Herceptin Resistance in HER2-Moderately Expressed Breast Cancer

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Abstract

HER2 overexpression occurs in approximately 15-20% of all breast cancers and is associated with poor prognosis. The development of Herceptin, a monoclonal antibody against HER2, has been significant in prolonging survival of these patients. Herceptin is less efficient in patients who do not overexpress HER2, so-called HER2-negative, such that they are not eligible for treatment with this drug. An incomplete understanding of Herceptin action and HER2 classification means that these patients may unnecessarily be being denied targeted therapy. In this thesis I show that an inability of Herceptin to maintain decreased HER3 and AKT activation in a subset of HER2-negative breast cancer cell lines (those that are HER2 2+, or HER2-moderately expressed) is responsible for reduced response. Surprisingly HER3 activation may also have a role in propagating the effects of Herceptin in these cells, making this receptor a central player. This study also shows that HER2-moderately expressed cells can rely heavily on ADAM-mediated ErbB receptors for normal survival and that targeting these by means of ADAM or tyrosine kinase inhibitors may present a better therapeutic option for these patients.

Abbreviations

ADAM:	A Disintegrin and Metalloproteinase
ADAM17i:	ADAM17 inhibitor
ADCC:	Antibody-Dependent Cell Cytotoxicity
AKT /pAKT:	Protein Kinase B / phospho-AKT
AKTi:	AKT inhibitor
BSA:	Bovine Serum Albumin
BTC:	Betacellulin
CDK2:	Cyclin Dependent Kinase 2
CEP17:	Chromosome 17
CHF:	Congestive Heart Failure
CTA:	Clinical Trials Assay
DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribose Nucleic Acid
ECL:	Enhanced Chemiluminescence
EGF:	Epidermal Growth Factor
EGFR:	Epidermal Growth Factor Receptor
ER:	Oestrogen Receptor
ERBB:	Erythroblastic Leukaemia Viral Oncogene Homologue
FACS:	Fluorescent Activated Cell Sorting
FBS:	Foetal Bovine Serum
FcγR:	Fc Gamma Receptor

FDA:	Food and Drug Administration
FGF:	Fibroblast Growth Factor
FISH:	Fluorescence In Situ Hybridisation
FOXO:	Forkhead Box O
FRET:	Forster Resonance Energy Transfer
GRB2:	Growth factor Receptor-Bound protein 2
HER/pHER:	Human Epidermal growth factor Receptor / phospho-HER
HGF:	Hepatocyte Growth Factor
IGF:	Insulin Growth Factor
IGF-IR:	Insulin Growth Factor 1 Receptor
IHC:	Immunohistochemistry
IRS1:	Insulin Receptor Substrate
LDS:	Lithium Dodecyl Sulphate
LVEF:	Left Ventricular Ejection Fraction
MAPK:	Mitogen Activated Protein Kinase
MBC:	Metastatic Breast Cancer
MET:	Hepatocyte Growth Factor Receptor
PBS:	Phosphate-Buffered Saline
PBST:	Phosphate-Buffered Saline with Tween
PFA:	Paraformaldehyde
PI3K:	Phosphatidylinositol 3-kinase
PTB:	Phosphotyrosine Binding
PTEN:	Phosphatase and Tensin Homologue
RNAi:	Ribose Nucleic Acid interference

RTK:	Receptor Tyrosine Kinase
SDS:	Sodium Dodecyl Sulphate
SH2:	Src Homology 2
SHC:	Src Homology 2-domain Containing
siRNA:	Small Interfering Ribose Nucleic Acid
TGFα:	Transforming Growth Factor alpha
TKI:	Tyrosine Kinase Inhibitor
TNFα:	Tumour Necrosis Factor alpha
VEGF:	Vascular Endothelial Growth Factor

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1 Introduction

Accounting for approximately 12,000 deaths a year in the UK alone, breast cancer remains significant among the female population. Despite rigorous research, the complex heterogeneities that exist among breast cancers means that clinical treatment relies heavily on non-specific chemotherapy and radiotherapy. While able to prolong survival, these often result in harmful side-effects due to destruction of normal as well as cancerous cells. The increasing presence of targeted therapy is more attractive since these drugs target attributes unique to cancer cells, such as over-expression of a receptor protein. Endocrine therapies and trastuzumab (Herceptin), a monoclonal antibody, are available for breast cancer patients who are oestrogen receptor (ER) or HER2 positive, respectively.

Herceptin is only prescribed for the 15-20% of breast cancer patients who overexpress HER2, i.e. are HER2-positive. Due to the lesser effect in breast cancers that do not overexpress HER2, so-called HER2-negative, these patients are not eligible for Herceptin treatment. Current methods for determining HER2 status rely on immunohistochemistry (IHC) and fluorescence in situ hybridisation (FISH) but both are subject to ambiguities meaning that patients who might respond are denied treatment, and vice versa.

Despite their higher efficacy compared to conventional treatments, current targeted therapies are not without the problems of side effects and resistance. One option to delay the onset of resistance is to administer Herceptin in combination with chemotherapy. If we are able to understand the mechanism behind Herceptin resistance in HER2-negative breast cancers we might be able to improve the targeted therapy regimen to make these patients eligible and reduce the reliance on chemotherapy.

1.1 The Molecular Mechanisms of Cancer

1.1.1 Oncogenes and Tumour Suppressor Genes

In a landmark review, Hanahan and Weinberg proposed that during transformation from normal to malignant state, cells adopt six specific alterations, including limitless replicative potential and evading cell-death (1). The cause of these changes can be attributed to aberrant genetic activity of two classes of genes, oncogenes and tumour suppressor genes (2). Oncogenes are mutated variants of genes that can potentiate cell signalling, so called proto-oncogenes; their mutations exhibit dominant characteristics. Tumour suppressor genes are concerned with guarding cells against transformation thus mutations that cause loss-of-function frequently lead to cancer (3). Being recessive in nature they are more common among inherited cancer genotypes (4).

A tumourigenic mutation in a proto-oncogene will result in either overexpression or increased activation of its protein product (5). The converse occurs for the products of mutated tumour suppressor genes. The subsequent excessive signalling of pathways that control cell survival, proliferation and migration can co-operate and further increase the potential for transformation (2).

1.1.2 Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are encoded by proto-oncogenes and are situated in the cell membrane. All members have sequence homology, comprising a ligand-binding extracellular domain, a single transmembrane helix and an intracellular tyrosine kinase domain (6). They are key mediators of various cell signalling processes such as survival,

proliferation and differentiation. This diversity is made possible by the panoply of signalling pathways they can activate.

In general, activation of RTKs requires growth factor-induced dimerisation (7). Dimerisation occurs in various forms depending on the receptor, with the ligand sometimes contributing to the dimerisation interface (6). Upon activation, intracellular tyrosine residues are autophosphorylated, producing docking sites for SH2 and PTB-containing signalling proteins, such as GRB2 (8, 9). These binding events activate signal transduction pathways such as MAPK and PI3K/AKT, key processes for cell survival and proliferation (10, 11). PI3K can interact directly, through its p85 regulatory subunit, or indirectly, via adaptor proteins such as GRB2 and IRS1, with receptor tyrosine kinases as well as other membrane receptors (11). Activating mutations of PI3K have been characterised that lead to transformation of cells (12, 13).

The multiple phosphotyrosine sites that arise on the C-terminal tails of RTKs, and the many docking proteins they can recruit, explain why they can have such a significant effect on cell signalling (14, 15). Aberrant RTK activity can lead to transformation and makes them a key family of oncogenes. Members of the ErbB family, IGF, FGF and Met receptors are frequently overexpressed or mutated in various cancers (16-20).

1.1.3 The ErbB Family

The ErbB protein family comprises four closely related, type I receptor tyrosine kinases: EGFR (also called HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) (10). Both homodimerisation and heterodimerisation can occur between members, in various combinations (21). The specific tyrosine residues that are phosphorylated vary for different

dimer combinations and the ligands that activate them, determining which signalling pathway is stimulated. The two major downstream transduction pathways are the MAPK and PI3K/AKT pathways (22).

EGFR

EGFR was the first member of the ErbB family to be shown to have a role in cancer; since then the other members have also been implicated in the disease (23). Whilst common in many tumours, EGFR overexpression is not sufficient for transformation. Studies have shown that the presence of a ligand or an activating mutation in the extracellular domain is also required, the latter of which is seen is in a variety of cancers. (22, 24). An inability to degrade EGFR by the Cbl pathway may also contribute to transformation (25).

EGFR binds a variety of ligands, including EGF, BTC and TGFα (26). Its preferred dimerisation partner is HER2 and it undergoes endocytosis much more rapidly than other ErbB members (27). EGFR can activate both the MAPK and PI3K pathways via GRB2 (6, 28).

HER2

Unlike the other receptors, there is no known ligand for HER2 (29). Instead it exists in a constitutively active, ‘open’ conformation, requiring no ligand for its dimerisation (30). The lack of intracellular binding sites for the PI3K regulatory subunit, p85, means HER2 homodimers are more prominently associated with activation of the MAPK pathway but are able to indirectly activate the former via GRB2 (10). Being the preferred dimer partner of

the other ErbB receptors, it is readily involved in activation of the PI3K/AKT pathway by means of HER3 or HER4 (31).

HER2 amplification and overexpression occurs in 18-20% of breast cancers as well as a host of other tumours (10). Its overexpression increases the formation of homo- and heterodimers, stimulating cell survival and proliferation pathways (22). HER2 overexpression is an early occurrence in transformation and correlates with poor prognosis in breast cancers (16, 32). This has been useful in the development of the drug Herceptin, as discussed in section 1.2.

HER3

HER3 is unique to the ErbB family in that it has a defective tyrosine kinase due to the alteration of three of its internal residues (33). These alterations prevent HER3 from binding the necessary adenosine triphosphate (ATP) (34). HER3 is stimulated by neuregulin 1 (also known as heregulin) and neuregulin 2 but its inactive tyrosine kinase means it relies more heavily on heterodimerisation than other members of the family (10). HER3 has been shown to activate the MAPK pathway via dimer formation as well as being a strong activator of the PI3K/AKT pathway, owing to its six docking sites for the p85 subunit (34, 35).

HER2-HER3 is considered to be the most active dimer within the family (36). The lack of ligand binding for HER2 and the absence of an active kinase domain in HER3 allows these receptors to exploit the benefits of a horizontal signalling network through dimerisation (21, 22, 33). The increase in membrane-bound HER3 in correlation with overexpression of HER2 in cell lines was shown to drive oncogenic signalling (37). These oncogenic properties can be successfully inhibited by preventing HER2-HER3 dimer formation with

the drug pertuzumab (38). Importantly, HER3 has been shown to play a role in breast cancers (39, 40) and is believed to underlie acquired resistance to small-molecule tyrosine kinase inhibitors (37, 41). Its importance in cancer is comparative to that of HER2, showing a much more significant role than EGFR (38).

HER4

HER4 has an extracellular structure similar to HER3, with an ability to bind the neuregulins (26), and an intracellular structure and function similar to EGFR (42). Whilst the presence of HER4 has been observed in breast carcinomas (43, 44), its expression levels are moderate to low in comparison to those of EGFR and HER2 (45, 46) but show a correlation with the expression of oestrogen receptor, unlike other members of the family (47). Docking sites for p85, SHC and GRB2 allow HER4 to activate both the PI3K/AKT and MAPK pathways (48-50).

1.1.4 The ADAMs Family

A Disintegrin and Metalloproteinase (ADAM) proteins are transmembrane and all contain the two eponymous domains. Among the 29 members only 17 have an active metalloproteinase and the function and distribution varies across the family [reviewed in 51]. Some ADAMs exhibit a sheddase function whereby they cleave membrane bound proteins; these include enzymes, receptors and growth factors. ADAMs can therefore have a strong influence on various signalling pathways.

One of the most studied ADAMs with sheddase activity is ADAM17; also named TNF-α Converting Enzyme (TACE) due to its ability to cleave and activate the cytokine TNF-α. Subsequently it has also been shown to cleave ErbB ligands, including Betacellulin and Heregulin (51, 52). The consequent activation of ErbB receptors drives dimer formation and increases signalling promoting cell survival. Activation by either AKT (52) or MAPK (53) makes ADAM17 integral to feedback loops that can reduce the efficacy of drugs targeting the ErbB family (52). Its role in various types of cancer has been reported (52, 54).

ADAM17 is initially inhibited by its pro-domain which is later cleaved by Furin, allowing for activity (55). Activity can be further regulated by means of phosphorylation. In the case of MAPK, phosphorylation of ADAM17 at threonine-735 is required for ectodomain shedding of ligands (53). Substrate recognition by ADAM17 is determined by residues in its active site, particularly valine-440 (56), as well as the sequence and position of the cleavage site of the substrate (57, 58).

1.2 HER2 in Breast Cancer

1.2.1 Classification

IHC

HER2-overexpression occurs in approximately 20% of all breast cancers (16). This overexpression is based on a classification system that uses immunohistochemistry (IHC). Tumours with uniform intense staining of HER2 in 30% or more cells are considered HER2 3+ and classed as HER2-positive (59). HER2 0 (no staining or membrane staining in less than 30% of cells) and 1+ (faint membrane staining in greater than 30% of cells) tumours are classed as HER2-negative. HER2 2+, or HER2-moderately expressed, tumours are considered equivocal and sent for further analysis by fluorescence in situ hybridisation (FISH) (60).

FISH

In FISH, fluorescent probes bind to regions of DNA with which they have high affinity. By specifying construction of these probes, the amplification of a specific gene can be analyzed. Gene copy number, and ratio of the gene with its resident chromosome are both determined for HER2 testing. Tumours are designated positive if the HER2 gene copy number is greater than 6 or if the HER2/CEP17 ratio (named for HER2's location on chromosome 17) is greater than 2.2 (61-63). Amplification of the HER2 gene has been shown to be directly linked to protein expression (64).

1.2.2 Importance of Classification

Having a classification system for HER2 is important because it is a strong predictor of prognosis and response in breast cancer (16). Patients with positive HER2 gene amplification and protein overexpression have shorter disease-free and overall survival due to more aggressive tumour behaviour (65-67). However, these patients respond significantly better to the drug Herceptin (discussed in detail in Section 1.3) than HER2–negative patients and thus only the former group are prescribed the drug (68-70). The same responses are seen in patients with high levels of HER2 extracellular domain in their serum (71, 72).

