Understanding the Mode of Action of Trastuzumab to Design a Better Therapy for ErbB2-positive Human Breast Cancer

by

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Abstract

Overexpression of ErbB2 occurs in about 25-30% of breast cancer cases and therefore, represents an attractive therapeutic target for treating breast cancer. There are two models for ErbB2 inhibitors that are currently in clinical use: humanized antibodies directed against ErbB2 and small molecule tyrosine-kinase inhibitors. Patients with ErbB2-positive breast cancer have significantly lower survival rates and a shorter period before relapse than patients without ErbB2 overexpression. Trastuzumab (Herceptin) is a humanized recombinant mAb that binds to the extracellular domain of ErbB2 protein. Although, the exact antitumor mechanisms for trastuzumab are not precisely known, several possibilities have been proposed.

Here, we show that trastuzumab is able to block pErbB2 and pEGFR in a ligand-dependent manner. Trastuzumab was able to inhibit both pEGFR and pErbB2 in CHO cell lines that express each receptor alone. Moreover, we show that trastuzumab is able to inhibit the phosphorylation of AKT and Erk that are activated by EGFR and ErbB2 in a ligand-independent manner. Pharmacologically, we found trastuzumab was neither able to significantly inhibit cell proliferation and survival nor enhance the cytotoxic effect of doxorubicin.

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List of abbreviations

ADCC	**Antibody dependent cell cytotoxicity**
ATCC	**American type culture collection**
AR	**Amphiregulin**
BSA	**Bovine serum albumin**
CBL	**E3 ubiquitin protein ligase**
CHK	**Csk homologous kinase**
DMEM	**Dulbecco modified eagle medium**
DMSO	**Dimethyl sulfoxide**
DTSSP	**3,3'-dithiobis(sulfosuccinimidyl propionate)**
DUSP5	**Dual specificity phosphatase 5**
ECD	**Extracellular domain**
EGF	**Epidermal growth factor**
EGFR	**Epidermal growth factor receptor**
EPG	**Epigen**
ERK	**Extracellular signal regulated kinases**
FBS	**Fetal bovine serum**
GRB2	**Growth factor receptor bond protein 2**
GRB7	**Growth factor receptor bond protein 7**
IGR-1R	**Insulin-like growth factor insulin**
mAb	**Monoclonal antibody**
MAPK	**Mitogen activated protein kinase**

mTOR	**Mammalian target of rapamycin**
MTT	**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide**
NK	**Natural killer**
PBS	**Phosphate buffered saline**
PF	**Paraformaldehyde**
PI3K	**phosphatidylinositol 3-kinase**
PTEN	**Phosphatase and tensin homolog**
SDS	**Sodium dodecyl sulfate**
SF	**Serum free**
SGK3	**Serum/glucocorticoid regulated kinase**
SHC	**Src homology 2 domain**
STAT	**Signal transducer and activator of transcription**
TEMED	**Tetramethylethylenediamine**
TGF α	**Transforming growth factor α**
VEGF	**Vascular endothelial growth factor**

Chapter 1 Introduction

1.1. ErbB/Her protein tyrosine kinases

The ErbB/human epidermal growth factor (Her) family of protein tyrosine kinases and the epidermal growth factor receptor (EGFR) was the first member to be discovered as a part of this family (Stanley Cohen, 1962; S Cohen et al., 1982; Stanley Cohen, 1983; G. C. and S. Cohen, 1990). Protein kinases are classified based on the amino acid that is phosphorylated and there are currently three known types of protein kinases; protein-tyrosine kinases/90 members (which contains the ErbB family), protein-serine/threonine kinases/385 members and tyrosine-kinases like proteins/43 members (Roskoski, 2013). Within the 90 tyrosine-kinases proteins there are 32 non-receptor kinases and the rest are receptor kinases. The phosphorylated state of the protein is usually regulated by a process known as dephosphorylation and is carried out by several types of phosphatases (Roskoski, 2013).

ErbB receptors are documented as the following four members: EGFR/ErbB1, ErbB2/Her2, ErbB3/Her3 and ErbB4/Her4. In general, all of these receptors contain three intensely studied structural domains: the extracellular ligand-binding domain, the transmembrane domain, and the cytoplasmic tail that contains the tyrosine-kinase domain (Y Yarden & Sliwkowski, 2001) (Fig 1.1). ErbB receptor activation occurs through ligand binding, which induces the formation of homo/hetero-dimers between the ErbB receptors. Moreover, activation of the intrinsic kinase activity leads to the phosphorylation of the tyrosine residues within the ErbB cytoplasmic tail. These phosphorylated residues act as a downstream response to specific stimuli and serve as docking sites for several proteins, activating a range of intracellular pathways. When activated, ErbB receptors can bind to a wide range of signaling proteins, including proto-oncogene tyrosine-protein kinase Src, growth factor receptor bound protein-7 (GRB7), Src homology 2

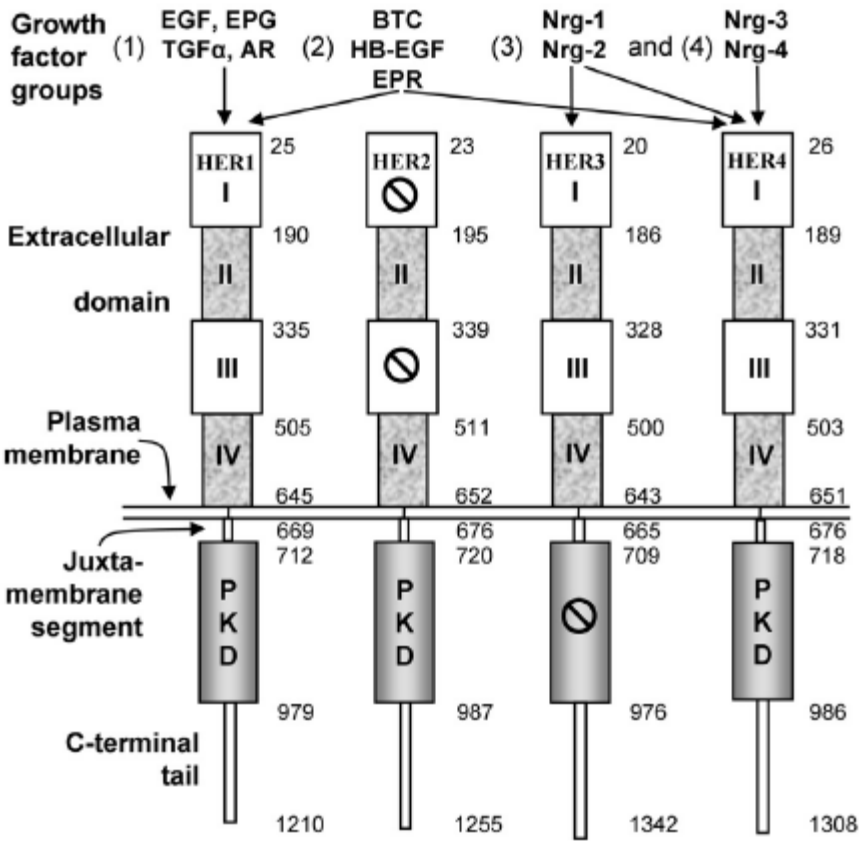

Fig 1.1. General schematic for EGFR/ERBB receptor domains. Growth factor groups 1, 2, 3 and 4 categorize the growth factors that bind to specific tyrosin-kinase receptors. The extracellular domain contains 4 parts: leucine-rich domains (I and III), which play a role in ligand binding, and cysteine residues containing domains (II and IV) which play a role in disulfide bond formations. Domain II also plays a role in heter/homo dimerization. The cytoplasmic domain contains the C-terminal tail and the phospho-kinase activity sites. The ErbB2 receptor has no ligand-binding domain and ErbB3 has no kinas domain.

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domain containing transforming protein (SHC), E3 ubiquitin protein ligase (Cbl),and growth factor receptor bound protein-2 (GRB2) (Hynes & Lane, 2005) (Fig 1.2). ErbB receptors can be specific to certain signaling proteins. For example, the ErbB2 and EGFR receptors contain Grb2/Shc binding sites, which leads to activation of the Ras-extracellular signal regulated kinases (Erk) pathway, resulting in cellular proliferation and differentiation (Hynes & Lane, 2005). On the other hand, the ErbB3 receptors are more likely to bind to phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) leading to PI3K-AKT pathway activation (Burgess, 2008).

1.2. ErbB receptors and Cancer

ErbB receptors play an important role in maintaining cellular activity and processes such as apoptosis, cellular proliferation and differentiation, cell cycle progression, immune response and development, transcription and nervous system function (Y Yarden & Sliwkowski, 2001; Manning et al., 2002; Holbro & Hynes, 2004). Mice with mutant ErbB4 receptors frequently die within the first weeks postnatal because of several heart development defects (Tidcombe et al., 2003). As well, inhibiting the ErbB receptors with antagonists causes a range of side effects, for example diarrhea, interstitial pneumonia and pulmonary fibrosis in murine models (Baselga & Swain, 2009; Camus et al., 2004). It has been well documented that aberrant activities caused by amplifications and/or mutations of ErbB receptors can lead to human cancers (Holbro & Hynes, 2004; Maruyama, 2014) (Fig 1.3.A). Accordingly, these receptors have been investigated and studied for their role in cancer biology and physiology and targeted for better therapeutic strategies (Fig 1.3.B) (Yarden & Pines, 2012; Hynes & Lane, 2005). The mechanisms and the pathways that participate in tumor progression and metastasis which involve ErbB receptor activation are complex. However, although many intracellular signaling pathways are stimulated, there are preferential pathways that are activated based on either the ligand or the

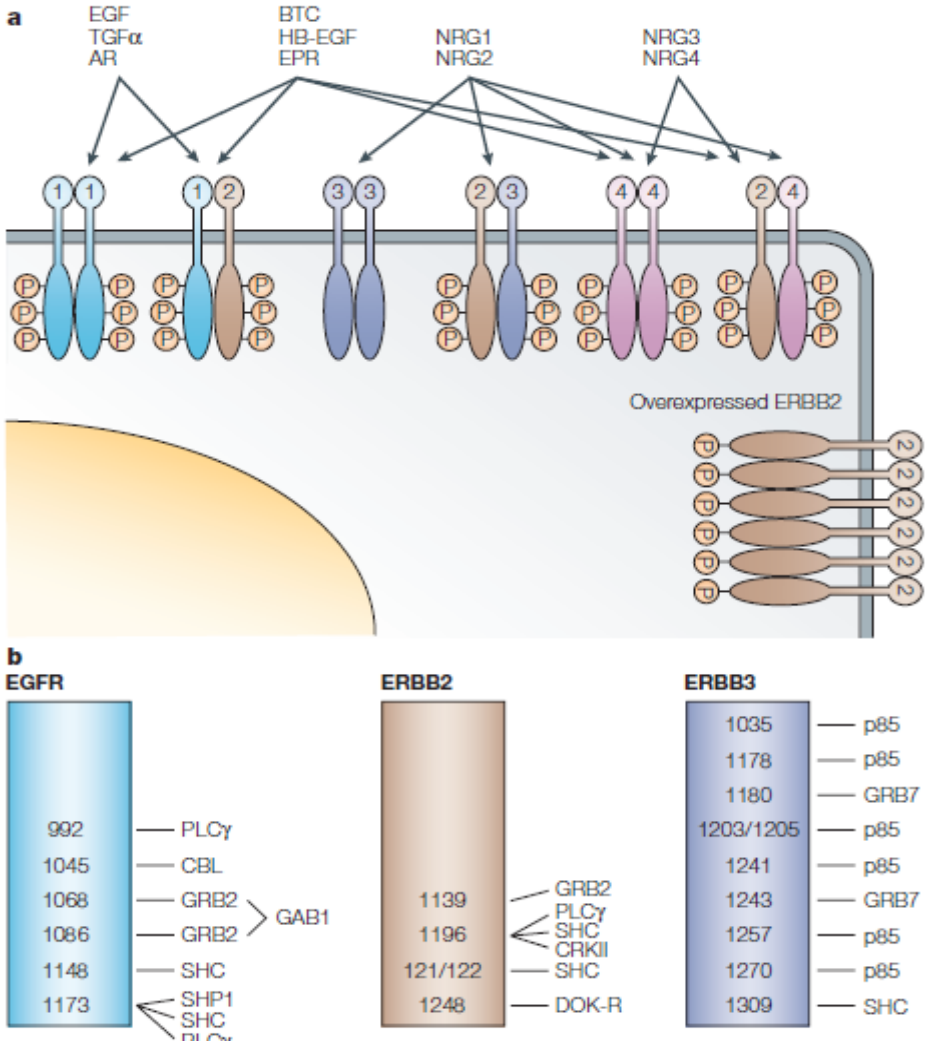
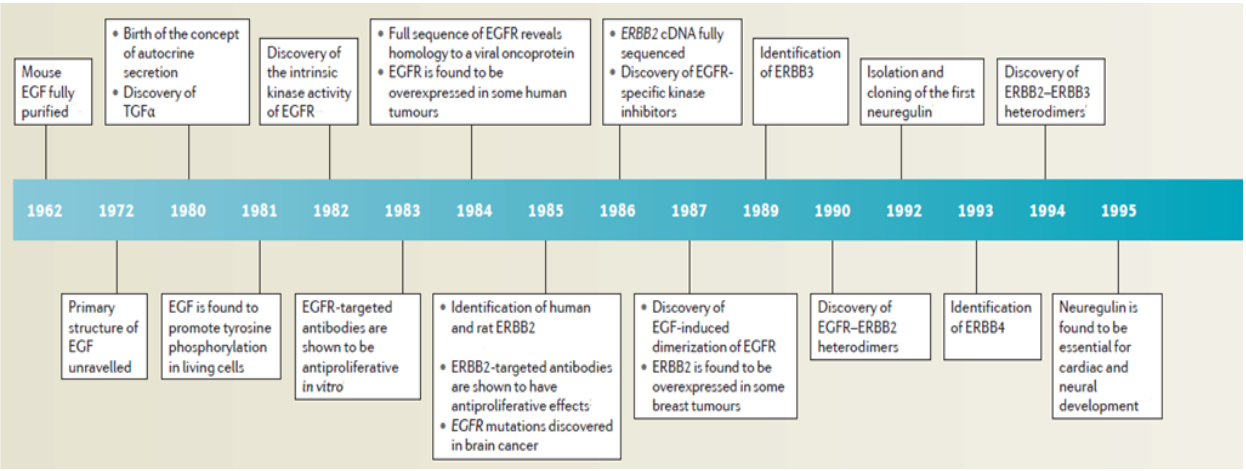


Fig 1.2. ErbB receptors and their signaling proteins. **a**. A representative schematic of ErbB receptor activation in response to external stimuli (ligands) and the formation of hetero/homo dimers. These ligands bind specifically to their target receptors, however some of these ligands have the ability to activate several receptors at the same time. Nevertheless, none of these ligands or other ligands have the ability to bind to ErbB2. For this reason ErbB2 receptors are considered as the most preferable receptor for homo/hetero-dimer formation. 1, 2, 3 and 4 represent the ErbB receptor types respectively**. b.** A schematic representation of potential sites for auto-phosphorylation in each of the receptors among the ErbB family and the docking proteins that these sites activate.

A



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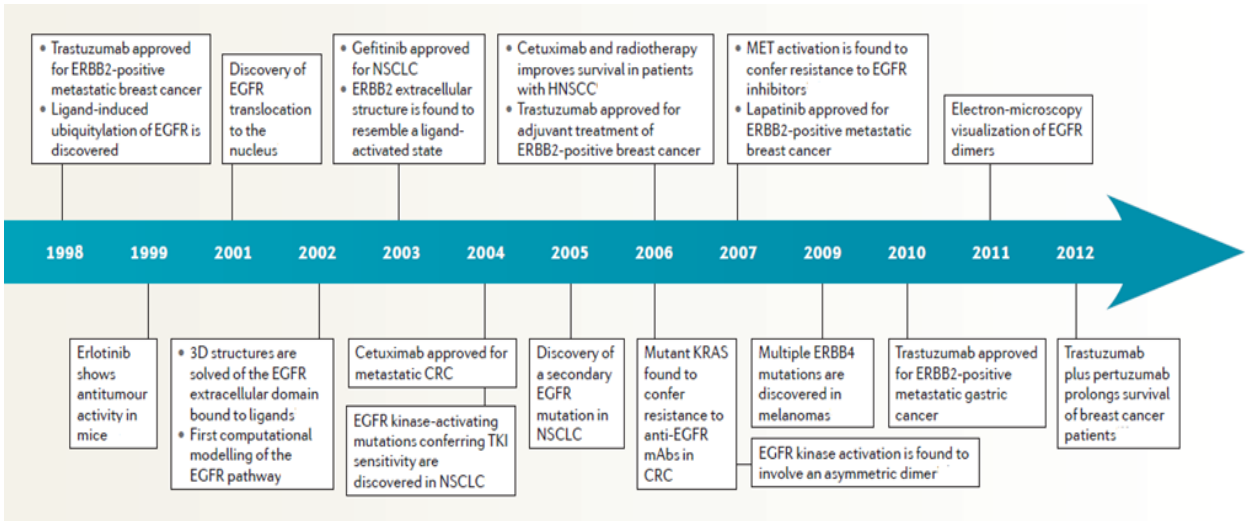


CRC, colorectal cancer; EGF, epidermal growth factor; EGFR, EGF receptor; HNSCC, head and neck squamous cell carcinoma; mAb, monoclonal antibody; NSCLC, non-small-cell lung cancer; TGFα, transforming growth factor-α; TKI, tyrosine kinase inhibitor.

Fig 1.3. ErbB receptors in cancer and their therapeutic targets. (A) A historical timeline of the discovery of ErbB receptors, ErbB receptor kinase activity, dimerization, overexpression, and role in several cancers and malignancies. (B) A historical timeline of drug development that target ErbB receptors in several types of cancer, and the ErbB mutations that may confer resistance for these drugs. Reprinted with permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Yosef Yarden & Pines, 2012), copyright (2012).

receptor (Hynes & Lane, 2005) (Fig 1.2). Two main pathways are usually involved in tumors with ErbB receptor activation; the phosphatidylinositol 3-kinase (PI3K)–AKT and the mitogen-activated protein kinase (MAPK) pathways (Schlessinger, 2004). Beyond these pathways other effectors may be involved. For example, the signal transducer and activator of transcription (STAT) proteins, the serine/threonine kinase mammalian target of rapamycin (mTOR), which is activated downstream of PI3K/AKT pathway, the SRC tyrosine kinases and many others (Yu & Jove, 2004; Bjornsti & Houghton, 2004) (Fig 1.4).

1.3. ErbB2 and breast cancer

Based on the Canadian Cancer Society study of 2015, the leading cancer for females is breast cancer (25,000 expected new cases, or 26% of all new female cases) (Canadian Cancer Statistics, 2015). Breast cancer is classified into three molecular subtypes, ErbB2- positive breast cancer (20 % of all cases), triple negative-breast cancer (10 % of all cases), and luminal-breast cancer (70 % of all cases) (Braunstein & Taghian, 2015). It is expected that there will be approximately 5000 new ErbB2-postive breast cancer cases in Canada by 2015 (Canadian Cancer Statistics, 2015). The ErbB2 (Her2/Neu) receptor (a member of the ErbB receptor family) gene is located on chromosome 17 and the molecular weight for the protein is 185 kD (Roskoski, 2013). ErbB2 is structurally different from the rest of the ErbB receptor family members because it lacks a known ligand-binding domain (Wilson et al., 2009; Maruyama, 2014). Generally, ErbB receptors can be activated upon ligand binding, these ligands bind to the extracellular domain of the receptor, inducing a major conformation change. This new conformation allows the formation of homodimers or induces the formation of heterodimers with other members of the family (Nevoltris & Chames, 2015). The absence of the ligand-binding activity in ErbB2 receptors makes it one of the preferential receptors for homo/hetero-dimerization (Karunagaran et al., 1996;

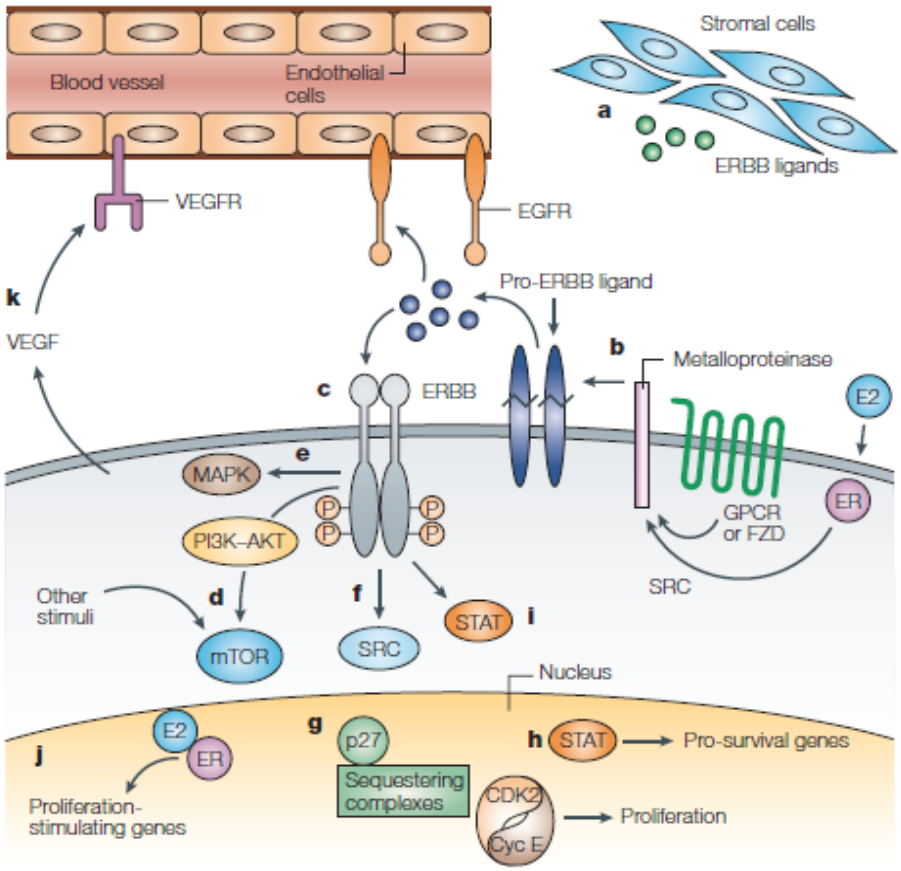


Fig 1.4. Active ErbB receptors and their associated downstream pathways and effectors in tumor cells. ErbB receptor activation might be caused by several mechanisms including: mutations, overexpression and abnormal paracrine/autocrine production of epidermal growth factor**. a, b, c**, and **e** represent the activation of MAPK and PI3K pathways in response to paracrine/autocrine stimuli that lead to the phosphorylation of several docking sites within the cytoplasmic domain of ErbB receptors. **d, f** and **i** together represent other proteins that can be involved with ErbB activation in tumor cells. These effector/modulators may involve other stimuli activation**. j, g** and **h** represent the downstream signaling activated once ErbB receptors are active. Aberrant activation of these pathways allow tumor cells to be more aggressive and metastatic. **K** represents angiogenesis signaling which has been associated with ErbB receptor activation (Yen et al., 2002) and this occurs through the activation of vascular endothelial growth factor (VEGF) as a result of MAPK pathways activity. Reprinted with permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Hynes & Lane, 2005), copyright (2005).

Graus-porta et al., 1997). Indeed, the active form of ErbB2 is always found in dimerization with ErbB2 receptors (homo) or other ErbB receptors (hetero) through the dimerization loop found in domain II . This dimerization loop is always exposed in ErbB2, while the other ErbB receptors require the ligand binding activity for the dimerization loop to be exposed (Garrett et al., 2008; Cho et al., 2003). Aberrant activation of the ErbB2 receptor kinases can lead to tumorigenesis or tumor progression in breast cancer. The clinical studies do not provide direct evidence that increased ErbB2 expression is sufficient for tumour initiation in the mammary gland. However, transgenic and mouse models studies have shown that overexpression of activated ErbB2 receptors is sufficient to efficiently transform mammary epithelial cells in transgenic mice (Ursini-Siegel et al., 2007). Indeed, Guy et al., (1996) showed that by using in situ hybridization and ribonuclease protection assays activated neu (ErbB2) transgene is able to convert normal mammary epithelium to have a malignant phenotype in three independent strains of mice. ErbB2 overexpression occurs in about 25-30% of primary breast cancer cases, as a result, patients with ErbB2-positive breast cancer have significantly lower survival rates and accelerated relapse than patients without ErbB2 overexpression (Spector & Blackwell, 2009; Fiszman & Jasnis, 2011; Hynes & Lane, 2005; Burgess, 2008).

Crosstalk and complex signaling pathways are always found with ErbB2 activation. For example, ErbB2 interacts with the insulin-like growth factor receptor (IGF-1R), which is considered a key regulator of cell growth and survival, and the membrane estrogen associated receptor (Harris et al., 2007; Yuhong Lu et al,. 2001). In addition, the PI3K and MAPK cellular signaling pathways are the two main pathways that are usually involved in ErbB2 activation (Spector & Blackwell, 2009). These pathways play important roles in cellular development; they regulate expression of

p53 and cyclin-dependant kinases through several transcription factors that control cellular proliferation and survival. As a result, patients whose breast tumors overexpress the ErbB2 have a significantly lower survival rate and a shorter period before relapse than patients without ErbB2 overexpression (Vu & Claret, 2012).

