

**NOVEL MONOCLONAL ANTIBODIES TARGETING
CONFORMATIONAL ERBB2 EPITOPES**

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

**BY
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OCTOBER 2012**

*To my loving, understanding, caring, self-sacrificing,
smart, wise, imaginative, helpful and inspiring mother, Reyhan*

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

NOVEL MONOCLONAL ANTIBODIES TARGETING CONFORMATIONAL ERBB2 EPITOPES

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ERBB2 is a tyrosine kinase receptor which can act as homodimers or heterodimers with other members of the ERBB family. Nearly 30% of breast cancers overexpress ERBB2, which can be effectively targeted by anti-ERBB2 monoclonal antibodies. Trastuzumab directed against an epitope on subdomain IV of the extracellular domain (ECD) of ERBB2 is a clinically used therapeutics but the response rate is poor and acquired resistance is frequent. Pertuzumab that binds to subdomain II and inhibits receptor dimerization is another promising therapeutics under clinical trials. Anti-ERBB2 antibodies directed to novel epitopes are potentially useful tools for replacement and combinatorial therapies. We produced five new anti-ERBB2 antibodies, all directed against epitope(s) present only on the native ECD. They performed selective growth inhibitory effects depending on the level of ERBB2 expression and cellular background. When used alone, novel anti-ERBB2 antibodies displayed modest but significant growth inhibition on SK-BR-3, BT-474 and MDA-MB-361 cells with ERBB2 overexpression; while no detectable inhibition was observed on MCF-7 and T47D cells lacking *ERBB2* amplification. When the antibodies were tested in combination with TNF- α , they acted synergistically on SK-BR-3 cells, producing upto 80% growth inhibition; but performed antagonistically on BT-474 cells. Detailed investigation of a representative antibody indicated G1-arrest as the main mechanism of the anti-proliferative effects exerted on SK-BR-3 cells. Antibody treatment induced permanent inhibition of DNA synthesis, leading to accumulation of cells at G1-phase; an effect which was accelerated in the presence of TNF- α . In addition, treated SK-BR-3 cells displayed inhibition of Akt and ERK1/2 phosphorylation leading to cyclin D1 accumulation and growth arrest, independently from TNF- α . Novel antibodies against conformational epitopes present on the extracellular domain of ERBB2 receptor may serve as new analytical and diagnostic tools, in addition to being potent anti-cancer bioactive molecules. Cell-dependent synergy and antagonism between anti-ERBB2 antibodies and TNF- α provide evidence for a complex interplay between ERBB2 and TNF- α signaling pathways. Such complexity may drastically affect the outcome of ERBB2-directed therapeutic interventions.

ÖZET

KONFORMASYONAL ERBB2 EPİTOPLARINI HEDEF ALAN ÖZGÜN MONOKLONAL ANTİKORLAR

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Bir tirozin kinaz alması olan ERBB2, gerek homodimer yapıda, gerekse ERBB ailesinin diğer üyeleriyle heterodimer oluşturarak etki gösterir. Meme kanserlerinin % 30'unu oluşturan ve ERBB2 alması ifadesi yüksek olan vakalar, hedefe yönelik tedavi kapsamında anti-ERBB2 antikorlarıyla başarılı bir şekilde tedavi edilebilmektedir. ERBB2'nin hücre dışı bölgesindeki IV. alt-bölgeye karşı geliştirilmiş Trastuzumab klinikte kullanılan bir terapötik olmasına karşın, elde edilen başarılı yanıt oranı zayıftır ve hastalarda sıklıkla tedaviye karşı bağışıklık gelişir. II. alt-bölgeye bağlanarak alması dimerizasyonunu engelleyen Pertuzumab, klinik çalışmaları yürütülmekte olan diğer bir ümit verici terapötiktir. Yeni epitoplara karşı geliştirilen anti-ERBB2 antikorları, replasman ve kombine tedavilerde kullanılabilecek etkin araçlardır. ERBB2 almasının yalnızca doğal haldeki hücre dışı bölgesine bağlanan beş adet özgün anti-ERBB2 antikoru ürettik. Bunlar, hücredeki ERBB2 ifade düzeyine ve hücrenin yapısal özelliklerine bağlı olmak üzere değişik oranda çoğalmayı baskılayıcı özellik gösterdiler. Tek başlarına kullanıldıklarında, SK-BR-3, BT-474 ve MDA-MB-361 gibi ERBB2 ifadesi yüksek hücrelerin çoğalmasını mütevazî ancak anlamlı ölçüde baskılayarak, MCF-7 ve T47D gibi normal ifade düzeyine sahip hücrelerde belirgin etki göstermediler. Antikorlar TNF- α ile birlikte kullanıldıklarında, SK-BR-3 hücrelerinde sinerjik bir etki göstererek hücre çoğalmasını % 80'e varan oranlarda baskılayarak, BT-474 hücrelerinde antagonistik etki gösterdiler. Antikorlardan seçilen bir tanesiyle yapılan detaylı inceleme, SK-BR-3 hücrelerinde görülen çoğalma baskılayıcı etkinin ana mekanizmasının hücre döngüsünün G1-evresinde durma ile ilgili olduğunu ortaya koymuştur. TNF- α ile birlikte kullanıldığında artan antikor etkisi, DNA sentezini kalıcı olarak engelleyerek hücrelerin G1-evresinde birikmesini sağlamıştır. Buna ek olarak, antikor tedavisine tâbi tutulan SK-BR-3 hücrelerinde TNF- α 'dan bağımsız olarak Akt ve Erk1/2 proteinlerinin fosforlanmasındaki azalmayı takiben Cyclin D1 birikmesi gözlenmiş ve hücre çoğalması engellenmiştir. ERBB2 almasının hücre dışı bölgesindeki üç boyutlu yapıya bağlı oluşan epitoplara karşı geliştirilen özgün antikorlar, anti-kanser özellik taşıyan biyoaktif ajan olma potansiyellerinin yanı sıra, yeni analitik ve tanısal araçlar olarak da kullanılabilir. Anti-ERBB2 antikorları ve TNF- α arasında hücre yapısına bağlı olarak görülen sinerji ve antagonizm, ERBB2 ve TNF- α arasında karmaşık bir etkileşim olduğunu göstermektedir. Böyle karmaşık etkileşimlerin, ERBB2 hedefli tedavilere gösterilen yanıtı ciddi ölçüde etkilemesi mümkündür.

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ABBREVIATIONS

Amp	Ampicillin
AP	Alkaline Phosphatase
APS	Ammonium persulphate
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CaCl ₂	Calcium Chloride
cDNA	complementary DNA
c-Met	Mesenchymal-epithelial transition factor
CO ₂	Carbon dioxide
CNS	Central nervous system
CTD	Carboxy terminal domain
CTF	Carboxy terminal fragment
DAPI	4',6-diamidino-2-phenylindole
ddH ₂ O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dsDNA	Double stranded DNA
ECD	Extracellular domain
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
ErbB	Epidermal growth factor receptor
ErbB1	Epidermal growth factor receptor 1
ERBB2	Epidermal growth factor receptor 2
ErbB3	Epidermal growth factor receptor 3
ErbB4	Epidermal growth factor receptor 4
EtOH	Ethanol

FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FPLC	Fast protein liquid chromatography
g	gram
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IgG	Immunoglobulin G isotype
K	Potassium
Kan	Kanamycin
Kb	Kilobase
kDa	Kilodalton
lt	Litre
MAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
µg	microgram
mg	milligram
MgCl ₂	Magnesium chloride
min	minute
µl	microliter
ml	milliliter
µm	micrometer
µM	micromolar
mM	millimolar
mRNA	messenger RNA
MUC4	Mucin4
N-terminal	Amino terminal
Na	Sodium
NaCl	Sodium chloride
Na-DOC	Sodium deoxycholate
NaOH	Sodium hydroxide
NEAA	Non-essential amino acid
ng	Nanogram
nm	Nanometer

NTD	N-terminal domain
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PI	Protease Inhibitor Cocktail
PI3K	Phosphatidylinositol-3-kinase
pmol	Picomole
PNPP	<i>p</i> -Nitrophenyl phosphate disodium salt
P/S	Penicilline/Streptomycin
PTEN	Protein tyrosine phosphatase
PVDF	Polivinylidene flouride
RNA	Ribonucleic acid
RNAse A	Ribonuclease A
rpm	Revolutions per minute
RIPA	Radioimmunoprecipitation assay
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	Second
SFM	Serum free media
SRB	Sulforhodamine B
TBS	Tris buffered saline
TBS-T	Tris-buffered saline with Tween-20
TCA	Trichloroacetic acid
TF	Transcription factor
TGF- α	Transforming growth factor alpha
TGF- β 1	Transforming growth factor beta
TK	Tyrosine kinase domain
TNF- α	Tumor necrosis factor alpha
Tris	Tris (Hydroxymethyl)- Methylamine
Tzm	Trastuzumab

U	Unit
UV	Ultraviolet
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
w/v	Weight/volume

1 INTRODUCTION

1.1 Breast Cancer

With a lifetime risk of ~12%, breast cancer is the most common cancer in women worldwide [1], ranking second overall. Although the survival rates were increased due to the recent developments in breast cancer therapy, it is still the most frequent cause of cancer-related death in women worldwide (with 458,000 estimated deaths in 2008), and fifth overall [2]. More than 225,000 new cases of female-breast cancer are estimated to occur in 2012, in the United States alone, and 40,000 female deaths are expected as a result [3].

Breast cancers can be divided into two main subtypes according to their estrogen dependency. Estrogen-dependent breast cancers can further be divided into Luminal A and Luminal B types. Luminal A type represents 58% of all breast cancer cases [4]. Luminal B type which accounts for 16% of all breast cancer cases, overexpresses ERBB2 in addition to the common hormone receptors, shows greater proliferative potential and lower frequency of relapse-free survival [5]. Estrogen-independent breast cancers which account for ~26-30% of all breast cancer cases, are more aggressive than estrogen-dependent cases, tend to have poorly differentiated phenotypes, higher recurrence rate and decreased overall survival and can be further divided into basal-like and ERBB2-positive subtypes [5]. Of all breast cancer cases, ~16% are basal-like mostly with a triple-negative phenotype, where the expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and ERBB2 is either low or absent. The rest, which comprise ~6% of all breast cancer cases have an estrogen-independent, ERBB2-positive phenotype [4]. Therefore ~25-30% of all breast cancer cases are associated with ERBB2 overexpression. [6, 7]

1.2 ERBB2

ERBB2 (also known as HER2) is a Receptor Tyrosine Kinase (RTK) identified in the early 1980s which is shown to be amplified in breast cancer cells [8]. The gene contains 27 exons spanning 38 kilobases and is located on chromosome 17q21 [9].

The 4.8 kb mRNA product is translated into a polypeptide of 1255 amino acids [10] and through extensive glycosylation, the precursor protein of 170 kDa is further processed and matured into 185 kDa glycoprotein [11]. In addition to the 185 kDa holoreceptor, breast cancer cell lines were documented to express two ERBB2 peptides of lower molecular mass [12]. The presence of the 68 kDa and 95 kDa peptides were very significant in the ERBB2-overexpressing cell line SK-BR-3 [12].

Being the human ortholog of the rat *Neu* protooncogene [10, 13], ERBB2 belongs to the ERBB transmembrane receptor family together with EGFR (HER1 or ERBB1), ERBB3 (HER3) and ERBB4 (HER4) [14]. Each family member contains four extracellular subdomains at their N-terminal, a transmembrane domain and an intracellular domain containing tyrosine residues that are used for signal transduction (**Figure 1.1 A**). In addition, except for ERBB3, all ERBB receptors have intracellular tyrosine kinase domains and intrinsic kinase activity. Extracellular subdomain I and III were documented to take role in the establishment of receptor conformations that allow dimerization through subdomain II [15] (**Figure 1.1 B**). In the absence of the ligand, the receptor is found on a closed-conformation, where subdomain II makes an intramolecular contact with subdomain IV and is not available for dimerization [16, 17]. Upon ligand binding by subdomains I and III, conformation of the receptor is changed exposing the dimerization domain [18, 19] and allowing dimerization of two receptors present in this open-conformation (**Figure 1.1 B**). ERBB2 is unique in the family in two ways; first it has no identified ligands upto date [20]. Second, the structure of its extracellular region is radically different from other family members; resembling the ligand-activated state of other ERBB receptors, it provides the receptor with a fixed open-conformation [17, 18], negating the requirement of ligand-induced activation. Due to its constitutive open-conformation, ERBB2 is permanently available for heterodimerization with other ligand-activated ERBB-family receptors and is frequently documented as their preferred partner [14] (**Figure 1.1 B**). Since their dimerization arm is permanently exposed, ERBB2 receptors readily homodimerize and activate each-other when they are overexpressed. This is a key mechanism in cell proliferation observed both in ERBB2 overexpressing cells in vitro [21] and ERBB2-positive breast cancers [6, 22]. Recently, an autoinhibition mechanism was revealed, where the α C helix and β 4 sheet in its kinase domain form a loop inactivating ERBB2 kinase. Since many kinase domain mutations observed on

human cancers were localized on this loop, the loss of this autoinhibition was suggested to cause oncogenic ERBB2 activation [23].

Upon dimerization, ERBB receptors cross-phosphorylate one another on multiple intracellular tyrosine residues and provide docking sites for adaptor proteins (**Figure 1.1 C**). In addition, via phosphorylating the adaptor proteins, they create additional docking sites available for the down-stream signalling transduction pathway and alter the activity of intracellular signalling proteins by phosphorylating them (**Figure 1.2**). Main auto- and trans-phosphorylation residues present on the intracellular region of ERBB receptors, and the adaptor molecules that interact with these docking sites are summarized on **Figure 1.1 C** [14]. Being the main autophosphorylation sites on ERBB2; Tyr-1221/1222 and Tyr-1248 interact with Shc through Src homology 2 (SH2) domain and participate in Ras-Raf-MAPK signal transduction pathway [24–26]. In addition, phosphorylated Tyr-1196 and Tyr-1248 interact with Shc through a phosphotyrosine binding (PTB) domain [24]. Also associated with ERBB2 overexpression, phosphorylation of Tyr-1139 induces GRB2 and GRB7 interaction through SH2 domain contributing to a subset of breast tumors [27]. Phosphorylation of Tyr-877 is performed by Src family kinases and was initially suggested to influence the intrinsic kinase activity of ERBB2 [28], however, Tyr-877→Phe substitution of ERBB2 did not alter its biological and/or biochemical properties [25], refuting its role in ERBB2 activation [23]. Tyr-1028 on rat NEU corresponding to Tyr-1023 on human ERBB2, was shown to be a negative regulator of NEU-mediated transformation [29]. Upon EGF stimulation, EGFR heterodimerizes with ERBB2; PI3K interacts with phosphorylated Tyr-1196 on ERBB2 [30] and Ser-1113 is phosphorylated by CaMK II which serves as an indicator of poor prognosis [31]. In addition, neuregulin mediated heterodimerization of ERBB2 and ERBB3 induces phosphorylation of Thr-686 on ERBB2 by PKA; and in turn stimulates the crosstalk between PKA and cAMP leading to activation of PI3K-Akt and MEK-Erk pathways [32]. c-Cbl ubiquitin ligase is recruited to the phosphorylated Tyr-1112 on ERBB2 and induces poly-ubiquitination and proteolytic degradation of ERBB2 [33].

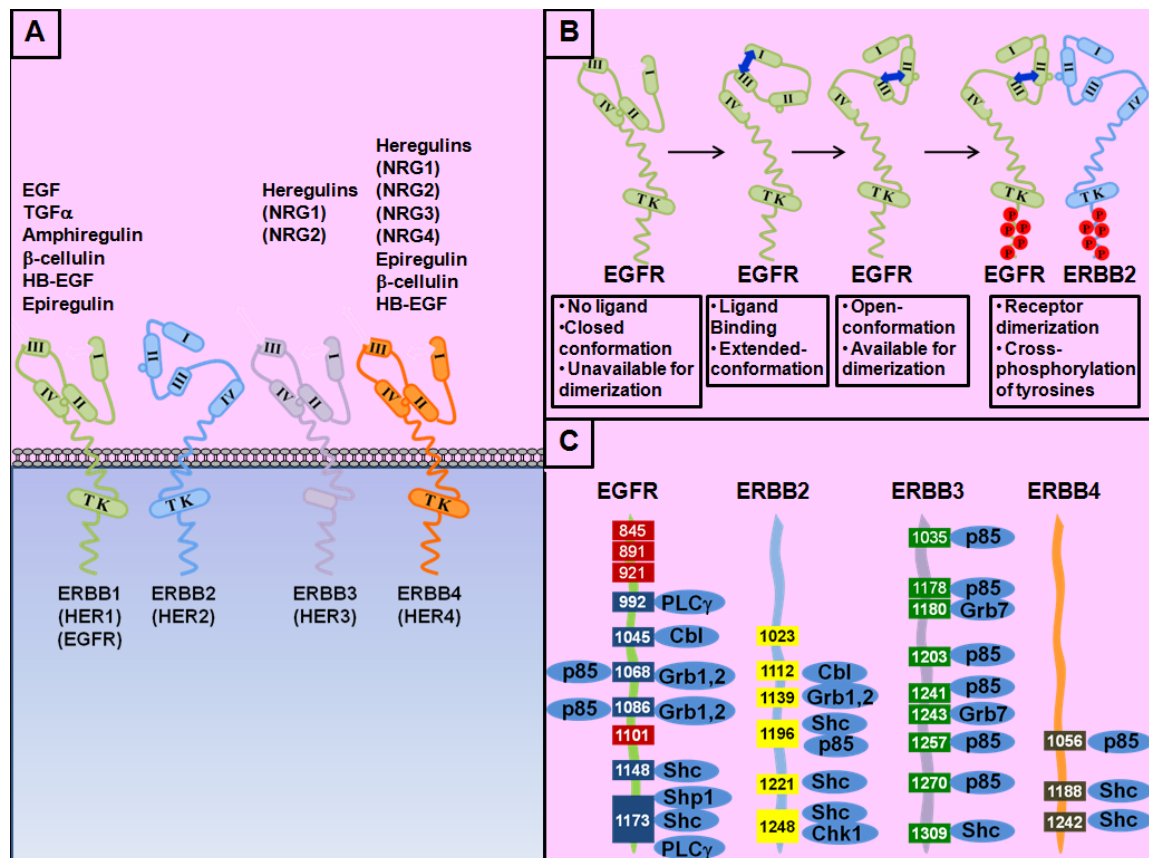


Figure 1.1 ERRB family of tyrosine kinase receptors. **A)** ERRB family of tyrosine kinase receptors are shown in their ligand-free conformation; while ERBB1, ERBB3 and ERBB4 are found in closed-conformation without a bound ligand, ERBB2 is constitutively present in the open-conformation. Each receptor contains four extracellular subdomains, a transmembrane domain and an intracellular C-terminal tail. All except ERBB3 also have an intracellular tyrosine kinase domain. Identified ligands of the receptors are listed above each receptor. **B)** Proposed mechanism of receptor dimerization is illustrated. Upon ligand binding (dark blue arrow), a conformational change occurring at the extracellular subdomains of EGFR exposes the previously buried subdomain II. ERBB2 and ligand-activated EGFR interact through their exposed subdomain II, and heterodimerize. **C)** Main auto- and trans-phosphorylation sites on the intracellular C-terminal tail of each ERRB receptor and the adaptor molecules interacting with the respective phosphorylated tyrosine residues are shown.

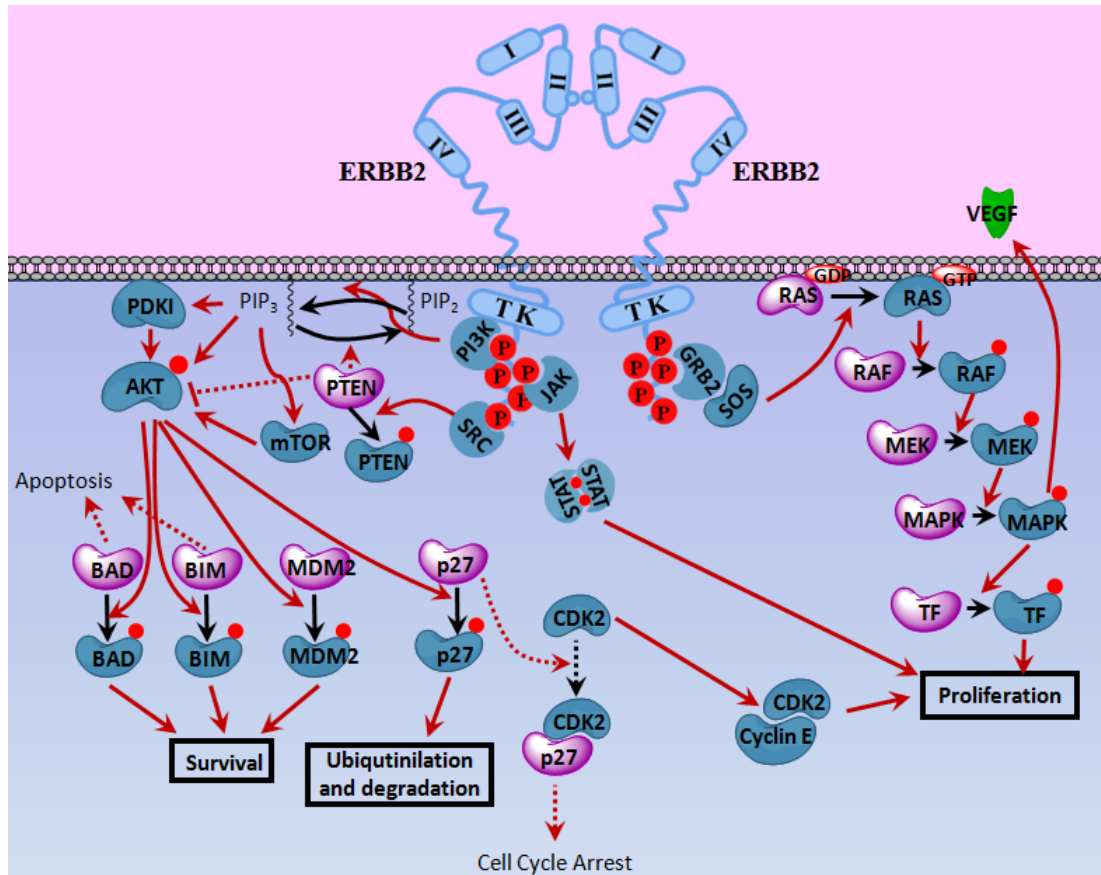


Figure 1.2 ERBB2 signaling. Upon homodimerization, tyrosine residues (in addition to few threonine and serine residues) on the cytosolic tail of ERBB2 are phosphorylated activating the downstream effectors mainly on PI3K-Akt, Ras-Raf-MAPK and JAK/STAT pathways. As a result, proliferative signals are received, pro-apoptotic signals are blocked and cell cycle arrest is inhibited leading to cell survival and proliferation. Effectors shown in dark blue are the molecular forms induced upon ERBB2-dimerization and participate in proliferation. Effectors shown in pale purple are the molecular forms which participate in cell cycle arrest and apoptosis that are decreased upon ERBB2-dimerization. $\rightarrow/\rightarrow$: molecular transitions/signal transductions in action. $\cdots\rightarrow$: inhibited signal transductions. $\bullet$: phosphorylated residues. TK: Tyrosine kinase domain. TF: Transcription factor.

Upon dimerization, receptors transphosphorylate each other and recruit downstream effectors such as PI3K, Src, Grb2 [29, 34–36]. When Src is recruited to intracellular phospho-tyrosine residues on activated ERBB2, it phosphorylates PTEN downregulating its activity. Therefore PTEN can no longer antagonise PI3K pathway. Activated ERBB2 further recruits and activates PI3K, facilitating Akt activation through PI3K-mediated phosphorylation. Activated Akt further phosphorylates pro-apoptotic proteins Bad, Bim and MDM2, leading to cell-survival [37]. It also phosphorylates p27 leading its ubiquitination and degradation. Since p27 is no longer active to block CDK2, CDK2 interacts with CyclinE, leading to cell

proliferation [38, 39] (**Figure 1.2**). As an additional mechanism for silencing tumor suppression, ERBB2 was shown to interfere with TGF β -induced cytostasis and oncogene-induced senescence by deregulating C/EBP β isoform translation [40]. Cell culture conditions were reported to induce a switch between the two main signalling pathways downstream of ERBB2; PI3K and MAPK pathways [41].

It was shown that ERBB2-overexpression in NIH3T3 cells leads to malignant cellular transformation and tumorigenesis [21, 42]. Numerous reports have demonstrated that ERBB2 amplification and receptor overexpression was observed in several human cancers including breast [6, 22], ovarian [43], endometrial [44], gastric [45], lung [46], esophageal [47], oropharyngeal [48], bladder [49]. Relation between ERBB2 status and breast pathology is described by Ross et. al. and shown on **Table 1.1** [50].

Pathologic condition	HER-2 status
DCIS	High-grade subtype, multifocal disease, and cases with comedonecrosis are positive
ILC versus IDC	ILC lower HER-2-positive rate (<10%) restricted to the pleomorphic ILC subtype
Tumor grade in IDC and ILC	HER-2-positive status in low-grade tumors extremely rare
Paget's disease	Virtually all Paget's disease cases are HER-2 positive
Inflammatory carcinoma	No association between HER-2 status and inflammatory carcinoma confirmed to date
Mucinous (colloid) carcinoma	Rare HER-2-positive mucinous carcinomas pursue an aggressive clinical course
Medullary carcinoma	Typical medullary carcinomas are HER-2 negative
Primary versus metastatic carcinoma	A near uniform consensus of multiple published studies states that the HER-2 status of matched primary and metastatic breast cancer samples maintain the same HER-2 status throughout the course of the disease in (at least) 70%–80% of cases
<i>BRCA1/BRCA2</i> mutation-associated carcinomas	Hereditary breast cancer consistently features a lower incidence of HER-2 positivity than sporadic disease
Breast sarcomas	These tumors are HER-2 negative
Male breast carcinoma	No consistent association of HER-2 status or response to anti-HER-2 targeted therapy for male breast cancer; the low number of cases limits confidence in these observations
Benign breast conditions	A near significant association with low-level HER-2 expression in benign breast biopsies with subsequent development of invasive breast cancer has been reported
Abbreviations: DCIS, ductal carcinoma in situ; HER-2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.	

Table 1.1 Relationship between ERBB2 status and breast pathology. Reprinted with permission from "Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN: The HER-2 Receptor and Breast Cancer: Ten Years of Targeted Anti-HER-2 Therapy and Personalized Medicine. The Oncologist 2009, 14:320–68." [50]. Copyright © AlphaMed Press 2009.

A Val664→Glu point mutation found on the transmembrane domain of rat ERBB2/NEU (corresponding to Val659→Glu in humans) was shown to be sufficient to transform cells [51, 52], where the glutamic acid variant was suggested to stabilize dimerization leading to conversion of pro-oncogenic NEU to an oncogenic protein [53]. Through examination of transgenic mice overexpressing ERBB2/NEU, single point missense mutations and in-frame deletions were detected at the juxtamembrane domain; and the cysteine-rich juxtamembrane region was suggested to have an important role in regulation of NEU catalytic activity [54, 55]. While the human equivalent Val659→Glu has been shown to constitutively activate ERBB2 *in vitro* [56], up to date it has never been observed in human breast cancers [57]. Through the computational analysis of the transmembrane regions of the ERBB2-homodimer, it was shown that Val664→Glu mutation stabilizes the active conformation of the dimeric RTK, whereas Ile-655→Val destabilizes the active conformation [58].

ERBB2 polymorphisms/mutations found on human tumors are summarized on **Figure 1.3**. The common Ile655→Val polymorphism that is suggested as a risk factor for breast cancer development is studied by various groups. While some groups reported increased risk of predisposition with Val-allele [59–63], many others observed no-association [64–68] or even decreased risk [69].

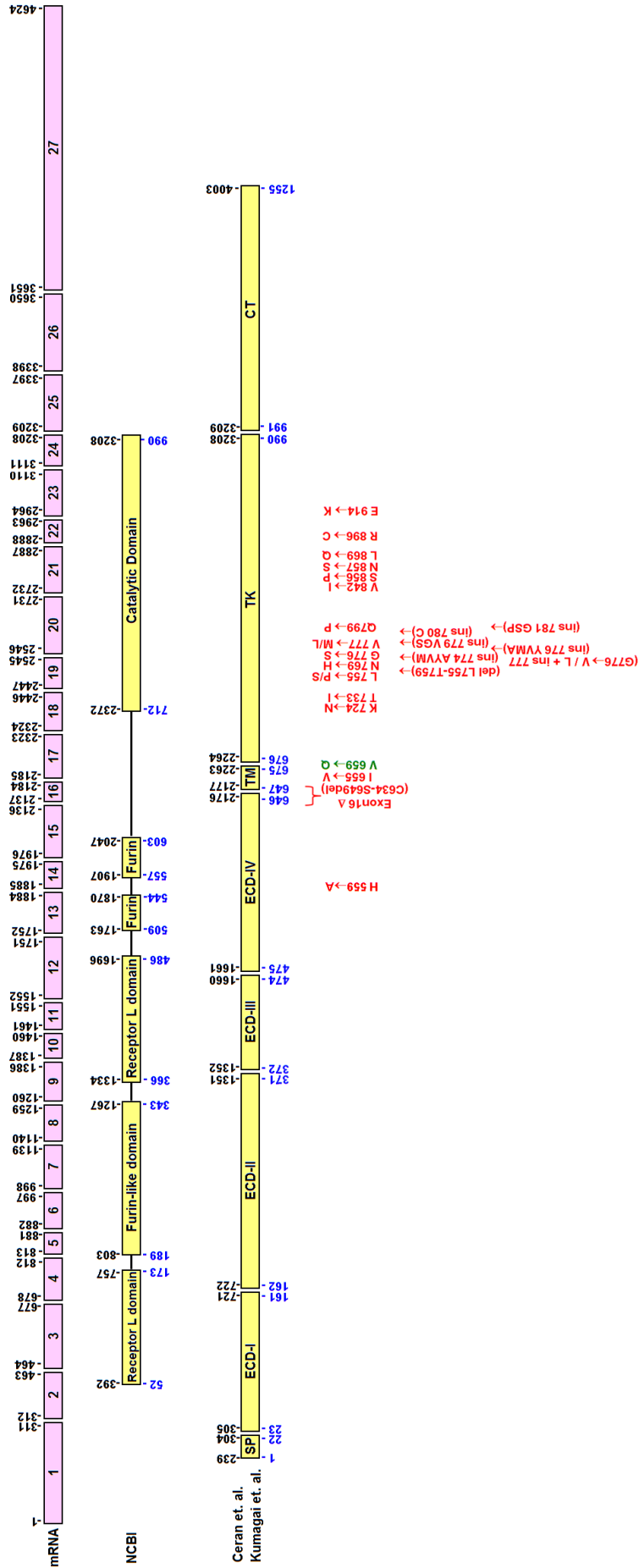
Screening kinase domain of ERBB2 in metastatic breast cancer patients, Gori et. al. have detected one insertion in exon 20 (ins780 (C)) and a missense mutation in exon 21 (Ser856→Pro) [70]. Through the investigation of breast, gastric and colorectal tumors, missense mutations in exon 18 (Lys724→Asn, Thr733→Ile), exon 19 (Leu755→Ser, Asp769→His) and exon 20 (Val777→Leu, Val777→Met, Glu799→Pro), exon 21 (Val842→Ile, Leu869→Glu), exon 22 (Arg896→Cys) and one in-frame deletion on exon 19 (Leu755-Thr759del) was detected on ERBB2-kinase domain [71]. Another study analysing exon 18-24 of ERBB2 on non-small cell lung cancers (NSCLC) identified four main types of insertion at exon 20 (ins776(TyrValMetAla), ins781(GlySerPro), Gly-776→Val + ins777(C), Gly-776→Leu + ins777(C)) [72].

Screening ERBB2 in various tumour types, Stephens et. al. detected further somatic mutations and in frame insertions in NSCLC, glioblastoma, gastric and ovarian tumours. These ERBB2 mutations which were located on exon 19 (Leu755→Pro), exon 20 (ins774(AlaTyrValMet), Gly776→Ser, ins779(ValGlySer)),

exon 21 (Asn857→Ser) and exon 23 (Glu914→Lys) of the kinase domain were not associated with ERBB2 overexpression in these tumours [73]. Kinetic analysis demonstrated that, the catalytic activity of ERBB2 dramatically increases when Gly776 and Gly778 are mutated [23].

Point mutations at the transmembrane domain of ERBB2 was detected in malignant central nervous system (CNS) tumors [74]. As a rare event, His559→Ala point-mutation located on the Trastuzumab-binding site was detected on the juxtamembrane domain of ERBB2 in invasive human breast cancers overexpressing ERBB2 [75].

In a recent study, Zito et al. resequenced every individual amplicon in human breast cells with ERBB2 amplification; the high-resolution analysis of the total *ERBB2* coding sequence on five cell lines and two breast tumors did not reveal any activating mutations on *ERBB2* [76]. This suggests that, receptor overexpression and ERBB2 activating mutations may have mutually exclusive roles in breast cancer development.



ERBB2 amplification and overexpression is tested either at gene level by fluorescence in-situ hybridization (FISH) or at protein level by immunohistochemistry (IHC) respectively. While IHC is easier to perform, faster and less expensive; lack of standardization in fixation and paraffin-embedding protocols, sensitivity of the antigen epitope, and the subjectivity in measurement of staining intensity may cause variation, leading to false-positive and false-negative results. On the contrary, FISH is a standardized procedure providing quantitative more reliable and reproducible results but more expensive, complex and slower than IHC. IHC based HercepTest (DakoCytomation Denmark A/S, Glostrup, Denmark), and FISH based PathVysion (Vysis Inc., Downers Grove, IL) kits are approved by FDA for the verification of HER2 status of breast tumors. Although there is strong correlation (upto 90%) between IHC and FISH results for strong positive cases (IHC +3), tumors with IHC scores of +2 are recommended to be confirmed by FISH analysis [45, 48, 77, 78].

While normal breast cells express ~20,000 ERBB2 receptors on their membrane (IHC score: 0), the protein expression in tumor cells was documented to reach as high as 2,000,000 ERBB2 molecules per cell (IHC score: +3) [79]. ERBB2 levels which were selectively much higher in cancer cells than the normal tissues were shown to strongly correlate with breast cancer prognosis and pathogenesis [22]. *ERBB2* gene amplification is observed in 20–25% of invasive breast cancers and is highly correlated with protein expression [6, 22, 78, 80]. ERBB2 overexpression which was detected both in primary lesions and metastatic sites was found to be relatively homogeneous among ERBB2 overexpressing tumor cells [81]. In addition to above, being a cell surface receptor, ERBB2 was an ideal candidate target for monoclonal antibody (MAb) based breast cancer therapy [82]

1.3 Anti-ERBB2 Antibody Based Targeted Therapy of ERBB2 Overexpressing Breast Cancers

ERBB2 amplification alone was shown to cause cell transformation [21] and was linked to TNF- α /macrophage resistance in vitro which was reversed upon treatment by monoclonal antibodies (MAbs) targeting extracellular domain (ECD) of ERBB2 [83, 84]. In addition, 25% of all breast cancers have ERBB2 overexpression

which was linked with a more aggressive type of breast cancer [6, 22], therefore ERBB2 was acknowledged as a significant therapeutic target in breast cancer treatment. A large panel of MAbs targeting ERBB2-ECD was developed [85] and shown to inhibit monolayer proliferation and revert anchorage independent phenotype [86]. In vivo data also supported the findings that indicate anti-ERBB2 antibody therapy as a treatment regime for ERBB2-overexpressing tumors. Radiolabeled antibodies against ERBB2-ECD were localised to ERBB2-overexpressing mammary tumors in NEU-transgenic mice; suggesting a potential for a successful therapeutics [87].

More than 100 monoclonal antibodies were developed against extracellular region of human ERBB2 by various groups [88]. Among this wide panel of anti-ERBB2 antibodies, mouse monoclonal 4D5 was shown to have anti-tumor effects both in vitro and in vivo [84, 89–91].

In order to overcome the human anti-mouse antibody response that limits the therapeutic efficiency of murine monoclonal 4D5 (MuMAb4D5), the heavy and light chain variable regions (V_H and V_L) of the antibody are humanized by gene conversion mutagenesis. In addition, 5 aminoacids on framework region 3 (FR3) were changed by site-directed mutagenesis to increase the affinity and the therapeutic efficiency of the humanized antibody named as Trastuzumab (Herceptin[®]) [92]. Trastuzumab is the first FDA approved humanized MAb therapeutics targeting ERBB2. Having received first FDA approval in 1998, Trastuzumab is now used both for metastatic and for early-breast cancer treatment mostly in adjuvant settings [93]. When Trastuzumab was used as a single agent therapy for ERBB2-overexpressing, metastatic breast cancer (MBC), ~66-88% of the patients showed primary resistance [94–96]. While combined therapy was better than Trastuzumab monotherapy for MBCs, the majority of patients developed resistance within one year even if they had an initial response [82]. In addition, although Trastuzumab led to promising results in the treatment of ERBB2-overexpressing early-stage breast cancers, ~15% of the patients still developed metastasis [97], demonstrating the clinical limitations caused by the development of Trastuzumab-resistance in the long run.

Recently another monoclonal antibody (2C4) developed at the same time with 4D5 is being investigated as an alternative therapeutics for the treatment of ERBB2-overexpressing breast tumors. 2C4 which initially displayed growth inhibitory

activity weaker than 4D5 was later found promising due to its inhibitory effects on ERBB2 dimerization. The therapeutic activity of Pertuzumab, the humanized form of 2C4 is being tested in clinical trials [98].

Although numerous additional anti-ERBB2 antibodies were developed up to date, Trastuzumab and Pertuzumab are the two main antibodies extensively studied so far.

Through crystal structure analysis, Trastuzumab was found to interact with ERBB2 through three regions (557–561, 570–573, 593–603) on the ERBB2 extracellular domain IV, located juxtamembrane of the receptor [17]. The dimerization inhibitory antibody Pertuzumab binds to the dimerization domain on ECD-II [99].

1.3.1 Mechanism of Action of anti-ERBB2 Antibody Treatment

Being the first monoclonal antibody developed for selective targeting of ERBB2 overexpressing tumors, Trastuzumab provided most of the information gathered on the mechanism of anti-ERBB2 treatment. As summarized on **Figure 1.4**, anti-ERBB2 treatment acts mainly by inhibiting growth inducing signals transduced through MAPK and PI3K pathways. Antibody treatment stimulates tumor cell elimination by further activating antibody dependent cellular cytotoxicity (ADCC) mechanism and apoptotic pathway. In addition, it was suggested that antibody interaction with ERBB2 receptor may decrease receptor shedding, influence endocytic receptor downregulation and enhance the chemotherapeutic response.

Phosphorylation of Tyr-1112 of ERBB2 induces recruitment of c-Cbl to the receptor [33]. Binding of anti-ERBB2 antibodies were shown to stimulate ubiquitinylation of the receptor by activating E3 ubiquitin ligase c-Cbl, leading to ERBB2 degradation [103]. While receptor internalization and degradation upon anti-ERBB2 treatment (mostly by Trastuzumab) is demonstrated by several groups as a means to explain the inhibition of ERBB2 kinase activity and downstream pathways [84, 101, 102, 104–106], others failed to show a decrease in ERBB2 levels [107–110].

1.3.1.2 Growth Inhibition Modulated Through PI3K and MAPK Pathways

ERBB2 overexpressing cells induce Src which phosphorylates and deactivates PTEN. Following Trastuzumab treatment of ERBB2-overexpressing cells, Src is dissociated from ERBB2; PTEN which is left active upon Src-inhibition is translocated to the plasma membrane [111], where it dephosphorylates and deactivates Akt [108]. Since phosphorylation of the internal tyrosine residues on ERBB2 is reduced by anti-ERBB2 treatment [100], anti-ERBB2 treatment blocked PI3K-docking necessary to activate the kinase. Through inhibition of PI3K, the membrane localization of PTEN is increased and phosphorylation level of Akt is decreased leading to increased GSK3 β stability, decreased Cyclin D1 expression and G1-arrest [111–114]. Inducing the association between p27 and CDK2, anti-ERBB2 treatment decreases CDK2/Cyclin E mediated cell cycle progression. The decrease in CDK2/Cylin E mediated phosphorylation and degradation of p27 further stabilizes p27 and leads to a negative feedback resulting in G1-arrest [39, 115, 116]. Cyclin G2 which belongs to the G-cyclins that are upregulated during cell cycle arrest and apoptosis is also induced by Trastuzumab [117]. Decreasing the phosphorylated MAPK levels, the treatment also inhibited the MAPK pathway [113, 118].

1.3.1.3 Inhibition of ERBB2 Shedding

Truncated ERBB2 receptors lacking extracellular N-terminal region were detected in human breast tumors. Through cleavage by matrix metalloproteases, the

wild type 185kDa ERBB2 was reported to be cleaved into two fragments; 110 kDa ERBB2-ECD circulates in the blood [119, 120], while the 95 kDa C-terminal fragment (CTF) is left attached to the cell membrane being constitutively activated [56, 121–124]. Elevated levels of p110 circulating in the serum was shown to be associated with poor prognosis in advanced breast cancer patients [125–128]. CTFs are also detected in a subset of breast cancer patients that developed nodal metastasis, leading to worse prognosis and poor clinical outcome [129, 130].

Trastuzumab decreased ERBB2 shedding by inhibiting ADAM metalloproteinase activity; an effect which is not shared by Pertuzumab [119, 120]. Trastuzumab not only decreased serum ERBB2-ECD levels, but also the intact ERBB2 levels; suggesting the presence of another mechanism in addition to sheddase inhibition [131]. At least ~20% reduction in serum ERBB2 (p110) levels has been proposed as a marker of better prognosis upon Trastuzumab therapy (patients with elevated pre-treatment ECD levels had higher response rates to Trastuzumab) [132–134].

1.3.1.4 ADCC

Antibody dependent cellular cytotoxicity (ADCC) is one of the mechanisms of tumor elimination induced by antibody binding and conducted by natural killer (NK) cells, macrophages and monocytes expressing Fc γ receptors that recognize IgG molecules bound to target cells. Anti-ERBB2 antibodies were shown to elicit apoptosis in breast cancer cell lines through ADCC [86, 92] and significant lymphoid infiltration was observed in Trastuzumab treated patients [107]. Kute et. al. demonstrated that ERBB2 overexpressing breast cancer cells that developed resistance to anti-proliferative effects of anti-ERBB2 treatment *in vitro* were still sensitive to ADCC response and were successfully eliminated by the effector-mediated cytotoxicity; emphasizing the importance of ADCC mechanism on anti-ERBB2 dependent tumor elimination [135].

Fc γ RIII receptor activates effector-mediated target cell lysis whilst Fc γ RIIB performs as an ADCC inhibitory receptor. While mice engineered to express only the activating Fc γ RIII receptors were capable of a successful cytotoxic response, those lacking the activating receptors were insufficient to arrest tumor growth *in vivo*. In

addition, anti-ERBB2 antibodies lacking the Fc region (Fab molecules) were unable to trigger significant anti-tumorigenic response; indicating ADCC as one of the main mechanism of anti-ERBB2 treatment *in vivo* [136, 137].

Antibody molecules were modified to enhance the ADCC response; non-fucosylated oligosaccharide chains on Fc region dramatically enhanced apoptosis both *in vitro* and *in vivo* [138, 139]. In addition, Fc regions engineered for higher FcγR affinity enhanced the *in vitro* effector functions and cytotoxicity [140]. Bispecific antibodies targeting both the ERBB2 receptors present on cancer cells and the immuno-effector cells were shown to significantly induce the apoptotic response [137, 141]. In addition to their ERBB2 specificities, Ertumaxomab target CD3 [142], 2B1 target FcγRIII [143], MDX-H210 target FcγRI [144] (**Figure 1.6**). Adjuvant lymphokines such as interleukin-2 (IL-2) intensified NK-induced ADCC upon anti-ERBB2 treatment both *in vitro* and *in vivo* [145, 146].

1.3.1.5 Inhibition of DNA Repair (after chemotherapy or radiation)

In order to overcome the tumor relapse occurring after the cessation of anti-ERBB2 treatment, the antibody therapy was combined with chemotherapeutics inducing DNA damage such as doxorubicin and cisplatin or with radiotherapy. DNA damage and DNA adducts induced by the chemotherapeutics and radiotherapy stimulates repair pathways. Addition of anti-ERBB2 antibody to the therapeutic cocktail inhibited p21 expression that participates in DNA repair, blocked unscheduled DNA synthesis and DNA-adduct repair leading to enhanced tumor elimination [147, 148].

1.3.1.6 Inhibition of Angiogenesis

Since tumor cell survival partly depends on angiogenesis that provide nutrients, growth factors and oxygen to the tumor microenvironment; cancer cells secrete factors to induce angiogenesis. ERBB2 overexpression was shown to stimulate angiogenesis through PI3K-Akt mediated induction of pro-angiogenic factors like vascular endothelial cell growth factor (VEGF) and interleukin-8 (IL-8) while decreasing the levels of anti-angiogenic factor thrombospondin-1 (TSP-1) [149–151].

Downregulating the PI3K-Akt pathway, anti-ERBB2 treatment counteracted ERBB2-mediated regulation of angiogenic factors, decreased vascular permeability and inhibited angiogenesis [151, 152]. Combination of Trastuzumab treatment with a microtubule-inhibitor (paclitaxel) further enhanced the dephosphorylation of Akt, downregulation of VEGF secretion, reduced endothelial cell migration and decreased the tumor microvessel density (MVD) [153].

1.3.2 Response to Trastuzumab

In phase II clinical trials where Trastuzumab was used as a single agent therapy for ERBB2-overexpressing, metastatic breast cancer, ~66-88% of the patients developed primary resistance to Trastuzumab monotherapy [94–96].

Later on in phase III trials, trastuzumab was combined with paclitaxel or docataxel for treatment of ERBB2-overexpressing metastatic breast cancers (MBCs) [154]. The response rates, time-to-disease progression and overall survival of the combined therapy were superior to Trastuzumab monotherapy, leading to FDA approval of Trastuzumab-based combined treatment regime in 1998 for ERBB2-overexpressing metastatic breast cancers. MBC patients receiving first-line treatment with chemotherapy in combination with Trastuzumab had ~20% lower risk of death than patients treated with chemotherapy alone [155]. Median time-to-disease-progression was 4.9 months for patients that received trastuzumab monotherapy without any prior chemotherapy [95]; however, it was 7.4 months for patients that received Trastuzumab in combination with chemotherapy [155]. While combined therapy was better than monotherapy for MBCs, the majority of patients still developed resistance within one year even if they had an initial response [82].

Afterwards, other clinical trials were established where Trastuzumab was tested for the treatment of early stage breast cancers with ERBB2-overexpression. Disease-free and over-all survival rates were found to be improved by Trastuzumab therapy either in combination with or following chemotherapy [156–158]. These results brought about FDA approval of Trastuzumab in 2006 as part of a treatment regimen containing doxorubicin, cyclophosphamide, and paclitaxel for the adjuvant treatment of patients with early-stage ERBB2-positive, node-positive breast cancer. In 2008, Trastuzumab received further FDA approvals to be used in early stage ERBB2-positive settings. Multiple reports suggest that, a trastuzumab-containing

treatment regime should be recommended to early-stage breast cancer patients carrying ERBB2-amplification and/or overexpression [159–162]. Although Trastuzumab led to promising results in the treatment of ERBB2-overexpressing early-stage breast cancers, ~15% of the patients still developed metastasis [97], demonstrating the clinical limitations caused by the development of trastuzumab-resistance in the long run.

Trastuzumab treatment was most beneficial for ERBB2-positive patients whose tumors displayed gene amplification by FISH or intensive membrane staining (3+) as detected by IHC [95, 155, 156, 158, 163, 164].

1.3.3 Mechanisms of Trastuzumab Resistance

In order to tackle with these limitations faced in clinic, numerous groups have endeavoured to elucidate the mechanisms of trastuzumab resistance *in vitro*, *ex-vivo* and *in-vivo*.

Mechanisms of Trastuzumab-resistance are being studied and reviewed extensively by various groups [82, 97, 165, 166]. Overexpression of other ERBB receptors (i.e. EGFR) and/or their ligands (i.e. EGF, TGF- α , heregulins) and heterodimerization with other receptors types (i.e. IGF-1R) were reported to increase downstream signalling through heterodimerization with ERBB2. Activating PI3K mutations, PTEN deletion, p27 downregulation and increased Akt activity also induce constant activation of downstream pathways stimulating cell proliferation and survival, and lead to Trastuzumab resistance. MUC4-ERBB2 interaction and circulating ERBB2-ECD (p110) in serum was shown to block Trastuzumab binding. Mutations on Fc-receptors were identified as the possible reason of hampered ADCC-response.

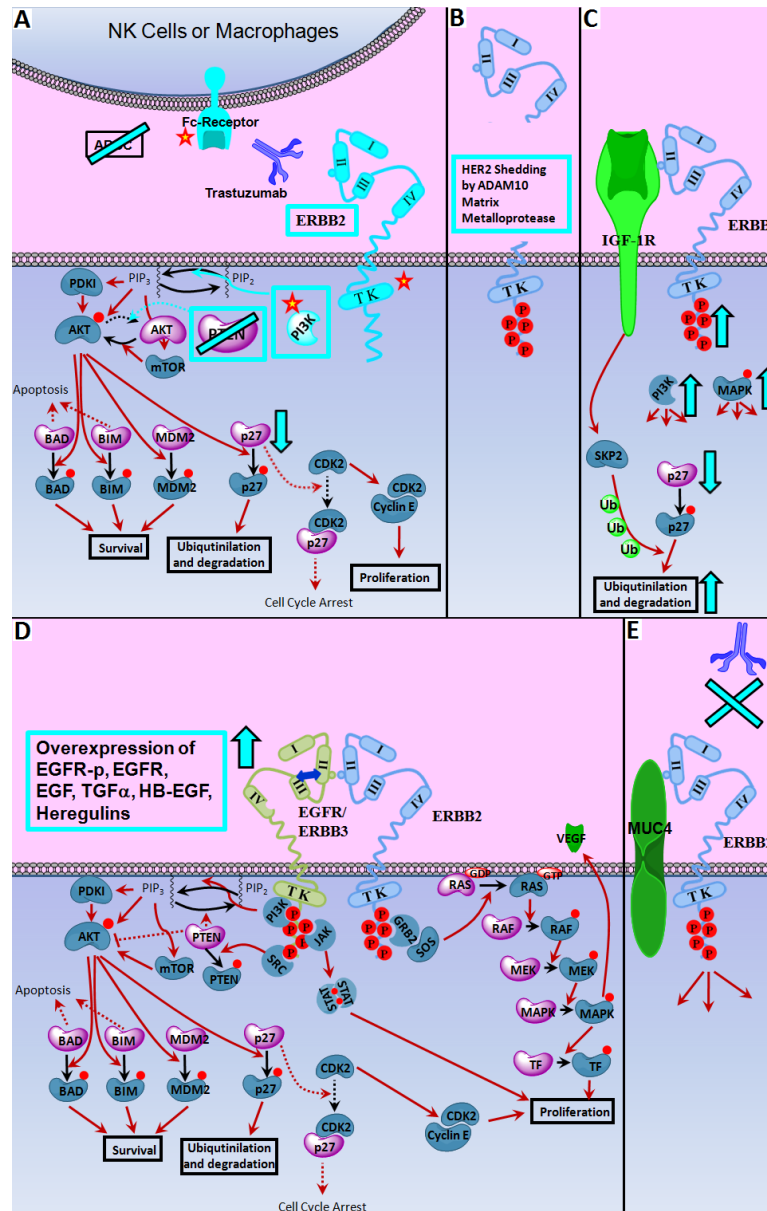


Figure 1.5 Mechanisms of Trastuzumab Resistance. Potential culprits of anti-ERBB2 resistance are shown. Mutations on intracellular region of ERBB2 receptor (mainly at Tyrosine Kinase region), activating PI3K mutations and PTEN deletion is shown to cause constitutive activation of Akt induced signaling, leading to p27 degradation (**A**). Mutations on antibody-binding epitope (**A**), receptor shedding by ADAM proteases (**B**) and receptor masking through ERBB2-MUC4 interaction (**E**) prevents antibody binding. Mutations on Fc receptor hamper the ADCC response (**A**). In addition cross-talk between ERBB2 and other growth factor receptors (**C** and **D**) activate the downstream proliferative pathways. Effectors shown in dark blue are the molecular forms demonstrated or suggested to be active in Trastuzumab resistance, participating in proliferation and survival pathways. Effectors shown in pale purple are the molecular forms demonstrated or suggested to be decreased in Trastuzumab resistance, participate in anti-proliferative pathways. $\rightarrow$ / $\rightarrow$: molecular transitions/signal transductions in action. $\cdots\rightarrow$: inhibited signal transductions. $\uparrow$: upregulation, activation or stabilization. $\downarrow$: downregulation, inactivation or degradation. $\times$: inhibition of receptor binding. $\star$: mutation or polymorphisms. $\bullet$: phosphorylated residues. TK: Tyrosine kinase domain.

1.3.3.1 Variations in ERBB2-ECD

Since Trastuzumab binds to a juxtamembrane position on the extracellular domain of ERBB2 receptor tyrosine kinase, any variation in that region will interfere with Trastuzumab-response. Missense mutations and in-frame deletions at the juxtamembrane domain of ERBB2 were found in transgenic mice overexpressing ERBB2/NEU and shown to be capable to oncogenic transformation when introduced into wild type ERBB2/NEU [55]. Yet, common point mutations in ERBB2-ECD have not been documented in human breast tumors (Nahta et al. 2006). A rare His-559-Ala point-mutation identified on the Trastuzumab-binding site on the juxtamembrane domain of ERBB2 might account for some resistance observed in ERBB2-overexpressing invasive human breast cancers, but far from explaining the high frequency of Trastuzumab-resistance observed in clinic [75].

Truncated ERBB2 receptors lacking extracellular N-terminal region were detected in human breast tumors. Since they lack the Trastuzumab binding extracellular domain IV, it is not surprising that they are irresponsive to Trastuzumab treatment. ERBB2 Carboxy-Terminal-Fragments (CTFs) were suggested to be produced either through proteolytic receptor shedding (as in the case of p95) [121–124] or by alternative ERBB2 translation (either starting from methionine-611 located upstream of the transmembrane domain as in the case of 611-CTF, or from methionine-687 downstream of the transmembrane domain) [167, 168]. CTFs were detected in a subset of breast cancer patients that developed nodal metastasis, leading to worse prognosis and poor clinical outcome [129, 130]. In addition, ERBB2-overexpressing patients were shown to be less responsive to Trastuzumab if they expressed p95; the response rate was 51.4% for tumors expressing full-length ERBB2, while it was 11.1% for patients expressing p95 [169]. HSP90 inhibitors which block downstream signalling induced by p95-ERBB2, were suggested as a therapeutic option for p95-overexpressing, Trastuzumab resistant breast cancer types [170].

ERBB2 N-terminal fragments (p100 and p110) circulating in the serum that are produced either through the cleavage of matrix metalloproteases [119, 120] or by alternative splicing also participate in Trastuzumab resistance [171, 172]. Identical to full-length ERBB2 sequence at its 5' end, the alternative transcript diverged from the wild type sequence due to an in frame stop codon 61 nucleotides upstream of the

transmembrane domain. The truncated ERBB2 of 100 kDa is found both intracellularly at the perinuclear cytoplasm and secreted into the conditioned media [171]. It has been reported that, p110 competed with full-length ERBB2 present on cell surface for binding to anti-ERBB2 antibodies [123, 173], and elevated levels of p110 circulating in the serum was shown to be associated with poor prognosis in advanced breast cancer patients [125–128].

Another alternative splicing event deleted the entire exon 16 which is coding for amino acids immediately preceding the transmembrane domain of the receptor on ERBB2 ECD-IV. Associated with constitutive ERBB2 activation [174] and stronger transformation capacity [175], this ERBB2 variant ($\Delta 16$ ERBB2) with a 16 amino acid in-frame deletion (C634-S649del) was detected in human breast tumors [174, 176]. A later report indicated that, the oncogenic ERBB2 $\Delta 16$ was found in 89% of the lymph-node positive breast tumors with ERBB2-overexpression [177]. Although C634-S649 is not involved in Trastuzumab-ERBB2 interaction [17], tumor cell lines with this variant were found to be resistant to Trastuzumab [174, 177].

One other alternative splicing event produces a 68 kDa Herstatin which only retains the total extracellular domain I (ECD-I) and most of extracellular domain II (ECD-II); replacing the remaining sequence [178]. Through its unique C-terminal tail which substitutes the extracellular domain III (ECD-III) of the wild type ERBB2, Herstatin was shown to bind ERBB2 ECD-II [179], EGFR, Her4, IGF-IR [180], prevent ERBB2 dimerization with Her3 and EGFR [181] and inhibit TGF- α , EGF and heregulin mediated growth of breast cancer cells [182, 183] similar to the dimerization inhibitor antibody Pertuzumab [99, 184]. On the contrary to other alternative splicing products of ERBB2 which are suggested to be associated with worse prognosis, Herstatin which acts as an endogenous pan-HER inhibitor was suggested as a therapeutic regimen to prevent ERBB2 mediated growth [185]

1.3.3.2 Activating Mutations on ERBB2 Transmembrane and Kinase Domain

Being the earliest detected mutation on NEU; Val664→Glu was commonly observed in *Neu* transgenic mice and was shown to constitutively activate NEU favouring its homo- and hetero-dimerization [51–53, 186]. While the human

equivalent Val659→Glu has been shown to constitutively activate ERBB2 *in vitro* [56], up to date it has never been observed in human breast cancers [57]. However, point mutations at the transmembrane domain was detected in malignant central nervous system (CNS) tumors [74]. Although rarely detected in invasive human breast cancers with ERBB2 overexpression, His559→Ala point-mutation located on the Trastuzumab-binding site may lead to Trastuzumab resistance [75]. In addition, kinase domain mutations mentioned above may cause constant activation of the downstream effectors, decreasing the response to Trastuzumab treatment.

1.3.3.3 Inhibition of Receptor Downregulation

Since downregulation of the ERBB2 receptor is one of the mechanisms suggested for the action mechanism of anti-ERBB2 treatment, it is not surprising to observe antibody resistance upon blockage of receptor downregulation.

During endocytosis, the endocytic vesicle is known to rapidly acidify following the internalization of the receptor. Since TGF- α is more basic than EGF, it quickly dissociates from the receptor, allowing EGFR to recycle to the cell surface instead of being degraded. This leads to the prolonged stimulation of both EGFR, and its partners in heterodimerization [187]. Since TGF- α did not interfere with Trastuzumab binding to ERBB2 receptor, Valagebra et. al. have suggested that, TGF- α may induce Trastuzumab-resistance by hampering the endocytic pathway through uncoupling ERBB2 from the Cbl ubiquitin ligase. They demonstrated that, exogenous TGF- α expression in breast cancer cells reduced Trastuzumab-mediated ERBB2 endocytosis, receptor downregulation and cell growth inhibition [188]. In addition they also observed TGF- α induction *in-vivo* following Trastuzumab therapy.

Through the action of fatty acid synthase (FAS), ERBB2 was shown to colocalize with lipid rafts in breast cancer cells, inhibiting antibody internalization [189]. Pharmacological or RNAi-mediated FAS blockage was demonstrated to sensitize ERBB2 overexpressing breast cancer cells to the Trastuzumab induced growth inhibition and apoptosis [190].

Rac-1, a Ras-like small GTPase was also demonstrated to cause Trastuzumab resistance by inducing cytoskeletal disorganization and impairing anti-ERBB2 mediated endocytic down-regulation of the receptor [191].

1.3.3.4 Receptor Masking

Through heterodimerization with ERBB2 receptor, the sialomucin complex MUC4 was shown to block interaction of anti-ERBB2 antibody Trastuzumab with the receptor; leading to therapeutic resistance [192]. Interestingly, anti-MUC4 antibodies decreased the anti-ERBB2 binding instead of restoring the interaction; substantiating the role of steric hindrance [193].

ERBB2 receptor in Trastuzumab resistant JIMT-1 cells [192, 194] were found to physically interact with the overexpressed CD44 receptor [195]. CD44 which is also accepted as a breast cancer stem cell marker [196] was shown to aid in internalization and downregulation of ERBB2 upon anti-ERBB2 treatment. The authors suggested that 6 months after the cessation of Trastuzumab treatment, anti-ERBB2 antibodies will be cleared from the circulation and CD44 cells which have downregulated their ERBB2 will re-express the receptor, displaying lower Trastuzumab/ERBB2 ratio on their membrane, leading to relapse [195]. They also recommended that screening for CD44 positivity will be useful when ERBB2 overexpressing cells are to be targeted with radioactive isotopes or chemotherapeutics that need to be internalised. Apart from its role in ERBB2 downregulation, CD44 pathway leads to anti-ERBB2 resistance through a totally different mechanism. When CD44 was bound by its bulky ligand hyaluronan, access of Trastuzumab to its epitope on ERBB2 was blocked due to steric hindrance; an effect similar to MUC4 [195].

1.3.3.5 Crosstalk Among Other ERBB Family Members

While ERBB2 is a ligand-less receptor, other ERBB family members are stimulated by a number of ligands, which induce their homo/heterodimerization with ERBB family members, preferentially with ERBB2 [197]. Several groups have indicated that activation of the downstream pathways through these ligands is one of the mechanisms responsible for Trastuzumab resistance.

Investigating Trastuzumab resistant cell lines that were established by continuous Trastuzumab treatment of BT-474 xenografts, Ritter et. al. detected

EGFR dependent phosphorylation of ERBB2. Trastuzumab resistance was shown to be correlated with overexpression of HER ligands (TGF- α , EGF, heregulins, betacellulin and heparin-binding EGF) in addition to increased levels of EGFR, phosphorylated EGFR and EGFR/ERBB2 heterodimers; suggesting EGFR and its ligands as potential inducers of Trastuzumab resistance [198–200]. In addition, as discussed in **section 1.3.3.3**, TGF- α was suggested to decrease Trastuzumab sensitivity by interfering with receptor degradation and downregulation [188]. TGF- β receptor stimulation by TGF- β binding was demonstrated to activate TACE/ADAM17 which in turn induce the secretion of TGF- α , amphiregulin, and heregulin secretion stimulating anti-ERBB2 resistance [201]. An independent study demonstrated that Trastuzumab-mediated deactivation of Akt further stimulated TACE/ADAM17-induced release of ERBB ligands such as heregulin and β -cellulin creating a negative feedback loop and resulting in anti-ERBB2 resistance [202].

Lacking a tyrosine kinase domain itself, once stimulated by its ligand, HER3 heterodimerizes with EGFR or ERBB2 and activates the downstream effectors through the tyrosine kinase of its partners [203, 204]. Trastuzumab was shown to be inefficient in preventing heregulin mediated ERBB2/HER3 heterodimerization, ERBB2 phosphorylation and Akt activation, suggesting HER3 receptor and its ligands as potential inducers of Trastuzumab resistance [184, 198]. Prevention of ERBB2 heterodimerization by Pertuzumab or inactivation of downstream effectors by adjuvant tyrosine kinase inhibitors were shown to inhibit growth of Trastuzumab-resistant cancer cells [184, 199, 205, 206]. Another study demonstrates that, interacting with ERBB2/ERBB3 heterodimer, IGF1-R induces the formation of heterotrimeric ERBB2/ERRB3/IGF1-R complex which activates downstream effectors in Trastuzumab resistant cells [207].

