies, CHER-LOB and NSABP B-41, showed similar results in operable breast cancer (Guarneri et al., 2012; Robidoux et al., 2013). The synergism achieved by the combination of lapatinib and trastuzumab was suggested to be caused by the complementary mechanism of actions of lapatinib and trastuzumab, resulting in a more complete blockade of HER2 (Konecny et al., 2006; Wang et al., 2011), and the accumulation of HER2 receptors on cell surface and the subsequent augmentation of trastuzumab-induced ADCC (Maruyama et al., 2011; Scaltriti et al., 2009).

In individual neoadjuvant studies, there was no statistical difference between the efficacies of lapatinib with chemotherapy and trastuzumab with chemotherapy in early breast cancer (Baselga et al., 2012a; Guarneri et al., 2012; Robidoux et al., 2013). However, a meta-analysis of six randomised trials concluded that lapatinib in combination with chemotherapy achieved a lower pCR rate and a higher risk of toxicity compared to trastuzumab with chemotherapy in the neoadjuvant setting (Valachis et al., 2012). Furthermore, a phase III study presented in the 2012 American Society of Clinical Oncology Annual Meeting found that, metastatic HER2-positive breast cancer patients receiving lapatinib and chemotherapy had shorter PFS than those who received trastuzumab and chemotherapy (Gelmon et al., 2012).

There are currently a few models to explain the resistance to lapatinib in HER2-overexpressing breast cancer: Lapatinib derepresses FOXO3a, a transcription factor that

transcriptionally upregulates ER and caveolin-1. Caveolin-1 also regulates the transcriptional activity of ER; hence it further enhances ER signalling. Breast cancer cells then switch their sole dependency on HER2 signalling to a co-dependency upon ER and HER2 signalling pathways (Figure 1-12C) (Xia et al., 2006). In HER2 and ER-positive cell lines, lapatinib induces the overexpression of Axl (UFO) RTK through ER. When co-activated with HER2, Axl can bind to the p85 subunit of PI3K, activating Akt signalling and enhancing cancer cell survival (Figure 1-12D) (Liu et al., 2009a). The inhibition of PI3K/Akt pathway caused by lapatinib derepresses FOXO3a, which also transcriptionally upregulates HER3. HER3 produced in this feedback mechanism is then phosphorylated by the residue activity of HER2 as its TKD activity is incompletely inhibited by lapatinib (Figure 1-12E) (Garrett et al., 2011). Lapatinib triggers a cytoprotective stress responses that activates RelA in a calcium-dependent manner (Figure 1-12F). RelA is a prosurvival subunit of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). This complex prevents the cell from undergoing lapatinib-induced apoptosis (Xia et al., 2010). In colorectal cancer, acquired lapatinib-resistant HCT116 cell line has an increased expression of mitochondrial protective proteins MCL-1 and BCL-XL and a decrease in the expression of mitochondrial toxic protein BAX. The cells become less susceptible to apoptosis because of the loss of BAX and the inhibition of BAK caused by MCL-1 (Figure 1-12G) (Martin et al., 2008). Hepatocyte growth factor (HGF) stimulates Met protein, which activates downstream MAPK and Akt pathway and renders gastric cancer cells resistant to lapatinib (Figure 1-12H) (Chen et al., 2012).

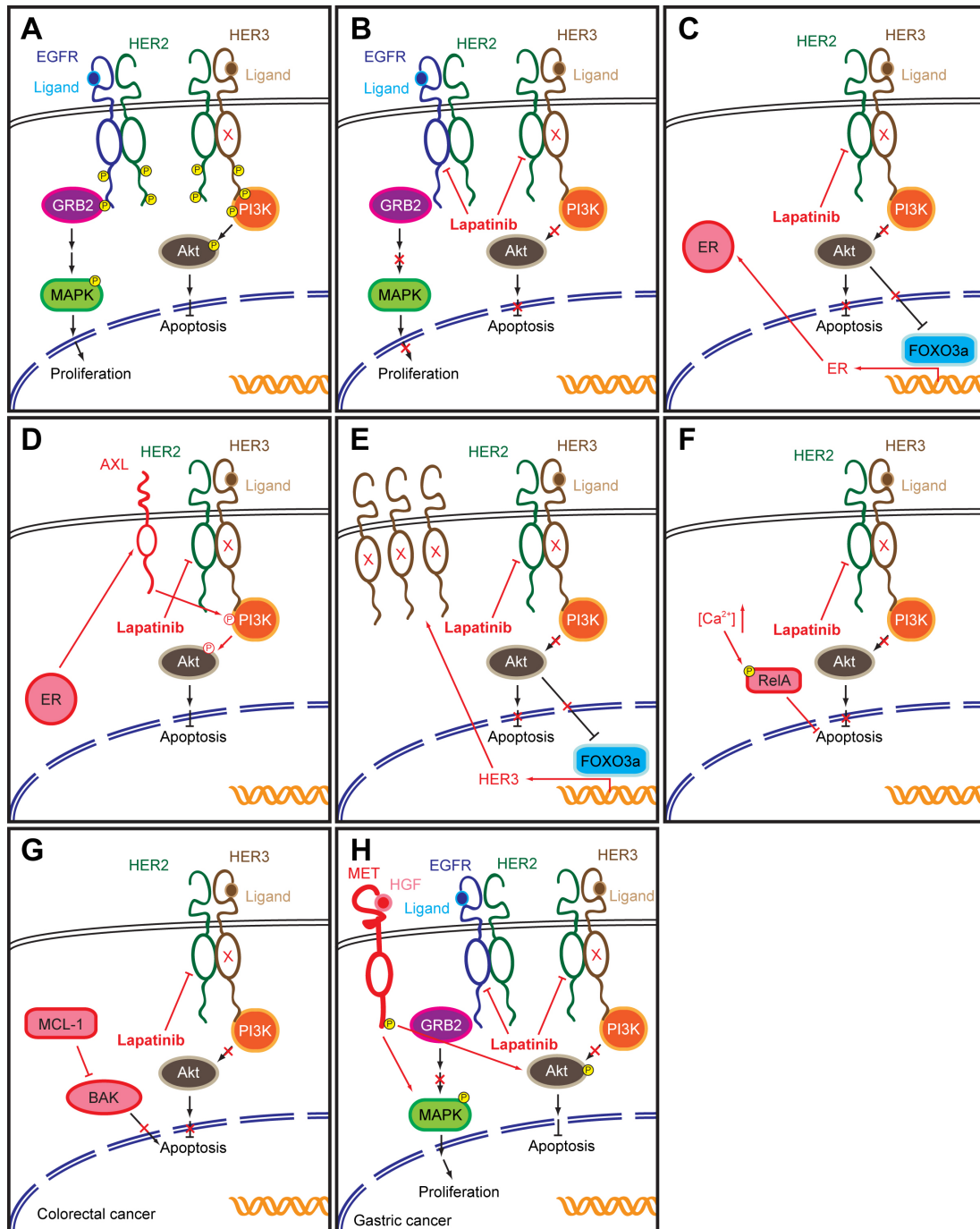


Figure 1-12 Model mechanisms of lapatinib resistance

(A) Ligands stimulate HER receptors dimerisation and the downstream MAPK and Akt pathways (black arrows), promoting proliferation and preventing apoptosis. HER3 is kinase defective (cross on the tyrosine kinase domain). (B) Lapatinib inhibits EGFR/HER2 tyrosine kinase activity and hence downstream pathways. This process causes the following adaptation mechanisms (red arrows and crosses). (C) Lapatinib derepresses FOXO3a that stimulates ER transcription, leading to the co-dependence on ER and HER2 signalling. (D) Lapatinib triggers ER-induced overexpression of AXL, which stimulates downstream PI3K/Akt pathway. (E) Derepression of FOXO3a also causes HER3 upregulation. (F) Lapatinib induces calcium stress response that activates RelA, which in turn inhibits apoptosis. (G) In colorectal cancer, lapatinib-adapted cells overexpress MCL-1, which inhibits BAK, an apoptotic promoter. (H) In gastric cancer, HGF stimulates Met phosphorylation and downstream MAPK and Akt pathways.

1.5 Previous work of the Kong group

Previously, Kong *et al.* showed that treatment with the EGFR TKIs, AG 1478 and gefitinib, for two hours induced the production of active NRG1 and BTC ligands in the HER2-overexpressing SK-BR-3 cell line and the HER2-negative MCF-7 cell line. They also found that these ligands contributed to gefitinib resistance in the SK-BR-3 cell line. In addition, gefitinib treatment dephosphorylated downstream MAPK and Akt in SK-BR-3 cells, but the phosphorylation recovered within 48 hours (Kong et al., 2008).

Gijsen *et al.* later demonstrated that trastuzumab treatment triggered increased expression and release of the HER ligands, NRG1 and BTC, in the HER2-overexpressing BT-474 cell line after one hour. Short-term trastuzumab treatment for one hour inhibited HER3 phosphorylation. However, NRG1 and BTC reactivated HER3 phosphorylation within 48 hours of trastuzumab treatment as well as constitutive activation of EGFR and HER4. They suggested that this process was mediated by a negative feedback mechanism whereby inhibition of Akt stimulated ADAM17, which cleaved and activated HER ligands (Gijsen et al., 2010).

Since other HER receptor-targeted therapies commonly activate the autocrine production of HER ligands, it is hypothesised that lapatinib would stimulate HER ligands in a similar way and this would mediate lapatinib resistance in HER2-overexpressing breast cancer cells.

1.6 Specific aims and objectives

As discussed in Section 1.4.2.3, there are several models explaining the mechanisms of resistance against lapatinib in cancer cells; however, these models only describe the events after 24 hours or with prolonged lapatinib treatment. It is important to understand how breast cancer cells escape drug actions and develop resistance. Our laboratory has previously shown that HER2-overexpressing breast cancer cells responded to trastuzumab by expressing and releasing autocrine ligands within one hour of treatment. Therefore our first hypothesis is that HER ligands expression and release may mediate the acute response to escape lapatinib treatment in HER2-overexpressing breast cancer cells. It is also hypothesised that lapatinib resistance is mediated by a change in the cell signalling profile. The ultimate goal of this project is to develop further strategies to overcome resistance to lapatinib in HER2-overexpressing breast cancer patients.

To achieve the above aims, this thesis has the following objectives:

1. Study the short-term response (within 24 hours) of HER2-overexpressing breast cancer cells to lapatinib treatment
 - Assess the protein level and activation status of the HER family of proteins and downstream pathways
 - Determine the expression levels and release of HER ligands, such as NRG1
2. Study the changes in the cell signalling profile of breast cancer cells that are resistant to lapatinib
 - Generate acquired lapatinib-resistant breast cancer cell lines
 - Compare the cell signalling profile between lapatinib-sensitive and -resistant breast cancer cell lines

3. Identify possible therapeutic strategies to tackle lapatinib resistance
4. Confirm the results from cell lines experiments in xenograft studies.

2. Materials & Methods

2.1 Materials

2.1.1 Human cell lines

SK-BR-3 is a breast adenocarcinoma cell line derived from the metastatic site of a 43-year-old Caucasian female. BT-474 is a breast ductal carcinoma cell line derived from the primary site of a 60-year-old Caucasian female. A-431 was an epidermoid carcinoma derived from the skin of an 85-year-old female. All the above cell lines were obtained from the Cell Services at Cancer Research UK (Lincoln's Inn Fields laboratory). MDA-MB-231 is a breast adenocarcinoma derived from the metastatic site of a 51-year-old Caucasian female. MDA-MB-231 was purchased from American Tissue Culture Collection (ATCC). The cell line authenticity was validated using short-tandem repeat profiling (performed by Dr. Abul Azad).

2.1.2 Antibodies

Name	Species	Application	Source
Polyclonal anti-phospho-EGFR (Tyr1173)	Rabbit	WB 1:1000 IHC-P 1:50	Santa Cruz Biotechnology (sc-101668)
Monoclonal anti-EGFR (D38B1)	Rabbit	WB 1:5000 IHC-P 1:50	Cell Signaling Technology (4267)
Monoclonal anti-phospho-HER2 (Tyr1221/22) (6B12)	Rabbit	IHC-P 1:320	Cell Signaling Technology (2243)
Polyclonal anti-phospho-HER2 (Tyr1221/22)	Rabbit	WB 1:1000	Cell Signaling Technology (2249)
Monoclonal anti-HER2 (29D8)	Rabbit	WB 1:10000 IHC-P 1:400	Cell Signaling Technology (2165)

Monoclonal anti-phospho-HER3 (Tyr1289) (21D3)	Rabbit	WB 1:1000	Cell Signaling Technology (4791)
Monoclonal anti-phospho-HER3 (Tyr1289) (D1B5)	Rabbit	IHC-P 1:1600	Cell Signaling Technology (2842)
Polyclonal anti-HER3	Rabbit	WB 1:10000 IHC-P 1:1000	Abcam (ab34641)
Polyclonal anti-phospho-HER4 (Tyr1056)	Rabbit	WB 1:1000	Santa Cruz Biotechnology (sc-33040)
Monoclonal anti-phospho-HER4 (Tyr1162) (EP2270Y)	Rabbit	IHC-P 1:100	Novus Biologicals (NBP1-40419)
Polyclonal anti-HER4	Rabbit	IHC-P 1:200	Thermo Scientific (RB-9045)
Monoclonal anti-phospho-Akt (Ser473) (D9E)	Rabbit	WB 1:1000	Cell Signaling Technology (4060)
Polyclonal anti-Akt	Rabbit	WB 1:5000	Cell Signaling Technology (9272)
Monoclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204) (D13.14.4E)	Rabbit	WB 1:1000	Cell Signaling Technology (4370)
Polyclonal anti-p44/42 MAPK	Rabbit	WB 1:5000	Cell Signaling Technology (9102)
Monoclonal anti-Cleaved Caspase-3 (Asp175) (5A1E)	Rabbit	IHC-P 1:400	Cell Signaling Technology (9664)
Monoclonal anti-Ki67 (SP6)	Rabbit	IHC-P 1:400	Vector Laboratories (VP-RM04)
Polyclonal anti-NGR1 type 1	Rabbit	IHC-P 1:100	Abcam (ab27303)
Polyclonal anti-NGR1 (H-210)	Rabbit	IHC-P 1:50	Santa Cruz Biotechnology (sc-28916)
Monoclonal anti-NGR1 (7D5)	Mouse	IHC-P 1:100	Neomarkers (MS-272-P1ABX)

Monoclonal anti-NRG1 (147724)	Mouse	IHC-P 1:100	R&D Systems (MAB377)
Monoclonal anti- β -actin (13E5)	Rabbit	WB 1:2500	Cell Signaling Technology (4970)
HRP conjugated anti-mouse IgG (H+L)	Goat	WB 1:10000	Life Technologies (62-6520)
HRP conjugated anti-rabbit IgG (H+L)	Goat	WB 1:10000	Life Technologies (65-6120)
Normal IgG	Human	Drug control at 20 μ g/ml	R&D systems (1-001-A)

Monoclonal anti-NRG1 antibody (R&D Systems) was reconstituted at 0.5 mg/ml in sterile PBSA and stored at -20°C . Human normal IgG was reconstituted at 20 mg/ml stock in sterile PBSA and stored at -20°C . All other antibodies were stored as original stock according to manufacturers' recommendation.

2.1.3 Growth factors

Name	Species	Application	Source
Neuregulin-1 β 1	Human	10 ng/ml	Sigma-Aldrich (SRP3055)

Neuregulin-1 β 1 (NRG1 β 1) was dissolved in sterile PBSA to 100 μ g/ml stock and stored at -20°C .

2.1.4 Drugs

Generic name	Trade name	Typical dose	Company
Cetuximab	Erbitux	N/A	Eli Lilly
Lapatinib	Tyverb	100 nM	GlaxoSmithKline
Pertuzumab	Perjeta	20 μ g/ml	Roche

Phorbol 12-myristate 13-acetate	N/A	1 μ M	Sigma-Aldrich (P8139)
SGP1	N/A	20 μ g/ml	Cancer Research Technology

Cetuximab was purchased from the Oxford Churchill Hospitals pharmacy and stored at 4°C as original stock. Lapatinib was purchased from the Oxford Churchill Hospitals pharmacy in tablet form, ground and dissolved in DMSO (Sigma-Aldrich) to 10 mM stock for *in vitro* experiments and in TWEEN80 to 10 mg/ml for *in vivo* experiments. Both lapatinib stock solutions were stored at –20°C. Pertuzumab was generously donated by Roche and stored at 4°C as original stock. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich, dissolved in DMSO to 10 mM stock and stored at –20°C. SGP1 was obtained from Cancer Research Technology and stored at 4°C as original stock.

2.1.5 Small interfering RNA

Name	Sequence	Source
ON-TARGET plus SMARTpool, Human NRG1	Mixture of the four sequences below	Thermo Scientific
siNRG1-09	5'-UUUCAAAACCCUCGAGAU-3'	Life Technologies
siNRG1-10	5'-UUGUAAAAUGUGCGGAGAA-3'	
siNRG1-11	5'-GGGGAGUGCUUCAUGGUGA-3'	
siNRG1-12	5'-ACAUCCACCACUGGGACAA-3'	
AllStars Negative Control	Not provided by manufacturer	Qiagen

The single siRNAs to NRG1 were synthesised by Life Technologies using the sequence information provided by Thermo Scientific.

2.1.6 Primers

Primer	Sequence
Actin_Fwd	5'-ATTGGCAATGAGCGGTTC-3'
Actin_Rev	5'-GGATGCCACAGGACTCCAT-3'
NRG1_Fwd	5'-GCTTCATGGTGAAAGACCTTTCA-3'
NRG1_Rev	5'-ATTACGTAGTTTTGGCAGCGATC-3'

The primer sequences were obtained from previous studies (Forster et al., 2011; Gijzen et al., 2010) and synthesised by Life Technologies.

2.1.7 Commercial Reagents

Application	Reagent	Source
Cell culture	Dulbecco's modified Eagle medium (DMEM)	Life Technologies
	Penicillin/Streptomycin	
	Roswell Park Memorial Institute (RPMI) medium 1640	
	Phosphate buffered saline (Dulbecco A) tablets	Ovoid
	Foetal bovine serum (FBS)	PAA Laboratories
	Trypsin-EDTA (10×)	
	Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
	Insulin solution (10 mg/ml)	
	Propan-2-ol, absolute	VWR International
Cellular protein extraction	cOmplete™ protease inhibitor cocktail tablets	Roche
	Phosphatase inhibitor cocktail 2	Sigma-Aldrich
	Phosphatase inhibitor cocktail 3	

	Sodium deoxycholate	
	Sodium dodecyl sulphate	
	Sodium pyrophosphate	
	Triton X-100 (t-octylphenoxy-polyethoxyethanol)	
Cell proliferation assay	Coulter Isoton II diluent	Beckman Coulter
	CyQuant™ NF cell proliferation assay kit	Life Technologies
Cell pellet preparation	Agarose power	Sigma-Aldrich
	16% Formaldehyde solution (w/v)	Thermo Scientific
Clonogenic assay	Methylene blue	Sigma-Aldrich
ELISA	Human NRG1-β1/HRG1-β1 DuoSet	R&D Systems
	Substrate reagent pack: hydrogen peroxide and tetramethylbenzidine	
	Stop solution 2N sulphuric acid	
	10% Normal goat serum (w/v)	Life Technologies
	Probumin bovine serum albumin (BSA) diagnostic grade powder	Millipore
Flow cytometry	Annexin V binding buffer (10×)	BD Pharmingen
	Annexin V antibody-Alexa Fluor 647 Conjugate	Life Technologies
	Propidium iodide solution (1 mg/ml)	Sigma-Aldrich
	Ribonuclease A, from bovine pancreas	
<i>In vivo</i> experiment	IsoFlo™ (isoflurane, USP)	Abbott Laboratories
	Baytril™ 10% oral solution	Bayer Animal Health
	β-Estradiol	Sigma-Aldrich
	TWEEN80	

Immunohistochemistry	EDTA pH8.0 buffer	Abcam
	Tris-EDTA pH9.0 buffer	
	Target retrieval solution concentrate (10×)	Dako
	Aquatex™ mounting agent	Merck
	Histo-clear™	National Diagnostics
	Hydrogen peroxide (30%)	Sigma-Aldrich
	Haematoxylin QS	Vector Laboratories
	ImmPACT™ DAB peroxidase substrate	
	ImmPRESS™ Anti-Mouse Ig (peroxidase) polymer detection kit	
	ImmPRESS™ Anti-Rabbit Ig (peroxidase) polymer detection kit	
Medium concentrating	Amicon™ Ultra-15, Ultracel 10 kDa centrifugal filter	Millipore
Protein concentration assay	Bio-Rad protein assay	Bio-Rad
	BSA solution (50 mg/ml)	Sigma-Aldrich
Real-time PCR	SensiMix™ SYBR Hi-ROX kit	Bioline
Reverse transcription	High capacity cDNA reverse transcription kit	Life Technologies
RNA extraction	DNase I	Bio-rad
	Ethanol, absolute, molecular biology grade	Fisher Scientific
	RNeasy Mini kit	Qiagen
RNA silencing	Opti-MEM reduced serum medium	Life Technologies
	DharmaFECT™ 1 transfection reagent	Thermo Scientific
RTK array	Proteome Profiler™ human phospho-RTK array kit	R&D Systems

SDS-PAGE	MagicMark™ XP western protein standard	Life Technologies
	Novex™ Sharp pre-stained protein standard	
	NuPAGE™ 4-12% bis-tris gel	
	NuPAGE™ LDS sample buffer (4×)	
	NuPAGE™ MOPS SDS running buffer (20×)	
Western blotting	Medical x-ray film	Fuji Film
	Amersham™ ECL western blotting detection reagents	GE Healthcare
	Immobilon-P™ 0.45 µm PVDF membrane	Millipore
	Bovine serum albumin	Sigma-Aldrich
	Glycine	
	TWEEN20 (Polyoxyethylene [20] sorbitan monolaurate)	
	Methanol, absolute	VWR International

2.1.8 Solutions

Agarose mixture: 2% (w/v) Agarose and 4% (w/v) formaldehyde in PBS

Annexin V staining solution: 1× Annexin V binding buffer, 0.2% (v/v) Annexin V-Alexa Fluor 647 conjugate antibody, 10 µg/ml propidium iodide

Cell lysis buffer for phospho-protein analysis: 20 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0), 150 mM NaCl, 10 mM Na₂H₂P₂O₇, 100 mM NaF, 1% (v/v) Triton X-100, supplemented with 1% (v/v) phosphatase inhibitor cocktail 2, 1% (v/v) phosphatase inhibitor cocktail 3 and 1 cComplete protease inhibitor cocktail tablet per 10 ml of cell lysis buffer

4% (w/v) formaldehyde solution: 10 ml of 16% formaldehyde solution in 30 ml of PBS

LDS loading buffer: 10% (v/v) β -mercaptoethanol in 4 $\times$ NuPAGE LDS sample buffer

Methylene blue solution: 0.2% (w/v) methylene blue in 60% (v/v) methanol

PBS: 136.8925 mM NaCl, 3.3534 mM KCl, 12.0199 mM NaHPO₄, 1.8371 mM KH₂PO₄, pH 7.2

PBSA: Phosphate buffered saline tablet dissolved in 100 ml of Milli-Q water per tablet

Propidium iodide staining solution: 40 μ g/ml propidium iodide, 100 μ g/ml RNase A in PBSA

RIPA buffer for NRG1 analysis: 20 mM Tris (pH 7.4), 1 mM EDTA (pH 8.0), 150 mM NaCl, 0.5% (w/v) Na-deoxycholate, 0.1% (w/v) SDS, 5% (v/v) glycerol, 1% (v/v) Triton X-100, supplemented with 1 cOmplete protease inhibitor cocktail tablet per 10 ml of buffer

Semi-dry blotting transfer buffer: 25 mM Tris (pH7.5), 192 mM glycine, 20% (v/v) methanol

TBS: 136.8925 mM NaCl, 2.6827 mM KCl, 24.7647 mM Tris, pH 7.5

TBST: 0.2% (v/v) Tween 20 in TBS

0.25% Trypsin-EDTA: 5 ml of 10 $\times$ Trypsin-EDTA in 45 ml of sterile PBSA

2.2 Methods

2.2.1 Human cell culture

2.2.1.1 Maintenance

A-431, MDA-MB-231 and SK-BR-3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. BT-474 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 10 µg/ml insulin, 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C.

2.2.1.2 Routine passage

Cells were washed with PBSA twice and then detached by incubating with 0.25% trypsin-EDTA solution at 37°C for 3 minutes. Detached cells were washed with complete medium and then reseeded.

2.2.1.3 Cryopreservation

Cells were cryopreserved in 10% dimethyl sulfoxide (DMSO) in FBS, frozen at -80°C in a propan-2-ol filled Cryo 1°C freezing container (Nalgene) overnight before being transported to liquid nitrogen.

2.2.1.4 Frozen cell recovery

Frozen stock cells were recovered by rapid thawing to 37°C and then transferred to complete medium. Culture medium was replaced after the cells had attached to base, typically from 4 hours to 16 hours.

2.2.2 Development of lapatinib-resistant cell lines

Lapatinib-resistant SK-BR-3 and BT-474 cell lines were generated by chronic exposure of increasing concentrations of lapatinib from 25 nM, 50 nM, 100 nM to 250 nM for ten months. Fresh lapatinib was replaced every three days. Lapatinib-resistant cell lines were named SK250LR and BT250LR respectively.

2.2.3 Proliferation/growth assays

2.2.3.1 Total cell counting

Approximately 10,000 cells per well (for SK-BR-3 and SK250LR) or 20,000 cells per well (for BT-474 and BT250LR) were seeded in triplicate on a 24-well tissue culture plate (Corning), allowed to adhere and enter log phase overnight. Each triplicate was treated with the indicated agents for three days. Culture medium was discarded. Cells were washed with PBSA twice and then detached from the well by incubating with 500 μ l of 0.25% trypsin-EDTA solution. 400 μ l of trypsinised cells were diluted into 20 ml using Coulter Isoton II diluent and counted using a Coulter Z2 particle counter (Beckman Coulter); only the cell population with the size of 13–27 μ m was counted. Percentage cell growth was calculated as the change in cell number relative to the number of cells seeded. The cell number was normalised against the untreated control as 100% and the highest lapatinib dose as 0%, then plotted against the log of drug concentration, and the IC₅₀ values were interpolated from the four-parameter sigmoid fit curve.

2.2.3.2 CyQuant NF assay

Approximately 5,000 cells per well were seeded in octuplicate on a 96-well, black, clear bottom tissue culture plate (Corning) and allowed to adhere and enter log phase overnight. Each octuplicate was treated with the indicated agents for three days. Culture medium was discarded. CyQuant dye reagent was diluted in Hank's balanced salt solution (available

from CyQuant kit, Life Technologies) according to manufacturer's protocol. 100 μ l of dye solution was aliquoted into each well. The plates were incubated at 37°C for an hour to allow dye binding. The fluorescence intensity was measured using Spectramax m2e microplate reader (Molecular Devices) with excitation at 485 nm and emission detection at 530 nm. The fluorescence reading from the first and last columns and rows were discarded to avoid variation caused by medium evaporation.

2.2.3.3 Real-time live cell imaging

Approximately 10,000 cells per well (for SK-BR-3 and SK250LR) or 20,000 cells per well (for BT-474 and BT250LR) were seeded in triplicate on a 24-well tissue culture plate, allowed to adhere and enter log phase overnight. Each triplicate was treated with the indicated agents. Cell proliferation was monitored by IncuCyte kinetic live cell imaging system (Essen BioScience). High quality phase-contrast images of 16 fields in each well were taken in 6-hour interval for four days (SK-BR-3 and SK250LR) or seven days (BT-474 and BT250LR). Culture medium and drugs were replaced on day 2 and day 5. The metric of monolayer confluence was measured by IncuCyte software package Confluence v1.5 and used as a surrogate for cell number.

2.2.4 Clonogenic assay

Approximately 150,000 cells per well were seeded on a 6-well tissue culture plate (Corning), allowed to adhere and enter log phase overnight. Each well was treated with the indicated agents for three days, and then the culture medium was discarded. Cells were washed with PBSA twice and then detached by incubating with 0.25% trypsin-EDTA solution at 37°C for 3 minutes. Detached cells were washed with 4 ml of complete medium. The density of cells was measured by cell counting as previously described (Section 2.2.3.1) and reseeded in triplicate on 6-well plates at the density of 1,000 cells per well (for SK-BR-3 and SK250LR) or 2,000 cells per well (for BT-474 and BT250LR). The

cells were allowed to grow into colonies for 10 days (for SK250LR), 14 days (for SK-BR-3) or 17 days (for BT-474 and BT250LR). Culture medium and insulin (for BT-474 and BT250LR) were replaced every alternate day. After incubation, the plates were washed with PBS twice and stained with methylene blue solution for one hour. Methylene blue solution was recycled after staining and the plates were washed with tap water three times and then air-dried. The colonies were counted using a GelCount colony counter (Oxford Optronix).

2.2.5 Protein extraction

Approximately 150,000 cells per well (for SK-BR-3 and SK250LR) or 300,000 cells per well (for BT-474 and BT250LR) were seeded on a 6-well tissue culture plate, allowed to adhere and enter log phase overnight. After the treatments indicated in experiments, the culture medium was removed. The cells were washed twice with ice-cold PBSA and then scraped into ice-cold lysis buffer (Section 2.1.8) for western blotting or ice-cold RIPA buffer (Section 2.1.8) for ELISA. The lysates were centrifuged at 17,200 g for 10 minutes at 4°C. The supernatants were obtained and the pellets were discarded. The protein samples were immediately used in further experimental procedures or stored at -80°C.

2.2.6 Protein concentration assay

Protein standards were prepared by diluting 50 mg/ml BSA solution to 2, 4, 6, 8 and 10 mg/ml using deionised water. Protein assay stock solution was diluted 1:5 in deionised water. Diluted protein assay solution was aliquoted at 500 µl per reaction tube. 2 µl of each sample and standard BSA solution was added to individual reaction tubes. A 200-µl duplicate of each sample mixture was transferred to a 96-well transparent microplate and the optical density (OD) at 595 nm was measured using µQuant microplate reader (Bio-tek Instruments). Diluted protein assay solution without sample protein was used as blank. The

concentration of the protein samples was calculated using the standard curve, example shown in Figure 2-1.

2.2.7 Western blotting

After protein quantification, the protein samples were diluted into equal concentration using the lysis buffer (Section 2.1.8). One part of LDS loading buffer was added to every three parts of diluted protein sample. Equal amount of protein samples (typically 20 µg) was resolved by SDS-PAGE on 4-12% Bis-Tris gels, and then electrophoretically transferred to 0.45 µm PVDF using Trans-blot SD semi-dry transfer cell (Bio-rad) at 12V for two hours. Membranes were blocked with 5% BSA in PBST for one hour and then incubated with the primary antibodies (Section 2.1.2) in 5% BSA in PBST overnight at 4°C. After four 5-minute washes in PBST, primary antibodies were probed with secondary goat horseradish peroxidase-linked anti-rabbit or mouse IgG diluted in 5% non-fat dry milk in PBST at room temperature (RT) for one hour. After four 5-minute washes in PBST, protein-antibody complexes were detected by chemiluminescence with the Amersham ECL Detection Reagents using medical X-ray film or the ImageQuant LAS 4000 mini system (GE healthcare).

2.2.8 Western blot densitometry

Developed medical X-ray films were scanned by Photosmart C4780 All-in-one Printer (Hewlett-Packard). The intensity of bands was quantified using ImageJ software. Digital images obtained using the ImageQuant LAS 4000 mini system were analysed by ImageQuant TL version 7.0 software (GE Healthcare).

2.2.9 RNA interference

siRNA (Section 2.1.5) was diluted in Opti-MEM medium to the final concentration as indicated in the experiments for each reaction. DharmaFECT 1 Transfection Reagent was

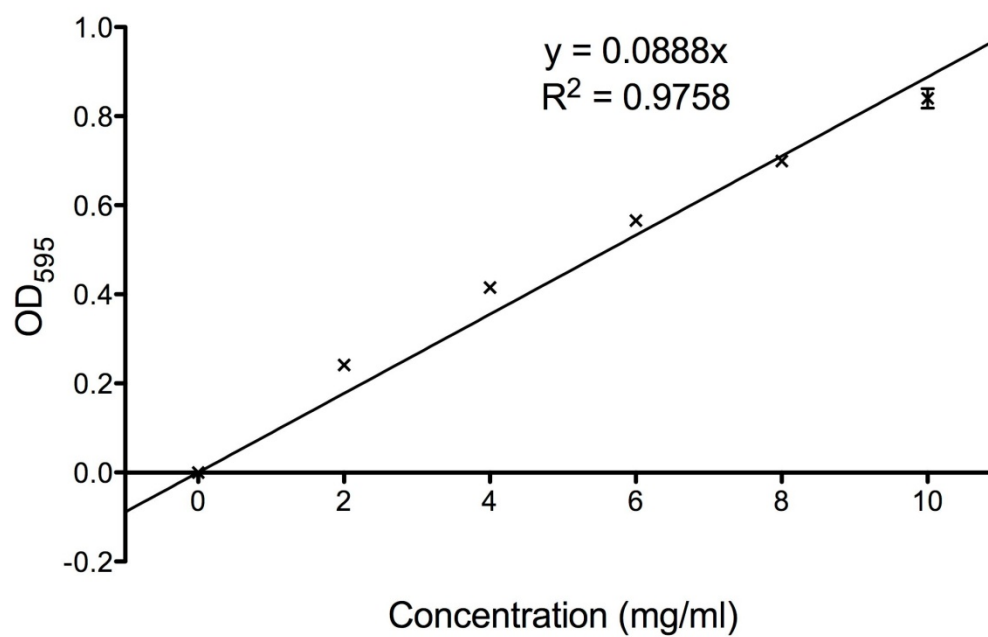


Figure 2-1 Sample standard curve of protein concentration assay

diluted in Opti-MEM medium (5 μ l reagent in 15 μ l medium for each reaction) and incubated at RT for 5 minutes. Diluted siRNA was mixed with DharmaFECT 1 solution and then incubated at RT for another 20 minutes. Approximately 150,000 cells maintained in antibiotics-free medium supplemented with 10% FBS were seeded on a 6-well plate with the DharmaFECT 1-siRNA complexes. The culture medium was replaced 24 hours post-transfection. Cells were cultured for the indicated time and/or treated with the indicated agents, and then subjected to RNA extraction or total cell counting.

2.2.10 Total RNA extraction

Cells were washed with ice-cold PBSA twice. Total RNA was then extracted using the RNeasy Mini kit (Qiagen) according to manufacturer's protocol. Genomic DNA was removed by DNase I (Bio-rad) treatment according to manufacturer's guideline. RNA was eluted using RNase free water and stored at -80°C .

2.2.11 Reverse transcription

RNA concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific). 2 μ g of RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription kit according to manufacturer's protocol. The cDNA products were then stored at -20°C .

2.2.12 Quantitative real-time PCR

Primers for quantitative real-time PCR (qPCR) (Section 2.1.6) were designed by Roche Applied Science Universal ProbeLibrary System (<http://lifescience.roche.com>) as previously described (Gijssen et al., 2010) or adopted from literature (Forster et al., 2011), and synthesized by Life Technology. 20 ng of cDNA was mixed with 300 nM of forward and reverse primers, and the SensiMix SYBR Hi-ROX qPCR master mix. Triplicate of the sample mixtures were analysed using Applied Biosystems 7900HT Fast Real-Time PCR

System (Life Technologies) under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A dissociation stage was performed after complete amplification: 15 seconds at 95°C, 20 seconds at 60°C and 15 seconds at 95°C. The threshold cycle for each triplicate was determined in the exponential phase of the amplification curve. Relative expression level of the gene of interest was calculated and normalised using the housekeeping gene β -actin.

2.2.13 Preparation of culture medium and cell lysate for ELISA

Approximately 3,000,000 cells were seeded to 10-cm culture dishes, allowed to adhere and enter log phase overnight. Cells were washed with serum-free medium three times prior to treatment. Cells were treated with the indicated concentration of lapatinib or DMSO control in serum-free medium for eight hours. Culture medium was collected and centrifuged at 1,231 g for three minutes to remove dead cells. Then the supernatants were concentrated by centrifugation in an Amicon Ultra-15, Ultracel 10 kDa centrifugal filter at 3,273 g for 35 minutes. The residues were collected and stored at -80°C. Cells were washed and scraped into RIPA buffer as previously described (Section 2.2.5).

2.2.14 Enzyme-linked immunosorbent assay (ELISA)

Cell lysate samples were assayed for protein concentration as previously described (Section 2.2.6). ELISA detection of NRG1 β 1 was performed using the Human NRG1- β 1/HRG1- β 1 DuoSet kit (R&D Systems) and the procedures were done according to manufacturer's protocol. Duplicates of 100 μ l of concentrated medium were loaded onto each well. 50 μ l of lysate was diluted to 250 μ l in reagent diluent (1% BSA in PBS) and duplicates of 100 μ l were loaded onto each well. A six-point standard curve was produced using a 2-fold serial dilution of a standard of 4000 pg/ml NRG1 β 1 and plotted using fourth order polynomial function in GraphPad Prism 6 (Figure 2-2 as an example). The

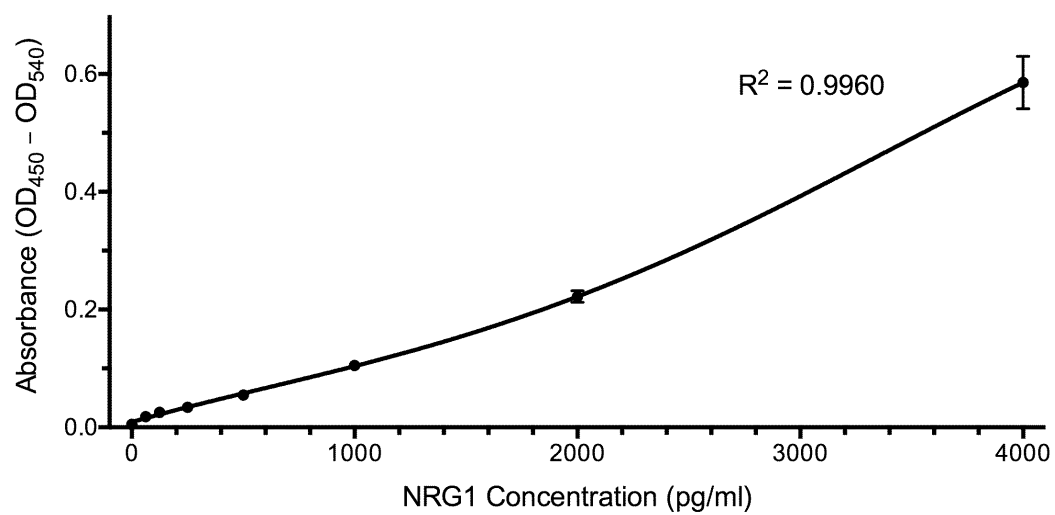


Figure 2-2 Sample standard curve of NRG1 β 1 ELISA

concentration of NRG1 β 1 in each sample was calculated using the standard curve and normalised with the protein concentration as a surrogate for cell number.

2.2.15 Flow cytometry

2.2.15.1 Apoptosis analysis

Approximately 150,000 cells per well were seeded on a 6-well tissue culture plate, allowed to adhere and enter log phase overnight. Each well was treated with the indicated agents for two days. Culture media were collected in 5 ml polypropylene round-bottom tubes (BD Falcon). The cells were washed twice with PBSA and the PBSA was retained to collect non-adhered cells. The cells were detached by incubating with 0.25% trypsin-EDTA solution at 37°C for three minutes. Detached cells were washed and resuspended with their respective culture medium-PBSA mixture. Along with the non-adhered cells, cells were pelleted by centrifugation at 233 g for three minutes and washed with PBSA twice. The washed cell pellet was then resuspended in 350 μ l of Annexin V (AV) staining solution (Section 2.1.8) and incubated in the dark at RT for 15 minutes. The stained cells were analysed using a CyAn ADP analyser (Beckman Coulter). AV-Alexa Fluor 647 was measured using excitation at 642 nm and emission detection at 665 nm. Propidium iodide (PI) was measured using excitation at 405 nm and emission detection at 675 nm.

2.2.15.2 Cell cycle analysis

Approximately 150,000 cells per well were seeded on a 6-well tissue culture plate, allowed to adhere and enter log phase overnight. Each well was treated with indicated agents for one day. The cells were collected and washed as previously described (Section 2.2.15.1). The washed cell pellet was then resuspended in 250 μ l of PBSA and fixed by a drop-wise addition of 2 ml of pre-chilled (-20°C) absolute methanol during gentle vortex, and then incubated at -20°C overnight. The fixed cells were pelleted by centrifugation at 335 g for

five minutes and washed twice with PBSA. The cell pellets were then resuspended in PI staining solution and incubated in dark at 37°C for 40 minutes. The stained cells were analysed using a CyAn ADP analyser. PI was measured using excitation at 405 nm and emission detection at 675 nm.

All the flow cytometry data were processed and analysed using FlowJo 7.6 software package.

2.2.16 Paraffin-embedded cell line pellet preparation

Cells were seeded on 10-cm culture dishes and treated as indicated in the experiments. After treatment, the cells were washed with PBSA twice and detached by incubating with 0.25% trypsin-EDTA solution at 37°C for three minutes. Detached cells were washed and resuspended with their respective culture medium. Cell suspension was spun at 1,000 rpm for three minutes and the medium was discarded. The cell pellet was resuspended and fixed in 4% formaldehyde solution with agitation at 4°C overnight. Fixed cells were pelleted at 1,000 rpm for three minutes and the formaldehyde solution was discarded. Fixed cell pellets were resuspended in 600 µl of agarose mixture (Section 2.1.8) at 60°C and immediately spun at 13,560 rpm for 10 seconds. After the agarose set, the gelled pellets were removed from microtubes and placed in paraffin processing cassettes. The cassettes were processed using a tissue processor at the Oxford Centre for Histopathology Research. The processed pellets were embedded in paraffin blocks and frozen at -20°C overnight. The samples were then sectioned to 4 µm using a microtome (Leica) and put on microscope slides (VWR). The slides were dried at 37°C overnight.

2.2.17 *In vivo* experiment

BalB/c-nude mice were purchased from Charles River. At the age of 5–6 weeks, approximately 5×10^6 BT-474 cells in 1:1 matrigel/RPMI 1640 medium were injected

subcutaneously into the mammary fat pad of the mice under general anaesthesia induced by isoflurane (performed by Jordan Tanner). The diet of the mice was supplemented by 5 µg/ml β -estradiol and 0.04% (v/v) Baytril in drinking water from two days before tumour cell implantation until the end of experiment. The xenograft tumour size was monitored twice a week before treatment and three times a week after treatment commenced. When the tumour size of more than 40% of mice reached 100-150 mm³ (length $\times$ width $\times$ height $\times$ 0.52), the mice were randomised into four treatment groups: a) TWEEN80 + PBSA, b) lapatinib + PBSA, c) TWEEN80 + pertuzumab, d) lapatinib + pertuzumab, and they received treatment for three weeks. Lapatinib was administered five times per week at 50 mg/kg body weight by oral gavage. Pertuzumab was administered once per week at 12 mg/kg body weight for the first week and 6 mg/kg body weight afterwards by intraperitoneal injection. The mice were euthanised when the geometric mean diameter ($[\text{length} + \text{width}] \div 2$) of the tumour exceeded 15 mm or animal welfare was compromised. The mice were euthanised by cardiac puncture under isoflurane general anaesthesia and their death was confirmed by neck dislocation (performed by Kelly Frater).

The blood was placed into EDTA tubes (Sarstedt) and placed on ice. Blood serum was collected after centrifugation at 3000 rpm at 4°C for 15 minutes, then snap-frozen in liquid nitrogen and stored at -80°C.

Residue tumours were quickly removed and weighed. Half of the tumour was snap-frozen in liquid nitrogen and stored at -80°C. The other half was fixed in 4% paraformaldehyde at 4°C for 48 hours and then stored in 70% ethanol at 4°C until being processed at Oxford Centre for Histopathology Research. The processed tumours were embedded in paraffin blocks and frozen at -20°C overnight. The samples were then sectioned to 4 µm using a microtome and put on microscope slides. The slides were dried at 37°C overnight.

2.2.18 Immunohistochemistry

2.2.18.1 Deparaffinising and rehydration

The slides were incubated at 60°C for 10 minutes and then immediately washed twice in histo-clear solution for five minutes. Then the slides were washed with 100% ethanol for five minutes, followed by 70% ethanol for five minutes, 50% ethanol for five minutes and finally tap water for five minutes.

2.2.18.2 Antigen retrieval

The rehydrated slides were put in a vessel containing 1× Target retrieval solution, EDTA pH8.0 buffer or Tris-EDTA pH9.0 buffer (according to antibody manufacturer's protocol). The vessel was heated to 125°C for two minutes in a pressure cooker. Once the cooker depressurised, the slides were rinsed in water.

2.2.18.3 Staining

The samples were blocked with normal horse serum (available from ImmPRESS kit, Vector Laboratories) at RT for 30 minutes. Primary antibodies diluted in normal horse serum were applied to the slides and incubated at 4°C overnight. The slides were washed in PBS for five minutes. Anti-mouse or rabbit secondary bodies (available from ImmPRESS kit, Vector Laboratories) were applied and incubated at RT for 30 minutes. The slides were washed in PBS for five minutes. ImmPACT DAB peroxidase substrate (Vector Laboratories) was diluted according to the manufacturer's protocol and applied to the slides for 1 to 10 minutes (depending on the primary antibody used), then washed with tap water. Haematoxylin QS counterstain was applied to the slides for 30 seconds and then washed with tap water. A cover glass was mounted on the stained samples slides using Aquatex mounting agent.

2.2.18.4 Scoring

Scoring of IHC staining was performed independently by the thesis author and a consultant breast pathologist, based on the intensity of staining and the percentage of cells stained with a particular intensity. Table 2-1 shows the scoring system of IHC staining intensity. The score of each intensity band was calculated by multiplying the intensity score with the percentage of cells in such intensity (e.g. if 20% of cells have 3+ intensity, the score would be 60). The total score of a sample is the sum of the scores of individual intensity bands; for example, a tumour sample, which has 60% of tumour cell with strong (3+) intensity, 20% of cells with moderate (2+) intensity and 20% of cells with weak (1+) intensity, has a total score of $3 \times 60 + 2 \times 20 + 1 \times 20 = 240$. The maximum score of a sample is 300 and the minimum is 0.

2.2.19 RTK Array

Cells were grown on 10-cm culture dishes to approximately 70% confluency. After the treatments of indicated agents, the cells were washed twice with ice-cold PBSA and lysed with ice-cold Lysis Buffer 17 provided in the RTK array kit. Sample preparation and subsequent array procedures were performed according to manufacturer's protocol using 100 µg of each sample. The array membranes were digitised using ImageQuant LAS 4000 mini system and quantified with ImageQuant TL version 7.0 software.

2.2.20 Statistical analysis

Statistical analysis was performed using Prism 6 software (GraphPad). For experiments involving multiple treatment groups and technical replicates (e.g. total cell counting and ELISA), the data were analysed using matched two-way ANOVA with Bonferroni post-hoc correction. For experiments involving multiple treatments without technical replicates (e.g. western blot densitometry and FACS), the data were analysed using matched one-way ANOVA with Bonferroni post-hoc correction. To compare dose-dependent effect on

Intensity	
Strength	Score
Strong	3+
Moderate	2+
Weak	1+
Negative	0

Table 2-1 **Scoring system of IHC staining**

different groups (e.g. lapatinib-sensitive and -resistant cell lines), the parameters of the best-fit line/curve were compared using extra sum-of squares F test. Comparison of two groups (regression vs. non-regression) was performed using unpaired t-test.

The symbols for p-values are as follow unless otherwise specified: *p <0.05, **p <0.01, ***p <0.001 and ****p <0.0001.

3. Acute and long-term responses to lapatinib of HER2-overexpressing cell lines

3.1 Introduction

As discussed in the introduction, there are several proposed mechanisms to explain lapatinib resistance. These models illustrate the changes in the breast cancer cells after 24 hours of lapatinib treatment, but the immediate response after lapatinib treatment within 24 hours has not been addressed. The acute response to lapatinib is very important, as it was previously shown that HER ligands are released within one hour of treatment, which mediates trastuzumab resistance (Gijsen et al., 2010). Therefore, the aim of this chapter is to address the acute as well as long-term response to lapatinib in HER2-overexpressing breast cancer cell lines. Cells were treated with lapatinib for a short period from 1 hour to 24 hours and tested for their responses in the HER signalling pathway. Acquired lapatinib-resistant cell lines were developed and the degree of resistance was confirmed using multiple methods. Resistant cells were subjected to HER signalling profiling to identify the changes in the cells when they were treated with lapatinib for a prolonged period of more than ten months. Finally, the lapatinib-resistant cell lines were tested to study whether the resistance to lapatinib was reversible, and to investigate if there were further changes after lapatinib was removed from the resistant cell lines.

3.2 Results

3.2.1 Assessing the growth inhibition caused by lapatinib treatment

SK-BR-3 and BT-474 are two HER2-overexpressing breast cancer cell lines that have been used as the model cell lines in previous research on HER2-targeted therapies (Baselga et al., 1998; Konecny et al., 2006; Sergina et al., 2007). They are sensitive to lapatinib treatment, as previously reported (Konecny et al., 2006; O'Brien et al., 2010; Rusnak et al., 2007). The concentrations of lapatinib that produced 50% of maximal inhibitory effect (IC_{50}) in SK-BR-3 and BT-474 ranged from 32-54 nM and 16-25 nM respectively based on the 3-to-7-day proliferation assays (Table 3-1).

To verify the above reports, total cell counting was performed after a 3-day treatment of lapatinib to SK-BR-3 and BT-474 cell lines. Results showed that lapatinib inhibited both cell lines in a concentration-dependent manner, while BT-474 cells were more sensitive to lapatinib than SK-BR-3 cells (Figure 3-2). The total cell number was normalised by taking the cell number at DMSO control as 100% and the highest dose of lapatinib as 0%, and then a four-parameter sigmoid curve was fitted as Figure 3-1. The IC_{50} values of lapatinib obtained from three independent experiments were 21.9 ± 4.5 nM and 8.1 ± 0.9 nM for SK-BR-3 and BT-474 respectively.

Analysing the change in total cell number by IC_{50} measures only the inhibitory effect on the whole population, but it does not describe the effect on growth inhibition. Therefore, the forward increase in cell number was computed by subtracting the number of cells seeded from the total number of cells counted, then an approximation of cell growth and the percentage growth inhibition were calculated and plotted (Figure 3-2). In SK-BR-3, 10 nM to 100 nM lapatinib significantly reduced cell growth (All $p < 0.0001$ compared to DMSO). A net cell-killing was observed at 250 nM and 500 nM (Both $p < 0.0001$

Authors	Duration	SKBR3 Lapatinib IC ₅₀	BT474 Lapatinib IC ₅₀
Rusnak et al.	3 days	32 ± 5 nM (SEM)	25 ± 4 nM (SEM)
O'Brien et al.	5 days	54 ± 8 nM (SD)	16 ± 11 nM (SD)
Konecny et al.	7 days	37 ± 31 nM (SEM)	22 ± 20 nM (SEM)
Leung	3 days	21.9 ± 4.5 nM (SD)	8.1 ± 0.9 nM (SD)

Table 3-1 Summary of IC₅₀ concentrations of lapatinib in HER2-overexpressing breast cancer cell lines

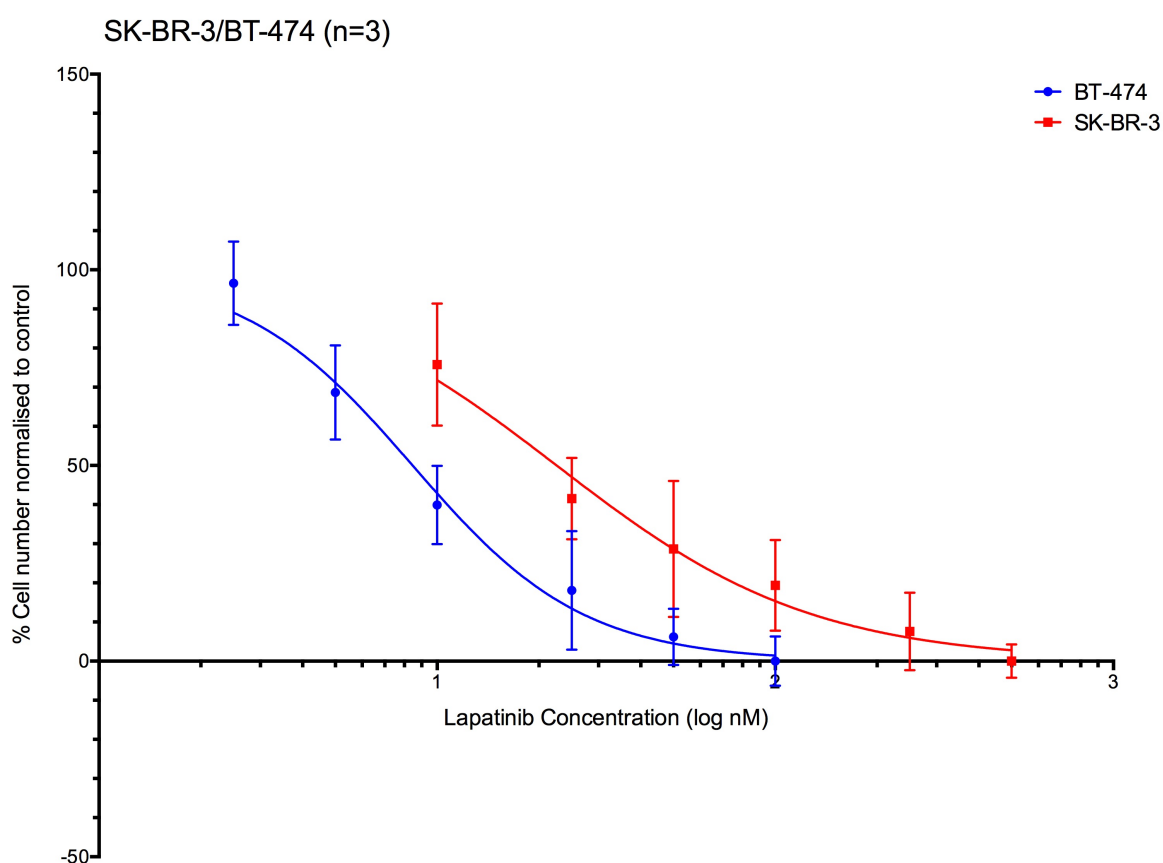


Figure 3-1 Dose-dependent effect of lapatinib on total cell number of HER2-overexpressing cell lines

SK-BR-3 (red) and BT-474 (blue) cell lines were treated with the indicated concentration of lapatinib or DMSO control for three days. Cells were then trypsinised and counted. Three independent experiments were done with three technical replicates. The percentage cell was normalised against the DMSO control as 100% and the highest lapatinib dose as 0%, then plotted against the log of drug concentration and fitted with a four-parameter sigmoid curve. (Error = SD)

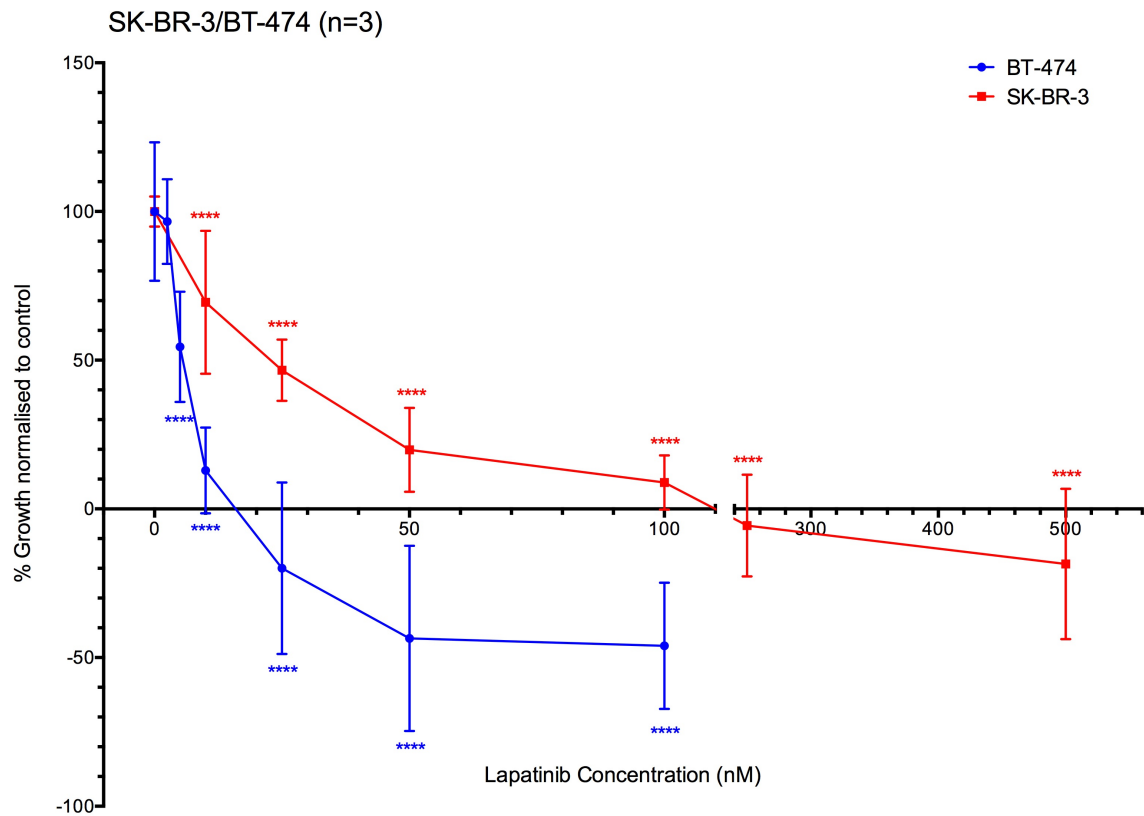


Figure 3-2 Dose-dependent effect of lapatinib on cell growth of HER2-overexpressing cell lines measured by total cell counting

Cell growth from the experiment in Figure 3-1 was calculated as the change in cell number relative to the number of cells seeded. The percentage growth was normalised to the cell growth in DMSO control. SK-BR-3 and BT-474 cell lines were plotted in red and blue respectively. (Error = SD)

compared to DMSO). In BT-474, 5 nM and 10 nM lapatinib reduced cell growth significantly ($p < 0.0001$ compared to DMSO). From 25 nM to 100 nM, lapatinib produced a net cell-killing effect to BT-474 cells (All $p < 0.0001$ compared to DMSO). The concentrations of lapatinib that caused 50% of growth inhibition in SK-BR-3 and BT-474 measured by total cell counting are 19.0 ± 9.6 nM and 5.5 ± 0.9 nM respectively.