1.4. ErbB2 and Trastuzumab (Herceptin)

In 1989 before trastuzumab approval by the FDA, Hudziak et al (1989) studied the efficacy of Her185 mouse monoclonal antibody 4D5 for blocking ErbB receptors and preventing metastasis in several breast carcinoma cell lines. Since then, several studies have been published on the effect of monoclonal antibodies (mAb) against breast cancer cells that over expressing ErbB2 receptors (Shepard & Mendelsohn, 1991; Santã et al., 1992; Snider et al., 1996). Two models for ErbB2 inhibitors are currently in clinical use: humanized antibodies directed against ErbB2 and small molecule tyrosine-kinase inhibitors (Hynes & Lane, 2005). In preclinical models, both inhibitors rapidly downregulated PI3K, AKT, Erk, SRC and STAT signaling, which consequently blocked the proliferation of tumor cells in human xenografts in nude mice (Petit et al., 1997; Motoyama et al., 2002). Trastuzumab (Herceptin) is a humanized recombinant mAb that binds to the extracellular domain of ErbB2 protein, and it was approved by the FDA for ErbB2-positive metastatic breast cancer treatment (Tripathy et al., 2004). Regardless, the exact antitumor mechanisms of trastuzumab are not precisely known, but several possibilities have been proposed (Fig 1.5) (Lewis et al., 1993; Petit et al., 1997; Motoyama et al., 2002; Spector & Blackwell, 2009; Fiszman & Jasnis, 2011).

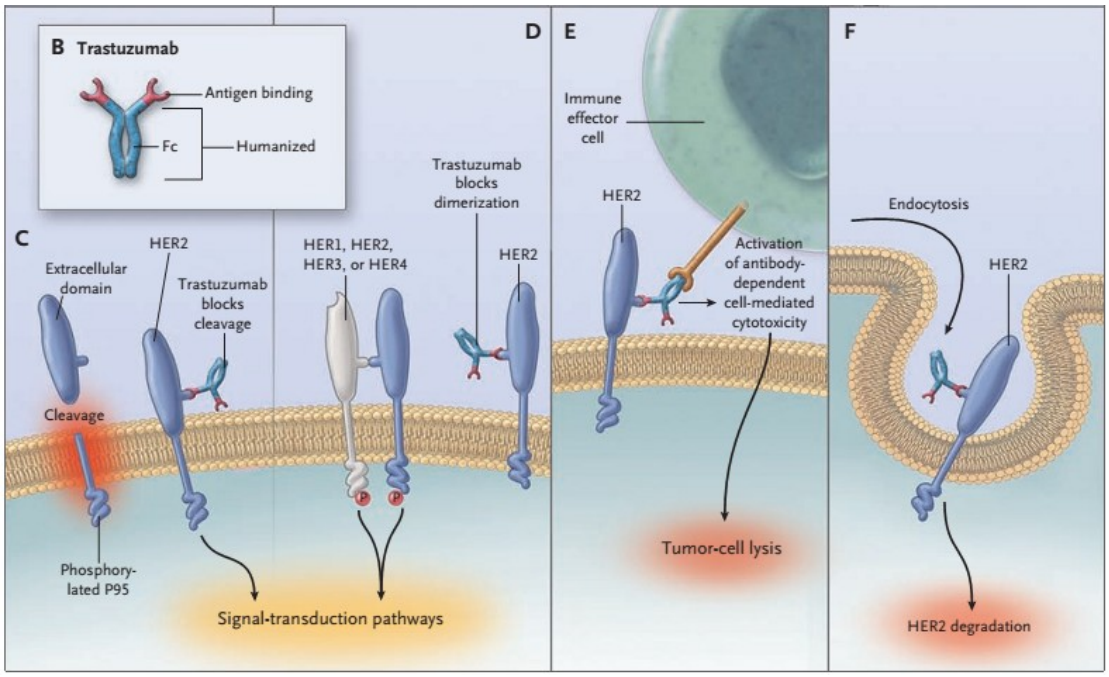


Fig 1.5. The potential mechanisms of the mode of action of trastuzumab. B. Structural representation of the trastuzumab mAb. **C.** Cleavage of the extracellular domain of ErbB2 leaves a membrane-bound phosphorylated p95, which can activate signal-transduction pathways. Binding of trastuzumab to a juxtamembrane domain of ErbB2 reduces shedding of the extracellular domain, thereby reducing p95. **D.** Trastuzumab may reduce ErbB2 signaling by physically inhibiting either homodimerization or heterodimerization. **E**. Trastuzumab may recruit Fc-competent immune effector cells and other components of antibody-dependent cell-mediated cytotoxicity, leading to tumor-cell death. **F**. Additional mechanisms such as receptor down-regulation through endocytosis have been postulated**.** (Adapted from Hudis, 2007)**.**

1.4.1. Trastuzumab; mode of action

As mentioned, the role of ErbB2 in cancer, specifically breast cancer, has been well studied and investigated. However, trastuzumab may target one or multiple cellular activities related to ErbB2 in breast cancer cells (Fig 1.6). Therefore, several mechanisms have been proposed for the action of trastuzumab towards metastatic breast cancer with ErbB2 receptor overexpression, but the exact mechanism of action is still not fully understood (Fiszman & Jasnis, 2011; Spector & Blackwell, 2009). However, both intracellular and extracellular modes of action have been proposed for trastuzumab and have been supported with several points of view, these mechanisms include: 1) the activation of the immune response (natural killer cells) against the breast cancer cells in a process known as Antibody Dependent Cell Cytotoxicity (ADCC) (Varchetta et al., 2007; Beano et al., 2008; Bianchini & Gianni, 2014); 2) inhibition of several downstream pathways that play roles in cellular proliferation and differentiation like PI3K and MAPK (Eichhorn et al., 2008); 3) downregulation of ErbB2 signaling by endocytosis (Ben-Kasus et al., 2009); 4) induction of apoptosis (Cuello et al., 2001) and 5) prevention of DNA repair activity and inhibition of angiogenesis (Spector & Blackwell, 2009) (Fig 1.7).

1.4.1.1. ADCC

Trastuzumab has been shown to act extracellularly to activate the immune system through ADCC and several studies have been conducted to examine the ADCC mechanism.

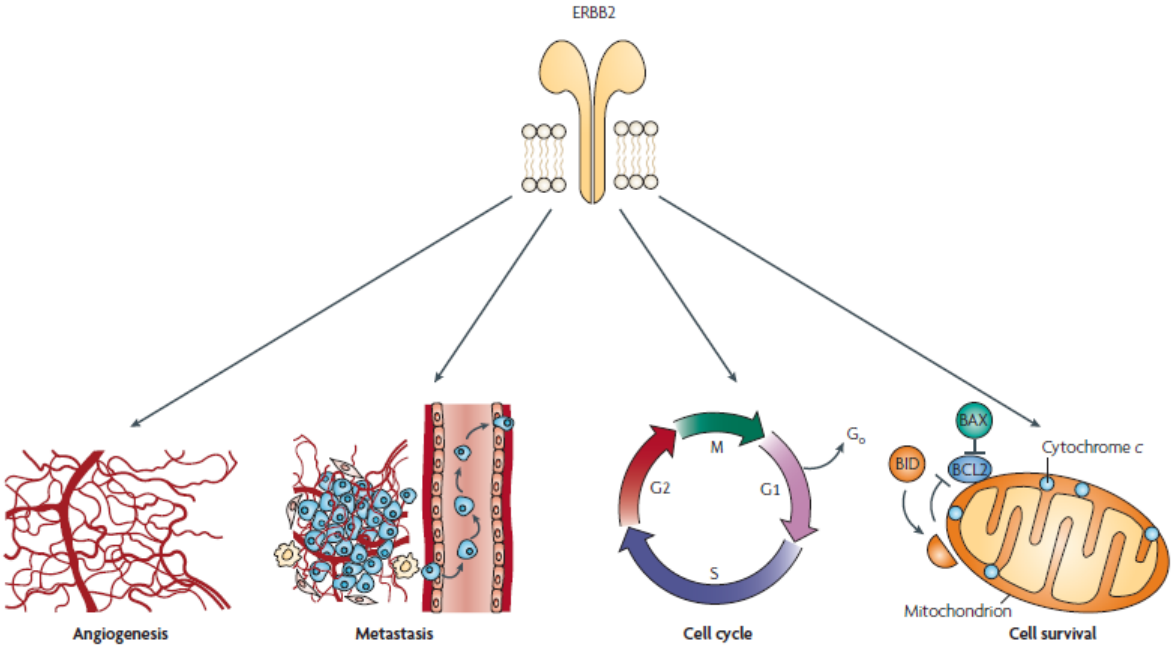


Fig 1.6. The outcomes of downstream activated pathways in ErbB2- overexpressing breast cancer cell lines. ErbB2 receptors play a role in cellular proliferation and survival, cancer cells take advantage of this and use it for their benefit leading to uncontrolled growth and proliferation. This includes increasing the ability of cells to survive and metastasize from one site to another in the human body via uncontrolled cell cycle and angiogensis. It has been suggested that trastuzumab targets and inhibts one or many of these ErbB2 activities in breast cancer cells.

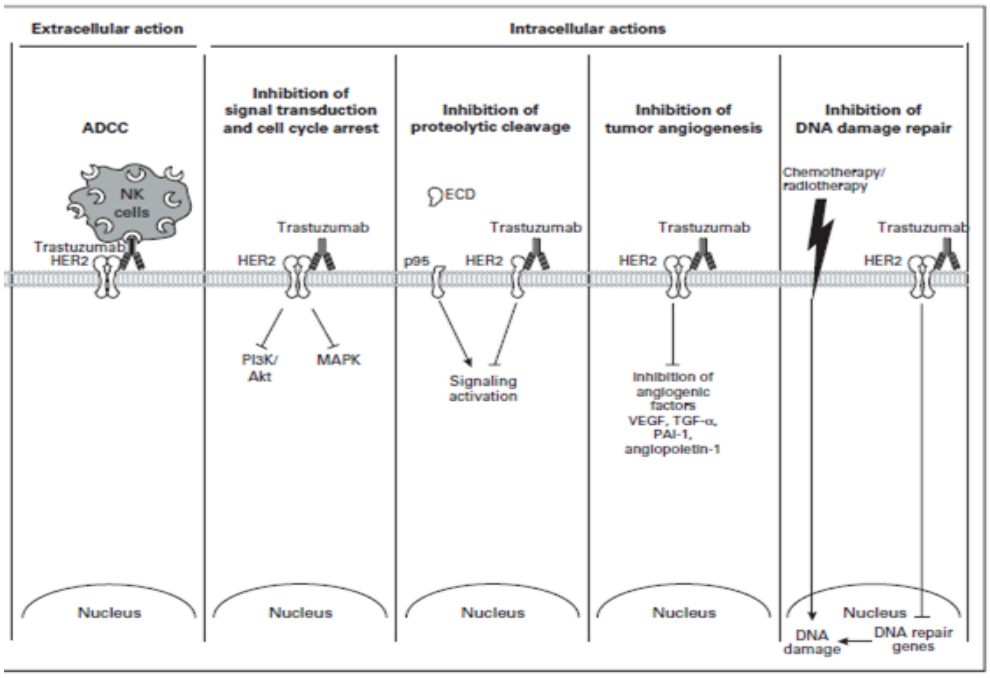


Fig 1.7. Representation of proposed intracellular/extracellular actions of trastuzumab. Binding of trastuzumab to ErbB2 in ErbB2 overexpressing breast cancer cells lead to recognition by and activation of natural killer (NK) cells, which then play a role in the immune response known as ADCC to destroy the cancerous cells. This occurs when the Fcγ receptors, present on the NK cells, recognize and bind the Fc domain on trastuzumab. This mode of action is described as an extracellular action. In terms of intracellular action, trastuzumab binds to ErbB2 and downregulates the PI3K and MAPK pathways that play a role in cell proliferation and survival. Trastuzumab is also able to prevent the production of the cleaved form of ErbB2, p95, which plays a role in signaling activation. Moreover, trastuzumab may also function to inhibit the vascular endothelial growth factor (VEGF), and the transforming growth factor α (TGF-α) receptor binding, through inhibiting the MAPK pathway, which consequently reduces angiogenesis. Finally, trastuzumab has been found to inhibit the DNA repair mechanisms that lead to accumulation of DNA damage caused by chemo/radio-therapy. However, the full mechanism for trastuzumab is still unknown and there are several controversial explanations regarding these proposed actions.

Spector & Blackwell: J Clin Oncol Vol. (27), 2009: 5838-5847.

Specifically, trastuzumab has an IgG1 Fc domain that allows immune cells to bind and activate natural killer cells through their Fcγ receptors (Moore et al., 2010). Indeed, Varchetta et al., (2007) found that ADCC was induced in 15 out of 18 ErbB2 overexpressing breast cancer patients treated with trastuzumab and they noticed an up-regulation of CD69 and CD107a levels compared to non treated patients, suggesting that natural killer cells and CD56+ T cells were involved in this immune response. Similar results were obtained by Arnould et al., (2006), Lazar et al., (2006), and Mimura et al., (2005), where immunohistochemically stained patient samples showed that activated natural killer cells were elevated approximately four times more in patients treated with trastuzumab than patients without trastuzumab treatment. It has been hypothesized that the ADCC mechanism happens through the interaction between Fcγ receptors on immune cells with the Fc domain of trastuzumab. This hypothesis was supported by Clynes et al., (2000) who showed that after treatment with trastuzumab or the 4D5 antibody there was a decrease in ErbB2-overexpressing tumor size in wild type mice compared to Fcγ receptor deleted/mutant mice. Repka et al., (2003) and Mimura et al., (2005) suggested that the ADCC response is proportional to the level of ErbB2 expression. Some studies suggested that ADCC is the exclusive mode of action of trastuzumab. For example, when Gennari et al., (2004) conducted a study on 11 patients with ErbB2 overexpressing breast cancer that had been treated with trastuzumab, they found only one patient with complete response and four patients with partial response. Interestingly, they did not find that ErbB2 levels, activity of downstream pathways or cellular proliferation was reduced and the mode of action was only through the ADCC mechanism as detected by high levels of lymphoid cells in all cases (Gennari et al., 2004).

1.4.1.2. Intracellular signaling pathways (PI3K and MAPK)

The PI3K and MAPK pathways are two of the main pathways activated by ErbB2 and are both very important for cancer cell proliferation and metastasis (Yakes et al., 2002; Junttila et al., 2009). Neve et al., (2002) demonstrated the role of the MAPK and PI3K pathways in breast cancer cells using different ErbB receptor inhibitors such as small molecules inhibtors and mAbs. They found that MAPK controls the cell cycle and cell survival by inducing activity of the transcription factor *c-Myc*, a proto-oncogene that up-regulates the expression of p53. This upregulation controls the level of cyclin-dependent kinase inhibitor p21/WAFI which plays a role in the cell cycle and cellular proliferation (Neve et al., 2002).

The crosstalk between the PI3K-AKT-mTOR pathways in addition to the MAPK pathways is complicated and therefore the action of trastuzumab in these pathways is not fully understood. For instance, Wenle Xia et al., (2002), and Wenle Xia et al., (2006) showed that trastuzumab does not affect cellular proliferation and survival to a large degree *in vitro*. However, there are several other studies that support the inhibitory effect of trastuzumab on cellular survival. Yakes et al., (2002) showed that trastuzumab downregulates the PI3K-MAPK pathway and cyclin D, which is a kinase regulator that plays a key role in the cell cycle. It has also been shown that treating patients with trastuzumab causes downregulation of the PI3K-AKT and MAPK-Erk pathways and consequently increases apoptosis by reducing the levels of survivin, an apoptosis inhibitor-like survival protein (Asanuma et al., 2005).

Normally, high expression of ErbB2 reduces the level of p27kip, a cyclin-dependant kinase inhibitor that plays a role in cell cycle arrest, and increases cellular proliferation (Fig 1.4). However, it has been reported by X.-F. Le et al., (2003) and X. Le, Pruefer, & Bast (2005) that p27kip1 activity was upregulated after trastuzumab treatment and this was conducted through the inactivation of the PI3K-AKT pathway.

Recently, Sahin et al., (2014) reported that trastuzumab treatment in both breast cancer cell lines and animal models that express high levels of ErbB2 and low levels of Phosphatase and Tensin homolog (PTEN), a negative regulator of the PI3K pathway, upregulates both the PI3K/mTOR and the MAPK-AKT pathway. They suggested that the upregulation of MAPK and PI3K after trastuzumab treatment in this study was based on loss of PTEN activity (Sahin et al., 2014). Moreover, when Green et al., (2014) immunohistochemically stained several breast cancer patient samples for several biomarkers involved with ErbB2 overexpression, and found that poor prognosis and high resistance for trastuzumab was associated with reduced levels of p21.

1.4.1.3. Blocking angiogenesis and proteolytic cleavage of ErbB2 extracellular domain (ECD)

In tumor cells, cleavage of the ErbB2 receptor extracellular domain (ECD) leads to p95 phosphorylation (Molina et al., 2001). Several studies have shown that trastuzumab blocks cleavage of the ErbB2 ECD. Molina et al., (2001) showed that generation of p95 was reduced due to blocking of ErbB2 ECD cleavage. On the other hand, Wenle Xia et al., (2004) showed that trastuzmab indeed blocks ErbB2 ECD cleavage however, they found that trastuzumab did not inhibit p95 phosphorylation or the expression of downstream pAKT and pErk . Altogether, trastuzumab plays role in blocking the ErbB2 cleavage but the exact mechanism is still poorly understood.

Angiogenesis is an essential process during tumorigenesis and allows cancer cell growth and metastasis (Jain, 2014). It has been shown that ErbB2 overexpression and vascular endothelial growth factor (VEGF), one of the key regulators of angiogenesis, are linked (Hynes & Lane, 2005) (Fig 1.4). VEGF plays a role in breast cancer cell survival and invasiveness (Klos et al.,

2003). Moreover, it has been suggested that PI3K and MAPK signaling pathways regulate VEGF expression in breast cancer cells (Chelouche-Lev et al., 2004). Inhibition of angiogenesis through inhibition of PI3K and MAPK pathways which in return reduce VEGF expression has been suggested as a mode of action of trastuzumab (Wen et al., 2006; Klos et al., 2003).

1.4.2. Trastuzumab; reasons behind resistance

The frequent occurrence of trastuzumab resistance after treatment in patients with metastatic breast cancer that overexpress ErbB2 receptor was noticed and is now considered one of the obstacles toward trastuzumab efficacy (Hudis, 2007). Both primary and secondary resistance were observed, approximately 35% and 70% respectively (Narayan et al., 2009; Devika Gajria And Sarat Chandarlapaty, 2001). Several mechanism have been proposed for trastuzumab resistance; loss of PTEN (Fig 1.8.b), signalling through other tyrosine kinase receptors, specifically, the insulin like growth factor receptor (IGFR) (Fig 1.8.c), and impaired trastuzumab binding to ErbB2 (Fig 1.8.a).

Impaired binding activity of trastuzumab was proposed as a mechanism of trastuzumab resistance for several reasons. First; Molina et al., (2002) and Scaltriti et al., (2007) showed that production of constitutive truncated p95, through ECD cleavage, cannot bind to trastuzumab (Fig 1.8.a). Also, Nagy et al., (2005) and Bourguignon et al., (2001) illustrated that

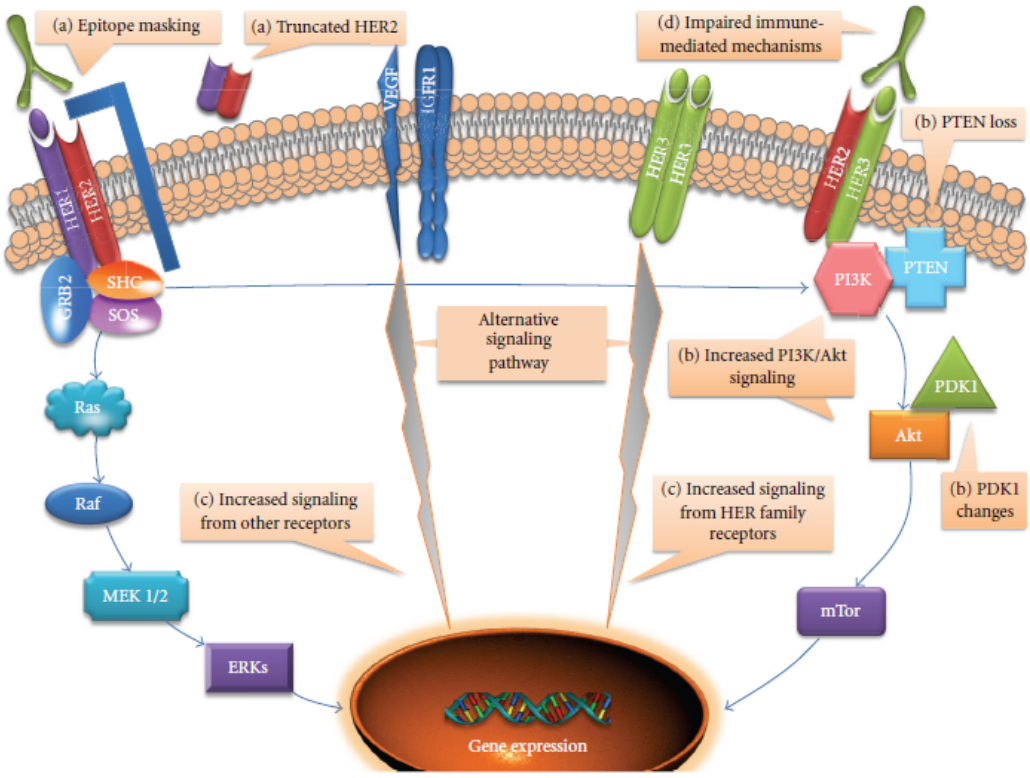


Fig 1.8. Possible mechansims implicated in trastuzumab resistance. a. The consequences of ErbB2 mutation or masking which prevents trastuzumab binding, leading to ErbB2 receptor activity. b. The upregulation of ErbB2 downstream proteins; pAKT, specifically through loss of PTEN and inactivation of PDK1. c. Shows the activation of cellular proliferation and growth through alternative signalling pathways, for example IGFR and VEGF. d. Loss of ADCC activity due to the lack of trastuzumab binding.

MUC4 (a membrane associated protein that plays a role in forming barriers on epithelial cells) and CD44 (a transmembrane receptor for hyaluronan that plays a role in cell-cell interaction and migration) have the ability to mask ErbB2 receptors and prevent recognition by immune cells. Loss of PTEN through mutation or inactivation is recognized in approximately 50% of breast cancers (Pandolfi & Ph, 2004) (Fig 1.9). Nagata et al., (2004), Pandolfi & Ph, (2004), and Dave et al., (2011) showed the loss of PTEN increased the activity of the PI3K-AKT pathway, which in return increased the rate of cell growth. Moreover, they found that the tumors associated with PTEN loss had a higher chance of developing trastuzumab resistance (Nagata et al., 2004; Dave et al., 2011).

Finally, many studies have uncovered a role for other receptors in trastuzumab resistance. Trastuzumab acts to downregulate ErbB2 receptors, however this may also lead to activation of other receptors in breast cancer cells. Indeed, crosstalk between EGFR/ErbB3 and/or ErbB2/ErbB3 (Motoyama et al., 2002), and ErbB2/the Insulin-like Growth factor 1 receptor (IGF-1R), a transmembrane receptor usually expressed in the breast cancer (Yuhong et al., 2001;Lu, Zi, & Pollak, 2004; Nahta et al., 2005), were proposed to activate the MAPK and PI3K pathways even with trastuzumab treatment. As a result, ErbB2 crosstalk activities with other receptors have been documented as one of the reasons behind trastuzumab resistance.

