

Assessing the role of ADAM10 in Trastuzumab treatment and resistance

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M.Sc. by Research

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To my Dad

Acknowledgments

I was allowed to do this M.Sc. by Research at the Weatherall Institute of Molecular Medicine, University of Oxford. I would like to thank several people without whom I would not have been able to pursue this dream.

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Abstract

Overexpression of the HER2 receptor occurs in about 15-20% of all breast cancers and is correlated with poor prognosis. Trastuzumab (Herceptin®), a monoclonal antibody targeting HER2, was shown to prolong survival of these patients. However, primary or acquired resistance remains a drawback for the drug. Understanding the mechanisms behind Herceptin resistance is crucial in order to increase the efficacy of the treatment. Many factors have been shown to contribute to resistance, including the protein family of the ADAMs (a disintegrin and metalloproteinase). One of them, ADAM10, cleaves several pro-ligands for the HER-family, such as betacellulin and HB-EGF. It is also responsible for shedding the extracellular domain of HER2. The objective of my M.Sc. is to understand the role of ADAM10 in relation to Herceptin treatment and resistance. In this thesis, it was shown that Herceptin treatment increased ADAM10 levels via an AKT/PKB feedback loop in HER2 positive cells and xenograft tumour samples and that ADAM10 was increased in Herceptin resistant cell lines. The increase of ADAM10 was correlated with an upregulation of the ligand betacellulin in the media. Inhibition of ADAM10 enzymatic activity or knockdown of the protein decreased the activation of HER family members and downstream markers in sensitive and resistant cell lines and was additive to Herceptin treatment in decreasing cell viability in sensitive cells. The TMA samples from a cohort of HER2 positive breast cancer patients have been stained for ADAM10 expression and positive staining was significantly correlated to poorer relapse free as well as overall survival. Both *in vitro* and *in vivo* data of this project suggested an important role of ADAM10 in Herceptin treatment of HER2 positive breast cancer.

Abbreviations

ADAM	A disintegrin and metalloproteases
ADCC	Antibody dependent cellular cytotoxicity
BSA	Bovine serum albumin
cDNA	Complementary DNA
DAB	3.3‘-Diaminobenzidine
DMEM	Dulbecco’s Modified Eagle Medium
dNTP	Deoxyribonucleoside triphosphate
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF (R)	Epidermal growth factor (receptor)
ELISA	Enzyme-Linked Immunosorbent Assay
FAB, FAC	Fragment Antigen Binding, Fragment crystallisable
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
GPCR	G-protein coupled receptor
HER	Human Epidermal Growth Factor Receptor
HERA	HERceptin Adjuvant
IHC	Immunohistochemistry
LDS	Lithium dodecyl sulphate
P/S	Penicillin / Streptomycin
PBS	Phosphate buffered saline
PVDF	Polyvinylidene difluoride
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SDS	Sodium dodecyl sulphate

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1 Introduction

1.1 The HER family

The HER (human epidermal growth factor receptor) family of receptor tyrosine kinases comprises four distinct receptors, the epidermal growth factor receptor (EGFR) or ErbB1, HER2 (or ErbB2), HER3 (or ErbB3), and HER4 (or ErbB4) [1]. The receptors share a similar structure as depicted in figure 1.1.

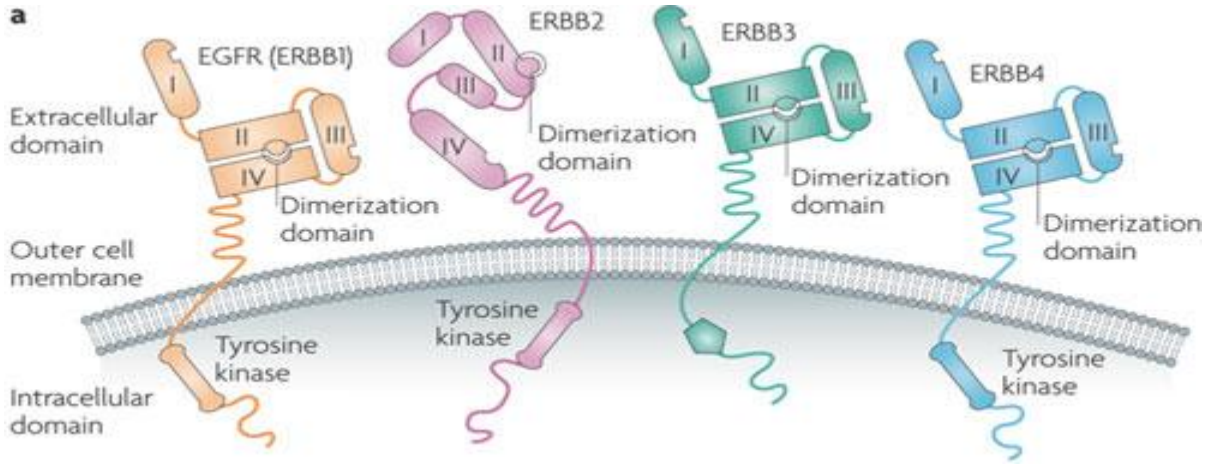


Figure 1.1 Structure of the HER receptors. Figure taken from Baselga and Swain (2009) [1]

Commonly, HER receptors are type I transmembrane proteins that comprise a glycosylated and disulfide bonded ectodomain with four subdomains, a single transmembrane domain, and a cytoplasmic region encoding a tyrosine kinase as well as multiple phosphorylation sites [2]. Several structurally related ligands are known to bind to these receptors, as shown in table 1.1. However, HER2 does not have any known ligands [3].

Table 1.1 List of ligands binding to HER receptors. Table taken from Linggi and Carpenter (2006) [2]

	ErbB-1	**ErbB-2**	**ErbB-3**	**ErbB-4**
EGF	+	−	−	−
TGF-α	+	−	−	−
HB-EGF	+	−	−	+
Amphiregulin	+	−	−	−
Betacellulin	+	−	−	+
Epigen	+	−	−	−
Epiregulin	+	−	−	+
Neuregulin-1	−	−	+	+
Neuregulin-2	−	−	+	+
Neuregulin-3	−	−	−	+
Neuregulin-4	−	−	−	+

In the absence of a ligand, the receptors are present in a tethered conformation. As shown in figure 1.2, upon ligand binding to the extracellular subdomains I and III, the receptor undergoes a conformational change, leading to the exposure of subdomain II, the binding domain, on the cell surface [4]. This dimerisation arm (a 10-residue sequence) was shown to be important but not exclusively essential for ectodomain contacts. Mutations in this arm do not affect ligand binding but abrogate receptor activation [5, 6].

Although there is no ligand known for HER2, the crystal structure shows a constitutively "open" conformation, mimicking a ligand-bound receptor [7]. Therefore, HER2 is able to form active homodimers and it was also shown to be the preferred dimerisation partner for the other HER family members [8]. HER3 lacks an active tyrosine kinase domain but most commonly dimerises with HER2 [9].

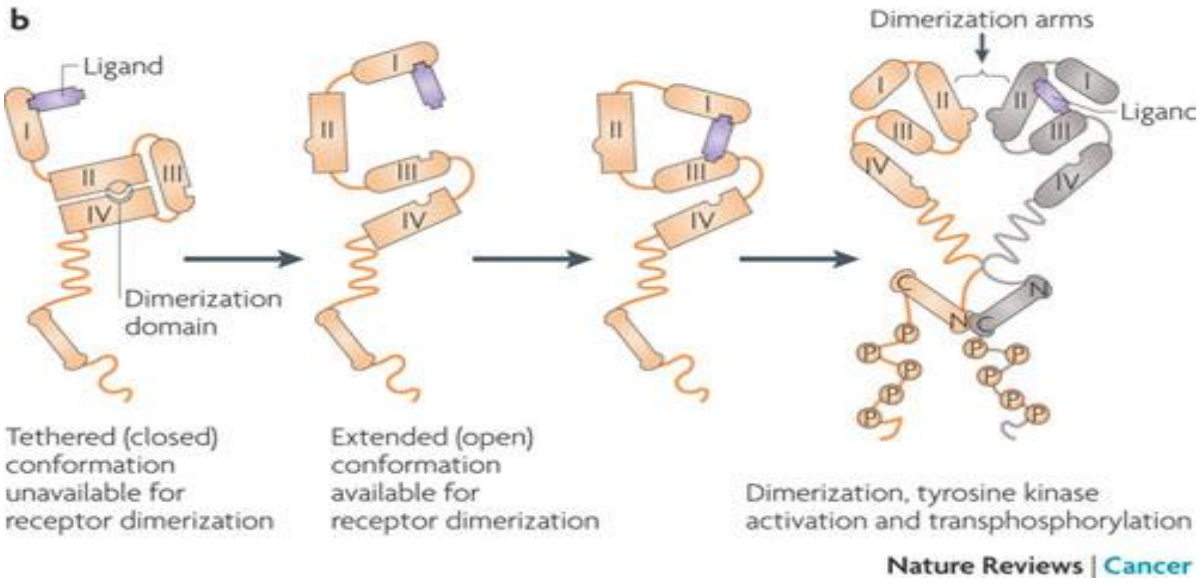


Figure 1.2 Dimerisation is triggered by ligand binding. Figure taken from Baselga and Swain (2009) [1]

Dimerisation is thought to rearrange two cytoplasmic domains such that trans-phosphorylation is activated. Zhang et al. (2006) [10] postulated that the kinase domain of EGFR is intrinsically auto-inhibited and this conformation is similar to that of Src and CDKs. Mutational analysis showed the importance of this resemblance and that asymmetric dimer formation is relevant for EGFR activation. During activation, the C-lobe of one kinase contacts the N-lobe of a second kinase thereby repositioning the activation loop in order to facilitate catalysis.

Post-receptor signals trigger the activation of several pathways including phospholipase Cγ, and signal transducer and activation of transcription (STATs) [11].

As seen in figure 1.3, binding of the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K), relieves the p110 catalytic subunit, which activates the PI3K/AKT signalling pathway and subsequently downstream molecules such as mTOR [12]. Moreover, the phosphorylation of tyrosine residues in the cytoplasmic tails of EGFR, HER2, and HER4 provides binding sites for proteins with a Src Homology 2 (SH2) domain. This facilitates binding of Growth Factor Receptor-Bound Protein 2 (Grb2) which in turn leads to the

activation of downstream signalling through the Mitogen-Activated-Protein-Kinase (MAPK) pathway [13].

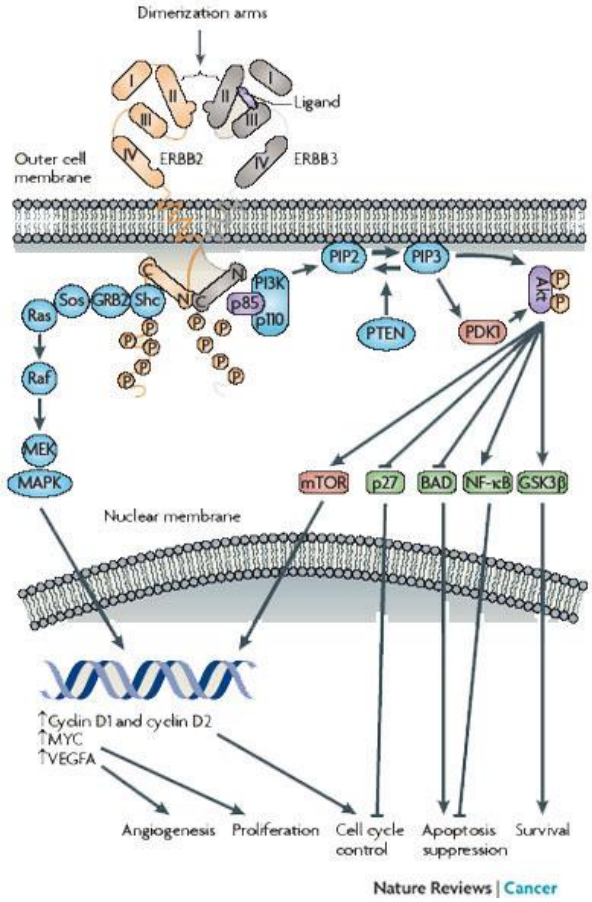


Figure 1.3 HER-family downstream signalling. Dimerisation leads to cross-phosphorylation of dimer partner and to the activation of the PI3K as well as the MAPK pathway. Figure taken from Baselga and Swain (2009) [1].

The activation of HER receptors and the subsequent downstream pathways stimulate different cell responses such as cell motility, survival and proliferation, and differentiation [14].

1.2 HER2

HER2 is a 185kDa oncoprotein encoded by the HER2/neu gene located on chromosome 17 [15]. HER2 is the preferred dimerisation partner within the HER network, however, it is also

able to form homodimers [8]. The intracellular domain of HER2 lacks binding sites for the p85 subunit of PI3K and HER2 homodimers are associated with the activation of the MAPK pathway. Nevertheless, HER2 is able to activate the PI3K pathway through heterodimerisation with HER3/HER4 thereby also attracting GRB2 [16].

The HER2 protein was also shown to comprise a nuclear localization sequence (NLS) [17], which interacts with importin β1, nuclear pore protein Nup358, and other mediators to facilitate translocation into the nucleus [18]. In the nucleus, HER2 can act as modulator of transcription with COX2 as one main target [19]. However, little is known about the functions of nuclear HER2.

HER2/neu was first identified in a neuro/glioblastoma model in rat [15, 20] and was later on related to an aggressive biological behaviour in breast cancer and associated with poorer prognosis for patients [21]. HER2 amplification and overexpression is diagnosed in about 15-20% of breast cancers as well as in other tumour types [1]. The overexpression was linked with increased homo- and heterodimerisation, enhancing cell survival and proliferation [11].

1.3 HER2 in breast cancer

1.3.1 Classification

The current methods in the clinical setting to assess HER2 over-expression are immunohistochemistry (IHC) or determination of gene amplification by fluorescent in situ hybridization (FISH)

IHC

IHC requires staining of a tumour biopsy for HER2 protein expression. As a standard, the HercepTest (Dako, Carpinteria, CA) is used to reduce inter-lab variations. Intensity of HER2 staining determines the score as outlined in table 1.2.

Table 1.2 IHC scoring criteria for HER2 staining. Table adopted from Vincent-Salomon et al. (2003) [22]

Scoring	Criteria
0+	Absence of membranous staining
1	> 10% stained cells with a weak staining intensity
2+	> 10% stained cells with moderate staining intensity
3+	> 10% stained cells with a strong intensity

However, according to the American Society of Clinical Oncology, staining of 30% of the tissue should be considered.

A 3+ scoring is classed HER2 positive. On the contrary HER2 0 (no membrane staining) or HER2 1+ (weak staining) are considered HER2 negative. Moderate staining for HER2 results in the classification HER 2+ and those samples are sent for further analysis by FISH [23].

FISH

With FISH, the gene expression of HER2 is determined. Specifically designed fluorescent probes bind to regions of DNA, where the gene of interest is located and the amplification of this gene can be analyzed. For HER2, the gene copy number as well as the ratio of the gene to its resident chromosome is tested. A sample is considered positive if the HER2 copy number is greater than 6 or if the HER2/CEP17 ratio is greater than 2.2 [24, 25]. Protein expression and gene amplification usually correlate with each other [26].

1.4 Trastuzumab

Trastuzumab (Herceptin®, Genentech, San Francisco, CA) is a humanized IgG1 kappa light chain monoclonal antibody whose complementary-determining regions (CDR) amino acids recognise subdomain IV of the HER2 ectodomain [7, 27, 28]. Initial data showed that the antibody was most effective in the treatment of cell lines with HER2 gene

amplification/protein overexpression [27, 29]. Encouraged by these results, several clinical studies investigated the effects of Herceptin in the treatment of HER2 positive breast cancer.

In metastatic breast cancer (MBC) single-agent treatment with Herceptin was shown to be successful and well tolerated in a first-, second-, or third line setting [30, 31]. As monotherapy in a first-line setting Herceptin induced an overall response rate of 19-26% in MBC [30, 32].

However, Herceptin is more effective when combined with chemotherapy.
A phase 3 trial compared chemotherapy (doxorubicin plus cyclophosphamide or paclitaxel) versus chemotherapy with first-line Herceptin in a cohort of 469 women with HER2 positive MBC. The Herceptin arm in comparison with chemotherapy alone had a significantly prolonged time to disease progression (TTP, 7.4 vs. 4.6 months), a better objective response rate (ORR, 50% vs. 32%), a longer median duration of response (9.1 vs. 6.1 months), and a reduced 1-year mortality rate (22% vs. 33%) [33].
The effects of Herceptin in early breast cancers (EBC) were later assessed in various studies, including the Herceptin Adjuvant (HERA) and the FinHER study. Overall more than 13,000 patients with HER2+ EBC have been enrolled in those studies. Despite the differences in the patient populations, chemotherapy regiments, and median follow up times, adjuvant therapy with Herceptin consistently showed a 50% reduction of the risk of reoccurrence. Furthermore, one study determined a significantly longer overall survival time and in other studies, with a shorter follow-up, a clear trend towards better survival became apparent [34].

Despite these promising results, adverse events (AE) have also been reported. The most frequent AE is cardio-toxicity. In the above mentioned study in MBC, cardiac dysfunction

was reported in 27% of patients treated with Herceptin in combination with doxorubicin vs. 8% of patients receiving chemotherapy alone (13% vs. 1% in the groups treated with Herceptin in combination with paclitaxel vs. paclitaxel alone respectively) [33].

Patients enrolling for the trials conducted in the setting of EBC were required to have no previous or current cardiac disease. The rate of cardiac events in the Herceptin arm was similar across all studies, with an overall incidence between 0.6 and 3.3%. Moreover, the majority of patients with symptomatic congestive heart failure (CHF) improved with treatment or when Herceptin treatment was stopped [34]. On the contrary, results of the FinHer study, where Herceptin was only given for nine weeks and prior to anthracyclin treatment, showed that patients receiving a combination treatment had better left ventricular ejection fraction (LVEF) than patients treated with chemotherapy only [35].

Although the mechanisms of action of Herceptin are not fully understood, the U.S. Food and Drug Administration (FDA) approved the drug for treatment of HER2-overexpressing breast cancer in adjuvant and metastatic settings due to the results of several clinical studies that showed improved survival of patients [31, 33, 36, 37].

As Herceptin is an IgG1, it is likely that the different parts of the antibody may mediate different effects. The Fab-fragment recognizes the HER2 epitope and may be responsible for most of the pharmacodynamic effects of Herceptin.

Herceptin was shown to be unable to block ligand induced HER2/HER3 dimerisation [38], but to be effective in the treatment of breast cancers driven by HER2 homodimers [39]. Binding of Herceptin to the extracellular domain of HER2 was found to induce membrane localization and activation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a negative regulator of PI3K and therefore of AKT signalling [40].

Moreover, Herceptin treatment leads to cell cycle arrest in G1 by upregulation of the cyclin-dependent kinase 2 (CDK2) inhibitor p27 [41] and upregulation of p21, which inhibits DNA synthesis [42, 43].

Inhibition of HER2 extracellular domain cleavage [44], receptor internalization [1], as well as regulation of angiogenesis by decreasing VEGF [45] are also proposed mechanisms of action. The Fc-fragment of the antibody is relevant for both pharmacodynamic and pharmacokinetic properties of Herceptin. The Fc-fragment is important for the antibody-dependent cell cytotoxicity (ADCC) by interacting with natural killer T-cells [46, 47].

1.4.1 Herceptin resistance

Although the results of the clinical studies are remarkable, it should not be neglected that the response rate to Herceptin monotherapy is less than 30%. Primary resistance is a major problem and most of the patients, who initially respond well, progress within one year of treatment. Various mechanisms of resistance are briefly described below. It is important to understand the underlying molecular mechanisms in order to improve treatment and patients' outcome.

Loss of PTEN

One mechanism of action of Herceptin is the accumulation of active PTEN at the cell membrane. However, mutation or loss of this protein occurs in up to 50% of breast cancers and these tumours therefore lack an important mechanism of PI3K/AKT regulation [48].

Depletion of PTEN in xenograft models was shown to reduce the response to Herceptin in comparison to controls. In concordance with this, patients with PTEN-deficient breast tumours show a lower clinical response rate when treated with Herceptin [40].

PI3K

PI3K is a downstream protein of PTEN and important in the regulation of cell survival as it positively regulates AKT signalling. Mutations in the alpha subunit of PI3K are present in about 25% of breast cancers and were correlated with HER2 positivity [49].