1.3.3.6 Constitutive Activation of Effector Proteins in PI3K Pathway

Since PI3K pathway was known to be effective in ERBB2-mediated signalling, and Trastuzumab was shown to exert its action through blockage of the signal-transducers in this pathway, it was not surprising that PTEN was one of the suspects of Trastuzumab resistance. In addition, it has long been known that, almost half of all breast cancers carried PTEN mutations, PTEN haploinsufficiency (LOH) or

epigenetic downregulation [208–210]. In order to demonstrate the effect of PTEN loss in Trastuzumab resistance, Nagata et. al. induced Trastuzumab resistance by anti-sense-oligonucleotides against PTEN [111]. In addition, to prove the necessity of Akt inhibition in overcoming Trastuzumab resistance, they have rescued the resistant phenotype (induced by PTEN anti-sense oligonucleotides) by using PI3K inhibitors. PI3K inhibitors were also proven useful to treat ERBB2-overexpressing cells which developed resistance following continuous exposure to Trastuzumab and had elevated levels of phosphorylated Akt [211]. Furthermore, breast cancer patients with PTEN-deficiency were reported to respond poorer to Trastuzumab therapy [111, 212]. On the contrary, patients having elevated levels of PTEN were reported to have better Trastuzumab responses [213, 214].

PI3K gene amplification and gain-of-function mutations on its catalytic subunit (p110) rendering the kinase constitutively active are commonly found in many types of human cancers [215–218]. These PI3K mutations disabled the modulation of the proliferative pathway causing resistance to anti-ERBB2 therapies [212, 214, 219]. Constitutive Akt activation was also shown to rescue proliferative loss of ERBB2 signaling [220]; causing resistance to anti-ERBB2 treatment [114, 211, 221].

Decreased p27 levels and elevated CDK2 activity were observed in Trastuzumab resistant cells that also displayed higher S-phase fraction and increased doubling speed. Induction of p27 expression by transfection or inhibition of the proteasome complex restored anti-ERBB2 sensitivity [222]. Inhibition of nuclear localization of p27 induced a similar resistance [223]. Downregulation of p27 was also shown in Trastuzumab-resistance caused by the heterodimerization of ERBB2 and IGF1R [222, 224, 225]. Consequently, p27 was suggested as a therapeutic target in treating anti-ERBB2 resistance caused by aberrant signalling upstream [82].

Recently, Scaltriti et. al have demonstrated the role of abnormal Cyclin E level in Trastuzumab-resistance. A genomewide copy-number variation analysis on the Trastuzumab-resistant cells has revealed cyclin E gene-amplification. In addition, comparing Trastuzumab-treated breast cancer patients according to the level of Cyclin E expression on their tumors, the group has shown an association between Cyclin E amplification/overexpression and worse clinical outcome. To confirm their hypothesis, they have further demonstrated decreased tumor growth of Trastuzumab-resistant xenografts upon CDK2 inhibition [226].

1.3.3.7 Increasing Signalling From Other Receptors

In addition to heterodimerization with other ERBB receptors, ERBB2 was found to interact with other receptors leading to activation of oncogenic pathways and anti-ERBB2 resistance.

IGF-1 mediated IGF-1R induction and subsequent IGF-1R/ERBB2 heterodimerization was demonstrated to decrease p27 levels and p27/CDK2 association leading to attenuation of anti-ERBB2 mediated G1-arrest in Trastuzumab resistant breast cancer cells [224, 225]. Clinical data indicate that, membrane IGF-1R expression was associated with a lower response to Trastuzumab/vinorelbine therapy [227]. While the dimerization inhibitory anti-ERBB2 antibody Pertuzumab was inefficient in preventing the interaction between ERBB2 and IGF-1R, anti-IGF-1R antibody α -1R3 sensitized the cells to anti-proliferative effects of Trastuzumab [228]. In addition, as mentioned in **section 1.3.3.5**, IGF1-R was shown to be present in a heterotrimeric ERBB2/ERBB3/IGF1-R complex in Trastuzumab resistant cells [207].

Met receptor that is induced by its ligand hepatocyte growth factor (HGF) was also shown to trigger Trastuzumab resistance in ERBB2 overexpressing breast cancer cells by inhibiting upregulation of p27. Similar to IGF signalling, both Met receptor and HGF was found to be upregulated in patients resistant to Trastuzumab/vinorelbine therapy [229].

Elevated levels of the receptor tyrosine kinase EphA2 and cleaved form of MUC1 (MUC1*) were also detected in Trastuzumab resistant breast cancer cell lines. Trastuzumab-induced EphA2 phosphorylation was shown to cause Src-mediated activation of PI3K-Akt and MAPK pathways, leading to anti-ERBB2 resistance. EphA2 overexpression was correlated with worse prognosis and lower survival of ERBB2-overexpressing breast cancer patients [230]. Sublethal doses of Trastuzumab exposure induced overexpression of MUC1*; a growth factor receptor that is also elevated on embryonic stem cells and correlated with drug resistance [231].

1.3.3.8 Fc Receptor Status

ADCC was suggested as one of the main anti-oncogenic mechanisms of anti-ERBB2 treatment. The antibody bound to the target tumor cells engage with IgG receptors present on the effector cells and induce effector-mediated cytotoxicity. As explained above, strong antibody-effector cell interaction is necessary for a successful cytotoxic response. There are three groups of Fc receptors found on human leukocytes; FcγRI, FcγRII and FcγRIII. While FcγRIIa and FcγRIIIa are activating receptors, FcRIIb and FcγRIIIb have inhibitory effects on ADCC. Analyses of the correlation between Fc receptor polymorphisms and clinical response to Trastuzumab treatment indicated that FcγRIIIa 158V/V and FcγRIIa 131H/H phenotypes either alone or in combination significantly enhanced the effector response both in vitro and in vivo, leading to stronger ADCC response and better clinical outcome [232, 233].

1.3.3.9 DNA Methylations

Microarray analysis of paired breast cancer samples indicated significant epigenetic regulations in breast cancer etiology and prognosis which can be used as biomarkers and therapeutic targets [234]. Although anti-ERBB2 resistant and sensitive subtypes are not selectively compared in this study, it suggests that, not only genetic but also epigenetic regulations might have a role in drug responses of certain tumor types.

1.3.3.10 Other Factors

Microarray analysis of Trastuzumab resistant cells revealed protein kinase A (PKA) network as one of the signalling mechanisms responsible for the constant activation of PI3K/Akt signalling. Darrp-32, suppressor of both PKA and protein phosphatase-1 (PP-1) was elevated in Trastuzumab resistant cells [235, 236].

Heat shock proteins (HSPs) were shown to be important in Trastuzumab response. When upregulated, HSP27 was shown to increase ERBB2 protein stability and induce Trastuzumab resistance [237]. HSP90 level was inversely correlated with

survival rates of breast cancer patients and HSP90 inhibitors were shown to reduce Trastuzumab resistance *in vitro* [170, 238].

1.3.3.11 Complexity Accompanying ERBB2 gene amplifications

As reviewed by Vanden Bempt et. al., the genes co-amplified with ERBB2 depends on the amplicon size, in turn influencing the clinical outcome of Trastuzumab therapy [239]. In addition, as reviewed above, there are other genes whose expression and activity critically affect the ERBB2 pathway, although they are not located on the same amplicon with ERBB2. It appears that resolving the aberrant expression pattern of each patient is key to designing a tailored therapeutic cocktail. In the near future, progressions in personal genomics technology would allow clinicians unveil genomic signature of every individual patient and offer personalized therapy [240–243]

1.4 Alternative Therapeutics for Treatment of ERBB2 Overexpressing Breast Cancer

A subset of ERBB2 positive tumors was inherently resistant to Trastuzumab treatment due to the constitutive activation of the proliferative pathways regardless of ERBB2 activation status. Others were shown to develop Trastuzumab resistance after prolonged treatment. Since cytostatic growth inhibition was suggested as the main effect of Trastuzumab treatment [39, 116, 244, 245], the clonal selection and proliferation of once dormant cells that are activated by other genetic or epigenetic factors appears to be one of the main reasons of the acquired clinical resistance [246]. Whether anti-ERBB2 resistance is inherent or *de novo*, there are two main approaches to revert this phenotype; a) blocking the initial activation of additional proliferative pathways by targeting their receptors, b) preventing the final message by targeting the molecular effectors down the signal transduction pathways. While former includes MAbs targeting ERBB2 dimerization domain (Pertuzumab) or other growth factor receptors such as EGFR (Cetuximab), IGF-1R (α -IR3), VEGF (Bevacizumab); the latter mainly includes kinase inhibitors targeting either the

receptor tyrosine kinases such as ERBB2 and VEGF (Lapatinib, Erlotinib, Neratinib, Sunitinib, etc) or intracellular kinases such as PI3K and mTOR (Dasatinib, Perifosine, Everolimus etc) [50, 247–250] (**Figure 1.6**).

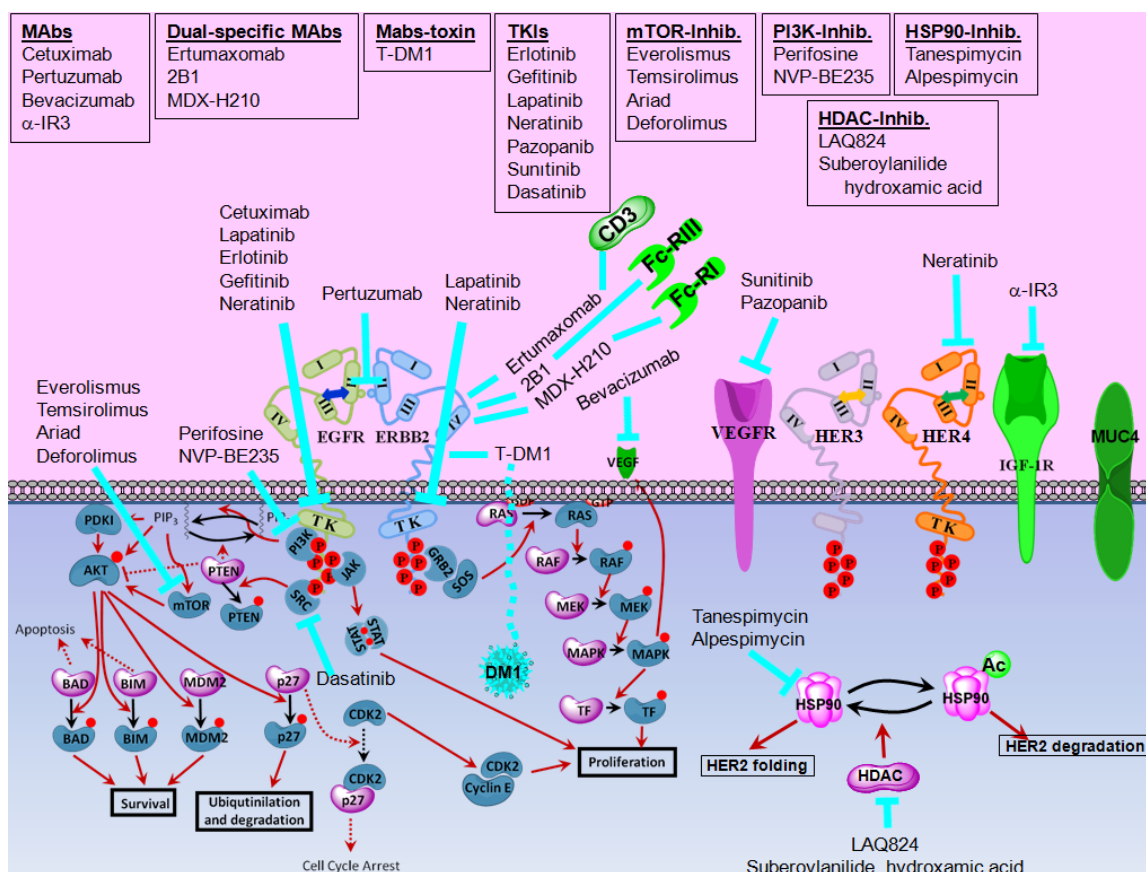


Figure 1.6 Alternative Therapeutics Agents targeting the molecular effectors on ERBB2 signaling pathway are shown. Effectors shown in dark blue are the molecular forms demonstrated or suggested to be active in Trastuzumab resistance, participating in proliferation and survival pathways. Effectors shown in pale purple are the molecular forms which participate in anti-proliferative pathways and are demonstrated or suggested to be decreased in Trastuzumab resistance. $\rightarrow$ / $\rightarrow$: molecular transitions/signal transductions in action. $\cdots$ / $\cdots$: inhibited molecular transitions/signal transductions. $\bullet$: phosphorylated residues. TK: Tyrosine kinase domain. $\perp$: inhibition.

Alternative therapeutics developed for the treatment of ERBB2-overexpressing breast cancers are summarized on **Figure 1.6**. Besides growth factor receptors and intracellular kinases, the molecular chaperon HSP90 which participates in the stabilization of survival mediators such as ERBB2, PDGF, Akt and p53; and histone deacetylases (HDACs) that activate HSP90 are also accepted as therapeutic targets and tested in clinical trials together with Trastuzumab [251, 252].

In addition, monoclonal antibody Pertuzumab (originally 2C4) developed simultaneously with Trastuzumab (originally 4D5) as a weaker growth inhibitor was later discovered to prevent heterodimerization between ERBB2 and other growth factor receptors such as EGFR, ERBB3 [184], IGF-1R [228]. Clinical trials suggest that Pertuzumab may be used to overcome Trastuzumab resistance induced by the crosstalk between ERBB2 and other growth factor receptors [228, 253].

Recently, therapeutic agents are being developed that target more than one signalling molecule at the same time. These include dual specificity kinase inhibitors such as Lapatinib, Neratinib and some modified anti-ERBB2 antibodies that interact with the receptors on immunoeffector cells in addition to ERBB2 on target cells. Bispecific antibodies Ertumaxomab (targeting ERBB2 and CD3) [142], 2B1 (targeting ERBB2 and FcγRIII) [143] and MDX-H210 (target FcγRI in addition to ERBB2) [144] are currently being tested in clinical trials. In addition, anti-ERBB2 antibodies fused to chemotherapeutics (T-DM1) are developed to specifically target ERBB2-overexpressing cells, limiting the overall toxicity of chemotherapy [254].

Although promising anti-oncogenic activities were demonstrated, each of the alternative therapeutics mentioned above has a limited selectivity, targeting only a small subset of Trastuzumab resistant conditions. In fact, most of the preclinical and clinical trials are performed with combinational regimens containing at least two agents with distinct target specificities in order to increase the application range and therapeutic efficacy [50, 247, 255].

It should also be noted that, even antibody combinations targeting distinct epitopes of the same receptor provide significantly stronger anti-oncogenic effect than either of the individual agents both *in vitro* and *in vivo* [253, 256]. Aiming to generate antibodies targeting unique epitopes with better therapeutic outcome, a number of groups developed novel anti-ERBB2 antibodies whose therapeutic efficiencies as single agent and/or in multidrug combinations are still being examined in preclinical and clinical studies [257–271].

In the very near future, with the developments in personalised medicine, it will be possible to unveil molecular signature of every single patient, and to offer the correct therapeutic combination individually. In order for this to come true, it is critically important to have a wide variety of therapeutics available in market offering different target selectivity and therapeutic efficacy.

1.5 TNF- α and ERBB2

TNF- α can act either as a pro-apoptotic and an anti-apoptotic agent depending on the receptor type, adaptor proteins and cellular context [272]. TNF- α -induced caspase-8 activation (mostly through TNF-R1) leads to apoptosis, whereas NF κ B activation causes downstream activation of Bcl-x_L and caspase inhibitors such as c-FLIP, c-IAP1 and c-IAP2, counteracting TNF- α -induced apoptotic pathway and leading to proliferation [273–276].

As an indication of its anti-apoptotic effect, TNF- α was shown to induce breast cancer growth through p42/44, JNK, Akt and NF κ B pathways [277]. Studying ERBB2-overexpressing breast cancer cells, the same group later demonstrated that TNF- α transactivates ERBB2 by inducing its autophosphorylation and c-Src mediated ERBB2-Tyr877 phosphorylation, leading to cell proliferation through NF κ B activation, and suggested that the presence of TNF- α in tumor environment is a factor in development of trastuzumab resistance [278].

On the other hand, TNF- α was best known for its pro-apoptotic effect. Therefore it had been striking when it was first shown that tumor cells developed TNF- α -resistant phenotype due to increased signalling through growth factor receptors [279], and overexpression of ERBB2 was demonstrated to render NIH3T3 cells resistant to cytotoxic effects of TNF- α treatment [83]. The TNF- α -resistant phenotype of ERBB2-overexpressing NIH3T3 cells was reversed by the help of monoclonal antibodies targeting the extracellular domain of ERBB2 [84]. The mechanism of the additive effect seen between TNF- α and anti-ERBB2 therapy was studied by a number of groups and illustrated on **Figure 1.7**. Zhou et. al. have shown that TNF- α -induced apoptosis was blocked by ERBB2 overexpression through Akt and NF κ B [280]. ERBB2-mediated PI3K-induction of Akt was demonstrated to activate IKK α /IKK β and in turn NF κ B, leading to TNF- α -resistance. Therefore equilibrium between pro-apoptotic/anti-apoptotic pathways downstream of TNF- α was suggested to shift towards anti-apoptosis upon ERBB2-overexpression. Later, Trastuzumab was demonstrated to inhibit PI3K and Akt in order to exert anti-tumoral activity on ERBB2-overexpressing cells [113], therefore Trastuzumab may be suggested to sensitize ERBB2-overexpressing cells to TNF- α -induced apoptosis

through deactivation of Akt/NF κ B pathway. PI3K-mediated activation of ILK was further suggested as an alternative pathway causing activation of Akt and IKK α /IKK β conferring resistance to TNF- α -induced apoptosis upon ERBB2-overexpression [281]. Trastuzumab treatment of ERBB2 overexpressing cells BT-474 and SK-BR-3 demonstrated induction of TNF- α -mediated NF- κ B activity and I κ B α phosphorylation only in BT-474; emphasizing the role of cell-specific responses [278]. On the other hand, I κ B phosphorylation which was associated with increased NF κ B activity was observed in Trastuzumab resistant clones of both SK-BR-3 and BT-474 [243].

A direct effect of Trastuzumab in enhancing TRAIL-mediated apoptosis was demonstrated in ERBB2-overexpressing ovarian and breast cancer cell lines upon downregulation of ERBB2 [104]. Blocking Akt pathway by dominant negative Akt or PI3K-inhibitors were shown to sensitize ERBB2-overexpressing cells to TNF- α -mediated [280] and TRAIL-mediated [104] apoptosis respectively. In addition, PARP-inhibitors were shown to be effective in triggering TNF- α -sensitivity in ERBB2-overexpressing cells [282], and interestingly, while PARP inhibitors reversed the TNF- α -resistant phenotype in ERBB2-overexpressing cells, they inhibited TNF- α -response in TNF- α -sensitive cells that has lower ERBB2 expression.

While these studies help to unveil the potential mechanisms by which the anti-ERBB2 antibody Trastuzumab enhanced the effect of TNF- α , the mechanism of the opposite route was to be answered. Although TNF- α treatment was suggested to decrease ERBB2 mRNA synthesis [283] and ERBB2 protein expression [284], the effect on transcriptional level was controversial [272] and the exact mechanism by which TNF- α increased the anti-oncogenic potential of anti-ERBB2 therapy remained unclear until TNF- α was shown to stimulate caspase-8 mediated ERBB2 cleavage which is counteracted by NF κ B [272].

TNF- α pro-apoptotic pathway acts through caspase-8, which was further shown to be active in ERBB2 cleavage. On the contrary, TNF- α anti-apoptotic pathway acts through NF κ B which in turn increases c-FLIP expression leading to inhibition of caspase-8 [272]. Upon ERBB2 overexpression, NF κ B is activated through either a PI3K $\rightarrow$ Akt activation, or through ILK $\rightarrow$ Akt activation [281], which might lead to caspase-8 inhibition and further stabilization of ERBB2. Therefore,

ERBB2 might produce a positive-feedback deactivating its own cleavage and increasing cellular ERBB2 protein level. However, it can be argued that, when ERBB2 is deactivated either by Trastuzumab, ERBB2 kinase inhibitors or anti-sense oligonucleotides, NF κ B is no-more activated [278, 280], rendering caspase-8 free to cleave ERBB2, adding up to the pro-apoptotic effect of TNF- α -stimulated caspases [272]. On the other hand, NF κ B-negative background is prone to TNF- α mediated caspase-8 dependent ERBB2 cleavage even in the absence of Trastuzumab, such as observed in NF κ B defective MCF-7 A/Z cells [272].

On its own, Trastuzumab was able only to block downstream signalling of wild-type full-length ERBB2, however signalling could continue through mutant or ECD-shed ERBB2. On the other hand, dependent on Trastuzumab-mediated NF κ B deactivation, TNF- α may lead to downregulation of total ERBB2-tyrosine kinase activity through induction of ERBB2 cleavage on 6 different intracellular sites, in turn causing a stronger anti-proliferative and apoptotic result. Although weak, a positive correlation was later shown between serum ERBB2 and pro-apoptotic TNF-R1 and TNF-R2 cytokines in patients with heart-failure, suggesting a clinical indication of association between TNF- α and ERBB2-cleavage [285].

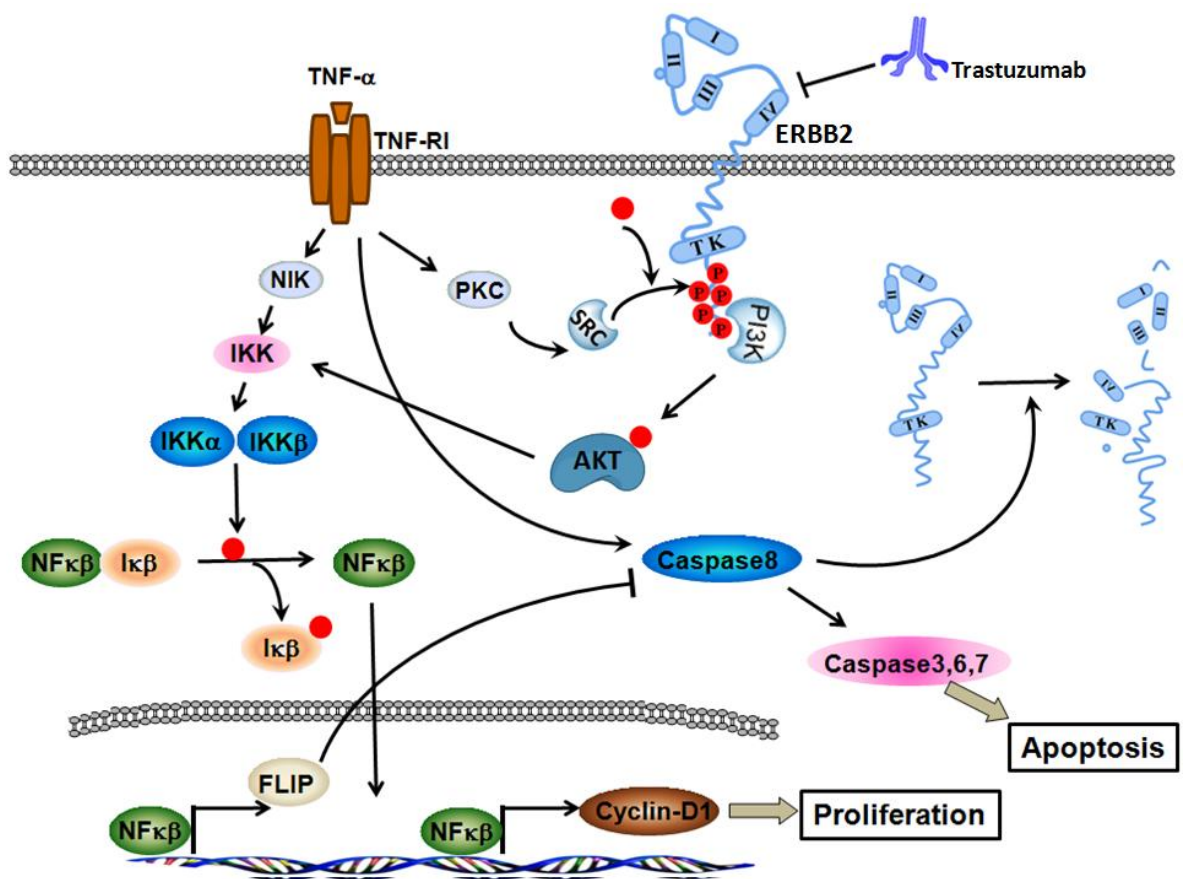


Figure 1.7 Interaction between TNF- α and ERBB2 TNF- α can act as a pro-apoptotic or anti-apoptotic agent. Through activation of NF κ B pathway, TNF- α transactivates Cyclin D1 and FLIP, leading to cell proliferation. Through activation of Caspase8 cascade, TNF- α induces apoptosis. TNF- α and ERBB2 pathways interact through Src and Akt; both inducing anti-apoptotic pathways. On the other hand TNF- α induced Caspase8 induces ERBB2 degradation, counteracting with the ERBB2 pathway. Through deactivation of Akt/NF κ B pathway, anti-ERBB2 treatment may sensitize ERBB2-overexpressing cells to TNF- α -induced apoptosis.

2 OBJECTIVES AND RATIONALE

2.1 Aim of the Study

As described above, there is no single therapeutics available to efficiently treat all the ERBB2 positive breast cancers. In addition, drug resistance against Trastuzumab (the anti-ERBB2 therapeutics widely used in clinics) stands in the way of long-term clinical success during prolonged treatment. In order to provide alternative therapeutics selectively targeting ERBB2-overexpressing cancer cells, we wanted to develop novel anti-ERBB2 antibodies having different epitope preferences and anti-proliferative efficiencies than the antibodies already used or being tested in the clinic. Since anti-ERBB2 treatment was previously suggested to increase TNF- α sensitivity of ERBB2-overexpressing cancer cells, we also intended to analyse the drug interactions between novel anti-ERBB2 antibodies and TNF- α .

2.2 Rationale

Immunizing BALB/c mice with recombinant “human ERBB2 ECD/Fc” fusion protein, we have developed five hybridoma clones producing novel anti-ERBB2 antibodies (BH-antibodies). Biochemical properties and biological activities of the antibodies were extensively analysed.

The affinities of the novel antibodies for recombinant and cellular ERBB2 were examined by ELISA, flow cytometry and indirect immunofluorescence assays. While all novel antibodies successfully detected native ERBB2, they failed to interact with ERBB2 proteins denatured during SDS-PAGE. Immunofluorescence and immunoprecipitation/western analyses of various ERBB2 deletion constructs revealed that all novel antibodies reacted with conformational ERBB2 epitopes.

Biological activities of novel anti-ERBB2 antibodies were tested *ex-vivo* on breast cancer cell lines having different ERBB2 expression levels. Effect of BH-antibodies on breast cancer cell growth either alone, in pairs or in combination with TNF- α was tested by Sulforhodamine B (SRB) based proliferation assay. BH-antibodies displayed anti-proliferative effects on tested cell lines with ERBB2

amplification. Drug interactions between anti-ERBB2 antibodies and TNF- α are analysed by two distinct methods; Bliss model of independence and Loewe model of additivity. While BH-antibodies and TNF- α had synergistic effects on SK-BR-3 background, they were antagonistic on BT-474 cells.

In order to reveal the mechanism of the growth inhibition observed by BH-antibodies in the presence or absence of TNF- α , BrdU-incorporation, SA β -gal positivity and cell-cycle distribution of the treated SK-BR-3 cells were analysed. In addition, molecular changes induced on PI3K and MAPK pathways were also examined.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 General Chemicals

General chemicals and reagents (i.e. NaCl, glycine, trisma-base, ethanol, bacto-tryptone) were purchased from Sigma-Aldrich (St. Louis, USA) or Calbiochem Merck Millipore (Darmstadt, Germany). Specific reagents are listed on **Table 3.1** and **Table 3.2**.

All tissue culture flasks, tissue culture plates, cryotubes, serological pipettes, bacterial petri-dishes, filter tips, and other general plastic-ware that were used throughout the project were purchased from Costar Corp. (Cambridge, England) or Greiner Bio-one (Austria).

Reagent Name	Catalogue Number	Company	
Trichloroacetic acid	1008100250	Merck	(Schucdarf, Germany)
Bovine serum albumin (BSA)	A7906	Sigma-Aldrich	(St. Louis, USA)
Sulforhodamine B (SRB) sodium salt	341738	Sigma-Aldrich	(St. Louis, USA)
p -Nitrophenyl Phosphate Disodium Salt (PNPP Tablet Set)	N2770	Sigma-Aldrich	(St. Louis, USA)
4',6-diamidino-2-phenylindole (DAPI)	D8417	Sigma-Aldrich	(St. Louis, USA)
insulin		Sigma-Aldrich	(St. Louis, USA)
5-Bromo-2'-deoxyuridine (BrdU)	B5002	Sigma-Aldrich	(St. Louis, USA)
Propidium Iodide (PI)	P4864	Sigma-Aldrich	(St. Louis, USA)
Protease inhibitor cocktail tablet (complete EDTA free)	11873580001	Roche Ltd	(Basel, Switzerland)
PhosSTOP	4906837001	Roche Ltd	(Basel, Switzerland)
Rnase A	EN0531	Thermo Scientific Inc.	(IL, USA)
PageRuler Prestained Protein Ladder	SM0671	Thermo Scientific Inc.	(IL, USA)
PageRuler Plus Prestained Protein Ladder	SM1811	Thermo Scientific Inc.	(IL, USA)
NotI	ER0592	Thermo Scientific Inc.	(IL, USA)
XhoI	ER0691	Thermo Scientific Inc.	(IL, USA)
SacI	ER1135	Thermo Scientific Inc.	(IL, USA)
EcoRI	ER0275	Thermo Scientific Inc.	(IL, USA)
XmnI	ER1532	Thermo Scientific Inc.	(IL, USA)
T4 DNA Ligase	EL0014	Thermo Scientific Inc.	(IL, USA)
Taq DNA polymerase	EP0402	Thermo Scientific Inc.	(IL, USA)
Phusion® High-Fidelity DNA Polymerase	F-530L	Finnzymes	(Espoo, Finland)
DyNAzyme™ II DNA Polymerase	F-501L	Finnzymes	(Espoo, Finland)
SYBR Green I nucleic acid gel stain	50513	FMC BioProducts	(ME, USA)
QIAquick Gel Extraction Kit	28706	QIAGEN	(CA, USA)
QIAquick PCR Purification Kit	28106	QIAGEN	(CA, USA)
QIAprep Spin Miniprep Kit	27106	QIAGEN	(CA, USA)
QIAGEN Plasmid Midi Kit	12145	QIAGEN	(CA, USA)
BCA Protein Assay Kit	23227	Thermo Scientific Inc.	(IL, USA)
ECL Plus Western Blotting Detection System	RPN2132	GE Healthcare	(WI, USA)
Monoclonal Antibody Isotyping Kit	37502	Thermo Scientific Inc.	(IL, USA)
SuperSignal West Dura Extended Duration Substrate	34075	Thermo Scientific Inc.	(IL, USA)
SuperSignal West Femto Maximum Sensitivity Substrate	34095	Thermo Scientific Inc.	(IL, USA)
SuperSignal West Pico Trial Kit	34079	Thermo Scientific Inc.	(IL, USA)
ProLong Gold Antifade Reagent with DAPI	P36935	Invitrogen	(CA, USA)
GeneRuler™ DNA Ladder Mix, 100-10,000 bp	SM0331	Thermo Scientific Inc.	(IL, USA)
GeneRuler™ 1 kb DNA Ladder, 250-10,000 bp	SM0311	Thermo Scientific Inc.	(IL, USA)
PageRuler™ Prestained Protein Ladder	SM0671	Thermo Scientific Inc.	(IL, USA)
PageRuler™ Plus Prestained Protein Ladder	SM1811	Thermo Scientific Inc.	(IL, USA)
Ampicillin	A9393	Sigma-Aldrich	(St. Louis, USA)
96-well polysorb ELISA plates	475094	NUNC	(Roskilde, Denmark)
Immobilon-P transfer (PVDF) membrane	IPVH00010	Millipore	(MA, USA)
3MM Cellulose Chromatography Papers (Filter Paper)	3030-917	Whatman International Ltd.	(Madison, USA)
Kodak medical x-ray film general purpose blue/E	7710783	Kodak	

Table 3.1 List of specific chemicals, enzymes and commercial kits used during the project.

Reagent Name	Catalogue Number	Company
Constructs, Recombinant Proteins, Antibodies		
Full length human ErbB2 plasmid	OCABo5050G0219D-pDEST26	Source BioScience LifeSciences (Nottingham, UK)
Recombinant Human ErbB2/Fc Chimera	1129-ER	R&D Systems (MN, USA)
Recombinant Human EGF R/Fc Chimera	344-ER	R&D Systems (MN, USA)
Recombinant human tumor necrosis factor- α (TNF- α)	300-01A	PeproTech (NJ, USA)
Trastuzumab (Herceptin)		Roche Ltd (Basel, Switzerland)
anti-ErbB2 (CB11) antibody	ab8054	Abcam (MA, USA)
anti-phospho ErbB2 antibody	sc-1011694	Santa Cruz (California, USA)
anti-Akt antibody	9272	Cell Signaling (MA, USA)
anti-phospho Akt antibody	9271	Cell Signaling (MA, USA)
anti-Erk1/2 antibody	9107	Cell Signaling (MA, USA)
anti-phospho Erk1/2 antibody	9106	Cell Signaling (MA, USA)
anti-CyclinD1 antibody	sc-246	Santa Cruz (California, USA)
IgG1 isotype control antibody	MAB002	R&D Systems (MN, USA)
anti-mouse IgG (H+L) Alexa Fluor® 488	A11029	Invitrogen (CA, USA)
anti-human IgG Alexa Fluor® 488	A11013	Invitrogen (CA, USA)
anti-mouse IgG (H+L) Alexa Fluor® 568	A11031	Invitrogen (CA, USA)
anti-BrdU (Bu20a) antibody	M0744	Dako (Glostrup, Denmark)
anti-mouse Immunoglobulins/AP	D0314	Dako (Glostrup, Denmark)
anti-Calnexin antibody	C4731	Sigma-Aldrich (St. Louis, USA)
anti-human IgG (Fc specific)?Peroxidase antibody	A0170	Sigma-Aldrich (St. Louis, USA)
anti-human IgG (Fc specific)?Alkaline Phosphatase antibody	A9544	Sigma-Aldrich (St. Louis, USA)
anti-mouse IgG (Fc specific)–Peroxidase antibody	A0168	Sigma-Aldrich (St. Louis, USA)
anti-rabbit IgG (whole molecule)–Peroxidase antibody	A6154	Sigma-Aldrich (St. Louis, USA)
FPLC Columns and Protein Purification Material		
Protein G coupled sepharose gel slurry	R801-H226	East Coast Biologics (ME, USA)
Hi-Trap rProtein A FF (1 ml)	17-5079-01	GE Healthcare (WI, USA)
Hi-Trap Protein A HP (5 ml)	17-0403-01	GE Healthcare (WI, USA)
Hi-Trap Protein G HP (1 ml)	17-0404-01	GE Healthcare (WI, USA)
Hi-Trap Protein G HP (5 ml)	17-0405-01	GE Healthcare (WI, USA)
Amicon Ultra-15 Ultracel-10k concentrators	UFC901024	Millipore (MA, USA)
Polyacrylamide Electrophoresis Ready Gels and Reagents		
NuPAGE 10% Bis-Tris Gel 1.5mm x 10 well	NP0315BOX	Invitrogen (CA, USA)
NuPAGE 4-12% Bis-Tris Gel 1.5mm x 10 wel	NP0335BOX	Invitrogen (CA, USA)
NuPAGE® MOPS SDS Running Buffer (for Bis-Tris Gels only) (20X)	NP0001	Invitrogen (CA, USA)
NuPAGE® Transfer Buffer (20X)	NP0006	Invitrogen (CA, USA)
NuPAGE® LDS Sample Buffer (4X)	NP0007	Invitrogen (CA, USA)

Table 3.2 List of constructs, recombinant proteins, protein purification and analysis reagents used during the project.

3.1.2 Instruments

Instruments used throughout the project and their application fields are listed on **Table 3.3**.

Instrument Name	Application	Company
XCell SureLock® Mini-Cell	Poly Acrylamide Gel Electrophoresis (PAGE)	Invitrogen (CA, USA)
XCell II™ Blot Module	Wet Transfer following PAGE	Invitrogen (CA, USA)
Mini-PROTEAN® 3 Cell	Poly Acrylamide Gel Electrophoresis (PAGE)	BioRad (CA, USA)
Mini Trans-Blot Cell	Wet Transfer following PAGE	BioRad (CA, USA)
Sub-Cell GT System	Agarose Gel Electrophoresis	BioRad (CA, USA)
Power-PAC300	Power Supply	BioRad (CA, USA)
Power-PAC200	Power Supply	BioRad (CA, USA)
ÄKTAexplorer™ FPLC system	Fast Protein Liquid Chromatography (FPLC) for antibody purification	GE Healthcare (WI, USA)
Beckman Spectrophotometer Du640	Spectrophotometry	Beckman Instruments Inc. (CA, USA)
Nanodrop ND-1000 Full-spectrum UV/Vis Spectrophotometer	Spectrophotometry	Thermo Fisher Scientific Inc. (IL, USA)
BioTek µQuant	Microplate Reader	BioTek (VT, USA)
FACSCalibur Flow Cytometer and CellQuest Software	Flow Cytometry	Becton Dickinson (NJ, USA)
Chemi-Smart 2026WL	Imaging System	Vilber Lourmat France
Hyperprocessor	Development of films used for autoradiography	GE Healthcare (WI, USA)
Axio Imager.A1	Fluorescent Microscopy	Zeiss (Jena, Germany)

Table 3.3 List of instruments used during the project.

3.1.3 Cell Lines and Tissue Culture Reagents

Human breast cancer cell lines SK-BR-3, MDA-MB-361, T-47D, MCF-7, human hepatocellular carcinoma cell line Huh7 and mouse myeloma cell line SP2/0 was obtained from Bilkent University Molecular Biology and Genetics department cell line stocks. Human breast cancer cell line BT-474 was a kind gift of Assoc. Prof. Dr. Elif Erson (Middle East Technical University). Authenticity of all mentioned cell lines was checked periodically by STR-analysis. The most recent analysis was performed on 09.02.12 and all cell lines were STR-proven to be authentic. Expression profiles of the breast cancer cell lines used throughout the project were examined by a number of groups [286–289] and the genomic amplifications and deletions they contain are listed on **Table 3.4**.

Cytoband	P-Border (nt)	Q-Border (nt)	Size (kB)	Cell Lines [Ⓔ]	Significant DNA-RNA Correlations [#]	Other notable genes [Ⓗ]
AMPLIFICATION						
1p32.2	56946690	57156366	210	EFM192A		
1p22.1-1p21.3	93549298	97052934	3504	SUM44*	DR1, FNBP1L, ARHGAP29, ALG14	
1p13.3	107738670	109306637	1568	HCC2688	C1orf59, PRPF38B, STXPB3, GPSM2, CLCC1	VAV3
1p13.2	114220960	115183599	963	MCF7, UACC812	AP4B1, DCLRE1B, HIPK1, TRIM33, BCAS2, CSDE1, NRAS	
1q21.2	148738080	148885763	148	HCC1143	TARS2, MCL1 , ENSA, GOLPH3L	
1q21.2-q21.3	149460307	150130540	670	HCC712, UACC812	PIP5K1A, PSMD4, ZNF687, PI4KB, PSMB4, POGZ, SNX27, MRPL9	
1q21.3	151000411	151885402	885	HCC712		
1q22	153424958	153999982	575	UACC812	MUC1, C1orf2, CLK2, HCN3, PKLR, C1orf104, RUSC1, ASH1L, YY1AP1	
1q23.3	159283361	159357995	75	SUM190	KLHDC9	
1q32.1	204736293	205144756	408	UACC812	MAPKAPK2	IKBKE
3p14.2-p14.1	61765808	64574645	2809	MCF7		
3q26.32	178223920	180535525	2312	HCC2185	TBL1XR1, ZNF639	PIK3CA
3q29	194971434	195513283	542	HCC1937		
3q29	196883266	196931777	49	HCC1937		
4q12	53304442	54084198	780	HCC1007	SCFD2, FIP1L1	
5p15.33	712977	2811691	2099	HCC1954	ZDHHC11, PDCD6, MRPL36, NDUFS6	TERT
6p12.1	55358212	57236103	1878	HCC1007	KIAA11586, ZNF451, BAG2	
6q16.3-q21	104858272	109112665	4254	HCC2185	HACE1, ATG5, C6orf203, PDSS2, SEC63, OSTM1, SNX3, FOXO3A	
6q21-q22.31	111961945	123089199	11127	HCC2185	C6orf225, HDAC2, DSE, GOPC, NUS1, ASF1A, HSF2, SERINC1	
7p15.2	26557965	27107611	550	HCC1007		
7p11.2	54595526	55931398	1336	BT20, MDA468	EGFR	
7q21.13-q21.2	90779687	91868629	1089	SUM52	MTERF, AKAP9, CYP51A1, KRIT1, ANKIB1	
7q21.3	95239813	96489919	1250	SUM52	SLC25A13, SHFM1	
7q22.1	100294293	100421513	127	SUM52	SLC12A9	
8p21.3	21593811	21966432	373	MDA134	XPO7	
8p12-p11.21	32328805	41907423	9579	BT483, HCC1500, HCC1599, MDA134, SUM44*, SUM52	FUT10, C8orf41, MAK16, ZNF703, ERLIN2, PROSC, BRF2, RAB11FIP1, EIF4EBP1, ASH2L, LSM1, BAG4, DDHD2, WHSC1L1, LETM2, FGFR1 , TACC1, PLEKHA2, TM2D2, ADAM9, GOLGA7, AGPAT6	IKBKB
8q12.2-q12.3	61817956	62960675	1143	SUM190	CHD7	
8q13.3	71707355	72999610	1292	SKBR3		
8q21.11-q21.13	79781799	85260376	5479	EFM192A, HCC1419, HCC1599, SKBR3	HEY1, TPD52, ZBTB10	
8q21.3-q22.1	89113344	95233478	6120	EFM192A, HCC1419, SKBR3	OSGIN2, NBN, DECR1, OTUD6B, RBM12B, TMEM67	
8q22.2-q22.3	100879473	101995283	1116	HCC1419, HCC2185	COX6C, POLR2K	
8q22.3	104311423	104550566	239	HCC1419	FZD6	
8q23.1-q24.21	108267427	131134620	22867	EFM192A, HCC1419, HCC1599, HCC2185, SKBR3, ZR75-30	EIF3E, TRPS1, EIF3H, C8orf53, RAD21, TAF2, DSCC1, MRPL13, MTBP, DERL1, WDR67, C8orf76, ZHX1, ATAD2, C8orf32, FAM91A1, TMEM65, TRMT12, RNF139, TATDN1, NDUFB9, SQLE, KIAA0196, NSMCE2, FAM84B	MYC
8q24.22	133917771	134337653	420	ZR75-30	PHF20L1	
8q24.3	141658961	143348731	1690	HCC1419, MDA436, ZR75-30	GPR20, FLJ43860	
8q24.3	144310706	144753628	443	MDA436, ZR75-30	ZFP41, GLI4, ZNF696, C8orf51, RHPN1, MAFA	

Cytoband	P-Border (nt)	Q-Border (nt)	Size (kB)	Cell Lines ^e	Significant DNA-RNA Correlations [#]	Other notable genes ^g
AMPLIFICATION						
8q24.3	145137850	146252219	1114	BT483, HCC1419, MDA436, ZR75-30	GRINA, OPLAH, SHARPIN, KIAA1833, FBXL6, CPSF1, VPS28, KIFC2, ZNF252	
9p13.3-p13.2	33876876	38058023	4181	HCC2185	UBE2R2, UBAP2, WDR40A, KIF24, KIAA1161, DCTN3, GALT, IL11RA, VCP, FANCG, PIGO, STOML2, RUSC2, TESK1, CD72, C9orf100, TLN1, CREB3, RGP1, HINT2, CLTA, RNF38, MELK, ZCCHC7, GRHPR, ZBTB5, POLR1E, FBXO10, RG9MTD3, WDR32, MCART1	
9q33.3	128307884	129195638	888	SUM44*	RALGPS1	
10q21.1-q21.2	72507196	73797267	1290	HCC2157	DNAJB12	
10q22.2-q22.3	76461776	82106491	5645	EFM19, HCC2157	SAMD8, VDACC2, DLG5, POLR3A, RPS24, LOC283050, ZMIZ1, PPIF, SFTPA1, FAM22E, C10orf57, ANXA11	
10q24.33-q25.1	105307581	106054698	747	EFM19	SH3PXD2A	
10q26.13	124598599	124962466	364	SUM52	IKZF5, BUB3	
11p13	33062705	35600197	2537	HCC1806*	HIPK3, FBXO3, CAPRIN1, NAT10, ABTB2, CAT, APIP, PDHX, CD44	
11q13.2	66874536	67198753	324	MDA134, ZR75-1	RAD9A, RPS6KB2, CORO1B, TMEM134	
11q13.3-q13.4	68427956	70812048	2384	HCC1143, HCC1500, HCC1954, MDA134, MDA175, MDA361, SUM44*, SUM190,	IGHMBP2, FADD, PPFA1, CTTN, SHANK2	CCND1
11q13.4	73316198	73649077	333	BT474, MDA134, SUM190	UCP2, C2CD3, PPME1	
11q13.4-q14.1	74648813	77963474	3315	MDA134, SUM44*, SUM52, SUM190	ARRB1, PRKRIR, EMSY , PHCA, PAK1, AQP11, CLNS1A, C11orf67, INTS4, NDUFC2, ALG8, GAB2, NARS2	
12p12.3	18727378	19246201	519	HCC1500		
12q21.31-q21.33	88265969	88443930	178	SUM52	WDR51B, GALNT4	
13q22.2-q31.1	74756931	78096263	3339	UACC812	UCHL3	
13q31.3-q32.1	90798074	93942902	3145	UACC812		
16q12.2	51800892	53524601	1724	EFM19, SUM44*	CHD9, FTO	
17p12	12611513	13636592	1025	EFM192A	ELAC2	
17q11.2	23686912	24013273	326	ZR75-30	POLDIP2, TREM199, SLC46A1, PIGS, SPAG5, FLJ25006, KIAA0100, SDF2	
17q11.2	24894649	25818484	924	HCC202	TAOK1, LOC116236, GIT1, ANKRD13B, CPD	
17q11.2	27727543	28293356	566	SUM190	ZNF207	
17q12	31206068	31649844	444	MDA361	FLJ12120	
17q12-q21.2	32627885	36209712	3582	BT474, EFM192A, HCC202, HCC1419, HCC1569, HCC1954, HCC2218, MDA361, SKBR3, SUM190, UACC812, UACC893, ZR75-30	ACACA, TADA2L, DDX52, SOCS7, MLLT6, CISD3, PCGF2, PSMB3, PIP4K2B, CCDC49, RPL23, LASP1, CACNB1, FAM153C, RPL19, LOC90110, FBXL20, MED1, PPP1R1B, STARD3, TCAP, PERLD1, ERBB2 , C17orf37, GRB7, IKZF3, GSDML, ORMDL3, PSMD3, MED24, MSL-1, CASC3, CDC6, RARA, SMARCE1	
17q21.31	38419019	38738864	320	SUM190	RND2	
17q21.32-q25.1	43329972	50826668	7497	BT474, EFM192A, HCC202, HCC712, HCC1419, HCC2218, ZR75-30	SP2, PNPO, CDK5RAP3, SNX11, HOXB13, CALCOCO2, ATP5G1, UBE2Z, SNF8, ZNF652, PHB, SPOP, SLC35B1, FAM117A, MYST2, PDK2, XYLT2, MRPL27, LRRC59, EME1, ACSF2, RSAD1, EPN3, SPATA20, ABCC3, ANKRD40, CROP, TOB1, NME1, TOM1L1, COX11, STXBP4	
17q23.2-q24.2	53282667	63106134	9823	BT474, HCC712, HCC2218, MCF7, MDA361, ZR75-30	SFRS1, DYNLL2, MKS1, SUPT4H1, MTMR4, RAD51C, TRIM37, FAM33A, C17orf71, YPEL2, DHX40, CLTC, PTRH2, TMEM49, TUBD1, RPS6KB1 , RNFT1, HEATR6, USP32, APPBP2, PPM1D , BRIP1, INTS2, MED13, METTL2A, TLK2, TANC2, CYB561, WDR68, CCDC44, MAP3K3, LYK5, CCDC47, DDX42, PSMC5, SMARCD2, DDX5, CCDC45, SMURF2, GNA13, HELZ	

Cytoband	P-Border (nt)	Q-Border (nt)	Size (kB)	Cell Lines [©]	Significant DNA-RNA Correlations [#]	Other notable genes [®]
AMPLIFICATION						
17q25.1	69755691	71418122	1662	HCC2218, MDA361, MDA453, UACC893	GPRC5C, SLC9A3R1, NAT9, TMEM104, FDXR, C17orf28, CDR2L, ICT1, KCTD2, SUMO2, NUP85, GGA3, MRPS7, MIF4GD, SLC25A19, GRB2, CASKIN2, TSEN54, MYO15B, SAP30BP, H3F3B, UNK, WBP2	
18q21.32-q21.33	55178911	57628085	2449	HCC1500		
19p13.2	14932742	15602448	670	HCC1143	ILVBL, BRD4, AKAP8L	
19q12-q13.11	33966349	38052482	4086	HCC1569, HCC1599	UQCRF51, POP4, PLEKHF1, C19orf2, DPY19L3, ANKRD27	
19q13.11	39866832	40146793	280	HCC1599		
19q13.42	60551045	60898029	347	EFM19	FIZ1, ZNF784, CCDC106	
19q13.43	63208125	63774724	567	HCC1806*	ZNF329, ZNF274, ZNF8, ZSCAN22, ZNF324, TRIM28, CHMP2A, UBE2M	
20p12.2	10224083	10433564	209	HCC2185	MKKS	
20q11.22	32363269	33563203	1200	BT474	DYNLRB1, NCOA6, UQCC	
20q13.12	42493067	43286511	793	BT474, SUM52	SERINC3	
20q13.12-q13.13	45234836	48636574	3402	BT474, HCC1419, MCF7	NCOA3, PREX1, ARFGEF2, STAU1, DDX27, ZNFX1, SLC9A8, SPATA2, PTPN1	
20q13.13-q13.32	49139330	57334442	8195	BT474, HCC1419, MCF7, SKBR3	ZFP64, ZNF217 , BCAS1, PFDN4, C20orf108, CSTF1, C20orf43, TFAP2C, BMP7, RAE1, RBM38, RAB22A, VAPB, STX16, NPEPL1, GNAS, TH1L, ATP5E, SLMO2	AURKA
20q13.33	61801252	62370522	569	HCC1419	PRR17, OPRL1	
22q11.21	18256420	19686015	1430	SUM190	COMT, HTF9C, PI4KA	
22q12.1	24895479	25885840	990	HCC202	HPS4	
Xp11.23-p11.22	48635684	51225253	2590	HCC712		
Xp11.22	52255712	54236019	1980	HCC202	TMEM29, PHF8	
Xq28	148368959	149592006	1223	HCC202		
DELETION						
6q16.3-q21	102493055	105832848	3340	HCC1395	HACE1	
7q11.23-q21.11	77246720	77484743	238	HCC1806*	TMEM60, PHTF2	
8p23.3	604200	2080787	1477	HCC2688	ERICH1	
9p24.3-p24.2	958704	3213008	2254	HCC2185	VLDLR, KIAA0020	
9p21.2-p21.1	26894518	29207861	2313	BT474, EFM19	PLAA, IFT74	CDKN2A
13q14.3-13q21.2	52175620	60001053	7825	HCC1395		
15q24.3	74984799	75116728	132	HCC1806*	RCN2	
17p12	11405197	11987872	583	EFM19	MAP2K4	
17q21.31	38252285	38419019	167	HCC1806*		BRCA1
18q11.2-q12.1	22256956	23913060	1656	HCC2185		
21q21.1	18342236	21590772	3249	ZR75-30		
Xp11.3	46208136	46345060	137	HCC2157		
Xq25	122657657	123338533	681	HCC1806*		

[©]For aberrations spanning multiple lines, inclusive interval indicated.

[#]DNA but not RNA profiled.

^{*}Only named genes listed, ordered by genome position; bold text indicates select known cancer genes.

[®]Within or immediately flanking interval.

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Table 3.4 High-amplitude amplifications and deletions of breast cell lines.
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Tissue culture media and reagents used throughout the project were purchased from GIBCO (CA, USA), Sigma-Aldrich (St. Louis, MO) or HyClone (IL, USA) and are listed on **Table 3.5**.

Reagent Name	Catalogue Number	Company	
Incomplete Freund's Adjuvant	F5506	Sigma-Aldrich	(St. Louis, USA)
Complete Freund's Adjuvant	F5881	Sigma-Aldrich	(St. Louis, USA)
HAT Media Supplement (50×) Hybri-Max	H0262	Sigma-Aldrich	(St. Louis, USA)
HT Media Supplement (50×) Hybri-Max™	H0137	Sigma-Aldrich	(St. Louis, USA)
Polyethylene glycol solution - Hybri-Max™	P7181	Sigma-Aldrich	(St. Louis, USA)
L-Glutamine	G7513	Sigma-Aldrich	(St. Louis, USA)
	25030024	Invitrogen/GIBCO	(CA, USA)
Penicilline/Streptomycin	P4333	Sigma-Aldrich	(St. Louis, USA)
	PST999	Invitrogen/GIBCO	(CA, USA)
Fetal Bovine Serum	F7524	Sigma-Aldrich	(St. Louis, USA)
	10270106	Invitrogen/GIBCO	(CA, USA)
Non Essential Amino Acids (NEAA)	M7145	Sigma-Aldrich	(St. Louis, USA)
	11140-035	Invitrogen/GIBCO	(CA, USA)
High Glucose Dulbecco's Modified Eagle Medium (DMEM)	D5796	Sigma-Aldrich	(St. Louis, USA)
	41965-039	Invitrogen/GIBCO	(CA, USA)
RPMI 1640	31870-074	Invitrogen/GIBCO	(CA, USA)
Serum Free Media (SFM)	SH30513	Hyclone	(IL, USA)
McCoy's 5A medium	M4892	Sigma-Aldrich	(St. Louis, USA)
0.25% Trypsin-EDTA (1X), Phenol Red	25200-056	Invitrogen/GIBCO	(CA, USA)
Lipofectamine 2000 Reagent	11668-027	Invitrogen	(CA, USA)
OPTIMEM	11058021	Invitrogen	(CA, USA)
Geneticin G418 Sulphate	11811-031	Invitrogen/GIBCO	(CA, USA)
AQUAGUARD-1	01-867-1B	Biological Industries	Israel
AQUAGUARD-2	01-916-1E	Biological Industries	Israel
DMSO		Sigma-Aldrich	(St. Louis, USA)

Table 3.5 List of cell culture solutions and reagents used during the project.

3.1.4 Animal Experiments

Animal experiments approved by Bilkent University Animal Ethical Review Panel were performed according to the regulations determined by the same committee, following the protocols stated in Bilkent University's guidelines on the humane care and use of laboratory animals. All procedures complied with the guidelines of the Ministry of Agriculture (Official Gazette. No. 25464). BALB/c mice used were obtained from and maintained at Bilkent University Animal Housing Facility.

3.2 SOLUTIONS AND MEDIA

3.2.1 Cell Culture Solutions

Foetal Calf Serum (FCS)

FCS that was stored in -20°C upon arrival was first thawed in water bath at 37°C. When FCS was totally thawed, the water bath was adjusted to 56°C and the complement factors in FCS were inactivated by 45-60 minute incubation. The FCS is then filtered to remove the particulate material, aliquoted and stored at -20°C.

Complete Media

	<u>Final Concentration</u>	<u>for 580 ml</u>
DMEM/RPMI/McCoy's 5A	1 X	500 ml
FCS	10%	58 ml
NEAA	1%	5.8 ml
P/S	1%	5.8 ml
L-Glutamine	1%	5.8 ml

SF-Media (Serum Free Media)

	<u>Final Concentration</u>	<u>for 515 ml</u>
DMEM/RPMI/McCoy's 5A	1 X	500 ml
NEAA	1%	5.2 ml
P/S	1%	5.2 ml
L-Glutamine	1%	5.2 ml

P/S(-)-Media (Antibiotic Free Media)

	<u>Final Concentration</u>	<u>for 570 ml</u>
DMEM/RPMI/McCoy's 5A	1 X	500 ml
FCS	10%	57 ml
NEAA	1%	5.7 ml
L-Glutamine	1%	5.7 ml

HAT-DMEM

	<u>Final Concentration</u>	<u>for 650 ml</u>
High Glucose DMEM	1 X	500 ml
FCS	20%	130 ml
NEAA	1%	6.5 ml
P/S	1%	6.5 ml
L-Glutamine	1%	6.5 ml
50X HAT	1 X	13 ml

HT-DMEM

	<u>Final Concentration</u>	<u>for 650 ml</u>
High Glucose DMEM	1 X	500 ml
FCS	20%	130 ml
NEAA	1%	6.5 ml
P/S	1%	6.5 ml
L-Glutamine	1%	6.5 ml
50X HT	1 X	13 ml

Freezing Media

	<u>Final Concentration</u>	<u>for 10 ml</u>
Complete DMEM/RPMI/McCoy's 5A	1 X	8 ml
FCS	20%	1 ml
DMSO	10%	1 ml

Hybridoma Freezing Media

	<u>Final Concentration</u>	<u>for 10 ml</u>
FCS	90%	9 ml
DMSO	10%	1 ml

TNF- α Stock

50 μ g lyophilized TNF- α (PeproTech, NJ, USA) with a specific activity of 2×10^7 Unit/mg was reconstituted in 500 μ l filter-sterilized ddH₂O to make a TNF- α stock of ~2000 Unit/ μ l. The stock was aliquoted and stored at -20°C.

IgG₁ Isotype Control Antibody Stock

500 μ g lyophilized IgG₁ Isotype Control Antibody (R&D Systems, MN, USA) was reconstituted in 500 μ l sterile PBS to make a IgG₁ Isotype Control Antibody stock of 1 μ g/ μ l. The stock was aliquoted and stored at -20°C.

1000X BrdU Stock

100 mg BrdU was reconstituted in 10 ml sterile PBS to make a BrdU stock of 10 mg/ml. The stock was aliquoted and stored at -20°C.

3.2.2 General Solutions

1 M KCl

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
KCl	74.55 g/mol	1 M	7.45 g
dH ₂ O			100 ml

1 M MgCl₂

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
MgCl ₂ •6H ₂ O	203.30 g/mol	1 M	20.3 g
dH ₂ O			100 ml

10 N NaOH

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
NaOH	40 g/mol	10 M	40 g
dH ₂ O			100 ml

5 M NaCl

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 250 ml</u>
NaCl	58.44 g/mol	1 M	73.05 g
dH ₂ O			250 ml

10X PBS

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
NaCl	58.44 g/mol	1.37 M	8 g
KCl	74.55 g/mol	27 mM	0.2 g
KH ₂ PO ₄	136.09 g/mol	180 mM	0.24 g
Na ₂ HPO ₄ •2H ₂ O	177.96 g/mol	0.1 M	1.78 g
dH ₂ O			100 ml

pH of the 10X buffer was not adjusted (initially ~pH: 6.7).

When the buffer was diluted to 1X, the pH automatically became 7.4.

PBS

	<u>for 1 L</u>
10X PBS Buffer	100 ml
dH ₂ O	900 ml

PBS-T (0.5% Tween)

	<u>for 1 L</u>
PBS	1 L
Tween-20	5 ml

10X TBS (pH:8.0)

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
Trisma-Base	121.14 g/mol	0.1 M	1.21 g
NaCl	58.44 g/mol	1.5 M	8.76 g
dH ₂ O			80 ml
pH was adjusted by concentrated HCl (37%) and 1 N HCl			
Volume was adjusted with dH ₂ O ⇒			100 ml

TBS

	<u>for 1 L</u>
10X TBS	100 ml
dH ₂ O	900 ml

TBS-T (0.5% Tween)

	<u>for 1 L</u>
TBS	1 L
Tween-20	5 ml

1 M Tris-HCl

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
Trisma-Base	121.14 g/mol	1 M	12.1 g
dH ₂ O			90 ml
pH was adjusted by concentrated HCl (37%) and 1 N HCl			
Volume was adjusted with dH ₂ O ⇒			100 ml

25 X Protease Inhibitor Cocktail (25 X PI)

	<u>Final Concentration</u>	<u>for 2 ml</u>
Protease Inhibitor Cocktail Tablet	25 X	1 tablet
dH ₂ O		2 ml

1 N HCl

	<u>Final Concentration</u>	<u>for 10 ml</u>
dH ₂ O		9.167 ml
37%(w/w) HCl (12N)	1N	833 µl
Acid was added onto dH ₂ O!		

0.5 M EDTA

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
EDTA•2H ₂ O	372.24 g/mol	0.5 M	18.6 g
dH ₂ O			70 ml

Note: EDTA is insoluble in water. dH₂O was initially added only as much as 70% of the solution volume (700 ml for a 1L solution). 10N NaOH was added with constant mixing until the pH was adjusted to 8.0. As the pH was close to pH:8.0, the insoluble EDTA dissolve, which decreased back the pH. 10N NaOH was continued to be added until all of the EDTA was dissolved and pH was 8.0.

Volume was adjusted with dH₂O ⇒ 100 ml

2.5 M CaCl₂

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 50 ml</u>
CaCl ₂ •2H ₂ O	147 g/mol	2.5 M	18.375 g
dH ₂ O			50 ml

TE

	<u>Final Concentration</u>	<u>for 100 ml</u>
1M Tris pH:8.0	10 mM	1 ml
0.5 M EDTA (pH:8.0)	1 mM	200 µl
dH ₂ O		98.8 ml

0.5 M Pipes

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
Pipes	302.36 g/mol	0.5 M	15.12 g
dH ₂ O			100 ml

1 M MnCl₂

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
MnCl ₂	161.87 g/mol	1 M	16.2 g
dH ₂ O			100 ml

1 M KOH

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
KOH	56.11 g/mol	1 M	14 g
dH ₂ O			100 ml

Na Azide (NaN₃)

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 10 ml</u>
Na Azide	g/mol	2% (w/v)	250 mg
dH ₂ O			10 ml

Note: NaN₃ is extremely toxic and was weighed under laminar flow.
The working solution is 2/10.000 (used at 1/100 dilution).

3.2.3 Cell Lysis Buffers**RIPA Lysis Buffer**

	<u>Final Concentration</u>	<u>for 1ml</u>
5M NaCl	150mM NaCl	30 µl
1M Tris-Cl (pH: 7.4)	50 mM Tris-HCl (pH: 7.4)	50 µl
0.5M EDTA	1 mM EDTA	2 µl
Triton-X100	1% Triton-X100	10 µl
10% Na-DOC	1% Na-DOC	100 µl
10% SDS	0.1% SDS	10 µl
25 X PI	1 X PI	40 µl
dH ₂ O		758 µl

NP40 Lysis Buffer

	<u>Final Concentration</u>	<u>for 1ml</u>
5M NaCl	150mM NaCl	30 µl
1M Tris-Cl (pH: 8)	50 mM Tris-HCl (pH: 8)	50 µl
NP40	1% NP40	10 µl
25 X PI	1 X PI	40 µl
dH ₂ O		870 µl

3.2.4 SDS-PAGE Solutions

For SDS-PAGE, either commercial ready systems or conventional method was followed. When commercial systems were used, ready gels, buffers, running and transfer apparatus were purchased from Invitrogen (CA, USA). When the conventional method was used, conventional buffers listed below were utilised.

3.2.4.1 Conventional SDS-PAGE Gel Buffers

1 M Tris-HCl, pH: 6.8

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
Trisma-Base	121.14 g/mol	1 M	12.1 g
dH ₂ O			90 ml
(for Stacking Gel of the SDS-PAGE, pH was adjusted to 6.8) pH was adjusted by concentrated HCl (37%) and 1 N HCl Volume was adjusted with dH ₂ O ⇒			
			100 ml

1.5 M Tris-HCl, pH: 8.8

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
Trisma-Base	121.14 g/mol	1.5 M	18.1 g
dH ₂ O			90 ml
(for Resolving Gel of the SDS-PAGE, pH was adjusted to 8.8) pH was adjusted by concentrated HCl (37%) and 1 N HCl Volume was adjusted with dH ₂ O ⇒			
			100 ml

30% Acrylamide (29% w/v Acrylamide, 1% w/v Bisacrylamide)

	<u>Final Concentration (w/v)</u>	<u>for 250 ml</u>
Acrylamide	29.2%	73 g
N'-N'-Bis-methylene acrylamide	0.8%	2 g
dH ₂ O		200 ml
Volume was adjusted with dH ₂ O ⇒		250 ml
The solution was heated to 37°C to dissolve the chemicals. The solution was filtered and de-gased for ~20 min. The solution was cover with aliminum foil and stored at 4°C.		