1.5. Project objective and research questions

After its approval by the FDA in 1998, trastuzmab has become a standard treatment for breast cancer patients who overexpress ErbB2, even though the mechanisms behind its resistance are not well understood. Many studies were conducted to answer the questions that

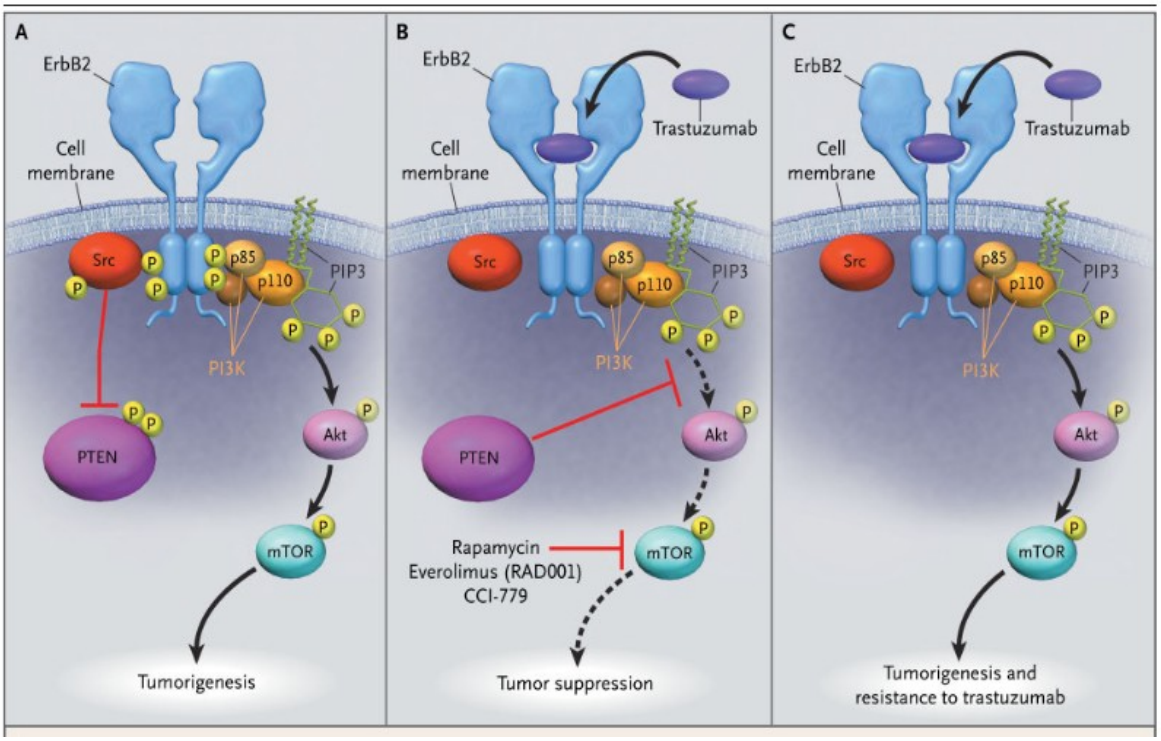


Fig 1.9. The activity of PTEN and its effect on the PI3K pathway. a. Normally, ErbB2 activation recruits and phosphorylates Src and PI3K subunits (p85 and p110), leading to inactivation of PTEN. b. Inactivation of ErbB2 by trastuzumab decreases activity of the PI3K pathway and Src, a negative regulator for PTEN. Consequently, PTEN inhibit the activity of AKT which plays a role in cellular proliferation. While, rapamycin is a mTOR antagonist. c. The loss of PTEN function restores AKT activity since there is no more dephosphorylation of AKT by PTEN, this leads to the activation of the PI3K-AKT-mTOR pathway which plays a role in trastuzumab resistance.

Reproduced with permission from (Pandolfi, 2004), copyright Massachusetts Medical Society.

arose concerning trastuzumab resistance, however, some basic biochemical questions still remain unanswered and needs more investigation. Specifically, it would be interesting to study whether the binding of trastuzumab is specific only to ErbB2 or other ErbB receptors as well. The ability of trastuzumab to block the homodimerization of ErbB2, the heterodimerization of ErbB2 with other ErbB receptors, and the heterodimerization of EGFR/ErbB3 is also unclear and need more investigation. Finally, it would be interesting to examine the major signaling pathways affected by trastuzumab and how these pathways might contribute to its resistance.

At the pharmacological level, there are as well a couple of questions that needs answering. Currently a combination therapy of trastuzumab with another drug such as pertuzumab or paclitaxel is the most effective in treatment of ErbB2-positive breast cancer (Swain et al., 2015). The role of trastuzumab is likely to inhibit excess signals for cell proliferation and survival and therefore a combined therapy is needed where another drug is required to kill the cancer cells. However the mechanism by which each drug contributes to the eradication of breast cancer, the reason behind the positive effects of a combined therapy is still not fully understood.

I believe that understanding the mode of action of trastuzumab will increase the understanding towards its resistance and contribute to the overall improvement of breast cancer therapy. Therefore, the goal of my project is to **elucidate the molecular mechanisms underlying the action of trastuzumab and combined trastuzumab therapy in breast cancer**. This was conducted through 1) studying the effect of short and long-term trastuzumab treatment on ErbB2 and EGFR receptors in a ligand-dependent and independent manner in breast cancer cell lines and CHO cell lines that stably express either ErbB2 or EGFR. 2) Examining the MAPK and PI3K pathways' activities after trastuzumab treatment. 3) Examining the homo-dimerization

activity of ErbB2 after trastuzumab treatment. 4) Assessing the viability of breast cancer cell lines after trastuzumab treatment or trastuzumab combined with chemotherapy drugs.

Chapter 2 Materials and Methods

2.1. Materials

All Chemicals, enzymes and kits were used according to the manufacturers' specifications and in accordance with protocols set out by the Environmental Health and Safety of the University of Alberta and Workplace Hazardous Materials Information System (WHMIS).

2.1.1. List of Chemicals and Reagents

Chemicals and Reagents	Vendor
Acrylamide/bis	Biorad
Ammonium persulfate	BDH
Anti-Anti (antibiotic-antimycotic)	Life Technologies
Bis (sulfosuccinimidyl) suberate (BS3)	Calbiochem
b-mercaptoethanol	Sigma
Bromophenol blue	Biorad
Coomasie brilliant blue, G250	Biorad
4',6-diamidino-2-phenylindole (DAPI)	Sigma
Dimethyl sulfoxide (DMSO)	Fisher
Dulbecco's modified eagle medium (DMEM)	Sigma
Epidermal growth factor (EGF)	Upstate
Ethanol, 70%	Fisher
Diaminoethanetetraacetic acid (EDTA)	Sigma
Fetal bovine serum (FBS)	Sigma
G418	Invitrogen
Glucose	EM science
Glycerol	BDH
Glycine	Biorad

Methanol	Fisher
MTT 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenylt etrazolium bromide	Fisher
Non-essential amino acids	Gibco
Paraformaldehyde (PF)	Sigma
Phosphate buffered saline, 10x	OmniPur
Sodium chloride	BDH
Sodium dodecyl sulfate (SDS)	Biorad
Tetramethylethylenediamine (TEMED)	Gibco
Trastuzumab (Herceptin)	Roche
Tris (hydroxymethyl) aminomethane	Biorad
Triton X-100	BDH
Tween 20	Fisher

2.1.2. Antibodies

Primary Antibodies	**Vendor**
Goat anti-Actin	Santa Cruz
Rabbit anti-EGFR (1005)	Santa Cruz
Rabbit anti-ErbB2 (C 18)	Santa Cruz
Rabbit anti-ErbB3 (C 17)	Santa Cruz
Rabbit anti-Erk (1/2)	Santa Cruz
Rabbit anti-phospho-AKT (Ser 473)	Santa Cruz
Rabbit anti-phospho-EGFR (992)	Biosource
Rabbit anti-phospho-EGFR (1086)	Santa Cruz

Rabbit anti-phospho ErbB2 (1248)	Santa Cruz
Rabbit anti-phospho ErbB2 (1196)	Santa Cruz
Rabbit anti-phospho ErbB2 (1222)	Santa Cruz
Rabbit anti-phospho ErbB3 (1328)	Santa Cruz
Rabbit anti-phospho PLCγ (1253)	Santa Cruz
Mouse anti-phospho Erk (1/2)	Santa Cruz
Mouse anti-Tubulin	Santa Cruz
Secondary Antibodies	**Vendor**
HRP-conjugated anti-Goat	Biorad
HRP-conjugated anti-Mouse	Biorad
HRP-conjugated anti-Rabbit	Biorad

2.1.3. Molecular Size Markers

Prestained marker for SDS-PAGE	Sigma

Table 2.1 Buffers and other solutions used in this thesis

Solution	**Composition**
Phosphate-buffered saline (PBS)	137 mM NaCl, 2.7mM KCl, 10 mm phosphate buffer
SDS-loading buffer	250mM Tris-Cl, 40% glycerol, 8% sodium dodecyl
Transfer buffer	48 mM Tris-Cl, 39 mM Glycine, 20% methanol, 0.03% sodium dodecyl sulfate
Tris-EDTA buffer	10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.5
Stripping buffer (Western-blot)	PBS-0.05% Tween 20, 2% SDS, 100mM β-mercaptoethanol

Protease inhibitor cocktail	0.5mM Na3VO4, 0.1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin and 1μM pepstatin A

2.1.4. Other Materials

Transblot Nitrocellulose	Biorad
Dehydrated milk for WB blocking	Pacific
Whatman Chromatography Paper	Fisher
Medical X-ray Film	Fuji
the Vybrant MTT Cell Proliferation Assay Kit	Fisher

2.1.5. Machines used in this thesis

SpectraMax 190 microplate reader	Molecular Devices
Delta vision Olympus	Applied precision

2.2. Methods

2.2.1. Cell Lines

The following cell lines were used in this thesis: BT-20 cells (human breast cancer cells, ATCC HTB-19); SKBR3 (human breast cancer cells, ATCC HTB-30); MDA-MB-453 (human breast cancer cells, ATCC HTB-131), ErbB2 is overexpressed in SKBR3 and MDA-MB-453, while it is moderately expressed in BT20 (Fig. 3.1); hamster ovary CHO cells (gift from Dr. Luc Berthiaume, University of Alberta); CHO cells stably expressing wtEGFR, (stable cell lines were selected previously by Dr. Qian Wang in our lab (Wang et al., 2007); CHO cells stably expressing ErbB2; CHO-ErbB2 (K6) and CHO-Her2 (K13) (these cell lines were a generous gift from Dr. Christian J Bucholz, Paul-Ehrlich-Institute (Münch et al., 2011).

2.2.2. Cell Culture

All cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin, and streptomycin (100 U/ml), and were maintained in a 5% CO_2 atmosphere. For CHO-EGFRwt, G418 was added to a final concentration of 500 μg/ml. For CHO-ErbB2 (K6) and CHO-ErbB2 (K13), G418 was added to a final concentration of 1.2 mg/ml and 0.5 mg/ml respectively.

2.2.3. Cell Treatment with Trastuzumab

2.2.3.1. Breast cancer cell lines (BT20, SKBR3, MD-MB-453) treatment

In this thesis, 10 μg/ml and 40 μg/ml of trastuzumab were used as a final concentrations for treatment as previously described (Gijsen et al., 2010; Yakes et al., 2002; Junttila et al., 2009; Dokmanovic et al., 2014).

Cells were supplemented with 10% FBS and protein total lysate was collected after the following conditions: C: control (no treatment); E: 50 ng/ml Epidermal growth factor (EGF) stimulation for 15 min at 37°C, (this dosage was used since previous dose response experiments already conducted in our lab showed that this concentration and this time point were able to activate EGFR (Wee et al., 2015)); T: trastuzumab treatment for 1 hr with 40 μg/ml final concentration at 37°C and T+E: Trastuzumab treatment for 1 hr followed by EGF stimulation for 15 min at 37°C with the previous mentioned concentrations. Then the same experiment of design was conducted but this time with 24 hr prior to serum starvation, to prevent any activation that might be caused by the growth factors that FBS contains.

Treatment of 10 μg/ml of trastuzumab was used following the same design used for the 40 μg/ml final concentration experiment. However, in this experiment, two more conditions were added to the previous design; 48 hr treatment of trastuzumab alone or followed by EGF stimulation for 15 min. All samples were 24 hr serum starved.

2.2.3.2. CHO cell lines (CHO-EGFR, CHO-ErbB2-K6 and CHO-ErbB2-k13) treatment

In order to have a better idea of how trastuzumab affects or does not affect ErbB receptors and its signaling pathways; a time course experiment treatment was conducted on the three CHO cell lines.

Two methods for the treatment were applied. Firstly, CHO-EGFR, CHO-ErbB2 (K6), and CHO-ErbB2 (K13) were treated after 24 hr serum starvation with 10 μg/ml of trastuzumab alone for 1 hr, 4 hr, 8 hr, 16 hr, 24 hr, and 48 hr. Secondly, the same time courses were used, but were followed by EGF stimulation for 15 min. For both methods, the controls used were CHO parental cells (not expressing either EGFR or ErbB2) as negative controls, and CHO-EGFR, CHO-ErbB2 (K6), and CHO-ErbB2 (K13) samples that were not treated with trastuzumab, either serum starved or supplemented with 10% FBS or stimulated with EGF alone as additional controls. CHO-ErbB2 (K6) and CHO-ErbB2 (K13) cell lines were stably transfected with ErbB2 and have a $2.1x10^5$ and $3.7x10^4$ ErbB2 surface density (number of ErbB2 molecules per cell) respectively (Münch et al., 2011)

2.2.4. Preparation of total lysate

To obtain total lysates from SKBR3, BT20, MDA-MB-453, CHO, CHO-EGFR, CHO-ErbB2 (K6), and CHO-ErbB2 (K13), cells were lysed in ice-cold Mammalian Protein Extraction Reagent (Pierce, Rockford, Illinois) containing a protease inhibitor cocktail. The lysates were

then centrifuged at 4°C for 15 min at 21,000 x *g*. The supernatant was collected, and protein was quantified using the Bradford protein dye assay (Bradford, 1976). Absorbance at λ=595 nm was measured by a Beckman DU 640 spectrophotometer (Beckman Instrument, Fullerton, CA). Bovine Serum Albumin (BSA) was used as a standard.

Following protein quantification, protein samples were boiled in SDS-loading buffer for 5 min and stored at -80°C for SDS-PAGE applications.

2.2.5. SDS-PAGE and Immunoblotting

Aliquots of protein from each sample were used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) assay. For the staining of total cell lysates, aliquots containing 20 µg of protein from each cell lysate were used. Protein samples were separated by electrophoresis through 8.5-10% SDS-polyacrylamide gels at 180 volts for 48 minutes. Pre-stained protein markers (Sigma) were used for molecular weight standards.

Following SDS-PAGE, Proteins were electrophoretically transferred onto Trans-blot nitrocellulose membranes (BioRad, Hercules, CA). Transfer was done using a semi-dry blotting apparatus (Model SD transfer cell, BioRad) at 15 mA per minigel for 90 min in transfer buffer. Blots were blocked with 3% skim milk in 0.05% Triton X -TBS (blocking buffer) for 25 min to reduce the background. Membranes were then probed with the respective primary antibody in blocking buffer at 4°C overnight. after washed twice with 0.05% Triton X –TBS for 10 min, membranes were then incubated with HRP-conjugated IgG secondary antibody for 1 hour at room temperature, washed with TBS buffer for 10 min. Secondary antibodies were detected by enhanced chemiluminescence, with SuperSignal ECL Western Blotting Detection Reagents (Pierce Chemical) and light detection with Fuji Super RX x-ray film (Tokyo, Japan).

All antibodies used for immunoblotting with their respective dilutions are listed in **Table 2.2.**

Table 2.2. Antibodies and their dilutions used for Western Blotting

Primary Antibodies	**Dilution**
Goat anti-Actin	1:500
Rabbit anti-EGFR (1005)	1:500
Rabbit anti-ErbB2 (C 18)	1:1000
Rabbit anti-ErbB3 (C 17)	1:1000
Rabbit anti-Erk (1/2)	1:1000
Rabbit anti-phospho-AKT (Ser 473)	1:2000
Rabbit anti-phospho-EGFR (992)	1:1000
Rabbit anti-phospho-EGFR (1086)	1:1000
Rabbit anti-phospho ErbB2 (1248)	1:1000
Rabbit anti-phospho ErbB2 (1196)	1:1000
Rabbit anti-phospho ErbB2 (1222)	1:1000
Rabbit anti-phospho ErbB3 (1328)	1:1000
Rabbit anti-phospho PLCγ (1253)	1:1000
Mouse anti-phospho Erk (1/2)	1:1000
Mouse anti-Tubulin	1:1000
Secondary Antibodies	**Dilution**
HRP-conjugated anti-Goat	1:1000
HRP-conjugated anti-Mouse	1:1500
HRP-conjugated anti-Rabbit	1:2000

2.2.6. BS3 Cross-linking assay

SKBR3, MDA-MB-453, and CHO-ErbB2 (K6) cells were cultured to subconfluency. Cells were then starved for 24 hr. After treatment either with1 hr trastuzumab (10 μg/ml) at 37°C, or 15 min EGF (50 ng/ml) at 37°C, or both 1 hr trastuzumab followed by 15 min EGF (50 ng/ml) at 37°C, the cells were collected in 0.2~0.5 ml PBS. BS3 (Calbiochem) was then added to a final concentration of 1.0~2.5 mM and the reaction was incubated on ice for 2 hr. After that, quench solution (1 M Tris, pH 7.5) was added to a final concentration of 10 mM and incubated on ice for 15 min. Finally, the cells were lysed with NP-40 (1:100 dilution) for 1 hr on ice and then centrifuged at 4°C for 15 min at 21,000 x *g*. Samples were stored at 80°C and receptor dimerization analysis was conducted by PAGE and immunoblotting.

2.2.7. Fluorescence Microscopy

In order to examine the efficacy of binding of trastuzumab to ErbB2 on the plasma membrane, indirect immunofluorescence microscopy was carried out as described previously (Liu et al., 2011). MDA-MB-453 and CHO-ErbB2 (K6) cells were grown in 24 wells plate on a glass coverslips. After 24 hr of serum starvation, cells were treated with trastuzumab (10 μg/ml) for 1 hr at 37°C. After treatment, the cells were fixed by -20°C methanol and permeabilized with 0.2% TBS-Triton X-100. Then, the cells were incubated with indicated primary antibodies at room temperature for 1 hr, followed by FITC/TRITC-conjugated secondary antibodies for 1 hr at room temperature. The stained cells were then observed and photographed with inverted Olympus 1X71 microscope (Applied Precision) with standard filters, and the data were analyzed using Delta Vision softWoRx software. A list of antibodies and their dilutions used for immunofluorescence are given in **Table 2.3.**

Table 2.3. Antibodies used in immunofluorecence

Primary Antibodies	**Dilution**	**Vendor**
Mouse anti ErbB2	1:100	Santa Cruz
Trastuzumab	1:8000*	Roche
Secondary antibody		
FITC- conjugated anti mouse	1:200	Bio-Rad
Rhodamin-conjugated anti human IgG	1:200	Sigma

*Trastuzumab's stock concentration is 80 mg/ml

2.2.8. Cell viability assay

SKBR3, MDA-MB-453, BT20, CHO-ErbB2 (K6), CHO-ErbB2 (K13) cells were plated onto 96-well plates at 30,000**,** 50,000, 10,000, 10,000, 10,000 cells/well, respectively. 24 hr later, the culture medium was replaced by fresh medium containing different concentrations of either doxorubicin, or trastuzumab, or both combined doxorubicin and trastuzumab simultaneously. Using the Vybrant MTT Cell Proliferation Assay Kit, 48 hr after the drug treatment, the culture media was replaced with fresh media contains 12 mM MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) and incubated for 4 hr at 37°C. After labeling the cells with MTT, 50 µl of DMSO was added to each well and incubated at 37°C for 10 min. Then, the percentages of viable cells were then determined by measuring the absorbance intensity using SpectraMax 190 microplate reader (Molecular devices) at 540 nm.

2.2.9. Statistical analysis

In this thesis, Student's t-test was applied for statistical analysis. For western blotting experiments, two tailed student's t-test was applied. All samples were normalized to loading control. Treated samples with trastuzumab were compared to control samples (not treated with trastuzumab) that were either supplemented with 10% FBS or serum starved. Regarding samples that were treated with trastuzumab and followed by EGF stimulation, they were compared to samples that were only stimulated with EGF. For MTT assay, one tailed student's t-test was applied. Treated samples with either trastuzumab or doxorubicin were normalized to control samples (not treated with trastuzumab or doxorubicin respectively). For the synergetic treatment of trastuzumab with doxorubicin samples, they were compared to either trastuzumab only treated samples or doxorubicin only treated samples or control samples (not treated with trastuzumab or doxorubicin).

Chapter 3 Results

3.1. EGFR, ErbB2, and ErbB3 expression level in breast cancer cell lines

SKBR3, BT20, and MDA-MB-453 cells lines have different expression level of ErbB2, EGFR and ErbB3 proteins in (Table 3.1, Fig 3.1). I hypothesized that this difference could provide us a better understanding of the mode of action of trastuzumab on all ErbB receptors, and not just ErbB2. As a result, these cell lines were selected out of 38 breast cancer cell lines that are provided by the American Type Culture Collection (ATCC) (Table 3.1). I first determined the expression level of EGFR, ErbB2, and ErbB3 in the SKBR3, BT20, and MDA-MB-453 cell lines using immunoblotting (Fig 3.1). ErbB2 is overexpressed in SKBR3 and MDA-MB-453, while it is moderately expressed in BT20 (Fig 3.1.A). On the other hand, EGFR is overexpressed in BT20, and moderately expressed in SKBR3. MDA-MB-453 lacks EGFR expression (Fig 3.1.B). Regarding ErbB3, it is expressed in MDA-MB-453 and SKBR3, while BT20 lacks the expression of ErbB3 (Fig 3.1.B).

3.2. Trastuzumab is not able to block ErbB2 and EGFR phosphorylation in a ligand-independent or -dependent manner

After examining the expression level of ErbB proteins in the breast cancer cell lines, immunoblotting experiments were conducted on treated samples with trastuzumab.

Treatment with only 1 hr of trastuzumab (40 μg/ml) did not inhibit ErbB2 phosphorylation in SKBR3, BT20, and MDA-MB-453, and instead upregulated the phosphorylation of the receptor compared to control samples in SKBR3 and BT20 (Fig 3.2.). On the other hand, 1 hr trastuzumab treatment had an inhibitory effect on the phosphorylation of ErbB3 in MDA-MB-453 compared to control sample (Fig 3.2). 1 hr trastuzumab treatment in SKBR3 was able to

Table 3.1. The expression level of EGFR, ErbB2, and ErbB3 in 38 breast cancer cell lines provided by the ATCC. Modified from (Neve et al., 2006)**.**

Cell line	ER	PR	EGFR	ErbB2	ErbB3
184A1N4	-	-	++++	-	-
184B5	-	-	+++	-	+
600MPE	-	-	-	+	++
AU565	-	-	++	+++++	++
BT20	-	-	+++	-	-
BT474	+	+	+++	++++	++++
BT483	+	+	-	+	++
BT549	-	-	+++	-	-
CAMA1	+	-	-	+	+++
HBL100	-	-	++	-	-
HCC1143	-	-	++++	+	++
HCC1187	-	-	++++	++++	+
HCC1500	-	-	++++	-	-
HCC1569	-	-	+++	++++	++
HCC1599	-	-	-	+	++++
HCC202	-	-	+++++	+++++	+++
HCC38	-	-	+++	++	+++
HCC70	-	-	+++	+	-
HS578TT	-	-	+++	-	-
LY2	-	-	-	+	+++
MCF10A	-	-	-	-	-
MCF12A	-	-	+	-	-
MCF7	+	+	-	-	+++
MDAMB134VI	+	-	-	+	++
MDAMB157	-	-	++++	++	-
MDAMB175VII	+	-	-	++++	++
MDAMB231	-	-	++++	++	-
MDAMB361	+	-	+	+++	+++
MDAMB415	+	-	-	++	++
MDAMB435	-	-	-	+	++
MDAMB453	-	-	-	++	++
SKBR3	-	-	++	+++++	++
SUM225CWIN	-	-	++++++	++++++	+++
T47D	+	+	-	++	+++
UACC812	+	-	++	++++	++
ZR751	+	-	-	++	++
ZR7530	+	-	++++	++++++	+++++
ZR75B	-	-	-	++	+++

	EGFR	ErbB2	ErbB3
• BT20	+++	+	-
• SKBR3	++	+++++	++
• MDA-MB-453	-	+++	++

(Modified from Neve *et al.*, 2006)

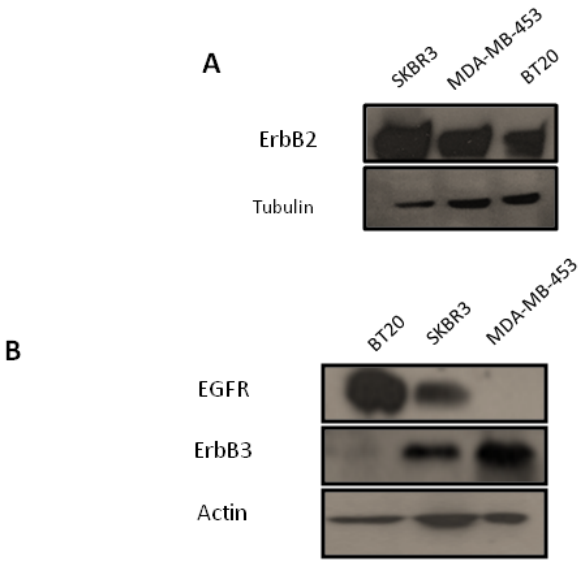


Fig 3.1. The expression level of EGFR, ErbB2, and ErbB3 in SKBR3, MDA-MB-453, and BT20 breast cancer cell lines. Immunoblotting experiments were performed on SKBR3, BT20, and MDA-MB-453 cells after they were cultured in 10% FBS medium to confluency. (A) An antibody that detect ErbB2 (C 18) was used. (B) Antibodies that detect EGFR (1005) and ErbB3 (C 17) were used. Actin and Tubulin were used as loading controls. Reproducible representative results are shown (n = 2).

Fig 3.2. The effect of 1hr Trastuzumab treatment in 10% FBS on ErbB2, EGFR, and ErbB3 in breast cancer cell lines. Immunoblotting experiments were preformed on SKBR3, BT20 and MDA-MB453 after 1 hr trastzumab treatment (40 μg/ml). Antibodies that detect the phosphorylated tyrosine residues 1248, 1328, 1086 for ErbB2, ErbB3, and EGFR respectivly were used. Cells were supplemented with 10% FBS. **C**: Control; **E**: EGF stimulation (15 min) (50 ng/ml)**; T**: Trastuzumab treatment 1hr (40 μg/ml); and **T+E**: 1hr trastzumab treatment (40 μg/ml) follwed by EGF stimulation (15 min) (50 ng/ml). Actin was used as a loading control. Reproducible representative results are shown (n = 2). Student's t-test was applied for statistical analysis. Error bars represent standard error (SE). n.s. not significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

reduce ErbB3 phosphorylation in the presence of a ligand compared to EGF-stimulated samples (Fig 3.2).

