Understanding the effects of therapeutic HER2 antibodies trastuzumab and pertuzumab on HER2-mediated cell signaling

by

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Abstract

The ErbB family of receptor tyrosine kinases, also called the HER receptors, consists of four members including epithelial growth factor receptor (EGFR), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4). HER2 overexpression has been reported in 20-30% of breast cancer cells and is responsible for lower overall survival rates in HER2-positive breast cancer cells. Trastuzumab as the first HER2-targeted therapy for the treatment of HER2-positive breast cancer patients was introduced in 1998. Although several mechanisms have been suggested for the mode of action of trastuzumab, the findings regarding these mechanisms are unclear and controversial. In addition, a considerable number of patients with HER2-positive breast cancer do not respond, or become resistant, to trastuzumab. In recent years, several new chemotherapeutic and targeted-therapeutic drugs have been introduced to increase the efficacy of targeted therapy with trastuzumab. Pertuzumab was specifically designed as a humanized recombinant monoclonal antibody to block both homodimerization and heterodimerization of HER2. Therefore, the lack of a clear understanding of the mechanisms of both trastuzumab and pertuzumab action may limit their application and efficacy.

Significant proportions of HER2-positive breast cancer cells co-express multiple types of HER receptors. Therefore, it is hard to investigate the possible effects of trastuzumab and pertuzumab on HER2 homodimer formation and HER2 homodimer-mediated HER2 phosphorylation. To overcome this limitation, we studied the effects of these antibodies in Chinese hamster ovary (CHO) cells transfected with genes encoding different HER receptors. Our time course and dose response results revealed that trastuzumab and pertuzumab only bind to HER2 with high affinity and specificity. Moreover, we showed that when used as single agents trastuzumab and

pertuzumab have no inhibitory effects on HER2 homodimerization and phosphorylation in CHO cells overexpressing HER2 (CHO-K6). However, the combination of trastuzumab and pertuzumab can inhibit the phosphorylation of HER2 at specific sites suggesting the possible inhibitory effects of combination therapy on HER2-mediated signaling.

I further studied the mechanism of action of trastuzumab in HER2-positive breast cancer cells including SKBR3 and BT474 cells. I determined the effects of trastuzumab on HER2 signaling under various conditions including treatment with DMEM, EGF, HRG, and 10% FBS. The HER2 signaling was assessed by its heterodimerization, phosphorylation, and activation of the major downstream signaling proteins including Erk and Akt. Results demonstrated two different mechanism of action for trastuzumab in these two different HER2-positive breast cancer cell lines. In SKBR3 cells, we found that trastuzumab reduced cell viability in all treated conditions; however, it only arrested cell cycle at the G1 phase in the absence of EGF and HRGα ligands. It is possible that trastuzumab can reduce the viability of the SKBR3 cells through inducing both cell cycle arrest and apoptosis and ligands suppress the inhibitory effects of trastuzumab on cell cycle progression. Indeed, our results showed that trastuzumab has inhibitory effects on PI3K/Akt and MAPK signaling pathways, which may act through inhibition of HER receptors phosphorylation. On the other hand, in BT474 cells we showed that trastuzumab reduced viability and arrest the cell cycle at G1 in the presence or absence of EGF but not in the presence of HRGα ligands. However, trastuzumab did not show any detectable inhibitory effects on HER receptors' phosphorylation, heterodimerization, and MAPK activation under these conditions. Interestingly, we showed that trastuzumab inhibited Akt phosphorylation at both threonine and serine phosphorylation sites. Previous studies have shown that lipid raft localization of HER receptors in breast cancer cell lines can promote HER kinase-independent activation of Akt. In addition, it has been shown that

trastuzumab can affect HER2 localization in the breast cancer cell membrane. Therefore, we hypothesis that trastuzumab inhibits the lipid raft localization of HER2, which leads to the inhibition of Akt phosphorylation. Our results showed that trastuzumab blocked HER2 and EGFR localization to lipid rafts. Moreover, we found that trastuzumab significantly reduced the phosphorylation of c-Src which plays an important role in HER-mediated Akt phosphorylation in lipid rafts.

Overall, my results showed that trastuzumab can exert its anti-proliferative effects through different mechanisms which is cell line dependent. Most importantly, we identified a novel mechanism underlying the action of trastuzumab. By this novel mechanism, trastuzumab targets a HER2 kinase-independent signaling pathway by hindering the movement of HER2 homodimers and HER2-EGFR heterodimers into the lipid rafts.

Preface

This thesis is an original work by Hamid Maadi under supervision of Dr. Zhixiang Wang and co-supervision of Dr. Sarah Hughes at the Department of Medical Genetics, Faculty of Medicine and Dentistry, University of Alberta.

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Chapter 6 is an original work with unpublished data carried out by Hamid Maadi. One paragraph of discussion has been published in "Cancers" journal with this citation information: Nami, B., H. Maadi, and Z. Wang, Mechanisms Underlying the Action and Synergism of Trastuzumab and Pertuzumab in Targeting HER2-Positive Breast Cancer. Cancers, 2018. 10(10).

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List of abbreviations

ABL   Abelson murine leukemia

ADAM   A disintegrin and metalloproteinase

ADCC   Antibody-dependent cell-mediated cytotoxicity

ADC   Antibody-drug conjugate

APS   Ammonium persulfate

ATCC   American Type Culture Collection

ATM   Ataxia telangiectasia mutated

BAD   BCL2-associated agonist of cell death

BC   Breast cancer

BRCA-1   Early onset breast cancer 1

BRCA-2   Early onset breast cancer 2

BS^3 Bissulfosuccinimidyl suberate

BSA   Bovine serum albumin

CHEK2   Checkpoint kinase 2

CHK   Csk-homologous kinase

CHO   Chinese hamster ovary

DAPI   4′,6-diamidino-2-phenylindole

DMEM   Dulbecco's Modified Eagle's Medium

DMSO   Dimethyl sulfoxide

ddH2O   Double-distilled water

ECD   Extracellular domain

ECL   Enhanced chemiluminescence

EGFR	Epithelial growth factor receptor
ER	Estrogen receptors
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FKBP	FK506 Binding Protein
FoxO	Forkhead box O transcription factors
GSK3	Glycogen synthase kinase 3
Grb2	Growth factor receptor-bound protein 2
HDAC	Histone deacetylase
HER	Human epidermal growth factor receptor
HRG	Heregulin
Hsp	Heat-shock protein
IGF	Insulin-like growth factor
IP	Immunoprecipitation
IgG	Immunoglobulin G
MAPK	Mitogen-activated protein kinases
MCL	Mantle cell lymphoma
MMP	Matrix metalloproteinase
mTOR	Mammalian Target Of Rapamycin
mTORC	mTOR Complex

MTT   3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide

MβCD   Methyl-β-cyclodextrin

NK   Natural killer

NRF2   Nuclear Factor Erythroid 2-Related Factor 2

NSCLC   Non-small cell lung cancer

PAGE   Polyacrylamide gel electrophoresis

PALB2   Partner and localizer of BRCA2

PBS   Phosphate-buffered saline

PD-L1   Programmed death-ligand 1

PDK1   Pyruvate Dehydrogenase Kinase 1

PH   Pleckstrin homology

pH   Potential hydrogen

PI   Propidium iodide

PI3K   Phosphatidylinositol-4,5-Bisphosphate 3-Kinase

PIK3CA   Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PIP₃   Phosphatidylinositol (3,4,5)-trisphosphate

PKB (Akt)   Protein kinase B

PLC-γ   Phosphoinositide phospholipase C gamma

PM   Plasma membrane

PP1   Protein phosphatase 1

PTEN   Phosphatase and tensin homolog

Ptp-2c   Protein Tyrosine Phosphatase

RTKs   Receptor tyrosine kinases

Ras   Rat Sarcoma Viral Proto-Oncogene

SDS	Sodium dodecyl sulfate
SFKs	Src family protein tyrosine kinases
SHC	Src Homology 2 Domain Containing Transforming Protein 1
SHP-1	Src homology region 2 domain-containing phosphatase-1
STAT	Signal Transducer And Activator Of Transcription
Src	SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TIGIT	T Cell Immunoreceptor With Ig And ITIM Domains
TKIs	Tyrosine kinase inhibitors
TNBC	Triple-negative breast cancer
TP53	Tumor protein p53
TR-FRET	Time-resolved Fluorescence Resonance Energy Transfer
TRITC	Tetramethylrhodamine
Thr	Threonine
VAV2	Vav Guanine Nucleotide Exchange Factor 2
Y	Tyrosine

Chapter 1. Introduction

1.1. Breast cancer

Breast cancer (BC) is the most common type of cancer in women [1]. The incidence of this type of cancer is increasing every year with the rate of 3.1% worldwide [2]. Various genetic and environmental factors can predispose a person to develop BC. Approximately 1 in 10 patients with BC has genetic mutations inherited from their parents [3]. Mutations in early onset BC 1 (BRCA1), BRCA2, tumor protein p53 (TP53), partner and localizer of BRCA2 (PALB2), ataxia telangiectasia mutated (ATM), phosphatase and tensin homolog (PTEN), and checkpoint kinase 2 (CHEK2) genes are the most frequent mutations which have been significantly correlated to the incidence of inherited BC [1]. In addition, it has been demonstrated that different non-genetic factors including obesity, alcohol consumption, and sedentary lifestyle can increase the possibility of BC occurrence in women [4].

Generally, BC can be classified into five subtypes based on their gene expression profiles determined by microarray: luminal-like subtypes A and B (expression of hormone receptors and luminal cytokeratins 8 and 18), basal-like (also called triple-negative BC (TNBC), typically with no expression of estrogen receptors (ER), progestin receptors, and HER2), HER2-positive (HER2+), and normal-like [5, 6].

Based on histological grade and molecular subtypes of BC, various treatment strategies can be used to treat BC patients. The surgical resection of tumor bulk is often recommended as the first step in the treatment of BC. However, most of the patients with BC receive systematic chemo- or targeted therapies before or after the surgery to reduce the mass of the tumor or decrease the chance of disease recurrence respectively [1]. In BC with the positive expression of ER or progesterone, endocrine therapy is the standard adjuvant therapy after surgery. Moreover, in HER2-positive BC patients with tumor size equal or less than 2 cm without metastasis to lymph nodes, chemotherapy with trastuzumab is concurrently used as a standard adjuvant therapy for 1 year. However, in patients with HER2-positive BC with tumor size more than 2 cm and presence of tumor cells in lymph nodes, chemotherapy and combination of trastuzumab and pertuzumab is used as standard therapy. In this case, if pathologic complete response (pCR) has been achieved, the treatment of patients with hormone receptor-negative tumors and positive lymph nodes will be continued with combination of trastuzumab and pertuzumab for 1 year. In other cases, patients will be treated with

trastuzumab only as a second line of treatment. On the other hand, if pCR have not been happened, trastuzumab emtansine (T-DM1) will be used in patients with HER2-positive BC who did not respond to the first line of treatment. Finally, using chemo drugs like anthracycline, taxane, and docetaxel have been shown to be effective in treatment of TNBC [1, 7].

1.2. HER Receptors and Cell Signaling

There are more than 60 receptor tyrosine kinases (RTKs) that have been identified in the human genome [8]. The ErbB family of receptor tyrosine kinases consists of four members including epithelial growth factor receptor (EGFR), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4). Like other RTKs, HER receptors are single transmembrane proteins which have an N-terminal extracellular domain, a transmembrane helix, and a cytoplasmic domain [9]. The extracellular domain contains four subdomains, including the ligand binding subdomains (domain I and III), and receptor dimerization subdomains (domain II and IV). The intracellular domain is composed of a tyrosine kinase domain and a C-terminal regulatory domain **(Figure 1.1)** [10].

EGFR, a 170 kD single polypeptide chain, is the prototype of the HER family receptor [11-16]. While EGFR and HER4 are fully functional RTKs capable of signaling both as a homo- and heterodimers following the binding to various ligands, the other two members, including HER2 and HER3, are different. HER2 is an orphan receptor without a ligand and HER3 lacks kinase activity. However, through ligand-induced heterodimerization, all HER receptors could be fully activated to mediate cell signaling [17-33].

Besides EGF, another ten ligands have been identified that bind to and stimulate HER receptors. These ligands form the EGF family of peptide growth factors and are subdivided into three groups based on their binding partners **(Figure 1.1)**. EGF, epigen (EPG), amphiregulin (AR), and transforming growth factor (TGF) form one group that specifically binds to EGFR. HB-EGF, epiregulin (EPR), and betacellulin (BTC) form the second group that binds to both EGFR and HER4. The four neuregulin, including NRG1, NRG2, NRG3, and NRG4, form the third group that binds to HER4. However, NRG1 and NRG2 also bind to HER3 **(Figure 1.1)** [34-39]. Through the distinctive binding specificity and affinity, each ligand contributes in a unique manner to regulate the activation and signaling of the four HER receptors [17].

Our understanding of HER receptor dimerization has been greatly enhanced due to the determination of the structures of the HER receptor extracellular domains. So far, the structures of all HER receptors without ligand have been determined [42]. In addition, the structures of ligand-bound EGFR and HER4 have also been determined. Many structures of HER receptors binding to antibodies or antibody mimics have also been revealed [9, 40, 41]. With the support of other evidence, a comprehensive picture regarding ligand-receptor interaction and HER receptor dimerization have emerged. In total, ten different homo- and heterodimers are formed by four HER receptors **(Figure 1.1)** [42, 43]. Structural studies indicate that the conformations of the receptors can only exist in two forms: a tethered form and an extended form. In the tethered form, the receptor is unable to dimerize due to the buried dimerization element. However, in the extended form the dimerization elements of the receptor are fully exposed to allow the receptor dimerization. It has been demonstrated that the rigid nature of the receptor extracellular domains restricted or "clicked" the receptors only to these two forms [9, 41].

It is significant and interesting to find that HER2 extracellular domains are already in an extended form in the absence of ligands. The subdomains I and III of HER2 extracellular domain interact directly to stabilize HER2 to the extended form. The close interaction between subdomain I and III leaves no space for a ligand in between. Therefore, HER2 is an orphan receptor by nature [43]. HER2 maintains a ligand-independent and constitutively activated conformation. Indeed, HER2 spontaneously forms homodimers when overexpressed in cells, and all the other HER receptors dimerize preferably with HER2 [35, 42]. Moreover, the overexpression of HER2 (but not the other HER receptors) transforms cells, and HER2 overexpression is associated with poor prognosis in BC [43]. On the other hand, HER3 homodimer is generally viewed as non-functional due to lack of kinase activity. However, HER3 possesses very low kinase activity (1/1000th the kinase activity of EGFR) and thus it is still possible that HER3 homodimers may be functional [35].

Through homo- or heterodimerization, all HER receptors are activated, which induces the phosphorylation of multiple tyrosine residues in the C-terminal regulatory region. Various studies including large-scale phosphoproteomic screening has identified more than 100 proteins that potentially bind to HER receptors **(Figure 1.1)** [35, 44-53]. Both EGFR and HER4 bind to many different downstream proteins. EGFR binds to Grb2, Shc, Src, PLC-γ1, Crk, Stat5, Ptp-2c, and SHP1. HER4 binds to Syk, RasA1, Abl, Crk and Vav2, and Grb2. However, the signaling

pathways linked to HER2 and HER3 are very specific and limited. HER3 contains multiple phosphor tyrosine residues that bind to p85, and as such, HER3 strongly activate the PI3K-Akt pathway. On the other hand, HER2 is mostly engaged in Shc/Grb2-mediated activation of Erk pathways. Due to the distinctive binding to various downstream signaling proteins, the heterodimerization of HER receptors allows the activation of more signaling cascades than the homodimer of HER receptors. Based on the binding specificity, it is likely that the HER2 homodimer will mostly stimulate the activation of the Ras-Erk pathway, and the HER2-EGFR heterodimer may function similarly to EGFR homodimer. However, the HER2-HER3 heterodimer could be much more powerful than either the HER2 homodimer because the HER2-HER3 heterodimer could strongly activate the PI3K-Akt pathway in addition to the Ras-Erk pathway.
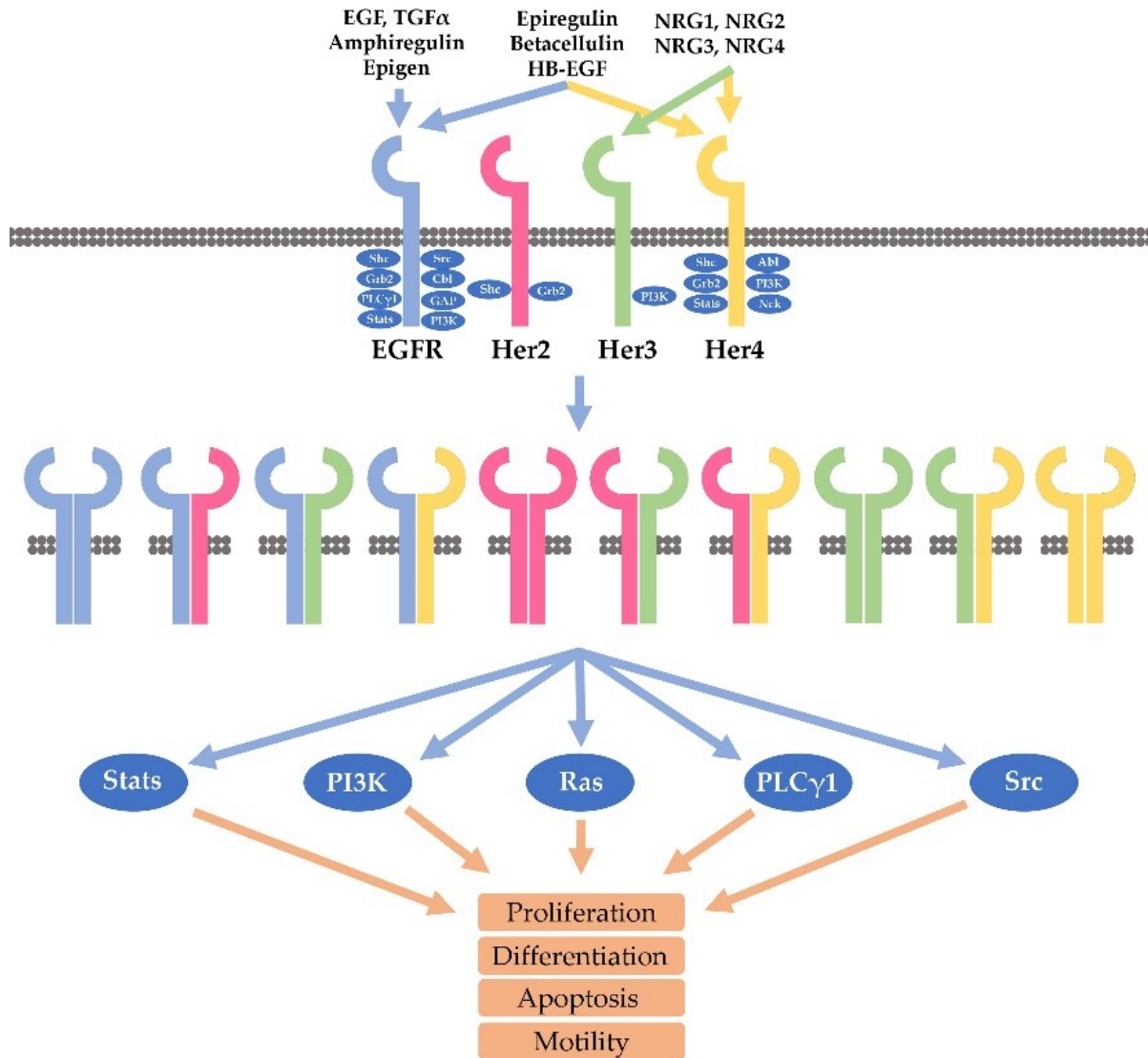
Figure 1.1 The activation of HER receptors and the downstream signaling cascades. Four members of HER receptors interact with 11 ligands, which results in the formation and the activation of 10 different homo- and heterodimers. Activated HER receptors promote many signaling cascades affecting many key biological outcomes.

1.3. The Roles of HER Receptors in BC

HER receptors have been implicated in the development of many types of human cancers, especially BC. Over activation of HER receptors is mostly due to overexpression driven by gene amplification, but also could be due to the truncation of the extracellular domain, mutation in kinase domain, or co-expression of HER receptor ligands. The over activation of HER receptors drives cancer development [34-36].

Overexpression of EGFR is observed in 20–30% of breast carcinoma. While high percentage of HER2-positive BC cells also overexpress EGFR, approximately 50% of TNBC cells overexpress EGFR [35, 44, 54]. Overexpression of EGFR has been frequently associated with large tumor size and poor clinical outcomes [35, 54-56].

HER2 overexpression occurs in 20–30% of BCs and ovarian cancers [18, 35, 57-61]. HER2 mutations are observed in approximately 1.6% of BC patients [62]. Patients whose breast tumors overexpress HER2 have a significantly lower survival rate and a shorter period before relapse than patients without overexpression [58, 61, 63]. Moreover, among different types of BC, HER2-positive BC has the worst prognosis and is responsible for considerable proportion of death in BC patients [64]. One of the reasons for poor prognosis of patients with HER2-positive BC is higher metastasis rate in these patients [65, 66]. HER2 overexpression has been positively correlated with lymph node metastasis in BCs [67, 68]. Moreover, several *in vitro* and *in vivo* studies have indicated that overexpression of HER2 elevates metastasis incidence rate [68-70] and increases tumorigenicity [71-73]. It has been shown that overexpression of HER2 in MCF10A cells increase the invasive properties of these cells via increasing matrix metalloproteinase and vimentin expression [74]. In addition, HER2 increase the expression of C-X-C chemokine receptor type 4 (CXCR4) which plays an important role in cancer cell invasion and metastasis [75]. The ability of HER2 to transform the cells is higher than other members of ErbB family of receptor tyrosine kinases [75]. Structural analysis of HER2 extra cellular domain have suggested an open conformation for HER2 receptors which can form ligand-independent homo- and heterodimers [76]. Therefore, HER2 receptors when overexpressed in the cells can easily dimerize with HER2 receptors as well as other members of ErbB family of receptor tyrosine kinases and activate multiple downstream signaling pathways involved in cancer cells proliferation and metastasis.

Overexpression of HER3 occurs in about 20% of BCs [60]. Overexpression is mostly due to increased transcription [60, 77]. Overexpression of HER3 alone does not promote anchorage-independent growth; however, when expressed together with HER2, HER3 strongly stimulates cell growth [35, 78]. Different from other HER receptors, both oncogenic and tumor-suppressor functions have been reported for HER4 [79-82].

1.4. Mechanisms Underlying the Action of Trastuzumab

HER2 is an attractive therapeutic target for the treatment of HER2-positive BC [83, 84]. A humanized recombinant monoclonal antibody to HER2, trastuzumab binds to HER2 domain IV that is close to the HER2 juxtamembrane region. Trastuzumab is the first HER2-targeted therapy that was approved by the FDA for the treatment of metastatic BC. It selectively exerts antitumor effects in HER2-positive BC patients [83, 84].

Although many mechanisms have been proposed for its antitumor activity, the exact mechanisms remain unknown. As summarized by several earlier reviews [83-85], several mechanisms including both intracellular and extracellular mechanisms have been proposed for the action of trastuzumab **(Figure 1.2).**

Antibody-dependent cell-mediated cytotoxicity (ADCC) has been identified as the extracellular action of trastuzumab. The Fc receptor on immune effector cells, principally natural-killer (NK) cells, recognizes the Fc portion of trastuzumab in the targeted cancer cell and attacks the cancer cells. Many studies have shown that action through ADCC is a major mechanism of trastuzumab [86-94]. Recent studies have focused on how to enhance the ADCC mediated by trastuzumab. It is reported that trastuzumab-induced ADCC could by augmented by enhancing NK cell activities [95, 96], by modifying the antibody itself [97-99], and by using inhibitors to various proteins including caspases [100], CD112R and TIGIT [101], histone deacetylase (HDAC), and a disintegrin and metalloproteinases (ADAMs) [102]. Moreover, chemotherapeutic drugs including taxanes [103] and tyrosine kinase inhibitors [104, 105] are also found to enhance trastuzumab-mediated ADCC.

Many intracellular mechanisms have been proposed for trastuzumab action; however, these data are controversial [48]. One of the main reasons behind the controversy of these results regarding trastuzumab effects on HER2 signaling is using different *in vitro* and *in vivo* models. Using

different concentrations of trastuzumab, treating the cells with trastuzumab for various time periods, and using different methods to investigate the effect of trastuzumab on HER2 signaling are the other factors which affect the results regarding the mode of action of trastuzumab.

The proposed intracellular mechanisms include: (1) inhibition of intracellular signal transduction leading to cell proliferation. Most experimental data indicate that trastuzumab does not inhibit, but under certain conditions stimulates HER2 phosphorylation [83-85, 106, 107]. Although, the data regarding the effects of trastuzumab on HER2 dimerization and HER2-mediated signaling pathways [108-111] are all controversial, several studies have shown that trastuzumab can significantly suppress Akt phosphorylation and arrest cell cycle at G1 phase through not well-understood mechanisms [106, 112-115]. (2) Inhibition of the proteolytic cleavage of HER2 extracellular domain. p95HER2 fragments are truncated HER2 proteins characterized by the lack of extracellular domain (ECD), but still possessing tyrosine kinase activity [116-120]. p95HER2 fragments arise by two different mechanisms: (i) proteolytic shedding/cleavage of p185HER2 by zinc-containing metalloproteinase, including A disintegrin and metalloproteinases (ADAM) and matrix metalloproteinase (MMP) family members [121-123]; and (ii) Alternative initiation of translation. p95HER2 could be generated by alternative initiation of translation from methionines located near the transmembrane domain of the full-length molecule [124]. Breast cancer patients that express p95HER2 are more likely to develop nodal metastasis [125] and have worse prognoses than those predominantly expressing the full-length receptor [126]. It was reported that trastuzumab blocked both basal and induced cleavage of p95HER2 [117]. (3) Inhibition of DNA repair. Chemo- and radiotherapies induce DNA damage in treated cancer cells and cancer cells may minimize this damage by repairing the damaged DNA. Some early studies suggest that trastuzumab partially blocks the repairing of damaged DNA [127-129]. However, all these data were published in the 1990s from one research group. (4) Inhibition of angiogenesis. Cancer cells promote angiogenesis to support tumor growth. Trastuzumab was shown in a preclinical murine xenograft tumor model to inhibit the angiogenesis [130, 131]. (5) Induction of HER2 endocytosis/downregulation [108-111]. Although findings about the effects of trastuzumab on HER2 internalization is controversial, using drug-conjugated trastuzumab clearly shows that trastuzumab is internalized into target cells. For instance, upon binding of T-DM1 (trastuzumab conjugated with emtansine) to HER2, the complex of HER2 and T-DM1 is internalized and

degraded in endosomes. During this processes emtansine is released into cell cytoplasm and bind to tubulin to inhibit microtubules formation [132, 133].