The total cell counting method measured only the end-point of an experiment. Since the cancer cells grow exponentially, measuring only the number of cells at the end of an experiment might not provide an accurate estimation of growth rate. Therefore, a real-time monitoring approach was employed to assess the growth inhibition by lapatinib using the IncuCyte imaging system, aiming to more accurately estimate the growth rate of cells. The advantages and disadvantages of using IncuCyte will be discussed in Section 3.3.2.

SK-BR-3 and BT-474 cells were treated with DMSO control or increasing doses of lapatinib from 5 nM to 1 μ M. Figure 3-3 shows the growth of SK-BR-3 and BT-474 cell lines under the treatment of different doses of lapatinib along the time axis. Representative microscopic images of the cells were shown in Figure 3-4. The number of SK-BR-3 cells treated with DMSO increased during the 4-day course of imaging, while cell growth was inhibited in those cells treated with 250 nM lapatinib. BT-474 cells showed retarded growth in 10 nM lapatinib and the cell number decreased in 250 nM lapatinib, indicating the drug was killing the cells in higher dose.

When the culture media and drug were replaced (indicated by the arrows in Figure 3-3), there was a sudden increase in the confluency of SK-BR-3 cells under all treatment conditions and in BT-474 cells with lapatinib concentration up to 10 nM. Contrarily, the confluency of BT-474 cells under lapatinib treatments from 50 nM decreased. The increase in the confluency of SK-BR-3 and BT-474 cells under lower lapatinib concentrations was contributed by the increased size of the cells after media and drug

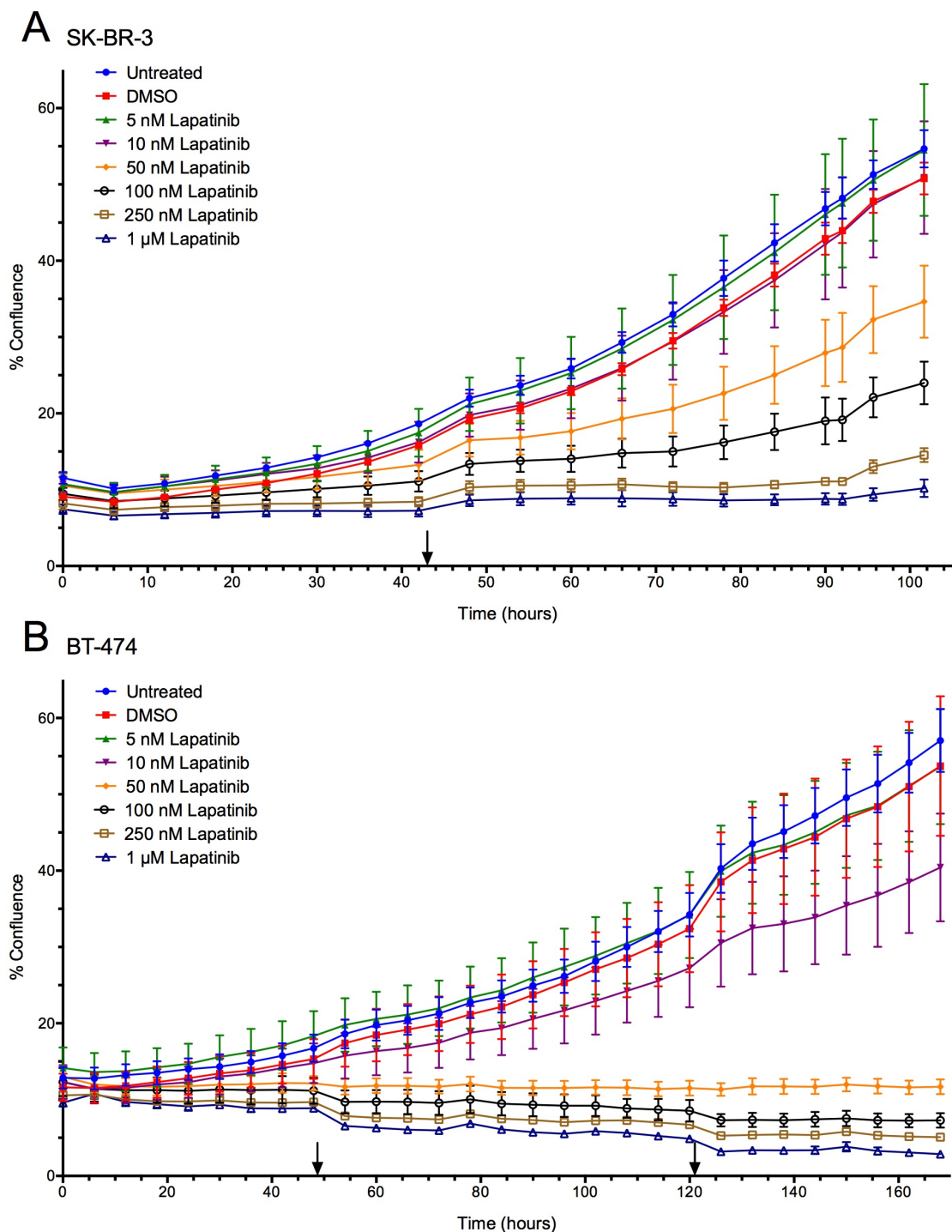
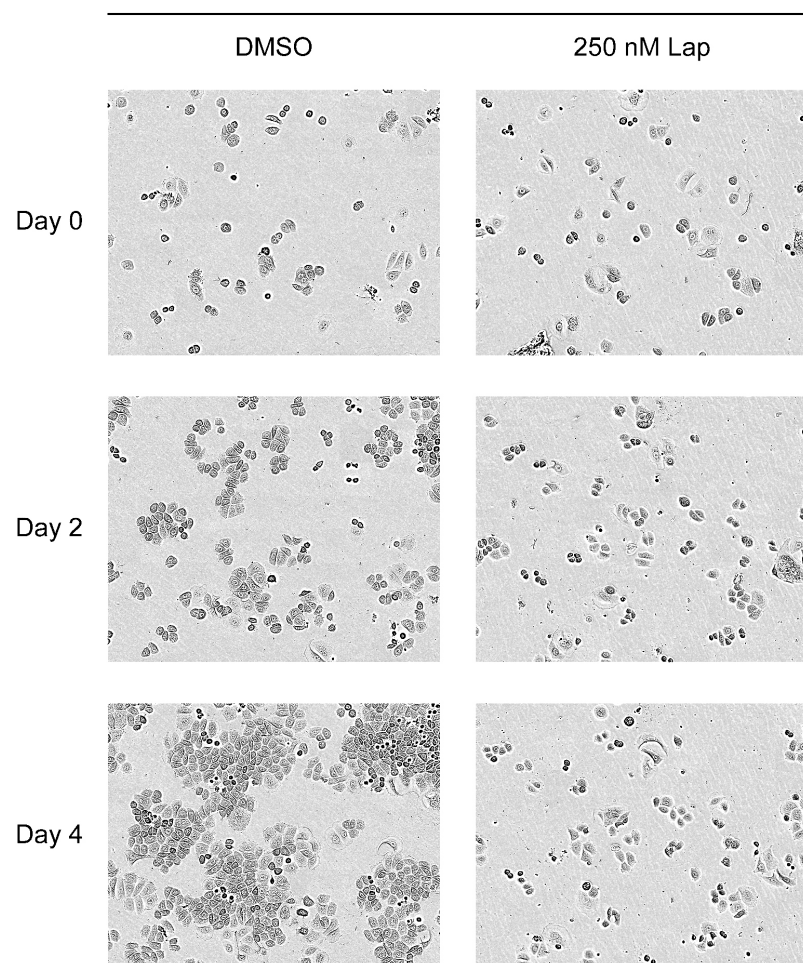


Figure 3-3 Real-time monitoring of growth inhibition caused by lapatinib in HER2-overexpressing cell lines

(A) SK-BR-3 and (B) BT-474 cell lines were treated with the indicated concentrations of lapatinib. The growth of cells was monitored using IncuCyte real-time imaging system over (A) four days or (B) seven days, with 16 views captured in each well at least every six hours. The drug and culture medium were replaced on day 2 and day 5. Three independent experiments were done with three technical replicates, and the confluency of the wells was calculated using manufacturer's algorithm Confluence v1.5. Reproducible representative results of are shown above. Arrow: Time of culture medium and drug replacement (Error = SD)

A

SK-BR-3



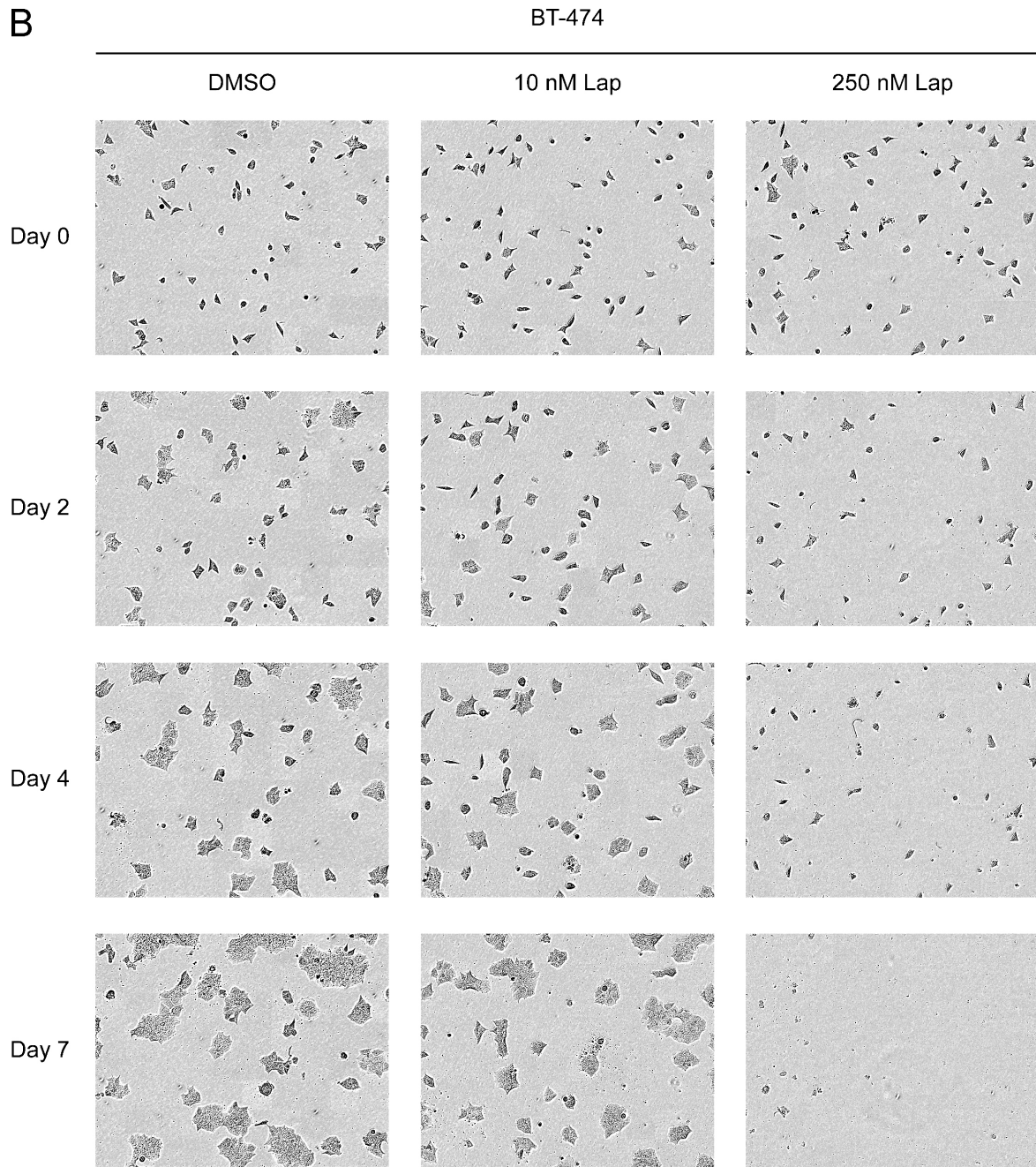


Figure 3-4 Microscopic images of lapatinib-sensitive cells under lapatinib treatment

(A) SK-BR-3 and (B) BT-474 were treated with the indicated concentrations of lapatinib or DMSO control for (A) four or (B) seven days respectively. Representative images from three independent experiments were shown.

replacement (Figure 3-5A and B). The decrease in the confluency of BT-474 under higher lapatinib concentrations was caused by the removal of dead cells during media and drug replacement (Figure 3-5C).

The growth rate of the cell lines was computed and plotted against the log of the concentrations of lapatinib (Figure 3-6). According to the growth rate computed using the data from real-time monitoring, lapatinib caused dose-dependent growth inhibition in SK-BR-3 and BT-474 cells (Figure 3-6). Significant growth inhibition occurred from 5 nM for SK-BR-3 cells ($95 \pm 1\%$, $p = 0.0031$) and 10 nM for BT-474 cells ($76 \pm 11\%$, $p < 0.0001$). From 50 nM lapatinib, the drug caused a negative growth rate in BT-474 cells, suggesting the dose above 50 nM produced a net cell-killing effect in this cell line. A four-parameter non-linear curve was fitted to the data, and the concentration of lapatinib required to cause 50% growth inhibition (GI_{50}) was interpolated from the curve. The GI_{50} of lapatinib for SK-BR-3 and BT-474 are 91.2 ± 10.9 nM and 18.7 ± 4.0 nM respectively.

3.2.2 Acute effect of lapatinib on HER2 signalling pathway

The acute effect of lapatinib on the HER signalling pathway and its downstream effectors was studied using 100 nM lapatinib, a concentration close to the GI_{50} value in SK-BR-3 cells. In SK-BR-3 cells, lapatinib readily dephosphorylated EGFR, HER2 and HER3 to an undetectable level after four hours of treatment (Figure 3-7A). However, the phosphorylation of EGFR, HER2 and HER3 started to recover from the eighth hour of lapatinib treatment, and further increased after 24 hours of treatment. Nevertheless, the level of phosphorylation of these proteins was still inhibited by lapatinib, as the phosphorylation level was lower than the DMSO control.

To demonstrate that the recovery of phosphorylation was not due to the degradation of lapatinib at the tissue culture condition, the efficacy of fresh-defrosted lapatinib was

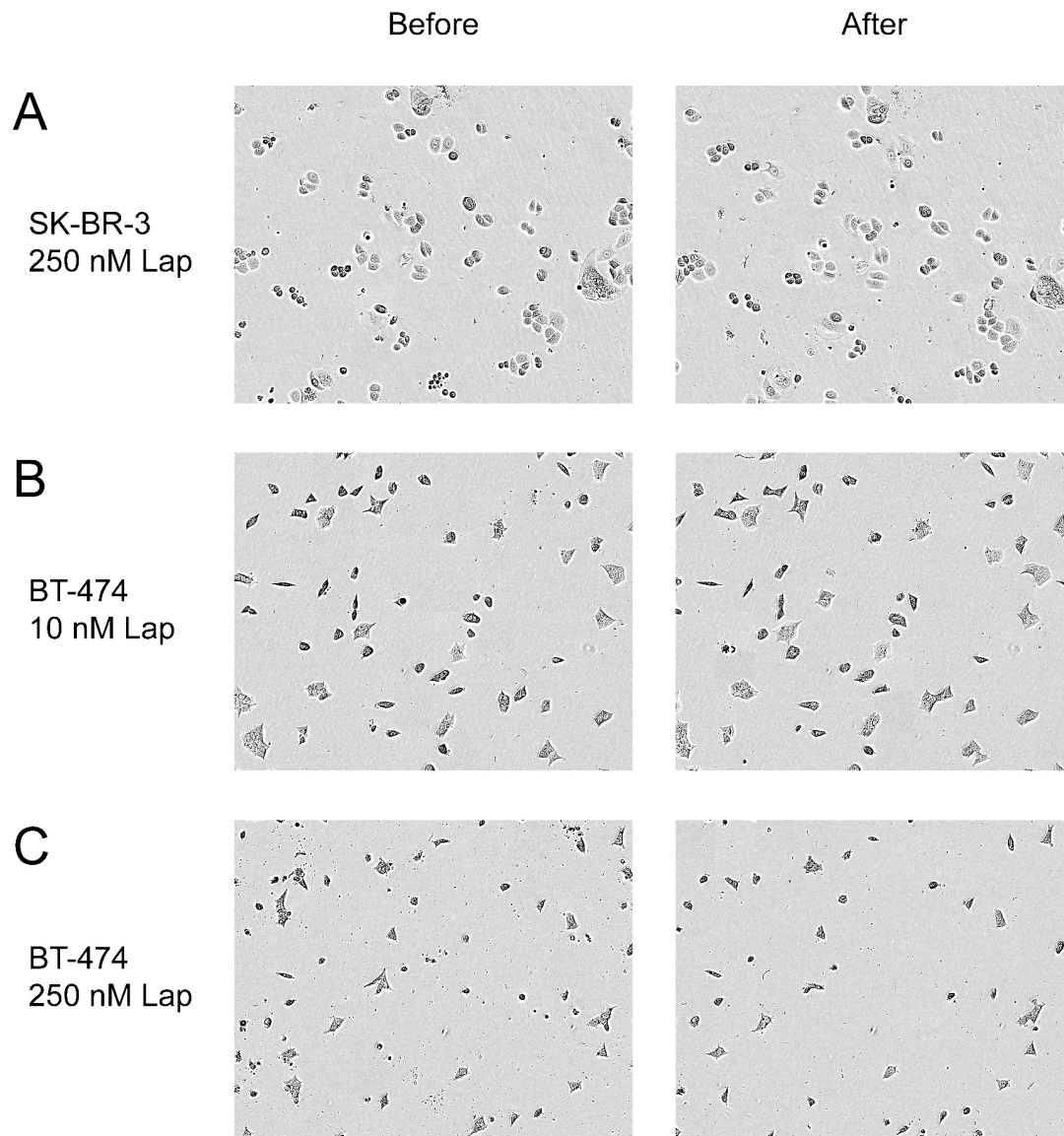


Figure 3-5 Microscopic images of lapatinib-sensitive cells before and after the replacement of culture medium and drug

Selected microscopic images of the cells to illustrate the events one hour before and five hours after the replacement of culture media. (A) SK-BR-3 in 250 nM lapatinib and (B) BT-474 in 10 nM lapatinib represented the increase in confluency after replacement. (C) BT-474 in 250 nM lapatinib represented the decrease in confluency after replacement.

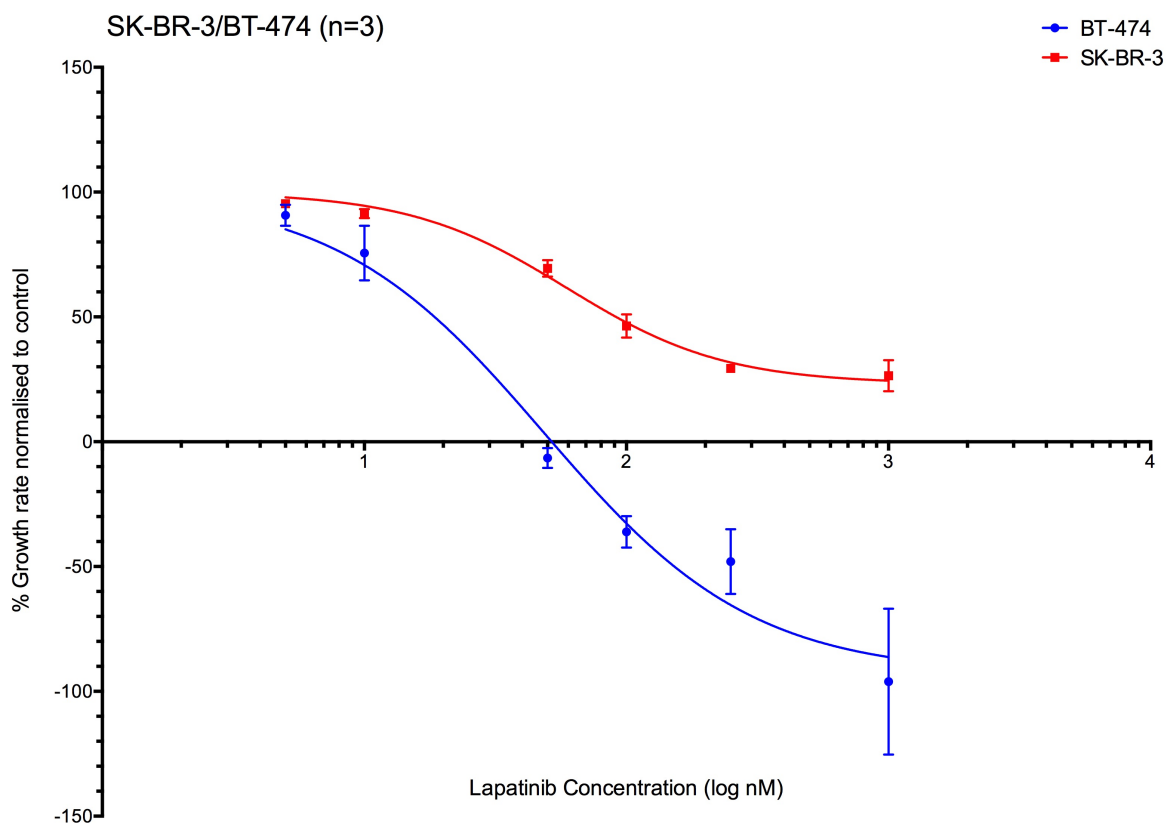


Figure 3-6 Dose-dependent effect of lapatinib on growth rate of lapatinib-sensitive HER2-overexpressing cell lines measured by real-time monitoring

The log of confluency of cells in the experiments of Figure 3-3 was plotted against time, and the slope of their linear regression was taken as the magnitude of growth rate. The percentage growth rate with respect to DMSO control was plotted against the log of lapatinib concentration as above. Four-parameter sigmoid curve fit was employed to interpolate the concentration of lapatinib that causes 50% decrease of growth rate (GI_{50}). (Error = SD)

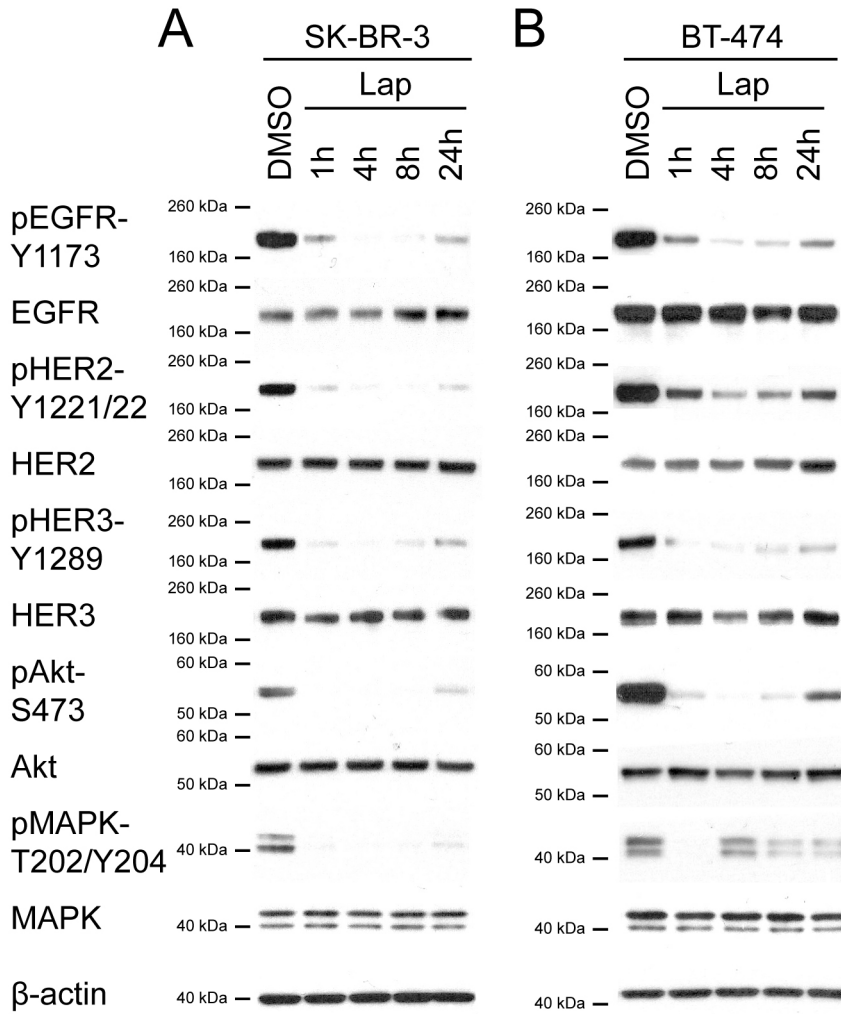


Figure 3-7 Effect of lapatinib on the phosphorylation status of HER receptors and downstream signalling

(A) SK-BR-3 and (B) BT-474 cells were treated with 100 nM lapatinib for indicated time or DMSO solvent control for 24 hours. Complete cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted for Tyr1173-phosphorylated EGFR (pEGFR-Y1173), total EGFR (EGFR), Tyr1221/22-phosphorylated HER2 (pHER2-Y1221/22), total HER2 (HER2), Tyr1289-phosphorylated HER3 (pHER3-Y1289), total HER3 (HER3), Ser473-phosphorylated Akt (pAkt-S473), total Akt (Akt), Thr202/Tyr204-phosphorylated p44/42 MAPK (pMAPK-T202/Y204) and total p44/42 MAPK (MAPK). β -actin blot served as loading control in all experiments. Reproducible representative results of at least three independent experiments are shown.

compared with lapatinib that was incubated at 37°C for 24 hours before use (Figure 3-8). Pre-incubated and fresh-defrosted lapatinib treatment of one hour did not produce detectable difference in HER2 phosphorylation, indicating the pre-incubated lapatinib was as effective as the fresh-defrosted one. However, the above experiment does not take into account the effect of cell metabolism on the efficacy of lapatinib. In another experiment, lapatinib was incubated with cells for 24 hours before the conditioned medium was used to treat a new batch of cells. Fresh lapatinib and lapatinib-conditioned medium also did not cause any detectable difference in HER2 phosphorylation (Figure 3-9).

There were no observable differences in total protein levels of EGFR, HER2 and HER3 in SK-BR-3 cells (Figure 3-7A). Although the phosphorylation of Akt and MAPK dropped to undetectable levels after one hour of lapatinib treatment, it recovered at 24 hours to a below-basal level. The total protein levels of Akt and MAPK were unchanged throughout the course of treatment. The dephosphorylation of downstream elements Akt and MAPK correlated with the inhibition of upstream receptors EGFR, HER2 and HER3, while the reactivation of downstream proteins lagged behind that of the receptors.

In BT-474 cells, the phosphorylation of EGFR, HER2, HER3 and Akt followed a similar pattern as in SK-BR-3 cells, with the minimum phosphorylation after four hours of treatment and recovery from eight hours (Figure 3-7B). The dephosphorylation and reactivation of Akt correlated with upstream receptors in the same way as in SK-BR-3, but phospho-MAPK followed a different pattern. The phosphorylation of MAPK decreased to an undetectable level after one hour of treatment; however, it rose to near basal level at four hours and decreased again from 8 to 24 hours. From 4 hours to 24 hours, the level phospho-MAPK inversely correlated with that of phospho-Akt, suggesting that there might be a crosstalk between MAPK and Akt in BT-474. Recently, there is a report showing that the inhibition of Akt/mTOR pathway activates ras/MAPK pathway in hepatocellular

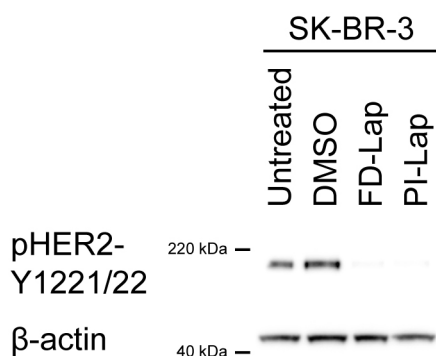


Figure 3-8 Effect of incubation of lapatinib in tissue culture condition for 24 hours

SK-BR-3 cells were treated with 100 nM fresh-defrosted (FD) or 24 hour pre-incubated (PI) lapatinib or DMSO solvent control for one hour. Complete cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted for Tyr1221/22-phosphorylated HER2 (pHER2-Y1221/22). β-actin blot served as loading control in all experiments. Reproducible representative results of at least three independent experiments are shown.

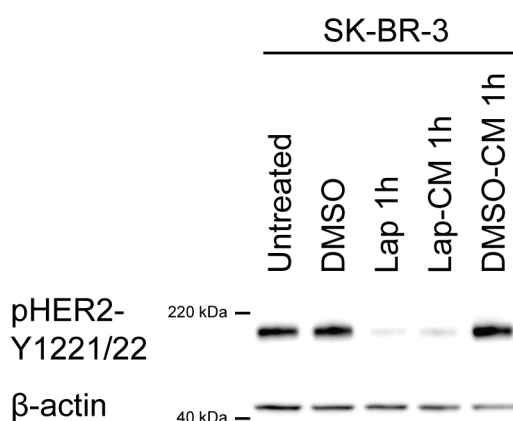


Figure 3-9 Effect of incubation of lapatinib with cells for 24 hours

SK-BR-3 cells were treated with DMSO solvent control or 100 nM lapatinib or the conditioned media (CM), which were from SK-BR-3 treated for 24 hours with same dose of DMSO or lapatinib, for one hour. Complete cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted for Tyr1221/22-phosphorylated HER2 (pHER2-Y1221/22). β-actin blot served as loading control in all experiments. Reproducible representative results of at least three independent experiments are shown.

carcinoma, although the mechanism was yet unclear (Wang et al., 2013a). Previous studies also found that Akt inhibits Raf (Zimmermann and Moelling, 1999) and GAB2 (Lynch and Daly, 2002) upstream of MAPK. Activation of MAPK might be caused by the derepression of Raf and GAB2 via inhibition of Akt. The total protein level of EGFR, HER2, HER3, Akt and MAPK did not change in BT-474 upon acute lapatinib treatment.

In summary, lapatinib inhibited the phosphorylation of all the HER receptors investigated and the downstream effectors Akt and MAPK after four hours, but the phosphorylation recovered from eight hours of treatment. Except in BT-474 cells, the phosphorylation of MAPK decreased to minimum after one hour of lapatinib treatment, recovered at four hours but reduced again after eight hours.

3.2.3 Development of lapatinib-resistant cell lines

In order to study the effect of prolonged lapatinib treatment, the two lapatinib-sensitive cell lines SK-BR-3 and BT-474 were chronically exposed to an increasing dose of lapatinib from 25 nM to 250 nM for ten months. The resistance of the cell lines was assessed by growth inhibition assay and cell viability assay.

The growth inhibitory effect of different concentrations of lapatinib on lapatinib-resistant SK-BR-3 and BT-474 cells (from here on designated as SK250LR and BT250LR respectively) was monitored on the IncuCyte real-time monitoring system. The resulting growth curves showed that lapatinib could still impose a growth inhibition effect in both resistant cell lines (Figure 3-10); however, the cells were much less sensitive to the drug. Representative microscopic images of the cells were shown in Figure 3-11. Similar to

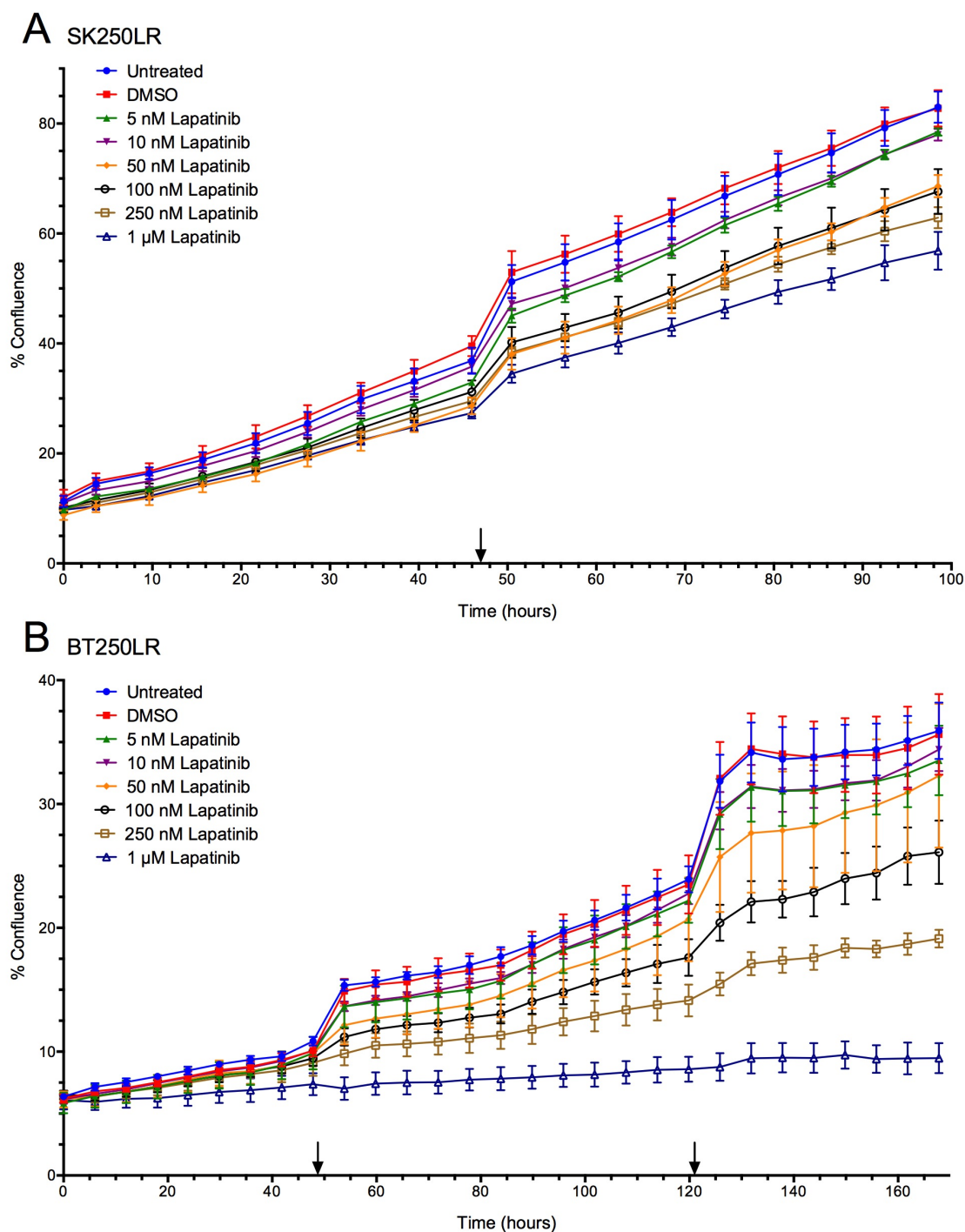


Figure 3-10 Real-time monitoring of growth inhibition caused by lapatinib in lapatinib-resistant cell lines

(A) SK250LR and (B) BT250LR cells were treated with the indicated concentration of lapatinib. The growth of cells was monitored using IncuCyte real-time imaging system over four days (A) or seven days (B), with 16 views captured in each well at least every six hours. The drug and culture medium were replaced on day 2 and day 5. Three independent experiments were done with three technical replicates, and the confluency of the wells was calculated using manufacturer's algorithm Confluence v1.5. Reproducible representative results of are shown above. Arrow: Time of culture medium and drug replacement (Error = SD)

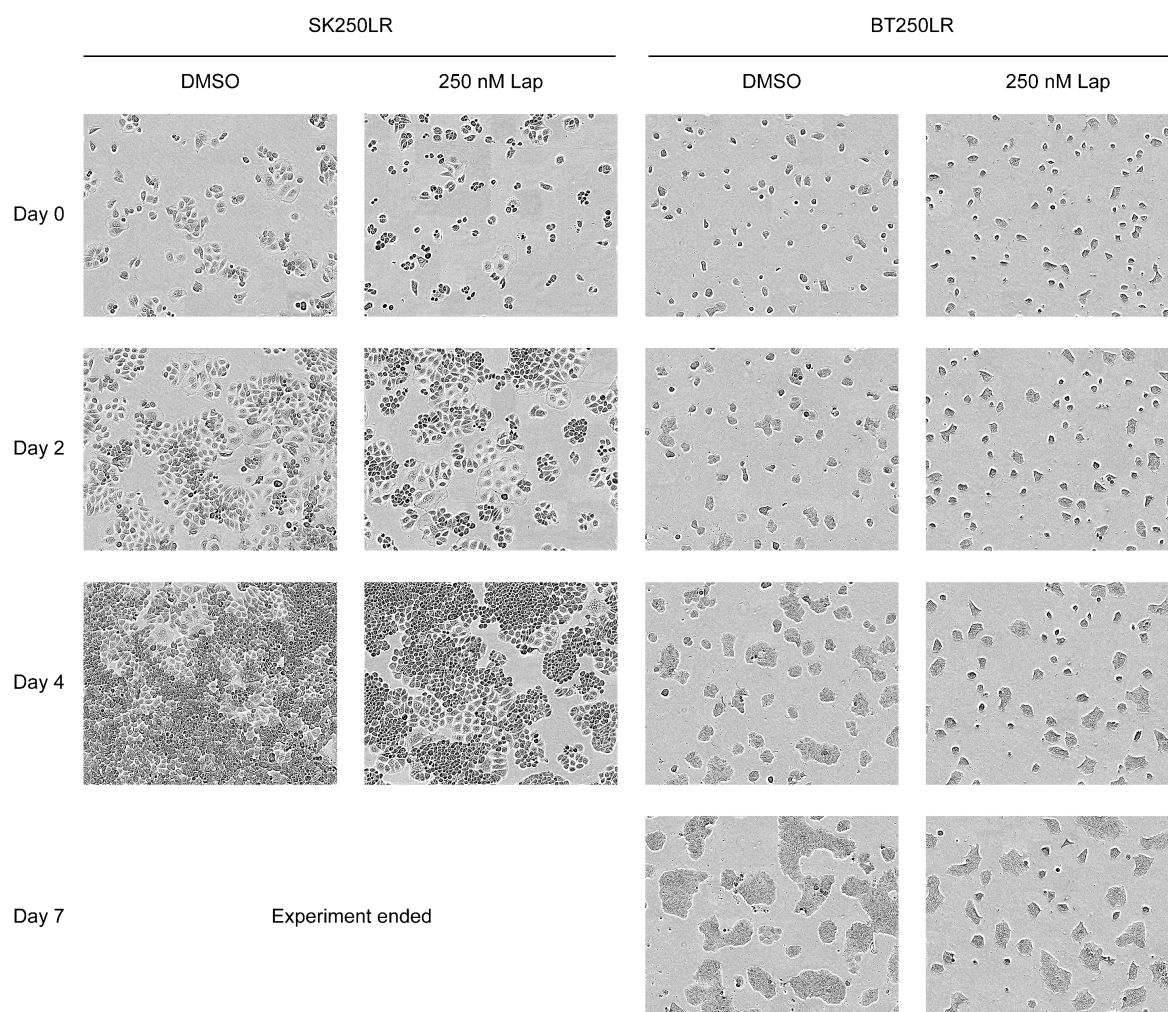


Figure 3-11 Microscopic images of lapatinib-resistant cells under lapatinib treatment

SK250LR and BT250LR cells were treated with 250 nM lapatinib or DMSO control for four and seven days respectively. Representative images from three independent experiments were shown.

lapatinib-sensitive cell lines, the replacement of media and drug (Figure 3-10, arrows) caused an increase in the size of the cells and increased the confluency sharply (Figure 3-12).

Only 250 nM and 1 μ M lapatinib reduced growth rate of SK250LR cells to $93\pm6\%$ ($p = 0.0118$) and $90\pm4\%$ ($p = 0.0004$) respectively. In BT250LR cells, 5 nM and 10 nM lapatinib did not significantly affect the growth rate, but growth inhibition occurred from 50 nM ($91\pm4\%$, $p = 0.0006$) to 1 μ M (30 ± 6 , $p<0.0001$) lapatinib. The resistance of lapatinib in SK250LR and BT250LR cell lines was confirmed by comparing the effect of lapatinib on growth inhibition between the sensitive and resistant cell lines. Figure 3-13 showed that the dose-response effect of lapatinib on growth inhibition in both lapatinib-resistant cell lines were statistically different from their respective parental cell lines (Both $p < 0.0001$). For SK250LR cells, the GI_{50} concentration of lapatinib could not be derived from the sigmoid curve fit because the highest dose of lapatinib used (1 μ M) was not adequate to inhibit 50% of the growth rate (Figure 3-13A). Therefore, the concentration of lapatinib that caused 10% growth inhibition, GI_{10} , was used to compare between SK-BR-3 and SK250LR cell lines. The GI_{10} of lapatinib for SK-BR-3 and SK250LR cell lines were 17.2 ± 3.8 nM and 235.4 ± 18.5 nM respectively, and this was statistically significant ($p = 0.0002$). BT-474 and BT250LR cell lines were compared between their GI_{50} value of lapatinib, which were 18.7 ± 4.0 nM and 365.0 ± 10.9 nM respectively ($p < 0.0001$) (Table 3-2).

To further confirm the lapatinib resistance in the cell lines, clonogenic assay was performed in all the four cell lines after lapatinib treatment at different concentrations for three days. Figure 3-14 summarises the dose-dependent relationship with the number of colony formed after treatment. Statistical differences were recorded between SK-BR-3 and SK250LR cell lines ($p = 0.0008$) (Figure 3-14A) and between BT-474 and BT250LR cell

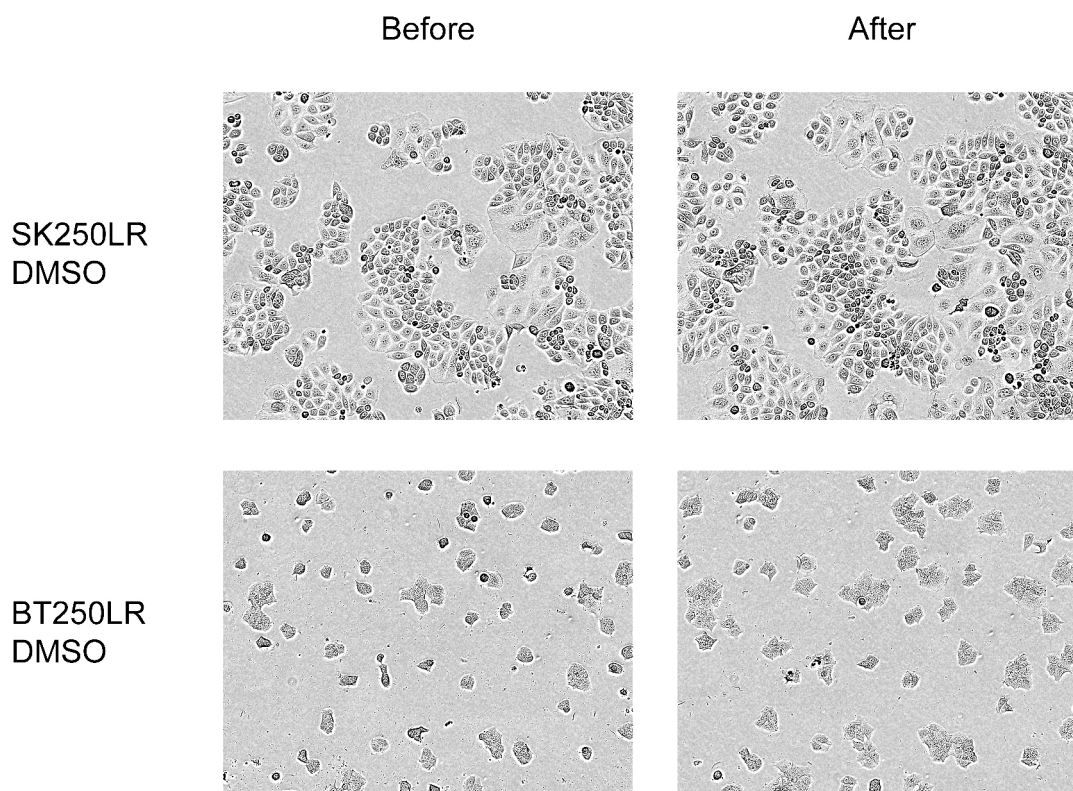


Figure 3-12 Microscopic images of lapatinib-resistant cells before and after the replacement of culture medium and drug

Selected microscopic images of the cells to illustrate the events one hour before and five hours after the replacement of culture media.

GI₁₀ value		
SK-BR-3	SK250LR	P value
17.2 ± 3.8 nM	235.4 ± 18.5 nM	0.0002
GI₅₀ value		
BT-474	BT250LR	P value
18.7 ± 4.0 nM	365.0 ± 10.9 nM	<0.0001

Table 3-2 Comparison of GI values between lapatinib-sensitive and -resistant SK-BR-3 and BT-474 (Error = SD; Unpaired t-test)

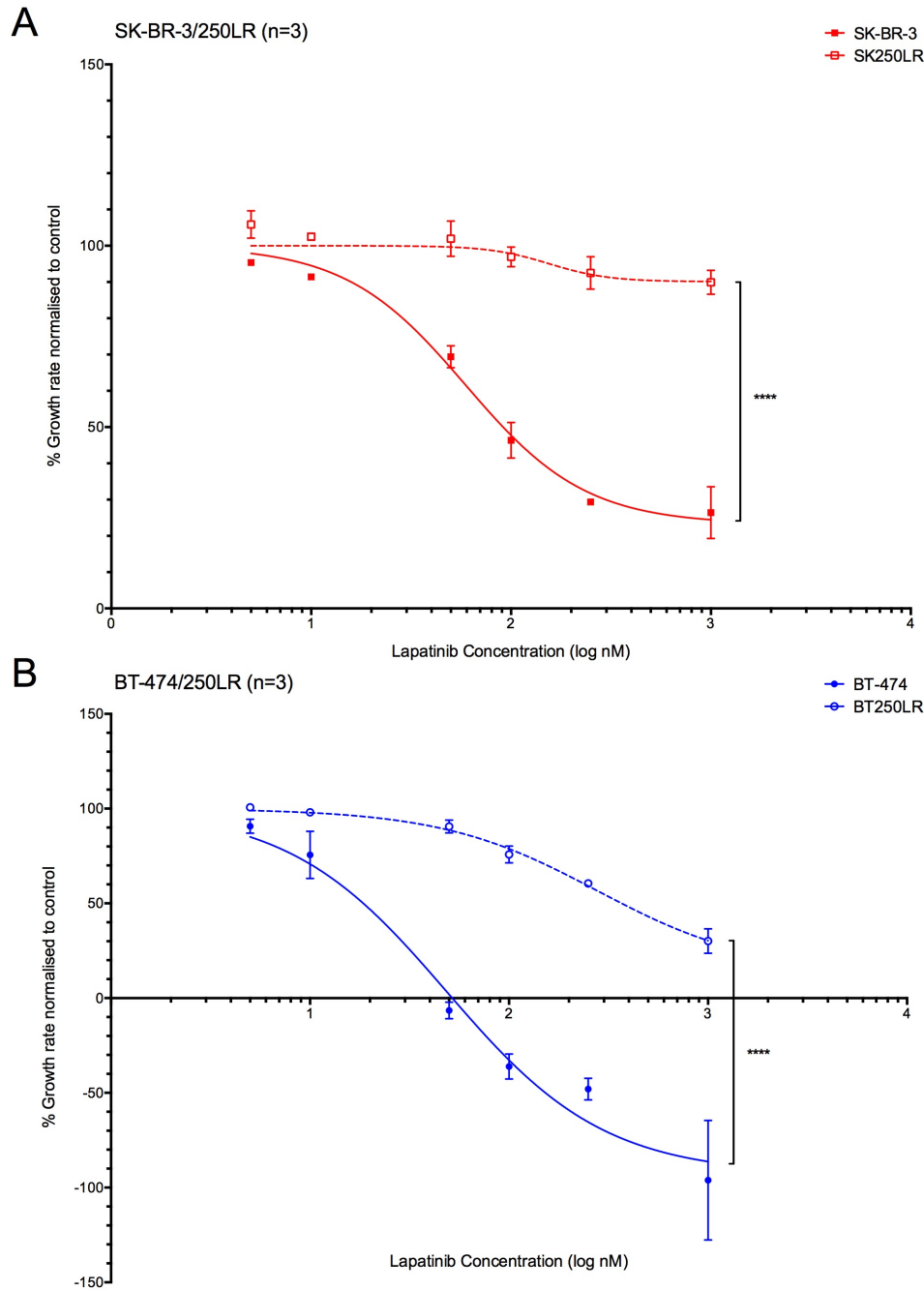


Figure 3-13 Dose-dependent effect of lapatinib on growth rate of lapatinib-sensitive and -resistant HER2-overexpressing cell lines measured by real-time monitoring

(A) SK-BR-3 (solid) and SK250LR (dashed), (B) BT-474 (solid) and BT250LR (dashed) cell lines were treated with the indicated dose of lapatinib for (A) four days or (B) seven days (Experiments shown in Figure 3-3 and Figure 3-10). The log of confluency of cells was plotted against time, and the slope of their linear regression was taken as the magnitude of growth rate. The percentage growth rate with respect to DMSO control was plotted against the log of lapatinib concentration using a four-parameter sigmoid curve fit. (Error = SD; Extra sum-of-squares F test, **** $p < 0.0001$)

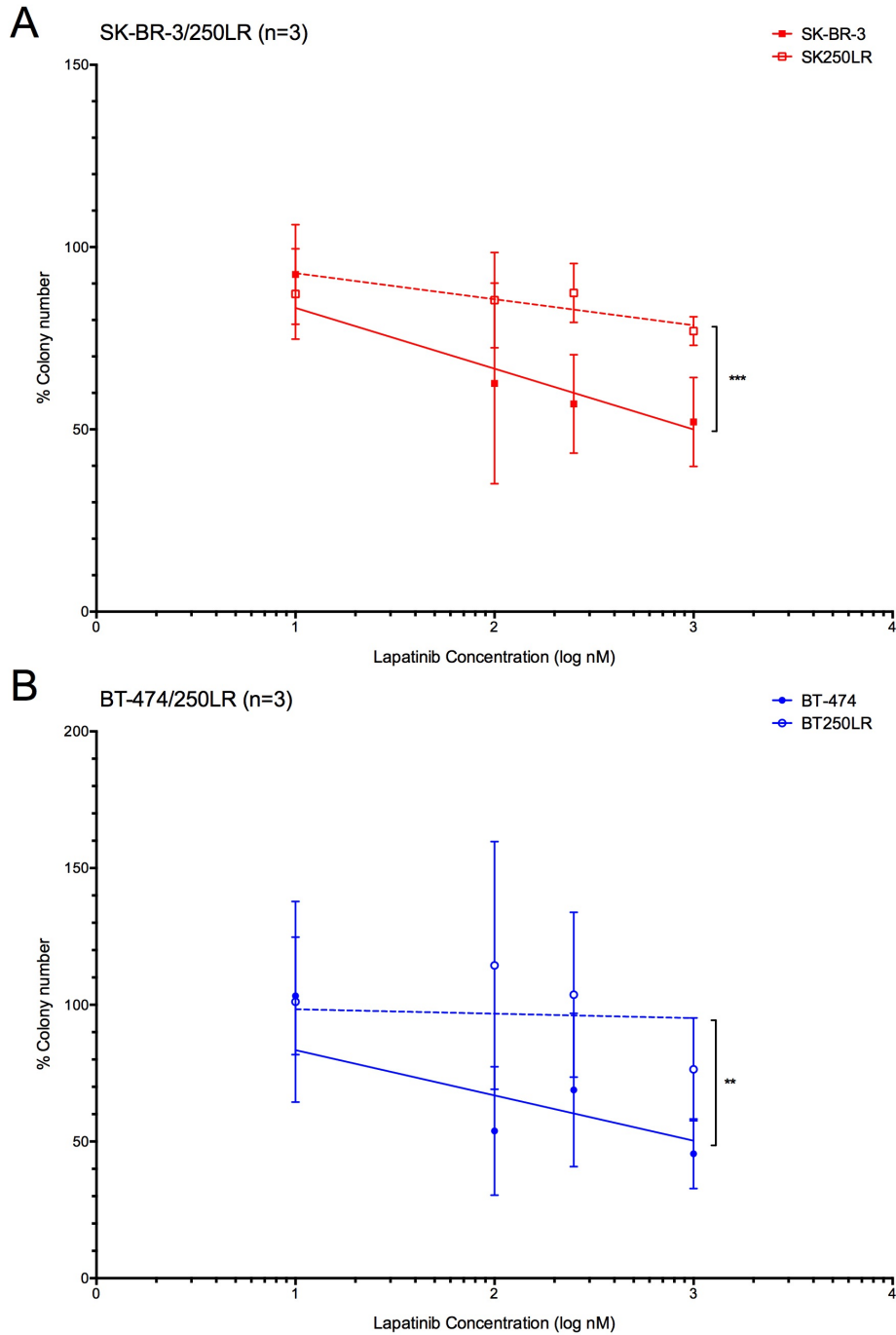


Figure 3-14 Dose-dependent effect of lapatinib on clonogenicity of lapatinib-sensitive and -resistant HER2-overexpressing cell lines

(A) SK-BR-3 (solid) and SK250LR (dashed), (B) BT-474 (solid) and BT250LR (dashed) cell lines were treated with the indicated concentrations of lapatinib for three days. Cells were then trypsinised, reseeded and allowed to grow into colonies. The number of colony was normalised to DMSO control. Three independent experiments were performed with three technical replicates. The percentage colony number was plotted against the log of lapatinib concentration. (Error = SD; Extra sum-of-squares F test, * $p < 0.05$, ** $p < 0.01$)

lines ($p = 0.0085$) (Figure 3-14B). Representative experiments were shown in Figure 3-15 (for SK-BR-3 and SK250LR cells) and Figure 3-16 (for BT-474 and BT250LR cells).

3.2.4 HER signalling in lapatinib-resistant cell lines

In order to understand the response to lapatinib treatment in lapatinib-resistant cell lines, the HER signalling profiles of resistant cell lines were compared to the sensitive cell lines upon acute treatment. The lapatinib concentration used here was the same concentration used to produce the resistant cell lines, i.e. 250 nM. In addition, lapatinib was withdrawn from the resistant cell lines to see whether the cells would revert their phenotype. In SK-BR-3 cell line, lapatinib inhibited the phosphorylation of all HER receptors as well as downstream effectors MAPK and Akt (Figure 3-17A). In the SK250LR cells, the phosphorylation of HER receptors was still significantly suppressed even upon withdrawal of the drug, suggesting a permanent alteration to the cell biology. Interestingly, there were also changes in the total levels of the HER receptors, which may be involved in the resistance mechanism. The levels of total EGFR and HER3 increased in SK250LR cell line whereas HER2 levels decreased. This change remained constant for EGFR and HER2 even after the removal of lapatinib for one day, but HER3 levels decreased. Unfortunately, western blotting detection of total HER4 was unsuccessful with no bands observed using two different HER4 antibodies. Phospho-Akt and phospho-MAPK levels were reduced by lapatinib treatment in both cell lines, but to a greater extent in the parental line. Withdrawal of lapatinib from SK250LR triggered an increase in phospho-Akt and phospho-MAPK levels, but they were still less than the untreated cells. The anti-MAPK antibody used in this study detects both p44-MAPK (ERK1/MAPK3) and p42-MAPK (ERK2/MAPK1). There was an increase in p44-MAPK in SK250LR cell line and this change was maintained after lapatinib withdrawal.