Not only has the ability to predict disease progression and eligible candidates for Herceptin been extremely beneficial for clinical treatment but also for drug trial selection and design (73). Before revised definitions, HER2 2+ patients by IHC were automatically classed as HER2-positive (now FISH-positivity is also required) (59). They, along with HER2 3+ patients, were selected for the initial pivotal trials with Herceptin (69, 70, 74). This initial IHC was done using the Clinical Trial Assay (CTA). For Herceptin therapy selection, the more stringent FDA-approved Dako HercepTest and Ventana Pathway are now used for quantification (73).

It is important to consider FISH as well when designing clinical trials and selecting eligible patients. Although there is a high correlation between HER2 and FISH statuses (one study found a significant 82% concordance (73)), the disagreements are important to consider. Studies on tissue samples have shown that for 0, 1+ and 3+, the concordance with FISH is almost always above 90% (73, 75). However, in one particular study approximately 12% of patients who were IHC 3+ were FISH-negative (73). Since FISH is considered to be more sensitive and accurate than IHC (76), patients unlikely to respond to Herceptin could be

included in a trial resulting in an underestimation of the drug's efficiency. Conversely approximately 10% of the HER2 0 and 1+ patients were shown to be FISH-positive and thus may have responded to Herceptin but would not be considered purely on IHC. The same study showed that for HER2 2+ IHC tumour samples, only 24% returned a positive FISH result, highlighting the ambiguity of this class and the extended need for FISH analysis (73). It also highlights that approximately three quarters of HER2-moderately expressed (HER2 2+) breast cancer patients are not eligible for Herceptin treatment.

1.2.3 The problems with IHC and FISH

Whilst figures from the study mentioned above (73) are quite remarkable, it should be noted that this is a single-lab study. Testing at multiple labs can take into account inter-lab variability which has been seen to reduce false-positives by greater than 10-fold (77); this is due to the equivocal nature of IHC and FISH. Upon retesting, results from two trials found that 9.7% of tumours initially defined as HER2-positive were in fact HER2-negative for both IHC and FISH classification (78). Therefore whilst concordance between IHC and FISH is important, it is not a fail-safe for selection criteria (79).

IHC results can vary depending on the methods used for tissue fixation and processing. IHC assays were found to be less accurate for paraffin-embedded samples compared to fresh frozen specimens (16). IHC also suffers due to the fact that it relies on human interpretation and scoring, which can vary from one person to next (73). Staining will also vary depending on the area of the tumour analyzed, the type of sample taken and between primary and secondary metastases.

Despite being considered the “gold standard”, FISH is prone to generating false negatives. The HER2/CEP17 ratio is uninformative in cases of chromosome 17 polysomy (8%-27% of all breast cancers) where gene amplification may appear normal despite more copies than in a normal breast cells (80). Further to this, the HER2 FISH probe spans a DNA region twice the length of the gene, in a region that is considered a hot-spot for genomic instability; this may give more generalised results on the instability than simply HER2 (81).

As a result of HER2-negative patients slipping through the net, the trials revealed that these patients can also respond to Herceptin (78). This suggests that our previous understanding of Herceptin response is denying some women from receiving the drug despite a potential benefit (80). General concern about excluding patients who may benefit from Herceptin treatment is becoming more apparent (80), so much so that plans to specifically test HER2-negative breast cancer patients with Herceptin have been planned (81).

A preclinical experiment has confirmed that HER2-negativebreast tumours may respond to Herceptin, by inducing expression of heregulin in MCF-7 cells and then treating with Herceptin and chemotherapy (82). Therefore, there is a need to further understand the mechanisms of resistance to Herceptin in HER2-negativebreast cancer cells so that we can propose suitable strategies to overcome drug resistance to benefit breast cancer patients.

1.3 Treatment

1.3.1 Herceptin

Trastuzumab (Herceptin, Genentech, South San Francisco, CA) is a humanised monoclonal antibody 4D5 against juxtamembrane domain IV of HER2 (83). Early work on Herceptin showed that it was most effective against cell lines with HER2 gene amplification and protein expression (83, 84). Significant clinical responses were observed in patients with HER2-positive breast cancer when Herceptin was applied in the adjuvant setting. (69, 74, 85, 86). As a single agent in first-line treatment, Herceptin induced an overall response rate of 19-26% in metastatic breast cancer (MBC) (70, 87). As part of adjuvant first-line treatment, a 20% reduction in risk of death was observed (69). Herceptin was later assessed in early breast cancers and found to increase time to disease progression, reduce risk of recurrence and increase pathological complete response and overall survival (86-89).

Although not fully understood, various mechanisms of Herceptin have been postulated. Physical actions against HER2 include internalization and degradation of the receptor (90) as well as inhibition of extracellular domain cleavage (91). Induction of the cell-cycle inhibitors PTEN and $p27^{kip1}$ allows Herceptin to prevent cell proliferation and survival by antagonising PI3K and CDK2, respectively (92, 93). By upregulating p21 activity, Herceptin inhibits DNA synthesis (94, 95). Extracellularly, Herceptin can induce antibody-dependent cell cytotoxicity (ADCC) by interacting with Natural Killer T cells (96, 97). It can also reduce angiogenesis by downregulating VEGF (98, 99).

Despite the successes seen with Herceptin it is not free from side-effects. Four major Herceptin trials observed cardiotoxic events including congestive heart failure (CHF) and a

decrease in left ventricular ejection fraction (LVEF) (100). A long-term treatment of metastatic breast cancer saw 28% of patients develop some form of cardiotoxicity. Most of these events were however reversible (101).

Resistance to Herceptin has also impeded its success in the clinic. This is something that is discussed in detail in Section 1.4.

1.3.2 Other monoclonal antibodies

Of the other anti-cancer monoclonal antibody treatments, pertuzumab (2C4; Genentech, South San Francisco, CA) stands out as having a potential use for HER2-positive breast cancer. Its ability to bind subdomain II of HER2 inhibits dimerisation of this receptor with other ErbB receptors, and subsequent signalling (102, 103). Pertuzumab has been shown to inhibit cell morphogenesis and induce tumour regression in HER2-amplified breast xenograft models (38). Its ability to prevent HER2/HER3 dimer formation is considered more relevant than inhibition of EGFR/HER2 dimers. One study showed that whereas HER3 knockdown decreases proliferation of breast cancer cells to a similar extent as HER2 knockdown, EGFR inhibition had relatively little effect. Pertuzumab therefore harbours more clinical benefit for HER2-positive breast cancer patients than monoclonal antibodies that target EGFR, such as cetuximab (38). There is currently no drug that targets HER3 but pertuzumab has been shown to be synergistic with Herceptin (104). Phase II trials of the drug in HER2-positive ovarian cancers have improved progression-free survival (105).

1.3.3 Tyrosine kinase inhibitors

The development of small molecule inhibitors that target the tyrosine kinase domains of the ErbB receptors has presented another therapeutic approach. Lapatinib, a reversible tyrosine kinase inhibitor (TKI) against EGFR and HER2, has been shown to induce growth arrest and cell death in a variety of cancer cell lines (106). It was also shown to improve response rates and time to progression in a phase III trial with MBC patients (107). This and its ability to be effective against Herceptin-resistant patients (108) have led to FDA-approval for its use in conjunction with capecitabine following a trastuzumab-containing regimen (109). Since EGFR and HER2 are the two main trans-activators of HER3 and HER4, lapatinib exhibits a pan-HER dephosphorylation. Based on previous discussion, this helps us to understand its success in targeting HER2-positive breast cancers.

Both *in vitro* and *in vivo*, lapatinib has been shown to inhibit the MAPK and PI3K/AKT signalling pathways (110). Its direct dual-HER and indirect pan-HER effects may explain why it is able to induce responses in cancers that are resistant to TKIs targeting just one receptor such as the EGFR-specific gefitinib (Iressa) (111). Various TKIs have shown additive or synergistic effects with Herceptin *in vivo* and *in vitro* (52, 112-115), and presented a way to overcome Herceptin resistance (116). Unfortunately cases of resistance to TKIs are already being observed (37, 41).

Relatively recently, the benefits of the drug Neratinib have become apparent. This irreversible panHER TKI has been show to be a direct potent inhibitor of both EGFR and HER2 activity, and their downstream signalling pathways (117). This study also showed a significantly strong ability of the drug to inhibit tumour growth in xenograft models.

Neratinib has also been shown to act synergistically with Herceptin in HER2-positive xenograft models (52). A clinical trial has shown that Neratinib successfully resulted in a 16-week progression-free survival in 59% of patients with advanced HER2-driven breast cancer with prior Herceptin treatment, and 78% with no-prior Herceptin treatment (118). Its irreversibility and relatively high potency makes it a good candidate for investigating HER2-negative breast cancers.

1.4 Herceptin Resistance

Despite its notable success in the clinic, primary resistance to Herceptin is observed in approximately 70% of HER2-positive patients who receive the drug alone, and between 20%-50% of patients receiving combination therapy (59, 119). Further to this, almost all HER2-positive patients that show an initial response develop resistance if treatment continues as monotherapy (120). Various mechanisms of resistance have been investigated and are described below. Elucidation of these mechanisms has helped us understand second-line therapies that may benefit patients with these underlying causes.

p95HER2

Clinical trials have shown that almost all patients expressing an amino-truncated HER2, p95HER2, do not respond to Herceptin due to its lack of the Herceptin-binding domain IV (121). Basal proteolytic cleavage of HER2, producing p95HER2, is observed *in vivo* and potentially highlights the importance of early Herceptin treatment since the drug has been shown to inhibit this extra-cellular domain shedding (122). Similar resistance patterns were observed in HER2-negative cells transfected with p95HER2 and in corresponding xenograft models. These tumours were however significantly responsive to lapatinib due to p95HER2 still possessing its tyrosine kinase domain (121). This suggests that these resistant cells still heavily rely on HER2 signalling for cell proliferation. A separate study reports that tyrosine kinase activity of truncated HER2 is higher than its full-length counterpart (123).

EGFR/HER3

Interestingly, cell lines exhibiting primary Herceptin resistance showed higher levels of EGFR and HER3 when exposed to the drug for a 12-week period, compared to the parental cell lines (124). This shows that although significant cell death did not occur, the cells were still responding to Herceptin. Importantly these cells were more responsive to the anti-EGFR drugs gefitinib and cetuximab (124). Similar results have been shown using anti-HER3 monoclonal antibodies (125). Whilst this pattern of expression was only investigated in primary resistance, it may explain why patients in a study into secondary Herceptin resistance responded significantly to lapatinib (107). However, the targeting of HER2 as well as EGFR by lapatinib is also likely to contribute.

IGF-IR

A study by Nahta et al. (126) showed that unique to Herceptin-resistant cells, insulin-like growth factor-I receptor (IGF-IR), an unrelated receptor tyrosine kinase, can heterodimerise with HER2. Importantly, these cells could be resensitised to Herceptin by the addition of anti-IGF-IR drugs or pertuzumab. Subsequent studies have confirmed the presence of IGF-IR/HER2 dimers by immunoprecipitation (127). Increased expression or stimulation of IGF-IR has been shown to reduce the ability of Herceptin to induce $p27^{kip1}$ and subsequent G1 arrest (128, 129). More recently it has been proposed that HER2, HER3 and IGF-IR exist in a heterotrimeric complex and that jointly targeting all 3 receptors could be a more efficient way to overcome Herceptin resistance (130).

Met Receptor

The Met receptor tyrosine kinase has been shown to be highly expressed in HER2-positive breast cancers (17). Its role in resistance was shown when both knockdown and inhibition of the receptor could individually resensitise the cells to the Herceptin. Furthermore, ligand-stimulation of Met with hepatocyte growth factor (HGF) enhanced the resistance by disrupting the induction of p27. Interestingly, exposure to Herceptin resulted in the cells upregulating Met, therefore exacerbating their resistance.

PI3K

Mutations in exons 9 and 20 of the PIK3CA gene, encoding p100, have been identified in approximately 25% of breast cancers. The resultant increase in PI3K signalling is responsible for shorter progression-free survival when these patients are treated with Herceptin (131).

PTEN

Loss or mutation of PTEN has been described in up to 50% of breast cancers (132) since this will allow for uncontrolled activation of the PI3K/AKT pathway (133). Knockdown of PTEN in tumour xenografts was shown to significantly reduce the response of tumours to Herceptin, compared to controls. In agreement with this, patients with PTEN-deficient breast tumours also show a reduced clinical response to Herceptin. Due to the effects of PTEN being antagonistic to PI3K, inhibition of the latter was sufficient for restoring

Herceptin sensitivity in both PTEN-knockdown cells and xenograft models (92), presenting a potential second-line treatment for these patients.

P27^{kip1}

Knockdown of p27^{kip1} blocks Herceptin-mediated growth arrest in HER2-positive cell lines (134). Its role in Herceptin resistance was confirmed by a study showing that levels of p27^{kip1} were lower in two pools of Herceptin-resistant cells compared to the parental cell line (135). The reduced levels of p27^{kip1} were shown to be responsible for an increase in CDK2 activity and proliferation rate. Sensitivity to Herceptin could be restored by ectopic expression of p27^{kip1}. The resistant pools were also shown to be more sensitive to a pharmacological induction of p27^{kip1} compared to the parental cell line. This therefore presents a possible therapy for patients displaying p27^{kip1}-mediated resistance.

ADAM17

A study (52) on ADAM17 found that levels of this metalloproteinase increase as a result of Herceptin-mediated downregulation of AKT phosphorylation in resistant cells. Importantly this increased activity of ADAM17 led to shedding of the ErbB ligands betacellulin and heregulin, presenting a way to maintain cell proliferation in the presence of the drug. By treating with an inhibitor of ADAM17, these cells could be resensitised to Herceptin.

ADCC

Indirect factors, relating to ADCC, can also confer Herceptin resistance. In order to induce cytotoxicity, there must be sufficient interaction between the Fc region of the antibody and the Fc receptors. Polymorphisms of the activating IgG1 receptors on macrophages and natural killer cells, FcγRIIa and FcγRIIIa, were shown to reduce their contact with Herceptin and promoted resistance through diminished ADCC (136). Protein engineering of antibody Fc regions has been shown to increase effector function by increasing affinity for Fc receptors (137), and could provide a way to overcome this resistance.

1.5 Aims and Objectives

1.5.1 Aims

As mentioned in section 1.3, HER2-moderately expressed breast cancer patients frequently fall into the HER2-negative category and are denied Herceptin due to their poor response to the drug. The majority of previous studies on Herceptin resistance concern HER2-positive breast cancer. The overall aim of this thesis was to understand the mechanisms behind Herceptin resistance in HER2-moderately expressed breast cancer cell lines and use this in an attempt to propose a more effective targeted therapy against these tumours.