To examine the effect that FBS might contribute to ErbB receptor activity, cells were serum starved for 24 hr and then treated with trastuzumab (40 μg/ml) for 1 hr. The preliminary data shows that ErbB2 phosphorylation is reduced to almost no detectable level in control samples after starvation in SKBR3 and BT20 cells (Fig 3.3.) compared to (Fig 3.2.) that shows phosphorylation activity for ErbB receptors in control samples. Interestingly, 1h trastuzumab treatment by itself seems is not increasing pErbB2 in SKBR3 (Fig 3.3) compared to (Fig 3.2.) that shows upregulation of pEGFR, and pErbB2 after trastuzumab treatment. However, trastuzumab enhanced AKT phosphorylation compared to control (Fig 3.3). To confirm these preliminary findings, more experiments after serum starvation and 40 μg/ml trastuzumab treatment are required.

3.3. Trastuzumab blocks the phosphorylation of ErbB2 and EGFR after 48h in the presence of EGF in SKBR3.

I then examined the effect of a lower concentration of trastuzumab (10 μg/ml) on ErbB receptors using the same breast cancer cell lines. 1 hr and 48 hr trastuzumab treatment experiments were conducted. Trastuzumab treatment alone did not affect the level of ErbB2 in SKBR3 (Fig 3.4.A). Moreover, 1 hr trastuzumab treatment did not block the ability of EGF to induce the phosphorylation of ErbB2 (Y1248, Y1196) in SKBR3 cells, however, interestingly 1 hr (10 μg/ml) trastuzumab treatment was able to reduce the phosphorylation of EGFR (Y992) compared to the EGF-only stimulated sample (Fig 3.4.C). Strikingly, 48 hr trastuzumab

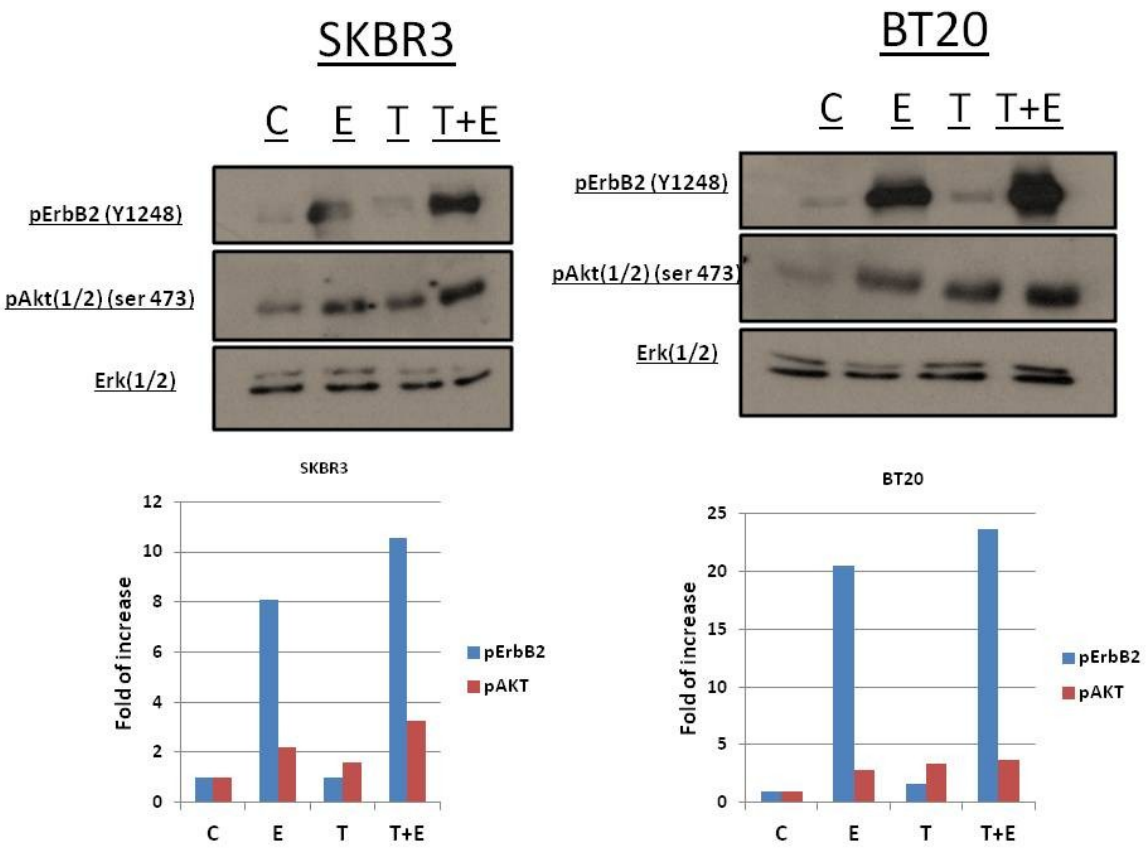
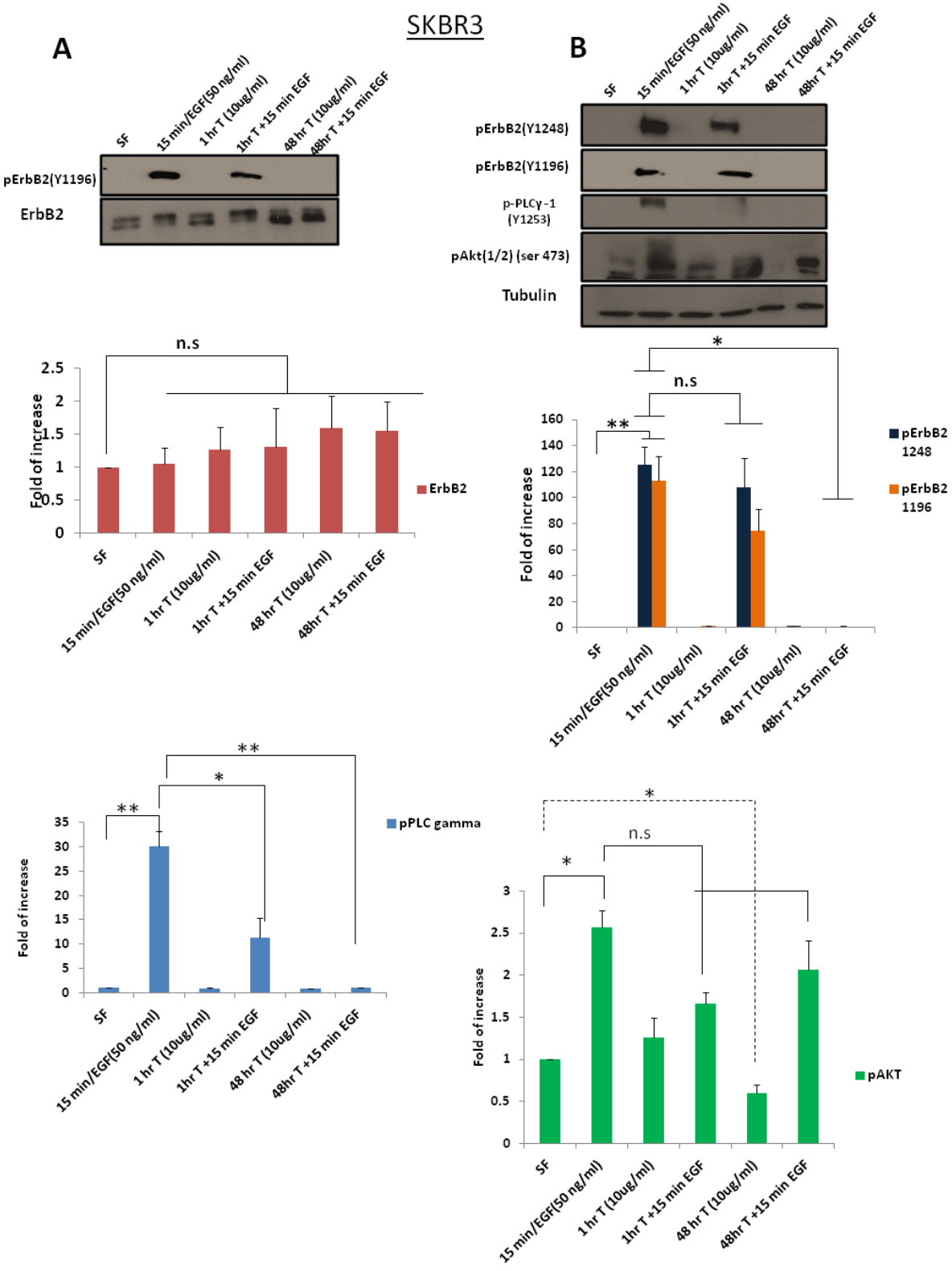


Fig 3.3. The effect of 1 hour Trastuzumab treatment in 24 hour serum starvation conditions on breast cancer cell lines. Immunoblotting experiments were performed on SKBR3 and BT20 after 1 hr trastuzumab treatment (40 µg/ml). Antibodies that detect phosphorylated Akt (Ser 473) and the phosphorylated ErbB2 (Tyr 1248) were used. Samples were serum-starved for 24hr. **C**: Control, **E**: EGF stimulation (15 min) (50 ng/ml),**T**: Trastuzumab treatment 1hr (40 µg/ml) and **T+E**: 1hr trastzumab treatment (40 µg/ml) follwed by EGF stimulation (15 min) (50 ng/ml)**.** Erk 1/2 was used as a loading control (n = 1).


SKBR3
A
SF
15 min/EGF(50 ng/ml)
1 hr T (10ug/ml)
1hr T +15 min EGF
48 hr T (10ug/ml)
48hr T +15 min EGF
pErbB2(Y1196)
ErbB2
B
pErbB2(Y1248)
pErbB2(Y1196)
p-PLCγ-1 (Y1253)
pAkt(1/2) (ser 473)
Tubulin
n.s
Fold of increase
ErbB2
*
**
pErbB2 1248
pErbB2 1196
pPLC gamma
pAKT

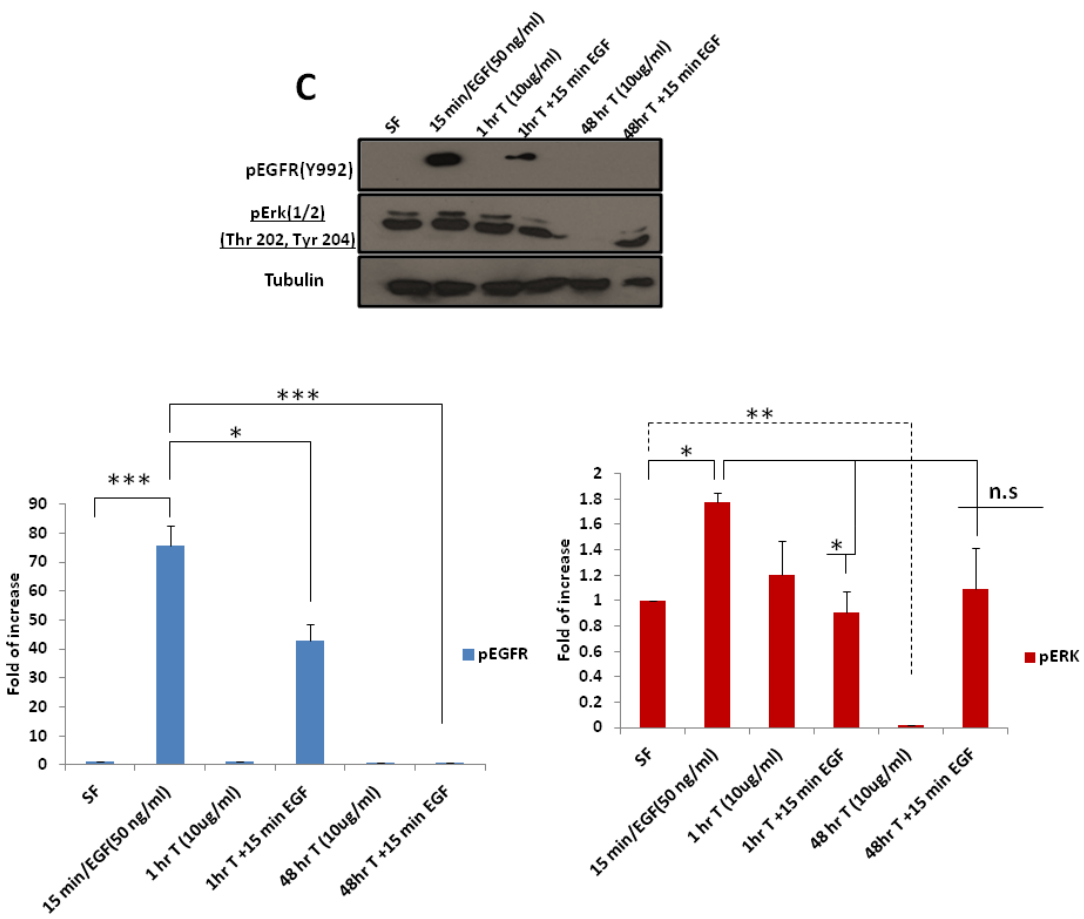


Fig 3.4. Trastuzumab blocks the phosphorylation of ErbB2, EGFR, but not Erk and AKT after 48h in the presence of EGF. Immunoblotting experiments were performed on SKBR3 samples treated with 1 hr and 48 hr trastzumab (10 μg/ml) as indicated. Antibodies that detect the phosphorylated Akt (Ser 473), the phosphorylated Erk 1/2 (Thr 202, Tyr 204), the phosphorylated PLC-γ1 (Tyr 1253), and the phosphorylated residues for ErbB2 (Tyr 1248, Tyr 1196) and the phosphorylated EGFR (Tyr 992) were used. Samples were serum-starved for 24 hr. **SF** (serum free) sample was used as a negative control and **EGF** (50 ng/ml) sample was used as a positive control. Tubulin was used as a loading control. Reproducible representative results are shown (n = 3). Student's t-test was applied for statistical analysis. Error bars represent standard error (SE). n.s. not significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

treatment did block the EGF-induced phosphorylation of both ErbB2 and EGFR receptors to almost undetectable levels compared to EGF only stimulated sample (Fig 3.4.B and 3.4.C).

I then examined the effect of trastuzumab treatment after 1 and 48 hr on the downstream signaling pathways that are activated by ErbB receptors in SKBR3 cells. For this purpose, I examined the phosphorylation of PLC-γ1 (Raimondi et al., 2012), as well as Erk and AKT, two downstream proteins that can be activated by ErbB receptors and that have been suggested to play a role in trastuzumab resistance (Neve et al., 2002; Asanuma et al., 2005).

Interestingly, and consistent with the reduction of pEGFR activity after 1 hr and 48 hr trastuzumab treatment in the presence of EGF ligand (Fig 3.4.C), trastuzumab was able to reduce the activity of PLC-γ1 phosphorylation compared to EGF-stimulated sample (Fig 3.4.B). pAkt (Fig 3.4.B) was not inhibited after 1hr and 48 hr trastuzumab treatment in the presence of EGF compared to EGF-only stimulated sample. However, 48 hr treatment of trastuzumab in the absence of EGF was able to reduce pAKT activity compared to SF control sample. 1 hr trastuzumab treatment did inhibit pErk activity, whereas 48 hr treatment did not significantly inhibit pErk activity in the presence of EGF compared to EGF-stimulated sample (Fig 3.4.C). 1 hr trastuzumab treatment only and in the absence of EGF failed to inhibit pErk compared to SF control sample, however, 48 hr treatment was significantly able to inhibit its activity when compared to the same control (Fig 3.4.C).

3.4. Trastuzumab upregulates the phosphorylation of ErbB2 after 1hr and prevents Erk phosphorylation after 48 hr treatment in MDA-MB-453.

An immunofluorescence experiment was conducted to see the localization of trastuzumab on the plasma membrane in MDA-MB-453. 1hr treatment was sufficient for trastuzumab to bind ErbB2

(Fig 3.5). 1 hr trastuzumab treatment in MDA-MB-453 cells was able to upregulate pErbB2 (Y1248 and 1196), but not 48 hr treatment (Fig 3.6.B and 3.6.C). EGF stimulation did not induce pErbB2 activation as expected, since it lacks EGFR expression (Fig 3.1). 1hr trastuzumab treatment did not inhibit Erk phosphorylation and instead it was upregulated when compared to control; however, 48 hr treatment was able to inhibit pErk activity (Fig 3.6.C). pAkt activity remained unaffected after 1 hr or 48 hr trastuzumab treatment (Fig 3.6.B).

3.5. Trastuzumab blocks the phosphorylation of EGFR after 48h in the presence of EGF, and does not activate the phosphorylation of EGFR or its binding protein PLC-γ1 in the absence of EGF in CHO-EGFR cell line

I then decided to work on CHO-derived cell lines that only express either ErbB2 or EGFR to understand the molecular mechanisms that trastuzumab might have on their signaling pathway. I found that in the CHO-EGFR cell line which constitutively overexpresses EGFR, trastuzumab did inhibit pEGFR in the presence of EGF after 1 and 48 hr (Fig 3.7). This is consistent with the results I have shown in (Fig 3.4) regarding pEGFR inhibition in SKBR3 and after 48 hr in the presence of EGF. I did not detect any pErk inhibition after 48 hr treatment in the presence of EGF in SKBR3 cells (Fig 3.4), however; trastuzumab was able to reduce pErk activity after 1 hr and 48 hr in the presence of EGF in CHO-EGFR cells (Fig 3.7). Neither EGFR nor EGFR's binding protein PLC-γ1 were phosphorylated after trastuzumab treatment alone and without EGF stimulation (Fig 3.8). pErk activity, however, was upregulated after trastuzumab treatment (16-24hr) when compared to SF control sample, but not after 48 hr trastuzumab treatment (Fig 3.8).

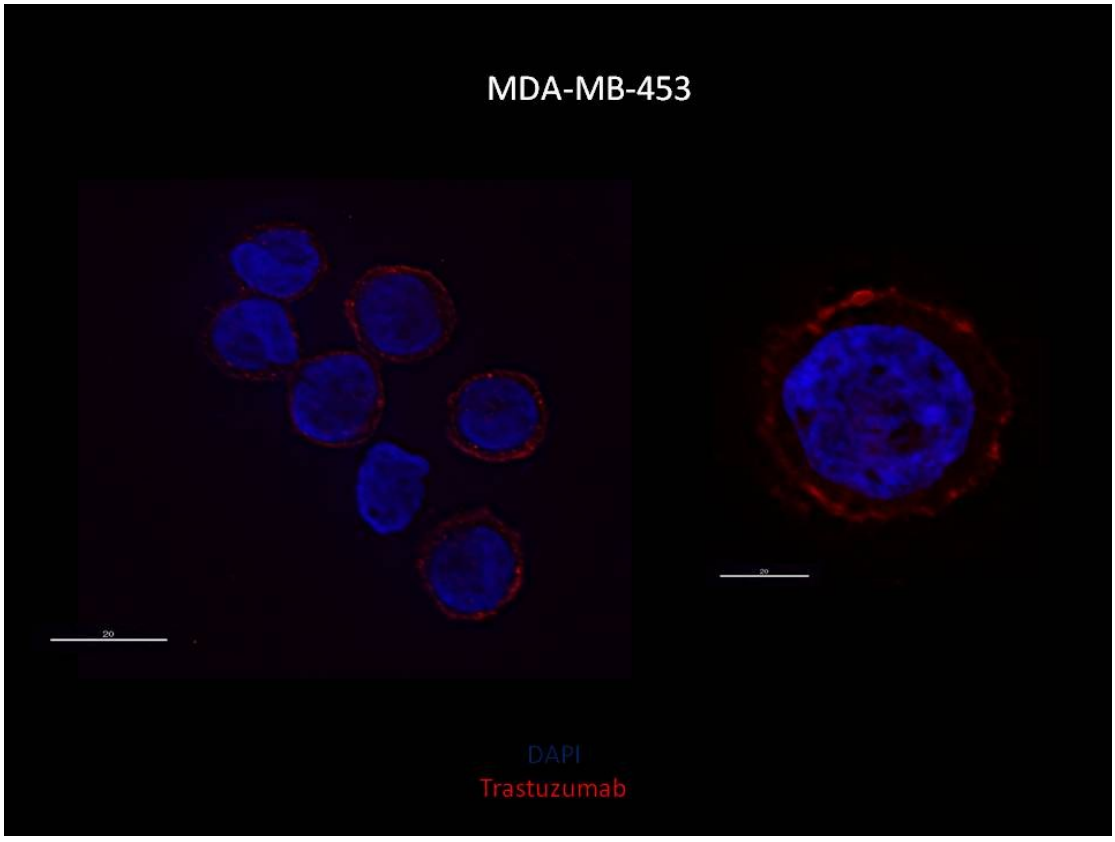
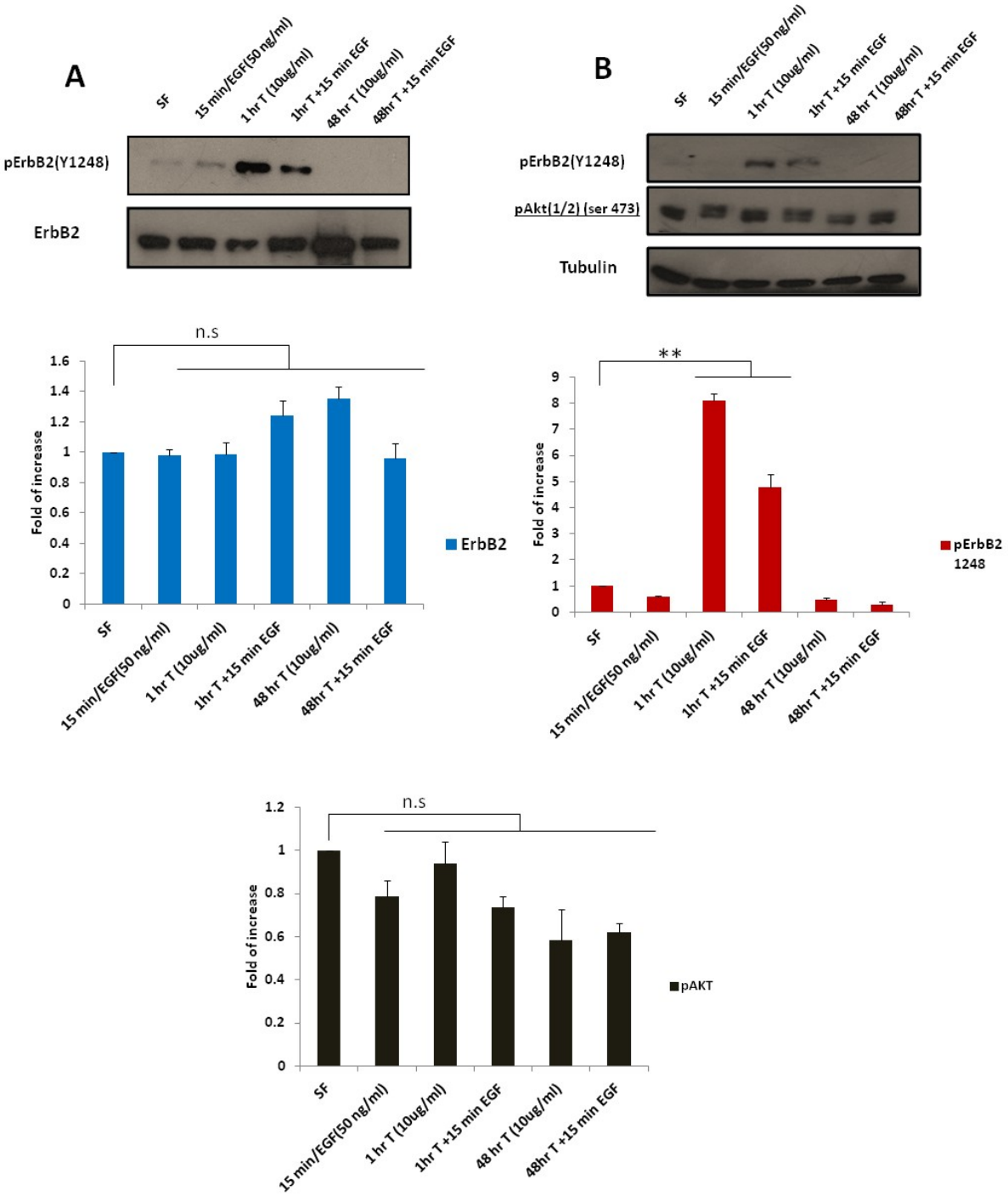


Fig 3.5. Trastuzumab binds to the plasma membrane of MDA-MB-453 cell after 1 hr treatment. MDA-MB-453 cells were treated with 10 µg/ml of trastuzumab for 1hr. Cells were then fixed and stained with DAPI and Rhodamine (TRITC) conjugated anti-human IgG that binds to the humanized IgG portion of trastuzumab. Scale bar 20 µm

MDA-MB-453


A
SF
15 min/EGF(50 ng/ml)
1 hr T (10ug/ml)
1hr T +15 min EGF
48 hr T (10ug/ml)
48hr T +15 min EGF
pErbB2(Y1248)
ErbB2
B
SF
15 min/EGF(50 ng/ml)
1 hr T (10ug/ml)
1hr T +15 min EGF
48 hr T (10ug/ml)
48hr T +15 min EGF
pErbB2(Y1248)
pAkt(1/2) (ser 473)
Tubulin
n.s
Fold of increase
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
SF
15 min/EGF(50 ng/ml)
1 hr T (10ug/ml)
1hr T +15 min EGF
48 hr T (10ug/ml)
48hr T +15 min EGF
ErbB2
**
Fold of increase
9
8
7
6
5
4
3
2
1
0
SF
15 min/EGF(50 ng/ml)
1 hr T (10ug/ml)
1hr T +15 min EGF
48 hr T (10ug/ml)
48hr T +15 min EGF
pErbB2 1248
n.s
Fold of increase
1.2
1
0.8
0.6
0.4
0.2
0
SF
15 min/EGF(50 ng/ml)
1 hr T (10ug/ml)
1hr T +15 min EGF
48 hr T (10ug/ml)
48hr T +15 min EGF
pAKT