PTEN loss and PI3K mutation are associated with poorer prognosis [50].

AKT/PI3K inhibitors are currently under investigation. She et al. (2008) [51] demonstrated that breast cancers with HER2 overexpression (but primary Herceptin resistant) or

PI3K/PTEN mutation were sensitive to AKT inhibition *in vitro* and *in vivo*. Moreover, PI3K inhibition was able to restore Herceptin sensitivity in PTEN-loss cells and xenograft models [40].

p27kip

The cell cycle control protein p27kip is regulated by AKT signalling. Active AKT phosphorylates threonine 157 of p27, thereby inhibiting nuclear translocation [52]. HER2 overexpression is related to increased cytoplasmic p27 and faster tumour growth [53].

Herceptin treatment leads to an upregulation of nuclear p27 by downregulation of AKT activity. Reversely, Herceptin resistant cells show lower p27 levels compared to parental cell lines. The reduced level of p27 was correlated with an increased activity of CDK2 and proliferation [53]. Moreover, induction of p27 in resistant cells enhanced response to Herceptin [54].

Reduced binding of Herceptin to HER2 and signalling through p95

Mucin 4 (Muc4) is a transmembrane glycoprotein that is associated with epitope masking, which has been investigated as a resistance mechanism to Herceptin, and knockdown of Muc4 could reverse resistance [55]. Another protein complex associated with epitope masking is CD44/hyaluronan. Inhibition of hyaluronan synthesis reduced its levels *in vitro* and *in vivo* and increased binding of Herceptin to HER2 and Herceptin effects [56].

Herceptin binds to subdomain IV in the extracellular domain of HER2. However, about 30% of all HER2 overexpressing breast cancers express a HER2 variant lacking the extracellular domain [57]. Herceptin therefore cannot target the remaining p95HER2 and patients expressing p95HER2 were found to respond less to Herceptin treatment [58]. The p95HER2 is able to undergo dimerisation with other HER family members. A HSP90 inhibitor, which counteracts the chaperon important for HER2 stabilization, could inhibit p95HER2 signalling [59].

Upregulation and activation of HER receptors or other tyrosine kinase receptors

Although Herceptin targets the HER2 receptor, the treatment does not abolish its activation [60]. IGF-IR overexpression, heterodimerisation of this receptor with HER2 and increased activation of the latter due to IGF-I stimulation were associated with Herceptin resistance [50, 61, 62].

The c-Met receptor is often co-expressed with HER2 and HER2 overexpressing cell lines treated with Herceptin show an increased expression of c-Met, which was also associated with resistance [63].

Long-term treatment of cell lines with Herceptin led to an increase of EGFR and HER3 and Geyer et al. (2006) [64] showed that treatment with the tyrosine kinase inhibitor Lapatinib was beneficial in patients who progressed on Herceptin. However, it was later published [65] that 60% of all patient had received Herceptin until 8 weeks before Lapatinib and a possible overlap of both treatments due to the long half-life of Herceptin in patients‘ serum could not be excluded. Nevertheless, Baselga et al. (2012) [66] presented the neoALTTO trial, in which the combination of Herceptin with Lapatinib was superior to Herceptin alone in the setting of EBC, indicating a role for EGFR during Herceptin treatment.

Gijsen et al. (2010) [67] found an AKT feedback loop, which triggered ligand release through the increased expression of ADAM17. This was associated with an increased heterodimerisation of HER receptors and is a possible resistance mechanism to Herceptin.

1.5 The ADAMs protein family

The ADAMs (a disintegrin and metalloproteinase) and related ADAMTS, which have additional thrombospondin domains, belong to the super family of metzincins, as do class III snake venom metalloproteinases [68].

ADAM1 and 2 were the first ones to be identified and are important for sperm-egg fusion which emphasized the similarity of ADAMs to viral proteins involved in cell fusion [69].

Generally, an ADAM protein consists of various domains and as depicted in figure 1.4, the protein is thought to have a C-shape.

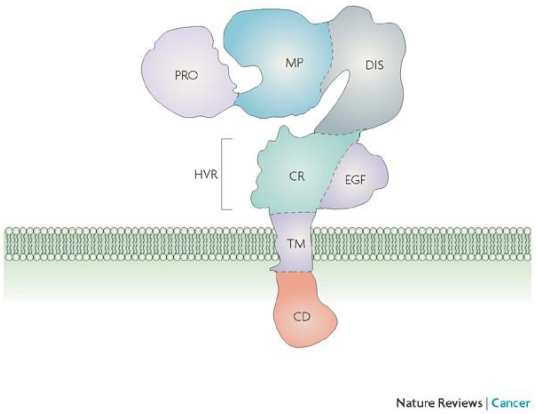


Figure 1.4 Structure of ADAMs, PRO – propeptide, MP – metalloproteinase domain, DIS – disintergrin domain, CR – cystein-rich region, EGF – epidermal growth factor-like repeat, TM – transmembrane domain, CD – Cytoplasmic domain. Figure taken from Murphy (2008) [68]

ADAMs are synthesized in the endoplasmic reticulum (ER) and are then processed in a late Golgi compartment. The folding of the protein as well as disulphide bond formation is heavily dependent on the presence of the N-terminal pro-domain, which acts as a chaperone [70]. The metalloproteinase domain identified in about half of all family members displays a distinct HExG HxxG xxHD consensus sequence that was predicted to be the catalytic site of active zinc-dependent metalloproteinases. It is assumed that all members with this sequence will have proteolytic activity [71]. This active site contains zinc and water atoms, that were shown to be necessary for the hydrolytic processing of distinct substrates, tightly controlled by three histidine residues and another water molecule [72].

The disintegrin domain present in ADAMs is able to mediate cell-cell and cell-matrix interactions by binding integrins, especially α9β1 [73]. However, other integrins are also able to bind the disintegrin domain [74].

The cysteine-rich domain was proposed to be important for binding of ECM constituents independently from a disintegrin-integrin interaction. Binding of ADAM12 to proteoglycans such as syndecan was found to be dependent on this region [75].

The cytoplasmic domain of different ADAMs varies considerably in length and sequence. However, several ADAMs display PxxP motifs, which act as a binding site for Src-homology (SH3) domain-containing proteins [76].

1.5.1 Regulation of ADAMs

It is thought that ADAMs are mostly regulated at the post-transcriptional level. Interactions of the pro-domain with the catalytic domain render ADAMs inactive whilst in the Golgi [77]. Cleavage of the pro-domain by pro-protein convertases, eg furin, leads to the maturation of several ADAMs, whereas other ADAMs may auto-activate [78].

It seems that the localization of ADAMs and their substrates is also of importance. ADAM10 substrates are present at adherens junctions of polarized epithelial cells and trafficking of ADAM10 to these sites is necessary for E-cadherin cleavage as well as cell migration [79]. Mature ADAM17 is enriched in lipid rafts and PMA stimulation leads to co-localization and enhanced shedding of TNFα [80].

ADAM activity is also influenced by various proteins and signalling mechanisms.

ADAM17 has been shown to be transiently upregulated by protein kinase C after the addition of phorbol esters, although long-term application led to a decrease of ADAM17 on the cell surface [81]. The MAPK pathway was found to play a role in the constitutive shedding of TGFα, an ADAM17 substrate [82], as well as in the release of membrane pro-HB-EGF in response to osmotic or oxidative stress in crosstalk with various ADAMs [83]. This pathway [84] also mediates CD44 shedding by ADAM10 in EGF treated glioblastoma cells.

Ca^{2+} influx was shown to enhance the shedding of the chemokines CX3CL1 and CXCL16 by ADAM10 [85] and ionomycin or a Ca^{2+}/calmodulin inhibitor triggered shedding of EGFR-ligands by ADAM10 although levels of mature ADAM10 did not change in this setting [86]. Various G-protein-coupled receptors (GPCR) activate cross-signalling partly through EGFR

via the generation of ligands by several ADAMs [87]. Beside other factors, Ca^{2+} was also found to be involved in the crosstalk between GPCRs and ADAMs [88]. Stimulation of bombesin receptors (GPCR) led to enhanced ligand cleavage by ADAM10 and it was shown that cross-activation of EGFR was dependent on this metalloproteinase activity [89]. Moreover, the redox status may be of importance. The thiol isomerase protein disulfide isomerase (PDI) may block ADAM17 activation and its downregulation enhanced ADAM17 activity by modulation of its disulfide bonds [90].

However, many ADAMs are also regulated at the gene level and alternative splicing, producing soluble ADAM proteins, has been reported. An example of this is ADAM9 that is present in a soluble and a membrane bound form due to alternative splicing. Interestingly, the membrane bound form inhibits cell migration dependent on the integrin binding domain, whereas the soluble form of ADAM9 promotes migration, the action of which is dependent on the metalloproteinase activity [91]. This brief summary shows that the action of ADAMs is tightly regulated and it may depend on various factors as well as structural conditions.

1.5.2 Shedding of proteins by ADAMs

Protein shedding via various ADAMs is important for cell fate determination, migration, wound healing, proliferation and several other mechanisms. About 4% of cell-surface proteins undergo ectodomain shedding [92]. ADAMs also play a major role in regulated intra-membrane proteolysis (RIPping). After ectodomain shedding via an ADAM, the presenilin-containing γ-secretase complex leads to the release of the intracellular domain (ICD) of the protein. This was shown for several proteins such as Notch, CD44, HER4, and amyloid precursor protein (APP) and the ICDs may function as transmembrane chemokines [93] or may translocate to the nucleus and act as transcription factors [94, 95].

1.5.3 ADAM10

Knockout studies showed that expression of ADAM10 is necessary for development and physiological processes [96, 97]. ADAM10 was implicated in the shedding of a number of proteins and was associated with several tumour diseases:

- N-cadherin cleavage [98]
- E-cadherin shedding induces β-catenin translocation and subsequent activation of target genes such as cyclin D1 [99]
- Extracellular domain shedding of L1-CAM promotes metastasis in colon cancer [100] and shedding of CD44 may promote migration of cancer cells [101]
- Fas ligand shedding by ADAM10 [102] can have pro- and anti-apoptotic effects
- Notch receptor and its ligand Delta 1 are also substrates [103] for ADAM10
- Ephrin-A2 shedding via ADAM10 is highly regulated [104]
- ADAM10 is upregulated in a Hif-1 dependent manner during hypoxia, which leads to shedding of MHC class I-related chain A (MICA). This counteracts the response of natural killer cells to tumour cells [105, 106]
- ADAM10 overexpression was found in leukemia [107] and in tumours of gastric [108], ovary [109], prostate [110], and colon [111]
- ADAM10 mRNA was increased in response to androgens, IGF1, and EGF stimulation in prostate cancer cell lines; nuclear and perinuclear staining for ADAM10 in prostate tumour tissue was found [110]
- In relation to the HER-family, the ligands EGF and betacellulin [112] as well as the HER2 receptor [113] are shed by ADAM10 as shown in figure 1.5. The level of HER2 ECD in blood samples of cancer patients was found to correlate with a poorer prognosis [114]

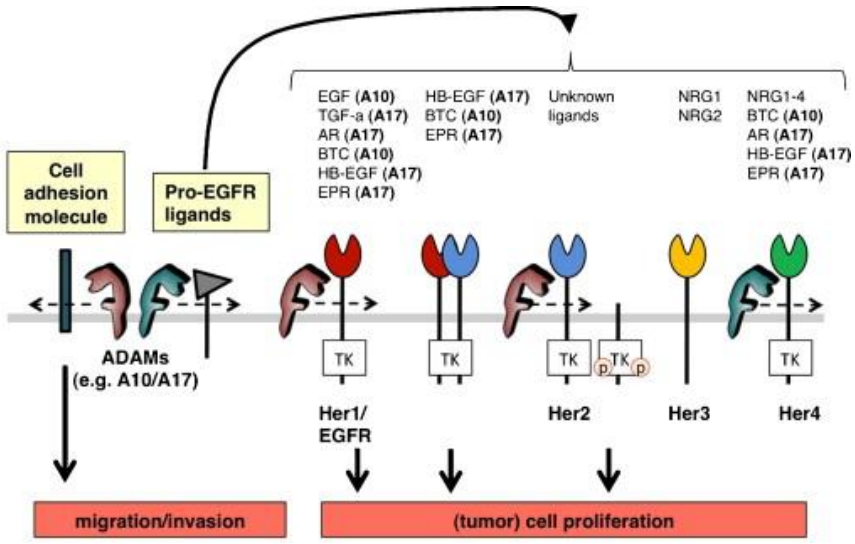


Figure 1.5 The relation of ADAM10/17 to the HER family. Figure taken from Saftig and Reiss (2011) [115]

1.5.4 ADAM inhibitors

Tissue inhibitors of metalloproteinases (TIMPs) are natural but weak inhibitors of ADAMs. ADAM10 was found to be a target of TIMP 1 and TIMP 3 and in contrast to ADAM17 inhibition, the isolated N-terminal domains of these two proteins are not sufficient for the inhibition of ADAM10 [116]. Earlier attempts to inhibit metalloproteinases/ADAMs failed due to the low specificity of the compounds leading to various side effects [117]. In order to overcome this problem, compounds that are more specific are needed.

Newer ADAM inhibitors are hydroxamic acid-derived and show better specificity as depicted in table 1.3. In this project a dose of 5µM of the ADAM10 inhibitor (INCB8765) was used in all experiments as advised by the manufacturer.

Table 1.3 Overview of ADAM inhibitors. Table taken from Zhou et al (2006) [118]

Enzyme	INCB3619 (IC50, nM)	Marimastat (IC50, nM)	INCB3420 (IC50, nM)	INCB8765 (IC50, nM)	INCB4298 (IC50, nM)
ADAM10	22	78	5000	97	>5000
ADAM17	14	12	1720	2045	<5
ADAM33	1026	155	>5000	4125	145
ADAM9	>5000	247	>5000	>5000	1100
MMP1	>5000	4	>5000	>5000	>5000
MMP2	35	3	20	>5000	>5000
MMP3	3560	30	>1000	>5000	3179
MMP7	>5000	<5	>5000	>5000	122
MMP9	304	<5	96	>5000	>5000
MMP12	17	<5	8	564	334
MMP14	772	<5	700	>5000	>5000
MMP15	60	<5	73	>5000	>5000

Friedman et al. [119] reported a phase I/II trial using the compound INCB7839, an ADAM10/17 inhibitor, to test drug safety and preliminary efficacy. One of the aims was to monitor the levels of HER2 ECD and p95HER2 in patients with HER2+ metastatic breast cancer during treatment with INCB7839 in combination with Herceptin or Herceptin/docetaxel. It was shown that a reduction of HER2 ECD could be achieved by INCB7839 and the reduced level was associated with the response to treatment. Moreover, the authors stated that the new drug was well tolerated.

1.6. Aim and Objectives

1.6.1. Aim

Primary or acquired resistance to Herceptin remains a drawback of this treatment option for patients diagnosed with HER2 overexpressing breast cancer. An understanding of the underlying pathology is needed to improve therapy and outcome for these patients.

Kong et al. [120] showed that resistance to EGFR tyrosine kinase inhibitors (TKI) was mediated by activation of alternative HER receptors and was triggered by the ligands heregulin and betacellulin. Gijsen et al. [67] presented data implicating a role for ADAM17 in Herceptin resistance. The main focus of this project was to investigate the role of ADAM10 during Herceptin treatment, but also in acquired resistance. Furthermore, the effects of ADAM10 inhibition were elucidated.

1.6.2. Objectives

- Investigate the levels of ADAM10 during Herceptin treatment using qRT-PCR and western blot
- Analysis of the expression of the ligand betacellulin during Herceptin treatment and the impact of ADAM10 inhibition using western blot and ELISA
- Assessment of the effect of ADAM10 inhibition using an inhibitor or knockdown in sensitive and resistant cell lines
    - Protein expression (using western blot)
    - Cell viability (using cell count, apoptosis assays and clonogenic assay)
- Assessment of the level of ADAM10 after Herceptin treatment of BT474 xenograft mice in comparison to control
- Analysis of ADAM10 expression in TMA samples in relation to clinical and survival data of patients

2 Materials and Methods

2.1 Materials

2.1.1. Cell lines

BT474 and SKBR3 cell lines used in this work were provided by cell services at Cancer Research UK (Lincoln's Inn Fields laboratory). Trastuzumab resistant cell lines have been generated in Dr. Kong's laboratory as previously described [67]. Briefly, native BT474 and SKBR3 cells were treated with increasing concentrations of Herceptin until 40µg/ml. Thereafter, both cell lines have been kept in medium supplemented with this dose of Herceptin for more than 6 months.

2.1.2 Solutions

PBS	137mM NaCl, 2.7mM KCl, 4.3mM Na2HPO4, 1.4mM KH2PO4, Ca2+ and Mg2+, pH 7.3
PBS-T	0.2% (v/v) Tween 20 in PBS
Trypsin EDTA 1x	2.5% (w/v) trypsin (10x) diluted in PBS to a 1x concentration
Complete Mini Protease Inhibitor Cocktail	1 tablet in 1ml of lyses buffer
Cell lysis buffer	20mM Tris-HCl (pH 7.5), 150mM NaCl, 100nM NaF, 100mM Na2P2O7, 10nM EDTA, supplemented with1% (v/v) Triton X-100, 10% (v/v) Complete Mini Protease Inhibitor Cocktail solution, 1% (v/v) Phosphatase inhibitor cocktail 2 and 3
4xLDS sample buffer	4x LDS sample buffer supplemented with 10% (v/v) β-mercaptoethanol
Transfer buffer	25mM Tris-HCl, 192mM glycine, 20% (v/v) methanol

2.1.3 Reagents

	Material	Source
Cell culture	RPMI 1640 DMEM Trypsin EDTA 2.5% in PBS	Invitrogen
	Foetal calf serum Penicillin/Streptavidin	PAA
Cell counting	ISOTON® II diluent	Beckmann Coulter

Transfection	Opti-MEM® I Reduced serum Medium GlutaMAX TM Oligofectamine™ Reagent	Invitrogen
	ADAM10 siRNAs and universal negative control	Sigma Aldrich
Cell lysis	Triton X -100 Phosphastase Inhibitor cocktail II Phosphatase inhibitor cocktail III	Sigma
	Complete Mini Protease Inhibitor cocktail tablets	Roche
Antibody - Biotin conjugation	Lightning-Link® Biotin conjugation kit Type B	Innova Biosciences
Co-Immunoprecipitation	Dynabeads® M-270 Streptavidin	Invitrogen
Bradford assay	BioRad Protein Assay	Biorad
SDS-Page gel and Western blot	4x LDS sample buffer	Invitrogen
	NuPAGE® 4-12% Bis-Tris Gels	
	Novex® sharp protein standard	
	20x MOPS SDS running buffer	
	Blotting Filer paper	
	Immobilon®	Millipore
	Beta-mercaptoethanol	Sigma Aldrich
	Bovine serum albumin	
	Tween 20®	
	ECL detection reagents	GE Healthcare
	Medical X-Ray film	Fujifilm
	Restore™ PLUS western blot stripping buffer	Thermo Scientific
MTT assay	Thiazolyl Blue Tetrazolium Bromide	USB corporation
RNA extraction	RNeasy Mini kit	Qiagen
	DNase and DNase dilution solution	BioRad
cDNA	High Capacity cDNA Reverse Transcription kit	Applied Biosystems
PCR	SensimixTM SYPR Mastermix (20x)	Bioline
FACS	APC-Annexin V Antibody Annexin V Binding buffer Propidium Iodide	BD Pharmingen
IHC	2.5% Horse serum blocking solution DAB	Vector Laboratories

2.1.4 Drugs

Generic name	Trade name	Dose	Source
Trastuzumab	Herceptin	40 µg/ml	Roche
INCB8765	ADAM10-inhibitor	5µM	Incyte
	Camptothecin	6 µM	Sigma Aldrich
	Akt/PKB inhibitor	2.5µM	
MG132	Proteasome inhibitor	25µM	

2.1.5 Primer sequences used for qRT-PCR

Primer		Oligonucleotide sequence
ADAM10	Forward	5′-ATATTACGGAACACGAGAAGCTG-3′
	Reverse	5'-TCAATCGCTTTAACATGACTGG-3'
ADAM17	Forward	5'-CCTTTCTGCGAGAGGGAAC-3'
	Reverse	5'-CACCTTGCAGGAGTTGTCAG-3'
Actin	Forward	5'-AAT GGC AAT GAG CGG TTC-3'
	Reverse	5'-GGA TGC CAC AGG ACT CCA T-3'

2.1.6 Antibodies used for Western blot

Antibody	Source	Concentration
Primary Antibodies		
EGFR	Santa Cruz Biotechnology	1:500
pEGFR (Tyr 1173)		
HER2 (21D3)	Cell Signalling Technology	1:1000
pHER2 (Tyr1221/22)		
HER4	Santa Cruz Biotechnology	1:500
pHER4 (Tyr 1056)		
AKT	Cell Signalling Technology	1:1000
pAKT (Ser 473)		
P44/42 MAPK ERK (1/2)		
p-p44/42 MAPK (Thr 202/Tyr 204) (ERK 1/2)		
Rabbit IGG		
Actin (13E5)		1:5000
Cleaved Caspase 7		1:500
Albumin		1:500
ADAM10	Calbiochem	1:10 000

Betacellulin	Abcam	1:500
Secondary Antibodies		
ECL Anti-rabbit	GE HealthCare	1:2500
ECL Anti-mouse	GE HealthCare	

2.1.7 Antibodies for IHC

Antibody	**Source**
ADAM10, concentration: 1:200	Abcam
ImmPress^{TM} Reagent Anti-Rabbit IgG (Peroxidase)	Vector Laboratories

2.2 Methods

2.2.1 Cell culture

General growth conditions

Cells were grown in 75cm^2 or 175cm^2 flasks (Cornig, USA) with vented cap and kept at 5% CO_2 at 37°C. BT474 and BT474 Herceptin resistant cell lines were cultured in Roswell Park Memorial Institute Medium (RPMI 1640), supplemented with 10% FCS, 100 IU/ml penicillin, and 100 mg/ml streptomycin.

