EGFR- and HER2-Binding Affibody Molecules

Cellular studies of monomeric, dimeric and bispecific ligands

LINA EKERLJUNG
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Abstract

Abnormal expression and signaling of the ErbB receptors is associated with the development and progression of several forms of cancer. In this thesis, new ErbB-targeting affibody molecules are evaluated regarding their cellular effects in vitro. Since ligand binding to an ErbB receptor might have an impact on the cell it is important to be aware of these effects as they may have consequences for the continued growth of the tumor when used in vivo. The affibody molecules are intended for tumor targeting with the prospect of clinical use in imaging or therapy.

Three types of affibody molecules were studied, HER2-binding, EGFR-binding and bispecific binders that target both EGFR and HER2. The HER2-targeting (ZHER2:342) showed promising characteristics. It sensitized SKBR-3 cells to irradiation and decreased cell growth to the same extent as the clinically approved antibody Herceptin. The monomeric version, ZHER2:342, did not induce any large effects on intracellular signaling or biological outcome. This makes (ZHER2:342)2 interesting for therapy purposes, while ZHER2:342 may be better suited for imaging.

The bispecific affibody molecules were all able to simultaneously bind to both EGFR and HER2, but none of the six constructs resulted in any large effects on cellular outcome. Interestingly, all three monovalent binders are more functional when positioned at the N-terminal part of the construct and the (S,G) linker renders higher affinity of the bispecific binders compared to (G,S)2. Tumors that co-express several ErbB receptors are often more aggressive and associated with a worse prognosis, suggesting that the total ErbB expression pattern might be more informative than the expression level of one receptor regarding cancer prognosis and prediction of response to targeted therapies. Bispecific ligands could thus be used as imaging agents with prognostic value. Another aspect of dual targeting is the possibility of increased tumor specificity since tumors are more likely than healthy tissue to express high amounts of two receptors.

Keywords: EGFR, HER2, Affibody molecule, cell signaling, receptor dimerization

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III **Ekerljung, L.**, Lennartsson, J., Gedda, L. (2011) The HER2-Binding Affibody Molecule \(Z_{\text{HER2:342}}\)\(_2\) Increases Radiosensitivity in SKBR-3 cells. *Manuscript*


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### Abbreviations

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<tr>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>ECD</td>
<td>Extracellular domain</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>Erk</td>
<td>Extracellular signal regulated kinase</td>
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<tr>
<td>Gy</td>
<td>Gray</td>
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<td>HER2</td>
<td>Human EGFR (ErbB2)</td>
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<tr>
<td>IB</td>
<td>Immunoblotting</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>K_d</td>
<td>Dissociation equilibrium constant</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>NRG</td>
<td>Neuregulin</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-OH kinase</td>
</tr>
<tr>
<td>PLC_γ</td>
<td>Phospholipase γ</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog deleted on chromosome 10</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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1 Populärvetenskaplig sammanfattning  
(Summary in Swedish)

Avhandlingen beskriver olika ErbB-bindande affibody-molekylers inverkan på cancerceller. Här följer en kort introduktion till området samt en sammanfattning av de fem delarbeten som ingår i avhandlingen.

Introduktion


Affibody-molekylerna är små ligander som är framtagna för att binda till exempelvis EGFR och HER2 med avsikten att de ska kunna användas kliniskt för diagnostik och behandling av tumörer. I och med att affibody-molekylerna binder tillväxtreceptorn finns möjligheten att de har inverkan på cellen. Om dessa ligander ska användas i människokroppen är det viktigt att vara medveten om deras effekter eftersom de kan påverka den fortsatta tillväxten av tumören.
Arbete I-III


Arbete IV

Arbete V

Dubbelmålsökning är intressant ur flera aspekter. Dels för att det visat sig att när flera olika ErbB-receptorer (t.ex. EGFR och HER2) finns på cancer-
2 Introduction

This thesis is based on \textit{in vitro} studies of EGFR- and HER2-binding affibody molecules, particularly focusing on their effect on cell growth and intracellular signaling. The included papers will be presented in the section “the present study”.

2.1 Cancer

Cancer is one of the most common diseases in the world, and approximately one in every three people will receive a cancer diagnosis during their lifetime. There are about 200 different types of cancer, classified by the type of cell the tumor originates from, and the differences between two types can be very large. Most cancers occur in epithelial cells and are classified as carcinomas. Common to all cancers is the disturbance of the normal balance between proliferation and apoptosis, resulting in dysregulated cell growth. Another defining feature of malignant tumors is the ability to invade and spread from the site of origin. A benign tumor, which is not evidence of cancer, can be large but does not infiltrate surrounding tissue or metastasize. Metastases are the major cause of death from cancer [1].