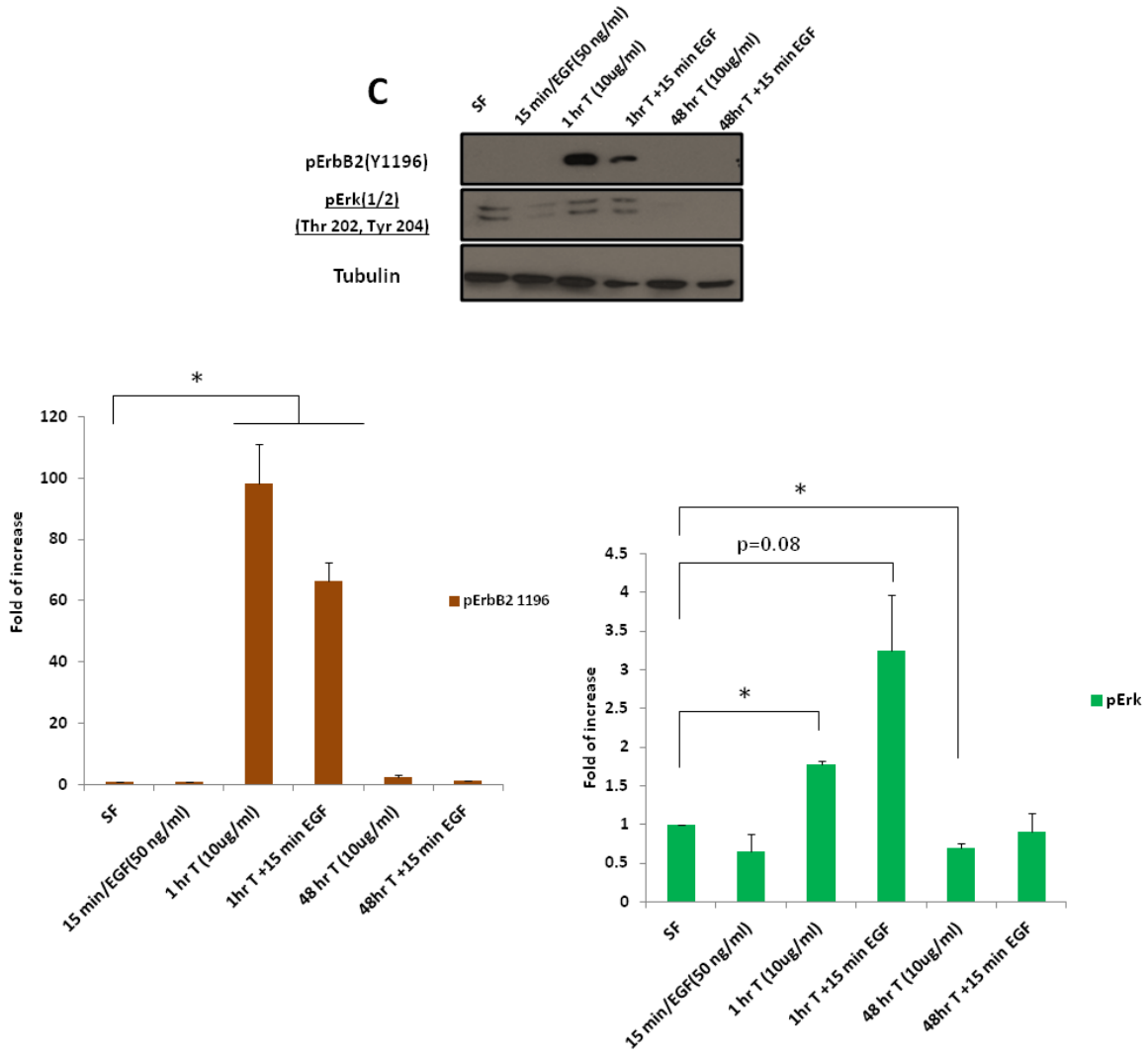


Fig 3.6. Trastuzumab upregulates the phosphorylation of ErbB2 after 1hr and prevents Erk phosphorylation after 48 hr treatment. Immunoblotting experiments were preformed on MDA-MB-453 after 1 hr and 48 hr trastzumab treatment (10 µg/ml) as indicated. Antibodies that detect the phosphorylated Akt (Ser 473), the phosphorylated Erk 1/2 (Thr 202, Tyr 204), and the phosphorylated residues for ErbB2 (Tyr 1248, Tyr 1196) were used. Samples were serum starved for 24 hrs. **SF** (serum free) sample was used as a negative control. Tubulin was used as a loading control. Reproducible representative results are shown (n = 3). Student's t-test was applied for statistical analysis. Error bars represent standard error (SE). n.s. not significant. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

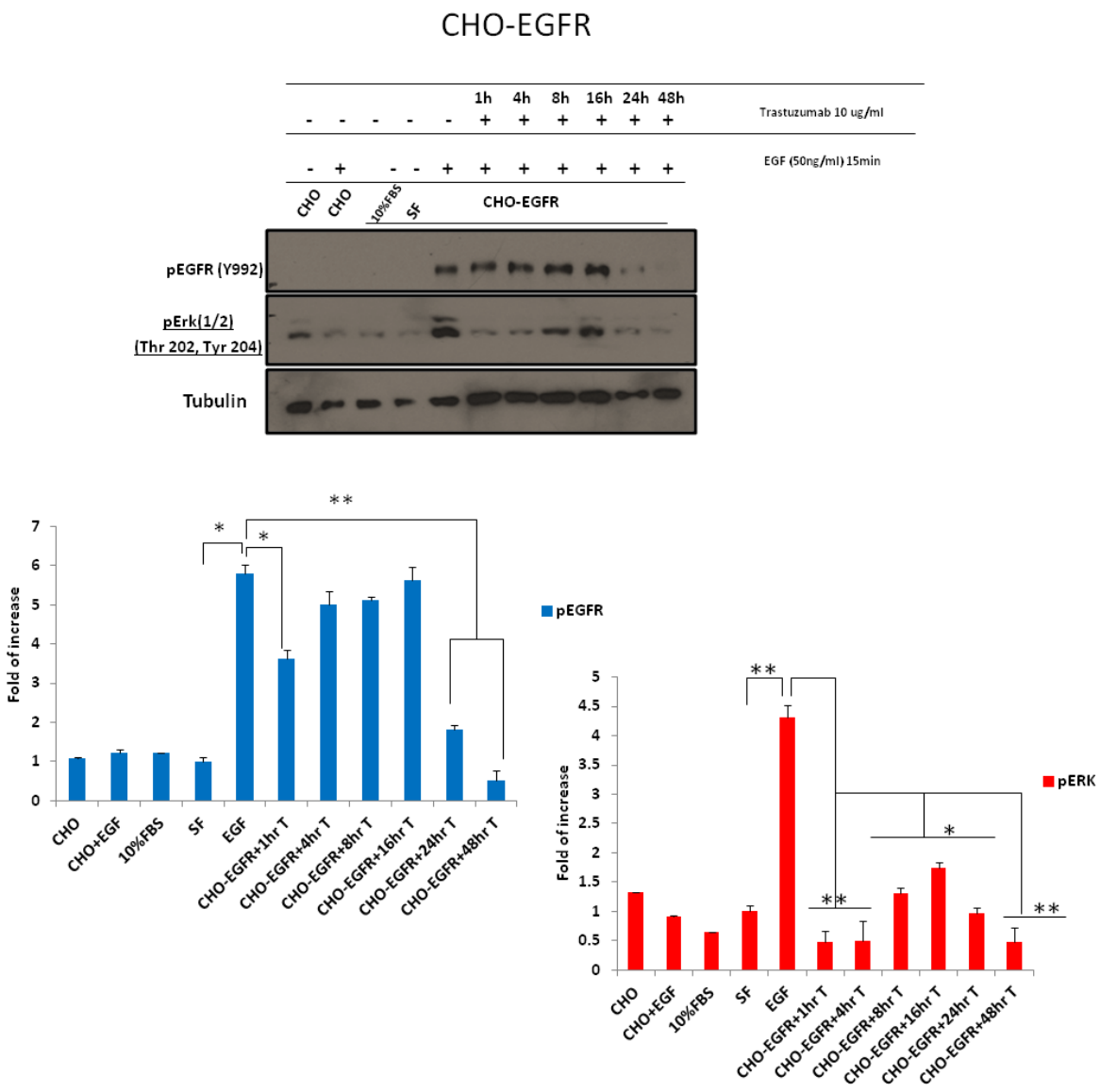


Fig 3.7. Trastuzumab blocks the phosphorylation of EGFR after 48h in the presence of EGF in CHO-EGFR cell line. Erk protein activation is also affected by Trastuzumab. Immunoblotting experiments were preformed on CHO-EGFR after a time course of trastuzumab treatment (10 µg/ml) as indicated. Antibodies that detect the phosphorylated tyrosine residue of EGFR (992), the phosphorylated Erk 1/2 (Thr 202, Tyr 204) were used. Samples were 24 hr serum starved. CHO parental cells were used as negative control. EGF (50 ng/ml) sample was used as a positive control. Tubulin was used as a loading control. Reproducible representative results are shown (n = 3). Student's t-test was applied for statistical analysis. Error bars represent standard error (SE). n.s. not significant. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

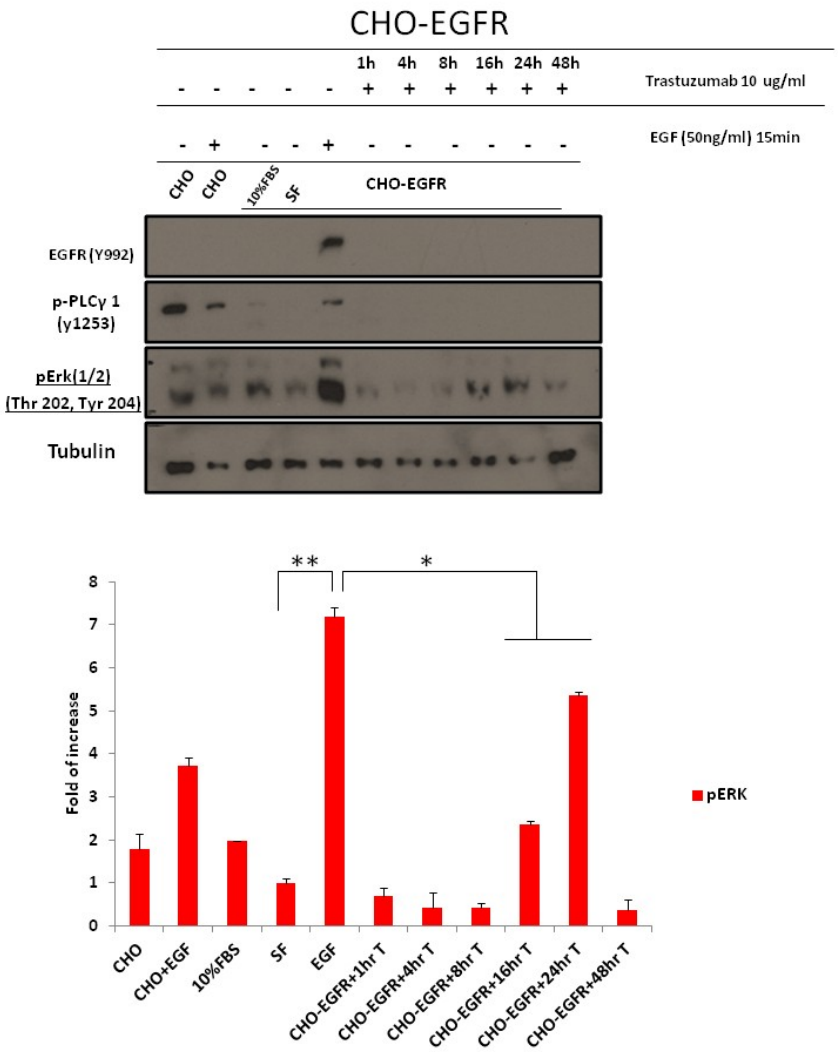


Fig 3.8. Trastuzumab does not activate the phosphorylation of EGFR or its binding protein PLC-γ1 in the absence of EGF in CHO-EGFR cell line. Immunoblotting experiments were preformed on CHO-EGFR after a time course of trastuzumab treatment (10 µg/ml) as indicated. Antibodies that detect the phosphorylated tyrosine residue of EGFR (992), the phosphorylated Erk 1/2 (Thr 202, Tyr 204), and phosphorylated tyrosine residue 1253 for PLC-γ1 were used . Samples were 24 hr serum starved. CHO samples were used as negative control. EGF-treated (50 ng/ml) sample was used as a positive control. Tubulin was used as a loading control. Reproducible representative results are shown (n = 2). Student's t-test was applied for statistical analysis. Error bars represent standard error (SE). n.s. not significant. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

3.6. Trastuzumab blocks the phosphorylation of ErbB2 on sepecfic tyrosine residues in CHO-ErbB2 (K6).

After I confirmed by immunofluorescence that trastuzumab binds to ErbB2 in CHO-ErbB2 (k6) using a trastuzumab concentration of 10 μg/ml (Fig 3.9.), immunoblotting experiments were conducted. In contrast to the results I showed in SKBR3 (Fig 3.4.), trastuzumab failed to block the phosphorylation of ErbB2 (Y1248) or pErk in CHO-ErbB2 (K6) even after 48 hr in the presence of EGF. Since CHO-ErbB2 (K6) lacks the expression of EGFR, EGF stimulation was used to keep the experimental design consistent (Fig 3.10). However, trastuzumab increased the phosphorylation of pErbB2 (Y1222) after 4-8 hr treatment then decreased the phosphorylation at 16 hr treatment (Fig 3.10). Similar preliminary results were obtained using the same cell line, but not stimulated with EGF after trastuzumab treatment (Fig 3.11). PLC-γ1 phosphorylation was upregulated after 4 hr of trastuzumab treatment (Fig 3.11). Interestingly, PLC-γ1 phosphorylation was reduced to undetectable levels in CHO-ErbB2 (K6) non treated samples (control samples) compared to CHO (parental cells) non treated samples (control samples) (Fig 3.11). CHO-ErbB2 (K6) is stably transfected with ErbB2 and have a $2.1X10^5$ ErbB2 surface density (number of ErbB2 molecules per cell) (Münch et al., 2011), while CHO (parental cells) lack ErbB2 expression.

3.7. Trastuzumab blocks the phosphorylation of ErbB2 in CHO-ErbB2 (K13) after 48 hr.

Immunoblotting experiments were conducted on the CHO-ErbB2 (K13) cell line that has a lower density of ErbB2 on its surface than K6 cells (Münch et al., 2011). Trastuzumab was able to upregulate pErbB2 (Y1196) and (Y1248) after 1-8 hr and 1-4 hr treatment respectively. Interestingly, and in contrast to the results I showed in (Fig 3.10 and Fig 3.11), trastuzumab

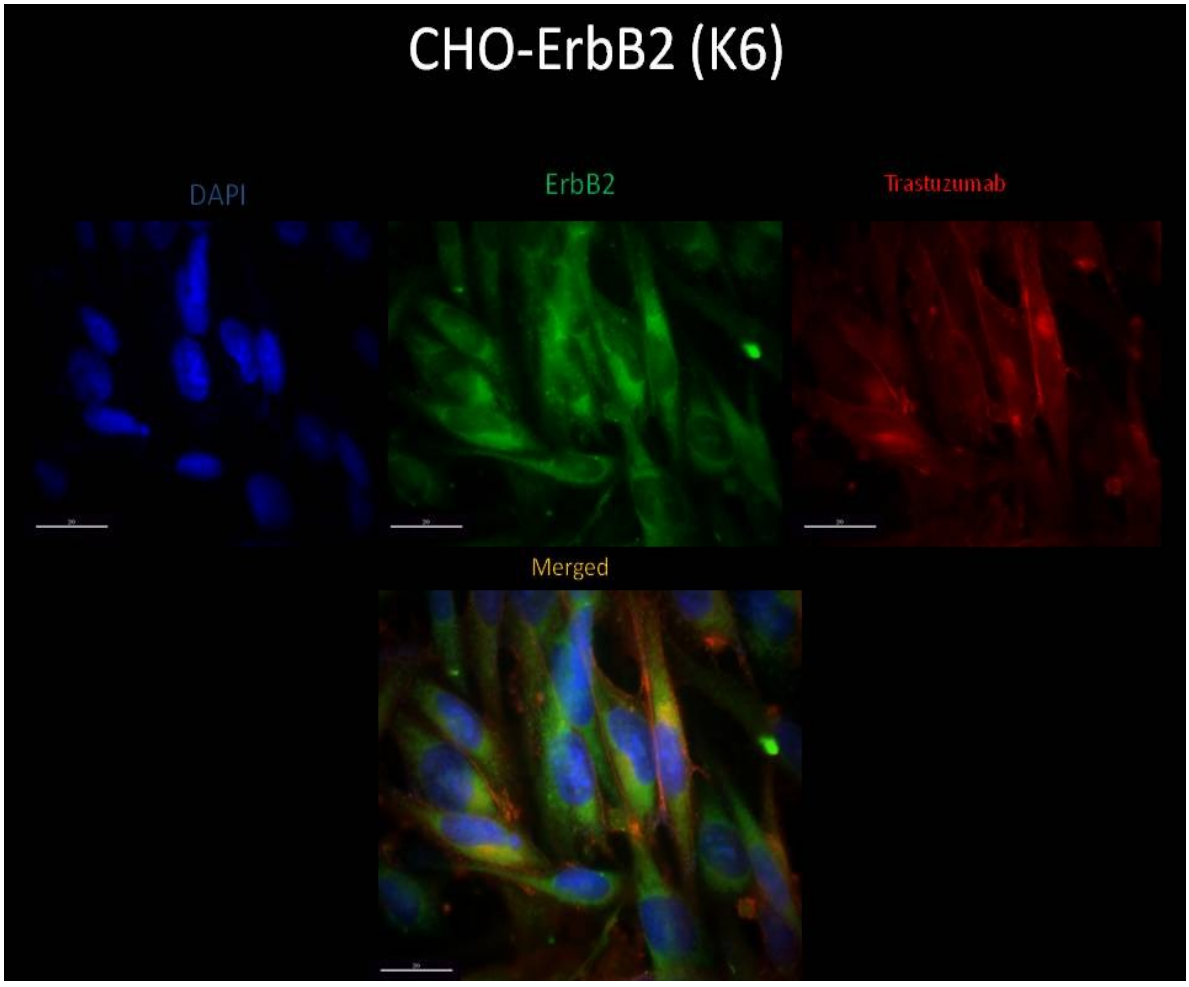
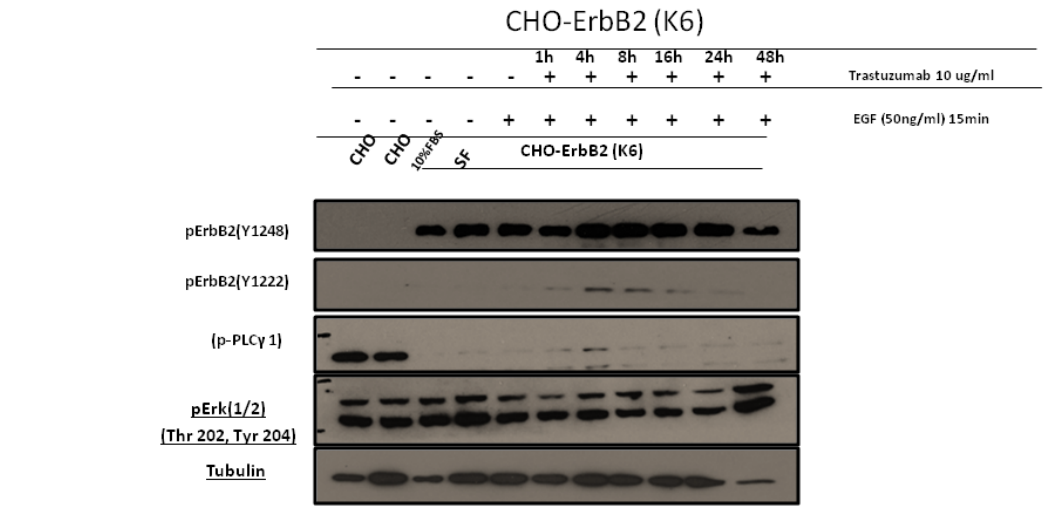
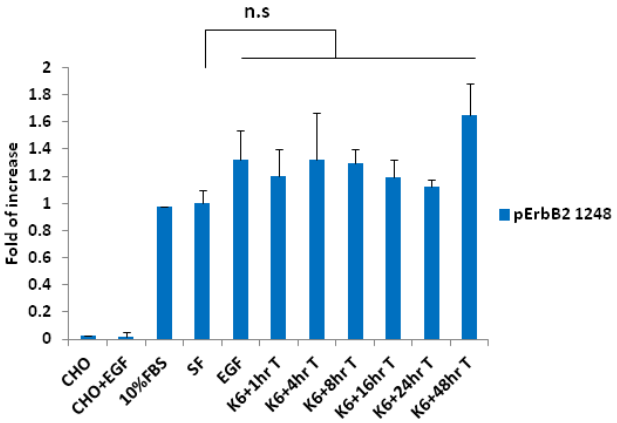


Fig 3.9. Trastuzumab binds to the plasma membrane of CHO-ErbB2 (K6) cell after 1 hr treatment. CHO-ErbB2 (K6) cells were treated with 10 µg/ml of trastuzumab for 1hr. Then cells were fixed and blocked with DAPI, Rhodamine (TRITC) conjugated anti-human IgG antibody that binds to the humanized IgG portion of trastuzumab and anti rabbit FITC antibody that binds to anti-ErbB2. Scale bar 20 µm.