1.5. Mechanisms Underlying Trastuzumab Resistance

Since its introduction in 1999, trastuzumab has changed the paradigm of treating metastatic HER2-positive BC patients. While it significantly improved the treatment, the resistance, both innate and acquired, has posed big challenges [134, 135]. The overall response rate is about 50% with a significant percentage (approximately 40%) of metastatic patients demonstrating primary resistance. Moreover, most of the patients who initially responded to trastuzumab treatment quickly acquired resistance. The scientific community has been studying the mechanisms underlying the resistance in the hope of overcoming this [135-137].

Many resistance mechanisms have been identified. The resistance may arise due the altered HER2 expression status of the cancer cells [138, 139]. The resistance may also arise due the alteration of HER2 molecular structures, such as proteolytic truncation of HER2 extracellular domain, which prevents the binding of trastuzumab to the truncated but constitutively activated HER2 [118, 119, 140, 141]. Activation of other HER receptors such as EGFR, which compensate for the lost HER2 signaling due to trastuzumab inhibition [142, 143], or activation of HER2 through a mechanism that is not sensitive to trastuzumab [144, 145]. Constitutive activation of downstream signaling pathways due to mutations are also a major mechanism for trastuzumab resistance. The most prominent cause is the constitutive activation of PI3K-Akt-mTor pathway due to gain of function mutation of PI3K, and the loss of function of PTEN [146-152]. Some other mechanisms are also reported, including FCγ receptor polymorphism [153, 154], miRNAs [155, 156], and Mucin 4 expression induced by TNFα [157].

As HER2/HER3 heterodimer-mediated activation of PI3K-Akt-mTor has been considered the most important signaling pathway driving the development of BC, and constitutive activation of this pathway identified as a major resistant mechanisms for trastuzumab resistance, combined inhibition of both HER2 and PI3K-Akt-mTor has been explored to overcome trastuzumab resistance [158, 159]. Most research has demonstrated that additional inhibition of PI3K-Akt-mTor could overcome trastuzumab resistance in HER2-positive BCs [149, 160-163].

1.6. Mechanisms Underlying the Action of Pertuzumab

As a fully humanized recombinant monoclonal antibody, pertuzumab represents a new class of agents that inhibit HER2 dimerization [164] **(Figure 1.2)**. Pertuzumab was approved by the FDA in 2012 to be used in combination with trastuzumab and docetaxel for treating metastatic BC patients. This approval is based on the clinic trial results reported the same year by the CLEOPATRA Study Group [160, 165]. This treatment regime has significantly improved the outcome of the patients with metastatic HER2-positive BC and is now the standard first-line treatment for HER2-positive metastatic BC [57, 164, 166-169].

Pertuzumab specifically interacts with the subdomain II of HER2 extracellular domain, sterically blocking a binding pocket necessary for receptor dimerization, thus blocking HER2 dimerization mediated by the HER2 dimerization domain [40]. Indeed, the same research showed that pertuzumab blocked heregulin-induced heterodimerization between HER2 and HER3 [40]. Inhibition of dimerization will lead to the blocking of HER2 activation and HER2-mediated downstream signaling [164]. This understanding is mostly based on important early research [170]. This research showed that pertuzumab blocks the association of HER2 and HER3, diminishes ligand-activated HER2 signaling including Erk activation, and inhibits the growth of human BC cell lines only in the presence of ligand (heregulin) [170]. This research was conducted with BC cell lines that co-express both HER2 and HER3 in the context of heregulin stimulation. Subsequent brief research suggests the synergistic effect of trastuzumab and pertuzumab on BC survival but showed that pertuzumab alone is less effective in blocking Akt phosphorylation than trastuzumab and both antibodies have no effect on Erk phosphorylation in BT474 cells [171]. It was further reported that pertuzumab disrupts EGF-induced heterodimerization of HER2 and EGFR in ovarian cancer cells, expressing both EGFR and HER2. Pertuzumab also inhibits in vitro and in vivo growth of the same ovarian cancers [172]. Moreover, pertuzumab can abrogate the inhibitory effect of HER2 on the degradation of HER3 [173]. A recent study showed that both trastuzumab and pertuzumab inhibit NRF2 function in ovarian cancers and the combination of the antibodies produces more potent effects than a single antibody alone [174]. In summary, while the data regarding the mode of action of pertuzumab is quite limited, the available data mostly support the role of pertuzumab in blocking the heterodimerization of HER2, which in turn blocks the activation

of HER2- and HER3-mediated signal transduction pathways leading to cancer cell proliferation and survival.

1.7. Mechanisms Underlying Pertuzumab Resistance

Pertuzumab was approved by the FDA in 2012 to be used in combination with trastuzumab and docetaxel for treating metastatic BC patients. This approval is based on the clinic trial results reported the same year by the CLEOPATRA Study Group [160]. This treatment regime has significantly improved the outcome of the patients with metastatic HER2-positive BC and is now the standard first-line treatment for HER2-positive metastatic BC [57, 164, 166-169]. It has also recently been shown that adding pertuzumab to adjuvant trastuzumab and chemotherapy results in better outcomes among patients with HER2-positive early BC [175]. As pertuzumab has only been introduced for a short period, there is not enough data and research regarding the resistance. However, the persistent high death toll and lower response rate among patients previously treated with trastuzumab suggests the presence of resistance and poses challenges [176, 177].

1.8. Other Anti-HER2 Strategies

In addition to trastuzumab and pertuzumab discussed above, many other agents and strategies have also been developed to target HER2. We have discussed the combination of targeting HER2 and PI3K-Akt-mTor above. The other agents and strategies developed simultaneously include the anti-HER2 agents that target the signaling pathways downstream of HER2 include the tyrosine kinase inhibitors (TKIs) such as neratinib and lapatinib, and the antibody-drug conjugate trastuzumab emtansine (T-DM1) [166, 169, 178, 179]. The recent trends and development in this field include the immunotherapy agents such as anti-PD-L1 agent pembrolizumab [180], the bispecific antibodies such as MCLA-128, which targets both HER2 and HER3 [181], and ZW25, which targets different epitopes on the HER2 extracellular domain, the antibody-drug conjugates (ADCs) such as SYD-0985 and DS-8201a, and new anti-HER2 antibodies and pan-HER TKIs [177]. These new agents and novel strategies have resulted in a multitude of opportunities to capitalize on the biology of HER2-positive BC and ultimately improve responses to HER2-targeted therapy.
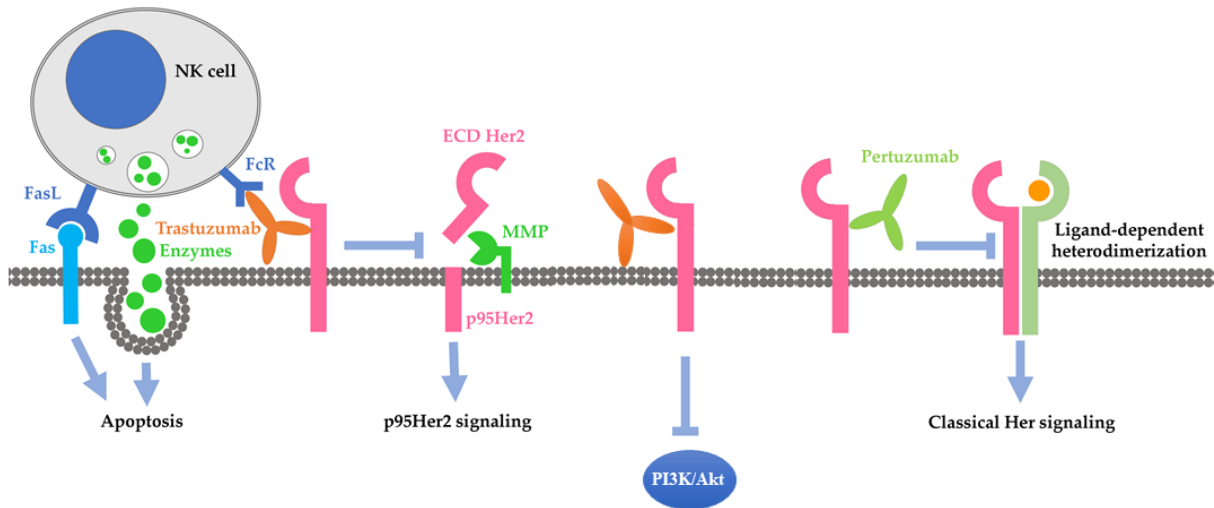
Figure 1.2. A model summarizing mode of action of trastuzumab and pertuzumab. Pertuzumab acts to inhibit the classical HER2-mediated cell-signaling cascades by blocking HER2 dimerization. Trastuzumab acts through ADCC and the inhibition of HER2 cleavage. Fas: Fas Cell Surface Death Receptor, FasL: Fas ligand, FcR: Fragment Crystallizable Receptor, ECD: Extracellular domain, MMP: Matrix metalloproteinase, PI3K: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Akt: Protein kinase B

1.9. Lipid raft and its role in cancer

The structure of the cell membrane is not homogeneous and the lipids are distributed unevenly throughout the membrane. It has been shown that some parts of membrane are highly dynamic and enriched with sphingolipids and cholesterol [182]. These regions form specific microdomains throughout the membrane which are known as lipid rafts. According to the formal definition suggested by Keystone Symposium on Lipid Rafts and Cell Function in 2006 "membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions" [183].

The lipid rafts can regulate downstream signaling pathways, and therefore cellular functions by recruiting or excluding certain proteins to the lipid rafts through mostly unknown mechanism(s) [184, 185]. These membrane microdomains provide efficient signal transduction by concentrating signal proteins in specific regions of membrane and facilitating the signal proteins interactions [186]. Caveolin-1 and flotillins are two main scaffolding proteins which exist in lipid rafts and play an important role in lipid raft formation and lipid raft-mediated signal transduction [187-189]. It has been shown that Caveolin-1 scaffolding domain can interact with several signal proteins including Src family protein tyrosine kinases (SFKs) and protein phosphatase 1 (PP1) in the lipid rafts [187, 190]. The interaction of these scaffolding domains with signal proteins can regulate the organization of signaling complexes which ultimately activate or inhibit certain signaling pathways.

The significant roles of lipid rafts in regulating cancer-related signaling pathways have been demonstrated by various studies. Li *et al*. have shown that cancer cells have more lipid rafts in comparison to normal cells and disruption of these regions in cell membrane can significantly reduce cancer cell viability through inducing apoptosis and inhibition of survival signaling pathway [191].

Insulin-like growth factor-1 (IGF-1) belongs to IGF system which can bind to IGF-1 receptors (IGF-1R) at the cell surface. The IGF system can activate several signaling pathways involved in cancer cell growth, survival, and metastasis [192-194]. It has been revealed that presence of lipid rafts in cell membrane is essential for the activation of IGF-1R-mediated downstream signaling

pathways [195]. Several studies have shown that the Akt phosphorylation modulated by IGF-1R is regulated by lipid rafts [196-198].

Moreover, it has been demonstrated that lipid raft plays a crucial role in activating PI3K/Akt pathway via other tyrosine kinase receptors and disruption of lipid rafts using specific small molecules like methyl-β-cyclodextrin (MβCD) can significantly inhibit Akt phosphorylation [199-201]. Reis-Sobreiro *et al*. have shown that phosphorylated Akt, phosphorylated PDK1, and mTOR is mainly localized in lipid rafts of mantle cell lymphoma (MCL) cells in comparison to non-rafts regions of cell membrane which suggest that lipid rafts regulate PI3K/Akt pathway by providing a specific platform for close interactions of the proteins involved in this pathway [199]. The results of this paper also showed that alteration of the lipid rafts composition using edelfosine as a synthetic antitumor lipid can suppress Akt phosphorylation by displacing the main components of PI3K/Akt signaling pathway from lipid rafts to the non-rafts region [199]. Autophosphorylation of focal adhesion kinase (FAK) at tyrosine 397 phosphorylation site can induce translocation of FAK to the lipid rafts. In the lipid rafts, FAK proteins are phosphorylated at tyrosine 861 and 925 after interaction with Fyn proteins as a member of SFKs and activate PI3K/Akt signaling pathway [202]. Moreover, Staubach *et al.* have indicated that certain heat-shock proteins (Hsps) which have significant role in cancer-associated signaling pathways are specifically localized to the lipid raft microdomains of cancer cells membrane [203-208].

Taken together, the findings of several studies show that lipid rafts play a crucial role in regulation and activation of various signaling pathways involved in cancer cells growth, survival, metastasis, and angiogenesis.

1.10. References

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Chapter 2. Rationales and aims

Trastuzumab (Herceptin), the first HER2-targeted therapy for the treatment of HER2-positive BC patients, was approved by FDA in 1998 [1, 2]. As a humanized monoclonal antibody, trastuzumab was designed to target the extracellular domain of HER2 [3]. Although several mechanisms have been suggested for the mode of action of trastuzumab the findings regarding these mechanisms are unclear and controversial. Previous studies have indicated that trastuzumab can exert its anticancer effects through inducing HER2 degradation [4] and MAPK and PI3K/Akt signaling pathways repression [5]. In addition, it has been shown that trastuzumab can increase the immune system activity against the cancerous cells through antibody-dependent cellular cytotoxicity (ADCC) [6, 7]. Although trastuzumab has opened a new window toward the treatment of patients with HER2-positive BC and even other types of cancer, some patients do not respond or become resistant to this treatment [8]. Activating and inactivating mutations in the p110α subunit of PI3K and phosphatase and tensin homolog (PTEN) respectively, allow continuous activation of PI3K/Akt signaling pathway and make the cells resistant to trastuzumab [9-11]. In addition, some HER2-positive BC cells can truncate the HER2 receptors to prevent trastuzumab binding [8, 12, 13]. Furthermore, overexpression of other tyrosine kinase receptors can compensate for any HER2 modulated downstream signaling pathway which is blocked by trastuzumab [14, 15]. In recent years, several new chemotherapeutic and targeted-therapeutic drugs have been introduced to increase the efficacy of targeted therapy with trastuzumab [16]. Pertuzumab, a humanized recombinant monoclonal antibody, has been selected specifically to block both homodimerization and heterodimerization of HER2 through sterically blocking a binding pocket necessary for receptor dimerization [17, 18]. However, only a few studies have shown that pertuzumab blocked the association of HER2 and HER3, diminished ligand-activated HER2 signaling including Erk activation, and inhibited the growth of human BC cell lines only in the presence of ligand (HRG) [19, 20]. Moreover, the NEOSPHERE clinical trial results showed that, the rate of complete pathological remission (pCR) for HER2 positive BC patients treated with the combination of trastuzumab and docetaxel was higher than pCR for patients treated with pertuzumab and docetaxel [21]. To unravel the mechanism of action of trastuzumab and pertuzumab, we designed two experimental aims.

2.1. Aim 1: Determine the effects of trastuzumab and pertuzumab on HER2 homodimerization and phosphorylation.

A significant proportion of HER2-positive BC cells co-express EGFR and HER3 [22]. HER2-mediated cell signaling, and cell function is closely related to and impacted by other HER receptors, including EGFR and HER3 [23]. It is not clear how the formation of various HER homo- and heterodimers impact the efficacy of trastuzumab and pertuzumab in treating BC. Although several studies have investigated the effect of trastuzumab and pertuzumab on HER2 heterodimerization and HER2 heterodimer-mediated downstream signaling, no study has used cell models lacking endogenous HER expression to unravel the effects of these drugs on HER2 homodimerization and phosphorylation in the absence of other members of the HER family. To have a better understanding about the effects of trastuzumab and pertuzumab on HER2-mediated cell signaling in various types of BC cells, we studied the effects of these antibodies in CHO cells stably expressing different HER receptors. Three different types of CHO stable cell lines have been used in this study: 1) CHO cells stably expressing HER2 receptors (CHO-K6); 2) CHO cells stably expressing EGFR receptors (CHO-EGFR); 3) CHO cells stably expressing HER3 receptors (CHO-HER3). The cells were treated with different concentrations of trastuzumab and pertuzumab including 0.1, 1, and 10 µg/ml for 1 hour and 10 µg/ml concentration was chosen to evaluate a time course of HER2 activities. The binding of trastuzumab and pertuzumab to these receptors and the effect of these drugs on HER2 homodimerization and HER2 phosphorylation have been evaluated by immunofluorescence and immunoblotting.

2.2. Aim 2: Determine the effects of trastuzumab on HER2 dimerization, phosphorylation, and HER2-mediated cell signaling in HER2-positive BC cells

The effect of trastuzumab on HER2-regulated downstream signaling pathways has been extensively investigated; however, the results are controversial. It has been shown that trastuzumab is able to inhibit Akt phosphorylation through a not well-established mechanism [24-27]. Several studies have suggested that trastuzumab inhibits phosphorylation of HER [24, 26]. On the other hand, other studies showed that trastuzumab can increase HER2 and Erk phosphorylation [28,29]. Although several studies have shown that trastuzumab can inhibit the phosphorylation of Akt, few studies have investigated the exact mechanism through which trastuzumab inhibits Akt phosphorylation. In addition, it is not clear how Akt phosphorylation is decreased after increasing

of HER2 phosphorylation by trastuzumab. Moreover, few studies have thoroughly evaluated the effect of trastuzumab on ligand-dependent and ligand-independent HER2 dimerization, phosphorylation, and HER2-mediated downstream signaling.

To determine the effects of trastuzumab on HER2 heterodimerization, phosphorylation, and HER2-mediated downstream signaling, I treated the HER2-positive BC cells with trastuzumab in presence and absence of EGF and HRGα ligands. First, I studied the effects of trastuzumab on BC cells viability and cell cycle progression. Next, the heterodimerization and phosphorylation of HER2 receptors were examined by immunoprecipitation (IP) followed by immunoblotting, and by immunoblotting, respectively. Finally, I determined the effects of trastuzumab on the activation of major signaling pathways regulated by HER2 including PI3K/Akt and MAPK.

2.3. References

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Chapter 3. Materials and methods

3.1. Buffers and solutions

Table 1. The name and composition of buffers and solutions used in this project.

Buffer or solution name	Composition
1X Phosphate-Buffered Saline (PBS)	The contents of one pouch of "Thermo Scientific BupH Phosphate Buffered Saline Packs" were dissolved in 500 ml distilled water to obtain PBS solution with 0.1M sodium phosphate and 0.15M sodium chloride, pH 7.2
Protein extraction solution	The "Thermo Scientific M-PER Mammalian Protein Extraction Reagent" was used to purify the nuclear and cytoplasmic proteins from the cultured cells
4X Laemmli buffer (protein loading buffer)	4.4 ml 0.5 M Tris Base (pH 6.8) was added to 4.4 ml glycerol, 2.2 ml 20% Sodium Dodecyl Sulfate (SDS) solution, 0.5 ml 1% Bromophenol Blue, and 0.5 ml 2-mercaptoethanol
5% separating SDS-PAGE gel	In 15 ml tube 0.625 ml acrylamide/bis-acrylamide 40% solution was added to 3.125 ml double-distilled water (ddH2O), 1.25 ml 1.5 M Tris-HCl buffer with pH 8.8 (lower gel buffer), 25 µl ammonium persulfate (APS), and 2.5 µl tetramethylethylenediamine (TEMED)
8% separating SDS-PAGE gel	In 15 ml tube 0.94 ml acrylamide/bis-acrylamide 40% solution was added to 2.8 ml ddH2O, 1.25 ml 1.5 M Tris-HCl buffer with pH 8.8 (lower gel buffer), 25 µl APS, and 2.5 µl TEMED
4% stacking SDS-PAGE gel	In 15 ml tube 250 µl acrylamide/bis-acrylamide 40% solution was added to 1.6 ml ddH2O, 625 µl 0.5 M Tris-HCl buffer with pH 6.8 (higher gel buffer), 25 µl 10% SDS solution, 12.5 µl APS, and 2.5 µl TEMED
Protein running buffer	10X Tris-Glycine-SDS buffer solution from Bio Basic Canada Inc. was diluted to 1X buffer solution which contains 0.025 M Tris Base, 0.192 M Glycin, and 0.1% SDS
Protein transfer buffer	To prepare 500 ml transfer buffer, 400 ml ddH2O was added to 2.91 g Tris (48 mM), 1.47 g glycine (39 mM), 1.88 ml 10% SDS solution and 100 ml methanol
1X Tris-buffered saline (TBS)	475 ml ddH2O was added to 25 ml 1 M Tris Base solution (pH 7.5), 4.4 g NaCl
1X Tris-buffered saline Tween 20 (TBST)	250 µl Tween 20 was added to 500 ml 1X TBS
Nitrocellulose blocking buffer	2.5 g BSA and 1 g skimmed milk was dissolved in 50 ml or 30 ml TBS respectively

3.2. Cell lines and culture

Chinese Hamster Ovary (CHO), SKBR3, and BT474 cell lines were purchased from ATCC (Manassas, VA, USA). CHO-K6 cells (stably overexpressing human HER2) [1], CHO-K13 (stably expressing human HER2) [1], and CHO-HER3 (stably expressing human HER3) [2] were obtained as gifts from Drs. Hitt and Buchholz labs (University of Alberta, Edmonton, AB, Canada). CHO-EGFR (stably expressing human EGFR) was previously generated [3]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained at 37ºC in a 5% CO2 atmosphere. Transgenic selection was maintained by adding of G418 (200 µg/mL) for CHO-K6, CHO-K13 and CHO-EGFR, and hygromycin (200 µg/mL) for CHO-HER3 to the culture medium. The cells were starved overnight at DMEM or DMEM containing 1% FBS before the treatments.

3.3. Chemicals and Antibodies

HER2 kinase inhibitor CP-724714 was purchased from Selleckchem (Houston, TX, USA). Pertuzumab (Perjeta®) and trastuzumab (Hercepton®) were purchased from Hoffmann-La Roche (Basel, Switzerland). Mouse monoclonal anti-human HER2 (9G6) (sc-08), anti-human HER2 (A-2) (sc-393712), anti-human EGFR (A-10) (sc-373746), anti-human HER3 (RTJ.2) (sc-415), α-tubulin, (B-7), Akt1/2 (H-136), Erk1/2 (k-23), pT202/Y204 Erk1/2, c-Src (N-16), p-c-Src (Thr 420)-R and rabbit polyclonal anti-human pY1248 HER2 (sc-12352-R) antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Rabbit polyclonal anti-human pY1005, pY1112, pY1127, pY1139, and pY1196 HER2 were purchased from FroggaBio (Toronto, ON, Canada). Mouse monoclonal anti-human pY1221/1222 HER2 (6B12), rabbit monoclonal phospho-Akt (Ser473), and rabbit monoclonal phospho-Akt (Thr308) were from Cell Signaling Technology (Danvers, MA, USA). Fluorescein isothiocyanate (FITC)- and Tetramethylrhodamine (TRITC)-conjugated donkey anti-mouse and anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Anti-rabbit and anti-mouse RDye® 800CW and RDye® 650 secondary antibodies were purchased from LI-COR biotechnology Inc. (Lincoln, NE, USA). Isotype control human IgG, Paclitaxel, Vinorelbine, recombinant human EGF, protein A conjugated with agarose, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.4. Western Blotting

Total protein was extracted as described previously [3]. Protein samples were prepared by boiling in half volume of 4X Laemmli buffer for 5 min. Twenty micrograms of total protein was run in 8% polyacrylamide gel by vertical electrophoresis at 100 V electric potential for 100 min, and then transferred on nitrocellulose membrane at 15 V electric potential for 90 min using a semi-dry protein transfer system (Bio-Rad Laboratories, Berkeley, CA, USA). The membranes were blocked by incubation in Odyssey® Blocking Buffer (LI-COR biotechnology Inc., Lincoln, NE, USA) or skimmed milk for 60 min and then were incubated overnight in primary antibody solution. After washing with Tris-buffered saline (TBS) containing 0.05% tween-20, the membranes were incubated in 25 ng/mL RDye® or 0.2 µg/ml HRP-conjugated secondary antibody solution for 60 min. The membranes incubated with RDye® were visualized by using Odyssey® CLx imaging system (LI-COR biotechnology Inc., Lincoln, NE, USA) after washing with TBS-T buffer. The membranes incubated with HRP-conjugated secondary antibody were washed and incubated with enhanced chemiluminescence (ECL) solution (Thermo Fisher Scientific) for 5 minutes and then visualized using X-ray films. Query protein bands intensity were quantified and normalized to intensity of relevant loading control protein bands.

3.5. Immunofluorescence Assay

The indirect double-immunofluorescence staining was done as described previously [4]. A number of 10^5 cells were seeded on 15 mm round cover glass in 24 well-plates and were cultured in standard culture condition for 48 h and then were starved overnight in 1% FBS culture medium. After treatment, the coverslips were washed with ice-cold PBS and the cells were fixed by incubation in -20ºC methanol for 5 min. Then, the coverslips were washed with TBS and were blocked in 1% bovine serum albumin (BSA) solution in TBS for 60 min. After blocking, the coverslips (except those treated with isotype human IgG, pertuzumab and trastuzumab) were incubated in 2 µg/mL of primary antibody for 60 min. The coverslips were washed and then were incubated in 1 µg/mL FITC-conjugated (for anti-HER2 or anti-EGFR or anti-HER3 antibodies) and/or 1 µg/mL rhodamine-conjugated (for pertuzumab or trastuzumab) secondary antibodies solutions for 60 min in dark. Afterwards, the coverslips were washed with TBS and then were incubated in 1 µg/mL DAPI solution for 5 min. The coverslips were mounted on microscope slides and were observed under a fluorescence microscope.