SKBR3 and SKBR3 Herceptin resistant cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with the same supplements.

Subculture

Cells were subcultured at 80%-90% confluency. Briefly, the medium was aspirated and cells were washed once with PBS. An appropriate volume of trypsin/EDTA was added and cells were allowed to detach at 37°C. An appropriate amount of medium (10x volume of trypsin/EDTA) was then added to the cells to obtain a single cell suspension.

2.2.2 Experimental set-up

400µl of cell suspension were mixed with 19.6 ml of isotonic buffer and counted using a cell counter machine. An appropriate number of cells depending on the cell line, the size of the

dish as well as the planned duration of treatment were seeded in full media and left overnight to adhere.

For experiments with treatments up to 48 hours, cells were washed with serum free media once. Drugs/compounds were pre-mixed with serum-free media and applied. For experiments with treatment longer than 48 hours (e.g. FACS, cell counting, and MTT assay), the same was done with serum-reduced media (5% FCS). Resistant cell lines were continuously treated with Herceptin unless stated otherwise.

Appropriate controls were applied in the same concentration as the respective drug/compound.

2.2.3 Co-Immunoprecipitation

Antibody-Biotinylation

Isotype specific IgG and EGFR antibodies were biotinylated using the Biotin Conjugation kit and used in combination with streptavidin beads for co-immunoprecipitation. In accordance with the manufacturer's instructions, 1µl of LL Modifier per 10µl of antibody were added to the Lightning-Link^{TM} mix and left at RT overnight. The next day 1µl of Quencher per 10µl of antibody was added and the solution stored at 4°C.

Pull down

Protein was extracted as described in the next section. Samples were pre-cleaned with 4µg isotype control IgG and 60µl of streptavidin beads for 1 hour at 4°C. The tubes were centrifuged and placed on a magnetic stand. Protein content was measured and normalized using Bradford assay as outlined below. Samples were then incubated overnight with biotinylated EGFR antibody or isotype control IgG at 4°C. 10µg of antibody was used per 1000µg of protein in each experiment. Beads were then washed four times with full lyses buffer and boiled in 40µl of 2X loading buffer at 70°C for 15 min before loaded on a gel.

2.2.4 Western Blotting

Protein extraction

Cells were placed on ice, medium was taken off and either collected (see following section) or discarded. Cells were washed once with ice-cold PBS and treated with lyses buffer for at least 10 min. Cells were then scraped and the lysate transferred to 1.5ml tubes. A centrifugation step at 13,500 rpm and 4°C for 10 min was followed by collection of the supernatant which was further processed or stored at -80°C.

Media Procedures

If media was needed for Western blot analysis, the media was collected and centrifuged to remove dead cells. The supernatant was transferred into filter centrifugation tubes (Amicon® Ultra) and centrifuged at 2000 rpm at 4°C until about 80µl remained. The sample was collected, protein content normalized, and each sample mixed with 4x sample buffer.

Protein quantification

Protein amount was determined using Bio-Rad Protein Assay according to the manufacturer's instructions. Serial dilutions of bovine serum albumin (2, 4, 6, 8, and 10 µg/ml) were used as standard. Bio-Rad dye reagent was diluted with water (1:5) and 500µl of the solution was mixed with an appropriate amount (between 2-4 µl) of sample or 1µl of standard. After a 5 minute incubation at room temperature (RT) two lots of 200µl from each tube was added to a 96-well plate. Absorbance at wavelength 595nm was measured using a plate-reader and samples were normalized.

SDS – polyacrylamide gel electrophoresis (SDS-page)

Samples were mixed with sample buffer in a 1:4 ratio and boiled for 8 min. Equal amounts of protein were loaded on precast gels and the tanks were filled sufficiently with running buffer. The initial voltage for running the gels was 100V, which was increased to 130V after the first 10 min of the run.

Semi-dry transfer

After separation in the gel, proteins were transferred using a Biorad Trans-Blot Semi-Dry Transfer machine. For each gel two filter pads were soaked in transfer buffer and a PDVE-membrane was equilibrated in methanol. The gel was placed on the membrane between the two filter pads. The transfer was achieved by applying a voltage of 12V for 2 hours.

Blotting procedure

At the end of the transfer, membranes were blocked in 3% BSA in PBS-T (w/v) for either 1 hour at RT or overnight at 4°C. Membranes were cut according to molecular markers and primary antibodies were applied either for 3 hours at RT or overnight at 4°C. Membranes were subjected to four 10-minute washes using 1% milk in PBS-T (w/v) followed by 1 hour incubation at RT with secondary antibody in 5% milk PBS-T (w/v). After another washing step of four times 15 min with the above mentioned washing solution, membranes were reassembled and covered with ECL developing solution for 2 minutes. For development membranes were exposed to medical X-ray films.

2.2.5 Transient knockdown – reverse transfection

In order to knockdown ADAM10, reverse transfection method was used. OptiMEM medium was mixed with siRNAs or scramble to give a final volume of 185µl. Untreated and mock samples did not contain any siRNA. Oligofectamine was mixed with OptiMEM in a 1:4 ratio and allowed to incubate for 5 min. 15µl of this mixture was applied to each sample, except the “untreated” and incubated for 20 min. Cells were trypsinised and after a centrifugation step at 1000 rpm for 4.5 min, 200,000 cells/ml for mRNA/western blot analysis and 40,000 cells/ml for cell counting studies were taken up in serum-only media. 0.8ml of cell suspension was added to each previously prepared tube and cells plated. The next day, media was replaced by serum-only media before the cells were incubated for 48h to assess mRNA levels; for 72h to do western blot analysis; and for 5 days to investigate the effect of

knockdown on cell number. Resistant cell lines were co-cultured with Herceptin for the duration indicated in distinct experiments.

2.2.6 mRNA analysis using qRT-PCR

Prior to procedures involving RNA, all surfaces and pipettes were cleaned with RNAse ZAP RNAse decontamination solution (Ambion).

RNA extraction from cultured cells

RNA was extracted using the RNeasy Mini Kit according to the manufacturer's instructions. Cells were placed on ice, the media and two washes of PBS were discarded. Cells were then lysed using 350µl of the provided RNA complete lysis buffer. This was mixed with the same volume of 70% ethanol and applied to a RNA binding column. Columns were centrifuged at 8000g for 30 min. This was followed by a washing step and incubation with DNAse I for 15 min at RT. Columns were washed twice and were centrifuged three times before being transferred to new microcentrifugation tubes for RNA elution. 80µl of RNAse free water was used for elution and samples stored -80°C.

Generation of cDNA

After RNA extraction the amount of nucleic acid was quantified. A 2µl sample of each extracted RNA was measured in duplicate. An absolute amount of 2000ng of RNA was diluted in 50µl of nuclease-free water.

The High Capacity cDNA Reverse Transcription Kit was used for cDNA synthesis. 50µl of a master-mix (10µl 10X RT buffer, 10µl 10X Random Primers, 4µl 25X dNTPs, 5µl MultiScribe Reverse Transcriptase, and 21µl Nuclease-Free water) was added to each 50µl RNA sample. The mix was incubated for 10 min at 25°C followed by 2 hours at 37°C in a Thermal Cycler (GeneAmp, PCR System 9700, Applied Biosystems). Samples were then stored at -80°C.

qRT-PCR

Primers for qRT-PCR were taken up in RNAse-free water in a final concentration of 100µM. Working dilutions had a concentration of 10µM of forward and reverse primers.

For qRT-PCR 15µl of master mix (10µl Sensimix SYBR Mastermix, 2µl of primers and 3µl of nuclease-fee water) were mixed with 5µl of cDNA in triplicate. The housekeeping gene actin was used to normalize samples to each other.

qRT-PCR was carried out using Rotor Gene RG 3000 thermal cycler (Corbett Research) which was set at the following profile for all reactions: hold phase at 95°C for 15 min, 40 cycling runs (95°C for 10 seconds and 60°C for 1 minute) and a melt phase (from 50-99°C).

2.2.7 Cell viability studies

Three different techniques were used to assess the effect of ADAM10 inhibition in HER2 positive breast cancer cells at various settings. The media was not replaced in either setting.

MTT assay

500 cells/well were seeded in a 96-well plate and allowed to settle overnight. 200µl of previously prepared serum-reduced media with the respective treatments were applied in triplicate including controls. At the end of treatment 50µl of MTT solution was added and incubated for 30-45 min at 37°C. When a sufficient change in colour was observed, the media was discarded and 100µl of DMSO added. Intensity of colour was measured by a plate reader at 560 and 670nm.

Cell counting

1.5 x 10^4 cells were seeded in 24-well plates and allowed to settle overnight. The next day, media was replaced by serum reduced media. Treatment was done in triplicate for each setting. At the end of treatment, the media was discarded and cells washed with PBS. 500µl of Trypsin/EDTA was added and cells allowed to detach for 10 to 15 min depending

on the cell line. 400µl of each sample were added to 19.6ml of isotonic buffer and cells were counting using a cell counter.

Assessment of level of cleaved caspase 7

The protein family of caspases (cystein aspartate protease) comprises several members. Caspases are inactive pro-enzymes that are activated by proteolytic cleavage. Sequential activation of caspases is necessary during apoptosis. The level of cleaved caspase 7 is a significant marker for apoptosis. Cells were treated for 72h in serum-reduced medium and the level of cleaved caspase 7 was assessed using western blot.

FACS analysis

In early stages of apoptosis the negatively charged phophatidylserine flips from the inside of the cell membrane to the outside, and can be recognized by Annexin V. This makes Annexin V positivity a good indicator for apoptosis. Propidium Iodide binds to DNA but is excluded in viable cells because of the intact cell membrane. Therefore, cells negative for Annexin V and Propidium Iodide were considered viable and those that were positive were the apoptotic cells. The degree of apoptosis induced by various treatments was investigated by staining cells for Annexin V and Propidium Iodide. Briefly, 1.5 x 10^5 cells were seeded in 10cm dishes and the next day media was replaced with serum-reduced media and cells were treated. 5 to 6 hours before analysis 6µM of Camptothecin was added to one plate for a positive control.

For analysis the media as well as the PBS used for washing the cells were collected and used to take up trypsinised cells. Subsequently, cells were washed twice with ice cold PBS. An absolute number of 10^6 cells was taken up in 1ml of 1X Annexin V binding buffer and 150µl of each sample were transferred to FACS tubes. Samples were incubated with 5µl of Annexin V antibody and 2µl of Propidium Iodide for 20 min in the dark. 500µl of Annexin V binding buffer was added and samples placed on ice. Analysis was carried out within 1 hour of staining using Summit 4.3 software. In each experiment unstained and single stained

samples determined the voltage-settings for both stainings and compensation was adjusted manually when necessary.

Clonogenic assay

Clonogenic assays were performed as advised by Franken et al. (2006) [121]. Briefly, cells were pre-treated in serum-reduced medium for 5 days before re-plating. In each condition 1000 cells were seeded in duplicate in full medium and cells left to form colonies for 12 days. Afterwards, the media was discarded and cells washed once with PBS. Cells were stained with 0.2% methylene blue in 50% ethanol for 30 min and carefully washed with water. Colonies were counted by eye and number of colonies compared between different treatment conditions.

2.2.8 Immunohistochemistry (IHC)

Preparation of Cell pellet

BT474 cells were reverse transfected as outlined in section 2.2.5. Cells were trypsinised after 72h and after centrifugation cells were re-suspended in 4% PFA and left overnight at 4°C. After a second centrifugation step cells were stored in PBS at 4°C.

Subsequently, samples were spinned down, the PBS removed and the cells dissolved in 2% Formalin-agarose. The resulting cone was processed using an automatic tissue processor to ensure paraffin infiltration and embedded in paraffin blocks. 4µm sections were cut and fixed on X-tra Adhesive slides (Leica).

IHC

Samples for immunohistochemistry were placed in an oven and submerged for 5 min each in Citroclean (twice), 100% ethanol (twice), 50% ethanol, and tap water. For antigen retrieval slides were placed in Tris-EDTA buffer pH 6 and pressure cooked. Afterwards slides were washed in PBS twice, blocked for 30’ at RT with horse serum and the primary antibody was applied in 1% BSA PBS overnight at 4°C. Slides were again washed with PBS twice and secondary antibody was applied for 30’ at RT. Following another washing step,

the slides were incubated with DAB (3,3'-Diaminobenzidine) until brown staining was visible, before they were counterstained with haematoxylin solution and mounted using Aquatex. Slides were scored according to the intensity (0, no staining, 1, weak staining, 2 moderate staining, 3 dark staining) and area (%) of staining

2.2.9 ELISA

For ELISA a kit from R&D systems was used. Briefly, a 96-well microplate was coated with 100µl/ well of mouse anti-human betacellulin and incubated at RT overnight.

The next day, wells were blocked with 300µl 1% BSA in PBS for 1 hour, before 100µl of samples or standards were applied. After incubation for 2 hours at RT, the detection antibody and the working solution were added and incubated for 20 min in the dark. Between these steps, wells were washed with 0.05% Tween®/PBS. After incubation with the working dilution, the substrate solution was applied for 20 min in the dark before the reaction was stopped with the provided stop solution. Plates were measured using a plate reader.

2.2.10 Statistical analysis

Prism 6 software (GraphPad software) was used for statistical analysis. Statistical significance was calculated using Kruskal-Walis Test if more than two conditions were compared. For comparison of two conditions, a Mann-Whitney-U Test was used. Correlation of patients' data and ADAM10 staining was calculated using Fischer's Exact Test and correlation with survival data was performed using Log-rank Test.

3 Results

As discussed in the introduction, ADAM10 is a major sheddase of HER-family ligands, including betacellulin. Moreover, the protein has been shown to be relevant in various cancers. Here, the role of ADAM10 in relation to treatment with and resistance to Herceptin, was assessed by looking at the level of ADAM10 and the ligand betacellulin during Herceptin treatment and the effects of ADAM10 enzyme inhibition or knockdown were investigated.

3.1 Herceptin treatment increases ADAM10 in HER2 overexpressing cell lines

To assess the effect of Herceptin treatment on ADAM10, BT474 and SKBR3 cells were treated with two doses of Herceptin for 24 hours. The level of ADAM10 was determined on mRNA as well as protein level.

Results from qRT-PCR experiments showed that ADAM10 mRNA levels were increased in a dose-dependent manner in both cell lines after 24 hours of treatment: a 3.5–fold increase in BT474 cells and 2.5-fold increase in SKBR3 after 40µg/ml Herceptin treatment was found (figure 3.1A). This correlated with the findings in the ADAM10 protein levels, which were increased 7–fold in BT474 and 5–fold in SKBR3 cells respectively (figure 3.1B).

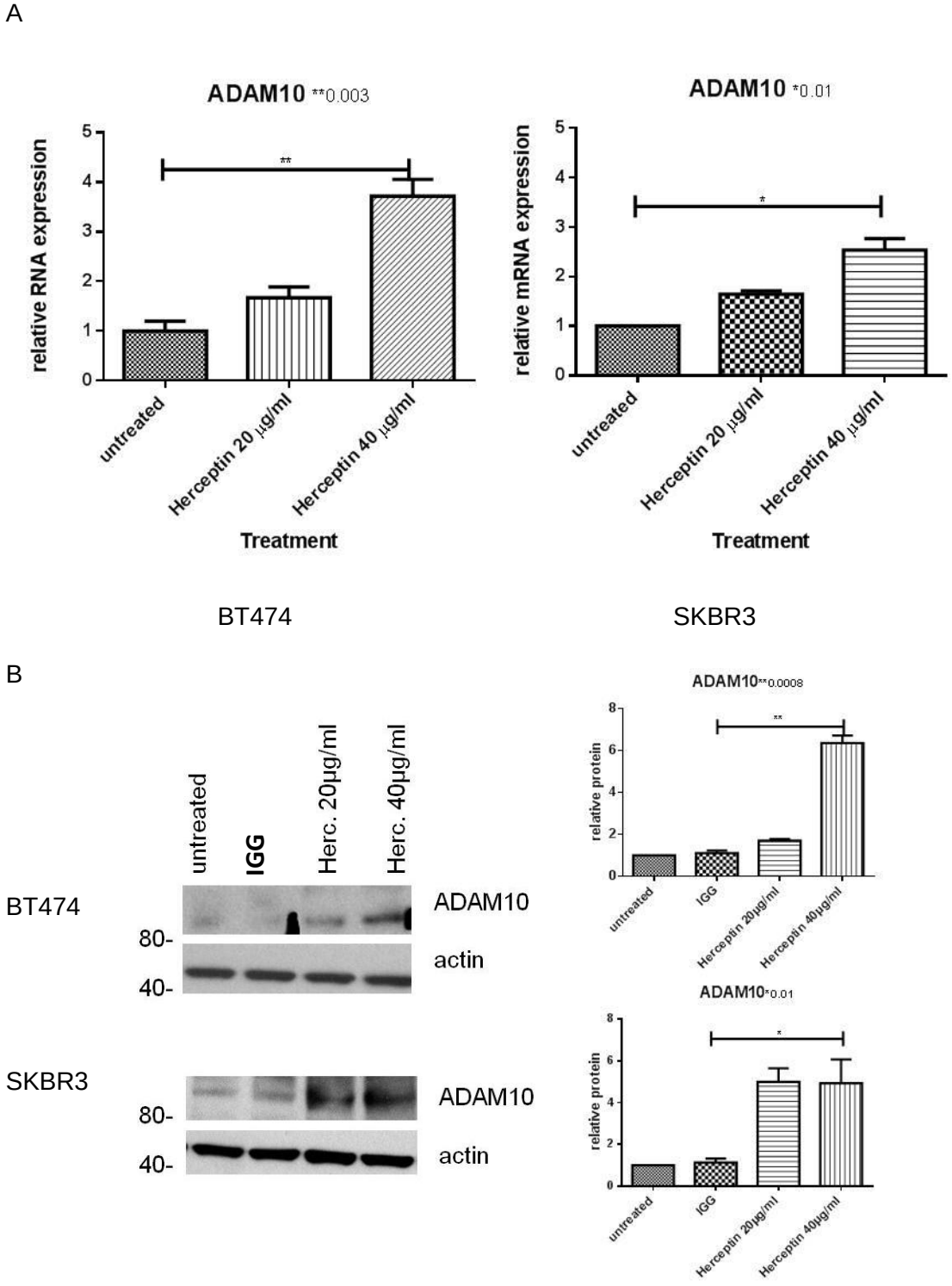


Figure 3.1 Herceptin treatment increases ADAM10 mRNA and protein level. SKBR3 and BT474 cells were treated for 24h with the indicated doses of Herceptin before being lysed and assessed for ADAM10 (A) mRNA and (B) protein levels. Three representative western blots were quantified as shown in B. Column bar graphs show means ± standard deviation, the differences between groups were assessed using Kruskal-Wallis-Test and p values are shown (*p ≤ 0.05, ** p ≤ 0.01).