1.5.2 Objectives

The first objective was to see how two HER2-moderately expressed cell lines (MDA-MB-453 and MDA-MB-361) responded to varying concentrations of Herceptin, in comparison to a HER2-positive cell line (SK-BR-3) and a cell line with negligible HER2 expression (MCF-7). After treatment with Herceptin, cell viability was assessed by cell counting and compared to an untreated control. In parallel to these experiments, western blot analysis at the same concentrations of Herceptin but for various, often shorter, time points showed how the PI3K and MAPK signalling pathways initially responded.

Differential effects on these pathways in the presence of Neratinib allowed me to further understand their role in cell survival. Furthermore, Neratinib is a targeted therapy significantly different in action to Herceptin and thus suitable for investigating as an additive drug. Analogous to Herceptin experiments, cell lines were treated with varying

concentrations of Neratinib and cell viability was assessed. Western blot analysis was again used to determine the response in key signalling pathways.

The second objective was to build on observations of Herceptin resistance by manipulating proteins thought to be essential, in an attempt to confirm initial hypotheses. This was achieved by using either small molecule inhibitors or by knockdown of protein expression using targeted siRNA. For inhibitors, a range of concentrations was investigated in order to get an observable effect without killing the cells. Western blot analysis was used to determine the efficacy of the inhibition or knockdown and cell viability assays assessed the survival ability of cells. The hope was to observe additive effects between the inhibitors and Herceptin and potentially resensitise cells to Herceptin.

My final objective was to ascertain whether Herceptin and Neratinib can have an additive or even synergistic effect on HER2-moderately expressed cells. A synergistic effect would suggest an increase in the efficacy of Herceptin in these cell lines and provide a novel targeted therapy regimen that patients with similar tumours could be eligible for. This objective was carried out in much the same way as the first. Cell viability in the presence of each drug, as well as the combination, was analyzed by cell counting and signalling pathways were observed by western blots. Western blots helped validate hypotheses about key signalling pathway, from the first objective.

The three objectives can be summarised as follows:

1. To observe the response of HER2-moderately expressed cell lines to Herceptin and Neratinib treatments and the molecular mechanisms behind them.

2. To ascertain the role of apparently key signalling molecules in Herceptin resistance by disrupting their function and assessing the effects.

3. To determine the effect of Herceptin in combination with Neratinib on cell viability of HER2-moderately expressed cells.

2 Materials and Methods

2.1 Materials

2.1.1 Mammalian Cell Lines

SK-BR-3 and MCF-7 cells were provided by the services at Cancer Research UK. MDA-MB-453 and MDA-MB-361 were provided by American Type Culture Collection (ATCC).

2.1.2 Antibodies

Name	Species	Application	Source
EGFR (intracellular)	Rabbit	Western blot (1:1000)	Cell Signalling
Phospho-EGFR (Y1173)	Rabbit	Western blot (1:1000)	Cell Signalling
HER2 (intracellular)	Rabbit	Western blot (1:1500)	Cell Signalling
Phospho-HER2 (Y1221/1222)	Rabbit	Western blot (1:1000)	Cell Signalling
HER3 (intracellular)	Rabbit	Western blot (1:1000)	Cell Signalling
Phospho-HER3	Rabbit	Western blot (1:1000)	Cell Signalling
AKT	Rabbit	Western blot (1:3000)	Cell Signalling
Phospho-AKT (S473)	Rabbit	Western blot (1:1000)	Cell Signalling
p42/44 (Erk1/2)	Rabbit	Western blot (1:3000)	Cell Signalling
Phospho-p42/44 (T202/Y204)	Rabbit	Western blot (1:1000)	Cell Signalling
β-Actin	Rabbit	Western blot (1:3000)	Cell Signalling
ADAM17	Rabbit	Western blot (1:3000)	Abcam

2.1.3 Inhibitors and drugs

Drugs	Trade name	Typical dose	Company
Trastuzumab	Herceptin	80µg/mL	Roche
HKI-272 (panHER inhibitor)	Neratinib	10nM	Pfizer
AKT Inhibitor	AKT Inhibitor VIII	2.5µM	Merck
ADAM17 inhibitor	INCB4298	10µM	Incyte

2.1.4 Solutions

PBS: 130.37mM NaCl, 3.19 mM KCL, 9.6mM Na_2PO_4, 1.1mM $CaCl_2$, 1mM $MgCl_2$, pH 7.2

PBST: 0.2% (v/v) Tween 20 (polyoxyethylene-sorbitan monolaurate) in PBS

Trypsin: 2.5% (w/v) trypsin (10x), 146.55mM NaCl, no EDTA, no phenol red

Cell lysis buffer: 20mM Tris-HCL (pH 7.4), 150mM NaCl, 100mM NaF, 10mM $Na_2P_2O_7$, 10nM EDTA, supplemented with 1% (v/v) Triton X-100 (t-Octylphenoxypoly-ethoxyethanol) and 1 protease inhibitor cocktail tablet per 10mL of cell lysis buffer

4xLDS sample buffer: 250mM Tris-Cl (pH 6.8), 20% (w/v) glycerol, 4% (w/v) LDS, 0.01% (w/v) Bromophenol Blue, 50mM β-mercaptoethanol

20xSDS Running buffer: 50mM MOPS, 50mM Tris Base, 0.1% (w/v) SDS, 1mM EDTA, pH7.7

Tris-glycine transfer buffer: 25mM Tris, 192mM glycine, 20% (v/v) methanol

4% PFA (paraformaldehyde): 8g in 200mL PBS

2.1.5 Reagents

Application	Reagents	Source
Cell culture	Foetal Bovine Serum	PAA
	Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen PAA
	OptiMEM	Invitrogen
	Trypsin	Invitrogen
	Penicillin/Streptomycin	PAA
RNAi	FlexiTube siRNA Hs ERBB3 (5'-UUUAUGACCAGAU ACCGUGGT-3')	Qiagen
	AllStars Neg. Control siRNA (20nmol)	
Cell lysis	Protease inhibitor cocktail tablets	Roche
	Triton X-100	Sigma
Protein assay	Bio-Rad Protein Assay	Biorad
SDS-PAGE	4-12% pre-cast gels	Invitrogen
	NuPAGE 4xLDS sample buffer	
	Novex® Sharp Pre-stained Protein Standard	
	NuPAGE® MOPS SDS running buffer (20x)	
Western blotting	Immobilon®-P PVDF transfer membrane	Millipore
	Bovine Serum Albumin (BSA)	Sigma
	Medical X-ray film	Fujifilm GE Healthcare

2.2 Methods

2.2.1 Mammalian cell culture

SK-BR-3, MDA-MB-453, MDA-MB-361 and MCF-7 were cultured as monolayers in DMEM supplemented with 9% (v/v) FBS in flasks sizes appropriate for the number of cells. The flasks were incubated at 37˚C in a 5.0% CO_2 humidified atmosphere. Cells were usually split when they reached confluence to reduce the risk of transformation; a given cell culture was never grown for more than 40 passages. To split the cells old media was removed and cells were washed with PBS to remove dead cells and unwanted products. For medium-sized flasks cells were incubated with 1mL (3mL for large flasks; 0.5mL for small flasks) 0.25% trypsin (w/v) until the majority of cells had detached from the base of the flask. The trypsin was then diluted by a minimum of 1:10 with DMEM to inhibit the process. Depending on confluence, new cells were seeded at dilutions from 1:5 - 1:10.

2.2.2 Treatments

For western blots approximately 800,000 cells were seeded per well in a 6-well plate and left to settle for 24 hours. The desired drug concentrations were made up by diluting the stock in the required volume of DMEM. For concentration-dependent experiments, media was removed and wells were washed with PBS. A different concentration of drug was then applied to each well, all at the same time. For time-dependent experiments, each time point was calculated backwards from the end of the experiment. Only the longest treatment was applied at the start of the experiment, all other wells were supplied with untreated DMEM.

When the required time point was reached, that well had its media replaced with DMEM containing the drug.

2.2.3 RNAi

Cells in antibiotic-free DMEM were transfected with siRNA that had been incubated with oligofectamine in OptiMEM for half an hour. These cells were then seeded at approximately 200,000 cells per well in a 6-well plate and incubated for 24 hours. After this time, media was replaced with fresh antibiotic-free medium and cells were incubated for another 24 hours. Treatment was initiated after this second 24-hour time point.

2.2.4 Determination of protein concentration

Following completion of treatment, media was removed from the wells and cells were washed in PBS cooled to 4˚C. Once the PBS was removed, 130µL lysis buffer was added to each well and cells were collected using a cell scraper, centrifuged and the lysate retained. The protein concentration of each sample was determined using Bio-Rad's protein assay and a standard curve made up of known concentrations of bovine serum albumin (2, 4, 6, 8 and 10µg/mL); the Bio-Rad dye reagent was used as a blank. After a 1:5 dilution with water, 500µL of the dye was added to each of a number of eppendorf tubes (1 for each sample). 1 or 2µL of each sample was added to a different eppendorf (compared to 1µL for the protein standard), estimated based on size of pellet observed after centrifugation. After a 10-minute incubation at room temperature, two lots of 200µL from every sample were each added to a

well of 96-well plate. Absorbance of light at wavelength 595nm was analysed in each sample using a plate-reader. Values were compared to the standard curve.

2.2.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

4X sample buffer, with β-mercaptoethanol (in a 9:1 ratio), was mixed with each sample in a 1:3 ratio. The mixtures were then boiled at 90˚C for 10 minutes before being loaded on a pre-cast SDS-PAGE gel. The volume to load for each sample was determined based on the Bradford Assay to ensure equal protein concentrations. 10- and 12-well gels were run at 100V for 15 minutes before the voltage was increased to 130V and the gel was left to run until it reached the bottom of the chamber. For 15-well gels, the voltage was increased to 150V after an initial 130V for 15 minutes.

2.2.6 Protein transfer

Separated proteins from the gel were transferred to membrane using a Biorad Trans-Blot Semi-Dry Transfer machine. Before transfer, the membrane was equilibrated with methanol and sandwiched with the gel between two pieces of equal sized blotting paper that had been immersed in transfer buffer. After transfer, the membrane was used for western blot analysis.

2.2.7 Western blot analysis

Immediately after transfer the membrane was blocked overnight at 4˚C using 3% BSA (w/v) in PBST. The next day, the membrane was cut along the appropriate molecular-weight bands and each segment was incubated with the required antibody for 3 hours on rollers at room temperature. After this time each membrane was subjected to four 10-minute washes using 1% milk (w/v) in PBST. The membranes were then incubated for 1 hour with the appropriate secondary antibody at a concentration of 1:2500 in 5% milk (w/v). Following this, four more washes were done, this time for 15 minutes each. The membrane was subsequently re-assembled as one. ECL developing solution was added all over and left for two minutes. The membrane was enclosed in cling-film and secured in a development cassette before being developed on medical X-ray film at various time points.

2.2.8 Cell Counting

For the first half of my masters, cells were grown in 24-well plates for the purpose of cell viability. Approximately 20,000 cells were seeded per well, depending on growth rate of the cell line and time length of treatment. Cells were left to settle for 24 hours before treatment with different drugs at different concentrations. Treatment was conducted in an identical manner to that described in 2.2.2. For experiments consisting of solely 5-day treatments, the drugs were not replaced. However for 6- and 9-day treatments, drugs were replaced at every 3 day time point for all wells.

Each row of the well-plate constituted a replicate such that conditions varied along the columns. When cells were treated with drugs dissolved in DMSO, a DMSO control at the equivalent dilution was included. After completion of treatment cells were trypsinised for

between 10-15 minutes, depending on the cell line, and were pipetted thoroughly to ensure detachment. 400µL (of the total 500µL) from each well was added to separate vials containing 20mL of isotonic buffer. The cell count was measured using the cell counting machine. Due to machine inaccuracies at low cell counts (particularly relevant when effective drugs were used), 6-well plates were used for the second half of my masters. In this instance approximately 100,000 cells were seeded per well; each well plate accounted for one replicate.

2.2.9 Statistical Analysis

For cell viability, significance of results was measured using the Mann-Whitney U test. This compares medians of groups against each other and thus is non-parametric. This test was chosen since there was never more than nine data points in each set and normal distribution could not be assumed.

3 A role for AKT signalling in the response of HER2-moderately expressed cells to Herceptin and Neratinib

3.1 Introduction

Whilst there has been extensive research into Herceptin resistance in cells, the majority of this work has been conducted on HER2-positive cell lines. It has been reported that HER2-moderately expressed tumours show a greater degree of resistance to Herceptin than the former group (68-70). In an attempt to overcome this resistance, this chapter of the thesis proposes ideas why cells with this classification of HER2 expression respond in the way they do. Since it was previously shown that the combination of Herceptin with a panHER inhibitor is more effective than either agent alone in HER2-positive cells (52), in this project I would like to assess the effects of Herceptin and Neratinib, alone and in combination, in HER2-moderately expressed breast cancer cells.

3.2 Results

3.2.1 HER2-moderately expressed cell lines are more resistant to Herceptin than a HER2-positive cell line, but are susceptible compared to a negative control cell line

MDA-MB-453 and MDA-MB-361 breast cancer cell lines were chosen as models of HER2 2+ (moderately expressed) tumours. SK-BR-3 cells (HER2 3+ / HER2-positive) were used as a positive control. MCF-7 cells, which express HER2 at a negligible level, were used as a negative control. Figure 3.1 shows that these cell lines act as appropriate models for this

purpose. This figure also shows that there is considerable heterogeneity for EGFR and HER3 expression between cell lines, even comparing MDA-MB-453 and MDA-MB-361. Perhaps of most importance, the former has noticeably higher EGFR and HER3 expression than the latter but slightly lower HER2 expression. Although MDA-MB-453 and MDA-MB-361 cells were both chosen as central models for this thesis, the majority of the work has been done on MDA-MB-453 alone due to the slow growth and low proliferation rates of MDA-MB-361, and time restraints.

When treated with Herceptin for 5 days, the cell lines responded in a manner that corresponds to the literature and clinical trials (68-70) (figure 3.2). SK-BR-3 cells were relatively susceptible, displaying an IC_{50} around the standard dose of 40µg/mL. MDA-MB-453 and MDA-MB-361 cells responded less well despite a significant response for the initial 10µg/mL increase. No further significant change was observed for MDA-MB-453. MDA-MB-361 cells did not show another significant reduction in cell number until a concentration as high as 160µg/mL was used, compared to 10µg/mL. For MCF-7, treatment with Herceptin appears to stimulate proliferation, reaching a maximum at 80µg/mL within the range used. This could be an interesting course for follow-up in a relevant investigation but is beyond the scope of this project.