3.6. Cross-linking assay

The cross-linking assay was done as described previously [5]. Cross-linking assay was employed to determine the dimerization of the receptors. Cells were cultured to subconfluency in 60 mm dishes. Following the treatment with EGF, trastuzumab, pertuzumab, and normal human IgG of indicated concentrations for 1 h at 37 °C, the cells were collected and suspended in 0.2~ 0.5 ml PBS. BS^3 [bis(sulfosuccinimidyl)suberate] was then added to a final concentration of 1.0~ 2.5 mM. The cross-linking reaction was conducted in 4°C for 2 h. To stop the reaction the quench solution (1 M Tris, pH 7.5, 1:100 dilution) was added and incubated for 15 min on ice. The final concentration of the quench solution was 10 mM. Afterwards, the cells were lysed with 1% NP-40 on ice for 1 h. The dimerization was analyzed by SDS-PAGE followed by immunoblotting. A gel of 5% was run to better separate the dimers from the monomers.

3.7. MTT Proliferation Assay

Cells were seeded in 96-well plates and cultured in 200 μL DMEM medium containing 10% FBS. After overnight culture the cells were treated in fresh DMEM or DMEM containing 1% FBS for 72 h. Cell proliferation was determined by MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) assay Vybrant MTT Cell Proliferation Assay Kit from Invitrogen (Grand Island, NY). Briefly, 10 μL of 12 mM MTT solution was added to the wells and then the plates were incubated at 37°C for 4 h until the formazan crystals form. Afterwards, the solution was removed and replaced with 50-100 μL dimethyl sulfoxide (DMSO). The plates then were incubated at 37°C for 10 min. The color intensity was measured at 540 nm wavelength using a microplate reader. The absorbance values were normalized to those of blank wells.

3.8. Antibody-dependent cellular cytotoxicity (ADCC)

ADCC of trastuzumab in CHO cells expressing HER2 or EGFR was determined by using Promega ADCC Bioassay kit according to Manufacturer's instruction. Generally, immune effector cells including NKs cells can detect the Fc portion of antibody and bind it through FcγRIIIa receptors. Upon binding of immune effector cells to antibody, several downstream signaling including nuclear factor of activated T-cells (NFAT) pathway are activated in immune cells to mediate ADCC which ultimately kill the target cell. The NFAT pathway modulate the expression of genes

involved in immune response against antibody-bound target cells. The expression of these genes is controlled by a promoter containing NFAT response elements. To assess this process *in vitro* models, Jurkat cells have been transfected with FcγRIIIa receptors and a gene containing firefly luciferase under the control of NFAT response elements. Upon the binding of FcγRIIIa receptors to the Fc portion of antibody, NFAT signaling pathway can be activated in these cells and induce the expression of luciferase. Finally, the luciferase activity can be measured using microplate reader as an indicator of ADCC activation. Cultured cells were plated at a density of 15,000 cells per well in complete culture medium overnight before the bioassay. On the day of the bioassay, the series of concentrations of trastuzumab were added to the cells, followed by addition of ADCC Bioassay Effector Cells. The E:T (Effector: Target) ratio was 5:1. After 6 h of induction at 37°C, Bio-Glo™ Luciferase Assay Reagent was added and luminescence was determined.

3.9. Cell cycle analysis

Cells were seeded in 6-cm plates in DMEM medium containing 10% FBS. After overnight culture the cells were treated in fresh DMEM for 72 h and then cell cycle was analyzed by flow cytometry. Briefly, the harvested cells were fixed in cold methanol for 20 minutes. The fixed cells were treated with 0.1% (v/v) Triton X-100 and 100 µg/mL RNase A in 1 ml PBS. The cells were centrifuged and resuspended in 1 ml PBS containing 0.1% (v/v) Triton X-100 and 15 µg/mL propidium iodide (PI) staining solution. The samples were kept in dark for 30 minutes and DNA contents were measured with BD FACSCanto™ II. Finally, the results were analyzed with FlowJo software.

3.10. Immunoprecipitation

Immunoprecipitation experiments were carried out as described previously [6]. IP buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 100 mM NaF, 5 mM MgCl2, 0.5 mM Na3VO4, 0.02% NaN3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 mg/ml aprotinin, and 1 mM pepstatin A] were used to lysis the cells. The cell lysates were centrifuged, and the supernatants were incubated with 1 µg of primary antibody at 4°C. After overnight incubation the protein A conjugated with agarose was added to samples and incubated at fridge for 2 h. The samples were boiled in 2X Laemmli buffer for 5 min and analyzed by SDS-PAGE followed by western blotting.

3.11. Lipid rafts associated proteins isolation

The protein present in lipid rafts were isolated from BT474 cells by Bio-Rad ReadyPrep™ Protein Extraction Kit (Signal) kit according to the instruction provided by manufacture. Generally, due to the presence of high amount of cholesterol and sphingolipids in the lipid rafts, these regions of the cell membrane are insoluble in nonionic detergents. Therefore, treating the cells with nonionic detergents provided by the kit will solubilize hydrophilic regions of the cells and leave the lipid rafts microdomains and its associated proteins insoluble. This insoluble fraction can be solubilized in a specific buffer named protein solubilization buffer (PSB) provided by the company.

3.12. Statistical analysis

Blot band intensity was quantified by ImageJ software and normalized to tubulin level or total expression of protein of interest. Data were statistically analyzed by one-way analysis of variance (ANOVA) using Prism software (GraphPad Software, La Jolla, CA, USA). Data was presented as mean and standard deviation. $p < 0.05$ was considered as statistically significant.

3.13. References

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Chapter 4. The effects of trastuzumab on HER2-mediated cell signaling in CHO cells expressing human HER2

4.1. Abstract

Targeted therapy with trastuzumab has become a mainstay for HER2-positive BC without a clear understanding of the mechanism of its action. While many mechanisms have been suggested for the action of trastuzumab, most of them are not substantiated by experimental data. It has been suggested that trastuzumab functions by inhibiting intracellular signaling initiated by HER2, however, the data are very controversial. A major issue is the different cellular background of various BC cells lines used in these studies. Each BC cell line has a unique expression profile of various HER receptors, which could significantly affect the effects of trastuzumab. To overcome this problem, in this research we adopted a cell model that allow us to specifically examine the effects of trastuzumab on a single HER receptor without the influence of other HER receptors. Three CHO cell lines stably expressing only human EGFR (CHO-EGFR), HER2 (CHO-K6), or HER3 (CHO-HER3) were used. Various methods including cytotoxicity assay, immunoblotting, indirect immunofluorescence, cross linking, and antibody-dependent cellular cytotoxicity (ADCC) were employed in this research. We showed that trastuzumab did not bind EGFR and HER3, and thus did not affect the homodimerization and phosphorylation of EGFR and HER3. However, overexpression of HER2 in CHO cells, in the absence of other HER receptors, resulted in the homodimerization of HER2 and the phosphorylation of HER2 at all major potential pY residues. Trastuzumab bound to HER2 specifically and with high affinity. Trastuzumab inhibited neither the homodimerization of HER2, nor the phosphorylation of HER2 at most potential phosphotyrosine sites. Moreover, trastuzumab did not inhibit the phosphorylation of Erk and Akt in CHO-K6 cells and did not inhibit the proliferation of CHO-K6 cells. However, trastuzumab induced strong ADCC in CHO-K6 cells. We concluded that, in the absence of other HER receptors, trastuzumab exerts its antitumor activity through the induction of ADCC, rather than the inhibition of HER2-homodimerization and phosphorylation.

Keywords: HER receptors, EGFR, HER2, HER3, Trastuzumab, Dimerization, Phosphorylation, ADCC, CHO cells

4.2. Introduction

The most controversial mechanism regarding trastuzumab function is its effect on the inhibition of HER2 activation. A major reason behind this controversy is the different cellular background of various BC cells lines used in those studies. Each BC cell line has a unique expression profile of various HER receptors, which could significantly affect the effects of trastuzumab. To overcome this problem, in this research we adopted a cell model that allow us to specifically examine the effects of trastuzumab on a single HER receptor without the influence of other HER receptors. We aimed to conclusively determine if trastuzumab specifically binds only to HER2, and blocks HER2 homodimerization and activation. To achieve our objective, we adopted a CHO cell model. The expression of ErbB tyrosine kinase receptors in CHO cells is well reviewed in paper published by Hosseinzadeh Colagar *et al.* [1]. It has been shown that CHO cells do not, or less, express HER receptors. Three stable CHO cell lines that stably express only a single HER receptor including EGFR (CHO-EGFR), HER2 (CHO-K6), and HER3 (CHO-ErbB3) were employed in this research. We demonstrate that overexpression of HER2 in CHO cells resulted in the homodimerization of HER2 and the phosphorylation of HER2 at all major potential pY residues. Trastuzumab bound to HER2 specifically and with high affinity. Trastuzumab neither inhibited the homodimerization of HER2, nor inhibited the phosphorylation of HER2 at most potential phosphotyrosine sites. Moreover, trastuzumab did not inhibit the phosphorylation of Erk and Akt in CHO-K6 cells and did not inhibit the proliferation of CHO-K6 cells. However, trastuzumab induced strong ADCC in CHO cells overexpressing HER2.

4.3. Results

4.3.1. Stable CHO cell lines expressing EGFR, HER2 and HER3

HER2 heterodimerizes with EGFR and HER3 in response to ligand stimulation [2-4]. HER2 also homodimerizes and activates in cells with over-expressed HER2 [3, 5, 6]. Most HER2-positive BC cells also express either EGFR, HER3 or both, which makes it difficult to explain the observed effects of trastuzumab. Thus, to understand the effects of trastuzumab on HER2-mediated cell signaling in BC cells, we plan first to study the effects of trastuzumab in CHO cells that selectively express a single HER receptor. The results from these CHO cells will unambiguously define the role of trastuzumab on HER2-mediated cell signaling under various expression profiles of HER

receptors. Thus, these data could be used to accurately interpret observation in BC cells. We have established CHO cell lines stably expressing EGFR (CHO-EGFR) [7]. CHO cells expressing HER2 (CHO-K6) or HER3 (CHO-HER3) were obtained from other labs [8, 9]. Parental CHO cells are used as control. We confirmed the expression of HER receptors in these cell lines by immunoblotting and immunofluorescence. As shown in **Figure 4.1**, CHO-K6 cells expressed high level of HER2. CHO-EGFR cells expressed high level of EGFR. CHO-HER3 cells expressed high level of HER3 and the parental CHO cells did not express detectable HER2, EGFR and HER3.
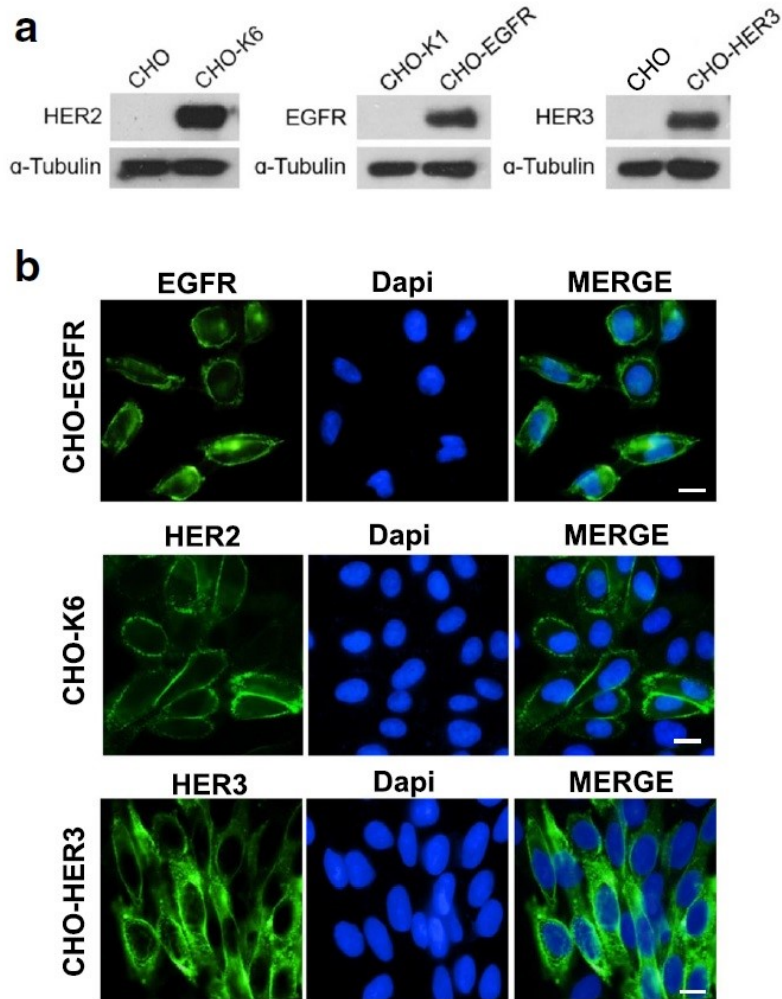
Figure 4.1. The expression of HER receptors in CHO cells stably transfected with a single HER receptor including CHO-EGFR, CHO-K6, and CHO-HER3. **(a)** Immunoblotting. The lysates of various CHO cells were separated by gel electrophoresis and immunoblotted with antibodies to HER receptors as indicated. The parent CHO cells (CHO) were used as control. **(b)** Immunofluorescence. Various CHO cells were fixed and stained with antibodies to HER receptors as indicated. The expression of HER receptor was revealed by FITC-conjugated secondary antibody (Green). Cell nuclei were counterstained with DAPI. Size bar: 10 μm (This experiment has been carried out by Babak Nami)

4.3.2. Binding of trastuzumab to HER receptors

While trastuzumab is an antibody to HER2, it is possible that it may weakly interact with EGFR and HER3 due the sequence homology among these receptors. Thus, we next examined the binding of trastuzumab to HER2, EGFR and HER3. We showed by immunofluorescence that trastuzumab only bound to HER2, but not EGFR and HER3 **(Figure 4.2)**. As shown in **Figure 4.2**, at the dosage ranging from 0.1 μg/ml to 10 μg/ml, trastuzumab showed strong binding to HER2 in CHO-K6 cells. HER2 was localized to the plasma membrane (PM) in CHO-K6 cells under all conditions as expected. Trastuzumab was also located to PM, co-localizing with HER2, which indicates the binding of trastuzumab to HER2 **(Figure 4.2a)**. PM localization of trastuzumab was increased with the increased dosage. We also determined the time course of trastuzumab binding to HER2 in CHO-K6 cells. As shown in **Figure 4.2b**, at 5 min following trastuzumab addition, trastuzumab had already been well localized to the PM, indicating a rapid binding between trastuzumab and HER2. Longer incubation only slightly increased the PM localization of trastuzumab. However, even at the high dosage of 10 μg/ml, no binding of trastuzumab to EGFR and HER3 was detectable in CHO-EGFR and CHO-HER3 cells, respectively **(Figures 4.2c & d)**. These results indicate that trastuzumab binds to HER2 specifically with high affinity.
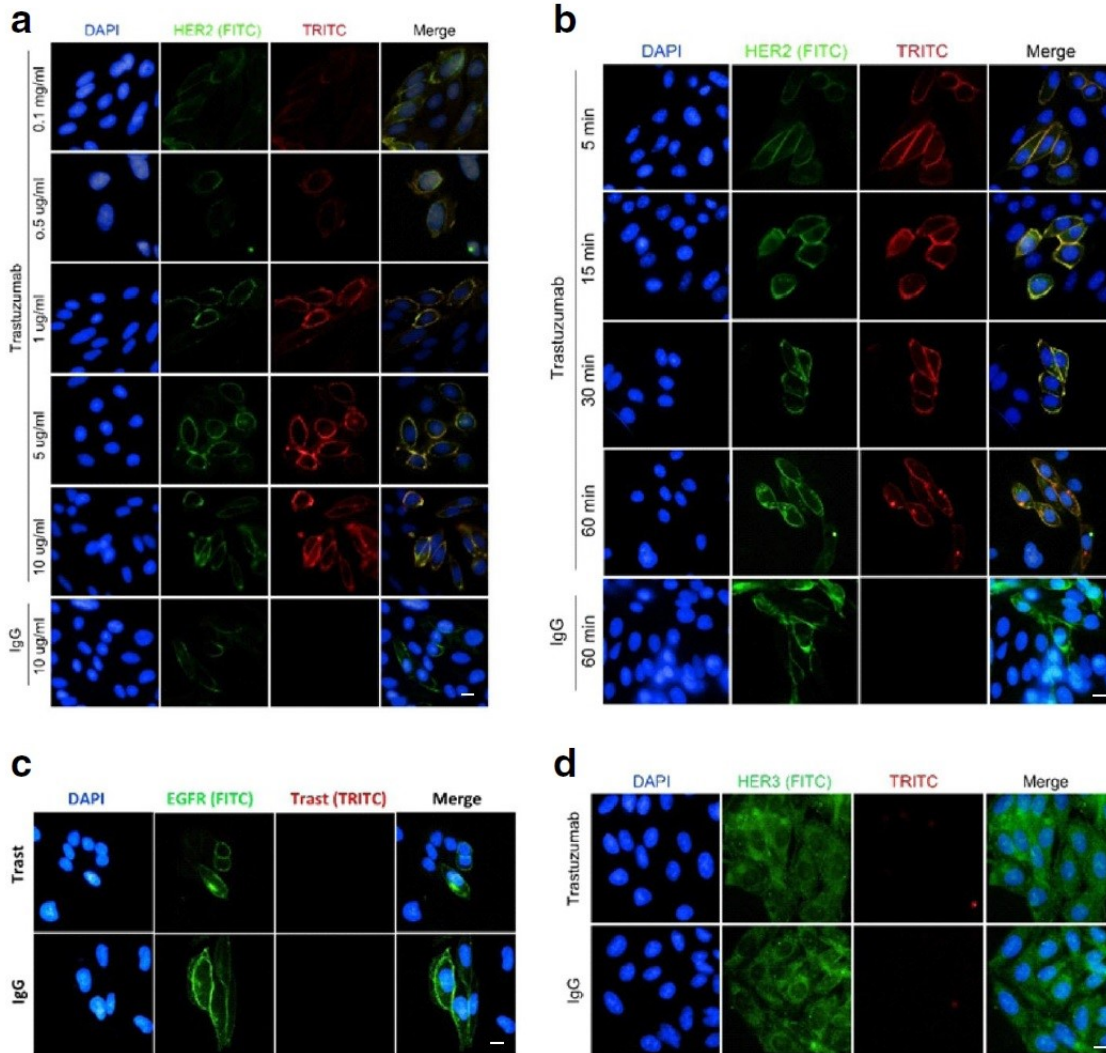
Figure 4.2. Binding of trastuzumab to HER receptors in CHO-K6, CHO-EGFR and CHO-HER3 cells as revealed by immunofluorescence. (**a**) and (**b**) The binding of trastuzumab to HER2 in CHO-K6 cells. CHO cells were treated with trastuzumab at various concentrations for 1 h (**a**) or at various time period at 10 μg/ml (**b**) as indicated. The membrane localization (binding) of trastuzumab was revealed by TRITC-conjugated donkey anti-human IgG. The localization of HER2 was revealed by rabbit anti-HER2 antibody followed by FITC-conjugated donkey anti-rabbit IgG. The cell nuclei were counter stained with DAPI. Yellow indicated the co-localization of trastuzumab and HER2. **(c)** The binding of trastuzumab to EGFR in CHO-EGFR cells. The membrane localization (binding) of trastuzumab was revealed by TRITC-conjugated donkey anti-human IgG. The localization of EGFR was revealed by rabbit anti-EGFR antibody followed by FITC-conjugated donkey anti-rabbit IgG. The cell nuclei were counter stained with DAPI. **(d)** The binding of trastuzumab to HER3 in CHO-HER3 cells. The membrane localization (binding) of trastuzumab was revealed by TRITC-conjugated donkey anti-human IgG. The localization of HER3 was revealed by rabbit anti-HER3 antibody followed by FITC-conjugated donkey anti-rabbit IgG. The cell nuclei were counter stained with DAPI. Size bar: 10 μm (This experiment has been carried out by Babak Nami)

4.3.3. The effects of trastuzumab on the homodimerization of HER2

So far, the reports are controversial regarding the effects of trastuzumab on the dimerization or HER2. Here we examined the effects of trastuzumab on the homodimerization of HER2 by crosslinking and immunoblotting **(Figure 4.3)**. As shown in **Figure 4.3**, overexpression of HER2 by itself resulted in high level of HER2 homodimerization. Clearly trastuzumab did not block the homodimerization of HER2. Interestingly, with the increase of the dosage from 0.1 µg/ml to 10 µg/ml, trastuzumab slightly induced the dimerization of HER2 **(Figure 4.3a)**. The induction of homodimerization of HER2 by trastuzumab was even more visible in the time course experiments **(Figure 4.3b)**.
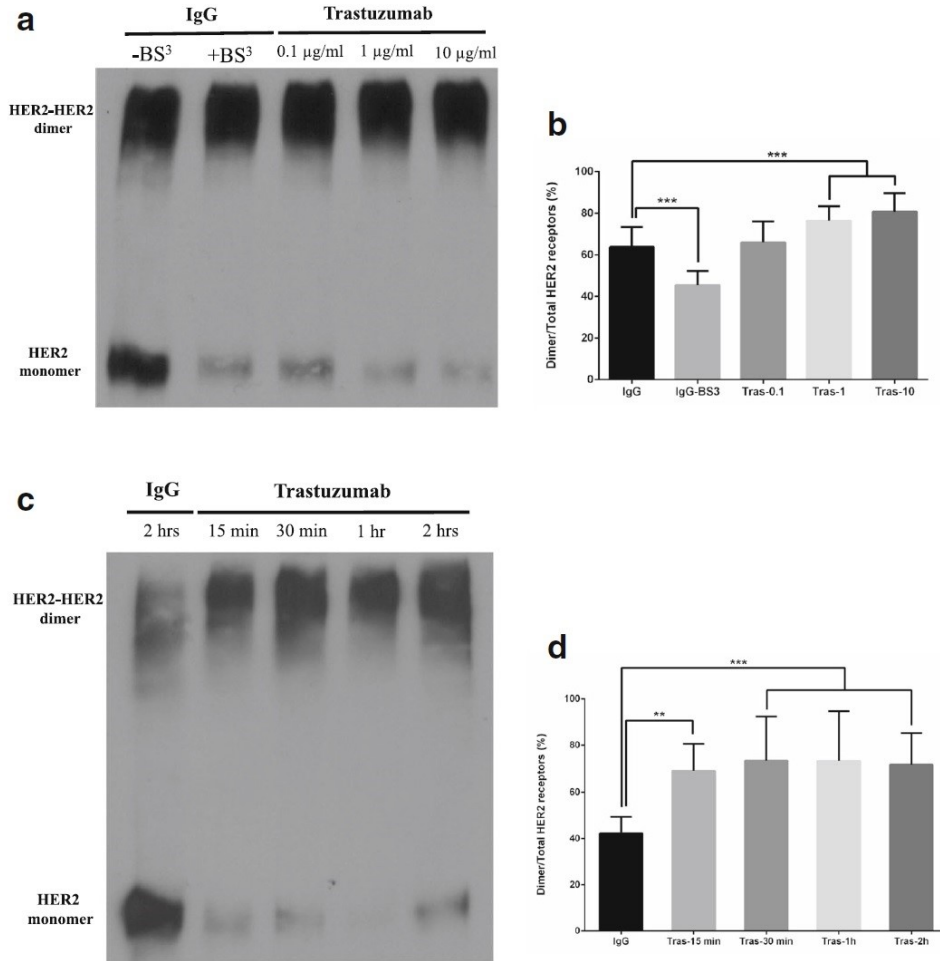
Figure 4.3. The effects of trastuzumab on HER2 homodimerization in CHO-K6 cells as revealed by crosslinking. Following trastuzumab treatment as indicated, CHO-K6 cells were treated with BS3 and the homodimerization of HER2 was revealed by immunoblotting. **(a)** CHO-K6 cells were treated with trastuzumab at various concentrations ranging from 0.1–10 µg/ml for 1 h. **(b)** Quantification of the data in **(a)**. **(c)** CHO-K6 cells were treated with 10 µg/ml trastuzumab for various time period as indicated. Cells treated with normal human IgG were used as control. **(d)** Quantification of the data in **(b)**. The level of HER2 homodimerization were quantitated by densitometry and expressed as the ratio of dimer/total HER2. Each value is the average of at least three experiments and the error bar is standard error. **: $p < 0.01$, ***: $p < 0.001$

4.3.4. The effects of trastuzumab on the phosphorylation of HER2

Activated HER2 phosphorylates multiple tyrosine (Y) residues at its C-terminus. We have examined the phosphorylation of following six tyrosine residues including Y1005, Y1112, Y1127, Y1139, Y1196, and Y1248 **(Figures 4.4, 4.5 and 4.6)**. As shown by immunoblotting, for the control cells treated with normal IgG, HER2 was well phosphorylated in all of the pY residues examined **(Figures 4.4a & b)**. The phosphorylation is likely due to the homodimerization induced by the overexpression of HER2. Treatment with trastuzumab at the dosage ranging from 0.1 µg/ml to 10 µg/ml did not significantly alter the phosphorylation levels of most phosphotyrosine residues including Y1005, Y1127, Y1196, and Y1248. However, trastuzumab partially inhibited the phosphorylation of Y1139. Similar to normal IgG, EGF did not have any effects on the phosphorylation of all the pY residues of HER2, which is not surprising as HER2 does not bind to EGF **(Figures 4.4a & b)**. These results were confirmed by time course experiments **(Figure 4.4c)**. Treatment from 15 min up to 2 h, did not change the phosphorylation levels of all pY residues except for pY1139 that is partially inhibited **(Figure 4.4c)**.