The results demonstrated that Herceptin treatment leads to an upregulation of ADAM10 mRNA and protein levels. However, it was also found previously that in SKBR3 ADAM10 protein level was increased even after one hour of Herceptin treatment, which was dose dependent. It was investigated whether this could be due to decreased degradation via the proteasome pathway as this would be a possible explanation for the early upregulation of ADAM10 level. Using SKBR3 cells, a proteasome inhibitor was applied for 1h and Hif-1, a protein known to be degraded via this pathway, was used as a positive control. As visible in figure 3.2, proteasome inhibition facilitated the detection of Hif-1. However, ADAM10 level was not increased. This suggests that the observed upregulation of ADAM10 at early time points is not due to impaired degradation via this pathway.

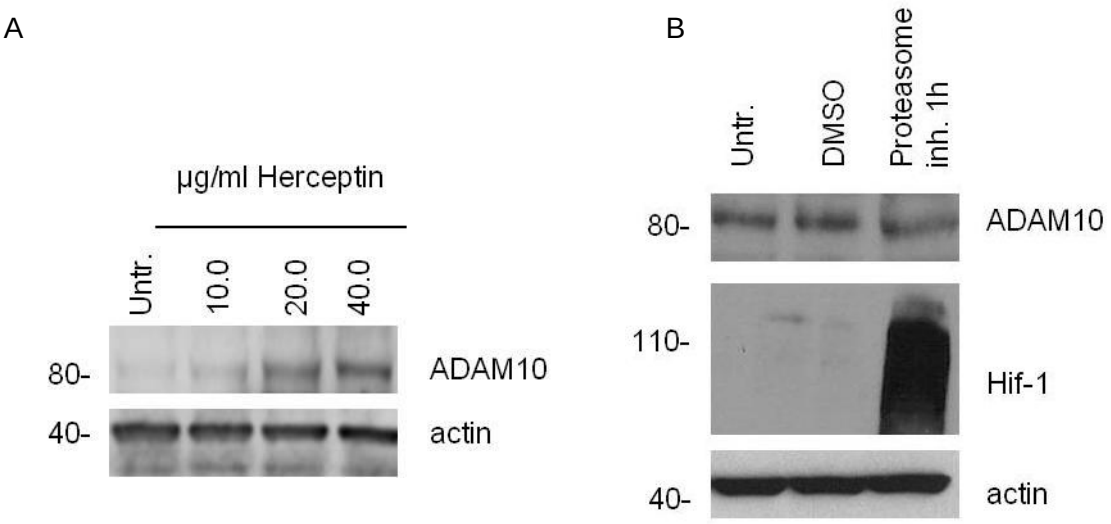


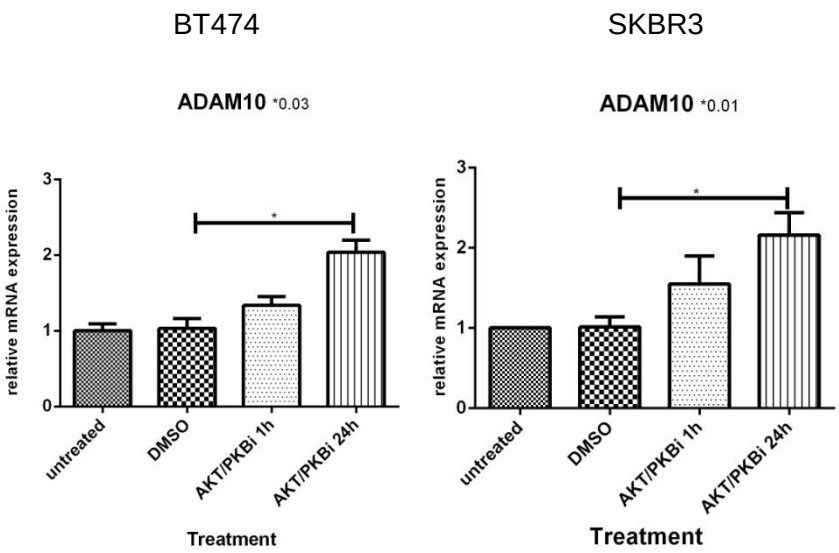
Figure 3.2 Proteasome inhibition does not lead to an upregulation of ADAM10 protein expression. SKBR3 cells were treated with (A) the indicated doses of Herceptin or (B) 25µM of a proteasome inhibitor for 1h prior to lysis and western blot. ADAM10 level was assessed and in (B) Hif-1 level was used as a positive control.

3.1.1 The increase of ADAM10 in Herceptin treatment is related to the decrease in pPKB

Previous publications have shown that Herceptin treatment leads to an accumulation and activation of PTEN and subsequent decrease of PKB phosphorylation [40]. It was presented in our laboratory that a decrease in pPKB leads to an increase of ADAM17 level during Herceptin treatment [67].

In order to assess the effect of PKB inhibition on ADAM10 protein expression, BT474 and SKBR3 cells were treated with an allosteric AKT (PKB) inhibitor. The results showed that a significant increase of ADAM10 mRNA as well as protein levels could be induced in a time-dependent manner. As shown in figure 3.3, mRNA and protein levels were increased between 2- to 3-fold in BT474 and SKBR3 cells.

A



B

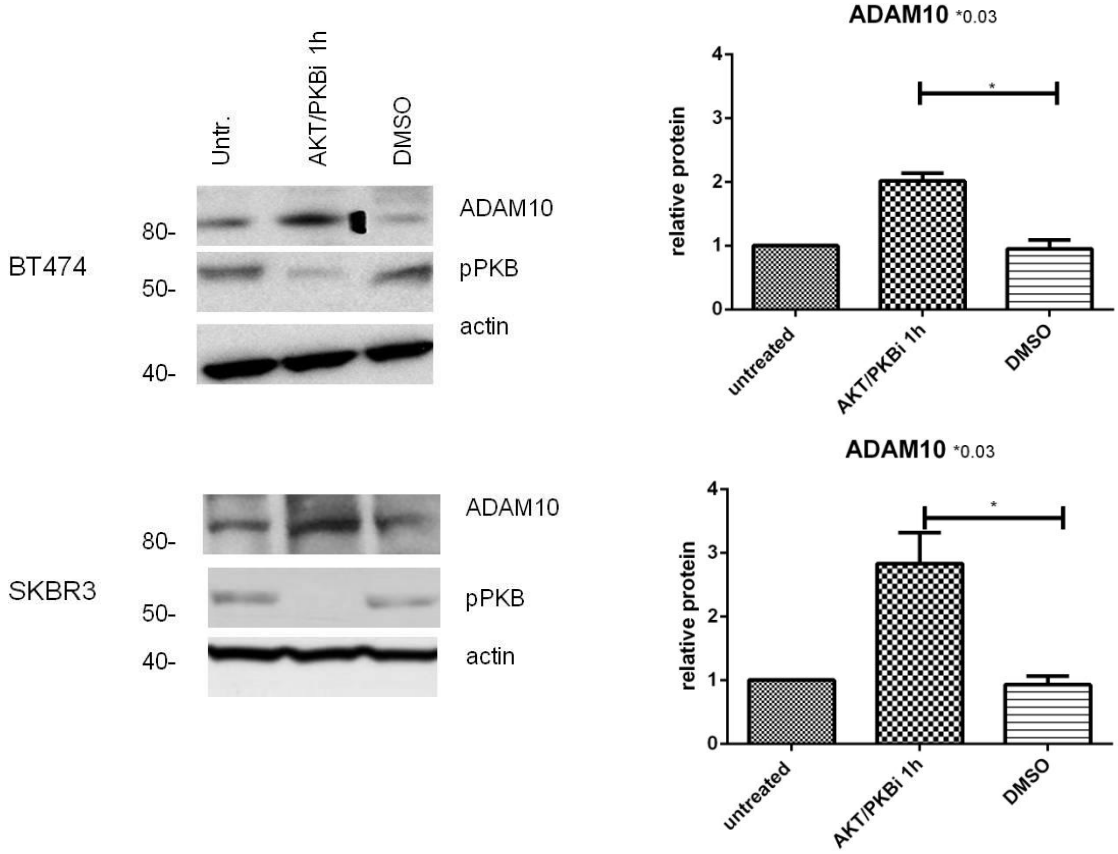


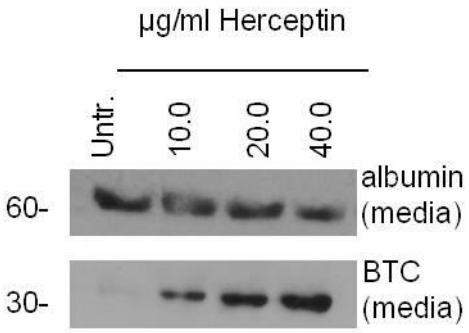
Figure 3.3 Inhibition of AKT/PKB leads to an increase of ADAM10 mRNA and protein levels. Cells were treated for the indicated time points with an allosteric AKT/PKB inhibitor (2.5µM) before lyses and (A) mRNA and (B) protein levels assessed. Three representative western blots were quantified as shown in B. Column bar graphs show means ± standard deviation. The differences between the groups were assessed by Kruskal-Wallis-Test and p-values are shown (*p ≤ 0.05).

Collectively, the above results suggest that AKT/PKB inhibition by Herceptin triggers a feedback mechanism, which induces the upregulation of ADAM10 level.

3.1.2 Herceptin treatment leads to an increase of the ligand betacellulin

One of the putative substrates for ADAM10 is the ligand betacellulin. Therefore, the level of betacellulin during Herceptin treatment was assessed as this could also be used as a response marker. The effect of Herceptin on betacellulin shedding had been assessed by ELISA when I was a visiting student in Dr. Kong's lab. The increase of betacellulin shedding in the medium was further confirmed by western blotting (Figure 3.4A).

A



B

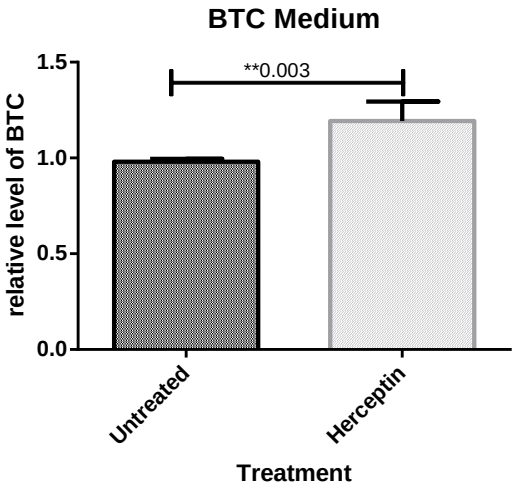


Figure 3.4 Herceptin treatment leads to an increased shedding of the release of ligand betacellulin (BTC) (A) BT474 cells were treated with increasing doses of Herceptin in serum-free media for 24h before the media was concentrated by filtration centrifugation. The protein content was measured and normalized using Bradford assay before the same concentrations of proteins were loaded. Albumin was used as an internal control. (B) The cells were treated with 40µg/ml Herceptin in serum-free media for 24h before the media samples from treated cells were subjected to ELISA analysis (please note that ELISA result was previously included in my BSc project report after a 6 month internship in Dr. Kong's lab). Graphs show means ± standard deviation; the differences between the groups were assessed by Man-Whitney-U test and a p-value is shown.

3.2 Inhibition of ADAM10

In order to assess the role of ADAM10 in the two examined breast cancer cell lines, the protein was inhibited using a compound negatively affecting the enzyme activity. Moreover, ADAM10 transient knockdown experiments using two different siRNAs (and a combination) were performed.

3.2.1 The effects of ADAM10 inhibition on betacellulin as well as EGFR and HER4 activation

Treatment of BT474 cells with INCB8765, an ADAM10 enzymatic inhibitor, led to a decreased level of betacellulin in the medium as shown by western blot and ELISA (figure 3.5 A and B respectively). Moreover, the increase initially seen in Herceptin treatment could be abolished, when cells were co-treated with the ADAM10 inhibitor.

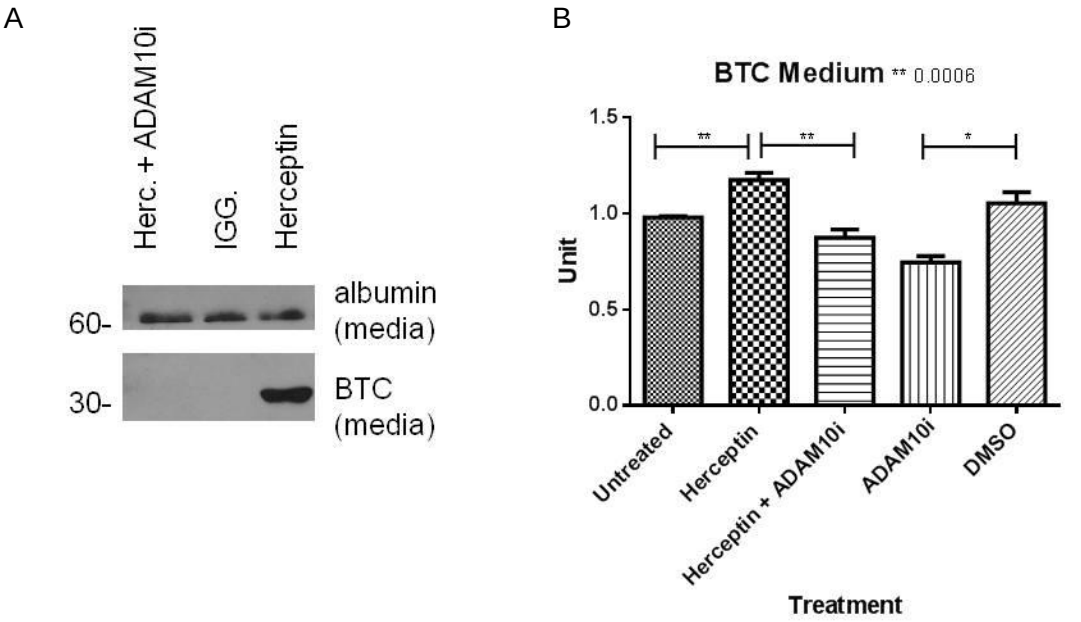


Figure 3.5 Inhibition of ADAM10 leads to a decrease of betacellulin levels in the media. BT474 cells were treated with 40µg/ml of Herceptin, 5µM of the ADAM10 inhibitor INCB8765 (ADAM10i), or combination in serum-free media as indicated for 24h. (A) Media samples were concentrated by filtration centrifugation. The protein content was measured and normalized using Bradford assay before the same concentrations of proteins were loaded. Albumin was used as an internal control. (B) Media samples were used for ELISA experiments to assess the level of the ligand betacellulin (BTC) (please note that the ELISA result was previously included in my BSc project report after a 6 month internship in Dr. Kong's lab). Column bar graphs show means ± standard deviation, the differences between groups were assessed using Kruskal-Wallis-Test and p values are shown (*$p \leq 0.05$, ** $p \leq 0.01$).

Since betacellulin is a ligand binding to EGFR and HER4, the status of the activation of these receptors during Herceptin treatment with or without ADAM10 inhibitor was further evaluated. As shown by western blot analysis, treatment with Herceptin led to an increased phosphorylation of both receptors at a 24 hour time point, whereas total protein was not affected (see figure 3.6). Herceptin treatment was not able to decrease pHER2 level in both cells lines (figure 3.6A, lane 3) at a 24h time point.

However, inhibition of ADAM10 using the inhibitor alone could decrease EFGR, HER2 and HER4 phosphorylation in comparison to control. Moreover, the addition of the ADAM10 inhibitor to Herceptin also led to a decreased phosphorylation status of these proteins, counteracting the increase seen with Herceptin treatment alone (figure 3.6A, lanes 4 and 5 respectively). Furthermore, Co-immunoprecipitation experiments in BT474 cells showed that Herceptin treatment led to an increase in EGFR/HER2 dimerisation, which could be decreased by co-treatment with the ADAM10 inhibitor (figure 3.6B).

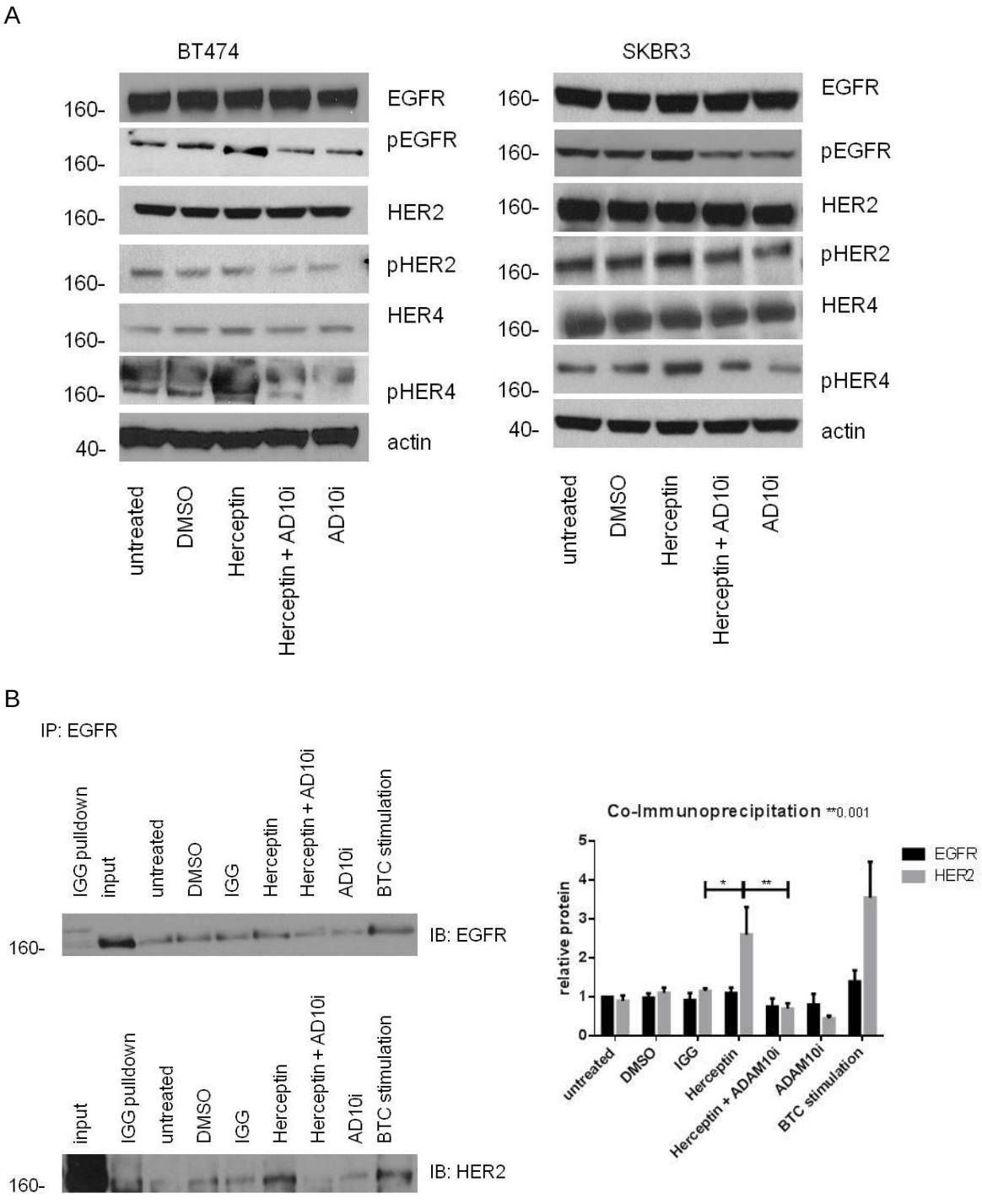


Figure 3.6 The effects of Herceptin and/or ADAM10 inhibition on HER receptor activation. (A) BT474 and SKBR3 cells were treated with 40µg/ml of Herceptin, or 5µM ADAM10 inhibitor INCB8765 (ADAM10i), or their combination for 24h in serum-free media before the cells were lysed and subjected to western blot analysis. (B) BT474 cells were treated with 40µg/ml of Herceptin, 5µM of ADAM10 inhibitor for 24h, or 200ng of betacellulin (BTC) for 20 minutes before co-immunoprecipitation (IP). The cell lysates were pre-cleaned with IgG before an anti-EGFR or an IgG control pull down was carried out. The IP sample was then blotted for EGFR and HER2 and quantification of the blots is shown as mean ± SD. The differences in HER2 were calculated using Kruskal-Wallis-Test and p values are shown (*p ≤ 0.05, ** p ≤ 0.01).