3.2.2 HER2-moderately expressed cell lines are more susceptible to Neratinib than Herceptin

Since previously our lab has shown that activation of alternative ErbB receptors mediates acquired resistance to Herceptin in HER2-positive cells, I hypothesised that the panHER inhibitor Neratinib could be effective in HER2-moderately expressed cells. My results

showed that all three HER2-negativecell lines responded better to Neratinib than they did to Herceptin (figure 3.2 & 3.3A). MDA-MB-453 reached IC_{50} at a concentration less than 10nM; for MDA-MB-361 it was between 10 and 40nM. The drug continued to have a significant reduction on cell number at concentrations above 10nM in a dose-dependent manner, in the HER2-moderately expressed cells (figure 3.3A). For MCF-7 cells, very little change was observed above this concentration; IC_{50} was never reached, even at 200nM.

Since Neratinib appeared as a better alternative to Herceptin in a concentration-dependent manner, time-dependent effects of both drugs were analysed in MDA-MB-453 cells (figure 3.3B). Herceptin has a marginal effect on the cell count of MDA-MB-453 cells but failed to reduce it to 50% of the untreated control (figure 3.3B). Furthermore, it lost its ability to decrease MDA-MB-453 cell number after 6 days, with no decrease observed between 6 and 9 days. Neratinib on the other hand continued to cause a decrease beyond the 6 day time point suggesting that its effects are longer-lasting, as well as more potent, than Herceptin (figure 3.3B).

3.2.3 Signalling by the PI3K/AKT pathway is a marker for response of HER2-moderately expressed cells to Herceptin and Neratinib.

Western blots analysing the PI3K/AKT and MAPK signalling pathways were examined in the HER2-moderately expressed cell lines and compared to the positive control in an attempt to understand the reasons for the observed responses to both drugs. When subject to identical increases in Herceptin concentration as the cell count experiments, SK-BR-3 cells showed an incremental decrease in PI3K/AKT pathway activation, represented by phospho-AKT (pAKT) levels (figure 3.4A & 3.4C). MDA-MB-453 and MDA-MB-361 on the other

hand both showed an initial decrease at 10µg/mL but minimal change at concentrations above this. Interestingly this trend is almost identical to that seen for cell number (figure 3.2) and may be causative of these observations.

To test this hypothesis further, the signalling pathways were analysed in response to Neratinib (figure 3.5A, B & C). As Neratinib concentration was increased, there was a steady reduction in the level of pAKT for MDA-MB-453 and MDA-MB-361, with SK-BR-3 cells showing higher sensitivity to the drug. MCF-7 cells showed relatively reduced changes in pAKT levels, even at concentrations as high as 200nM. This also correlates with cell count assays, supporting the previous hypothesis.

Interestingly, assessment of MAPK phosphorylation revealed an increase in SK-BR-3 cells in response to Herceptin. This observation has been reported elsewhere (52) but the its significance in this cell line is still not fully understood. Although MAPK activation does not correlate with cell count to the same degree as AKT, figures 3.4B and 3.5B show that this pathway is also affected by both Herceptin and Neratinib. Therefore, in order to confirm a specific role for the AKT pathway in survival, it was important to examine whether direct inhibition of AKT could cause a corresponding reduction to HER2-moderately expressed cell number. This inhibition was found to be very effective at 2.5µM, completely reducing pAKT levels after just one hour (figure 3.6B). This caused a strongly significant decrease in the cell count of MDA-MB-453 cells over a 5-day period (figure 3.6A).

Trends observed when various concentrations of AKT inhibitor (AKTi) were used showed that incremental increases in AKTi concentration determined cell survival in a similar manner (figure 3.6C). Interestingly there appeared to be an additive effect between the inhibitor and Herceptin when 250nM AKTi was used. This data, in conjunction with

observations that Neratinib can downregulate PI3K/AKT signalling, pointed towards a potential additive effect for Herceptin and Neratinib.

Phospho-AKT levels analysed at the end of the 5-day AKTi treatments (figure 3.6D), help to explain the trends seen in cell number. In the presence of 25nM AKTi, pAKT levels were similar to the DMSO control; cell number at this concentration remained relatively unaffected. After a 5-day treatment with 250nM AKTi, pAKT was negligible; this can account for the greater reduction in cell count at this concentration, compared to 25nM.

3.3 Figures

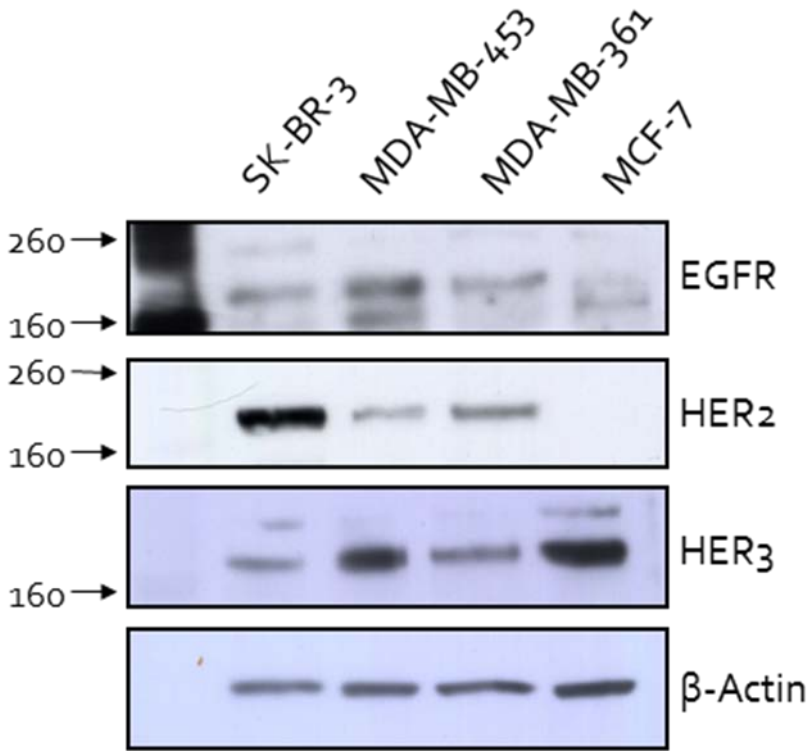


Figure 3.1: Different expression levels of ErbB family proteins between the four cell lines. Cells were incubated in 6-well plates for 24 hours before being collected and subject to western blotting.

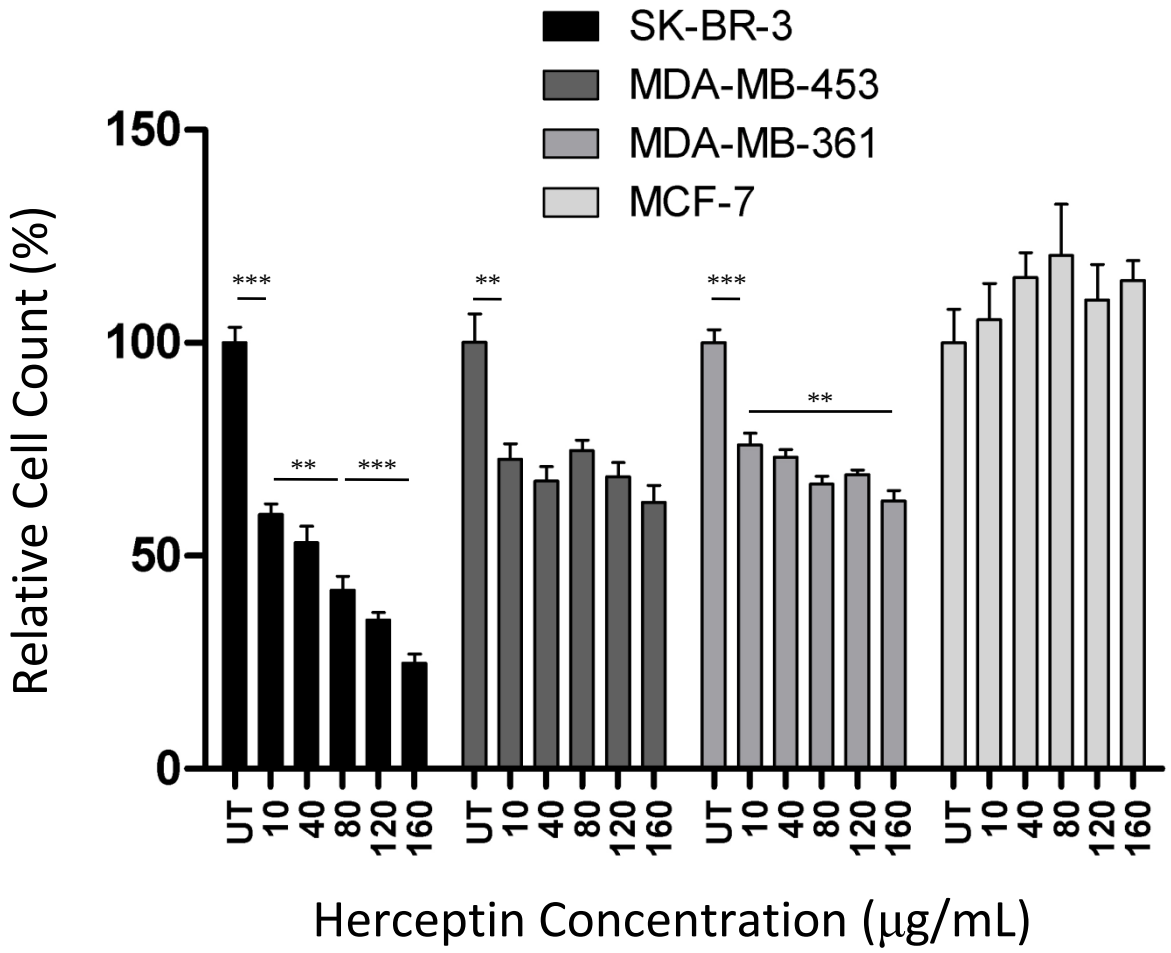
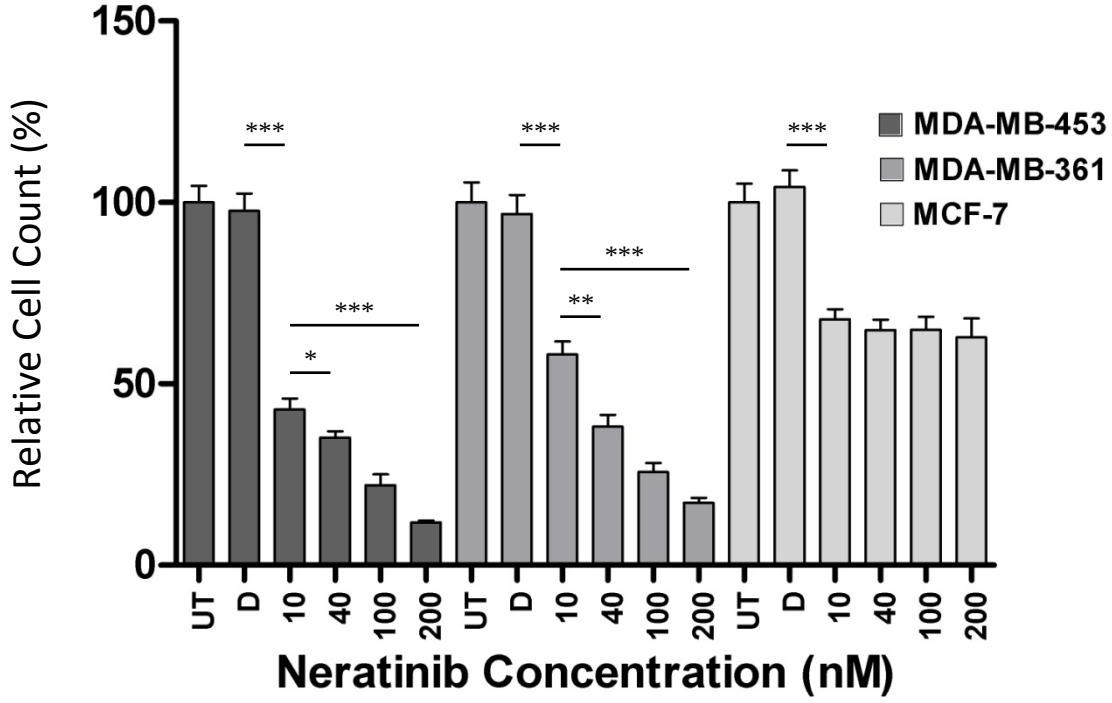


Figure 3.2: The effect of Herceptin on cell numberof cell lines with varying HER2 expression. Cell lines were treated with varying concentrations of Herceptin for five days. After this time, cells were collected and counted. Values have been normalised relative to the untreated control. Error bars represent three independent experiments, each as a triplicate. **UT = Untreated; ** = p<0.01; *** = p<0.001.**

A


Relative Cell Count (%)
150
100
50
0
***
***
***
*
***
**
MDA-MB-453
MDA-MB-361
MCF-7
UT
D
10
40
100
200
Neratinib Concentration (nM)

B

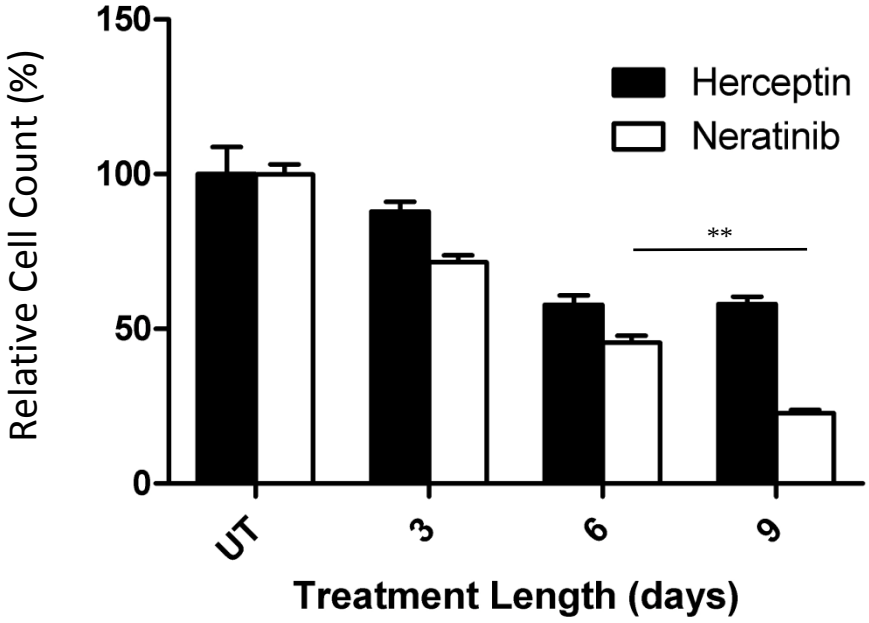

Relative Cell Count (%)
150
100
50
0
Herceptin
Neratinib
**
UT
3
6
9
Treatment Length (days)