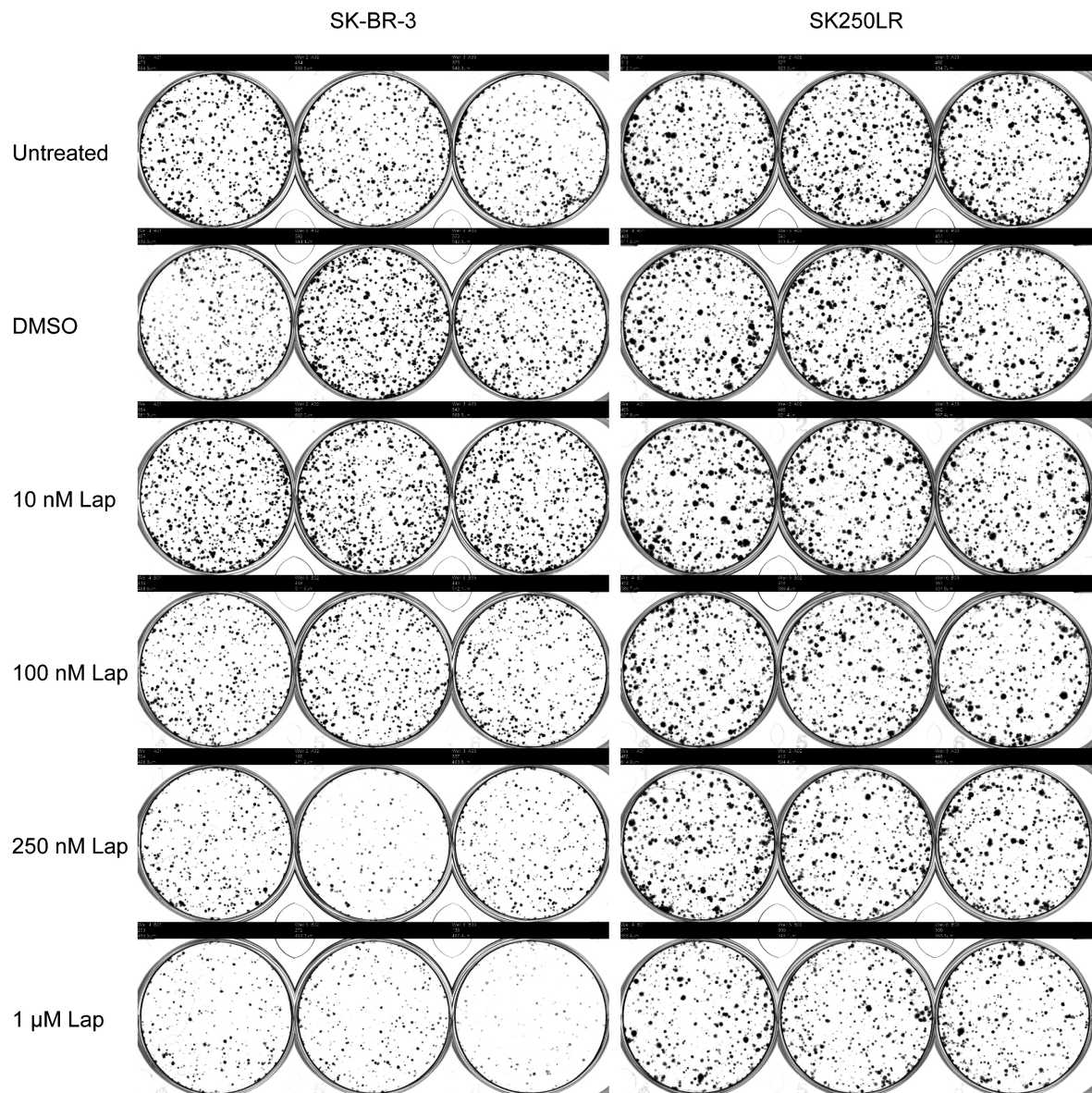


Figure 3-15 Representative experiment for dose-dependent effect on clonogenicity of SK-BR-3 and SK250LR

SK-BR-3 and SK250LR were treated with the indicated concentration of lapatinib or DMSO control for three days. Cells were then trypsinised, reseeded and allowed to grow for 14 days. Plates were then washed with PBS and stained with methylene blue.

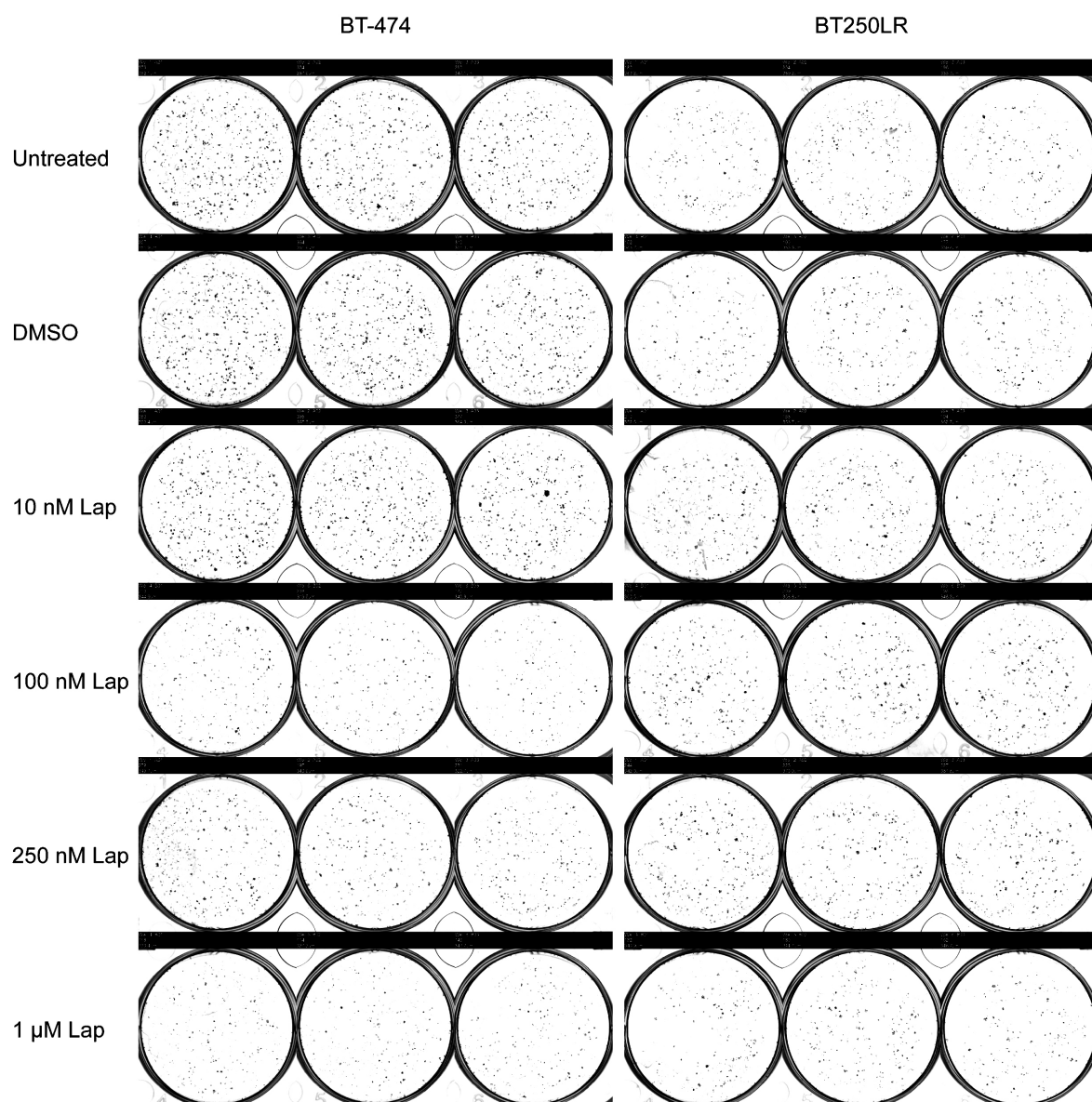


Figure 3-16 Representative experiment for dose-dependent effect on clonogenicity of BT-474 and BT250LR

BT-474 and BT250LR were treated with the indicated concentration of lapatinib or DMSO control for three days. Cells were then trypsinised, reseeded and allowed to grow for 17 days. Plates were then washed with PBS and stained with methylene blue.

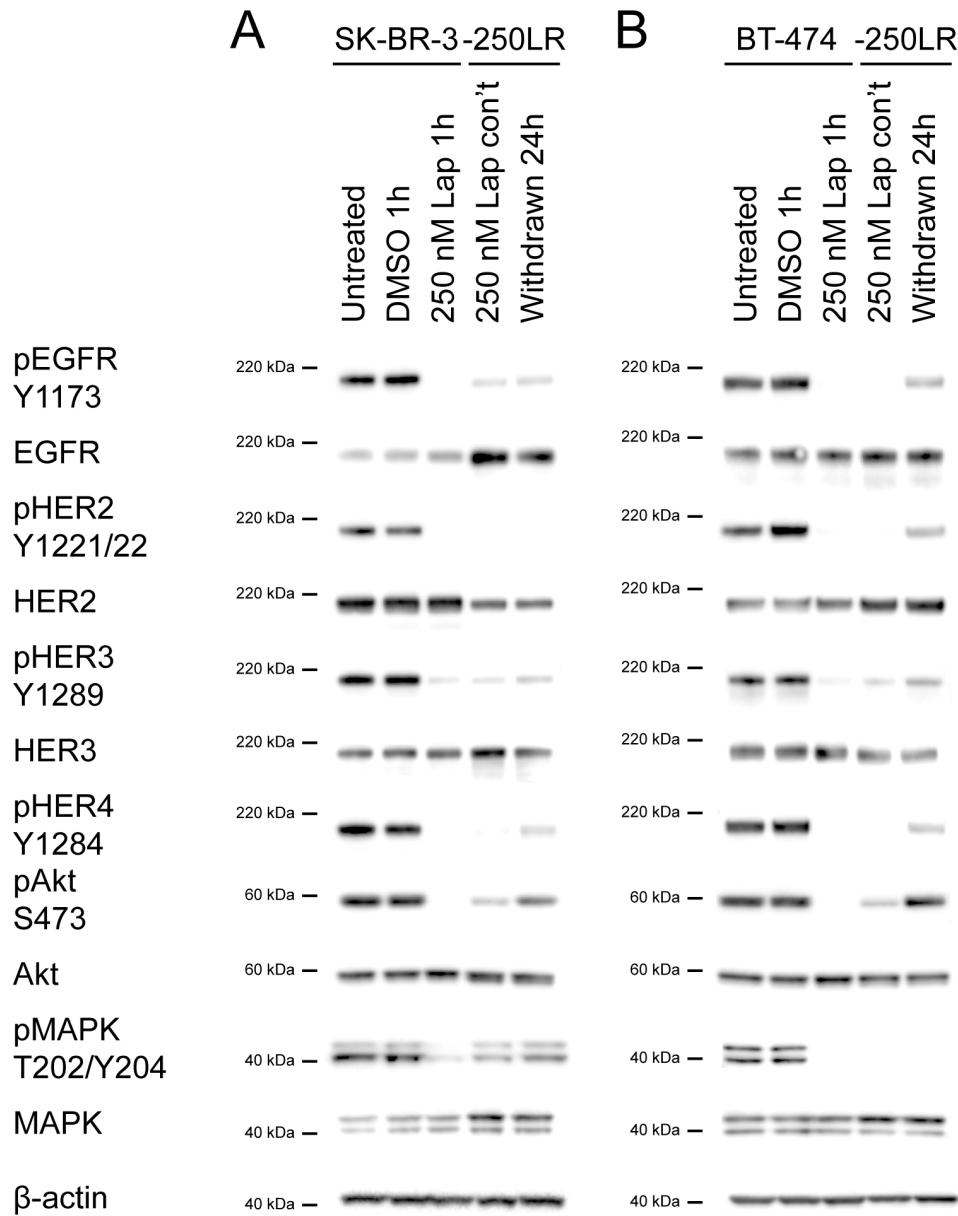


Figure 3-17 HER signalling pathway in lapatinib-resistant cell lines

(A) SK-BR-3 and (B) BT-474 cell lines were treated with 250 nM lapatinib or DMSO solvent control for one hour. (A) SK250LR and (B) BT250LR were continuously treated with 250 nM lapatinib or had the drug withdrawn for 24 hours. Complete cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted for Tyr1173-phosphorylated EGFR (pEGFR-Y1173), total EGFR (EGFR), Tyr1221/22-phosphorylated HER2 (pHER2-Y1221/22), total HER2 (HER2), Tyr1289-phosphorylated HER3 (pHER3-Y1289), total HER3 (HER3), Tyr1284-phosphorylated HER4 (pHER4-Y1284), Ser473-phosphorylated Akt (pAkt-S473), total Akt (Akt), Thr202/Tyr204-phosphorylated p44/42 MAPK (pMAPK-T202/Y204) and total p44/42 MAPK (MAPK). β-actin blot served as loading control in all experiments. Reproducible representative results of at least three independent experiments are shown.

The density of western blotting bands was quantified and the densitometry data of three experiments were plotted and analysed (Figure 3-18). The EGFR levels in SK250LR cells were 4.8 ± 1.7 -fold of untreated SK-BR-3 cells across three experiments, but it was statistically insignificant compared to DMSO treatment. The decrease in total HER2 in the resistant cell line was also not significant. The insignificant results may be due to the large variation between the experiments and a small number of repeats ($n = 3$). Generally, the densitometry plots did not show statistically significant results in most of the blots apart from total HER3 level, which decreased in SK250LR line after one day of lapatinib withdrawal. Lapatinib withdrawal also resulted in the recovery of phospho-Akt and phospho-MAPK levels, however not statistically significant.

In BT-474 cell line, lapatinib was capable of inhibiting the phosphorylation of all the HER receptors and downstream MAPK and Akt proteins (Figure 3-17B). The HER receptors remained inhibited in BT250LR cell line; however, their phosphorylation recovered after lapatinib withdrawal. These results suggested an adaptive mechanism to counter-balance the inhibition of HER signalling, instead of a permanent change in cell biology like SK250LR cells. There was also an increase in the total HER2 and HER3 levels in BT250LR cells and these changes maintained after drug withdrawal. Phospho-Akt was still inhibited in BT250LR cells, but in a lesser extent than the parental line. Moreover, the level of phospho-Akt in BT250LR cells, after withdrawal of the drug, returned to a similar level to untreated BT-474 cells. Phospho-MAPK was inhibited in both BT-474 and BT250LR cell lines and it did not recover even when the drug was removed, suggesting MAPK may become redundant in the resistant cell line. The level of total p44-MAPK increased in the BT250LR cell line and it was maintained even after the withdrawal of lapatinib.

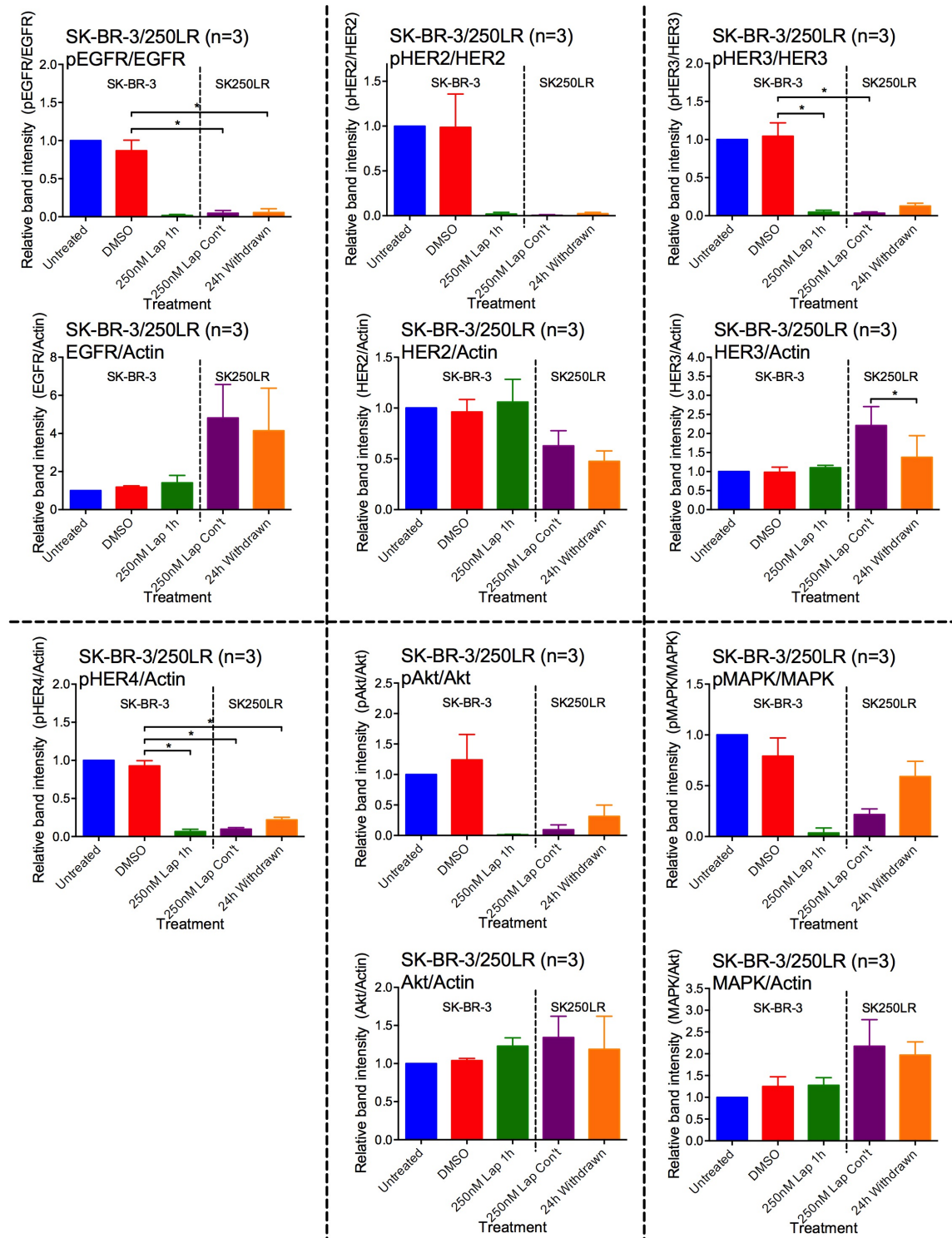


Figure 3-18 Densitometry results of HER signalling in lapatinib-sensitive and -resistant SK-BR-3

The density of western blotting bands of SK-BR-3 and SK250LR in the three experiments in Figure 3-17 was quantified by ImageQuant system and normalised with their respective total proteins (for phosphorylated proteins) or β -actin (for total proteins). The average fold change compared to untreated control was plotted. (Error = SD; Matched one-way ANOVA with Bonferroni correction, * $p < 0.05$)

In densitometry, there was a significant increase ($p = 0.0313$) in the ratio of phospho-EGFR/EGFR when lapatinib was withdrawn from BT250LR (Figure 3-19). Phospho-HER4 level also increased significantly ($p = 0.0082$) after lapatinib withdrawal; however, the ratio phospho-HER4/HER4 was not available because of the failure in HER4 immunodetection. Therefore, it was unknown whether the increase in phospho-HER4 was due to reactivation of HER4 or increased expression of HER4. There was a statistically insignificant increase in the levels of phospho-EGFR, phospho-HER2, phospho-HER3 and phospho-Akt.

SK250LR cells had an elevated expression of EGFR compared to the parental cells (Figure 3-17 and Figure 3-18), which might contribute to lapatinib resistance in this cell line. To test this hypothesis, SK250LR cells were treated with lapatinib in combination with an increasing doses of cetuximab, a monoclonal antibody against EGFR that downregulates the receptor from the cell surface (Maurizi et al., 1996). Unfortunately, the addition of cetuximab to lapatinib did not result in any difference in growth inhibition compared to the non-specific IgG control (Figure 3-20).

3.2.5 Reversion of lapatinib resistance

In order to understand whether the acquired resistance to lapatinib in SK250LR and BT250LR cell lines is reversible, lapatinib was withdrawn from the resistant cell lines for seven days. The lapatinib-withdrawn cells were compared to those continuously treated with lapatinib in terms of their sensitivity to growth inhibition by lapatinib.

In SK250LR cells, there were no significant differences between any of the treatment groups (Figure 3-21A). After seven days of drug removal, SK250LR cells did not become sensitive to lapatinib, suggesting that SK250LR cells became permanently adapted to lapatinib. The percentage cell number of SK250LR-7DW (7-day withdrawal) was higher

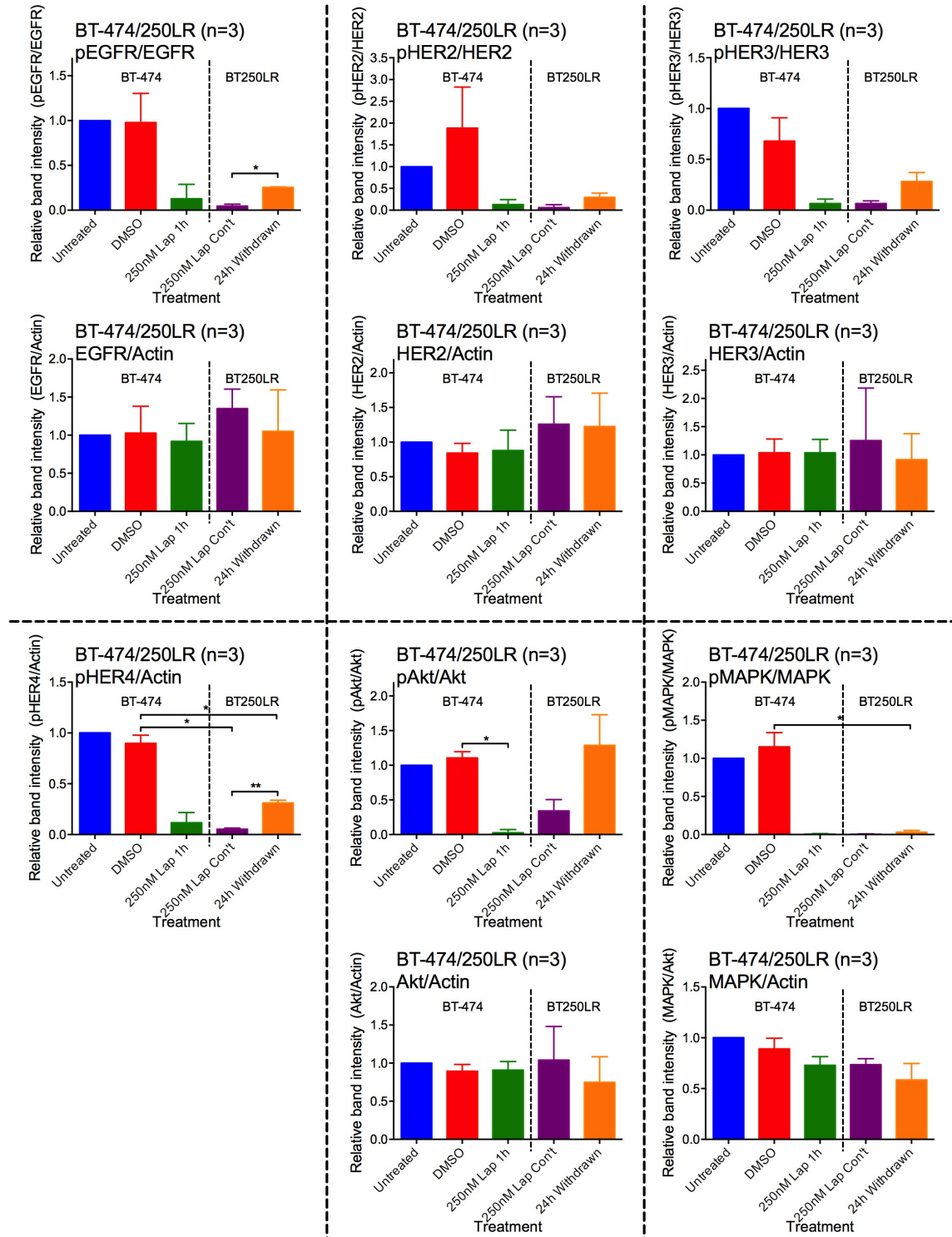


Figure 3-19 Densitometry results of HER signalling in lapatinib-sensitive and -resistant BT-474

The density of western blotting bands of BT-474 and BT250LR in the three experiments in Figure 3-17 was quantified by ImageQuant system and normalised with their respective total proteins (for phosphorylated proteins) or β -actin (for total proteins). The average fold change compared to untreated control was plotted. (Error = SD; Matched one-way ANOVA with Bonferroni correction, *p<0.05, **p<0.01)

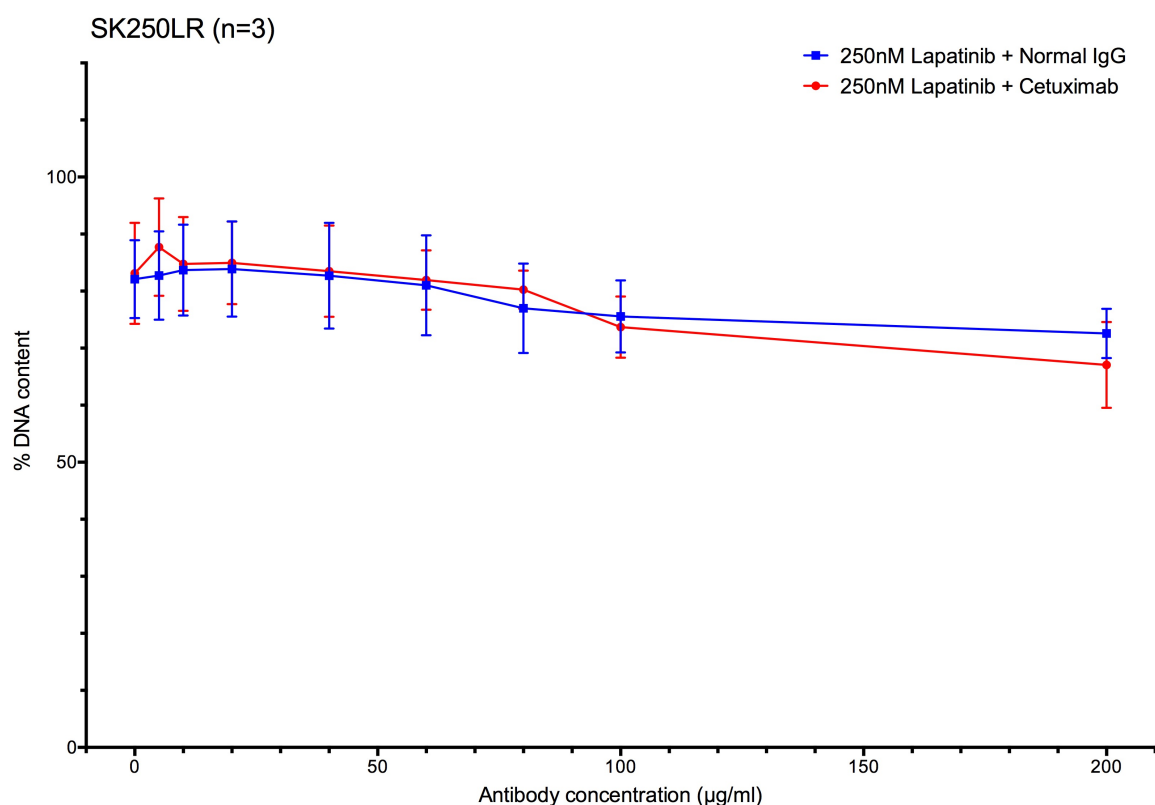


Figure 3-20 Effect of lapatinib and cetuximab combination on SK250LR

SK250LR cells were treated with 250 nM lapatinib in the combination with the indicated dose of cetuximab (red) or normal IgG (blue). After three days of treatment, the media was discarded and the cells were incubated with CyQuant reagent for one hour. Fluorescence measurements were made with excitation at 485 nm and emission detection at 530 nm. Three independent experiments were done with six technical replicates. The percentage DNA content was calculated with respect to fluorescence reading of DMSO control in each experiment. (Error = SD)

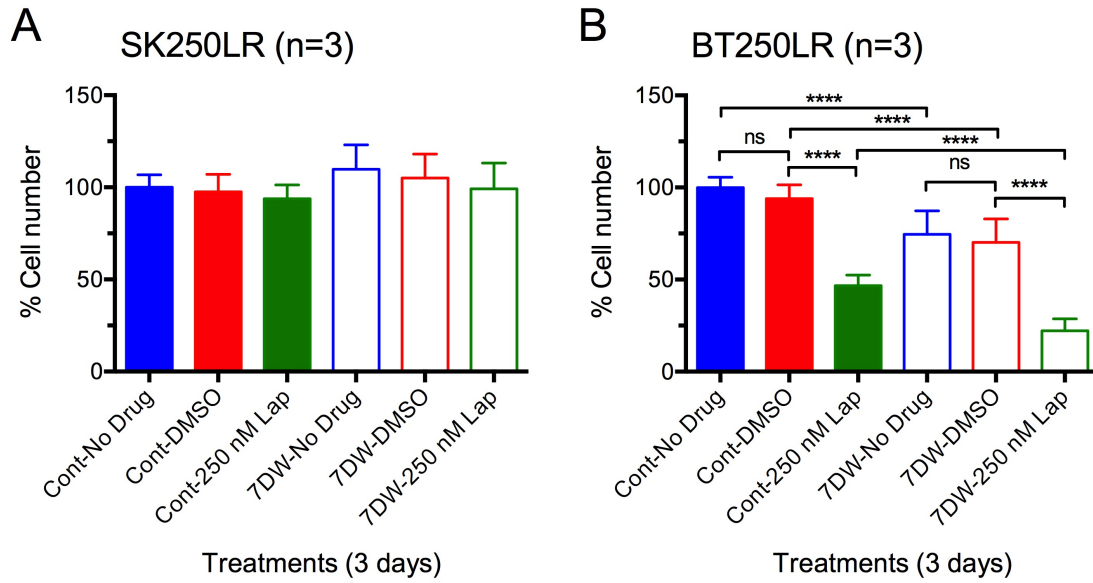


Figure 3-21 Effect of lapatinib withdrawal from lapatinib-resistant cell lines on their sensitivity to lapatinib

(A) SK250LR and (B) BT250LR were grown with continuous 250 nM lapatinib treatment (solid bars, Cont) or without drug (hollow bars, 7DW) for seven days before they were reseeded to 24 well plates. Cells were treated with DMSO or 250 nM lapatinib for three days, and then trypsinised and counted. The percentage cell number was normalised to the cells treated with lapatinib continuously and then no drug for three days (Cont-No drug). Three independent experiments were performed with three technical replicates. (Error = SD; Matched two-way ANOVA with Bonferroni correction, **** $p < 0.0001$)

than those with continuous treatment, but the difference was statistically insignificant.

In the case of BT250LR cell line, even when they were constantly treated with 250 nM lapatinib, the cell number decreased significantly ($p < 0.0001$) in 250 nM lapatinib treatment compared to DMSO control (Figure 3-21B), indicating the cells were still sensitive to the growth inhibition effect of lapatinib. In another words, BT250LR grew faster when lapatinib was removed from the medium. When lapatinib was just removed after continuous treatment for three days (Cont-No drug), the cell number was significantly greater ($p < 0.0001$) than those cells that were under lapatinib withdrawal for seven days (7DW-No drug). Meanwhile, 250 nM lapatinib caused a decrease in the cell number of BT250LR-7DW, compared to DMSO control ($p < 0.0001$). Lapatinib reduced cell number of BT250LR (compared to DMSO control) irrespective to the fact whether or not lapatinib was removed for seven days; however BT250LR cells was more sensitive to lapatinib after the drug was removed for seven days, indicating that the cells were only transiently adapted to lapatinib. Removing lapatinib from BT250LR cell line reverted its resistance and BT250LR-7DW became more sensitive to lapatinib.

In order to study the difference in resistance reversion between SK250LR and BT250LR cell lines, the HER signalling profile of both lapatinib-resistant cell lines after 7-day drug withdrawal was analysed using western blotting. In SK250LR cells, phospho-EGFR, phospho-HER2 and phospho-HER4 did not regain activity after lapatinib was removed from the cells (Figure 3-22A). Their ratio of phospho/total protein (except HER4 since it could not be detected) remained very close to zero (Figure 3-23). In fact, after 7-day withdrawal of lapatinib, EGFR and HER2 expression decreased. The activity of Akt recovered slightly after seven days without lapatinib, but it was still reduced relative to untreated SK-BR-3 cells. MAPK was inhibited in SK250LR cells; however, after removal of lapatinib for seven days, it was phosphorylated more than that in untreated SK-BR-3

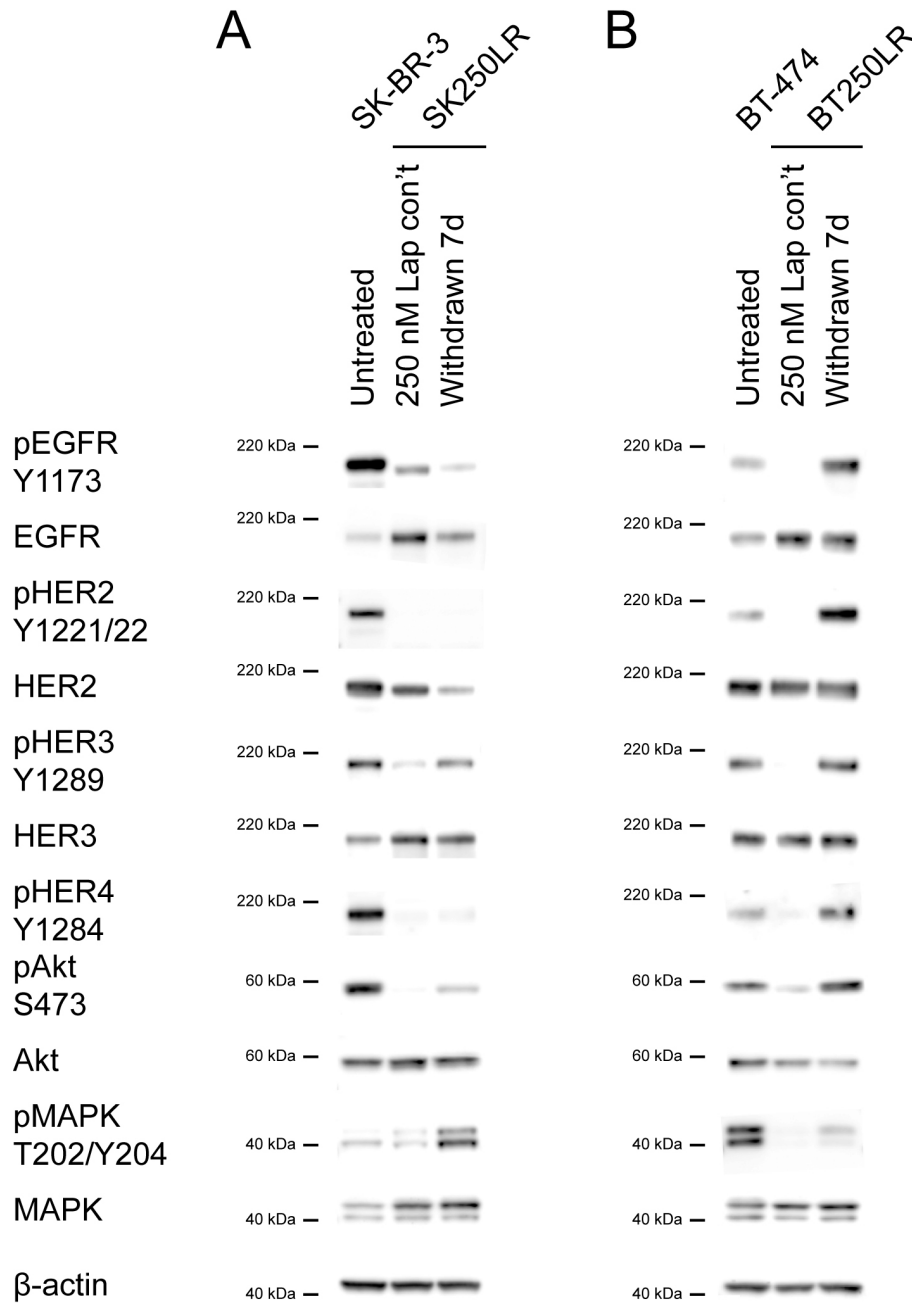


Figure 3-22 HER signalling pathway in lapatinib-resistant cell lines after 7-day drug withdrawal

(A) SK250LR and (B) BT250LR cells were continuously treated with 250 nM lapatinib or had the drug withdrawn for seven days. Complete cell lysates were resolved, alongside with the untreated parental cells, by SDS-PAGE, transferred to PVDF membrane and immunoblotted for Tyr1173-phosphorylated EGFR (pEGFR-Y1173), total EGFR (EGFR), Tyr1221/22-phosphorylated HER2 (pHER2-Y1221/22), total HER2 (HER2), Tyr1289-phosphorylated HER3 (pHER3-Y1289), total HER3 (HER3), Tyr-1284-phosphorylated HER4 (pHER4-Y1284), Ser473-phosphorylated Akt (pAkt-S473), total Akt (Akt), Thr202/Tyr204-phosphorylated p44/42 MAPK (pMAPK-T202/Y204) and total p44/42 MAPK (MAPK). β-actin blot served as loading control in all experiments. Reproducible representative results of at least three independent experiments are shown.

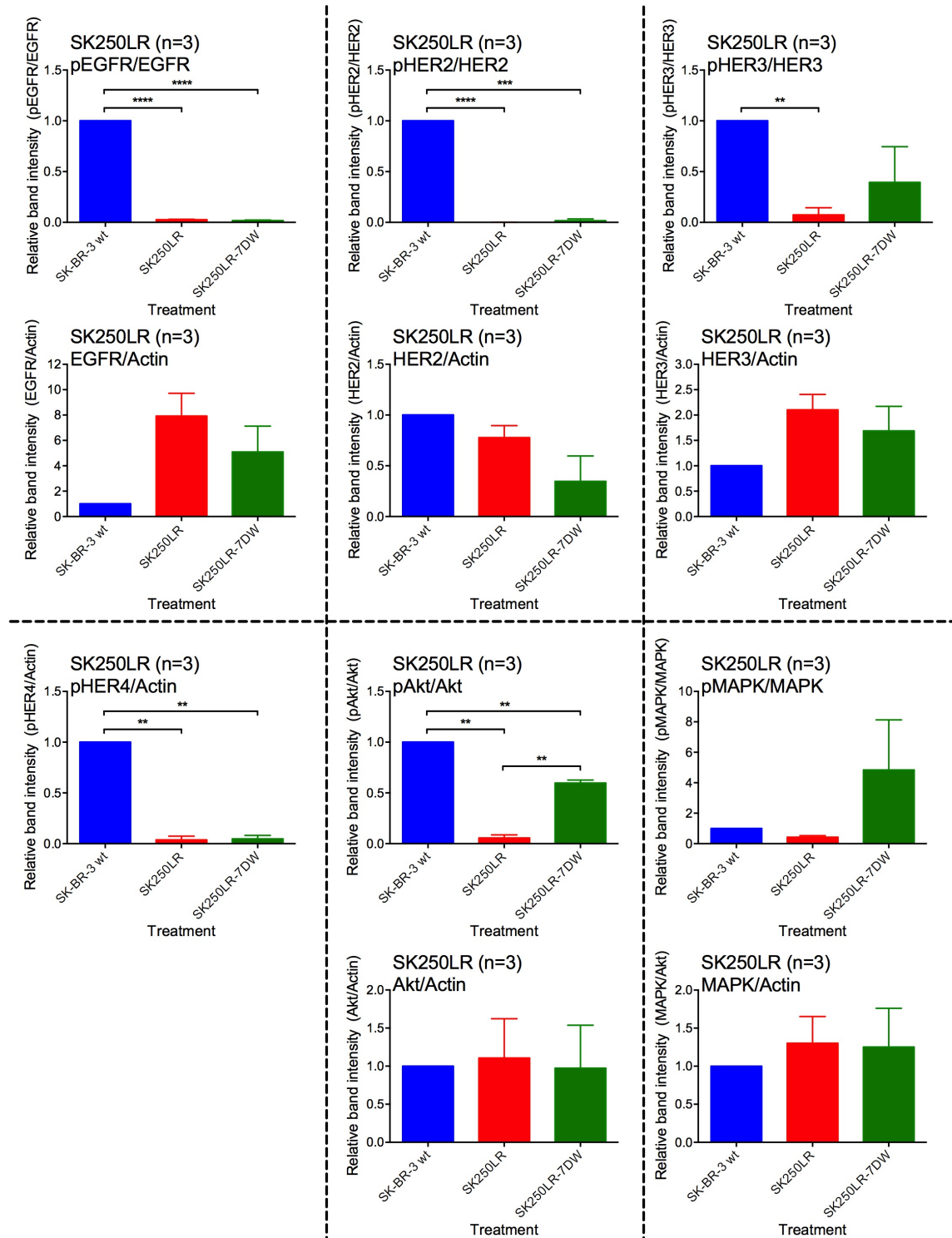


Figure 3-23 Densitometry results of HER signalling in SK250LR after 7-day drug withdrawal

The density of western blotting bands of SK-BR-3 and SK250LR in the three experiments in Figure 3-22 was quantified by ImageQuant system and normalised with their respective total proteins (for phosphorylated proteins) or β -actin (for total proteins). The average fold change compared to untreated control was plotted. (Error = SD; Matched one-way ANOVA with Bonferroni correction, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

cells. There was no observable difference in Akt expression between untreated SK-BR-3 and SK250LR cells with or without lapatinib; however, the increased levels of p44-MAPK in SK250LR were maintained after lapatinib being removed for seven days.

In BT250LR cells, all phosphorylated proteins were inhibited under continuous lapatinib treatment (Figure 3-22B), but all of them recovered after lapatinib was removed for seven days. The levels of phospho-EGFR, phospho-HER2, phospho-HER4 and phospho-Akt in BT250LR after 7-day withdrawal were even higher than those in untreated BT-474 cells. Phospho-HER3 recovered to similar level of untreated BT-474 cells, but phospho-MAPK was still below the basal level. Total EGFR level increased in BT250LR cells and it was maintained after lapatinib withdrawal. Total Akt decreased in BT250LR cells after lapatinib was removed for seven days. The level of p44-MAPK increased in BT250LR cells and was maintained after 7-day lapatinib withdrawal.

Densitometry analysis showed that the differences in BT250LR cells before and after lapatinib removal were not statistically significant (Figure 3-24).

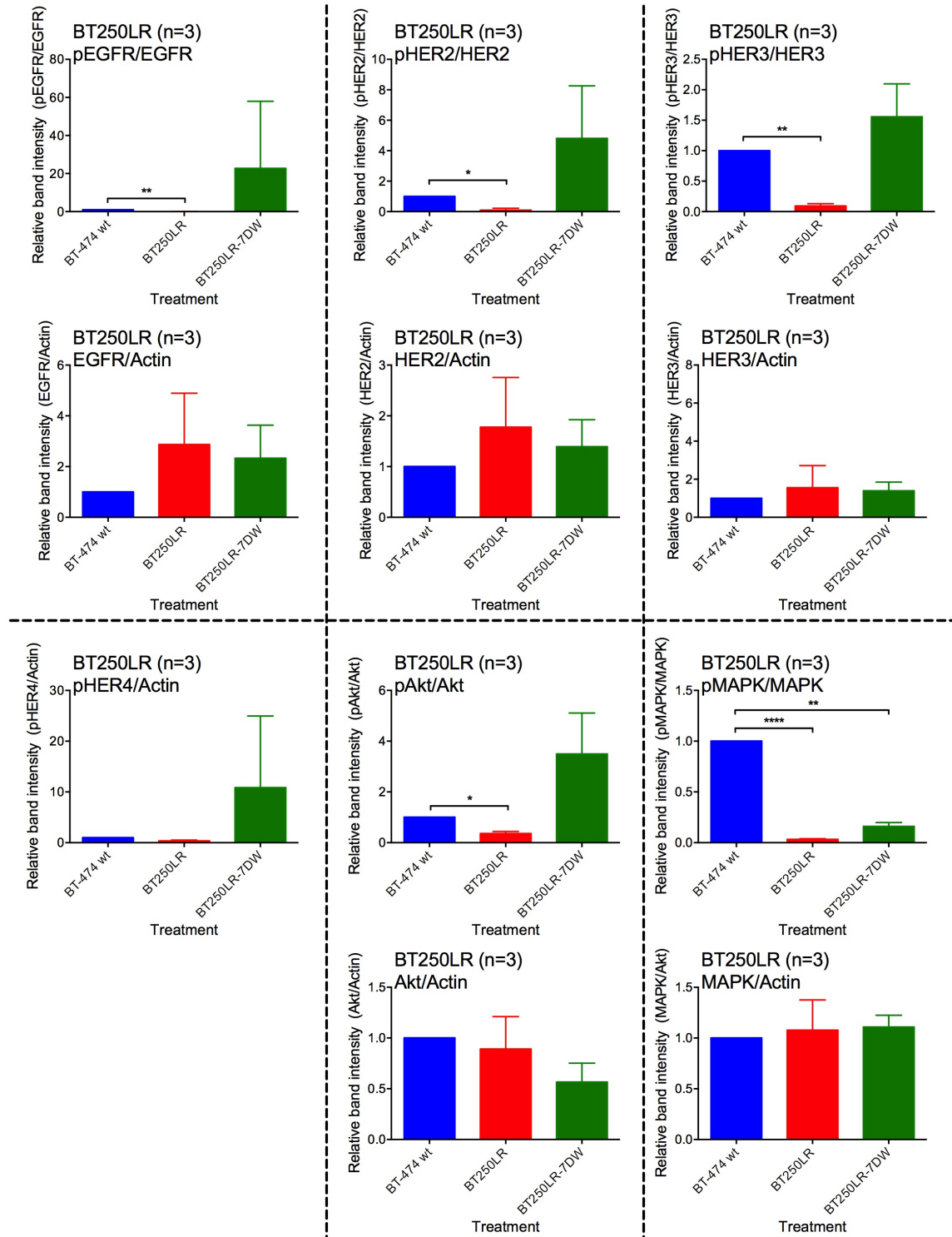


Figure 3-24 Densitometry results of HER signalling in BT250LR after 7-day drug withdrawal

The density of western blotting bands of BT-474 and BT250LR in the three experiments in Figure 3-22 was quantified by ImageQuant system and normalised with their respective total proteins (for phosphorylated proteins) or β -actin (for total proteins). The average fold change compared to untreated control was plotted. (Error = SD; Matched one-way ANOVA with Bonferroni correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

3.3 Discussion

3.3.1 Discrepancy in IC_{50} experiments with previous studies

The experimental IC_{50} values of the current study were lower than the previously reported values in Table 3-1. The experimental IC_{50} of SK-BR-3 was around 40%–68% of the reported values and that of BT-474 was around 32%–50%. The following might be the underlining reasons of the lower experimental IC_{50} values.

O'Brien *et al.* and Konecny *et al.* used the same experimental method of total cell counting, but they carried out their experiments for different length of time (five days and seven days respectively). As aforementioned in Section 3.2.1, cancer cell grows exponentially and the length of the experiment causes differences in the forward change of cell numbers, which may affect the calculation of percentage cell number.

Rusnak *et al.* carried out the experiment for three days as the current study did, but they used a different methodology. They stained the cells with methylene blue, released the colour using salted 1.0% N-lauroylsarcosine solution and then measured the colour intensity using a spectrometer (Rusnak *et al.*, 2007). As different experimental methods use different readouts as the surrogate for the number of cells, the resulting data may vary.

As a final note, the maximum concentration of lapatinib used in this study (500 nM for SK-BR-3 and 100 nM for BT-474) might not be high enough to achieve the plateau of inhibition, which could cause an overestimation of minimal cell number and hence an underestimation of IC_{50} .

3.3.2 Advantages and disadvantages of using IncuCyte real-time monitoring

IncuCyte™ (Essen Bioscience) is a microscope that can be placed in an incubator. Its camera is remote-controlled by computer to photograph the contents of a tissue culture vessel. IncuCyte can perform real-time imaging of cells over a long period to assess cell growth, migration, wound healing and morphological changes. IncuCyte software has a Confluence v1.5 algorithm to measure the area in a picture covered by cells and compute the confluency of the cells.

The advantage of IncuCyte is that it can be set to take multiple images of a tissue culture well, for instance, 4–36 images in each well of a 24-well plate. Then the programme will calculate the confluency in each picture and report the average confluency. It is also possible to retrieve the images at different time point to analyse the behaviour of the cells, to understand whether the increase in confluency is due to an increase in cell number or a change in morphology like cell expansion.

The disadvantage of this technology is that the algorithm only measures cell coverage, but it cannot differentiate whether the change in confluency is caused by the change of cell number or morphology, which requires manual analysis. Cell quiescence can induce error in confluency measurement.

3.3.3 Reactivation of HER signalling after acute lapatinib treatment

Previous studies of the response to lapatinib in cancer cells often included only one fixed time treatment (Rusnak et al., 2001; Xia et al., 2002), which failed to address the dynamic changes in cell biology, especially for rapid processes like phosphorylation. The current study suggested that the recovery of HER receptors phosphorylation started after eight hours of lapatinib treatment (Figure 3-7). The reactivation of HER receptors is crucial to

cell survival as they stimulate downstream survival pathways, allowing cancer cells to escape from the inhibition by the drug. Surviving in the presence of drug then allows time for the cancer cells to develop mechanisms of drug resistance. Therefore, it is very important to understand the mechanism of HER receptors reactivation in order to prevent the development of drug resistance.

3.3.4 HER signalling profile in lapatinib-resistant cell lines

Lapatinib-resistant HER2-overexpressing breast cancer cell lines SK250LR and BT250LR were generated. Their resistance to lapatinib was confirmed by growth inhibition assay and clonogenic assay.

HER signalling pathway analysis showed that SK250LR cells had an increased expression of EGFR, suggesting a possibility of combining EGFR-targeted therapy with lapatinib to tackle lapatinib resistance. However, the combination of cetuximab, a monoclonal antibody to EGFR used in bowel and head and neck cancers (Baselga and Arteaga, 2005; Bernier, 2008; Scaltriti and Baselga, 2006), with lapatinib did not have any effect on SK250LR cells. In fact, EGFR and HER2 remained inhibited in SK250LR (Figure 3-18A and Figure 3-25B) and their expression levels decreased after withdrawing lapatinib for seven days (Figure 3-23A and Figure 3-25C), indicating that EGFR and HER2 might be redundant proteins produced via negative-feedback mechanism in response to the inhibitory effect of lapatinib. There was no evidence to show that EGFR contributed to lapatinib resistance in SK250LR. However, the role of EGFR can be further tested with EGFR knockdown in the SK250LR cell line.

In SK-BR-3 cells, MAPK was inhibited to an undetectable level in western blotting (Figure 3-7); however, MAPK was slightly activated in SK250LR cells, although no mechanism is yet found (Figure 3-25C). Together with the increased expression of MAPK

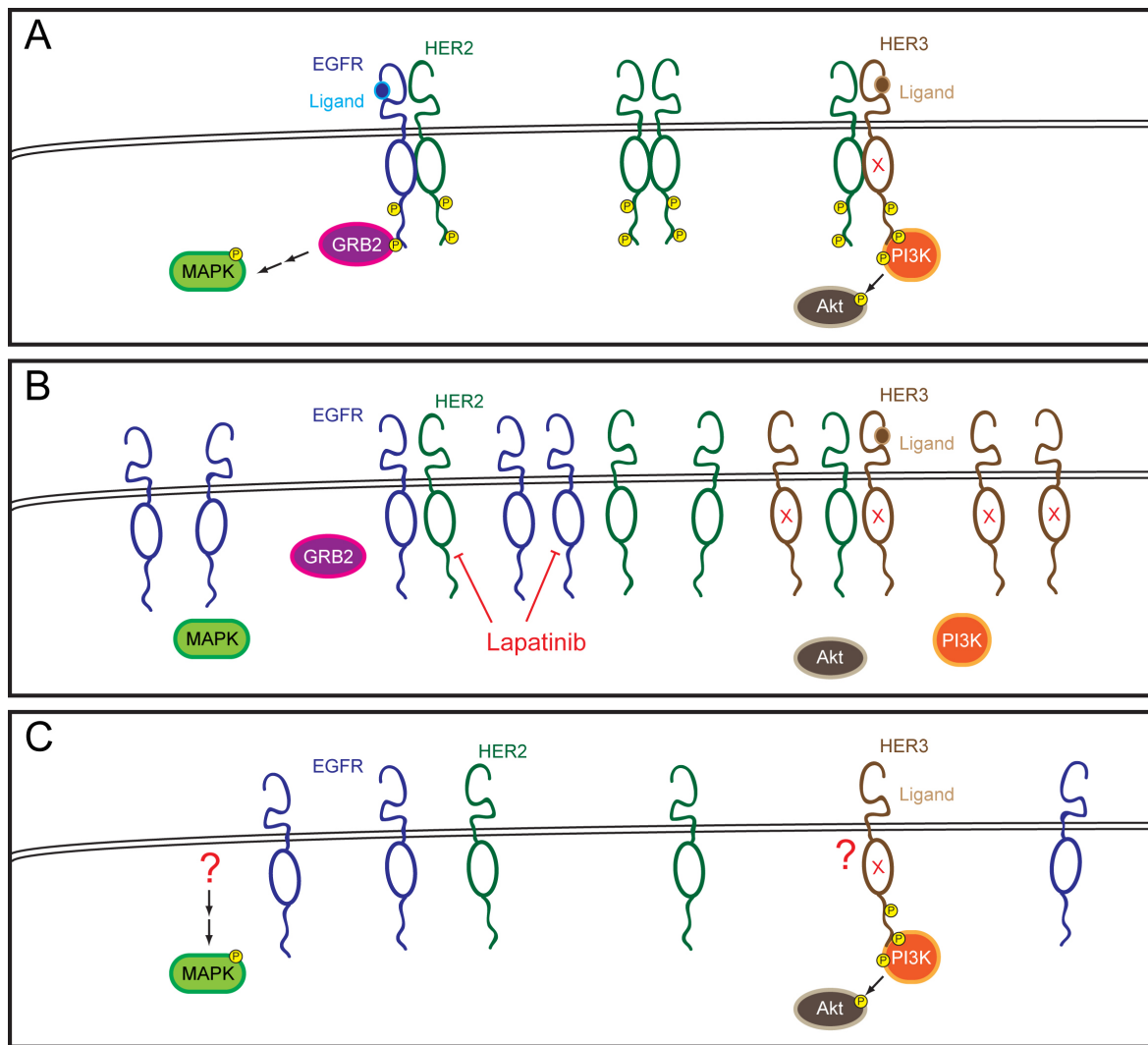


Figure 3-25 Schematic diagram of the HER signalling profiles in SK250LR cell line

(A) In SK-BR-3 cells, ligands stimulate HER receptor dimerisation and the downstream MAPK and Akt pathways when the cells are not treated with lapatinib. HER3 is kinase defective (cross on the tyrosine kinase domain). (B) In SK250LR cells, the protein expression levels of EGFR and HER3 increased, but the receptors were still inhibited by lapatinib. (C) When lapatinib was withdrawn from SK250LR cells for seven days, EGFR and HER2 expression levels decreased. HER3, Akt and MAPK were rephosphorylated, but the mechanisms are still unknown.

in SK250LR cells, these results suggested MAPK as another possible target for treating lapatinib-resistant breast cancer. Further experiments are needed to investigate the inhibitory effect of MAPK inhibitors in the combination with or without lapatinib.

In BT250LR cells, although the HER receptors and downstream proteins were inhibited in lapatinib treatment (Figure 3-17 and Figure 3-26B), the phosphorylation of all HER receptors and Akt recovered readily when lapatinib was removed from one day (Figure 3-17) to seven days (Figure 3-22 and Figure 3-26C). This finding suggests that BT250LR cell line employed strategies to increase HER signalling to counterbalance the inhibition from lapatinib. These mechanisms may include elevated expression of receptor (in case of EGFR) and increased secretion of HER ligands to stimulate HER receptors phosphorylation. As these mechanisms only involve HER signalling pathway, BT250LR cells still responded to lapatinib when they were re-exposed to the drug after initial withdrawal.

After three days of no drug treatment, the cell number of BT250LR previously in continuous lapatinib treatment was higher than those cells with lapatinib removed for seven days (Figure 3-21). This observation may be due to less cells being seeded for BT250LR-7DW, or the initial boost on cell growth after lapatinib removal levelled off after a prolonged period without drug.

As the lapatinib-resistant cell lines, especially SK250LR cells, may have changes in cell biology and switch to depend on different signalling pathways. Receptor tyrosine kinase array was performed in both SK250LR and BT250LR. Results will be discussed in Chapter 6.