As controls, we have examined the effects of trastuzumab on EGFR phosphorylation in CHO-EGFR cells. As shown in **Figure 4.5a**, EGFR was not phosphorylated in CHO-EGFR cells, and addition of EGF stimulated the phosphorylation of EGFR. Treatment with trastuzumab was not able to inhibit EGF-induced EGFR phosphorylation. Moreover, trastuzumab by itself did not have any detectable effects on EGFR phosphorylation in CHO-EGFR cells. We also examine the effects of chemical inhibitor of HER2, CP-724714 on HER2 phosphorylation in CHO-K6 cells. As shown in **Figure 4.5b**, various concentrations of CP-724714 ranging from 1 to 100 µM significantly block the phosphorylation of HER2 at Y1005, which is in stark contrast from trastuzumab as shown in **Figure 4.4**. Furthermore, CP-724714 also significantly inhibited the phosphorylation of HER2 pY1139, however, trastuzumab only partially inhibited pY1139. These results indicated that trastuzumab has little, if any, inhibitory effects on HER2 activation/ phosphorylation.

We further examine the effects of trastuzumab on the phosphorylation of HER2 by indirect immunofluorescence **(Figure 4.6)**. CHO-K6 cells either treated with trastuzumab or control IgG were double stained for both trastuzumab (TRITC, red) and phosphor-HER2 (FITC, green). Antibodies specific to six HER2 pY residues including Y1005, Y1112, Y1127, Y1139, Y1196, and Y1248 were used to determine the effects of trastuzumab on HER2 phosphorylation. As shown

in **Figure 4.6**, HER2 was well phosphorylated on all of these six pY residues in the absence of trastuzumab, indicating the autophosphorylation due to overexpression. Treatment with trastuzumab at the concentration ranging from 0.1 μg/ml to 10 μg/ml had no effects on the phosphorylation levels of these HER2 pY residues including Y1005, Y1112, Y1127, Y1196, and Y1248 **(Figures 4.6a, b, c & e)**. However, for pY1139, trastuzumab at 1–10 μg/ml showed some inhibitory effect **(Figure 4.6d)**.

Together, our results indicated that overexpression of HER2 resulted in strong HER2 phosphorylation in all its pY residues studied here. Addition of trastuzumab, in general, did not inhibit the phosphorylation of HER2. The only possible exception is that trastuzumab at higher dosage (1–10 ng/ml) slightly reduced the phosphorylation of HER2 at pY1139.

Figure 4.4. The effects of trastuzumab on HER2 phosphorylation in CHO-K6 cells by immunoblotting. **(a)** CHO-K6 cells were treated with trastuzumab at various concentrations for 1 h. The phosphorylation of HER2 at Y1005, Y1127, Y1139, Y1196, and Y1248 were then examined by immunoblotting. Cells treated with normal human IgG or EGF were used as controls. **(b)** Quantification of the results in **(a)**. The phosphorylation level of each HER2 pY residue was normalized against the expression level of tubulin. Each value is the average of at least three experiments and the error bar is standard error. ******: $p < 0.01$. **(c)** Time course experiments. CHO-K6 cells were treated with trastuzumab (10 μg/ml) for various time as indicated. The phosphorylation of various HER2 pY residues were examined by immunoblotting.
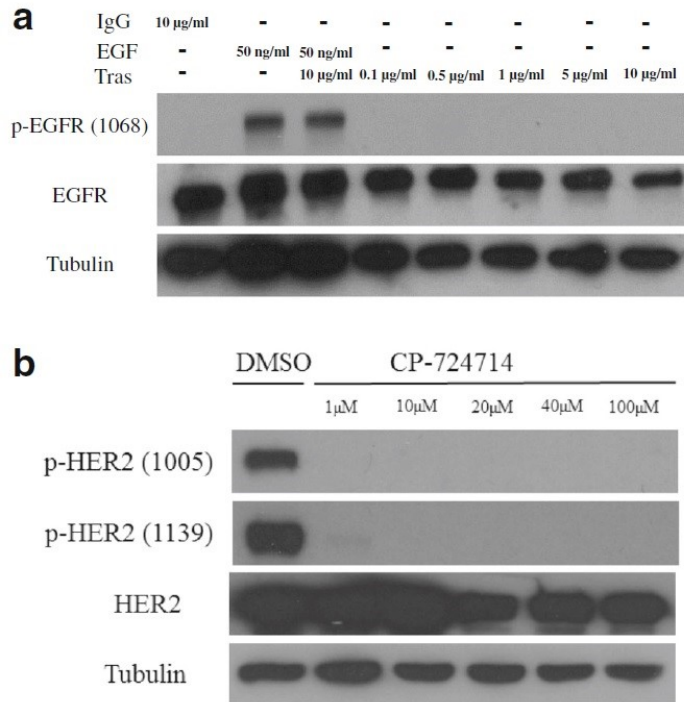
Figure 4.5. Control experiments to show the effects of trastuzumab on EGFR phosphorylation in CHO-EGFR cells and the effects of CP-724714 on HER2 phosphorylation in CHO-K6 cells. **(a)** The effects of trastuzumab on EGFR phosphorylation or EGF-induced EGFR phosphorylation in CHO-EGFR cells. Cells were treated with EGF and/or trastuzumab at various concentrations as indicated. The phosphorylation of EGFR was determined by immunoblotting with antibody to EGFR pY1068. **(b)** The effects of chemical inhibitor of HER2, CP-724714 on HER2 phosphorylation in CHO-K6 cells. Cells were treated with CP-724714 at various concentrations as indicated for 1 h. The phosphorylation of HER2 was examined by immunoblotting with antibodies against HER2 pY1005 and pY1139.
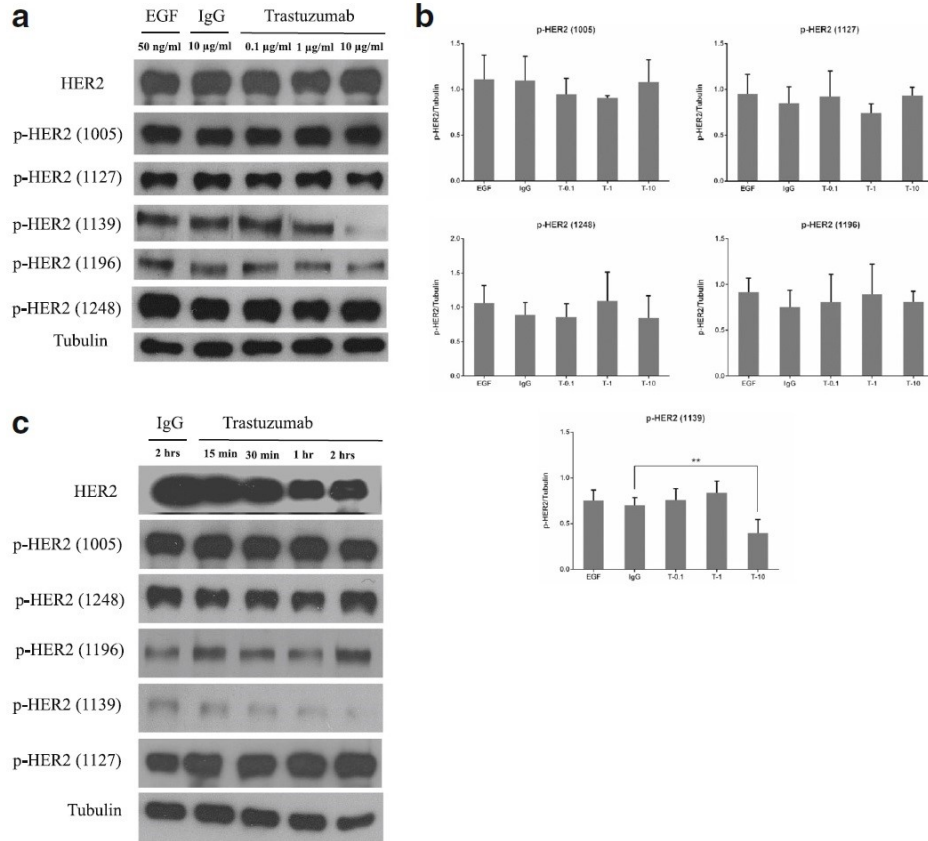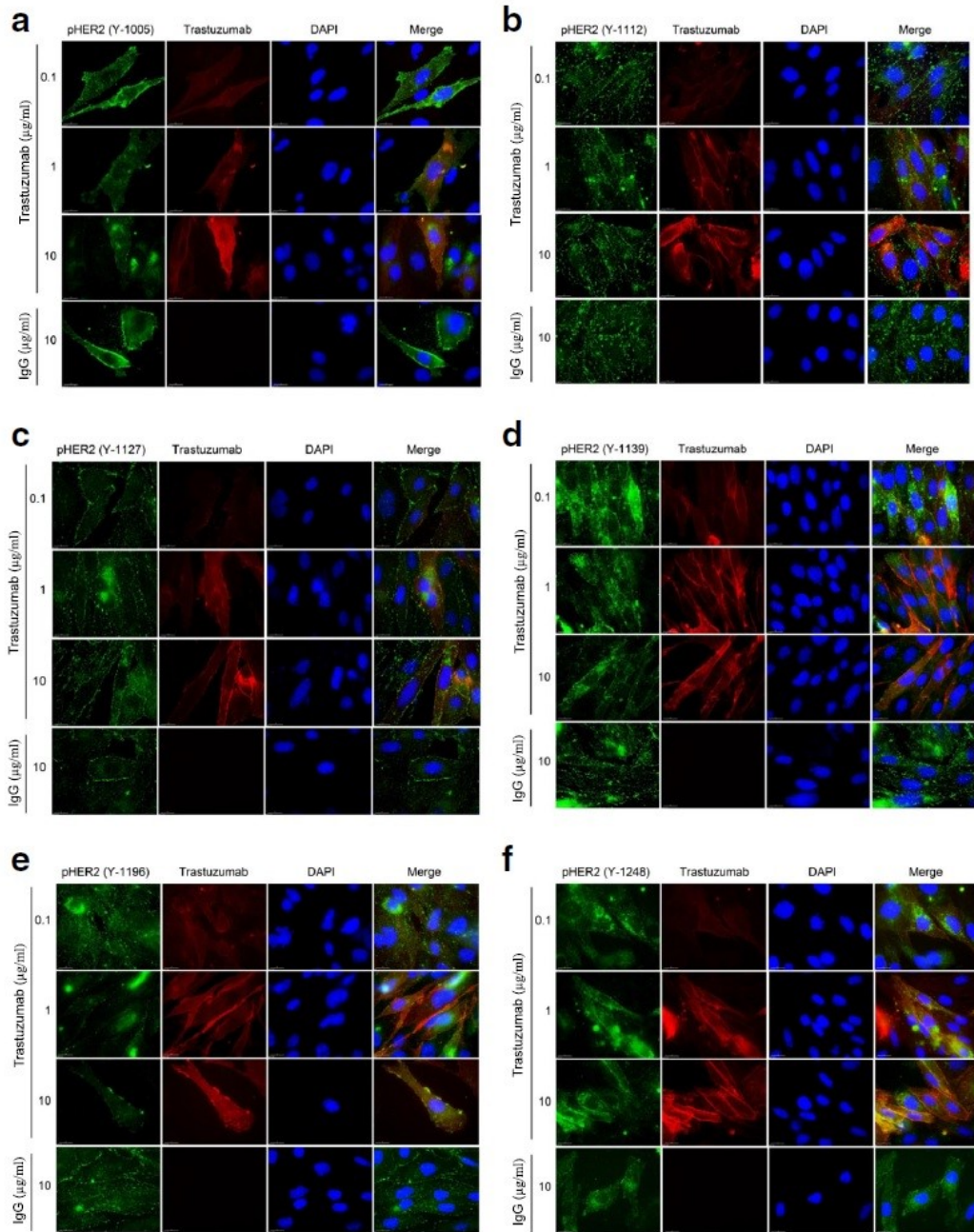
Figure 4.6. The effects of trastuzumab on HER2 phosphorylation in CHO-K6 cells by immunofluorescence. CHO-K6 cells were treated with trastuzumab at concentrations ranging from 0.1–10 µg/ml for 1 h. The phosphorylation of HER2 at Y1005 **(a)**, Y1112 **(b)**, Y1127 **(c)**, Y1139 **(d)**, Y1196 **(e)**, and Y1248 **(f)** were then examined by immunofluorescence. The localization of trastuzumab was revealed by TRITC-conjugated donkey anti-human IgG. The localization of HER2 was revealed by rabbit anti-phosphoHER2 antibody followed by FITC-conjugated donkey anti-rabbit IgG. The cell nuclei were counter stained with DAPI. Yellow indicated the co-localization of trastuzumab and phosphoHER2. The cells treated with normal human IgG (10 µg/ml) were used as negative controls. Size bar: 10 µm (This experiment has been carried out by Babak Nami)

4.3.5. The effects of trastuzumab on the activation of Erk and Akt

We finally examined the activation of Erk and Akt. The Erk and Akt activation was measured by their phosphorylation. As shown in **Figure 4.7a**, the Erk phosphorylation level is higher in CHO-K6 cells than the control CHO cells, which suggests that overexpression of HER2 increased Erk activation. However, we did not observe the increase in Akt phosphorylation, which is not surprising as HER2 homodimer has very limited effects on the activation of PI3K-Akt pathway. We next examined the effects of trastuzumab on the phosphorylation of Erk and Akt in CHO-K6 cells. As shown in **Figure 4.7b**, treatment with trastuzumab did not block the phosphorylation of Erk and Akt.
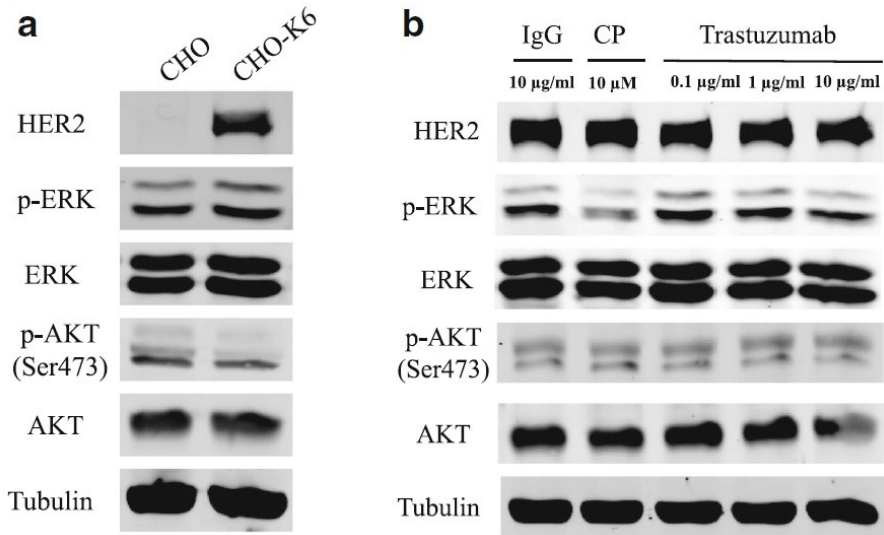
Figure 4.7. The effects of trastuzumab on the phosphorylation of Erk and Akt in CHO-K6 cells. The phosphorylation of Erk and Akt was revealed by immunoblotting. **(a)** The phosphorylation of Erk and Akt in CHO parental cells and in CHO-K6 cells. **(b)** The effects of trastuzumab on the phosphorylation of Erk and Akt in CHO-K6 cells. Cells were treated with trastuzumab with indicated concentrations for 7 h. Cells treated with normal human IgG was used as negative control and cells treated with CP-714724 (CP) was used as positive control.

4.3.6. Trastuzumab-induced ADCC

Above results suggests that trastuzumab did not inhibit HER2 dimerization and phosphorylation. Thus, it is interesting to find out if trastuzumab can induce ADCC in cells overexpressing HER2. Trastuzumab-induced ADCC in CHO-K6 and CHO-EGFR cells was determined by using Promega ADCC Bioassay kit. As shown in **Figure 4.8**, trastuzumab induced very strong ADCC in CHO-K6 cells, but not in CHO-EGFR cells.
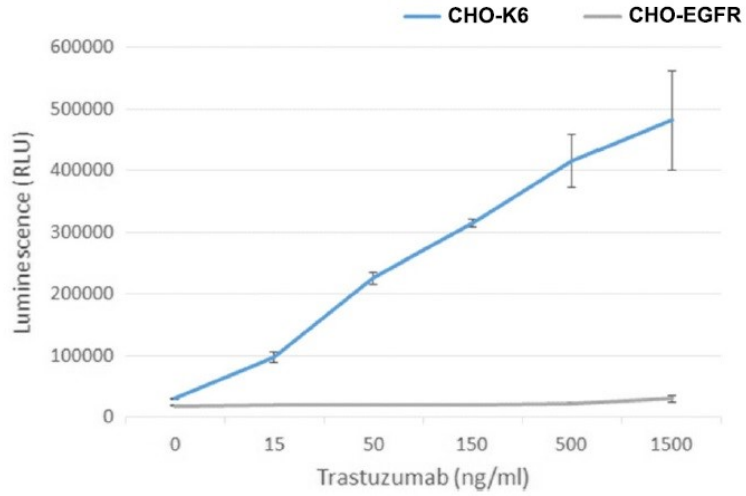
Figure 4.8. Trastuzumab-induced ADCC in CHO-K6 and CHO-EGFR cells. Trastuzumab-induced ADCC was examined in both CHO-K6 and CHO-EGFR cells by using Promega ADCC Bioassay kit. (This experiment has been carried out by Junfeng Tong).

4.3.7. The effects of trastuzumab on the proliferation of CHO-K6 cells

We next determined if trastuzumab inhibits the proliferation of cells with overexpressed HER2. MTT cell proliferation kit was used to assess the proliferation of various CHO cells including CHO parental cells, CHO-EGFR, CHO-K6, and CHO-HER3 cells. Non-treated cells were used as negative controls, and the cells treated with vinorelbine (an anticancer drug) were used as positive controls.

We first determined if overexpression of HER2 in CHO cells stimulates cell proliferation by comparing CHO-K6 cells with the parental CHO cells. As shown in **Figure 4.9a**, the proliferation rate of CHO-K6 cells is much higher than that of CHO cells, which indicates that overexpression of HER2 stimulates cell proliferation. We next examined the effects of trastuzumab on cell proliferation. It is not surprising that treatment with trastuzumab for either 24 or 48 h had no effects on the proliferation of CHO, CHO-EGFR and CHO-HER3 cells as these cells did not express HER2 **(Figures 4.9b-d)**. Interestingly, even for CHO-K6 cells that overexpressed HER2, trastuzumab at high dosage did not have any effect on their proliferation **(Figure 4.9e)**. However, vinorelbine significantly inhibited the proliferation of all these CHO cells following 24 or 48 h incubation **(Figures 4.9b-e)**. Moreover, HER2 kinase inhibitors including Lapatinib and CP714724 significantly inhibited the proliferation of CHO-K6 cells when at high dosage **(Figure 4.9f)**. Our data indicated that trastuzumab did not inhibited the proliferation of CHO-K6 cells that overexpressed HER2.
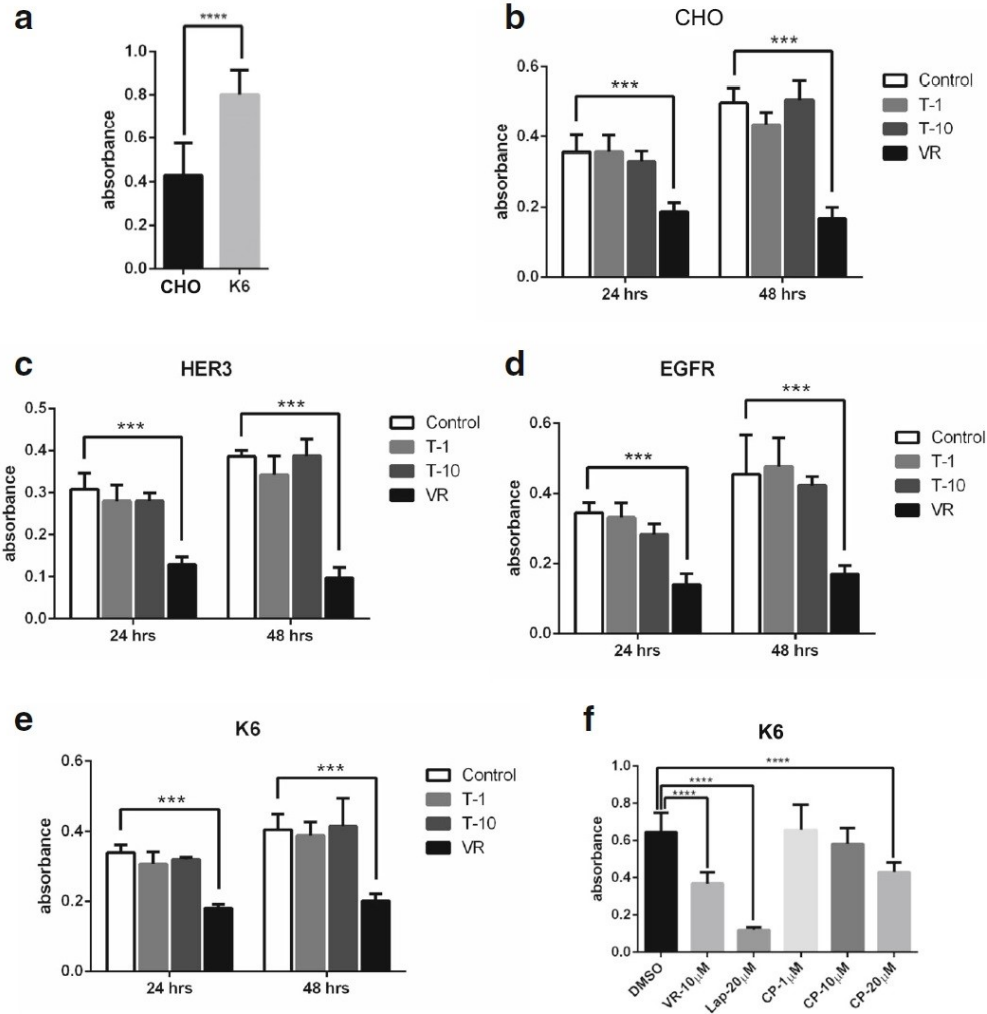
Figure 4.9. The effects of trastuzumab on the proliferation of CHO, CHO-EGFR, CHO-K6, and CHO-HER3 cells. The cell proliferation was examined by MTT assay. **(a)** The effects of HER2 overexpression on the proliferation of CHO cells. Cell proliferation of both CHO parental cells and CHO-K6 cells was examined. **(b-e)** The effects of trastuzumab on the proliferation of CHO, CHO-EGFR, CHO-K6 and CHO-HER3 cells. Cell were treated with various concentration of trastuzumab as indicated for 24 and 48 h. Non treated cells were used as negative control and the cells treated with vinorelbine (VR) were used as positive controls. **(b)** CHO cells. **(c)** CHO-HER3 cells. **(d)** CHO-EGFR cells. **(e)** CHO-K6 cells. **(f)** The effects of other HER2 inhibitors on the proliferation of CHO-K6 cells. CHO-K6 cells were treated with HER2 kinase inhibitors lapatinib (Lap) and CP714724 of indicated concentration. Each value is the average of at least three experiments and the error bar is standard error. ***: $p < 0.001$. ****: $p < 0.0001$

4.4. Discussion

The most controversial mechanism regarding trastuzumab function is its effect on the inhibition of HER2 activation. A major reason behind this controversy is the different cellular background of various BC cells lines used in those studies. Each BC cell line has a unique expression profile of various HER receptors, which could significantly affect the effects of trastuzumab due to the heterodimerization among HER receptors. In this research we adopted a CHO cell model. Besides the parental CHO cells that do not express any detectable HER receptors, three stable CHO cell lines that stably express only a single HER receptor including EGFR (CHO-EGFR), HER2 (CHO-K6), and HER3 (CHO-ErbB3) were employed in this research. Our cell model system avoided the interference of other HER receptors and is very suitable to study the effects of trastuzumab on the homodimerization of HER2 and the phosphorylation of HER2 homodimers. We aim to conclusively determine if trastuzumab specifically binds only to HER2, and blocks HER2 homodimerization and activation.

We showed that trastuzumab only bound to HER2 specifically and with high affinity. Trastuzumab did not bind to EGFR and HER3 even at high dosage (10 ng/ml) **(Figure 4.2)**. Most HER2-positive BC cells also express EGFR and HER3, our finding suggests that any trastuzumab effects on these cells must be initiated through the interaction between trastuzumab and HER2.

We next examined the effects of trastuzumab on HER2 dimerization. HER2 is an orphan receptor and does not have a ligand. However, HER2 are heterodimerized with EGFR in response to EGF stimulation and heterodimerized with HER3 in response to HRG [10]. HER2 is also homodimerized when overexpressed in cells. CHO-K6 cells only expresses a single HER receptor HER2, not EGFR, HER3 or HER4. Thus, our results are regarding the effects of trastuzumab on the homodimerization of HER2.

We showed that in CHO-K6 cells HER2 was mostly dimerized, likely due to the overexpression **(Figure 4.3)**. This is not surprising. As revealed by crystal structures of the HER2 extracellular region, HER2 adopts an extended configuration, which resembles the configuration of EGFR seen in each molecule of an EGFR dimer. Thus, ErbB2 possesses a constitutive, or ligand independent, activated conformation, which allows the HER2 homodimerization when overexpressed [10-12].

We also showed that trastuzumab did not block the homodimerization of HER2 **(Figure 4.3)**. While it is originally proposed that trastuzumab acts to block HER2 dimerization, so far, no research has been done to determine the effects of trastuzumab on the homodimerization of HER2. Given the fact that trastuzumab binds to the juxtamembrane region of HER2 [11], which is not essential for HER2 dimerization, our results are not surprising. What surprising is that our data suggest that trastuzumab at high dosage actually enhanced the homodimerization of HER2 **(Figure 4.3)**. While we are not certain how trastuzumab stimulates the homodimerization of HER2, it is possible that it functions through HER2 transmembrane domain. Many data support the role of HER2 transmembrane domain in HER2 dimerization and activation [11]. Parts of juxtamembrane region has also been implicated in HER2 dimerization and activation [13-15]. As trastuzumab binds to the extracellular juxtamembrane region of HER2, it will likely affect the function of HER2 transmembrane domain and juxtamembrane region in terms of HER2 dimerization. It is possible that somehow the specific effects of trastuzumab enhanced the interaction between two HER2 transmembrane domains and thus increased HER2 homodimerization as we observed here.