Figure 3.3: The effect of Neratinib on non-HER2-overexpressed cell count. A) Cells were treated with varying concentrations of Neratinib for five days. After this time, cells were collected and counted. Values are been normalised relative to the untreated control. Error bars represent two independent experiments, each as a quadruplicate. **B)** MDA-MB-453 cells were treated with 10nM Neratinib for different lengths of time. The resultant cell countswere compared to the same treatment regimen but with 80µg/mL Herceptin instead of Neratinib. Drugs were replaced for all treatments at every 3-day time interval. Values are normalised relative to the untreated control. Error bars represent two individual experiments, each as a quadruplicate. **UT = Untreated; D = 1:50000 DMSO; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.**

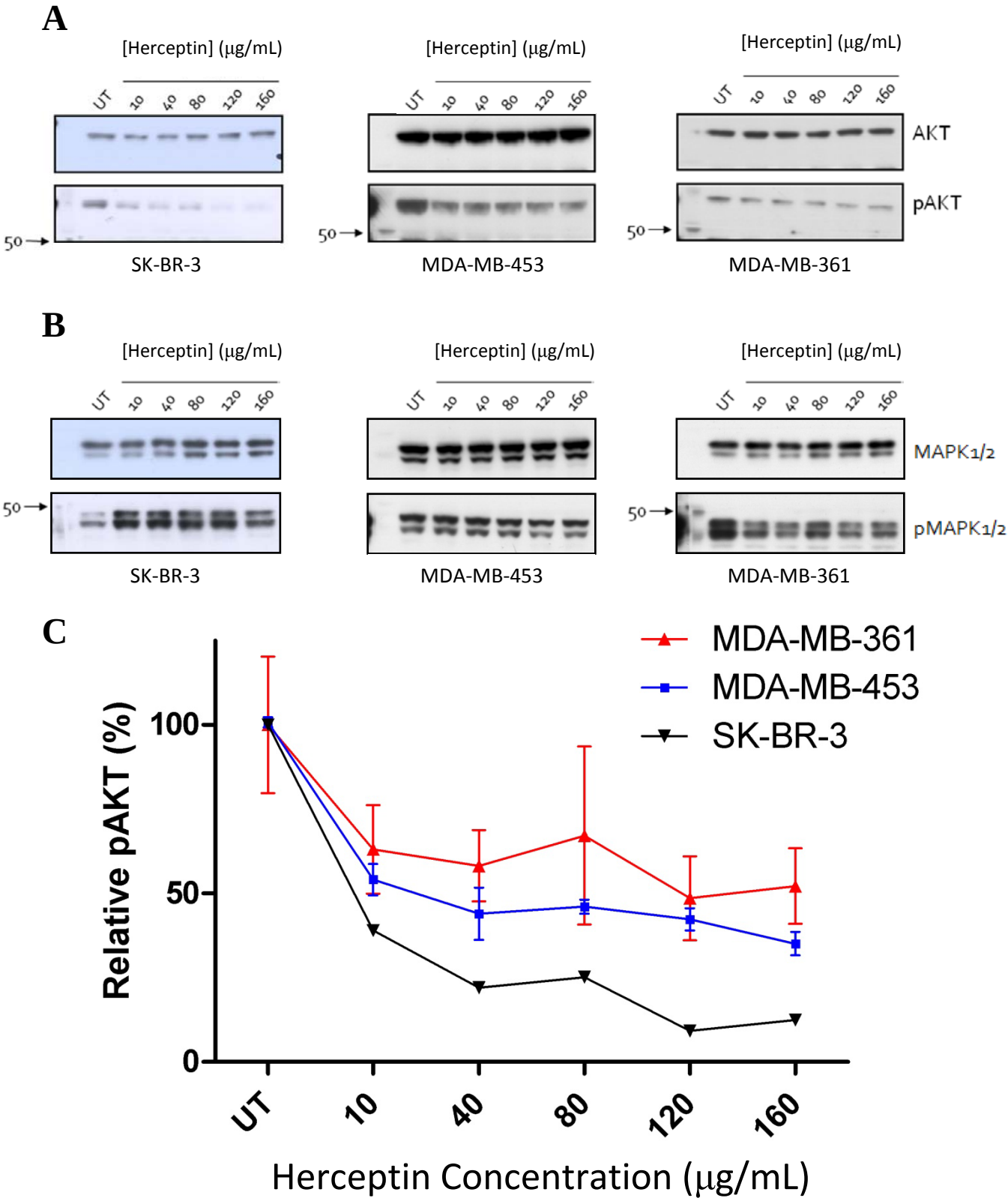
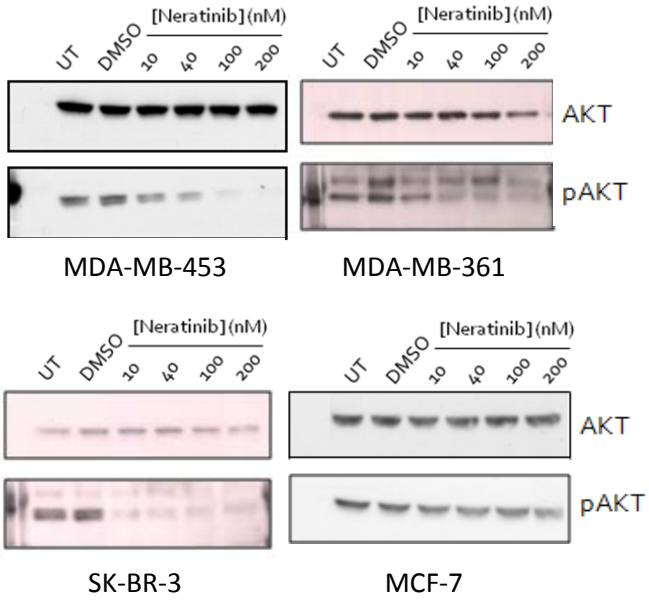
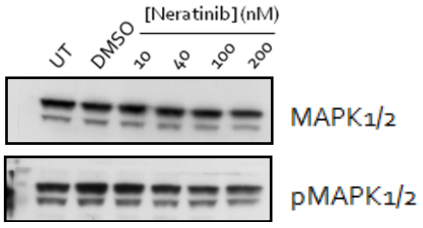

A
[Herceptin] (µg/mL)
UT
10
40
80
120
160
AKT
pAKT
50
SK-BR-3
MDA-MB-453
MDA-MB-361
B
MAPK1/2
pMAPK1/2
C
MDA-MB-361
MDA-MB-453
SK-BR-3
Relative pAKT (%)
100
50
0
Herceptin Concentration (µg/mL)

Figure 3.4: Western blot analysis of pAKT levels in response to Herceptin. A) Cell lines were subjected to a one hour treatment with Herceptin at varying concentrations and the effects on pAKT levels were analyzed. **B)** Cell lines were subjected to a one hour treatment with Herceptin at varying concentrations of Herceptin and the effects on pMAPK levels were analyzed. **C)** Quantification of western blots analysing pAKT, including repeats for HER2-moderately expressed cell lines. The error bars represent three independent experiments for each cell line, performed under identical conditions, as described in (A). Levels of pAKT are normalised relative to the mean average of the three untreated controls for a given cell line. **UT = Untreated.**

A


UT
DMSO
[Neratinib](nM)
10
40
100
200
AKT
pAKT
MDA-MB-453
MDA-MB-361
SK-BR-3
MCF-7

B


UT
DMSO
[Neratinib](nM)
10
40
100
200
MAPK1/2
pMAPK1/2
MDA-MB-453

C

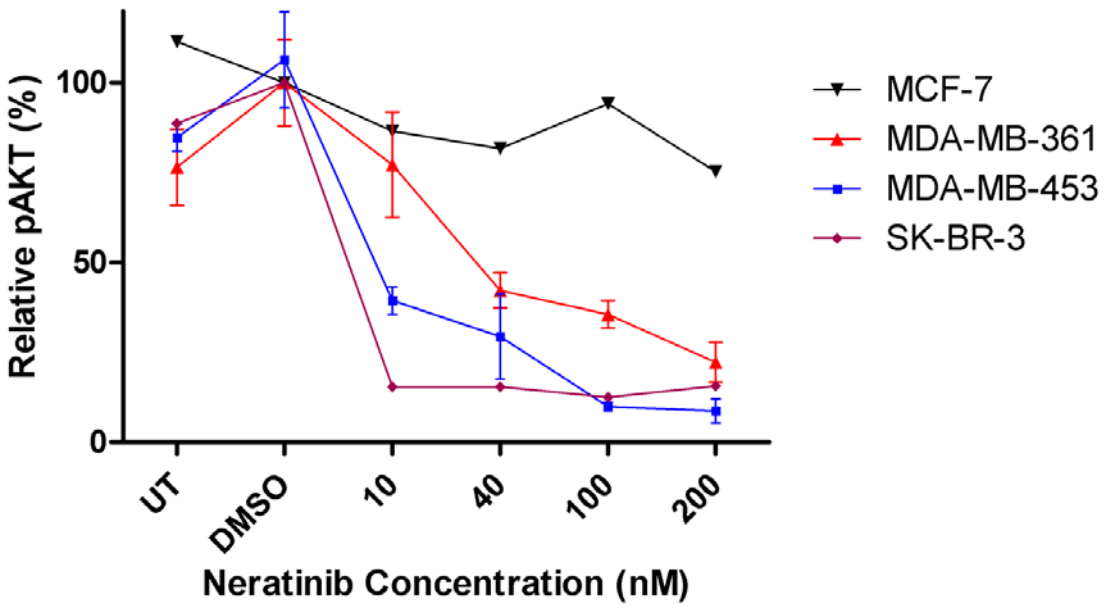
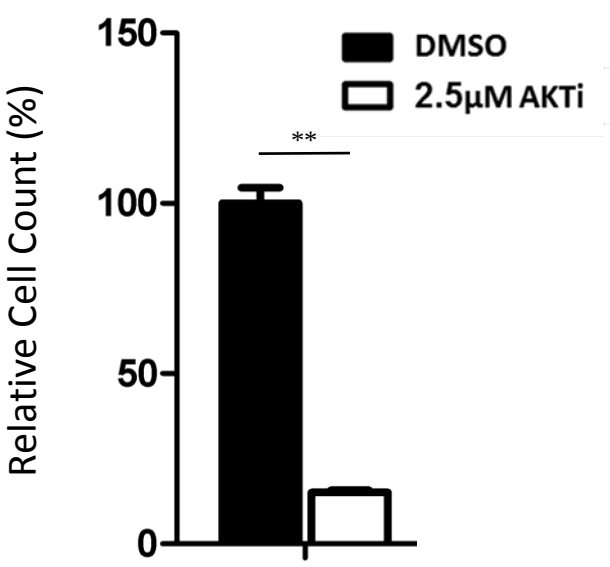
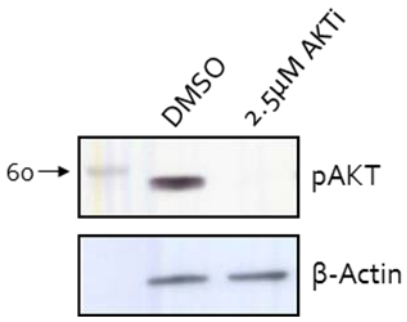

Relative pAKT (%)
100
50
0
UT
DMSO
10
40
100
200
Neratinib Concentration (nM)
MCF-7
MDA-MB-361
MDA-MB-453
SK-BR-3

Figure 3.5: Effect of Neratinib on pAKT and pMAPK levels in cell lines of different HER2 classification. A, B) Cells were treated for one hour in the presence of different concentrations of Neratinib. After this time, cells were collected and subjected to western blot analysis. **C)** Quantification of the western blots analysing pAKT, with repeats for HER2-moderately expressed cell lines. Error bars represent three independent experiments conducted under identical conditions, as in (A). Levels of phospho-AKT were normalised relative to the mean DMSO control, for each cell line. **UT = Untreated.**

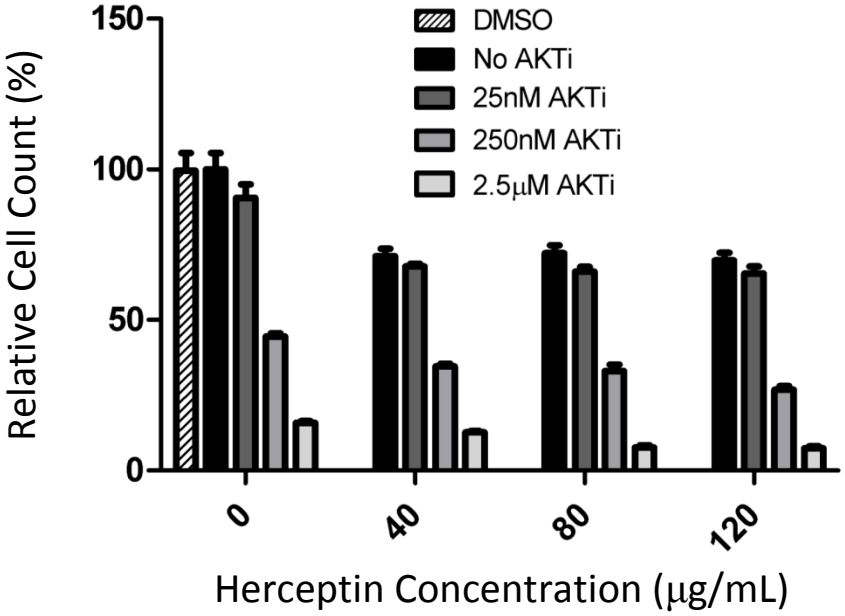
A


DMSO
2.5μM AKTi
**
Relative Cell Count (%)
150
100
50
0

B


DMSO
2.5μM AKTi
60
pAKT
β-Actin

C


DMSO
No AKTi
25nM AKTi
250nM AKTi
2.5μM AKTi
Relative Cell Count (%)
150
100
50
0
0
40
80
120
Herceptin Concentration (μg/mL)