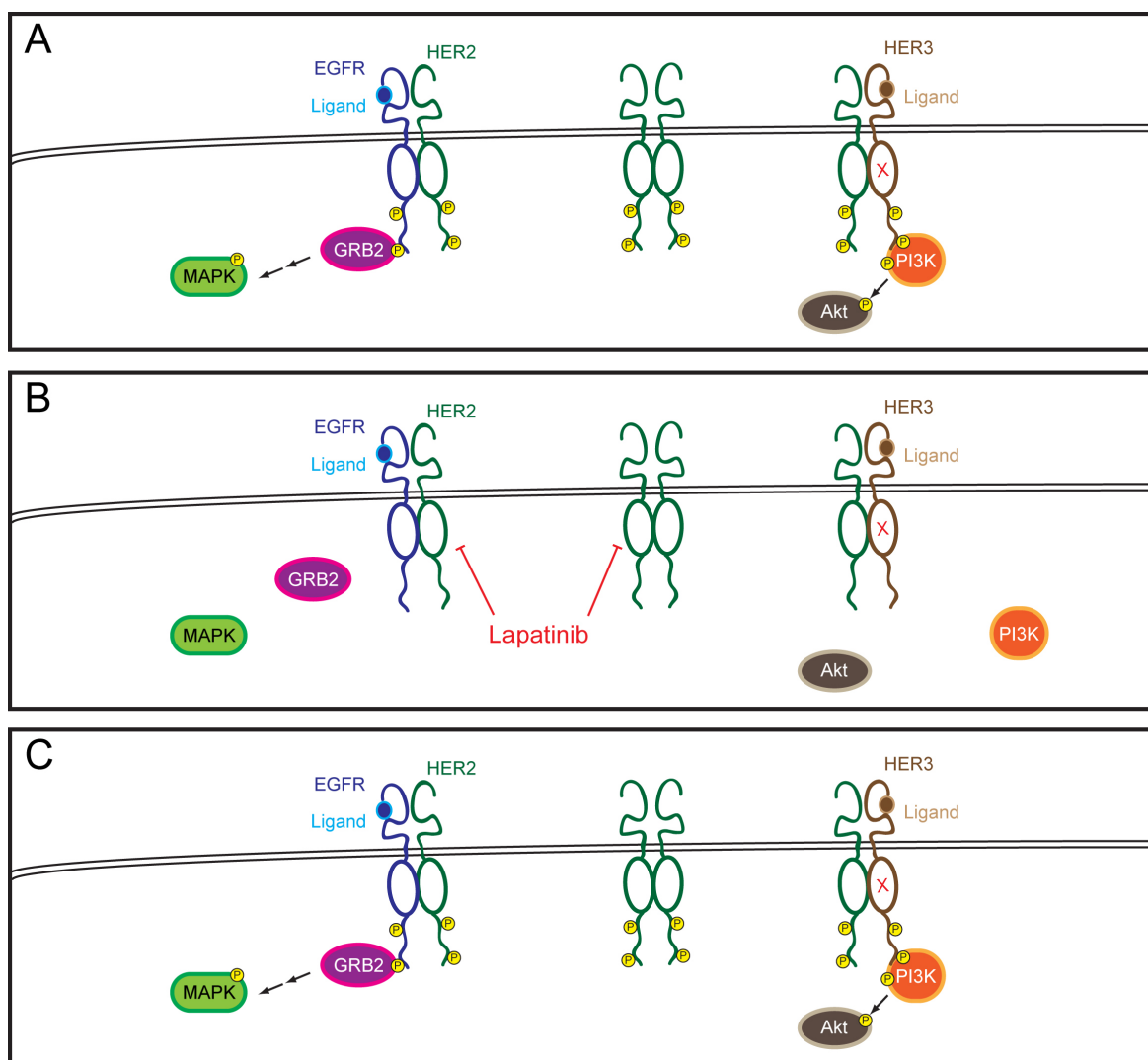


Figure 3-26 Schematic diagram of the HER signalling profiles in BT250LR cell line

(A) In BT-474 cells, ligands stimulate HER receptors dimerisation and the downstream MAPK and Akt pathways when the cells are not treated with lapatinib. HER3 is kinase defective (cross on the tyrosine kinase domain). (B) In BT250LR cells, lapatinib inhibited the phosphorylation of all the HER receptors and the downstream MAPK and Akt. (C) When lapatinib was withdrawn from BT250LR cells for seven days, the phosphorylation of all the HER receptors and the downstream MAPK and Akt recovered.

3.3.5 MAPK and lapatinib resistance

The expression levels of p44-MAPK, also called ERK1 and MAPK3, in lapatinib-resistant cell lines increased and maintained even after withdrawal of the drug. Both p42- and p44-MAPK have been implicated in cellular proliferation and differentiation (Yarden and Sliwkowski, 2001). p42- and p44-MAPK were isolated in 1991 and 1990 respectively (Boulton and Cobb, 1991). They have 84% amino acid sequence identity; however, only p42-MAPK knockout was embryonic lethal in mice (Yao et al., 2003), but not p44-MAPK, suggesting p44-MAPK may be dispensable and can be compensated by the p42 isoform (Pages et al., 1999). To date, all known MAPK stimulants simultaneously activate both p42- and p44-MAPK, and it is yet unknown if there is any cellular function that is unique to or prefer either one of the isoforms (Roskoski, 2012). The differential increase in p44-MAPK expression in lapatinib-resistant cell lines indicates that there may be some cellular functions in these cell lines that preferentially interact with p44-MAPK.

In non-small cell lung cancer, epithelial-mesenchymal transition (EMT) and mesenchymal differentiation are related to EGFR-targeted therapy resistance. Chronic inhibition of MAPK prevents EMT and sensitises lung cancer cells to EGFR inhibition (Buonato and Lazzara, 2014). MAPK was activated in SK250LR cells (Figure 3-18) and might mediate lapatinib resistance through EMT process and differentiation. Chronic treatment with MAPK inhibitors such as SCH772984 (Morris et al., 2013) or upstream MEK inhibitors such as U1026 (Buonato and Lazzara, 2014) may be useful to re-sensitise SK250LR to lapatinib.

3.3.6 Chapter summary

HER2-overexpressing breast cancer cell lines SK-BR-3 and BT-474 responded to lapatinib by rapidly dephosphorylation of HER receptors and downstream MAPK and Akt signalling molecules. However, phosphorylation recovered as soon as eight hours after

treatment, suggesting that the cells have certain ways to stimulate HER signalling in response to the inhibitory effect of lapatinib. In previous report, these cell lines were shown to overcome trastuzumab treatment using a feedback release of autocrine ligands such as NRG1 and BTC (Gijzen et al., 2010). These cells may behave in a similar manner in response to lapatinib.

Lapatinib-resistant strains of the above cell lines were generated. Initial studies revealed that SK250LR and BT250LR cell lines have different mechanisms to adapt to lapatinib treatment. SK250LR cells might have changed their cell biology and become independent of EGFR and HER2. BT250LR cells only transiently adapted to the drug, probably through increased HER signalling as HER receptors rephosphorylated readily after lapatinib withdrawal. Only phospho-HER3 recovered in both cell lines after removal of lapatinib, indicating that HER3 signalling might be a common mechanism to mediate survival in prolonged lapatinib treatment. Therefore, in the next chapter, the role of HER3 and its ligands will be investigated.

4. The role of NRG1 in lapatinib resistance in HER2-overexpressing breast cancer

4.1 Introduction

As described in the previous chapter, HER2-overexpressing breast cancer cell lines were able to rapidly respond to lapatinib treatment and reversed the effects of treatment after eight hours. In SK250LR cells, there was an increased expression of the HER3 receptor and phospho-HER3 recovered rapidly after lapatinib was removed for seven days. These results indicated that HER3 signalling might have a crucial role in lapatinib resistance.

HER3 has been shown to have an important role in HER2-overexpressing breast cancer, as HER3 knockdown decreases tumour cell proliferation as much as HER2 knockdown (Lee-Hoeflich et al., 2008). Furthermore, HER3 has been described in the resistance mechanism of a number of HER receptors-targeted therapies including gefitinib (Sergina et al., 2007), trastuzumab (Gijzen et al., 2010) and lapatinib (Garrett et al., 2011). In the case of lapatinib, HER3 was transcriptionally upregulated through the derepression of FOXO3a due to Akt inhibition, and subsequently activated by residual HER2 activity (Garrett et al., 2011). Although there are numerous reports on the role of HER3 in targeted therapies, the role of HER3 ligands is not clearly understood.

HER3 has two known ligands, NRG1 and NRG2 (Olayioye et al., 2000). They belong to a family of EGF-like ligands called neuregulins (NRGs) and are expressed in various developing and adult tissues (Hayes and Gullick, 2008). NRG1 has a crucial role in the development and the functions of the nervous system (Esper et al., 2006), circulation system (Iivanainen et al., 2007) and in mammary gland development (Wansbury et al., 2008). Loss of NRG1 expression results in embryonic lethality in mice (Meyer and

Birchmeier, 1995). NRG2 is involved in the development of central nervous system (Longart et al., 2004) as well as the lungs, heart and bladder (Hayes and Gullick, 2008). Unlike NRG1, the deficiency of NRG2 is not lethal in the mouse model; however, it causes early growth retardation and reduced reproductive ability (Britto et al., 2004).

High levels of NRG1 and NRG2 expression were found in certain breast cancer cell lines and patient samples (Dunn et al., 2004; Marshall et al., 2006), suggesting that HER3 ligands may have a role in breast cancer. Moreover, rearrangement of the NRG1 gene was found in a few breast cancer cell lines (Adelaide et al., 2003) and in around 6% of breast cancer patients (Huang et al., 2004). However, there has been limited clinical research on the NRG family and hence their association with clinical outcome and relapse remains unexplored (Hayes and Gullick, 2008).

Despite the lack of validation in clinical samples, NRG1 has been reported to mediate resistance to HER receptors-targeted therapies. ADAM17-mediated NRG1 autocrine ligand stimulation on HER3 signalling was shown accountable for the resistance to gefitinib in some non-small cell lung cancer cell lines (Zhou et al., 2006) and trastuzumab in some HER2-overexpressing breast cancer cell lines (Gijssen et al., 2010). In another study, NRG1 was upregulated in trastuzumab-resistant BT-474 cell line (Ritter et al., 2007).

On the contrary, overexpression of NRG1 in innate trastuzumab-resistant MCF-7 cells, a cell line that expresses low level of HER2, resulted in trastuzumab sensitivity *in vivo* (de Alava et al., 2007). In addition, the same study showed that patients with high NRG1 levels but low HER2 levels had better relapse-free and overall survival than those with low levels of both, when they were treated with trastuzumab. Moreover, a recent study reported that NRG1-mediated autocrine signalling contributes to sensitivity to lapatinib in a subset of human cancers (Wilson et al., 2011).

Since HER3 ligands have a crucial role in the resistance of some HER receptors-targeted therapies, this section continues to investigate the role of HER3 ligands, in particular NRG1, in the response and resistance to lapatinib in HER2-overexpressing breast cancer cell lines.

4.2 Results

4.2.1 Effect of blocking HER3 ligand stimulation and its combination with lapatinib

To test the hypothesis that HER3 ligands are important in mediating lapatinib resistance in HER2-overexpressing breast cancer, the anti-HER3 monoclonal antibody SGP1, which binds to the ligand-binding domain of HER3 and prevents ligand stimulation (Blackburn et al., 2012; Rajkumar and Gullick, 1994), was used. In the following experiments, lapatinib-resistant cell lines were treated with 250 nM lapatinib and the sensitive cell lines were treated with a dose close to their GI_{50} concentration, i.e. 100 nM for SK-BR-3 cells and 10 nM for BT-474 cells.

In SK250LR cells (Figure 4-1A), while continuous lapatinib treatment plus non-specific IgG did not cause any significant reduction in total cell number, addition of SGP1 to lapatinib significantly reduced cell number compared to the control ($p < 0.0001$) or continuous lapatinib treatment alone ($p < 0.0001$). SGP1 alone also reduced cell number compared to the control ($p = 0.0001$), but there was no significant difference between the combined treatment and SGP1 alone (lapatinib discontinued). These results indicated that SK250LR cells responded to SGP1 and the growth inhibition in this experiment was solely due to SGP1 treatment, independent of whether lapatinib treatment was continued in the presence of acquired resistance.

As shown in Figure 4-1C, SGP1 alone reduced cell number of the parental SK-BR-3 ($p = 0.0005$ compared to control). Lapatinib alone and the combination of lapatinib and SGP1 decreased cell number of the parental SK-BR-3 significantly (Both $p < 0.0001$ compared to control); however, there was no significant difference between these two treatment groups, indicating that the inhibition was mainly caused by lapatinib, and SGP1 did not cause

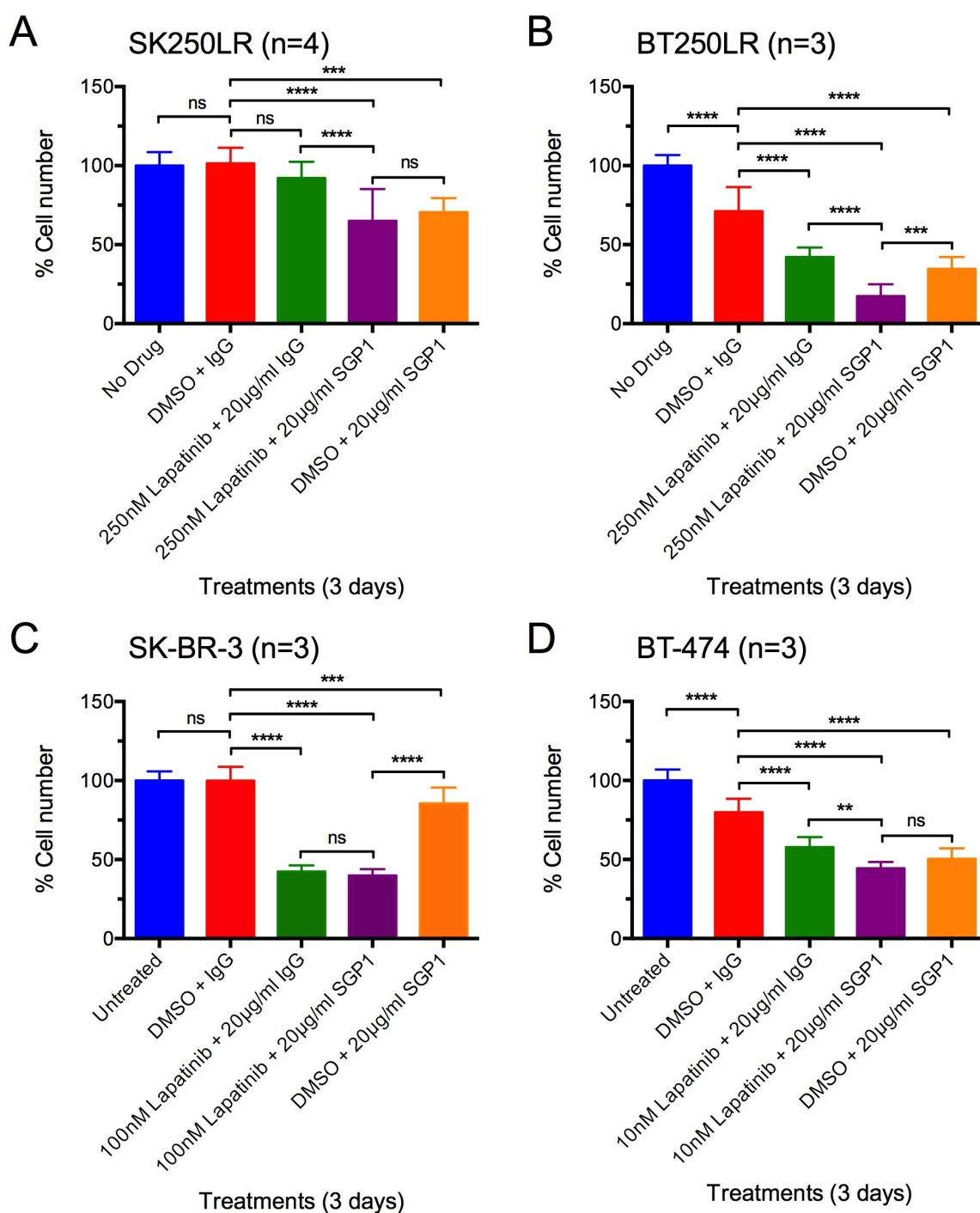


Figure 4-1 Effect of lapatinib and/or SGP1 on HER2-overexpressing breast cancer cell lines

(A) SK250LR and (B) BT250LR cells were seeded and maintained in continuous lapatinib treatment the night before experiment started. On the next day, the drug was taken off. Then these cells, (C) SK-BR-3 and (D) BT-474 cells were treated with the indicated agents for three days, then trypsinised and counted. The percentage cell number was normalised to the untreated/no drug control. At least three independent experiments were done with three technical replicates. (Error = SD; Matched two-way ANOVA with Bonferroni correction, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

further inhibition was mainly caused by lapatinib, and SGP1 did not cause further reduction of growth or cell death. The small magnitude of change in SK-BR-3 cell number caused by SGP1 alone ($14 \pm 10\%$) indicated that the effect of this antibody, although statistically significant, might lack biological importance, in which it would be unlikely to influence the cell biology of cancer cells.

In BT250LR cells (Figure 4-1B), DMSO and IgG control (without continuous lapatinib treatment) significantly reduced cell number compared to no drug treatment ($p < 0.0001$). As shown in Figure 3-21, DMSO alone (lapatinib discontinued) did not cause any significant cell number reduction in BT250LR, suggesting that BT250LR cells might be affected by the non-specific IgG. Continuous lapatinib treatment and IgG further decreased cell number, which was significant compared to the DMSO/IgG control (lapatinib discontinued) ($p < 0.0001$). BT250LR cells also responded to SGP1 alone without continuous lapatinib treatment and the cell number dropped significantly ($p < 0.0001$ compared to control). The combination of lapatinib and SGP1 caused a further drop in the cell number of BT250LR. The combination of lapatinib and SGP1 generated a significant difference in the cell number compared to lapatinib alone ($p < 0.0001$) or SGP1 alone ($p = 0.0006$). However, the determination of synergism between these two agents will require isobologram analysis to help visualise the combined drug effects (Tallarida, 2001).

Similar to BT250LR cells, DMSO and IgG control also caused a significant decrease in the cell number ($p < 0.0001$) of the parental BT-474 cells compared to no treatment (Figure 4-1D). Both lapatinib and SGP1 single treatment significantly reduced cell number compared to the DMSO/IgG control (Both $p < 0.0001$). Although the combination of lapatinib and SGP1 decreased cell number ($p < 0.0001$ compare to control), this reduction was only significantly different from lapatinib treatment alone ($p = 0.0021$) but not from

SGP1 alone. As there was no difference between the combination and SGP1 alone, the decrease in cell number in combination treatment might be solely caused by SGP1.

4.2.2 Exogenous NRG1 rescued cancer cells from growth inhibition caused by lapatinib

The previous section showed that blocking HER3 ligand stimulation caused a growth inhibition in lapatinib-resistant SK250LR and BT250LR cell lines, and produced an additive effect with continuous lapatinib treatment in BT250LR cells. These results suggested that ligand-dependent HER3 signalling may be crucial to the proliferation of lapatinib-resistant cell lines. Of the two HER3 ligands, the function of NRG1 is better described (Olayioye et al., 2000). Previous studies also showed that NRG1 is the important mediator of HER3 signalling in ovarian (Sheng et al., 2010) and head and neck cancers (Wilson et al., 2011). Our group previously reported the role of ADAM17 and NRG1 in the resistance of another HER2-targeted therapy, trastuzumab (Gijzen et al., 2010). Therefore, the following study focused on the role of NRG1-driven ligand-dependent HER3 signalling.

Initially, exogenous NRG1 was used to study its effect on the cancer cell lines' response to lapatinib. SK-BR-3 and BT-474 cells were treated with lapatinib at a concentration close to their GI_{50} doses, which were 100 nM and 10 nM respectively, with or without 10 ng/ml NRG1, a standard dose used in other studies (Ethier et al., 1996; Hutcheson et al., 2007; Yang et al., 2008). The cell growth was measured using total cell counting. In this particular experiment, cells were grown in 1% FBS to reduce the presence of growth factors in the culture medium. The cells were unable to survive for three days in FBS-free conditions.

4.2.2.1 Total cell counting

The results showed that lapatinib decreased SK-BR-3 cell number significantly ($p < 0.0001$ compared to DMSO), but the addition of NRG1 to lapatinib treatment reversed the inhibitory effect of the drug ($p < 0.0001$ compared to lapatinib alone) (Figure 4-2A). Interestingly, NRG1 stimulation alone significantly reduced cell number ($p < 0.0001$ compared to DMSO). In fact, NRG1 caused a dose dependent decrease in the cell number of SK-BR-3 (Figure 4-3). The lowest concentration of NRG1 used in this experiment, 0.156 ng/ml, caused a significant decrease of cell number to $78 \pm 13\%$ ($p < 0.0001$). The percentage cell number reached a plateau from 5–10 ng/ml NRG1, indicating the cells had either stopped growing or there were no further cells being killed at this range of NRG1 concentration.

BT-474 cells showed a similar effect when being treated with lapatinib and NRG1 (Figure 4-2B). Lapatinib caused a significant decrease in the cell number ($p < 0.0001$ compared to DMSO); however, addition of NRG1 to lapatinib treatment reversed the inhibition and increased cell number to an extent that was even higher than the control. The difference between lapatinib only and lapatinib with NRG1 was significant ($p < 0.0001$). NRG1 stimulation alone, unlike in SK-BR-3 cells, promoted cell proliferation in BT-474 cells ($p < 0.0001$ compared to DMSO).

The above cell counting experiments provided the first evidence that NRG1, a HER3 ligand, rescues HER2-overexpressing cell lines SK-BR-3 and BT-474 cells from the inhibitory effect of lapatinib. In order to further confirm this observation, clonogenic assays were performed.

4.2.2.2 Clonogenic assay

Clonogenic assays were performed to confirm the effect of lapatinib and NRG1 on cell viability. In SK-BR-3 cells, lapatinib treatment, NRG1 stimulation and their combination

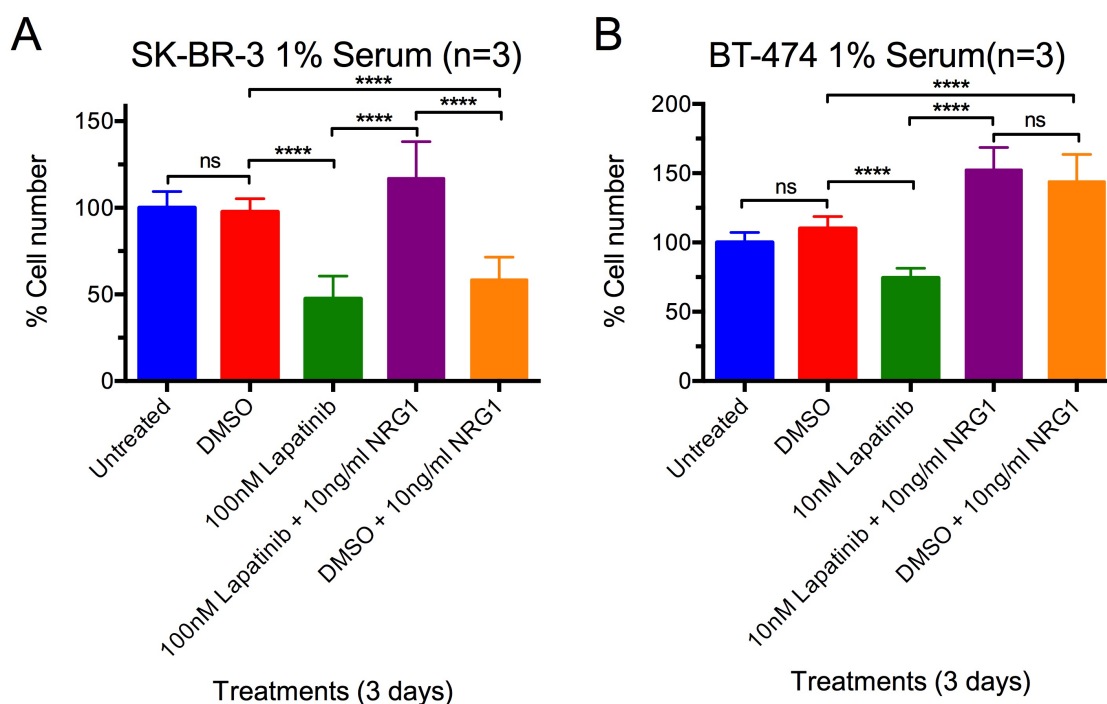


Figure 4-2 Effect of NRG1 on lapatinib response in HER2-overexpressing cell lines

(A) SK-BR-3 and (B) BT-474 cells were grown in culture medium supplemented with 1% serum. Cells were treated with the indicated agents for three days, then trypsinised and counted. The percentage cell number was normalised to untreated control. Three independent experiments were performed with three technical replicates. (Error = SD; Matched two-way ANOVA with Bonferroni correction, **** $p < 0.0001$)

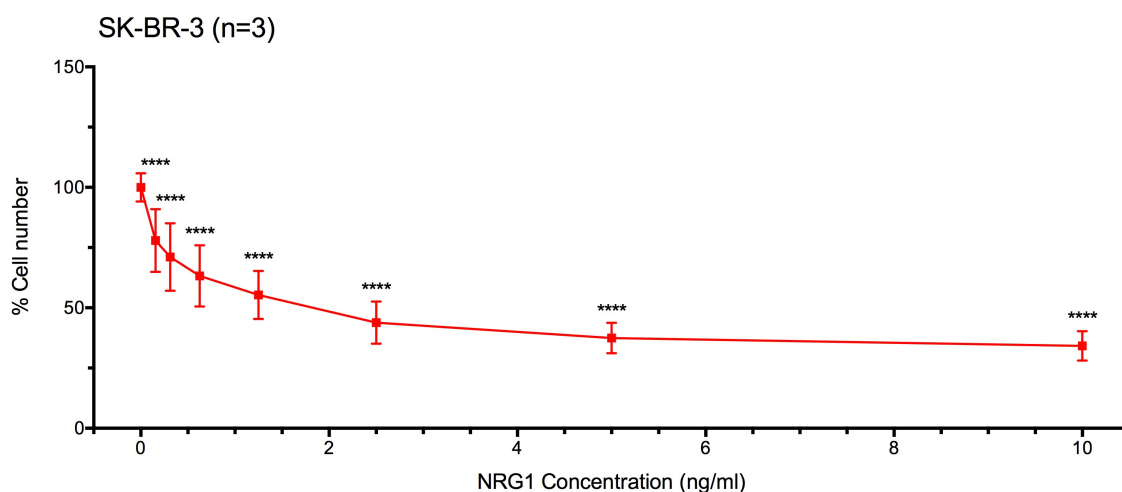


Figure 4-3 Dose dependent effect of NRG1 on SK-BR-3

SK-BR-3 cells were grown in culture medium supplemented with 1% serum. Cells were treated with the indicated concentrations of NRG1 for three days, then trypsinised and counted. The percentage cell number was normalised to untreated control. Three independent experiments were performed with three technical replicates. (Error = SD; Matched two-way ANOVA with Dunnett correction, **** $p < 0.0001$ compared to control)

produced a similar trend to the total cell counting experiments in the clonogenic assay (Figure 4-4A, Figure 4-5A). Lapatinib alone or NRG1 stimulation with DMSO treatment significantly reduced colony number (Both $p < 0.0001$ compared to DMSO), suggesting that both agents decreased the cell viability of SK-BR-3. The combination of lapatinib and NRG1, however, returned the colony number to a level that was similar to the DMSO control, and the differences with either agent alone were significant (Both $p < 0.0001$).

However, the results in BT-474 cells were unexpected. Lapatinib treatment significantly increased colony number compared to DMSO ($p = 0.0442$) (Figure 4-4B, Figure 4-5B). In fact, the previous dose-dependent experiment showed that 10 nM lapatinib might be too low to cause reduction in the colony number in clonogenic assay (Figure 3-14B). Furthermore, NRG1 stimulation significantly decreased colony number ($p < 0.0001$). The combination of lapatinib and NRG1 had no significant difference in colony number from NRG1 alone, indicating that the decrease might be solely caused by NRG1.

In both of the total cell counting and clonogenic experiments, BT-474 received the same treatment, but the results from the two methods were not coherent. This suggests that the decrease in cell number might be caused by a rapid cell death mechanism, for example apoptosis. The cell viability of the remaining cells after three-day treatment was not affected by 10 nM lapatinib but only at a higher dose. The limitation of growth assays in BT-474 cell line will be discussed in detail in Section 7.4.

4.2.3 Exogenous NRG1 stimulated HER signalling pathway despite the presence of lapatinib

This section describes the experiments performed to try to understand the mechanism by which NRG1 decreased the inhibitory effect of lapatinib. Western blotting was employed

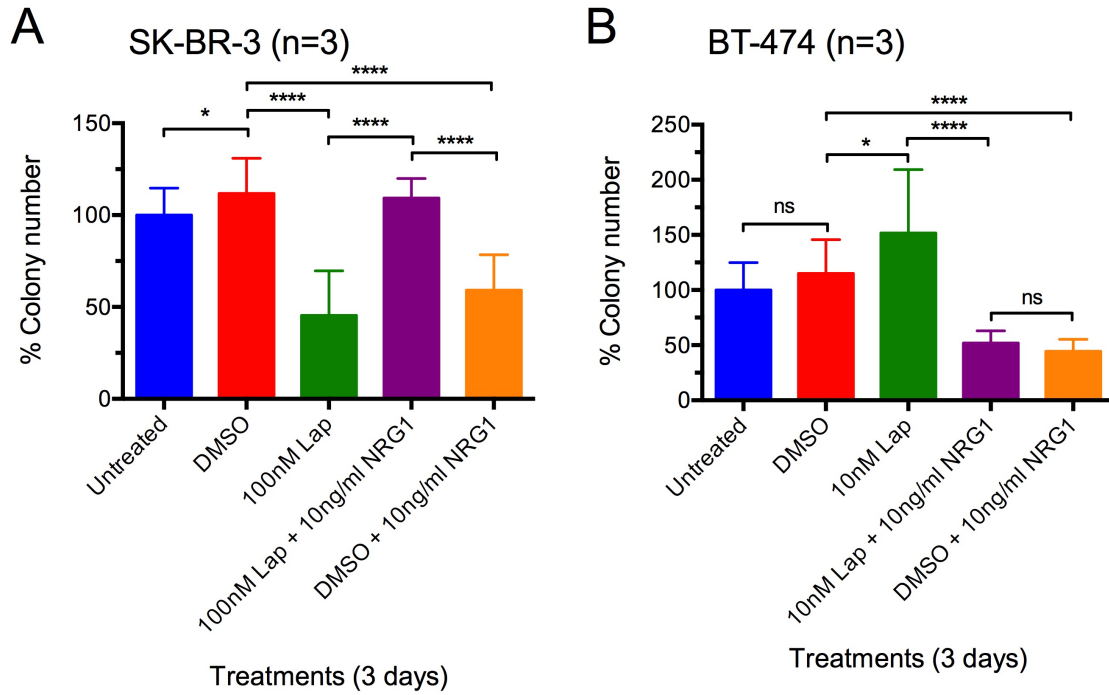


Figure 4-4 Effect of lapatinib and/or NRG1 on the clonogenicity of HER2-overexpressing cell lines

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated agents for three days. Cells were then trypsinised, reseeded and allowed to grow into colonies. The number of colony was normalised to untreated control. Three independent experiments were performed with three technical replicates. (Error = SD; Matched two-way ANOVA with Bonferroni correction, * $p < 0.05$, **** $p < 0.0001$)

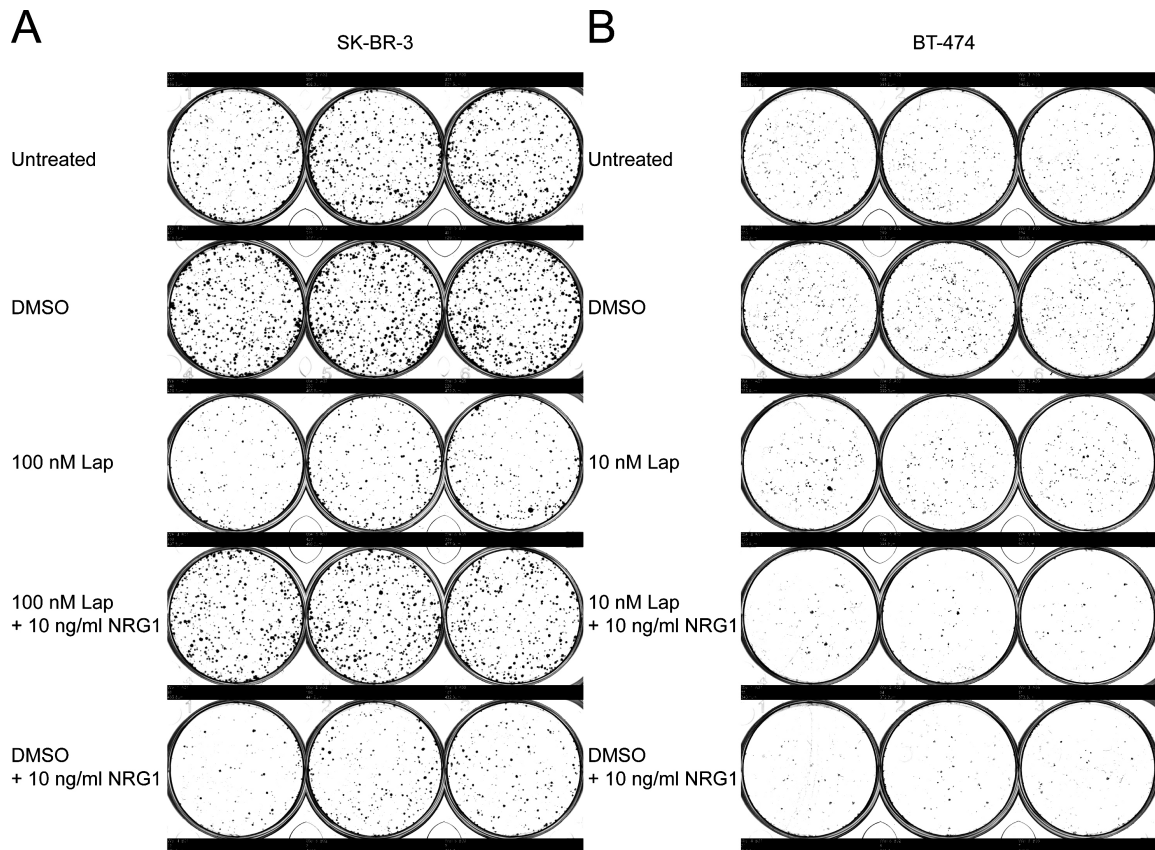


Figure 4-5 Representative experiment for the effect of lapatinib and NRG1 combination on clonogenicity of SK-BR-3 and BT-474

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated agents for three days. Cells were then trypsinised, reseeded and allowed to grow for (A) 14 days or (B) 17 days. Plates were then washed with PBS and stained with methylene blue.

to determine the HER signalling pathway of HER2-overexpressing breast cancer cells whilst being treated with lapatinib and NRG1.

Although NRG1 is the ligand for HER3 and HER4 (Olayioye et al., 2000), it only increased phosphorylation of HER3 and the downstream Akt and MAPK in both SK-BR-3 and BT-474 cells in this study when lapatinib was absent (Figure 4-6). Interestingly, NRG1 stimulation decreased phosphorylation of EGFR, HER2 and HER4. The binding of NRG1 to HER3 triggers dimerisation of HER3 to other members of the HER family (Harari and Yarden, 2000). HER3 acts as an allosteric activator for its dimerisation partner and activates their tyrosine kinase domain (Baselga and Swain, 2009). EGFR, HER2 or HER4 phosphorylates the C-terminal tail of HER3; however, having a defective kinase domain (Yarden and Sliwkowski, 2001), HER3 was not able to phosphorylate its binding partner. This might explain the inhibitory effect of NRG1 on phospho-EGFR, phospho-HER2 and phospho-HER4. However, their phosphorylation was still be present, possibly via dimerisation with HER4 after NRG1 stimulation (Harari and Yarden, 2000) or the very low kinase activity of HER3 (Shi et al., 2010; Steinkamp et al., 2013).

In both cell lines, lapatinib inhibited the phosphorylation of all HER receptors and the downstream Akt and MAPK in both cell lines (Figure 4-6). In the presence of both lapatinib and NRG1, lapatinib could still inhibit phosphorylation of EGFR and HER2; however, there was an increase in the phosphorylation of HER3, HER4, MAPK and Akt compared to lapatinib alone. These results suggested that NRG1 could partially reverse the inhibition caused by lapatinib.

The above experiments showed that lapatinib could not effectively inhibit phospho-HER3, phospho-HER4 and their downstream MAPK and Akt pathways in the presence of NRG1 stimulation. This mechanism would explain how NRG1 rescued SK-BR-3 and BT-474 cells from the growth inhibition and the reduced cell viability caused by lapatinib.

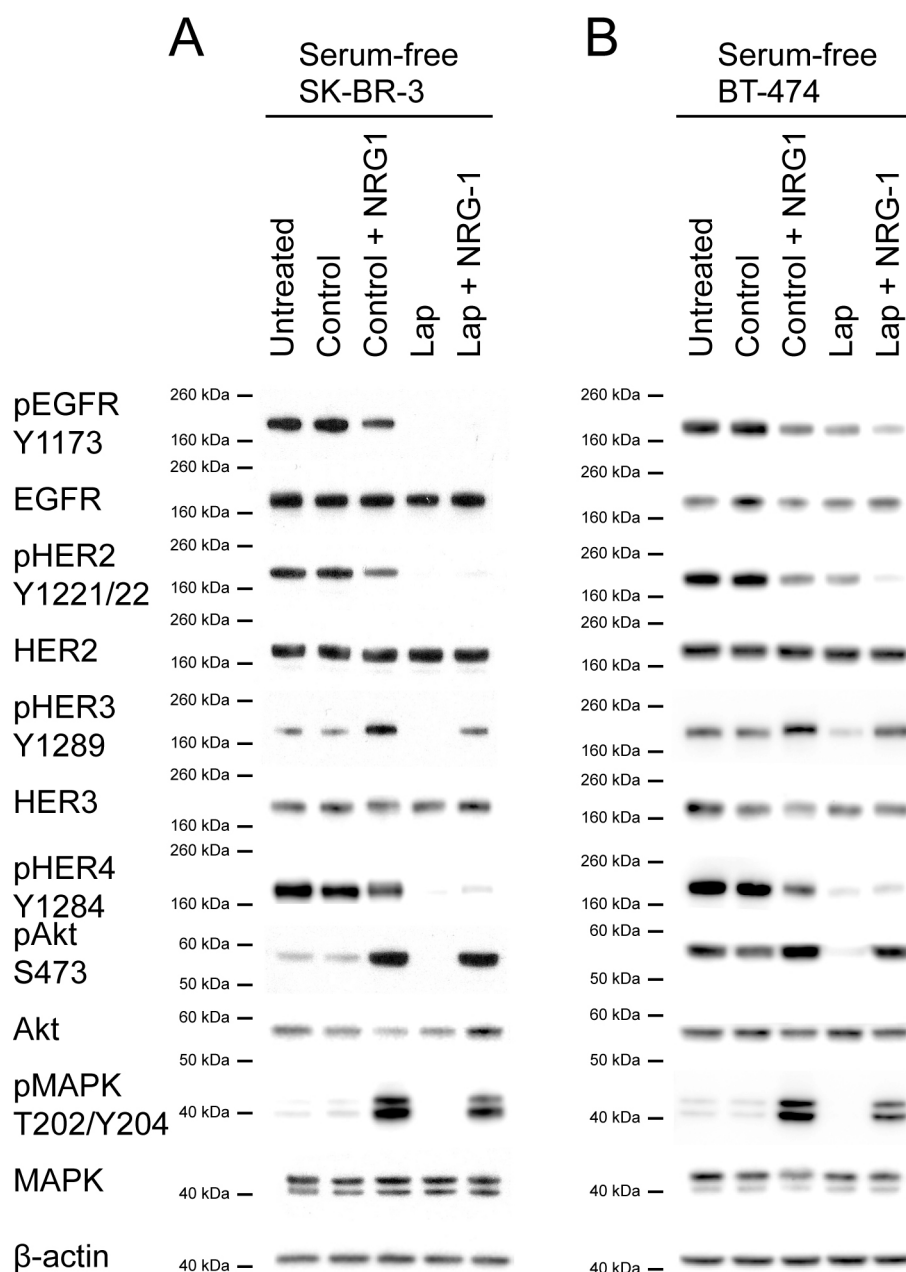


Figure 4-6 Effect of NRG1 on the inhibitory action of lapatinib

(A) SK-BR-3 and (B) BT-474 were grown in serum-free culture media for 24 hours, and then treated with 100 nM lapatinib or DMSO control for one hour. In the last ten minutes of the treatments, 10 ng/ml NRG1 was added as indicated. Complete cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted for Tyr1173-phosphorylated EGFR (pEGFR-Y1173), total EGFR (EGFR), Tyr1221/22-phosphorylated HER2 (pHER2-Y1221/22), total HER2 (HER2), Tyr1289-phosphorylated HER3 (pHER3-Y1289), total HER3 (HER3), Tyr1284-phosphorylated HER4 (pHER4-Y1284), Ser473-phosphorylated Akt (pAkt-S473), total Akt (Akt), Thr202/Tyr204-phosphorylated p44/42 MAPK (pMAPK-T202/Y204) and total p44/42 MAPK (MAPK). β-actin blot served as loading control in all experiments. Reproducible representative results of at least three independent experiments are shown.

4.2.4 Effect of lapatinib treatment on NRG1 expression and endogenous autocrine release

Previous experiments showed that NRG1 reversed the inhibitory effect of lapatinib in HER2-overexpressing breast cancer cell lines SK-BR-3 and BT-474; however, these experiments were based on exogenous ligand, i.e. NRG1 was added to the culture medium but not produced by the cells. It was hypothesised that lapatinib treatment could cause an increase in NRG1 expression and release; therefore, this was investigated. The expression of NRG1 was determined by quantitative real-time polymerase chain reaction (qPCR) of complementary DNA (cDNA) and enzyme-linked immunosorbent assay (ELISA) detection in complete cell lysate. The release of NRG1 was measured by ELISA detection in culture media.

4.2.4.1 NRG1 mRNA expression

4.2.4.1.1 Basal NRG1 expression

First, the basal mRNA expression level of NRG1 was quantified by qPCR and was compared to MDA-MB-231, a triple negative breast adenocarcinoma cell line that is reported to highly express NRG1 (Atlas et al., 2003; Tsai et al., 2003), and A431, an EGFR-overexpressing epidermoid carcinoma cell line that is commonly used as positive control for NRG1 antibody (SCBT, N. D.). The NRG1 primers used in this thesis was designed and reported in previous publication (Forster et al., 2011). The primers were validated by performing qPCR with a serial dilution of NRG1 expression vector (Golding et al., 2004) provided by Bill Gullick's group at the University of Kent. Results showed that these primers were sensitive to the change in the quantity of NRG1 vector and the relationship between threshold cycle (Ct) and the log of NRG1 copy number was linear ($R^2 = 0.9889$) (Figure 4-7A). The dissociation curve shows that the NRG1 primers specifically amplified NRG1 from the cDNA sample (Figure 4-7B).

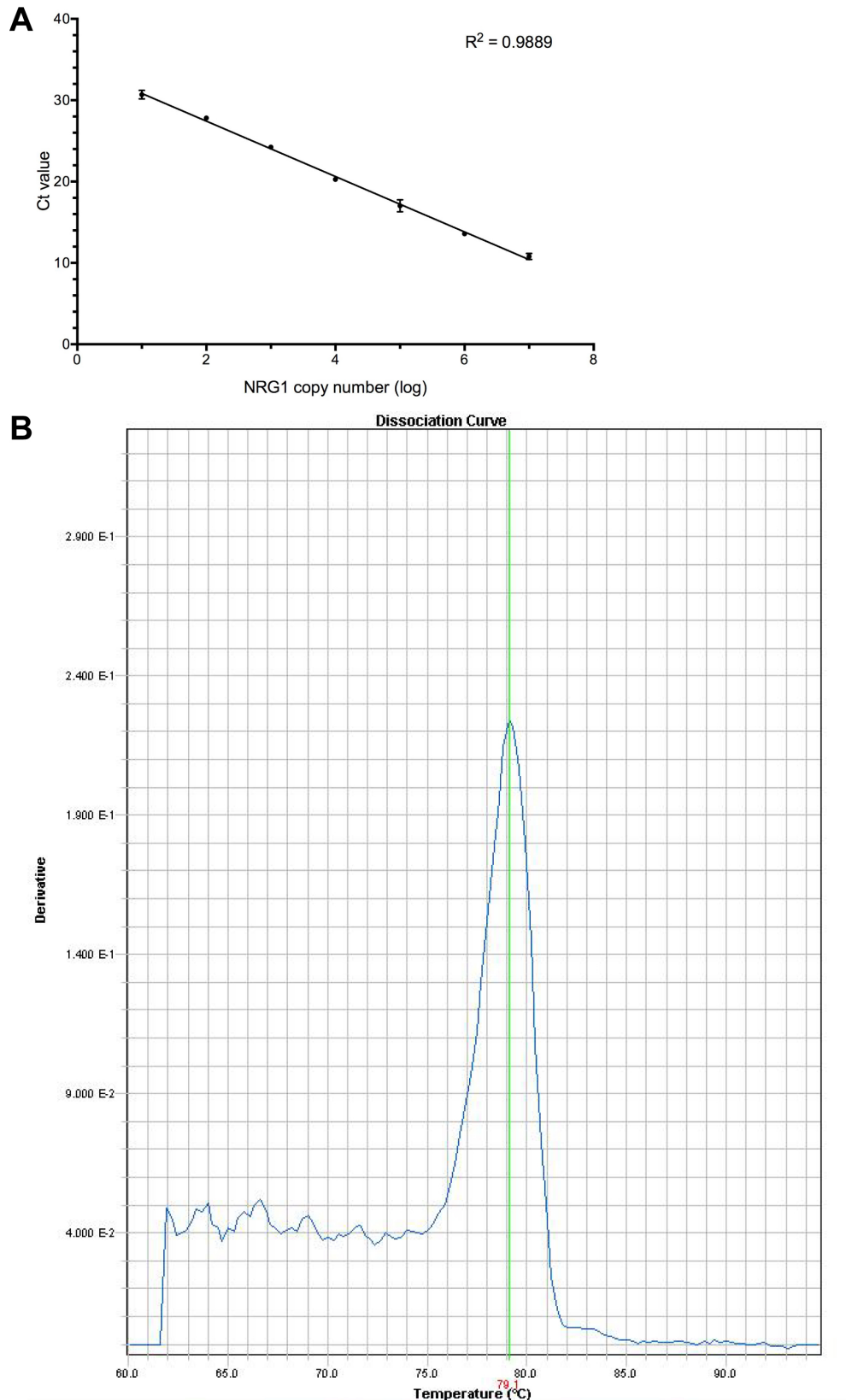


Figure 4-7 Validation of NRG1 primers for qPCR

(A) A 10-fold serial dilution of NRG1 expression vector was prepared and the Ct value was measured using qPCR. Three technical replicates were performed. (B) A representative dissociation curve of the amplicon after a qPCR reaction using the NRG1 primers.

MDA-MB-231 and A-431 cells had the highest levels of NRG1 mRNA as expected (Figure 4-8). SK-BR-3 and BT-474 cells had much lower levels of NRG1 mRNA, with Ct values of 33.65 ± 2.08 and 35.32 ± 1.00 respectively. This range of Ct value (33-36) is very high and indicates that NRG1 mRNA was barely detectable in SK-BR-3 and BT-474 cells.

4.2.4.1.2 NRG1 expression upon acute lapatinib treatment

The effect of lapatinib treatment on NRG1 mRNA expression was further determined. Two doses of lapatinib, 100 nM and 250 nM were used. In previous experiment (Figure 3-7), 100 nM lapatinib was found to cause rapid dephosphorylation of HER signalling pathway in both SK-BR-3 and BT-474 cell lines, but phosphorylation recovered after eight hour of drug treatment. The lapatinib concentration of 250 nM was used to generate lapatinib-resistant cell lines. The cells were treated with lapatinib for eight hours, which was the time point that re-phosphorylation occurred (Figure 3-7).

Both 100 nM and 250 nM lapatinib induced an increasing trend of NRG1 expression in SK-BR-3 cells (Figure 4-9A); however, the increase was not statistically significant compared to the DMSO control. In BT-474 cells (Figure 4-9B), NRG1 mRNA level increased after the cells were treated with 250 nM lapatinib for eight hours ($p = 0.0108$ compared to the DMSO control). The above results showed that short-term lapatinib treatment might induce an increase of NRG1 mRNA level.

4.2.4.1.3 NRG1 expression in lapatinib-resistant cell lines

The next step was to determine whether long-term lapatinib exposure would change the NRG1 mRNA level in the cells. Lapatinib-resistant cell lines SK250LR and BT250LR cells were continuously treated with 250 nM lapatinib. The levels of NRG1 mRNA expression of the lapatinib-resistant cell lines were compared with DMSO-treated parental cells. Lapatinib was also removed from the resistant cells for one day or seven days to determine if the changes in NRG1 mRNA expression were permanent.

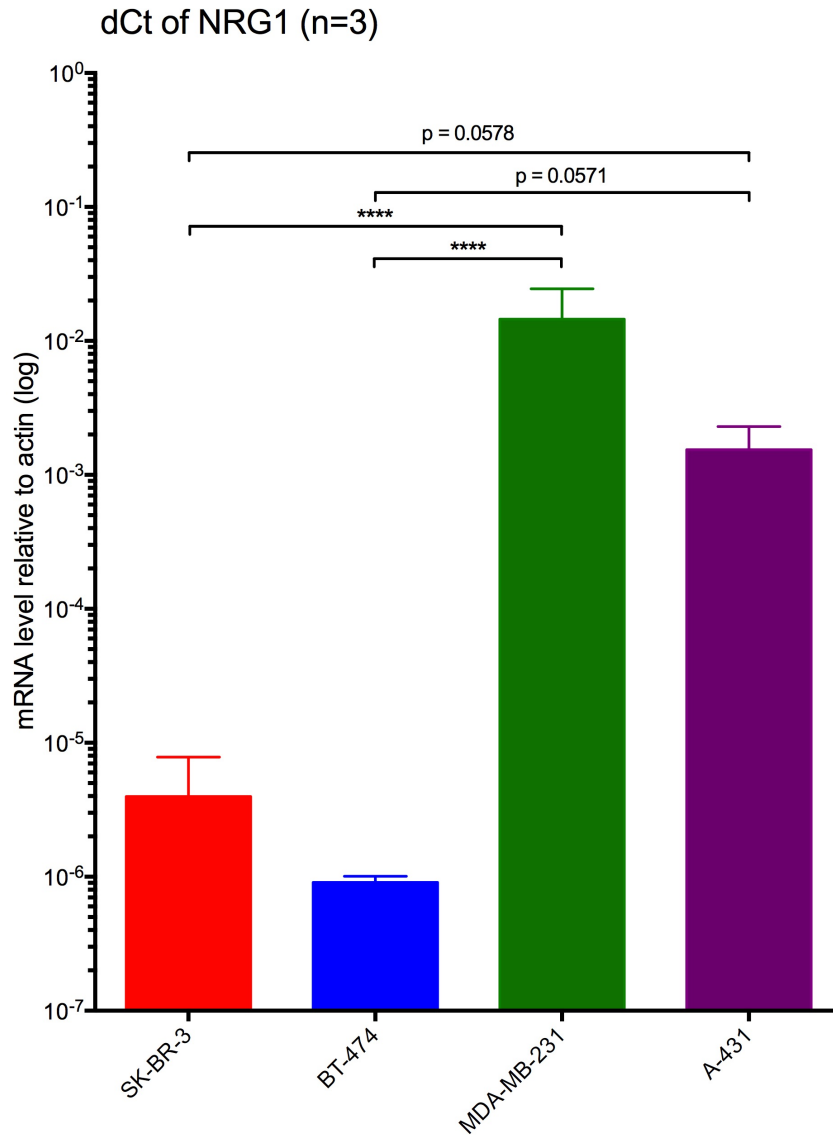


Figure 4-8 Basal mRNA expression level of NRG1

mRNA was extracted from four cell lines: SK-BR-3 (red), BT-474 (blue), MDA-MB-231 (green) and A-431 (purple), and reverse-transcribed into cDNA. NRG1 cDNA was detected by qPCR. The relative expression level of NRG1 with respect to the β -actin control was calculated using dCt method. Three independent experiments were performed with three technical replicates each time. (Error = SD; Matched two-way ANOVA with Bonferroni correction, **** $p < 0.0001$)

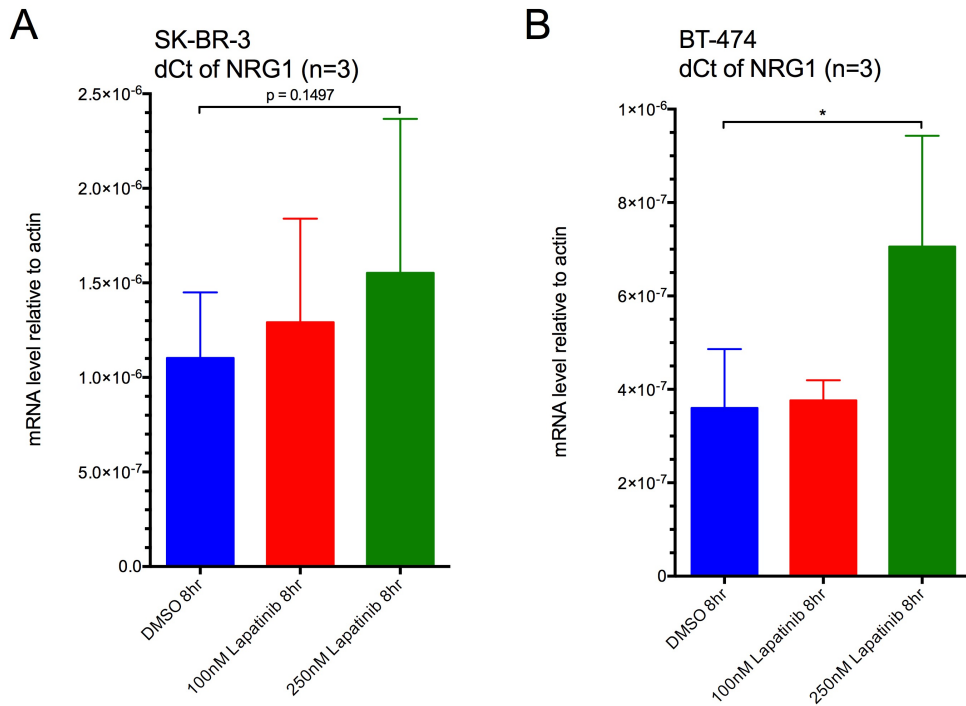


Figure 4-9 Effect of short-term lapatinib treatment on NRG1 mRNA expression

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated concentrations of lapatinib or DMSO control for eight hours. mRNA was extracted and reverse-transcribed into cDNA. NRG1 cDNA was detected by qPCR. The relative expression level of NRG1 with respect to the β -actin control was calculated using dCt method. Three independent experiments were performed with three technical replicates each time. (Error = SD; Matched two-way ANOVA with Dunnett correction, * $p < 0.05$)

Figure 4-10A shows that NRG1 mRNA level in SK250LR cells was significantly higher than that in the parental SK-BR-3 cells ($p < 0.0001$). When lapatinib was withdrawn, NRG1 mRNA expression decreased from day 1 and to a significant ($p = 0.0034$) decrease on day 7.

BT250LR cells showed a similar trend of NRG1 mRNA expression level (Figure 4-10B). NRG1 mRNA expression increased significantly in BT250LR cells compared to the DMSO-treated parental BT-474 cells ($p < 0.0001$). Removal of lapatinib caused a decreasing trend of NRG1 mRNA level in seven days, with an insignificant decrease on day 1 but a significant decrease on day 7 ($p < 0.0001$). These experiments revealed that prolonged lapatinib treatment caused an increase in NRG1 mRNA expression in the lapatinib-resistant cell lines. This increase was lapatinib-dependent as the removal of drug decreased the NRG1 mRNA levels; however, on day 7, the NRG1 mRNA levels were still higher than those in the lapatinib-sensitive parental cell lines.

4.2.4.2 NRG1 protein expression

Four commercially available antibodies to NRG1 were tested in western blotting but none of them produced clear bands (Data not shown). Therefore, the protein expression of NRG1 was measured using ELISA. Cells were treated with different concentrations of lapatinib or DMSO control for eight hours, as previously described (Section 4.2.4.1).

In SK-BR-3 cells, NRG1 expression decreased in the cell lysate after eight hours of DMSO treatment compared to untreated cells ($p < 0.0001$) (Figure 4-11A). Both 100 nM and 250 nM lapatinib significantly increased NRG1 expression in the cell lysates (Both $p < 0.0001$ compared to DMSO). Interestingly, SK250LR cells had less NRG1 expression in the cell lysate than the DMSO-treated parental SK-BR-3 cells ($p < 0.0001$).