It has been thought that trastuzumab functions to inhibit HER2 activation/phosphorylation and HER2- mediated cell signaling [16-18]. However, our data indicated that trastuzumab only had very limited effects on HER2 phosphorylation. Among six pY residues examined in this research, HER2 had no effects on the phosphorylation of Y1005, Y1112, Y1027, Y1196, and Y1248 **(Figures 4.5 and 4.6)**. While HER2 decreased the phosphorylation of Y1139, this is a much weaker inhibition when compared with CP-724714 **(Figures 4.5, 4.6 and 4.7)**. In general, this is consistent with our observation regarding the role of trastuzumab in HER2 dimerization. Trastuzumab did not block HER2 dimerization, thus it did not block HER2 phosphorylation. It is not clear how the effects of HER2 transmembrane domain on HER2 dimerization affect the phosphorylation of HER2. Some research indicated the presence of an alternative dimerization mode of HER2. In this model, HER2 dimerization is mediated by both transmembrane domain and the cytoplasmic juxtamembrane region of HER2. Such a dimerization model exerts inhibiting effects on the HER2 kinase activity [13-15]. Thus, in theory, the enhanced dimerization through the interaction of transmembrane domain and the juxtamembrane region could result in the inhibition of certain HER2 phosphorylation including pY1139. Recently, some research with various BC cell lines have shown that trastuzumab did not significantly alter HER2

phosphorylation [18-22]. Moreover, one research shows the enhanced phosphorylation of pY1248 in response to trastuzumab[17].

Our results suggest that trastuzumab has, if any, limited effects on HER2-mediated intracellular signaling. Indeed, when we examined the effects of trastuzumab on the phosphorylation of Erk and Akt, we showed that trastuzumab did not block the phosphorylation of both Erk and Akt in CHO-K6 cells **(Figure 4.7)**. Together, our data indicate that trastuzumab did not significantly alter HER2 activation and HER2 mediated intracellular signaling in the absence of other HER receptors. However, we need to be cautious to apply these findings to BC cells. CHO cell is derived from hamster ovary, thus the expressed human HER2 may not be coupled well with downstream signaling cascades.

We then examined if trastuzumab induces ADCC in CHO-K6 cell. We showed that trastuzumab indeed induces strong ADCC in CHO-K6 cells **(Figure 4.8)**. This is specifically due to the expression of HER2 in CHO-K6 cells as there is no ADCC observed in CHO-EGFR cells **(Figure 4.8)**. The role of trastuzumab in the induction of ADCC in HER2-positive BC cells have been consistently well supported by many researches [23-30]. Our results confirmed the role of trastuzumab in the induction of ADCC in a simple but specific cell setting.

We also showed that trastuzumab did not affect cell proliferation in CHO-K6 cells **(Figure 4.9)**. Some reports indicated that trastuzumab had little effect on proliferation and survival [31, 32]. However, other reports indicated that trastuzumab inhibited ErbB2 activation, and decreased the activation of Erk and PI3K-Akt pathways, which leads to reduced cell proliferation [33]. Given that trastuzumab has little effects on the phosphorylation of HER2, it is likely that trastuzumab has no effects on HER2-mediated cell signaling leading to cell proliferation. Although trastuzumab induces ADCC in CHO-K6 cell, under the culture conditions used for the MTT assay, no effector cells were present and no ADCC response are expected. It is interesting to note that our above finding is different from the observation by Ghosh [34]. Ghosh *et al*. reported that trastuzumab inhibited HER2 homodimer-mediated Erk phosphorylation and cell growth. The difference could be due to the different model system used in these two studies. The HER2 receptor used in the research by Ghosh *et al*. is fused with FKBP, and the receptor homodimerization is induced by a chemical linker AP1510 that dimerizes the receptor intracellularly through the fused FKBP.

It should be noted that while we observed strong inhibition of HER2 phosphorylation by CP-724714 at 1 μM **(Figure 4.5b)**. We only observed the inhibition of CHO-K6 cell proliferation at much higher CP-724714 concentration **(Figure 4.9f)**. We are not sure what cause this discrepancy, however, there are several possible explanations. Firstly, at 1 μM, CP-724714 may not completely inhibit Her2 phosphorylation, we can see weak phosphorylation of Y1139 in **Figure 4.5b**. There could be weak phosphorylation of HER2 at other pY residues that were not examined. A weak HER2 phosphorylation may still be sufficient to support cell growth. Secondly, there could be the existence of kinase-independent effects of HER2 receptors. There are many reports supporting the existence of kinase-independent cell signaling of various receptor tyrosine kinases including EGFR and Insulin receptor [35-38].

4.5. References

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Chapter 5. The Effects of Pertuzumab and Its Combination with Trastuzumab on HER2 Homodimerization and Phosphorylation

5.1. Abstract

Pertuzumab (Perjeta) is an anti-HER2 monoclonal antibody that is used for treatment of HER2-positive BC in combination with trastuzumab (Herceptin) and docetaxel and showed promising clinical outcomes. Pertuzumab is suggested to block heterodimerization of HER2 with EGFR and HER3 that abolishes canonical function of HER2. However, evidence on the exact mode of action of pertuzumab in homodimerization of HER2 are limited. In this chapter, we investigated the effect of pertuzumab and its combination with trastuzumab on HER2 homodimerization, phosphorylation and whole gene expression profile in Chinese hamster ovary (CHO) cells stably overexpressing human HER2 (CHO-K6). CHO-K6 cells were treated with pertuzumab, trastuzumab, and their combination, and then HER2 homodimerization and phosphorylation at seven pY sites were investigated. Results showed that pertuzumab had no significant effect on HER2 homodimerization, however, trastuzumab increased HER2 homodimerization. Interestingly, pertuzumab increased HER2 phosphorylation at Y1127, Y1139, and Y1196 residues, while trastuzumab increased HER2 phosphorylation at Y1196. More surprisingly, combination of pertuzumab and trastuzumab blocked the phosphorylation of Y1005 and Y1127 of HER2. Our findings confirm that pertuzumab is unable to inhibit HER2 homodimerization but induces HER2 phosphorylation at some pY sites that abolishes HER2 effects on cell cycle progress. These data suggest that the clinical effects of pertuzumab may be mostly through the inhibition of HER2 heterodimers, rather than HER2 homodimers and that pertuzumab binding to HER2 may inhibit non-canonical HER2 activation and function in non-HER-mediated and dimerization-independent pathway(s).

Keywords: pertuzumab; trastuzumab; breast cancer; HER2; homodimer; phosphorylation

5.2. Introduction

Pertuzumab (originally known as 2C4 and commercially known as Perjeta®, Hoffmann-La Roche, Basel, Switzerland), is a fully humanized recombinant anti-HER2 monoclonal antibody. Pertuzumab is approved by FDA to be used as neoadjuvant in combination with trastuzumab (Herceptin®, Hoffmann-La Roche, Basel, Switzerland), another anti-HER2 monoclonal antibody, and docetaxel for the treatment of early stage and metastatic HER2-positive BC [1-3]. Adding pertuzumab to trastuzumab and docetaxel has produced better outcome than treatment with trastuzumab and docetaxel alone, including significant improvement in progression-free and overall survival rates [4-6]. Binding of pertuzumab to HER2 of HER2-positive tumor cells induce immune response against the Fc domain of the antibody. This mechanism provokes the immune cells to attack and destroy the tumor cells by releasing cytotoxic enzymes and apoptosis induction the process called antibody-dependent cellular cytotoxicity (ADCC) [7-10]. In addition to induction of ADCC, pertuzumab was also shown to inhibit HER2-positive cancer cell proliferation in the absence of immune cells, implicating the anti-cancer effects of the pertuzumab through alteration of HER2-mediated signaling pathways [11-13]. Pertuzumab binds to the dimerization pocket in the domain II of the extracellular region of HER2 that is believed to inhibit HER2/EGFR [14] and HER2/HER3 heterodimerizations [15-18]. Since the heterodimerization between HER2 and EGFR/HER3 is induced by ligand-binding, pertuzumab is believed to block ligand-dependent activation of HER2 and downstream signaling [14, 17-19].

Given the better outcome of pertuzumab treatment in combination with trastuzumab, there seems to be a synergism between the two therapeutics [20]. Trastuzumab binds to extracellular domain IV close to the transmembrane region of HER2 [1, 21]. Trastuzumab is reported to block the homodimerization of HER2, and to inhibit ligand-independent HER2-mediated signaling as HER2 is an orphan receptor, but could homodimerize when overexpressed [20, 22, 23]. However, we have previously shown that trastuzumab does not inhibit HER2 homodimerization, phosphorylation and downstream signaling [24]. So far evidences on exact mode of action of pertuzumab, particularly its role in blocking HER2 homodimerization, HER2-mediated cell cycle progression and cell death still remains controversial. In the present study we investigated the effects of pertuzumab and its combination with trastuzumab on homodimerization and tyrosine phosphorylation of HER2 in an HER2 overexpressing cell line model.

5.3. Results

5.3.1. Specific Binding of Pertuzumab to HER2

In this study we used Chinese hamster ovary (CHO) cells stably expressing human HER2 (HER2-K6 [24, 25]) as a HER2 overexpressing cell model. The expression level of HER2 in CHO K6 cells was significantly higher than that in BC cell lines including SKBR-3, BT-474, MCF-7, and MDA-MB-231, as well as another clone of HER2-overexpressing CHO cell line HER2-K13 cells [24, 25] **(Figure 5.1A)**. To examine binding of pertuzumab to HER receptors, we treated CHO cells stably overexpressing EGFR (CHO-EGFR), HER2 (CHO-K6) and HER3 (CHO-HER3) with 10 μg/ml pertuzumab for 60 min. As shown in **Figure 5.1B**, pertuzumab specifically bound to HER2 in cell membrane, but not to EGFR and HER3. Dose-response experiments (0.1, 0.5, 1, 5, and 10 μg/mL for 60 min) indicated that pertuzumab strongly bound to HER2 even at a low concentration of 0.1 μg/mL **(Figure 5.1C)**. Moreover, time-course (10 μg/mL for 5, 15, 30, and 60 min) treatment of CHO-K6 cell showed that 5 min incubation is enough to result in strong binding between pertuzumab and HER2 **(Figure 5.1D)**. Together, these results confirm that pertuzumab specifically binds to HER2 receptor with high affinity.
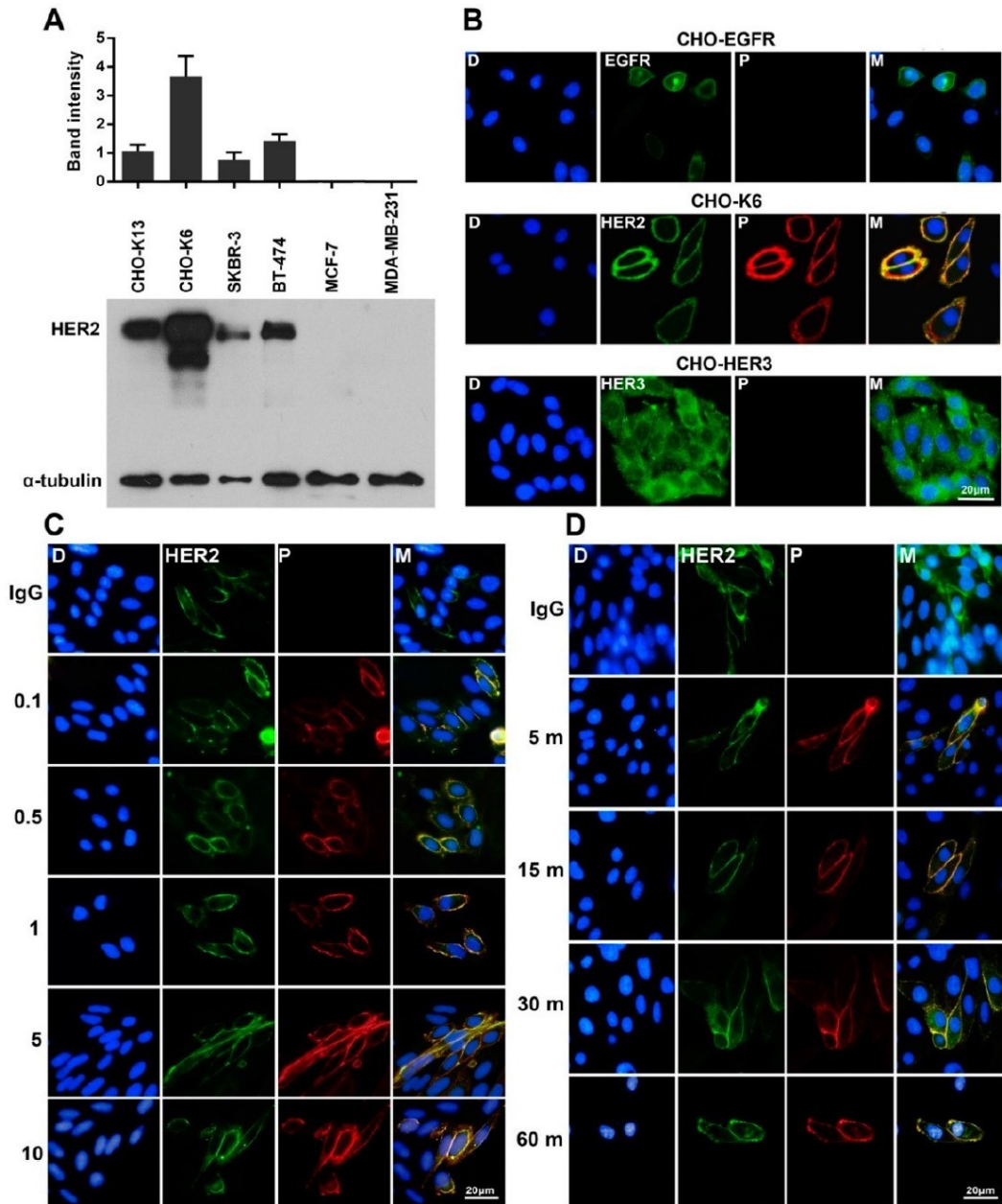
Figure 5.1. Specific binding of pertuzumab to HER2. **(A)** The expression levels of HER2 in CHO cell lines stably overexpressing HER2 (CHO-K13 and CHO-K6), two HER2-positive (SKBR-3 and BT-474) and two HER2-negative (MCF-7 and MDA-MB-231) BC cell lines. **(B)** Double-immunofluorescence staining of EGFR, HER2, and HER3 (green) and pertuzumab (P; red) in CHO-EGFR, CHO-K6, and CHO-HER3 cell lines. Pertuzumab binds specifically to HER2 but not to EGFR and HER3. **(C,D)** Double-immunofluorescence staining of HER2 (green) and pertuzumab (red) in the cells **(C)** treated with 0.1, 0.5, 1, 5, and 10 μg/mL pertuzumab for 60 min (dose-course treatment) and **(D)** treated with 10 μg/mL pertuzumab for 5, 15, 30, and 60 min (time-course treatment). Treatment with 10 μg/mL of human IgG for 60 min was used as a mock control. (This experiment has been carried out by Babak Nami)

5.3.2. The Effects of the Pertuzumab on HER2 Homodimerization

We next examined the effects of pertuzumab, trastuzumab and their combination on the homodimerization of HER2. We previously showed the specific binding of trastuzumab to HER2 [24]. For dose-response experiments, the CHO-K6 cells were treated with 0.1, 1, and 10 μg/mL pertuzumab, trastuzumab, or their combination for 60 min. For time-course treatment the cells were treated with 10 μg/mL pertuzumab for 15, 30, 60, and 60 min. Ten μg/mL human IgG and 20 μM HER2 tyrosine kinase inhibitor agent CP-724714 were used as mock and positive controls respectively. HER2 monomer and homodimer was assessed by BS^3-based protein cross-linking assay. As result, the mean ratio of quantified HER2 homodimer to HER2 monomer in the cell treated with CP-724714 was under 1, significantly lower than that of the cells treated with IgG in the dose-response experiments **(Figure 5.2A)** and the time-course experiments **(Figure 5.2B)**. This indicates higher level of monomer HER2 than homodimer HER2, confirming that CP-724714 significantly inhibited HER2 homodimerization. In contrast, there was no significant difference between HER2 dimer/monomer ratio of IgG treated cells and that of the cells treated with single-agent pertuzumab in both dose- and time-course treatment experiments. While, the cells treated with trastuzumab as well as those treated with combination of pertuzumab and trastuzumab showed higher HER2 dimer/monomer ratios compared to IgG treated cells **(Figure 5.2A)**. These results implicate that pertuzumab had no significant effect on HER2 homodimerization. However, trastuzumab increased HER2 homodimerization, which is consistent with our previous observation [24].
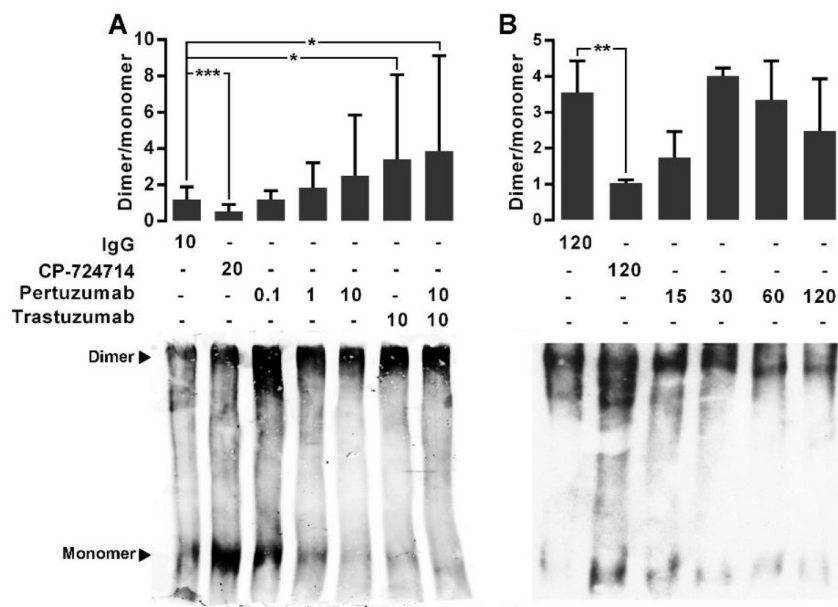
Figure 5.2. The effect of pertuzumab on HER2 homodimerization. **(A)** Immunoblot expression of monomer (185 kDa) and dimer (360–380 kDa) HER2 and quantified dimer/monomer ratios in CHO-K6 cells treated with 0.1, 1 and 10 µg/mL pertuzumab, 10 µg/mL trastuzumab or their combination for 60 min (dose-course treatment). **(B)** Time-course treatment of CHO-K6 with 10 µg/mL pertuzumab for 15, 30, 60 and 120 min. Ten µg/mL human IgG and 20 µM CP-724714 were used as mock and HER2 inhibitor controls. CP-724714 reduced dimer HER2. Trastuzumab increased dimer HER2 and pertuzumab had no significant effect on HER2 homodimerization. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.3. The Effects of the Pertuzumab on the HER2 Phosphorylation

We have shown previously that HER2 is strongly phosphorylated in CHO-K6 cells [24]. Here, to examine whether pertuzumab inhibits HER2 phosphorylation, we investigated the phosphorylation of seven pY sites on HER2 C-terminal including Y1005, Y1112, Y1127, Y1139, Y1196, Y1221/1222, and Y1248. CHO-K6 cells were treated with pertuzumab at final concentrations of 0.1, 1 and 10 μg/mL, 10 μg/mL trastuzumab, and a combination of 10 μg/mL pertuzumab and trastuzumab for 60 min, and then the levels of total HER2 and the phosphorylated HER2 were analyzed by Western blotting. A final concentration of 10 μg/mL human IgG and 20 μM CP-724714 were used as mock and positive controls, respectively.

As shown in **Figure 5.3A**, none of treatments showed change in the protein expression levels of total HER2. However, CP-724714 dramatically reduced the phosphorylation of HER2 at all the pY sites **(Figure 5.3)**. Surprisingly, the cells treated with 10 μg/mL pertuzumab showed an increased level of HER2 phosphorylation at Y1127 **(Figure 5.3C)**, Y1139 **(Figure 5.3D)** and Y1196 **(Figure 5.3E)**, but not at other pY sites. On the other hand, treatment with 10 μg/mL trastuzumab only increased HER2 phosphorylation at pY1196 **(Figure 5.3E)**, but not at any other pY sites. More interestingly, the treatment with combined pertuzumab and trastuzumab reduced the phosphorylation of HER2 at pY1005 **(Figure 5.3B)** and pY1127 **(Figure 5.3C)**.
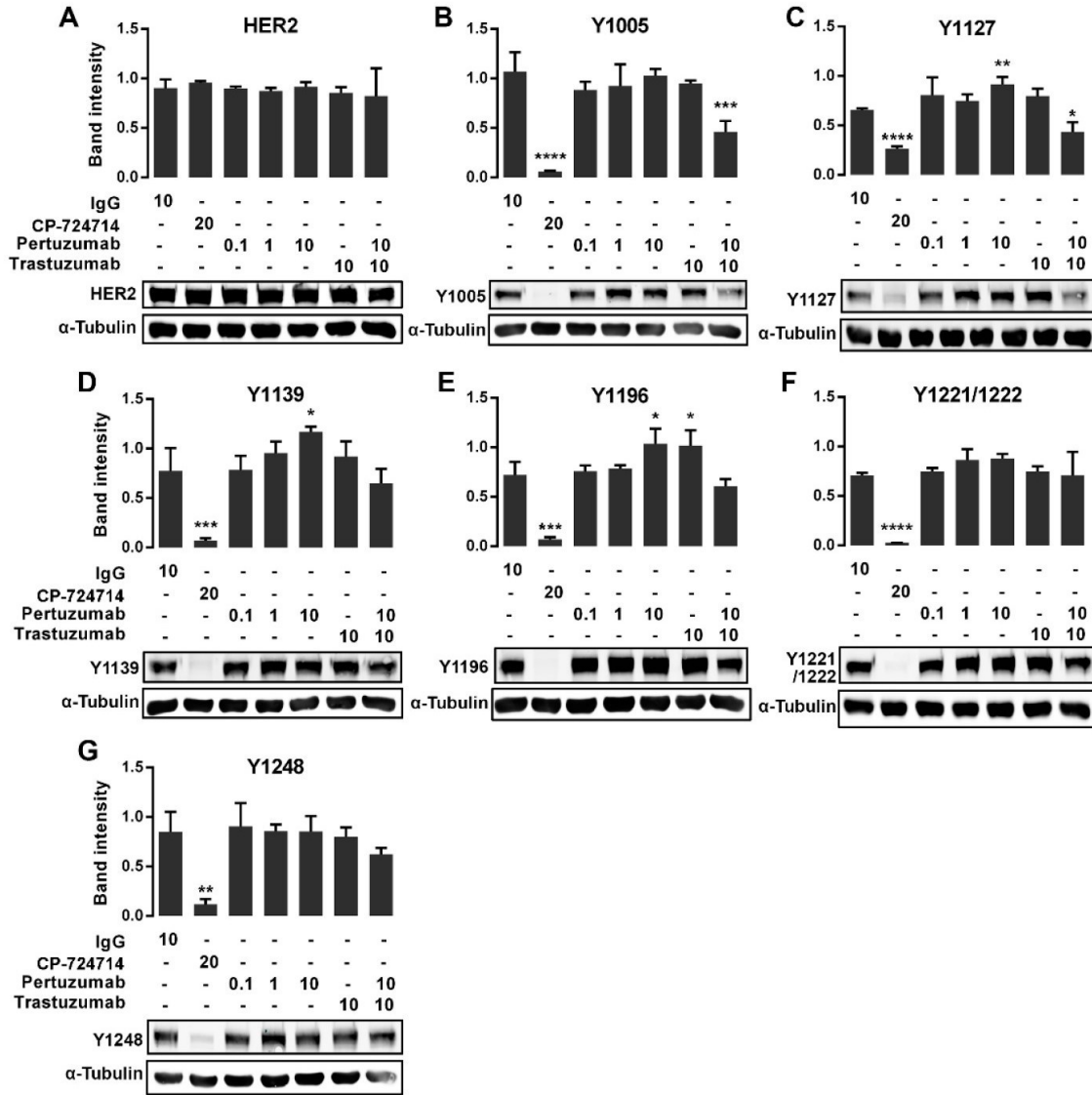
Figure 5.3. Immunoblot expression of pHER2 in pertuzumab treated CHO-K6 cells. CHO-K6 cells were treated with 0.1, 1 and 10 μg/mL pertuzumab, 10 μg/mL trastuzumab and their combination for 60 min. Ten μg/mL human IgG and 20 μM CP-724714 were used as mock and positive controls respectively. The expression of **(A)** total HER2, **(B)** pY1005, **(C)** pY1127, **(D)** pY1139, **(E)** pY1196, **(F)** pY1221/1222 and **(G)** pY1248 HER2 was monitored by immunoblotting. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The effects of pertuzumab on the phosphorylation of HER2 were further examined by immunofluorescence staining. CHO-K6 cell were treated with 0.1, 1, and 10 μg/mL pertuzumab for 60 min. The phosphorylation of HER2 at Y1005, Y1112, Y1122, Y1139, Y1196, and Y1248 was examined by immunofluorescence staining with specific antibodies as indicated. As shown in **Figure 5.4**, CP-724714 treatment dramatically decreased HER2 phosphorylation at all the pY sites. Similar to the western blotting results, pertuzumab increased HER2 phosphorylation at pY1127 **(Figure 5.4C)**, pY1139 **(Figure 5.4D)** and pY1196 **(Figure 5.4E)** compared to IgG treated cells. Pertuzumab had no significant effects on HER2 phosphorylation at other pY sites **(Figure 5.4)**. Taken together, pertuzumab did not inhibit HER2 phosphorylation, but induced phosphorylation of HER2 at Y1127, Y1139, and Y1196 residues.