D

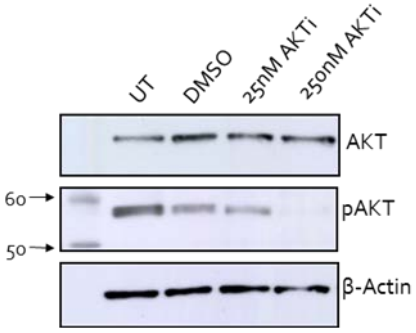

UT
DMSO
25nM AKTi
250nM AKTi
AKT
60
pAKT
50
β-Actin

Figure 3.6: The effect of AKT inhibition on MDA-MB-453 cell count. A) Cells were treated with 2.5µM AKTi for a 5-day period, after which cells were collected and counted. Cell numberwas normalised relative to the untreated control. Error bars represent two independent experiments, each conducted as a triplicate. **B)** Cells were treated for 1 hour in the presence of 2.5µM AKTi and subjected to western blot analysis where pAKT levels were analysed to assess efficiency of the inhibitor. **C)** Cells were subjected to various concentrations of AKTi either alone or with Herceptin at 40, 80 or 120µg/mL, for 5 days. Cells were then collected and counted. Cell countwas normalised relative to the untreated control. Error bars represent one experiment, conducted as a triplicate. **D)** The relative levels of pAKT were investigated at the end of the 5-day treatment, for 25nM and 250nM AKTi, using western blots analysis. **AKTi = AKT inhibitor; UT = Untreated; ** = $p<0.01$**

3.4 Discussion

The use of cell lines with various basal HER2 expressions provided a reliable basis for investigating why HER2-moderately expressed cells do not respond to Herceptin as well as HER2-positive cells. Although treatment with Herceptin was shown to have an effect on both MAPK and PI3K/AKT signalling in these cells, the correlation between AKT activation and cell survival was striking, implicating it as a cause for resistance. Although observations of the pathway changes were for 1-hour treatments, and cell survival were over the course of 5 days, the initial changes in response to the drug relative to the other concentrations are likely reflected as treatment progresses. The idea of AKT underlying observed cell counts also helps to explain why HER2-positive cells responded better to Herceptin. AKT activation in these cells was more efficiently inhibited compared to the HER2-moderately expressed.

A significant role for the PI3K/AKT-pathway in cell survival is further supported by the fact that treatment with Neratinib also showed a correlation between this pathway and cell viability. The importance of AKT was further confirmed by direct AKT inhibition.

4 Assessing the role of HER3 signalling in resistance of HER2-moderately expressed breast cancer cells to Herceptin

4.1 Introduction

Previously it has been shown that there is strong correlation between the PI3K/AKT pathway and cell survival (10, 11). This supports data from the previous chapter that implicates an inability of Herceptin to downregulate this pathway as the cause of resistance in HER2-moderately expressed cells. Specifically relating to breast cancer cell lines, AKT signalling has been shown to be the key mediator of feedback loops that confer survival in the acquisition of resistance (52, 138). Taken together, this provided a good basis for further investigation into the role of AKT in Herceptin resistance in HER2-moderately expressed breast cancer.

4.2 Results

4.2.1 The inability of HER2-moderately expressed cells to maintain reduced HER3 activation in the presence of Herceptin confers similar observations in the AKT signalling pathway.

In the previous chapter it was shown that an inability to decrease AKT signalling contributed to Herceptin resistance in HER2-moderately expressed breast cancer cells. Due to the dependence of HER3 on HER2 and the close association between HER3 and the p85

subunit of PI3K, the role of these receptors was investigated. Interestingly, not only did Herceptin fail to abolish HER2 signalling in HER2-moderately expressed cell lines, it was seen to cause an increase (figure 4.1). This is similar to HER2-positive cell lines as has previously been reported in our lab (52). Herceptin decreased HER3 phosphorylation (pHER3) after 1 hour at various concentrations with the optimum dose of pHER3 inhibition at 80µg/mL. However, above this dose, a downregulation of HER3 was accompanied by reactivation of HER3 i.e. increased pHER3 (figure 4.1).

Since reactivation occurred as Herceptin concentration was increased, a time course was run using 80µg/mL to see if the observed increase could be induced in a time-dependent manner (figure 4.2A); this concentration was chosen because it was the most effective at inhibiting HER3 (figure 4.1). Reactivation of HER3 was witnessed, after an initial decrease at the 1-hour time point; by 24 hours, signalling had returned to basal level. An almost identical trend in AKT activation heavily implicates a direct role for HER3 in this pathway, in HER2-moderately expressed cells. Based on results from the previous chapter, this would suggest that HER3 is a key player in mediating Herceptin resistance in these cells. This was further supported by a correlative trend between HER3 and AKT activation in the presence of Neratinib (figure 4.2B). 10nM Neratinib, which was very effective in these cell lines (figure 3.3), was able to efficiently downregulate pHER2, pHER3 and pAKT in a time-dependent manner in MDA-MB-453 cells (figure 4.2B).

4.2.2 Knockdown of HER3 does not resensitise HER2-moderately expressed cells to Herceptin

To test this hypothesis, a knockdown of HER3 using RNAi was performed on MDA-MB-453 cells to analyse its role in Herceptin resistance (figure 4.3). Efficient knockdown of HER3 led to an expected decrease in pHER3 and pAKT levels compared to the control siRNA (figure 4.3A). There was no further decrease of pAKT when Herceptin was added to siHER3 transfected cells, although the basal level of pAKT was very low (figure 4.3A). HER3 knockdown seems to have a dramatic effect on the cell count of MDA-MB-453 cells, which correlated with the low basal pAKT. However, Herceptin only minimally enhanced the effect of HER3 knockdown on cell survival, which was not significant compared to HER3 knockdown alone ($p = 0.0022$) (figure 4.3B).

4.2.3 ADAM17 has a significant role in basal function of HER2-moderately expressed cells but appears to have minimal role in the observed resistance to Herceptin

It has previously been reported that ADAM17 can mediate a feedback loop in HER2-positive cells whereby inhibition of AKT activation increases ADAM17, and therefore ErbB ligand release such as heregulin (52). I decided to assess whether such a mechanism also occurs in HER2-moderately expressed cells. As shown above, knockdown of HER3 could not enhance the effects of Herceptin. However, inhibiting ADAM17 presented a way to reduce ligand-mediated HER3 activation and thus provided an alternative method to investigate the role of this receptor in resistance.

Initially, an AKT-mediated HER3 feedback mechanism was investigated by direct inhibition of AKT; this caused inhibition of upstream HER3 activation after one hour (figure 4.4A).

Additionally, this AKT inhibition did not affect ADAM17 expression. With Herceptin treatment however, there was a slight increase in ADAM17 as concentrations were increased from 0-120µg/mL; at 160µg/mL there was a slight decrease compared to 120µg/mL (figure 4.4B).

To investigate this observation further, cells were treated with a combination of Herceptin and ADAM17 inhibitor. Based on previous experiments conducted on HER2-positive cells, a concentration of 10µM was initially chosen to investigate cell survival (figure 4.5A). Surprisingly this caused almost complete cell death in MDA-MB-453, an outcome that is not seen in HER2-positive cells.

A less potent concentration, 1µM, of ADAM17i was used so that the effect in conjunction with Herceptin could be observed. At this concentration there was no synergy observed when it was combined with Herceptin for a 5-day treatment (figure 4.5B) despite ADAM17i being able to reduce pHER3 levels (figure 4.5C); a weakly significant additive effect was observed ($p = 0.0499$). In fact, the addition of Herceptin to ADAM17i-treated cells caused a comparatively smaller reduction in cell number from 0-40µg/mL, compared to the addition of Herceptin to untreated cells.

4.2.4 Neratinib has an additive effect when combined with Herceptin but its addition does little to resensitise HER2-moderately expressed cells to Herceptin

In order to explore the role of HER3 further, Neratinib was used because its panHER inhibition successfully reduces HER2 and HER3 signalling directly and indirectly, respectively, without interfering with expression of the native receptor (figure 4.2B). A combination of Herceptin and Neratinib additively reduced pHER2, pHER3 and pAKT in

MDA-MB-453 (figure 4.6A) and pHER2 and pAKT in MDA-MB-361 (figure 4.6B). When Herceptin was combined with Neratinib there was a significant additive effect on the reduction of cell number ($p = 0.0087$) (figure 4.6C). Interestingly this additive effect was more significant at the earlier time point of 3 days, compared to 6 days. Herceptin still had a strongly significant effect on its own between 3 and 6 days ($p = 0.0022$).

4.3 Figures

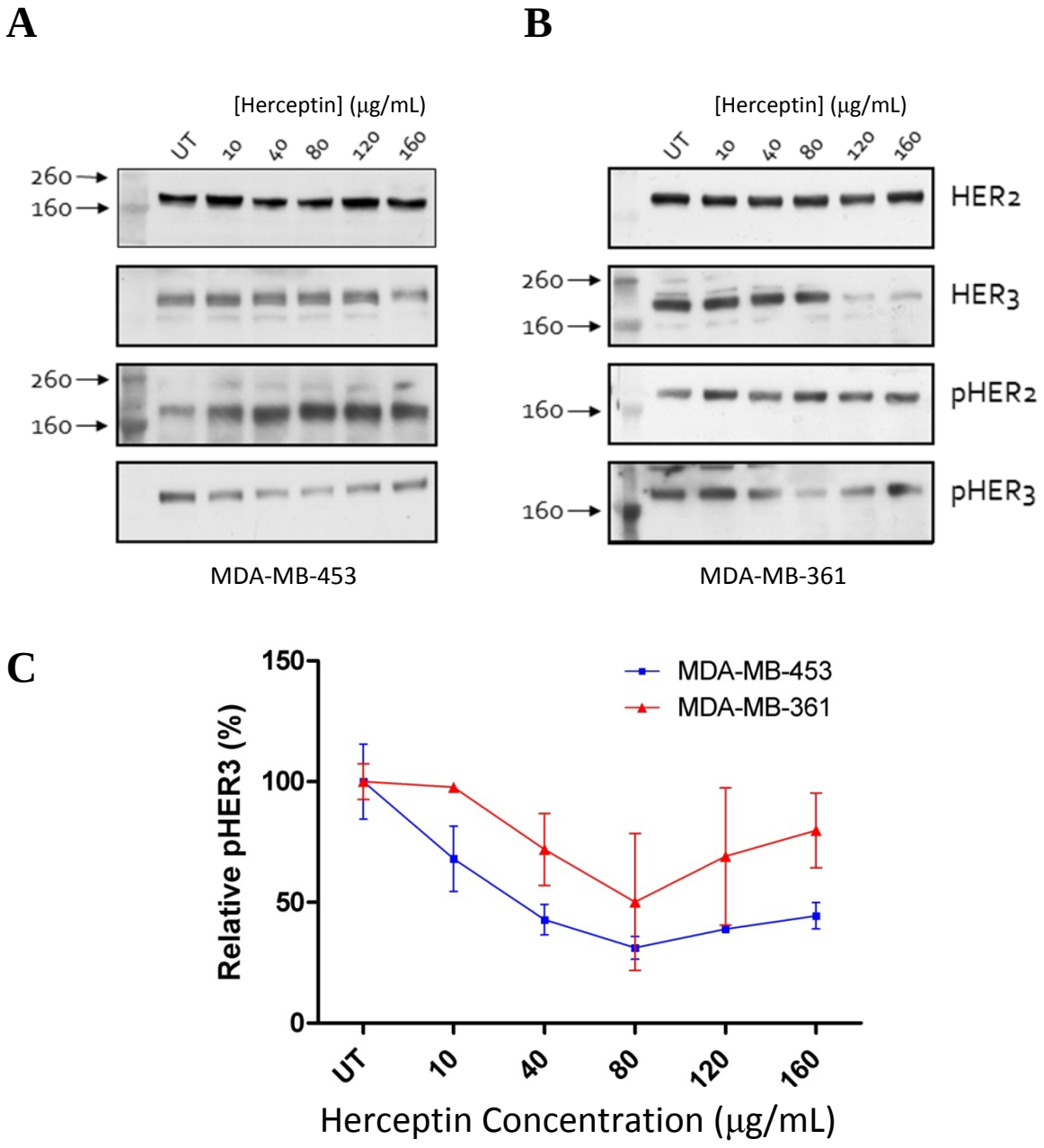


Figure 4.1: The concentration-dependent effects of Herceptin on HER2 and HER3 signalling in HER2-moderately expressed cell lines. A) MDA-MB-453 and **B)** MDA-MB-361 cells were treated with varying concentrations of Herceptin for one hour and signalling of HER2 and HER3 receptors was assessed by western blotting. **C)** Quantification of HER3 signalling in both cell lines. Error bars represent three independent experiments conducted under identical conditions. Levels of pHER3 are normalised relative to the mean average of the three untreated controls for a given cell line. **UT = Untreated.**

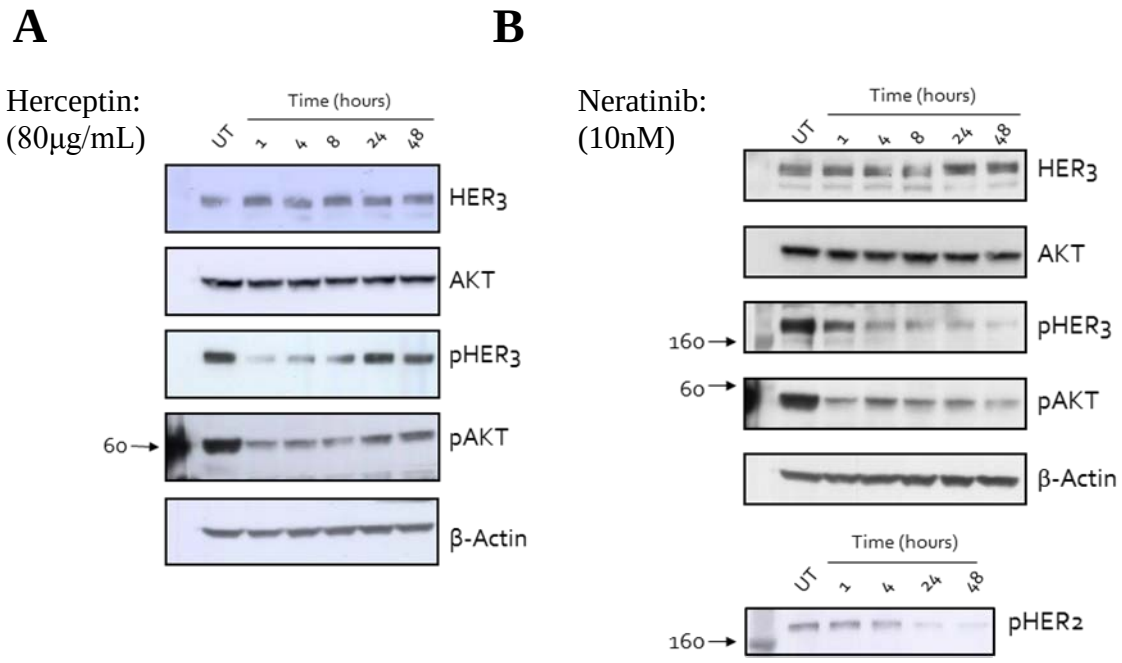


Figure 4.2: The correlative trend of HER3 and AKT signalling in MDA-MB-453 cells. A) Western blot analysis of cells treated with 80µg/mL Herceptin at the designated time points. **B)** Western blot analysis of protein signalling in cells treated with 10nM Neratinib in a similar setup to (A). Phospho-HER2 is shown as an example of how Neratinib indirectly targets HER3 via the former.