3.2.2 The effects of ADAM10 knockdown in HER2 positive cell lines

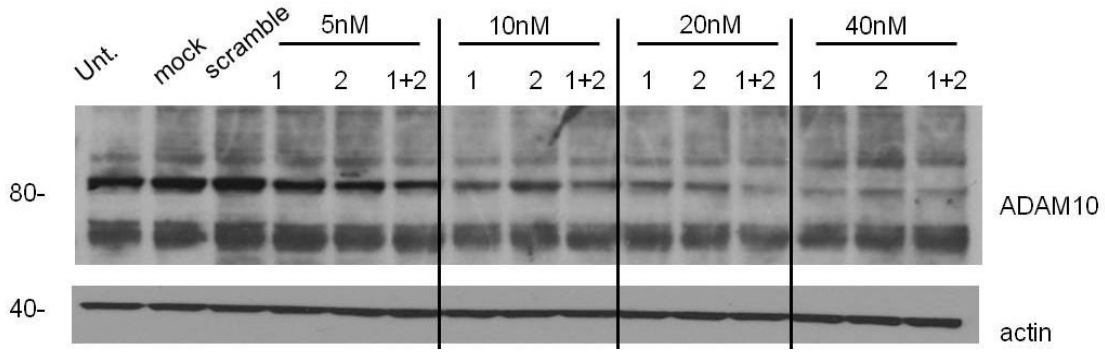
In order to investigate the biological relevance of ADAM10 further, knockdown of the protein was performed using transient siRNA method. Later on, studies using the ADAM10 inhibitor or knockdown assessed the effect on cell viability

Optimization of ADAM10 knockdown

Experiments were performed to optimize the siRNA-concentration for ADAM10 knockdown. Two different siRNAs against ADAM10 and their combination were tested and compared to control (scramble siRNA), mock, or naive cells. Cells were analysed at a 72h time point to verify the knockdown on protein level.

A significant knockdown was achieved using a siRNA-concentration of 20nM or 40nM in BT474 (figure 3.7A) and SKBR3 cells (not shown). However, the 40nM concentration was found to decrease cell number greatly in comparison to scramble transfected cells. Therefore, a concentration of 20nM was used in further experiments. This concentration was also used to verify the knockdown on mRNA level at a 48h time point after transfection. Knockdown with either siRNA or combination led to a more than 5 –fold decrease in ADAM10 mRNA levels in BT474 (figure 3.7B) and SKBR3 cells (not shown).

A



B

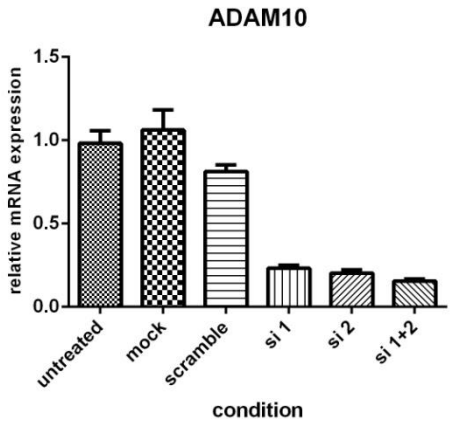
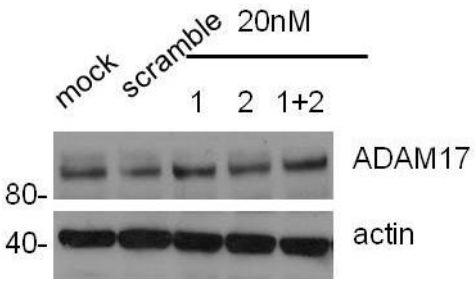


Figure 3.7 Optimization of ADAM10 knockdown. BT474 cells were transfected with different concentrations of two siRNAs or their combination. (A) Western blot analysis was performed after 72h and (B) mRNA levels were assessed after 48h.

To control for a possible off-target effect, mRNA and protein levels of ADAM17 were assessed in this setting since ADAM10 and ADAM17 are similar in their sequences. However, it seems that the ADAM10 knockdown using a siRNA-concentration of 20nM did not significantly affect ADAM17 protein or mRNA levels (figure 3.8 A and B respectively).

A



B

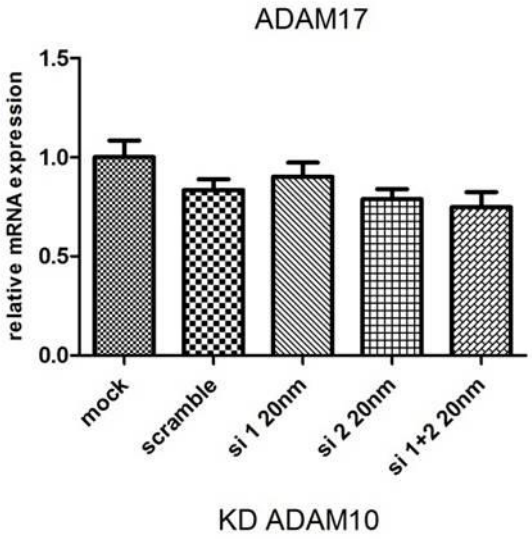


Figure 3.8 Control for off-target effect. BT474 cells were transfected with 20nM of siRNA 1, 2, or a combination. (A) The protein levels of ADAM17 were assessed after 72h. (B) mRNA levels were determined after 48h post transfection.

Effects of ADAM10 knockdown

In BT474 as well as SKBR3 cells, ADAM10 knockdown using either separate siRNAs or their combination led to a decreased activation of EGFR and HER4 as well as HER2 to a lesser extent in comparison to mock or scramble transfected cells. The decrease in HER receptor phosphorylation was correlated with a decreased activation of the downstream proteins ERK and AKT (figure 3.9).

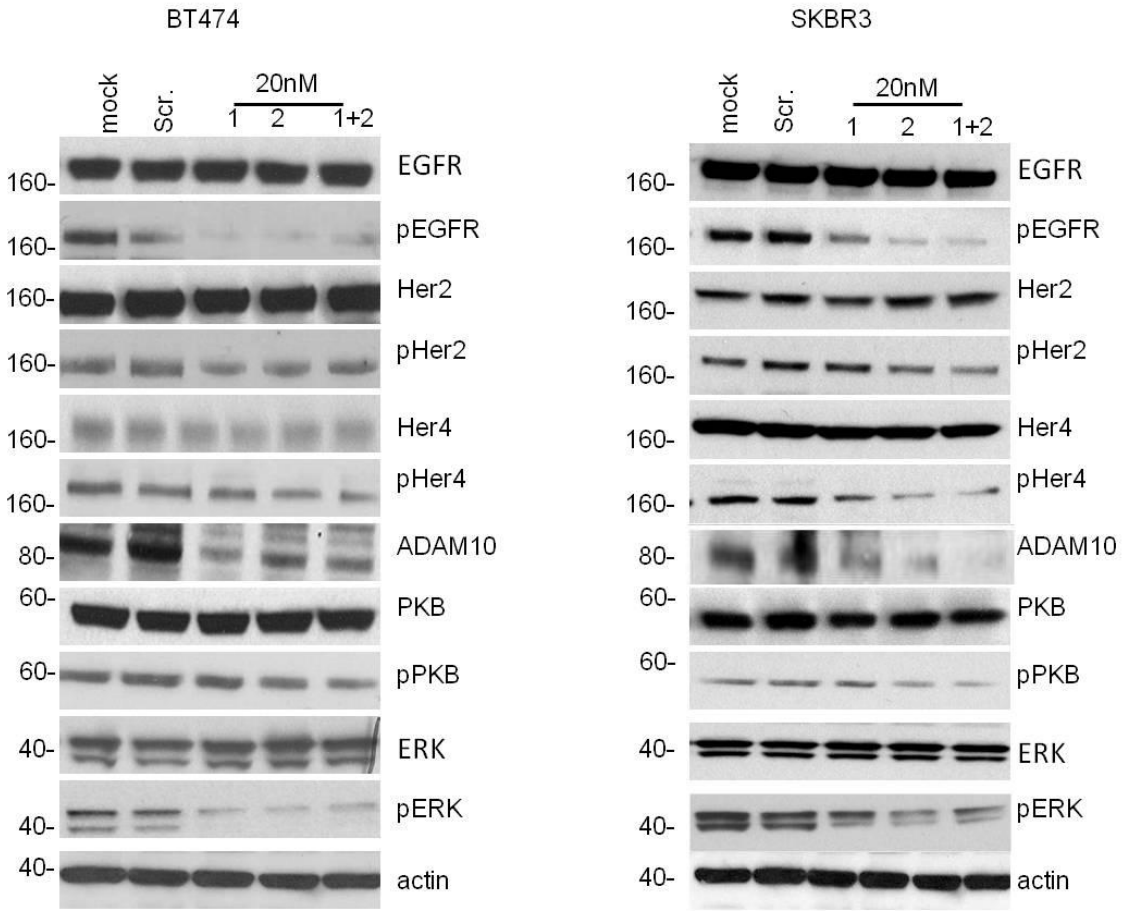


Figure 3.9 Effect of ADAM10 knockdown in HER2 overexpressed breast cancer cells. Transfection with 20nM against ADAM10 was performed in SKBR3 and BT474 cell lines and proteins of interest were analysed by western blot 72h after transfection.

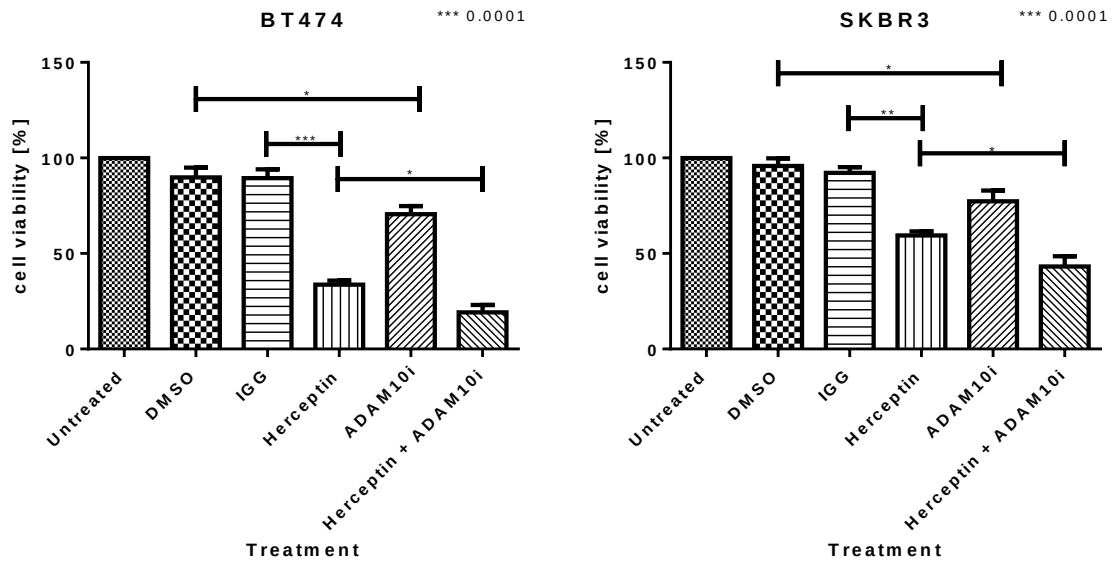
3.2.3 Effects of ADAM10 inhibition or knockdown on cell number

To further assess the role of ADAM10 in relation to Herceptin treatment, SKBR3 and BT474 cells were treated with either Herceptin or/and ADAM10 inhibitor/knockdown before being assessed for cell viability using MTT or cell counting assays.

The ADAM10 inhibitor could slightly decrease cell number in both cell lines in comparison to DMSO control. Interestingly, ADAM10 inhibition was able to potentiate Herceptin sensitivity in both cell lines and cell number was further decreased in comparison to Herceptin treatment alone (figure 3.10A). Knockdown of ADAM10 in both cell lines (figure 3.10B) also led to a decrease in cell number that was more significant than the inhibitor

experiments shown in figure 3.10A (when compared to DMSO or scramble respectively). Again, the combination of ADAM10 knockdown and Herceptin treatment was additive in decreasing cell number (figure 3.10B).

A



B

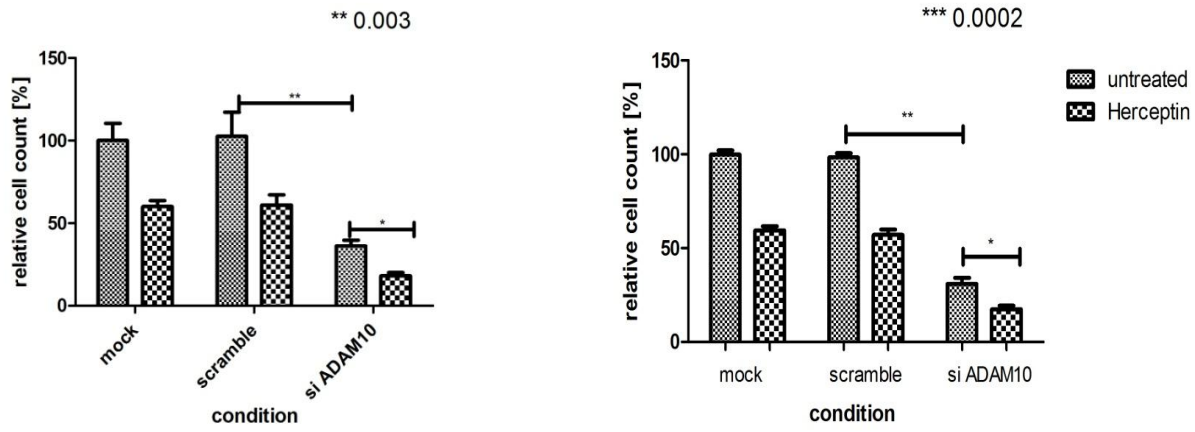


Figure 3.10 The effects of ADAM10 inhibition on cell number. (A) For MTT assays 500 cells were seeded in 96-well plates in triplicate and treated with 40µg/ml of Herceptin, or 5µM ADAM10 inhibitor INCB8765 (ADAM10i), or their combination in serum-reduced media for 5 days before analysis. (B) In cell counting assays 10 000 transfected cells were seeded in triplicate and treated the next day with 40µg/ml Herceptin if applicable. Cell numbers were analyzed after 5 days. Graphs show data from three independent experiments. Column bar graphs show means ± standard deviation, the differences between groups were assessed using Kruskal-Wallis-Test and p values are shown (*$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.2.4 Effect of the ADAM10 inhibitor or knockdown on apoptosis and proliferation

To further investigate the decrease in cell number seen in cell counting and MTT experiments (see previous section, figure 3.10), apoptosis as well as proliferation assays were performed in cells treated with Herceptin with or without ADAM10 inhibitor.

The contribution of apoptosis to the decrease in cell number was assessed by cleaved caspase 7 using western blot as well as fluorescence assisted cell sorting (FACS).

As seen in figure 3.11, the protein level of cleaved caspase 7 was increased after 72h treatment with either Herceptin or ADAM10 inhibitor, but the highest level was seen in the combination treatment in both cell lines.

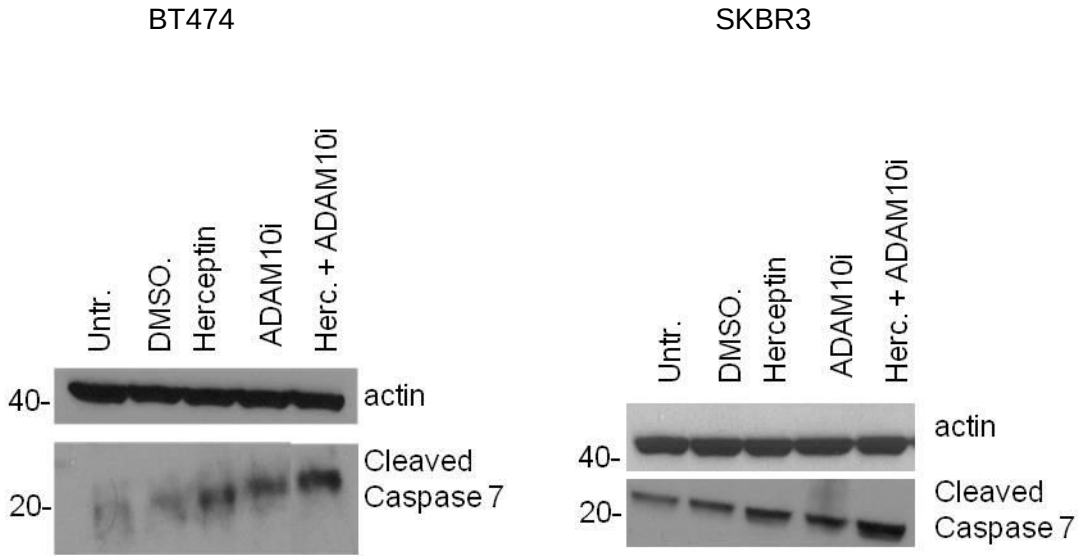
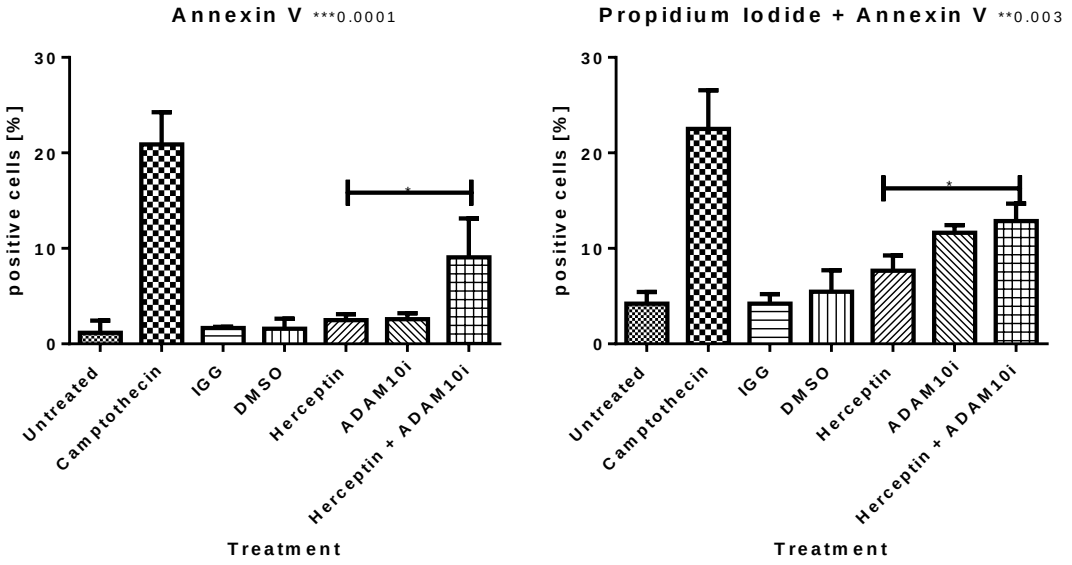


Figure 3.11 Induction of cleaved caspase 7. BT474 and SKBR3 cells were treated with 40µg/ml of Herceptin, or 5µM ADAM10 inhibitor INCB8765 (ADAM10i), or their combination for 72h in serum-reduced media before western blot analysis.

To further elucidate the role of ADAM10 inhibition or knockdown in apoptosis, the effects of Herceptin with or without ADAM10 inhibitor on apoptosis in BT474 cells was further assessed using FACS analysis.