5.3.4. The Effect of Pertuzumab on Cell Proliferation

We next investigated the effects of pertuzumab on cell proliferation in CHO-K6 cells. We cultured CHO-K1, CHO-K6, CHO-K13, CHO-EGFR and CHO-HER3 cells in the presence of 10 μg/mL pertuzumab or 10 μg/mL trastuzumab or their combination for 72 h and then evaluated viable cells by MTT assay. Human IgG (10 μg/mL) was used as mock control for the monoclonal antibodies. CP-724714 (10 μM) was used as positive control of HER2 inhibition. Paclitaxel (5 μM) was used as positive control of cell proliferation inhibition.

The results showed that the proliferation rates of all the paclitaxel treated cell lines were significantly lower than that of relevant untreated cells. Among the cell lines treated with CP-724714, CHO-K1, CHO-K6 and CHO-EGFR but not CHO-K13 and CHO-HER3 cells showed lower proliferation rates compared to the relevant untreated cells **(Figure 5.5)**. None of the cells treated with pertuzumab, trastuzumab, and their combination showed significant changes in the proliferation rates in comparison with relevant IgG treated cells **(Figure 5.5)**. These results indicate that treatment with pertuzumab, trastuzumab and their combination did not have significant effect on the proliferation of HER2, HER3, and EGFR overexpressing CHO cells.
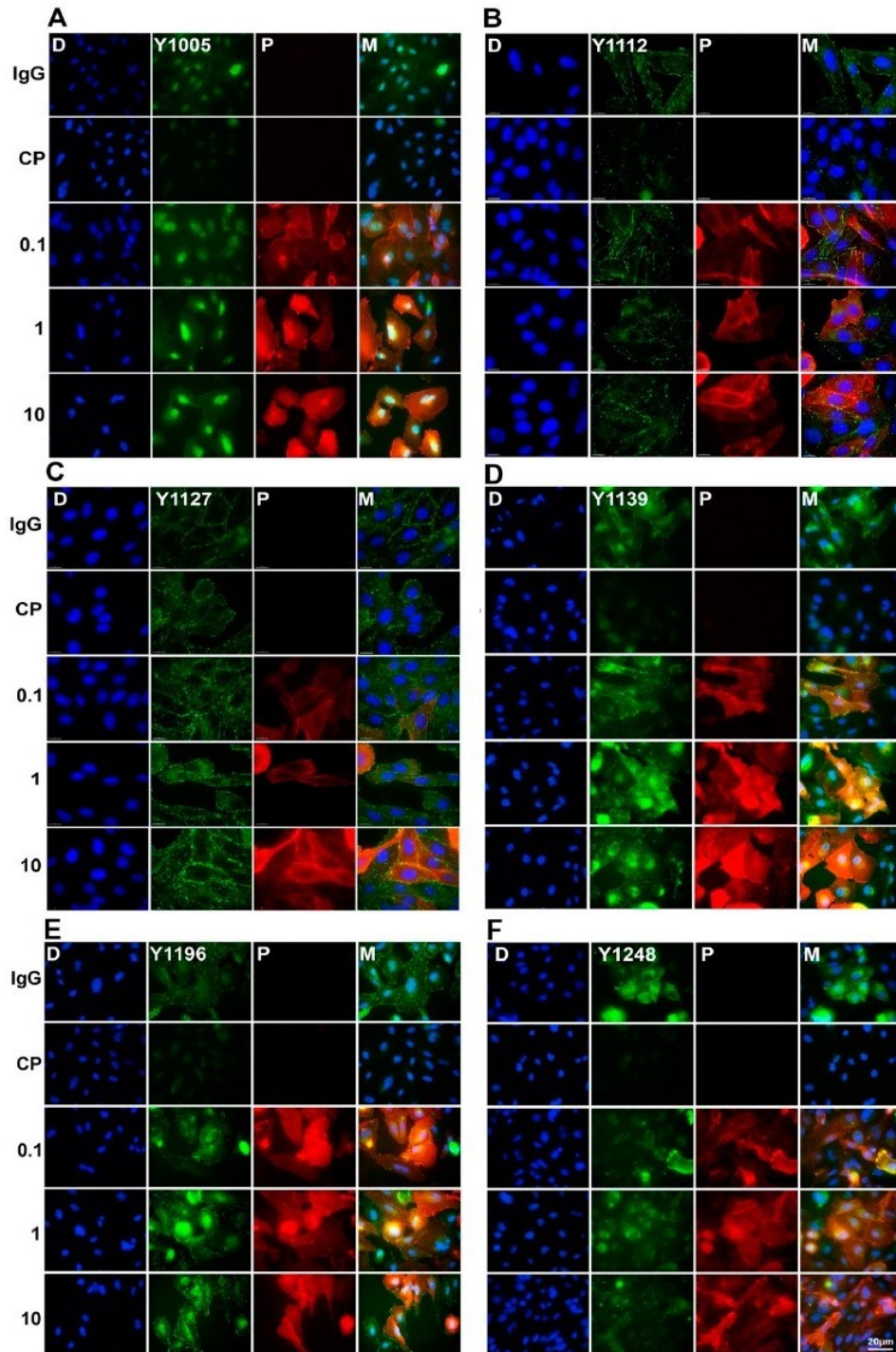
Figure 5.4. Double-immunofluorescence staining of pHER2 and pertuzumab in pertuzumab treated CHO-K6 cells. CHO-K6 cells were treated with 0.1, 1 and 10 μg/mL pertuzumab for 60 min then pHER2 at **(A)** pY1005 **(B)** pY1112, **(C)** pY1127, **(D)** pY1139, **(E)** pY1196, and **(F)** pY1248 HER2 (all green) and pertuzumab (red) were stained. Ten μg/mL human IgG and 20 μM CP-724714 (CP) were used as mock and positive controls, respectively. (This experiment has been carried out by Babak Nami)
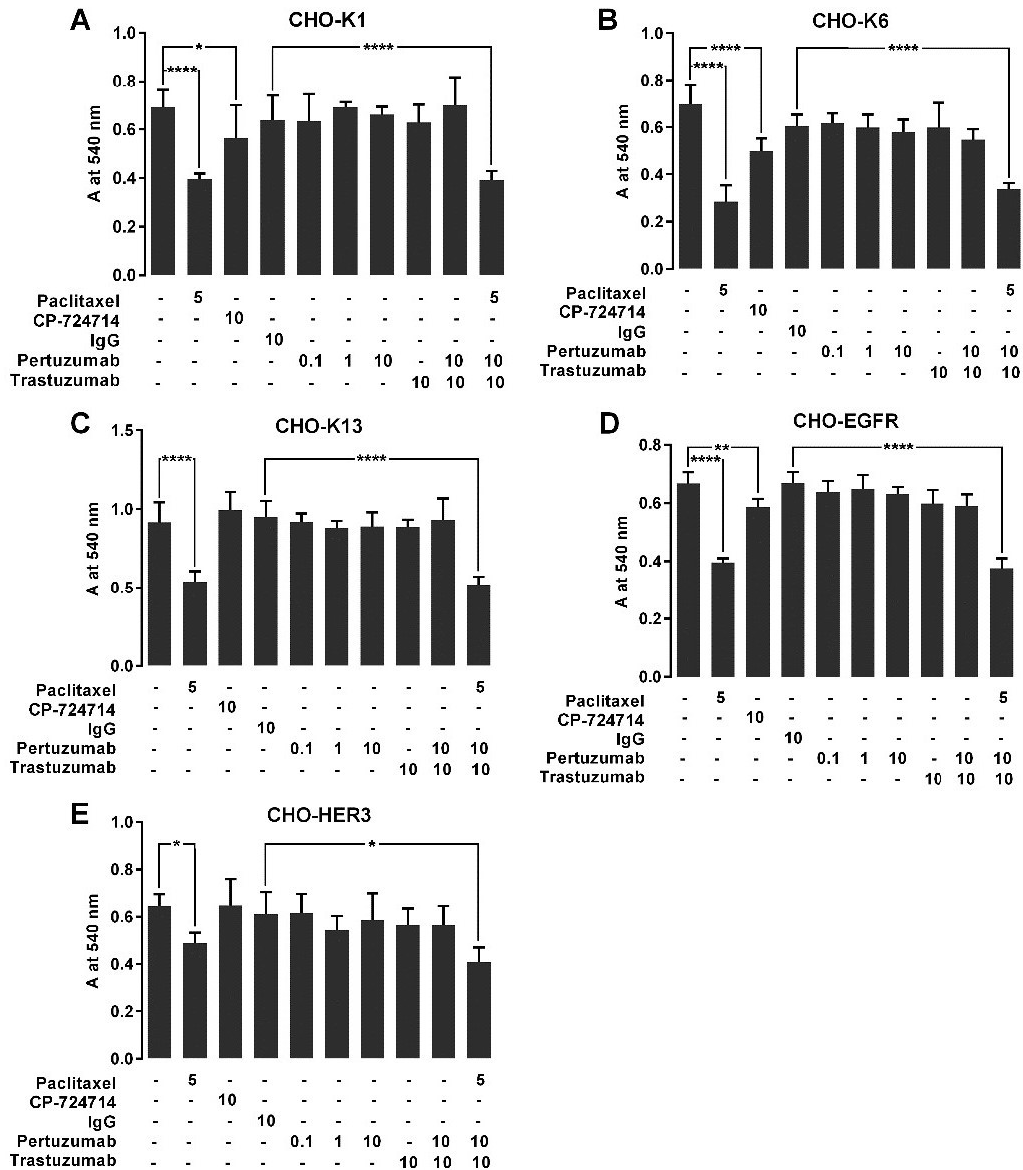
Figure 5.5. The effect of pertuzumab on the proliferation of cells overexpressing EGFR, HER2 and HER3. **(A)** CHO-K1, **(B)** CHO-K6, **(C)** CHO-K13, **(D)** CHO-EGFR and **(E)** CHO-HER3 cell lines were treated with 0.1, 1 and 10 µg/mL pertuzumab, 10 µg/mL trastuzumab and their combination for 72 h, and then the living cell mass was evaluated by MTT assay. Five µM paclitaxel, 10 µg/mL human IgG and 20 µM CP-724714 were used as respectively antiproliferative, mock and HER2 inhibitor controls. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. (This experiment has been carried out by Babak Nami)

5.4. Discussion

In this study we investigated the effect of pertuzumab on the function of HER2 homodimers. We also compared the effects of pertuzumab with trastuzumab and the combination of pertuzumab and trastuzumab. Three CHO cell lines stably overexpressing human HER2 (CHO-K6), human EGFR (CHO-EGFR) and human HER3 (CHO-HER3) as well as parental CHO (CHO-K1) cells were used in our study. The parental CHO cell line dose not express any of HER family receptors per se. CHO-K6 provides appropriate model cell to study HER2 receptor function that allows monitor HER2 function without interaction by other HER receptors. Although pertuzumab specifically binds only to HER2, studying HER2 homodimerization and its subsequent effects on the cellular biology is not quite feasible in BC cell lines. Since HER2-positive BC cell lines express also at least one other member of HER receptor family particularly EGFR and HER3 [26-28]. Endogenous EGFR and HER3 could mediate HER2 heterodimerization and significantly affect the HER2 phosphorylation. On the other hands, the CHO cell lines allow us to study each HER receptor independently. CHO-K6 cells show high rates of HER2 homodimerization and phosphorylation that can be inhibited by CP-724714. Although canonical downstream signaling pathways of HER2 (PI3K/Akt and MAPKpathways) do not work in CHO-K6 cells, the cell proliferation rate is higher than that of parental CHO cells (CHO-K1) [24]. This HER2-mediated increased proliferation can be inhibited by lapatinib and CP-724714. These confirm that CHO-K6 cell line is sensitive to anti-HER2 agents. This sensitivity is revealed by inhibition of HER2 dimerization, phosphorylation and CHO-K6 cell proliferation in response to treatment with the agents. This further confirms that CHO-K6 is a suitable cell model for studying HER2 homodimerization and phosphorylation but is not an appropriate model for studying HER2-mediated PI3K/Akt and MAPK pathways. Furthermore CHO-K6 cells provides a valuable cell model for studying the oncogenic function of HER2 via non-canonical mechanism(s) independently of PI3K/Akt and MAPK signaling pathways that deserves more attention.

We showed that pertuzumab specifically bound to HER2 with high affinity on CHO-K6 cells. No pertuzumab binding was detected on CHO-EGFR and CHO-HER3 cells. Pertuzumab binds at the dimerization pocket of HER2 located in its extracellular domain II [16], while trastuzumab binds to a site located in extracellular domain IV [29]. Thus, it is suggested that the binding of pertuzumab but not trastuzumab to HER2 disrupts its heterodimerization with EGFR [14] and

HER3 [15-18, 20]. However, there is no independent research focusing on the effect of pertuzumab on HER2 homodimerization and phosphorylation. Structurally, conformation of monomer HER2 resemble to ligand-bound EGFR receptor. Therefore HER2, when overexpressed, is able to form homodimer in the absence of ligand, which also resulted in the phosphorylation of HER2 [16, 24, 29]. In the present study, HER2 was highly dimerized in CHO-K6 cells, most likely due to the overexpression. We showed that pertuzumab does not have a significant effect on HER2 homodimerization, while trastuzumab and the combination of pertuzumab and trastuzumab increase HER2 homodimerization. Homodimerization of HER2 takes place through interaction of domain II of one HER2 receptor with the C-shaped pocket formed by domain I, II, and III of the adjacent HER2 receptor [30]. A previous study shows that pertuzumab but not trastuzumab inhibits HER2 homodimerization and increases the antiproliferative effect of trastuzumab on HER2-positive BC cells in combination with trastuzumab [31]. In another study Hu *et al*. [30] report that pertuzumab binding to its epitope on domain II of HER2 prevents interaction of C-shaped pocket from adjacent HER2 with dimerization arm of HER2 by masking the dimerization pocket [30]. According to this study, pertuzumab abolishes HER2 homodimerization in COS-7 cell expressing extracellular domains of HER2, however, trastuzumab had a negligible effect on the HER2 homodimerization [30]. However, our results do not support these reports. In addition, pertuzumab is not able to abrogate the positive effect of trastuzumab on HER2 homodimerization. There are some reasons that show our results are more reliable compared to previous opposite results. Firstly; in this study we used an originally HER2-negative cell line that stably expresses full-length human HER2, while Hu *et al*. [30] used a partial extracellular domain of HER2 (residues 1–624). It is quite possible that partial HER2 has a structure distinct from the structure of full-length HER2. Aberrant conformation of partial HER2 most likely effects the binding and function of the monoclonal antibody on HER2. Second; Diermeier-Daucher *et al*. [31] used BT-474 and SKBR-3 cell lines to study homodimerization. Both of the cell lines express EGFR, HER3 and HER4 in addition to HER2. The HER receptors have similar molecular size and can form 10 different dimers with each other. Distinction of HER2 homodimer from heterodimers and homodimers of the other three HER receptors based on molecular weight may be highly erroneous.

We showed that trastuzumab increases HER2 homodimerization, which is consistent with our previous results [24]. The epitope of trastuzumab is located near transmembrane domain of HER2 [29]. Several experimental and molecular dynamics simulation studies support the critical role of

transmembrane and juxtamembrane domains in HER2 homo- and heterodimerization [32-40]. HER2 transmembrane has two GxxxG-like motifs, one in the N-terminal close to the extracellular domain and one in the C-terminal close the intracellular domain [34, 39, 40]. The N-terminal GxxxG-like motif mediates in heterodimerization, whereas the C-terminal motif had a role in the formation of homodimer [40]. It has been shown that a mutation at valine 664, such as Val664Glu which is located in the transmembrane domain between the GxxxG-like motifs, leads to constitutive HER2 activation by enhancing the tendency to dimerize [41]. Moreover, substitution of isoleucine 655 of the HER2 transmembrane with valine is found to increase BC risk. This mutation changes the conformation of the receptor that causes constitutive activation of HER2 tyrosine kinase domain [42]. Further, phosphorylation of threonine 677 in the juxtamembrane domain of HER2 is shown to inhibit HER2/EGFR heterodimerization [43]. This evidence demonstrates that conformation change in the HER2 transmembrane can alter HER2 receptor dynamics resulting in an altered tendency for dimerization. Indeed, binding of trastuzumab [29] and pertuzumab [16] significantly changes HER2 conformation. It is possible that somehow trastuzumab binding confers a new conformation that facilitates homodimerization of HER2, a function that pertuzumab is not able to perform.

We showed that pertuzumab did not inhibit HER2 phosphorylation but induced phosphorylation of HER2 at Y1127, Y1139, and Y1196 residues. Trastuzumab did not have a significant effect on HER2 phosphorylation except at Y1196. This result is consistent with our previous report that shows trastuzumab does not affect HER2 phosphorylation at any pY sites [24]. We showed that combination of pertuzumab and trastuzumab inhibits pY1005 and pY1127. Lack of inhibitory effect of pertuzumab on phosphorylation is expected since it had no inhibitory effect on dimerization. The function of pY1127 is not described yet. pY1005 have been shown to bind SHC [44]. pY1139 is an important pY for HER2 function. pY1139 is a binding site for EGFR and PI3K [45, 46]. Phosphorylation of Y1139 activates RAS through GRB2 and increases transcriptional activity of STAT3 [47, 48]. Phosphorylation of Y1196 enhances activation of Erk through a RAS-independent pathway and increase the binding affinity of HER2 to CRK which is a member of an adapter protein family. CRK is required for HER2 to increase RAC-dependent cell motility and HER2-mediated inhibition of apoptosis [49-51]. It is interesting to note that pertuzumab did not change the phosphorylation level of HER2 Y1248. We did not observe any significant effect of trastuzumab on pY1248 in CHO-k6 cells in our previous research [24]. However, two recent

studies indicate that trastuzumab increases the phosphorylation level of pY1248 in several BC cell lines, and the enhanced pY1248 phosphorylation is linked to the inhibitory role of trastuzumab on cell proliferation [52, 53].

Phosphorylation of HER2 at canonical pY sites takes place by HER receptor tyrosine kinases following homo- and heterodimerization. HER2 is not phosphorylated only by HER family receptor tyrosine kinases, but it can be also phosphorylated by other kinases in a dimerization-independent manner at tyrosine residues. For example, SRC phosphorylates HER2 at tyrosine 877 located in the P-loop of the kinase domain and increases the kinase activity of HER2 [54]. It is possible that pertuzumab has a ligand-like function that confers HER2 a new conformation providing Y1127, Y1139, and Y1196 residue binding sites for non-HER family tyrosine kinases. However, there is no evidence yet supporting or abrogating this hypothesis. Taken together we suggest that pertuzumab may have roles in non-canonical HER2 activation in a dimerization-independent manner. To test this hypothesis, the effect of pertuzumab and trastuzumab on phosphorylation of non-canonical HER2 phospho-sites and the identity of potential kinases involved in pertuzumab-induced phosphorylation will be investigated in the future research.

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Chapter 6. The effects of trastuzumab on HER2-mediated cell signaling in HER2-positive BC cells

6.1. Abstract

Several studies have shown that trastuzumab has inhibitory effects on HER2-mediated downstream signaling pathways; however, the exact mechanism(s) through which trastuzumab inhibit these signaling pathways is greatly unknown. In SKBR3 cells, our results showed that trastuzumab suppresses the cell viability in presence and absence of HER-specific ligands and arrests the cell cycle at G1 phase when cells treated in DMEM or DMEM supplemented with 10 percent FBS. Moreover, we showed that trastuzumab has inhibitory effects on PI3K/Akt and MAPK signaling pathways which may through inhibition of HER receptor's phosphorylation. In BT474 BC cells, our results revealed that trastuzumab is able to decrease the cell viability and arrest cell cycle at all phases in presence and absence of EGF ligands. In addition, we showed that trastuzumab does not inhibit HER receptors dimerization and phosphorylation. Although, our results demonstrated that trastuzumab can inhibit Akt phosphorylation, no inhibitory effect was found on MAPK signaling pathway. Interestingly, we revealed that, trastuzumab may inhibit Akt phosphorylation through blocking the lipid raft localization of HER receptors and inhibition of c-Src phosphorylation. Altogether, our results show that the mechanism of action of trastuzumab can vary in different types of trastuzumab-sensitive HER2-positive BC cells. This may suggest that the combination therapy with trastuzumab should be carefully selected to increase the efficiency of treatment as well as reduce the possibility of disease recurrence in patients with HER2-positive BC.

Keywords: Trastuzumab, HER receptors, PI3K/Akt pathway, Heterodimerization, Phosphorylation, Lipid raft

6.2. Introduction

The effect of trastuzumab on downstream signaling pathways regulated by HER2 receptors have been investigated in various studies. Many intracellular mechanisms have been suggested for trastuzumab mode of action; however, the controversial results of these studies make it hard to identify the exact mechanisms of action of trastuzumab. Previous studies have shown the inhibitory effects of trastuzumab on PI3K/Akt signaling pathway [1-5]. It has been shown that trastuzumab is able to inhibit Akt phosphorylation at both serine and threonine phosphorylation sites. Moreover, various studies have revealed that trastuzumab can significantly downregulate the expression of the genes involved in cell cycle progression and arrest the cell cycle at G1 phase [3, 6, 7]. It seems that trastuzumab through inhibition of Akt phosphorylation can suppress cell cycle; however, the exact mechanism through which trastuzumab suppress PI3K/Akt signaling pathway is mostly unknown. Some studies suggested that trastuzumab inhibits Akt phosphorylation through inhibition of HER3 receptor phosphorylation [1, 4]. It has been demonstrated that HER3 receptor can provide several binding sites for p85 subunit of PI3K upon phosphorylation by mainly HER2 receptors [8]. On the other hand, several studies showed that trastuzumab can increase the HER2 and Erk phosphorylation which may suggest that trastuzumab increases the tyrosine kinase activity of HER2 receptors [9, 10]. Higher tyrosine kinase activity of HER2 can affect other HER receptors' phosphorylation in cancer cells with HER2 overexpression. Although several studies showed that trastuzumab can inhibit the phosphorylation of Akt, few studies have investigated the exact mechanism through which trastuzumab inhibit Akt phosphorylation. In addition, it is not clear how Akt phosphorylation is decreased after increasing of HER2 receptors phosphorylation by trastuzumab. In present study we investigated the effects of trastuzumab on cell viability, cell cycle progression, HER2 heterodimerization, HER receptors phosphorylation, and HER2-mediated downstream signaling in two trastuzumab-sensitive HER2-positive BC cells including BT474 and SKBR3 cells.