CHO-ErbB2 (K6)
1h 4h 8h 16h 24h 48h
- - - - - + + + + + + Trastuzumab 10 ug/ml
- - - - + + + + + + + EGF (50ng/ml) 15min
CHO CHO 10%FBS SF CHO-ErbB2 (K6)
pErbB2(Y1248)
pErbB2(Y1222)
(p-PLCγ 1)
pErk(1/2)
(Thr 202, Tyr 204)
Tubulin


n.s
Fold of increase
pErbB2 1248
CHO
CHO+EGF
10%FBS
SF
EGF
K6+1hrT
K6+4hrT
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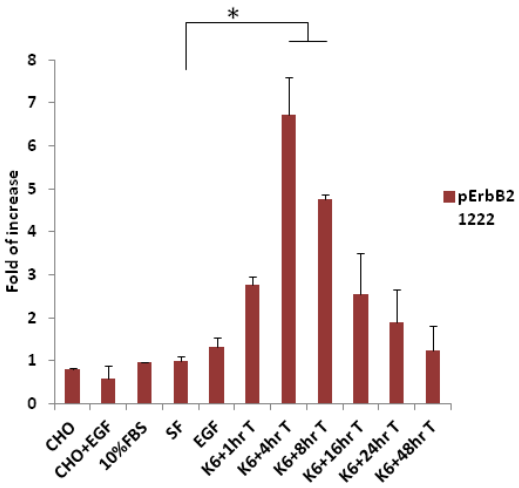
K6+16hrT
K6+24hrT
K6+48hrT


*
Fold of increase
pErbB2 1222
CHO
CHO+EGF
10%FBS
SF
EGF
K6+1hrT
K6+4hrT
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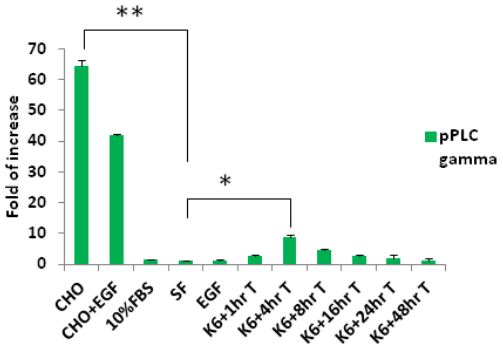
K6+16hrT
K6+24hrT
K6+48hrT


**
*
Fold of increase
pPLC gamma
CHO
CHO+EGF
10%FBS
SF
EGF
K6+1hrT
K6+4hrT
K6+8hrT
K6+16hrT
K6+24hrT
K6+48hrT

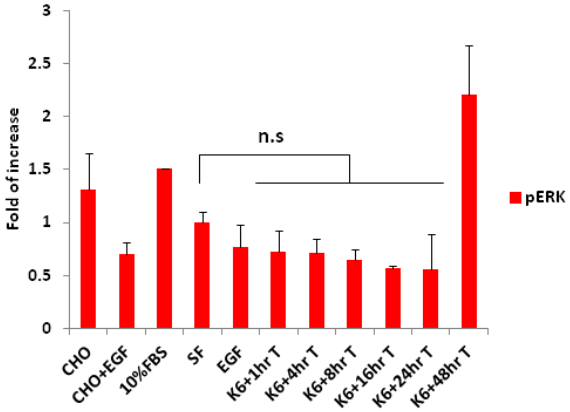

n.s
Fold of increase
pERK
CHO
CHO+EGF
10%FBS
SF
EGF
K6+1hrT
K6+4hrT
K6+8hrT
K6+16hrT
K6+24hrT
K6+48hrT

Fig 3.10. Trastuzumab blocks the phosphorylation of ErbB2 on certain tyrosine residues rather than others in CHO-ErbB2 (K6). Erk protein phosphorylation is not inhibited. Immunoblotting experiments were preformed on CHO-ErbB2 (K6) after a time course of trastzumab treatment (10 μg/ml) as indicated. Antibodies that detect the phosphorylated tyrosine residue of ErbB2 (1248, 1222), the phosphorylated Erk 1/2 (Thr 202, Tyr 204), phosphorylated tyrosine residue 1253 for PLC-γ1 were used . Samples were 24 hr serum starved. Tubulin was used as a loading control. Reproducible representative results are shown (n = 2). Student's t-test was applied for statistical analysis. Error bars represent standard error (SE). n.s. not significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

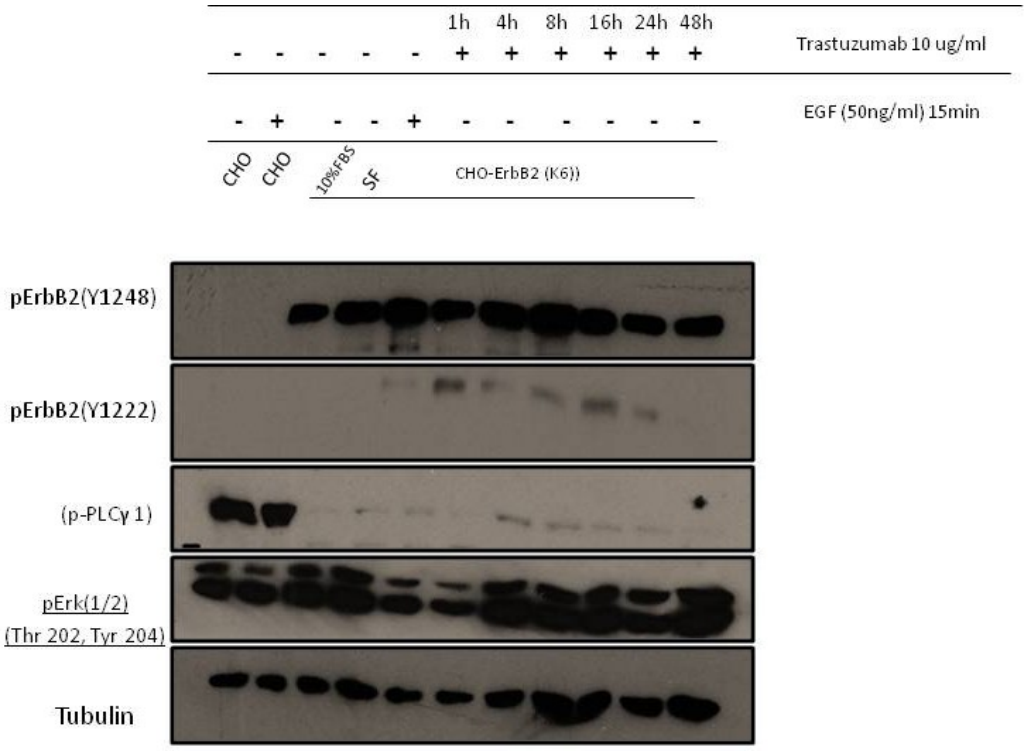


Fig 3.11. Trastuzumab blocks the phosphorylation of ErbB2 on certain tyrosine residues rather than others in CHO-ErbB2 (K6). Erk protein phosphorylation is not inhibited. Immunoblotting experiments were preformed on CHO-ErbB2 (K6) after a time course of trastzumab treatment (10 µg/ml) as indicated. Antibodies that detect the phosphorylated tyrosine residue of ErbB2 (1248, 1222), the phosphorylated Erk 1/2 (Thr 202, Tyr 204), phosphorylated tyrosine residue 1253 for PLC-γ1 were used. Samples were serum starved for 24 hr. Tubulin was used as a loading control. (n = 1).

blocked pErbB2 (Y1248) after 48 hr treatment (Fig 3.13). This was consistent with the results I showed in (Fig 3.4) where trastuzumab inhibited pErbB2 after 48 hr treatment in SKBR3 cell line. Moreover, trastuzumab was also able to block the phosphorylation of the ErbB2 tyrosine residue 1196 along with 1248 (Fig 3.12). Since CHO-ErbB2 (K13) lacks the expression of EGFR, EGF stimulation was used to keep the experimental design consistent as described previously for similar experiments with CHO-ErbB2 (K6) (Fig 3.13.). However, ErbB2 level remained unchanged after trastuzumab treatment (Fig 3.12). Regarding PLC-γ1 phosphorylation, I was not able to detect any activity of PLC-γ1in CHO-ErbB2 (K13) (Fig 3.13). Interestingly, PLC-γ1 phosphorylation was reduced to undetectable level in CHO-ErbB2 (K13) non-treated samples (control samples) compared to CHO (parental cells) non-treated samples (control samples) (Fig 3.13). CHO-ErbB2 (K13) is stably transfected with ErbB2 and have a $3.7X10^4$ ErbB2 surface density (number of ErbB2 molecules per cell) (Münch et al., 2011), while CHO (parental cells) lack ErbB2 expression. Similar preliminary results were obtained using the same cell line with no EGF stimulation (Fig 3.13).

3.8. Trastuzumab is not able to block ErbB2 dimerization in the presence or absence of EGF in SKBR3, MDA-MB-453, and CHO-ErbB2 (K6) cell lines.

BS3 crosslinking assays were conducted on SKBR3, MDA-MB-453, and CHO-ErbB2 (K6). 10 µg/ml of trastuzumab treatment alone for 1hr failed to block the homo or/and hetero-dimerization of ErbB2 in SKBR3 and MDA-MB-453 cell lines that overexpress ErbB2 (Fig 3.13). Moreover, trastuzumab failed to block their dimerization in the presence of EGF in both SKBR3 and MDA-MB-453 cell lines (Fig 3.14). Similar results were obtained in CHO-ErbB2 (K6) that stably expressed ErbB2 (Fig 3.14), where trastuzumab failed to block ErbB2 homo-dimerization in the presence or the absence of EGF.

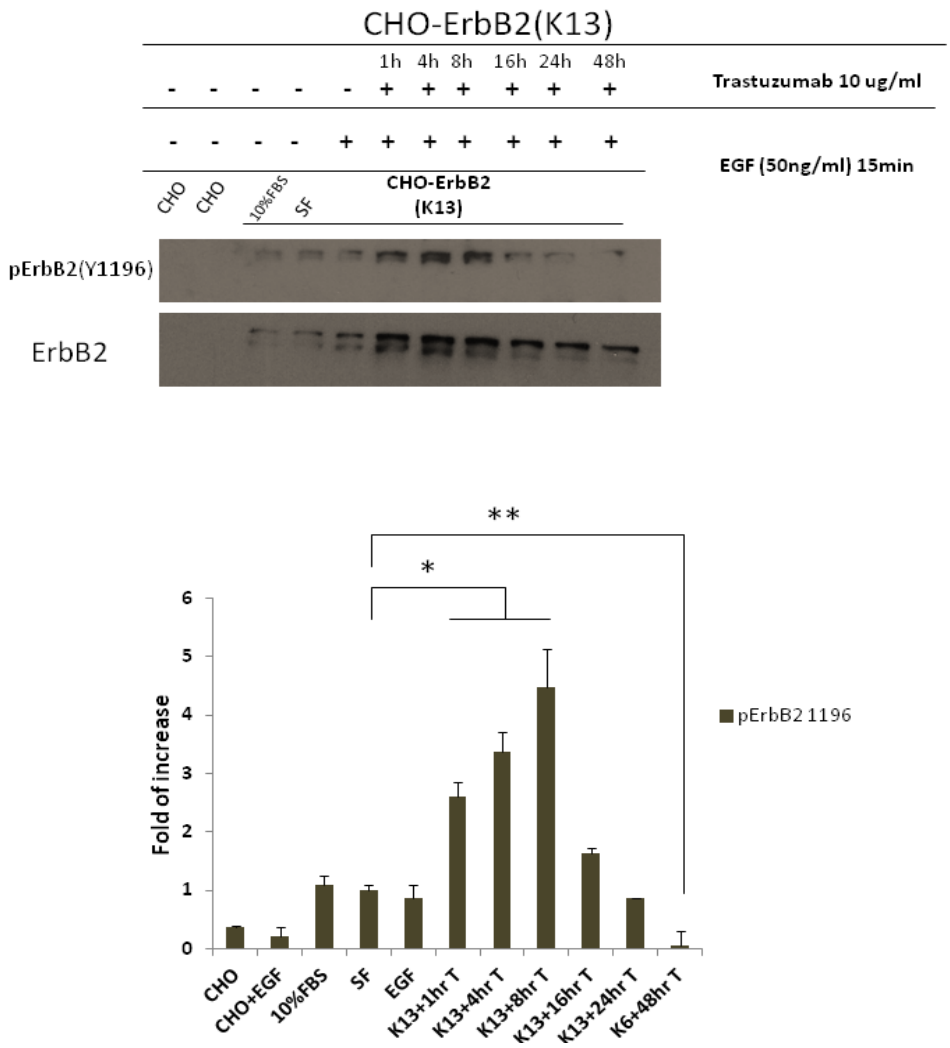


Fig 3.12. Trastuzumab blocks the phosphorylation of ErbB2 Y1196 in CHO-ErbB2 (K13) after 48 hr. Immunoblotting experiments were preformed on CHO-ErbB2 (K13) after a time course of trastzumab treatment (10 µg/ml) as indicated. Antibodies that detect the phosphorylated tyrosine residue of ErbB2 (1196) and ErbB2 (C-18) were used. Samples were serum starved for 24 hr. CHO samples and EGF (50 ng/ml) were used as negative control. Reproducible representative results are shown (n = 2).

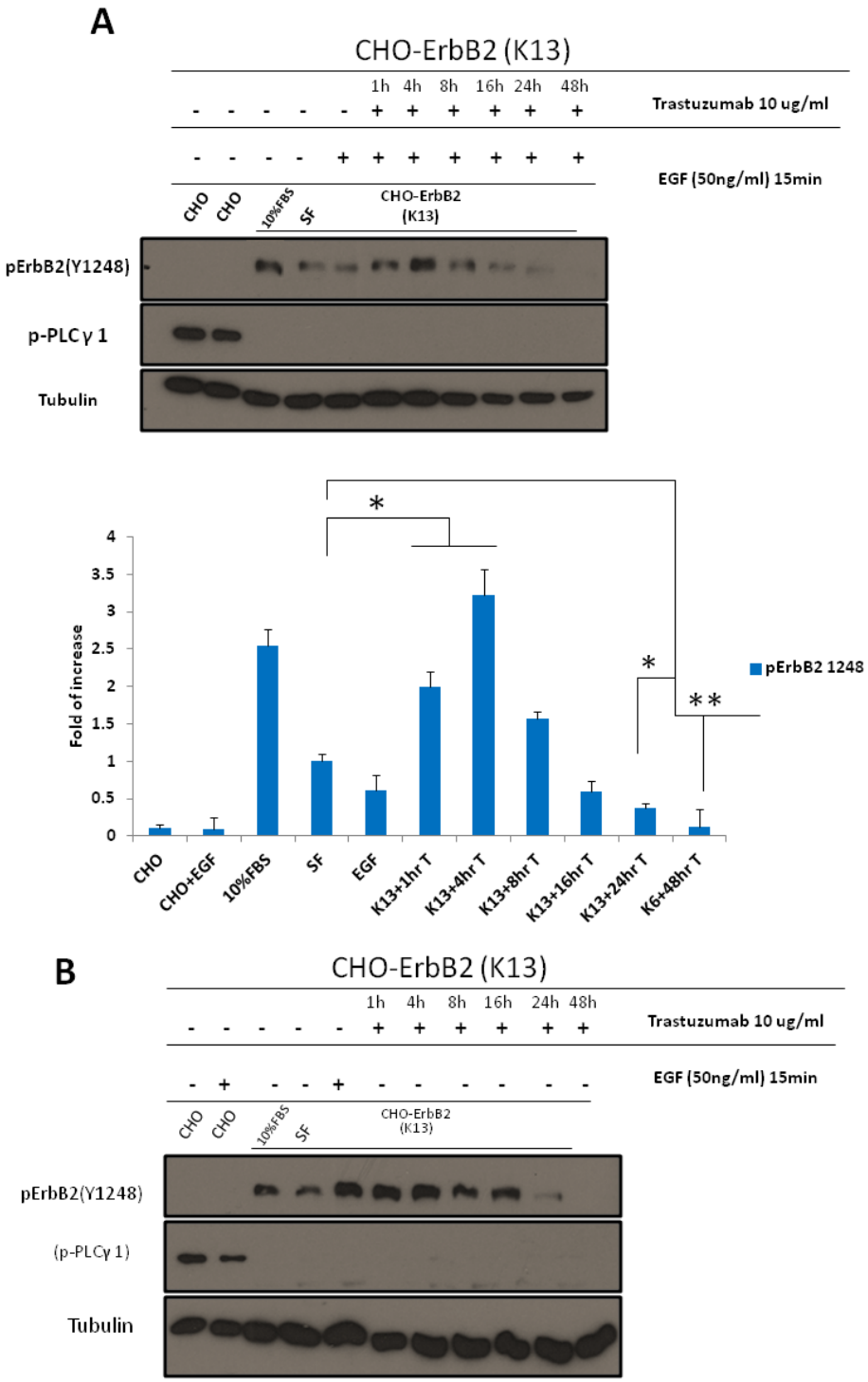
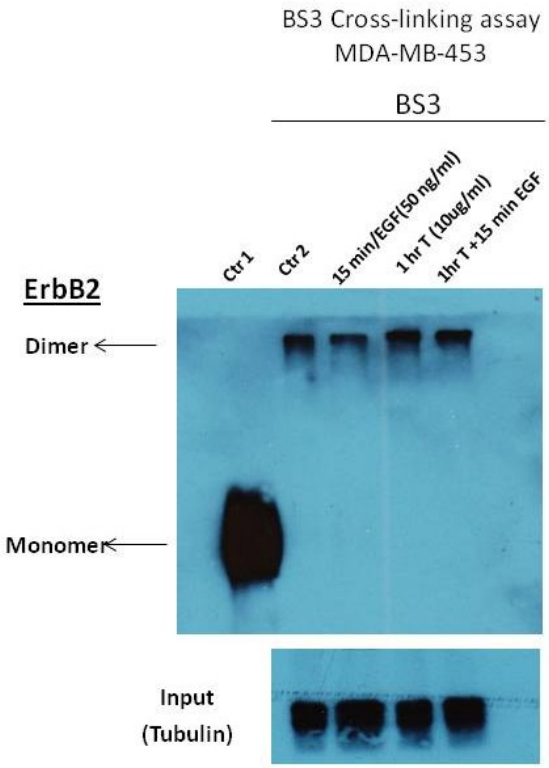
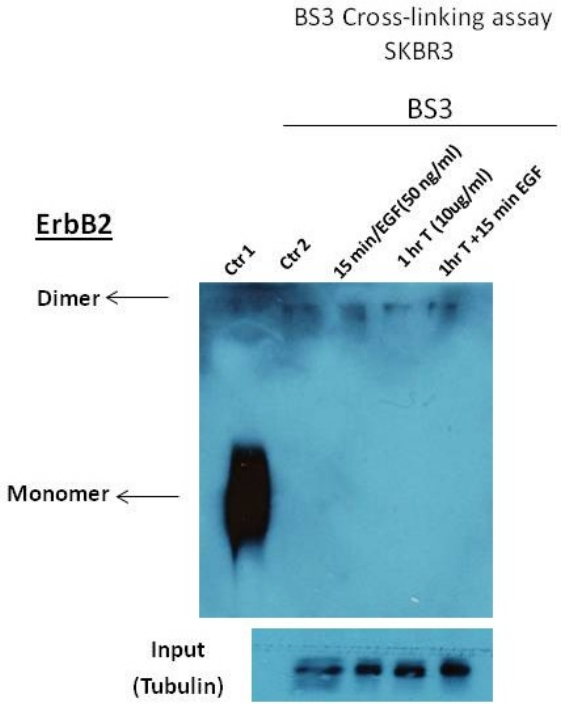

A
CHO-ErbB2 (K13)
1h 4h 8h 16h 24h 48h
Trastuzumab 10 ug/ml
EGF (50ng/ml) 15min
CHO
CHO
10%FBS
SF
CHO-ErbB2 (K13)
pErbB2(Y1248)
p-PLC γ 1
Tubulin
Fold of increase
pErbB2 1248
CHO
CHO+EGF
10%FBS
SF
EGF
K13+1hrT
K13+4hrT
K13+8hrT
K13+16hrT
K13+24hrT
K6+48hrT
B
CHO-ErbB2 (K13)
1h 4h 8h 16h 24h 48h
Trastuzumab 10 ug/ml
EGF (50ng/ml) 15min
CHO
CHO
10%FBS
SF
CHO-ErbB2 (K13)
pErbB2(Y1248)
(p-PLCγ 1)
Tubulin

Fig 3.13. Trastuzumab blocks the phosphorylation of ErbB2 Y 1248 in CHO-ErbB2 (K13) after 48 hr. Immunoblotting experiments were preformed on CHO-ErbB2 (K13) after a time course of trastzumab treatment (10 μg/ml) as indicated. Antibodies that detect the phosphorylated tyrosine residue of ErbB2 (1248) was used. Tubulin was used as a loading control. (A) EGF stimulation (50 ng/ml) for 15 min after trastuzumab treatment. Reproducible representative results are shown (n = 2). (B) No EGF stimulation after trastuzumab treatment (n = 1). Student's t-test was applied for statistical analysis. Error bars represent standard error (SE). n.s. not significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.


BS3 Cross-linking assay
MDA-MB-453
BS3
Ctr1
Ctr2
15 min/EGF(50 ng/ml)
1 hr T (10ug/ml)
1hr T +15 min EGF
ErbB2
Dimer
Monomer
Input
(Tubulin)


BS3 Cross-linking assay
SKBR3
BS3
Ctr1
Ctr2
15 min/EGF(50 ng/ml)
1 hr T (10ug/ml)
1hr T +15 min EGF
ErbB2
Dimer
Monomer
Input
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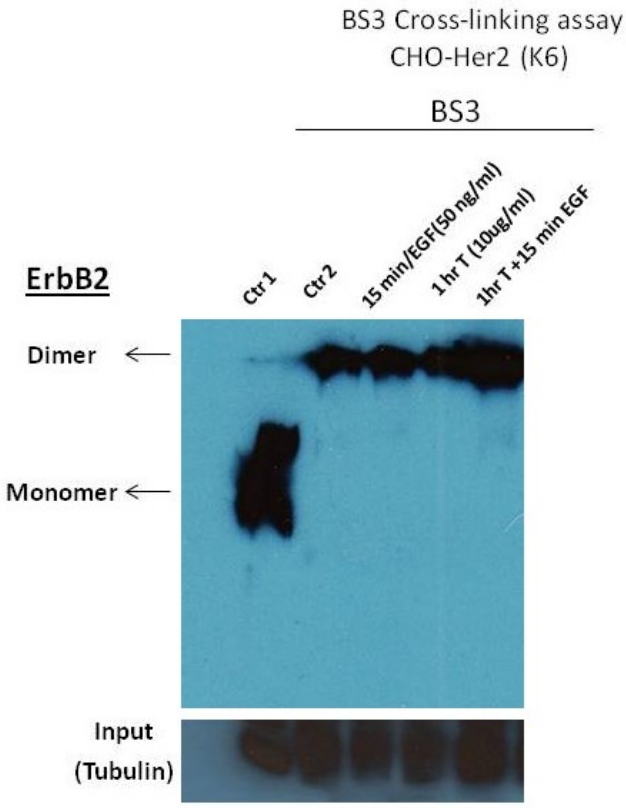
BS3 Cross-linking assay
CHO-Her2 (K6)
BS3
Ctr1
Ctr2
15 min/EGF(50 ng/ml)
1 hr T (10ug/ml)
1hr T +15 min EGF
ErbB2
Dimer
Monomer
Input
(Tubulin)

Fig 3.14. Trastuzumab is not able to block ErbB2 homo/hetero-dimerization in the presence or absence of EGF in SKBR3, MDA-MB-453, and CHO-ErbB2 (K6) cell lines. Cells were treated with trastuzumab 10 μg/ml alone for 1 hr or trastuzumab followed by EGF (50 ng/ml) stimulation. Then after the treatment, BS3 was used for the crosslinking assay. Lysates were applied to SDS-PAGE. Antibodies against ErbB2 (C-18) was used. Ctr1 is a negative control sample with no BS3 crosslinker, Ctr2 is a positive control sample with BS3 crosslinker. Samples were serum starved. Tubulin was used as a loading control.

3.9. Trastuzumab has a limited effect on cell proliferation and survival in SKBR3 and MDA-MB-453.

To examine the effect of trastuzumab concentration on cell viability, I performed MTT assays to examine the dose response of cells treated with trastuzumab for 48 hr as mentioned in (2.2.8). Trastuzumab had a significant inhibitory effect on cell viability in SKBR3 after a dose of 2.5 μg/ml ($p < 0.05$). In contrast, a Trastuzumab dose of 10 μg/ml did not significantly inhibit the cell viability in SKBR3 (p value of 0.1) (Fig 3.15). The dose of trastuzumab had no effect on cell viability after it was applied on and MDA-MB453 (Fig 3.15).

Moreover, trastuzumab has no significant inhibitory effect on CHO cell lines, CHO-ErbB2 (K6), and CHO-ErbB2 (K13) after it was applied for 48 hr (Fig 3.16).

3.10. Trastuzumab does not enhance the effect of doxorubicin in breast cancer cell lines

SKBR3 and MDA-MB-453 cells were treated with either doxorubicin alone or doxorubicin and trastuzumab simultaneously as mentioned above in (2.2.8).

Doxorubicin had an inhibitory effect on SKBR3 and MDA-MB-453 cells in a dose dependent manner with an IC50 of approximately 0.5 μM (Fig 3.17). I then examined the synergetic effect of trastuzumab and doxorubicin on SKBR3 and MDA-MB-453. Trastuzumab did not enhance the effect of doxorubicin in SKBR3 (Fig 3.18), and neither did MDA-MB-453 (interestingly, except 5 and 10 μg/ml) (Fig 3.19).