10% SDS

	<u>Final Concentration (w/v)</u>	<u>for 250 ml</u>
SDS	10%	25 g
dH ₂ O		50 ml

10% Ammonium Persulfate

0.1 g APS was dissolved in 1 ml dH₂O. (Prepared freshly prior to the experiment.)

Tris-Glycine SDS-PAGE Running Buffer (5X)

	<u>MWt</u>	<u>Final Concentration of 5X</u>	<u>for 250 ml</u>
Glycine	75.07 g/mol	1.25 M	23.75 g
Trisma-Base	121.14 g/mol	125 mM	3.78 g
SDS (from 10%)		0.5 %	12.5 ml
dH ₂ O			200 ml
Volume was adjusted with dH ₂ O ⇒			250 ml
Buffer was stored at 4°C.			

Tris-Glycine SDS-PAGE Transfer Buffer 5X

	<u>MWt</u>	<u>Final Concentration of 5X</u>	<u>for 250 ml</u>
Glycine	75.07 g/mol	195 mM	3.6 g
Trisma-Base	121.14 g/mol	240 mM	7.25 g
SDS (from 10%)		0.185%	4.625 ml
dH ₂ O			225 ml
Volume was adjusted with dH ₂ O ⇒			250 ml

Buffer was stored at 4°C.

When using, the buffer was diluted down to 1X with ddH₂O and Methanol was added to a final concentration of 20%.

5X SDS-PAGE Gel-Loading Buffer

	<u>Final Concentration of 5X</u>	<u>for 10 ml</u>
1M Tris-HCl (pH. 6.8)	250 mM	2.5 ml
Glycerol	50 % (v/v)	5 ml
SDS	10% (w/v)	1 g
1% BPB	0.1 % (w/v)	1 ml
dH ₂ O		1 ml
β-mercapto-ethanol (BME)	5% (w/v)	0.5 ml

5X loading buffer was prepared without BME.

BME was added just before using.

3.2.5 Solutions for Staining the Proteins on SDS-Polyacrylamide Gels**Coomassie Staining Solution**

	<u>for 250 ml</u>
Coomassie Brilliant Blue G-250	25 mg
95% Ethanol	12.5 ml
Phosphoric Acid (85%)	25 ml
dH ₂ O	200 ml

Volume was adjusted with dH₂O ⇒ 250 ml

The solution was filtered through Whatman No: 1.

Coomassie Destaining Solution (Slow Destain)

	<u>Final Concentration</u>	<u>for 250 ml</u>
Methanol	20% (v/v)	50 ml
Acetic Acid	7% (v/v)	17.5 ml
dH ₂ O		182.5 ml

3.2.6 Solutions for Staining Total Proteins on PVDF Membranes

Ponceau-S Staining Solution

	<u>Final Concentration</u>	<u>for 50 ml</u>
Acetic Acid (100%)	10% (v/v)	5 ml
Ponceau-S	5 % (w/v)	2.5 g
dH ₂ O		45 ml

Destaining after Ponceau-S staining was done simply washing with dH₂O.

3.2.7 Western Blotting Solutions

Western Blocking Buffer (TBS-T 5% Milk)

	<u>Final Concentration</u>	<u>for 10 ml</u>
TBS-T		10 ml
Milk Powder (Marble)	5% (w/v)	0.5 g

½ Blocking Buffer (TBS-T 2.5% Milk)

	<u>Final Concentration</u>	<u>for 10 ml</u>
TBS-T		10 ml
Milk Powder (Marble)	2.5% (w/v)	0.25 g

3.2.8 ELISA Solutions

0.1 M Sodium Bicarbonate (NaHCO₃)

	<u>MWt</u>	<u>Final Conc.</u>	<u>for 100 ml</u>
Sodium Bicarbonate (Anhydrous) NaHCO ₃	84.01 g/mol	0.1 M	0.84 g
dH ₂ O			100 ml

0.1 M Sodium Carbonate Na₂CO₃

	<u>MWt</u>	<u>Final Conc.</u>	<u>for 100 ml</u>
Sodium Carbonate (Anhydrous) Na ₂ CO ₃	105.99 g/mol	0.1 M	0.53 g
dH ₂ O			100 ml

Coating Buffer for ELISA (0.1M Sodium Bicarbonate Buffer, pH: 9.4)

	<u>for 100 ml</u>
0.1 M Sodium Bicarbonate (Anhydrous) NaHCO ₃	70 ml
0.1 M Sodium Carbonate (Anhydrous) Na ₂ CO ₃	30 ml

ELISA Blocking Buffer (TBS-T 1% BSA)

	<u>Final Concentration</u>	<u>for 10 ml</u>
TBS-T		10 ml
BSA	1% (w/v)	0.1 g

PNPP (p-Nitrophenyl Phosphate Disodium) Substrate Solution

One tablet of Sigma Fast™ Tris-Buffer was dissolved in 20 ml dH₂O. One tablet of Sigma Fast™ PNPP was later dissolved in the prepared Tris buffer. PNPP solution was freshly prepared prior to the experiment.

3.2.9 Immunofluorescence (IF) Solutions

2% Paraformaldehyde (PFA)

	<u>Final Concentration (w/v)</u>	<u>for 100 ml</u>
Paraformaldehyde	2 %	2 g
PBS		100 ml

The solution was mixed overnight on a heat block to dissolve the reagent.
The solution was filtered, de-gased, aliquoted and stored at -20°C.

Permeabilization Buffer

	<u>Final Concentration (w/v)</u>	<u>for 100 ml</u>
Triton X-100	0.1 %	100 µl
PBS		100 ml

IF Blocking Buffer (PBS 10% FCS)

	<u>Final Concentration (v/v)</u>	<u>for 10 ml</u>
Foetal Calf Serum (FCS)	10 %	1 ml
PBS		9 ml

3.2.10 Agarose Gel Electrophoresis Solutions

50X TAE

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 250 ml</u>
Trisma-Base	121.14 g/mol	2 M	60.5 g
0.5 M EDTA pH:8.0		0.05 M	25 ml
Glacial Acetic Acid			14.275 ml
dH ₂ O			211 ml

6X Agarose Gel Loading Dye Solution

	<u>Final Concentration</u>	<u>for 25 ml</u>
1M Tris-HCl pH:7.6	10 mM	250 µl
1% Bromophenol Blue	0.03% (w/v)	750 µl
1% Xylene Cyanol FF	0.03% (w/v)	750 µl
Glycerol	60%	15 ml
0.5 M EDTA (pH:8.0)	60 mM	3 ml
dH ₂ O		5.25 ml

BPB and Xylene Cyanol Stock solutions were prepared dissolving in dH₂O

3.2.11 Bacterial Culture Media and Solutions

LB (Luria-Bertani) Broth

	<u>for 250 ml</u>
Bacto-tryptone	2.5 g
Yeast Extract	1.25 g
NaCl	2.5 g
dH ₂ O	250 ml

pH was adjusted to 7.5 with 10 N NaOH.

Volume was adjusted with dH₂O ⇒ 250 ml

After preparation, LB Broth was autoclaved right away!

2XTY Broth

	<u>for 250 ml</u>
Bacto-tryptone	4 g
Yeast Extract	2.5 g
NaCl	1.25 g
dH ₂ O	250 ml

pH was adjusted to 7.5 with 10 N NaOH.

Volume was adjusted with dH₂O ⇒ 250 ml

After preparation, 2XTY Broth was autoclaved right away!

LB (Luria-Bertani) Agar

	<u>for 250 ml</u>
Bacto-tryptone	2.5 g
Yeast Extract	1.25 g
NaCl	2.5 g
dH ₂ O	225 ml
Bactoagar	3.75 g

Before adding bactoagar, pH was adjusted to 7.5 with 10 N NaOH.

After adjusting the pH, bactoagar was added, agar was melted into solution in the microwave

Volume was adjusted with dH₂O ⇒ 250 ml

After preparation, LB Agar was autoclaved right away!

Ampicilline 1000X

Ampicilline was dissolved in dH₂O as 100 mg/ml stock solution. The solution was filter-sterilized and stored at -20°C in 500µl aliquots.

Kanamycin 1000X

Kanamycin was dissolved in dH₂O as 25 mg/ml stock solution. The solution was filter-sterilized and stored at -20°C in 500µl aliquots.

Transformation Buffer (TB)

	<u>Final Concentration</u>	<u>for 100 ml</u>
0.5 M Pipes	10 mM Pipes	2 ml
2.5 M CaCl ₂	15 mM	600 µl
1 M KCl	250 mM	25 ml
dH ₂ O		63 ml
1 M MnCl ₂	55 mM	5.5 ml
Before adding MnCl ₂ , pH was adjusted to 6.7 with 10 N KOH.		
Volume was adjusted with dH ₂ O ⇒		100 ml
The solution was filter sterilized and stored at 4°C.		

3.2.12 Sulforhodamine (SRB) Based Growth Assay Solutions

100% Trichloroacetic Acid (TCA)

	<u>Final Concentration (w/v)</u>	<u>for 100 ml</u>
Trichloroacetic Acid (TCA)	100%	100 g
dH ₂ O		100 ml

The solution is aliquoted, covered with aluminium foil and stored at 4°C.
Stock solution was diluted down to 10% with dH₂O when to be used.
Direct contact was avoided since TCA is extremely toxic.

Sulforhodamine (SRB) Stain

	<u>Final Concentration (w/v)</u>	<u>for 10 ml</u>
Sulforhodamine (SRB)	0.4%	0.04 g
dH ₂ O		10 ml

The solution is freshly prepared before use and covered with aluminium foil.

1% Acetic Acid

	<u>Final Concentration (v/v)</u>	<u>for 100 ml</u>
Acetic Acid	1%	1 ml
dH ₂ O		99 ml

10mM Tris Buffer (pH: 10.5)

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 100 ml</u>
Trisma-Base	121.14 g/mol	10 mM	0.121 g
dH ₂ O			100 ml

pH was 10.5 right away.
The solution was kept at 4°C.

3.2.13 Antibody Purification Buffers using Protein G Columns

While purifying antibodies by FPLC method using Protein G columns, 20 mM phosphate buffer (pH: 7) is used as the binding buffer and 0.1 M Glycine-HCl (pH: 3.7) is used as the elution buffer.

Phosphate Buffer will be prepared mixing Na-Phosphate Monobasic Buffer and Na-Phosphate Dibasic Buffer solutions in specific proportions (as indicated below) to obtain a solution with a given pH.

200 mM Na-Phosphate Monobasic (NaH₂PO₄) Stock

	<u>MWt</u>	<u>Final Conc.</u>	<u>for 250 ml</u>
Na-Phosphate Monobasic Monohydrate	138 g/mol	200 mM	6.90 g
dH ₂ O			250 ml

200 mM Na-Phosphate Dibasic (Na₂HPO₄) Stock

	<u>MWt</u>	<u>Final Conc.</u>	<u>for 250 ml</u>
Na-Phosphate Dibasic Dihydrate	177.96 g/mol	200 mM	8.90 g
dH ₂ O			250 ml

Binding Buffer (20 mM Phosphate Buffer, pH: 7)

	<u>for 500 ml</u>
200 mM Na-Phosphate Monobasic (NaH ₂ PO ₄)	19.5 ml
200 mM Na-Phosphate Dibasic (Na ₂ HPO ₄)	30.5 ml
dH ₂ O	450 ml

Elution Buffer (0.1 M Glycine-HCl, pH: 2.7)

	<u>MWt</u>	<u>Final Concentration</u>	<u>for 250 ml</u>
Glycine	75.07 g/mol	0.1 M	1.875 g
dH ₂ O			200 ml
Continue adding HCl until the pH is 2.7			
Finally adjust the volume with dH ₂ O ⇒			250 ml

3.2.14 Flow Cytometry and Cell Cycle Analysis Buffers

Flow Blocking Buffer

	<u>Final Concentration (w/v)</u>	<u>for 100 ml</u>
BSA	1%	1 g
NaN ₃	0.05%	0.05 g
PBS		100 ml

Propidium Iodide (PI) Staining Buffer

	<u>Final Concentration (w/v)</u>	<u>for 5 ml</u>
1 mg/ml PI Stock	50 µg/ml	250 µl
10 mg/ml RNaseA	0.1 mg/ml	50 µl
Triton X-100	0.05%	25 µl
PBS		4675 µl

3.2.15 SAβ-Gal Staining Solutions

3.7 % Formaldehyde

	<u>Final Concentration (w/v)</u>	<u>for 100 ml</u>
Concentrated Formaldehyde (37 %)	3.7 %	10 ml
ddH ₂ O		90 ml

200 mM Citric Acid (Trisodium Salt)

	<u>MWt</u>	<u>Final Conc.</u>	<u>for 100 ml</u>
Trisodium citrate•dihydrate	294.12 g/mol	0.2 M	5.88 g
ddH ₂ O			90 ml
pH was adjusted to 6.0 by adding Citric Acid			
Finally adjust the volume with dH ₂ O ⇒			100 ml

1 M Phosphate Buffer (pH:6.0)

	<u>for 100 ml</u>
1 M Na-Phosphate Monobasic (NaH ₂ PO ₄)	74.5 ml
1 M Na-Phosphate Dibasic (Na ₂ HPO ₄)	25.5 ml

0.1 M K₄Fe(CN)₆•3H₂O (Potassium Ferrocyanide) (Sigma P-9387)

	<u>MWt</u>	<u>Final Concentration</u>	<u>50 ml</u>
K ₄ Fe(CN) ₆ •3H ₂ O	422.39 g/mol	0.1 M	2 g
ddH ₂ O			50 ml

The solution was light sensitive, and covered with aluminum foil.

0.1 M K₃Fe(CN)₆ (Potassium ferricyanide) (Sigma P-8131)

	<u>MWt</u>	<u>Final Concentration</u>	<u>50 ml</u>
K ₃ Fe(CN) ₆	329.25 g/mol	0.1 M	1.646 g
ddH ₂ O			50 ml

The solution was light sensitive, and covered with aluminum foil.

100 mM X-gal (40 mg/ml)

	<u>MWt</u>	<u>Final Concentration</u>	<u>25 ml</u>
X-gal	408.64 g/mol	100 mM	1 g
ddH ₂ O			25 ml

The solution was covered with aluminum foil, aliquoted and kept at -20°C.

Adriamycin (Doxorubicin HCl) Stock (1 mg/ml)

	<u>MWt</u>	<u>Final Concentration</u>	<u>100 ml</u>
Adriamycin	580 g/mol	1 mg/ml	0.1 g
ddH ₂ O			100 ml

The solution was covered with aluminum foil, aliquoted and kept at -20°C.

Camptothecin Stock (20 mM)

	<u>MWt</u>	<u>Final Concentration</u>	<u>20 ml</u>
Camptothecin	348.35 g/mol	20 mM	0.139 g
DMSO			20 ml

The solution was covered with aluminum foil, aliquoted and kept at -20°C.

SAβ-gal Staining Solution

	<u>Final Concentration</u>	<u>for 20 ml</u>
200 mM Trisodium Citric Acid pH:6.0	40 mM	4 ml
1M Phosphate Buffer pH:6.0	200 mM	4 ml
100 mM Potassium Ferricyanide	5 mM	1 ml
100 mM Potassium Ferrocyanide	5 mM	1 ml
5M NaCl	150 mM	600 µl
1M MgCl ₂	2 mM	40 µl
40 mg/ml X-gal	1 mg / ml	0.5 ml
dH ₂ O		8.86 ml

3.3 METHODS

3.3.1 Cell Culture

SK-BR-3 cells were grown in McCoy's 5A medium. MDA-MB-361, T47D, MCF-7, BT-474 and Huh-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM). SP2/0 mouse myeloma cells were grown in DMEM, and generated hybridoma lines were grown in RPMI 1640, DMEM or Serum Free Media (SFM). Unless otherwise stated, all cell culture media was supplemented with 10% foetal calf serum (FCS), 1% penicillin/streptomycin (P/S), 1% non-essential amino acids and 1% L-glutamine. BT-474 cell growth medium was supplemented additionally with 10 µg/ml insulin. All cell lines were cultured as monolayers in humidified CO₂ incubators at 37°C.

3.3.1.1 Thawing Cell Lines

1. Cryotubes full of cell culture stocks were taken from liquid nitrogen tank and processed immediately.
2. The cells were rapidly thawed at 37°C (either by hand or in water bath).
3. The contents of each vial was aspirated by a 5 ml serological pipette and dispensed into a 15 ml falcon tube containing 10 ml fresh complete DMEM.
4. The falcon tube was inverted immediately to dilute the DMSO in the freezing medium and centrifuged at 1000 rpm for 5 minutes.
5. The supernatant was aspirated and the tube was flicked to resuspend the cell pellet.
6. The cells were resuspended with 10 ml of "Thawing Media" and seeded on fresh 100 mm tissue culture dishes (alternatively, resuspended with 13 ml media and plated on fresh 75 mm culture flasks)
7. The plate (or the flask) was placed inside a CO₂ incubator at 37°C and kept overnight.
8. When the thawed cells attach the plate surface properly (~24 to 48 hours later depending on the cell type), the cell media is aspirated and replaced with fresh complete media.

3.3.1.2 Expansion of Adherent Cell Lines

1. Cells attached to the 100 mm plate (or 75 mm flask) surface were grown until 80% confluency inside a CO₂ incubator at 37°C.
2. When the cells reached 80% confluency (still at the logarithmic phase), the medium was aspirated and the cells were washed with 5 to 10 ml sterile PBS.
3. PBS was aspirated thoroughly and 0.5 to 1 ml of trypsin was dispensed onto the plate surface and allowed to cover the total surface by tilting the plate.
4. Plate is placed back inside the incubator and kept 3 to 5 minutes until the cells are detached (cell detachment was inspected with an inverted microscope.)
5. When all the cells were detached from the plate and from each other, trypsin was inactivated by adding 10 ml fresh complete media. The suspension was thoroughly pipette back and forth to prevent cell clumps.
6. The suspension was placed in a 15 ml falcon tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was aspirated and cells were resuspended by flicking.
7. The cells were resuspended in 10 ml fresh complete media and splitted 1/4 to 1/10 depending on the cell type. The volume is brought up to 10 ml with fresh complete media and cells were seeded on a new tissue culture plate. Flasks with newly splitted cells were placed back in CO₂ incubator at 37°C.
8. Medium of the cells was renewed every 2 to 3 days until they were ready for next round of splitting (old medium was aspirated and fresh complete media was dispensed).

3.3.1.3 Expansion of Suspension Cell Lines

1. Suspension cultures were grown in upright flasks. Generally, the cells were grown in 15 to 25 ml complete media in 75 mm flasks.
2. To check the health and confluency of suspension cells, the flasks were bent down. When the cells settled on the bottom of the flask, they were inspected with an inverted microscope. Healthy suspension cell cultures would appear round and shiny.
3. When it was not able to distinguish individual cells in the crowd, the cells were splitted. Alternatively, the cells were splitted when their media started to turn yellow (when they were still at the logarithmic phase).

4. The flask was placed upright and the cells were resuspended in the solution by pipetting the cells up and down. When the suspension became homogeneous, the cells were aspirated leaving 1/5 to 1/10 of the cell suspension in the flask.
5. The volume was completed upto 15 to 25 ml by adding fresh complete media and the flask was placed back in CO₂ incubator at 37°C.

3.3.1.4 Freezing Cell Lines

1. When the cells reached highest confluency at their logarithmic phase, they were collected in 15 ml falcon tubes. In order to collect adherent cells, they were trypsinized as explained above. Suspension cells were collected directly.
2. The cells were centrifuged at 1000 rpm for 5 minutes, the supernatant was aspirated and the cells were resuspended by gentle flicking.
3. The cells were resuspended in freezing media (the volume of the freezing media was strictly dependent on the cell type and confluency. While 5 ml was adequate for 80% confluent Huh-7 cells grown on 75 mm flask, 3 ml was enough for SK-BR-3 cells), aliquoted into previously labelled cryotubes and immediately placed at -20°C.
4. After 1 to 2 hour incubation at -20°C, the cryotubes were placed at -80°C.
5. After overnight incubation at -80°C, the cryotubes were transferred to liquid nitrogen tanks for longer storage.

3.3.1.5 Transient Transfection of Huh-7 Cells

Cell Seeding (Day 0)

1. 3×10^5 cell/ml Huh-7 cell suspension was prepared in P/S(-) DMEM and 2 ml was seeded on each well in 6-well tissue culture plates. Plates were incubated overnight at 37°C in CO₂ incubator for cell attachment.

Transfection (Day 1)

1. The next morning, the medium in all wells was aspirated and replaced with 2 ml OPTIMEM medium (Invitrogen/GIBCO, CA, USA). (It was essential to avoid serum and antibiotics); the plates were transferred back to the incubator.

2. In the mean time, DNA and Lipofectamine 2000 (Invitrogen/GIBCO, CA, USA) was prepared in OPTIMEM medium as follows:

- a) 5 µg DNA was diluted in 250 µl OPTIMEM medium in 1.5 ml tube.
- b) Tube b: 10 µl Lipofectamine 2000 was diluted in 250 µl OPTIMEM medium in 1.5 ml tube.

3. Diluted Lipofectamine 2000 in 250 µl OPTIMEM medium was added onto the diluted DNA in 250 µl OPTIMEM medium, mixed by very gentle vortexing and incubated for 20 minutes at RT to allow the formation of DNA-Lipofectamine 2000 complex.

4. Plates were taken from the incubator and 500 µl DNA-Lipofectamine 2000 complex was added directly to each well drop by drop. The solution was mixed by gently shaking the plates, as if drawing “8” with the plates on the bench surface.

5. The plates were placed back in the incubator and incubated at 37°C for 8 hours.

6. 8 hours after the addition of transfection reagents, each well was aspirated, washed once with PBS and cells were fed with fresh complete DMEM. The plates were placed back in the incubator and incubated overnight at 37°C

Day 2

1. The next morning, media in all wells were aspirated, the wells were washed once with PBS and the cells were trypsinized.

2. Trypsinized cells were seeded as follows:

- a) Cells whose proteins would be collected and used for immunoprecipitation experiments were seeded on 100 mm culture dishes and grown at 37°C in CO₂ incubator for 2 to 3 days until they reached ~80% confluency. Upon reaching ~80% confluency, the cells were scraped and lysed as explained in 3.3.6.
- b) Cells that would be used for immunofluorescence experiments were seeded on sterile coverslips placed in 6-well dishes. Each transfectant was seeded on 8 wells and grown at 37°C in CO₂ incubator for 2 to 3 days until they reached ~80 % confluency.

3.3.2 Generation of anti-ERBB2 Monoclonal Antibodies

Anti-ERBB2 monoclonal antibodies were prepared as described previously [290]. The detailed protocol is explained below.

3.3.2.1 Immunization of BALB/c mice against human ERBB2

1. $5-10 \times 10^6$ pathogen-free SK-BR-3 cells were washed three times with SF-DMEM and finally resuspended in 125 μ l PBS.
2. Complete Freund's Adjuvant (CFA) solution was resuspended completely.
3. 125 μ l cell suspension was mixed with 125 μ l homogenous CFA and the mixture was drawn up into a sterile 1 ml syringe. (Since the antigen was a cell-surface protein, extreme care was taken not to lyse the SK-BR-3 cells during the process.)
4. CFA/cell emulsion was injected intraperitoneally to five BALB/c mice.
5. BALB/c mice were re-immunized at three-week intervals by intraperitoneal injection of live SK-BR-3 cells mixed 1:1 with Incomplete Freund's Adjuvant (IFA).
6. In the following week after each booster injection, blood was collected from a tail bleed of all immunized animals and the sera were screened by ELISA for the presence of anti-ERBB2 antibodies.
7. After the fourth booster immunization, the last immunization was performed with recombinant human ERBB2 ECD/Fc fusion protein (R&D Systems, MN, USA). For the final immunization, 20 μ g recombinant human ERBB2 ECD/Fc fusion protein resuspended in 125 μ l PBS was mixed with 125 μ l IFA and injected intraperitoneally to each BALB/c mice.
8. On the third day of last immunization, mice were sacrificed by cervical dislocation (anaesthetics were avoided) and their spleens were harvested and placed in 50 ml sterile falcon tubes with SF-DMEM.

3.3.2.2 Harvesting splenocytes and preparation of cells

Preparation of SP2/0 Myeloma Cells

1. At least one week prior to fusion, SP2/0 cells were started to be expanded.
2. One day before the fusion, SP2/0 cells were splitted into fresh complete DMEM.

Preparation of Feeder Layer

1. One day prior to fusion, a spleen from an un-immunized mice was harvested, brought to tissue culture facility and processed under aseptic conditions (in biological safety cabinet) hereafter.
2. The spleen was transferred to a 100 mm petri dish with 10 ml SF-DMEM and teased into single cell suspension by chopping with and squeezing between two sterile needles.
3. Dissociated splenocytes were transferred to 50 ml falcon tubes filled with SF-DMEM and centrifuged at 1500 rpm for 5 minutes.
4. The supernatant was aspirated, the cell pellet was resuspended initially by gentle flicking and the tube was topped up with SF-DMEM to re-wash the splenocytes.
5. Cell suspension was centrifuged at 1500 rpm for 5 minutes, the supernatant was aspirated and the cell pellet was resuspended in 100 ml HAT-DMEM.
6. Cell suspension was gently aspirated by 10 ml pipette and 2 drops (~100 μ l) cell suspension is dispensed to each well of a 96-well flat-bottom plate.
7. Plates with the feeder layer cells were placed inside a CO₂ incubator at 37°C.

Preparation of Splenocytes from Immunized Mice

1. On the day of fusion, spleens from immunized mice were harvested; the splenocytes were dissociated and washed as explained above.
2. After the second wash with SF-DMEM, the cell suspension was centrifuged at 1500 rpm for 5 minutes, the supernatant was aspirated, the cell pellet was resuspended in SF-DMEM and the cells were counted.

3.3.2.3 Preparation of Hybridoma Clones and Subcloning

Fusion of Splenocytes and SP2/0 Myeloma Cells

1. SF-DMEM, HAT-DMEM and PEG were prewarmed in a 37°C water bath. 2 beakers were prepared containing ~100-200 ml H₂O at 37°C (to be used as a water bath in the laminar flow during the next steps).
2. Cultured SP2/0 cells were washed twice with SF-DMEM, resuspended in SF-DMEM and counted.
3. SP2/0 cells and splenocytes were combined in 4:1 ratio.

4. The SP2/0 – splenocyte cell mixture was washed once with SF-DMEM. The cells were centrifuged at 1500 rpm for 5 minutes, the supernatant was aspirated well and the cell pellet was resuspended by gentle flicking.

Note: Proteins and HEPES were avoided since PEG would precipitate the proteins and HEPES could be toxic during cell fusion.

5. Water bath beakers were placed inside the laminar flow. Falcon tubes having SF-DMEM and PEG were placed in one the beakers.

6. The falcon tube with the cells was gently flicked again to prevent cell pellet chunks and placed in the other water bath beaker.

7. Drop-by-drop, 1 ml prewarmed PEG was added on to the mixed cell suspension (with a rate of ~1 ml/min). Cells were occasionally stirred by the pipette tip very gently. Finally, the cells were stirred for an additional minute.

Note: Since the cells were extremely gentle during fusion, they were never pipetted.

8. Drop-by-drop, 1 ml prewarmed SF-DMEM was added on to the PEGylated cell suspension (with a rate of ~1 ml/min). Cells were occasionally stirred by the pipette tip very gently. The step was repeated with an additional ml of prewarmed HAT-DMEM.

9. Additional 10 ml prewarmed SF-DMEM was added on to the cell suspension over 2-3 minutes with occasional stirring.

10. The cells were centrifuged at 1500 rpm for 5 minutes, the supernatant was aspirated and the cell pellet was resuspended initially by gentle flicking.

11. 10 ml HAT-DMEM was forcefully discharged onto the cells to disrupt the cell pellets. Cell clumps were allowed to settle down and disrupted with pipette tip.

12. The cells were resuspended in total ~100 ml prewarmed HAT-DMEM and aliquoted (~100 μ l /well) onto 96-well plates with feeder layer.

13. In order to feed the cells, in every 2 to 3 days, ~100 μ l of media was aspirated above each well and 2 drops (~100 μ l) of fresh HAT-DMEM was dispensed.

14. Around 14th day, aminopterin should have killed all unfused SP2/0 cells, therefore feeding medium was changed to HT-DMEM.

15. After three rounds of feeding with HT-DMEM, the aminopterin left in the wells would be diluted out, therefore feeding was carried out with complete DMEM.

16. The supernatant of the hybridomas were screened by ELISA when most of the wells with growing cells reach upto ~25% confluency (their media turned yellow within 2 days after re-feeding).

17. 100 µl supernatant were collected from each well to be screened by ELISA and the wells were topped up with fresh complete DMEM.

18. Hybridoma clones were screened by ELISA and positive clones were splitted to fresh plates and expanded. Half of the cells in each clone were frozen Hybridoma Freezing Media. The other half was subcloned.

ELISA Screening of Hybridoma Clones

ELISA assay which is comprehensively explained in 3.3.3 was utilized to screen hybridoma clones that produce antibodies against extracellular domain of human ERBB2 protein. Briefly, 96-well polysorb microtiter plates were coated overnight at 4°C with recombinant human ERBB2 ECD antigen (~50 ng/well). 100 µl of each hybridoma supernatant was applied onto separate wells as the primary antibody and incubated for 1 to 2 hours at 37°C. Wells were further incubated with 1/1000 diluted AP-conjugated anti-mouse IgG antibodies for one hour at 37°C to detect bound antibodies.

Subcloning Hybridoma Cells for Establishing Monoclonal Cell Lines

The ELISA positive hybridoma clones were subjected to subcloning by limiting dilution. In order to provide the necessary growth factors, the hybridoma cells were subcloned on a feeder layer.

1. One day prior to subcloning, the feeder layer was prepared as explained above. (additionally, conditioned supernatant from SP2/0 cell cultures grown in complete DMEM could be filtered and used instead of feeder layer)

2. Each selected hybridoma clone that has been expanded privously was collected and washed once with complete DMEM. The cell pellet was resuspended in fresh complete DMEM and cells were counted.

3. The hybridoma cells were diluted in complete DMEM in order to give one cell per 100 µl and the cells were stirred thoroughly to make a homogenous suspension.

4. 100 µl of cell suspension was seeded onto each well containing feeder layer.

5. Before feeding, the monoclonal wells were noted, to avoid picking polyclonal cell populations. When the media of the wells containing growing cells started to turn yellow, the monoclonality of the wells was inspected with an inverted microscope.
6. Monoclonal wells were feeded every 2 to 3 days with fresh complete DMEM.
7. When the wells were confluent enough, the subclones were ELISA screened.
8. ELISA positive clones were further propagated and cell stocks were prepared.

3.3.3 Indirect ELISA

1. Antigen was diluted in “Coating Buffer” to make 250-500 ng/ml solution and 100 µl solution was aliquoted into each well of Nunc Polysorb ELISA plate.
2. Plate was covered with parafilm and incubated overnight at 4°C.
3. The wells were emptied by finally tapping the plate onto paper towel.
4. Each well was washed 3 times with 250 µl TBS.
5. The wells were emptied by finally tapping the plate onto paper towel.
6. 100 µl “ELISA Blocking Buffer” was added onto each well, plate was covered with parafilm and incubated 1 hour at 37°C.
7. The wells were emptied and washed three times with 250 µl TBS-T.
8. Primary antibody was either applied directly (i. e. hybridoma supernatant), or was diluted in TBS (i.e. concentrated pure antibody) and 100 µl antibody solution was added onto the each well. The plate was covered and incubated 1-4 hour at 37°C.
9. The wells were emptied and washed four times with 250 µl TBS-T.
10. AP-conjugated secondary antibody was diluted 1/1000 in TBS and 100 µl antibody solution was added onto the each well. The plate was covered and incubated 1 hour at 37°C.
11. The wells were emptied and washed six times with 250 µl TBS-T.
12. 100 µl “PNPP Substrate Solution” was added onto each well. The plate was covered and incubated ~30 min (or until color development is sufficiently) at RT.
13. The signal intensity at 405 nm was measured using BioTek µQuant Elisa Reader.

3.3.4 Antibody Isotyping and Purification of anti-ERBB2 Antibodies

Hybridoma cell cultures secrete monoclonal antibodies into the culture media. This culture media which contain 1 to 50 µg secreted antibody per ml can be collected and used for antibody purification. Alternatively, hybridoma cultures can be used for production of ascites in mice which contain antibodies at much higher concentrations (1 to 5 mg of secreted antibody per ml). Since 1 ml ascites can yield upto 5000 times more concentrated antibodies than ordinary hybridoma culture supernatants, ascites are commonly preferred when higher amounts of antibodies are needed to be produced. On the other hand, when grown in special serum free media, hybridoma supernatants are devoid of any contaminating immunoglobulins which may be present in ascites solutions.

3.3.4.1 Collecting Antibodies from Hybridoma Supernatants

1. Hybridoma cell cultures that were grown initially in Complete DMEM were adapted to Serum Free Media (SFM) through multiple dilution steps.
 - a) Initially they were transferred to SFM containing 10% FCS (SFM10).
 - b) When they grow in SFM10 enough to be splitted, they were transferred into SFM containing 5 % FCS (SFM5)
 - c) Next they were splitted into SFM containing 2.5 % FCS (SFM2.5)
 - d) This dilution was continued until the cells grew healthy in SFM containing 0.3% FCS. Antibody production efficiency of SFM adapted hybridoma cultures were monitored by ELISA assay.
2. Hybridoma cells were grown for 5 to 7 days densely without renewing the media.
3. When the culture media became highly acidic (yellow), the media was culture media was collected and centrifuged at 1500 rpm for 10 minutes.
4. The supernatant was transferred into fresh 50 ml falcon tubes and pH was neutralized by adding 1 M Tris solution at pH:8.
5. Neutralized supernatants were stored at -20°C.

3.3.4.2 Preparation and Collection of Ascites from Mice

1. 6 to 11 weeks old five BALB/c mice were initially primed by intraperitoneal injection of 250 μ l IFA.
2. 7 days after IFA injection, 5×10^6 hybridoma cells resuspended in 250 μ l PBS were injected to each mouse intraperitoneally.
3. Abdominal distention of each mouse was monitored every other day. When ascetic fluid volume increased to a level that would discomfort and impair movement of mice (at days 7 to 15 depending on the mouse), they were anesthetized and the ascites was harvested using 20 gauge needle.
4. Warm saline was injected intraperitoneally at a volume equal to the harvested ascites in order to prevent hypovolemic shock. The mice were transferred back to their cages and the tap date was recorded on the cage cards.
5. Mice were monitored for few more weeks and tapped twice more, last one being performed after euthanasia (by servical dislocation).
6. The collected ascites was incubated 30 to 60 minutes in a water bath at 37°C and centrifuged at 2500 rpm for 5 minutes at 4°C.
7. Serum supernatant was transferred to 1.5 ml tubes and centrifuged at 13,000 rpm for 5 minutes at 4°C to remove fat.
8. The supernatant was transferred to fresh 1.5 ml tubes and stored at -20°C.

3.3.4.3 Isotyping of Monocolonal Antibodies

ProteinA and ProteinG beads have different affinities for different mouse immunoglobulin isotypes. In order to use the purification system with highest affinity, the isotype of each of the antibody clone was determined by an ELISA based method using a commercial Monoclonal Antibody Isotyping Kit (Thermo Thermo Fisher Scientific Inc., IL, USA) according to the protocol provided by the manufacturer.

3.3.4.4 Purification of Monoclonal Antibodies by FPLC using ProteinG

Columns

All of the samples and buffers including ddH₂O and 20% ethanol were filtered and degassed before applying on to instrument in order to prevent system clogging and introduction of bubbles.

1. In order to protect the ÄKTAexplorer™ FPLC instrument, initially a pressure alarm of 0.6MPa was set up.
2. 5 ml Hi-Trap Protein G HP column was connected to the system and the system was washed first with 20% ethanol, then with ddH₂O (10 column volume each).
3. Instrument probes were transferred into solution bottles that would be used during purification. Probe A was placed in Binding Buffer (20 mM Phosphate, pH:7) and Probe B was placed in Elution Buffer (0.1 M Glycine-HCl, pH:2.7). Tubing system was washed with these buffers using “Pump Wash Basic” comment.
4. The column was equilibrated with at least 10 column volumes of Binding Buffer.
5. In the mean time, the filtered sample was loaded into the superloop while the valves were in “LOAD” position.
6. The valves were transferred into “INJECT” position and the sample stored in the superloop was injected onto the column. Flow Through containing the sample unbound to the column was collected in 15 ml falcon tubes at the fraction collector.
7. The column was washed with 5 column volumes of Binding Buffer to remove the unbound proteins.
8. Bound antibodies were eluted with 5 to 10 column volumes of Elution Buffer and the eluates were collected in 5 ml falcon tubes.
9. Antibodies eluted in Elution Buffer were immediately neutralized by addition of 1 M Tris Buffer, pH:9 (1 ml Tris buffer was added per 5 ml eluate).
10. All the elution tubes containing antibodies were pooled and concentrated using Amicon Ultra-15 Ultracel-10k concentrators as follows:
 - a) Antibody eluates were loaded onto concentrators and centrifuged at 2500 g (~3200 rpm) at 4°C until the volume above the concentrator filter was decreased down to ~1.5 ml.

- b) Eluted buffer at the bottom of the concentrator tube was discarded and ~10 ml sterile cold PBS was added onto the concentrated antibody solution; the tube content was mixed and centrifuged at 2500 g (~3200 rpm) at 4°C until the volume above the concentrator filter was decreased down to ~1.5 ml.
 - c) PBS collected at the bottom of the concentrator tube was discarded and buffer exchange was repeated once more with ~10 ml sterile cold PBS.
 - d) The tube was centrifuged at 2500 g (~3200 rpm) at 4°C until the volume above the concentrator filter was decreased down to ~1 ml.
- 11.** The antibody concentration of the solution was measured on nanodrop at 280 nm. (5 volume Elution Buffer was mixed with 1 volume 1 M Tris pH:9 and used as blank)
- 12.** The concentrated antibody solution was transferred into fresh 0.5 ml tubes and stored at -20°C as 50 µl aliquotes.

3.3.5 Cloning ERBB2-deletion Constructs

3.3.5.1 Preparation of ERBB2-deletion Constructs

Human ERBB2 constructs containing various combinations of the extracellular subdomains I – IV were created using the human ERBB2 full length construct OCABo5050G0219D-pDEST26 purchased from Source BioScience LifeSciences (Nottingham, UK) (will be mentioned as p219-D hereafter). p219-D is a pDEST26 based vector where human ERBB2 cDNA was cloned 3-terminal to a 6-His tag between attB1 and attB2 TOPO-cloning sites.

Initially, the total extracellular ERBB2 region was excised from p219-D and two additional cloning sites (NotI and XhoI sites) were introduced between the signal peptide and transmembrane (TM) domain (**Figure 3.1**). A nucleotide was added before the NotI site in order to prevent frameshift, and six additional nucleotides (corresponding to amino acids Val and Leu) were inserted between the NotI and XhoI sites in order to enable enough space for restriction enzyme binding during double digestion. This new plasmid was named pCC2001 and used as a backbone to

insert various extracellular ERBB2 subdomains amplified by PCR. Insertion of PCR-amplified extracellular subdomain I into pCC2001 and generation of pCC2002 is demonstrated on **Figure 3.2** as an example. Details on fragment preparation for other ERBB2 constructs can be seen on **Table 3.6**, **Table 3.7** and **Table 3.8** which provide information about the oligos used, fragments produced and plasmids constructed respectively. All plasmids were sequence verified using oligonucleotides listed on **Table 3.9** as schematized on **Figure 3.3**. Both of the template plasmids (p219-D and pCC2001) were extensively sequenced. Since plasmids pCC2002 to pCC2014 were produced by insertion of the PCR amplified extracellular subdomains into the sequence verified pCC2001, only the newly inserted extracellular ERBB2 regions and the transmembrane domain (TM) of pCC2002 to pCC2014 were sequenced (starting prior to first ATG, to the end of transmembrane domain, covering all the restriction sites used for each cloning).

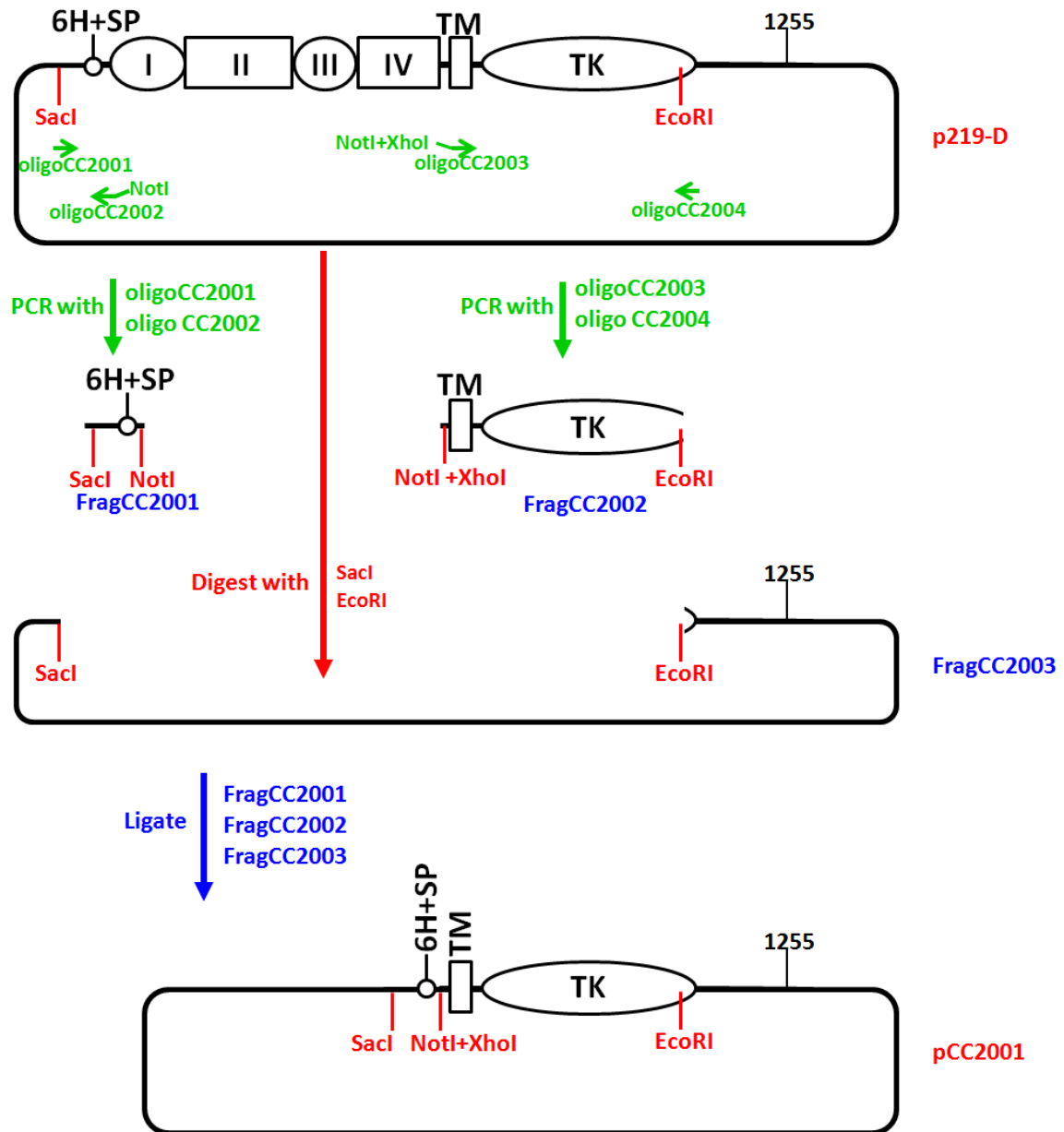


Figure 3.1 Cloning of pCC2001. Through SacI-EcoRI digestion of p219-D, the backbone fragment FragCC2003 was prepared. 6xHis tag and the N-terminal 22 amino acids of the human ERBB2 cDNA forming the signal peptide (6H+SP) were amplified using PCR primers oligoCC2001 and oligoCC2002; producing the FragCC2001 with an inserted NotI restriction site at the 3' end. Region between the 647th (beginning of TM) and 976th (EcoRI site inside TK) amino acids of the human ERBB2 cDNA was amplified using PCR primers oligoCC2003 and oligoCC2004; producing the FragCC2002 with an inserted NotI and XhoI restriction site at the 5' end. The boxes named as I, II, III, IV represent four subdomains of ECD. 6H+SP: 6xHis tag and signal peptide domain, TM: transmembrane domain, TK: tyrosine kinase domain, 1255: last amino acid on human ERBB2 cDNA.

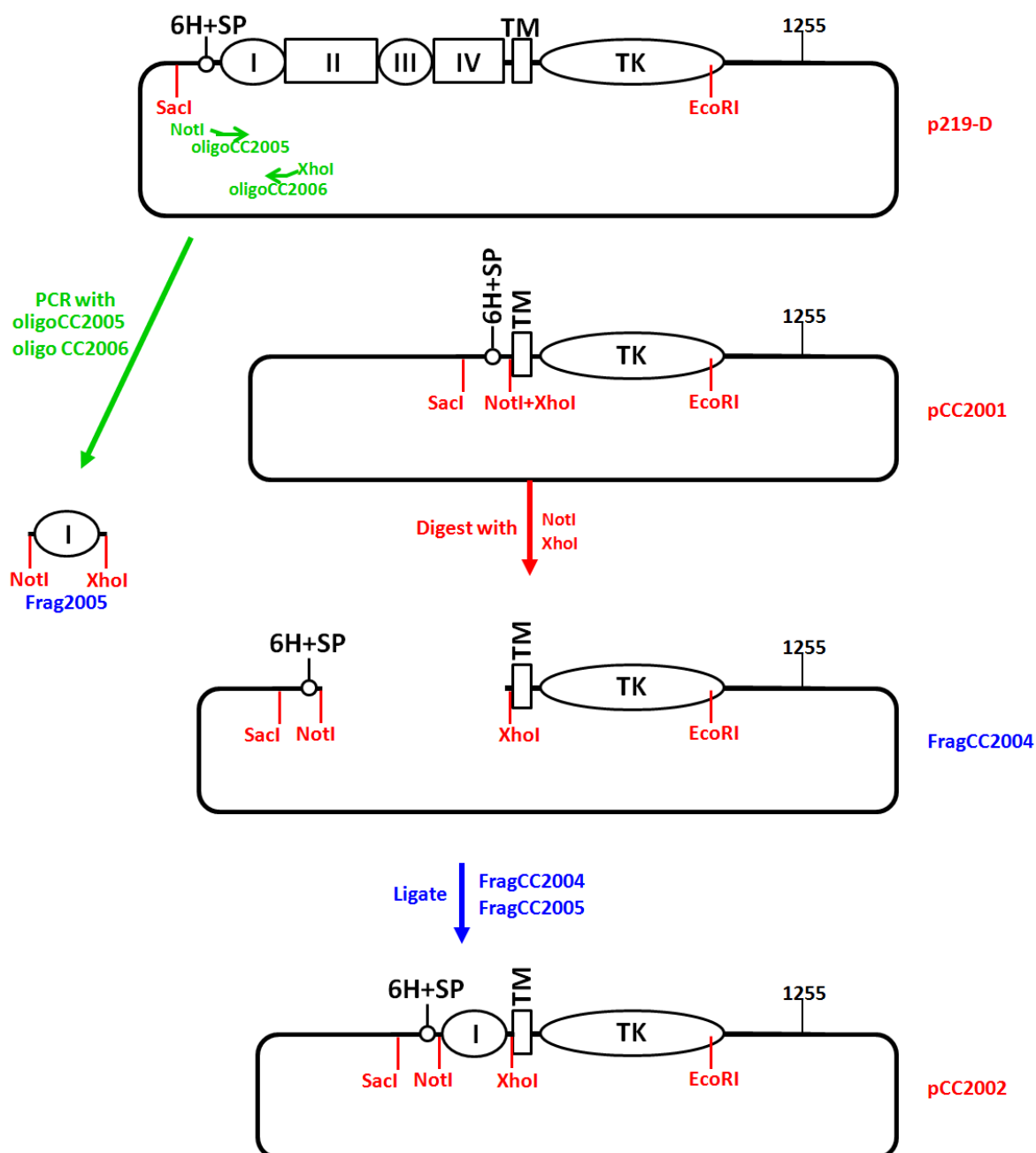


Figure 3.2 Cloning of pCC2002. Through NotI-XhoI digestion of pCC2001, the backbone fragment FragCC2004 was prepared. Extracellular subdomain I located between the 23rd and 162nd amino acids of the human ERBB2 cDNA was amplified using PCR primers oligoCC2005 and oligoCC2006; producing the FragCC2005 with inserted NotI and XhoI restriction site at the 5' and 3' ends respectively. The boxes named as I, II, III, IV represent four subdomains of ECD. 6H+SP: 6xHis tag and signal peptide domain, TM: transmembrane domain, TK: tyrosine kinase domain, 1255: last amino acid on human ERBB2 cDNA.

Oligo	Sequence	Size	Aim		Location on p219-D
			F/R	Product	
oligoCC2001	5'-AGCAGAGCTCGTTTAGTGAACC-3'	22 bp	F	FragCC2001	514(SacI)-535
oligoCC2002	5'- GCGGCCGC TGCTCGCGGCTCCGGG-3'	25 bp	R	FragCC2001	(6xHis+SP)763-778+N+ NotI
oligoCC2003	5'-TT GCGGCCGC GTATT CTCGAG AGAGCCAGCCCTC-3'	35 bp	F	FragCC2002	NotI +Val+Leu+ XhoI +2651-2663(TM)
oligoCC2004	5'-CGGGAGAATTCAGACACCAACTCC-3'	24 bp	R	FragCC2002	(TK)3622-(EcoRI)3645
oligoCC2005	5'-AA GCGGCCGC ACCCAAGTGTGCACCGGC-3'	28 bp	F	FragCC2005	N+N+ NotI +779-796(Subdomain I)
			F	FragCC2009	
			F	FragCC2012	
			F	FragCC2016	
oligoCC2005	5'-AA GCGGCCGC ACCCAAGTGTGCACCGGC-3'	28 bp	F	FragCC2019	
			F	FragCC2005	
			F	FragCC2006	
			F	FragCC2010	
oligoCC2006	5'-TT CTCGAG GAGCTGGGGTTCCGCT-3'	25 bp	R	FragCC2005	(Subdomain I)1179-1195+ XhoI +N+N
oligoCC2007	5'-TT GCGGCCGC TGCTACCAGGACACGAT-3'	27 bp	F	FragCC2006	N+N+ NotI + 1196-1212(Subdomain II)
			F	FragCC2010	
			F	FragCC2015	
			F	FragCC2017	
oligoCC2008	5'-CG CTCGAG AAAGATCTTCTTGACGCC-3'	26 bp	R	FragCC2006	(Subdomain II)1808-1825+ XhoI +N+N
			R	FragCC2009	
oligoCC2009	5'-TT GCGGCCGC GGGAGCCTGGCATTCTG-3'	28 bp	F	FragCC2007	N+N+ NotI +1826-1843(Subdomain III)
			F	FragCC2011	
oligoCC2010	5'-TT CTCGAG GAGGTGGGTGTTATGGAGG-3'	27 bp	R	FragCC2007	(Subdomain III)2116-2134+ XhoI +N+N
			R	FragCC2010	
			R	FragCC2013	
			R	FragCC2016	
oligoCC2011	5'-TT GCGGCCGC TGCTTCGTGCACACGG-3'	26 bp	F	FragCC2008	N+N+ NotI +2135-2150(Subdomain IV)
oligoCC2012	5'-TT CTCGAG CTGCTCGGCGGGGAG-3'	24 bp	R	FragCC2008	(Subdomain IV)2635-2650+ XhoI +N+N
			R	FragCC2011	
			R	FragCC2014	
			R	FragCC2017	
oligoCC2012	5'-TT CTCGAG CTGCTCGGCGGGGAG-3'	24 bp	R	FragCC2018	
			R	FragCC2018	
oligoCC2013	5'-TT ACTAGT GAGCTGGGGTTCCGCT-3'	25 bp	R	FragCC2012	(Subdomain I)1179-1195+ SpeI +N+N
oligoCC2014	5'-TT ACTAGT GGGAGCCTGGCATTCTG-3'	26 bp	F	FragCC2013	N+N+ SpeI +1826-1843(Subdomain III)
			F	FragCC2018	
oligoCC2015	5'-TT ACTAGT TGCTTCGTGCACACGG-3'	24 bp	F	FragCC2014	N+N+ SpeI +2135-2150(Subdomain IV)
oligoCC2016	5'-CG ACTAGT TAAAGATCTTCTTGACGCC-3'	26 bp	R	FragCC2015	(Subdomain II)1808-1825+ SpeI +N+N
			R	FragCC2019	

Table 3.6 Oligonucleotide primers used during cloning of ERBB2 constructs.

Sequence and size of each primer are shown on “Sequence” and “Size” columns. Highlighted sequences in color denote the introduced restriction sites (red: NotI, blue: XhoI, green: SpeI). The orientation (F/R) and amplification aims of each designed primer are shown on the “Aim” column. Location of each primer on the template plasmid p219-D is shown on the “Location” column. Location of each primer on the human ERBB2 cDNA is highlighted and shown in brackets; the numbers indicate the complementary nucleotides on the template plasmid p219-D. NotI (red), XhoI (blue) and SpeI (green) sites introduced next to the complementary sequences are shown. SacI and EcoRI sites that were already present on the complementary plasmid sequence were shown in brackets and highlighted in pink. Six additional nucleotides (corresponding to amino acids Val and Leu) were inserted between the NotI and XhoI sites on oligo2003. F: forward, R: reverse, N: random nucleotide.

Fragment	Synthesis			Size	Elements
	Method	by	template		
FragCC2001	PCR	oligoCC2001-CC2002	p219-D	259 bp	SacI-6xHis-SP-NotI
FragCC2002	PCR	oligoCC2003-CC2004	p219-D	1003 bp	NotI-XhoI-TM-TK-EcoRI
FragCC2003	digestion	SacI-EcoRI	p219-D	6508 bp	SacI-Backbone-CT-TK-TM-EcoRI
FragCC2004	digestion	NotI-XhoI	pCC2001	7757 bp	NotI-SP-6xHis-SacI-Backbone-CT-TK-EcoRI-TM-XhoI
FragCC2005	PCR	oligoCC2005-CC2006	p219-D	424 bp	NotI-Subdomain I-XhoI
FragCC2006	PCR	oligoCC2007-CC2008	p219-D	637 bp	NotI-Subdomain II-XhoI
FragCC2007	PCR	oligoCC2009-CC2010	p219-D	316 bp	NotI-Subdomain III-XhoI
FragCC2008	PCR	oligoCC2011-CC2012	p219-D	523 bp	NotI-Subdomain IV-XhoI
FragCC2009	PCR	oligoCC2005-CC2008	p219-D	1054 bp	NotI-Subdomain I-II-XhoI
FragCC2010	PCR	oligoCC2007-CC2010	p219-D	946 bp	NotI-Subdomain II-III-XhoI
FragCC2011	PCR	oligoCC2009-CC2012	p219-D	832 bp	NotI-Subdomain III-IV-XhoI
FragCC2012	PCR	oligoCC2005-CC2013	p219-D	426 bp	NotI-Subdomain I-SpeI
FragCC2013	PCR	oligoCC2014-CC2010	p219-D	315 bp	SpeI-Subdomain III-XhoI
FragCC2014	PCR	oligoCC2015-CC2012	p219-D	522 bp	SpeI-Subdomain IV-XhoI
FragCC2015	PCR	oligoCC2007-CC2016	p219-D	637 bp	NotI-Subdomain II-SpeI
FragCC2016	PCR	oligoCC2005-CC2010	p219-D	1363 bp	NotI-Subdomain I-II-III-XhoI
FragCC2017	PCR	oligoCC2005-CC2012	p219-D	1462 bp	NotI-Subdomain II-III-IV-XhoI
FragCC2018	PCR	oligoCC2014-CC2012	p219-D	831 bp	SpeI-Subdomain III-IV-XhoI
FragCC2019	PCR	oligoCC2005-CC2016	p219-D	1054 bp	NotI-Subdomain I-II-SpeI

Table 3.7 Production of fragments used for construction various ERBB2 constructs. The method of synthesis for each fragment and the fragment lengths are shown on “Synthesis” and “Size columns respectively. From 5’ end to the 3’ end, the components of each fragment are given on “Elements” column. Highlighted sequences in color denote the restriction sites (red: NotI, blue: XhoI, green: SpeI, pink: SacI or EcoRI).

Plasmid	Ligation of	Size	Elements
p219-D		9621 bp	6xHis-SP-Subdomain I-II-III-IV-TM-TK-CT
pCC2001	FragCC2001 + FragCC2002 + FragCC2003	7770 bp	6xHis-SP-TM-TK-CT
pCC2002	FragCC2005 + FragCC2004	8181 bp	6xHis-SP-Subdomain I-TM-TK-CT
pCC2003	FragCC2006 + FragCC2004	8394 bp	6xHis-SP-Subdomain II-TM-TK-CT
pCC2004	FragCC2007 + FragCC2004	8073 bp	6xHis-SP-Subdomain III-TM-TK-CT
pCC2005	FragCC2008 + FragCC2004	8280 bp	6xHis-SP-Subdomain IV-TM-TK-CT
pCC2006	FragCC2009 + FragCC2004	8811 bp	6xHis-SP-Subdomain I-II-TM-TK-CT
pCC2007	FragCC2010 + FragCC2004	8703 bp	6xHis-SP-Subdomain II-III-TM-TK-CT
pCC2008	FragCC2011 + FragCC2004	8589 bp	6xHis-SP-Subdomain III-IV-TM-TK-CT
pCC2009	FragCC2012 + FragCC2013 + FragCC2004	8496 bp	6xHis-SP-Subdomain I-III-TM-TK-CT
pCC2010	FragCC2012 + FragCC2014 + FragCC2004	8703 bp	6xHis-SP-Subdomain I-IV-TM-TK-CT
pCC2011	FragCC2015 + FragCC2014 + FragCC2004	8916 bp	6xHis-SP-Subdomain II-IV-TM-TK-CT
pCC2012	FragCC2016 + FragCC2004	9120 bp	6xHis-SP-Subdomain I-II-III-TM-TK-CT
pCC2014	FragCC2012 + FragCC2014 + FragCC2004	9012 bp	6xHis-SP-Subdomain I-III-IV-TM-TK-CT

Table 3.8 Fragment components of each ERBB2 construct. Except for p219-D, all listed plasmids are produced by the ligation of fragments listed on “Ligation of” column and size of each plasmid is listed on “Size” column. From 5’ end to the 3’ end, the protein coding regions on each plasmid are given on “Elements” column.

Oligo	Sequence	Size	F/R	Position on pCC2001
oligoCC2017	5'-TGAACCGTCAGATCGCCTGGAG-3'	22 bp	F	530-551 (after SacI, before ATG)
oligoCC2018	5'-CTCATTCTGGATGACCAC-3'	18 bp	R	1820-1837 (after EcoRI at TK)
oligoCC2019	5'-CTCCTCTCGCCTCTTG-3'	18 bp	F	743-760 (inside SP, before NotI)
oligoCC2020	5'-GAATGCCAACCACCGCAGAG-3'	20 bp	R	826-845 (after XhoI site, inside TM)
oligoCC2021	5'-CAGAGCTCGTTTGTGAACC-3'	20 bp	F	516-535 (after SacI, before ATG)
oligoCC2022	5'-GTGTAATTCGGATCTTCTG-3'	20 bp	R	899-918 (after XhoI site, inside TM)
oligoCC2023	5'-GGGCCAAACCTTACGATGGG-3'	20 bp	F	1617-1636 (inside TK, before EcoRI at TK)
oligoCC2024	5'-CACATACTCTCAGCATCCAC-3'	21 bp	R	1913-1930 (after EcoRI site at TK, inside CT)
oligoCC2025	5'-GAGAGCTATGACGTCGATGCAC-3'	23 bp	R	2672-2694 (after stop codon)

Table 3.9 Oligonucleotides used for sequence verification of the ERBB2 constructs. Sequence and size of each primer used for sequence verification are shown on “Sequence” and “Size” columns. The orientation (F/R) and location of each primer on the template plasmid pCC2001 are shown on the “F/R” and “Location” columns respectively.

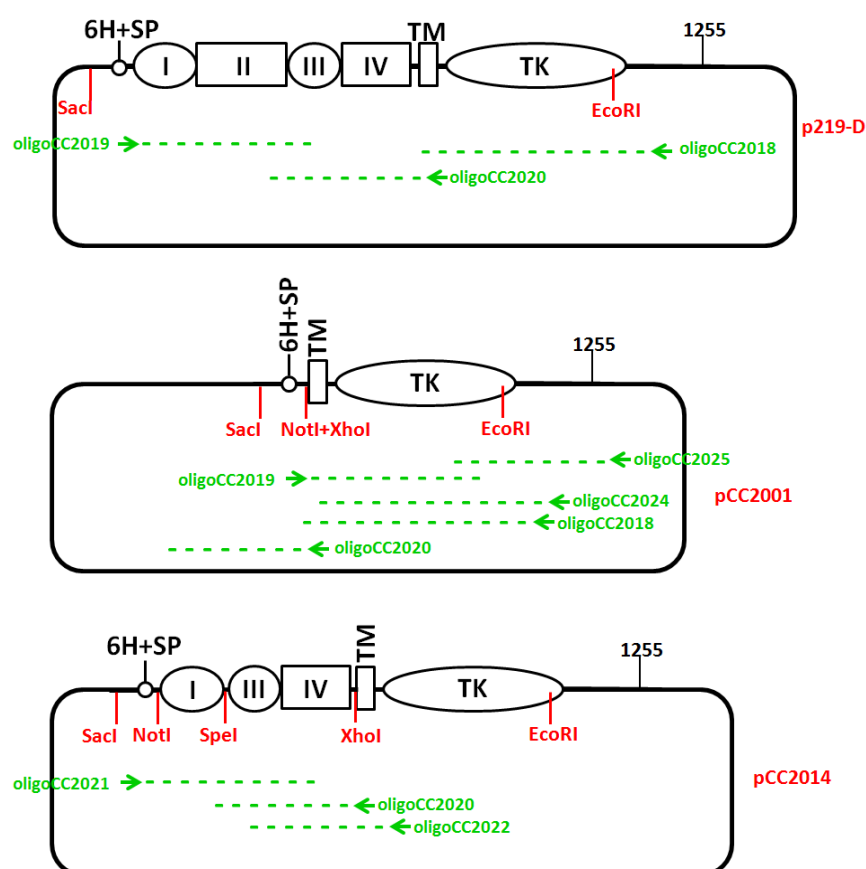


Figure 3.3 Sequence verification of ERBB2 constructs. Template plasmid p219-D was sequence verified from the signal peptide (SP) to the end of Tyrosine Kinase (TK) domain including the EcoRI site used for cloning purposes. Template plasmid pCC2001 was sequence verified extensively starting upstream of the initial ATG codon prior to the 6H+SP site to downstream of the STOP codon after 1255th amino acid of ERBB2 cDNA. Sequencing of pCC2014 is shown as an example of ERBB2 constructs containing various combinations of ERBB2 extracellular subdomains. To verify the sequences of their PCR-amplified inserts, these constructs were sequenced using three primers shown on figure. Green arrows indicate the sequencing start sites and green dots indicate the verified sequences by each primer. The boxes named as I, II, III, IV represent four subdomains of ECD. 6H+SP: 6xHis tag and signal peptide domain, TM: transmembrane domain, TK: tyrosine kinase domain, 1255: last amino acid on human ERBB2 cDNA.