6.3. Results

6.3.1. The effect of trastuzumab on the viability of HER2-positive BC cells

I determined if trastuzumab inhibits the proliferation of HER2-positive BC cells. For this purpose, MTT cell proliferation kit was used to assess the proliferation of BT474 and SKBR3 HER2-

positive BC cells. The cells were treated with trastuzumab for 72 hours in four different conditions including: 1) DMEM only without adding any supplements 2) DMEM supplemented with 10% FBS 3) DMEM supplemented with EGF ligand, and 4) DMEM supplemented with HRGα ligand. It is worth mentioning that even though culturing the cells in the medium without 10% FBS reduced the cell growth, it did not have adverse effect on the health of the cells. Our results showed that trastuzumab inhibited BT474 cell viability in absence and presence of 10% FBS and EGF ligand but not HRGα. In addition, the MTT results revealed that trastuzumab can significantly reduce SKBR3 cell viability in all conditions. As a positive control, we treated the cells with HER2-specific small molecule tyrosine kinase inhibitor, CP-724714. Our results demonstrated that CP-724714 reduced the viability of BT474 and SKBR3 cells in all treated conditions **(Figure 6.1)**. As shown in **figure 4.5b**, the phosphorylation of HER2 receptors in CHO-K6 cells treated with different concentrations of CP-724714 ranging from 1μM to 100 μM was assessed by immunoblotting. The results showed that treating the cells with CP-724714 at concentration as low as 1 μM is sufficient to inhibit HER2 phosphorylation. Therefore, the lowest concentration of CP-724714 tested in this experiment was used for the rest of experiments in this chapter to minimize the possible non-specific activities of CP-724714.
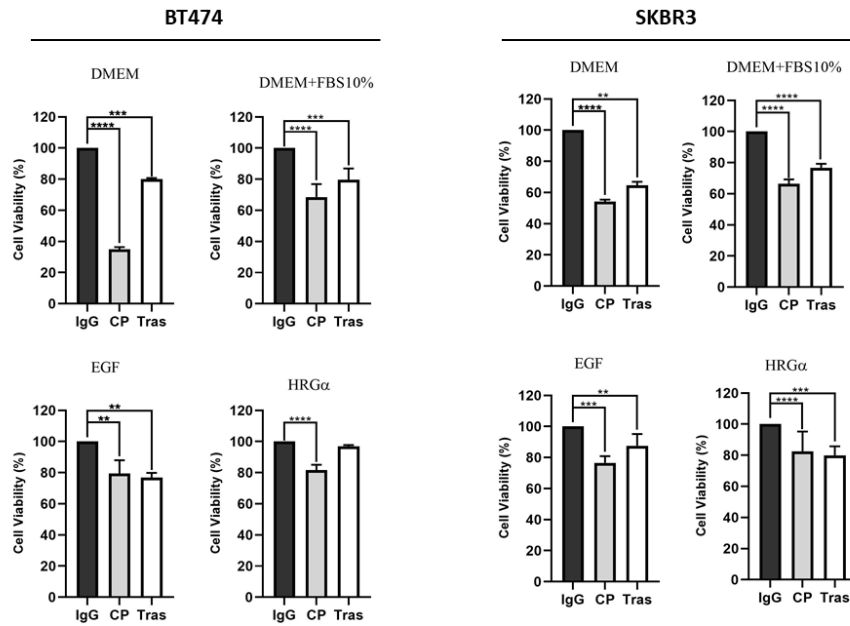
Figure 6.1. The effects of trastuzumab (Tras) on the viability of BT474 and SKBR3 BC cells in absence and presence of 10% FBS, EGF, and HRGα. Cells were treated with 10 μg/ml concentration of trastuzumab for 72 hours. Cells treated with normal human IgG was used as negative control and cells treated with CP-714724 (CP) at 1 μM concentration was used as positive control. Each value is the average of at least three experiments and the error bar is standard error. Each value is the average of at least three experiments and the error bar is standard error. **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

6.3.2. The effect of trastuzumab on the cell cycle progression of HER2-positive BC cells

To identify the mechanism through which trastuzumab reduces cell viability, I investigated the effect of trastuzumab on cell cycle progression of HER2-positive BC cell lines. For this purpose, I treated the cells with trastuzumab at 10 µg/ml concentration in the presence and absence of 10% FBS, EGF, and HRGα for 72 hours. Our result showed that trastuzumab arrest BT474 BC cell cycle at G1 phase and reduce the population of the cells in S and G2 phase in presence and absence of 10% FBS and EGF. Consistent with MTT results, the cell cycle analysis results showed that trastuzumab is not able to inhibit BT474 cell cycle progression in presence of HRGα ligand. In addition, our results revealed that trastuzumab can arrest the SKBR3 cell cycle at G1 phase in presence and absence of 10% FBS. However, our results showed that treatment of the SKBR3 cells with EGF and HRGα can overcome the inhibitory effects of trastuzumab on cell cycle progression. Interestingly, similar results have been obtained when SKBR3 cell treated with CP **(Figure 6.2)**.
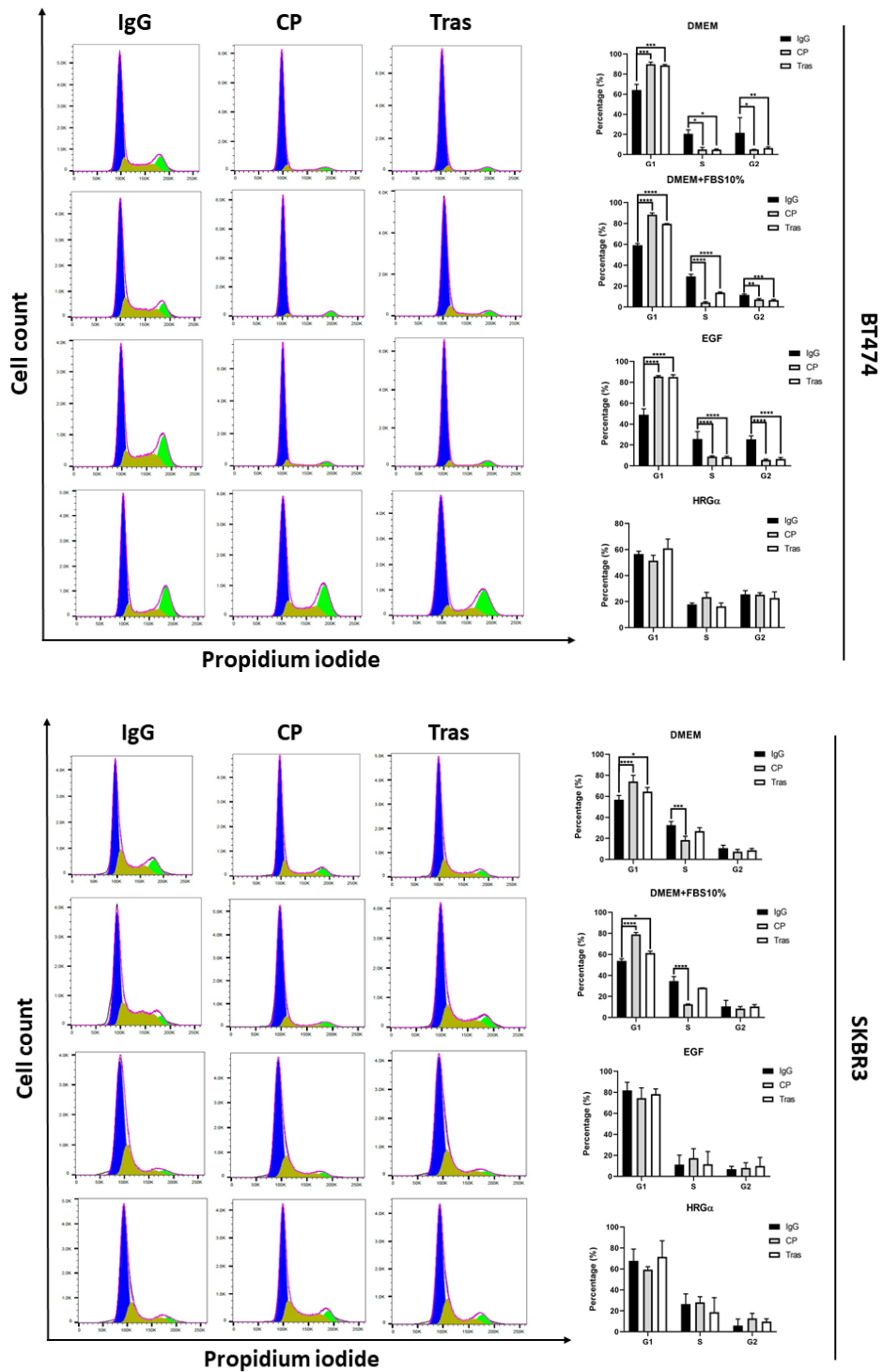
Figure 6.2. The effects of trastuzumab on the cell cycle progression of BT474 and SKBR3 cells in presence and absence of 10% FBS, EGF, and HRGα. Cells were treated with 10 µg/ml concentration of trastuzumab for 72 hours. Cells treated with normal human IgG was used as negative control and cells treated with CP-714724 (CP) at 1 µM concentration was used as positive control. Each value is the average of at least three experiments and the error bar is standard error. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

6.3.3. The effect of trastuzumab on HER2-HER3 and HER2-EGFR heterodimer formation

To assess the effect of trastuzumab on HER2 heterodimer formation, I used immuneprecipitation (IP) assay in BT474 and SKBR3 HER2-positive BC cells. For this purpose, HER2 antibody was used as a primary antibody to precipitate HER2 receptors and heterodimers formation was identified by immunoblotting of EGFR and HER3 receptors. Our results showed no inhibitory effects on HER2 heterodimer formation after treatment of BT474 and SKBR3 BC cells with trastuzumab **(Figure 6.3)**.
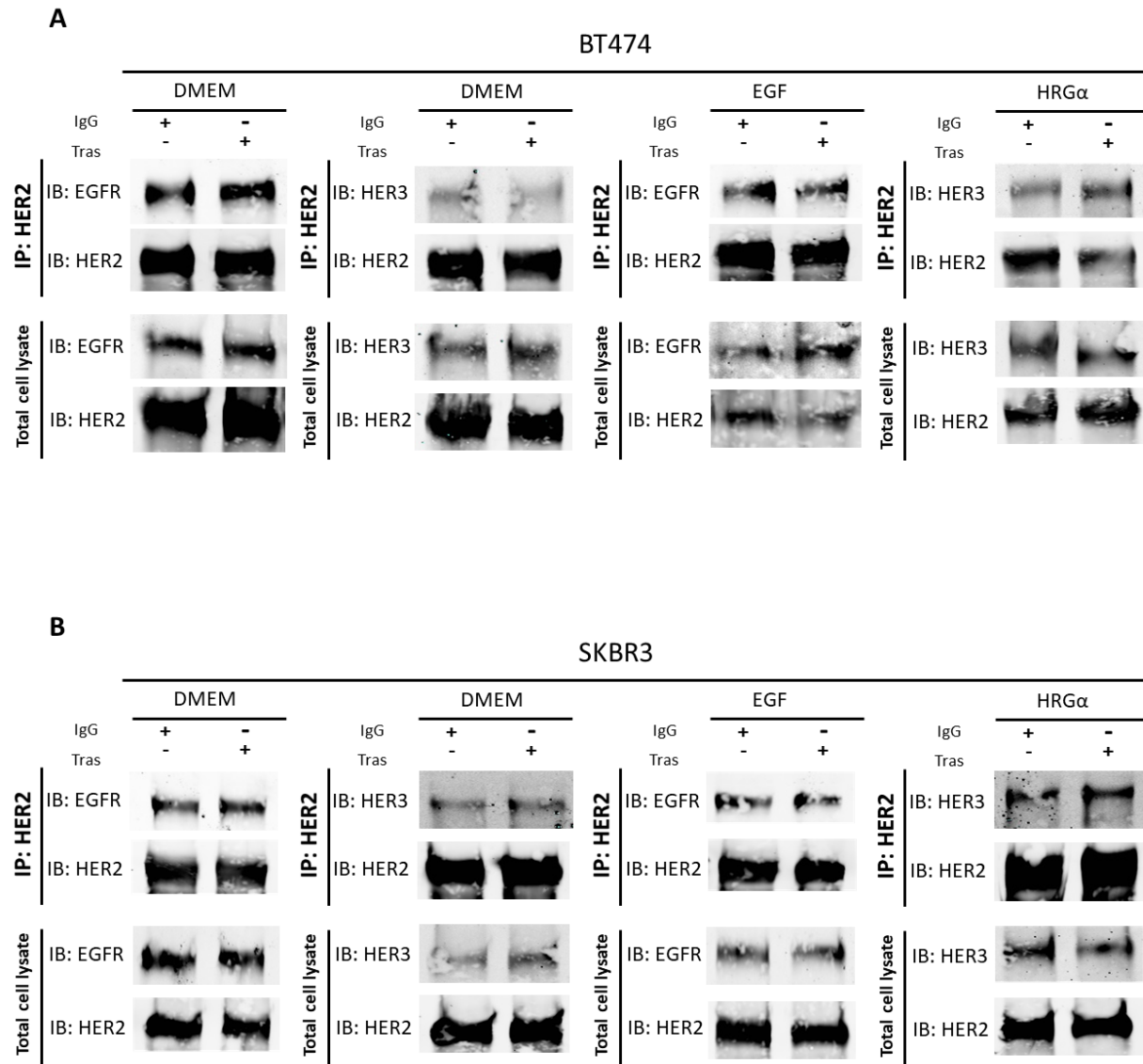
Figure 6.3. The effect of trastuzumab on HER2 heterodimer formation. The effect of trastuzumab at concentration of 10 μg/ml on HER2-EGFR and HER2-HER3 heterodimers in **A)** BT474 and **B)** SKBR3 BC cells for 1 hr. HER2-EGFR and HER2-HER3 heterodimers formation were assessed in the presence and absence of EGF and HRGα at 50 ng/ml concentration respectively. The expression of HER receptors in both IP and total cell lysate (TCL) samples was revealed by immunoblotting. HER2 receptors were precipitated using HER2-specific antibody as primary antibody followed by immunoblotting with the indicated antibodies. Cells treated with normal human IgG was used as negative control.

6.3.4. The effect of trastuzumab on HER receptors' phosphorylation

To determine the effect of trastuzumab on HER receptors phosphorylation, I treated the BT474 and SKBR3 HER2-positive BC cells with IgG (10 µg/ml) as negative control, CP-714724 (1 µM) as positive control, and trastuzumab (10 µg/ml) in presence and absence of EGF and HRGα for 1 hr. Our result showed that trastuzumab increases the HER receptors' phosphorylation with specific patterns in BT474 BC cells. The immunoblotting results revealed that in the absence of any ligands trastuzumab is able to induce the phosphorylation of all HER receptors. However, when the cells treated with EGF or HRGα ligands, trastuzumab elevates the phosphorylation of HER2-EGFR and HER2-HER3 receptors respectively. It seems that binding of trastuzumab to HER2 receptors increases the tyrosine kinase activity of these receptors which can subsequently increase other HER family receptors phosphorylation after formation of heterodimers with HER2 receptors **(Figure 6.4A)**. On the other hand, the effect of trastuzumab on HER receptors' phosphorylation in SKBR3 BC cells is opposite. Our results showed that trastuzumab can suppress the phosphorylation of EGFR and HER3 receptors in the absence of ligands, phosphorylation of HER3 in the presence of EGF ligand, and phosphorylation of all HER receptors in presence of HRGα ligand **(Figure 6.4B)**.
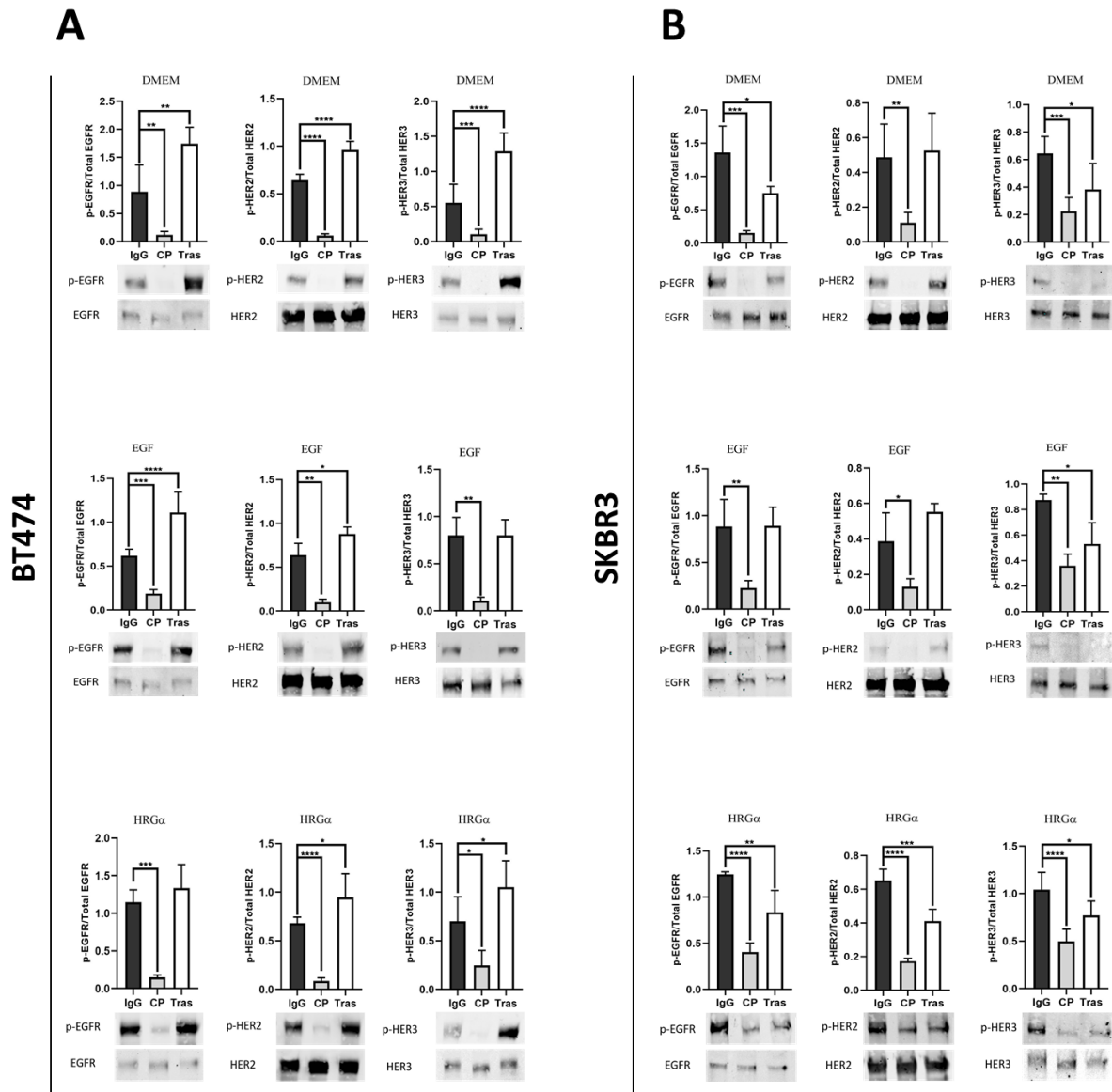
Figure 6.4. The effects of trastuzumab on HER receptors phosphorylation. The cells treated with trastuzumab at concentration of 10 µg/ml in the presence and absence of EGF and HRGα at 50 ng/ml concentration. The phosphorylation of HER receptors in **A)** BT474 and **B)** SKBR3 HER2-positive BC cells were revealed by immunoblotting. Cells treated with normal human IgG was used as negative control and cells treated with CP-714724 (CP) at 1 µM concentration was used as positive control. Each value is the average of at least three experiments and the error bar is standard error. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

6.3.5. The effect of trastuzumab on HER2-mediated downstream signaling pathway in HER2-positive BC cells

To determine the effect of trastuzumab on HER2-mediated downstream signaling, I treated the cells with IgG (10 µg/ml) as negative control, CP-714724 (1 µM) as positive control, and trastuzumab (10 µg/ml) in presence and absence of EGF and HRGα for 1 hr. Our immunoblotting results in BT474 BC cells showed that trastuzumab can suppress Akt but not Erk phosphorylation in presence and absence of EGF ligand. Interestingly, our results demonstrated that trastuzumab is unable to inhibit Akt phosphorylation when BT474 BC cells treated with HRGα ligand **(Figure 6.5A)**. On the other hand, our results in SKBR3 BC cells revealed that trastuzumab can inhibit the Akt phosphorylation at both serine and threonine phosphorylation sites in all treated conditions. In addition, lower Erk phosphorylation has been shown in this study when SKBR3 BC cells are treated with EGF and HRGα ligands **(Figure6.5B)**.
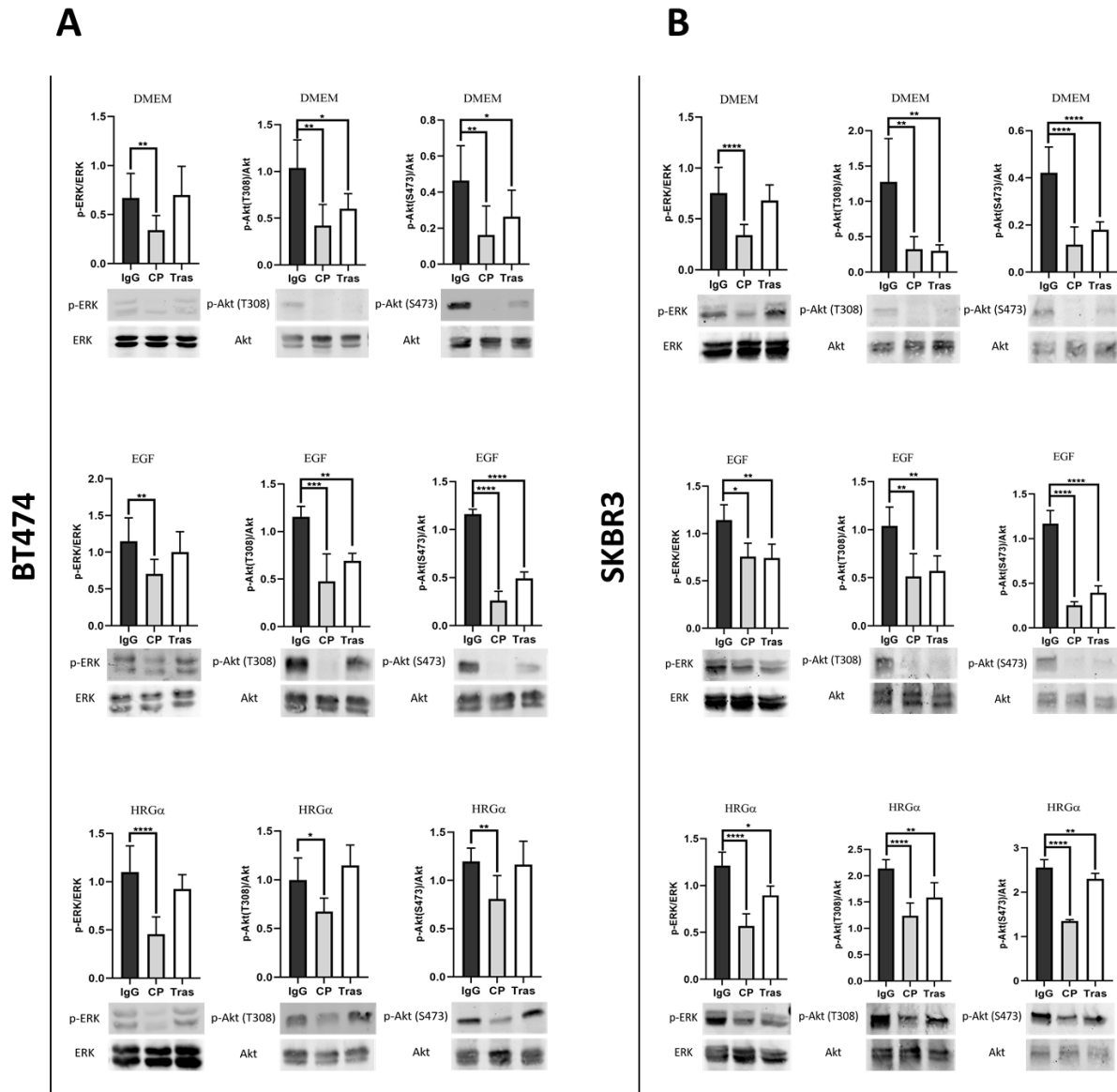
Figure 6.5. The effects of trastuzumab on HER2-mediated downstream signaling pathway. The cells treated with trastuzumab at concentration of 10 µg/ml in the presence and absence of EGF and HRGα at 50 ng/ml concentration. The phosphorylation of Akt at threonine 308 and serine 473 phosphorylation sites as well as Erk phosphorylation in **A)** BT474 and **B)** SKBR3 HER2-positive BC cells were revealed by immunoblotting. Cells treated with normal human IgG was used as negative control and cells treated with CP-714724 (CP) at 1 µM concentration was used as positive control. Each value is the average of at least three experiments and the error bar is standard error. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

6.3.6. The possible role of lipid rafts on trastuzumab-mediated Akt phosphorylation inhibition

We demonstrated that trastuzumab can inhibit Akt phosphorylation without inhibition of HER receptors' phosphorylation or dimerization in BT474 BC cells. This result prompted us to find out if trastuzumab is able to inhibit HER2-mediated downstream signaling through noncanonical pathways. Previous studies have been shown that lipid raft localization of HER receptors in BC cell lines can promote HER-dependent, HER kinase-independent activation of Akt [11]. In addition, it has been shown that trastuzumab can affect HER2 receptors localization and arrangement in BC cell membrane [9, 12]. Therefore, we hypothesis that trastuzumab inhibits the lipid raft localization of EGFR-HER2 heterodimer, which leads to the inhibition of Akt phosphorylation. It has been demonstrated that methyl-β-cyclodextrin (MβCD) can disrupt cell membrane lipid raft through depletion of cell membrane cholesterol [13]. To determine if disruption of lipid raft can suppress Akt phosphorylation in BT474 BC cells, I treated the cells with different concentrations of MβCD. Our results showed that MβCD can suppress Akt phosphorylation in BT474 BC cells which is comparable to the effect of trastuzumab **(Figure 6.6)**. For the next step, we investigated the possible effect of trastuzumab on HER2 and EGFR localization to the lipid raft microdomains of the cell membrane. To do this we treated the cells with IgG (10 µg/ml) as negative control and trastuzumab (10 µg/ml) in presence and absence of EGF for 1 hour and isolated the proteins associated with lipid rafts using Bio-Rad ReadyPrep™ Protein Extraction Kit (Signal). The level of HER2 and EGFR receptors in isolated lipid rafts were examined by immunoblotting. Our results showed that localization of HER2 and EGFR to the lipid rafts was decreased after treatment of BT474 BC cells with trastuzumab **(Figure 6.7A)**. Moreover, we showed that trastuzumab can inhibit the phosphorylation of c-SRC in the lipid rafts **(Figure 6.7B)**. In addition, our IP results revealed that trastuzumab can inhibit binding of c-SRC to HER2 receptors which may affect the phosphorylation of c-SRC **(Figure 6.7C)**.
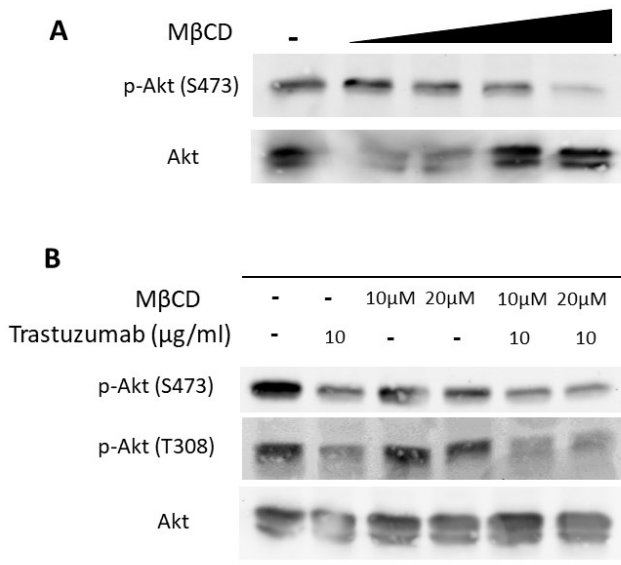
Figure 6.6. The effects of MβCD on Akt phosphorylation in BT474 BC cells. **A)** BT474 BC cells were treated with MβCD at 0-20 μM concentration for 4 hours. The phosphorylation of Akt was then examined by immunoblotting. **B)** BT474 BC cells were treated with MβCD alone at 10 and 20 μM concentrations or in combination with trastuzumab at 10 μg/ml for 3 hours. The phosphorylation of Akt was then examined by immunoblotting.
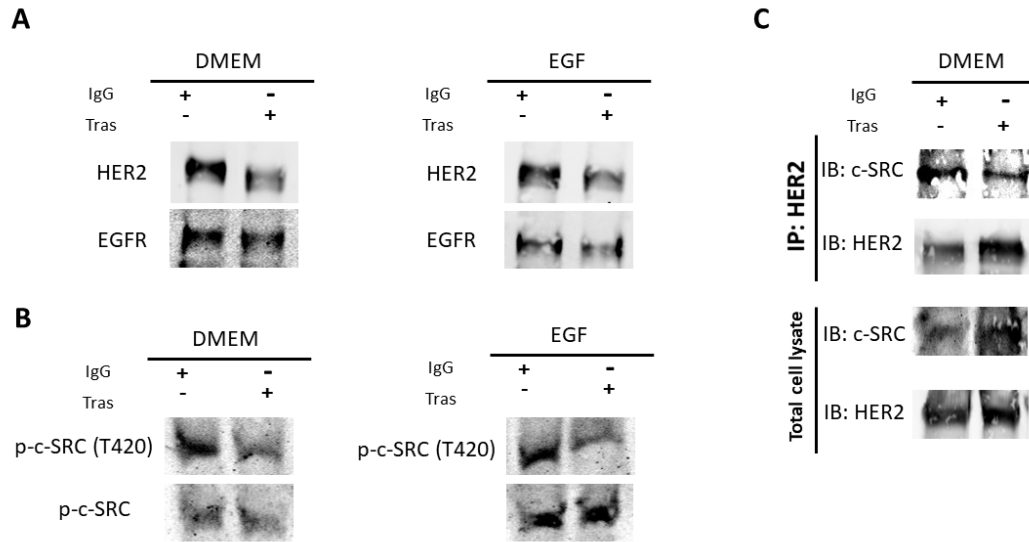
Figure 6.7. The effects of tratuzumab on localization of HER2 and EGFR to the lipid rafts in BT474 BC cells. **A)** The level of HER2 and EGFR and **B)** c-SRC phosphorylation in lipid rafts after treatment of BT474 BC cell with with IgG (10 µg/ml) as negative control and trastuzumab (10 µg/ml) in presence and absence of EGF for 1 hour. **C)** The effect of trastuzumab at concentration 10 µg/ml on association of HER2 and c-SRC BT474 BC cells. The HER2 and c-SRC association were assessed in the presence and absence of EGF at 50 ng/ml concentration. The expression of HER2 receptor and c-SRC in both IP and total cell lysate (TCL) samples was revealed by immunoblotting. HER2 receptors were precipitated using HER2-specific antibody as primary antibody followed by immunoblotting with the indicated antibodies. Cells treated with normal human IgG was used as negative control.