A



B

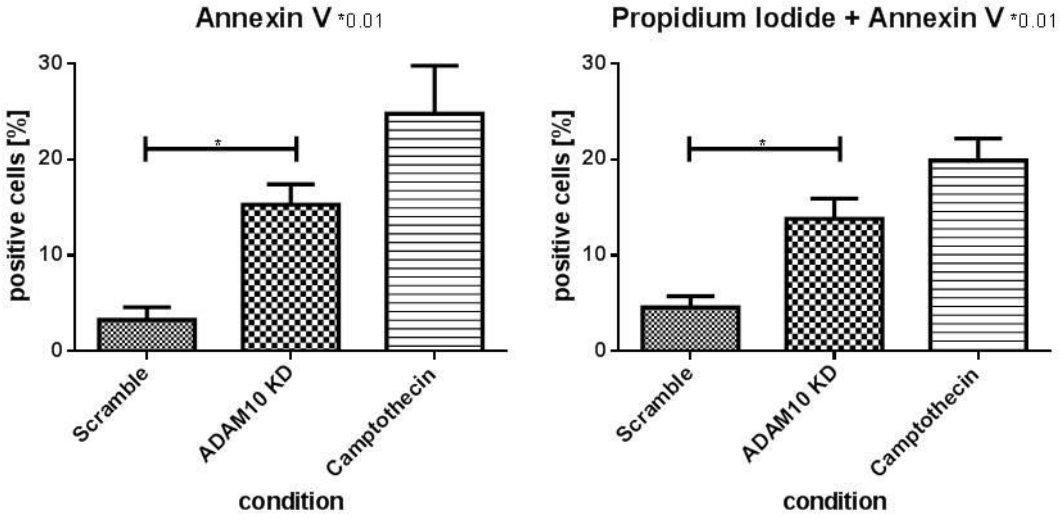


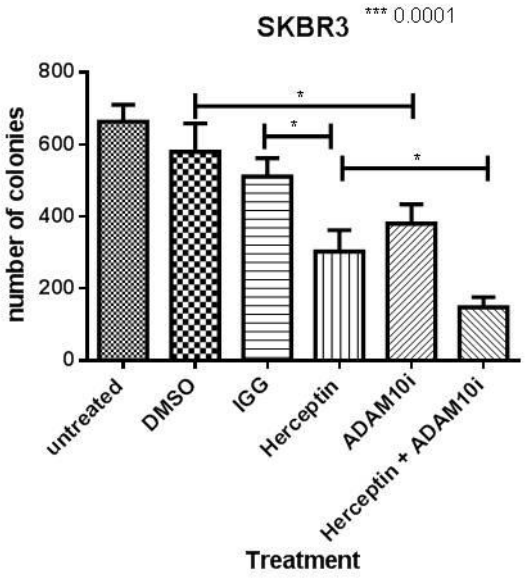
Figure 3.12 ADAM10 inhibition enhances apoptosis in Herceptin treated cells. (A) BT474 cells were treated with 40µg/ml of Herceptin, or 5µM ADAM10 inhibitor INCB8765 (ADAM10i), or their combination for 5 days in serum-reduced media. (B) BT474 were transfected with 20nM of siRNA against ADAM10 for 72h. 6µM of camptothecin was added for 4-6h to generate a positive control. Cells were stained with APC-Annexin V and Propidium Iodide and the number of positive cells was assessed using FACS analysis. Graphs show data from three independent experiments. Column bar graphs show means ± standard deviation, the differences between groups were assessed using Kruskal-Wallis-Test and p values are shown (*p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001).

In the inhibitor studies, treatment with Herceptin or ADAM10 inhibitor monotherapy induced apoptosis to a slight extent but the combination treatment was most effective ($p < 0.05$). Moreover, ADAM10 knockdown also induced apoptosis.

However, the degree of apoptosis could not account for the drop in cell count seen in figure 3.10. Therefore, a clonogenic assay was further performed to elucidate the impact of ADAM10 inhibition on cell proliferation and the ability of cells to form colonies.

First, the effect of Herceptin and/or ADAM10 inhibitor on colony formation was further assessed. Herceptin or ADAM10 inhibitor treatment decreased the number of colonies but the combination treatment was shown to have an additive effect (figure 3.13A). Next, the effect of ADAM10 knockdown on the ability of cells to form colonies was assessed. Figure 3.13B shows that ADAM10 knockdown decreased the cell colonies.

A



B

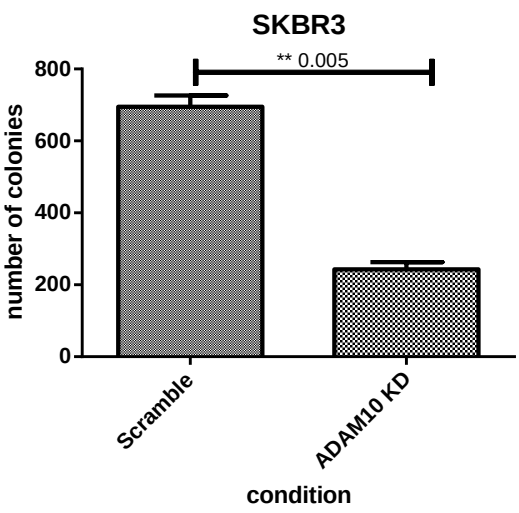


Figure 3.13 The effect of ADAM10 inhibition or knockdown on cell proliferation and colony formation. SKBR3 cells were (A) treated with 40µg/ml of Herceptin, or 5µM ADAM10 inhibitor INCB8765 (ADAM10i), or their combination for 5 days in serum-reduced media or (B) transfected with 20nM of siRNA against ADAM10 for 72h before cells were re-plated in duplicate and left for colony-formation. Cells were then stained with 0.2% methylene blue and counted. The overall plating efficiency was 63.4% in experiments using the inhibitor (A) and 64.9% in assays following ADAM10 knockdown (B). Each graph reflects three independent experiments. Column bar graphs show means ± standard deviation, the differences between groups were assessed using (A) Kruskal-Wallis-Test and (B) Mann-Whitney-U-Test and p values are shown (*$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.3 The role of ADAM10 in Herceptin resistant cell lines

Since ADAM10 was upregulated during short term Herceptin treatment and ADAM10 inhibition enhanced response to Herceptin in sensitive HER2 positive breast cancer cells, its role in Herceptin resistant cells was further investigated.

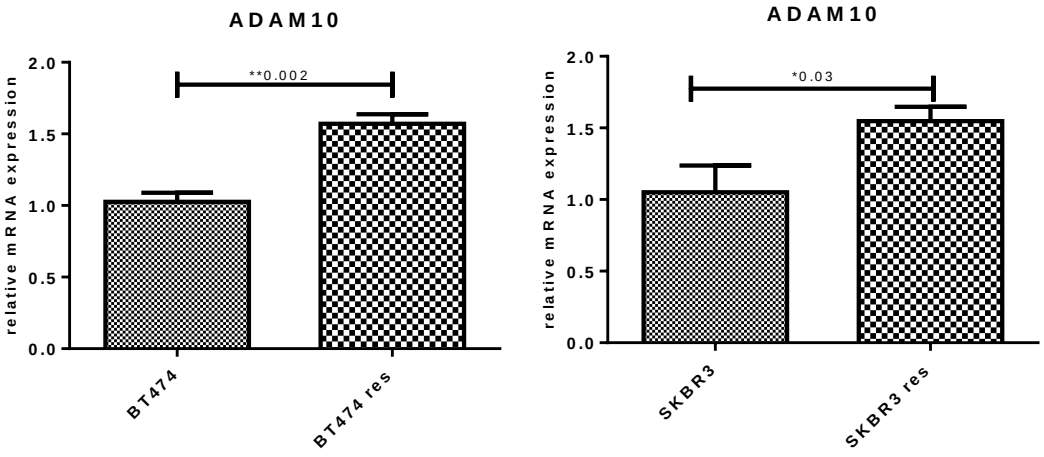
The lab has previously generated Herceptin resistant BT474 and SKBR3 cell lines (see Methods section).

3.3.1 Comparison of ADAM10 and betacellulin levels in sensitive versus resistant HER2 positive cell lines

The basal levels of ADAM10 and betacellulin (which acts as a response marker) were compared between sensitive and resistant cell lines.

The ADAM10 levels were significantly increased at the mRNA (figure 3.14A) and the protein levels (figure 3.14B) in the resistant cell lines in comparison to the parental cell lines. Moreover, the results showed that media of resistant cell lines continuously treated with Herceptin contained more betacellulin than media of parental cell lines (figure 3.14C).

A



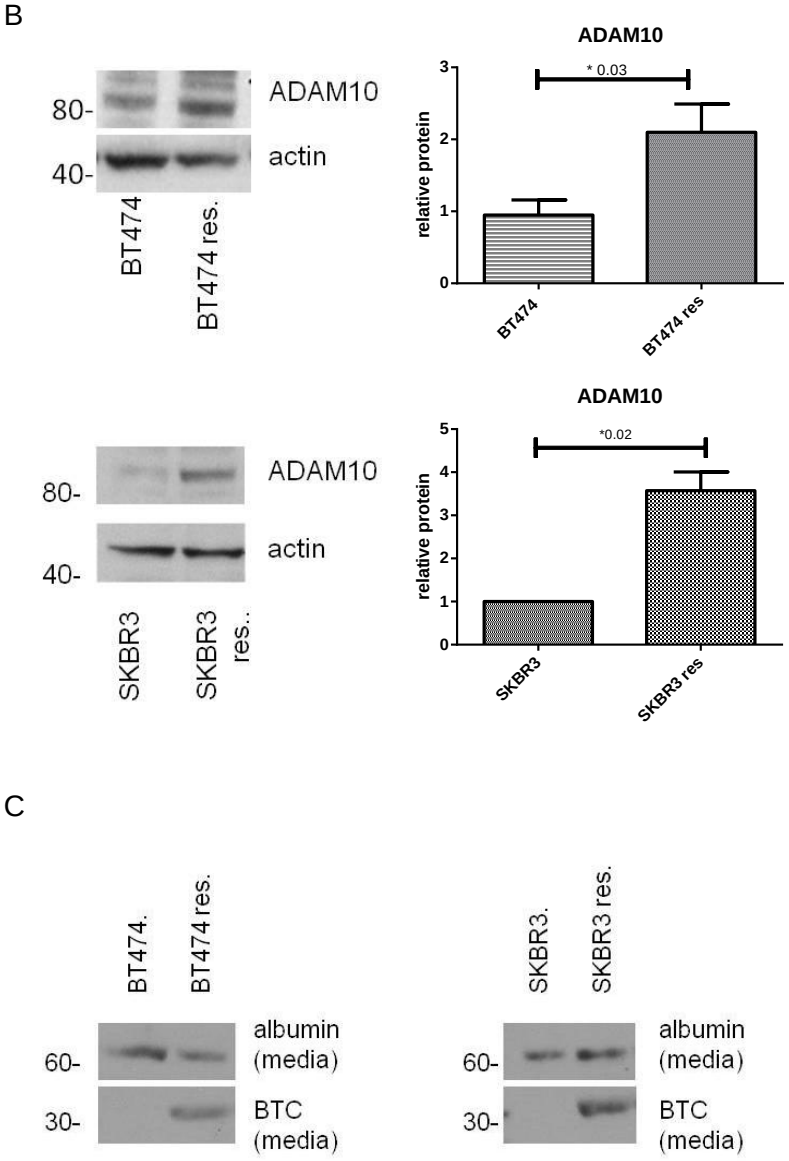


Figure 3.14 Comparison of ADAM10 and betacellulin levels between sensitive and resistant cell lines. Sensitive and resistant cells were seeded and left overnight to settle. Cell lysates were prepared and ADAM10 (A) mRNA as well as (B) protein levels assessed. (C) For the comparison of the ligand betacellulin, sensitive and resistant cells were seeded and the media was replaced the next day by serum free media for 24h. Resistant cells were continuously treated with Herceptin. Media samples were normalized, filter centrifuged and used for western blot analysis. Column bar graphs show means ± standard deviation, the differences between groups were assessed by Mann-Whitney-U-Test and a p-value is shown.

3.3.2 The effects of ADAM10 inhibition in Herceptin resistant HER2 positive cell lines

ADAM10 inhibition using an inhibitor

As ADAM10 was increased in the resistant cell lines, the effect of ADAM10 inhibition was investigated. An ADAM10 inhibitor or DMSO control was applied for 24h to Herceptin resistant HER2 positive BT474 and SKBR3 cell lines, while these cells were continuously treated with 40µg/ml Herceptin. Remarkably, inhibition of ADAM10 in the resistant cell lines led to a decrease of EGFR, HER2, and to a lower extent HER4 activation. Moreover, ERK and PKB activation seemed to be decreased. Total protein levels were not affected as seen in figure 3.15.

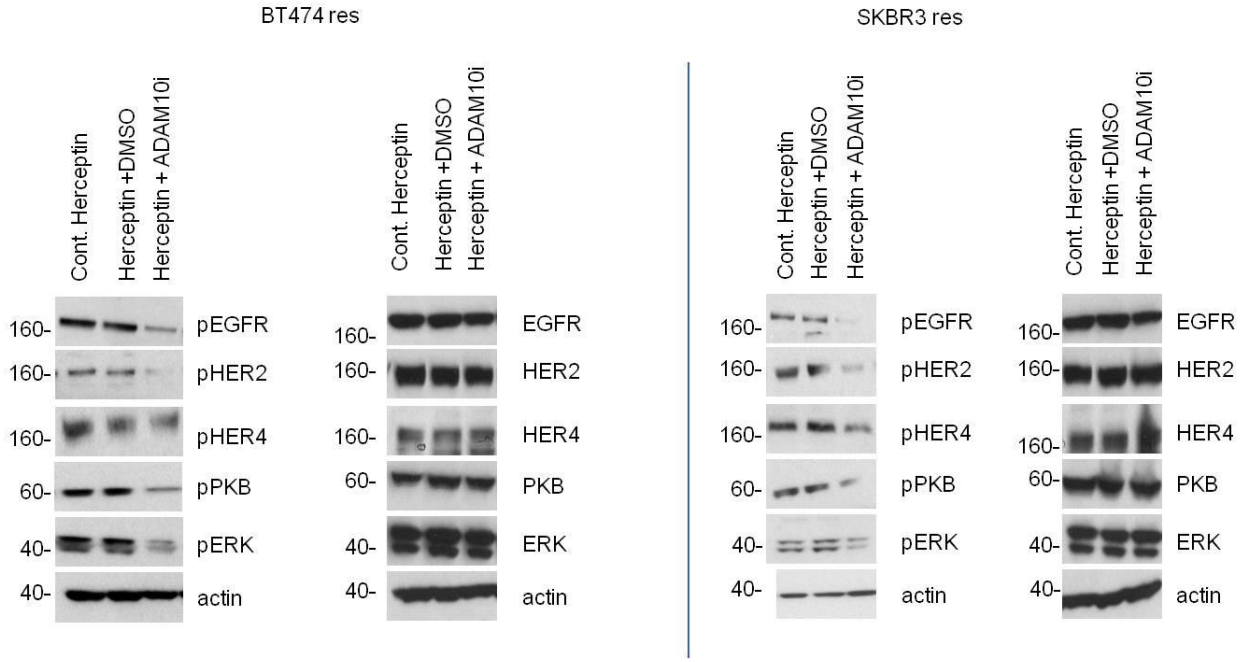


Figure 3.15 The effect of ADAM10 inhibition on Herceptin resistant cell lines. Resistant cells were continuously treated with 40µg/ml Herceptin. For experiments, the media was replaced with serum-free media and cells were treated with the depicted 5µM ADAM10 inhibitor or control for 24h. Cell lysates were used for western blot.

ADAM10 knockdown in Herceptin resistant cell lines

To further assess the role of ADAM10 in Herceptin resistant cells, ADAM10 was knocked down in the cells that were continuously treated with Herceptin for 72h hours.

Western blot analysis of theses samples showed a picture consistent with the studies using the inhibitor; EGFR and HER4 activation as well as HER2 and downstream phosphorylation were decreased due to knockdown of ADAM10 in both cell lines (figure 3.16).

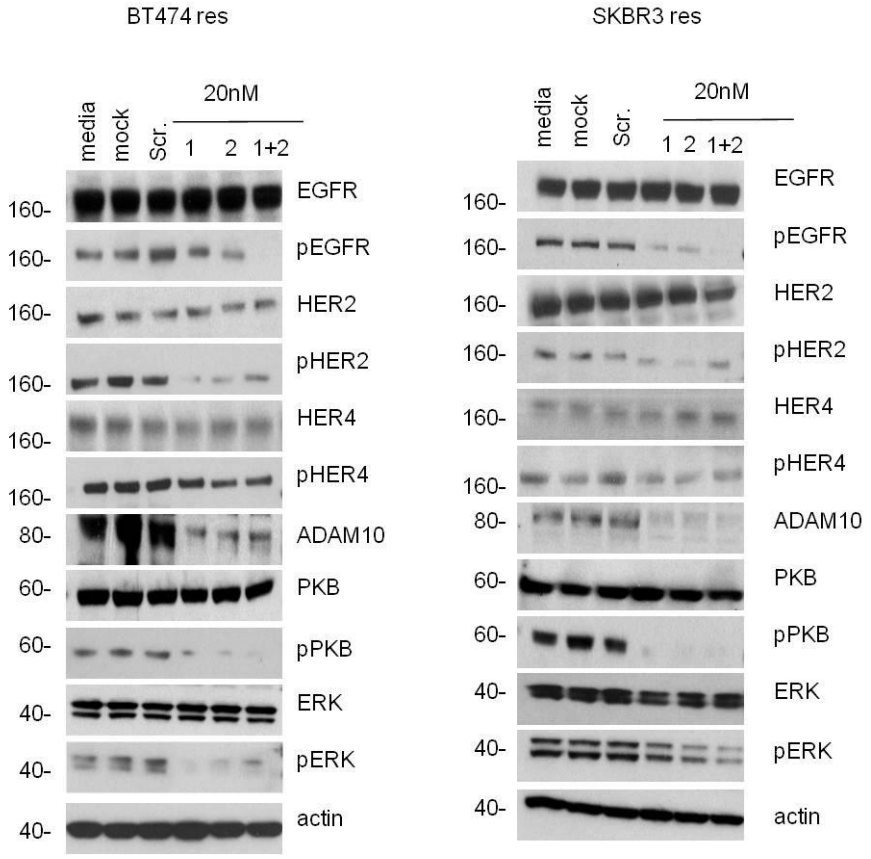


Figure 3.16 The effect of ADAM10 knockdown on Herceptin resistant cell lines. Cells were transfected with 20nM of siADAM10 as described in the methods section and were continuously treated with Herceptin (40µg/ml). 72h post transfection cells were lysed and protein levels assessed.

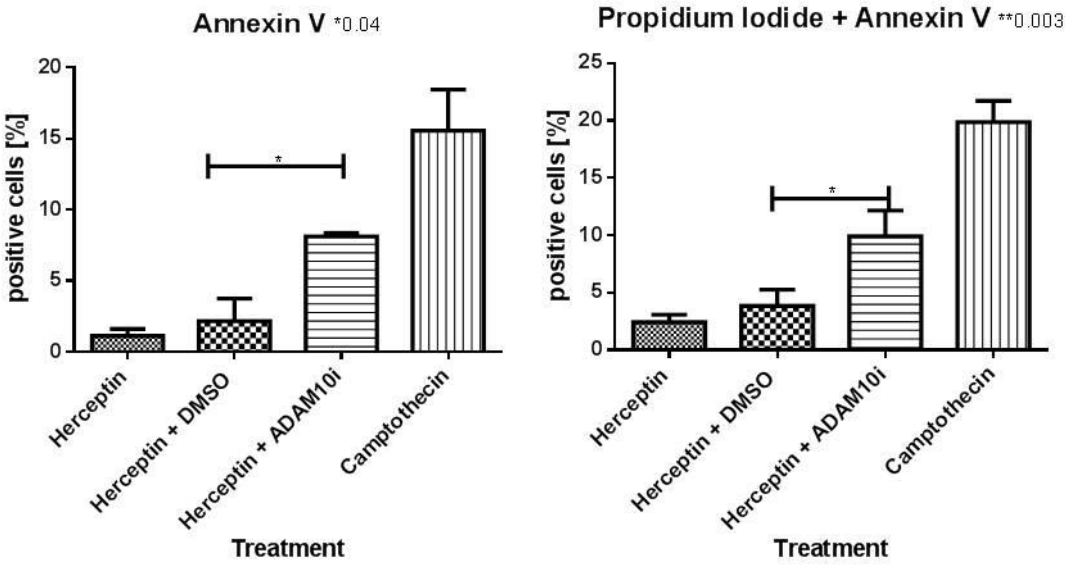
3.3.3 ADAM10 inhibitor and knockdown decrease cell number

The effect of ADAM10 inhibition on proliferation and apoptosis

FACS analysis was performed using BT474 Herceptin resistant cells to assess the effect of the ADAM10 inhibitor. The result showed that ADAM10 inhibition significantly increased the level of apoptosis in comparison to DMSO control (figure 3.17A).