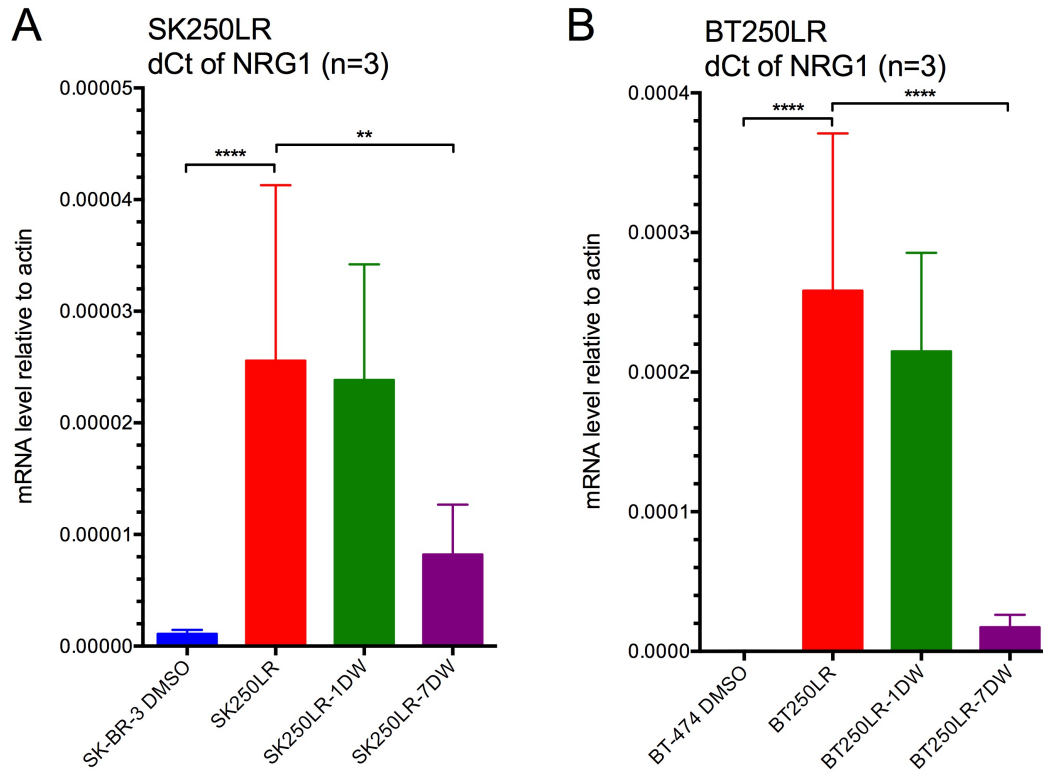


Figure 4-10 Effect of long-term lapatinib treatment on NRG1 mRNA expression

(A) SK-BR-3 and (B) BT-474 cells were treated with DMSO for eight hours. (A) SK250LR and (B) BT250LR cell lines were continuously treated with 250 nM lapatinib or had the drug removed for one day (1DW) or seven days (7DW). mRNA was extracted and reverse-transcribed into cDNA. NRG1 cDNA was detected by qPCR. The relative expression level of NRG1 with respect to the β -actin control was calculated using dCt method. Three independent experiments were performed with three technical replicates each time. (Error = SD; Matched two-way ANOVA with Dunnett correction, ** $p < 0.01$, **** $p < 0.0001$)

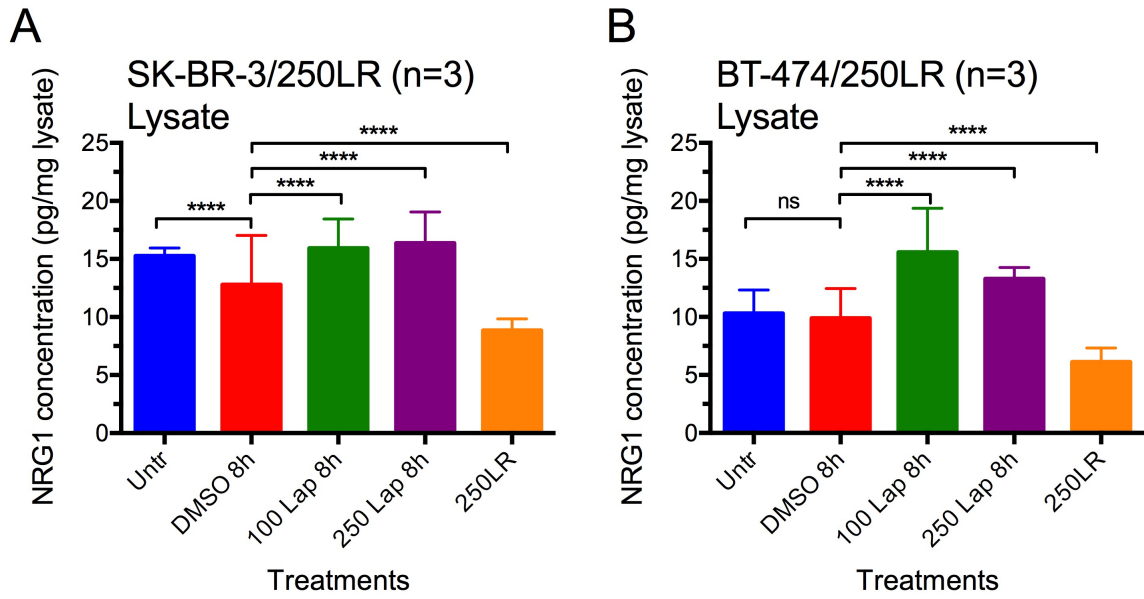


Figure 4-11 Effect of lapatinib treatment on NRG1 protein level

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated concentrations of lapatinib or DMSO control for eight hours. Lapatinib-resistant cells (250LR) were continuously treated with 250 nM lapatinib. Equal amount of complete cell lysates were applied to NRG1 ELISA kit according to manufacturer protocol. Three independent experiments were performed with two technical replicates for ELISA. (Error = SD; Matched two-way ANOVA with Dunnett correction, **** $p < 0.0001$)

BT-474 cells followed a similar trend to SK-BR-3 cells, except that there was no difference between DMSO-treated and untreated cells (Figure 4-11B). Both concentrations of lapatinib significantly upregulated NRG1 protein expression in the cell lysates (Both $p < 0.0001$ compared to DMSO). BT250LR cells also had reduced NRG1 protein in the cell lysate compared to DMSO-treated BT-474 cells ($p < 0.0001$).

The decrease in NRG1 protein concentration in the cell lysates of SK250LR and BT250LR contradicted to the increase in NRG1 mRNA expression measured in Section 4.2.4.1. This will be discussed in Section 4.3.4.4.

4.2.4.3 NRG1 release

NRG1 is normally released from the cell membrane to activate auto/paracrine signalling (Blobel, 2005; Hayes and Gullick, 2008); therefore, the amount of NRG1 released to the culture medium upon lapatinib treatment was estimated. Cells were treated with different doses of lapatinib or DMSO control in serum-free medium, to avoid interference from any possible NRG1 present in the serum, for eight hours. Lapatinib-resistant cells were incubated with or without lapatinib to address the amount of NRG1 released in the same period of time and whether the release is induced by lapatinib. Phorbol 12-myristate 13-acetate (PMA) treatment for one hour served as a positive control since it was reported to stimulate ADAM17 (Hahn et al., 2003; Herrlich et al., 2008), which in turn cleaves and releases NRG1 (Blobel, 2005; Montero et al., 2000). After eight hours of incubation, the conditioned media were concentrated and loaded onto an NRG1 ELISA kit.

ELISA of the conditioned media from SK-BR-3 and BT-474 cells did not show any trend of increase or decrease in NRG1 level after lapatinib treatment (Figure 4-12A and B). In fact, 25 out of 42 readings of media from SK-BR-3 cells (Figure 4-12C) and 30 out of 42 readings of media from BT-474 cells (Figure 4-12D) were below the lower limit of detection of the NRG1 ELISA kit. The PMA positive control did not produce detectable

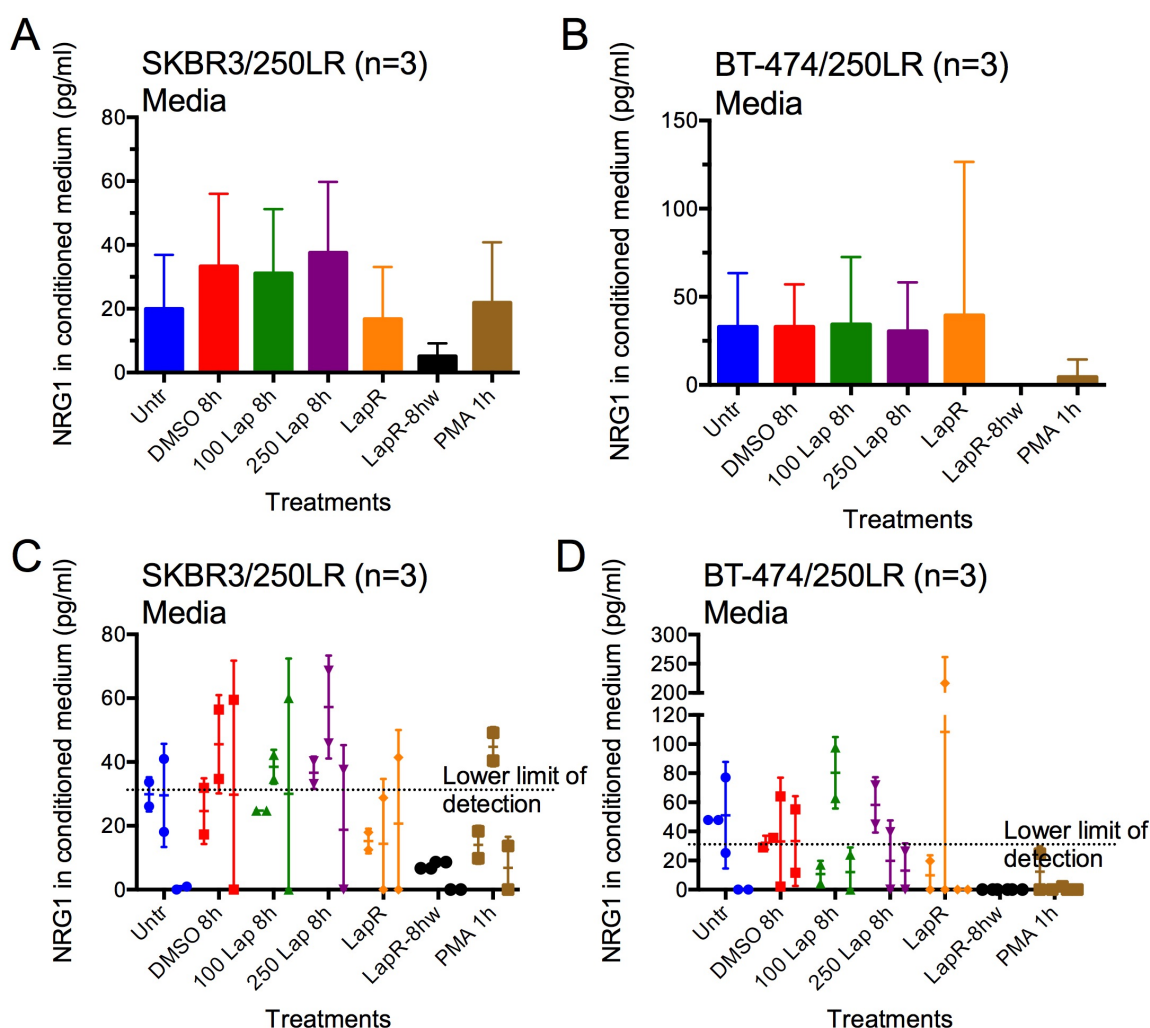


Figure 4-12 Effect of lapatinib treatment on NRG1 release

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated doses of lapatinib or DMSO control for eight hours in serum-free culture medium. One-hour PMA treatment served as a positive control. Lapatinib-resistant cells (LapR) were treated with 250 nM lapatinib or no drug for eight hours. Conditioned media were collected, concentrated and an equal volume was applied to the NRG1 ELISA kit according to manufacturer protocol. The concentration of NRG1 in condition media was normalised with the cell lysate concentration as a surrogate of cell number. Three independent experiments were performed with two technical replicates for ELISA. The results of individual experiments in (C) SK-BR-3 and (D) BT-474 cells and the lower limit of detection of the NRG1 ELISA kit were plotted. (Error = SD)

NRG1 release in any except one experiment. Although this ELISA kit could capture NRG1 protein in the cell lysates as depicted in Section 4.2.4.2, the above observations indicated that the amount of NRG1 released to media might be too low to be detected by this ELISA kit. Therefore, no conclusion could be drawn on the effect of lapatinib on NRG1 release.

4.2.5 Knocking down NRG1 in the lapatinib-resistant cells

Finally, the role of NRG1 in the lapatinib-resistant cell lines was determined using knockdown with RNA interference. Small interfering RNAs (siRNAs) against NRG1 were validated in MDA-MB-231 cells as they have high expression levels of this gene. Figure 4-13A showed that both 25 nM and 50 nM NRG1 siRNA pool resulted in good and comparable mRNA knockdown over three days. To avoid excessive toxicity, 25 nM was used in later experiments. Next, all the single siRNA in the NRG1 pool were tested in MDA-MB-231 cells (Figure 4-13B) and the two lapatinib-resistant cell lines (Figure 4-13C and D). From the above experiments, siNRG1-9 and siNRG1-11 gave a consistently good knockdown efficiency in the three cell lines, except siNRG1-11 in BT20LR (Figure 4-13D). Hence, these two single siRNA were used to knockdown NRG1 in further experiments.

NRG1 knockdown using siNRG1-9 and siNRG1-11 reduced cell number of SK250LR by around 30% (both $p < 0.0001$ compared to scramble) whether or not the cells were continuously treated with lapatinib (Figure 4-14A). There was no additive effect in the combination of siNRG1 and continuous lapatinib treatment, indicating the reduction in the cell number may be due to NRG1 knockdown alone in the lapatinib-resistant SK250LR cells.

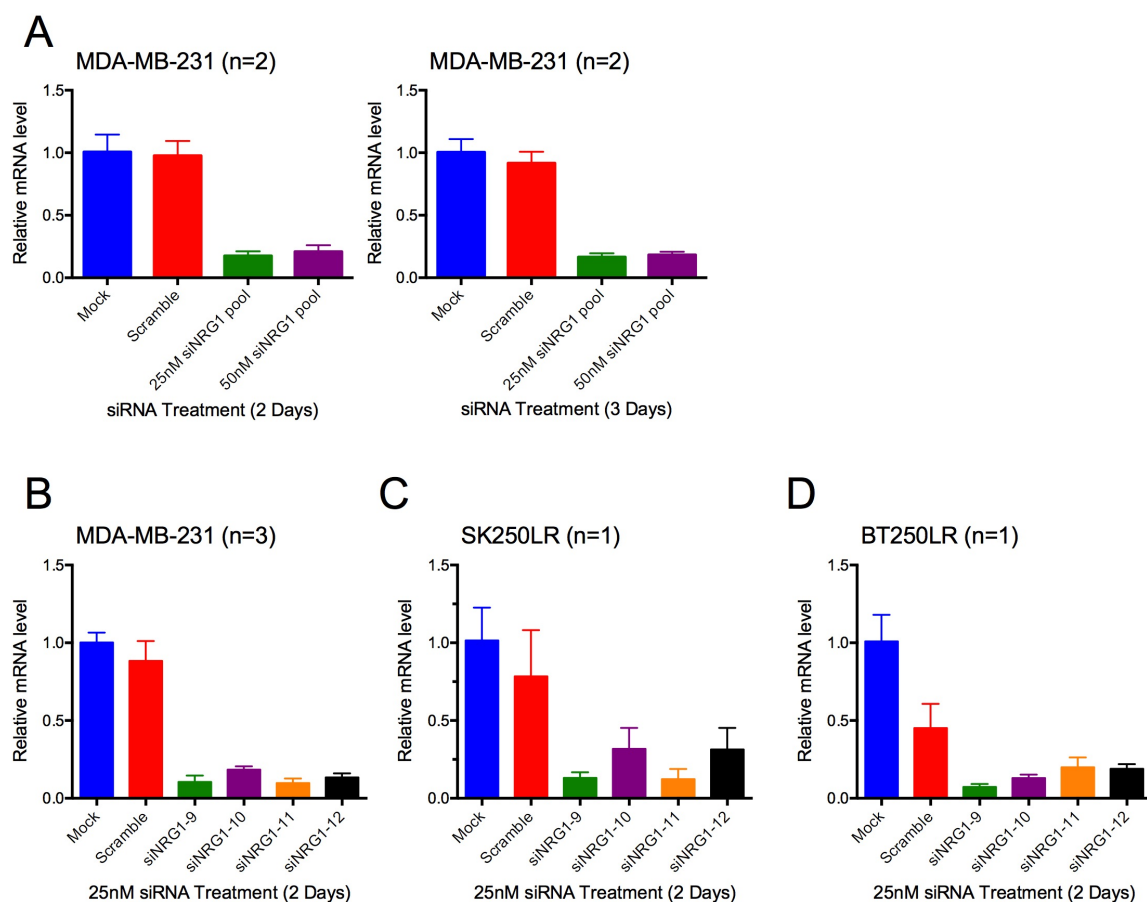


Figure 4-13 Validation of NRG1 siRNA

(A) MDA-MB-231 cells were treated with transfecting agent only (mock) or together with 50 nM non-specific scramble siRNA or the indicated concentrations of NRG1 siRNA pool for two days (left) or three days (right). (B) MDA-MB-231, (C) SK250LR and (D) BT250LR cells were treated with transfecting agent only (mock) or together with 25 nM non-specific scramble siRNA or 25 nM of the indicated NRG1 single siRNA for two days. mRNA was extracted and reverse-transcribed into cDNA. NRG1 cDNA was detected by qPCR. The relative expression level of NRG1 normalised to the mock treatment was calculated using ddCt method. (A) Two independent experiments, (B) three independent experiments or (C and D) one experiment were/was performed in three technical triplicates each time. (Error = SD)

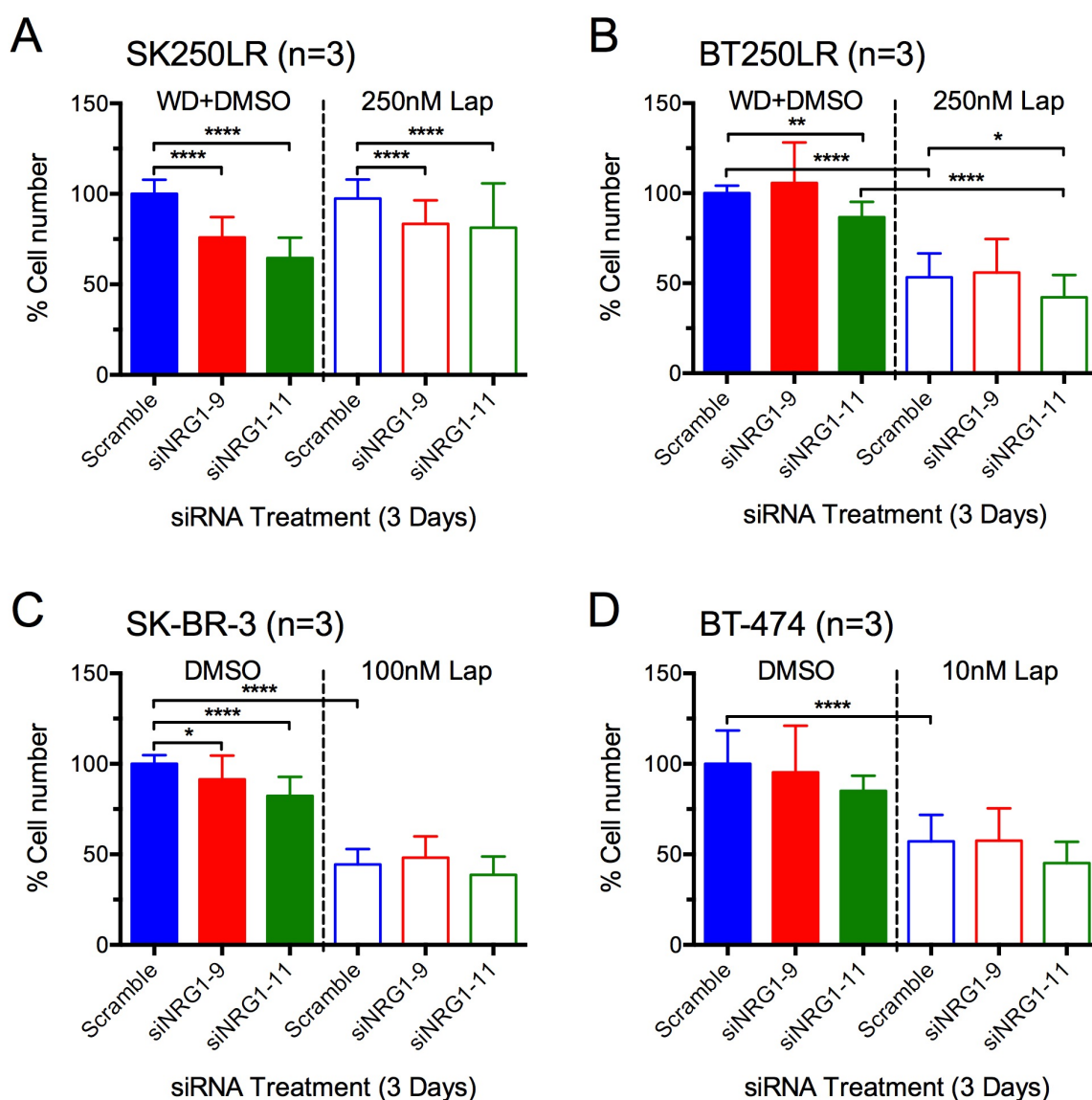


Figure 4-14 Effect of NRG1 knockdown and/or lapatinib in lapatinib-resistant and -sensitive HER2-overexpressing cell lines

(A) SK250LR and (B) BT250LR cells were treated with 25 nM of the indicated siRNA or non-specific scramble control, and either with 250 nM lapatinib (250nM Lap) or had the drug withdrawn (WD+DMSO) for three days. (C) SK-BR-3 and (D) BT-474 cells were treated with 25 nM of the indicated siRNA or non-specific scramble control, and either with the indicated dose of lapatinib (100nM or 10nM Lap) or DMSO control for three days. The cells were then trypsinised and counted. The percentage cell number was normalised to the scramble plus DMSO control. Three independent experiments were performed with three technical replicates each time. (Error = SD; Matched two-way ANOVA with Bonferroni correction, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$)

NRG1 knockdown caused significant decrease in the cell number of parental SK-BR-3 (Figure 4-14C). The decreases in cell number caused by both NRG1 siRNAs were significant (siNRG1-9: $p = 0.0329$, siNRG1-11: $p < 0.0001$ compared to scramble) in SK-BR-3 cells; however, the decrease was small (10-20%) that it was unlikely to have any biological importance. While lapatinib reduced cell number significantly compared to the DMSO control ($p < 0.0001$), the combination of NRG1 knockdown with lapatinib treatment did not significantly reduced cell number compared to the scramble plus lapatinib control, suggesting that the combination might not be additive (Figure 4-14C). The NRG1 knockdown experiments in SK250LR and SK-BR-3 cells had the similar trend of cell number decrease as produced by SGP1 antibody (Figure 4-1A and C).

In summary, these results suggested that the growth of these two cell lines depended on NRG1 expression to a certain extent. While NRG1 knockdown had a small inhibitory effect on the parental SK-BR-3 cells, the effect of lapatinib treatment and NRG1 knockdown was not additive. In the SK250LR cells, NRG1 knockdown reduced cell number significantly in the presence of acquired lapatinib resistance, but the effect was independent of whether lapatinib treatment was continued in these resistant cells.

As previously described, BT250LR cell line was still responsive to lapatinib but to a lesser extent than the parental BT-474 cell line (Figure 3-13B). When the drug was withdrawn, BT250LR cells grew faster than those that were continuously treated with lapatinib (Figure 3-21B). Therefore, continuous 250 nM lapatinib treatment still decreased the cell number of BT250LR significantly ($p < 0.0001$ compared to WD+DMSO) (Figure 4-14B). When lapatinib was withdrawn, siNRG1-11 treatment significantly reduced the cell number ($p = 0.0017$ compared to WD+DMSO and scramble). With the continuous treatment of 250 nM lapatinib, knockdown by siNRG1-11 also significantly decreased cell number ($p = 0.0146$ compared to lapatinib plus scramble control; $p < 0.0001$ compared to WD+DMSO control

plus siNRG1-11), indicating the effect of lapatinib and NRG1 knockdown may be additive. However, siNRG1-9 treatment produced no significant difference from the scramble siRNA. These results were also different from that with SGP1 antibody, which reduced cell number significantly and was additive with lapatinib. The contradicting results suggested that no definite conclusion could be drawn on the BT250LR cell line yet until more experiments are performed, such as knocking down NRG1 with the reserve siNRG1-10 and siNRG1-12.

In BT-474 cells, NRG1 knockdown did not cause any significant change in the cell number with or without lapatinib (Figure 4-14D). The limitations concerning total cell counting experiments with BT-474 and BT250LR cell lines will be discussed in Section 7.5.3.

4.3 Discussion

4.3.1 HER3 ligand blocking and HER2-targeted therapy

The present study showed that the HER3 ligand-blocking antibody, SGP1, inhibited the growth of the acquired lapatinib-resistant HER2-overexpressing cell lines SK250LR and BT250LR. Bill Gullick's group, who produced this antibody, reported that SGP1 cooperated with trastuzumab in inhibiting the growth of HER2-expressing breast cancer cell lines SK-BR-3 and MDA-MB-361 (Blackburn et al., 2012). Such cooperation was not found in the combination treatment of SGP1 and lapatinib in the parental SK-BR-3 and BT-474 cells. Although this combination inhibited SK250LR and BT-474 cells more than lapatinib alone, the growth inhibition was contributed by SGP1 alone (Figure 4-1). An additive effect of the lapatinib and SGP1 combination was observed only in BT250LR cells, suggesting these two drugs were cooperative only in a particular case.

4.3.2 Effect of NRG1 on lapatinib treatment and HER signalling

The current study demonstrated that NRG1 rescued HER2-overexpressing cells from the anti-cancer activity of lapatinib, which agreed with the results of a recent report (Wilson et al., 2012). However, the work of Wilson *et al.* did not propose any mechanism. The present study demonstrated that NRG1 activated HER3 and HER4 signalling, and the downstream MAPK and Akt pathways, despite the presence of kinase inhibitor. These results suggested that HER3 and HER4 signalling might be one of the mechanisms that contributed to the rescue from lapatinib (Figure 4-15B). In order to confirm the importance of these two pathways, elements in these pathways, for instance, HER3, HER4, MAPK and Akt, could be knocked down to understand whether the lack of these pathways would reverse the rescue.

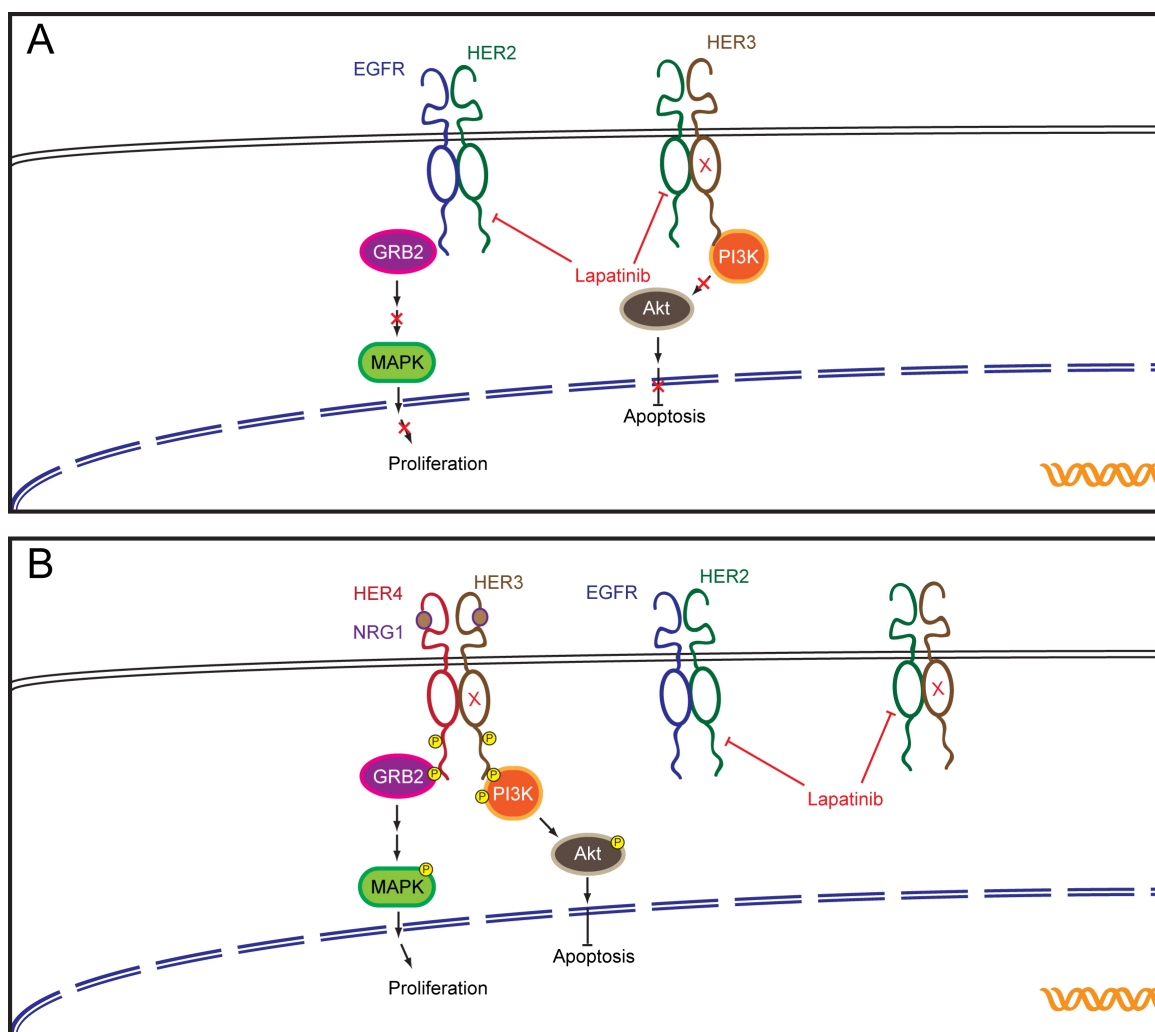


Figure 4-15 Schematic diagram of HER signalling in response to lapatinib treatment and NRG1 stimulation

(A) Lapatinib inhibits EGFR/HER2 tyrosine kinase activity, reducing the phosphorylation of the HER receptors and the downstream MAPK and Akt, thereby inhibiting proliferation and inducing apoptosis. (B) NRG1 stimulates HER3 and HER4 activities through homo- or heterodimerisation, recovering the phosphorylation of the downstream MAPK and Akt despite the presence of lapatinib. Hence, NRG1 rescued the cells from the cytostatic and cytotoxic effects of lapatinib.

4.3.3 Growth inhibitory effect of NRG1

In the present study, total cell counting experiments showed that NRG1 inhibited the proliferation of SK-BR-3 cells but not BT-474 cells; however, subsequent clonogenic assays revealed that BT-474 cells were also inhibited by NRG1 in the longer term. One of the reasons that total cell counting could not demonstrate growth inhibition in BT-474 cells may be the difference in doubling time between the two cell lines. The doubling time of SK-BR-3 and BT-474 cells was reported as around 15 hours (Lee et al., 2004) and 100 hours (Faltus et al., 2004) respectively. A slower growth rate means that the cells require longer time to exhibit the effect of growth inhibition. The effect of NRG1 on the growth rate of BT-474 cells may need to be studied by either increasing the length of treatment in the total cell counting experiments, or even better by performing real-time monitoring using IncuCyte.

Although NRG1 has been described as a growth factor that induces tumour growth and is associated with tumour aggressiveness (Lupu et al., 1996) and metastasis (Atlas et al., 2003; Cardillo et al., 1995), there were also reports showing that overexpression of NRG1 in HER2-overexpressing triggers growth arrest and apoptosis (Guerra-Vladusic et al., 1999). The mechanism of this phenomenon was later discovered to be the transcriptional and translational downregulation of HER2 and HER3 (Guerra-Vladusic et al., 2001). NRG1 promotes proliferation and tumour aggressiveness in a short period, while the model of receptors downregulation would explain the growth inhibition and the reduced cell viability in SK-BR-3 and BT-474 cells caused by long-term NRG1 stimulation. In general, the level of cell growth inhibition might depend on the balance between NRG1 and inhibitors (e.g. lapatinib) and also the length of NRG1 stimulation.

4.3.4 Difficulties in NRG1 detection

4.3.4.1 Low expression levels of NRG1

As shown in Section 4.2.4.1.1, the basal expression levels of NRG1 mRNA in SK-BR-3 and BT-474 cells were a few hundreds to thousands times lower than those in NRG1-positive cell lines MDA-MB-231 and A-431. These results agreed with previous studies showing very low NRG1 expression levels in SK-BR-3 (Dunn et al., 2004) and BT-474 cells (Chua et al., 2009). There were also reports stating that NRG1 expression in breast cancer cell lines and tumour biopsy samples was negatively correlated with HER2 expression (Atlas et al., 2003; Cardillo et al., 1995; Tsai et al., 2003). These studies may explain the low NRG1 levels in SK-BR-3 and BT-474 cells. The low expression levels of NRG1 in the HER2-overexpressing cell lines might be the reason of the difficulties in mRNA and protein detection.

4.3.4.2 Alternative splicing of NRG1 gene

NRG1 gene produces a great variety of products through an extensive alternative splicing mechanism (Hayes and Gullick, 2008), in which there are at least six transcription start sites controlled by probably nine promoters (Stein and Staros, 2006; Steinthorsdottir et al., 2004). Considering the combination of different exons spliced into the mRNAs, there are at least 15 reported NRG1 protein variants (Falls, 2003). These variants generate complications in the detection of NRG1; for example, immunodetection using western blotting would produce multiple bands, which are difficult to be identified from non-specific detection.

4.3.4.3 mRNA detection by qPCR

Detection of NRG1 mRNA in untreated SK-BR-3 and BT-474 cells was difficult because of the low expression levels of this gene as aforementioned. The Ct values of qPCR were

around 33.5 and 35.5 in SK-BR-3 and BT-474 respectively. In general, Ct values above 30 were considered low expression; however, these were still positive results as the negative control (MilliQ water) had Ct values >40.

The primers used in this thesis were previously published (Forster et al., 2011). It targets exons 8–10 of NRG1 gene and amplifies a number of splice variants (Figure 4-16), including NRG1 Type I variants $\beta 1$, $\beta 2$ and $\beta 3$ and NRG1 Type III SMDF (Sensory and motor neuron-derived factor). Another pair of primers that targets exons 4–6 (Chua et al., 2009), which is specific to Type I variants (Hashimoto et al., 2004), was tested; however, NRG1 mRNA was often undetectable in the lapatinib-sensitive cell lines using these primers (Data not shown).

4.3.4.4 Protein detection by ELISA

The ELISA kit for NRG1 was functional and was able to detect the NRG1 standard, giving a standard curve with good curve fit ($R^2 = 0.9960$) (Figure 2-2). There was no problem in detecting NRG1 in the cell lysates; however, detection of NRG1 in the concentrated conditioned media was poor, with 55 out of 84 (~65%) reading lower than the lower limit of detection. Unexpectedly, the positive control PMA, which was supposed to activate NRG1 release, did not increase NRG1 levels in the media. This indicated that NRG1 released by SK-BR-3 and BT-474 cells might be too low to be detected, or the protein was degraded during the medium concentration process.

As aforementioned, mRNA detection by qPCR may detect multiple NRG1 splice variants because the primers used were not specific to a particular NRG1 variant. On the contrary, the NRG1 ELISA kit from R&D systems detects a specific variant, NRG1 $\beta 1$. The difference in variants detection might be the reason of discrepancy between the NRG1 mRNA and protein levels in the lapatinib-resistant cell lines.

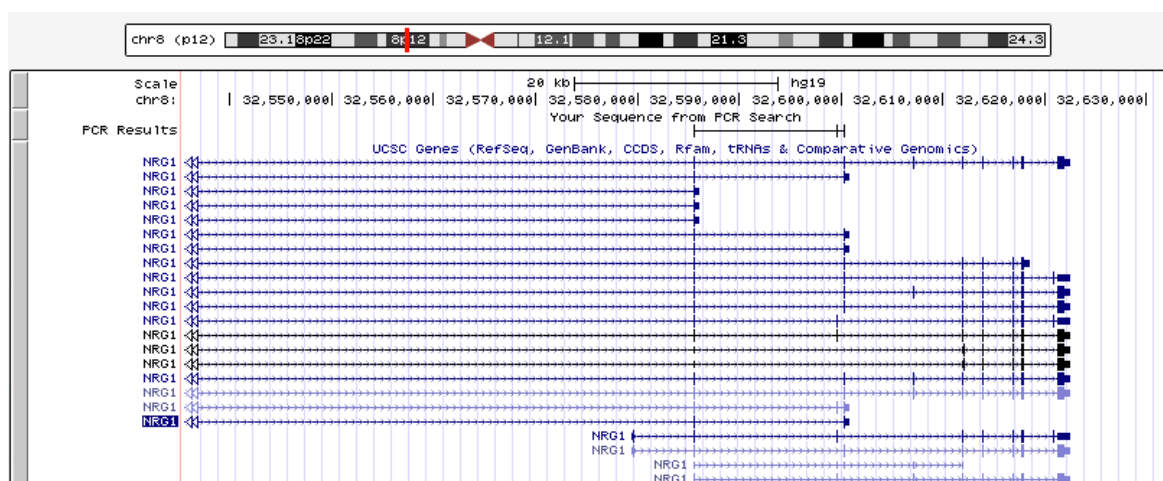


Figure 4-16 In-Silico PCR results using NRG1 primers

In-Silico PCR was performed using the UCSC genome browser (Karolchik et al., 2014). The cDNA sequence amplified by NRG1 primers was searched in the database of the Feb. 2009 (GRCh37/hg19) assembly in human genome, targeting UCSC Genes, i.e. transcribed sequence.

In addition, the discrepancy between mRNA and protein levels might be due to the different sensitivities of the experimental techniques. qPCR involves the exponential amplification of NRG1 cDNA, which enhances the sensitivity of detection. On the contrary, NRG1 protein detection is only amplified by secondary antibody, hence its sensitivity is limited. Some companies, such as Sigma-Aldrich and Abcam, also offer NRG1 ELISA kits, which may have a greater sensitivity and may be worthwhile trying in the future.

Our group has previously detected NRG1 in BT474 cells using an NRG1 antibody from Abcam (ab-27303) (Gijssen et al., 2010); however, the Western blotting results were not reproducible in this thesis and in fact, there was no change in any band when NRG1 was knocked down using siRNA (data not shown). In the recent publication from Xia *et al.*, they detected the bands in Western blotting using a fluorescence-probed secondary antibody (Xia et al., 2004), which increased the sensitivity of Western blotting.

4.3.5 NRG1 gene methylation

Chua *et al.* suggested that DNA methylation is one of the possible mechanisms to regulate NRG1 expression and it explains the low expression level of NRG1 in BT-474 cell line (Chua et al., 2009). They did not perform the same experiment on SK-BR-3 cell line, but it is an interesting direction to understand if lapatinib treatment would cause a relief in NRG1 gene methylation. Investigation of NRG1 gene methylation in lapatinib-resistant cell lines might also be able to explain the increase in mRNA level in those cell lines.

4.3.6 Clinical relevance of NRG1

Since NRG1 rescued HER2-overexpressing cell lines from the inhibitory effect of lapatinib, it might be a new biomarker to predict patients' response to the drug. A very recent study revealed that high NRG1 expression predicts a decreased recurrence-free

survival in breast cancer patients (Xia et al., 2013), suggesting the clinical importance of NRG1. Although this study aimed to demonstrate the role of NRG1-stimulated EGFR-HER3 autocrine signalling in acquired lapatinib resistance, they combined the data of all patients receiving chemotherapy, tamoxifen or systematically untreated; therefore, this analysis could not address the association between NRG1 expression and lapatinib response. To date, there is only one published clinical trial involving lapatinib (EGF104911) that has measured NRG1 level in tumours using IHC staining, but they did not mention any correlation between NRG1 expression and the clinical benefits from lapatinib monotherapy (Toi et al., 2009). There are three other completed clinical trials involving lapatinib (BRSADJ0002, EGF106988 and LCCC-0411) that aim to analyse gene expression data, but the data are not yet available for analysis.

Aside from gene expression analysis, NRG1 protein expression in tumour samples could be analysed using IHC staining, then scored (intensity and abundance) and correlated with lapatinib treatment outcome. The current study tried to validate commercial NRG1 antibodies for IHC application using NRG1 knockdown in MDA-MB-231 cells; however, none of them were specific to NRG1 (Figure 4-17).

4.3.7 Alternative strategies to assess the role of HER3 ligands in lapatinib resistance

The experiment involving SGP1, the HER3 ligand blocker, demonstrated that the growth and/or the survival of lapatinib-resistant HER2-overexpressing cell lines might depend on HER3 ligands. The current study and another report (Xia et al., 2013) have also shown the increase in NRG1 expression at mRNA level and protein level respectively in lapatinib-resistant cells; however, the NRG1 knockdown experiment in this current study did not provide strong enough evidence to justify the role of NRG1 in lapatinib resistance. At least in BT250LR cell line, NRG1 knockdown was not as effective as SPG1 antibody.

Inducible NRG1 overexpression experiments could be performed in SK-BR-3 and BT-474 cells using lentiviral stable transfection of a doxycycline-induced NRG1 overexpression vector. With this method, it is possible to understand if NRG1 overexpression would introduce lapatinib resistance to the sensitive cell lines.

NRG2 is another HER3 ligand but its function is not fully understood. A previous study showed that NRG2 but not NRG1 was predominantly expressed in SK-BR-3 cells (Dunn et al., 2004), suggesting that NRG2 might be a potential target in addressing the role of HER3 ligands in lapatinib resistance. NRG2 mRNA expression levels could be studied in the HER2-overexpressing breast cancer cell lines upon lapatinib treatment and in the lapatinib-resistant cell lines using qPCR. Furthermore, NRG2 siRNAs are available from companies such as Thermo Scientific (Dharmacon) and Origene, making it possible to perform NRG2 knockdown experiments to study its role.

4.3.8 Chapter summary

In this chapter, the lapatinib-resistant cell lines were found to be more sensitive to HER3 ligand-blocking antibody than the parental cell lines, suggesting the role of HER3 ligands in lapatinib resistance. NRG1 rescued HER2-overexpressing cell lines from the inhibitory effect of lapatinib. The mechanism of rescue might be NRG1-stimulated HER3 and HER4 signalling (Figure 4-15). Lapatinib triggered an increased expression of NRG1 mRNA and protein, and the mRNA expression levels of NRG1 in the lapatinib-resistant cell lines were highly upregulated; however, NRG1 β 1 protein level in the resistant cell lines was lower than that in the parental cell lines.

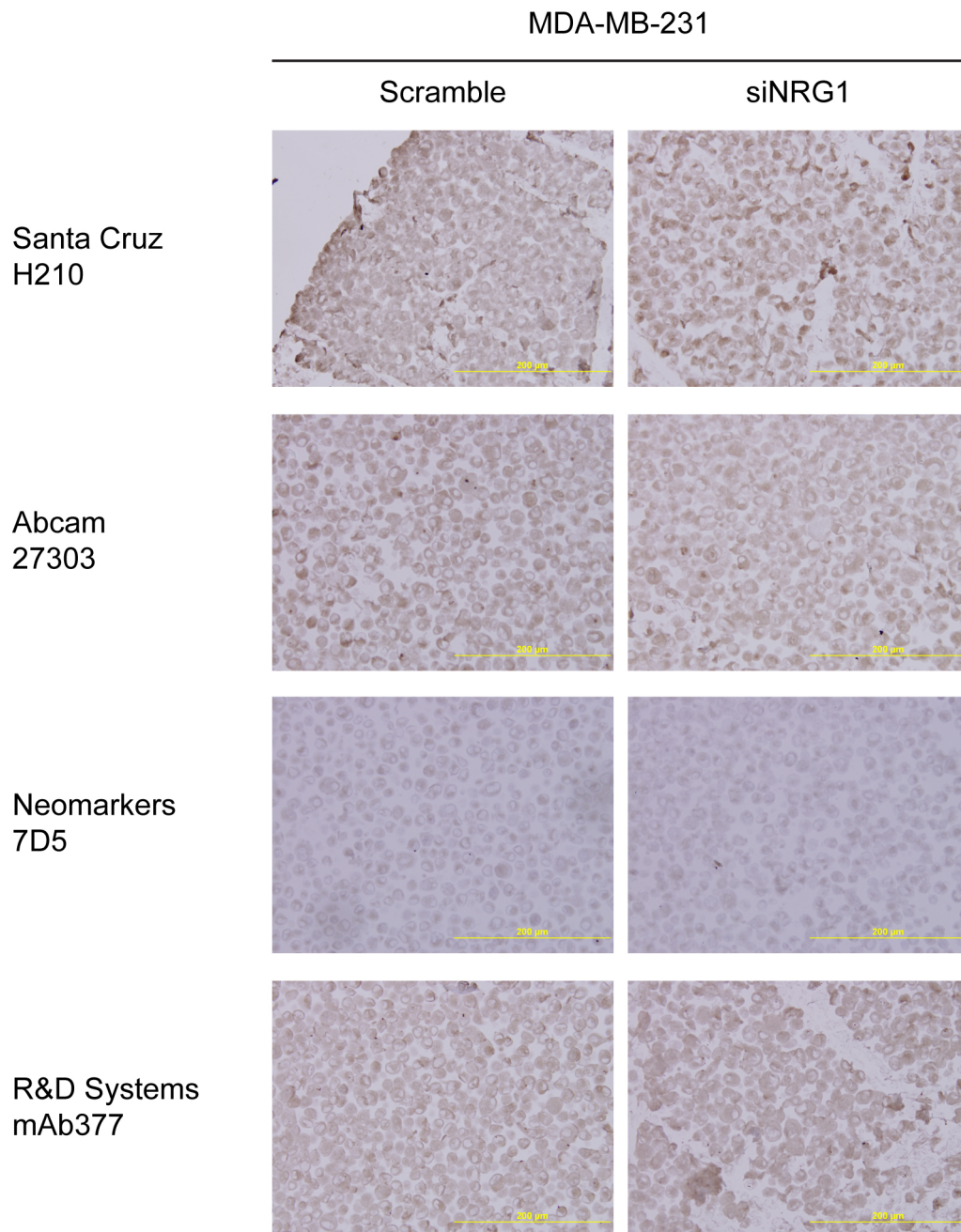


Figure 4-17 Validation of NRG1 antibody in immunohistochemistry staining

MDA-MB-231 cells were treated with 25 nM NRG1 siRNA pool or scramble siRNA for two days, then trypsinised and fixed with paraformaldehyde at 4°C overnight. Fixed cells were pelleted, processed by tissue processor and embedded in paraffin. Cell pellet sections were stained with indicated NRG1 antibody by immunohistochemistry technique.

NRG1 knockdown showed a role of this protein in the growth of SK-BR-3 and SK250LR cell lines; however, the effects of lapatinib and NRG1 knockdown were not additive. No conclusive results were found concerning the role of NRG knockdown in BT250LR and BT-474 until further experiments are performed.

Pertuzumab, a HER2 dimerisation-blocking antibody, was reported to inhibit ligand-dependent HER signalling in several cell lines, including breast, colon, lung, ovarian and prostate cancers (Adams et al., 2006). Since NRG1-stimulated HER signalling is important to the resistance to lapatinib, pertuzumab was used in combination with lapatinib to investigate whether this combination would produce a synergistic or additive therapeutic effect.

5. Novel combination of lapatinib and pertuzumab in HER2-overexpressing breast cancer

5.1 Introduction

In Chapter 4, NRG1 was found to stimulate HER3 and HER4 signalling despite the presence of lapatinib, which might be an important mechanism for HER2-overexpressing cell lines to escape the inhibitory effect of lapatinib as well as to acquire lapatinib resistance. In this chapter, experiments showed that the combination of lapatinib and pertuzumab successfully inhibited NRG1-stimulated HER3 and HER4 signalling pathway.

As discussed in Section 1.4.2.2, pertuzumab is a promising new candidate for HER2-targeted therapy as a combination with trastuzumab and chemotherapy. This drug is capable of blocking the NRG1 stimulated HER2/HER3 and HER2/HER4 signalling pathways, which contributes to lapatinib resistance. Therefore, it is useful to investigate the effect of combining lapatinib and pertuzumab on HER2-overexpressing breast cancer. First, the effects of the drug combination or either drug alone on growth inhibition, apoptosis and cell cycle were compared *in vitro*. The efficacy of the drug combination was then confirmed *in vivo* in a mouse xenograft model. Finally, the effect of the drug combination on the lapatinib-resistant cell lines was assessed.

5.2 Results

5.2.1 Effect of lapatinib and pertuzumab combination on HER signalling

As shown in Figure 4-6, NRG1 stimulated the activation of HER3 and HER4 receptors and the downstream Akt and MAPK pathways even in the presence of lapatinib inhibition. Pertuzumab was combined with lapatinib to understand if this combination could reverse the effect of NRG1 stimulation. Pertuzumab was used at the concentration of 20 µg/ml, a common dose used in other studies (DeFazio-Eli et al., 2011; Garrett et al., 2011; Jackson et al., 2004).

As discussed in Section 4.2.3, NRG1 stimulation alone reduced phosphorylation of EGFR, HER2 and HER4. Pertuzumab alone did not inhibit any HER receptors or the downstream targets in both SK-BR-3 and BT-474 cell lines (Figure 5-1); however, when the cells were stimulated with NRG1, pertuzumab inhibited the phosphorylation of all the HER receptors. These results indicated that pertuzumab could only inhibit ligand-induced HER signalling. Interestingly, the downstream phospho-Akt and phospho-MAPK were not inhibited. This was probably because pertuzumab could not inhibit NRG1-stimulated EGFR/HER3 and HER3/HER4 dimerisation, so these receptor dimers stimulated downstream signalling. The residual phosphorylation in the HER receptors could be explained by the fact that the HER receptors dimerise with each other but not only HER2.

In both cell lines, the combination of lapatinib and pertuzumab (Figure 5-1, blue boxes) inhibited phospho-HER3, phospho-HER4, phospho-Akt and phospho-MAPK more than lapatinib alone (Figure 5-1, red boxes). In fact, both phospho-HER3 and phospho-HER4 were inhibited to levels comparable to no NRG1 stimulation. In SK-BR-3 cells, phospho-Akt was at basal level and phospho-MAPK was higher than basal levels when

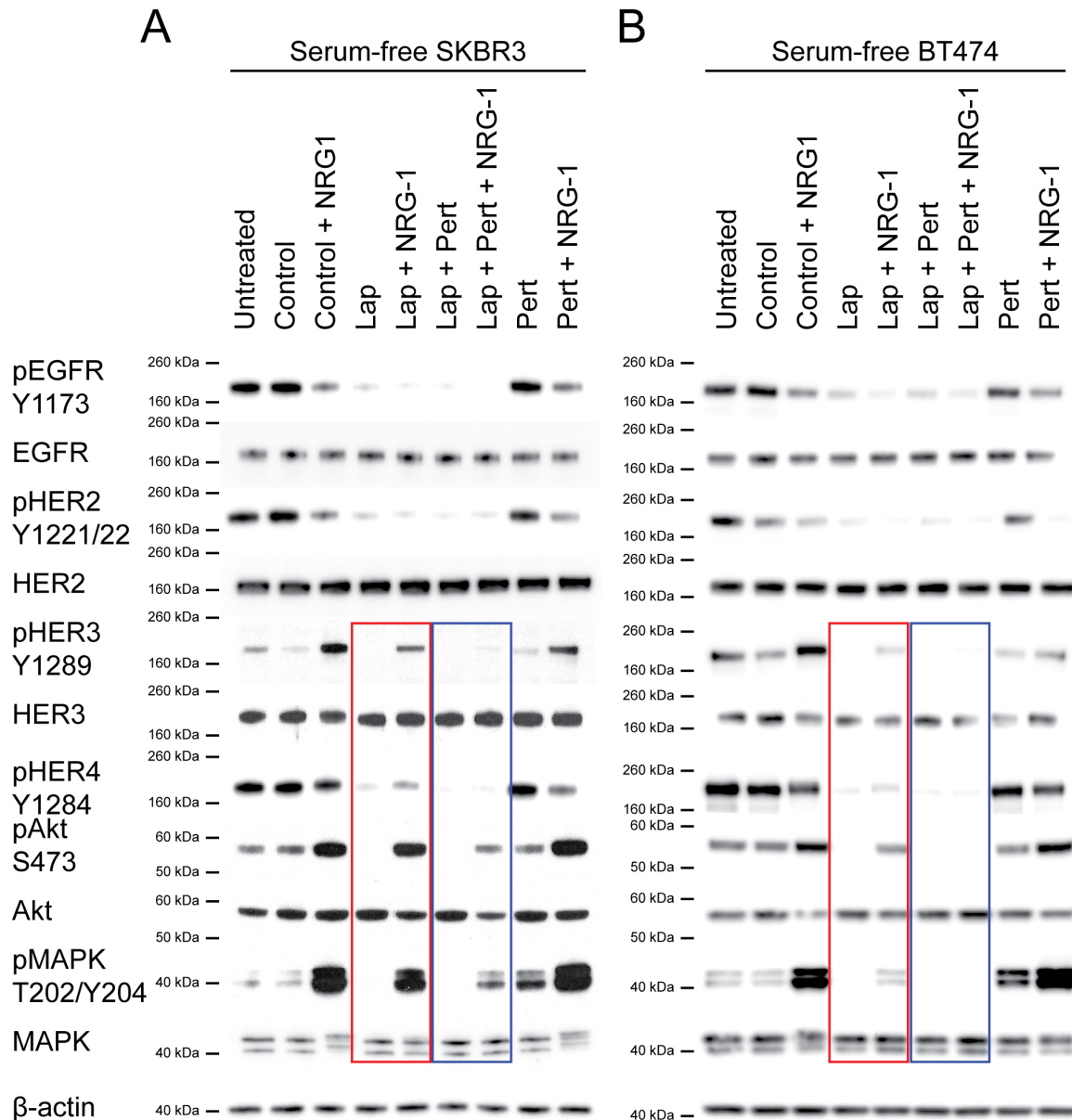


Figure 5-1 Effect of lapatinib/pertuzumab combination on NRG1 stimulated HER signalling

(A) SK-BR-3 and (B) BT-474 cells were grown in serum-free culture media for 24 hours, then treated with 100 nM lapatinib and/or 20 μ g/ml pertuzumab for one hour. DMSO and/or 20 μ g/ml non-specific IgG served as the treatment controls. In the last ten minutes of treatments, 10 ng/ml NRG1 was added as indicated. Complete cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane and immunoblotted for Tyr1173-phosphorylated EGFR (pEGFR-Y1173), total EGFR (EGFR), Tyr1221/22-phosphorylated HER2 (pHER2-Y1221/22), total HER2 (HER2), Tyr1289-phosphorylated HER3 (pHER3-Y1289), total HER3 (HER3), Tyr-1284-phosphorylated HER4 (pHER4-Y1284), Ser473-phosphorylated Akt (pAkt-S473), total Akt (Akt), Thr202/Tyr204-phosphorylated p44/42 MAPK (pMAPK-T202/Y204) and total p44/42 MAPK (MAPK). β -actin blot served as loading control in all experiments. Reproducible representative results of at least three independent experiments are shown.

NRG1-stimulated cells were treated with the combination (Figure 5-1A, blue box); however, in BT-474 cells, both downstream effectors were completely inhibited (Figure 5-1B, blue box).

5.2.2 Effect of lapatinib and pertuzumab combination *in vitro*

The combination of lapatinib and pertuzumab provided better inhibition on NRG1-stimulated HER signalling pathway than either drug alone. This additive effect on inhibition suggested that this combination might have improved anti-tumour activity. In this section, the effect of the drug combination on tumour cell growth and survival will be investigated using the following four methods: total cell counting, clonogenic assay, apoptotic assay and cell cycle analysis.

5.2.2.1 Cell growth and cell viability

In the total cell counting experiments, the cells were treated with an increasing dose of lapatinib with 20 µg/ml pertuzumab or non-specific human IgG control. The combination of lapatinib and pertuzumab (blue line) always reduced cell number more than lapatinib and normal IgG at any lapatinib concentration (red line) or pertuzumab alone (green dashed line) in both SK-BR-3 and BT-474 cells (Figure 5-2). The difference between lapatinib and pertuzumab combination and lapatinib alone was significant in both SK-BR-3 ($p = 0.0007$) (Figure 5-2A) and BT-474 ($p < 0.0001$) (Figure 5-2B) cells.