3.3.5.2 PCR-Amplification of human ERBB2 regions

For PCR amplification upto 1kb, DyNAzyme™ II DNA Polymerase (Finnzymes, Espoo, Finland) was used. For amplification of fragments longer than 1 kb, Phusion® High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was used. Oligonucleotide primers that were used in PCR amplification reactions are listed on **Table 3.6**. Iontek (İstanbul, Turkey) synthesized primers were resuspended in autoclaved ddH₂O to make 100 µM stock solutions and were stored at -20°C.

PCR Amplification using DyNAzyme™ II DNA Polymerase

	<u>Final Concentration</u>	<u>For 75 µl reaction</u>
10X DyNAzyme™ Buffer Mg(-)	1X	7.5 µl
10 ng/µl template DNA (p219-D)	2 ng/µl	15 µl
2 mM dNTP	125 µM	4.5 µl
5 µM Forward Primer	0.5 µM	7.5 µl
5 µM Reverse Primer	0.5 µM	7.5 µl
12.5 mM MgCl ₂	1 mM	6 µl
DyNAzyme™ II DNA Polymerase	0.04 U/µl	1.5 µl
dH ₂ O		25.5 µl

PCR Programme used with DyNAzyme™ II DNA Polymerase

Heated Lid		105°C
Heated Lid before programme		On
Pause before programme		Off
Initial Denaturation	02m00s	95.0°C
Hot Start		Disabled
Number Cycles		35
Seq MAX °C/s	00m30s	94.0°C
Seq MAX °C/s	00m10s	60.0°C
Seq MAX °C/s	01m30s	72.0°C
Final Extension	04m00s	72.0°C
Final Hold		10°C

PCR Amplification using Phusion® High-Fidelity DNA Polymerase

	<u>Final Concentration</u>	<u>For 75 µl reaction</u>
5X Phusion GC Buffer Mg(-)	1X	15 µl
10 ng/µl template DNA (p219-D)	0.2 ng/µl	1.5 µl
2 mM dNTP	200 µM	7.5 µl
5 µM Forward Primer	0.5 µM	7.5 µl
5 µM Reverse Primer	0.5 µM	7.5 µl
Phusion® High-Fidelity DNA Polymerase	0.02 U/µl	0.75 µl
dH ₂ O		35.25 µl

PCR Programme used with Phusion® DNA Polymerase

Heated Lid	105°C
Heated Lid before programme	On
Pause before programme	Off
Initial Denaturation	02m00s 98.0°C
Hot Start	Disabled
Number Cycles	35
Seq MAX °C/s	00m10s 98.0°C
Seq MAX °C/s	00m20s 60.0°C
Seq MAX °C/s	01m00s 72.0°C
Final Extension	05m00s 72.0°C
Final Hold	10°C

PCR products were mixed with 1/5000 SybrGreen and run on 0.7% agarose gels. Since ethidium bromide is known to induce thymidine-dimers upon UV exposure, it was strictly avoided for visualization of fragments that will further be purified from agarose gel and be used for ligation. Under UV light, the correct sized bands were excised from the gel by sterile blades; the fragments were purified by QIAquick Gel Extraction Kit (QIAGEN, CA, USA) according to the manufacturer's instructions, eluted in total 70 µl elution buffer and stored at -20°C. (Typical fragment yield: 50 µl of 2-15 µg/ml)

3.3.5.3 Restriction Digestion of PCR Products

The PCR amplified and gel purified fragments were digested from their 5' and 3' ends with the appropriate restriction enzymes to provide the sticky ends needed for fragment ligation. Restriction enzyme buffers were chosen to be compatible with both enzymes for the double digest reactions.

	<u>For 50 µl restriction reaction</u>	
10X Restriction Enzyme Buffer	5 µl	} Incubated at 37°C for 3 to 5 hours
Restriction Enzyme 1	1 µl (5-10x10 ³ Unit)	
Restriction Enzyme 2	1 µl (5-10x10 ³ Unit)	
Gel-purified DNA Fragment	30 µl	
ddH ₂ O	13 µl	

Digested fragments were purified by QIAquick PCR Purification Kit (QIAGEN, CA, USA) according to the manufacturer's instructions, eluted in total 70 µl elution buffer and stored at -20°C.

3.3.5.4 Ligation of Digested Fragments

Ligation reactions were performed using T4 DNA Ligase (Fermentas, Vilnius, Lithuania), where backbone fragments were combined with insert fragments at various ratios from 1:1 to 1:8 respectively. A typical ligation reaction is exemplified below.

<u>For 20 µl ligation reaction</u>		} Incubated at 16°C overnight
10X T4 DNA Ligase Buffer	2 µl	
T4 DNA Ligase	1 µl	
Backbone Fragment	N	
Insert Fragment a	4N	
Insert Fragment b	4N	
N: molar amount of Backbone fragment added		
Volume was adjusted with sterile dH ₂ O ⇒		20 µl

After overnight incubation at 16°C, the ligase was inactivated by 10 minute incubation at 65°C; evaporated contents were collected at the bottom of the tube by a quick spin and the tube was stored at -20°C until transformation.

3.3.5.5 Preparation of Competent DH5α *E. coli* Cells

1. Since no antibiotic selection would be applied, all steps were carried out under strictly aseptic conditions.
2. Using the DH5α glycerol stock stored at -80°C an LB-agar plate was streaked and incubated overnight at 37°C.
3. A single DH5α colony was selected on the plate, inoculated in 3 ml 2XTY Broth and incubated overnight at 37°C.
4. 0.5 ml from the liquid culture was taken, added into in a 500 ml flask containing fresh 100 ml 2XTY and incubated at 37°C until OD₆₀₀ reaches 0.45-0.5 (~4 to 5 hours).

5. The bacterial culture was transferred into two 50 ml falcon tubes and incubated 10 minutes on ice.
6. The tubes were centrifuged at 2500 g (~3000 rpm) for 10 minutes at 4°C and the supernatant was removed by gentle pouring (without disturbing the pellet).
7. The pellet was initially resuspended by flicking and then 20 ml ice cold TB was added onto each tube, cells were resuspended completely by pipetting and collected in a single 50 ml tube.
8. Cells in TB were incubated on ice for 10 minutes.
9. The tubes were centrifuged at 2500 g (~3000 rpm) for 10 minutes at 4°C and the supernatant was removed by gentle pouring (without disturbing the pellet).
10. The pellet was initially resuspended by flicking. 8 ml ice cold TB was added onto the cell suspension and cells were resuspended completely by pipetting.
11. While mixing the cells gently (when a slow vortex was not present, the cells were mixed either by constant flicking or mixing with the pipette tip), 560 µl DMSO was slowly added onto the cell suspension (to make final 7% DMSO).
12. The cell suspension was quickly aliquoted into sterile 1.5 ml tubes (500 µl per tube) and immediately frozen in liquid N₂.
13. The frozen competent cells were stored at -80°C until use.

3.3.5.6 Transformation of Ligated Plasmids to DH5α

1. 42°C water bath was prepared before the transformation.
2. Competent DH5α cells were taken out from -80°C and thawed on ice.
3. 100µl of competent DH5α was transferred into a fresh tube, 10 µl of the ligation reaction was added onto cell suspension and the solution was mixed by gentle flicking.
4. DH5α-plasmid mixture was incubated for 10 to 30 minutes on ice.
5. The tubes were transferred into 42°C water bath and the competent DH5α cells were heat shocked for 90 seconds for the plasmid to enter.
6. After 90 seconds heat shock, the tubes were immediately transferred onto ice and incubated for 1 to 3 minutes.
7. 1 ml fresh LB was added onto the heat shocked DH5α-plasmid mixture and cells were incubated 20 – 60 minutes at 37°C.

Note: Since ampicilline is bacteriostatic, cells will survive on ampicilline plates after recovery incubations in LB even as short as 30 minutes following heat shock. However, kanamycin is bacteriocidal and cells need to express resistance genes before being plated onto kanamycin plates, therefore these cells need to be incubated at least for 60 minutes in LB after heat shock.

8. The cells were spun down for 30 seconds at 3500 rpm, the supernatant was removed by sterile 1 ml pipette leaving 50 µl LB on the cells and the cells were resuspended by flicking.
9. The cells were resuspended gently by pipetting and transferred onto agar plates with antibiotics and spread homogeneously.
10. The plates were inverted and incubated at 37°C for 16 to 24 hours.

3.3.5.7 Plasmid Isolation from transformed DH5α

1. 6 to 12 colonies were selected on plates with DH5α cells transformed with ligated plasmids.
2. Each colony was picked individually, transferred into 3 ml LB broth and was grown overnight at 37°C.
3. The tubes were centrifuged at 4500 g for 15 minutes at 4°C and the supernatant was removed by gentle pouring (without disturbing the pellet).
4. The pellets were lysed using the lysis buffer in QIAGEN Mini-Prep Kit and plasmid was isolated according the manufacturer's instructions. (Typical plasmid yield: 50 µl of 100-500 µg/ml)

3.3.5.8 Selection of Clones by Diagnostic Digest

Using Clone Manager Suite 7 software, sequences of recently cloned plasmids and the backbone plasmid were compared and the restriction enzymes that would identify the correct insertion were selected. Newly constructed and backbone plasmids were digested with the same enzymes; each construct was checked by at least two different restriction enzyme digestions.

1 unit restriction enzyme is defined as the enzyme sufficient to digest 1 µg DNA in a 50 µl reaction at 1 hour. Assuming that the concentration of each plasmid

would at most be 500 µg/ml, we have used excess amounts (1000 to 2000 Units) of enzymes for digesting 2 µl plasmid in a 3 to 5 hour reaction.

	<u>Master Mix for 10 plasmids (20 µl reaction each)</u>
10X Restriction Enzyme Buffer	20 µl
Restriction Enzyme 1	2 µl (1000-2000 Unit/rxn)
Restriction Enzyme 2	2 µl (1000-2000 Unit/rxn)
ddH ₂ O	156 µl

1. 18 µl Master Mix was aliquoted into each tube containing 2 µl of different plasmid solutions.
2. Plasmids were incubated with the restriction enzymes at 37°C for 3 to 5 hours.
3. Digested plasmids were run on 0.7 to 1 % agarose gels and the band patterns were compared with the expected sizes.
4. Plasmids having the correct band patterns were sent to sequencing.

3.3.5.9 Sequence Verification of Constructed Plasmids

Plasmid clones selected by diagnostic digest were sequenced by Iontek (İstanbul, Turkey) using the primers listed on **Table 3.9**. Sequence-verified plasmids were stored at -20°C.

3.3.6 Cell Lysis and Protein Quantification

When gentle lysis was needed, cells were lysed with a mild lysis buffer containing non-ionic detergent (1% NP40). When stronger lysis conditions were needed, RIPA lysis buffer containing ionic detergents (SDS and Na-DOC) was used. In order to ensure membrane disruption, cell lysates were further sonicated gently. Since SDS would interfere with the Bradford protein quantification assay, we have used BCA protein quantification assay kit from Thermo Fisher Scientific Inc. (IL, USA).

Cell Lysis

1. Cells that were grown on 150 mm tissue culture plates until 80% confluency were washed twice with 10 ml PBS. The PBS was aspirated and the plates were placed on ice.
2. While the cells were on ice, the cells were scraped with 5 to 10 ml cold PBS and collected in 15 ml falcon tubes.
3. The cells were centrifuged at 1500 rpm for 5 minutes and the supernatant was aspirated completely and the cell pellet was kept at -80°C (which facilitates cell lysis).
4. The cell pellet was taken from -80°C, thawed, 3 to 5 volume of appropriate lysis buffer was added onto each pellet. Cell suspension was transferred to 1.5 ml tubes and incubated on ice for 30 minutes by occasional flicking.
5. If the cell suspension was not clear at the end of 30 minute incubation, the cell lysate was subjected to gentle sonication on an iced water bath in the cold room. The cells were sonicated 15 seconds at 40% amplitude. The tubes were kept on ice for at least 30 seconds and the sonication was repeated once more.
6. The cell lysate was centrifuged at 13,000 rpm for 30 to 40 minutes. The soluble fraction was collected, and the total protein concentration was determined by BCA assay (Thermo Scientific Inc., IL, USA).
7. The soluble lysate was aliquoted and stored at -80°C.

3.3.7 Immunoprecipitation (IP)

Even if the lysates were prepared by RIPA Lysis Buffer, the IP procedure was performed with the milder NP40 Lysis Buffer in order to preserve protein interactions.

Immunoprecipitation of Huh-7 Lysates transfected with ERBB2 constructs

1. 150 µg of Huh-7 total cell lysate was added onto 0.5 ml tubes and the volume was completed upto 600 µl with ice cold NP40 Lysis Buffer.
2. 15 µg of purified antibodies were added to each tube containing Huh-7 lysates and the tubes were incubated for 2 hours on a rotator at the cold room (4°C).
3. In the mean time, ProteinG-Agarose beads were equilibrated.

- N x 50 µl ProteinG-Agarose slurry was placed in a 1.5 ml tube and topped up with ice cold NP40 Lysis Buffer. (N: number of sample tubes)
 - Beads were centrifuged at 2000 rpm for 1 minute.
 - Supernatant was gently aspirated avoiding pellet disturbance.
 - Beads were washed two more times and finally resuspended in N x 50 µl NP40 Lysis Buffer.
4. Tubes containing lysate-antibody mixture was spun down and 50 µl of equilibrated ProteinG-Agarose slurry was added onto each tube.
 5. ProteinG-lysate-antibody mixture was incubated for 4 hours on a rotator at the cold room (4°C).
 6. Following 4 hour incubation, tubes filled with ProteinG-lysate-antibody mixture were centrifuged at 2000 rpm for 1 minute.
 7. Supernatant was gently collected using 200 µl pipette tips avoiding pellet disturbance and saved in fresh tubes (in case, unbound fraction was needed for analysis).
 8. Beads were washed three times with 600 µl NP40 Lysis Buffer, centrifuged at 2000 rpm for 1 minute and the supernatant was aspirated.
 9. Finally, 50 µl 5X SDS Loading dye (with 5% β-mercaptoethanol) was added onto the beads and the beads were boiled for 10 min, and placed on ice (or stored at -20°C).

3.3.8 SDS-PAGE

SDS-PAGE was either performed with conventional methods, or by NuPAGE[®] Bis-Tris ready gels and solutions from Invitrogen (CA, USA) were used.

3.3.8.1 Conventional SDS-PAGE

Preparation of Tris-Glycine SDS-PAGE Gels

When conventional SDS-PAGE system was used, the proteins in the sample were first stacked on a 5% stacking gel, and then separated on 8% resolving gel under denaturing conditions.

1. The Biorad Mini-PROTEAN[®] 3 Cell glass plates (BioRad, CA, USA) were assembled according to the manufacturer's instructions.
2. In two separate 50 ml tubes, resolving and stacking gel mixture were prepared as explained on **Figure 3.4** and **Figure 3.5** without TEMED and APS.
3. Acquired volume of APS and TEMED were added to resolving gel mixture, stirred and poured between inner and outer glass plates leaving sufficient space for stacking gel. A small volume was left in the tube to monitor polymerization status.
4. The acrylamide solution was overlaid with isopropanol.
5. When the resolving gel was polymerized completely, isopropanol was poured off and the top of the gel was washed several times with dH₂O to remove unpolymerized acrylamide. Remaining water was drained using a whatman paper (without touching the gel)
6. Acquired volume of APS and TEMED were added to stacking gel mixture, stirred and poured on polymerized resolving gel. The comb was immediately inserted into the stacking gel, removing all air bubbles. A small volume was left in the tube to monitor polymerization status.
7. When polymerization was complete, the gels were assembled on the running apparatus and inserted into the running tank.
8. The space between two gels was completely filled with 1X Tris-Glycine SDS-PAGE running buffer, the combs were removed carefully and the wells were washed with a syringe filled with running buffer.
9. Remaining running buffer was poured into the tank so that the bottoms of the gels were dipped into the buffer.

Solution components	Component volumes (mL) per gel for total gel volumes of							
	5mL	10mL	15mL	20mL	25mL	30mL	40mL	50mL
6%								
H ₂ O	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
30% acrylamide mix	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8%								
H ₂ O	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% acrylamide mix	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.004	0.008	0.009	0.012	0.015	0.018	0.024	0.03
10%								
H ₂ O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% acrylamide mix	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12%								
H ₂ O	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% acrylamide mix	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15%								
H ₂ O	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% acrylamide mix	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

Figure 3.4 Resolving gel for SDS-PAGE

Solution components	Component volumes (mL) per gel for total gel volumes of							
	5mL	10mL	15mL	20mL	25mL	30mL	40mL	50mL
H ₂ O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acrylamide mix	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% ammonium persulfate	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Figure 3.5 Stacking gel for SDS-PAGE

Resolving Proteins on Tris-Glycine SDS-PAGE Gels

1. The protein samples in 1X SDS-PAGE loading buffer (containing 5% β -mercaptoethanol) were boiled in water bath for 10 minutes.
2. Boiled samples were immediately placed on ice, spun down gently and loaded on the wells of the gel.
3. The gel apparatus was attached to power supply and run at 80 V until the proteins exit stacking gel. Once the proteins enter resolving gel, the voltage was increased upto 120 V.

4. As the dye-front (bromophenol blue) has left the bottom of the resolving gel, the gel run was stopped and the plates were removed from the electrophoresis apparatus.
5. Resolved proteins on the SDS polyacrylamide gel were either transferred to a PVDF membrane or stained with Coomassie Brilliant Blue R250.

Transfer of Resolved Proteins on SDS-Polyacrylamide Gels to PVDF

1. The running apparatus was disassembled, the gels were transferred into 1X Tris-Glycine Transfer Buffer and incubated for at least 10 minutes.
2. In the mean time, the PVDF membrane of size equal to the gel was labelled from the top and incubated in methanol for 1 minute, washed with ddH₂O and placed into 1X Tris-Glycine Transfer Buffer.
3. Two pieces of Whatmann paper at equal size and four transfer pads supplied by the transfer apparatus were also placed in 1X Tris-Glycine Transfer Buffer.
4. Two fiber pads, one Whatmann paper, the gel, PVDF, another Whatman paper, two more fiber pads were placed on top of each other in this order. The stack was placed into the Biorad Mini Trans-Blot Cell transfer apparatus (BioRad, CA, USA) full of 1X Tris-Glycine Transfer Buffer (gel facing the cathode, PVDF facing the anode)
5. Proteins were transferred for 90 minutes at 80 V in the cold room.
6. The transfer apparatus was disassembled; PVDF was soaked into Western Blocking Buffer and proceeded as explained in 3.3.9.

3.3.8.2 SDS-PAGE using Commercial NuPAGE™ Bis-Tris System

Resolving Proteins on Commercial NuPAGE™ Bis-Tris Gels

1. The ready 4-12% NuPAGE™ gels were removed from their plastic bags and assembled on the running apparatus according to the manufacturer's instructions.
2. The space between two gels was completely filled with 1X MOPS SDS Running Buffer, the combs were removed carefully and the wells were washed with a syringe filled with running buffer.
3. Remaining running buffer was poured into the tank so that the bottoms of the gels were dipped into the buffer. The tank is placed inside an ice bath to prevent melting of the gel during high voltage runs.

4. The protein samples prepared in 1X NuPAGE LDS Sample Buffer (containing 5% β -mercaptoethanol) were incubated at 70°C for 10 minutes.
5. Heated samples were immediately placed on ice, spun down gently and loaded on the wells of the gel.
6. The gel apparatus was attached to power supply and run at 200 V for 90 minutes.
7. As the dye-front (bromophenol blue) has left the bottom of the gel, the gel run was stopped and the plates were removed from the electrophoresis apparatus.
8. Resolved proteins on the SDS polyacrylamide gel were either transferred to a PVDF membrane or stained with Coomassie Brilliant Blue R250.

Transfer of Resolved Proteins on NuPAGE™ Bis-Tris Gels to PVDF

The transfer of proteins from NuPAGE™ Bis-Tris gels to PVDF membranes was similar to that of conventional Tris-Glycine system, except the NuPAGE™ transfer apparatus and transfer buffers were used.

3.3.8.3 Coomassie Staining of Total Proteins Resolved by Polyacrylamide Gel Electrophoresis

1. The running apparatus was disassembled; the gels were soaked in Coomassie Staining Solution and incubated for 45 to 60 minutes at RT on a slow shaker.
2. The staining solution was drained; gel was soaked in Coomassie Destaining Solution and incubated at RT on a slow shaker until the destaining solution was blue.
3. The staining solution was renewed few times until the gel background was totally destained but protein bands were adequately visible.

Note: If the gel was to be destained overnight, it was soaked in slow destain solution, the contained was covered with parafilm and placed at 4°C to prevent over destaining and gel drying.

3.3.9 Western Blotting

1. PVDF membrane was removed from the transfer apparatus, soaked into Western Blocking Buffer and incubated gently for 1 hour at RT on a slow shaker.

2. In the mean time, primary antibodies were diluted in “1/2 Western Blocking Buffer” at concentrations denoted in **Table 3.10**.
3. The blocking buffer was drained; the PVDF was soaked in the primary antibody solution and incubated overnight at 4°C.
4. Next morning, the primary antibody solution was drained and the PVDF was washed three times with TBS-T for a total of 30 minutes on a fast shaker.
5. In the mean time, HRP conjugated secondary antibodies were diluted in “1/2 Western Blocking Buffer” at concentrations denoted in **Table 3.10**.
6. TBS-T was drained; the PVDF was soaked in the secondary antibody solution and incubated gently for 1 hour at RT on a slow shaker.
7. The secondary antibody solution was drained and the PVDF was washed three times with TBS-T for a total of 30 minutes on a fast shaker.
8. TBS-T was drained well; the PVDF was placed on a glass plate; 1 ml SuperSignal West Femto Maximum Sensitivity Substrate was added on the PVDF membrane and incubated for 5 minutes.
9. PVDF membrane was drained, covered with stretch film and chemiluminescent signal was captured by Kodak films which were further developed in Hyperprocessor (GE Healthcare, WI, USA)

Reagent Name	Catalogue Number	Company	Dilution	Incubation
anti-ErbB2 (CB11) antibody	ab8054	Abcam (MA, USA)	1/500	overnight 4oC
anti-phospho ErbB2 antibody	sc-1011694	Santa Cruz (California, USA)	1/300	2 hour RT
anti-Akt antibody	9272	Cell Signaling (MA, USA)	1/200	2 hour RT
anti-phospho Akt antibody	9271	Cell Signaling (MA, USA)	1/200	2 hour RT
anti-Erk1/2 antibody	9107	Cell Signaling (MA, USA)	1/200	2 hour RT
anti-phospho Erk1/2 antibody	9106	Cell Signaling (MA, USA)	1/200	2 hour RT
anti-CyclinD1 antibody	sc-246	Santa Cruz (California, USA)	1/200	2 hour RT
BH1			6 ug/ml	overnight 4oC
BH2			6 ug/ml	overnight 4oC
BH5			6 ug/ml	overnight 4oC
BH6			6 ug/ml	overnight 4oC
BH7			6 ug/ml	overnight 4oC
anti-mouse IgG (Fc specific)–Peroxidase antibody	A0168	Sigma-Aldrich (St. Louis, USA)	1/2000 to 1/5000	1 hour RT
Trastuzumab (Herceptin)		Roche Ltd (Basel, Switzerland)	8 ug/ml	3 hour RT
anti-human IgG (Fc specific)?Peroxidase antibody	A0170	Sigma-Aldrich (St. Louis, USA)	1/2000 to 1/5000	1 hour RT
anti-Calnexin antibody	C4731	Sigma-Aldrich (St. Louis, USA)	1/10,000	1 hour RT
anti-rabbit IgG (whole molecule)–Peroxidase antibody	A6154	Sigma-Aldrich (St. Louis, USA)	1/5000	1 hour RT

Table 3.10 Antibodies and assay conditions used for immunoblotting.

3.3.10 Immunofluorescence (IF)

1. The cells were seeded at ~30-50% confluency on sterile coverslips placed in 6-well tissue culture dishes.
2. When the cell confluency reached ~70-80% (after 36 to 48 hours), the cells were washed twice with sterile PBS and the PBS was aspirated.
3. 2 ml 2% paraformaldehyde (PFA) was added onto the wells from the sides (avoiding direct drips onto the cells in order to prevent cell detachment prior to fixation.); the dishes were incubated for 15 minutes at RT at dark.
4. After 15 minute fixation, PFA was aspirated and cells were incubated in 2 ml of Permeabilization Buffer for 3 to 5 minutes.
5. The cells were washed three times with PBS (If necessary, the cells may be stored at 4°C for one week in PBS after this step)
6. PBS was aspirated and the cells were incubated for 30 to 60 minutes at RT in 2 ml IF Blocking Buffer.
7. In the mean time, purified antibodies to be used as primary antibodies were diluted in PBS-T at concentrations denoted in **Table 3.11** (hybridoma supernatants were used without dilution).
8. IF Blocking Buffer was aspirated; the cells were washed once with PBS-T for 5 minute and PBS-T was aspirated.
9. Primary antibodies were added at a volume enough to cover the coverslip surface and incubated for 1 to 3 hour at RT.
10. Primary antibody solution was aspirated; the cells were washed three times with PBS-T (5 minute each) and PBS-T was aspirated.
11. In the mean time, Alexa Fluor labelled secondary antibodies were diluted in PBS-T at concentrations denoted in **Table 3.11**.
12. Secondary antibodies were added at a volume enough to cover the coverslip surface and incubated for 1 hour at RT at dark.
13. Secondary antibody solution was aspirated; the cells were washed three times with PBS-T (5 minute each) and PBS-T was aspirated.
14. Finally, coverslips were washed once with dH₂O to remove salts that would crystallize after mounting and disturb the vision during the microscopic detection.
15. The coverslips were mounted onto the pre-labeled microscopy slides using ProLong Gold Antifade Reagent with DAPI (Invitrogen, CA, USA).

16. The fluorescent signal was detected using Axio Imager.A1 microscope (Jena, Germany).

Reagent Name	Catalogue Number	Company	Dilution	Incubation
mouse monoclonal anti-BrdU (Bu20a) antibody	M0744	Dako (Glostrup, Denmark)	1/500	1/500
anti-ErbB2 (CB11) antibody	ab8054	Abcam (MA, USA)	1/50	1-3 hour RT
BH1			10 ug/ml	1-3 hour RT
BH2			10 ug/ml	1-3 hour RT
BH5			10 ug/ml	1-3 hour RT
BH6			10 ug/ml	1-3 hour RT
BH7			10 ug/ml	1-3 hour RT
IgG1 isotype control antibody	MAB002	R&D Systems (MN, USA)	10 ug/ml	1-3 hour RT
anti-mouse IgG (H+L) Alexa Fluor® 488	A11029	Invitrogen (CA, USA)	1/500	1 hour RT
anti-mouse IgG (H+L) Alexa Fluor® 568	A11031	Invitrogen (CA, USA)	1/500	1 hour RT
Trastuzumab (Herceptin)		Roche Ltd (Basel, Switzerland)	5 ug/ml	1-3 hour RT
anti-human IgG Alexa Fluor® 488	A11013	Invitrogen (CA, USA)	1/500	1 hour RT

Table 3.11 Antibodies and assay conditions used for immunofluorescence.

3.3.11 Sulforhodamine (SRB) Based Growth Assay

Cell Treatment

1. Cells at their logarithmic phase were trypsinized and diluted to give $\sim 1-4 \times 10^4$ cell/ml in Complete Media.
2. 100 μ l of cell suspension was seeded on each well of 96-well tissue culture plate and incubated for 2 hours in a CO₂ incubator at 37°C.
3. In the mean time, purified antibodies were diluted in complete media (20 μ g/ml).
4. 50 μ l of diluted antibodies were added on each well (for 5 μ g/ml final antibody concentration in 200 μ l culture) and the plates were incubated for 4 hours in a CO₂ incubator at 37°C.
5. In the mean time, 4X TNF- α solutions ranging from 250 Unit/ml to 16,000 Unit/ml were prepared by diluting TNF- α stock (~ 2000 Unit/ μ l) in complete media.
6. After 4 hour incubation, 50 μ l 4X TNF- α was added onto each well to make a final TNF- α concentration of 62.5-4000 Unit/ml in 200 μ l culture. For the negative control wells, 50 μ l of complete media was added instead of 50 μ l 4X TNF- α .
7. The plates were placed back in the CO₂ incubator and incubated for 6 days at 37°C.
8. SRB analysis was performed as described by Vichai and Kirtikara [291].

Cell Fixation

1. Stock TCA was diluted down to 10% with cold dH₂O and kept on ice.
2. 96 well plates were gently washed twice with 200 µl PBS. Being careful not to detach the yet unfixed cells, plates were gently tapped on tissue paper to remove the excess liquid.
3. 100 µl 10% TCA solution was added onto each well; the plates were covered with aluminum foil and incubated at 4°C for 1 hour. (It was important not to exceed 1 hour in order to prevent false staining during SRB incubation)
4. After fixation, the plates were emptied, tapped on tissue paper to remove residual liquid and washed 4 times with ~200 µl dH₂O.
5. The plates were emptied, tapped on tissue paper to remove residual liquid and dried at RT at dark. (If necessary, dried plates may be stored at dark at RT and processed later.)

SRB-Staining

1. When plates with fixed cells were completely dried, 50 µl of freshly prepared SRB stain was added onto each well and incubated for 10 minutes at RT at dark.
2. After 10 minute staining, the plates were emptied, tapped on tissue paper to remove residual stain and washed 6 times with 1% Acetic Acid.
3. The plates were emptied, tapped on tissue paper to remove residual liquid and dried at RT at dark. (If necessary, dried plates may be stored at dark at RT and processed later.)

SRB Read-Out

1. When plates with stained cells were completely dried, exactly 200 µl cold 10 mM Tris Buffer was added onto each well and SRB stain was fully dissolved by thorough pipetting (Since this is a quantification assay, care was taken to pipette precisely the same volume to each well and to avoid evaporation).
2. The absorbances at 515 nm were read by µQuant Microplate Reader.

3.3.12 Flow Cytometry

- 1.** Cells at their logarithmic phase were trypsinized and resuspended in Complete Media ($3-10 \times 10^5$ cells per sample). Trypsinized cells were inspected with an inverted microscope to ensure complete cell detachment before inactivating trypsin.
- 2.** The cells were transferred into a 15 ml falcon tube, centrifuged at 800 rpm for 3 to minutes and the supernatant was aspirated.
- 3.** The cells were washed twice with at least 10 ml PBS each, the supernatant was aspirated and the cells were resuspended applying mechanical force to the bottom of the tube in only one direction (or simply by flicking).

Note: Care was taken to achieve a cell suspension with highest healthy single cell concentration, preventing both cell necrosis and cell clumps before fixation.

- 4.** 5 ml 2% PFA was added onto the resuspended cell pellet drop by drop (with constant flicking) and the cells were fixed for 15 minutes at dark.
- 5.** After fixation the cells were centrifuged at 1500 rpm for 5 to minutes and the supernatant was aspirated.
- 6.** The cells were washed 4 times with 5 ml Flow Blocking Buffer, centrifuged at 1500 rpm for 5 to minutes and the supernatant was aspirated.
- 7.** The cells were resuspended by flicking and blocked in 5 ml Flow Blocking Buffer for 30 to 60 minutes.
- 8.** The cells were centrifuged at 1500 rpm for 5 to minutes, the supernatant was aspirated; the pellet was resuspended by flicking and blocking step was repeated once more.
- 9.** In the mean time, the antibodies were diluted in 50 μ l Flow Blocking Buffer.
- 10.** Steps 7 – 8 were repeated and the cells were blocked once more.
- 11.** The cell pellet was flicked, resuspended in N x 50 μ l Flow Blocking Buffer and cell suspension was aliquoted into N x 1.5 ml tubes (N: the number of samples).
- 12.** Antibody solution prepared at step 9 was added onto each cell suspension and cells were incubated for 60 minute at RT (cells were flicked occasionally to resuspend cells and enable homogenous staining).
- 13.** 1 ml Flow Blocking Buffer was added onto each tube, tubes were centrifuged at 2000 rpm for 5 minutes.
- 14.** The supernatant was aspirated and the cells were washed twice with Flow Blocking Buffer.

15. The tubes were centrifuged at 2000 rpm for 5 minutes, the supernatant was aspirated and the cells were resuspended by flicking.
16. 100 μ l of 1/500 diluted Alexa conjugated secondary antibodies were added to each tube and cells were incubated for 60 minute at RT (cells were flicked occasionally to resuspend cells and enable homogenous staining).
17. Cells were washed as explained in Steps 13 – 14 and the cells were resuspended by flicking.
18. 500 μ l PBS was added onto each tube and cells were transferred into FACS tubes of size 12x75 mm.
19. Staining densities were read using FACSCalibur Flow Cytometer and analyzed by CellQuest Software (Becton Dickinson, NJ, USA)

3.3.13 Long-term bromodeoxyuridine (BrdU) incorporation assay

Long-term bromodeoxyuridine (BrdU) incorporation assay is used to assess the change in proliferative capacity of cells in the total population. When treated with BrdU for 24 hours, cells undergoing permanent cell cycle arrest cannot incorporate BrdU into nuclear DNA [292].

Cell Treatment (Day 0)

1. Sterile coverslips were placed inside each well of 12-well dishes and 400 μ l Complete McCoy's 5A was added onto each well.
2. SK-BR-3 cells at their logarithmic phase were trypsinized and diluted to give 2.5×10^4 cell/ml in Complete McCoy's 5A.
3. 400 μ l of cell suspension was seeded on each well and incubated for 2 hours in a CO₂ incubator at 37°C.
4. In the mean time, purified antibodies were diluted in Complete McCoy's 5A (50 μ g/ml).
5. 100 μ l of diluted antibodies were added on each well (for 5 μ g/ml final antibody concentration in 1 ml culture) and the plates were incubated for 4 hours in a CO₂ incubator at 37°C.
6. In the mean time, TNF- α stock was diluted in Complete McCoy's 5A (4000 Unit/ml).

7. After 4 hour incubation, 100 μ l 10,000 Unit/ml TNF- α was added onto each well to make a final TNF- α concentration of 1000 Unit/ml in 1 ml culture. For the negative control wells, 100 μ l Complete McCoy's 5A was added instead of TNF- α solution.

Note: Time point at TNF- α addition was taken as **t:0**

8. The plates were placed back in the CO₂ incubator and incubated for 6 days at 37°C.

BrdU Application (t:24 hr, t:48 hr, t:72 hr, t:96 hr, t:120 hr, t:144 hr)

Starting from 24th hour after TNF- α addition, different wells were chased with BrdU at every time point in 24th hour intervals. Each BrdU chase lasted 24 hours.

1. 1000X BrdU Stock was diluted down to 50X in Complete McCoy's 5A
2. 20 μ l 50X BrdU was added to each well to be examined and plate was gently shaken to enable homogenous solution (Final BrdU concentration was 10 μ g/ml).
3. The plates were placed back in the CO₂ incubator and incubated for 24 hours at 37°C.

Anti-BrdU Staining

Anti-BrdU staining was performed similar to immunofluorescence protocol explained in 3.3.10 except for minor variations explained below in details.

1. Cells on coverslips were washed twice with 1 to 2 ml PBS.
2. PBS was aspirated and the cells were fixed with 1 ml ice cold 70% Ethanol for 5 minutes on ice (Ethanol was dispensed from the side of the well, avoiding direct dripping onto the cells). Ethanol was aspirated and slides were air dried.
3. In order to denature dsDNA, the cells were treated with 1 ml 2N HCl for 20 minutes at RT and washed twice with PBS.
4. Cells were incubated in 1 ml IF Blocking Buffer for 30 to 60 minutes at RT.
5. The cells were washed twice with PBS-T and PBS-T was aspirated well to prevent antibody leak from the coverslip surface at the next step.
6. Anti-BrdU antibody was diluted 1/500 in PBS-T and 50-70 μ l of the antibody solution was added onto each coverslip. Cells were incubated with the antibody for 1 to 3 hours at RT (Coverslips were inspected occasionally to prevent leak).

7. The cells were washed twice with PBS-T and PBS-T was aspirated well.
8. Alexa488 conjugated anti-mouse secondary antibody was diluted 1/500 in PBS-T; 50-70 μ l of the antibody solution was added onto each coverslip and incubated for 1 hour at RT at dark.
9. The cells were washed twice with PBS-T and PBS-T was aspirated well.
10. Finally, the coverslips were washed once with dH₂O.
11. The coverslips were mounted onto the pre-labeled microscopy slides using ProLong Gold Antifade Reagent with DAPI (Invitrogen, CA, USA).
12. The fluorescent signal was detected using Axio Imager.A1 microscope.
13. 3 to 5 frames per coverslip was captured and the percent BrdU incorporation was calculated after manual counting of BrdU⁺ and BrdU⁻ nuclei.

% BrdU Incorporation:
$$\frac{\%P \text{ of } 1^{\text{st}} \text{ frame} + \%P \text{ of } 2^{\text{nd}} \text{ frame} + \dots + \%P \text{ of } N^{\text{th}} \text{ frame}}{N}$$

(%P: percent positivity value for each frame, N: total number of frames)

3.3.14 Cell Cycle Analysis by Flow Cytometry

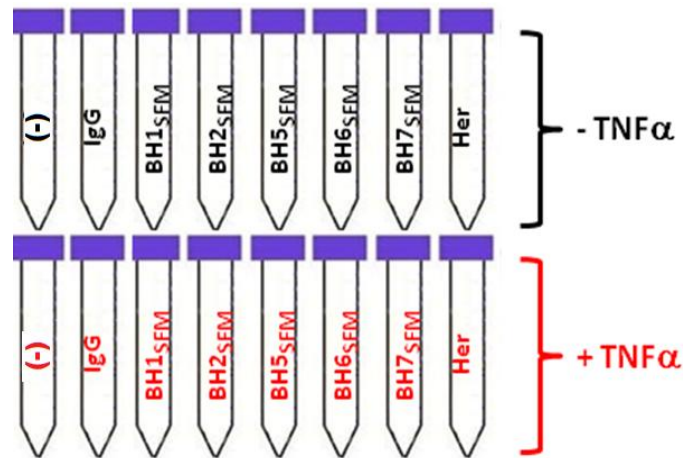
Cell Seeding

1. SK-BR-3 cells at their logarithmic phase were trypsinized and diluted in Complete McCoy's 5A media as follows:
 - ~12.5 x 10³ cell/ml for samples to be collected on 6th day.
 - ~50 x 10³ cell/ml for samples to be collected on 1st, 2nd and 3rd day.
2. 2 ml cell suspension was seeded on each well of 6-well tissue culture plate and incubated for 36 hours in a CO₂ incubator at 37°C to allow enough time for proper cell attachment.

Cell Treatment t:0

1. 2 ml fresh Complete McCoy's 5A media containing test samples was prepared for each sample (2 ml/sample/time point)
 - a) 2 ml complete Complete McCoy's 5A media was added into each tube for each time point.
 - b) 40 μ g of purified antibody per 2 ml Complete McCoy's 5A was added into each tube.

- c) 2000 Unit TNF- α per 2 ml Complete McCoy's 5A was added into each appropriate tube.



2. 36 hours after cell plating, the old media was aspirated, cells were washed with 2 ml PBS and PBS was aspirated
 - a) (-) control samples that were to be collected at t:0 were trypsinized and collected, fixed and stored as explained below.
 - b) Fresh media containing the appropriate reagents were added onto other wells and cells were incubated at 37°C in the CO₂ incubator until cell fixation.

Cell Fixation t:24, t:48, t:72, t:144

1. Since apoptotic cells detach from the plate surface and remain in the supernatant, the culture media in each well was collected into a separate falcon tube (labelled prior to the collection).
2. Each well washed with 2 ml PBS and PBS was transferred into the appropriate falcon tubes containing the detached cells.
3. Adherent cells in each well were trypsinized (3 drops of trypsin per well) for 3 to 5 minutes at 37°C. Trypsin was inactivated by 1 ml Complete McCoy's 5A media and single cell suspension was transferred into the appropriate falcon tubes containing the cells collected in previous steps.
4. Cell suspensions were centrifuged at 1500 rpm for 5 minutes; the supernatant was aspirated and the cells were resuspended by flicking.
5. 1 ml PBS was added onto each tube and cells were resuspended properly by gentle pipetting.

6. Cell suspensions were vortexed vigorously, and 2.5 ml of 100% ice-cold absolute EtOH was added dropwise to prevent cell clumps during fixation (final 70% EtOH).
7. Cell suspensions were incubated in fixative on ice for 30 min with occasional vortexing.
8. Fixed cell suspensions were stored at 4°C.

Cell Staining

1. Fixed cells were centrifuged at 1500 rpm for 5 minutes at 4°C.
2. The supernatant was aspirated and the cells were resuspended by flicking.
3. 500 µl PI Staining Solution was added onto each pellet and the suspension was pipetted gently couple of times to break cell clumps.

Note: Since PI was light-sensitive, tubes were kept at dark after this step.

4. Cells were incubated 40 minutes in PI Staining Solution at 37°C with occasional vortexing.
5. After 40 minutes staining, 3 ml PBS was added onto each tube and the cells were centrifuged at 1500 rpm for 5 minutes at 4°C.
6. The supernatant was aspirated and the cells were resuspended by flicking.
7. 200 to 500 µl PBS was added onto the cell suspension (according to the pellet size) and the cells were analysed by Flow Cytometer.

Cell Analysis

1. The instrument and software were prepared as follows:

Instrument

- Sheat Fluid was filled upto 80%
- Waste container was emptied
- Valve was turned on to provide vacuum
- First the Flow Cytometer, then the computer was turned on
- Flow Cytometer was kept at “Standby” mode at “Lo”

Software

- Cell Quest Pro was opened
- Acquire menu → Connect to cytometer
(“Acquisition Control” window should always be open)
- Cytometer menu → Status

(When the instrument is at “Standby” mode, it will read “Not Ready”)
 (“Sheat Fluid” and “Waste Tank” should read “OK”)

- Acquire menu → Parameter Description
 (“SetUp” should be “√” during setup, therefore the data will not be saved.)
 (When the samples are read, “SetUp” should be unchecked to save the data)
 (Directory and the file name for each data were changed from this menu before sample read)
- Acquire menu → Acquisition and Storage
 (The number of cells to be read were entered)
- Cytometer menu → Detectors/Amps
 Voltage (increases/decreases fluorescence intensity)
 E-1 (decreases signal of large events) (E-1 was used for PI analysis)

FL1 (for Annexin)

FL2 (for PI) (FL2 was used for PI analysis)

FL3 (for PI)

FL2-A (Area) for total fluorescence

FL2-W (Width)

FL2-H (Max Fluorescence Emission)

- Cytometer menu → Instrument Settings
 (Browsing the hard disk, previously saved setting file was selected)
 → Set → Done
 (Every time the settings were changed, “event count” was controlled from Acquire menu → Acquisition and Storage)

2. Before the cells were read, a template was opened to visualize the graphics on the screen (as seen on **Figure 3.6**)

3. Before the cells were read, following windows should remain open on the screen:

- Cytometer menu → Status
- Cytometer menu → Detectors/Amps
- Acquire menu → Counters
- Acquire menu → Acquisition Control

Running the Samples

1. Instrument setting was changed from “Standby” to “Run” and, “Status” on the screen was read “Ready”
2. While running the samples, SetUp is initially ticked, gate was selected as shown on **Figure 3.6 B**, in order to avoid multicellular clumps.
3. FL2-A levels were fine-tuned from “Detectors/Amps”.
 - First and second peaks seen on **Figure 3.6 C** should sit on 200 and 400. “Voltage” and “Amp Gain” on “Detectors/Amps” menu was fine-tuned for every sample before sample read.
 - “Voltage” setting shifts the total graph to right or left on the x-axis
 - “Amp Gain” setting changes the width between two peaks
4. When the setting were fine tuned → Pause → Abort
5. To read to samples, SetUp is unticked → Acquire
6. Once enough events were obtained, the instrument automatically stoped counting, saved the data and increased the number of the data file name by one.
7. After each data, SetUp was ticked not to save the runs in between.
8. Cells remaining in the tubing were washed with dH₂O and events were monitored on the screen (no events should be detected unless the system is clogged or the dH₂O tube was contaminated).
9. For each new sample, Steps 3 to 6 were repeated.

Data Analysis

1. An analysis file was prepared for each data as seen on **Figure 3.6**.
2. Initially the analysis template file was opened, the data folder was browsed and the same data file was selected for each graph.
3. On the histogram graph (**Figure 3.6 C**), gates M1, M2, M3 and M4 were set for apoptotic, G0/G1, G2/S and M-phase cells respectively.
4. The analysis file was saved.
5. The file is renamed with the next data number and data files of new data were selected for each graph in order to keep M1, M2, M3 and M4 gates constant. (M1, M2, M3 and M4 were adjusted slightly only when the voltage level during the

sample read was not set properly leading to deviation of G0/G1 and M-phase peaks from 200 and 400 respectively). The analysis file was saved.

6. Step 5 was repeated for each data file.

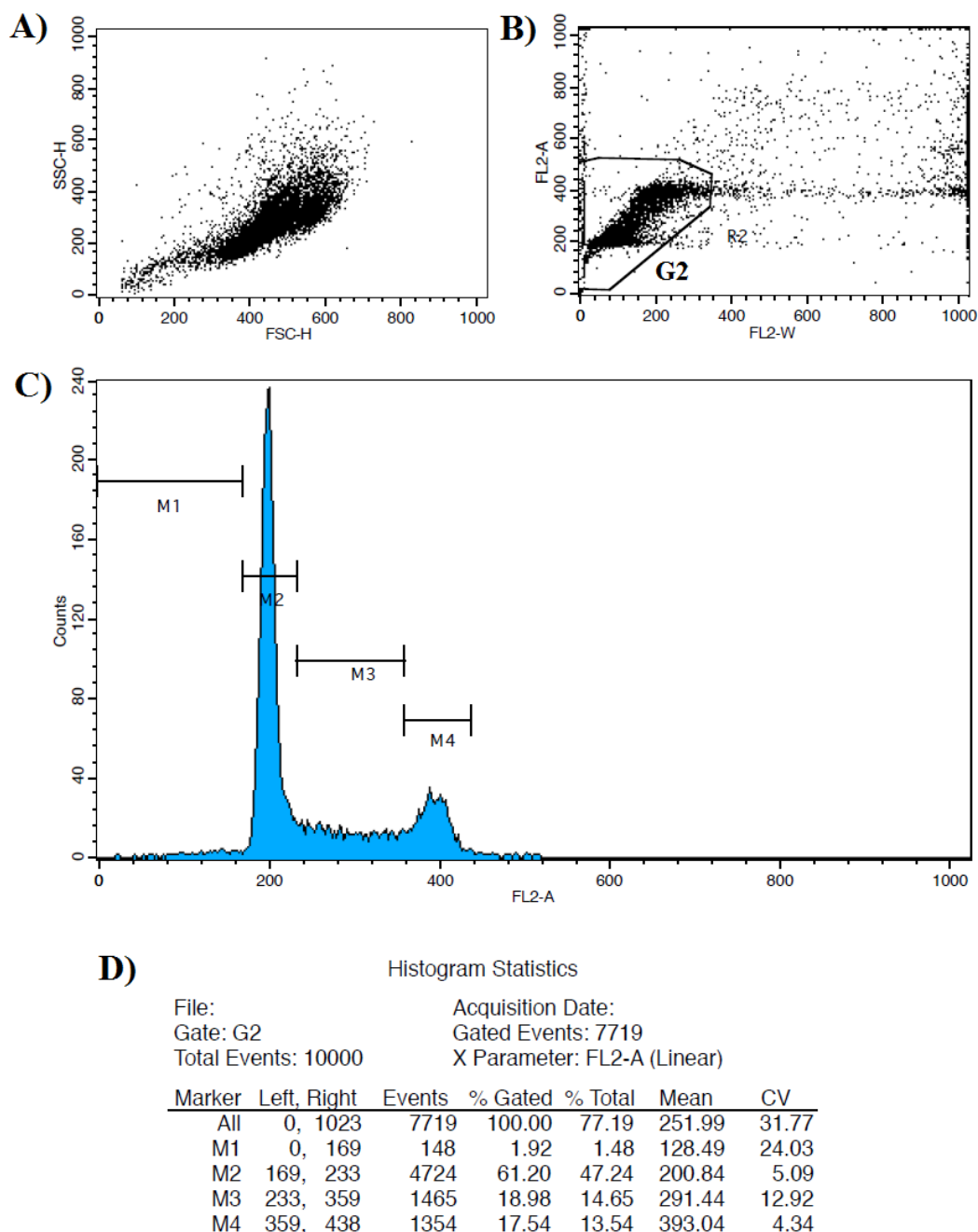


Figure 3.6 Typical read out of a PI stained cell suspension A) X-axis (FSC-H) shows the size of each cell, Y-axis (SSC-H) shows the complexity (granular, etc) of each cell. B) X-axis (FL2-W) shows the width of each cell, (FL2-A) indicates the area of total fluorescence observed on each cell. C) The histogram showing the number of cells (x-axis) with each fluorescence level (y-axis). D) Histogram statistics for each reading show number of apoptotic (M1), G0/G1 (M2), G2/S (M3) and M phase (M4) cells.

3.3.15 Senescence Associated β -Gal (SA β -gal) Assay

Senescence associated beta galactosidase activity is used as a biomarker to distinguish senescent cells from quiescent cells. While both cell types are unable to incorporate BrdU due to the cessation of DNA synthesis; only senescent cells were shown to display SA β -gal activity [293, 294].

Cell Treatment (Day 0)

1. Sterile coverslips were placed inside each well of 12-well dishes.
2. SK-BR-3 cells at their logarithmic phase were trypsinized and diluted to give 1.25×10^4 cell/ml in Complete McCoy's 5A.
3. 800 μ l of cell suspension was seeded on each well and incubated for 2 hours in a CO₂ incubator at 37°C.
4. In the mean time, purified antibodies were diluted in Complete McCoy's 5A (50 μ g/ml).
5. 100 μ l of diluted antibodies were added on each well (for 5 μ g/ml final antibody concentration in 1 ml culture) and the plates were incubated for 4 hours in a CO₂ incubator at 37°C.
6. In the mean time, TNF- α stock was diluted in Complete McCoy's 5A (4000 Unit/ml).
7. After 4 hour incubation, 100 μ l 10,000 Unit/ml TNF- α was added onto each well to make a final TNF- α concentration of 1000 Unit/ml in 1 ml culture. For the negative control wells, 100 μ l Complete McCoy's 5A was added instead of TNF- α solution.

Note: Time point at TNF- α addition was taken as **t:0**

8. The plates were placed back in the CO₂ incubator and incubated for 6 days at 37°C.

SA β -gal Staining (t:6 day)

1. Cells on coverslips were washed twice with 1 to 2 ml PBS.
2. PBS was aspirated and the cells were fixed with 1 ml 3.7% Formaldehyde for 15 minutes at RT (The plates were kept at dark).
3. Formaldehyde was aspirated and the cells were washed three times with 2 ml PBS.

4. PBS was aspirated SA β -gal Staining Solution was added onto each enough to cover cells completely.

NOTE: The SA β -gal solution should be FRESHLY prepared and FILTERED before each use.

5. Petri dishes containing coverslips were incubated in a 37°C incubator at DARK!!!.

NOTE: The petri dishes should be placed in a 37°C incubator without CO₂ circulation. !!!

- The presence of CO₂ will alter the pH of the staining solution, and nonspecific staining will result.
- Petri dishes should be incubated with lids closed to prevent evaporation.
- In addition, care should be taken to ensure that the plates are not tilted and the staining solution covers the whole area of the dish.

6. Development of a blue color in senescent cells was checked using a light microscope.

7. As soon as such coloration is detected, aspirate the staining solution and wash cells 3x with dH₂O.

NOTE: Time required for blue coloration to develop depends on cell type.

- Wait 2 to 4 hr after adding the staining solution before monitoring. Color development usually is complete after 16 hr.
- Some nonspecific staining, which is caused by loss of the buffering capacity of the staining solution, may be observed after a long staining incubation.
- Compare experimental sample with a negative control to detect color development.
- A proliferating cell population and/or a quiescent cell population may serve as good controls.

8. After more than 8 hr of incubation, some salt crystals may be observed. These crystals can be removed by repeated washing with water.

9. 1-2 ml nuclear-fast red was added onto each coverslip, and incubated for 4-6 min.

10. Cells on the coverslips were washed twice with dH₂O (incubation is not needed; just rinsed and aspirated).

11. The coverslips were mounted onto the glass slides using 80% glycerol or a commercial mounting media.

12. The coverslips on the slide were sealed with colorless nail polish.

3.3.16 Analyses of Multiple Drug Interactions

Drug interactions between BH-antibodies and TNF- α were analysed by two independent methods; Bliss model of independence [295] and Loewe model of additivity [296][297].

The Bliss independence model [295] is also called the effect multiplication or the fractional product and the interaction score is calculated by multiplying maximal effects of drug 1 and drug 2.

$$\text{Bliss Interaction Score} = \frac{f_{1,2}}{f_1 \times f_2}, \text{ where}$$

$f_{1,2}$: effect (relative cell survival) obtained by combination of drug 1 and drug 2

f_1 : maximum effect (minimal relative cell survival) obtained with drug 1

f_2 : maximum effect (minimal relative cell survival) obtained with drug 2

Drug 1 and 2 are accepted to be at “no interaction” (or acting “independently”), when the Bliss score is 1. For scores less than 1, drugs are accepted to behave “synergistic”, and for scores higher than 1, drugs are accepted to behave “antagonistic”.

Interaction score for Loewe additivity model is calculated as follows [296, 297]:

$$\text{Loewe Interaction Score} = \frac{d_1}{D_{y,1}} + \frac{d_2}{D_{y,2}}, \text{ where}$$

d_1 : dose of drug 1 in the mixture producing an effect (relative cell survival) “y”

d_2 : dose of drug 2 in the mixture producing an effect (relative cell survival) “y”

$D_{y,1}$: dose of drug 1 producing an effect (relative cell survival) “y” when used alone

$D_{y,2}$: dose of drug 2 producing an effect (relative cell survival) “y” when used alone

For Loewe score equal to 1, drug 1 and 2 are accepted to act “additive”. For scores less than 1, drugs are accepted to behave “synergistic”, and for scores higher than 1, drugs are accepted to behave “antagonistic”.

While cell survival data of treatments performed with different doses of drug 1, drug 2 and their combination are needed at for Loewe analysis, treatments performed only with the maximal effective dose are sufficient for analysing the data according

to Bliss method. Therefore cell treatments performed at single antibody (5 µg/ml) and TNF- α (1000 Unit/ml) concentrations were analysed only by Bliss method of independence. Corrccoef function of MatLab was used for the correlation analysis.

3.3.17 Statistical Analyses

Experimental data were presented as mean of samples tested at least in triplicates, with T Bars showing the standart deviation values. Statistical analysis of experiments that have been performed at least in triplicate was done using Student's *t* test.

4 RESULTS

4.1 Generation of Novel Monoclonal Antibodies Directed to Extracellular Domain of Human ERBB2

Using a combined immunization protocol explained in **Section 3.3.2**, we generated hybridoma clones producing novel monoclonal antibodies against extracellular domain (ECD) of human ERBB2. Briefly, five BALB/c mice were initially immunized with live SK-BR-3 human breast cancer cells that overexpress ERBB2 on their cell surface. After four intraperitoneal live SK-BR-3 injections, the final injection was performed with 20 µg recombinant human ERBB2 ECD/Fc fusion protein (R&D Systems, MN, USA) (**Figure 4.1**).

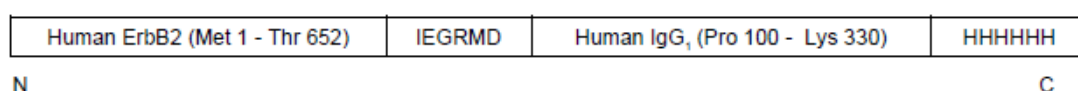


Figure 4.1 Structure of recombinant human ERBB2 ECD/Fc fusion protein used for immunization and ELISA assays. As explained by the manufacturer (R&D Systems, MN, USA), the recombinant protein has been constructed by fusing extracellular region (Met 1 – Thr 652) of human ERBB2 protein to the N-terminus of human IgG Fc region (Pro 100 – Lys 330) with a C-terminal 6-histidine tag. The final protein product was a disulfide-linked homodimeric protein devoid of ERBB2 signal peptide (Met 1 – Ser 22) which was removed by a proteolytic cleavage. The mature protein starting from Thr 23 was documented to migrate at ~131 kDa under denaturing conditions.

Hybridoma culture supernatants were screened for the presence of anti-ERBB2 antibodies using microtiter plates coated with recombinant human ERBB2 ECD/Fc fusion protein (will be called as “recombinant ERBB2-ECD” hereafter). Hybridoma clones that were found to produce reactive antibodies in this initial ELISA screen were expanded for further analysis and characterization.

4.2 Characterization of Novel anti-ERBB2 Monoclonal Antibodies

Expanded hybridoma clones were subcloned to assure monoclonality and re-screened by ELISA to select the clones producing anti-ERBB2 antibodies with highest reactivity.

4.2.1 Novel Antibodies React Selectively With Human ERBB2 ECD

Since EGFR and ERBB2 are known to possess sequence homology (**Figure 4.2**), and BALB/c mice were initially immunized with SK-BR-3 cell line known to overexpress both EGFR and ERBB2; we screened the hybridoma supernatants for EGFR-ECD cross-reactivity in addition to their reactivity to ERBB2-ECD. Five hybridoma clones (BH-antibodies; namely BH1, BH2, BH5, BH6 and BH7) displaying high reactivity for ERBB2, without any EGFR cross-reactivity on ELISA assay were selected for further analyses (**Figure 4.3**).

4.2.2 Comparison of Binding Strengths of Novel Antibodies for Human ERBB2

The immunoglobulin isotype of the five selected antibodies was detected by Monoclonal Antibody Isotyping Kit (Thermo Scientific Inc., IL, USA) and all were found to be IgG1 with κ -light chains.

In order to study their biochemical properties, BH-antibodies (BH1, BH2, BH5, BH6, BH7) were initially purified using Protein G affinity chromatography (**Section 3.3.4**) from culture supernatants of hybridoma clones grown in serum free media (SFM). For assays where antibodies were needed in higher quantities, BH-antibodies were also purified from mice ascites (**Section 3.3.4.2**).

Recombinant and cellular ERBB2 binding efficiencies of purified antibodies (**Figure 4.4**) were tested by ELISA, flow-cytometry and immunofluorescence assays.

Figure 4.2 Pairwise alignment of EGFR and ERBB2 amino acid sequences.

Amino acid sequences of human EGFR isoform a (gi|29725609|ref|NP_05219.2|) and human ERBB2 isoform a (gi|54792096|ref|NP_004439.2|) were aligned using Clustal W2. “*” indicates conserved residues. “.” indicates residues having strongly similar properties (> 0.5 in the Gonnet PAM 250 matrix.). “:” indicates residues having weakly similar properties (≤ 0.5 in the Gonnet PAM 250 matrix.). Small and hydrophobic amino acids (AVFPMILW) are shown in red. Acidic amino acids (DE) are shown in blue. Basic amino acids (RK) are shown in magenta. Amino acids with hydroxyl, sulfhydryl or amine groups (STYHCNGQ) are shown in green. Other amino and imino acids are shown in grey.

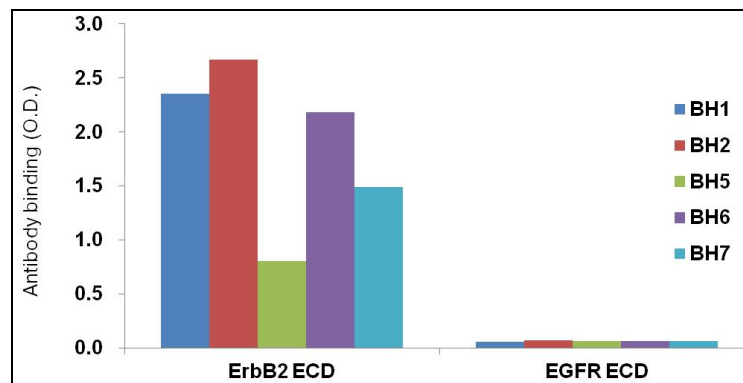


Figure 4.3 ERBB2 and EGFR reactivities of novel anti-ERBB2 monoclonal antibodies. 50 ng/well recombinant ERBB2-ECD or EGFR-ECD coated Nunc-polysorb microtiter plates were incubated with hybridoma culture supernatants containing secreted antibodies. Strength of antigen-antibody interaction was colorimetrically detected (OD 405 nm) by the alkaline phosphatase activity following the incubation with alkaline phosphatase-conjugated anti-mouse IgG antibodies. (Samples were tested in duplicates and the average antibody binding strengths are shown on Y-axis.)

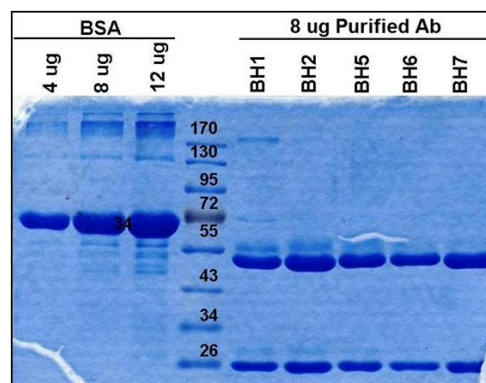


Figure 4.4 BH-antibodies purified by Protein-G affinity chromatography. 8 μ g purified BH-antibodies were run on SDS-PAGE. The gel was stained with coomassie brilliant blue G-250 to analyse the purity of the antibody preparations. Increasing amounts of bovine serum albumin (BSA) loaded on neighboring wells was used for quantification.

4.2.3 Comparison of Affinities for Recombinant ERBB2-ECD by ELISA

Affinities of BH-antibodies for recombinant ERBB2-ECD were compared by ELISA assay. BH1, BH6 and BH7 antibodies presented the strongest binding activity for recombinant human ERBB2-ECD. BH5 antibody displayed the lowest binding capacity, whereas BH2 had intermediate activity (**Figure 4.5**). These observations strongly suggested that we produced three anti-ERBB2 antibodies with high affinity, one with low and one with intermediate affinities.

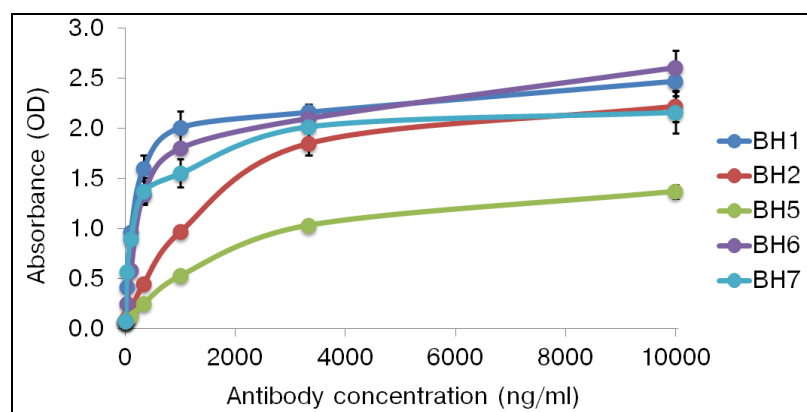


Figure 4.5 Analysis of BH-antibody affinities for recombinant ERBB2 by ELISA. 50 ng/well ERBB2 ECD-coated Nunc-polysorb plates were incubated with increasing concentrations of purified anti-ERBB2 antibodies. Strength of antibody binding was colorimetrically detected (OD 405 nm) by the alkaline phosphatase activity following the incubation with alkaline phosphatase-conjugated anti-mouse IgG antibodies. T Bars: SD.

4.2.4 Comparison of Affinities for Cellular ERBB2-ECD by Flow Cytometry

The affinity of BH-antibodies for cellular ERBB2 was tested by flow cytometry on breast cancer cell lines expressing low (T47D) and high (BT-474) levels of ERBB2 [171, 298, 299] (**Figure 4.6, Figure 4.7**).

When tested on T47D cells, BH1 and BH6 displayed highest shifts in staining intensity as compared to isotype control antibody. No BH5 binding was detectable at any tested condition; as illustrated by overlapping BH5 and IgG1 isotype control peaks at every concentration. BH2 displayed a weak binding that was more significant at higher antibody concentrations ($>10 \mu\text{g/ml}$) (**Figure 4.6, Figure 4.7**).