6.4. Discussion

In this study we investigated the effect of trastuzumab on various functions of HER2 receptors from HER2 heterodimerization to HER2-mediated downstream signaling in HER2-positive BC cells. Significant portion of HER2-positive BC cells co-express EGFR and HER3 [14], and HER2-mediated cell signaling, and cell function is closely related to and impacted by other HER receptors, including EGFR and HER3 [15]. It is not clear how the formation of various HER receptors homo- and heterodimers impact the efficacy of trastuzumab in treating BC. One of the significant limitations which make it difficult to determine trastuzumab precise mechanism of action is the formation of various HER dimers in HER2-positive BC cells. To limit the heterodimer formation of HER receptors, we treated the cells in three different conditions including: 1) DMEM only without adding any ligands which enable the cells to form various ligand-independent HER homo- and heterodimers, 2) DMEM containing 50 ng/ml EGF ligand which can increase the HER2-EGFR heterodimers, and 3) DMEM containing 50 ng/ml HRGα which can increase the HER2-HER3 heterodimers formation. It has been shown that different ligands can activate distinct signaling pathways in cells expressing HER receptors [16].

The majority of studies have investigated the effect of trastuzumab in serum-free medium and few studies assessed the possible effects of trastuzumab on HER-specific ligand-activated downstream signaling pathway. Moreover, due to the inhibitory effects of trastuzumab on Akt phosphorylation [1-5] and the significant role of HER3 receptors phosphorylation in activating PI3K/Akt pathway [8], most of these studies have focused on the effect of trastuzumab on HER2-HER3 heterodimer-mediated downstream signaling which can be activated by HRG ligands. In addition, our knowledge about the effect of trastuzumab on EGF ligand-mediated downstream signaling is very limited. Gosh *et al.* have transfected the MCF10A cells with plasmid containing human HER2 and FK506-binding protein (FKBP) sequences to generate a cell model suitable for studying the trastuzumab mode of action in three different conditions [16]. In this model, the AP1510 chemical was used as an artificial ligand to homodimerize HER2 receptors from c-terminal domain. Moreover, the cells were treated with transforming growth factor α (TGFα) and heregulin to induce HER2/EGFR and HER2/HER3 heterodimers respectively. The results showed that, although trastuzumab does not have any inhibitory effects on Akt phosphorylation, the drug can inhibit the HER2 homodimer-mediated Erk1/2 phosphorylation [16]. On the other hand, Bagnato *et al*.

showed that treatment of HER2-positive BC cell line, SKBR3, with trastuzumab can induce redistribution of HER2 receptors on cell's membrane and increase the phosphorylation of HER2 and Erk1/2 [9]. Although there is not any clear explanation about how increased HER2 and Erk1/2 phosphorylation mediated by trastuzumab is correlated with trastuzumab anti-tumor activity, similar results have been reported by other studies [4, 10]. Overall, the findings of these studies are controversial, and more investigations are needed to determine the exact mode of action of trastuzumab. According to the result of these studies, it seems that trastuzumab exerts its anti-tumor effect through various mechanisms which is greatly influenced by the role of HER2 receptors in each type of HER2-positive BC cells.

Our results demonstrated that trastuzumab can reduce BT474 BC cells viability in absence and presence of FBS and EGF. Interestingly, we showed that HRGα ligand can make the BT474 but not SKBR3 cells resistant to trastuzumab. This result suggests that, even though, BT474 and SKBR3 BC cells are both sensitive to trastuzumab, they respond differently to trastuzumab in various conditions. Previous studies have suggested that different expression level of ErbB tyrosine kinase receptors in BT474 and SKBR3 cells can affect the cell response to trastuzumab [17-19]. It has been shown that higher expression of EGFR is correlated with lower sensitivity to trastuzumab in BC cells overexpressing HER2 [17]. The data regarding the effects of trastuzumab on ligand-dependent cell growth is limited and controversial. Gosh *et al.* have shown that trastuzumab is unable to reduce the viability of MCF10A cells transfected with HER2 receptors when cells are treated with EGF and HRGα ligands [16]. In contrast, Yu *et al*. have demonstrated that trastuzumab reduces the viability of BT474 and SKBR3 cells in presence of EGF and HRGα ligands [20].

To unravel the exact mechanism through which trastuzumab exerts its anti-tumor effect, we analyzed the cell cycle of BC cells after treatment of cells with trastuzumab in absence and presence of EGF and HRGα ligands for 72 hours. Our results in BT474 cells showed that trastuzumab can significantly arrest cell cycle at G1 phase and reduce the cell population in S and G2 phase in presence and absence of FBS and EGF. Consistent with MTT results, the cell cycle analysis showed that trastuzumab does not have any effect on cell cycle progression when cells treated with HRGα ligands. In addition, our results showed that trastuzumab can completely suppress the stimulatory effects of EGF ligands on cell cycle progression which may suggest that

in BT474 BC cells trastuzumab has preferential inhibitory effects against HER2-EGFR heterodimer-mediated downstream signaling pathway. Although several studies have shown that trastuzumab can arrest the cell cycle at G1 phase [3, 5, 6, 21], to the best of our knowledge no study has assessed the effect of trastuzumab on BC cell cycle progression in presence of EGF and HRGα ligands. Le et al. have demonstrated that trastuzumab can decrease the expression level of genes involved in cell cycle, DNA replication, and DNA repair [3]. On the other hand, the SKBR3 cell cycle analysis showed that trastuzumab can arrest the cell cycle at G1 phase which is consistent with the findings of previous studies [5, 6]; however, our results revealed that trastuzumab does not have any inhibitory effects of on cell cycle progression when cells treated with EGF and HRGα ligands. Although, our MTT results demonstrated that trastuzumab is able to reduce the SKBR3 cells viability in presence of EGF and HRGα ligands, these inhibitory effects are attenuated by these ligands. It is possible that trastuzumab can reduce the viability of the SKBR3 cells through inducing both cell cycle arrest and apoptosis and ligands suppress the inhibitory effects of trastuzumab on cell cycle progression. Clinical studies have shown that trastuzumab can significantly induce apoptosis in breast tumors [22]. In addition, the findings of several studies have revealed that trastuzumab can downregulate the expression of genes with anti-apoptotic functions such as Bcl-2 and survivin [23-27].

Ligand binding domain of HER receptors, except for HER2 receptors, can bind to specific ligands to form either homodimer or heterodimer [8]. Moreover, overexpression of HER2 receptors can increase the formation of HER2 ligand-independent homo- or heterodimers [28, 29]. Upon dimerization of these receptors, certain tyrosine residues in intracellular domain can be autophosphorylated or transphosphorylated to activate several intracellular signaling pathways [8, 28, 30]. Our results showed that trastuzumab does not have any significant effects on ligand-dependant or -independent HER2 heterodimer formation in both HER2-positive BC cells. Similar results have been found by other studies [1, 10, 20]. We previously showed that trastuzumab is unable to inhibit the homodimerization of HER2 receptors in CHO cells overexpressing HER2 receptors [29]. Yu et al. have demonstrated that trastuzumab does not block EGF- and HRGα-mediated HER2 heterodimer formation [20]. On the other hand, the inhibitory effects of ligand-independent HER2 heterodimer formation have been indicated by few studies. Gaborit et al. have used Time-resolved Fluorescence Resonance Energy Transfer (TR-FRET) assay to quantify the HER2-EGFR heterodimer formation in SKOV-3 ovarian cancer cell line [31]. For this purpose,

the cells treated with trastuzumab in complete medium were incubated with anti-HER2 and anti-EGFR monoclonal antibodies conjugated with specific donor or acceptor fluorophores and the emission light was quantified. The results of this study have demonstrated that trastuzumab can significantly reduce the HER2-EGFR heterodimer formation [31]. In this study due to using the complete medium and presence of various ligands and growth factors in the medium, the EGFR-HER2 heterodimer formation can be both ligand-dependent and -independent. Furthermore, to assess the effect of trastuzumab on HER2-HER3 ligand-independent heterodimer formation, Junttila *et al.* have used a reversible cross-linkers to temporarily bind membrane proteins together [1]. They demonstrated that, although, trastuzumab does not block HER2-HER3 ligand-dependent heterodimer formation, it inhibits ligand-independent formation of this heterodimer. However, our results do not support the findings of these paper. One of the reasons that our results differ from these two studies is using different methods to investigate the ligand-independent HER2 heterodimer formation.

We showed that trastuzumab increases the HER receptors' phosphorylation of BT474 BC cell in various treated conditions. In the absence of any supplements, our results showed that trastuzumab can induce the phosphorylation of all HER receptors. However, when cell treated with EGF which increase the HER2-EGFR heterodimer formations, trastuzumab can enhance the phosphorylation of both HER2 and EGFR receptors without affecting HER3 receptors phosphorylation. Similar results have been found when cells treated with HRGα ligands. Several studies have shown that trastuzumab is able to increase the phosphorylation of HER2 receptors [9, 10]. We have previously shown that trastuzumab does not have any inhibitory effects on HER2 phosphorylation in CHO cells overexpressing HER2 receptors [29, 32]. Dokmanovic *et al.* have reported that treating the HER2-positive BC cells with trastuzumab can induce the phosphorylation of HER2 receptors at 1248 tyrosine phosphorylation sites which can increase the binding of Csk-homologous kinase (CHK) to HER2 receptors and reduce the cell growth [10]. These results may suggest that binding of trastuzumab to HER2 receptors can increase the tyrosine activity of these receptors. Indeed, it has been shown that trastuzumab can significantly increase the kinase activity of HER2 receptors in BT474 BC cells, but not SKBR3 cells, after treating the cells with this drug for 1 hour [10]. On the other hand, we demonstrated that trastuzumab has inhibitory effects on HER receptors' phosphorylation in SKBR3 cells which is in contrast to our findings in BT474 cells. Our results showed that trastuzumab suppress HER3 receptors phosphorylation in all treated conditions.

Previous studies have indicated that trastuzumab is able to decrease the phosphorylation of HER3 receptors in serum-free medium [1, 4, 10]. In addition, the immunoblotting results pointed out that trastuzumab can inhibit the phosphorylation of EGFR receptors in absence and presence of HRGα, but not EGF ligands. Since EGF ligands can induce the EGFR homodimer formation [33], EGFR can be phosphorylated via other EGF receptors and this may compensate the inhibitory effects of trastuzumab on EGFR phosphorylation. Overall, these results may suggest that the structure of HER2 receptors as well as the expression level of other HER receptors in BC cells can affect the trastuzumab mode of action in various HER2-positive BC cells. For instance, several studies have attributed the higher expression of exon 16 deleted HER2 (d16HER2) isoforms to the better responses of patients with HER2-positve BC to the trastuzumab [34, 35].

PI3K could be activated by HER receptors either directly through the interaction between its p85 subunit and HER receptor or indirectly through the activated Ras [36, 37]. A negative regulator of PI3K is the phosphatase and tensin homologue deleted on chromosome 10 (PTEN). The function of PI3K in cell survival is mediated by Akt, a serine threonine (Thr) kinase [38-40]. Akt contains an N-terminal Pleckstrin homology (PH) domain, a C-terminal regulatory domain, and a central kinase domain. Akt is recruited to the plasma membrane by the interaction of its SH3 domain with PIP3 (generated by PI3K), which induces the conformational change of Akt to allow the phosphorylation of its Thr 308 by membrane-localized 3-phosphoinositide-dependent kinase 1 (PDK-1). Following the additional phosphorylation of Ser 473 by rapamycin complex 2 (mTORC2), Akt is fully activated. Akt controls various cellular functions by phosphorylating several intracellular proteins, including the glycogen synthase kinase 3 (GSK3), the BCL2-associated agonist of cell death (BAD), and forkhead box O transcription factors (FoxO). Akt also activates mTORC1, which protects the cell from undergoing apoptosis [39-41]. We showed that trastuzumab has significant inhibitory effects on HER2-mediated downstream signaling pathways and its effects vary in different HER2-positve BC cells. Our results in BT474 BC cells demonstrated that trastuzumab can decrease the phosphorylation Akt at both serine and threonine phosphorylation sites in presence and absence of EGF ligands; however, it does not have any effects on Erk phosphorylation. Moreover, we revealed that trastuzumab is unable to inhibit Akt phosphorylation when cells treated with HRGα ligand. This result is in consistent with MTT and cell cycle analysis results which show that trastuzumab does not have any inhibitory effects on cell viability and cell cycle progression in presence of HRGα ligand. This may suggest that treatment

of the cells with HRGα ligand can reactivate the PI3K/Akt pathway and make the cells resistant to trastuzumab. On the other hand, in SKBR3 BC cells, trastuzumab reduce Akt phosphorylation in all treated conditions. In addition, we showed that trastuzumab can suppress ligand-dependent Erk phosphorylation. These results are consistent with MTT results which show that trastuzumab can reduce the viability of SKBR3 BC cells in presence and absence of EGF and HRGα ligands. Moreover, we showed that trastuzumab can inhibit HER3 receptors phosphorylation in all treated conditions. It has been demonstrated that HER3 receptors plays a crucial role in activating of PI3K/Akt pathway by providing five different binding sites for p85 subunit of PI3K [8]. Therefore, inhibition of HER3 receptors phosphorylation can significantly suppress Akt phosphorylation and inhibit the cancerous features of BC cells. Several studies have determined the inhibitory effects of trastuzumab on Akt phosphorylation [1, 3-5], although few studies showed opposite results [16, 42]. The effect of trastuzumab on MAPK signaling is unclear due to the controversial results. The findings of previous studies have identified both inhibitory and activatory effects of trastuzumab on MAPK signaling pathway [9, 10, 16, 20]. Our results showed that the effect of trastuzumab on Erk phosphorylation varies in different type of HER2-positive BC cells. Overall, we revealed that trastuzumab can significantly inhibit HER-mediated PI3K/Akt signaling pathway in HER2-positive BC cells.

Our results in BT474 BC cells raised this question of how trastuzumab suppress the Akt phosphorylation without affecting HER receptors' phosphorylation and heterodimerization. Finding the answer for this question became even harder when our results showed that trastuzumab increase the phosphorylation of HER receptors in different conditions. To answer this question, we examined the effect of trastuzumab on noncanonical signaling pathways through which HER2 receptors activate PI3K/Akt pathway. Previous studies have shown that HER receptors are significantly localized in lipid raft microdomains of BC cells [43-47]. Orr *et al*. have revealed that the cholesterol contents of lipid rafts can facilitate the mobility and heterodimerization of HER2 and EGFR receptors [48]. It has been shown that EGFR receptors can activate PI3K/Akt signaling pathway in BC cells after localization into lipid rafts [49]. In addition, it has been reported that the EGFR-mediated Akt activation which is independent of kinase activity of EGFR receptors is mediated by c-Src protein function [11]. On the other hand, the clinical studies have shown that higher HER2 expression is significantly associated with higher FAK expression as well as higher c-Src and Akt phosphorylation in BC patients [50]. Furthermore, *in vitro* studies have revealed

that the formation of HER2-Src-FAK complexes mediated by TGFβ ligands can increase the Akt phosphorylation [51]. Wang *et al*. have demonstrated that inhibiting the kinase activity of Src can disrupt the binding of HER2 receptors to integrins and FAK and suppress the FAK and Akt phosphorylation. It has been revealed that that phosphorylation of FAK can induce Akt phosphorylation through providing a docking site for p85 subunit of PI3K [52]. Therefore, it is possible that trastuzumab inhibit Akt phosphorylation via inhibition of HER receptors localization to lipid rafts and subsequent inactivation of c-Src activity. We showed that disruption of lipid rafts in BT474 BC cells can significantly reduce the Akt phosphorylation. In addition, our results demonstrated that trastuzumab can inhibit the localization of both HER2 and EGFR receptors into lipid rafts and decrease the phosphorylation of c-Src in presence and absence of EGF ligands. Interestingly, our IP experiments indicated that trastuzumab can suppress the binding of c-Src to HER2 receptors. Nagata *et al*. have shown that trastuzumab can activate PTEN function by inhibiting the binding of Src to HER2 receptors and phosphorylation of Src at 416 tyrosine phosphorylation sites [53]. Overall, our results may suggest that trastuzumab can inhibit Akt phosphorylation through HER2-mediated noncanonical pathways, although more investigations should be carried out to determine the exact mechanism.

6.5. References

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Chapter 7. Discussion and future directions

7.1. Discussion

Since approval of trastuzumab in 1998 [1, 2], many clinical and experimental investigations have been carried out to unravel the trastuzumab mechanism of action and find an appropriate combination therapy to enhance the treatment efficacy of trastuzumab. Several clinical trials have used trastuzumab in combination with other dugs to treat HER2-positive metastatic BC. For instance, it has been shown that anthracyclines do not increase the efficiency of trastuzumab [3]; however, administration of everolimus, a mTOR inhibitor, in combination with trastuzumab can significantly benefit the patients [4]. In addition, several clinical studies have been carried out to determine the correlation of certain proteins' expression with trastuzumab response and resistance. Moreover, *in vitro* and *in vivo* studies have suggested various intracellular and extracellular mechanisms for anti-cancer effects of trastuzumab. It has been demonstrated that trastuzumab can induce the immune response against HER2-poitive tumors through a mechanism known as ADCC [5, 6]. Furthermore, studies have suggested the inhibitory roles of trastuzumab on HER2 dimerization [7, 8], HER receptors' phosphorylation [8, 9], HER2-regulated downstream signaling [10, 11], and HER2 cleavages[12], even though the findings are controversial.

Although significant progress has been made in identifying the mode of action of trastuzumab and increasing the efficacy of trastuzumab in treating the patients with HER2-positive BC, many questions remain to be answered. According to clinical findings the majority of patients with HER2-positive BC do not respond to trastuzumab or become resistant to this targeted therapy. There is not any practical guideline for separating patients who are sensitive to trastuzumab from the resistant ones. In addition, our knowledge about the mechanism(s) which HER2-positive breast tumors activate to overcome trastuzumab antitumor effects is limited. Therefore, it is necessary to clarify how trastuzumab exerts its anti-tumor effects in various types of HER2-positive BC and how cancer cells become resistant to this therapy. Pertuzumab has been specifically designed to block HER2 dimerization and HER2-mediated signaling [13]. The combination of pertuzumab with trastuzumab and docetaxel was approved for treatment of patients with metastatic HER2-positive BC [14]. Although, it has been shown that pertuzumab can inhibit ligand-dependent HER2 heterodimerization [15, 16], our understanding about the effect of this drug on ligand-independent HER2 homo- and heterodimerization is very limited. In this thesis, we aimed to investigate the

possible effects of trastuzumab and pertuzumab on canonical and non-canonical HER2-regulated signaling using cell models lacking endogenous HER expression as well as HER2-positive BC cells.

HER2-positive BC cells express different members of HER receptors family which can form 10 different homo- and heterodimers. Although many studies have investigated the effects of trastuzumab and pertuzumab on HER2 heterodimerization and HER2 heterodimer-mediated downstream signaling, there is no study to evaluate the effects of trastuzumab on HER2 homodimerization and phosphorylation in absence of other HER receptors expression. Therefore, we adopted a cell model that allows us to specifically examine the effects of trastuzumab and pertuzumab on a single HER receptor without the influence of other HER receptors. We showed that trastuzumab and pertuzumab can specifically bind to HER2 receptors without any inhibitory effects on HER2 homodimerization and phosphorylation. Moreover, our results showed that CHO cell overexpressing HER2 receptors are not an appropriate cell model to investigate the effects of trastuzumab on HER2-mediated downstream signaling. Therefore, we used two trastuzumab-sensitive HER2-positive BC lines to evaluate the effect of trastuzumab on HER receptors heterodimerization, phosphorylation, and HER-mediated downstream signaling in absence and presence of HER-specific ligands. Our results demonstrated that trastuzumab mechanism of action can be different in various types of BC and treating the cells with different ligands can affect the trastuzumab mode of action. Although our findings suggest that trastuzumab can suppress HER receptors' phosphorylation and canonical HER2-mediated signaling pathway in SKBR3 BC cells, the mode of action of trastuzumab in BT474 BC cells is completely different. We revealed that treatment of BT474 BC cells with HRGα ligand makes cells resistant to trastuzumab; however, this ligand is unable to completely suppress the inhibitory effects of trastuzumab in SKBR3 cells. Our results in BT474 BC cells suggest that trastuzumab may exert its anti-cancer effect through a novel mechanism which is independent of HER heterodimerization and phosphorylation. We found that trastuzumab can block the HER2 and EGFR localization into lipid rafts in both the presence and absence of EGF ligands. Moreover, we showed that this process may have an inhibitory role on c-Src phosphorylation which have an important role on lipid raft-mediated PI3K/Akt signaling pathway activation. These results can help us to have a clearer understanding about trastuzumab mechanisms of action in different types of BC cells and possible roles of each HER2 homo- or heterodimer on the trastuzumab mode of action.

7.2. Future directions

Since CHO cells do not express ErbB family receptors [17], they are an appropriate cell model to study HER dimerization and phosphorylation. In chapter 4 we used CHO cells transfected with HER receptors to investigate the specificity of trastuzumab for HER2 and to study the effects of trastuzumab on HER2 homodimerization and phosphorylation. Previous studies have evaluated the effect of trastuzumab on HER2 dimerization and phosphorylation in cells co-expressing different members of HER receptors. To specifically activate single HER2 heterodimer and study the effects of trastuzumab on HER phosphorylation, CHO cells overexpressing HER2 receptors can be transfected with other EGFR or HER3 receptors. It will deepen our knowledge about the exact effects of trastuzumab on each type of HER2 heterodimer and HER2 heterodimer-mediated phosphorylation.

It has been reported that HER2 receptors have different isoforms which can be co-expressed with full-length HER2 and affect HER signaling pathway [18, 19]. Our knowledge about the expression of these isoforms in different HER2-positive BC cells and their possible effects on trastuzumab mode of action is very limited. Exon 16 deletion of extracellular domain of full-length HER2 can generate d16HER2 isoform of HER2 [18]. Higher expression of d16HER2 have been correlated with better response of patients with HER2-positive BCs to trastuzumab [20]. In addition, Palladini *et al*. have demonstrated that trastuzumab has significant inhibitory effect on tumors expressing d16HER2 and tumors without the expression of d16HER2 are resistant to this therapy[19]. It has been shown that d16HER2 receptors can activate several signaling pathways by direct binding and activation of Src non-receptors tyrosine kinase [21]. Since we indicated different mode of action of trastuzumab in two different HER2-positive BC, it worth to investigate if expression of d16HER2 receptors can affect trastuzumab anti-cancer effects in these BC cell lines. In addition, *in vivo* models can be used to assess the effects of trastuzumab on tumors overexpressing different isoforms of HER2.

Our preliminary results showed that trastuzumab is able to partially inhibit the localization of HER2 receptors into the lipid rafts. This finding has raised several questions which can be answered in future studies. What is the effect of HER receptors' localization to lipid rafts on HER2-positive BC responses to the treatments? What is the role of this process on activation of signaling pathways involved in trastuzumab resistance? Trastuzumab through which mechanism

inhibit HER2 receptors localization to lipid rafts? Which signaling pathways are regulated by HER2 receptors in lipid rafts and in different types of HER2-positive BCs? If trastuzumab inhibits the HER2-mediated signaling pathway in lipid rafts, which combination therapy can increase the efficiency of trastuzumab in a better way? Overall, our understanding about the role of HER2 receptors in regulating of downstream signaling pathways in lipid rafts is very limited and need to be investigated in detail. Such investigations will help us to improve our understanding about the mechanism of action of trastuzumab and increase the efficacy of trastuzumab by using a suitable combination therapy. For instance, due to higher amount of cholesterol in lipid rafts, combination of trastuzumab with drugs interfering the cholesterol metabolism may increase the efficacy of HER2-targeted therapy and improve the treatment outcomes in HER2-positive BC. In addition, these findings will help us to design novel therapies based on blocking HER2 activities in lipid rafts to efficiently target HER2-mediated signaling in HER2-positive BCs.

7.3. References

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