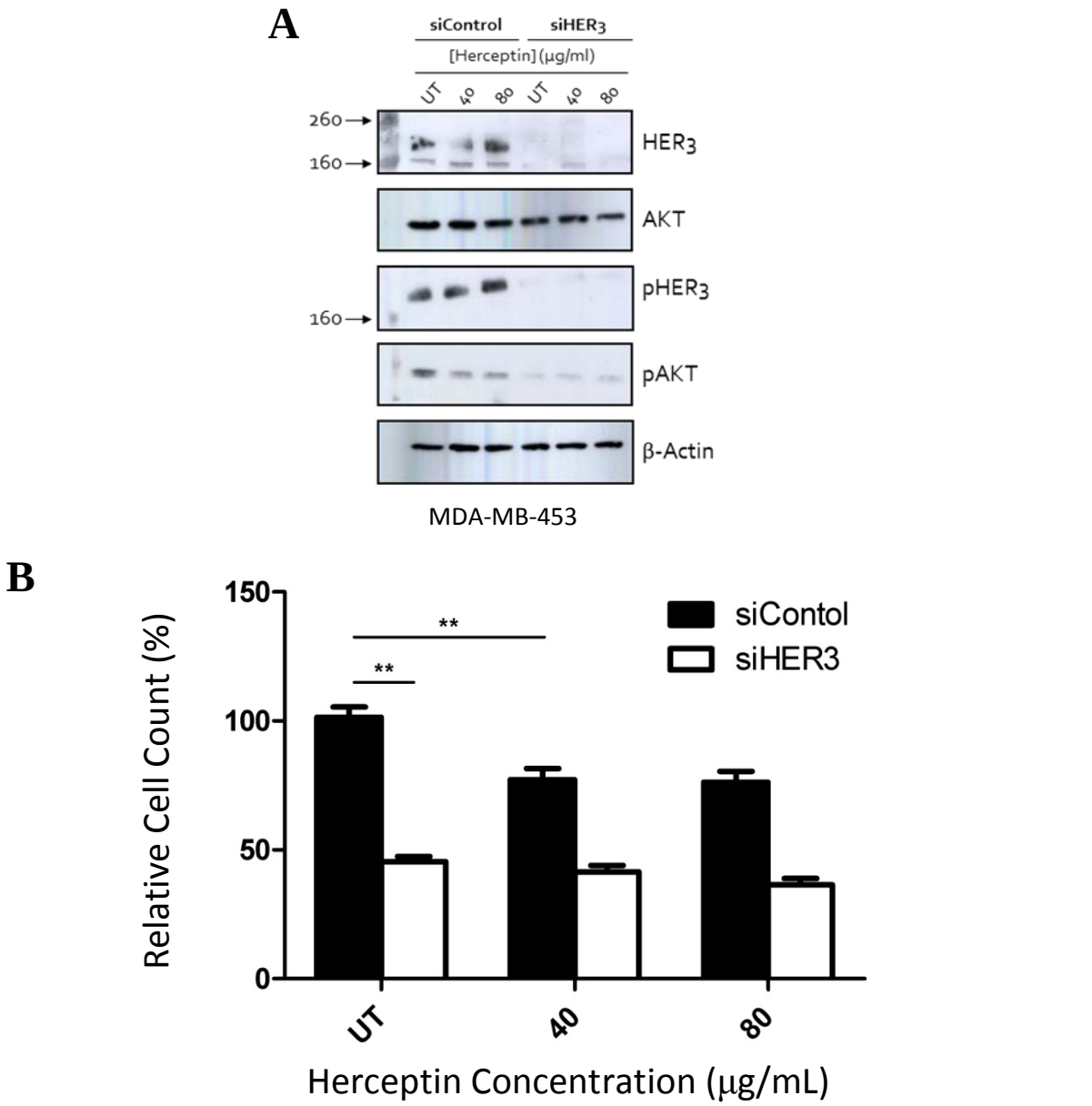


Figure 4.3: Herceptin treatment in HER3-knockdown MDA-MB-453 cells. A) Western blot analysis of protein signalling in response to Herceptin treatment in the presence and absence of HER3. Both HER3-knockdown and native cells were treated with 40 or 80µg/mL Herceptin for one hour. **B)** Cell survival analysis of the effect of HER3 knockdown on MDA-MB-453 cells, with and without Herceptin. Cells were treated for five days. Error bars represent three independent experiments each conducted as a triplicate. Values are normalised relative to the untreated control.

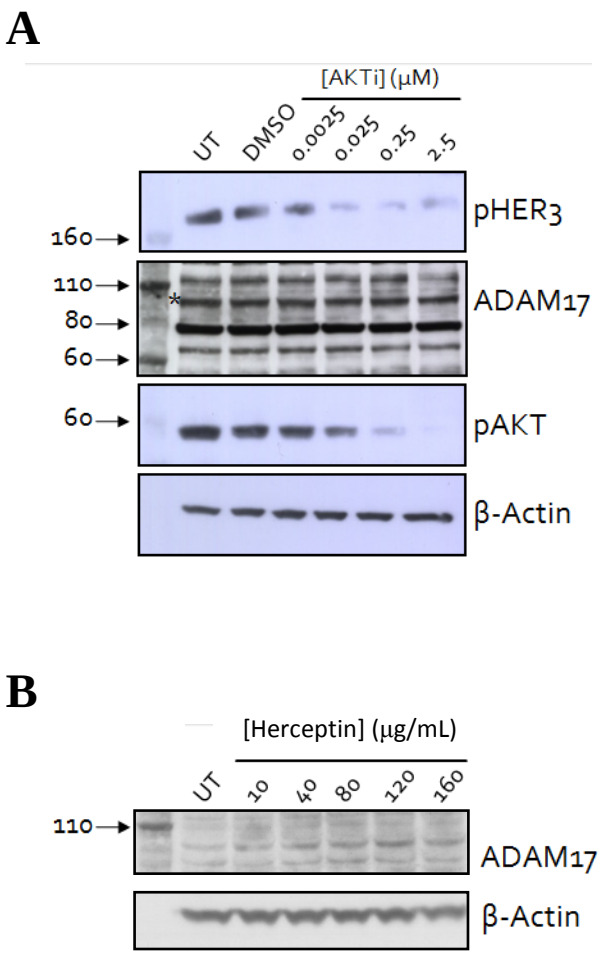
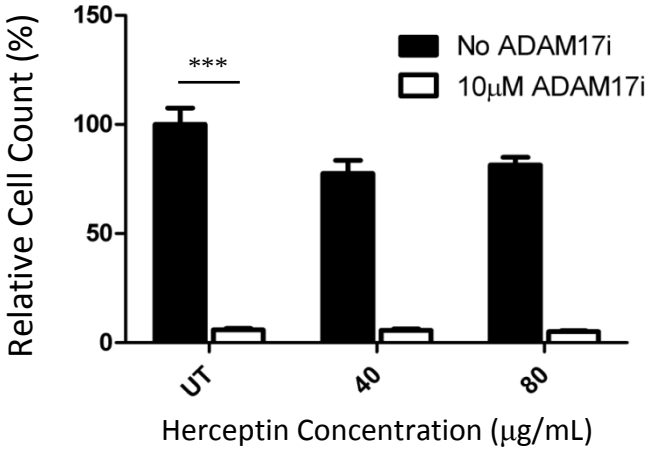
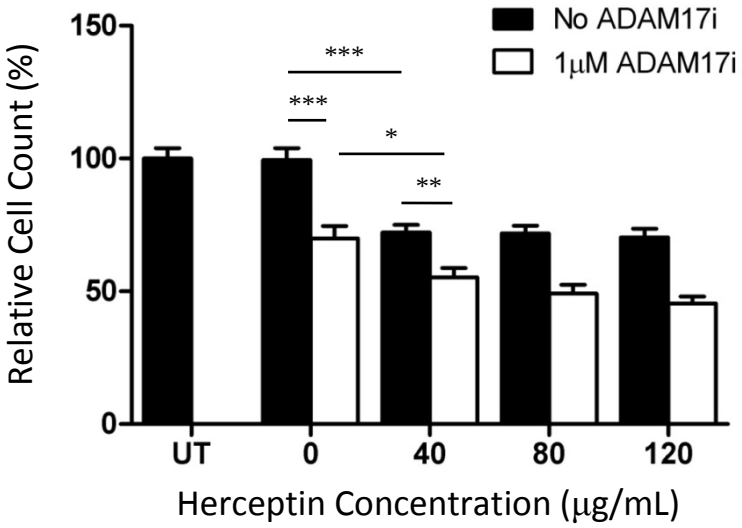


Figure 4.4: Analysis of ADAM17 and pHER3 in response to AKT inhibition and Herceptin, in MDA-MB-453 cells. A) Cells were treated for one hour with different concentrations of AKT inhibitor. * = ADAM17; documented weight = 93kDa. **B)** Cells were treated for one hour with different concentrations of Herceptin. **UT = Untreated.**

A


Relative Cell Count (%)
150
100
50
0
***
No ADAM17i
10μM ADAM17i
UT
40
80
Herceptin Concentration (μg/mL)

B


Relative Cell Count (%)
150
100
50
0
***
***
*
**
No ADAM17i
1μM ADAM17i
UT
0
40
80
120
Herceptin Concentration (μg/mL)

C


UT
DMSO
1μM ADAM17i
160
pHER3
β-Actin

Figure 4.5: The effect of ADAM17 inhibition on the action of Herceptin in MDA-MB-453 cells. A) Cells were treated with or without an ADAM17 inhibitor in the presence of 40 or 80μg/mL Herceptin or alone. Cell number was assessed after a 5-day treatment. Error bars represent seven replicates conducted as two individual experiments. Values are normalised relative to the untreated control. **B)** In the presence or absence of 1μM ADAM17i, cells were treated with 40, 80 or 120μg/mL Herceptin. Error bars represent two individual experiments, each conducted as a quadruplicate. Values are normalised relative to the untreated control. **C)** Western blot analysis confirming the ability of ADAM17i to decrease HER3 signalling after a 1-hour treatment at a concentration of 1μM. **UT = Untreated; * = p<0.05; ** = p<0.01; *** = p<0.05.**

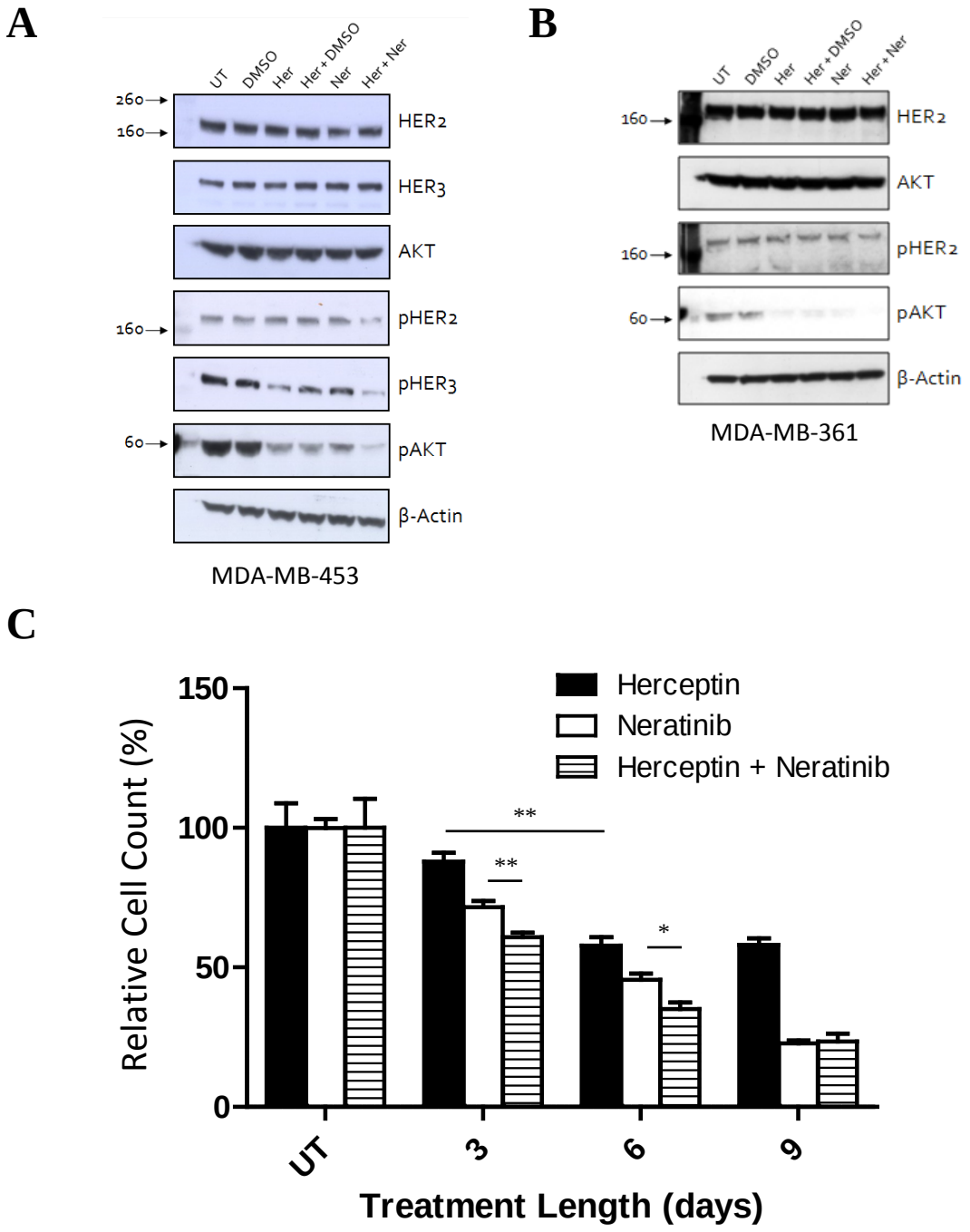


Figure 4.6: The effect of a combination of Herceptin and neratinib on HER2-moderately expressed cells. **A)** Western blot analysis of the effects of a combination of Herceptin and neratinib, relative to each drug on its own, on protein signalling in MDA-MB-453 and **B)** MDA-MB-361 cells. **C)** MDA-MB-453 cells were treated with 80µg/mL, 10nM neratinib or a combination of both for 3, 6 or 9 days. Cell number was analysed and normalised relative to the mean average of the untreated controls for a given treatment. Error bars represent two independent experiments each conducted as a triplicate. **UT = Untreated; * = p<0.05; ** = p<0.01.**