When tested on BT-474 cells, all BH-antibodies displayed strong intensity shifts at every antibody concentration, BH1 producing the best shift (**Figure 4.6, Figure 4.7**). In addition, two separate peaks with different fluorescence intensities were observed when BT-474 cells were incubated with BH2 and BH5 antibodies at concentrations below and above $20 \mu\text{g/ml}$ (**Figure 4.6**). This suggests that BH2 and especially BH5 might react with two distinct sub-populations of BT-474.

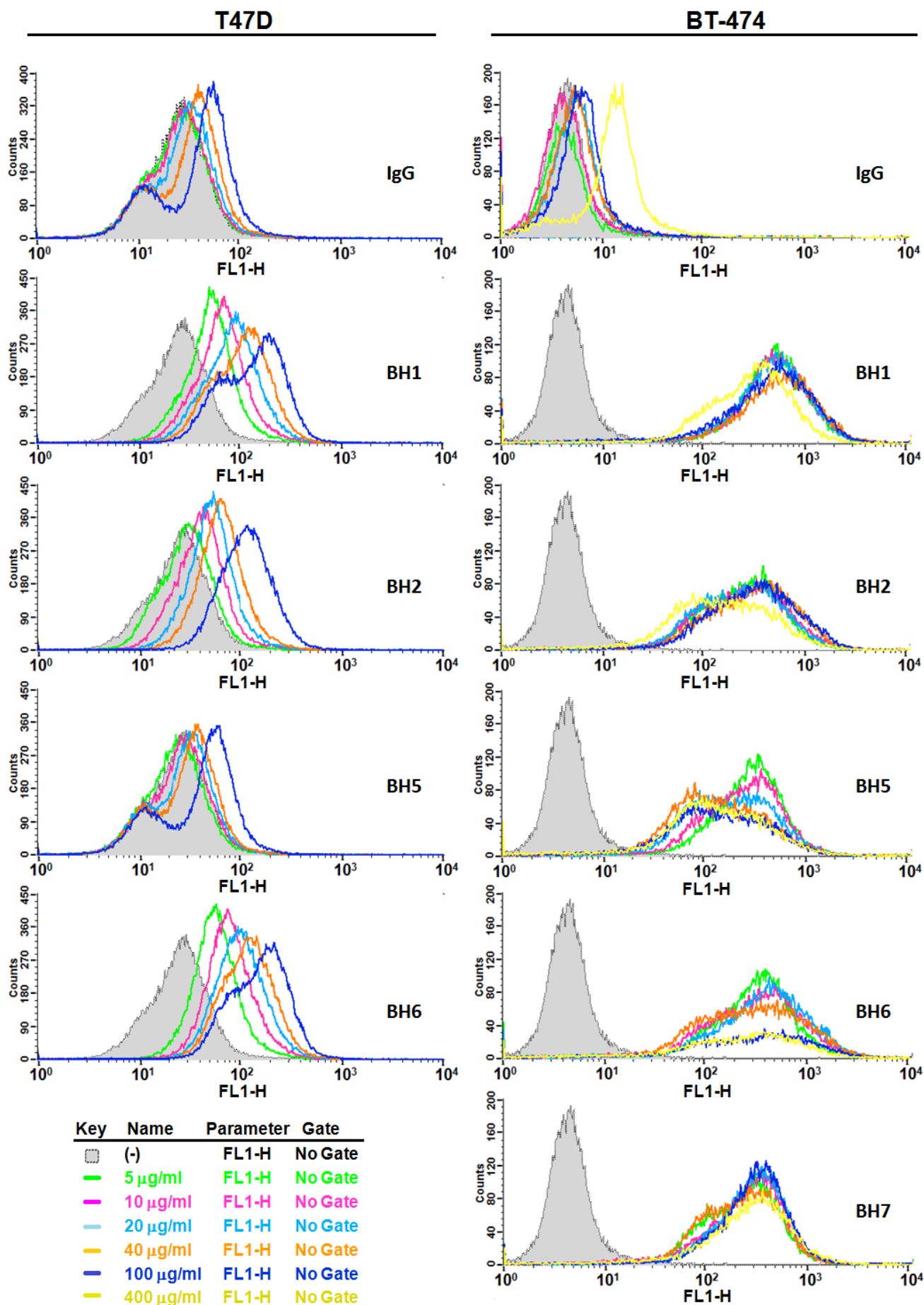


Figure 4.6 Analysis of BH-antibody affinities for cellular ERBB2 by Flow Cytometry (A). $\sim 3 \times 10^5$ T47D (left column) or $\sim 5 \times 10^5$ BT-474 (right column) cells were incubated with increasing amount of BH-antibodies (0.5 – 40 μg antibody/sample) in 100 μl . Anti-ERBB2 incubation of paraformaldehyde fixed single cell suspensions was followed by Alexa-488-conjugated secondary antibodies and fluorescence intensities were measured by flow cytometry. Each graph shows fluorescence intensities of cells stained with the same antibody at different concentrations. Maximum fluorescence emissions at 488 nm are shown on x-axis (FL1-H) and cell counts are shown on y-axis. IgG1-isotype control antibody (IgG) at equal concentrations were used as negative controls. (-) indicates the control samples which were incubated only with Alexa-488-conjugated secondary antibodies without any prior primary antibody incubation.

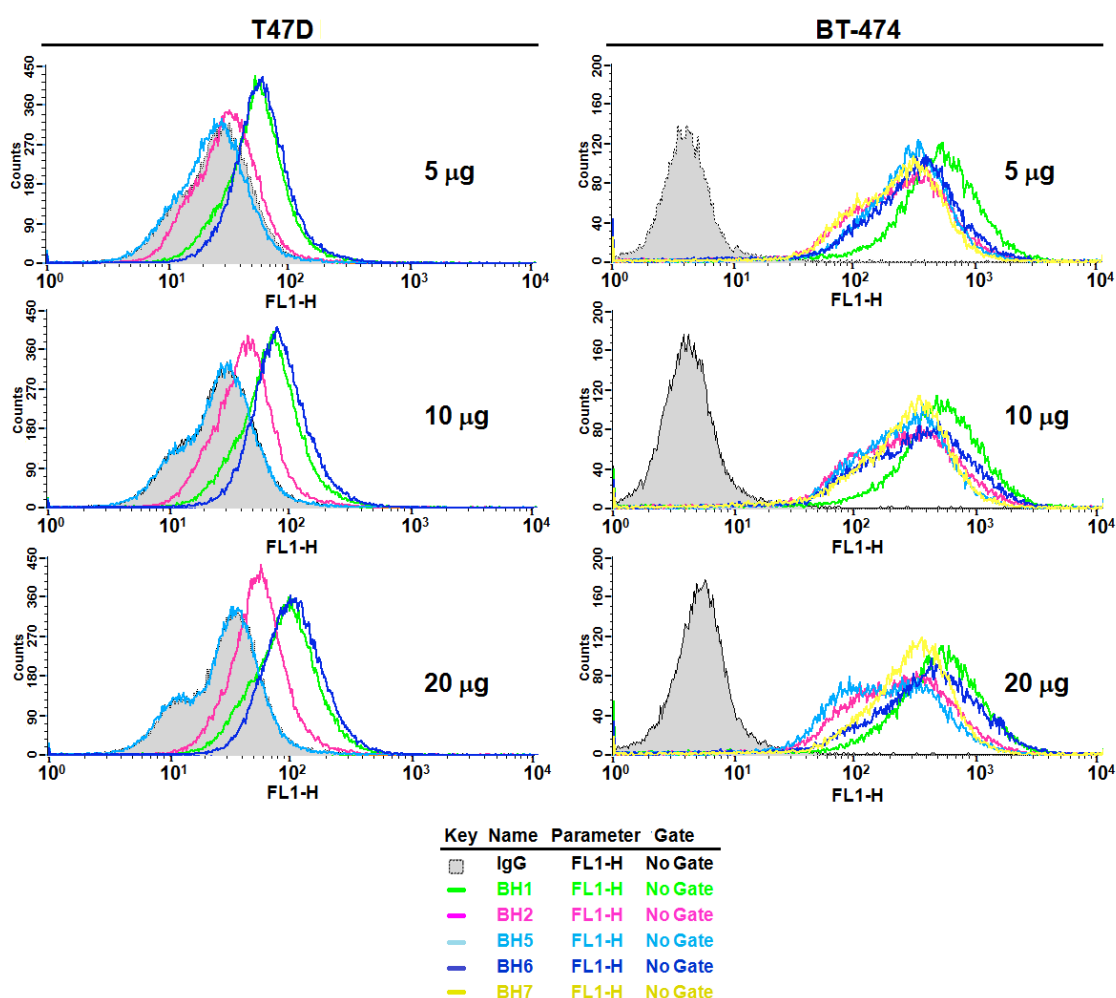


Figure 4.7 Analysis of BH-antibody affinities for cellular ERBB2 by Flow Cytometry (B). $\sim 3 \times 10^5$ T47D (left column) or $\sim 5 \times 10^5$ BT-474 (right column) cells were incubated with increasing amount of BH-antibodies (0.5 – 2 μg antibody/sample) in 100 μl . Anti-ERBB2 incubation of paraformaldehyde fixed single cell suspensions was followed by Alexa-488-conjugated secondary antibodies and fluorescence intensities were measured using flow cytometry. Each graph shows fluorescence intensities of cells stained with different primary antibodies used at same concentration. Maximum fluorescence emissions at 488 nm are shown on x-axis (FL1-H) and cell counts are shown on y-axis. IgG1-isotype control antibody (IgG) at equal concentrations were used as negative controls. (-) indicates the control samples which were incubated only with Alexa-488-conjugated secondary antibodies without any prior primary antibody incubation.

4.2.5 Comparison of Affinities for Cellular ERBB2-ECD by Indirect Immunofluorescence

Binding strengths of BH-antibodies to endogenous ERBB2 protein were compared by indirect immunofluorescence assay using human breast cancer cell lines expressing different levels of ERBB2 (**Figure 4.8**).

Fluorescence in situ hybridization (FISH) using probes against *ERBB2* gene and chromosome 17 centromere showed that MCF-7 had three chromosome 17 and two *ERBB2* signals. MDA-MB-361 provided 4-8 chromosome 17, and 8-12 *ERBB2* signals. Both SK-BR-3 and BT-474 displayed 5-6 chromosome 17 signals and highly amplified *ERBB2* signals (**Figure 4.8 A**). Most of T47D cells displayed three chromosome 17 and three *ERBB2* copies (data not shown). These findings were in perfect agreement with a previous study reporting 47, 43, 11 and 2.5 copies of the *ERBB2* gene in BT-474, SK-BR-3, MDA-MB-361 and MCF-7, respectively [299].

BH-antibodies displayed highly correlated immunoreactivities with respect to *ERBB2* gene copy numbers; all five antibodies displayed strong fluorescence intensities on cellular membranes of breast cancer cell lines expressing high (BT-474, SK-BR-3) and moderate (MDA-MB-361) levels of ERBB2. On the contrary, ERBB2 signals on MCF-7 cells were negative or barely detectable. Highest signal intensities were obtained by BH1 and BH6 antibodies, followed by BH2, BH7 and BH5 (**Figure 4.8 B-G**).

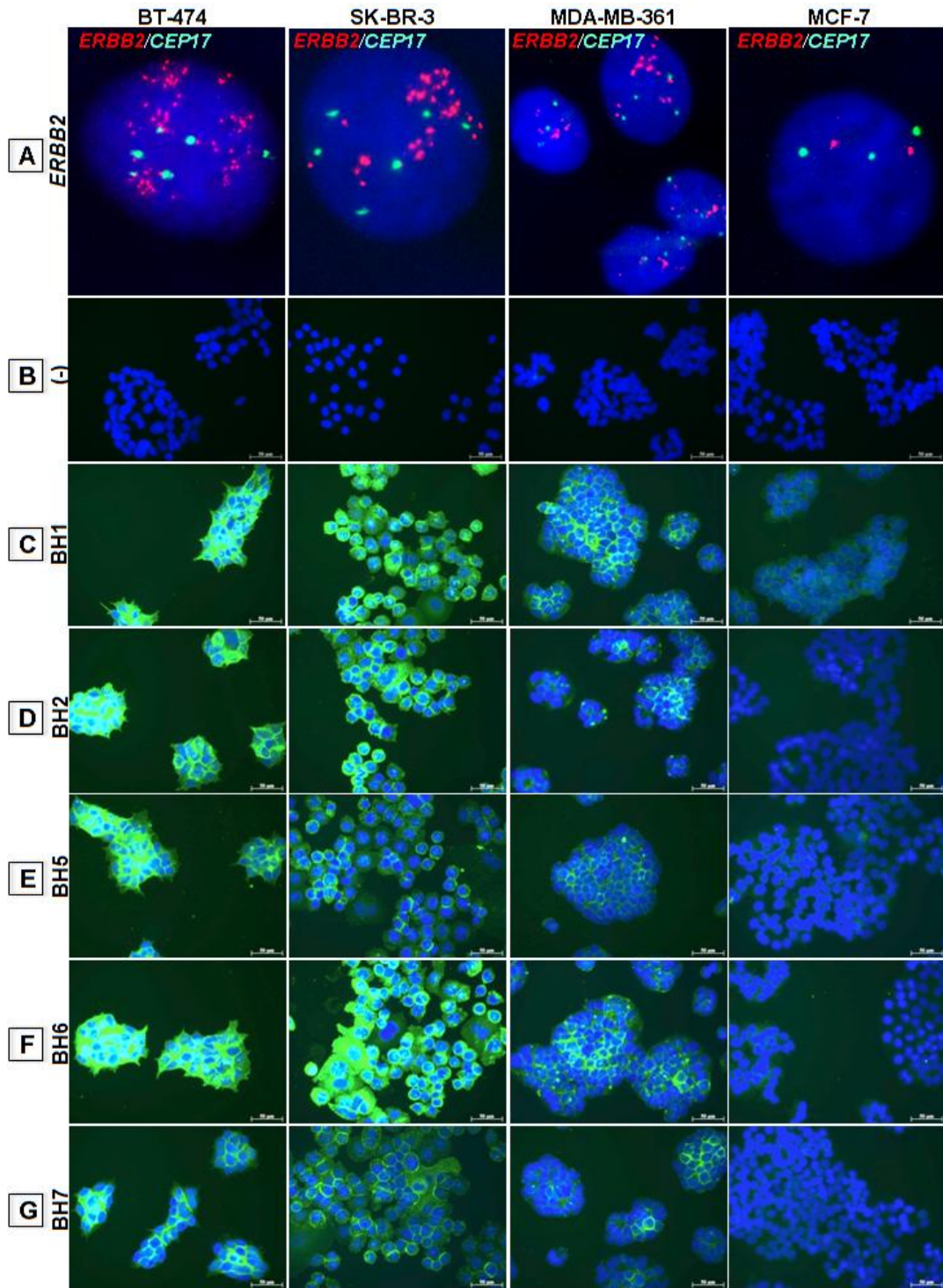


Figure 4.8 Analysis of cellular ERBB2 by indirect immunofluorescence using BH-antibodies. **A)** For FISH analysis, human breast cancer cells with different ERBB2 levels were harvested at confluence, fixed and subjected to dual-color FISH using Texas Red-labeled *ERBB2* (red) and FITC-labeled chromosome 17 α -satellite CEP17 (green) DNA probes. **B-G)** For immunofluorescence assays, cells were seeded on coverslips on 6-well plates and incubated 36 hours in cell culture medium at 37°C. Paraformaldehyde fixed and permeabilised cells were incubated with 20 μ g/ml BH-antibodies for two hours. Photographs are representative of merged images of BH-antibody binding detected using Alexa-488-conjugated anti-mouse IgG and nuclei counterstained with DAPI. (-) indicates the control samples where the cells were incubated only with Alexa-488-conjugated secondary antibodies without any prior primary antibody incubation.

4.3 Human ERBB2 Epitope(s) Recognized by BH-antibodies

4.3.1 The Role of Structural Integrity of ERBB2 in Antibody Binding

In order to confirm the specificity of BH-antibodies for ERBB2, we performed western blot assays using different breast cancer cell line lysates prepared at mild (NP40) or stringent (RIPA) conditions. NCL-CB11 (CB11 in short) antibody against intracellular ERBB2 epitope was used as a positive control. While western blot analysis of breast cancer cell line lysates with CB11 antibody provided positive signals (**Figure 4.9**) representing their ERBB2 amplification status [299], neither BH-antibodies, nor Trastuzumab detected ERBB2 in this experimental setting (data not shown).

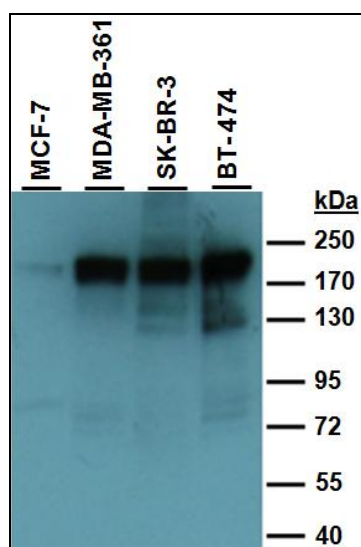


Figure 4.9 Western blot analysis of endogenous ERBB2 expression on different breast cancer cell lines. 30 μ g total cell lysates of RIPA lysed MCF-7, MDA-MB-361, SK-BR-3 and BT-474 breast cancer cell lines were subjected to western blot assay using CB11 antibody raised against an intracellular ERBB2 epitope. ERBB2 protein band is seen at 185 kDa. Calnexin signal was used to verify equal loading (data not shown)

Next, endogenous ERBB2 was immunoprecipitated by Trastuzumab to concentrate the protein before analysis (**Figure 4.10 A**). While ERBB2 present in the soluble fraction remained totally in the flow through fraction in the absence of Trastuzumab (**Figure 4.10 A, Lane 2, 4**), it was totally captured by Protein G-

conjugated beads in the presence of Trastuzumab (**Figure 4.10 A, Lane 1**), depleting ERBB2 from the flow through (**Figure 4.10 A, Lane 3**). Yet, no reactivity of BH-antibodies was detected in western blot assays with this ERBB2 enriched conditions either (**Figure 4.10 B**).

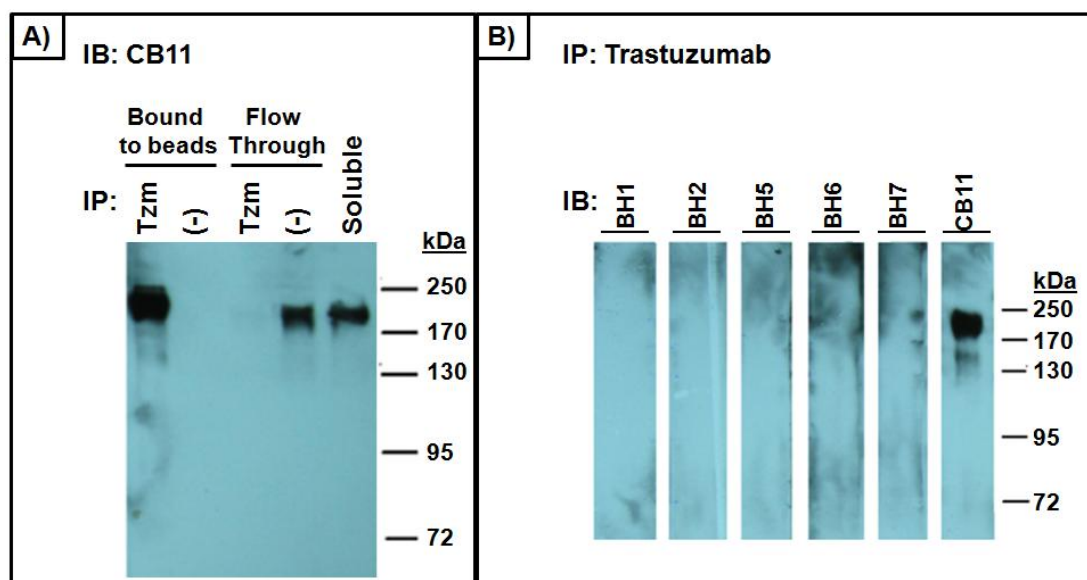


Figure 4.10 Analysis of antibody binding to endogenously expressed ERBB2 denatured under reducing conditions. Endogenously expressed ERBB2 present in 15 mg T47D RIPA lysate was immunoprecipitated by 240 μ g Trastuzumab. **A)** T47D cell lysate (**Lane 5**) was incubated with Protein G-conjugated beads in the presence (**Lane 1**) or absence (**Lane 2**) of Trastuzumab. ERBB2 protein captured by the beads (**Lane 1 and 2**) and left unbound in the flow-through fraction (**Lane 3 and 4**) are shown. Proteins resolved on SDS-PAGE was subjected to western blot assay using CB11 antibody. **B)** Endogenous ERBB2 enriched by Trastuzumab immunoprecipitation was subjected to western blot assay using BH-antibodies. CB11 antibody was used as a positive control to locate the 185 kDa ERBB2 band.

Based on these findings, we hypothesized that the epitope(s) recognized by BH-antibodies were unstable under denaturing conditions of SDS-PAGE, although they remained stable after paraformaldehyde fixation used in indirect immunofluorescence (**Figure 4.8**) and flow cytometry experiments (**Figure 4.6, Figure 4.7**).

Therefore, we tested antibody immunoreactivities for native ERBB2 protein by immunoprecipitating endogenous ERBB2 in the cell lysates by BH-antibodies. RIPA lysates of T47D cells were subjected to immunoprecipitation with different

antibodies, and antigen binding was tested by western blot assay using CB11 antibody. Although incapable of binding to ERBB2 proteins denatured during SDS-PAGE, all antibodies tested were able to immunoprecipitate ERBB2 in RIPA lysates (**Figure 4.11**). BH1 (with the highest affinity), BH2, BH6 and BH7 antibodies efficiently precipitated the native ERBB2 and almost depleted the protein in the lysate. Whereas, as expected from flow cytometry analyses, BH5 poorly precipitated the ERBB2 protein present in T47D lysates, leaving the protein mostly in the “flow-through (unbound) fraction”. These findings provided evidence that newly generated antibodies recognized conformation-dependent epitope(s) on endogenously expressed ERBB2 protein.

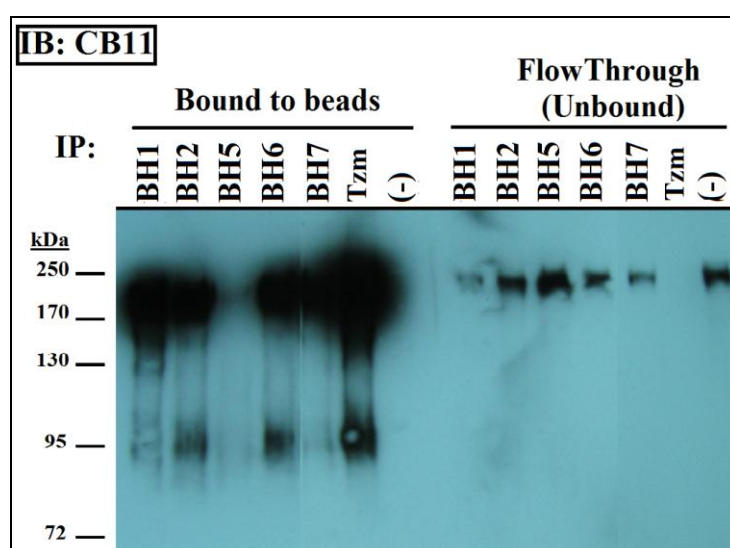


Figure 4.11 Immunoprecipitation of endogenous ERBB2 by BH-antibodies. Endogenously expressed ERBB2 present in 2 mg T47D RIPA lysate was immunoprecipitated by 20 μ g BH-antibodies or Trastuzumab (Tzm). Antigen-antibody complexes that were captured onto Protein G-conjugated beads were eluted by boiling in SDS-PAGE loading dye (**left panel**). ERBB2 proteins that were uncaptured by the antibody-bead complex remained in the flow through (**right panel**). Proteins resolved under denaturing conditions were subjected to western blot assay using CB11 antibody. Trastuzumab was used as a positive control for ERBB2 immunoprecipitation. (-) is the negative control where T47D lysate was incubated with Protein-G beads in the absence of any antibody. ERBB2 protein band is detected at 185 kDa.

4.3.2 BH-antibodies Do Not Compete for The ERBB2 Epitope Recognized by Trastuzumab

To compare epitopes targeted by BH-antibodies and Trastuzumab, we have performed ELISA-based and immunofluorescence-based competition experiments.

Two different approaches were pursued using ELISA technique to test competition between BH-antibodies and Trastuzumab for ERBB2-ECD [300]. For both approaches, the wells of an ELISA-plate were initially coated with recombinant ERBB2-ECD protein. In the first, we aimed to find out whether prior incubation with Trastuzumab would block epitopes on ERBB2-ECD that are to be recognized by BH-antibodies, and therefore decrease the signal received upon binding of BH-antibodies. As seen on **Figure 4.12**, prior incubation with excess Trastuzumab did not decrease the BH-antibody binding to the recombinant ERBB2-ECD protein. In the second approach, we wanted to see whether prior incubation with BH-antibodies would block the Trastuzumab-binding epitopes and decrease binding of Trastuzumab. As seen on **Figure 4.13**, prior incubation with excess amount (250ng) of BH-antibodies did not decrease the Trastuzumab signal. These reciprocal experiments suggested that BH-antibodies do not share the same epitope with Trastuzumab.

Competition between BH-antibodies and Trastuzumab for endogeneous ERBB2 protein was investigated by immunofluorescence using two approaches [257] where SK-BR-3 cells were incubated with combination of Trastuzumab and BH-antibodies. In the first, cells were incubated with increasing levels of Trastuzumab in addition to one of the BH-antibodies and the change in BH-antibody signal was analysed (**Figure 4.14 left panel**). As a second approach, SK-BR-3 cells were incubated with a combination of equal amounts of Trastuzumab and BH-antibodies and the change in Trastuzumab signal was analysed (**Figure 4.14 right panel**). None of the samples revealed a significant competition between Trastuzumab and BH-antibodies, confirming the absence of epitope sharing.

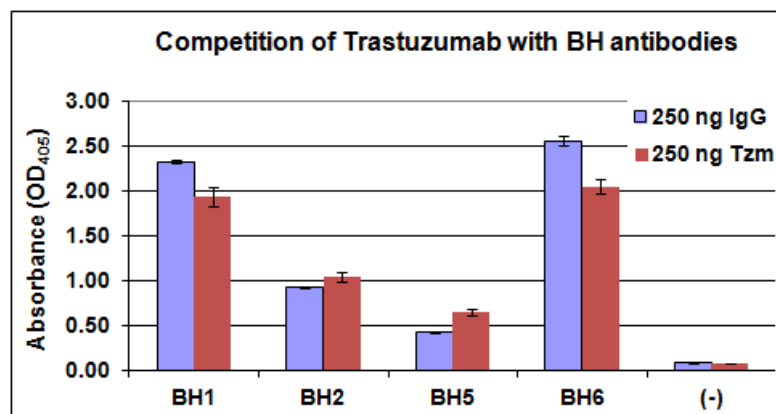


Figure 4.12 Trastuzumab does not compete with BH-antibodies for ERBB2-ECD binding on ELISA assays. Wells coated with 25 ng recombinant ERBB2-ECD protein were treated either with excess (250 ng) Trastuzumab (Tzm) or IgG1 isotype control antibody. Then 25 ng of BH-antibodies were added. Following the incubation with alkaline phosphatase-conjugated anti-mouse IgG antibodies, the colorimetric signal was measured at 405 nm. (-) represents negative control wells directly treated with alkaline phosphatase conjugated anti-mouse IgG antibodies, following competitor antibody incubation. Error bars represent standard deviation of samples tested in duplicates.

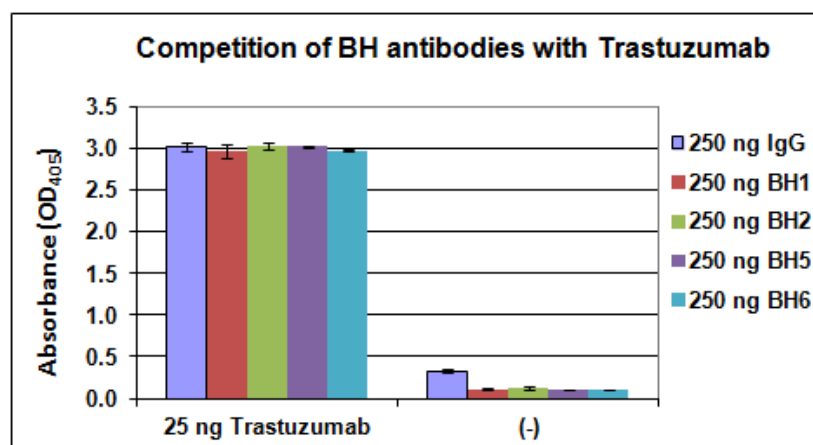


Figure 4.13 BH-antibodies do not compete with Trastuzumab for ERBB2-ECD binding on ELISA assays. Wells coated with 25 ng recombinant ERBB2-ECD protein were initially treated with HRP conjugated anti-human IgG in order to block the human Fc regions on the coated antigen. Wells were then treated either with excess (250 ng) BH-antibodies or excess IgG1 isotype control antibody. Then 25 ng of Trastuzumab was added. Following the incubation with alkaline phosphatase-conjugated anti-human IgG antibodies, the colorimetric signal was measured at 405 nm. (-) represents negative control wells directly treated with alkaline phosphatase conjugated anti-human IgG antibodies, following competitor antibody incubation. Error bars represent standard deviation of samples tested in duplicates.

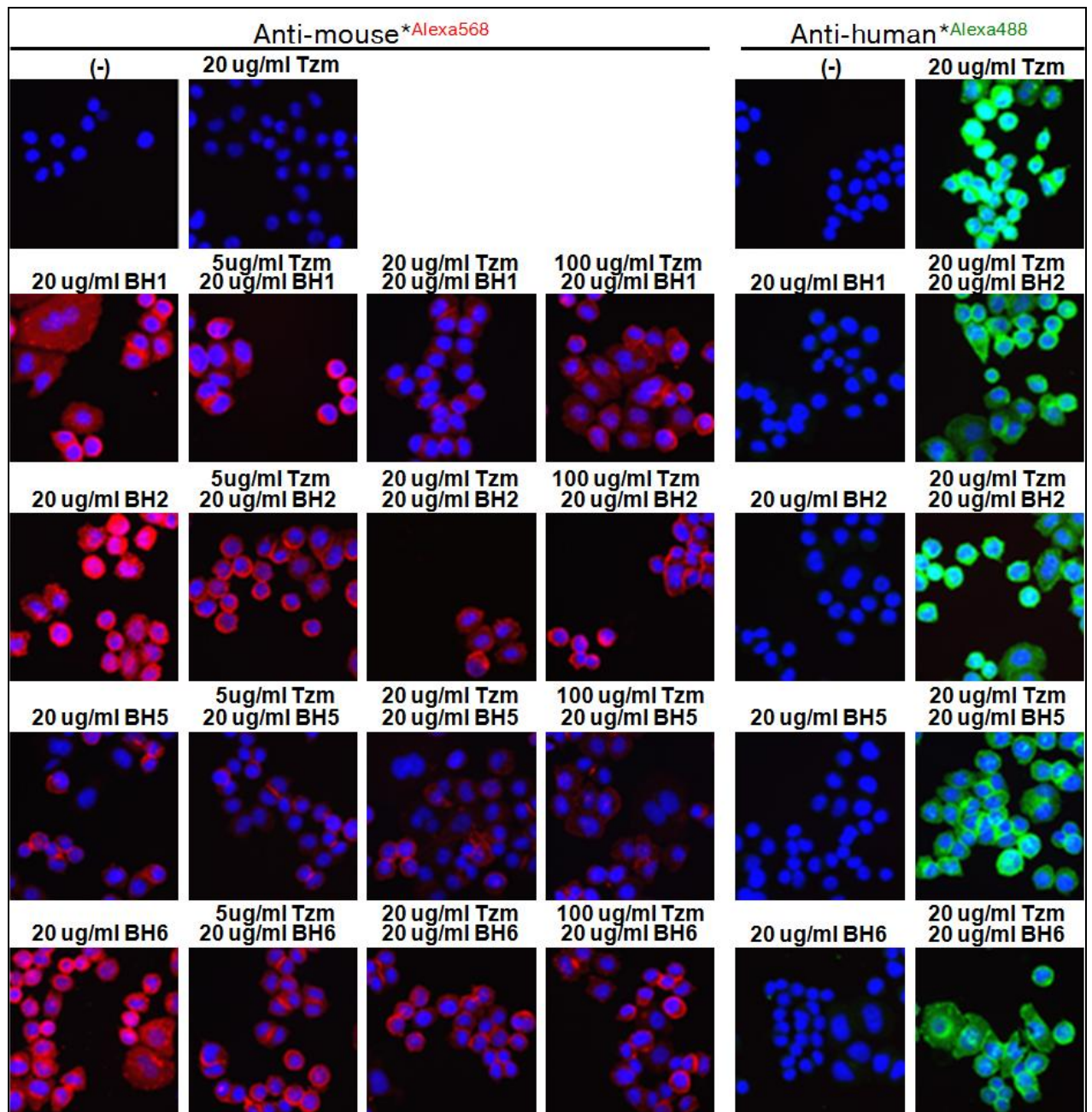


Figure 4.14 BH-antibodies and Trastuzumab do not share the same epitope on cellular ERBB2 protein. SK-BR-3 cells grown on coverslips were fixed by 2% paraformaldehyde, permeabilized and incubated with a combination of Trastuzumab (Tzm) and one of the BH-antibodies. **Left panel:** 20 μ g/ml BH-antibodies were competed with 5 μ g/ml, 20 μ g/ml or 100 μ g/ml Trastuzumab, and the binding was detected by Alexa568-conjugated anti-mouse IgG antibodies. **Right panel:** 20 μ g/ml Trastuzumab was competed with 20 μ g/ml BH-antibodies, and the binding was detected by Alexa488-conjugated anti-human IgG antibodies. Photographs are representative of merged images of anti-ERBB2 binding detected using Alexa-conjugated secondary antibodies and nuclei counterstained with DAPI.

4.3.3 Identification of Epitopes Recognized by BH-antibodies

In order to delineate the epitope(s) recognized by BH-antibodies, we constructed a series of plasmids to express truncated forms of ERBB2 at its ECD. Putative domains on human ERBB2 protein and their corresponding locations on the coding mRNA are shown on **Figure 4.15** [58, 99, 266, 301, 302]. Each construct, whose cloning was explained in details at **Section 3.3.5**, contains different combinations of ECD-subdomains surrounded by the signal peptide (SP) and transmembrane (TM) domains of the full length human ERBB2 (**Figure 4.16**).

Briefly, *ERBB2*-extracellular domain (ECD I-II-III-IV) was extracted from the plasmid vector expressing full-length human ERBB2 protein (OCABo5050G0219D; shortly called p219-D here), leaving the coding sequence for the N-terminal 22 amino acid signal peptide intact. pCC2001 was produced by inserting Not I and Xho I sites between the signal peptide sequence and the sequence encoding the C-terminal domain starting from the transmembrane region. DNA sequences allowing the inclusion of domains I, II, III and IV of ECD under different combinations were amplified by PCR and inserted into pCC2001 plasmid using the Not I, Spe I and Xho I sites (**Figure 4.16**). In addition to the vectors containing single ECD domains or multiple domains consecutively, we have designed various constructs that fuse distant subdomains, in order to mimic physiological folding (i.e subdomain I-IV). The integrity of cloned sequences has been verified by DNA sequencing. Attempts for cloning pCC2013 and pCC2015 constructs failed to produce plasmids with correct nucleotide sequences; therefore they were excluded from the ERBB2 construct panel and were not used in further analyses.

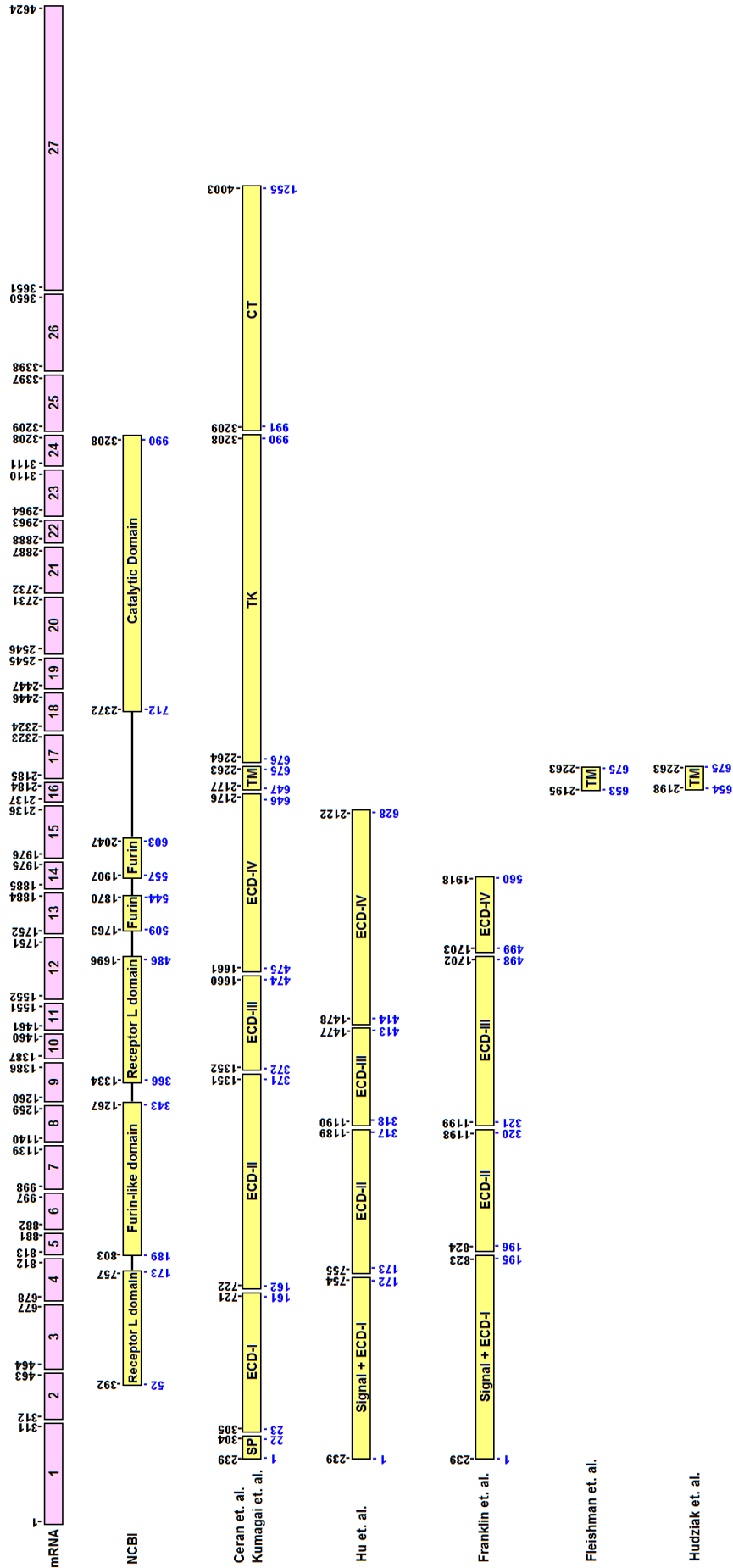


Figure 4.15 Human *ERBB2* mRNA and protein domains; and the corresponding nucleotides on *ERBB2* mRNA. Twenty seven exons forming the human *ERBB2* isoform-a mRNA are illustrated with numbered pink boxes drawn to scale; numbers indicate the nucleotides surrounding each exon. Putative protein domains are illustrated with yellow boxes drawn to scale according to the information presented on NCBI and indicated references. Blue numbers indicate the amino acid positions at the boundaries of each putative protein domain and black numbers indicate their corresponding nucleotides on the full length mRNA sequence.

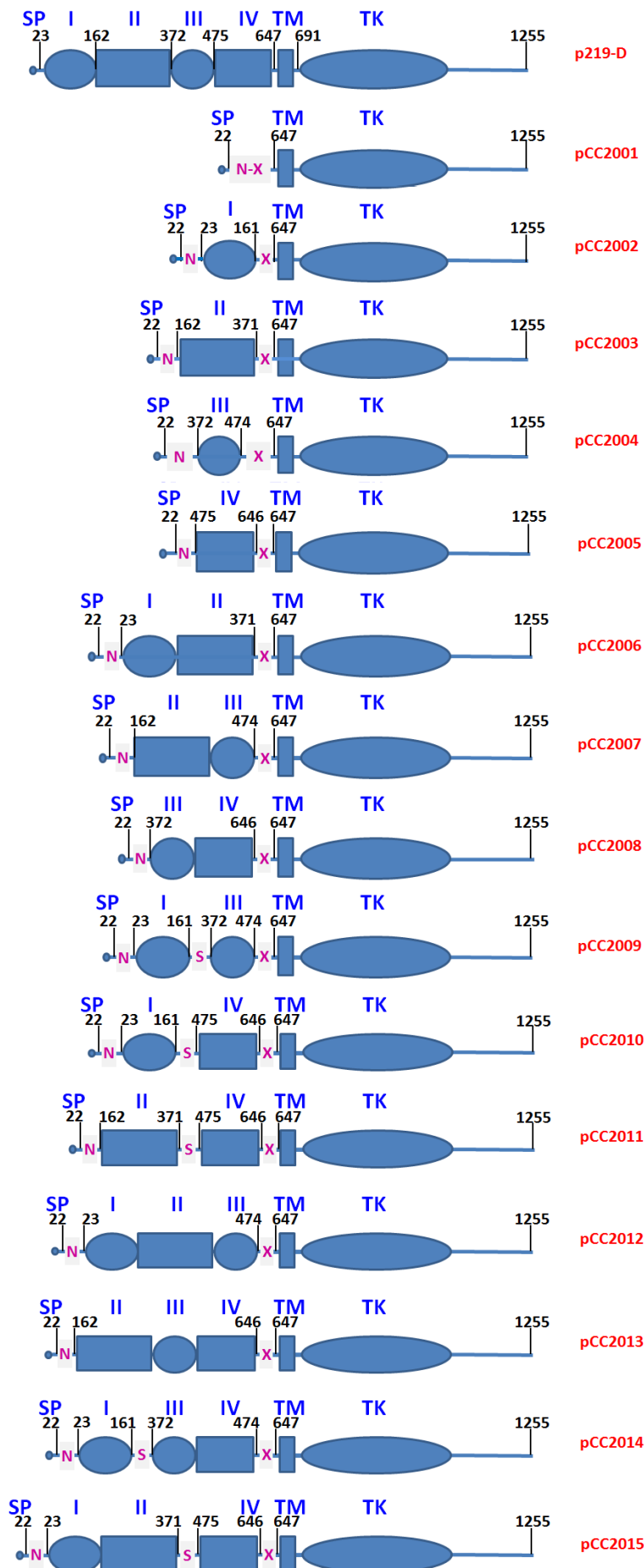


Figure 4.16 ERBB2 deletion constructs expressing different ECD-subdomains.

Extracellular subdomains (ECD I-II-II-IV) were deleted from p219-D plasmid coding full length human ERBB2 protein to construct pCC2001 backbone plasmid. PCR-amplified individual ERBB2 ECD subdomains (shown in blue boxes numbered from I to IV) were introduced between NotI and XhoI sites on pCC2001 at different combinations to produce other truncated ERBB2 constructs (pCC2002-pCC2015) shown in red. Numbers in black correspond to the amino acid positions on full length human ERBB2 protein. Letters in purple represent the restriction enzyme sites added between the inserted subdomains; N: Not I, S: Spe I, X: Xho I. SP: Signal Peptide, TM: Transmembrane domain, TK: Tyrosine Kinase domain. (pCC2013 and pCC2015 were excluded from the construct panel since their cloning did not produce plasmid clones with correct nucleotide sequences.)

Huh-7 cells that express low levels of endogenous ERBB2 protein [303] were transiently transfected with plasmids to express different forms of ERBB2, and immunoreactivity of each antibody was tested by immunofluorescence (**Figure 4.17**), and combined immunoprecipitation-western blot (**Figure 4.19**) assays. Huh7 cells transfected with backbone pDEST26 vector and pCC2001 vector expressing a truncated ERBB2 construct lacking all ECD domains were used as negative controls.

Huh7 transfection efficiency was lower than 100%. This enabled visualization of two distinct populations on the same slide; untransfected wild-type cells expressing endogenous levels of ERBB2, and transfected cells overexpressing ectopic ERBB2. Since ectopic ERBB2 signal was easily discriminated from endogenous ERBB2 signal, high affinity recognition of endogenous ERBB2 protein was not misinterpreted as low affinity recognition of truncated ERBB2 proteins.

CB11 antibody that is directed against an intracellular epitope shared by all constructs was used as a positive control to confirm the expression of full length and truncated forms of ERBB2 transgene. While cells transfected with empty vector (pDEST26) were unstained, all ERBB2 transfectants (pCC2001-pCC2014 and p219-D) were successfully detected with CB11, indicating that all constructs were efficiently transfected, transcribed and translated (**Figure 4.17**). Another control was Trastuzumab that is directed against an epitope located at domain IV of the extracellular region of ERBB2 [17]. All truncated forms of ERBB2 protein containing domain IV (pCC2005, pCC2008, pCC2010, pCC2011 and pCC2014) provided strongly positive signals with Trastuzumab (**Figure 4.17**), indicating that not only the intracellular domains but also the extracellular domains of the deletion constructs were expressed successfully. Western blot analysis of the transfectants also confirmed the expression of the deletion constructs at their expected sizes (**Figure 4.18**).

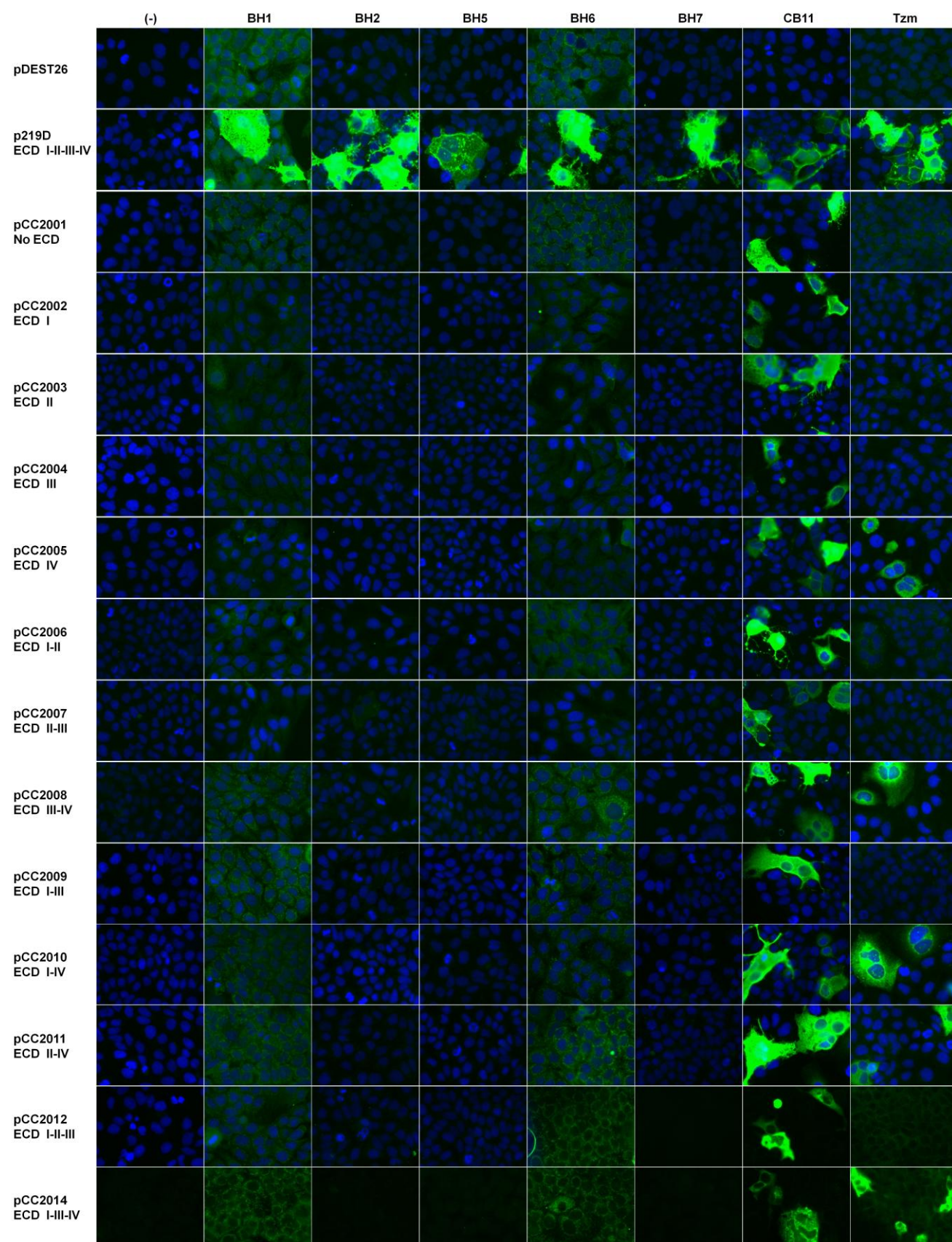


Figure 4.17 Epitope mapping of anti-ERBB2 antibodies using indirect immunofluorescence. Huh7 cells were transfected either with full-length ERBB2 construct (p219-D), empty vector (pDEST26), or truncated ERBB2 constructs (pCC2001-pCC2014) were cultivated for 48 hours, and subjected to indirect immunofluorescence as described in **Figure 4.8**. Briefly, transfectants were reacted with 10 µg/ml BH1, BH2, BH5, BH6, BH7, Trastuzumab (Tzm) or 1/50 diluted CB11. Photographs are merged images of anti-ERBB2 binding detected using Alexa488-conjugated secondary antibodies and nuclei counterstained with DAPI. (-): no primary antibody.

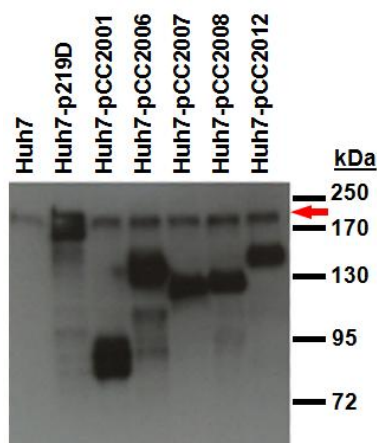


Figure 4.18 Verification of protein products of ERBB2 constructs. RIPA lysates of Huh7 cells that were transfected with ERBB2 constructs were resolved on SDS-PAGE and were subjected to western blot assay using CB11 antibody. Red arrow indicates the endogenous ERBB2 band.

BH1 and BH6 which have ERBB2 affinities higher than that of BH2, BH5 and BH7 as suggested by the previous indirect ELISA (**Figure 4.5**), flow cytometry and (**Figure 4.6**) indirect immunofluorescence (**Figure 4.8**) analyses, recognized the low level endogenous ERBB2 in Huh7 cells transfected with the empty vector (pDEST26), producing a faint background staining. All five BH-antibodies effectively recognized the cells expressing the full-length ERBB2 constructs (p219-D); producing strong staining that was easily distinguished from the endogenous ERBB2 signal. However, none of the BH-antibodies significantly reacted with truncated ERBB2 proteins containing individual ECD subdomains, alone or in combination (**Figure 4.17**). The immunofluorescence results indicated that BH-antibodies recognize conformational epitopes which are disturbed in the absence of any of the ERBB2 ECD-subdomains.

In order to see whether low-affinity recognition of the deletion constructs by BH1, BH2, BH5 and BH6 would enable them to pull down the products of pCC2006, pCC2007 or pCC2012, transfectant lysates were immunoprecipitated by BH-antibodies, Trastuzumab and CB11; and probed with CB11 (**Figure 4.19**). pCC2008-transfectant lysate (containing ECD-IV recognized by Trastuzumab) was also included in the test panel as an internal control. Total cell lysates were run next to immunoprecipitates, to show the exact location of the expected bands (indicated by arrows on **Figure 4.19**).

Intracellular ERBB2 domains were intact on all truncated ERBB2 proteins and CB11 successfully immunoprecipitated ERBB2 proteins in all tested lysates. Trastuzumab was documented to recognize a conformational epitope formed by the amino acids present totally on ECD-IV therefore presence of ECD-IV was sufficient to create that epitope [17]. Trastuzumab successfully immunoprecipitated the truncated ERBB2 protein present in pCC2008-lysates (containing ECD-IV), indicating the efficiency of the assay procedure. In addition, it also pulled down the endogenous ERBB2 present in Huh7 cells.

While all of the BH-antibodies successfully immunoprecipitated the ectopic full-length ERBB2 protein present in p219-D lysates, they failed to pull-down truncated ERBB2 proteins (**Figure 4.19**). These results, supporting the previous indirect immunofluorescence analyses with truncated ERBB2 transfectants (**Figure 4.17**), indicate that BH-antibodies are directed to an epitope, or closely related epitopes present only on structurally intact ECD of ERBB2, and the reactivity of antibodies was lost when any one of the four subdomains of the ERBB2 ECD was deleted. Therefore, all five antibodies require the structural integrity of ECD for recognition of ERBB2 receptor.

BH1 and BH6 stood out among the other BH-antibodies in that they stained the endogenous full-length ERBB2 in Huh7 cells in indirect immunofluorescence assays. High-exposure of the IP-immunoblots also demonstrated that, both BH1 and BH6 were able to pull-down low-level expressed endogenous ERBB2 in Huh7 cells, illustrating the strength of their affinity for ERBB2.

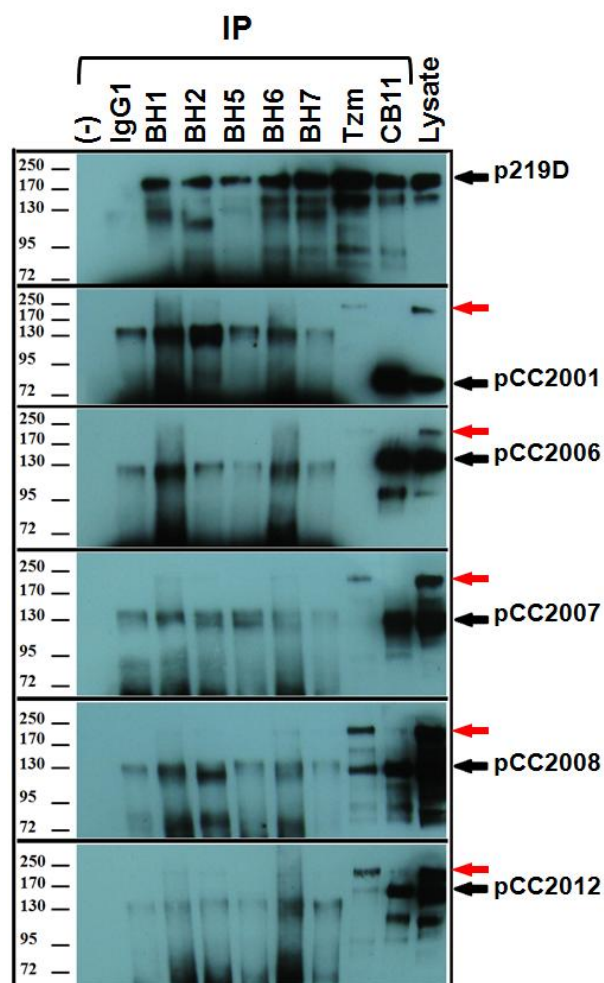


Figure 4.19 Epitope mapping of anti-ERBB2 antibodies using combined immunoprecipitation-western blot assay. Huh7 cells that were transfected with full length (p219-D) and truncated ERBB2 constructs were RIPA lysed and immunoprecipitated with BH-antibodies, Trastuzumab, CB11 or IgG1 isotype control antibody. Precipitated proteins were subjected to western blot assay using CB11 antibody. “(-)” is the negative control sample, where the lysates were incubated with protein G beads without any antibody. The input lysate is shown on the last lane of each blot. Red arrows indicate the endogenous ERBB2 band.

4.4 Biological Activity of anti-ERBB2 Antibodies

4.4.1 Effect of BH-antibodies on Breast Cancer Cell Growth

Novel monoclonal antibodies recognising conformational ERBB2 epitopes hold promise as breast cancer therapeutics. Therefore the biological activities of BH-antibodies were investigated on ERBB2-overexpressing human breast cancer cell line SK-BR-3.

Affinity purified anti-ERBB2 antibodies were added into SK-BR-3 culture media and relative cell growth was tested following 6 days of cell culture as explained above (**Section 3.3.11**). When SK-BR-3 cells were treated with anti-ERBB2 antibodies alone, all BH-antibodies significantly decreased the cell density upon 6-day treatment. BH6 and BH1 antibodies displayed stronger inhibitory effects on SK-BR-3 target cells, by lowering relative cell growth to 62% and 71% respectively, as compared to isotype control antibody (**Figure 4.20**).

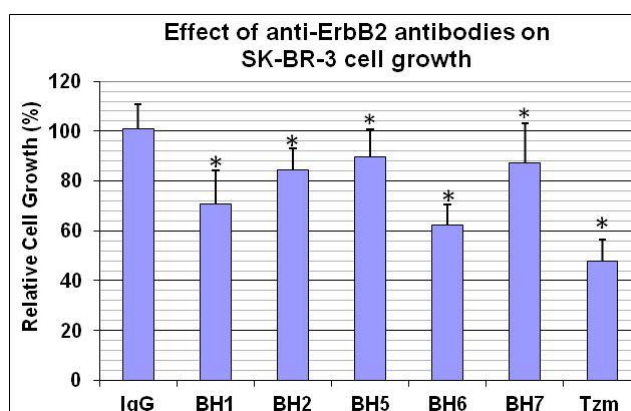


Figure 4.20 Effects of BH-antibodies on SK-BR-3 cell growth. SK-BR-3 cells were plated onto 96-well plates and incubated at 37°C with 5% CO₂. After 2 hours of incubation, BH-antibodies, Trastuzumab (Tzm) or IgG1 isotype control antibody (IgG) were added (5 µg/ml), and the cells were incubated for 6 days. After 6 days of treatment, the percentages of surviving cells to controls, defined as 100% survival, were determined by Sulforhodamine B assay. Each antibody was tested in triplicates in more than ten independent experiments and the average values are represented as SK-BR-3 relative cell growth (%). T Bars, SD. *, decrease in relative cell growth wrt IgG isotype control treatment (p<0.05).

We performed a dose-effect curve assay with anti-ERBB2 antibodies, to find the lowest concentration that significantly decreases SK-BR-3 cells growth (**Figure 4.21**). Under these conditions, as little as 1.25 µg/ml BH6 and 5 µg/ml BH1 was sufficient to cause significant growth inhibition (p=0.039 and p=0.005 respectively).

A panel of other breast cancer cell lines with different levels of ERBB2 expression was also tested for their sensitivity to BH-antibodies (**Figure 4.22**). While all BH-antibodies displayed significant growth inhibition on MDA-MB-361 cells, only BH1 performed a significant growth inhibition on BT-474 cells in comparison to isotype control antibody. As expected, none of the anti-ERBB2 antibodies tested induced a significant change in the relative cell growth of MCF-7 and T47D cell lines with low level ERBB2 expression.

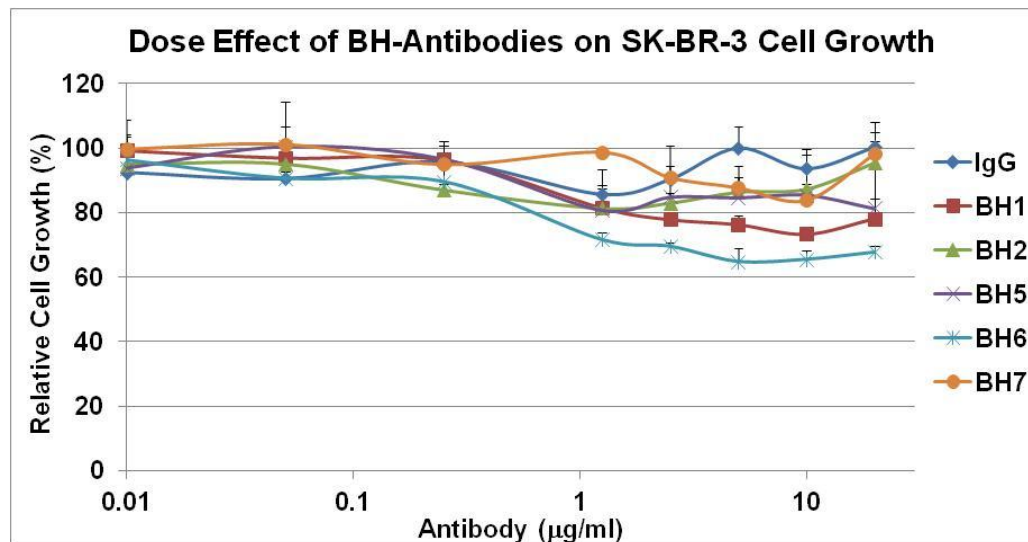


Figure 4.21 Dose effect of BH-antibodies on SK-BR-3 cell growth. SK-BR-3 cells were treated with increasing amounts of (0.01-20 µg/ml) BH-antibodies or IgG1 isotype control antibody (IgG) as explained in **Figure 4.20**. Each condition was tested in triplicates and the average values are represented as relative cell growth (%). T Bars, SD.

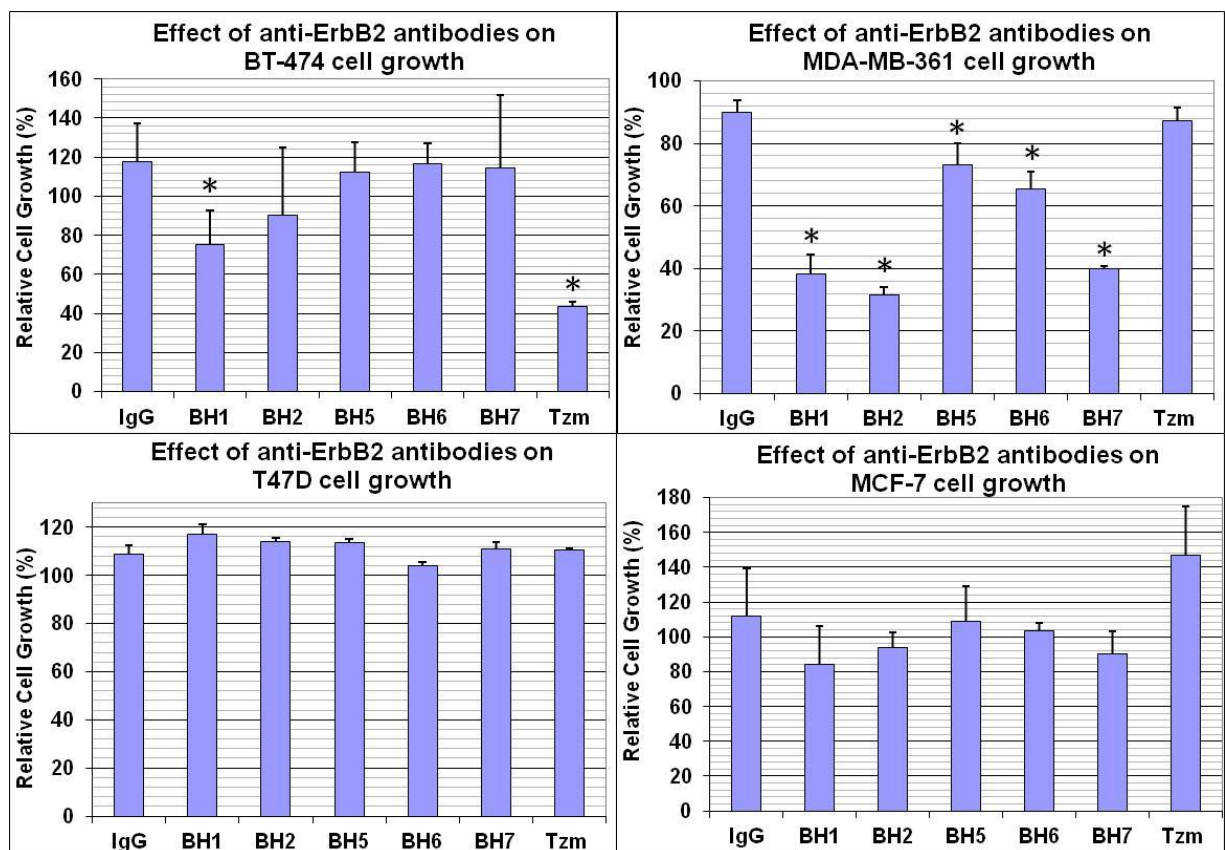


Figure 4.22 Effects of BH-antibodies on breast cancer cell panel with different levels of ERBB2 expression. Breast cancer cell lines expressing high levels of ERBB2; MDA-MB-361 and BT-474, and low levels of ERBB2; MCF-7 and T47D were treated with BH-antibodies as explained in **Figure 4.20**. Each treatment was tested in triplicates and the average values are represented as relative cell growth (%). T Bars, SD. *, decrease in relative cell growth wrt IgG isotype control treatment ($p < 0.05$).