Total cell counting experiments were unable to determine whether the drug effect was cytotoxic or cytostatic. Therefore, clonogenic assay was used to study cell viability after lapatinib and pertuzumab treatment. SK-BR-3 and BT-474 cells were treated with either drug or their combination for three days, trypsinised and then reseeded to allow them to grow into colonies. The drugs were removed during colony forming in order to determine whether the cells were committed to cell death.

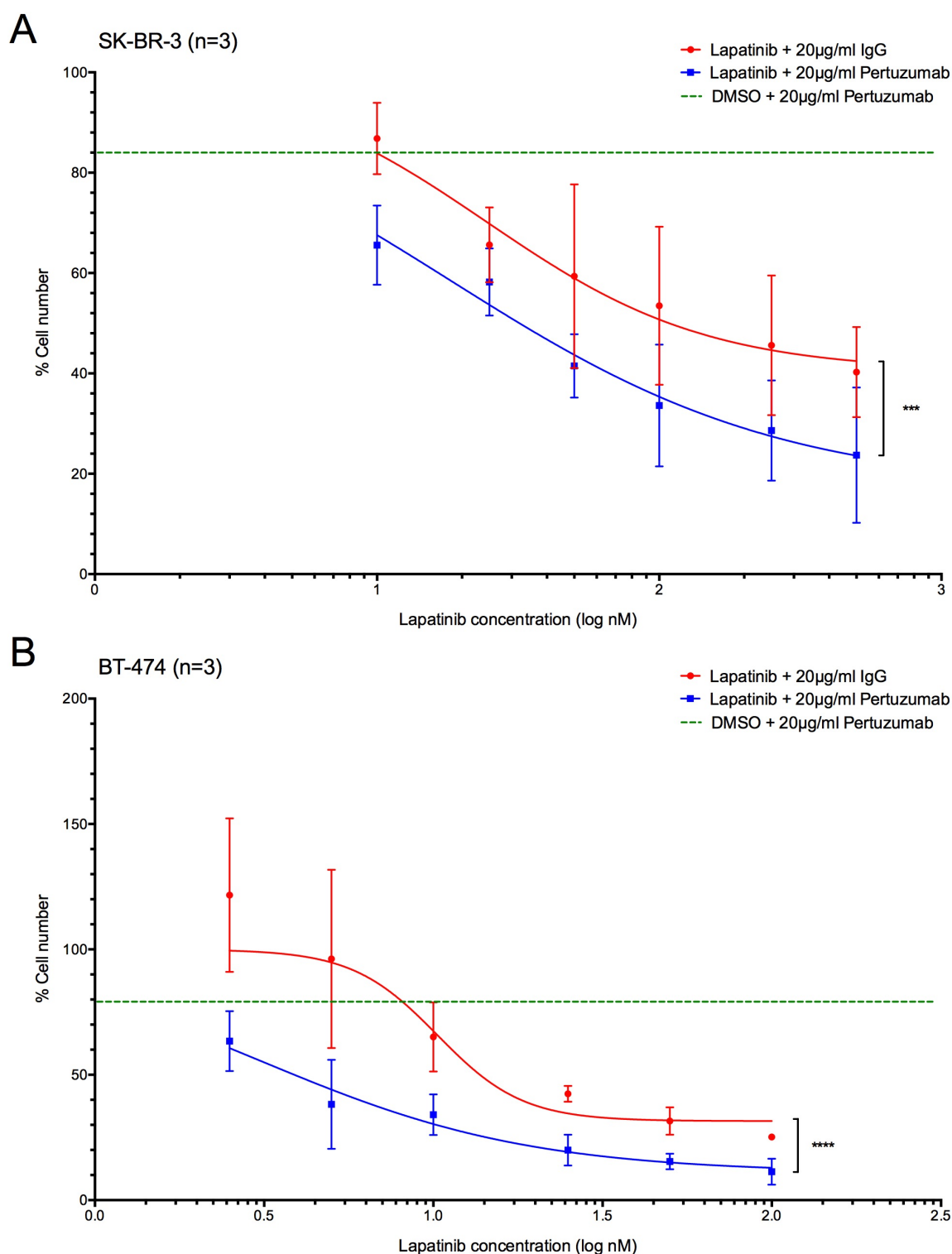


Figure 5-2 Effect of lapatinib and pertuzumab combination on the growth of HER2-overexpressing cell lines using total cell counting

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated dose of lapatinib or DMSO control and 20 µg/ml pertuzumab or non-specific human IgG control. Cells were trypsinised and counted. The percentage cell number was normalised with DMSO and IgG control. Three independent experiments were performed with three technical replicates. (Error = SD; Extra sum-of-squares F test, *** $p < 0.001$, **** $p < 0.0001$)

In SK-BR-3 cells, lapatinib alone significantly ($p < 0.0001$) reduced colony number compared to DMSO/IgG control, while pertuzumab alone had no significant effect (Figure 5-3A). The combination of lapatinib and pertuzumab also significantly ($p < 0.0001$) reduced colony number of SK-BR-3 when compared to DMSO/IgG control; however, there was no significant difference between lapatinib alone and the combination.

In BT-474 cells, there were no significant differences between any treatment except lapatinib alone had a higher colony number than DMSO/IgG control ($p = 0.0107$) (Figure 5-3B). Clonogenic assay did not pick up the differences among the treatments. The issues of performing clonogenic assay in BT-474-related cell lines will be discussed in Section 7.5.3.2.

The representative experiments were shown in Figure 5-4. Clonogenic assay did not show any additional effect in the combination of lapatinib and pertuzumab compared to either drug alone. Therefore, apoptotic assay and cell cycle analysis were performed to study the cytotoxic and cytostatic effect of the drug combination respectively.

5.2.2.2 Apoptosis

Apoptosis caused by drug treatment was analysed using Annexin V (AV)-Alexa Fluor 647 and propidium iodide (PI) staining. AV is a protein that binds to phosphatidylserine, which is exposed at the cell surface during apoptosis for the recognition by macrophages (Koopman et al., 1994). PI is a fluorescent dye that binds to DNA and RNA. As PI is impermeable to cell membrane, it is used to assess membrane integrity and identify dead cells (Moore et al., 1998). The cells were treated with the drugs for two days (Vermes et al., 1995) or camptothecin, a DNA topoisomerase I inhibitor that triggers apoptosis (Liu et al., 2000), for four hours as the positive control.

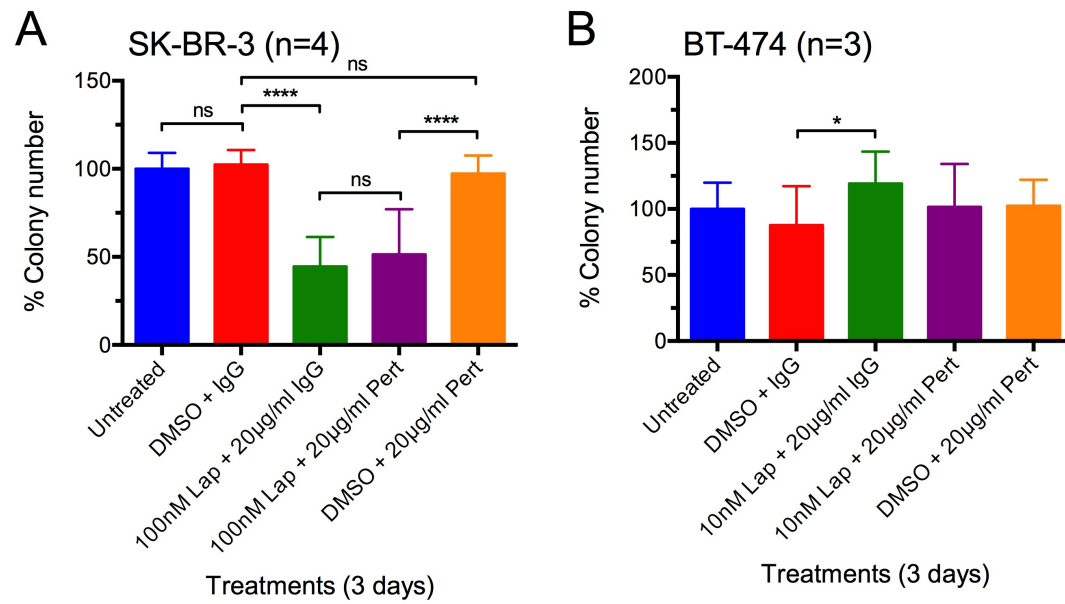


Figure 5-3 Effect of lapatinib and pertuzumab combination on the clonogenicity of HER2-overexpressing cell lines

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated agents for three days. Cells were then trypsinised, reseeded and allowed to grow into colonies. The number of colony was normalised to the untreated control. Three independent experiments were performed with three technical replicates. (Error = SD; Matched two-way ANOVA with Bonferroni correction, * $p < 0.05$, **** $p < 0.0001$)

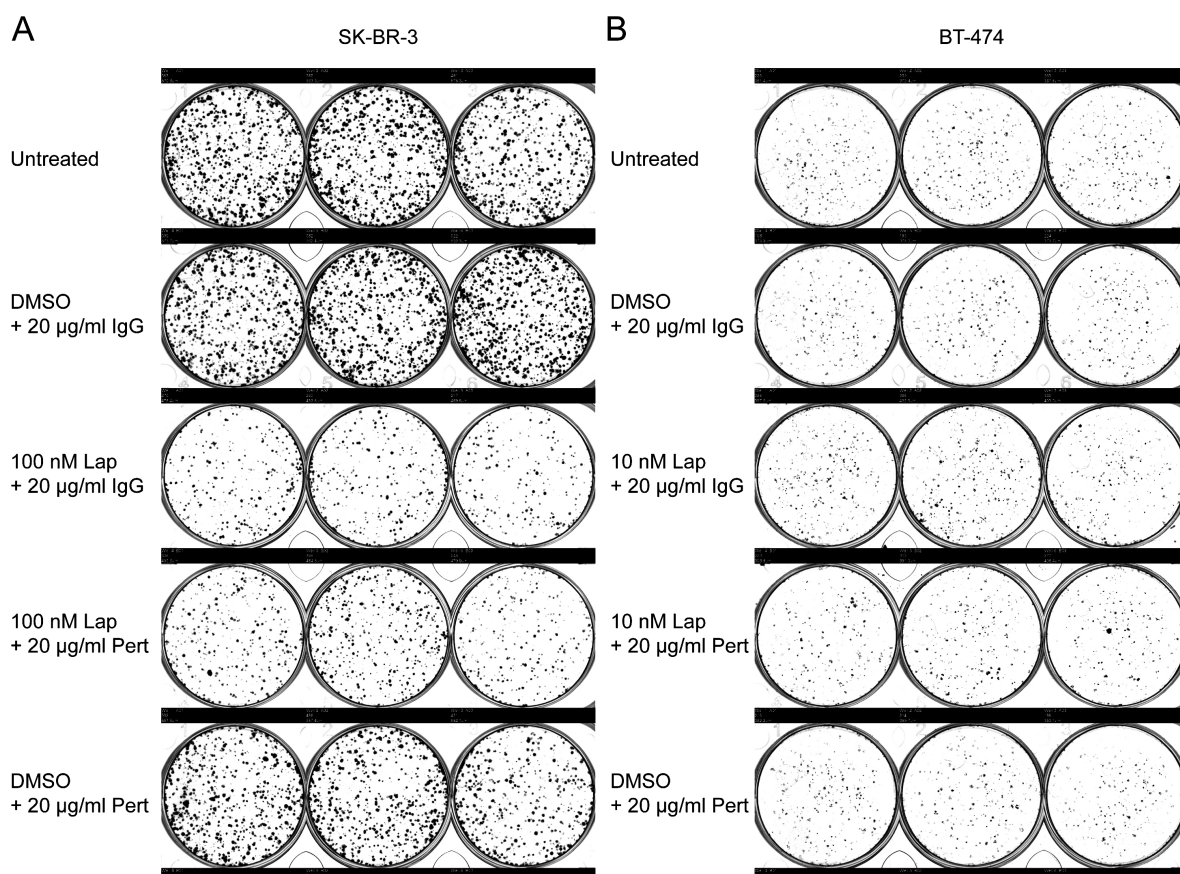


Figure 5-4 Representative experiment for the effect of lapatinib and pertuzumab combination on clonogenicity of SK-BR-3 and BT-474

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated agents for three days. Cells were then trypsinised, reseeded and allowed to grow for (A) 14 days or (B) 17 days. Plates were then washed with PBS and stained with methylene blue.

The dot plots of AV and PI staining is divided into four quadrants. The bottom-left quadrant is low AV and low PI, representing the healthy cells. The bottom-right quadrant is high AV but low PI, representing the apoptotic population. The top-right quadrant is high AV and high PI, representing the dead cells.

In SK-BR-3 cells, pertuzumab alone did not cause any changes in AV and PI staining compared to DMSO/IgG control (Figure 5-5B). Lapatinib alone caused around 2% cells to shift to the apoptotic region (Figure 5-5C). The combination of lapatinib and pertuzumab caused around 4% cells to undergo apoptosis (Figure 5-5D). Among all treatment groups, only the combination treatment achieved significant increase in the apoptotic population compared to DMSO/IgG control ($p = 0.0487$) (Figure 5-7A). Since the differences in the percentage of apoptotic cells were so small, clonogenic assay might not be sensitive enough to detect these differences. Moreover, the small increase in apoptosis in SK-BR-3 might not have much influence in the biology of cancer.

In BT-474 cells, lapatinib (Figure 5-6C), pertuzumab (Figure 5-6B) and their combination (Figure 5-6D) increased the apoptotic and dead populations compared to DMSO/IgG control. However, none of these increases were significant compared to the control (Figure 5-7B), probably because of the small number of repeats ($n=3$). The percentage of apoptotic cells after the combination treatment was, however, significantly higher than lapatinib alone ($p = 0.0139$) or pertuzumab alone ($p = 0.0388$). The proportion of dead cells caused by the combination treatment was also significantly greater than that caused by pertuzumab alone ($p = 0.0221$).

The above data showed that the combination of lapatinib and pertuzumab might have an additive effect in inducing apoptosis, but the combined effect of the two drugs will need synergism studies such as isobologram.

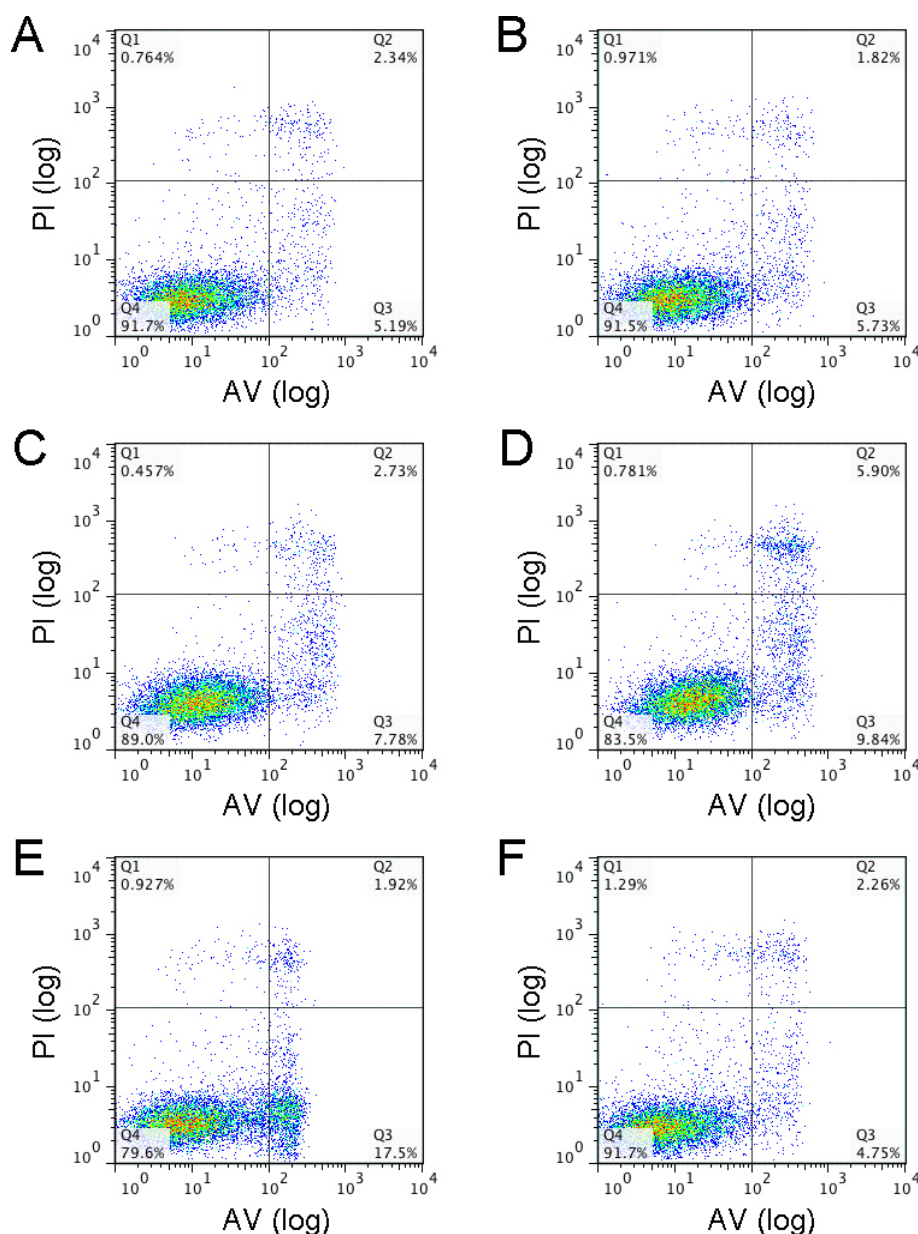


Figure 5-5 Apoptosis analysis of SK-BR-3 cell line after lapatinib and/or pertuzumab treatment

SK-BR-3 cells were treated with (A) DMSO and 20 µg/ml non-specific human IgG, (B) DMSO and 20 µg/ml pertuzumab, (C) 100 nM lapatinib and 20 µg/ml non-specific human IgG, (D) 100 nM lapatinib and 20 µg/ml pertuzumab, (E) 6 µM camptothecin or (F) no reagents for two days. Cells were trypsinised, collected and stained with Annexin V-Alexa Fluor 647 and propidium iodide. Stained cells were analysed using a CyAn FACS analyser. Plots are representative of three independent experiments. (PI: Propidium Iodide, AV: Annexin V)

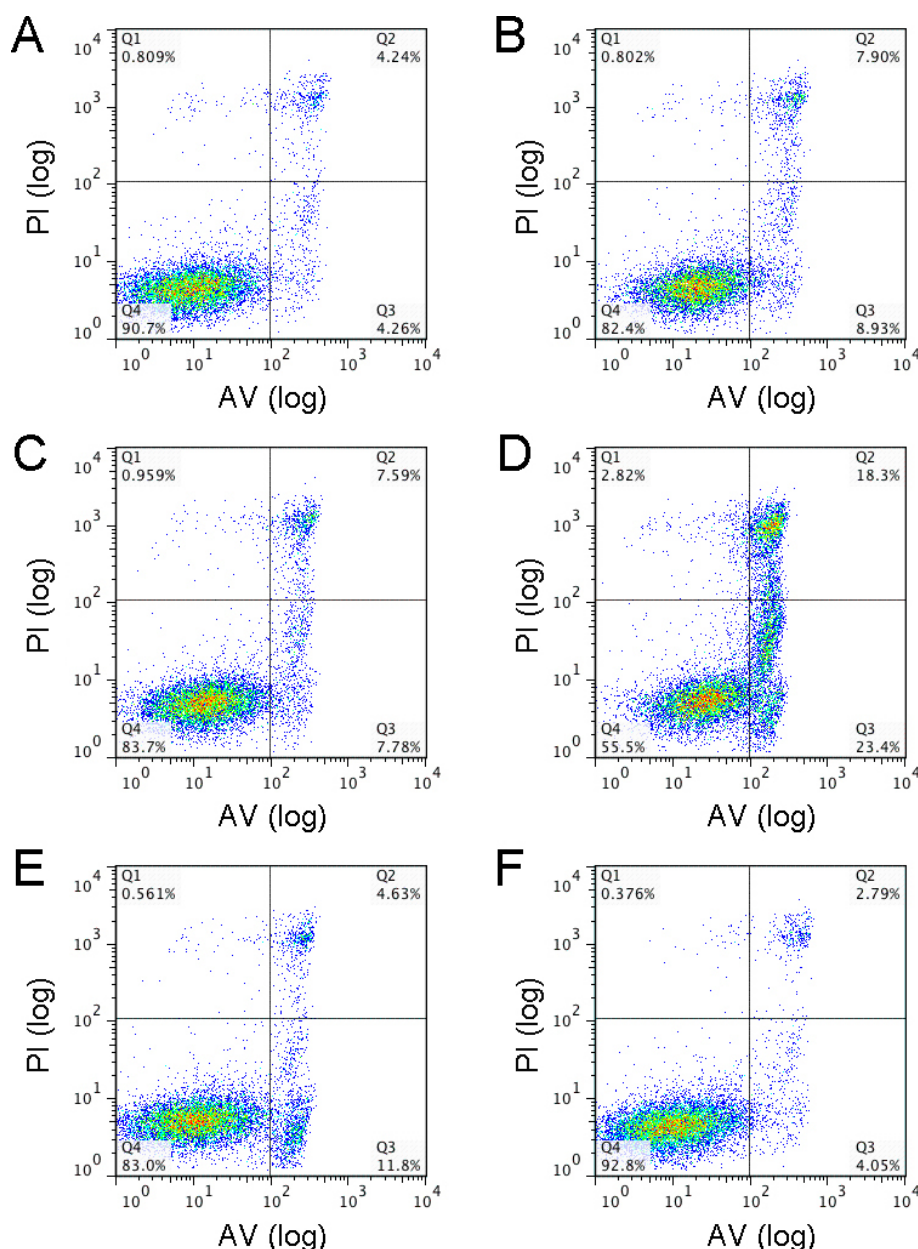


Figure 5-6 Apoptosis analysis of BT-474 cell line after lapatinib and/or pertuzumab treatment

BT-474 cells were treated with (A) DMSO and 20 μ g/ml non-specific human IgG, (B) DMSO and 20 μ g/ml pertuzumab, (C) 10 nM lapatinib and 20 μ g/ml non-specific human IgG, (D) 10 nM lapatinib and 20 μ g/ml pertuzumab, (E) 6 μ M camptothecin or (F) no reagents for two days. Cells were trypsinised, collected and stained with Annexin V-Alexa Fluor 647 and propidium iodide. Stained cells were analysed using a CyAn FACS analyser. Plots are representative of three independent experiments. (PI: Propidium Iodide, AV: Annexin V)

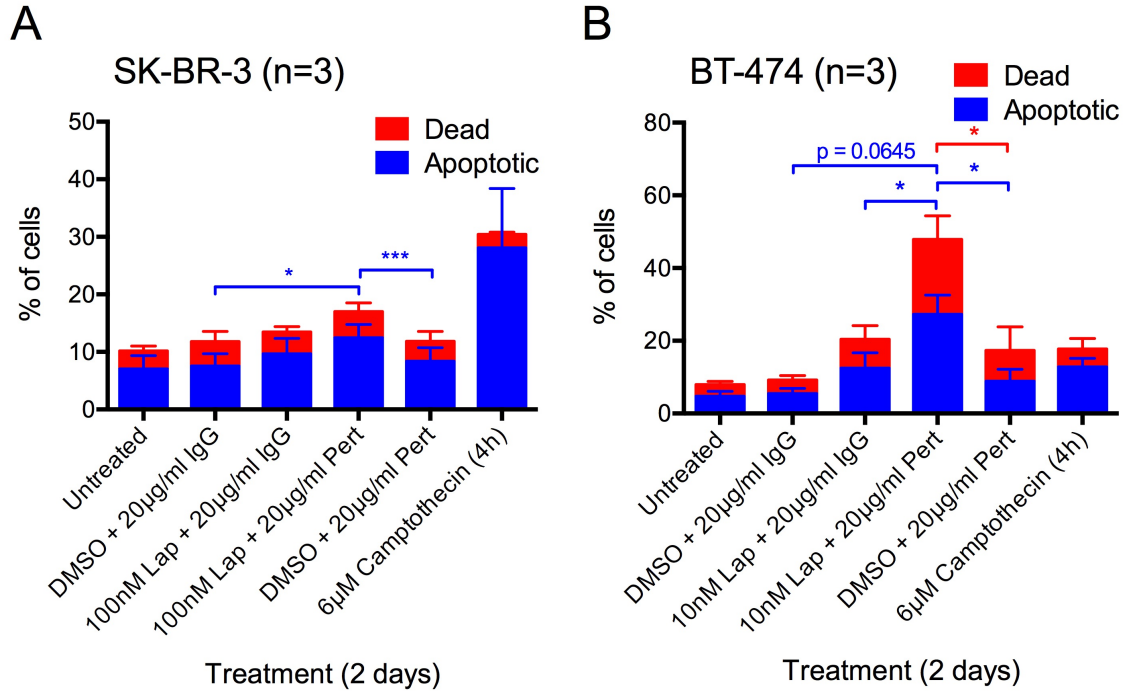


Figure 5-7 Effect of the combination of lapatinib and pertuzumab on apoptosis of HER2-overexpressing cell lines

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated agents for two days. Cells were then trypsinised, collected and stained with Annexin V-Alexa Fluor 647 and propidium iodide. Stained cells were analysed using CyAn FACS analyser. Three independent experiments were performed. (Error = SD; Matched one-way ANOVA with Bonferroni correction, * $p < 0.05$, *** $p < 0.001$)

5.2.2.3 Cell cycle

The cell cycle of the cells after drug treatment was analysed by propidium iodide staining after the cell membrane was permeabilised by methanol fixation.

Lapatinib and the combination of lapatinib and pertuzumab caused G1 arrest in SK-BR-3 cells (Figure 5-8C and D), suggesting cell cycle arrest might be one of the mechanisms of growth inhibition caused by lapatinib. However, the combination of lapatinib and pertuzumab did not trap more cells in G1-phase than lapatinib alone; therefore, there was no evidence to show that the combination was better than lapatinib alone. The shift to G1-phase was observed in all three experiments in SK-BR-3 cells; however, the changes were not statistically significant from DMSO/IgG (Figure 5-10A).

In BT-474 cells, lapatinib alone caused G1 arrest (Figure 5-9C) but the combination of the two drugs did not cause any change compared to DMSO/IgG control (Figure 5-9D). These results suggested that the combination of drug might work mainly by causing apoptosis but not growth inhibition. The statistical data from three experiments in BT-474 cells were not significant (Figure 5-10B), except the combination had lower percentage of cells in S-phase than the DMSO/IgG control ($p = 0.0472$).

The cell cycle analysis showed that the combination of lapatinib and pertuzumab did not cause more cell cycle arrest than either drug alone, hence it did not explain the decrease in cell number shown in Section 5.2.2.1.

5.2.3 Effect of lapatinib and pertuzumab combination *in vivo*

The previous section showed the efficacy of the lapatinib and pertuzumab combination *in vitro*. This section aims to confirm the anti-tumour activity of the combination *in vivo* using a BT-474 mouse xenograft model. SK-BR-3 cell line was not used for establishing xenograft tumour because it is non-tumourigenic in athymic mice (Tang et al., 2005).

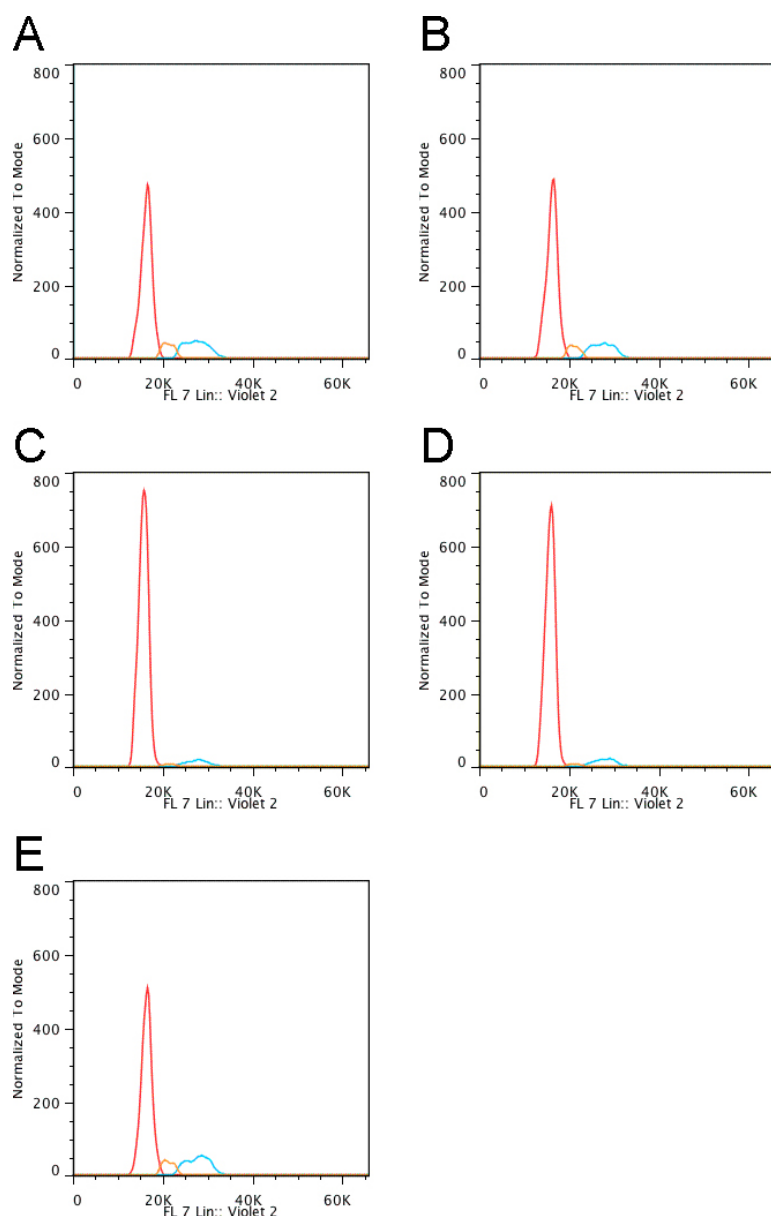


Figure 5-8 Cell cycle analysis of SK-BR-3 cell line after lapatinib and/or pertuzumab treatment

SK-BR-3 cells were treated with (A) DMSO and 20 $\mu\text{g/ml}$ non-specific human IgG, (B) DMSO and 20 $\mu\text{g/ml}$ pertuzumab, (C) 100 nM lapatinib and 20 $\mu\text{g/ml}$ non-specific human IgG, (D) 100 nM lapatinib and 20 $\mu\text{g/ml}$ pertuzumab or (E) no reagents for 24 hours. Cells were trypsinised, collected, fixed, treated with RNase A and stained with propidium iodide. Stained cells were analysed using a CyAn FACS analyser. Plots are representative of three independent experiments. (Red: G1-phase; Orange: S-phase; Blue: G2/M-phase)

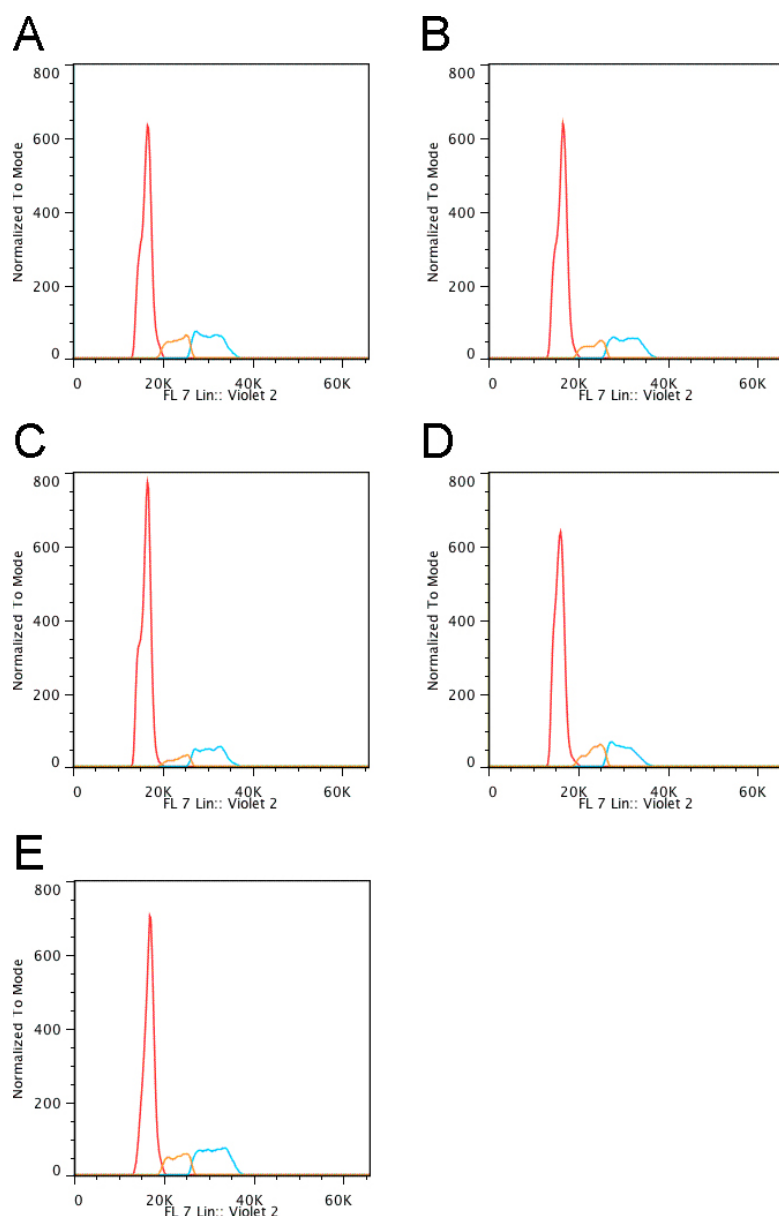


Figure 5-9 Cell cycle analysis of BT-474 cell line after lapatinib and/or pertuzumab treatment

BT-474 cells were treated with (A) DMSO and 20 $\mu\text{g/ml}$ non-specific human IgG, (B) DMSO and 20 $\mu\text{g/ml}$ pertuzumab, (C) 10 nM lapatinib and 20 $\mu\text{g/ml}$ non-specific human IgG, (D) 10 nM lapatinib and 20 $\mu\text{g/ml}$ pertuzumab or (E) no reagents for 24 hours. Cells were trypsinised, collected, fixed, treated with RNase A and stained with propidium iodide. Stained cells were analysed using a CyAn FACS analyser. Plots are representative of three independent experiments. (Red: G1-phase; Orange: S-phase; Blue: G2/M-phase)

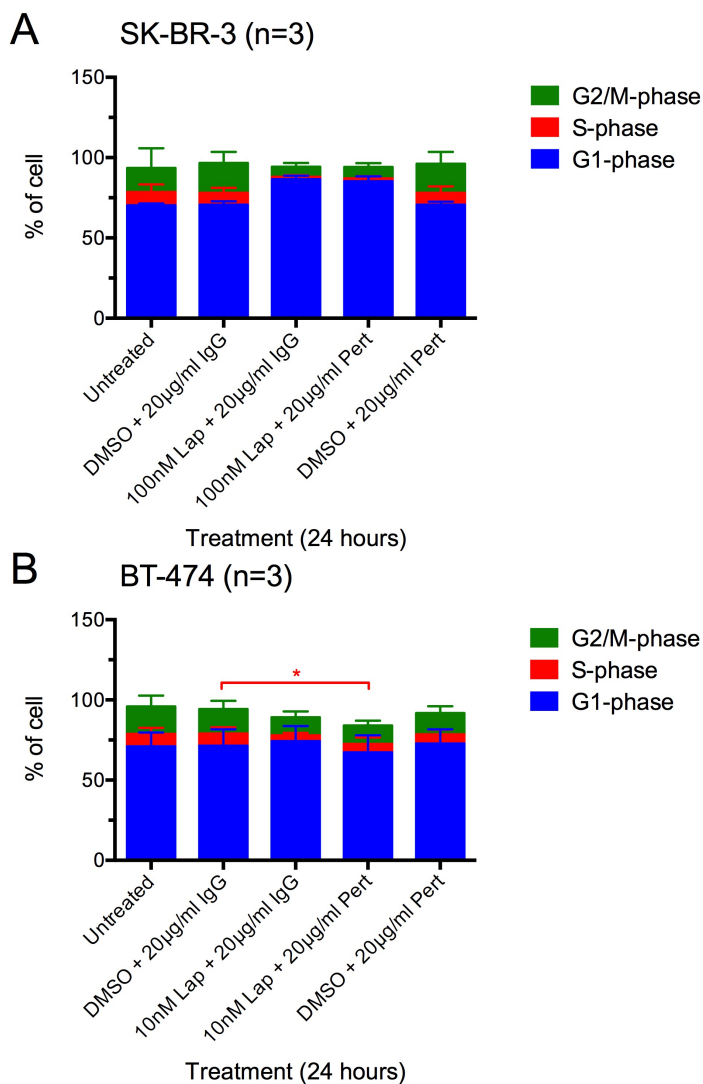


Figure 5-10 Effect of the combination of lapatinib and pertuzumab on cell cycle of HER2-overexpressing cell lines

(A) SK-BR-3 and (B) BT-474 cells were treated with the indicated agents for 24 hours. Cells were then trypsinised, collected and fixed. Fixed cells were treated with RNase A and stained with propidium iodide at 37°C for 40 minutes. Stained cells were analysed using a CyAn FACS analyser. Three independent experiments were performed. (Error = SD; Matched one-way ANOVA with Bonferroni correction, * $p < 0.05$)

BT-474 cells were injected subcutaneously into the mammary fat pad of 28 nude mice. Before the start of drug treatment, three mice were euthanised because animal welfare was compromised. Seven mice had no tumour at the start of treatment. The remaining 18 mice were randomised into four treatment groups: vehicle (TWEEN80) and PBS – four mice; lapatinib and PBS – five mice; vehicle and pertuzumab – four mice; and lapatinib and pertuzumab – five mice. Lapatinib was used at 50 mg/kg, which is half of the standard dose used in previous studies (Gril et al., 2008; Rimawi et al., 2011) to avoid masking the effect of the drug combination. The dose of pertuzumab, 12 mg/kg as a loading dose following by 6 mg/kg, was used in previous research (Arpino et al., 2007; Friess et al., 2005).

Five days after the start of treatment, one mouse in the combination group lost weight by more than 20% and was euthanised because of animal welfare reasons.

5.2.3.1 Tumour growth

The summary of average tumour size in the mice is shown in Figure 5-11. Lapatinib did not reduce the tumour size in this experiment, probably because the dose used was sub-optimal. The results in the pertuzumab-treated group varied, with two tumours regressing but another two progressing (Figure 5-12). The changes in tumour size in the pertuzumab group were therefore not significantly different from the vehicle group. All tumours in the combination treatment group regressed and these changes were significantly different from the vehicle group ($p = 0.0151$) and the lapatinib group ($p = 0.0057$) (Figure 5-13). The above results suggested that the combination of lapatinib and pertuzumab outperformed either monotherapy.

Table 5-1 shows the number of tumour regression across different treatment group. Tumour regression might be correlated with pertuzumab treatment and the combination further triggered tumour regression. However, statistical analysis could not be performed,

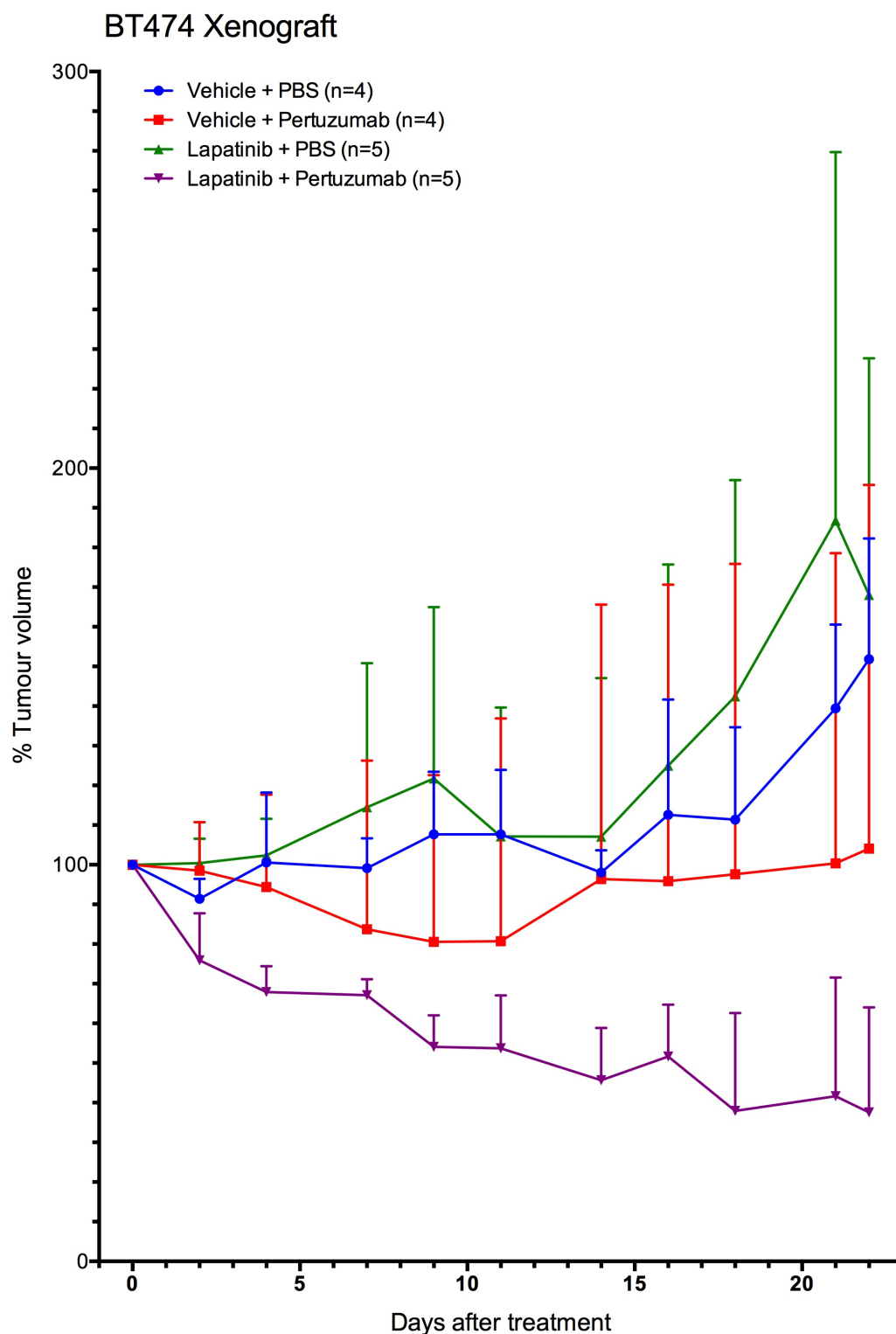


Figure 5-11 Effect of the combination of lapatinib and pertuzumab on tumour growth *in vivo*

Mice bearing BT-474 xenograft were treated with the indicated agents. (Lapatinib: 50 mg/kg five times per week; pertuzumab 12 mg/kg per week for loading, 6 mg/kg per week afterwards) Tumour volume was measured three times a week as indicated at each data point for 22 days. The percentage tumour volume was normalised to the tumour volume on day 0. (Error = SD)

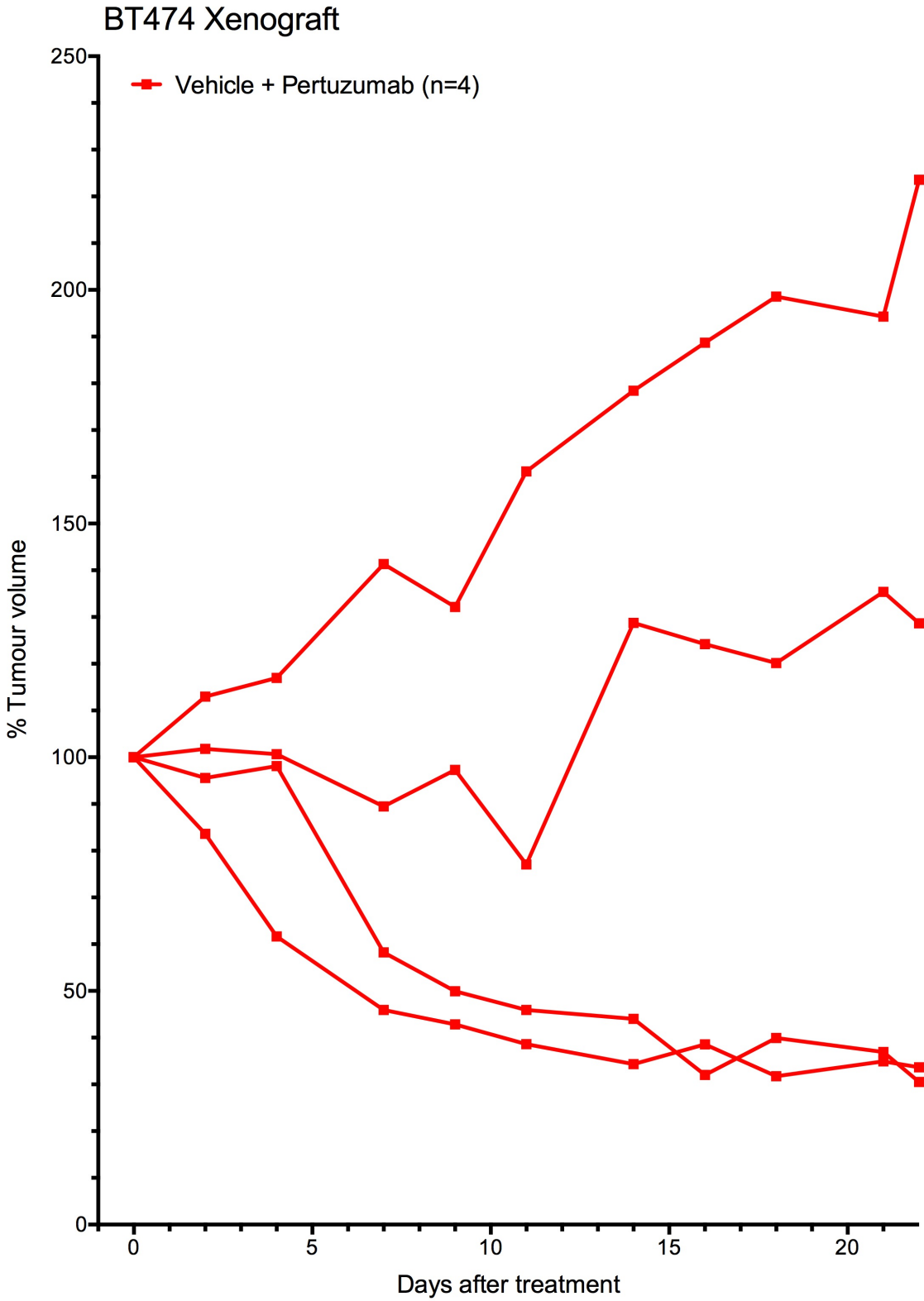


Figure 5-12 Individual plots of all tumours in the pertuzumab treatment group

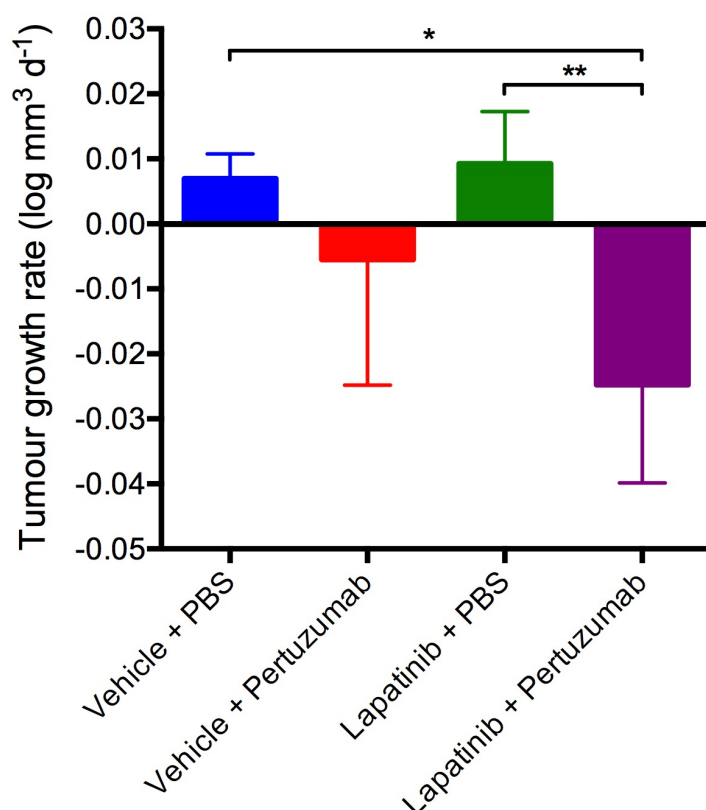


Figure 5-13 Statistical analysis of tumour growth

The log of tumour volume in each mouse was plot against time. Linear regression was fit to the data of each mouse to obtain the tumour growth rate as the slope of the line. The growth rates of the tumours were compared using one-way ANOVA with Bonferroni post-hoc analysis (* $p < 0.05$, ** $p < 0.01$).

Treatment group		Vehicle + PBS	Vehicle + Pertuzumab	Lapatinib + PBS	Lapatinib + Pertuzumab
Tumour regression	Yes	0	2	0	5
	No	4	2	5	0

Table 5-1 Tumour regression status in different treatment groups

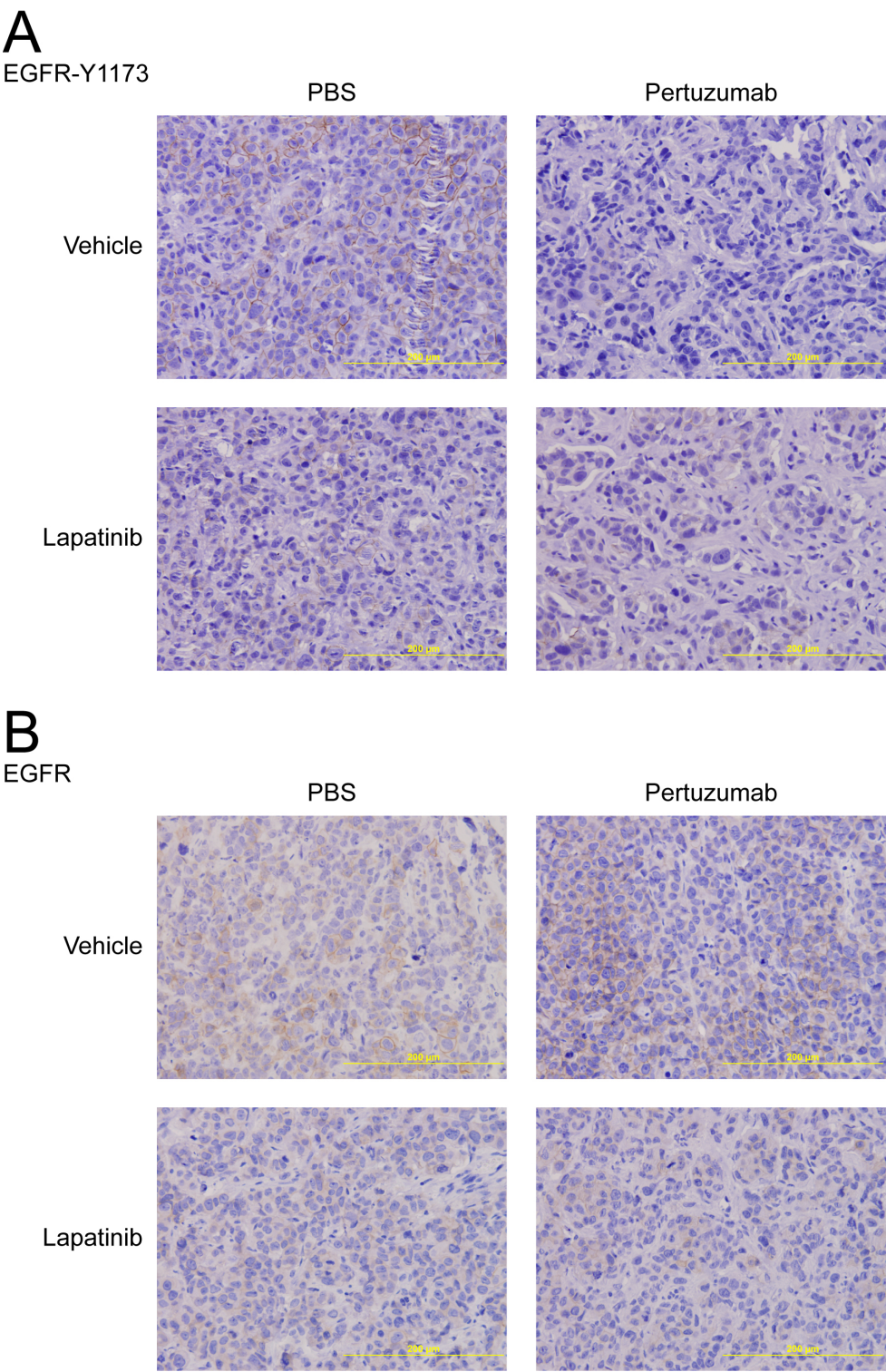
because the number of samples in the xenograft experiment was too small for binary analysis using chi-square test or Fisher's exact test.

5.2.3.2 Study of molecular markers

In order to understand the mechanisms of action of the drugs, the tumours were collected from the euthanised mice, paraffin-embedded and stained for molecular markers using immunohistochemistry staining (IHC). IHC for Ki-67, a proliferation marker, cleaved caspase-3, an apoptosis marker, and all the phospho- and total-HER receptors was performed. Percentage of necrotic area in a tumour was also assessed.

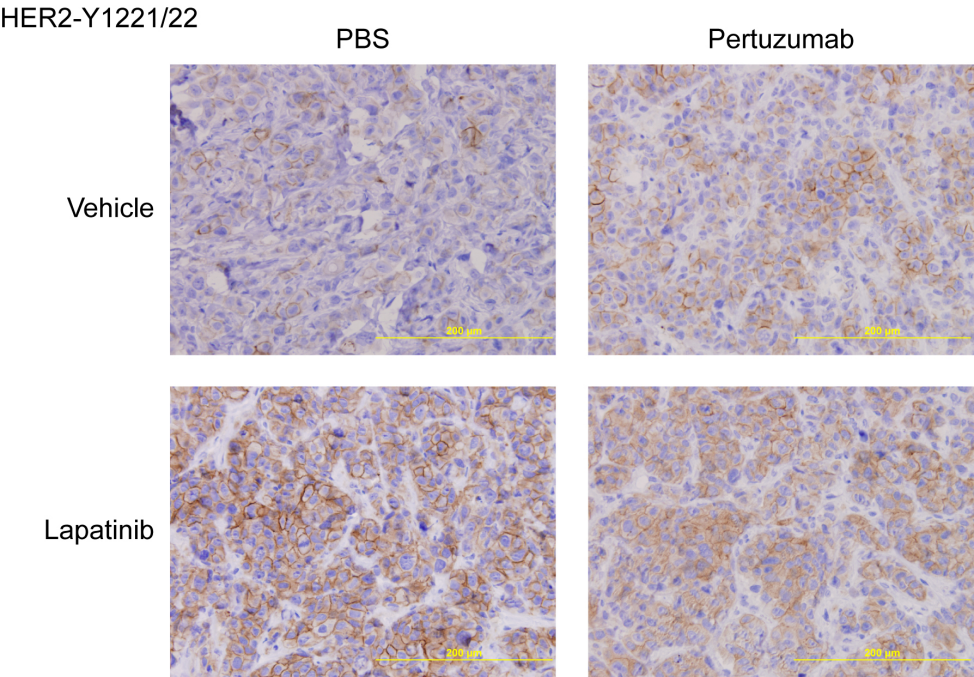
Figure 5-14 shows the representative images of IHC staining of all the above-mentioned proteins in each treatment group. The tumours that received combination treatment expressed significantly higher levels of HER4 than all other treatment groups (all $p < 0.05$) (Figure 5-15). There was no significant difference in phospho-HER4 levels among all treatment groups (not shown); however, since the combination treatment group had much higher HER4 expression, the ratio of pHER4:HER4 in the combination group was significantly lower than that of the vehicle control group ($p = 0.0217$). The above results suggested that the high amount of unphosphorylated HER4 might be an underlying mechanism of action of the combination treatment *in vivo*. There were no significant differences in the other protein markers between groups.