A clonogenic assay was also performed on Herceptin resistant SKBR3 cell lines. As shown in figure 3.17B, addition of the ADAM10 inhibitor significantly decreased the number of colonies (in comparison to DMSO control).

A



B

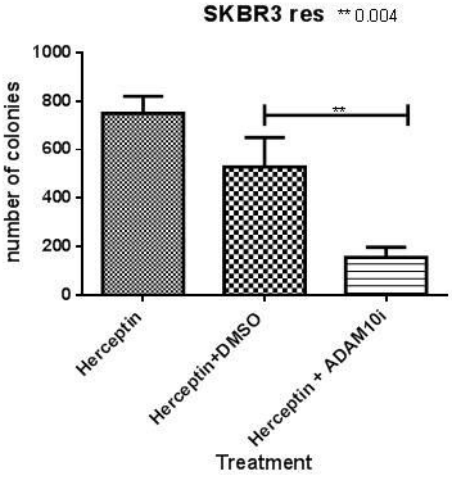


Figure 3.17 Effect of ADAM10 inhibition on apoptosis in Herceptin resistant cell lines. (A) For FACS analysis BT474 resistant cells were continuously treated with 40µg/ml Herceptin in serum-reduced media for 5 days. 6µM of camptothecin was applied to generate a positive control. Cells were stained with Annexin V and Propidium Iodide and analysed. (B) For the clonogenic assay, resistant SKBR3 cells were treated with 5µM of the ADAM10 inhibitor and 40µg/ml of Herceptin for 5 days in serum-reduced media and re-seeded with continuous Herceptin treatment in duplicate for colony-formation. Cells were counted and graphs represent data from three independent experiments. The overall plating efficiency was 75%. Column bar graphs show means ± standard deviation, the differences between groups were assessed using Kruskal-Wallis-Test and p values are shown (*$p \leq 0.05$, ** $p \leq 0.01$).

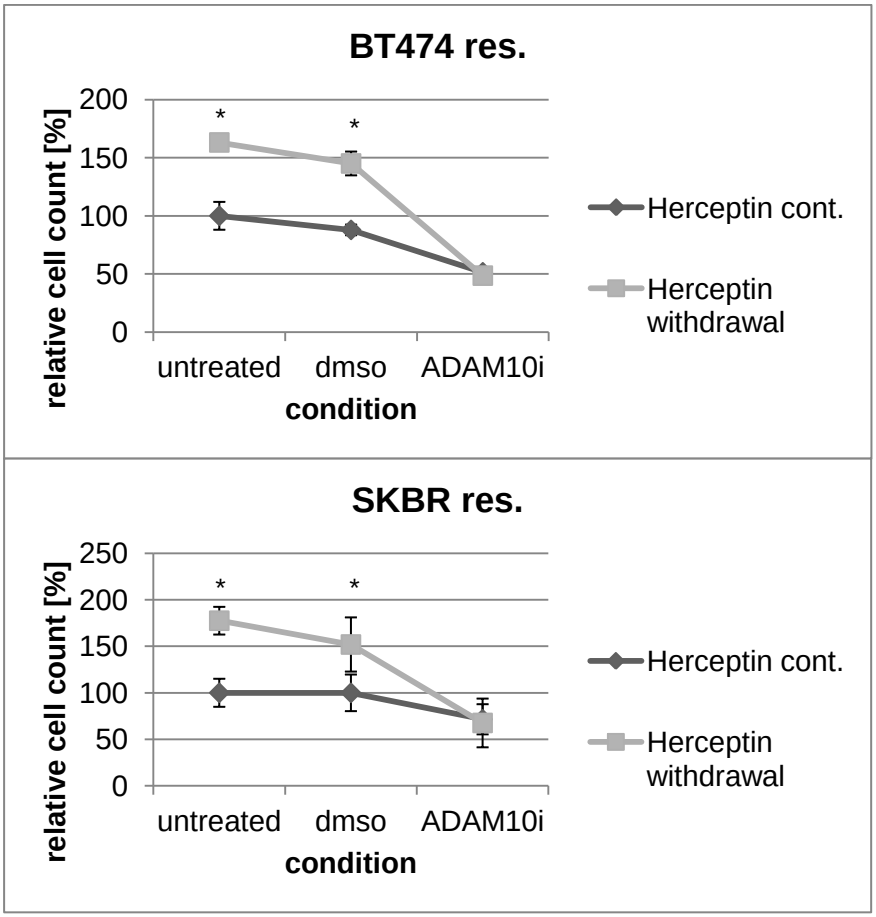
Inhibition or knockdown of ADAM10 and concomitant Herceptin withdrawal

Acquired resistance to Herceptin remains a big problem in the clinical setting. One question is whether Herceptin treatment should be continued even when patients do not seem to respond anymore.

Experiments were performed using either an ADAM10 inhibitor or ADAM10 knockdown. Herceptin resistant cells were either continuously treated with Herceptin in serum-reduced media for 5 days or Herceptin was withdrawn for this period. To analyse the importance of continuous Herceptin treatment in relation to ADAM10 knockdown, siRNA experiments were performed and resistant cells were either continuously treated with Herceptin or left in FCS only media.

The results showed that Herceptin withdrawal increased cell number in comparison to control (in this case continuously Herceptin treated) cells. However, ADAM10 inhibition or knockdown effectively reduced cell number, especially when combining two different siRNAs. In fact, continuous Herceptin treatment did not improve the effect seen with ADAM10 inhibition in either setting (figure 3.18).

A


BT474 res.
relative cell count [%]
200
150
100
50
0
*
*
untreated
dmso
ADAM10i
condition
Herceptin cont.
Herceptin withdrawal
SKBR res.
relative cell count [%]
250
200
150
100
50
0
*
*
untreated
dmso
ADAM10i
condition
Herceptin cont.
Herceptin withdrawal

B

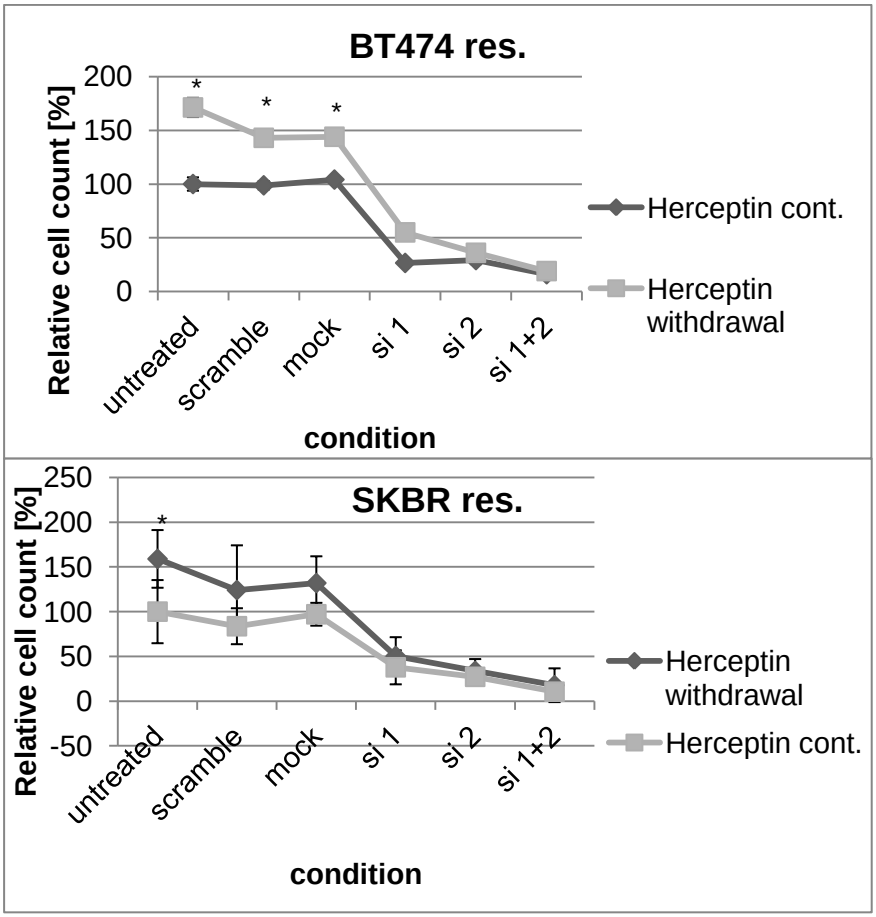


Figure 3.18 ADAM10 enzyme inhibition or knockdown with or without Herceptin continuation in resistant cell lines. (A) 10 000 cells were seeded in triplicate and treated for 5 days with 40µg/ml of Herceptin, or 5µM of ADAM10 inhibitor serum-reduced media as indicated. (B) 10 000 cells transfected with 20nM of siRNA against ADAM10 were seeded in triplicate and treated with 40µg/ml of Herceptin if applicable in serum-only media for 5 days. Afterwards cells were counted and each graph represents data from three independent experiments, showing means ± SD, * denotes a p value ≤ 0.05 (Kruskal-Wallis-Test).

3.4 Xenograft staining using Immunohistochemistry

After proving that Herceptin treatment leads to an increase of ADAM10 expression *in vitro*, its effect was also assessed in *in vivo* models.

ADAM10 staining - Antibody verification using a BT474 cell pellet

BT474 cell pellets first were used to verify the antibody used for immunohistochemistry. As shown in figure 3.19, knockdown of ADAM10 greatly reduced staining intensity of the cell pellet in comparison to scrambled siRNA and the secondary antibody control also did not show staining, demonstrating the specificity of the antibody.

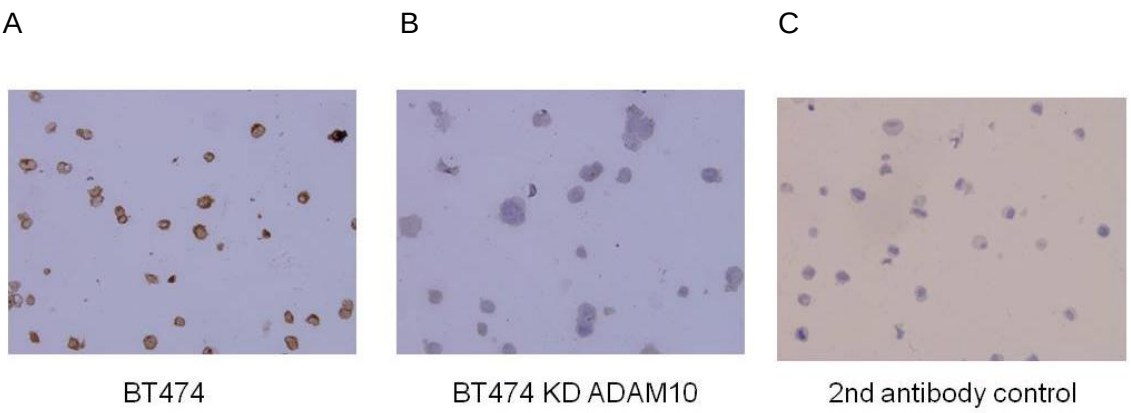


Figure 3.19 Antibody verification for IHC staining. BT474 cell pellets were generated from parental cells (A and C) or from cells transfected with siADAM10 for 72h (B). Cells were stained for ADAM10 expression (A and B) and a second antibody control is shown in C.

Herceptin treatment and treatment with an AKT inhibitor increased ADAM10 expression *in vivo*

Xenograft experiments had been previously carried out in collaboration with NCI, where mice, bearing BT474 derived tumours, were either treated with 5 doses of Herceptin or control vehicle [122]. Sections were prepared and stained for ADAM10 expression as described in the methods section.

Figure 3.20A shows two representative stainings. Staining for ADAM10 tended to be darker in tumour slides from mice that had previously been treated with Herceptin. The results

showed that a significant increase of ADAM10 protein expression occurred *in vivo* after Herceptin treatment in comparison to control (figure 3.2B).

The *in vitro* experiments suggested that the increase in ADAM10 during Herceptin treatment was associated with the suppression of PKB phosphorylation. Direct inhibition of PKB using an allosteric inhibitor was followed by an increase in ADAM10 mRNA and protein expression (see section 3.1.1).

Therefore, it was of interest to assess whether PKB inhibition would have a similar effect *in vivo*. Mice bearing BT474 xenograft tumours were treated with an AKT/PKB inhibitor or control for 24h and 4h and this experiment was done by our collaborator in Hong Kong, Prof Tzi Bun Ng. Interestingly, treatment with the PKB inhibitor greatly reduced tumour size at 24h and only inadequate samples were obtained from this group. Therefore, only slides from 4h experiments were stained. When staining sections of these tumours for ADAM10, it seemed that PKB inhibition led to an increase of ADAM10 protein expression *in vivo* (figure 3.20C). The representative examples of the staining from AKT/PKB inhibitor group and control groups (control vs. treatment) are shown next to the scoring of four samples with respective controls (figure 3.20D).

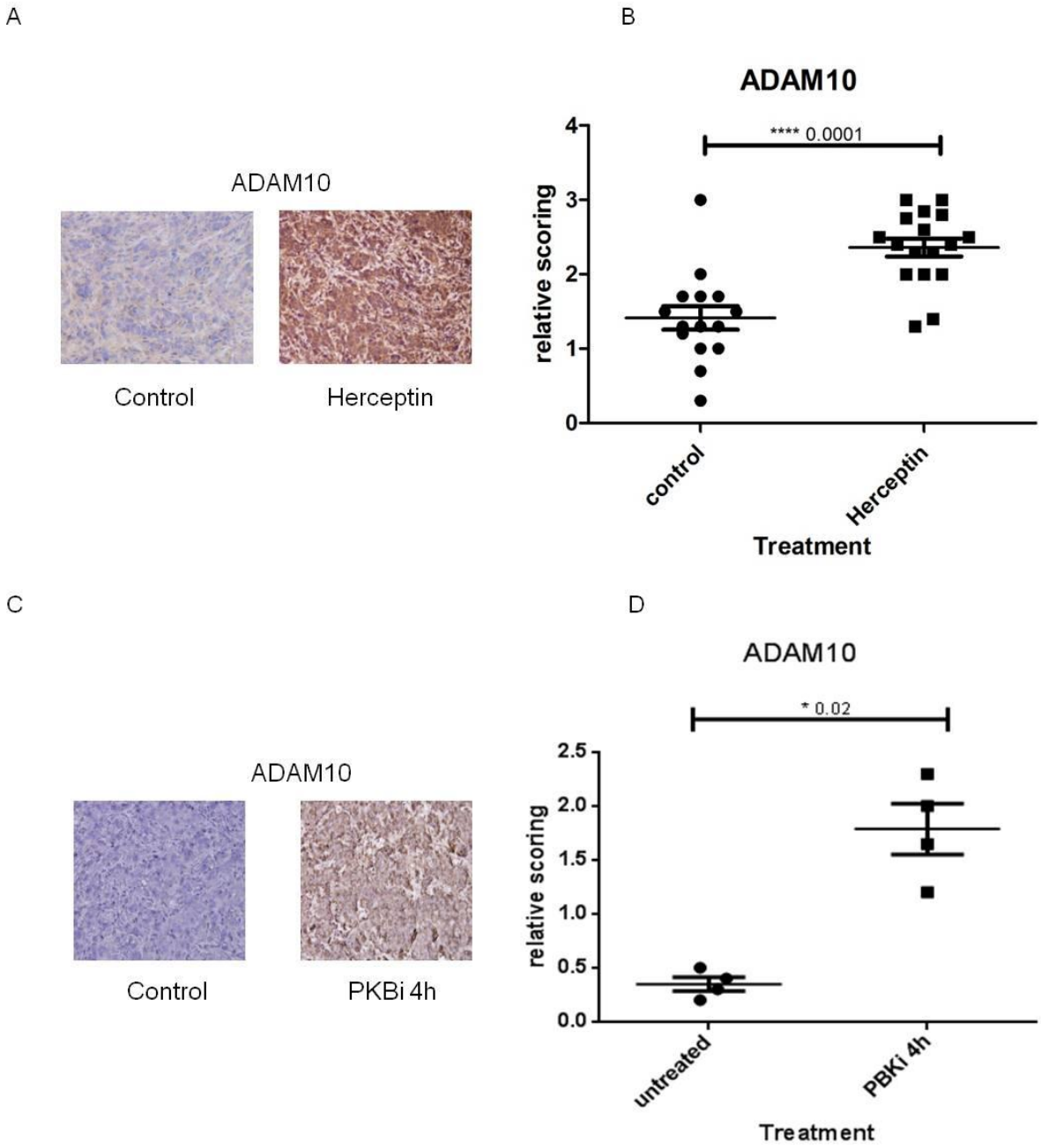


Figure 3.20 Herceptin and AKT/PKB inhibition leads to an increase of ADAM10 protein expression *in vivo*. Tissue samples from xenograft mice treated with Herceptin (A and B) or an AKT/PKB inhibitor (C and D) were stained for ADAM10 expression. Representative stainings are shown in A and C. Scoring (B and D) considered the intensity (0-3) as well as the amount of staining (%) visible. Graphs show means ± standard deviation, the statistical difference was calculated using a Mann-Whitney-U-Test and p- values are shown.

These results show that ADAM10 is also upregulated by Herceptin treatment through PKB inhibition *in vivo*.

3.5 TMA staining

The *in vitro* studies as well as the staining done on sections from BT474 xenograft mice treated with Herceptin indicated a role for ADAM10 in Herceptin treatment. A possible prognostic value of ADAM10 expression was further investigated in HER2 positive breast cancer patients. Specific questions were:

- Is ADAM10 expression related to any other clinical marker such as tumour stage or lymph node positivity?
- Is ADAM10 expression related to overall outcome?

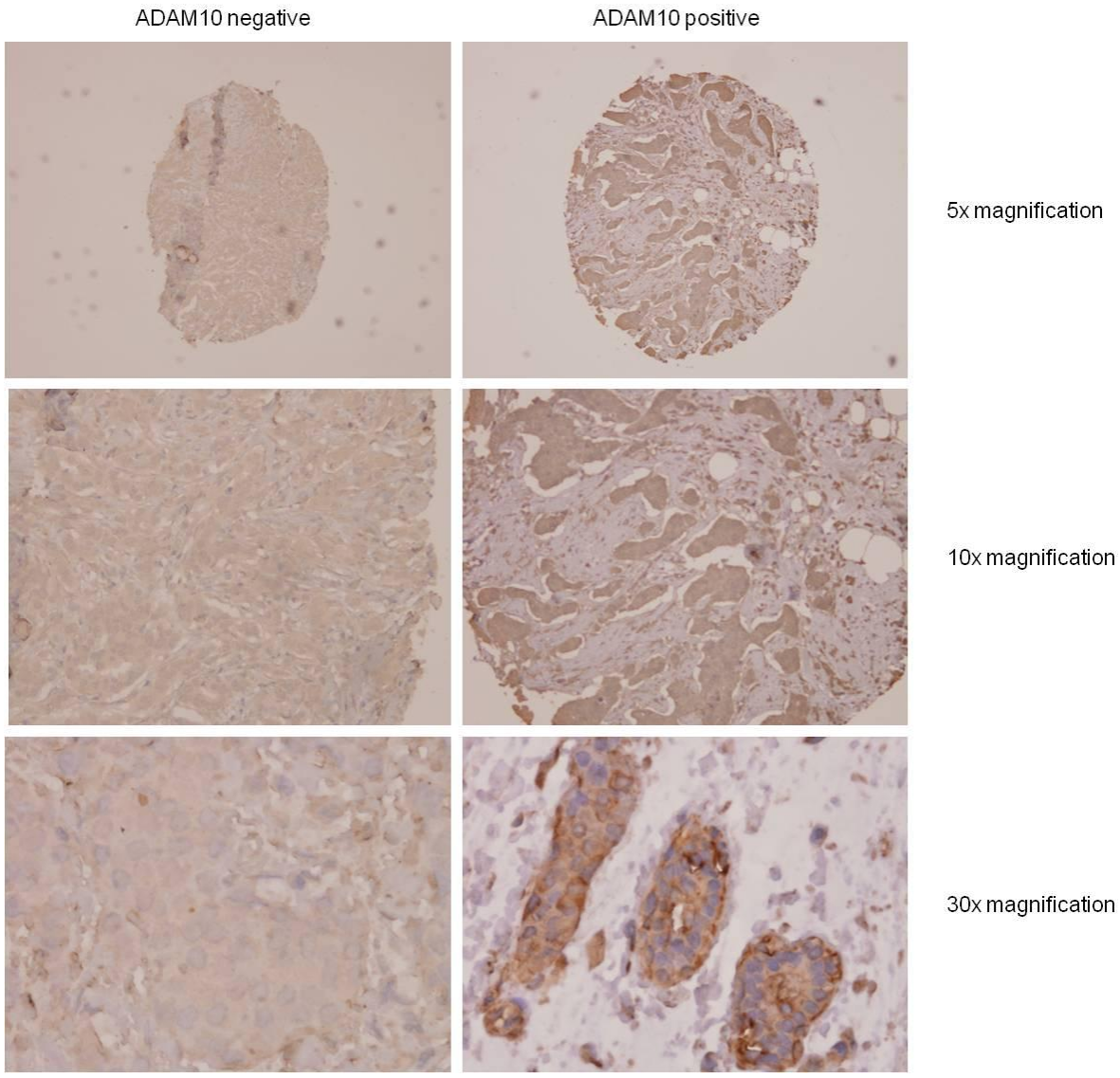
Oxford tissue bank

In this study, samples of HER2 positive tumour samples were stained for basal ADAM10 expression and scored. 69 (74 samples for survival analysis) samples were deemed suitable for further evaluation. Other samples had to be excluded due to poor tissue quality or missing data. Overall, a weak staining for ADAM10 was observed and 44 samples were scored as negative for ADAM10. Interestingly, staining of ADAM10 was more common in samples that were distinctive in their overall structure, e.g. showing small islands or ducts (see figure 3.21A). In addition, all three DCIS were positive for basal ADAM10 expression.
Patient and tumour characteristics were stratified by ADAM10 expression. However, ADAM10 expression could not be significantly correlated to patient age, tumour size, nodal status, ER status, or grade. However, there was a trend towards a correlation between ADAM10 expression and younger age (figure 3.21 B).