It has been previously reported that tumor cells develop TNF- α -resistance due to increased signalling through growth factor receptors [279], and amplified expression of ERBB2 in breast cancer cells induces resistance to TNF- α cytotoxicity [83, 280]. In order to determine whether anti-ERBB2 antibodies were able to sensitize SK-BR-3 cells to TNF- α , cells were treated with anti-ERBB2 antibodies for 6 days in the presence of TNF- α as explained before [84].

In the experimental setting where 1000 Unit/ml TNF- α was applied together with 5 μ g/ml anti-ERBB2 antibodies, all BH-antibodies caused a substantial decrease in SK-BR-3 growth rate, reversing TNF- α resistance (**Figure 4.23**). When SK-BR-3 cells were treated with TNF- α only, a partial growth inhibition (43 %) was observed. When BH2, BH5, BH6 and BH7 were used in combination with TNF- α , they triggered 70-80 % growth inhibition; a response rate similar to Trastuzumab. With 87 % growth inhibition, BH1 was far stronger than BH2, BH5, BH6, BH7 and Trastuzumab in the presence of TNF- α .

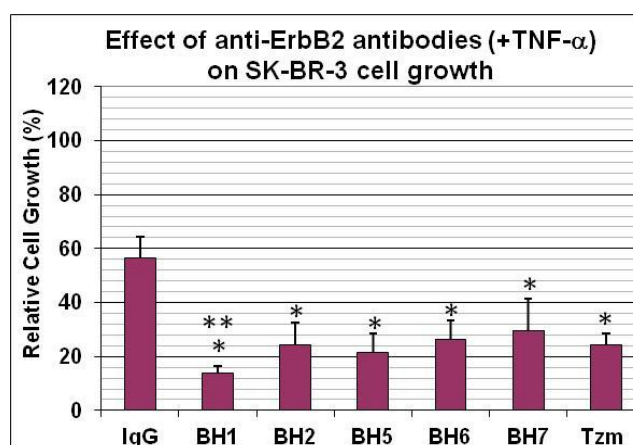


Figure 4.23 Effects of BH-antibodies together with TNF- α on SK-BR-3 cell growth. SK-BR-3 cells were plated onto 96-well plates and incubated at 37°C with 5% CO₂. After 2 hours of incubation, antibodies (5 μ g/ml) were added, and cells were incubated for 4 hours prior to 1000 Unit/ml TNF- α addition. After 6 days of incubation, the percentages of surviving cells to controls, defined as 100% survival, were determined by Sulforhodamine B assay. Each antibody was tested in triplicates in more than ten independent experiments and the average values are represented as SK-BR-3 relative cell growth (%). T Bars, SD. *, decrease in relative cell growth wrt IgG isotype control treatment in the presence of TNF- α ($p < 0.05$). **, decrease in relative cell growth wrt Trastuzumab (Tzm) treatment in the presence of TNF- α ($p < 0.05$).

We tested increasing concentrations of BH-antibodies in the presence of 1000 Unit/ml TNF- α to find the lowest antibody concentrations causing significant TNF- α sensitization of SK-BR-3 cells (**Figure 4.24**). Under these conditions, as little as 0.25 μ g/ml BH-antibodies were sufficient to significantly enhance TNF- α -mediated growth inhibition ($p < 0.005$). Only one antibody, namely BH7, displayed a slightly weaker enhancer activity, reaching significance at 1.25 μ g/ml concentration ($p < 0.02$). Enhancer effects of BH-antibodies were nearly saturated at 2.5 μ g/ml concentration ($p < 0.0007$), reaching to $>80\%$ growth inhibition. These effects were specific to BH-antibodies, since IgG1 isotype control antibody did not modify TNF- α response.

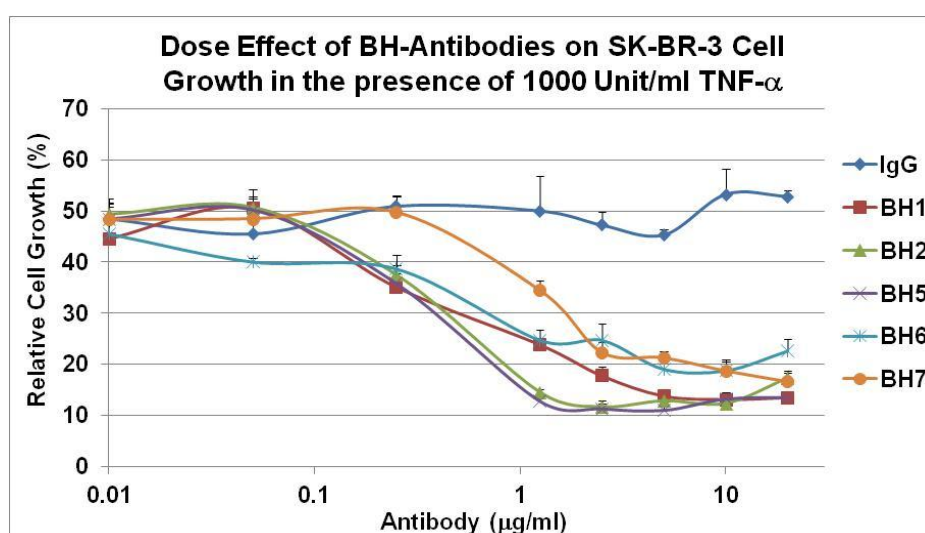


Figure 4.24 Dose effect of BH-antibodies on SK-BR-3 cell growth in the presence of 1000 Unit/ml TNF- α . SK-BR-3 cells were treated with increasing amounts of (0.01-20 μ g/ml) BH-antibodies or IgG1 isotype control antibody (IgG) in the presence of 1000 Unit/ml TNF- α as explained in **Figure 4.23**. Each condition was tested in triplicates and the average values are represented as relative cell growth (%). T Bars, SD.

Next, increasing concentrations of TNF- α were tested in the presence of 5 μ g/ml anti-ERBB2 antibodies to find the minimal TNF- α concentration that potentiates the growth inhibition of BH-antibodies (**Figure 4.25**). The lowest dose of TNF- α used in these experiments (62.5 U/ml) produced $27 \pm 5\%$ growth inhibition. Increasing doses of TNF- α resulted in a limited increase of growth inhibition reaching to only $46 \pm 2\%$ with the highest dose of TNF- α used in the experiments (4000 U/ml). When we repeated TNF- α treatments in the presence of 5 μ g/ml IgG1

isotype control antibody, cellular response to TNF- α was not affected. In contrast, all five BH-antibodies (5 μ g/ml) dramatically enhanced TNF- α -induced growth inhibition. Combination of 5 μ g/ml BH1, BH2, BH5 or BH7 antibodies with 62.5 U/ml TNF- α allowed more than 70% growth inhibition ($p < 0.005$). While BH6 had the highest growth inhibition effect on SK-BR-3 cells when used alone, enhancement of its growth inhibition by TNF- α was slightly lower than for other BH-antibodies; leading to 60% growth inhibition in the presence of 62.5 U/ml TNF- α ($p < 0.005$). At the highest TNF- α dose used (4000 U/ml); the presence of BH-antibodies increased the growth inhibition up to 85% ($p < 0.005$).

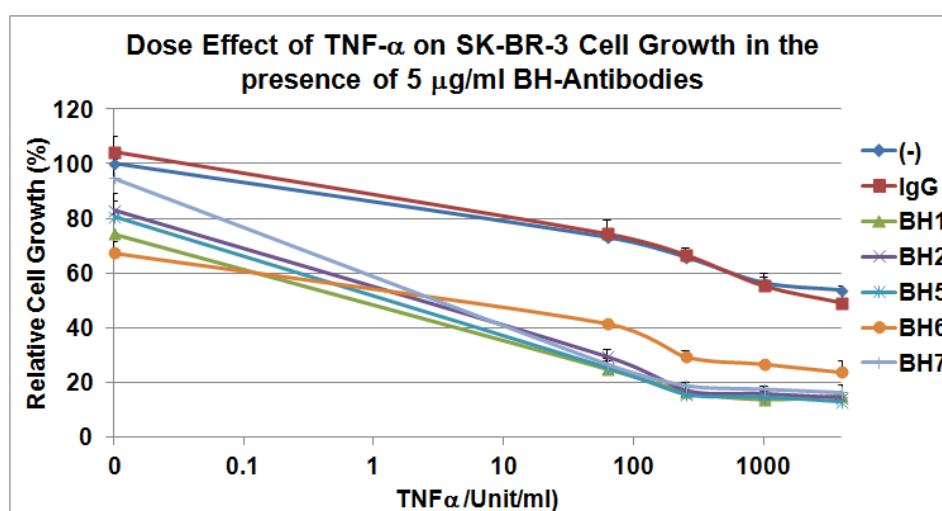


Figure 4.25 Dose effect of TNF- α on SK-BR-3 cell growth in the presence of 5 μ g/ml BH-antibodies. SK-BR-3 cells were treated with BH-antibodies or IgG1 isotype control antibody (IgG) (5 μ g/ml) in the presence of increasing TNF- α concentrations (0-4000 Unit/ml) as explained in **Figure 4.23**. Each condition was tested in triplicates and the average values are represented as relative cell growth (%). T Bars, SD.

A panel of other breast cancer cell lines with different levels of ERBB2 expression were also tested for their sensitivity to 5 μ g/ml BH-antibodies in the presence of 1000 Unit/ml TNF- α (**Figure 4.26**).

BT-474 cells which express high levels of ERBB2 displayed partial TNF- α resistance similar to SK-BR-3 cells. TNF- α had 60% growth inhibitory effect on BT-474 cells when used alone, and Trastuzumab sensitized the cells to TNF- α . On the contrary, while BH-antibodies increased TNF- α sensitivity on SK-BR-3 cells (**Figure 4.23**), they displayed an antagonistic effect on BT-474 cells; reaching to

total suppression of TNF- α -induced growth inhibition when used in combination with BH-antibodies (**Figure 4.26**).

TNF- α was almost totally toxic for MDA-MB-361 cells; causing 96% decrease in the relative cell density when used alone. This drastic TNF- α induced growth inhibition/toxicity was slightly decreased by addition of BH1, BH2, BH5 and BH7 to the regimen (95-92% growth inhibition). Trastuzumab further increased the TNF- α induced growth inhibition to 98% (**Figure 4.26**).

Previously, T47D cells were shown to be irresponsive to BH-antibodies (**Figure 4.22**); 1000 Unit/ml TNF- α alone or in the presence of BH-antibodies did not cause any significant growth inhibition either (**Figure 4.26**). BH1 and BH7 caused modest but significant increase in T47D relative cell growth under these conditions.

TNF- α at this concentration displayed stronger toxicity on MCF-7 cells than it displayed on MDA-MB-361 cells. While no anti-ERBB2 antibody related effect was expected on this low ERBB2-expressor, it was not possible to observe any potential additive/synergic interaction of BH-antibodies with TNF- α , due to the complete TNF- α toxicity. On the contrary to MDA-MB-361 cells, anti-ERBB2 antibodies had no suppressive effect on TNF- α toxicity; therefore it may be inferred that BH-antibodies don't function antagonistic to TNF- α on MCF-7 background under tested conditions (**Figure 4.26**).

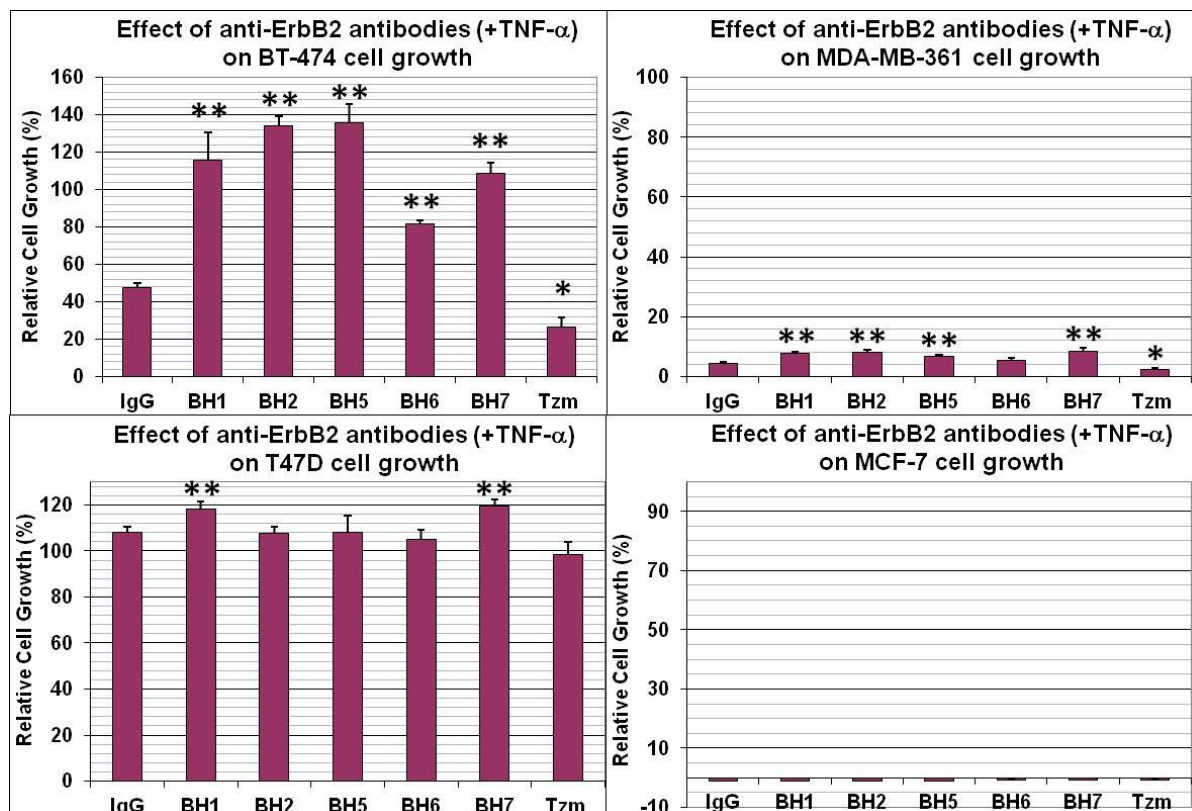


Figure 4.26 Effects of BH-antibodies together with TNF- α on breast cancer cell panel with different levels of ERBB2 expression. Breast cancer cell lines expressing high (MDA-MB-361 and BT-474), and low (MCF-7 and T47D) levels of ERBB2 were treated with BH-antibodies as explained in **Figure 4.23**. Each treatment was tested in triplicates and the average values are represented as relative cell growth (%). T Bars, SD. *, decrease in relative cell growth wrt IgG isotype control treatment in the presence of TNF- α ($p < 0.05$). **, increase in relative cell growth wrt IgG isotype control treatment in the presence of TNF- α ($p < 0.05$).

4.4.2 Effect of BH-antibodies in Combinatorial Treatments

In order to examine drug interactions between BH-antibodies, we used BH-antibodies in paired combinations and tested their effect on SK-BR-3-growth (**Figure 4.27**). In the absence of TNF- α , combinations of BH1+BH7 and BH2+BH6 were found to be significantly stronger than each of the individual antibodies alone ($p < 0.05$). When pairwise BH-antibody combinations were examined in the presence of 1000 Unit/ml TNF- α , BH7 combinations with BH2, BH5 and BH6 decreased SK-BR-3 cell growth more than any of the participant antibodies alone ($p < 0.05$). In the presence of TNF- α , BH1 was found to be the most potent antibody and none of the BH-antibody combinations could reach the growth suppression attained by BH1 alone (**Figure 4.27**). In order to investigate whether the observed decrease in cell

growth was a simple additive effect due to the increased total antibody concentration, we conducted another experiment. Here, SK-BR-3 growth responses to paired combinations of 2.5 $\mu\text{g/ml}$ BH-antibodies were compared with individual treatments with each BH-antibody at 5 $\mu\text{g/ml}$ concentration. Growth inhibitions obtained with BH2+BH7 and BH6+BH7 combinations in the presence of 1000 Unit/ml TNF- α were significantly higher than individual BH-antibodies used at 5 $\mu\text{g/ml}$ concentration (data not shown).

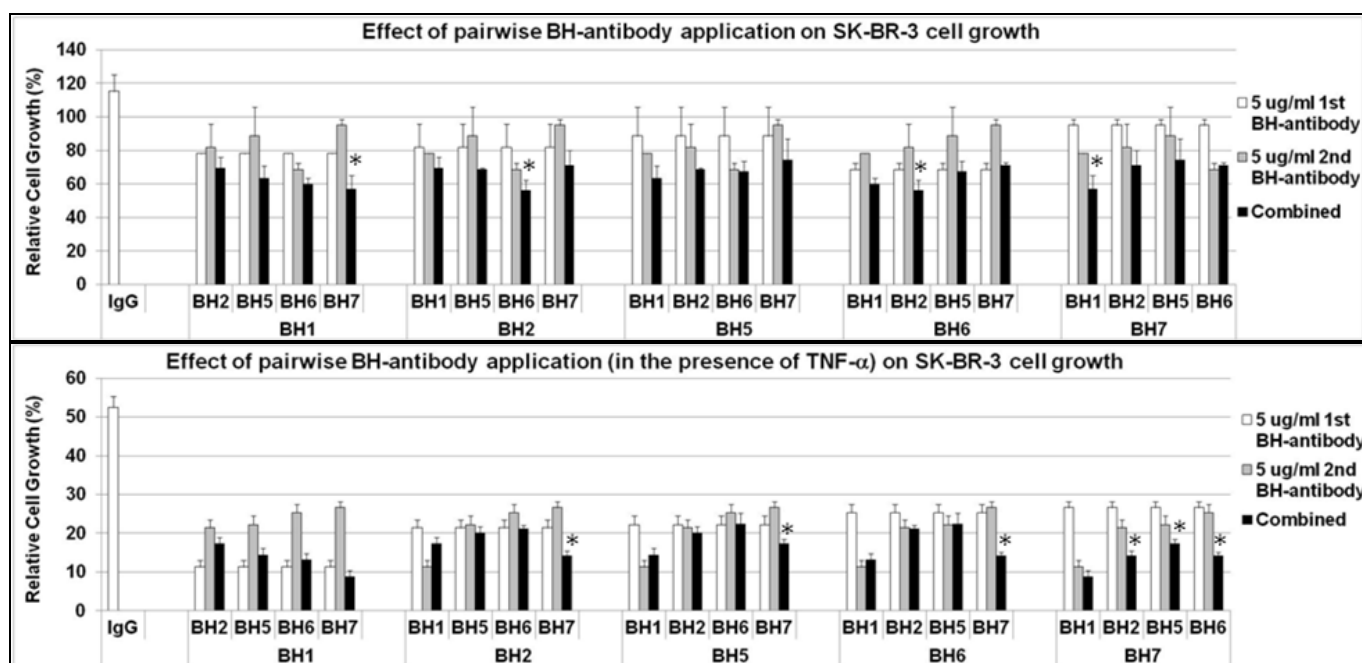


Figure 4.27 Effect of paired BH-antibody combinations on SK-BR-3 cell growth. SK-BR-3 cells were treated with BH-antibodies either alone or in paired combinations. Each individual antibody was used 5 $\mu\text{g/ml}$ per treatment and assays were performed as explained in **Figure 4.23**. IgG1 isotype control antibody treatment was used as negative control. Each condition was tested in triplicates and the average values are represented as relative cell growth (%). T Bars, SD. *, significant increase in the growth inhibitory effect of both agents ($p < 0.05$).

In order to examine the drug interactions between BH-antibodies and Trastuzumab, each of the BH-antibodies was paired with Trastuzumab and their effect on SK-BR-3 cell growth was determined (**Figure 4.28**). For this, the growth of SK-BR-3 cells treated with combinations of equal amounts (2.5 $\mu\text{g/ml}$) of BH antibodies and Trastuzumab were compared with individual antibody treatments. In the absence of TNF- α , Trastuzumab combinations with BH2, BH5 and BH6 were

significantly stronger in SK-BR-3 growth inhibition than individual antibody treatments at equal doses. When the effect of the combined treatments (2.5 µg/ml each) were compared with individual antibody treatments performed at concentrations equal to the total antibody concentration in the combinations (5 µg/ml), BH6+Trastuzumab was still significantly more potent than either of the individual antibodies. When used together with TNF-α, Trastuzumab combinations with BH2 and BH6 were significantly stronger in SK-BR-3 growth inhibition than individual antibody treatments both applied at equal doses (2.5 µg/ml) and twice doses (5 µg/ml). In the presence of TNF-α, BH1 alone was found to be the most potent antibody and neither Trastuzumab or BH-antibodies alone, nor other BH-antibodies combined to Trastuzumab could reach the growth suppression attained by BH1 alone.

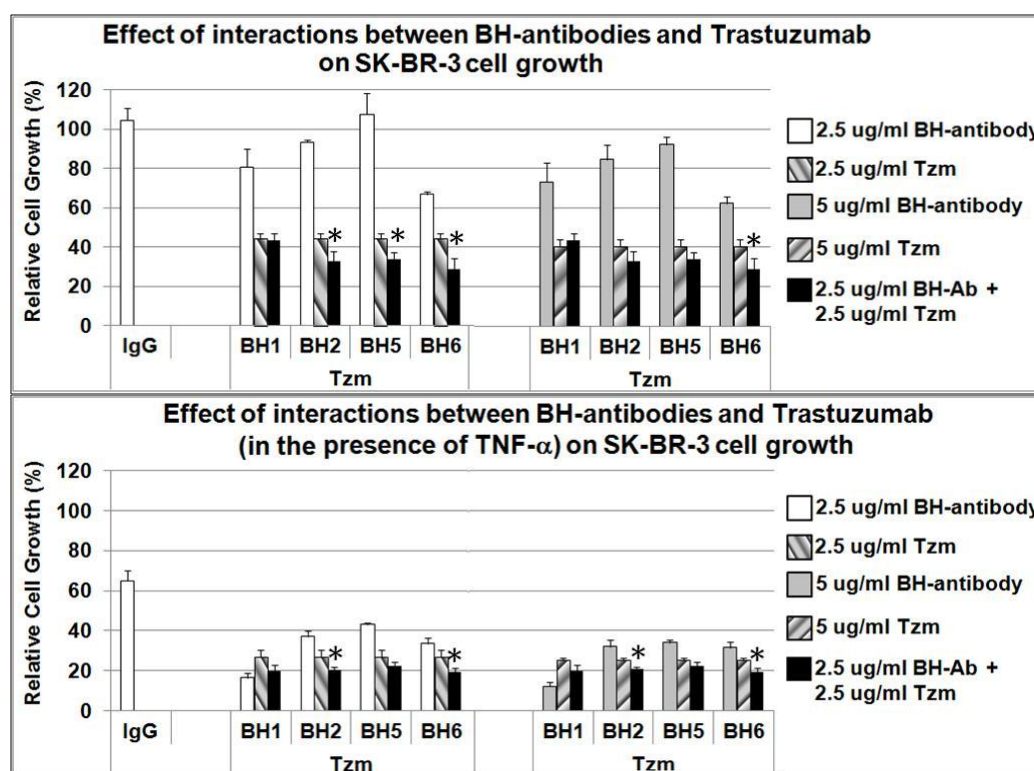


Figure 4.28 Effects of drug interactions between BH-antibodies and Trastuzumab on SK-BR-3 cell growth. SK-BR-3 cells were treated with BH-antibodies and Trastuzumab (Tzm) either alone or in combination and assays were performed as explained in (Figure 4.23). Combined treatments where BH-antibodies and Trastuzumab were both used at 2.5 µg/ml were compared with treatments performed with 2.5 µg/ml or 5 µg/ml individual antibodies. IgG1 isotype control antibody treatment (5 µg/ml) was used as negative control. Each condition was tested in triplicates and the average values are represented as relative cell growth (%). T Bars, SD. *, significant increase in the growth inhibitory effect of both agents ($p < 0.05$).

Previous results suggested that combining BH-antibodies with TNF- α has opposite effects on different cellular backgrounds. In order to investigate drug interactions between BH-antibodies and TNF- α in more details, we have analysed the growth responses of different breast cancer cell lines both according to Bliss model of independence and Loewe model of additivity.

Previously, SK-BR-3, BT-474, MDA-MB-361, T47D and MCF-7 cells were treated with maximal effective doses of anti-ERBB2 antibodies (5 μ g/ml), TNF- α (1000 Unit/ml) and their combination (**Figure 4.29**).

On SK-BR-3 background, TNF- α combinations of all anti-ERBB2 antibodies had significantly stronger growth inhibitory effects than antibody-only and TNF- α only treatments (denoted by “*” on **Figure 4.29**) The suggested synergy between TNF- α and BH-antibodies were confirmed by their Bliss scores significantly lower than 1 (0.35-0.77). However, while Trastuzumab+TNF- α combination had a significantly stronger growth inhibitory effect on SK-BR-3 cells than individual agents, it appeared “independent” on Bliss analysis, with a score of 0.95.

On BT-474 background, BH1, Trastuzumab and TNF- α had significant growth inhibitory effects when used alone. However addition of all BH-antibodies significantly eliminated growth inhibitory effect of TNF- α (denoted by “***” on **Figure 4.29**), suggesting suppression. For BH2, BH5 and BH6, the growth inhibition caused by the antibodies alone were also suppressed when they were used in combination with TNF- α . The suggested antagonism between TNF- α and BH-antibodies was confirmed by their Bliss scores significantly higher than 1 (1.40-3.08). While Trastuzumab+TNF- α had significantly stronger growth inhibitory effect than TNF- α and Trastuzumab alone (denoted by “*” on **Figure 4.29**), its Bliss score (1.21) was higher than 1, indicating an antagonistic interaction.

On MDA-MB-361 background, all BH-antibodies had significant growth inhibitory effects when used alone. In addition, under these conditions, TNF- α was almost completely toxic when used alone. When BH1, BH2, BH5 and BH7 were combined with TNF- α , they caused a modest but significant decrease on TNF- α toxicity (denoted by “***” on **Figure 4.29**). Bliss analysis generating scores higher than 1 (1.88-5.87) indicated antagonistic interactions between TNF- α and all BH-antibodies. While Trastuzumab appeared ineffective on MDA-MB-361 cells when used alone, it significantly increased the growth inhibitory effect of TNF- α (denoted

by “*” on **Figure 4.29**), generating a Bliss score significantly lower than 1 (0.60) indicative of synergy. Though Bliss analysis suggest synergistic or antagonistic interactions between TNF- α and anti-ERBB2 antibodies, a definitive conclusion was not possible due to the high TNF- α toxicity in MDA-MB-361 cells.

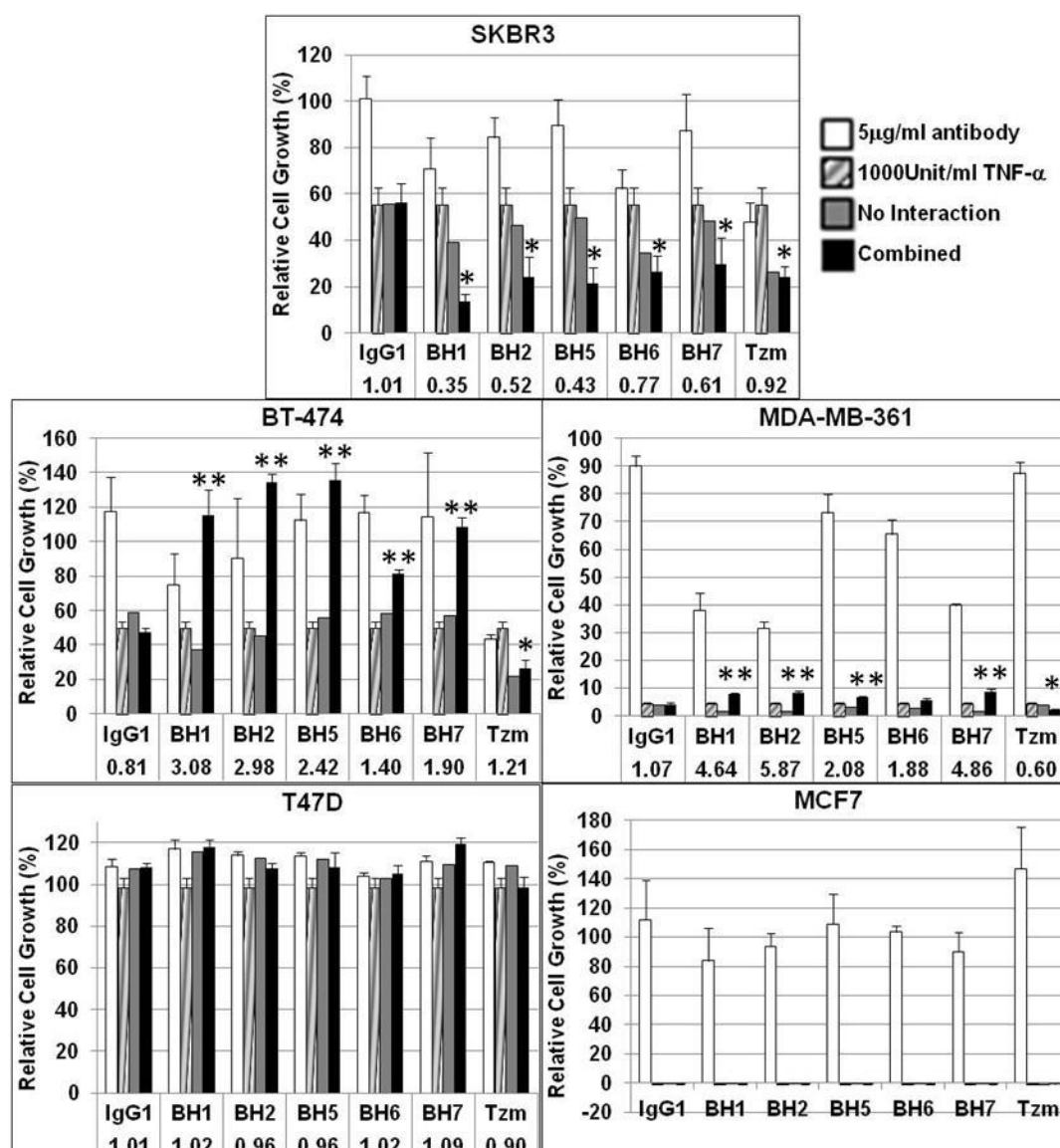


Figure 4.29 Effects of BH-antibodies on SK-BR-3 cell growth. Breast cancer cell lines expressing high (SK-BR-3, BT-474 and MDA-MB-361); and low (MCF-7 and T47D) levels of ERBB2 were treated with 5 μ g/ml anti-ERBB2 antibodies, 1000 Unit/ml TNF- α or their combination and assayed as explained in **Figure 4.23**. Each treatment was tested in triplicates and the average values are represented as relative cell growth (%). T Bars, SD. *, significant increase in the growth inhibitory effect of both agents, when they are used in combination ($p < 0.05$). **, significant decrease in the growth inhibitory effect of the most potent agent ($p < 0.05$). Bliss scores of each drug combination are shown below each treatments. Hypothetical growth inhibition values that would have been observed if tested drugs behaved independently are shown as “no interaction” bars.

Neither anti-ERBB2 antibodies, nor TNF- α had any growth inhibitory effect on T47D cells. Bliss scores close to 1 confirm their independence under these conditions (**Figure 4.29**).

Low ERBB2-expressing cell line MCF-7 was totally irresponsive to all of the anti-ERBB2 antibodies tested. On the contrary, TNF- α had a totally toxic effect on these cells when applied at a concentration of 1000 Unit/ml, therefore it was not possible to analyse drug interactions with anti-ERBB2 antibodies under tested conditions.

In order to investigate drug interactions in a detailed manner, we have concentrated on one of the most potent BH-antibodies; BH1. BH1 (0, 0.16, 0.312, 0.625, 1.25, 2.5 and 5 μ g/ml) and TNF- α (0, 8, 16, 32, 62.5, 125, 250 and 500 U/ml) were combined at various doses and their effect on the growth of five selected breast cancer cell lines were analysed (**Figure 4.30**, **Figure 4.31**). In addition to testing growth of each cell line in 42 concentration combinations of BH1 and TNF- α , cell responses to each drug alone at increasing doses were also determined in this setting, which were necessary for the analysis of drug interactions according to Loewe model of additivity. Interactions between BH1 and TNF- α were analysed by both Bliss and Loewe methods and the interaction scores according to each method are given on **Figure 4.30**.

It was previously shown that SK-BR-3 cells were sensitive to BH1 and TNF- α when used alone or in combination (**Figure 4.20**, **Figure 4.23**). Increasing doses of BH1 and TNF- α were found to decelerate the relative SK-BR-3 growth when used alone (**Figure 4.30**, **Figure 4.31**). Addition of BH1 at increasing doses potentiated the growth inhibition observed by TNF- α at every dose tested (**Figure 4.31**). Increasing doses of TNF- α also had a similar effect at every BH1 dose tested, suggesting synergy between BH1 and TNF- α (**Figure 4.31**). Analyses of the data by both Bliss and Loewe methods indicated synergy between BH1 and TNF- α on SK-BR-3 cells with interaction scores lower than 1 (**Figure 4.30**).

It was previously shown that BT-474 cells were sensitive to solo BH1 and TNF- α treatments to a certain extent (**Figure 4.22**, **Figure 4.26**). While their TNF- α sensitivity was found to be increased at increasing doses, sensitivity to BH1 treatment alone at increasing doses was masked by high standard deviation observed (**Figure 4.30**, **Figure 4.31**). Although this prevented calculation of Loewe scores,

strong suppression of TNF- α -mediated growth inhibition observed even at very low BH1 doses (0.16 μ g/ml) indicated significant antagonism strongly correlating with the previous results (**Figure 4.26**) and was confirmed by Bliss score higher than 1 (**Figure 4.30**).

Increasing doses of BH1 and TNF- α decreased the relative MDA-MB-361 growth when used alone (**Figure 4.30, Figure 4.31**). While Bliss scores implied modest antagonism on MDA-MB-361 cells, Loewe analysis indicated a synergic interaction (**Figure 4.30**). Analyses of the relative growth levels in more details (**Figure 4.31 right panel**) suggested that derivation of single interaction scores may be an oversimplification as a TNF- α threshold was observed; above and below which the agents showed opposite interaction patterns. Above TNF- α IC₅₀ concentration (125 Unit/ml), BH1 was found to decrease TNF- α toxicity, suggesting an antagonistic interaction. However below IC₅₀ concentration (125 Unit/ml), BH1 increased TNF- α toxicity, suggesting a synergic interaction.

Although standard deviation values were high for T47D cells, it was clear that these cells were irresponsive to both agents when they were used alone or in combination (**Figure 4.30, Figure 4.31**); therefore Loewe score was not determined for this cell line either. On the other hand, Bliss score higher than 1 indicated antagonistic interaction between BH1 and TNF- α (**Figure 4.30**).

While increasing doses of TNF- α decreased the relative MCF-7 growth when used alone (**Figure 4.30, Figure 4.31**), MCF-7 was irresponsive to BH1 treatment (**Figure 4.30 left panel**), therefore Loewe score was not determined for this cell line. On the other hand, with a Bliss score of 1.07, BH1 and TNF- α were found to act totally independent of each other on MCF-7 background.

BH1 and TNF- α have synergic growth inhibitory effects on SK-BR-3 and MDA-MB-361 cells, they act antagonistic on BT474 cells and independently on MCF-7 cells. T47D cells were irresponsive to both agents. Therefore it can be inferred that there is a cell-dependent interaction between BH1 and TNF- α .

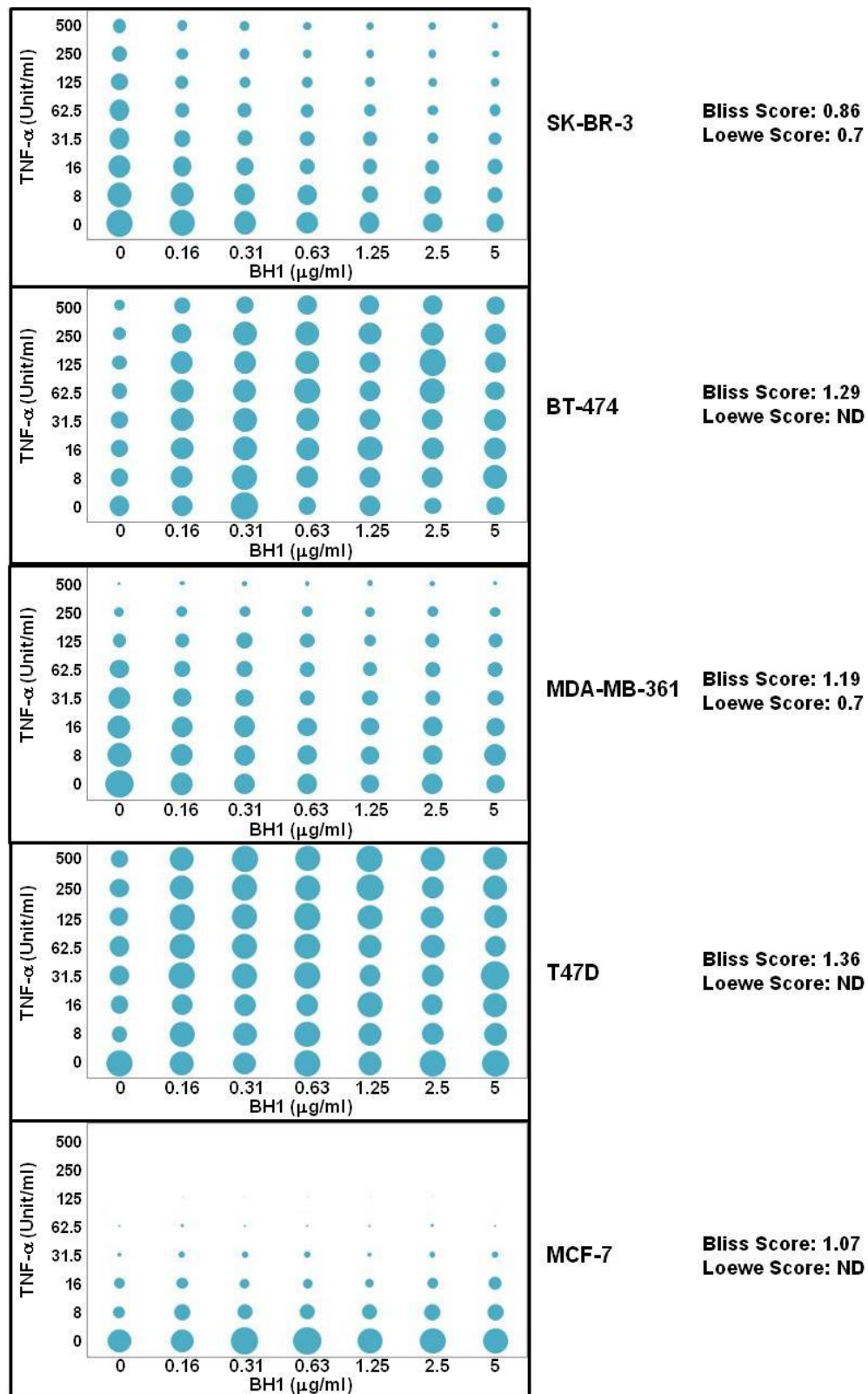


Figure 4.30 Interaction between BH1 and TNF- α on different breast cancer cell lines. SK-BR-3, BT-474, BDA-361, T47D and MCF-7 cells were treated with various combinations of BH1 (0, 0.16, 0.312, 0.625, 1.25, 2.5 and 5 $\mu\text{g/ml}$) and TNF- α (0, 8, 16, 32, 62.5, 125, 250 and 500 U/ml) for 6 days and assayed as previously explained on **Figure 4.23**. Average values representative of relative growth levels at each condition tested in triplicates are shown on the bubble graph. Bliss and Loewe interaction scores between BH1 and TNF- α are shown next to the name of each cell line. ND, not determined.

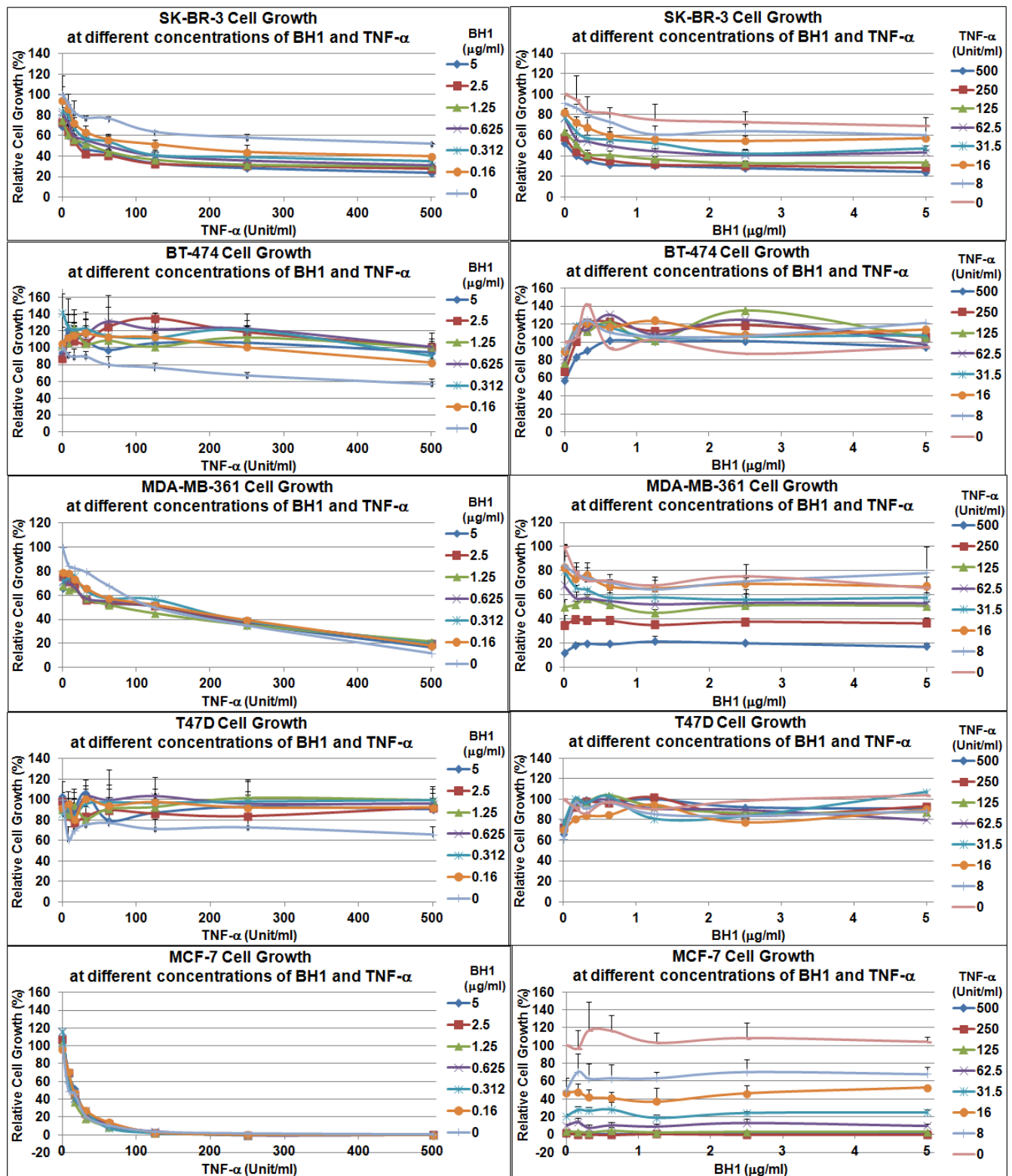


Figure 4.31 Linear analyses of interaction between BH1 and TNF- α on different breast cancer cell lines. SK-BR-3, BT-474, BDA-361, T47D and MCF-7 cells were treated with various combinations of BH1 (0, 0.16, 0.312, 0.625, 1.25, 2.5 and 5 μ g/ml) and TNF- α (0, 8, 16, 32, 62.5, 125, 250 and 500 U/ml) for 6 days and assayed as previously explained on **Figure 4.23**. Each treatment was tested in triplicates and the average values are represented as relative cell growth (%). T Bars, SD. (for visual clarity, T Bars are placed on one of the graphs for each cell line on which the data is displayed less overlapping).

4.4.3 Mechanism of Growth Inhibition Induced by BH-antibodies on SK-BR-3 Cells

BH-antibodies significantly enhanced TNF- α mediated growth inhibition of SK-BR-3 cells even at very low doses (0.25 $\mu\text{g/ml}$, $p < 0.005$), reaching to $>80\%$ growth inhibition at higher doses (2.5 $\mu\text{g/ml}$, $p < 0.0007$) (**Figure 4.24**). In addition, as little as 1.25 $\mu\text{g/ml}$ BH6 and 5 $\mu\text{g/ml}$ BH1 was sufficient to cause significant growth inhibition ($p = 0.039$ and $p = 0.005$ respectively) when used alone in SK-BR-3 cells (**Figure 4.21**). In order to understand the mechanism of these effects, we have analysed BrdU-incorporation, SA β -gal positivity and cell-cycle distribution of the treated cells.

Long-term bromodeoxyuridine (BrdU) incorporation assay allows the detection of cells with permanent withdraw from cell cycle [292, 304]. When asynchronously growing cells are subjected to 24 hour BrdU incorporation assay, proliferating cells undergoing S-phase are labeled positively. Non-proliferating cells remain BrdU negative. Therefore, we tested 24 hour BrdU incorporation of SK-BR-3 cells treated with anti-ERBB2 antibodies or isotype control antibody for 1-6 days.

When used alone, BH-antibodies displayed a modest but significant decrease in DNA-synthesis starting from day 2 ($p < 0.03$, **Figure 4.32**). Following 72 hour treatment with a BrdU chase during the last 24 hours (day 2), cells treated with IgG1 (5 $\mu\text{g/ml}$) control antibody displayed $95 \pm 3\%$ BrdU incorporation, indicating that most of the cells were under proliferative state. Treatment with different anti-ERBB2 antibodies (5 $\mu\text{g/ml}$) resulted in a modest, but statistically significant ($p < 0.03$) inhibition of BrdU incorporation (**Figure 4.32 Day2**). The highest inhibition effect was observed with BH1 antibody, reaching to 45 % decrease in BrdU incorporation on day 6, similar to Trastuzumab (**Figure 4.32 Day6**).

Treatment of cells with TNF- α (1000 U/ml) in association with IgG1 control antibody (5 $\mu\text{g/ml}$) caused progressive reduction in BrdU incorporation; increasing from 32 % inhibition on day 1 to 65 % inhibition on day 6. On the other hand, combination of anti-ERBB2 antibodies with TNF- α significantly accelerated this effect, leading to strong inhibition as early as day 1. The decrease in BrdU incorporation was 75 % on day 1 when TNF- α was combined with BH1 ($p < 0.0005$), even stronger than Trastuzumab which caused 68 % inhibition ($p < 0.005$) (**Figure 4.32 Day1**).

Since BrdU incorporation was progressively and significantly decreased in the cells treated with TNF- α alone, the significance of addition of anti-ERBB2 antibodies started to fade in time. In addition, a modest increase in BrdU incorporation was observed on later days with TNF- α combinations containing anti-ERBB2 antibodies, due to the selection and expansion of resistant clones in time (**Figure 4.32 Days 4-6**).

Taken together, these findings indicated that both anti-ERBB2 antibodies and TNF- α provoked permanent loss of DNA synthesis in a substantial fraction of SK-BR-3 cells. In addition, BH1 was persistently effective in inducing proliferation arrest both in the presence and absence of TNF- α , suggesting a potential for a putative anti-proliferative agent for the treatment of ERBB2-overexpressing cancers (**Figure 4.32**).

In order to understand the role of senescence in the observed proliferation inhibition, we have examined SA β -gal positivity of SK-BR-3 cells treated with 5 μ g/ml BH1, 1000 U/ml TNF- α or with their combination for 6 days (**Figure 4.33**).

No substantial senescence induction was observed on SK-BR-3 cells after 1 or 3 days of BH-antibody treatment (data not shown). When cells were analysed after 6 days, only a limited extent of spontaneous senescence was detected on untreated SK-BR-3 cells, which was modestly increased by 1000 U/ml TNF- α . However, 6 day treatment with 5 μ g/ml BH1 led to significant amount of SA β -gal positivity on SK-BR-3 cells both in the presence and absence of 1000 U/ml TNF- α (**Figure 4.33**). While BH1 treatment significantly increased the fraction of SA β -gal positive cells, its combination with TNF- α had a stronger effect on SK-BR-3 cells; drastically decreasing the colony sizes in addition to increasing the SA β -gal positivity. As seen on SRB (**Figure 4.23**, **Figure 4.24**) and BrdU incorporation assays (**Figure 4.32**), both the total cell amount and the proliferation index considerably decreased on 6th day of BH1+TNF- α treatment. SA β -gal assay additionally revealed that, most of the surviving cells entered senescence (**Figure 4.33**). Trastuzumab-mediated SA β -gal positivity was similar to BH1 both in the absence and presence of TNF- α (**Figure 4.33**).

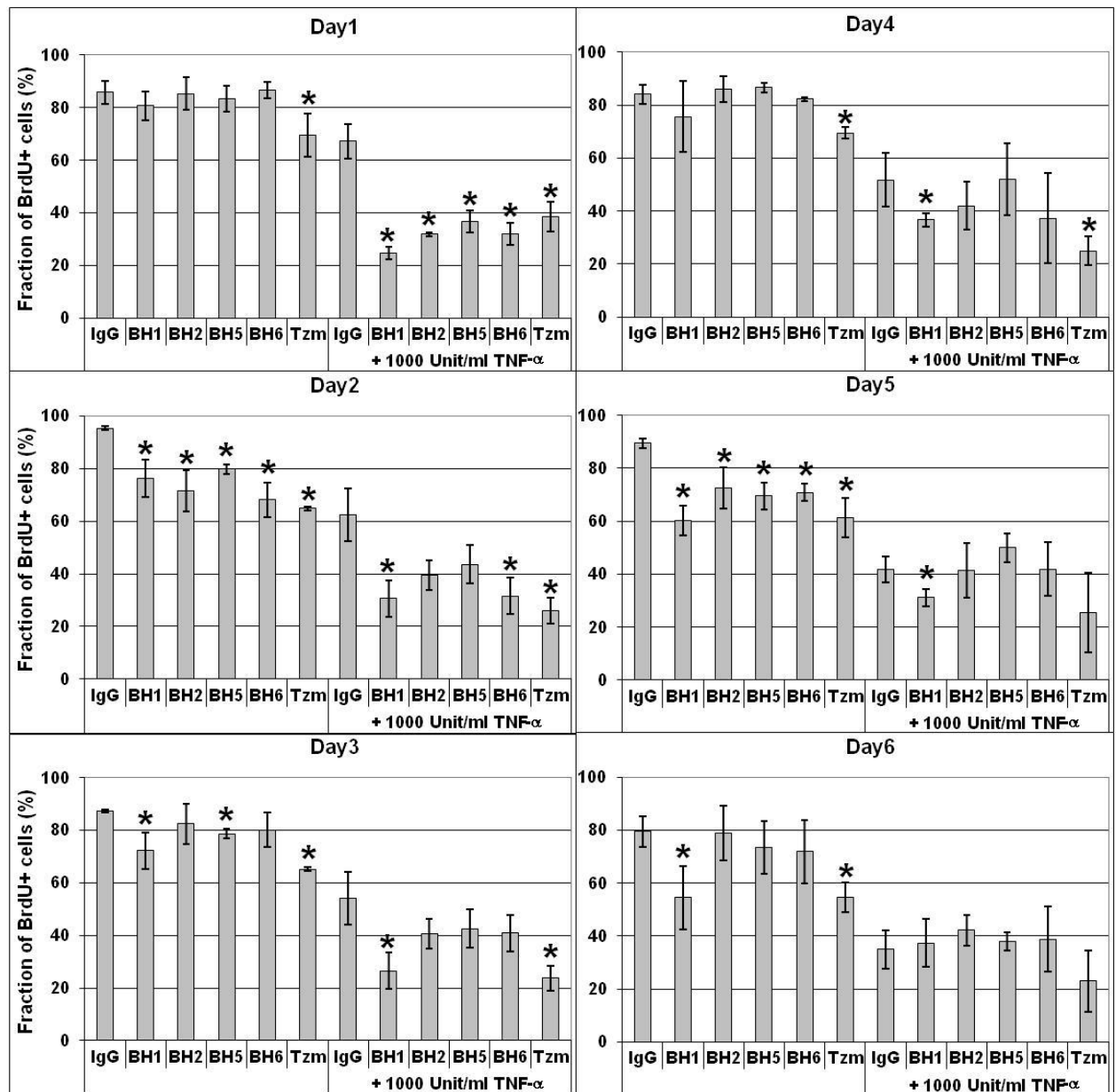


Figure 4.32 Effects of BH-antibodies on SK-BR-3 DNA synthesis in the presence or absence of TNF- α . SK-BR-3 cells plated on coverslips in 12-well dishes were treated with 5 μ g/ml BH-antibodies, Trastuzumab or IgG1 isotype control antibody with or without 1000 U/ml TNF- α . Cells were pulsed with BrdU on day 1-6 following the addition of TNF- α . Following 24 hour BrdU pulse, the cells were fixed and stained by anti-BrdU antibody. Signal detection was made by Alexa488-conjugated anti-mouse IgG antibody. Nuclei were counterstained with DAPI and examined using Zeiss Axio Imager.A1 microscope. Representative photographs (3 frames per coverslip) were acquired. BrdU-incorporation index = (BrdU-positive cells/Total cells) $\times$ 100. *, $p < 0.07$ with respect to IgG or IgG+TNF- α .

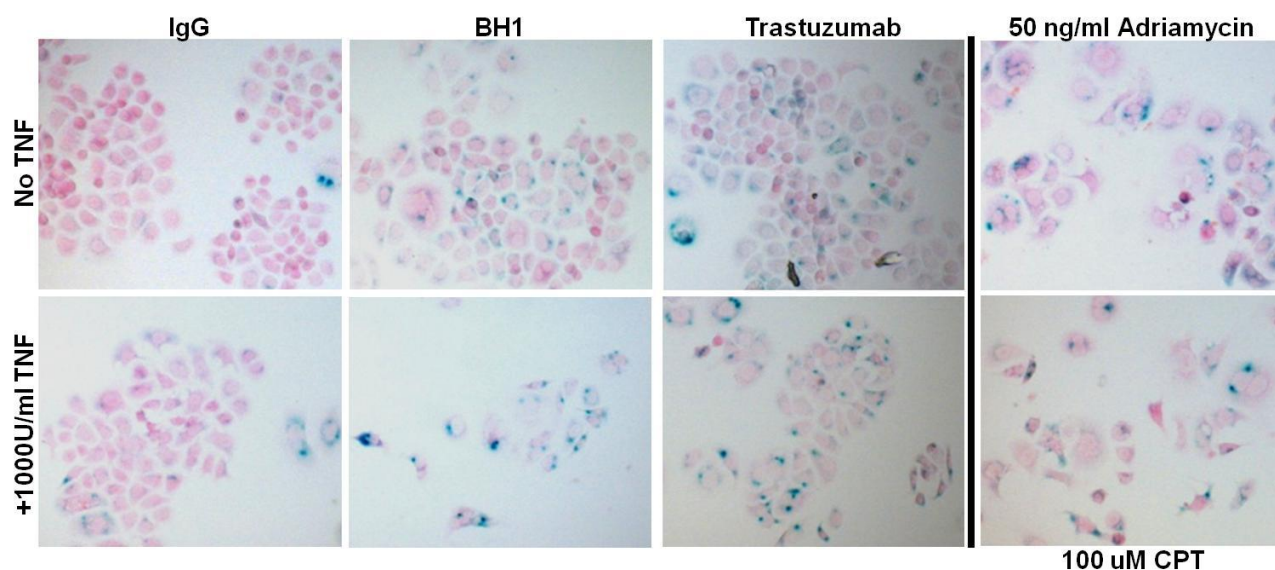


Figure 4.33 Analysis of senescence induction upon BH1 treatment in the presence or absence of TNF- α . SK-BR-3 cells plated on coverslips in 12-well dishes were treated with 5 μ g/ml BH1, Trastuzumab or IgG1 isotype control antibody with or without 1000 U/ml TNF- α . After 6 day treatment, cells were formaldehyde fixed and SA β -gal stained for 12 hours. Nuclei was counterstained with nuclear fast red and and cells were observed under light microscopy. SA β -gal positive cells contain dark blue spots suggesting a senescent phenotype. 100 μ M CPT (camptothecin) and 50 ng/ml Adriamycin were used as positive controls of senescence induction.

In order to investigate the changes occurring in cell cycle distribution upon treatment with anti-ERBB2 antibodies in the absence or presence of TNF- α , we have analysed propidium iodide (PI)-stained 10,000 SK-BR-3 cells by flow cytometry (**Figure 4.34**).

A time-dependent effect was observed on the distribution of cells between G1 (**Figure 4.34 C**) and G2/M-phases (**Figure 4.34 E**) of cell cycle when SK-BR-3 cells were treated with 20 μ g/ml anti-ERBB2 antibodies alone. While the fraction of G2/M-phase cells was increased on day 1 being accompanied with a decrease in the fraction of G1-cells, more pronounced effect of the antibody treatment on day 3 onwards was the significant increase of cells present at G1-phase; suggesting an initial G2/M-arrest followed by G1-arrest (**Figure 4.34 C and E**). In addition, all anti-ERBB2 antibodies caused a significant decrease in the fraction of S-phase cells as early as day 1 (**Figure 4.34 E**). On the other hand, when used in the absence of TNF- α , anti-ERBB2 antibodies only had a modest effect on the fraction of SubG1-cells (**Figure 4.34 A**).

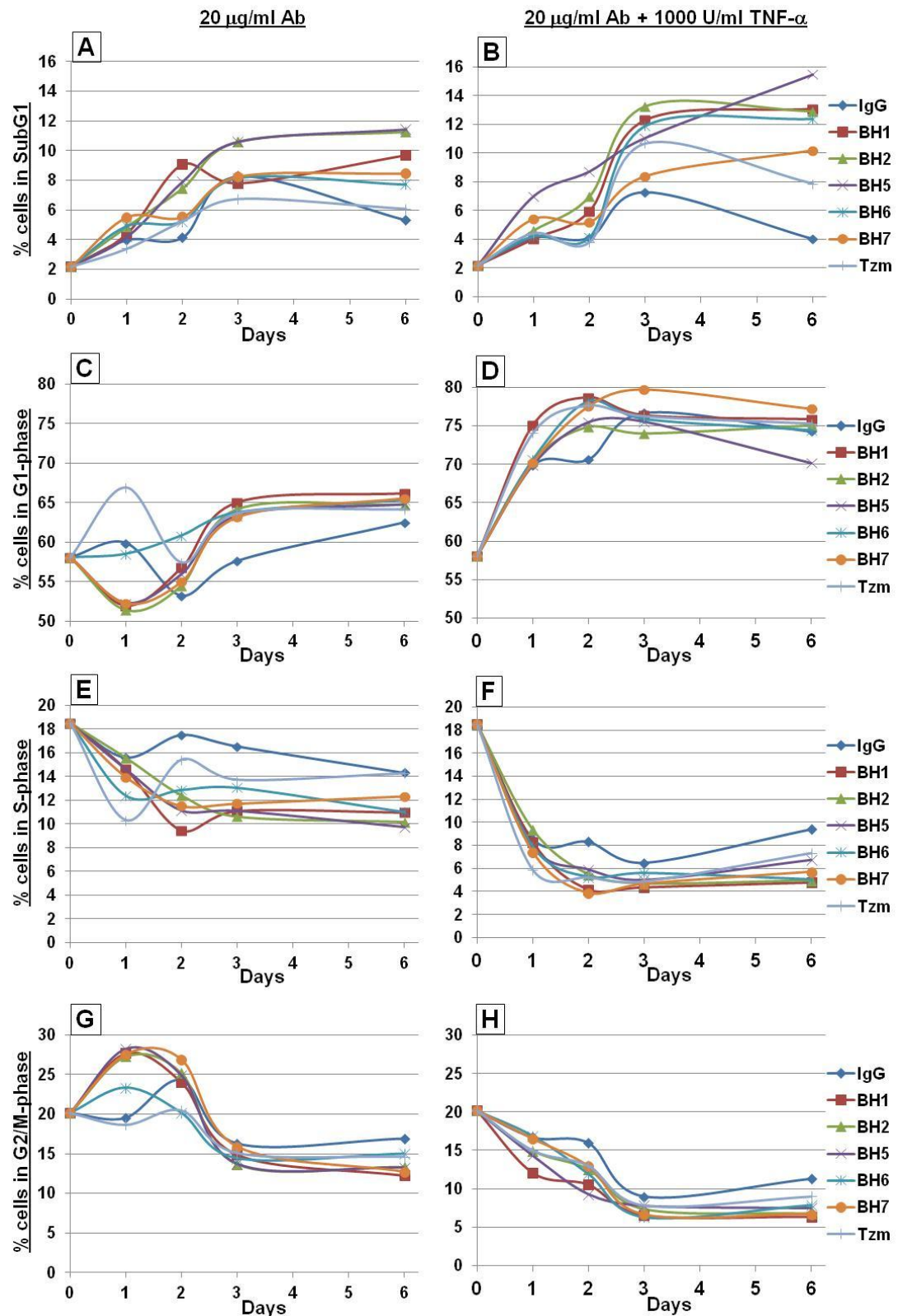


Figure 4.34 Effects of BH-antibodies on SK-BR-3 cell cycle. SK-BR-3 cells plated on coverslips in 12-well dishes were treated with 20 µg/ml BH-antibodies, Trastuzumab (Tzm) or IgG1 isotype control antibody ± 1000 U/ml TNF-α were fixed on day1, day2, day3 or day6. For each condition, propidium iodide stained 10,000 cells were analysed with flow cytometry. Day1 and day2 samples were seeded on the same day at 10^5 cells/well. Day3 and day6 samples were seeded on the same day at 10^5 cells/well and 2.5×10^4 cells/well, respectively.

When SK-BR-3 cells were treated with 1000 U/ml TNF- α , the fraction of cells at G1-phase significantly increased as early as day 1 (**Figure 4.34 D**), accompanied with a decrease in S-phase (**Figure 4.34 F**) and G2/M-phase cells (**Figure 4.34 H**), suggesting G1-arrest. The change in cell cycle distribution induced at day 1 continued throughout the 6 days analysed and was modestly enhanced by the addition of 20 μ g/ml anti-ERBB2 antibodies (**Figure 4.34 D, F, H**). While the addition of anti-ERBB2 antibodies had a modest influence on the fractional change of G1-phase, S-phase and G2/M-phase cells caused by 1000 U/ml TNF- α treatment, they significantly increased the fraction of SubG1-cells in the presence of TNF- α on day 3 onwards, suggesting a role for apoptosis induction (**Figure 4.34 B**).