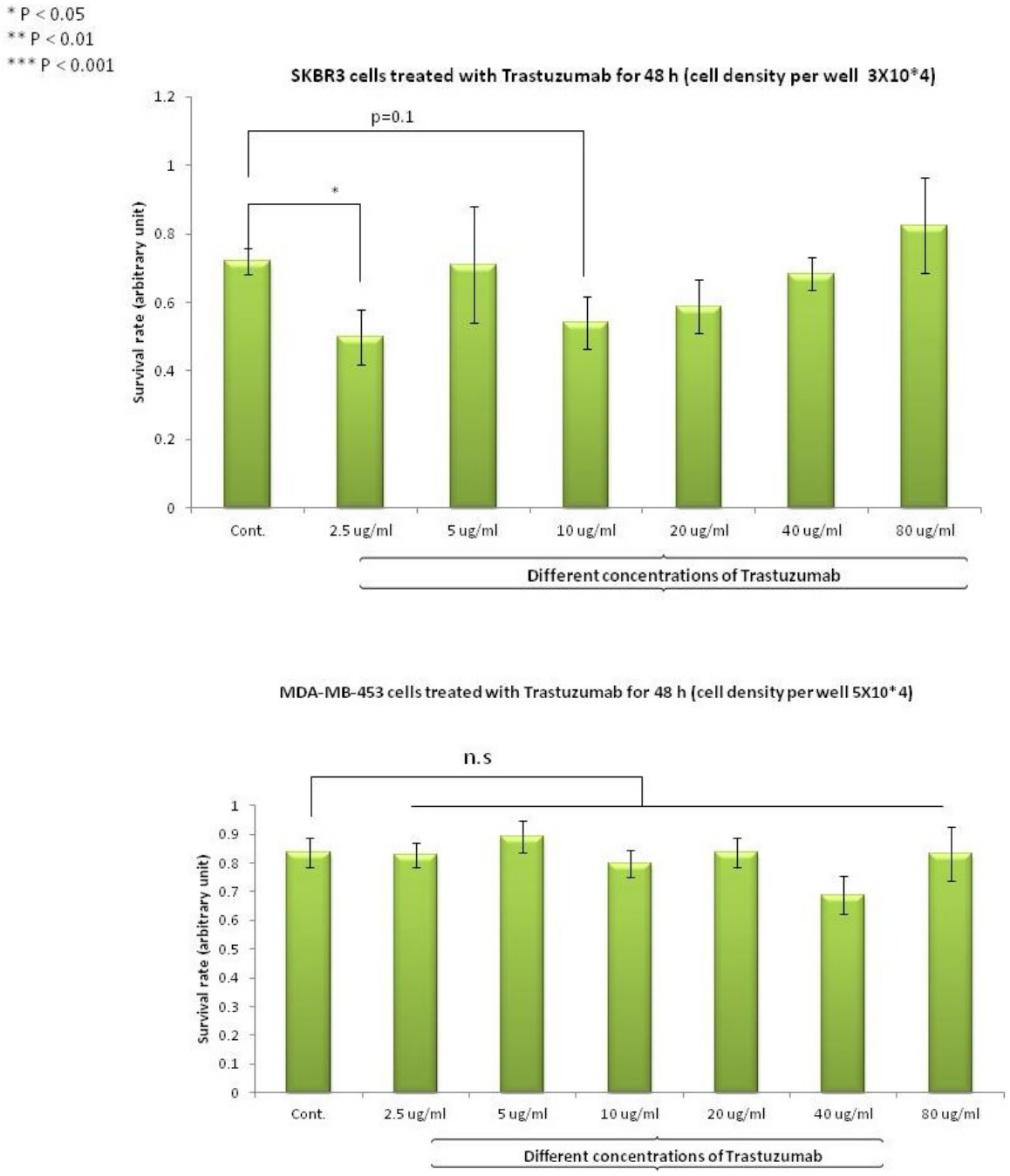

Fig 3.15. Trastuzumab effect is limited on cell proliferation or survival based on MTT assay. Cells were treated with different concentrations of trastuzumab as indicated for 48. Then MTT assay was applied and cell viability readings were conducted at 540 nm. Experiments were conducted in at least triplicates and student's t-test was applied for statistical analysis. Error bars represent standard error (SE). n.s: not significant. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

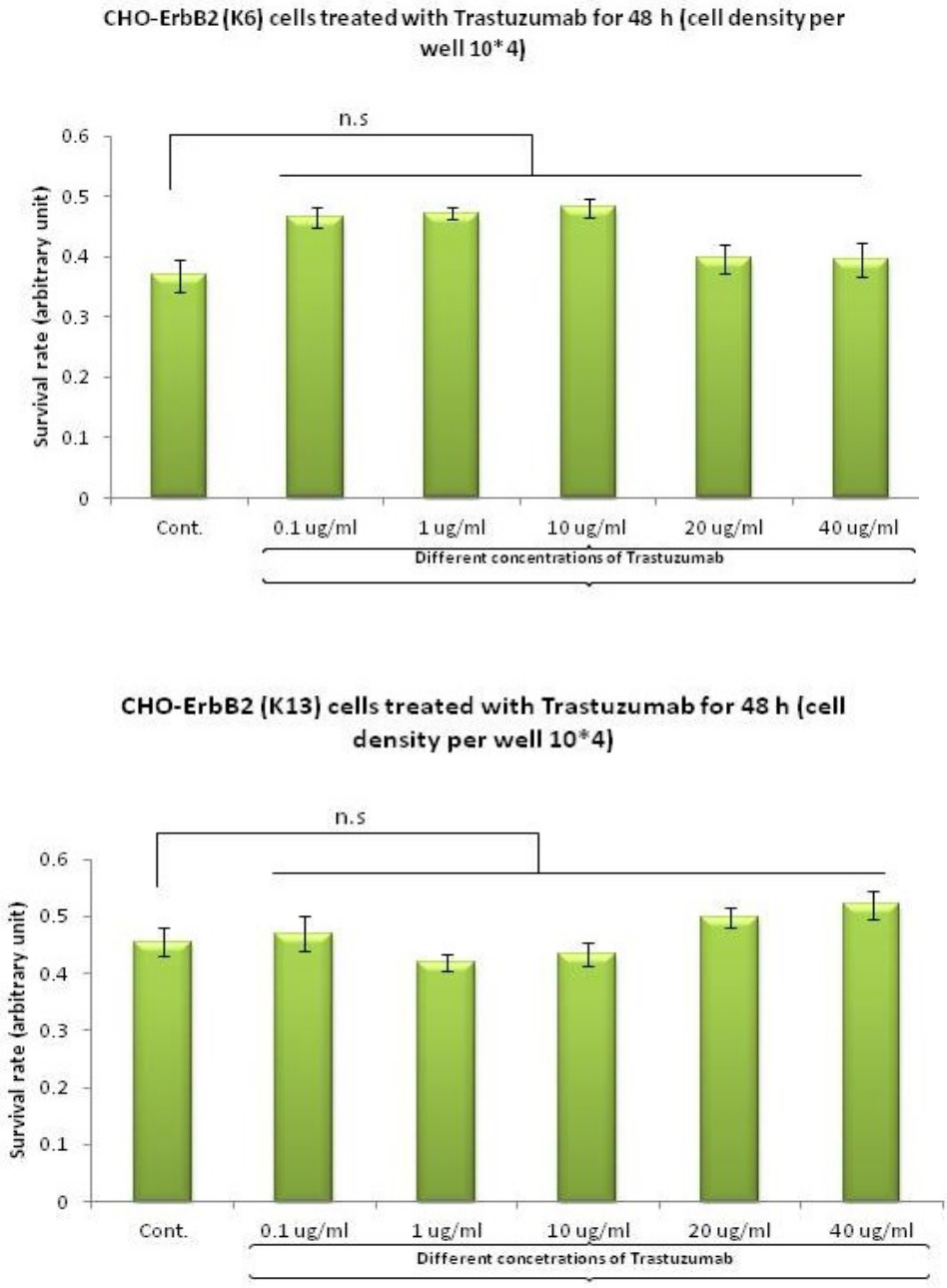

Fig 3.16. Trastuzumab effect is limited on cell proliferation or survival based on MTT assay. Cells were treated with different concentrations of trastuzumab as indicated for 48. Then MTT assay was applied and cell viability readings were conducted at 540 nm. Experiments were conducted in at least triplicates and student's t-test was applied for statistical analysis. Error bars represent standard error (SE). n.s: not significant.

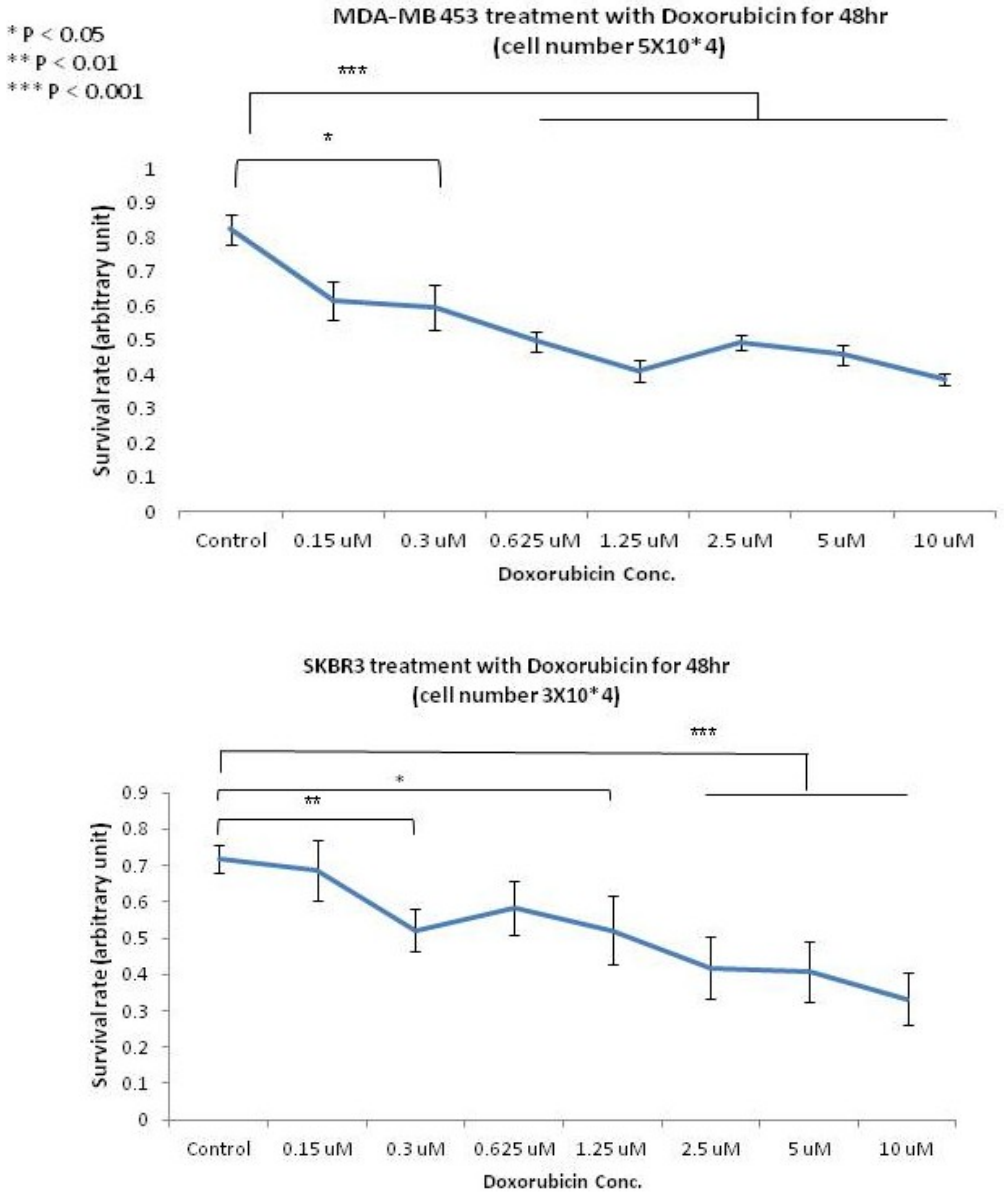

Fig 3.17. Doxorubicin has a dose response inhibitory effect on SKBR3, and MDA-MB-453. Cells were treated with different concentrations of doxorubicin as indicated for 48. Then MTT assay was applied and cell viability readings were conducted at 540 nm. Experiments were conducted in at least triplicates and student's t-test was applied for statistical analysis. Error bars represent standard error (SE). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

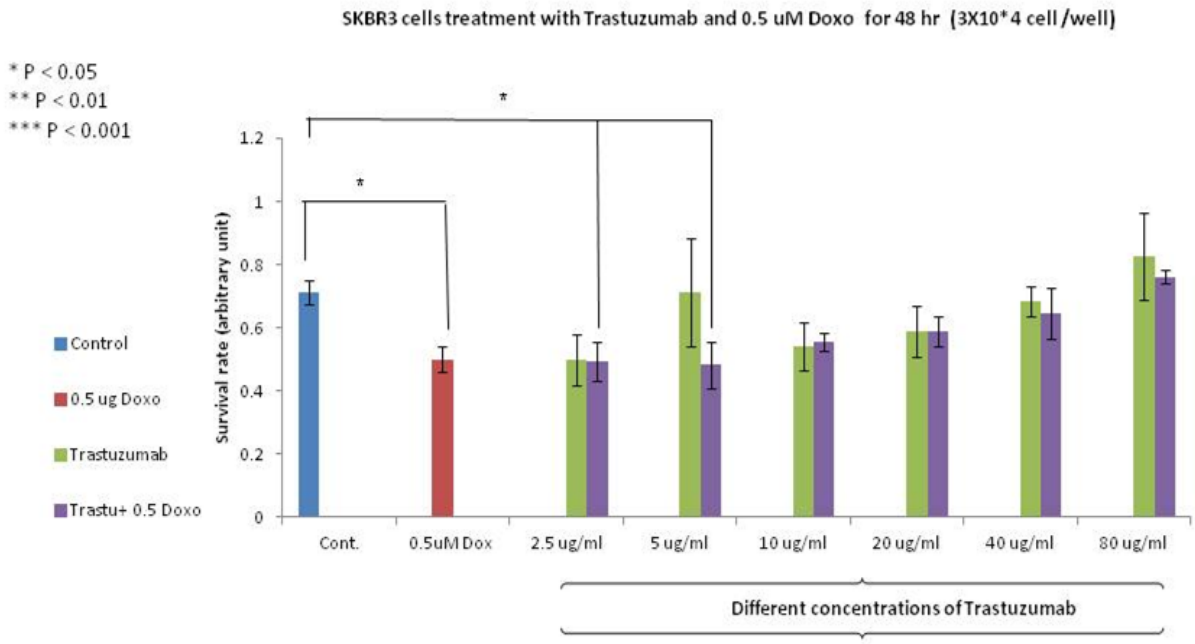

Fig 3.18. Trastuzumab do not enhance the effect of doxorubicin in SKBR3 cell lines. Cells were treated with either doxorubicin alone or trastuzumab alone or different concentrations of trastuzumab combined with 0.5 µM doxorubicin as indicated for 48hr. Then MTT assay was applied and cell viability readings were conducted at 540 nm. Experiments were conducted in at least triplicates and student's t-test was applied for statistical analysis. Error bars represent standard error (SE). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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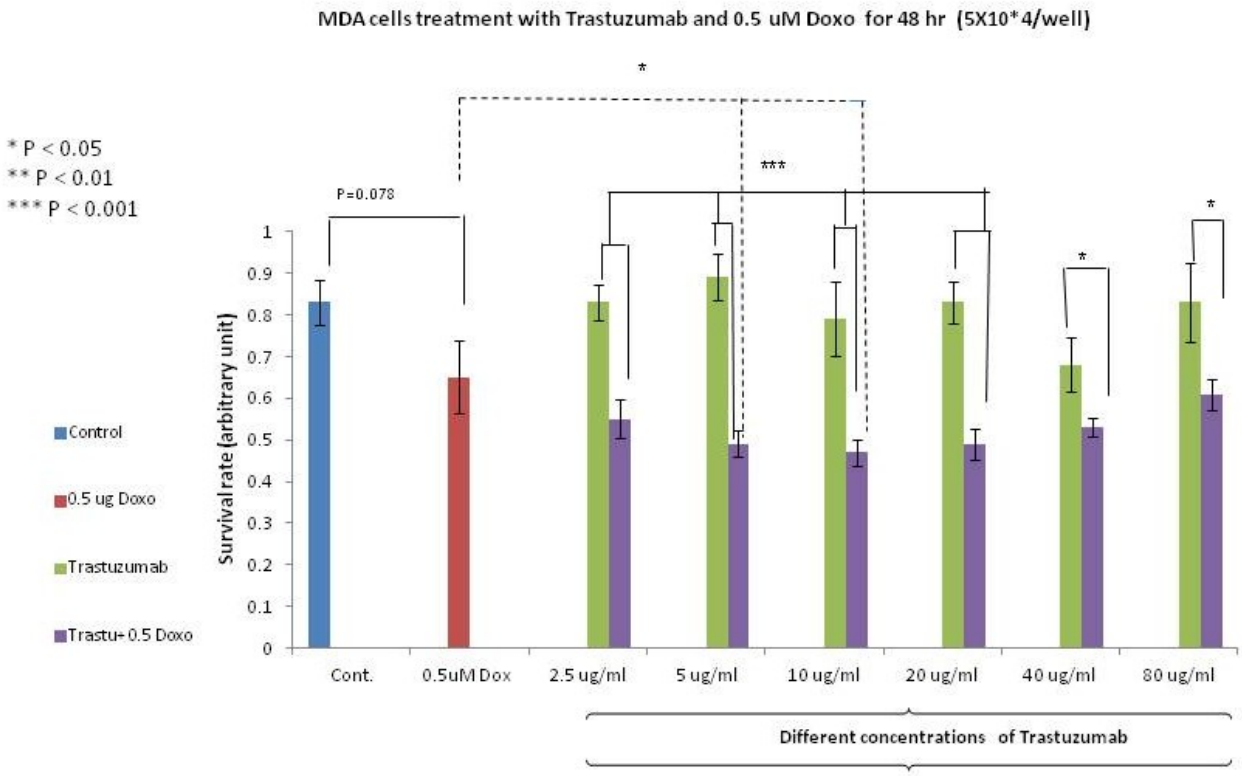

Fig 3.19. . Trastuzumab does not enhance the effect of doxorubicin in MDA-MB-453 cell lines. Cells were treated with either doxorubicin alone or trastuzumab alone or different concentrations of trastuzumab combined with 0.5 μM doxorubicin as indicated for 48hr. Then MTT assay was applied and cell viability readings were conducted at 540 nm. Experiments were conducted in at least triplicates and student's t-test was applied for statistical analysis. Error bars represent standard error (SE). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Chapter 4 Discussion

4.1. ErbB2 expression level and breast cancer cell lines

In this thesis, three cell line models of breast cancer were used (Fig 3.1). The SKBR3, MDA-MB-453 and BT20 cell lines have been used in numerous breast cancer studies in the last decade (Cuello et al., 2001; Yakes et al., 2002; Nagata et al., 2004; Wu et al., 2005; Ginestier et al., 2007; Eichhorn et al., 2008; Junttila et al., 2009; Gijsen et al., 2010; Dokmanovic et al., 2014). These cell lines vary in expression levels of ErbB2 and therefore I choose to use them in my study to understand the molecular mechanisms of trastuzumab and its association with ErbB2 expression (Table 3.1, Fig 3.1.A). I have shown that the SKBR3 cell line overexpresses ErbB2, has normal expression of ErbB3, and a moderate expression of EGFR (Fig 3.1), consistent with several past studies (Nagata et al., 2004; Junttila et al., 2009; Gijsen et al., 2010). Consistent with Cuello et al., 2001, we show that the MDA-MB-453 is an ErbB2 overexpressing cell line, whereas BT20 has rarely been used in trastuzumab studies due to low ErbB2 expression (Fig 3.1). Furthermore, BT20 is mostly used in triple negative breast cancer studies due to the fact that this cell line overexpresses EGFR and lacks ErbB2, ER, and PR overexpression (Madden & Mueller, 2014; Jin et al., 2014). For this reason I was interested in using BT20 to elucidate the effect of EGFR on the mode of action of trastuzumab in the presence of its ligand EGF and to examine if EGFR-ErbB2 hetero-dimerization plays a role in drug resistance. Our lab has previously characterized the expression level of ErbB2 among different breast cancer cell lines (Wang et al., 1999). I was able to validate these results in this thesis and confirm that SKBR3 and MDA-MB-453 overexpresses ErbB2 whereas BT20 express ErbB2 moderately (Fig 3.1.A).

4.2. Trastuzumab activity on ErbB receptors and their downstream proteins in breast cancer cell lines

Trastuzumab is a drug that binds to and blocks the ErbB2 receptor, which in turn downregulates major downstream pathways involved in cellular proliferation and differentiation (Spector & Blackwell, 2009; Fiszman & Jasnis, 2011). However, my findings (Fig 3.2.) suggest that trastuzumab (40 μg/ml) upregulated ErbB2 phosphorylation after 1 hr of treatment in the presence or the absence of EGF in SKBR3 and BT20. Upregulation of ErbB2 phosphorylation has been reported previously by other studies (Nagata et al., 2004; Gijsen et al., 2010; Dokmanovic et al., 2014). These studies show that upregulation of pErbB2 after trastuzumab treatment can be due to loss of PTEN, a negative regulator of the PI3K pathway, increase in the mRNA and protein levels of ADAM17, a protein that upregulates the ligand heregulin which leads to phosphorylation of the ErbB2 receptor. These studies also show that interaction of ErbB2 with the Csk homologous kinase (CHK), also known as C-Src kinase that phosphorylates and inhibits Src family kinase, which mimics the trastuzumab inhibitory effect and downregulates AKT. These factors might explain why I found increased phosphorylation of ErBb2 in SKBR3 and ErbB2 and EGFR in BT20 compared to EGF-only stimulated samples (Fig 3.2).

On the other hand, 1 hr of trastuzumab treatment reduced the phosphorylation of ErbB3 (Y1328) as compared to control in MDA-MB-453 cells (Fig 3.2), and as compared to EGF-only stimulated sample in SKBR3 (Fig 3.2). Trastuzumab's inhibitory effect on ErbB3 was suggested before by several studies. For example, Gijsen and his colleauges (2010), reported that trastuzumab is able to downregulate ErbB3 phosphorylation, but does not decrease ErbB2 phosphorylation. Moreover, (Garner et al., 2013) showed that Trastuzumab inhibited pErbB3

(Y1197) by approximately 40% and 50% in SKBR3 and MDA-MB-453 respectively. They found more growth inhibition in these cell lines after they combined treatment of trastuzumab with LJM716, an ErbB3 monoclonal antibody. Other studies (Motoyama et al., 2002; Yakes et al., 2002) reported that trastuzumab treatment decreased ErbB3 activity in ErbB2-amplified cells. Yakes and his colleauges (2002) described a robust initial increase in pErbB3 (Y1289) after 1 hr treatment with trastuzumab, followed by slow dephosphorylation after 12-24 hr. In contrast, Junttila et al., (2009) reported a rapid dephosphorylation of ErbB3(Y1289), this was readily visible after 10 min of treatment with trastuzumab in SKBR3 and BT474. However, no inhibition of ErbB2 phosphorylation was detected in their study. The authors concluded that trastuzumab causes an immediate and potent inhibition of the ErbB3/PI3K/AKT pathway that lasts for at least 4 days (Junttila et al., 2009).

I did not detect any phosphorylation in control cells after 24 hr serum starvation (Fig 3.3, 3.4, 3.6); whereas there was phosphorylation in control cells grown in media supplemented with 10% FBS (Fig 3.2). FBS contains EGF and some other cofactors (Ohyama et al., 2001) that can activate ErbB receptors and lead to their phosphorylation. This may explain the reduction of ErbB2 and EGFR phosphorylation in serum starved control cells compared to non-serum starved control cells. On the other hand, (Dokmanovic et al., 2014) showed pErbB2 in SKBR3 control cells after 24 hr serum starvation. Moreover, Ginestier and his colleauges (2007) found detectable pErbB2 specifically in tyrosine residue 1248 in SKBR3 control samples after 18 hr serum starvation. They linked this phosphorylation of this tyrosine residue to trastuzumab sensitivity.

Interestingly, my preliminary data showed that 1 hr (40 µg/ml) of trastuzumab treatment after 24 hr serum starvation did not enhance ErbB2 phosphorylation in SKBR3 and BT20 cells (Fig 3.3)

compared with 1 hr trastuzumab treatment (40 µg/ml) (Fig 3.2). And 1hr and 48 hr (10 µg/ml) of trastuzumab treatment in SKBR3 did not increase ErbB2 phosphorylation in SKBR3. This suggests that FBS might interfere with the trastuzumab mode of action, although further studies on this issue are required. However, SKBR3 cells treated with trastuzumab (4 µg/ml) following serum starvation did upregulate pErbB2 after 15, 45, and 60 min treatment (Dokmanovic et al., 2014).

I further examined the effect of 10 µg/ml of trastuzumab on SKBR3 and MDA-MB-453 cells for 1 and 48 hr treatment in the precense or absence of EGF (Fig 3.4, 3.6). This dosage of trastuzamab has been previosuly used in related studies (Junttila et al., 2009; Dokmanovic et al., 2014). As a generally accepted mechanism, trastuzumab is a humanized monoclonal antibody that binds to and blocks the post translational phosphorylation of the ErbB2 receptor. However, I found that the treatment of SKBR3 with 10 µg/ml of trastuzumab for 1 hr was not able to reduce phosphorylation of ErbB2 (Y1248, Y1196) (Fig 3.4.A, 3.4.B) compared to the EGF-only stimulated samples, whereas the level of total ErbB2 receptor remained the same (Fig 3.4.A). My findings (Fig 3.4) confirm the viewpoints on the molecular mechanisms of trastuzumab. For example, a slight activation or no inhibition of pErbB2 after short-term treatment with different concentration of trastuzumab has been previously reported (Benz et al., 1992; Nagata et al., 2004; Dokmanovic et al., 2014). Treatment of SKBR3 with 10 µg/ml of trastuzumab for 48 hr can inhibit pErbB2 (Y1248, Y1196) activity, in the presence of the ligand, to almost an undetectable level (Fig 3.4.B). Long term treatment of trastuzumab has been previously reported to reduce the activity of pErbB2 (Lee et al., 2002; Gijsen et al., 2010). However, the reason why trastuzumab long-term treatment inhibits pErbB2 more efficiently than short-term treatment in breast cancer cell lines remains unclear.

It was suggested that targeting ErbB2 by trastuzumab itself is not be enough to treat ErbB2-positive breast cancers (Hynes & Lane, 2005). Like other growth factor receptors, ErbB2 lies at the start of the signaling pathway, thus, inhibition of ErbB2 will only specifically inhibit ErbB2-mediated cell signaling. However, signaling pathways activated by ErbB2 could also be activated by many other growth factors, which may keep the cells viable. Our lab has shown previously that co-expression of EGFR or ErbB3 with ErbB2 highly induces phosphorylation of ErbB2 and renders cells more resistant to various anticancer drugs (Wang et al., 2000). EGFR overexpression has been linked to many malignancies, and its activity has been suggested to enhance breast cancer cell survival, migration, and proliferation (Masuda et al., 2012). Furthermore, it has been suggested that EGFR overexpression might lead to trastuzumab resistance, and targeting both ErbB2 and EGFR with lapatinib, afatinib or neratinib was investigated in the context of metastatic ErbB2 positive breast cancer that develop trastuzumab resistance (Geyer et al., 2006; Gradishar, 2013). However, EGFR overexpression was neither associated with trastuzumab sensitivity nor overall survival in ErbB2-postive metastatic breast cancer (Gori et al., 2009). In addition, a recent study suggested that EGFR overexpression does not link to trastuzumab resistance and might be considered as a predictive factor for trastuzumab response in ErbB2-postivie primary breast cancer, but not metastatic breast cancer (H. J. Lee et al., 2015). However, in contrast to Ye et al., (1999), who showed no effect of trastuzumab on EGFR activity in ovarian cancer cell lines, I show here that trastuzumab is able to inhibit the phosphorylation of EGFR (Y992) after 1 hr and along with ErbB2 after 48 hr treatment in SKBR3 cells in the presence of its ligand (EGF) (Fig 3.4.C). The mechanism of how trastuzumab inhibits EGFR phosphorylation in the presence of EGF, remain elusive and needs more investigation. Moreover, EGFR receptors can be activated upon the binding of EGF,

transforming growth factor (TGF)-α, amphiregulin (AR), and epigen (EPG) ligands to its extracellular domain (Hynes & MacDonald, 2009). In this thesis, I investigated the effect of trastuzumab on EGFR activity in the presence of EGF only, the mode of action of trastuzumab after activation of EGFR with other ligands will need to be studied further.