ADAM10 expression was also correlated with patient relapse free and overall survival data. Interestingly, patients with tumours which were ADAM10 positive seemed to have a shorter relapse free as well as overall survival than patients with negative ADAM10 tumours.

Moreover, a subgroup of patients with membranous ADAM10 staining in their tumours had the worst outcome (figures 3.21 C and D).

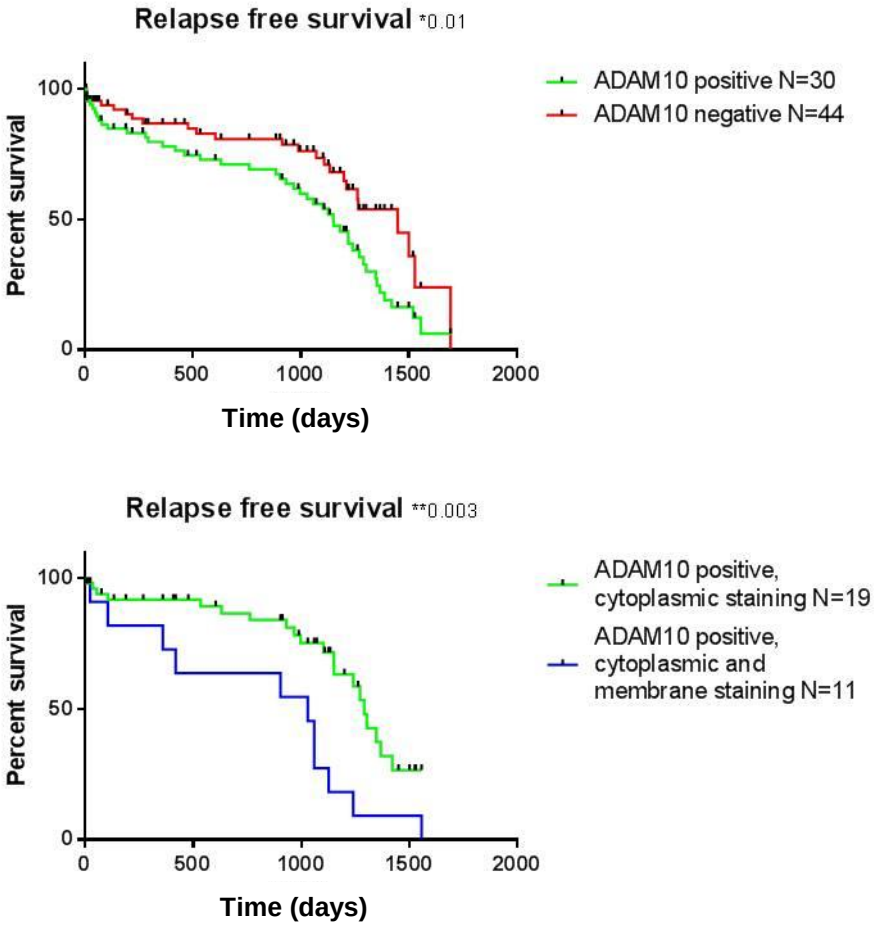
A



B

Features	**Total Number**	**ADAM10 expression**		**P-Value**
		Positive	**Negative**	
Tumour size				0.2
Smaller than 2cm	23	11 (44%)	12 (27%)	
2-5cm	37	11 (44%)	26 (59%)	
5cm or larger	9	3 (12 %)	6 (14%)	
Nodal status				1.000
Negative	35	15 (60%)	20 (45%)	
Positive	34	10 (40%9	24 (54%)	
ER status				0.5
Negative	18	5 (20%)	13 (30%)	
Positive	51	20 (80%)	31 (70%)	
Grade				0.2
3	52	17 (68%)	35 (80%)	
1-2	17	9 (32%)	9 (20%)	
Age				0.053
50 or above	55	17 (68%)	38 (86%)	
Under 50	14	8 (32%)	6 (14%)	

C


Relapse free survival *0.01
Percent survival
100
50
0
0
500
1000
1500
2000
Time (days)
ADAM10 positive N=30
ADAM10 negative N=44
Relapse free survival **0.003
Percent survival
100
50
0
0
500
1000
1500
2000
Time (days)
ADAM10 positive, cytoplasmic staining N=19
ADAM10 positive, cytoplasmic and membrane staining N=11

D

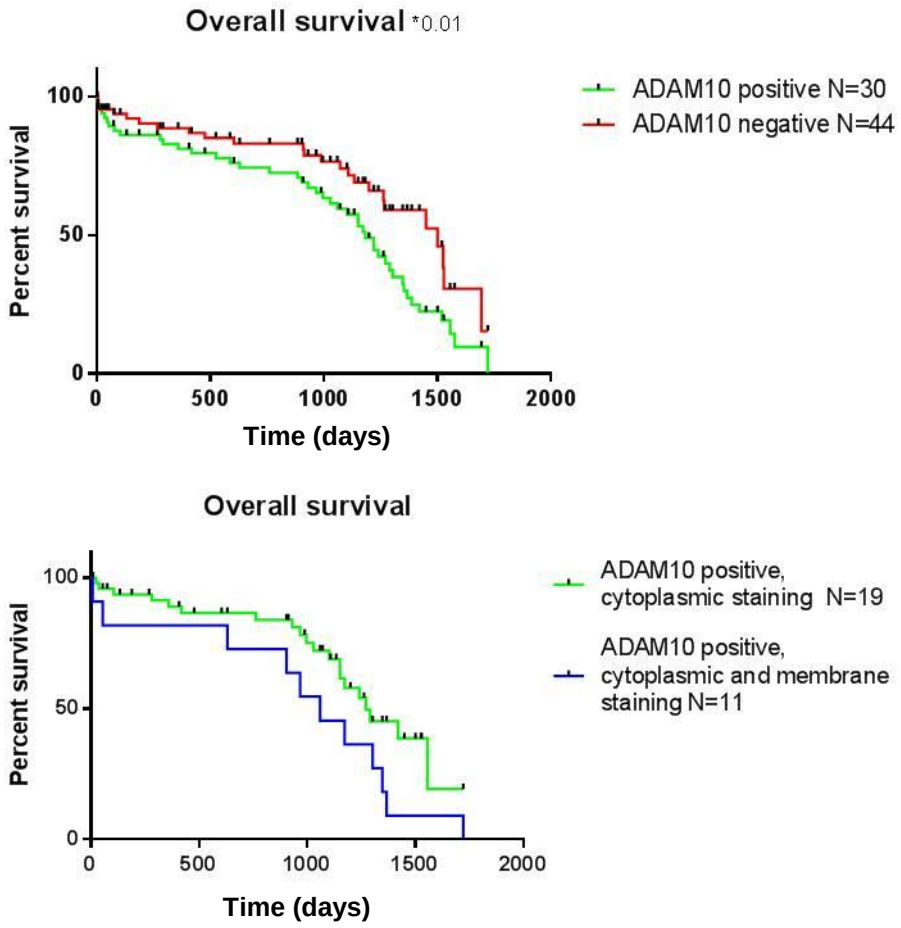


Figure 3.21 The correlation of basal ADAM10 expression with clinical markers. Tissue samples from patients diagnosed with a HER2 overexpression tumour from the Oxford tissue bank were stained for basal ADAM10 expression. Two samples are shown in (A). (B) Tumour and patient characteristics were stratified by ADAM10 expression and correlations calculated using Fischer's exact test. (C) Relapse free survival and (D) overall survival of patients was also plotted according to ADAM10 expression in their tumours. The overall outcome according to ADAM10 positivity/negativity was plotted as well as a comparison of outcome depending on ADAM10 membrane staining. The overall survival differences between the two groups of patients was assessed using Log-rank test and p values (*) are shown.

4 Discussion

Herceptin is the current standard treatment for patients diagnosed with HER2 overexpressing breast cancer. However, primary or acquired resistance is a big drawback during therapy. The aim of this project was to elucidate the role of ADAM10 in relation to Herceptin treatment and resistance.

The inhibition of PKB activity is one of the proposed mechanisms of action of Herceptin [40]. Previously it was shown by our laboratory that Herceptin induces an upregulation of ADAM17 through a PKB feedback loop [67]. The results from this report showed an upregulation of ADAM10 mRNA and protein due to short-term treatment with Herceptin in cell lines and xenograft models as a consequence of PKB inhibition as proven by the use of an allosteric AKT/PKB inhibitor. Thus the results suggested this feedback loop also results in an upregulation of ADAM10, a closely related family member of ADAM17. The upregulation of ADAM10 during short-term Herceptin treatment is in part due to increased transcription and thus translation of ADAM10 as shown in this thesis.

It is also known that ADAMs are tightly regulated to ensure response within a short time.

A known mechanism of rapid response is the proteasome pathway that regulates the protein level by degradation. If a fast response is needed, this degradation is inhibited. However, as treatment with a proteasome inhibitor for one hour did not increase ADAM10 protein level, (in contrast to Hif-1, a positive control) this level of regulation could be excluded. This is in concordance with results from Peiretti et al. (2005), who found that proteasome inhibition stimulated the ADAM17-dependent release of TNF-α and TNFR. However, they also found that ADAM17 levels did not change in this setting [123].

Other possible mechanisms of ADAM10 regulation would include endocytosis leading to lysosomal degradation. In this context, PKB has been implicated in the regulation of endocytosis in other models [124-126] and already one hour of PKB inhibition led to an

increase of ADAM10 protein levels which was not due to enhanced transcription of the protein. To investigate this option, dynasore or a lysosome inhibitor could be used.
Naturally, ADAMs are also regulated by TIMPs. For example, ADAM10 was shown to be controlled by TIMP1 and TIMP3. However, how these factors interact to upregulate the levels of ADAM10 as seen during Herceptin treatment is likely to be complicated and would need more investigation.

It was also shown in this thesis that Herceptin treatment led to increase shedding of the ligand betacellulin, a substrate of ADAM10. This shedding of betacellulin was demonstrated by two assays namely ELISA and western blot of filtered media. However, the increase of betacellulin seems to be more prominent in the western blot than in the ELISA.
This might be because the ELISA was performed using 96-well plates for cell culture and the coating might influence the results and give a lower readout. Moreover, the media was not concentrated for the ELISA experiments, whereas it was filter-centrifuged for western blot analysis. Nevertheless, both assays confirmed an increase of betacellulin during Herceptin treatment, which happened concomitantly with the previously shown increase of ADAM10, the protease of this ligand. Furthermore, increased activation of EGFR and HER4, the respective receptors for this ligand, was also seen during Herceptin treatment, confirming the functional significance of ADAM10 upregulation.

Treatment with the ADAM10 inhibitor was able to reduce the amount of betacellulin in the media in comparison to control as shown by ELISA. Unfortunately, this could not be proven by western blot as it seems that the basal betacellulin level was too low to be detectable by this method. However, it could be shown by both methods that ADAM10 inhibition was able to decrease the increased shedding of betacellulin in medium during Herceptin treatment. This correlated with a decrease of EGFR/HER2 dimerisation (as shown for BT474) as well

as a reduction of EGFR, HER4, and to lesser extent HER2 phosphorylation in sensitive BT474 and SKBR3 cells.

The combined data from both cell lines suggested an additive effect of ADAM10 inhibition to Herceptin treatment in decreasing cell viability and proliferation as suggested by apoptosis and proliferation assays. However, the latter could only be shown for SKBR3 as BT474 cells did not form colonies in the set up used. The data associate ADAM10 to Herceptin treatment and co-treatment with an ADAM10 inhibitor may be superior to Herceptin monotherapy.

The results from this thesis showed that ADAM10 and its substrate betacellulin were upregulated in resistant cells, implicating the role of ADAM10 in Herceptin resistance.

Interestingly, it seemed that the resistant cell lines were very sensitive to ADAM10 enzyme inhibition or knockdown, as demonstrated by the inhibitory effect on EGFR, HER4 and HER2 phosphorylation and activation of downstream pathways. This was further supported by cell counting, apoptosis and proliferation assays. The ADAM10 inhibitor or knockdown reduced cell number more in the resistant cell lines than in the sensitive ones when compared to the respective controls. Osipo et al. (2008) reported that Notch signalling is also related to Herceptin resistance [127]. Interestingly, ADAM10 is associated with Notch- and ligand cleavage that is needed to activate this pathway. Therefore, inhibition of this protease may also affect Notch signalling aiding the effects seen on cell viability.

There has been a continuous debate as to whether Herceptin should be continued when patients developed resistance and whether a new agent should be introduced upon resistance. A few studies suggested a benefit from Herceptin continuation in monotherapy [128] or combination treatments with capecitabine or Lapatinib that were shown to be superior in comparison to monotherapy with those agents alone in the setting of Herceptin resistance [129, 130].

Remarkably, the results from this report showed that withdrawal of Herceptin led to an increase in cell number after 5 days. However, ADAM10 inhibition or knockdown decreased cell numbers and this effect was independent of Herceptin treatment at a 5 day time point.

Only limited literature is available on ADAM expression in breast tumour tissue. Analysis of breast cancer tissue in comparison with normal breast tissue using RT-PCR and western blot showed that ADAM9 was increased in carcinoma tissue. Within carcinomas the expression of ADAM9 was higher in node-positive tumours in comparison to node-negative ones and correlated positively wit HER2 expression [131].
ADAM17 expression at both mRNA and protein level was upregulated in breast cancer compared with normal breast tissue and was highest in lymph-node metastasis. ADAM17 expression correlated with levels of urokinase plasminogen activator and proliferating cell nuclear antigen [132].

There is one other study about ADAM10 expression and its correlation to clinical markers. Previously, Wang et al. (2011) showed that ADAM10 is upregulated in gastric cancer tissue in comparison to normal tissue and ADAM10 was found to significantly correlate with prognosis. High levels of ADAM10 were associated with various other clinical markers such as patient age, tumour size, and location, depth of invasion, vessel invasion, lymph node status, distant metastasis, and TNM stage [133].

Staining of our retrospective TMA samples from 69 HER2 positive breast cancer patients showed a very low basal level of ADAM10 in general and the majority of the samples (44) were scored as negative. ADAM10 staining was heterogeneous and was confined to tumour islands or ducts. Although ADAM10 positivity could not be significantly correlated with any of the chosen clinical markers such as grade, nodal status or ER status and age, ADAM10 positive staining was statistically significantly correlated to poorer relapse free and

overall survival. Interestingly, ADAM10 membrane staining was correlated with the worst relapse and overall survival. As ADAM10 is a metalloproteinase it is tempting to speculate that ADAM10 on the cell membrane may influence cell migration and invasion and therefore metastasis. The current data suggest that ADAM10 might be correlated to younger age and it may be a prognostic marker in HER2 positive breast cancer. However, a larger set of samples would be needed to verify these trends.

Moreover, the samples do not allow to further analyse whether ADAM10 basal expression can predict benefit from Herceptin in these patients since the sample size is small and only a small number of patients in this cohort were treated with Herceptin since some of these samples were taken before Herceptin was introduced as routine clinical care.

The data suggest that ADAM10 inhibition may be beneficial in Herceptin treatment of HER2 positive breast cancer and may be useful in the treatment of Herceptin-resistant cells. This is highly relevant for clinical implications and *in vivo* experiments would be interesting to confirm the effects of an ADAM10 inhibitor in this setting.

Moreover, it would be interesting to see the role of ADAM10 in triple negative breast cancers as they often depend on EGFR signalling which was shown to be less phosphorylated due to ADAM10 inhibition. Furthermore, ADAM10 was shown to be relevant in other cancers, especially gastric cancer. Therefore, the findings of this report may be relevant for these cancer types.

In a small single arm study, Friedman et al. (2009) used an inhibitor of ADAM10/17 (INCB7839) in combination with Herceptin in patients with advanced breast cancer and an overall response rate of 40% (6/15 evaluable patients) with a higher response rate (6/11 or 55%) in patients with average plasma concentrations of above 320nM was observed [119]. This report as well as the paper from Gijsen et al. (2010) emphasized a role for ADAM17 [67] alongside ADAM10. In relation to the HER family, dual inhibition of both ADAMs might

be interesting as ligands for all receptors would be targeted (e.g. ADAM17 sheds heregulin, the ligand for HER3), influencing dimerisation and downstream pathways. This could even enhance the effects shown in this thesis. However, ADAM10 and 17 both act as α-secretase, which is important in the cleavage of the amyloid precursor protein, counteracting the formation of plaque that is related to Alzheimer's disease.

5 Conclusion and future prospective

The data presented here provide good evidence that the metalloproteinase ADAM10 plays a role during Herceptin treatment and resistance. It was also shown that inhibition or knockdown of ADAM10 might be beneficial in the treatment of sensitive and Herceptin resistant HER2 overexpressing breast cancer cells. Furthermore, ADAM10 may be prognostic in HER2 positive breast cancer patients.

Despite the promising result, the role of ADAM10 as a prognostic marker needs to be validated in a larger set of independent samples, preferably from randomised trial samples like HERA and FinHER. These trial samples could also help to answer whether pre-treatment ADAM10 level is predictive for Herceptin benefit in these patients.

It is also important to assess whether ADAM10 upregulation occurs during Herceptin treatment in patients and whether pre-treatment or post-treatment ADAM10 level is important to predict sensitivity and resistance to Herceptin. Therefore, pre and post Herceptin treatment samples derived from patients will help to answer these questions. These samples usually derive from window studies where targeted therapies are given before surgery. We have recently established a collaboration with Dr. Daniele Generali from the Instituti Ospital di Cremona, Italy. We have been given tissue slides from a cohort of 20 patients with pre and post Herceptin samples. This will be an important study to further establish the role of ADAM10 in relation to Herceptin treatment and resistance. It is also hoped that the prognostic and predictive role of ADAM10 in HER2 positive breast cancer patients can be elucidated. In future, it may also be important to establish the role of ADAM10 in other types of breast cancer (like triple negative breast cancer) and other cancers.

While most of the results were shown for two cell lines, some experiments could only be performed for one due to time constraints. Moreover, the study should also be moved to xenograft models to see whether the promising results seen *in vitro* can be translated.

Moreover, the regulation and biological activity of ADAM10 in benign and malignant tissue, as well as during Herceptin treatment, and its role in various pathways might uncover other potential targets that may be important for HER2 positive breast cancer and other cancers.

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