Since BH1-treatment have decreased both the relative cell growth (**Figure 4.20, Figure 4.21**) and the BrdU incorporation (**Figure 4.32**) of SK-BR-3 cells stronger than the other BH-antibodies, it was selected for further analysis. As the change in cell cycle distribution and BrdU-incorporation of SK-BR-3 cells treated with BH1 $\pm$ TNF- α were analysed together (**Figure 4.35**), the decrease in BrdU incorporation caused by BH1 antibody treatment was associated with a decrease in DNA-synthesis both in the presence and absence of TNF- α (**Figure 4.35 C and E**).

When BH1 was used alone, BrdU incorporation decreased progressively in 6 days (**Figure 4.35 E**). While the decrease in BrdU incorporation was coupled with G2/M-arrest on the first day of treatment (**Figure 4.35 D**), G1-arrest was dominant on day 2 onwards (**Figure 4.35 B**).

Inhibition of BrdU incorporation caused by 1000 U/ml TNF- α was slightly stronger than 5 μ g/ml BH1 treatment alone; both effects increased progressively in time in a parallel manner (**Figure 4.35 E**).

Addition of BH1 modestly enhanced the effects of TNF- α on SK-BR-3 cell cycle distribution. The drastic and immediate decrease observed in BrdU incorporation (that was already saturated on day 1) when BH1 treatment was combined with TNF- α did not have a similarly strong complement in a single phase of the cell cycle. Rather, it appeared that the strong inhibition of BrdU incorporation was the result of the combined changes in all cell cycle phases. On the other hand, BH1 + TNF- α almost doubled the SubG1 fraction of cells on day 3 onwards (**Figure 4.35 A**).

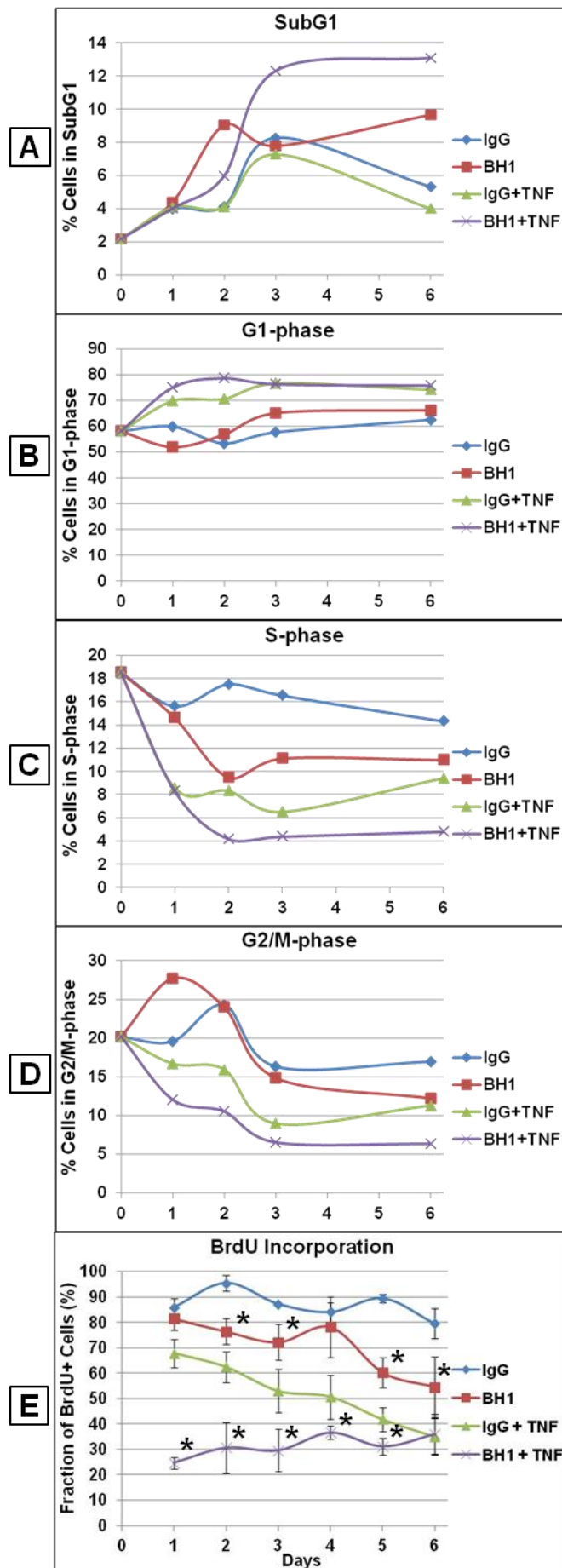


Figure 4.35 Change in cell cycle distribution and BrdU incorporation of SK-BR-3 cells upon BH1 treatment with or without TNF- α . A-D, SK-BR-3 treated with 20 μ g/ml BH1 or IgG1 isotype control antibody $\pm$ 1000 U/ml TNF- α were fixed on day1, day2, day3 or day6. Cell cycle distribution of 10,000 propidium iodide stained cells were analysed with flow cytometry. Day1 and day2 samples were seeded on the same day at 10^5 cells/well. Day3 and day6 samples were seeded on the same day at 10^5 cells/well and 2.5×10^4 cells/well, respectively. E, SK-BR-3 cells treated with 5 μ g/ml BH1 or IgG1 isotype control antibody with or without 1000 U/ml TNF- α were pulsed with BrdU on day 1-6 following the addition of TNF- α . Following 24 hour BrdU pulse, the cells were fixed and stained by anti-BrdU antibody. Signal detection was made by Alexa488-conjugated anti-mouse IgG antibody. Nuclei were counterstained with DAPI and examined using Zeiss Axio Imager.A1 microscope. Representative photographs (3 frames per coverslip) were acquired. BrdU-incorporation index = (BrdU-positive cells/Total cells) $\times$ 100. *, $p < 0.07$ with respect to IgG or IgG+TNF- α .

ERBB2-mediated cell proliferation is documented to be stimulated through two major signal transduction cascades; PI3K-Akt mediated and Ras-Erk mediated pathways. While Raf/MEK/Erk activation induces *Cyclin D1* promoter, increasing Cyclin D1 protein expression, leading to passage through restriction point [305]; PI3K mediated Akt activation is mainly responsible for phosphorylation and degradation of p27, in turn releasing CDK2 which will induce cell cycle progression upon interacting with Cyclin E [306]. Anti-ERBB2 antibodies Trastuzumab and Pertuzumab were shown to inhibit both Akt and Erk1/2 phosphorylation, leading to growth inhibition of cells that overexpress ERBB2 [102, 113, 206, 253].

In order to investigate the effects of BH1-treatment on effector pathways downstream ERBB2, we have treated SK-BR-3 cells with 10 µg/ml BH1 for different periods of time during 24 hours and analysed the changes in the phosphorylation status of ERBB2, Erk1/2 and Akt, in addition to the expression levels of Cyclin D1 (**Figure 4.36**). No significant change was observed in the expression levels of the proteins examined in untreated samples during 24 hours (**Figure 4.36 A**). While active Akt levels decrease starting from the 8th hour of BH1-treatment, there was a sudden increase in the level of phospho Erk1/phospho Erk2, starting as early as 1 hour, showing a partial decrease between 4 and 24 hours of treatment (**Figure 4.36 B**). In parallel with these changes, we also observed progressive accumulation of Cyclin D1 levels detected as early as 4 hours of treatment (**Figure 4.36 B**). BH1-treatment did not induce a significant change in ERBB2 phosphorylation (**Figure 4.36 B**). Co-treatment of SK-BR-3 cells with 10 µg/ml BH1 and 1000 U/ml TNF-α displayed essentially the same type of alterations, suggesting that TNF-α did not modify the effects of BH1 antibody on phospho-Akt, phospho-Erk1/2 and Cyclin D1 in SK-BR-3 cell line (**Figure 4.36 D**). Indeed, treatment of cells with 1000 U/ml TNF-α alone did not affect any of the parameters studied, except inducing a delayed inhibition of Akt phosphorylation at 24 hours (**Figure 4.36 C**).

Cyclin D1 levels in the cell were shown to be regulated in a cell cycle dependent manner. Necessary for the G1-checkpoint, Cyclin D1 remains elevated during G1-phase. Later, its expression is lost at the beginning of DNA-synthesis in S-phase, and induced back at G2-phase [307]. In a review discussing the bi-phasic transition of G0/G1 checkpoint, Jones and Kazlauskas suggested that activated

MEK/Erk which is responsible for Cyclin D elevation is necessary to push the cells from G0 to early-G1, but is insufficient to proceed through late-G1. On the other hand, PI3K that is dispensible for the early-G1 transition, is necessary to drive the cells through late-G1 [308]. In parallel with this review, roles of Raf/MEK/Erk and PI3K/Akt pathways in inducing Cyclin D1 promoter [305, 309, 310] and stabilization of Cyclin D1 protein through Akt-mediated inhibition of GSK-3 β [311] respectively are extensively documented in literature [312].

On the other hand, some groups demonstrated that, Erk phosphorylation is dispensible for the regulation of Cyclin D1 protein level [313], while PI3K is both necessary and sufficient for the induction of Cyclin D1 protein expression [314]. In addition, Wnt1-dependent GSK-3 β inhibition is suggested as a substitute for PI3K/Akt pathway for Cyclin D1 stabilization [315]. Alternatively, Akt was suggested to induce Cyclin D1 stabilization through a totally GSK-3 β -independent mechanism [316].

While required for G1/S transition, Cyclin D1 levels should decline in S-phase to allow DNA synthesis as elevated Cyclin D1 expression was shown to block DNA synthesis upon binding and inactivating PCNA [317]. Cyclin D1 accumulation in nucleus during S-phase was shown to disturb DNA replication, inducing double strand breaks leading to activation of DNA-PK, activation of Akt, in turn triggering a positive feedback loop of Cyclin D1 overexpression through suppression of GSK3 β -mediated Cyclin D1 degradation [318]. In addition, high amounts of Cyclin D1 was detected in senescence cells [319, 320].

Cyclin D1 induction after mitogenic induction is a widely accepted phenomenon driving cells through G1/S transition and is accompanied with active Erk and Akt. On the other hand, the observed increase in Cyclin D1 levels can not be attributed to the renewal of cell culture media at time:0, since the cells were not serum-starved and synchronized prior to treatment; in addition there was no detectable Cyclin D1 induction in SK-BR-3 cells that received fresh media without BH1 antibodies (**Figure 4.36 A**). Therefore the Cyclin D1 increase observed upon BH1-treatment (**Figure 4.36 B and D**) is an accumulation rather than mitogenic induction upon media renewal. In addition, while Cyclin D1 increase was expected to follow Akt activation upon mitogenic induction, the observed Cyclin D1

accumulation observed following BH1-treatment was accompanied with a decrease in the level of phosphorylated Akt, negating the role of media renewal.

In accordance with the literature cited above; decreased cellular growth (**Figure 4.20, Figure 4.21**), decreased BrdU-incorporation (**Figure 4.32, Figure 4.35**), increased SA β -gal staining (**Figure 4.33**), and Cyclin D1 accumulation (**Figure 4.36 B and D**) in addition to the changes in cell cycle distribution of BH1-treated SK-BR-3 cells (**Figure 4.34, Figure 4.35**), suggests a role for BH1-treatment in induction of G0/G1-arrest.

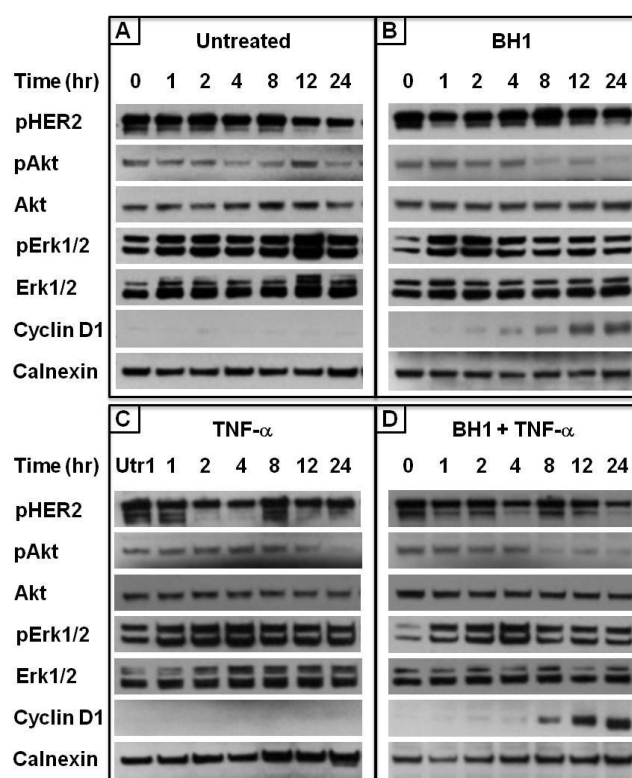


Figure 4.36 Effects of BH1±TNF- α treatment on molecular responses contributing to SK-BR-3 cell survival. SK-BR-3 cells were either untreated (**A**) or treated up to 24 hours with BH1 antibody (10 μ g/ml) (**B**), TNF- α (1000 U/ml) (**C**) or the combination of both agents (**D**). Cell lysates were prepared from cells harvested at indicated times (hr, hours) and expression of phospho-ERBB2 (pERBB2), phospho-Akt (pAkt), total Akt (Akt), phospho-Erk1/phospho-Erk2 (pErk1/2), total Erk1/Erk2 (Erk 1/2) and Cyclin D1 were analysed by western blotting. Calnexin was used as a loading control. Untreated cells were incubated in a parallel experiment in the absence of any treatment. (Utr1, lysates of untreated cells collected after 1 hour.)

5 DISCUSSION AND CONCLUSION

5.1 Novel anti-ERBB2 Antibodies Recognize Conformational Epitope(s) Restricted to Intact ERBB2-ECD

Aiming to develop alternative therapeutics targeting ERBB2 receptor, we have generated five monoclonal antibodies against extracellular region of human ERBB2. The ex-vivo characteristics of these novel antibodies were investigated; significant ERBB2-affinity and anti-proliferative effect was demonstrated.

All BH-antibodies efficiently reacted with human ERBB2 protein. While BH1 and BH6 were the antibodies with highest affinity both for recombinant (**Figure 4.5**) and endogenous ERBB2 (**Figure 4.6, Figure 4.8**), BH2 and BH5 were remarkable in that, when used at different antibody concentrations they produced two distinct BT-474 peaks with different fluorescence intensities (**Figure 4.6**). With no identified ligand, ERBB2 is a receptor known to homo-dimerize upon overexpression, suggesting the presence of different conformations in ERBB2-overexpressing cells [14]. It is possible that BH-antibodies have different affinities for distinct ERBB2 conformations (i.e monomeric or dimeric), and they may interact with the less favoured conformations only when they are supplied in high concentrations. On the other hand, since cancer cells are unstable in nature, it is acknowledged that, despite being clonogenic, they may still contain distinct sub-populations. Therefore, another possibility is that, BH2 and BH5 may selectively interact with distinct BT-474 sub-populations depending on the assay conditions. These sub-populations may diverge from each other in their level of ERBB2 expression, favoring homodimerization; alternatively they may express ERBB2 proteins with altered structure due to acquired genetic modifications. It should be noted that the recombinant ERBB2 protein used for the final booster immunization was a disulfide-linked ERBB2 homodimer which raises the possibility that BH-antibodies might be produced against conformational epitopes formed upon homodimerization. Supporting this possibility, the low affinity BH5 antibody interacted with ERBB2 proteins present on ERBB2-overexpressing BT-474 cells which were expected to contain high amounts of ERBB2 homodimers, but was inefficient in detecting ERBB2 proteins present on T47D cells (**Figure 4.7**).

Another indication for the recognition of conformational epitopes came from IP-western blot analyses. While BH-antibodies successfully pulled-down native ERBB2 (**Figure 4.11**), they failed to detect ERBB2 resolved under denaturing conditions of SDS-PAGE (**Figure 4.10**). The same feature which was also shared by Trastuzumab suggested that they all recognized conformational epitopes. Competition experiments indicated that BH-antibodies did not share the same epitope with Trastuzumab (**Figure 4.12**, **Figure 4.13**, **Figure 4.14**). In order to find which subdomains of ERBB2-ECD were responsible for the construction of each epitope, we aimed to mimic physiological folding and have cloned various ERBB2-constructs where the extracellular subdomains were combined in different combinations (**Figure 4.16**).

There was no significant interaction between BH-antibodies and the products of these artificial *ERBB2* constructs. None of the BH-antibodies recognized individual ECD subdomains when they were cloned alone. Even when they were cloned in pairs, the fluorescent signal detected by BH-antibodies was very faint suggesting that the proper conformation of their epitope(s) may contain amino acids from all four subdomains; absence of any subdomain interfering strongly with the antibody recognition (**Figure 4.17**). On the other hand, even if all the amino acids constructing the epitope(s) are located on a single subdomain, presence of all the other subdomains may still be necessary to build the precise conformation. Trastuzumab recognizes a conformational epitope as well. However, all amino acids participating in its epitope formation were localized on subdomain IV [17] and its presence was sufficient for Trastuzumab recognition as indicated by the positive signals obtained with all truncated ERBB2 products containing subdomain IV (**Figure 4.17**). On the contrary, as also suggested by immunofluorescence results, all BH-antibodies recognize conformational epitopes whose construction necessitate the presence of all four ERBB2 ECD-subdomains.

None of the BH-antibodies could immunoprecipitate ectopically expressed ERBB2 products other than the full length protein (**Figure 4.19**); confirming the immunofluorescence results (**Figure 4.17**) and supporting the necessity of all four subdomains for successful antibody interaction.

Huh-7 cells had low level endogenous ERBB2 expression. While Trastuzumab was able to pull-down endogenous ERBB2 in Huh-7 cells, only BH1

and BH6 managed weak immunoprecipitation (**Figure 4.19**). Though weakly, BH1 and BH6 also detected endogenous ERBB2 present in Huh-7 cells in immunofluorescence assays, unlike other BH-antibodies (**Figure 4.17**). The insensitivity of BH2, BH7 and especially of BH5 for ERBB2 in cells with low ERBB2 expression (**Figure 4.7**, **Figure 4.17**) supports the importance of overexpression-induced ERBB2 conformations for these antibodies.

Covalent modifications, protein-protein interactions and external factors such as ligand concentration, pH and ionic strength induce conformational changes on proteins, influencing their functionality *in vivo*. While certain conformations are needed for the normal cellular activities, other conformations may be responsible for activation of destructive pathways. For this reason, rather than targeting hot spots present in every conformation, recent drug development strategies have narrowed their focus on targeting specific protein conformations and therefore conformational epitopes [321]. For some molecules, antibodies were generated that would specifically target oligomeric forms without interacting with the monomers [322].

Bioactive antibodies that are most effective against ERBB2-overexpressing tumors recognize conformational epitopes located at ECD subdomains involved in either receptor activation [17] or receptor dimerization [99]. Thus, the bioactivity of anti-ERBB2 antibodies may be strongly dependent on both the critical regions they occupy on the receptor and the conformations favoured for antibody recognition. Clarification of whether BH-antibodies recognize conformations present on structurally different ERBB2 proteins or conformations specifically induced upon overexpression needs further investigation. In either case, one of the most important aims of developing cancer therapeutics is to minimize damage caused for healthy cells; and antibodies capable of selective targeting will prove useful in clinic where there is increasing need to limit side-effects.

5.2 Novel anti-ERBB2 Antibodies Perform Growth Inhibitory Effects on ERBB2-overexpressing Breast Cancer Cells and Influenced by TNF- α in a Cell Type Dependent Manner

Novel monoclonal antibodies recognising conformational ERBB2 epitopes hold promise as breast cancer therapeutics. Therefore *ex vivo* effects of novel anti-

ERBB2 antibodies were tested in five different breast cancer cell lines displaying different degrees of *ERBB2* amplification and ERBB2 expression (**Figure 4.8**).

Individually tested novel anti-ERBB2 antibodies (5 µg/ml) significantly decreased the relative growth of SK-BR-3 cells upon 6-day treatment and the strongest growth inhibition was obtained by BH6 and BH1 antibodies which lowered the relative cell growth to 62% and 71% respectively (**Figure 4.20**). When used alone, BH-antibodies were less effective as growth inhibitors, in comparison to Trastuzumab. This was not surprising. Indeed, even Trastuzumab has a modest growth inhibitory effect (56%), and Pertuzumab, the best therapeutic alternative to Trastuzumab, induces only 20% growth inhibition [84].

It has been previously reported that TNF- α resistance caused by the amplified ERBB2 expression [83, 280] was significantly decreased by anti-ERBB2 treatment [84]. In the experimental setting where 5 µg/ml anti-ERBB2 antibodies were combined with 1000 U/ml TNF- α , BH-antibodies caused a substantial decrease in relative SK-BR-3 cell growth. BH2, BH5, BH6 and BH7 had a response rate similar to Trastuzumab in the presence of TNF- α (**Figure 4.23**). With ~85 % growth inhibition in the presence of TNF- α , BH1 was far stronger than other BH-antibodies, Trastuzumab or their paired combinations (**Figure 4.23**, **Figure 4.27**, **Figure 4.28**). In addition, as little as 0.25 µg/ml BH-antibodies were sufficient to significantly enhance TNF- α -mediated growth inhibition ($p < 0.005$). TNF- α is one of the most potent anti-tumor factors when used at high concentrations, but its systematic toxicity hampers its clinical applications [323]. The sensitization of SK-BR-3 cells to TNF- α with anti-ERBB2 antibodies strongly suggests that combination with antibody treatment may help to achieve strong growth inhibition at TNF- α doses as low as 62.5 U/ml (**Figure 4.24**).

Biological activities of BH-antibodies were also tested on other breast cancer cell lines. When used alone, all BH-antibodies tested at 5 µg/ml displayed significant growth inhibition on MDA-MB-361 cells, while trastuzumab was ineffective as a single agent. On the other hand, only BH1 performed a significant growth inhibition on BT-474 cells in comparison to isotype control antibody. As expected, MCF-7 and T47D cell lines with low level ERBB2 expression were irresponsive to anti-ERBB2 treatment under tested conditions (**Figure 4.22**). Initial experiments where 5 µg/ml anti-ERBB2 antibodies were combined with 1000 U/ml TNF- α indicated that, BT-

474 cells displayed a response opposite to SK-BR-3 cells, where addition of BH-antibodies suppressed TNF- α -induced BT-474 growth inhibition (**Figure 4.26**). TNF- α was almost totally toxic for MDA-MB-361 and MCF-7 cells at 1000 U/ml, and addition of BH-antibodies did not alter this toxicity significantly under these conditions (**Figure 4.26**). Growth of T47D cells were unaffected by anti-ERBB2 treatment, TNF- α and their combinations (**Figure 4.26**).

Growth assays where BH-antibodies and TNF- α were tested in single dose combinations suggested that they had opposite modes of interaction on different cellular backgrounds; strongly synergic on SK-BR-3 and antagonistic on BT-474 (**Figure 4.29**). Trastuzumab on the other hand, significantly increased the growth inhibitory effect of TNF- α on both cell lines (**Figure 4.29**). The sensitization of SK-BR-3 cells to TNF- α cytotoxicity by mouse monoclonal antibody 4D5 (from which Trastuzumab has been humanized) was reported previously [84]. Though the Bliss analysis provided scores of 0.92 and 1.21 for their interaction on SK-BR-3 and BT-474 backgrounds respectively, since their combined effect were significantly stronger than either Trastuzumab or TNF- α alone on both cell lines, their mode of interaction were accepted as additive if not synergistic on both cell lines..

While 2% more growth inhibition than the most effective agent is enough to receive a significantly “synergistic” Bliss score when the cell is almost irresponsive to one of the agents (i.e 5 μ g/ml Trastuzumab + 1000 Unit/ml TNF- α on MDA-MB-361 cells; **Figure 4.29**), even 17% more growth inhibition than both of the agents may still yield an “antagonistic” Bliss score when the individual growth inhibitions of both agents are around 60% (i.e 5 μ g/ml Trastuzumab + 1000 Unit/ml TNF- α on BT-474 cells; **Figure 4.29**). In addition, Bliss analysis does not consider “additivity” which is a very important phenomenon of drug interactions [296]. In a review where different methods used for the determination of the drug interactions were compared, Lee et. al. highlighted the major differences between the Loewe additivity model and Bliss independence model; while the former was suggested to be used in mutually exclusive drug settings, the latter was suggested to be used in mutually nonexclusive drug settings [296]. Therefore, we used both Loewe additivity model and Bliss independence model in order to drive conclusions regarding the drug interactions between BH-antibodies and TNF- α on checkerboard assays.

Being the most potent of all five novel anti-ERBB2 antibodies, BH1 was selected to further investigate the drug interactions between BH-antibodies and TNF- α on different breast cancer cell lines in more details. The checkerboard assay results where growth of each cell line was examined on a 7x8 grid of BH1 antibody and TNF- α combinations were analysed by two separate drug interaction analysis methods; Bliss model of independence and Loewe model of additivity (**Figure 4.30**).

According to checkerboard analyses, synergy between BH1 and TNF- α on SK-BR-3 cells was confirmed by both methods. On the contrary, BH1 concentrations as low as 0.16 μ g/ml strongly suppressed TNF- α induced growth inhibition of BT-474 cells, confirming their antagonistic interaction on BT-474 background (**Figure 4.30**).

Since both of the cell lines had high degree of *ERBB2* amplification and *ERBB2* expression, it was interesting to observe opposing drug interactions on SK-BR-3 and BT-474. Gijzen et. al demonstrated opposing effects between anti-ERBB2 treatment and TNF- α on BT-474 model; Trastuzumab mediated decrease of phosphorylated Akt was shown to induce ADAM-mediated activation of TNF- α , creating a negative feedback loop and resulting in anti-ERBB2 resistance [202]. In addition, multiple other pathways participate in the effects observed downstream of *ERBB2* receptor and TNF- α (**Sections 1.2 and 1.5**); and various articles comparing the molecular signatures of breast cancer cell lines displayed numerous differences between the expression profiles of SK-BR-3 and BT-474 [286–289] (**Table 3.4**). Detailed molecular analysis is required to identify the exact players responsible for the opposing modes of interaction observed on SK-BR-3 and BT-474.

While it was not possible to calculate Loewe interaction scores on MCF-7 and T47D backgrounds due to their BH1-insensitivity, Bliss scores indicated independence and antagonism for MCF-7 and T47D respectively (**Figure 4.30**). Although Bliss scores implied modest antagonism on MDA-MB-361 cells, Loewe analysis indicated a synergic interaction. Linear analysis of the effect of BH1 and TNF- α combinations indicated a more complicated interaction; BH1 decreased TNF- α toxicity above IC₅₀ concentration of TNF- α (125 Unit/ml), while increasing its toxicity below IC₅₀ (**Figure 4.31**).

As a conclusion, BH-antibodies induced selective growth inhibition of breast cancer cell lines depending on their level of *ERBB2* expression and cellular background. When used alone, novel anti-ERBB2 antibodies significantly inhibited

the growth of SK-BR-3, BT-474 and MDA-MB-361 cells with ERBB2 overexpression; while no detectable effect was observed on MCF-7 and T47D cells lacking *ERBB2* amplification. When the antibodies were tested in combination with TNF- α , they acted synergistically on SK-BR-3 cells, while they had antagonistic effects on growth of BT-474 cells.

5.3 BH-antibodies Induce G0/G1 Arrest on ERBB2-overexpressing SK-BR-3 Cells

BH-antibodies had a substantial therapeutic effect on ERBB2-overexpressing SK-BR-3 cells ex-vivo, significantly decreasing the total number of cells upon 6 day treatment $\pm$ TNF- α . In order to understand whether they modulate proliferation, senescence and/or apoptosis mechanisms, we have analysed BrdU-incorporation (**Figure 4.32**), SA β -gal positivity (**Figure 4.33**) and cell-cycle distribution of the treated cells (**Figure 4.34**).

When BrdU incorporation was assessed, a modest yet significant inhibition was observed starting from the second day of anti-ERBB2 treatment; strongest response was obtained by BH1 antibody. When BH-antibodies were used in combination with TNF- α , BrdU incorporation was decreased down to 25 % starting from the first day, which was not present in IgG control samples (**Figure 4.32**).

The significance of the combined treatments started to decrease on the following days due to decelerating BrdU incorporation observed by TNF- α alone. In addition, expansion of the resistant clones survived from the devastating initial effect of the combined treatment caused a modest increase in the BrdU incorporation index of combined treatments on later time points. Despite these, inhibitory effect of BH1 + TNF- α was still significantly higher than TNF- α alone until the sixth day of treatment; suggesting limited resistance to BH1 in the presence of TNF- α (**Figure 4.32**). Indeed, microscopic analysis revealed that colony size was dramatically decreased after 6 days BH1 + TNF- α treatment, and the surviving cells were almost totally SA β -gal positive; suggestive of senescence induction (**Figure 4.33**). To summarize, BH1 was the strongest anti-proliferative agent among all BH-antibodies both in the presence and absence of TNF- α .

Anti-ERBB2 treatments were shown to have substantial effects on cell cycle distribution of SK-BR-3 and BT-474 cells. Significant decrease in BrdU incorporation after Trastuzumab and Pertuzumab treatments were accompanied with reduced percentage of S phase cells and increased percentage of G0/G1 cells [324, 325]. Anti-ERBB2 antibodies were also shown to induce G2-arrest [257].

In order to determine the cell cycle phases affected by BH1 treatments and to investigate whether the proliferation inhibition was also accompanied by apoptosis, the cell cycle distribution of the treated SK-BR-3 cells were analysed (**Figure 4.34**). In addition, expression of some of the effector proteins downstream ERBB2 were examined by western blotting (**Figure 4.36**).

BH1 treatment caused a modest decrease in S-phase cells on the first day of treatment; an effect more significant starting from day 2. While the decrease in S-phase was accompanied with an increase in G2/M-phase cells on the initial days of treatment; a significant G0/G1-arrest as well as increase in SubG1 cells was observed on the later days (**Figure 4.35**). In addition to these changes, phospho-Erk1/phospho-Erk2 levels were elevated as early as 1 hour. Although their phosphorylation was slightly decreased between 4 and 24 hours, it still remained higher than the pre-treatment levels. While phospho-Akt levels decreased starting from 8th hour, Cyclin D1 started to accumulate as early as 4 hour (**Figure 4.36**).

TNF- α signaling generates both cell survival and apoptosis signals, depending on the activation of NF κ B transcription factor [326]. Under tested conditions, TNF- α treatment caused a decrease in the fraction of S-phase cells throughout the analysis, together with an increase in G0/G1 fraction. The further increase observed in the fraction of cells at G0/G1-phase on day 6 was accompanied with strong inhibition of BrdU incorporation. No significant apoptosis induction was observed by TNF- α treatment alone (**Figure 4.35**). Of all the molecular effectors analyzed, only phospho-Akt levels were affected by TNF- α treatment; a delayed inhibition of Akt phosphorylation was observed at 24 hours (**Figure 4.36**).

The decrease in the fraction of S-phase cells obtained by BH1 or TNF- α alone was coupled with strong increase in G0/G1 fraction starting from day 1 when the two agents were combined, and accompanied with a drastic inhibition of BrdU incorporation. In addition, strong SA β -gal positivity was obtained on day 6, suggesting induction of senescence. Yet, proliferation inhibition coupled to

senescence induction was not the only route by which they led to decrease in colony size of ERBB2 overexpressing cancer cells. Cell cycle analysis also revealed that BH1-induced apoptosis was significantly substantiated by the addition of TNF- α . Since apoptotic cells do not remain intact in the culture media for long periods, the observed subG1-peaks represent the recent apoptosis instead of reflecting the total amount of cells that has entered apoptosis over time. Therefore samples collected on each day should be evaluated individually and the total apoptotic response should be envisioned additively. Apoptotic response upon BH-antibody $\pm$ TNF α treatment started to get stronger second day onwards, reaching highest levels on the sixth day (**Figure 4.35**). Complementation of TNF- α cytotoxicity suggests BH-antibodies as potential therapeutic agents for TNF- α resistant, ERBB2 overexpressing breast cancer cases. On the other hand, TNF- α did not modify the effects of the BH1 antibody on phospho-Akt, phospho-ERK1/2 and Cyclin D1 in SK-BR-3 cell line, suggesting involvement of other molecular effectors and/or pathways (**Figure 4.36**).

Information gathered on cell cycle distribution of cells analysed by PI-staining depends on the DNA-content of the cells. Since it is technically not feasible to distinguish regular G1-phase cells from the cells arrested at G0 (senescent) and G1 (quiescent), proliferation inhibition and senescence induction effects of a treatment maybe underestimated if only judged by cell cycle analysis. In order to overcome this limitation, both the fraction of non-proliferating cells and the extent of senescence-induction were considered in addition to the cell cycle distribution of the treated cells. Therefore, when accompanied with SA β -gal positivity, inhibition of DNA synthesis (decrease of S-phase cells and BrdU-incorporation) and G0/G1-accumulation strongly suggested senescence instead of a regular G1-arrest.

Signal transduction downstream ERBB2 proceeds mainly through PI3K and MAPK pathways and involves PI3K-dependent activation of Akt and Ras-dependent activation of Erk1/2; leading to cell survival and proliferation. Therapeutically relevant Anti-ERBB2 antibodies were previously shown to exert their effect through inhibition of Akt and Erk1/2 phosphorylations [102, 113, 206, 253]. In addition, Cyclin D1 levels were demonstrated to be elevated throughout the cell cycle except S-phase indicating that its degradation was required for the initiation of DNA synthesis. Therefore, Cyclin D1 accumulation was suggested to block DNA-synthesis and G1-S transfer; inducing G1-arrest [307]. Here we show Cyclin D1

accumulation was coupled to Akt deactivation when SK-BR-3 cells were treated with BH1 antibody either in the presence or absence of TNF- α ; suggesting accumulation of cells at G1 and G2/M phases.

The observations summarized on **Table 5.1** suggest that, the early influence of BH1 treatment was induction of G2/M-arrest, whereas its later effect was more pronounced as G0/G1-arrest and senescence induction. TNF- α on the other hand, appears to cause G0/G1-arrest throughout the treatment period without a significant apoptosis induction; and was accompanied with a modest senescence induction on day 6. Combination of BH1 with TNF- α induced stronger outcomes both macroscopically and on a molecular basis. BH1+TNF- α led to a dramatic G0/G1-arrest as early as day 1; this strong proliferation inhibition was significantly associated with a senescent phenotype and accompanied with apoptosis induction at later time points; suggesting profound multifunctional effects on elimination of ERBB2-overexpressing cells. Further molecular analyses of additional effectors will enable us understand the exact mechanism of these treatments and suggest alternative adjuvant settings in treatment of ERBB2-overexpressing breast cancers.

It should be noted that, stabilization of Cyclin D1 protein through Akt-mediated inhibition of GSK-3 β is extensively documented in literature [312], and on the contrary to our results, Trastuzumab-induced decrease of Akt phosphorylation was suggested to decrease Cyclin D1 levels through GSK-3 β stabilization [113]. In addition, anti-ERBB2 treatment was also documented to increase p27 stability [113] inhibit CDK2 [39] and decrease Cyclin E levels [327]. Examination of these additional molecular effectors will aid in the detailed verification of changes occurring on PI3K and MAPK pathways upon BH-antibody treatment.

Early Effects (24 hr)		BH1	TNF- α	BH1+TNF- α
Cell Cycle Analysis	Apoptosis	~	~	~
	G1	↓ (60% → 52%)	↑ (60% → 70%)	↑↑ (60% → 75%)
	S	~ (16% → 15%)	↓ (16% → 9%)	↓ (16% → 8%)
	G2/M	↑ (20% → 28%)	↓ (20% → 17%)	↓ (20% → 12%)
BrdU Incorporation		~ (86% → 81%)	↓ (86% → 68%)	↓↓↓ (86% → 25%)
Downstream Effectors	p-Akt	↓ (t:8hr)	↓ (t:24hr)	↓ (t:8hr)
	p-Erk	↑ (t:1hr) ↓ (t:4hr)	~	↑ (t:1hr) ↓ (t:4hr)
	Cyclin D1	↑ (t:4hr)	~	↑ (t:4hr)

Late Effects (6 days)		BH1	TNF- α	BH1+TNF- α
Cell Cycle Analysis	Apoptosis	↑↑ (5% → 10%)	~ (5% → 4%)	↑↑ (5% → 13%)
	G1	↑ (62% → 66%)	↑↑ (62% → 74%)	↑↑ (62% → 76%)
	S	↓ (14% → 11%)	↓ (14% → 9%)	↓↓ (14% → 5%)
	G2/M	↓ (17% → 12%)	↓ (17% → 11%)	↓↓ (17% → 6%)
BrdU Incorporation		↓ (79% → 55%)	↓↓ (79% → 35%)	↓↓ (79% → 36%)
SA β -gal positivity		++	+	+++
Colony size		~	↓	↓↓

Table 5.1 Summary of biological activities of BH1, TNF- α and their combination on SK-BR-3 cells. SK-BR-3 cells were treated with BH1 antibody, TNF- α or their combination. Macroscopic and molecular changes observed 24 hours (early effects) or 6 days (late effects) after each treatment are listed.

Besides PI3K and MAPK pathways, multiple other pathways either acting interactively or independently were documented to perform anti-ERBB2 and TNF- α responses of ERBB2-overexpressing cells. While NF κ B induced c-FLIP was shown to inhibit caspase-8-mediated ERBB2 cleavage decreasing TNF- α induced apoptosis [272], Akt inhibition was documented to sensitize these cells to TNF- α induced apoptosis through deactivation of NF κ B [280]. Treatment with anti-ERBB2 agents together with TNF- α was shown to down-regulate Akt-phosphorylation and induce apoptosis through cleavage of caspase-8, caspase-3 and poly(ADP)ribose polymerase (PARP) [328]. The advances in molecular biology rapidly unravel the resistance mechanisms faced in clinics during cancer therapy. Therefore, analysis of signalling pathways other than PI3K and MAPK pathways are also necessary to derive a more definitive conclusion regarding the exact mechanism of individual and combined treatments.

As a conclusion, we have developed five novel anti-ERBB2 antibodies, all reacting with conformational epitopes formed by the intact extracellular region of

native human ERBB2 protein. Although we do not yet know whether our antibodies recognize identical, overlapping or independent epitopes on the intact ECD domain, it is evident that their epitope(s) is different from those recognized by Trastuzumab and Pertuzumab. Antibodies directed to ECD epitope(s) different from other anti-ERBB2 therapeutic antibodies may serve as new analytical tools to study the structural organization of ERBB2 at the cell surface that may be present as monomers, homodimers and heterodimers depending on cell type and extracellular environment such as the availability of ligands that activate heterodimer partners of ERBB2. Dynamic changes at the receptor ECD in relation with transcriptional, translational and post-translational modifications can be monitored with these antibodies, both biochemically and biologically. In addition, regarding their growth inhibitory, senescence and apoptosis inducing effects, the novel antibodies may be potent anti-cancer agents. BH1, followed by BH6 stood out being the strongest anti-proliferative agents. Although BH2, BH5 and BH7 were weaker anti-proliferative agents, they reacted with ERBB2 epitopes selectively present on ERBB2-overexpressing cells which would be quite useful in clinic where there is increasing need to limit side-effects on healthy cells. Cell-dependent synergy and antagonism between anti-ERBB2 antibodies and TNF- α provide evidence for a complex interplay between ERBB2 and TNF- α signaling pathways. Such complexity may drastically affect the outcome of ERBB2-directed therapeutic interventions.

6 FUTURE PERSPECTIVES

Anti-proliferative effects of BH-antibodies suggest that it would be feasible to investigate these antibodies further as potential alternative therapeutics for ERBB2-overexpressing cancer therapy. Yet their efficiency should be validated *in vivo*; their exact action mechanism and individual epitopes preferred by each novel anti-ERBB2 antibody should be revealed through additional investigations; and any possible resistance should be determined.

As preliminary results suggest that some of these novel antibodies may be interacting with ERBB2 epitopes selectively present on dimerized receptors, it is judicious to investigate the affinities of each antibody for monomeric, homo- and heterodimeric ERBB2 molecules and complexes. Chemical cross-linkers such as bis(sulfosuccinimidyl) suberate selectively stabilize oligomeric complexes present in the cellular milieu, especially on the cell membrane. Analysis of the interaction between the antibodies and the dimerized receptor complexes stabilized by chemical crosslinking would enable detection of the preferred conformations by each antibody. A thorough MALDI-TOF mass spectrometric analysis may further be used for detailed epitope mapping [329]. It is suggested that antireceptor therapy by some monoclonal antibodies may affect oligomerization status [330], and internalization of ERBB2 receptors [258]. In this regard, exploration of the changes in cellular ERBB2 dimerization and membrane localization statuses upon treatment would provide invaluable information on therapeutic activity of these novel anti-ERBB2 antibodies.

Novel antibodies demonstrated cell-dependent synergy and antagonism between with TNF- α . Therapeutic potentials and selective targeting efficiencies of the BH-antibody treatments in presence and absence of TNF- α need to be validated on heterogeneous cell populations both *in vitro* and *in vivo*. Co-cultures of differentially labelled normal human mammary epithelial cells (HMECs) and ERBB2-overexpressing breast cancer cell lines would be useful to mimic the tumorigenic breast tissue. In addition, the anti-proliferative effects, effective dose ranges, potential side-effects and the tissue disposition need to be verified *in vivo* using mice xenografts. Furthermore, it will be useful to examine whether ADCC

induction is one of the mechanisms by which the novel anti-ERBB2 antibodies eliminate tumor cells *in vivo*.

The detailed analysis of downstream pathways activated by BH-antibodies will unveil the precise mechanism of their action and enable us to place them in the correct therapeutic combination according to the molecular signature of the tumor to be treated. Therefore, the changes in expression and phosphorylation levels and the localization of effector proteins in PI3K and MAPK pathways such as PI3K, Src, PTEN, GSK3 β , p70S6K, p27, CDK2, Cyclin E; and effector proteins in NF κ B pathway such as NF κ B, c-FLIP, caspase-8, caspase-3, PARP should be examined upon BH-antibody treatment both in the presence and absence of TNF- α .

Examination of downstream effectors on different cell lines will also reveal the mechanism of the opposite responses observed on SK-BR-3 and BT-474 cells upon BH-antibody treatment in combination with TNF- α .

It is long been known that, upto 88 % of the patients developed resistance to Trastuzumab therapy depending on the stage of the disease and the treatment regime. Numerous mechanisms participating in resistance development were revealed as explained above. Some of the resistance was due to the mutations or deletions of the effector molecules downstream of antibody binding; such as activating PI3K mutations, Cyclin E overexpression and PTEN deletion. However, other mechanisms inducing resistance could be by-passed through targeting different ERBB2 epitopes. For instance, it is possible to evade destructive effects caused by overexpression of other Her-family receptors (i.e. EGFR) and/or their ligands (i.e. EGF, TGF- α , heregulins) or other receptors types (i.e. IGF-1R) by preventing heterodimerization of ERBB2. Therefore growth inhibitory effects of BH-antibodies both in the presence and absence of TNF- α should be validated on cells overexpressing these additional factors. Part of the Trastuzumab resistance was documented to be caused by prevention of antibody binding either through blockage by MUC4 or by receptor shedding. Therefore, the effect of BH-antibody binding on prevention of receptor shedding should be studied. In addition, efficiency of BH-antibody antibody treatment should be examined on MUC4-overexpressing JIMT-1 cell line. It is also necessary to perform long-term BH-antibody treatments *in vitro*; in order to investigate potential resistance development.

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Novel anti-HER2 monoclonal antibodies: synergy and antagonism with tumor necrosis factor- α

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Abstract

Background

One-third of breast cancers display amplifications of the *ERBB2* gene encoding the HER2 kinase receptor. Trastuzumab, a humanized antibody directed against an epitope on subdomain IV of the extracellular domain of HER2 is used for therapy of HER2-overexpressing mammary tumors. However, many tumors are either natively resistant or acquire resistance against Trastuzumab. Antibodies directed to different epitopes on the extracellular domain of HER2 are promising candidates for replacement or combinatorial therapy. For example, Pertuzumab that binds to

subdomain II of HER2 extracellular domain and inhibits receptor dimerization is under clinical trial. Alternative antibodies directed to novel HER2 epitopes may serve as additional tools for breast cancer therapy. Our aim was to generate novel anti-HER2 monoclonal antibodies inhibiting the growth of breast cancer cells, either alone or in combination with tumor necrosis factor- α (TNF- α).

Methods

Mice were immunized against SK-BR-3 cells and recombinant HER2 extracellular domain protein to produce monoclonal antibodies. Anti-HER2 antibodies were characterized with breast cancer cell lines using immunofluorescence, flow cytometry, immunoprecipitation, western blot techniques. Antibody epitopes were localized using plasmids encoding recombinant HER2 protein variants. Antibodies, either alone or in combination with TNF- α , were tested for their effects on breast cancer cell proliferation.

Results

We produced five new anti-HER2 monoclonal antibodies, all directed against conformational epitope or epitopes restricted to the native form of the extracellular domain. When tested alone, some antibodies inhibited modestly but significantly the growth of SK-BR-3, BT-474 and MDA-MB-361 cells displaying *ERBB2* amplification. They had no detectable effect on MCF-7 and T47D cells lacking *ERBB2* amplification. When tested in combination with TNF- α , antibodies acted synergistically on SK-BR-3 cells, but antagonistically on BT-474 cells. A representative anti-HER2 antibody inhibited Akt and ERK1/2 phosphorylation leading to cyclin D1 accumulation and growth arrest in SK-BR-3 cells, independently from TNF- α .

Conclusions

Novel antibodies against extracellular domain of HER2 may serve as potent anti-cancer bioactive molecules. Cell-dependent synergy and antagonism between anti-HER2 antibodies and TNF- α provide evidence for a complex interplay between HER2 and TNF- α signaling pathways. Such complexity may drastically affect the outcome of HER2-directed therapeutic interventions.

Keywords

HER2, ERBB2, TNF- α , Monoclonal Antibodies, Epitope Mapping, Growth Inhibition, Breast Cancer, Synergy, Antagonism

Background

With a lifetime risk of ~12%, breast cancer is the most common cancer in women [1]. Although the survival rates of breast cancer patients are increasing due to the recently developed therapeutic approaches, breast cancer remains the most frequent cause of cancer-related death in women [2]. More than 225,000 new cases of female-

breast cancer are estimated to occur in 2012, in the United States alone, and 40,000 female deaths are expected as a result [3]. 25-30% of breast cancers display HER2 overexpression [4]. *ERBB2* gene amplification that was discovered in the early 1980s is the main cause of HER2 overexpression [5]. HER2 is a tyrosine kinase receptor belonging to the ErbB/HER family that also includes epidermal growth factor receptor (EGFR/HER1), ErbB3/HER3 and ErbB4/HER4. HER2 displays tyrosine kinase activity, but has no known ligand. ErbB family receptors are activated by homodimerization or heterodimerization. HER2 is activated either by ligand-independent homodimerization when it is overexpressed, or by heterodimerization with a ligand-dependent ErbB/HER member, in particular with ErbB3/HER3 [6].

Following the discovery of *ERBB2* gene amplifications in breast cancer, the HER2 receptor became an attractive target for monoclonal antibody-based therapies. Trastuzumab is the first clinically approved humanized monoclonal antibody targeting HER2. It produced 11-26% overall response rates as monotherapy for metastatic breast cancer. In addition, as adjuvant to chemotherapy, Trastuzumab significantly increased disease-free and overall survival in both early stage and metastatic breast cancers. Trastuzumab is effective only in HER2-amplified/overexpressing tumors and primary resistance to therapy is a challenge [7]. Moreover, the majority of metastatic breast cancer patients who initially respond to Trastuzumab begin to demonstrate disease progression again within 1 year [8]. Trastuzumab resistance was linked to many factors, including the overexpression of other ErbB/HER family receptors and/or their ligands [9,10], insulin-like growth factor receptors [11,12], MUC4 [13] and soluble extracellular domain (ECD) of HER2 circulating in the blood [14,15], activating PI3K mutations [16], PTEN deletions [17], downregulation of cyclin-dependent kinase inhibitor p27 [18] and increased Akt activity [19,20].

HER2-HER3 heterodimer is known to be the most potent activated form of HER2 in terms of strength of interaction, ligand-induced tyrosine phosphorylation and downstream signaling. Indeed, HER3 might be a necessary partner for HER2-mediated oncogenic activity in HER2-overexpressing tumors [6]. The ECD of HER2 is composed of four subdomains and Trastuzumab is directed to its subdomain IV [21]. The antibody inhibits HER2 activation [6], but cannot prevent HER2-HER3 heterodimerization [22]. Pertuzumab is another antibody directed to another epitope that is located at subdomain II, which is involved in receptor dimerization [23]. It appears to be more effective than Trastuzumab, in particular in low HER2-expressing tumor cells because of its ability to interfere with HER2-HER3 heterodimerization [23]. Pertuzumab and Trastuzumab were shown to act synergistically against breast cancer cell survival [24], suggesting that antibodies against different epitopes of the ECD are able to generate not only additive but also synergistic cellular responses. The limited efficacy of Trastuzumab for cancer therapy, problems of acquired resistance and the potential impact of novel anti-HER2 antibodies as therapeutic partners or alternative therapeutics justify more investment on HER2 targeting with novel monoclonal antibodies. Here, we describe five novel monoclonal antibodies directed against an epitope (or epitopes) restricted to the native conformation of HER2 ECD. *In vitro* studies provide evidence for anti-cancer activity of these antibodies. More interestingly, these novel antibodies appear to interact with tumor necrosis factor- α (TNF- α) in a cell-dependent manner, generating both synergistic and antagonistic growth responses.

Methods

Reagents

A disulfide-linked homodimer of histidine-tagged extracellular domain of HER2 fused to human IgG1 Fc domain (HER2 ECD), a similar construct based on EGFR extracellular domain (EGFR ECD), and IgG1 isotype control antibody were from R&D Systems (MN, USA). Alkaline phosphatase (AP)- and horseradish peroxidase (HRP)-conjugated secondary antibodies to mouse and human IgGs, propidium iodide, trichloroacetic acid (TCA), Sulforhodamine B (SRB), Incomplete Freund's Adjuvant, anti-calnexin antibody (#C4731), *p*-nitrophenyl phosphate, disodium salt (PNPP), 4', 6-diamidino-2-phenylindole (DAPI), insulin and Bromodeoxyuridine (BrdU) were from Sigma Aldrich (MO, USA). Anti-HER2 CB11 antibody (#ab8054) was from Abcam (MA, USA). Anti-BrdU antibody (#M0744) was from Dako (Glostrup, Denmark). Anti-Akt (#9272), anti-phospho-Akt (#9271), anti-Erk 1/2 (#9107), anti-phospho-Erk 1/2 (#9106) antibodies were from Cell Signaling (MA, USA). Anti-phospho-HER2 (#sc-1011694) and anti-Cyclin D1 (#sc-246) antibodies were from Santa Cruz (California, USA). Alexa Fluor 488-conjugated secondary antibodies to mouse and human IgGs and Lipofectamine 2000 were from Invitrogen (CA, USA). HiTrap Protein G HP columns and ECL plus western blot detection kit were from GE-Healthcare (WI, USA). Recombinant human TNF- α was from PeproTech (NJ, USA). RNase A and all restriction enzymes were from Fermentas (Vilnius, Lithuania). Trastuzumab (Herceptin), EDTA-free protease inhibitor cocktail tablets and phosSTOP (phosphatase inhibitor cocktail) tablets were from Roche Ltd (Basel, Switzerland). Antibody isotyping kit was from Thermo Fisher Scientific Inc (IL, USA). Protein G coupled Sepharose gel slurry was from East Coast Biologics (ME, USA).

Cell culture

SK-BR-3, MDA-MB-361, T47D, MCF-7 and BT-474 cell lines were obtained from ATCC. Huh-7 cell line is used in lab since 1995 and was last tested for authenticity in 2010 (originally from Jack Wands Lab at Massachusetts General Hospital, Boston, MA). All breast cancer cell lines have been tested and authenticated by short tandem repeat profiling in September 2009 and February 2012, as described previously [25]. SK-BR-3 cells were grown in McCoy's 5A medium. MDA-MB-361, T47D, MCF-7, BT-474 and Huh-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM), as described [25,26]. Hybridomas were grown in RPMI 1640, DMEM or Serum Free Media (SFM) from Hyclone (IL, USA). Cell culture media were purchased from GIBCO (CA, USA), Sigma or Hyclone, and were supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (P/S), 1% non-essential amino acids and 1% L-glutamine, unless otherwise stated. BT-474 cell growth medium was supplemented additionally with 10 μ g/ml insulin.

Florescent in situ hybridization analysis of *ERBB2* copy numbers

MCF-7, T47D, MDA-MB-361, SK-BR-3 and BT-474 cells were harvested at confluence and fixed in Carnoy's fixative (3:1 methanol/glacial acetic acid). A dual-color FISH was performed with Texas Red-labeled *ERBB2* and FITC-labeled

chromosome 17 α -satellite (D17Z1) DNA probes mix (LPS 001; Cytocell Ltd, Cambridge, UK), as described by supplier with minor modifications. The slides were treated at 72°C for 2 min in denaturation solution containing 70% formamide, 2XSSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). The probe mixture was applied onto slides; the area was covered with a 22X22 mm glass coverslip, sealed with rubber cement. Slides were placed on a hot plate and heated at 75°C for 5 min. The hybridization was done overnight at 37°C in humidified chambers. Post-hybridization washes were done once with 0.4XSSC at 72°C for 2 min and once with 2XSSC containing 0.05% Tween 20, at room temperature for 30 s. After post-hybridization washes, the slides were dehydrated in a series of 70%, 85%, and 97% ethanol. Air-dried slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in an anti-fade solution. Copy numbers in >100 nuclei of each cell line were counted under a Nikon Eclipse E600 epifluorescence microscope (Nikon Corp., Tokyo, Japan) equipped with filter sets appropriate for DAPI, FITC, and rhodamine. Individual single-color images of DAPI, FITC, and rhodamine were acquired through a high-sensitivity monochrome charge-coupled device (CCD) camera integrated to a Power Macintosh. Digitally acquired individual images were overlaid and operated with MacProbe image analysis software (PCI Scientific Systems League City, TX, USA). *ERBB2* copy numbers and ploidy level based on average counts of centromere 17 in interphase and metaphase FISH were evaluated according to international standard cytogenetic nomenclature [27].

Generation of anti-HER2 monoclonal antibodies

Animal experiments described here have been pre-approved by the Bilkent University Animal Experiments Ethical Review Panel, and conducted at Bilkent Animal House, which is certified by the Ministry of Agriculture of Turkey. All procedures complied with the guidelines of the Ministry of Agriculture (Official Gazette. No. 25464). BALB/c mice were immunized five times with live SK-BR-3 cells ($5\text{--}10 \times 10^6$ cells in PBS) by intra-peritoneal injection at three-week intervals. As a final booster, each mouse received 20 μg recombinant HER2 ECD protein injected with incomplete Freund's adjuvant. On the third day of last immunization, mice were sacrificed and their splenocytes were fused with SP2/O myeloma cells to produce hybridomas as previously described [28].

HER2 ECD ELISA assay

96-well Polysorb (NUNC, Roskilde, Denmark) microtiter plates were coated overnight at 4°C with HER2-ECD (25–50 ng/well) in carbonate buffer. Following saturation with 1% BSA for one hour, plates were incubated with hybridoma supernatants or purified primary antibodies for 2–4 h, and bound antibodies were detected by further incubation with AP-conjugated anti-mouse IgG antibodies. Trastuzumab binding was tested using AP-conjugated anti-human IgG antibodies. Phosphatase activity was measured using PNPP substrate with absorbance (OD) reading at 405 nm using μ Quant ELISA reader (BioTek, VT USA). The cross-reactivity tests with EGFR ECD were performed under the same conditions, except that ELISA plates were coated with EGFR ECD.

Antibody isotyping and affinity purification

The isotypes of monoclonal antibodies were determined using a commercial kit. Selected hybridoma clones were expanded and the antibodies were purified from the culture supernatants by FPLC using HiTrap Protein G HP columns (GE Healthcare, WI, USA).

Immunofluorescence

Cells were seeded on coverslips in 6-well plates. After 36 h of cell culture, they were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. Following saturation with 10% FCS, the fixed cells were incubated with anti-HER2 antibodies for 90 min. Primary antibody binding was detected using Alexa Fluor 488 conjugated goat anti-mouse IgG or Alexa Fluor 488 conjugated goat anti-human IgG antibodies, depending on the type of the primary antibody. Nuclei were counterstained with DAPI and cells were observed using Zeiss Axio Imager.A1 microscope (Jena, Germany).

Flow Cytometry

Cells ($3\text{--}4 \times 10^5$) were fixed with 2% paraformaldehyde and incubated 1 h with 5 $\mu\text{g/ml}$ anti-HER2 antibodies or IgG1 isotype control antibody (in 1% BSA and 0.5 mg/ml NaN_3 in PBS). Cells were then incubated 1 h with Alexa-488-conjugated anti-mouse IgG antibody and the percentage of positively stained cells were counted on FACSCalibur (BD Biosciences).

Immunoprecipitation

For detection of endogenous HER2 protein, cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH: 7.4, 1 mM EDTA, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 1X Protease Inhibitors) [29,30], 2 mg protein were diluted with milder NP40 buffer (150 mM NaCl, 50 mM Tris-HCl pH: 8, 1% NP40, 1X Protease Inhibitors) and incubated with 20 μg antibody for 2 h on a rotator at 4°C. Then, 200 μl Protein G-coupled Sepharose gel slurry was added, and the mixture was incubated 4 h at 4°C.

Western blot

Following immunoprecipitation experiments, antigen-antibody bound Protein G beads were boiled in SDS-loading buffer; solubilized proteins were run on 8% or 4-12% gradient SDS-PAGE. Proteins subjected to SDS-PAGE were transferred to PVDF membrane using XCell II Blot Module (Invitrogen, CA, USA). Unoccupied protein binding sites on the PVDF membrane were saturated by incubating with 5% dried milk (in TBS-T) for 1 h. Full length and truncated forms of HER2 protein were detected using CB11 antibody directed against intracellular domain. Briefly, membranes were incubated overnight with CB11 antibody (1/500 dilution), followed by 1 h incubation with HRP-conjugated secondary antibodies to mouse IgGs and bound antibody was detected using ECL plus western blot detection kit.

Epitope mapping

The experiment was based on testing of antibody binding to HER2 proteins that have been modified by deletion of different subdomains of its ECD. Mammalian expression plasmids were constructed and transiently transfected into Huh-7 cells. Antibody binding was tested by combined immunoprecipitation-western blot and indirect immunofluorescence assays. A pDEST26-derived plasmid vector expressing full-length human HER2 protein (OCABo5050G0219D; shortly called p219D here) was obtained from Source BioScience LifeSciences (Nottingham, UK). HER2 extracellular domain was extracted from p219D by Sac I/Eco RI digestion leaving the coding sequence for the N-terminal 22 amino acid signal peptide intact. pCC2001 was produced by inserting Not I and Xho I sites between this sequence and the sequence encoding the rest of HER2 protein starting from the transmembrane domain. DNA sequences allowing the inclusion of domains I, II, III and IV of ECD under different combinations were amplified by PCR and inserted into pCC2001 plasmid using the Not I and Xho I sites. The integrity of cloned sequences has been verified by DNA sequencing. Huh-7 cells (6×10^5) were transfected in 6-well plates with different HER2 plasmid constructs (5 μ g each) using Lipofectamine 2000, following a protocol provided by the manufacturer. Following 8-h Lipofectamine incubation, cells were washed with PBS and incubated overnight with fresh complete media (without P/S). Next day, the cells were trypsinized and replated. On the second day of cell-expansion, the cells were collected, lysed in RIPA buffer and subjected to immunoprecipitation and western blot analyses as described above, except that 150 μ g proteins were immunoprecipitated using 15 μ g antibody.

Cell growth assay

These assay was performed as described previously [31]. Briefly, cells (10^3) were seeded in 96-well plates in triplicate and allowed to attach by 2 h of preincubation. Antibodies were diluted in cell culture medium and added to preincubated cells to obtain 0–20 μ g/ml concentrations under a final volume of 200 μ l medium/well. Cells were then incubated up to 6 days. TNF- α treatment was performed similarly except that cells were preincubated for 6 h. For antibody and TNF- α co-treatment experiments and checkerboard assays, cells were preincubated for 2 h for attachment, followed by 4-h incubation with antibodies prior to TNF- α addition. At the end of each time point, culture supernatants were discarded. Attached cells were washed twice with PBS, fixed with ice-cold 10% TCA for one hour, followed by Sulforhodamine B-based reading, as described [32].

Drug interaction modeling

Drug interaction scores for pairwise combinations of TNF- α with individual antibodies at fixed concentrations were calculated as follows. Growth level in a drug condition was defined as the growth measurement in the drug condition normalized by growth measurement under no drug. To understand the interaction between an antibody and TNF- α at certain concentrations, growth levels under single antibody, TNF- α , antibody + TNF- α were compared. Expected growth level under no drug interaction was defined for each pairwise drug combination as the multiplication of the growth levels under each individual drug, following the Bliss Independence

Model for drug interactions [33]. The observed growth level for each combination was divided by the expected growth level to find an interaction score. According to this analysis, a score of 1 means no interaction, or independence, a score smaller than 1 means synergy, and a score larger than 1 means antagonism. For the drug interaction experiments where more than one concentration combination was experimentally tested, a single interaction score between two drugs was computed. For this, a modified version of the above algorithm and the data from all concentration combinations was used, as described previously [34]. The scores and the scale of these scores are equivalent to the single combination drug interaction experiment and are interpreted in the same way (<1 = synergy; >1 = antagonism). Each interaction experiment was done in triplicate, and the average growth levels were used for interaction score calculation.

Bromodeoxyuridine incorporation assay

Cells (10^4) were plated on coverslips in 12-well plates, and treated as described for cell growth assays. At 48th hour, BrdU (10 μ g/ml) was added into cell culture media and cells were further incubated for 24 h. The detection of BrdU incorporation was performed as described previously [35], except that Alexa Fluor 488-conjugated anti-mouse IgG antibody was used for visualization. Cell nuclei were counterstained with DAPI, examined using Zeiss Axio Imager.A1 microscope and representative photographs (3–5 frames per coverslip) were acquired. Percent BrdU incorporation was calculated after manual counting of BrdU-positive (BrdU⁺) and BrdU-negative (BrdU⁻) nuclei.

Cell cycle analysis

Analyses were performed as described previously [36]. Briefly, 2.5×10^4 (for 6-day samples) or 10^5 (for 1 and 3-day samples) SK-BR-3 cells were seeded in 6-well dishes in McCoy's 5A medium. After 36 h incubation, the medium was replaced with fresh McCoy's 5A medium including BH1 or IgG1 isotype control antibody (final 5 μ g/ml) $\pm$ 1000 Unit/ml TNF- α . On days 1, 3 and 6 after the addition of antibodies, both suspended and adherent cells were collected, fixed and stained with propidium iodide (PI). PI-stained cells were measured and analysed by flow cytometry.

Analysis of the downstream protein expression

In order to analyze the effect of BH1 treatment on the downstream pathway, SK-BR-3 cells were treated for 1–24 h with 10 μ g/ml BH1, 1000 Unit/ml TNF- α alone or their combination. Cells collected at each time point (1st, 2nd, 4th, 8th, 12th, 24th hour) were lysed with RIPA buffer (containing 1X PhosSTOP). Solubilized proteins were resolved by SDS-PAGE and analyzed by immunoblotting. PVDF membranes initially used for detection of phospho-Akt, phospho-Erk 1/2 and phospho-HER2 proteins were stripped once (62.5 mM Tris-Cl pH: 6.7, 2% SDS, 100 mM β -mercaptoethanol), washed thoroughly with TBS-T and re-blotted with anti-Akt, anti-Erk 1/2, anti-cyclin D1 and anti-calnexin antibodies. Following incubation with HRP-conjugated secondary antibodies to mouse or rabbit IgGs, bound antibodies were detected using ECL plus western blot detection kit.

Statistical analysis

Experimental data were presented as mean $\pm$ SD. Statistical analysis of experiments that have been performed at least in triplicate was done using Student's *t* test.