Two of the main pathways associated with ErbB2 activation are the PI3K pathway and the MAPK pathway, both of which are important for cancer cell proliferation and metastasis (Vu & Claret, 2012). Trastuzumab has been shown to disrupt these pathways and their signaling proteins but the exact role on how this is conducted is not fully understood and is controversial. It is generally accepted that the MAPK and PI3K pathways regulate the cell cycle which affect cell survival. The MAPK pathway induces levels of *c-Myc* (proto-oncogene), a transcription factor that up-regulates the expression of p53. In turn, p53 upregulation controls and upregulates the level of cyclin-dependent kinase inhibitor p21/WAFI, which plays a role in cell cycle and proliferation (Neve et al., 2002). As a result, a constitutive activation of the MAPK and PI3K pathways or mutations of p53 are hallmarks of cancer.

AKT and Erk are downstream proteins of PI3K and MAPK pathways respectively, that can be activated by ErbB receptors and thus play a role in trastuzumab resistance (Neve et al., 2002; Asanuma et al., 2005). In this thesis, I show that trastuzumab (10 µg/ml) failed to block AKT phosphorylation (Fig 3.4.B) but not Erk phosphorylation (Fig 3.4.C) after 1 hr treatment in the presence of EGF. This result differs from previous studies which reported that short-term treatment with different concentrations of trastuzumab was able to reduce the phosphorylation of AKT in the absence of a ligand in ErbB2 overexpressing cell lines (Nagata et al., 2004; Junttila et al., 2009; Gijsen et al., 2010; Dokmanovic et al., 2014).

However, the effects of long term trastuzumab treatment were consistent with previous results. Similar to the effect of long-term treatment on ErbB2 and EGFR phosphorylation activity that I have shown above; trastuzumab did block the phosphorylation of Erk (Fig 3.4.C) and AKT (Fig 3.4.B) and after 48 hr treatment in SKBR3 cells in the absence of EGF. This is consistent with results from (Eichhorn et al., 2008) who showed that trastuzumab (5 µg/ml) long-term (24 hr) treatment is able to reduce the phosphorylation of Erk and AKT in the presence of 10% serum.

Although 48 hr trastuzumab treatment was able to inhibit pErbB2 and pEGFR (Fig 3.4.B and 3.4.C) after EGF stimulation, it failed to block the phosphorylation of Akt (Fig 3.4.B) and Erk (Fig 3.4.C). This might suggest that the activity of negative regulators of pAkt and pErk (eg. PTEN and dual specificity phosphatase 5 (DUSP5) respectively) that can be involved between the inhibition of ErbB receptors and the activation of its downstream proteins after trastuzumab treatment. This issue needs to be further investigated.

Loss of PTEN through mutation or inactivation has been reported in approximately 50% of breast cancers (Pandolfi & Ph, 2004). Past studies have illustrated how the loss of PTEN activity upregulates the phosphorylation of the PI3K-AKT pathway and increases the rate of cell growth (Pandolfi & Ph, 2004). Nagata et al., (2004) and Dave et al., (2011) found that the tumors with loss of PTEN tend to be more susceptible to trastuzumab resistance. In addition, ErbB3 preferentially binds to PI3K to activate the PI3K-AKT pathway leading to cell survival (Hynes & Lane, 2005; Burgess, 2008). This might limit my data regarding the PI3K-AKT pathway, and as a result more investigation is needed. In particular, it would be interesting to investigate the PTEN and ErbB3 proteins after treatment of SKBR3 with 10 µg/ml of trastuzumab for 48 hr.

I further examined the effect of trastuzumab on another downstream protein, PLCγ1, activated by the ErbB receptors. I was interested in this protein in particular for several reasons. First, PLCγ1 can be activated as a result of RTK activation, including EGFR (Young Noh et al., 1995; Bunney & Katan, 2011). Second, it has been suggested that PI3K has the ability to activate PLCγ1 (Bae et al., 1998) but the exact role is not well understood. Third, some studies suggest a role of PLCγ1 in cancer metastasis and progression (Wells, Grandis, & C-, 2003; Sala et al., 2008). Lastly, the role of PLCγ1 in the mechanism of trastuzumab on breast cancer has rarely been investigated. Therefore, it was interesting to examine PLCγ1 activity after trastuzumab treatment. I show that when SKBR3 was subjected to short-term treatment with trastuzumab (1 hr), the phosphorylation of PLCγ1 was reduced compared to EGF stimulated only samples. Long term-treatment reduced PLCγ1 phosphorylation to almost an undetectable level in both EGF unstimulated and stimulated samples, suggesting that trastuzumab is able to inhibit PLCγ1 phosphorylation in the presence of EGF (Fig 3.4.B). It should be noted that some studies have suggested that the phosphorylation of PLCγ1 is critical, but not sufficient for the full activation of the protein, and other mechanisms for PLCγ1 full activation have been proposed (Sekiya et al., 2004; Maffucci & Falasca, 2007; Raimondi et al., 2012). Additional exploration of these other mechanisms in the cell lines I have studied might be very fruitful.

Several mechanisms have been proposed for trastuzumab resistance, one of these mechanisms suggested that resistance arises from impaired trastuzumab binding to ErbB2. Fiszman & Jasnis (2011) suggested that ErbB2 mutation or masking disrupts trastuzumab binding and leads drug resistance. In addition, Vu & Claret (2012) revealed a possible mutation within ErbB2 receptor that lead to several phenotypical changes affecting the binding affinity between ErbB2 and trastuzumab. Here in this thesis, I show that a trastuzumab concentration of 10 μg/ml is sufficient

for binding to ErbB2 receptors on the plasma membrane in MDA-MB-453 and CHO-ErbB2 (K6) cell lines (Fig 3.5, 3.8).

Regarding MDA-MB-453, the results I demonstrate in this thesis are different from SKBR3. I show that 1 hr trastuzumab treatment in MDA-MB-453 cells was able to increase phosphorylation of ErbB2 (Y1248 and 1196), but not with 48 hr treatment (Fig 3.6.B and 3.6.C). As expected, EGF stimulation did not induce pErbB2 activation (Fig 3.6), since MDA-MB-453 lacks EGFR expression (Fig 3.1). On the other hand, S. Lee and his colleagues (2002) showed no upregulation of ErbB2 phosphorylation after long-term (24-36hr) treatment of trastuzumab (10nM) in MDA-MB-453. However, as mentioned above several studies have shown increase in ErbB2 phosphorylation after short term of trastuzumab treatment (Nagata et al., 2004; Gijsen et al., 2010; Dokmanovic et al., 2014), which is consistent with what I show here with short-term trastuzumab treatment (Fig 3.6.B and 3.6.C). Several studies have shown that the ErbB2-overexpressing SKBR3 cell line is trastuzumab sensitive (Nagata et al., 2004; Junttila et al., 2009; Cuello et al., 2001; Gijsen et al., 2010; Dokmanovic et al., 2014). However, even though MDA-MB-453 is considered an ErbB2 overexpressing cell line as Ginestier and his colleauges (2007) have reported, it is considered a trastuzumab resistant model (Yakes et al., 2002, Junttila et al., 2009).

Currently, there are two hypotheses explaining MDA-MB-453 trastuzumab resistance. Ginestier et al., (2007) have reported a link between MDA-MB-453 resistance to trastuzumab with the lack of phosphorylation of Y1248 residues within the ErbB2 receptor. On the other hand, Junttila and his colleauges (2009) have described how PI3K mutations generate trastuzumab resistance and that trastuzumab-resistant cell lines commonly harbor activating PI3K mutations or are PTEN null. PI3K is classified into three classes based on structure and substrate specificity, class 1 is

further classified into class 1A and class 1B, where class 1A contains a catalytic domain known as p110 (p110α (encoded by *PIK3CA* gene), p110β, p110δ) (Mukohara, 2015). *PIK3CA* somatic mutations have been reported in 30% of breast cancer cases, these mutations are a gain of function mutations that occur within exon 9 (E542K or E545K) and exon 20 (H1047R or H1047L) which are known as "hot spots" (Samuels et al., 2004, Zhao et al., 2005, Mukohara, 2015). The MDA-MB-453 cell line has a *PIK3CA* H1047R activating mutation which leads to overexpression of the p110α protein which constitutively activates the PI3K-AKT pathway (Junttila et al., 2009). This may be the reason why pAKT remained unaffected after either 1 hr or 48 hr of trastuzumab treatment (Fig 3.6.B). On the other hand, 48 hr of trastuzumab treatment inhibited Erk phosphorylation activity in MDA-MB-453 cells when compared to control (Fig 3.6.C). Furthermore, it has been suggested that the inhibitory effect of trastuzumab on the MEK/ERK pathway is not sufficient for inhibition of proliferation (Junttila et al., 2009; Solit et al., 2006), which may explain why MDA-MB-453 remains trastuzumab-resistant after inhibition of pErk (Fig 3.14).

It has been suggested that measuring the phosphorylation activity of AKT is a useful way to assess the activity of class 1 PI3K (Costa et al., 2015). However, this technique might not be efficient in the presence of *PIK3CA* mutations present in certain types of breast cancer. This is due to its inability to examine upstream proteins in the PI3K-AKT pathway (e.g. p110α and PIP2/PIP3 ratio) which play a critical role in activating several downstream target of the PI3K-AKT pathway (Stemke-Hale et al., 2008). Moreover, *PIK3CA* mutations can regulate cell survival and viability in breast cancer independently of AKT protein through other downstream targets like serum/glucocorticoid regulated kinase (SGK3) (Vasudevan et al., 2009) or those involved in the Rac-Erk pathway (Ebi et al., 2013). Since in this thesis only AKT

phosphorylation activity was examined by immunoblotting after trastuzumab treatment, further studies on p110α activity in *PIK3CA* mutated cell lines are recommended in order to understand the effects of this mutation on the mode of action of trastuzumab.

4.3. Trastuzumab activity on ErbB receptors and their downstream proteins in CHO cell lines

As stated above, the mode of action of trastuzumab on ErbB receptors is complicated and controversial. As a result, I decided to work on CHO cell lines that expresses either ErbB2 [CHO-ErbB2 (K16) and CHO-ErbB2 (K13)] or EGFR (CHO-EGFR), to understand the molecular mechanisms that trastuzumab might have on its signaling pathway.

I show in this thesis that trastuzumab had an inhibitory effect on EGFR phosphorylation after long-term treatment in the presence of a ligand. Trastuzumab was able to inhibit the phosphorylation of EGFR in the presence of EGF after 1 hr and 48 hr in CHO-EGFR cell line (Fig 3.7). This is consistent with the results I found regarding pEGFR inhibition in SKBR3 after 48 hr treatment with trastuzumab in the presence of EGF (Fig 3.4). I did detect an inhibition of pErk after 1 hr of trastuzumab treatment in CHO-EGFR (Fig 3.7) which consistent with the inhibition of pErk in the presence of EGF in SKBR3 cells (Fig 3.4). On the other hand, I did not detect any pErk inhibition after 48 hr treatment with trastuzumab in the presence of EGF in SKBR3 cells (Fig 3.4); however, trastuzumab treatment was able to reduce pErk activity after 48 hr (Fig 3.7). As mentioned above, the mechanism behind how trastuzumab inhibits EGFR phosphorylation in the presence of its ligand (EGF) is elusive and needs further investigation.

I have showed in this thesis that trastuzumab treatment for short or/and long-term periods was not able to upregulate EGFR phosphorylation in the CHO-EGFR cell line (Fig 3.8). However, it

is interesting that trastuzumab was able to block pErk while pEGFR remained unaffected (4 hr treatment) (Fig 3.7). It is also interesting that trastuzumab alone without EGF stimulation was able to increase the phosphorylation of Erk at 16 hr and 24 hr (Fig 3.8). Since ERK is a downstream effector protein of the RAS/RAF/MEK pathway (Solit et al., 2006), it will be interesting to examine the activity of other proteins within this pathway after trastuzumab treatment either alone or in the presence of a ligand. This might explain why I found upregulation of pErk activity after trastuzumab treatment.

I further examined the effect of trastuzumab on ErbB2 receptors in CHO cell lines that stably express ErbB2, CHO-ErbB2 (K6), and CHO-ErbB2 (K13). In contrast to the results I showed in SKBR3 (Fig 3.4.), trastuzumab failed to block the phosphorylation of ErbB2 (Y1248) or pErk in CHO-ErbB2 (K6) even after 48 hr (Fig 3.10 and 3.11). However, trastuzumab increased levels of pErbB2 (Y1222) after short-term (4-8 hr) treatment then reduced the phosphorylation at 16 hr treatment (Fig 3.10 and 3.11). These findings suggest that trastuzumab might have an inhibitory effect on tyrosine residues rather than other residues on ErbB2 receptor. Indeed, a previous study suggested that breast cancer cell lines which express, in particular pErbB2 (Y1248), might have higher sensitivity to trastuzumab rather than those cell lines which do not express the phosphorylation for the same tyrosine residue (Ginestier et al., 2007). Moreover, a study by Dokmanovic et al., (2014) suggested that in order to induce its inhibitory effect, trastuzumab has to increase the phosphorylation of the ErbB2 receptor tyrosine residue of 1248. As a result, this increases the interaction of ErbB2 with CHK, which mimics the trastuzumab inhibitory effect and downregulates AKT. Which might explain the pErbB2 (Y1248) upregulation after 1-4 hr of trastuzumab treatment (Fig 3.13). On the other hand, trastuzumab blocked pErbB2 (Y1248) after 48 hr treatment in the CHO-ErbB2 (K13) cell line (Fig 3.13). This is consistent with the results

where trastuzumab inhibited pErbB2 after 48 hr treatment in the SKBR3 cell line (Fig 3.4). Moreover, trastuzumab was also able to block the phosphorylation of the ErbB2 tyrosine residue 1196 along with 1248 after 48 hr (Fig 3.12). Interestingly, PLC-γ1 phosphorylation was reduced to undetectable levels in CHO-ErbB2 (K6) and CHO-ErbB2 (K13) non treated samples (control samples) compared to CHO (parental cells) non treated samples (control samples) (Fig 3.10, 3.11, and 3.13). For this reason, the mode of action of trastuzumab on PLC-γ1 phosphorylation in K6 and K13 cell lines is still unclear and needs more investigation. CHO-ErbB2 (K6) and CHO-ErbB2 (K13) cell lines were stably transfected with ErbB2 and have a $2.1x10^5$ and $3.7x10^4$ ErbB2 surface density (number of ErbB2 molecules per cell) respectively (Münch et al., 2011), while CHO (parental cells) lacks ErbB2 expression. This leads to the following question; does ErbB2 overexpression play a negative role in PLC-γ1 activity or not?

In conclusion, the results obtained from CHO-ErbB2 (K6) and CHO-ErbB2 (K13) after trastuzumab treatment add to our understanding for ErbB2-positive breast cancer. *In vitro*, since both cell lines have different ErbB2 surface density, both responded to trastuzumab differently. It would be interesting to see if ErbB2 density contributes to trastuzumab resistance, whether or not it would affect the mode of action of trastuzumab and improve therapeutic assessment of ErbB2 overexpressing breast cancer.

4.4. Trastuzumab effect on ErbB2 dimerization

The ErbB2 receptor structure is different from the rest of the ErbB receptor family members since it does not require a ligand to be activated. The absence of ligand-binding in ErbB2 receptors makes it one of the preferential receptors for homo/hetero-dimerization (Garrett et al.,

2008; Cho et al., 2003). It is likely that in breast cancers overexpressing ErbB2, overexpression itself can cause ErbB2 activation, without ligand binding, due to homo-hetero dimerization (Junttila et al., 2009), and it is possible for ErbB2 receptors to form homo or hetero-dimmers on the plasma membrane without individual receptor activation through ligand binding (Liu et al., 2007; Tao & Maruyama, 2008) . Ligand independent ErbB2/ErbB3 complexes were readily detected in SKBR3 cells by using 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP) crosslinking (Junttila et al., 2009), which is consistent with my data regarding the trastuzumab non-treated control samples that were treated with BS3 (Ctr2), where ErbB2 dimers were detected in the absence of EGF ligand (Fig 3.14).

I show in this thesis that 10 µg/ml of trastuzumab treatment alone for 1hr failed to block the homo and/or hetero-dimerization of ErbB2 in SKBR3 and MDA-MB-453 cell lines that overexpress ErbB2 (Fig 3.14). Moreover, trastuzumab failed to block their dimerization in the presence of EGF in both SKBR3 and MDA-MB-453 cell lines (Fig 3.14). Similar results were obtained in CHO-ErbB2 (K6) that stably expressed ErbB2 (Fig 3.14), where trastuzumab failed to block ErbB2 homo-dimerization in the presence or the absence of EGF. My results are consistent with previous results that reported trastuzumab inability to block ErbB2 homo and/or hetero-dimerization (Scaltriti et al., 2009; Gijsen et al., 2010).

Pertuzumab is a humanized monoclonal antibody that target ErbB2 dimerization (Diermeier-Daucher et al., 2008; Hu et al., 2015), it binds to domain II of the ErbB2 receptor, the domain that contains the dimerization loop (Hynes & Lane, 2005). In contrast, trastuzumab binds to domain IV, which does not play a role in the dimerization loop and therefore, trastuzumab in combination with another drug that targets the ErbB2 homo-hetero dimerization (e.g. pertuzumab) may increase therapeutic efficacy. Indeed, several studies recently are trying to

examine the inhibitory effect of both drugs combined with docetaxel (Swain et al., 2015; Kawajiri et al., 2015).

4.5. Trastuzumab and cell viability

Consistent with other studies (Ginestier et al., 2007; Junttila et al., 2009), trastuzumab had a significant inhibitory effect on cell viability in SKBR3 at a dose of 2.5 μg/ml ($p < 0.05$). On the other hand, a trastuzumab dose of 10 μg/ml did not significantly inhibit cell viability in SKBR3 (p value of 0.1) (Fig 3.15). Furthermore, none of the experimental dose of trastuzumab had significant effect on cell viability in the MDA-MB453 cell line (Fig 3.15). This is consistent with studies by Ginestier et al., (2007) and Juntt ila et al., (2009) who suggested that MDA-MB-453 is a trastuzumab resistance cell line due to lack the phosphorylation of ErbB2 (Y1248) and *PIK3CA* mutations. Moreover, I showed that trastuzumab was able to inhibit pErk activity in MDA-MB-453 after 48 hr treatment, but not pAKT (Fig 3.6). It has been suggested that PI3K/AKT pathway activity is directly linked to the proliferation of ErbB2 overexpressing cells (Junttila et al., 2009). Moreover, it was suggested that the inhibitory effect of trastuzumab on the MEK/ERK pathway is not sufficient for inhibition of proliferation (Junttila et al., 2009; Solit et al., 2006). Collectively, this might explain why MDA-MB-453 remains trastuzumab-resistant after 48 hr treatment (Fig 3.15).

Clinical benefits from trastuzumab-based therapy in both early and advanced breast cancer have been demonstrated (Hudis, 2007). However, a combined treatment of trastuzumab with another cancer drug such as paclitaxel or doxorubicin is able to achieve an even better response (Spector & Blackwell, 2009). The role of trastuzumab is likely to inhibit excess signals for cell

proliferation and survival and therefore a combined therapy is needed where another drug is required to kill the cancer cells.

The standard therapy that has been used in clinical practices to treat metastatic ErbB2-positive breast cancer is to combine pertuzumab, trastuzumab and docetaxel (Swain et al., 2015). I was therefore interested to examine the synergetic effect of trastuzumab and doxorubicin. I found that doxorubicin had a significant inhibitory effect on SKBR3 and MDA-MB-453 cells in a dose dependent manner and with an IC_{50} of approximately 0.5 μM (a clinical relevant dose) (Cooper et al., 2015) (Fig 3.17). Trastuzumab did not enhance the effect of doxorubicin in SKBR3 (Fig 3.18) and MDA-MB-453 (interestingly, except at concentrations of 5 and 10 μg/ml) (Fig 3.19). This is consistent with Cooper et al., (2015) who found that 5 μg/ml of trastuzumab enhances the effect of doxorubicin, however, the concentration of doxorubicin was lower and the treatment duration was longer.

In summary, I show in this thesis that trastuzumab (40 μg/ml) is able to inhibit pErbB3 (Y1328) in MDA-MB-453 and SKBR3 without and with ligand respectively (Table 4.1). Trastuzumab (10 μg/ml) inhibits pErbB2 (Y1248, Y1196) and pEGFR (Y992) after 48 hr in SKBR3 in the presence of EGF (Table 4.1 and 4.2). Furthermore, I show that trastuzumab was able to inhibit the phosphorylation of downstream proteins (AKT and Erk) of EGFR and ErbB2 after 48 hr in SKBR3, but not in the presence of EGF. Trastuzumab (10 μg/ml) failed to inhibit pAKT after 1 hr for both SKBR3 and MDAMB-453 cell lines. Moreover, I show that trastuzumab had an inhibitory effect on EGFR phosphorylation when it was stably expressed in the CHO cell line after both 24 and 48 hr treatment. The effect of trastuzumab was different between CHO-ErbB2 (K6) and CHO-ErbB2 (K13) that stably expresses ErbB2 and have a $2.1x10^5$ and $3.7x10^4$ ErbB2 surface density (number of ErbB2 molecules per cell) respectively (Münch et al., 2011).

Trastuzumab was able to inhibit pErbB2 (Y1248) in CHO-ErbB2 (K13) but not in CHO-ErbB2 (K6). This emphasizes that there is variation in the action of trastuzumab that is dependent upon the density differences of ErbB2 between the two cell lines. Interestingly, trastuzumab after 48 hr of treatment was able to inhibit pErbB2 (Y1222), but not pErbB2 (Y1248), in CHO-ErbB2 (K6). This might suggest that trastuzumab might act on certain tyrosine residues rather than others within ErbB2 receptors.

In conclusion, I show that trastuzumab is able to block pErbB2 and pEGFR, however, its role on other receptors need more investigation. Since trastuzumab downregulates ErbB2/EGFR receptors, this does not eliminate the presence of other signals from other receptors that can compensate for the loss of ErbB2 or EGFR activity, for example, the Insulin-like Growth factor 1 receptor (IGF-1R). IGF-1R is a transmembrane receptor usually expressed in breast cancer and has been documented as one of the reasons behind trastuzumab resistance (Nahtaet al., 2005).Yuhong et,. (2001), and Nahtaet al., (2005) emphasized that breast cancer cells that express both ErbB2 and IGF-1R tend to be more resistant to trastuzumab due to IGF-1R activation of the PI3K-AKT pathway leading to p27 degradation, which causes breast cancer cells to proliferate uncontrollably. Moreover, the use of IGF-1R antagonists prevented trastuzumab resistance (Nahtaet al., 2005). In addition, the role of ErbB4 is not well documented in the context of trastuzumab mode of action. How this receptor might play a role in ErbB2 and its downstream protein activation, potentially through receptor hetero-dimerization, is still elusive and needs to be further studied.

Even though, the mode of action of trastuzumab appears to be more complex than previously assumed, further studies to elucidate the signaling pathways involved will allow us to improve therapy against ErbB2-overexpressing breast cancers. For example, the reason why trastuzumab

long-term treatment inhibits pErbB2 more efficiently than short-term treatment in breast cancer cell lines remains unclear and needs further investigation. In addition, the mechanism of how trastuzumab inhibits EGFR phosphorylation in the presence of EGF, and the mode of action of trastuzumab after activation of EGFR with other ligands remains elusive and needs to be studied further. Moreover, since in this thesis only AKT phosphorylation activity was examined by immunoblotting after trastuzumab treatment, further studies on p110α activity in *PIK3CA* mutated cell lines are recommended in order to understand the effects of this mutation on the mode of action of trastuzumab.

Table 4.1. Summary of ErbB2 and ErbB3 phosphorylation after short and long term of treatment trastuzumab in previous published studies and in this thesis

Study	Trastuzumab conc.	Effect on ErbBs receptors after short term of treatment	Effect on ErbBs receptors after long term of treatment
Lee *et al.*, 2002	20, 10 nM	-----	↓ pErbB2
Nagata *et al.*, 2004	2 µg/ml	↑ pErbB2	-----
Junttila *et al.*, 2009	10 µg/ml	No effect on pErbB2 ↓ pErbB3	No effect on pErbB2 ↓ pErbB3
Gijsen *et al.*, 2010	40 µg/ml	↑ pErbB2 ↓ pErbB3	↓ pErbB2
Dokmanovic *et al.*, 2014	4, 10, and 40 µg/ml	↑ pErbB2 ↓ pErbB3	-----
In this thesis	10 and 40 µg/ml	↑ pErbB2 ↓ pErbB3	↓ pErbB2

Table 4.2. Summary of EGFR phosphorylation after long term treatment of trastuzumab in previous published studies and in this thesis

Study	Trastuzumab conc.	Effect on EGFR receptor after short term of treatment	Effect on EGFR receptor after long term of treatment
Ye *et al.*, 1999	20 nM	-----	No effect on pEGFR
Kuwada *et al.*, 2004	10 µg/ml	-----	↓ EGFR level ↓pEGFR
In this thesis	10 µg/ml		↓pEGFR

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