The results of IHC staining were also compared with the regression status of the tumours (Figure 5-16). The regressed tumours had significantly reduced ratio of pEGFR:EGFR ($p = 0.0201$) and pHER4:HER4 ($p = 0.0004$) compared to the tumours without regression, suggesting the inhibition on EGFR and HER4 might be the cause of tumour regression. The regressed tumours also had higher HER4 levels than the non-regressed group ($p = 0.0207$). Interestingly, there was more necrosis in non-regressed tumours than regressed tumours ($p = 0.0456$). Regressed tumours were significantly smaller than those



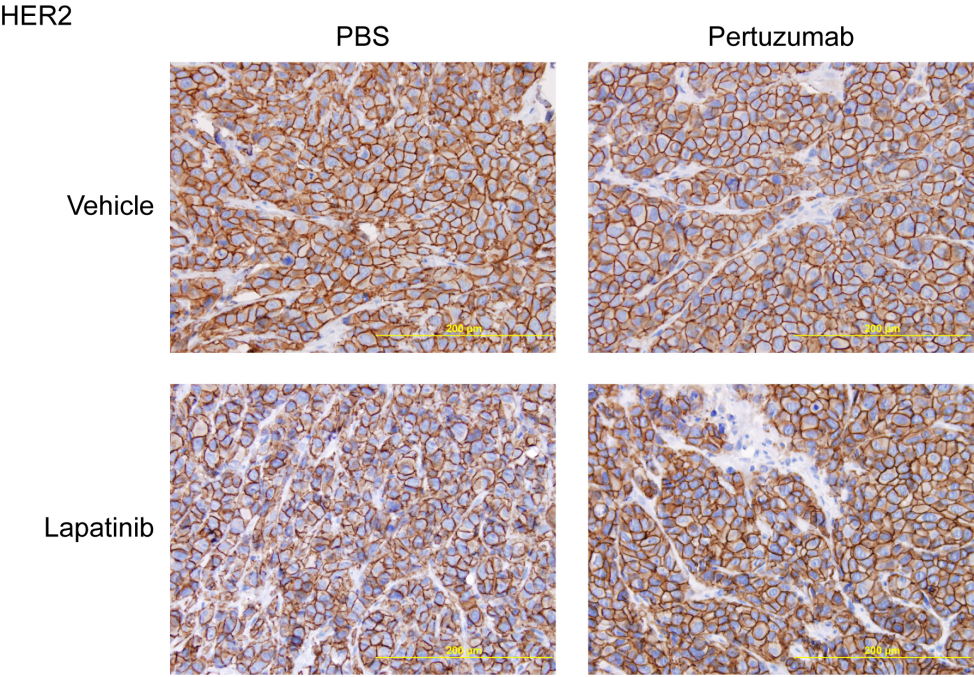
C

HER2-Y1221/22

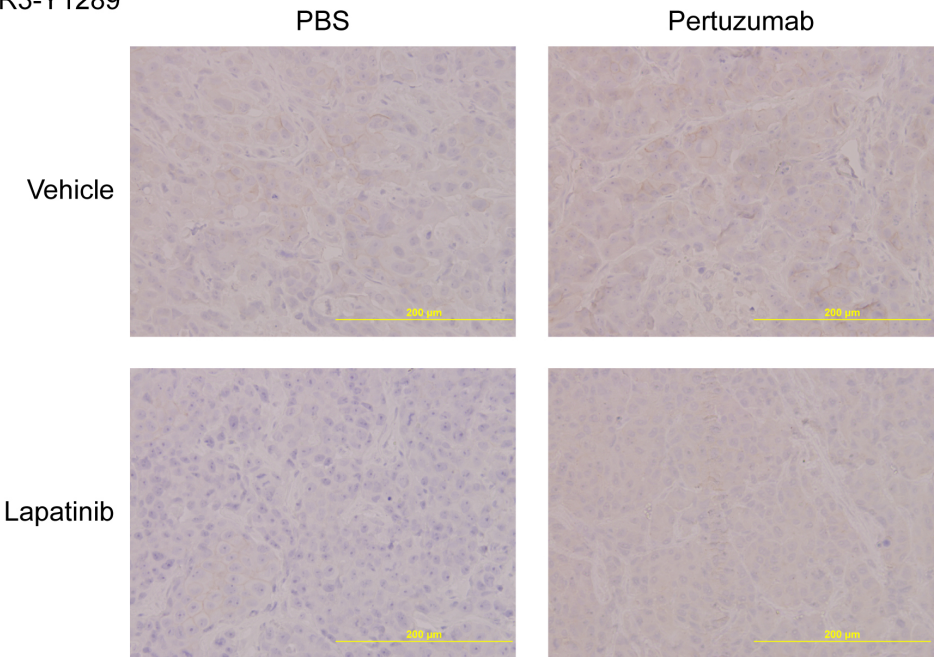


D

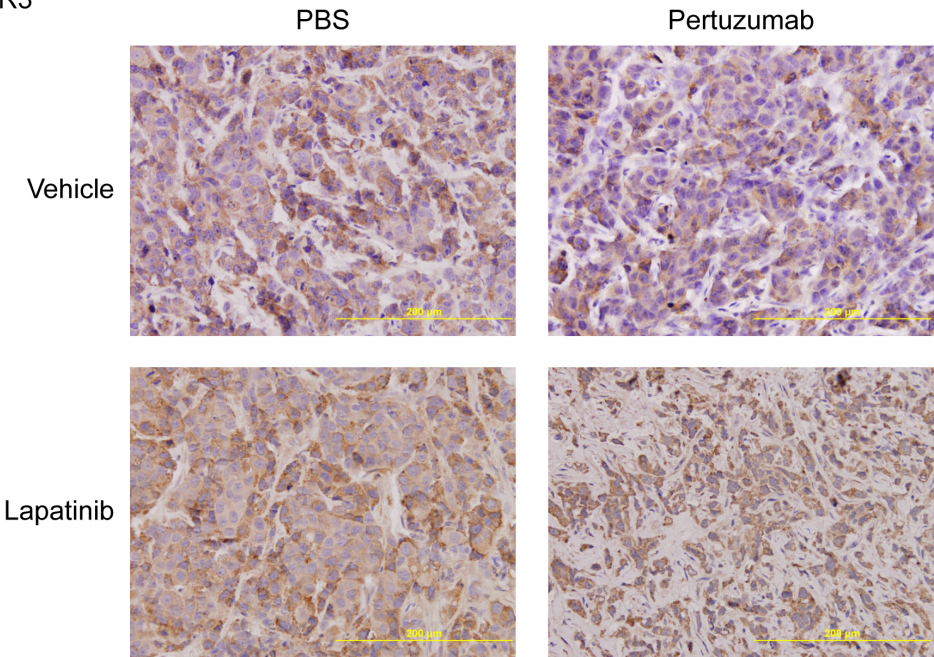
HER2



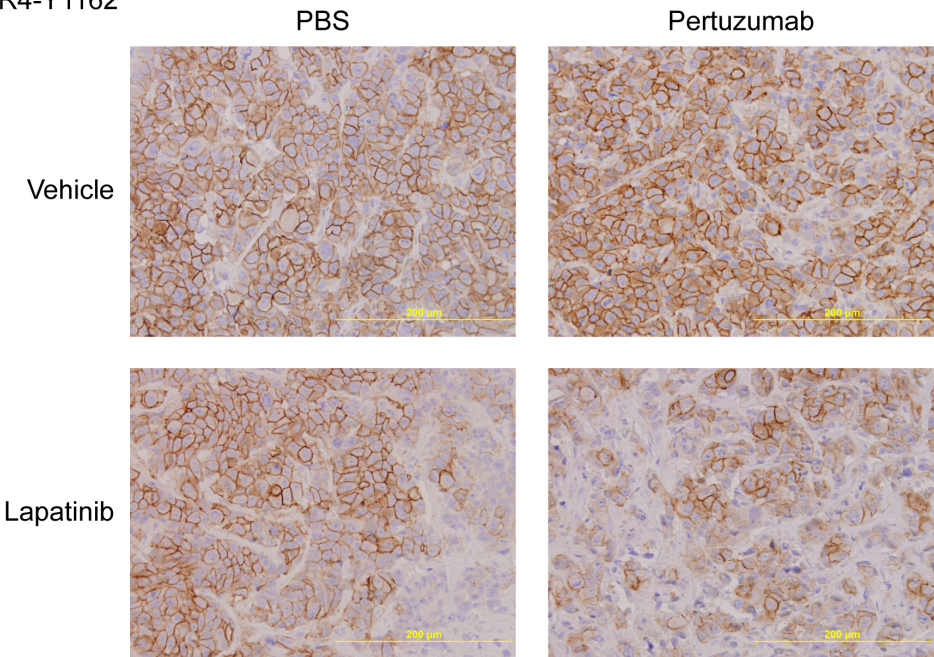
E
HER3-Y1289



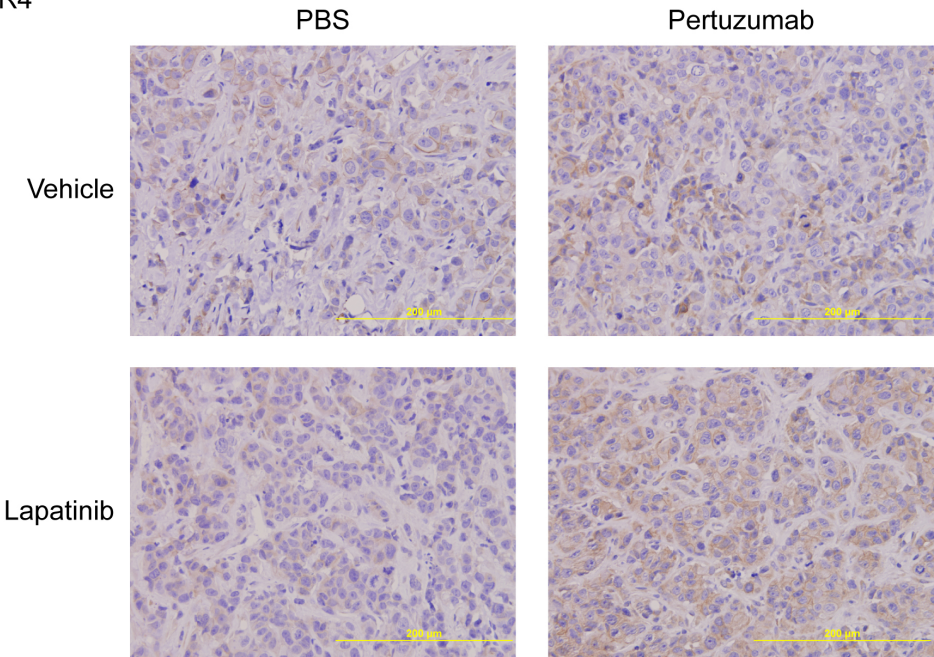
F
HER3



G
HER4-Y1162



H
HER4



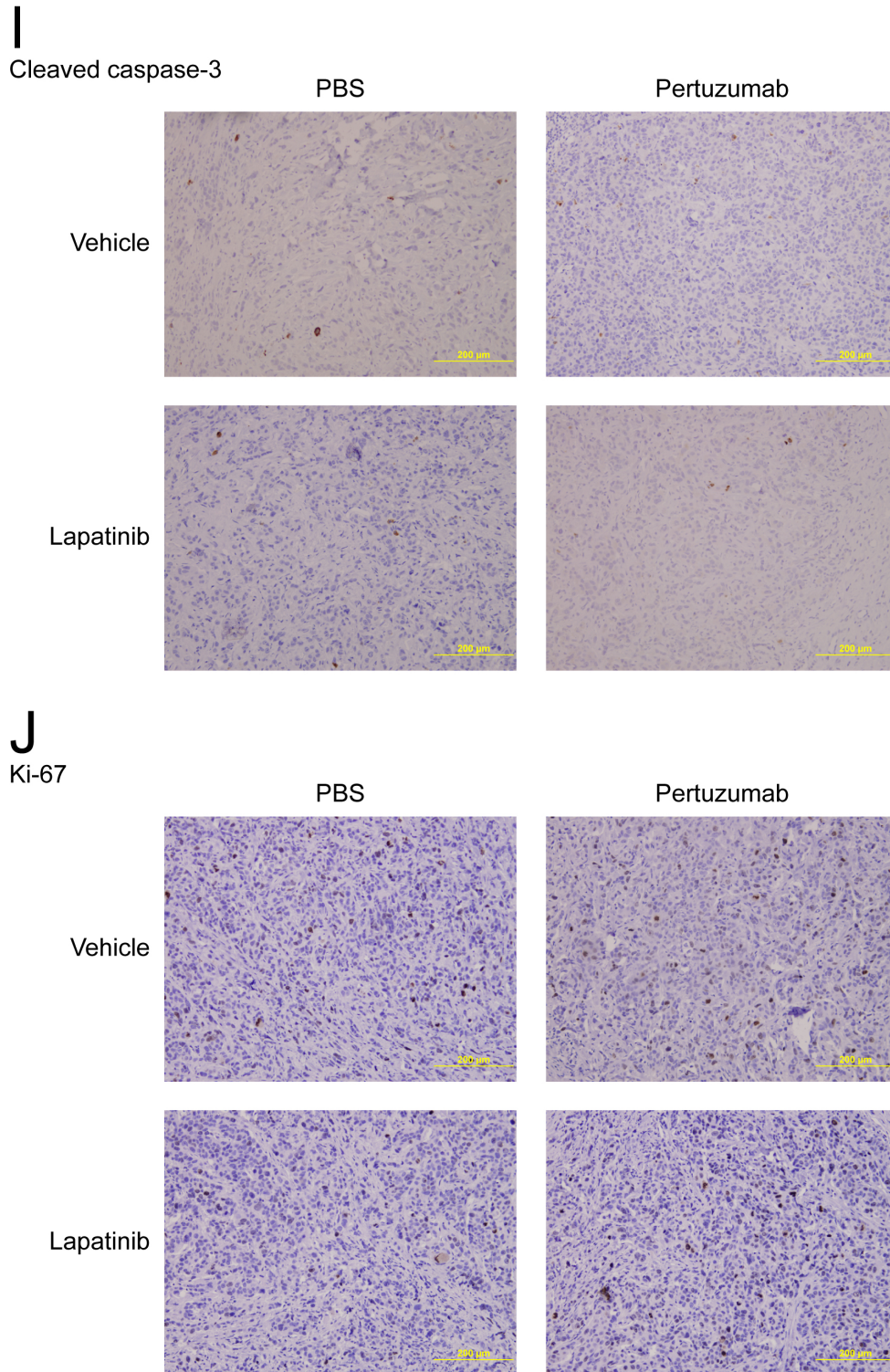
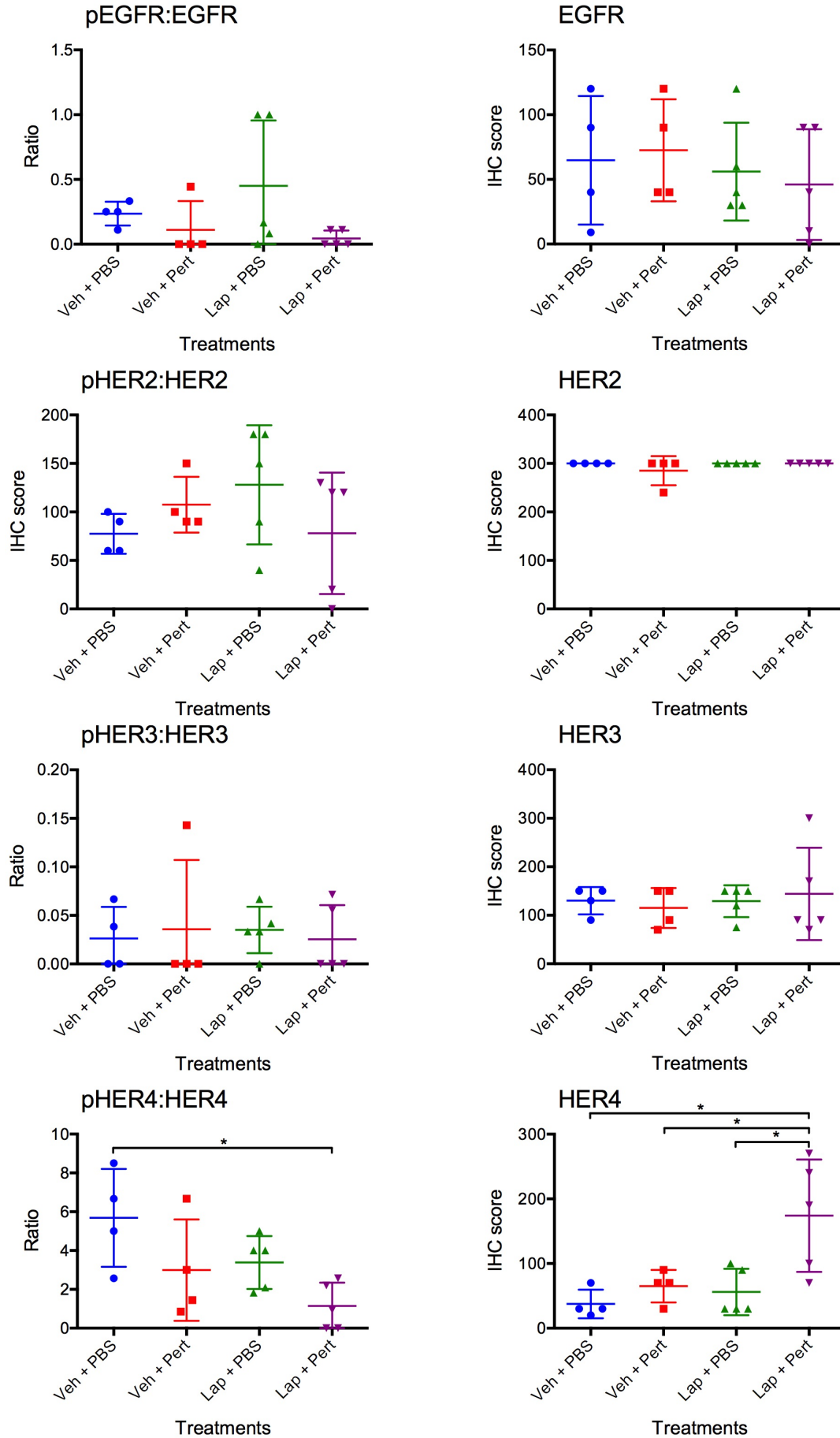


Figure 5-14 Representative images of immunohistochemistry staining of the mouse xenograft tumours

Mice were treated with the indicated agents. Mouse xenograft tumours were sectioned into 4 μ m slides. IHC staining was performed to detect (A) Tyr1173-phosphorylated EGFR, (B) total EGFR, (C) Tyr1221/22-phosphorylated HER2, (D) total HER2, (E) Tyr1289-phosphorylated HER3, (F) total HER3, (G) Tyr-1162-phosphorylated HER4, (H) total HER4, (I) cleaved caspase-3 or (J) Ki-67. Representative images of each treatment group were shown.



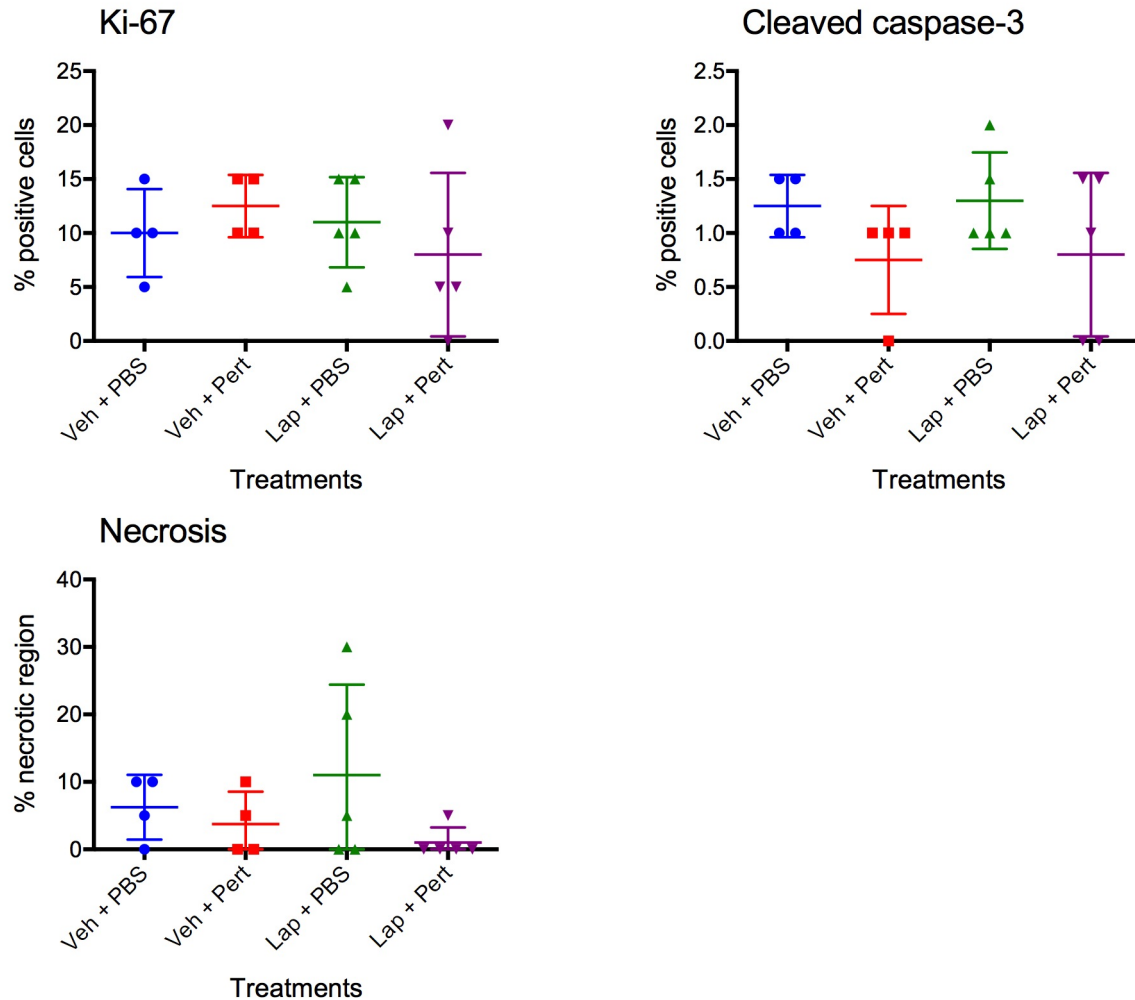
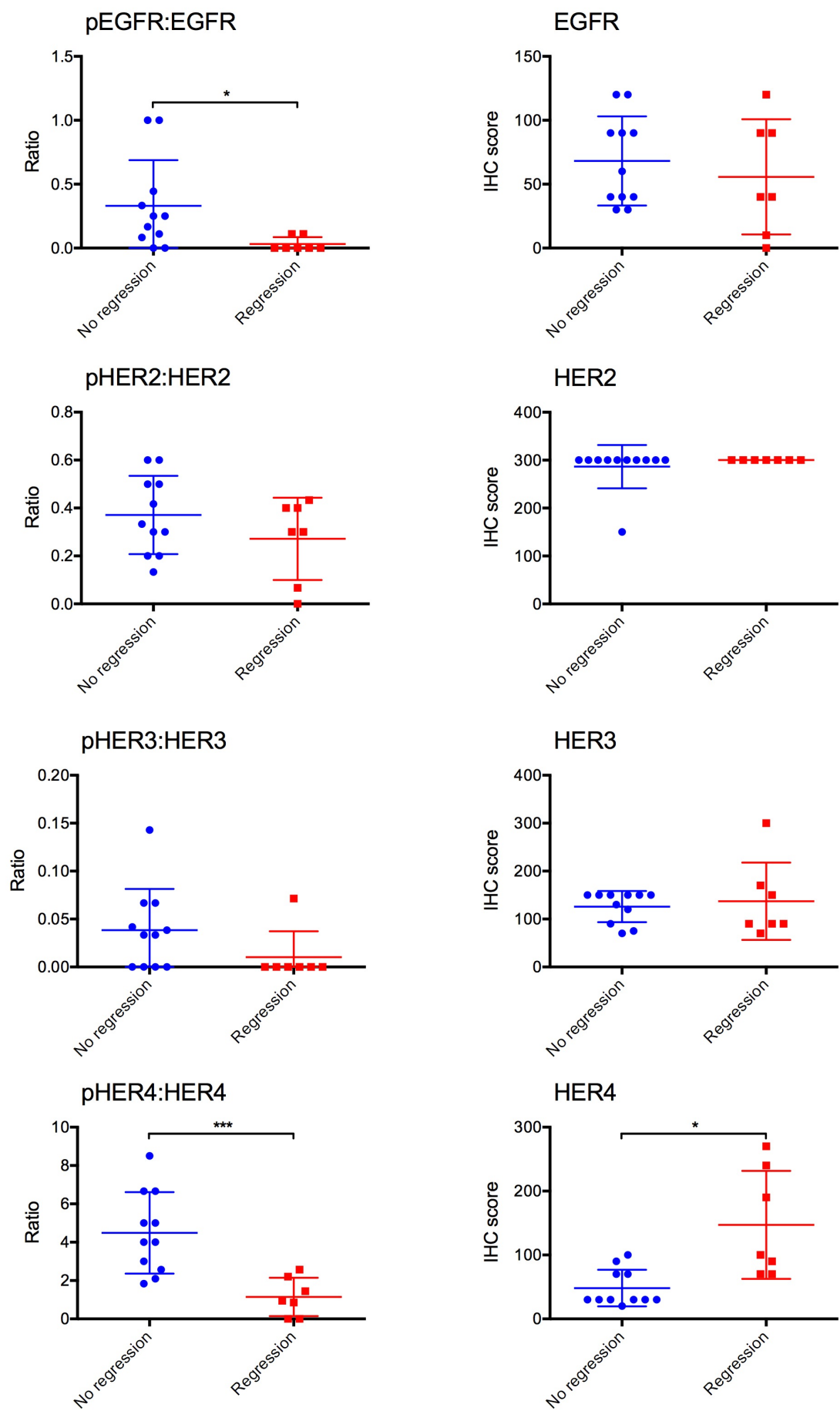


Figure 5-15 Effect of lapatinib and/or pertuzumab treatment on HER signalling, proliferation, apoptosis and necrosis *in vivo*

The HER receptors and their phosphorylated forms were scored in terms of the staining intensity and the percentage of cells stained with the respective intensity. Ki-67 and cleaved caspase-3 were scored in terms of the percentage of positive stained cells. Necrosis was scored in terms of the percentage of necrotic region in a tumour. The scores were then grouped according to the treatments that the mice received. All scorings were performed individually by the author and a consultant pathologist. (Error = SD; Unmatched one-way ANOVA with Bonferroni correction, * $p < 0.05$)



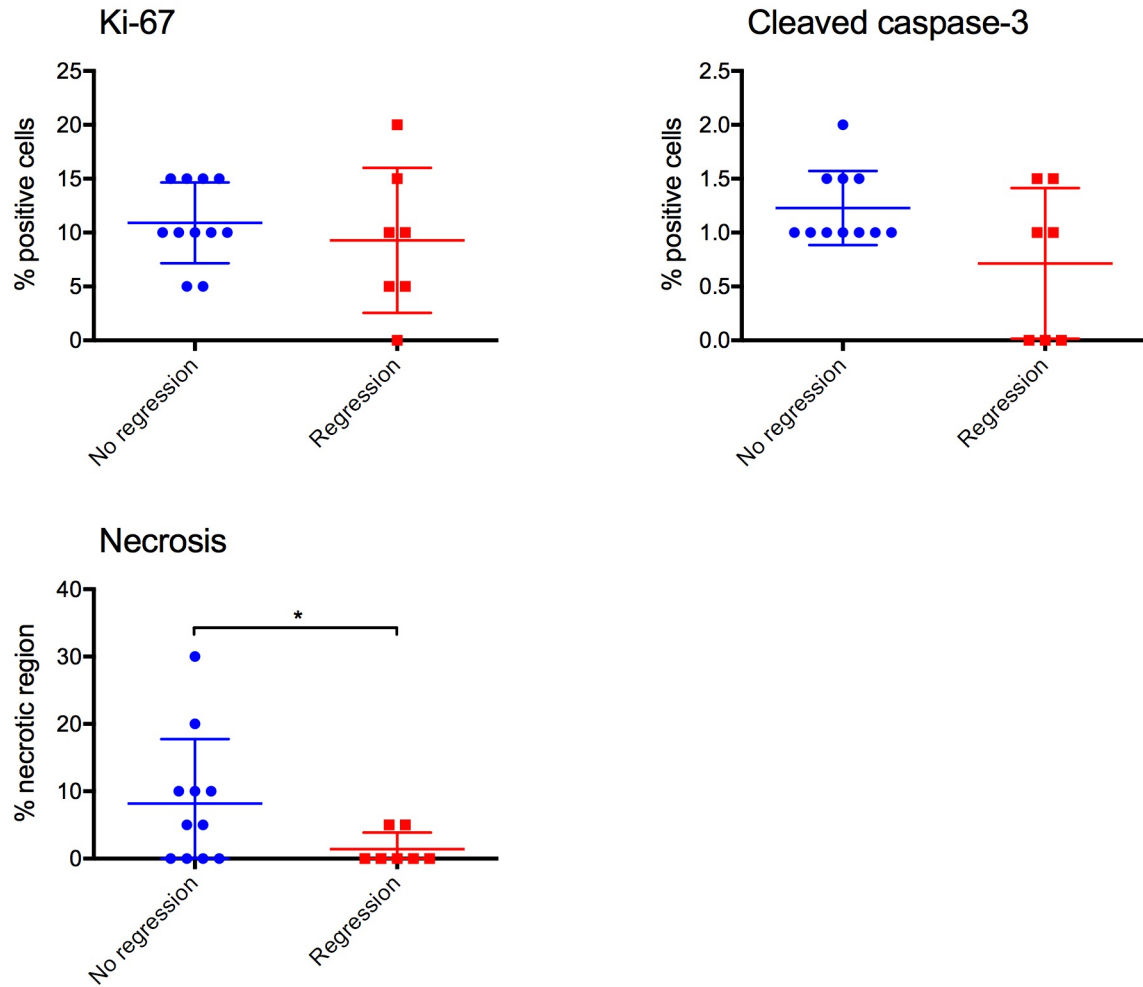


Figure 5-16 Comparison of IHC markers and the regression status of tumours

The IHC scores were grouped according to whether the tumour regressed or not. (Error = SD; Unpaired t-test, *p<0.05, ***p<0.001)

non-regressed ($p = 0.0167$) (Figure 5-17A) and there was a positive correlation between tumour size and necrosis ($p = 0.0139$) (Figure 5-17B). Therefore, the decreased necrosis in the regressed tumours might be due to their smaller sizes.

There were some special patterns of protein expression in the tumours. EGFR was expressed predominantly next to the necrotic area of the tumours (Figure 5-18A). EGFR expression was positively correlated with the percentage of necrotic area ($p = 0.0394$) (Figure 5-19). As necrosis is a hallmark of hypoxia in tumour (Shimizu et al., 1996), these results indicated that EGFR expression might be correlated with hypoxia. This can be confirmed using IHC staining of hypoxia markers such as HIF1 α and CA9. HER3 was preferentially expressed at the epithelial regions of the groups of tumour cells (Figure 5-18B) or at the single cells (Figure 5-18C). These expression patterns were independent from the treatments received by the mice.

There were no significant differences in Ki-67 and cleaved caspase-3 among the different treatment groups (Figure 5-15) and tumour regression (Figure 5-16). This might be because the differences between the groups were small and the number in each group was too low to detect a significant difference; or there might also be other mechanisms of cell toxicity, such as ADCC from pertuzumab (Scheuer et al., 2009), vessel growth inhibition or changes in vessel perfusion, that contributed to tumour regression.

5.2.4 Effect of the combination of lapatinib and pertuzumab on lapatinib-resistant cell lines

Finally, the drug combination was tested on the lapatinib-resistant cell lines generated in Chapter 3. The same dose of pertuzumab, 20 $\mu\text{g/ml}$, was used to investigate whether this antibody would re-sensitise the lapatinib-resistant cell lines to 250 nM lapatinib.

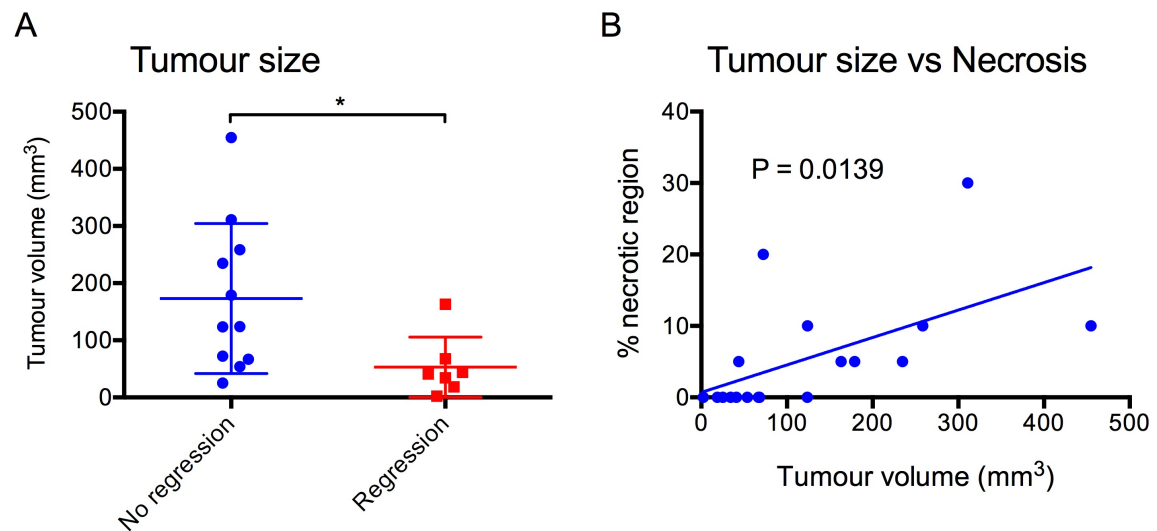


Figure 5-17 Correlations among tumour regression, size and necrosis

(A) The size of the tumours before the mice were euthanised was compared between regressed and non-regressed tumours. (Error = SD; Unpaired t-test, * $p < 0.05$) (B) The correlation between tumour size and necrosis was analysed using linear regression model. (Pearson correlation test)

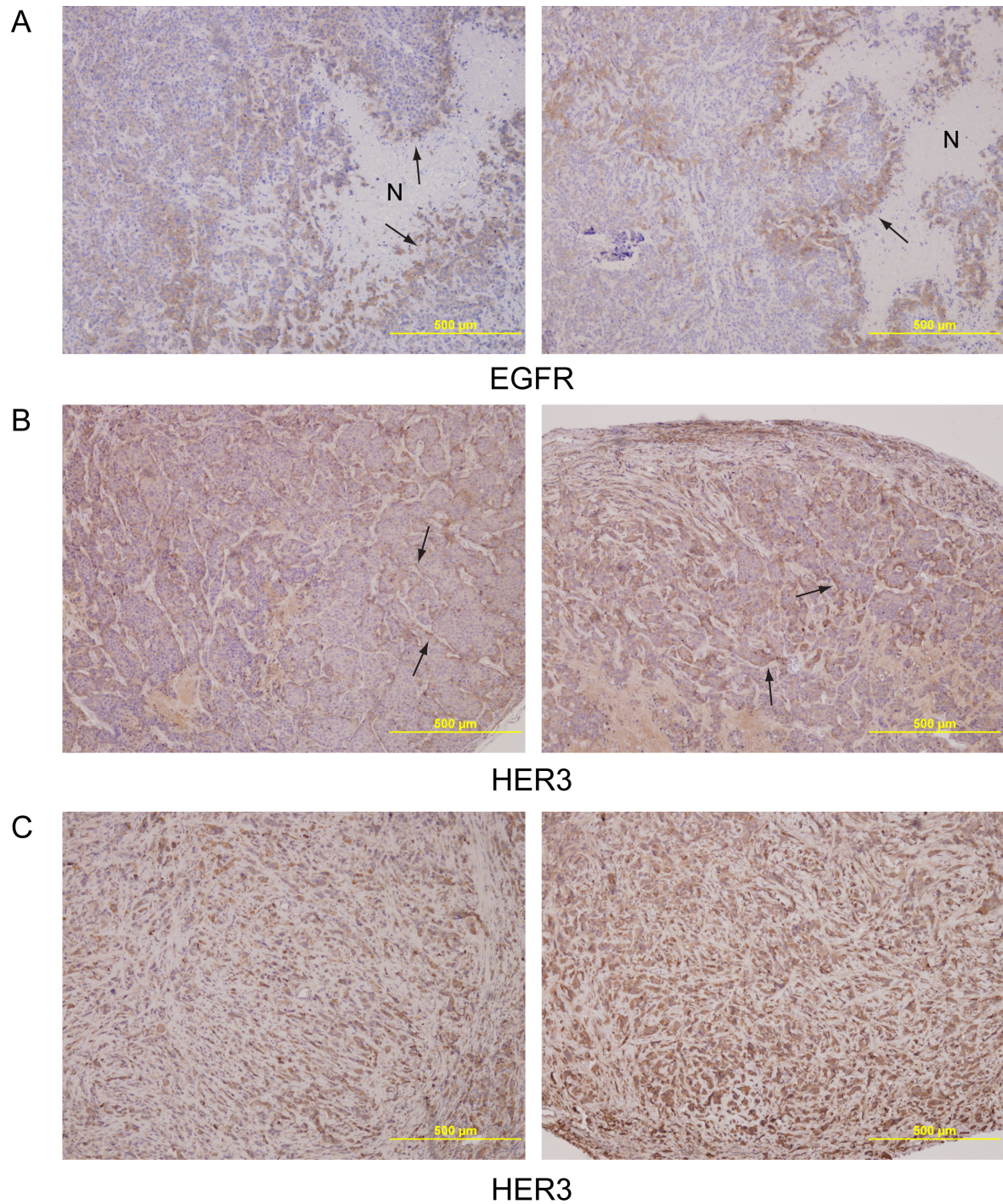


Figure 5-18 Special patterns of protein expression in tumours

(A) EGFR was expressed in the cells next to the necrotic region (marked by arrowheads; N: necrotic region) (B) HER3 was highly expressed in the cell at the epithelial (marked by arrowheads) and (C) in single cells.

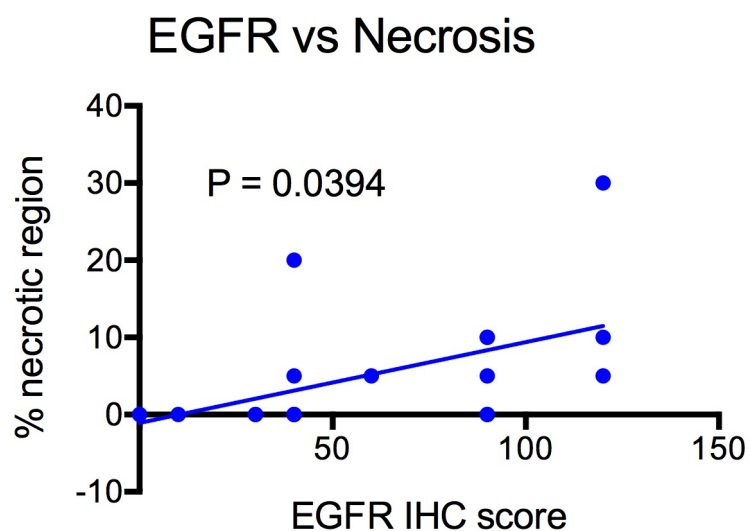


Figure 5-19 The correlation between EGFR expression and necrosis

The IHC scores of EGFR were correlated with the percentage of necrotic region in the tumours using a linear regression model. (Pearson correlation test)

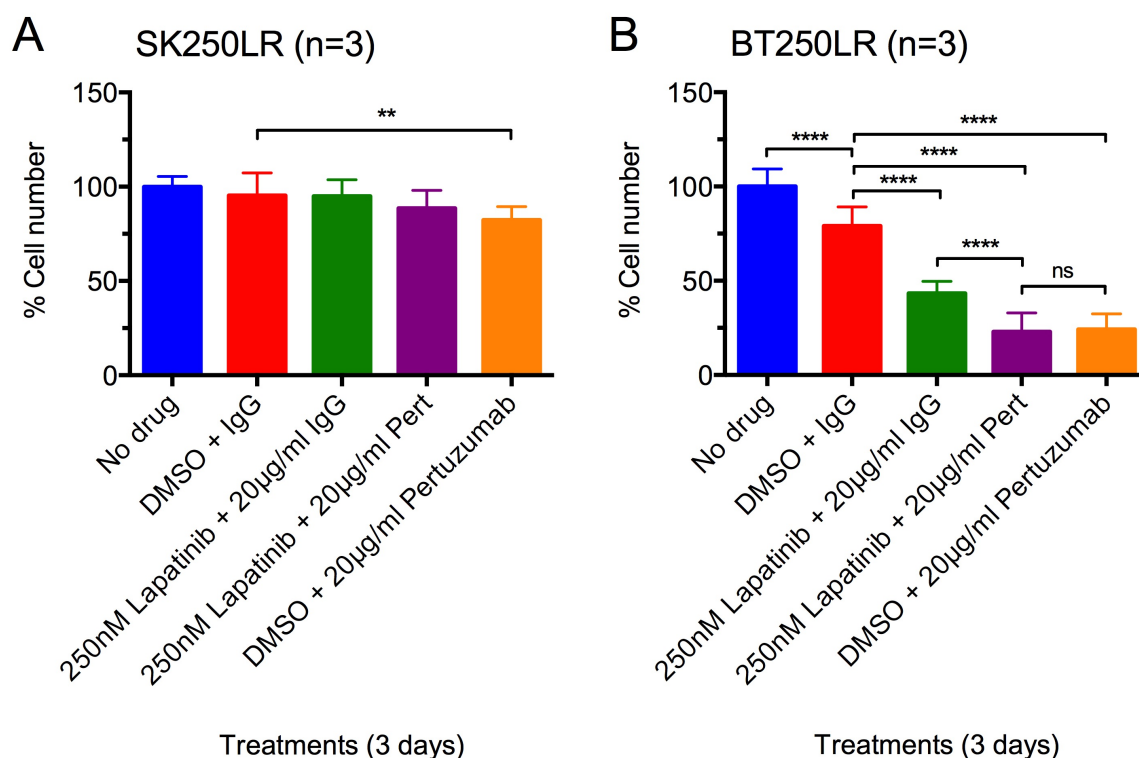


Figure 5-20 Effect of the combination of lapatinib and pertuzumab on cell number in lapatinib-resistant cell lines

(A) SK250LR and (B) BT250LR cells were seeded and maintained in continuous lapatinib treatment the night before experiment started. On the next day, the drug was taken off. Then these cells were treated with the indicated agents for three days. Cells were then trypsinised and counted. The percentage cell number was normalised with the no-drug control. Three independent experiments with three technical replicates were performed. (Error = SD; Matched two-way ANOVA with Bonferroni correction, ** $p < 0.01$, **** $p < 0.0001$)

5.2.4.1 Total cell counting

Pertuzumab without lapatinib reduced cell number of SK250LR by $18 \pm 7\%$ ($p = 0.0031$) (Figure 5-20A); however, lapatinib and pertuzumab together decreased cell number less than pertuzumab alone, and this decrease was not statistically significant from DMSO/IgG control. As previously described, removal of lapatinib caused downregulation of HER2 in SK250LR cells (Figure 3-22). It is possible that the decrease in HER2 levels mediated the sensitivity of SK250LR cells to pertuzumab.

As aforementioned in Section 3.2.5, BT250LR cells were still sensitive to 250 nM lapatinib and it reduced cell number compared to DMSO/IgG control ($p < 0.0001$) (Figure 5-20). The addition of pertuzumab to lapatinib further decreased cell number and it was statistically significant ($p < 0.0001$) compared to lapatinib/IgG. In fact, pertuzumab alone reduced cell number to the same extend as the combination ($p < 0.0001$) compared to DMSO/IgG and there was no statistical difference the cell number between lapatinib/IgG and lapatinib/pertuzumab.

The above experiments showed that the lapatinib-resistant cell lines were sensitive to pertuzumab; however, the combination of lapatinib and pertuzumab did not work better than pertuzumab alone.

5.2.4.2 Apoptotic assay

Treatment with lapatinib (Figure 5-21C), pertuzumab (Figure 5-21B) or their combination (Figure 5-21D) did not change the percentage of apoptotic cells in SK250LR cells. In BT250LR cells, all the drug treatments increased apoptosis. The combination of lapatinib and pertuzumab had the greatest effect on apoptosis (Figure 5-22D), followed by pertuzumab alone (Figure 5-22B). Statistical analysis showed that only pertuzumab single treatment increased apoptosis significantly ($p = 0.0361$) (Figure 5-23). Although the combination caused a higher mean of percentage apoptotic cells, the difference from

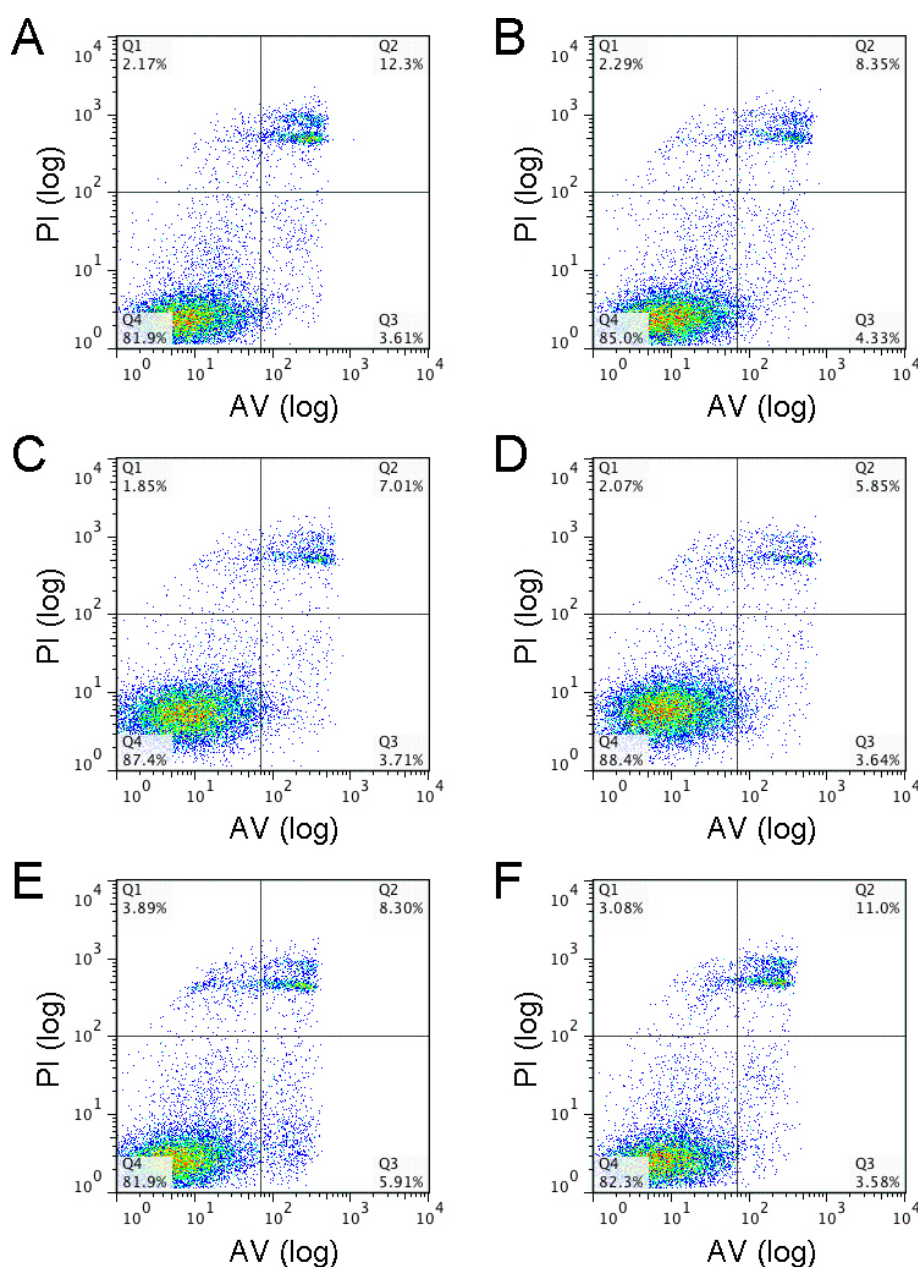


Figure 5-21 Apoptosis analysis of SK250LR cell line after lapatinib and/or pertuzumab treatment

After lapatinib was removed from the cells, SK250LR cells were treated with (A) DMSO and 20 μ g/ml non-specific human IgG, (B) DMSO and 20 μ g/ml pertuzumab, (C) 250 nM lapatinib and 20 μ g/ml non-specific human IgG, (D) 250 nM lapatinib and 20 μ g/ml pertuzumab, (E) 6 μ M camptothecin no (F) no reagents for two days. Cells were trypsinised, collected and stained with Annexin V-Alexa Fluor 647 and propidium iodide. Stained cells were analysed using a CyAn FACS analyser. Plots are representative of three independent experiments. (PI: Propidium Iodide, AV: Annexin V)

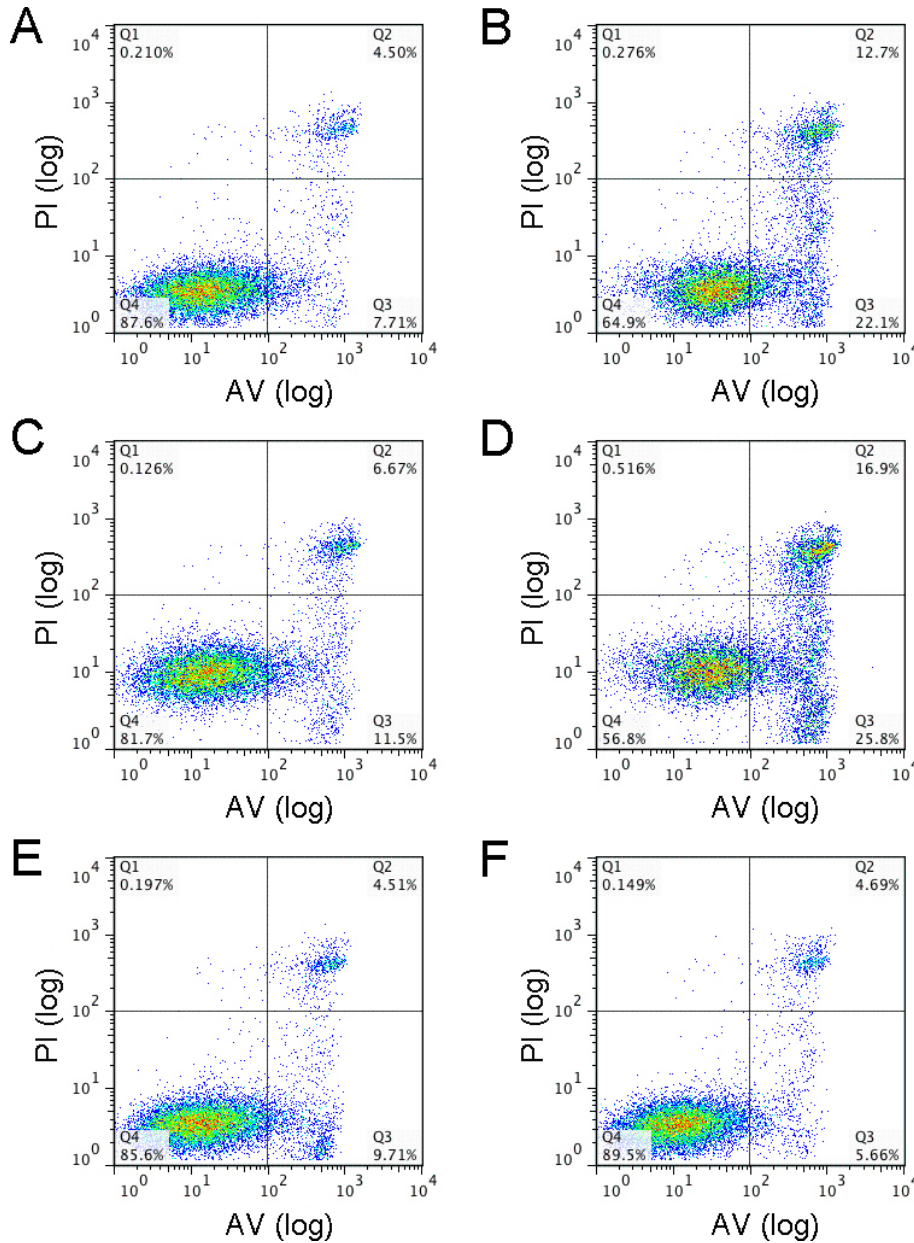


Figure 5-22 Apoptosis analysis of BT250LR cell line after lapatinib and/or pertuzumab treatment

After lapatinib was removed from the cells, BT250LR cells were treated with (A) DMSO and 20 μ g/ml non-specific human IgG, (B) DMSO and 20 μ g/ml pertuzumab, (C) 250 nM lapatinib and 20 μ g/ml non-specific human IgG, (D) 250 nM lapatinib and 20 μ g/ml pertuzumab, (E) 6 μ M camptothecin or (F) no reagents for two days. Cells were trypsinised, collected and stained with Annexin V-Alexa Fluor 647 and propidium iodide. Stained cells were analysed using a CyAn FACS analyser. Plots are representative of four independent experiments. (PI: Propidium Iodide, AV: Annexin V)

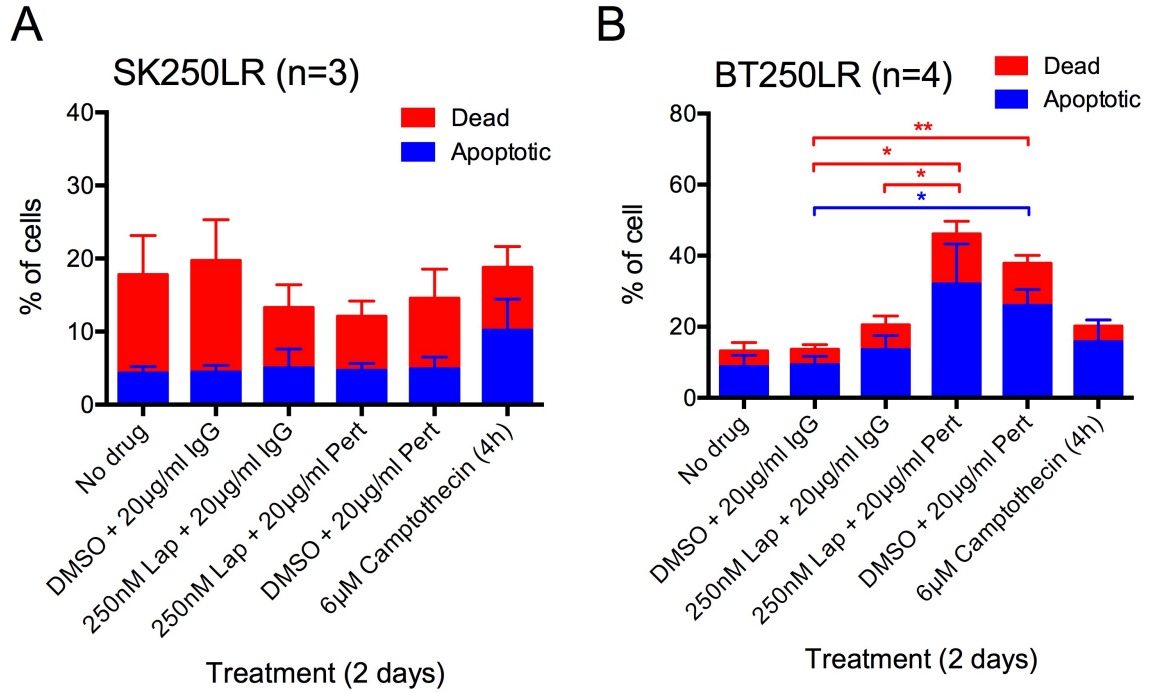


Figure 5-23 Effect of the combination of lapatinib and pertuzumab on apoptosis of lapatinib-resistant cell lines

After lapatinib was removed from the cells, (A) SK250LR and (B) BT250LR cells were treated with the indicated agents for two days. Cells were then trypsinised, collected and stained with Annexin V-Alexa Fluor 647 and propidium iodide. Stained cells were analysed using a CyAn FACS analyser. Three independent experiments were performed. (Error = SD; Matched one-way ANOVA with Bonferroni correction, * $p < 0.05$, ** $p < 0.01$)

DMSO/IgG control was not significant probably because of the huge variation and the small number of repeats ($n = 4$). Both pertuzumab and the combination caused more cell death in BT250LR cells compared to the DMSO/IgG control ($p = 0.0048$ and $p = 0.0259$ respectively). The combination caused more cell death than lapatinib alone ($p = 0.0447$); however, there was no statistical difference in cell death between pertuzumab alone and the combination.

Unfortunately, the apoptotic assay did not provide any evidence to show that pertuzumab or the combination of lapatinib and pertuzumab worked in SK250LR cells. However, BT250LR cells appeared to be sensitive to pertuzumab, although more experiments should be performed to attain statistical significance for the combination of lapatinib and pertuzumab.