Results

Novel monoclonal antibodies directed to extracellular domain of HER2

We used a combined immunization protocol with repeated injections of live SK-BR-3 cells to BALB/c mice, followed by a single injection of recombinant HER2 ECD protein. Hybridoma clones were screened by HER2 ECD ELISA assay. Five hybridoma clones (namely BH1, BH2, BH5, BH6 and BH7) were selected for further analyses, based on their reactivity with HER2 ECD, but not EGFR ECD (Additional File 1). All monoclonal antibodies were isotyped as IgG1 with κ -light chains. Antibodies were purified from hybridoma supernatants using Protein G affinity chromatography and used for further studies.

Antibody reactivities against breast cancer cell lines

Anti-HER2 antibodies were tested using five breast cancer cell lines with different *ERBB2* copy numbers. Fluorescence in situ hybridization (FISH) using probes against *ERBB2* gene and chromosome 17 centromere showed that MCF-7 had three chromosome 17 and two *ERBB2* signals. MDA-MB-361 provided 4–8 chromosome 17, and 8–12 *ERBB2* signals. Both SK-BR-3 and BT-474 displayed 5–6 chromosome 17 signals and highly amplified *ERBB2* signals (Figure 1, left panel). Most of T47D cells displayed three chromosome 17 and three *ERBB2* copies (data not shown). These findings were in perfect agreement with a previous study reporting 47, 43, 11 and 2.5 copies of the *ERBB2* gene in BT-474, SK-BR-3, MDA-MB-361 and MCF-7, respectively [37]. When tested by indirect immunofluorescence assay, all five anti-HER2 antibodies displayed strong fluorescence intensities with both BT-474 and SK-BR-3 cells. The signals obtained with MDA-MB-361 were positive but not strong, but those provided by MCF-7 were negative or barely detectable (Figure 1, BH1, BH2, BH5, BH6, BH7 panels).

Figure 1 Strong binding of anti-HER2 antibodies to *ERBB2*-amplified breast cancer cell lines. *ERBB2* amplification was analyzed by FISH analysis. Cells were harvested at confluence, fixed and subjected to dual-color FISH using Texas Red-labeled *ERBB2* (red) and FITC-labeled chromosome 17 α -satellite CEP17 (green) DNA probes (left panel). For generation of anti-HER2 monoclonal antibodies, mice were immunized with SK-BR-3 cells, followed by a recombinant protein composed of extracellular domain of HER2 fused to human IgG1 Fc domain (HER2 ECD). Antibodies were purified from conditioned hybridoma cell culture media by affinity chromatography. For immunofluorescence assay, cells were seeded on coverslips in 6-well plates, incubated 36 h in cell culture medium, fixed, and permeabilized. Fixed cells were incubated with anti-HER2 antibodies, and the primary antibody binding was detected using Alexa 488- conjugated anti-mouse IgG (BH1, BH2, BH5, BH6, BH7 panels; green). Nuclei were counterstained with DAPI (blue) and pictures were acquired using Zeiss Axio Imager.A1 microscope

The binding strengths of antibodies were compared using HER2 ECD ELISA and flow cytometry. ELISA test provided evidence of strong binding capacity for BH1, BH6 and BH7 antibodies. BH5 antibody displayed lowest binding capacity, whereas BH2 had intermediate activity. These observations strongly suggested that we produced three anti-HER2 antibodies with high affinity, one with low and one with intermediate affinities (Figure 2A). Flow cytometric analyses provided similar findings. When tested with T47D breast cancer cell line expressing low levels of HER2 [38], BH1 and BH6 displayed highest shifts in staining intensity as compared to isotype control antibody. The binding of BH5 was not detectable, whereas BH2 displayed a weak binding (Figure 2B). However, when tested with BT-474 that has *ERBB2* gene amplification (Figure 1, left panel), all antibodies displayed strong intensity shifts, BH1 producing the best shift (Figure 2C).

Figure 2 Binding affinities of anti-HER2 antibodies. (A) HER2 ECD ELISA assay showing the binding strengths of five monoclonal antibodies. HER2 ECD-coated plates were incubated with increasing concentrations of anti-HER2 antibodies, followed by alkaline phosphatase-conjugated anti-mouse IgG antibodies. Y axis shows the absorbance (OD) reading at 405 nm following incubation with alkaline phosphatase substrate. T-bars: SD. (B) and (C), Flow cytometric analysis of antibody binding to low HER2-expressing T47D (B) and high HER2-expressing BT-474 (C) breast cancer cells. Fixed single-cell suspensions were incubated with anti-HER2 antibodies, followed by Alexa-488-conjugated secondary antibodies and fluorescence intensities were measured using flow cytometry

Newly generated anti-HER2 antibodies recognize conformation-dependent epitopes

Bioactive antibodies that are most effective against HER2-overexpressing tumors recognize conformational epitopes located at ECD subdomains involved in either receptor activation [21] or receptor dimerization [23]. Thus, the bioactivity of anti-HER2 antibodies may be strongly dependent on how they occupy the critical regions on their target. We performed a series of analyses to identify epitopes recognized by newly raised monoclonal antibodies. In these studies, Mouse IgG1 isotype antibody and humanized Trastuzumab were used as negative and positive controls, respectively. NCL-CB11 (CB11 in short) antibody against intracellular domain of HER2 was also used. Initially performed western blot assays using different breast cancer cell line lysates provided positive signals with CB11 antibody, but not with others (data not shown). Based on these findings, we hypothesized that the epitope(s) recognized by our antibodies were unstable under denaturing conditions of SDS-PAGE, although they remained stable after paraformaldehyde fixation used in indirect immunofluorescence and flow cytometry experiments (Figures 1, 2). Next, we tested whether our antibodies were able to immunoprecipitate HER2 from cell lysates made in RIPA buffer. Cell lysates made from T47D cells were subjected to immunoprecipitation with our antibodies and antibody-bound proteins were tested by western blot assay using CB11 antibody. All antibodies tested were able to immunoprecipitate HER2 under these conditions. The signal obtained with BH5 antibody was weak, as expected (Additional File 2). These findings provided evidence that newly generated antibodies recognized conformation-dependent epitope(s) on endogenously expressed HER2 protein.

Discovery of a new epitope(s) restricted to the intact form of the extracellular domain of HER2

In order to delineate the epitope(s) recognized by the antibodies, we constructed a series of plasmids to express truncated forms of HER2 at its ECD (Figure 3). Huh-7 cells that express low levels of endogenous HER2 protein [39] were transiently transfected with plasmids to express different forms of HER2, and immunoreactivity of each antibody was tested by indirect immunofluorescence and western blotting following immunoprecipitation. In immunofluorescence assay, CB11 antibody that was used as a positive control for expression of full length and truncated forms of HER2 transgene identified strongly positive transfected cells with all plasmids tested (Figure 4, right panel). Another control was Trastuzumab that is directed against an epitope located at domain IV of the extracellular region of HER2 [21]. All truncated forms of HER2 protein containing domain IV provided strongly positive signals with Trastuzumab (Figure 4, Tzm panel). Surprisingly, all five of our antibodies recognized only full-length HER2. They did not react with recombinant HER2 protein forms lacking any one of the ECD subdomains (Figure 4, panels BH1, BH2, BH5, BH6, BH7). Immunoprecipitation-western blotting assays following transient expression of full length and four truncated forms of HER2 confirmed immunofluorescence findings (Additional File 3). We deduced that newly generated antibodies are directed to an epitope, or closely related epitopes present only on structurally intact ECD of HER2. In other words, the reactivity of antibodies was lost when any of the four subdomains of the HER2 ECD was deleted. Thus, all five antibodies described in the report required the structural integrity of ECD for recognition of HER2 receptor (Figure 3).

Figure 3 Schematic representation of antibody epitope mapping studies.

Mammalian expression plasmids expressing full-length HER2 (p219D) or partially deleted HER2 were transfected into Huh7 cells. Anti-HER2 antibody reactivity was tested by indirect immunofluorescence (Figure 4) and immunoprecipitation-western blotting (Additional File 3) techniques. The boxes named as I, II, III, IV represent four subdomains of ECD. Grey box: transmembrane domain, TK: tyrosine kinase domain, CT: carboxyl-terminal domain. Tzm: Trastuzumab.+: antibody reactivity, -: no antibody reactivity. Stars (*) near plasmid names indicate that immunofluorescence data were confirmed with immunoprecipitation-western blot assay

Figure 4 Epitope mapping of anti-HER2 antibodies using indirect immunofluorescence assay.

Huh7 cells were transfected in 6-well plates using a set of mammalian expression plasmids encoding full-length or N-terminally truncated HER2 protein. Transfected cells were cultivated for 48 h, and subjected to indirect immunofluorescence assay. Transfected cells that were recognized by specific antibodies displayed strong green fluorescence. The letters from (a) to (k) indicate the plasmids in the following order: pDEST26 (empty vector), p219D, pCC2001, pCC2005, pCC2004, pCC2003, pCC2002, pCC2008, pCC2007, pCC2006, pCC2012 (see Figure 3 for description of HER2-expression plasmids). (–) No primary antibody, Tzm: Trastuzumab, CB11: a monoclonal antibody recognizing an epitope at intracellular domain of HER2 that was used as a positive control for expression of transfected plasmids

Effect of anti-HER2 antibodies on cell growth

The bioactivity of antibodies was first tested against SK-BR-3 cells. Affinity purified anti-HER2 antibodies were added at increasing concentrations into the culture media and relative cell growth was tested following 6 days of cell culture. Only BH6 and BH1 antibodies displayed modest but significant inhibitory effect on target cells, by lowering relative cell growth to 65% and 73% respectively, as compared to isotype control antibody (Figure 5A; $p < 0.006$ at 10 $\mu\text{g/ml}$ antibody concentration). In contrast, BH2, BH5 and BH7 antibodies did not affect the growth of SK-BR-3 cells.

Figure 5 Anti-HER2 antibodies exhibit enhanced anti-growth effect in the presence of tumor necrosis factor- α in SK-BR-3 cells. (A) SK-BR-3 cells were plated onto 96-well plates and incubated at 37°C with 5% CO₂. After 2 h of incubation, antibodies (0–10 $\mu\text{g/ml}$) were added, and cells were incubated for 6 days. (B) Antibodies (0–10 $\mu\text{g/ml}$) were added as described in (A), and cells were incubated for 4 h prior to TNF- α (1000 U/ml) addition to the culture medium. Cells amount was determined by Sulforhodamine B assay after 6 days of treatment. T Bars, SD; * $p < 0.006$, ** $p < 0.0005$, (A, B)

Effects of anti-HER2 antibodies in combination with tumor necrosis factor- α

It has been previously reported that amplified expression of HER2 in breast cancer cells induces resistance to TNF- α cytotoxicity [40,41] that can be sensitized by Trastuzumab [42]. Therefore, we asked whether our anti-HER2 antibodies were able to sensitize SK-BR-3 cells to TNF- α . We performed antibody treatment experiments in the presence of 1000 U/ml of TNF- α (Figure 5B). In the absence of antibody, TNF- α alone induced nearly 50% growth inhibition. This effect was not modified significantly in the presence of increasing concentrations of IgG1 isotype control antibody (up to 10 $\mu\text{g/ml}$ tested). In sharp contrast, all five anti-HER2 antibodies strongly enhanced TNF- α -induced growth inhibition ($p < 0.0005$ at 10 $\mu\text{g/ml}$ antibody concentration). As little as 0.25 $\mu\text{g/ml}$ anti-HER2 antibody was sufficient to significantly enhance TNF- α -mediated growth inhibition, as compared to IgG1 isotype control antibody at the same concentration ($p < 0.005$). Only one antibody, namely BH7, displayed a slightly weaker enhancer activity, reaching significance at 1.25 $\mu\text{g/ml}$ concentration ($p < 0.02$). Enhancer effects of anti-HER2 antibodies were nearly saturated at 2.5 $\mu\text{g/ml}$ concentration, reaching to 80-90% growth inhibition depending on antibody (Figure 5B).

Cell-dependent interactions between anti-HER2 antibodies and tumor necrosis factor- α creating both synergistic and antagonistic growth effects

Based on observations with SK-BR-3 cells, we analyzed interactions between anti-HER2 antibodies and TNF- α , using five different breast cancer cell lines. We compared growth responses to (i) 5 $\mu\text{g/ml}$ of each individual antibody, (ii) 1000 U/ml of TNF- α , and (iii) 5 $\mu\text{g/ml}$ antibody and 1000 U/ml of TNF- α in combination. An interaction score for each combination was calculated as described in the Methods, where a score of 1 means no interaction, or independence; a score smaller than 1

means synergy, and a score larger than 1 means antagonism [32]. In Figure 6, we analyzed TNF- α and anti-HER2 antibody interactions as compared to IgG1 isotype control antibody. IgG1 control antibody interactions with TNF- α provided scores between 0.81-0.88. Therefore, we considered values between 0.8-1.2 as an indication of non-interaction. When tested with SK-BR-3 cell line, interaction scores obtained for TNF- α and new anti-HER2 antibody combinations ranged between 0.26 and 0.48 (Figure 6, top). Synergy scores obtained with BH1, BH2 and BH5 were particularly strong (scores: 0.35, 0.30 and 0.26, respectively). In contrast, there was no interaction between Trastuzumab and TNF- α (score = 0.95). Thus, all five new anti-HER2 antibodies acted synergistically with TNF- α to inhibit the growth of SK-BR-3 cells.

Figure 6 Synergy and antagonism between anti-HER2 antibodies and tumor necrosis factor- α . Anti-Her2 antibodies and TNF- α acted synergistically in SK-BR-3 cells (top), but antagonistically in BT-474 cells (bottom). Growth effects observed under antibody (white columns), TNF- α (striped columns), and antibody + 1000 TNF- α (black columns) were obtained as follows. Cells were plated onto 96-well plates and incubated at 37°C with 5% CO₂. After 2 h of pre-incubation, antibodies (5 μ g/ml) were added, and cells were incubated for 6 days to study the effects of antibodies alone (white columns). The effects TNF- α (1000 U/ml) alone (dark columns) were studied by adding this cytokine into cell culture medium after 4 h of pre-incubation. For combined treatments, antibodies (5 μ g/ml) and TNF- α (1000 U/ml) were added after 2 h and 4 h of pre-incubations, respectively. Cells amount was determined by Sulforhodamine B assay after 6 days of treatment. Growth level under a drug condition was defined as the growth under that condition normalized by growth under no drug condition. Expected growth level under no interaction (gray columns) was calculated by multiplying the growth levels under each individual drug. The observed growth level for each combination was divided by the expected growth level to find an interaction score according to Bliss Independence Model for drug interactions (displayed under each antibody label). The combination of TNF- α with isotype control antibody (IgG1) or Trastuzumab (Tzm) provided scores near 1, meaning no interaction or independence in both cell lines. New anti-HER2 antibodies provided scores less than 0.57 in SK-BR-3 cells, meaning synergy. In contrast their interaction scores were more than 1.40 in BT-474 cells, meaning antagonism

Further studies indicated that the interaction between anti-HER2 antibodies and TNF- α was cell-dependent. Indeed, we observed a completely opposite response with another breast cancer cell line, namely BT-474 (Figure 6, bottom). This cell line responded to a few anti-HER2 antibodies modestly albeit significantly (BH1, $p < 0.05$; Tzm, $p < 0.003$). The response to TNF- α alone was about 50% growth inhibition, as observed with SK-BR-3 cells. Novel anti-HER2 antibodies abolished TNF- α -induced growth inhibition either completely (BH1, BH2, BH5, BH7) or partially (BH6) in BT-474 cells. Interactions scores of novel anti-HER2 antibodies ranged between 1.40 and 3.08, all indicative of an antagonistic interaction with TNF- α . Again, Trastuzumab had no major interaction with TNF- α (score = 1.21). Data on interaction analyses performed with MDA-MB-361, MCF-7 and T47D were shown in Additional File 4. When tested alone with MDA-MB-361 cells, some antibodies (BH1, BH2, BH7) displayed significant growth inhibition (>50% inhibition, $p < 0.0003$). Trastuzumab had no detectable anti-growth effect, but BH5 and BH6

displayed only modest activity (27% and 35% growth inhibition, respectively; $p < 0.02$). TNF- α was highly toxic to this cell line causing >95% growth inhibition. Antibody co-treatments rescued TNF- α induced growth inhibition only slightly. Thus, drug interaction analysis was not conclusive. The growth of MCF-7 cells did not appear to be affected by antibody treatment. In contrast, these cells were the most sensitive ones to TNF- α with nearly 100% growth inhibition. Antibody co-treatments did not change their response to TNF- α . T47D cells were resistant to both anti-HER2 antibody and TNF- α treatments, either alone or in combination.

Detailed analysis of interaction between BH1 antibody and tumor necrosis factor- α

Drug interaction analyses described above were performed using fixed amounts of antibodies and TNF- α . We obtained clear-cut results with only SK-BR-3 and BT-474 cells. Therefore, we decided to test the effects of combined treatment of anti-HER2 antibody BH1 and TNF- α on four breast cancer cell lines using a checkerboard assay. BH1 was selected as a representative of newly generated anti-HER2 antibodies. Each cell line was grown on a 7×8 grid of BH1 antibody and TNF- α combinations. The concentration of TNF- α was increased on the x-axis (0, 8, 16, 32, 62.5, 125, 250 and 500 U/ml) and the concentration of BH1 antibody was increased on the y axis (0, 0.16, 0.312, 0.625, 1.25, 2.5 and 5 μ g/ml). Such an experimental setting allowed us to measure the growth of each cell type in 42 concentration combinations of BH1 antibody and TNF- α , in addition to the growth of cells under each ‘drug’ alone. This experiment was done in triplicate for each cell type. The average growth measures are shown in Figure 7. We analyzed the growth measures using the same definition of drug synergy based on Bliss Independence Model as described above. We found concentration combinations where BH1 and TNF- α are significantly synergistic or antagonistic, which are indicated as green or blue dots on Figure 7, respectively. For each of the drug interaction experiments, we next computed a single interaction score as described previously [33]. These scores, which are interpreted as described above, are shown in Figure 7. According to this, BH1 and TNF- α exhibited synergy in SK-BR-3 cells, while they were antagonistic in BT-474 and MDA-MB-361 cell types, when all concentration combinations are considered. Interestingly, BH1 antibody, even at a very low dose (0.16 μ g/ml), strongly suppressed the growth inhibition triggered by TNF- α in BT-474 cells. These observations confirm strong synergistic and antagonistic interactions of TNF- α and anti-HER2 antibodies in SK-BR-3 and BT-474, respectively (Figure 6). The interaction score for MCF-7 was very close to 1, suggesting independence.

Figure 7 Anti-HER2 antibody and tumor necrosis factor- α interaction is cell type-specific. The growth of breast cancer cell lines under various combinations of BH1 antibody (0, 0.161, 0.312, 0.625, 1.25, 2.5, 5.0 μ g/ml) and TNF- α (0, 8, 16, 32, 62.5, 125, 250, 500 U/ml) treatments was experimentally tested in triplicate and the average relative growth levels were calculated. Heatmap represents the cell growth (as measured by OD) under different conditions. Synergistic and antagonistic interactions are indicated by green and blue dots, respectively. Numbers associated with cell line names indicate interaction scores. According to this checkerboard assay, BH1 and TNF- α show synergy in SK-BR-3 cells, but antagonism in BT-474 and MDA-MB-361 cells. There is no interaction in MCF-7 cells

Mechanisms of growth inhibition by anti-HER2 antibodies and tumor necrosis factor- α

In cancer cells, overexpressed HER2 is believed to be constitutively active as homodimers and/or as heterodimers with ligand-dependent HER3 [6]. Downstream signaling from HER2 involves PI3K-dependent activation of Akt and Ras-dependent activation of ERK1/ERK2 proteins by phosphorylation, mediating cell survival and proliferation activities of HER2 [6]. Therapeutically relevant Trastuzumab and Pertuzumab antibodies inhibit both Akt and ERK1/ERK2 phosphorylation, leading to growth inhibition [24,43-45]. TNF- α signaling generates both cell survival and apoptosis signals, depending on the activation of NF κ B transcription factor [46]. We further investigated the mechanisms of growth inhibition in responsive SK-BR-3 cells. As shown in Figure 8 left panel, untreated SK-BR-3 cells displayed relatively stable expression of phospho-HER2, phospho-Akt, total Akt, phospho-Erk1/2 and total Erk1/2. Cyclin D1 levels were undetectable under this experimental condition. Cells treated with 10 μ g/ml BH1 antibody inhibited Akt phosphorylation that was clearly detectable between 8 h and 24 h of treatment. There was also a partial inhibition of Erk1/2 phosphorylation between 4 h and 24 h of treatment. In parallel with these changes, we also observed progressive accumulation of cyclin D1 levels detected as early as 4 h of treatment. In contrast, HER2 phosphorylation was not affected by antibody treatment (Figure 8 middle panel). Cells co-treated with 10 μ g/ml BH1 and 1000 U/ml TNF- α displayed essentially the same type of alterations (Figure 8 right panel). Thus, TNF- α appeared not to modify the effects of the anti-HER2 antibody on phospho-Akt, phospho-ERK1/2 and Cyclin D1 in SK-BR-3 cell line. Indeed, treatment of cells with 1000 U/ml TNF- α alone did not affect any of the parameters studied, except a delayed inhibition of Akt phosphorylation at 24 h (Additional File 5).

Figure 8 *In vitro* molecular responses of SK-BR-3 cell to anti-HER2 antibody BH1 and tumor necrosis factor- α . SK-BR-3 cells were treated up to 24 h with BH1 antibody (10 μ g/ml), TNF- α (1000 U/ml) or the combination of both agents. Cell lysates were prepared from cells harvested at indicated times (hr, hours) and the expressions of phospho-HER2 (pHER2), phospho-Akt (pAkt), total Akt (Akt), phospho-ERK1/phospho-Erk2 (pErk1/2), total Erk1/Erk2 (Erk 1/2) and Cyclin D1 were analyzed by western blotting. Calnexin was used as a loading control. Untreated cells were incubated in a parallel experiment in the absence of any treatment

Cyclin D1 accumulates in G1 and G2 phases of cycling cells with a loss of expression in S phase cells [47]. Accordingly, Cyclin D1 accumulation could indicate progressive accumulation of cells at G1 and G2/M phases under anti-HER2 antibody treatment. We tested this hypothesis by exposing BH1 and/or TNF- α -treated cells to BrdU labeling and cell cycle analysis experiments during 6 days of treatment. Tests were performed at days 1, 3 and 6 (Figure 9A). SK-BR-3 cells have doubling times varying between 15 h and 36 h [48,49]. In order to detect the ratio of cells at G1/G0 phase, we used a modified DNA labeling protocol by exposing cells to BrdU for 24 h prior to each experimental time. As compared to IgG1 isotype control antibody, BH1 induced a significant decrease in the number of BrdU⁺ cells at days 3 and 6 ($p \leq 0.05$). Treatment of cells with IgG1 and TNF- α resulted in a more

pronounced decrease of BrdU⁺ cells that dropped progressively to 35% at day 6. When compared to IgG1 + TNF- α -treated cells, BH1 + TNF- α -treated cells responded immediately by a radical decrease of BrdU incorporation rates to 25% and 26% at days 1 and 3, respectively ($p \leq 0.05$). However, this inhibition was not stable and reversed to 37% at day 6 (Figure 9A). These findings indicated that both BH1 and TNF- α significantly increased the number of cells at G1/G0 phase, although this effect was delayed in BH1-only treatment. Sharp and immediate decrease in BrdU (+) cells with BH1 + TNF- α co-treatment confirmed the synergy between BH1 and TNF- α in these cells. Cell cycle analyses confirmed these findings (Figure 9B-D). As compared to IgG1 isotype control antibody-treated cells, BH1-treated cells first showed an increase in G2 cells at day 1, followed by a progressive increase of G1 cells at days 3 and 6. This was accompanied with a decrease in S phase cells. TNF- α treatment resulted in an immediate and stable response with an increase in G1 cells, concomitant with a decrease of S phase cells at all time points tested. BH1 + TNF- α treatment exaggerated this response, leading to a stronger accumulation of G1 phase cells, associated with a depletion of G2 phase cells. We concluded that both BH1 antibody and TNF- α were able to induce sustained accumulation of SK-BR-3 cells at G1/G0 phase, and their combination amplified this response particularly in early times of co-treatment.

Figure 9 Effects of anti-HER2 antibody BH1 and tumor necrosis factor- α on DNA synthesis and cell cycle in SK-BR-3 cells. (A) Effects on DNA synthesis were tested by 24-h BrdU incorporation assay. Cells were plated on coverslips in 12-well plates, treated as described in Figure 5, and were exposed to BrdU during 24 h prior to indicated days. Nuclear BrdU was detected by indirect immunofluorescence assay. Nuclei were counterstained with DAPI, examined using Zeiss Axio Imager.A1 microscope. Representative photographs (3–5 frames per coverslip) were acquired. Percent BrdU incorporation was calculated after manual counting of BrdU⁺ and BrdU⁻ nuclei. * Denotes p -values ≤ 0.05 (BH1 vs. IgG1, BH1 + TNF- α vs. IgG1 + TNF- α). T-bars: SD. (B-D). Effects on cell cycle were analyzed by flow cytometry. SK-BR-3 cells were treated with 20 μ g/ml BH1 or IgG isotype control antibody in the presence or absence of 1000 U/ml TNF- α . Cells were fixed on day 1 (B), day 3 (C) and day 6 (D), stained with propidium iodide and the cell cycle distribution was analysed with flow cytometry. Fraction of cells present in G2/M-phase (G2/M), S-phase (S) and G1-phase (G1) are shown in purple, green and orange respectively. Fraction of apoptotic cells observed as a SubG1 peak is shown in black

Discussion

This report describes five novel anti-HER2 monoclonal antibodies that have been produced by immunizing mice with HER2-overexpressing whole cells, followed by a boost with recombinant HER2 ECD homodimer protein. Immunoprecipitation and western immunoblotting studies indicated that all five antibodies were directed against conformation-dependent epitope(s) present on the endogenous HER2 protein. Epitope mapping with recombinant HER2 protein forms that have been truncated at ECD domain indicated that all five antibodies react only with the native form of cellular HER2, being unable to recognize truncated proteins lacking any of the four subdomains. Although we do not yet know whether our antibodies recognize identical, overlapping or independent epitopes on the intact ECD domain, it is

evident that their epitope(s) is different from those recognized by Trastuzumab and Pertuzumab. Trastuzumab, a therapeutic antibody already approved for clinical use, recognizes an epitope restricted to subdomain IV, involved in receptor activation [21]. Pertuzumab, a therapeutic antibody tested in different clinical trials, recognizes a distinct epitope located on subdomain II, involved in receptor dimerization [23]. Antibodies directed to ECD epitope(s) different from other anti-HER2 therapeutic antibodies may serve as new analytical tools to study the structural organization of HER2 at the cell surface that may be present as monomers, homodimers and heterodimers depending on cell type and extracellular environment such as the availability of ligands that activate heterodimer partners of HER2. Dynamic changes at the receptor ECD in relation with transcriptional, translational and post-translational modifications can be monitored with these antibodies, both biochemically and biologically.

In vitro effects of novel anti-HER2 antibodies were tested against five different breast cancer cell lines displaying different degrees of *ERBB2* amplification and HER2 expression. Individually tested novel anti-HER2 antibodies were weakly effective as growth inhibitors (Figure 6). This was not surprising. Indeed, Trastuzumab (4D5) has a modest growth inhibitory effect (56%), and Pertuzumab (2C4), the best therapeutic alternative to Trastuzumab, induces only 20% growth inhibition [42]. Based on the synergy between Trastuzumab and Pertuzumab directed to different epitopes of HER2 [24], we performed studies to test combinatory anti-growth effects of our anti-HER2 antibodies with Trastuzumab on SK-BR-3 cells. Preliminary data suggested a synergy between them, raising the possibility that our antibodies may be combined with Trastuzumab or Pertuzumab to enhance anti-cancer activity. This will be the focus of further work.

The most striking feature of novel anti-HER2 antibodies was their differential interaction with TNF- α depending on cell type. They synergized with TNF- α on SK-BR-3 cells by raising growth inhibition from 50% to nearly 90%. In sharp contrast, the same antibodies displayed strong antagonism with TNF- α on BT-474 cells and MDA-MB-361 cells by abolishing TNF- α -induced growth inhibition either completely (BT-474 cells) or partially (MDA-MB-361 cells). Trastuzumab did not have any synergistic or antagonistic effects on TNF- α -mediated growth inhibition in these cell lines (Figure 6). The sensitization of SK-BR-3 cells to TNF- α cytotoxicity by mouse monoclonal antibody 4D5 (from which Trastuzumab has been humanized) has been reported [41], but there is no previous data on synergy or antagonism between anti-HER2 antibodies and TNF- α , to our best knowledge.

TNF- α is one of the most potent anti-tumor factors when used at high concentrations, but its systematic toxicity hampers its clinical applications [50]. The sensitization of SK-BR-3 cells to TNF- α with anti-HER2 antibodies provides experimental evidence for combination of low TNF- α doses with anti-HER2 antibody treatment to achieve effective anti-growth effects. Based on checkerboard analyses reported in Figure 7, a synergistic anti-growth response could be obtained from SK-BR-3 cells with as low as 32 U/ml TNF- α under *in vitro* conditions. But, one caveat is that the same antibody-TNF- α combination could produce completely opposite responses in other cells, as reported here for BT-474 and MDA-MB-361 cells. For example, as little as 0.1 mg/ml BH1 antibody was able to abolish TNF- α cytotoxicity in BT-474 cells.

The mechanisms of differential responses of breast cancer cell lines to combined treatment with anti-HER2 antibody and TNF- α merit further investigation. Here, we investigated the mechanisms of growth response of SK-BR-3 cells to combined treatment. The analysis of several downstream targets of HER2 signaling indicated that BH1 antibody did not inhibit HER2 phosphorylation, yet it was able to inhibit the phosphorylation of Akt and ERK1/2 kinases. These changes resulted in a progressive increase of cyclin D1 protein levels. DNA synthesis was inhibited and cells accumulated progressively at G1 phase of the cell cycle. The addition of TNF- α to BH1 treatment dramatically enhanced these responses leading to an immediate and stable growth arrest.

Conclusions

Newly generated anti-HER2 monoclonal antibodies allowed the identification of a novel epitope on HER2 receptor that is restricted to the intact form of its ECD. These antibodies displayed cell type-dependent interactions with TNF- α , resulting in synergistic, antagonistic or independent anti-growth effects. The synergistic activity was associated with inhibition of Akt and ERK1/2 phosphorylation leading to immediate and stable cell cycle arrest.

Abbreviations

AP, Alkaline phosphatase; BrdU, Bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle medium; ECD, Extracellular domain; EDTA, Ethylene diamine tetra-acetic acid; EGFR, Epidermal growth factor receptor; ELISA, Enzyme linked immunosorbent assay; ErbB2, Epidermal growth factor receptor 2; FCS, Fetal calf serum; FITC, Fluorescein isothiocyanate; FPLC, Fast protein liquid chromatography; HER2, Human epidermal growth factor receptor 2; HRP, Horse radish peroxidase; IgG1, Immunoglobulin G1 isotype; MUC4, Mucin4; PI3K, Phosphatidylinositol-3-kinase; OD, Optical density; PAGE, Polyacrylamide gel electrophoresis; PNPP, *p*-nitrophenyl phosphate disodium salt; P/S, Penicillin/streptomycin; PVDF, Polivinylidene fluoride; RIPA, Radioimmunoprecipitation assay; SD, Standard deviation; SDS, Sodium dodecyl sulphate; SFM, Serum free media; SRB, Sulforhodamine B; TBS-T, Tris-buffered saline + Tween-20; TCA, Trichloroacetic acid; TNF- α , Tumor necrosis factor- α ; Tzm, Trastuzumab

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

TY and MO designed the study. CC designed, performed and analyzed all experiments except FISH analysis. IT and CC performed checkerboard growth assays. MC analyzed growth effects by drug interaction modeling. SC performed

FISH analyses. MO, TY and CC wrote the manuscript. All authors read and approved the final manuscript.

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Additional files

Additional_file_1 as PDF

Additional File 1 Anti-HER2 antibodies did not react with EGFR. Mice were immunized with SK-BR-3 cells, followed by a recombinant protein composed of extracellular domain of HER2 fused to human IgG1 Fc domain (HER2 ECD). ELISA plates that have been coated with either HER2 ECD or EGFR ECD were incubated with antibody-containing hybridoma supernatants, followed by alkaline phosphatase-conjugated anti-mouse IgG antibodies. The Y axis shows the absorbance (OD) reading at 405 nm following incubation with alkaline phosphatase substrate (assays in duplicate).

Additional_file_2 as PDF

Additional File 2 Antibody binding to endogenously expressed HER2 protein as tested by immunoprecipitation-western blot assay. T47D-derived lysates were subjected to immunoprecipitation with different anti-HER2 antibodies. Antigen-antibody complexes were captured onto Protein G-conjugated beads, eluted and

subjected to western blot assay using CB11 antibody. Blots were overexposed to visualize the weakly positive HER2 immunoprecipitated by BH5 antibody. Tzm: Trastuzumab used as a positive control antibody. (-): No primary antibody.

Additional_file_3 as PDF

Additional File 3 Epitope mapping of anti-HER2 antibodies using combined immunoprecipitation-western blot assay. Huh7 cells were transfected in 6-well plates using a set of mammalian expression plasmids encoding full-length or N-terminally truncated HER2 protein. Transfected cells were cultivated for 48 h; cell lysates were subjected to immunoprecipitation with different anti-HER2 antibodies. Antigen-antibody complexes were captured onto Protein G-conjugated beads, eluted and subjected to western blot assay using CB11 antibody. . Arrows: immunoreactive bands specific for full-length and truncated HER2 protein forms. IP: immunoprecipitation; *: non-specific bands originating from antibodies used for immunoprecipitation.

Additional_file_4 as PDF

Additional File 4 Interaction between anti-HER2 antibodies and tumor necrosis factor- α . MDA-MB-361 cells were partially sensitive to anti-HER2 antibodies, but highly sensitive to TNF- α (top). MCF-7 cells were resistant to anti-HER2 antibodies, but highly sensitive to TNF- α (middle). T47D cells were resistant to both anti-HER2 antibodies and TNF- α (bottom). Growth measurements observed under 5 μ g/ml antibody (white columns), 1000 U/ml TNF- α (striped columns), and 5 μ g/ml antibody + 1000 U/ml TNF- α (black columns) were obtained experimentally, as described in Figure 6. Growth level under a drug condition was defined as the growth under that condition normalized by growth under no drug condition. Expected growth level under no interaction (gray columns) was calculated by multiplying the growth levels under each individual drug. The observed growth level for each combination was divided by the expected growth level to find an interaction score according to Bliss Independence Model for drug interactions Interaction scores were not calculated for these cell lines.

Additional_file_5 as PDF

Additional File 5 In vitro molecular responses of SK-BR-3 cells to tumor necrosis factor- α . SK-BR-3 cells were treated up to 24 h with TNF- α (1000 U/ml). Cell lysates were prepared from cells harvested at indicated times (hr, hours) and the expressions of phospho-HER2 (pHER2), phospho-Akt (pAkt), total Akt (Akt), phospho-ERK1/phospho-Erk2 (pErk1/2), total Erk1/Erk2 (Erk 1/2) and Cyclin D1 were analyzed by western blotting. Calnexin was used as a loading control.

Figure 1

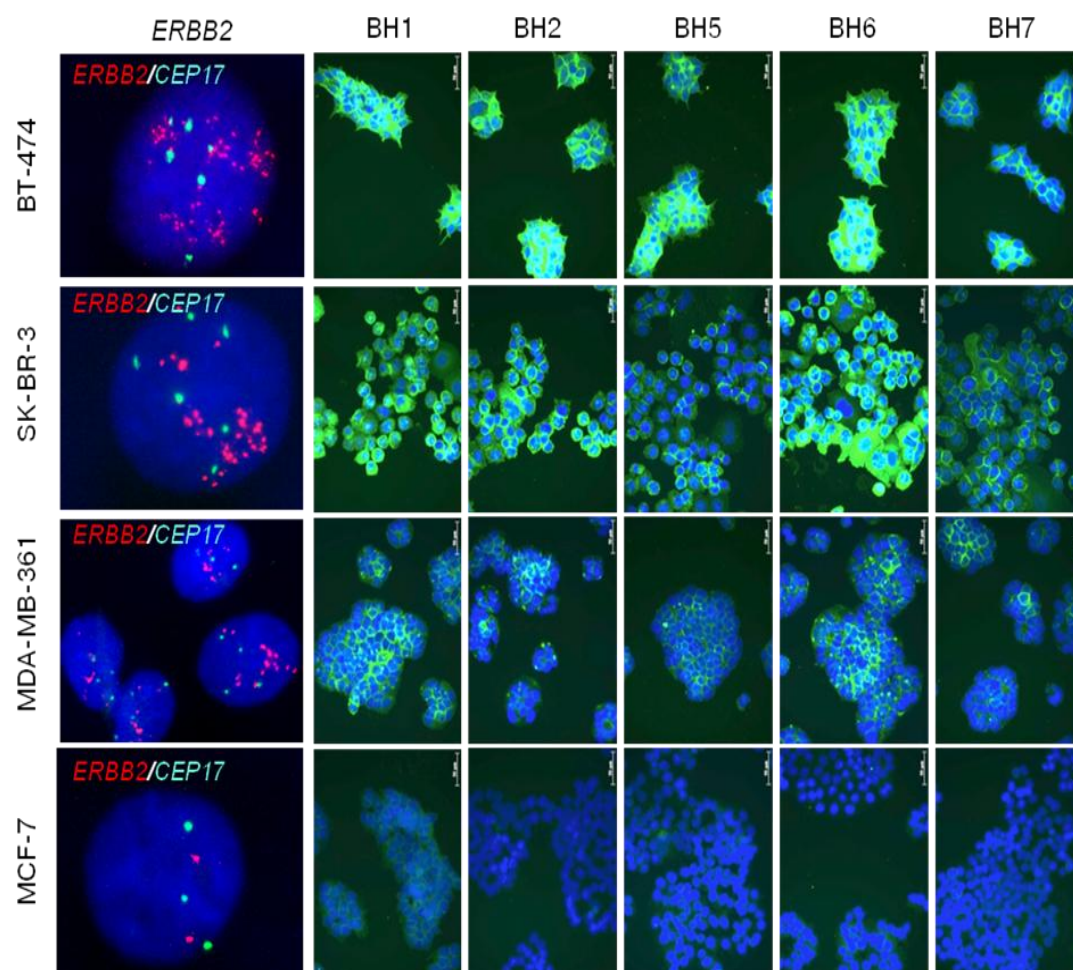


Figure 2

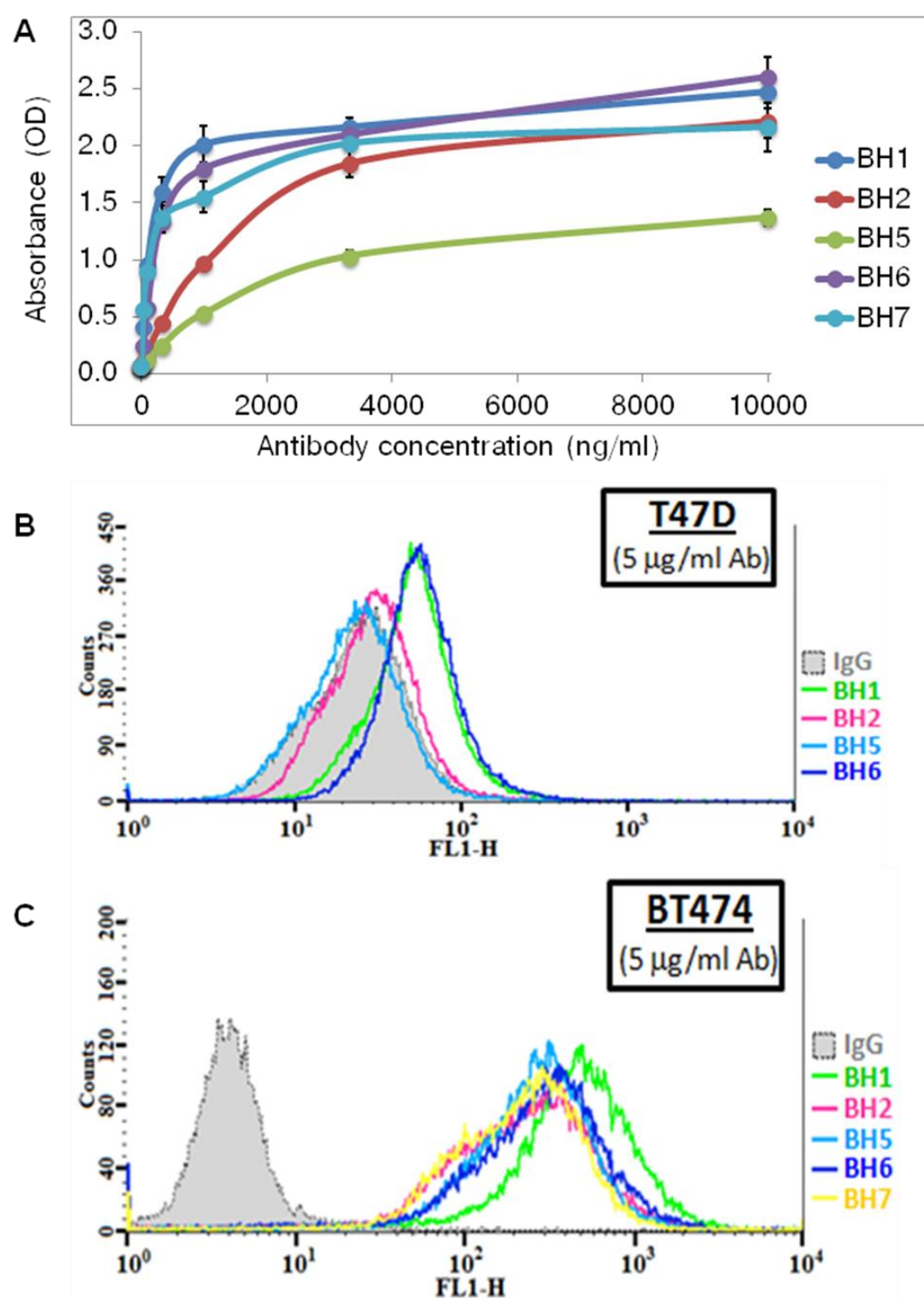


Figure 3

		BH1	BH2	BH5	BH6	BH7	Tzm	CB11
p219D*		+	+	+	+	+	+	+
pCC2001*		-	-	-	-	-	-	+
pCC2005		-	-	-	-	-	+	+
pCC2004		-	-	-	-	-	-	+
pCC2003		-	-	-	-	-	-	+
pCC2002		-	-	-	-	-	-	+
pCC2008*		-	-	-	-	-	+	+
pCC2007*		-	-	-	-	-	-	+
pCC2006*		-	-	-	-	-	-	+
pCC2012*		-	-	-	-	-	-	+

Figure 4

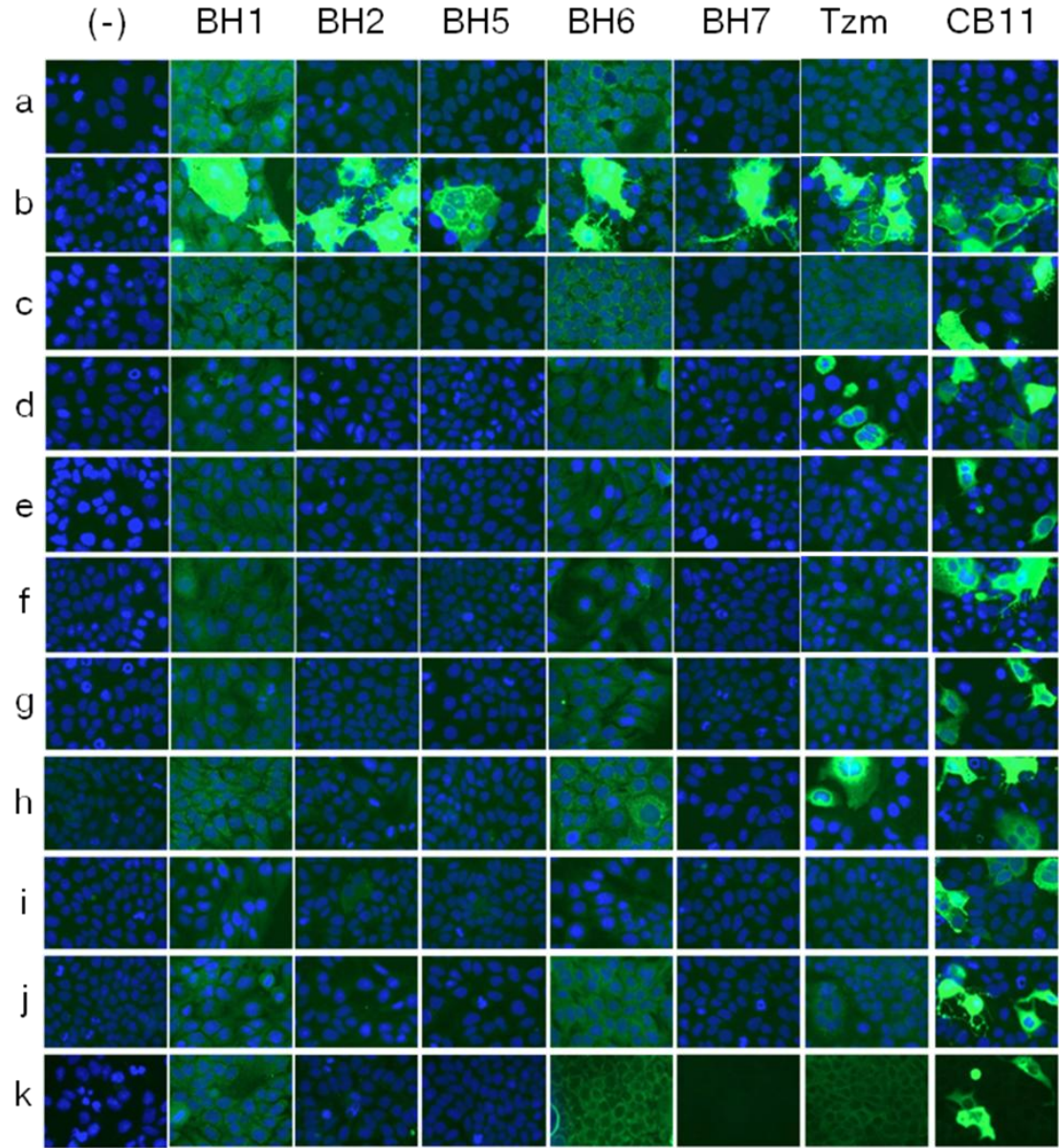


Figure 5

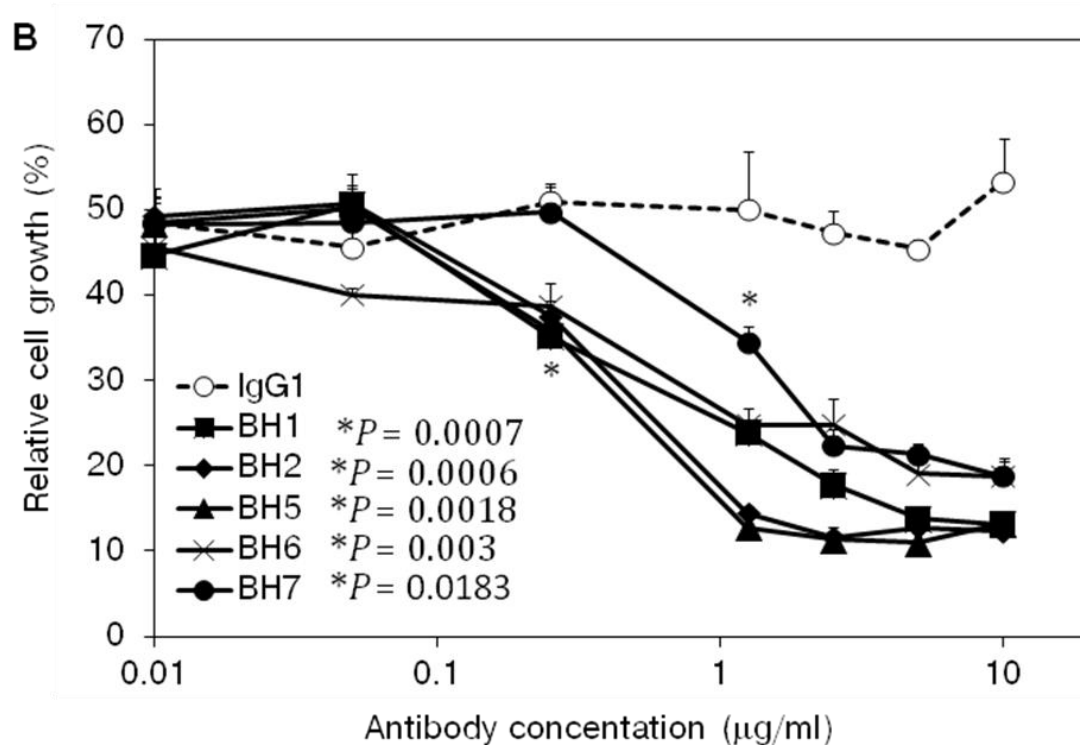
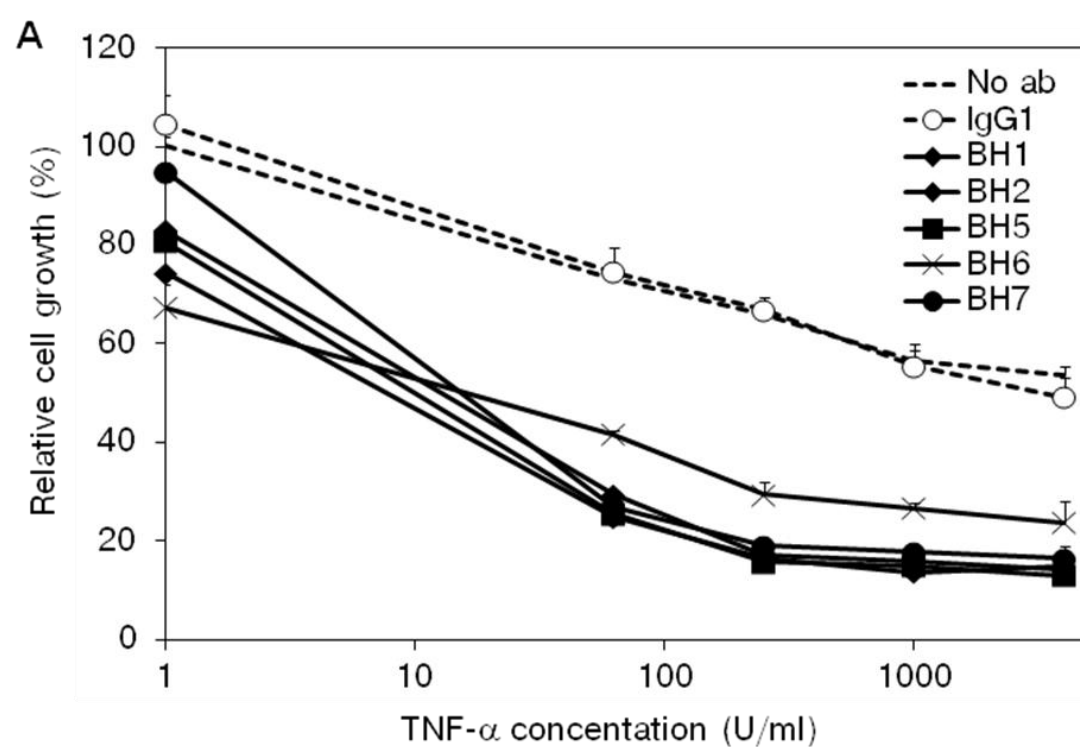


Figure 6

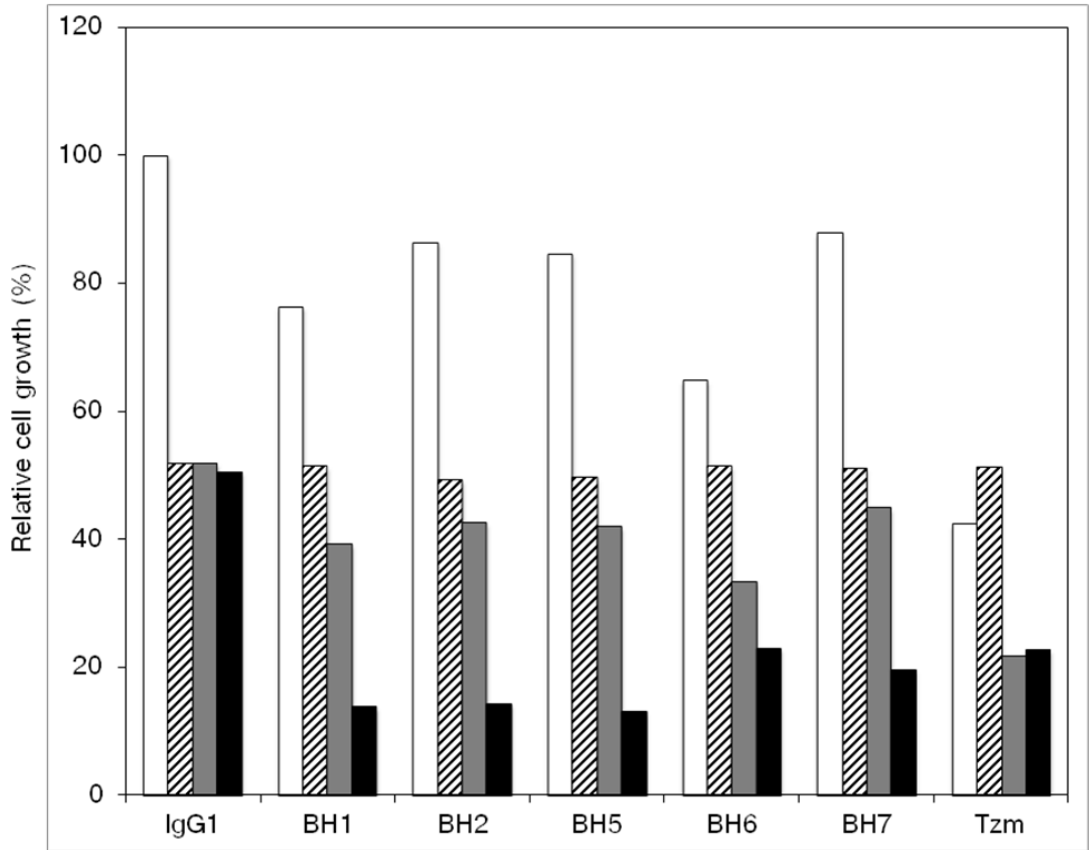


Figure 7

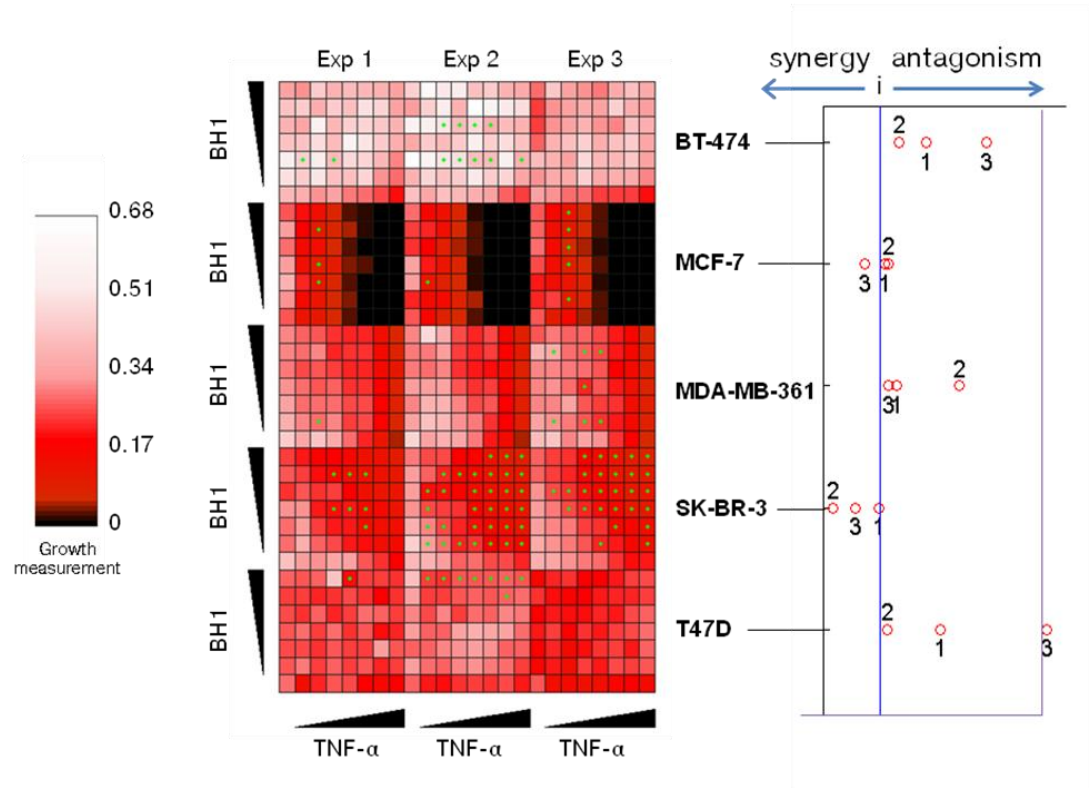
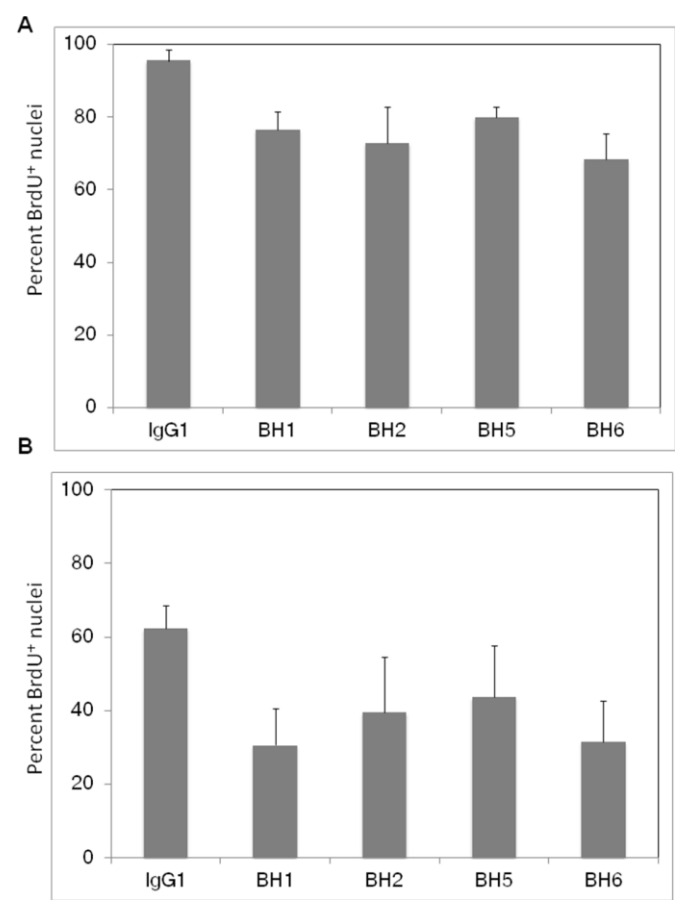
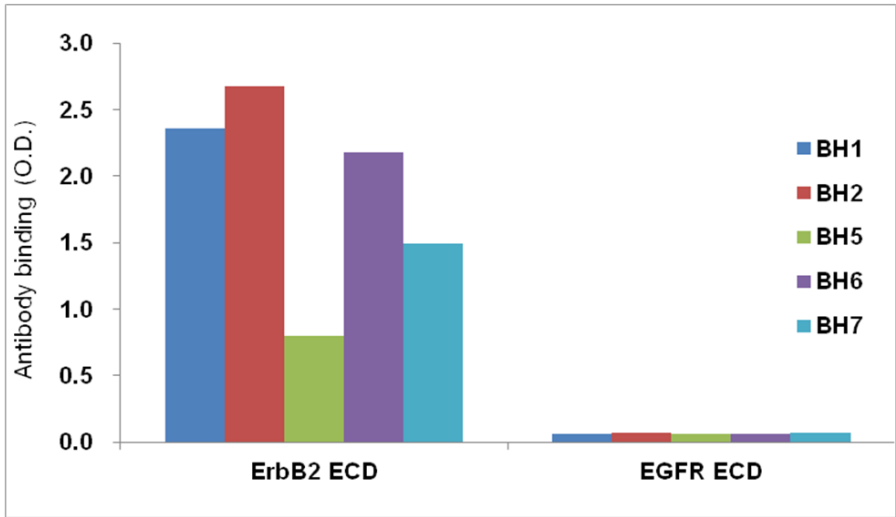


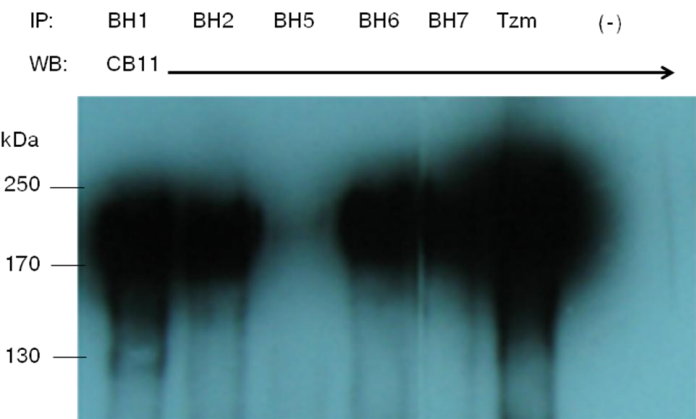
Figure 8



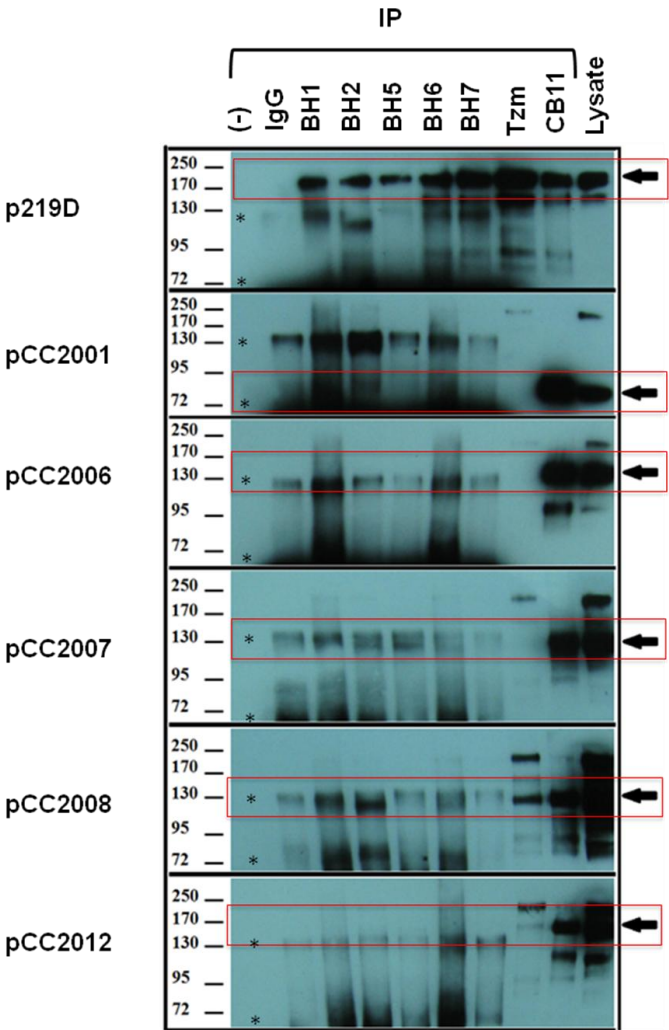
Additional File 1



Additional File 2



Additional File 3



Additional File 4