4.4 Discussion

Based on results from chapter 3, maintenance of AKT activation, and the role of upstream HER2 and HER3 receptors, in the presence of Herceptin was investigated further. Whilst initially counterintuitive, the increase of HER2 phosphorylation in response to Herceptin has been previously reported and investigated in HER2-positive cell (52); it is believed to be mediated by ADAM17 and reduced efficacy of Herceptin. Its occurrence also in HER2-moderately expressed cell lines may contribute to their relatively resistant phenotype. Despite a decrease in HER3 phosphorylation in the presence of Herceptin up 80µg/mL, above this concentration phosphorylation began to increase. There was a strong correlation between HER3 and AKT activation in both a concentration- and time-dependent manner in the presence of both Herceptin and Neratinib in HER2-moderately expressed cells. In the presence of Herceptin, an initial decrease in phospho-HER3 occurred over the first hour of treatment with 80µg/mL Herceptin. This was followed by an increase in pHER3 levels over the course of 48 hours; AKT phosphorylation showed a similar trend. This strongly suggests that HER3 plays a major role in the AKT activation that is responsible for resistance.

However, knockdown experiments on HER3, with the intention of showing its role in Herceptin resistance, did not enhance the effects of Herceptin. By observation of both AKT signalling and cell survival over a 5-day period, the effect of Herceptin on HER3-knockdown cells was significantly reduced compared to the control. It is possible that knockdown of HER3 was so effective that it masked the effect of Herceptin. However, cell number after siHER3 treatment was at 50% suggesting that there was still sufficient AKT activation. At this level, we might expect a more noticeable reduction in cell count when Herceptin is applied, as was seen for experiments involving AKTi. It could

therefore mean that HER3 contributes to Herceptin action in HER2-moderately expressed cells. From observing the AKT signalling we can also see that in the presence of HER3-knockdown, Herceptin is no longer causing the initial decrease in phospho-AKT that has been observed in every instance up until now.

The ability of Herceptin to cause a slight increase in ADAM17, albeit much less than in HER2-positive cells (52), implies that this protease could play a role in resistance of HER2-moderately expressed cells. However, its invariant levels in response to AKT inhibition, and the decrease in pHER3 implies that its role is different to the AKT-mediated negative feedback loop previously reported (52). An issue with these results is that the ADAM17 antibody is very unspecific, giving an unclear western blot (figure 4.4A). As well as testing with ADAM17 antibodies from other companies, it would be worth loading a positive control to be certain of the ADAM17 band. The apparent relative redundancy of ADAM17 in Herceptin resistance may explain why its inhibition only slightly increased sensitivity to Herceptin. Whilst ADAM17 appeared to have a limited role in Herceptin resistance in HER2-moderately expressed cells, a critical function for this protease in basal proliferation of these cells is clear, based on the strong reduction in cell number at the standard dose of ADAM17i.

The ability of Neratinib to inhibit HER2-moderately expressed cell number was described in the previous chapter. Another advantageous feature of Neratinib is the significantly additive effect when combined with Herceptin. The strong potency of Neratinib appears to limit this additive effect such that the addition is not vast compared to Neratinib as monotherapy. The data also suggests that there is no synergy between the two drugs in these cell lines.

5 Final Discussion

5.1 Findings from this study

This thesis aimed to assess the efficacy of, and resistance to, Herceptin in HER2-moderately expressed breast cancer cell lines. By understanding the mechanisms behind Herceptin resistance I aimed to investigate other targeted therapies that may be more effective than Herceptin or able to overcome resistance. To achieve my aims I used breast cancer cell lines with different HER2 expression: HER2-overexpressed SK-BR-3, HER2 moderately expressed MDA-MB-453 and MDA-MB361, and MCF-7, with minimal HER2.

The repeated correlation between AKT activation and cell viability suggests that the extent to which a cell line can inhibit this protein in response to a drug determines efficacy. Herceptin action was limited in HER2-moderately expressed cells due to persistence of AKT activation above 10µg/ml; reactivation of HER3 was found to be responsible for this maintenance. Further study into HER3 revealed that this receptor may also be crucial to the ability of Herceptin to exert its effects in these cells. However, more remains to be investigated to confirm this. The use of an ADAM17 inhibitor and the panHER inhibitor Neratinib presented two drugs that have significantly strong potency as monotherapy in HER2-moderately expressed cells. Although both drugs showed an additive effect when combined with Herceptin, combination only minimally enhanced the strength of either drug on its own.

5.2 Supporting literature

The decreased levels of HER2 and increased levels of HER3 in HER2-moderately expressed cells relative to HER2-positive cells help us to understand the above findings, when compared with previous studies. A study by Ghosh et al. (139) that induced various dimer combinations in non-transformed MCF10A cells by the addition of ligands showed that Herceptin was able to inhibit HER2-homodimer mediated growth significantly better than heterodimer-mediated growth. HER2-homodimer formation was induced by means of a chimeric HER2-FKBP molecule that could be activated by addition of the FKBP ligand AP1510. Their findings were consistent with clinical studies where HER2-positive breast cancer patients with high levels of HER2-homodimers exhibited longer time to progression when treated with Herceptin. The strong response of HER2-moderately expressed cells to ADAM17 inhibition, shown in this study, suggests a reduced ability of these cells to rely on dimers not under the control of ADAM17, i.e. HER2-homodimers, compared to HER2-positive cells, where this response is not observed. The study by Ghosh et al. therefore helps us to understand the first-line reasons why Herceptin works poorly in HER2-moderately expressed breast cancers.

Results from this thesis that find a role for HER3-mediated AKT maintenance in resistance are supported by other studies that show a close link between HER3 and AKT (34, 35), and its role in cell survival and breast cancer (10, 11, 38). It has previously been shown that reactivation of HER3 is a common mechanism employed by HER2-driven breast cancer cells to escape the activity of drugs. One study (37) showed that cells can evade the action of the tyrosine kinase inhibitors Gefitinib and Erlotinib by increasing membrane expression of HER3 thus causing a shift towards its phosphorylated state and increased activation of the PI3K/AKT pathway. A similar study (138) went further,

showing that HER3-mediated escape from the effects of TKIs was induced by AKT inhibition and subsequent upregulation of HER3 expression by FOXO transcription factors. Furthermore, a study (140) on previously Gefitinib-sensitive lung cancer cells described the acquisition of HER3-mediated resistance as a result of upregulation of the Met tyrosine kinase. This shows that HER3 can even be influenced by non-ErbB proteins making its role in resistance very complex. HER3's close association with the PI3K/AKT pathway makes it a good candidate for being involved in resistance that maintains cell proliferation. Indeed data from this thesis highlights it as a primary marker for Herceptin resistance. It is possible that the high-level of HER3 in HER2-moderately expressed cells makes them susceptible to more efficient establishment of HER3-mediated resistance in response to Herceptin. This could explain the onset of reduced sensitivity to Herceptin at lower concentrations compared to HER2-positive cells.

A study by Gijsen et al. (52) found that Herceptin treatment in HER2-positive cells could induce ADAM17-mediated up-regulation of ErbB ligands, specifically heregulin and betacellulin, and that this was initiated by a decrease in AKT activation. This negative-feedback loop reduced the efficiency of Herceptin in these cells since inhibition of ADAM17 was shown to have a synergistic effect in xenograft models. This implicates a role for ligand-dependent ErbB interactions in reducing the ability of Herceptin. However, whilst this helps us to understand a role for ADAM17-mediated dimer activation in Herceptin resistance, data from this thesis implicates that while ADAM17 may have a minimal role it may be a different mechanism in HER2-moderately expressed.

Based on work showing the preferential formation of HER2-HER3 heterodimers (10), and the levels of these two receptors in HER2-moderately expressed cells, we would expect there to be a relatively high level of this dimer pair compared to other

combinations. Therefore, while the presence of this dimer would appear to reduce the ability of Herceptin compared to HER2-homodimers, it is likely that any action of Herceptin occurs primarily through this dimer since the majority of HER2, the target of Herceptin, would be expected to be found in this form. If indeed knockdown of HER3 did reduce the ability of Herceptin it could be understood in terms of disrupting the necessary HER2-HER3 dimers. Interestingly, ligand-independent HER2-HER3 interactions have been shown to be key to the proliferative potential of breast cancer cells and disruption of these interactions by Herceptin led to significant reductions in pAKT and cell proliferation (141). Ligand-dependent signalling of this dimer pair has also been shown to affect Herceptin efficacy (82). This study engineered HER2-negative MCF-7 cells to overexpress heregulin. The increased phosphorylation of HER2-HER3 dimer pairs significantly sensitised these otherwise unresponsive cells to Herceptin.

The strong ability of ADAM17 inhibition alone to reduce cell viability in HER2-moderately expressed cells would suggest that a ligand-dependent ErbB dimer is responsible for basal function, and targeting of Herceptin in these cells. Analysis of the literature suggests this could be a HER2-HER3 heterodimer. If this is the case it presents another explanation for the relatively small additive effects seen when combining Herceptin with HER3 knockdown or drugs that inhibit HER3; HER3 inactivation by the secondary drug could limit the potential of Herceptin due to the drug's requirement for HER3 activation. This could also explain the reduction in the additive effects seen in time-dependent combination treatments despite frequent replacement of drugs, and observation that Herceptin alone is still strongly significant.

HER3-mediated Herceptin resistance in these cells appears to be ligand-independent with respect to ADAM17 substrates, and thus suggests a change in state of HER3 activation, from initial response to acquisition of resistance. In this manner it is possible to see how

HER3 could play a role in both susceptibility and resistance of HER2-moderately expressed cells to Herceptin.

5.3 Limitations

Cell viability was analysed using a cell counter that distinguishes cells based on a range of diameters. This mean some cells will inevitably not be included whilst other debris could be counted as a cell. Whilst the majority of dead cells will be removed before cell counting, this method cannot determine cells on the brink of apoptosis. However, since the same method was used for all cell viability experiments it is appropriate for comparing between experiments. Fluorescent-activated cell sorting (FACS) could be used for a more thorough evaluation.

RNAi experiments will always have the limitation of unspecific binding. Assessing function of a protein by RNAi knockdown is therefore never completely conclusive since off-target effects could influence the observed results.

Results from this study speculate a potential for the action of one drug to limit the effects of another based upon inhibition of HER3. It is not possible to conclude this from present data since the effects of one drug could be such that they epistatically mask the ability of the other.

5.4 Future Work

Based on speculations arising from this data it will be important to identify the method of HER3 maintenance in resistance. Although initial data suggests a reduced role for

ADAM17 in the resistant state, it should be investigated further, along with other ADAM proteases, by knockout and analysis of changes to ligand levels. It will also be worthwhile investigating proteins described by previous studies to have a role in HER3-mediated resistance, particularly those downstream. Their effects in response to Herceptin could be observed, and confirmed using RNAi or overexpression. Unfortunately, the complex network of interactions between ErbB family members, and the feedback loops that exist make it very difficult to accurately determine the specific role of one pathway within the network.

The role of HER3 in initial response to Herceptin requires further investigation. Whether this exists as a HER2-HER3 dimer could be assessed by either induction, via ligand overexpression, or knockout of this dimer, confirmed using immunoprecipitation or Forster Resonance Energy Transfer (FRET), and tested for its ability to affect response to Herceptin. Unfortunately, due to the critical role these receptors play in cell survival it is hard to avoid epistatic effects.

Whilst most of this work was conducted using MDA-MB-453 cells, it should be more thoroughly analysed in MDA-MB-361 cells in order to assess heterogeneity within the same class of HER2-driven breast cancer cells. The study should also be moved to xenograft models to see if the promising results seen when using ADAM17i or Neratinib translate to mammals.

5.5 Importance of this study

This study helps us gain a clearer understanding of Herceptin resistance in HER2-moderately expressed cells. While the exact mechanism behind HER3 reactivation remains uncertain, its contribution due to maintenance of AKT activation is clear.

The successful reduction in cell viability observed when treating with an ADAM17 inhibitor or Neratinib as monotherapy presents two exciting potentials for targeted therapy that could be beneficial for HER2-moderately expressed breast cancer patients, particularly those with increased HER3 expression. Although in combination with Herceptin the additive effects are minimal, if Herceptin action in these tumours does rely on HER2-HER3 dimers, potency of the drug could be increased by induction of this dimer. However, the success of both ADAM17i and Neratinib without the need to be administered as combination therapy suggests that these two drugs should strongly be considered for clinical trials in the near future.

5.6 Concluding Remarks

The common role of the HER3/PI3K/AKT pathway in breast cancer resistance is also responsible in HER2-moderately expressed breast cancer cells. The rapid induction of this pathway after initial treatment with Herceptin, establishes AKT activation that limits sensitivity to the drug. Whilst the mechanism behind this reactivation remains elusive, its reduced reliance on ADAM17 is clear. Conversely, basal survival of these cells relies heavily on ADAM17-mediated receptor activation making them suitable targets for ADAM17 and panHER inhibition. It will be important to see if these effects translate to

mammalian organisms and whether this ushers in a new targeted therapy for HER2-moderately expressed breast cancer patients.

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