5.3 Discussion

5.3.1 Combination of lapatinib and pertuzumab *in vitro*

As shown in Chapter 4, lapatinib only partially inhibited NRG1-stimulated HER3 and HER4 signalling (Figure 5-24A). The addition of pertuzumab to lapatinib further inhibited the phosphorylation of HER3 and HER4 (Figure 5-24B), suggesting that HER3 and HER4 still preferentially bound to HER2 even in the presence of lapatinib. However, the phosphorylation of downstream MAPK and Akt was not completely inhibited in SK-BR-3 (Figure 5-1A). This might be caused by the residue kinase activity of the HER receptors or the dimerisation of HER3 and HER4 with EGFR or each other (Figure 5-24B).

The efficacy of the combination of lapatinib and pertuzumab was demonstrated using total cell counting and apoptotic assay. The decrease in cell number correlated with increased apoptosis, suggesting the combination induced a stronger cytotoxic effect than either drug alone. However, Clonogenic assay did not show any difference between lapatinib alone and the combination, indicating that this assay might not be sensitive enough for the small differences shown in SK-BR-3 cells. There was also no difference in the cell cycle in SK-BR-3 cells between lapatinib alone and the combination, suggesting that the drug combination and lapatinib alone might have similar cytostatic potential. However, there was a statistically difference in the percentage of BT-474 cells in S-phase when they were treated with the combination, compared to the DMSO/IgG control. More experiments are needed to understand the effect on cell proliferation and viability caused by the combination. Real-time growth monitoring using IncuCyte and metabolic activity assay are suggested.

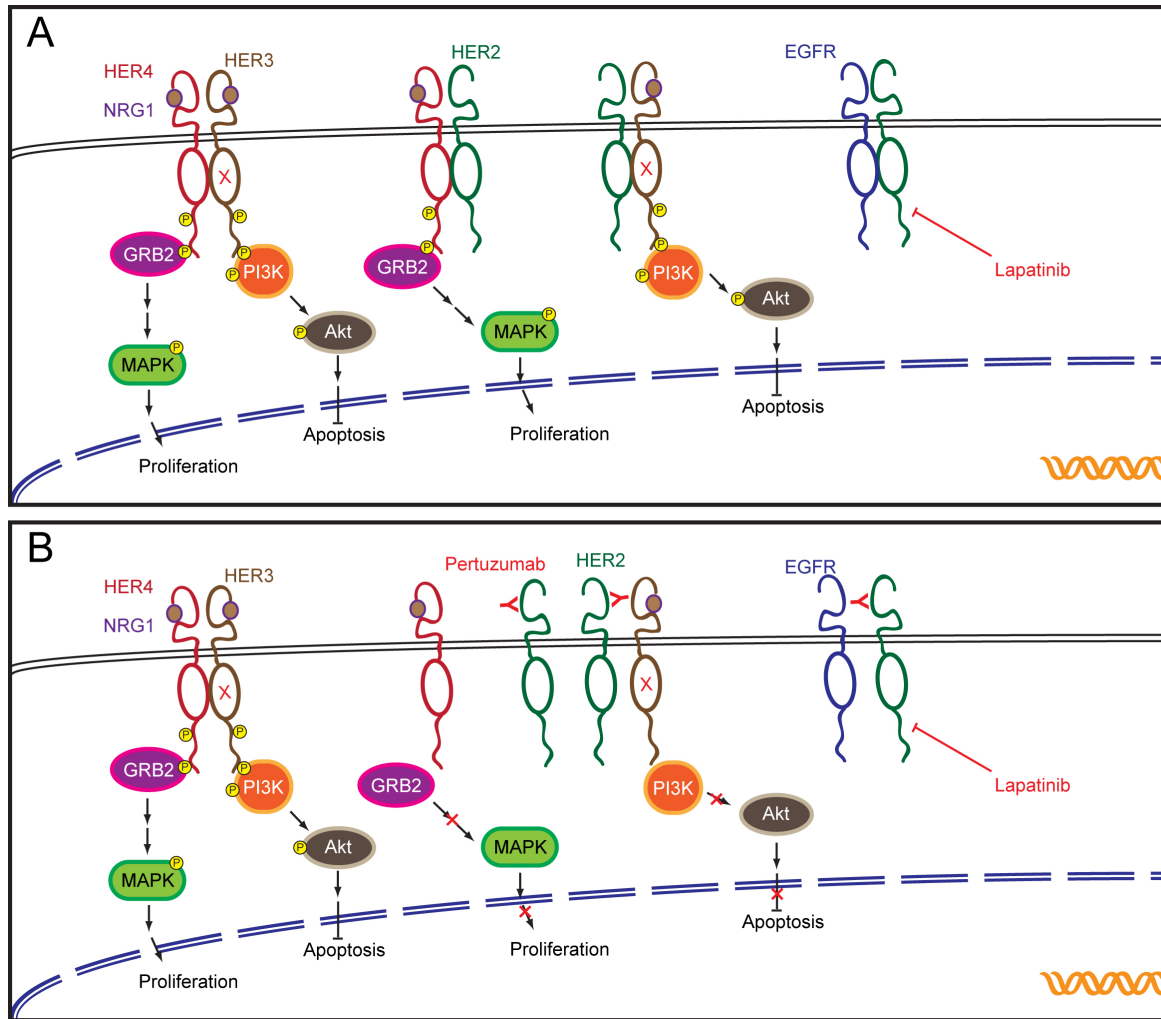


Figure 5-24 Schematic diagram of the mechanism of action of the combination of lapatinib and pertuzumab *in vitro*

(A) Revised model of the effect of NRG1 stimulation on lapatinib treatment. NRG1 stimulates HER2/HER3, HER2/HER4 and HER3/HER4 dimerisation and activation, which trigger the downstream MAPK and Akt pathways to promote proliferation and prevent apoptosis. (B) The addition of pertuzumab to lapatinib treatment prevents the NRG1-driven HER2/HER3 and HER2/HER4 dimerisation. Therefore, this combination inhibits the downstream MAPK and Akt to a certain extent. However, lapatinib and pertuzumab combination could not inhibit NRG1-stimulated HER2-independent pathways.

5.3.2 Combination of lapatinib and pertuzumab *in vivo*

5.3.2.1 Tumour growth in vehicle control group

The BT-474 xenograft tumour growth was slower than that in other studies, in which less cells were injected into mammary fat pad of nude mice (Liu et al., 2009b) or NSG mice (Iorns et al., 2012). Liu *et al.* injected 3×10^6 cells but their tumours in control group increased volume by around 4-fold after three weeks of treatment (Liu et al., 2009b). In this thesis, 5×10^6 cells were injected but the tumours in the control group only grew less than 2-fold bigger after three weeks. Moreover, their BT-474 xenograft tumours reached 100 mm^3 after 12 days, while the tumours reached 100 mm^3 only after 60 days in this study. Iorns *et al.* injected 2.5×10^6 cells and the tumours grew rapidly from less than 100 mm^3 to around 400 mm^3 from day 50 to 80 after injection (Iorns et al., 2012). In this study, the size of the largest tumour in the control group was only around 250 mm^3 . One possible reason is that the other studies implanted an oestrogen pellet into the mice to supply the tumour with the hormone, but we supplied oestrogen in the drinking water to avoid the development of kidney stones, which had been a problem previously that led to mouse fatalities.

5.3.2.2 Effect of lapatinib on xenograft tumour

Previous studies showed that lapatinib significantly inhibited the growth of BT-474 mouse xenograft tumours, but there was no tumour regression (Kodack et al., 2012; Konecny et al., 2006; Molina et al., 2008). The present xenograft experiment did not show that 50 mg/kg lapatinib daily caused any inhibition of tumour growth. This observation might be due to the suboptimal dose of lapatinib used in this study, as the previous studies used at least 2–4 times higher dose of lapatinib (Kodack et al., 2012; Konecny et al., 2006; Molina et al., 2008). It might also be because the tumour growth in the present study was already very slow and lapatinib would work better in faster growing cells.

After the xenograft experiment, it was found that pure TWEEN80 were mistakenly used to dissolve lapatinib for mouse treatment. TWEEN80 is normally used at the maximum concentration of 10% solution in mouse xenograft experiments (Bozzi et al., 2013; Cheng et al., 2005; Varvaresou et al., 2004). Pure TWEEN80 did not have obvious toxic effect on the mice as they did not lose weight nor had abnormal behaviour, but the effect of using pure TWEEN80 on lapatinib uptake by the mice was unknown. The statistical analysis on the changes in tumour size showed that the combination was the only treatment that caused significant tumour regression compared to vehicle control. There was also a significant difference between the combination and lapatinib-only treatment, suggesting that the results from this xenograft study were still valid, although the lapatinib dose in the mouse system might be lower than expected.

The plasma concentration of lapatinib in the mice can be estimated by mass spectrometry after high-performance liquid chromatography. The results can then be compared with published pharmacokinetics data of lapatinib in mice (Hudachek and Gustafson, 2013) to determine whether pure TWEEN80 had inhibited lapatinib uptake.

5.3.2.3 Implication of *in vivo* drug combination studies

This xenograft experiment showed that by combining lapatinib and pertuzumab, it was possible to reduce the dose of lapatinib required to see an effect. Lowering the dose of a TKI may reduce toxicity or side effects. In order to test the benefit of the drug combination compared with the use of full-dose lapatinib, another xenograft experiment should be performed. Mice harbouring BT-474 xenograft tumour will be treated with full-dose lapatinib (100 mg/kg) or half-dose lapatinib (50 mg/kg) combined with pertuzumab (12 mg/kg loading, 6 mg/kg maintaining) treatment. The primary endpoint of this experiment will be the change in tumour size and animal welfare such as weight loss.

5.3.2.4 Molecular marker studies

The staining of phospho-EGFR and phospho-HER3 in the xenograft tumours was quite weak, which might be due to the instability of phosphoproteins after the tumours were taken away from blood supply (Baker et al., 2005). Although the xenograft tumours were fixed as soon as it was removed from the mice, the time lagged between euthanasia and tumour removal might have caused the degradation of some phosphoproteins. The other possibility is that the antibodies used may not be good for IHC staining.

HER4 expression levels were high in the tumours treated with the combination of lapatinib and pertuzumab, suggesting HER4 might mediate the effect of the drugs on tumour regression. HER4 has been implicated in both favourable and poor prognosis (Koutras et al., 2010), but the cellular functions of HER4 vary among different isoforms (Sundvall et al., 2010). Further isoform analysis will be required to understand the correlation of HER4 expression and drug-induced tumour regression.

Ki-67 expression was low in all xenograft tumours. Only one tumour expressed Ki-67 in around 20% of the cells, while the majority expressed Ki-67 in less than 15% of the cells. According to latest St. Gallen International Breast Cancer Conference, tumours expressing Ki-67 in less than 20% of the cells are considered low to intermediate expression (Goldhirsch et al., 2013). The result correlates with the fact that BT-474 cells are slow-growing.

Expression of cleaved (active) caspase-3 was extremely low. All the xenograft samples had less than 5% cells expressing cleaved caspase-3. However, cleaved caspase-3 staining appeared at the necrotic region of the tumours, which might represent non-specific staining from dead cells.

There were no significant differences in Ki-67 and cleaved caspase-3 among different treatment groups, suggesting there might not be any difference in growth rate and apoptosis. The regression of tumours in the combination treatment group might be due to other mechanisms; for example, ADCC, which pertuzumab was reported to activate (Scheuer et al., 2009), vessel growth inhibition and changes in vessel perfusion.

ADCC is studied *in vitro* by incubating cancer cells with the drugs and NK cells isolated from the peripheral blood mononuclear cells of healthy donors. Then the cytotoxicity is determined using the Guava Cell Toxicity Kit (Millipore) (Collins et al., 2012). *In vivo* study of ADCC is now being developed by injecting isolated NK cells into the mice along with drug treatment (Shiokawa et al., 2010); however, the procedures may require more validation before the technique can be routinely used in animal models. Alternatively, mice harbouring BT-474 xenograft tumour can be treated with the combination of lapatinib and pertuzumab or the combination of lapatinib with the antigen-binding fragment [F(ab')₂] of pertuzumab. Previously research has shown that trastuzumab-F(ab')₂ was not capable of eliciting ADCC (Barok et al., 2007); therefore, with the lack of Fc region, pertuzumab-F(ab')₂ is also hypothesised not to trigger ADCC and can be used to compared ADCC effect *in vivo*. Pertuzumab-F(ab')₂ can be produced using pepsin digestion (Jones and Landon, 2002).

Immunohistochemistry staining of the xenograft tumour samples with mouse blood vessel markers CD31 or Factor VIII-related antigen can be used to assess vessel growth (Wang et al., 2008). To study vessel perfusion, mice can be injected with Hoechst 33342 or biotinylated tomato lectin before they are sacrificed. Then perfusion is detected using Hoechst 33342 fluorescence or IHC staining with anti-biotin antibody (Li et al., 2007).

5.3.3 Combination of lapatinib and pertuzumab in lapatinib-resistant cell lines

Total cell counting and apoptotic assay suggested that SK250LR cell line might not be sensitive to the combination treatment of lapatinib and pertuzumab. These results agreed with the previous finding that SK250LR cells had permanent change in cell biology and were no longer dependent on EGFR and HER2 signalling.

In the BT250LR cell line, total cell counting suggested that the combination of lapatinib and pertuzumab was effective; however, the decrease in cell number might be contributed by pertuzumab alone. In the apoptotic assay, both combined treatment and pertuzumab alone caused significant cell death compared to the DMSO/IgG control. Pertuzumab alone induced apoptosis in a significant portion of BT250LR cells compared to the control, but the combination only caused an insignificant increase. This insignificance in statistics was probably caused by the huge variations across the repeats. Again, the difference between the combination and pertuzumab alone was not significant, suggesting that the apoptotic effect might come from pertuzumab alone.

In contrast to SK250LR cell line, HER2 activation was only suppressed by lapatinib in BT250LR cell line. When the drug was removed, HER2 phosphorylation readily recovered, suggesting that BT250LR cell line still depended on HER2 signalling pathway. The difference in their dependence on HER2 signalling pathway might be the reason of the differential response to pertuzumab treatment in the lapatinib-resistant cell lines.

The above data showed that the combination of lapatinib and pertuzumab or pertuzumab alone might be effective in a subset of lapatinib-resistant cancers. The combination will require *in vivo* confirmation by testing in a BT250LR mouse xenograft model. Should the

combination be effective *in vivo*, it can be considered as an alternative treatment for patients who progress after lapatinib treatment.

5.3.4 Chapter summary

The combination of lapatinib and pertuzumab inhibited NRG1-stimulated HER3 and HER4 signalling more than either drug alone. This combination was tested in the two HER2-overexpressing cell lines and produced additive anti-cancer effect in total cell counting and apoptosis. The combination also had better anti-tumour effect *in vivo*, where the combination significantly induced tumour regression. The drug combination upregulated HER4 expression in the tumour; however, its link to tumour regression is still unclear. The drug combination still inhibited the lapatinib-resistant BT250LR cell line, but not the SK250LR cell line, indicating SK250LR cells were independent from HER2 signalling. Therefore, other signalling pathways that may mediate lapatinib resistance in SK250LR cell line should be investigated. A RTK array was used to study other RTK signalling pathways in the lapatinib-resistant cell lines.

6. Assessing receptor tyrosine kinases in lapatinib-resistant cell lines

6.1 Introduction

The previous chapters of this thesis discussed the changes in HER signalling in lapatinib-resistant HER2-overexpressing breast cancer cell lines, the role of NRG1 in lapatinib resistance and the novel combination of lapatinib and pertuzumab that can reduce NRG1-mediated HER signalling. Interestingly, SK250LR cells did not respond to the combination of lapatinib and pertuzumab; it also appeared to have loosen their dependence on HER2 signalling (Section 3.2.5). These results suggested that permanent changes have occurred in their signalling profile in response to prolonged lapatinib treatment.

To understand these changes, the phosphorylation of RTKs was monitored using the Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems). This kit is designed to monitor the relative phosphorylation of 49 different RTKs (Table 6-1), offering a useful screening method to detect upregulated signalling pathways in a given sample. BT250LR cells appeared to be still dependent on HER signalling pathways; therefore, the phosphorylation of other RTKs was also monitored using this RTK array to compare to SK250LR cells.

6.2 Results

In this experiment, the parental cell lines were treated with 250 nM lapatinib or DMSO as a control for one hour. Their RTK phosphorylation profile was compared with that of the lapatinib-resistant lines. The lapatinib-resistant cell lines were grown in continuous 250 nM lapatinib treatment or the drug was removed for 24 hours. Because of time and financial limits of this DPhil project, the RTK array experiment was only performed once. The results below represented only a one-off observation.

In both sensitive cell lines, lapatinib treatment readily dephosphorylated the HER receptors (Figure 6-1). In SK250LR cells, HER2 and HER3 remained dephosphorylated when the cells were continuously treated with lapatinib (Figure 6-1A). Although the array showed that there was more phospho-EGFR in SK250LR cells than SK-BR-3 cells under lapatinib treatment, it was previously shown that it was due to an upregulation of EGFR expression (Section 3.2.4). Ten RTKs were found activated in SK250LR cells and their phosphorylation was maintained after lapatinib was removed for 24 hours. These RTKs are ALK, Axl, Dtk, EGFR, EphA4, EphA10, FGFR3, MSPR, Tie-2 and RYK. These results agreed with those in Section 3.2.5 that SK250LR underwent permanent changes in its signalling profiles in response to prolonged lapatinib treatment.

In BT250LR cells, none of the RTKs except EGFR, HER2 and HER3 were activated when the cells were continuously treated with lapatinib (Figure 6-1B). However, a number of RTKs rephosphorylated readily after the removal of lapatinib, including ALK, EGFR, HER2, HER3, c-Ret and RYK. These findings were in agreement with the previous findings that BT250LR cells remained dependent on HER signalling (Section 3.2.5). Nevertheless, BT250LR cells may have developed co-dependency on other RTKs such as ALK, c-Ret and RYK when lapatinib was withdrawn.

ALK	EphA2	EphB1	FGFR3	IGF-1R	PDGFR β	Tie-2
Axl	EphA3	EphB2	FGFR4	InsulinR	c-Ret	TrkA
DDR1	EphA4	EphB3	Flt-3	M-CSFR	ROR1	TrkB
DDR2	EphA5	EphB4	HER2	Mer	ROR2	TrkC
Dtk	EphA6	EphB6	HER3	MSPR	RYK	VEGFR1
EGFR	EphA7	FGFR1	HER4	MuSK	SCFR	VEGFR2
EphA1	EphA10	FGFR2 α	HGFR	PDGFR α	Tie-1	VEGFR3

Table 6-1 List of RTKs detected by the Proteome Profiler Human Phospho-RTK Array Kit

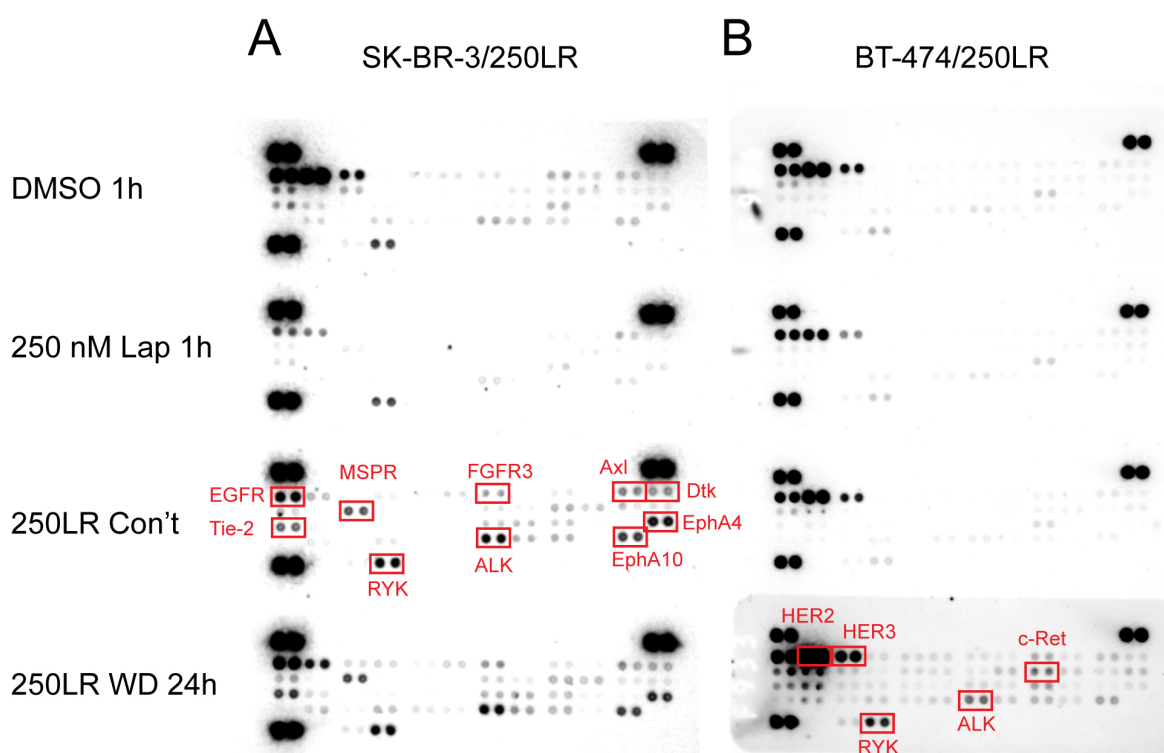


Figure 6-1 Phospho-RTK array of lapatinib-resistant HER2-overexpressing cell lines

(A) SK-BR-3 and (B) BT-474 cells were treated with 250 nM lapatinib or DMSO control for one hour. (A) SK250LR and (B) BT250LR cells were continuously treated with 250 nM lapatinib (Con't) or with the drug withdrawn for 24 hours (WD 24h). The cell lysates were analysed with phospho-RTK array (R&D systems) according to manufacturer's protocol. The experiment was performed once.

6.3 Discussion

As shown in the results sections, several RTKs were found activated in SK250LR cell line after prolonged lapatinib treatment and/or rephosphorylated readily in BT250LR cell line after the removal of drug. In this section, these RTKs are reviewed in terms of their roles in cancer and their reported interactions with the HER signalling pathways.

6.3.1 Anaplastic lymphoma kinase (ALK)

ALK has been reported as the receptor for pleiotrophin (PTN) and midkine (MK), but the stimulation of PTN and MK on ALK is still controversial (Hallberg and Palmer, 2013). A report showed that ALK has a crucial role in the development of nervous system (Iwahara et al., 1997), but its precise function in human is poorly understood.

ALK locates at a hotspot for translocation, forming constitutively active fusion proteins. To date, 22 ALK fusion proteins have been reported in non-small cell lung cancer (NSCLC), squamous cell carcinoma (SCC), renal cell carcinoma (RCC), etc. (Hallberg and Palmer, 2013) The echinoderm microtubule-associated protein-like 4-ALK (EML4-ALK) fusion gene has been identified in NSCLC (Soda et al., 2007) and breast cancer (Lin et al., 2009). Amplification of ALK gene has also been reported in various cancers including inflammatory breast cancer (Krishnamurthy et al., 2013; Robertson et al., 2013).

There are several inhibitors targeting ALK, including the FDA-approved crizotinib (FDA, 2011) and TAE684 that is originally designed to target nucleophosmin-ALK (NMP-ALK), another variation of ALK fusion protein (Galkin et al., 2007). In a recent report (Tanizaki et al., 2012), a TAE684-resistant NSCLC cell line harbouring EML4-ALK fusion has elevated levels of EGFR, HER2 and HER3 phosphorylation. While EGF stimulation rescues the sensitive cells from the ALK-TKI, the irreversible EGFR/HER2 TKI afatinib induces apoptosis in the ALK-TKI-resistant cells.

As ALK was phosphorylated in SK250LR cells, this lapatinib-resistant cell line may be responsive to ALK-TKI such as crizotinib and TAE684.

6.3.2 Axl and Dtk

Axl (also known as UFO) and Dtk (originally designated Tyro3) belong to the receptor tyrosine kinase family called TAM (Tyro3/Axl/Mer), which mediates the recognition of apoptotic cells and their clearance (Nguyen et al., 2013; Pierce and Keating, 2013). The ligands of TAM receptor family are growth arrest-specific gene 6 (Gas6), which binds to all TAM receptors, and protein S, which binds to Dtk and Mer (Linger et al., 2008). The loss of TAM receptors leads to autoimmune diseases, but they are often overexpressed in cancers and contribute to poor prognosis (Nguyen et al., 2013; Pierce and Keating, 2013).

The expression of Axl is dysregulated in various cancers such as breast, lung, gastric, liver cancers and different leukaemias (Linger et al., 2008), and its expression correlates with poor prognosis in lung, gastric cancers and acute myeloid leukaemia (AML). Axl promotes growth and survival via downstream Ras/Raf/MAPK and PI3K/Akt pathways (Fridell et al., 1996; van Ginkel et al., 2004). Axl also contributes to tumour migration, invasion (Vajkoczy et al., 2006) and angiogenesis (Holland et al., 2005). A recent study reported that Axl is responsible for the resistance against imatinib, a tyrosine kinase inhibitor specific to α -platelet-derived growth factor receptor, in gastric cancer (Mahadevan et al., 2007) and the resistance against lapatinib in breast cancer (Liu et al., 2009a).

Dtk was reported to be overexpressed in AML and multiple myeloma (Linger et al., 2008). The function of Dtk is poorly understood, but it was reported that its association with PI3K mediates the transforming ability of Dtk (Lan et al., 2000).

There are not many reports about the crosstalk between the TAM receptors and the HER receptors. Inhibition of Axl using an inhibitor or siRNA knockdown has been shown to

reduce phosphorylation of the Tyr-877 residue of HER2, the indicator of HER2 biological function, and sensitise oesophageal adenocarcinoma cells to lapatinib (Hector et al., 2010). An earlier study found that Gas6 and NRG1 synergistically stimulate growth of human Schwann cells (Li et al., 1996), suggesting a possible crosstalk among Axl, Dtk, HER3 and HER4.

6.3.3 Ephrin type-A receptor 4 and 10 (EphA4 and EphA10)

Ephrin receptor family (Eph) is the largest receptor tyrosine kinase subfamily, with 16 receptors and 9 ligands. The family is further divided into two subfamilies: EphA receptors, of which ligands (Ephrin-A) are bound to the cell membrane through glycosylphosphatidyl inositol moiety; and EphB receptors, of which ligands (Ephrin-B) are type I transmembrane proteins. As the Ephrin ligands are membrane bound, Eph/Ephrin system is capable for bidirectional signalling, where forward signals are carried through Eph receptors and reverse signals are transduced by the ligands (Kaenel et al., 2012; Miao and Wang, 2012).

Eph/Ephrin signalling can be oncogenic or tumour suppressing, depending on the context. For example, activation of EphA2 by its ligand inhibits the Ras/Raf/MAPK pathway (Miao et al., 2001) and suppresses growth of breast (Noblitt et al., 2004) and prostate cancers (Miao et al., 2001). On the contrary, overexpression of EphA2 results in the receptor being unligated due to insufficient ligands. Unligated EphA2 serves as the substrate of Akt and participates in signalling pathways that promote tumour migration and invasion (Miao et al., 2009).

Overexpression of EphA4 is reported to negatively correlate with recurrence-free and overall survivals in breast cancer patients (Brantley-Sieders et al., 2011), although the mechanism is still unclear. Interestingly, Ephrin-A5, one of the ligands of EphA4,

promotes the binding of Cbl, an ubiquitin E3 ligase, to EGFR, resulting in the ubiquitination and degradation of EGFR (Li et al., 2009). The activation of EphA4 in SK250LR may be caused by an increased expression of Ephrin-A5 via negative feedback to elevated EGFR levels.

EphA10 is kinase-defective, but it is highly expressed in metastatic breast cancer cell lines but not in normal breast epithelium, suggesting its possible role in tumourigenesis and invasiveness (Fox and Kandpal, 2004).

There have been reports showing the interaction of EphA2 receptor with EGFR (Larsen et al., 2007) and HER2 (Brantley-Sieders et al., 2008); however, no other crosstalk between the HER family and the Eph receptor family has been reported.

6.3.4 Fibroblast growth factor receptor 3 (FGFR3)

FGFR3 is one of the four members of the FGFR family. The FGFR family plays a key role in the development of the brain and limbs, angiogenesis, wound healing and the endocrine system in adults (Jain and Turner, 2012). There are 18 fibroblast growth factor ligands and their specificity depends on the isoforms of FGFR formed by alternative splicing of the third Ig loop of the ligand-binding domain (Kelleher et al., 2013). Upon ligand binding, FGFRs dimerise and phosphorylate FGFR substrate 2 (FRS2). Through some adaptor proteins like Grb2, phosphorylated FRS2 triggers downstream Ras/Raf/MAPK and PI3K/Akt pathways and mediates proliferation, differentiation and angiogenesis (Kelleher et al., 2013).

Somatic FGFR3 mutation occurs in around 50% of bladder cancers (Cappellen et al., 1999). FGFR3 mutations are also found in other cancer types such as cervical and prostate cancer (Turner and Grose, 2010); however, no common FGFR3 mutations are found in breast cancer (Jain and Turner, 2012). Gene amplification of FGFR3 is rarely found in

cancers (Nord et al., 2010), but the translocation of this gene to the highly active IgH promoter happens in 15% of multiple myelomas (Chesi et al., 1997).

Although amplification of FGFR1 and FGFR2 and single nucleotide polymorphism of FGFR2 and FGFR4 are well described in breast cancer (Jain and Turner, 2012), there are no reports about the role of FGFR3 in breast cancer and its interaction with the HER receptors. Activation of FGFR3 in SK250LR cells may present a novel therapeutic target to tackle lapatinib resistance. There are two pan-FGFR inhibitors, BGJ398 (Guagnano et al., 2011) and AZD4547 (Gavine et al., 2012); however, no FGFR3 specific inhibitors were discovered.

6.3.5 Macrophage-stimulating protein receptor (MSPR)

MSPR or the Recepteur d'Origine Nantais (RON) is the physiological receptor of macrophage-stimulating protein (also known as hepatocyte growth factor-like) (Yao et al., 2013). MSPR belongs to the same protein family of Met, the receptor of HGF and scatter factor (Ronsin et al., 1993). Aberrant activation of MSPR promotes tumorigenesis, malignancy, angiogenesis and resistance to chemotherapy (Yao et al., 2013). The phosphorylated C-terminal of MSPR serves as a docking site for adaptor proteins, for examples, Shc and Grb2, mediating downstream signalling pathways such as Ras/Raf/MAPK and PI3K/Akt pathways (Iwama et al., 1996).

A crosstalk between MSPR and EGFR was demonstrated in previous studies, which showed that EGFR directly associates with MSPR and is essential for the function of MSPR (Hsu et al., 2006; Peace et al., 2003). A recent study revealed that MSPR activation contributes to lapatinib resistance in SK-BR-3 cells, and MSPR inhibition using chemical inhibitors and receptor knockdown restores lapatinib sensitivity via the inhibition of PI3K/Akt pathway (Wang et al., 2013c).

6.3.6 Rearranged during transfection receptor (c-Ret)

c-Ret is activated by the binding of glial derived neurotrophic factor (GDNF) family ligands, including GDNF, neurturin, artemin and persephin. GDNF family- α receptors 1–4 act as the coreceptor of c-Ret (Morandi et al., 2011).

c-Ret overexpression and mutation have been reported in various cancers such as pancreas, prostate and thyroid carcinoma (Morandi et al., 2011). In breast cancer, c-Ret is found to be overexpressed in ER positive tumour samples (Esseghir et al., 2007) and cell lines (Boulay et al., 2008). The ligand of c-Ret, GDNF, promotes cancer cell proliferation, scattering (Esseghir et al., 2007) and tamoxifen resistance (Plaza-Menacho et al., 2010) in ER positive breast cancer cells. c-Ret knockdown has been shown to sensitise ER positive breast cancer cells to tamoxifen and restore tamoxifen sensitivity in tamoxifen-resistant cells (Plaza-Menacho et al., 2010). A recent study reported that high c-Ret expression is correlated with reduced metastasis-free and overall survivals (Gattelli et al., 2013). They also showed that inhibition of c-Ret reduces growth and metastatic potential of ER positive breast cancer cells by breaking the positive feed-forward loop between c-Ret and interleukin 6, an inflammatory cytokine.

So far, there are no reports about the crosstalk between c-Ret and HER signalling in the context of cancer; however, genetic variations in c-Ret and NRG1 contributes to additive risk of Hirschsprung disease, a genetic disorder characterised by the absence of enteric neuron along the intestine (Gui et al., 2013). They also demonstrated that the activation and expression of c-Ret reduces HER2 expression in neural crest cells.

BT-474 is an ER positive cell line that expresses c-Ret (Boulay et al., 2008). Although inactive in BT-474 cells, c-Ret was phosphorylated readily in BT250LR cells when lapatinib was removed. The correlation between lapatinib and c-Ret was unclear; however,

this result suggested that c-Ret might be important in keeping BT250LR cells survive in lapatinib.

6.3.7 Receptor related to tyrosine kinases (RYK)

RYK is a coreceptor of frizzled homolog proteins for the Wnt ligands. RYK is required for canonical Wnt signalling, which is the transcriptional activation mediated through β -catenin, and non-canonical Wnt/JNK pathway (Cheyette, 2004; Ueno et al., 2013).

Using chimeric protein assay, RYK was found to have impaired kinase activity; however, ligand stimulation of this chimeric protein triggers activation of MAPK (Katso et al., 1999). RYK may stimulate downstream pathways via coreceptors, which is the mechanism employed by the kinase defective HER3.

RYK was previously reported to co-immunoprecipitate with EphB2, EphB3 and EphA7 receptors, but only EphB2 and EphB3 could phosphorylate RYK; nevertheless, these results suggested a crosstalk between RYK and Ephrin receptors (Halford et al., 2000). The association between RYK and other Eph receptors is yet to be investigated (Halford and Stacker, 2001). EphA4 and EphA10, which were activated in SK250LR cells, may be potential coreceptors of RYK.

Previous studies reported that RYK overexpression is associated with decreased survival in ovarian cancer (Katso et al., 2000; Wang et al., 1996) and the RYK gene is rearranged in some atypical chronic myeloid leukaemia patients (Micci et al., 2009).

There have not been any reports stating the correlation between RYK and HER signalling. However, since RYK was strongly phosphorylated in SK250LR cells and RTK phosphorylation readily increased in BT250LR cells after the removal of lapatinib, RYK might be involved in lapatinib resistance.

6.3.8 Tyrosine kinase with immunoglobulin-like and EGF-like domains 2 receptor (Tie-2)

Tie-2 falls into the category of angiopoietin (Ang) receptors that regulate angiogenesis. The ligands of Tie-2 in human are Ang1, 2 and 4 (Huang et al., 2010a). The other member of this receptor family, Tie-1, has no known ligands and acts as the negative regulator of Tie-2 (Milner et al., 2009).

Ang1 is the agonist of Tie-2 and has a crucial role in maintaining the integrity of existing blood vessels (Thurston et al., 2000). It is constitutively expressed in numerous adult normal tissues. Although Huang *et al.* reported no clear correlation between Ang1 expression and cancer prognosis (Huang et al., 2010a), another group had recently found the correlation between increased Ang1 expression and improved disease-free survival in the head and neck SCC (Dhiwakar et al., 2010).

Ang2 normally acts as the antagonist of Ang1 and increases the permeability of vessels; however, Ang2 can be a partial agonist depending on the context (Huang et al., 2010a). For example, Ang2 functions as an agonist in the absence of Ang1 (Yuan et al., 2009) and in lymphangiogenesis (Gale et al., 2002). Previous studies revealed that increased expression of Ang2 and a high Ang2/Ang1 ratio correlated with poor prognosis in various cancers (Huang et al., 2010a), in particular with lymph node invasion and shorter survival in breast cancer (Sfiligoi et al., 2003). Overexpression of Ang2 was also shown to promote invasion and migration in breast cancer, increasing metastatic potential (Imanishi et al., 2007).

Studies showed that Ang2 expression is regulated by HER2 activity as trastuzumab and HER2 knockdown downregulates Ang2 expression via MAPK and Akt pathways (Carter

and Ward, 2000; Niu and Carter, 2007). In lapatinib-resistant SK250LR, Tie-2 might be derepressed because of Ang2 downregulation caused by HER2, MAPK and Akt inhibition.

6.3.9 Further studies RTKs in lapatinib-resistant cell lines

From the data obtained in these RTK arrays, ALK and RYK are promising targets for further validation and should be prioritised. It is because both ALK and RYK activities increased in SK250LR cell line regardless of lapatinib treatment, and they were also activated when lapatinib was withdrawn from BT250LR cell line for 24 hours.

The role of these RTKs can be studied *in vitro* using robust biochemical techniques, such as western blotting to detect the abundance and activation status of these RTKs in the lapatinib-resistant cell lines, and siRNA knockdown of RTKs to determine the proteins' role in cell growth and viability. In addition, specific inhibitors to these RTKs can be used, in combination with lapatinib, to understand the role of these RTKs in lapatinib resistance, such as crizotinib (ALK) (Hallberg and Palmer, 2013) and RWD1 antibody (RYK) (Halford et al., 2013).

The above results can then be verified *in vivo*. SCID mice will be implanted with BT250LR xenograft tumour, and then treated with specific inhibitors (e.g. crizotinib or RWD1) alone or in combination with lapatinib. The change in tumour size will indicate whether the above RTKs have a role in lapatinib resistance *in vivo*. Finally, the results will be confirmed using clinical material. Tumour biopsy samples from patients before and after treatment with lapatinib will be obtained from clinical trials. The expression and activation of RTKs can then be determined using IHC staining and compared between the samples before and after lapatinib treatment.

6.3.10 Chapter summary

In this chapter, more than ten RTKs were found to be potential targets that mediate lapatinib resistance in HER2-overexpressing breast cancer cell lines SK250LR and BT250LR. However, further confirmation of the role of these RTKs is required using robust biochemical techniques, such as western blotting and siRNA knockdown of RTKs. Besides, these models of resistance have to be validated in another lapatinib-resistant cell lines, as SK250LR and BT250LR cell lines have no common mechanisms. Due to the time constrain of this DPhil project, the investigation on the role of the above-mentioned RTKs in lapatinib resistance was not further pursued.

7. Discussion

7.1 Responses and resistance to lapatinib

In this study, complete inhibition of the phosphorylation of HER receptors and downstream pathways only lasted for several hours. After eight hours of lapatinib treatment, the phosphorylation of HER receptors started to recover. These results suggested there might be a rapid mechanism to rescue the HER2-overexpressing cell lines from lapatinib by re-phosphorylating the receptors.

After long-term lapatinib treatment, it was found that the SK-BR-3 cell line underwent a permanent change in cell biology. The lapatinib-resistant SK250LR cell line was no longer sensitive to lapatinib treatment. Removal of the drug for one week did not revert lapatinib resistance in SK250LR cells. In fact, EGFR and HER2 levels decreased after lapatinib withdrawal, indicating that EGFR and HER2 were redundant and dispensable. The SK250LR cell line had developed alternative signalling pathways to counteract the inhibition caused by lapatinib.

On the contrary, the BT250LR cell line only had a transient adaptation to survive lapatinib treatment. Growth of BT250LR cells was still inhibited, although to a smaller extent, by lapatinib treatment; however, lapatinib did not cause as much cell death as in the sensitive BT-474 cells. After withdrawal from lapatinib for one week, BT250LR cells became more sensitive to lapatinib, suggesting a reversion of lapatinib resistance. In addition, removal of lapatinib from BT250LR cell line resulted in rapid phosphorylation of HER signalling components.

7.2 Role of NRG1 in lapatinib resistance

The combination of HER3 ligand-blocking antibody, SGP1, with lapatinib showed that HER3 ligands could play a role in lapatinib resistance. Although NRG1 mRNA levels markedly increased in lapatinib-resistant cell lines, the ELISA did not show any increased levels of NRG1 β 1, suggesting the increase in mRNA levels might be due to other NRG1 isoforms or the NRG1 produced in the lapatinib-resistant cell lines is readily secreted. However, the NRG1 ELISA experiments could not detect any presence of NRG1 in the conditioned media from the lapatinib-resistant cell lines as previously discussed (Section 4.3.4.4). NRG1 knockdown experiments showed that the growth of SK250LR cell line was dependent on NRG1 expression, but the effect of NRG1 knockdown and continuous lapatinib treatment was not additive. No definite conclusion could be formed in BT250LR cell line concerning NRG1 knockdown because of the contradicting results from the two NRG1 siRNA used in this study, further knockdown experiments using other NRG1 siRNAs are required.

The role of NRG1 in the context of lapatinib may vary based on the expression level of HER2. In non-HER2 amplified cancer, NRG1 predicts the sensitivity of the cell lines to HER2 kinase inhibitor such as lapatinib (Wilson et al., 2011); however, in HER2-overexpressing breast cancer, NRG1 rescues the cells from lapatinib inhibition (Wilson et al., 2012) and mediates lapatinib resistance (Xia et al., 2013). The current study furthered the understanding of the mechanism that NRG1 selectively activated HER3 and HER4 receptors as well as their downstream pathways.

7.3 Combination of lapatinib and pertuzumab

This study reports a novel HER2-targeted therapeutic combination of lapatinib and pertuzumab to increase the drug efficacy in HER2-overexpressing cell lines. From

previous studies, one of the limitations of lapatinib is that it cannot completely inhibit NRG1-induced cell signalling, thus NRG1 rescues the cells from lapatinib treatment (Wilson et al., 2012). The new combination tackles this resistance mechanism by inhibiting NRG1-induced HER2/HER3 and HER2/HER4 signalling. The combination reduced cell number more than either single treatment alone, which was probably because apoptosis was promoted by the drug combination.

The effect of this combination was confirmed by a BT-474 mouse xenograft model, in which the combination of lapatinib and pertuzumab induced tumour regression more than either drug alone. The above results suggested that this combination might be worth pursuing in a clinical setting since blockade of NRG1-induced signalling by the combination may produce a better anti-tumour effect than lapatinib alone in patients whose tumour highly expresses NRG1.

Combined lapatinib and pertuzumab treatment induced apoptosis in lapatinib-resistant BT250LR cell line; however, no observable differences were seen in the cell number and apoptosis in SK250LR cell line. The resistance of SK250LR cells to pertuzumab may be because of the permanent change in cell biology that this cell line was no longer dependent on cell signalling involving HER2.

7.4 Insights to the current paradigm of lapatinib resistance

A report has shown that growth factors mediate resistance to various TKIs; in particular, NRG1 rescued eight cell lines from lapatinib in a panel of nine HER2-amplified cell lines (Wilson et al., 2012). Recently, a group suggested that NRG1 autocrine signalling mediates lapatinib resistance through EGFR/HER3 signalling (Xia et al., 2013). This thesis furthered the understanding of the role of NRG1 in lapatinib resistance. NRG1 stimulated HER2/HER3 and HER2/HER4 signalling even in the presence of lapatinib and therefore,

the addition of pertuzumab inhibited NRG1-driven phosphorylation of HER3 and HER4. Furthermore, the combination of lapatinib and pertuzumab was shown as a promising strategy to tackle NRG1-driven lapatinib resistance. There has been a report showing that lapatinib treatment induces increased expression of HER3 (Garrett et al., 2011). Together with the increase in NRG1 expression, the current study strengthened the theory of the central role of HER3 signalling axis in lapatinib resistance.

Liu *et al.* demonstrated that AXL mediates lapatinib resistance in the ER-positive BT-474 cell line (Liu et al., 2009a). The RTK array in this study showed that AXL might also mediate lapatinib resistance in the ER-negative SK-BR-3 cell line. However, the results need to be validated using robust biochemical methods and the ER expression levels in SK250LR cell line needs to be determined.

7.5 Limitations and recommendations

There were several limitations in the current study because of the methods and techniques used in the experiments, which are discussed as below. Recommendations for the issues are made, which should be considered in future studies where resource is available.

7.5.1 Total cell counting

The cell counter used in this study, Coulter Z2, was a particle counter that counts everything passing through the aperture. Although a gate was set up to count particles of relevant size, which was 13-27 μm for cells, to prevent the counting of debris, this machine could not differentiate dead cells from living cells. Therefore, the cell number might have included a number of dead cells. The major pitfall of total cell counting is that this technique cannot identify the cause of change in cell number; therefore, other techniques, such as real-time monitoring and apoptotic assay, were used to understand whether the change is caused by cell growth or cell death.

Traditional viable cell counting involves the use of trypan blue to distinguish living cells from dead ones, because trypan blue cannot penetrate the cell membrane of healthy cells. However, the use of haemocytometer is time-consuming, low-throughput and hence would limit the number of conditions, such as doses and agents, to be tested.

Recent technology allows the automation of viable cell counting. For example, Cellometer Auto T4 (Nexcelom Bioscience) has an algorithm to discriminate living and dead cells based on trypan blue staining, exclude debris based on particle size and recognise clumpy cells and able to count them individually.

7.5.2 Clonogenic assay

Clonogenic assay has been the gold standard to study the survival of cells after drug treatment because of the high sensitivity of the technique (Valiathan et al., 2012). However, this method is time-consuming and labour intensive, hence limiting the number of doses and agents that could be tested.

7.5.3 Growth assays for BT-474 cell line

7.5.3.1 Total cell counting in BT-474 cell line

The treatment length of three days may be too short to induce growth inhibition effect on BT-474 cell line because the doubling time of this cell line is around 100 hours (Faltus et al., 2004). In the future, other methods should be used to assess growth inhibition in BT-474 cells, for examples, immunodetection of proliferation marker Ki-67 protein and real-time growth monitoring using IncuCyte.

Since the growth of BT-474 cells is not measurable using total cell number after three days of treatment, the changes in cell number is probably caused by cell death. As mentioned in Section 7.5.1, total cell counting is not a good method to address cell death. Other cell

death assays such as apoptotic assays should be used in the future to study the effect on cell death.

In terms of NRG1 knockdown, since siRNA transfection only last for a few days, lentivirus could be employed to produce a stable cell line that processes an inducible knockdown system using shRNA. In conjunction with long-term monitoring using IncuCyte, the effect of NRG1 knockdown on tumour growth in BT-474 cell line can be studied.

7.5.3.2 Clonogenic assay in BT-474 cell line

From the experiments in this study, it was found that the colonies formed by BT-474 and BT250LR cell lines were very small and the plating efficiency, i.e. percentage of single cells seeded that form colony, was extremely small. On average, there were only around 200 colonies formed by seeding 2,000 untreated cells, giving a plating efficiency of around 10%. The small size of the colonies also caused problems in counting. The colonies might be missed out in manual counting. In automated counting, setting the size gate of counting too small would introduce false positive results.

BT-474 cell line is known to have a slow growth rate, with the doubling time of 100 hours (Faltus et al., 2004). The time required for BT-474 to grow into a colony of 50 cells, the cell number defined as a proper colony (Franken et al., 2006), would take more than 200 days. Therefore, clonogenic assay might not be a good assay to study cell viability of BT-474 cell line. Alternative methods such as live/dead discrimination by trypan blue staining and cell metabolism activity assay using metabolic dyes like 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) could be considered (Stoddart, 2011).

7.5.4 Detection of phosphorylated proteins

In this thesis, only one phosphorylation site was analysed for each protein; however, most of the proteins in this study, such as the HER receptors and Akt, have multiple phosphorylation sites. Assessing only one phosphorylation site in WB and IHC might not have revealed the whole picture of cell signalling after lapatinib treatment, especially for Akt, in which Ser473 and Thr308 are regulated by different upstream proteins (Manning and Cantley, 2007). In future studies, assessing more than one phosphorylated form of each protein is recommended.

7.5.5 Densitometry of western blots

Using digital imaging system, such as ImageQuant LAS 4000 in this study, allows quantification of band intensity of western blots with high sensitivity and standardised exposure. However, western blotting is only semi-quantitative and differences in band intensity are not linear. In addition, taken into account of the errors in the experimental procedure, such as uneven protein transfer, the results are highly variable. Therefore, it is hard to detect statistical significant results from only three experimental repeats. In fact, western blots quantification is not a standardised technique and different procedures of quantifying an identical blot result in a huge variation of p-value, from 0.000013 to 0.76 (Gassmann et al., 2009), suggesting that western blots densitometry might not be very reliable.

Mass spectrometry enables precise identification and quantification of proteins (Gillette and Carr, 2013); however, the major disadvantage of this technique is the high cost.

7.5.6 Immunohistochemistry staining

IHC is a semi-quantitative method to detect protein expression in tissue samples. The major limitations of this technique are the subjective interpretation on scoring, the

variation in antibody performance and the cut-off points used to define high expression from low expression. In this study, for example, the phospho-HER4 antibody performed better than the total HER4 antibody, giving confusion that phospho-HER4 level was higher than total HER4.

Automating the scoring process using computer algorithm could reduce subjective interpretation of scoring; however, the automation algorithm is still under development in the Oxford Centre for Histopathology Research.

7.5.7 Cell cycle analysis by propidium staining

Propidium staining only produced a one-dimensional analysis of cell cycle. Although the peaks of G1- and G2/M-phases are clear in the histogram, the resolution of S-phase is often not high enough to distinguish the overlapping regions between S-phase and G1- or G2/M-phases.

S-phase resolution can be increased by incubating the cells with a thymidine analogue, bromodeoxyuridine (BrdU), before cell harvesting. BrdU incorporate into DNA during DNA replication and can be detected by fluorophore-conjugated anti-BrdU antibody (Cecchini et al., 2012). By detecting the amount of BrdU in the cells, early and late-S phases can be distinguished from G1- and G2/M-phases respectively.

7.6 Future work

7.6.1 Role of HER3 ligands in lapatinib resistance

SGP1 antibody showed that lapatinib-resistant cell lines are dependent on HER3 ligands; however, subsequent ELISA and NRG1 knockdown experiments were not conclusive on the role of NRG1 in this context. Because of the low expression of NRG1 in SK-BR-3 and BT-474 cell lines and the presence of its multiple isoforms, the detection of NRG1 protein may require a more sensitive method such as mass spectrometry. Knockdown of NRG1

needs to be validated and optimised in the HER2-overexpressing breast cancer cell lines and their corresponding lapatinib-resistant lines. However, it should be also noted that the HER3 ligand involved in lapatinib resistance maybe NRG2 instead. Expression of NRG2 in response to lapatinib and in lapatinib-resistant cell lines should be accessed, and knocking down of NRG2 will be required to confirm the role of NRG2 in lapatinib resistance.

The recently published model of NRG1-induced lapatinib resistance suggested that the expression of this ligand predicts poor recurrence-free survival in breast cancer patients treated with conventional therapies (Xia et al., 2013). However, the role of NRG1 in lapatinib response in patients was not addressed. As NRG1 reverses the inhibitory effect of lapatinib, it is important to study the correlation between NRG1 expression and lapatinib response in clinical samples. The clinical trials of lapatinib such as NeoALTTO could provide valuable samples and the information on patient responses for this study. It is hoped that when the validation of NRG1 IHC staining is done, applications will be made to access breast tumour samples treated with lapatinib (e.g. NeoALTTO) to further validate the role of NRG1 in relation to lapatinib sensitivity and resistance in HER2-overexpressing breast cancer patients.

7.6.2 Combination of lapatinib and pertuzumab

In vitro and *in vivo* experiments on the combination of lapatinib and pertuzumab showed a possible additive effect of the two drugs. The combination inhibited ligand-dependent HER2/HER3 and HER2/HER4 signalling *in vitro*; however, the mechanism of action of this combination *in vivo* is not clearly understood. More work is to be done to investigate other possible mechanisms such as activation of ADCC, as it was reported before that lapatinib enhances trastuzumab-induced ADCC (Maruyama et al., 2011; Scaltriti et al., 2009), which may also be a mechanism of action of pertuzumab *in vivo*.

Pertuzumab was shown to inhibit BT250LR cell line and the combination produced a trend of increased apoptosis, suggesting the combination may be effective in some cases of lapatinib resistance. The effect of this combination on lapatinib-resistant cell lines should be assessed using BT250LR xenograft.

7.6.3 Lapatinib-resistant cell lines

Two lapatinib-resistant cell lines, SK250LR and BT250LR, were generated to study the model of acquired lapatinib resistance; however, the two cell lines might have developed different mechanisms to adapt to long-term lapatinib treatment. As evidence based on one cell line is not enough to justify any mechanism, more acquired lapatinib-resistant cell lines from different parental cell lines should be developed.

Candidate cell lines to generate lapatinib-resistant strains include SUM-190, SUM-255, UACC-812 and UACC-893, which are lapatinib-sensitive HER2-overexpressing breast cancer cell lines (Konecny et al., 2006).

7.6.4 Alternative signalling pathway mediating lapatinib resistance

The SK250LR cell line showed a different mechanism of acquired resistance from the BT250LR cell line. Having its cell biology permanently changed, SK250LR cells were not dependent on HER signalling pathway for growth and survival and they were not sensitive to pertuzumab treatment. The RTK array revealed the activation of nine other RTKs in SK250LR cells. Apart from Axl and MSPR, other activated RTKs found in this study were not implicated in lapatinib resistance; therefore, they are potential targets of further studies in acquired lapatinib resistance.

The role of these RTKs should be confirmed by assessing their expression and activation in lapatinib-resistant cell lines using robust methods such as western blotting. By knocking down these RTKs and using specific inhibitors against them, these studies may lead to the

development of further strategies to tackle lapatinib resistance. These alternative pathways should also be validated in other lapatinib-resistant cell lines.

Finally, when the role of the above RTKs are confirmed, further drug combination strategies to tackle acquired lapatinib resistance can be formed. Table 7-1 shows some selected RTK-targeted therapies that inhibit the RTKs listed in Section 6.3. There is usually more than one targeted therapy for each RTK, but only the drugs that are most specific and most developed in clinical stages are reported here.

7.7 Concluding remarks

In this investigation, the phosphorylation of the HER receptors and the downstream effectors in HER2-overexpressing breast cancer cells was rapidly inhibited by lapatinib treatment. However, these proteins started to rephosphorylate as soon as after eight hours of lapatinib treatment. The two lapatinib-resistant cell lines generated in this study, SK250LR and BT250LR, adapted to prolonged lapatinib treatment in different mechanisms. The resistance in SK250LR cell line involved a permanent change in cell biology and hence non-reversible, but the resistance in BT250LR cell line was only transient and the withdrawal of lapatinib reverted its resistance.

It was demonstrated that blocking HER3 ligands from binding inhibited lapatinib-resistant cell lines. NRG1, as one of the potent HER3 ligands, rescued breast cancer cells from the effects of lapatinib, because lapatinib could not fully inhibit NRG1-stimulated HER3 and HER4 signalling. The expression of NRG1 was found to be increased upon short-term and prolonged lapatinib treatment. Knocking down NRG1 decreased cell growth of lapatinib-resistant SK250LR cell line, but the effect was not additive to lapatinib. No conclusion on NRG1 knockdown in BT250LR cell line could be drawn without further experiments.

RTKs	Drug Name	Company	Development stage	Mechanism of action
ALK	Crizotinib	Pfizer	Approved in lung cancer	ALK and ROS1 TKI
Axl	BGB324	BerGenBio & Rigel Pharmaceuticals	Phase I clinical trial	Axl TKI
Dtk	BMS-777607	Bristol-Myer Squibb	Preclinical	Pan-TAM, Met and MSPR TKI
EphA4	2,5-Dimethylpyrrolyl benzoic acid derivatives	University of California, San Diego	Preclinical	EphA2 and EphA4 ligand binding inhibitor
EphA10	No drug available			
FGFR3	AZD4547	AstraZeneca	Phase I clinical trial	Pan-FGFR TKI
MSPR	Narnatumab	Eli Lilly and Company	Phase I clinical trial	Monoclonal antibody to MSPR
c-Ret	Sorafenib	Bayer and Onyx Pharmaceuticals	Approved in advanced renal cell carcinoma and unresectable hepatocellular carcinoma	VEGFRs, BRAF and c-Ret TKI
RYK	RWD1	The University of Melbourne	Preclinical	Monoclonal antibody to RYK
Tie-2	Trebananib	Amgen	Phase III clinical trial	Peptibody against Ang1 and Ang2

Table 7-1 Potential drugs that can be combine with lapatinib to tackle lapatinib resistance

Data from references (Amgen, 2013; FDA, 2011; Gavine et al., 2012; Halford et al., 2013; Morandi et al., 2011; Noberini et al., 2008; Sheridan, 2013; Suarez et al., 2013; Yao et al., 2013).

The combination of lapatinib and pertuzumab inhibited NRG1-induced signalling better than either drug alone. The combination also processed additive anti-cancer effect *in vitro* and *in vivo* compared to monotherapy. This study proposed a novel and promising strategy in drug combination in clinical HER2-targeted therapy.

Future work on the role of NRG1 in lapatinib-resistant BT250LR cell line requires the use of other siRNAs or an inducible knockdown in conjunction with real-time monitoring. The role of another HER3 ligand, NRG2, should also be studied in the context of lapatinib resistance. The lapatinib and pertuzumab combination will be tested *in vivo* to investigate its anti-tumour effect on the lapatinib-resistant BT250LR xenograft model. More lapatinib-resistant cell lines should be generated to confirm the findings in alternative signalling pathways that may mediate lapatinib resistance.

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