Cancer development is a multistep process through which normal cells evolve into invasive cancer. There are 8 hallmarks of cancers, which according to Weinberg and Hanahan, can be applicable to most cancers [2,3].

- Sustained proliferative signaling
- Insensitivity to growth inhibitory signals
- Invasion and metastasis
- Unlimited replication
- Angiogenesis
- Evasion of apoptosis
- Deregulated cellular metabolism
- Evading the immune system

Each of these features is a potential target for cancer targeting therapeutics. Additionally, genome instability and inflammation are two characteristics that facilitate acquisition of the hallmarks [3].

Nevertheless, there are many factors that can cause cancer. Approximately 30\% of all cancers could be prevented by avoiding key risk factors, most importantly; smoking, obesity, physical inactivity, exposure to sunlight and
alcohol consumption [1]. Some genomic changes that might lead to cancer can also be inherited, however most cancers are not inheritable but acquired randomly. Cancer can affect people at any age, but the risk of acquiring cancer increases with age, and more than 60% of people are above 65 years of age at the time of diagnosis [4].

The number of cancer cases diagnosed in Sweden has increased during the recent decades. The major reasons for this are the increasing age of the population together with improved diagnostic methods and more screening programs. There are about 50,000 new cases in Sweden each year, and cancer is responsible for almost 25% of all deaths. Next to heart disease it is the most common cause of death. The most common types of cancer in Sweden are prostate- and breast cancer, while lung cancer causes the most deaths. During the last 30 years, the overall survival and, in particular the life expectancy of cancer patients has increased [4]. This indicates that treatment, as well as diagnosis, have improved.

2.1.1 Detection and Treatment

An early diagnosis of cancer is very important for the potential survival of patients. If the tumor is detected at an early stage, i.e. before it has spread to other parts of the body, the effects of therapy and the prognosis of the disease are improved. Detection can be done for example by biopsies, lab tests and different visualization techniques such as x-rays, computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET).

Surgery, chemotherapy and external radiation are the most common therapies used to treat cancer, and different combinations are often used. A drawback with surgery, and to some extent radiotherapy, is that small metastases are not removed or killed. Chemotherapy on the other hand, affects all dividing cells, which includes metastases but also healthy tissues. These three conventional treatment methods cure approximately 60% of cancer patient [5]. For many patients with metastases or disseminated tumors the best effect of chemotherapy is prolonged survival and/or a palliative effect [6]. Other complementary methods that can improve survival are; immunotherapy, anti-angiogenesis and radionuclide therapy, among others.

2.1.2 Tumor Targeting

The optimal way to treat or detect cancer is to target the cancer cells only, i.e. tumor targeting. This can be accomplished by molecular recognition of structures present on cancer cells only. However, in reality these structures are also present in normal tissue but often to a much lower extent than in tumors. In theory, tumor targeting is a good method to find and treat disseminated tumor cells and metastases, but in order for the targeting to work they
need to express the target structure at sufficient levels. Examples of tumor targets are CEA, CD20 and the EGFR-family. Figure 1 shows the targeting principle.

The targeting agent can either have a therapeutic effect by itself, or an effector substance can be coupled to the targeting agent. Depending on the desired effect, different effectors can be used. Examples of such are cytotoxic drugs and radionuclides. The same concept is used for molecular imaging, which today is the most widely used application for the concept of targeting. It can be used for cancer diagnosis but also in other clinical areas like neurology and cardiology. Natural ligands, antibodies, antibody fragments and small peptides are examples of targeting agents.

![Figure 1. Tumor targeting. The targeting agent recognizes cell surface proteins on the tumor cells, and by selection of effector molecule the desired effect can be achieved. Radionuclides as effectors can be used for both imaging (a) and therapy (b).](image)

2.1.3 Molecular Imaging and Radionuclide Therapy Today

Molecular imaging is used to visualize, characterize and measure biological processes at molecular and cellular level [7]. In oncology it can for example be used to locate tumors, identify targets for tumor-targeted therapy and to monitor response to therapy.

Since there are many types and subtypes of tumors it is natural that they respond differently to different treatments. This creates a need for personalized therapy, meaning the tailoring of each patient’s treatment according to the characteristics of his/her disease. Personalized medicine demands a lot of tools for diagnosis and the rapidly evolving field of molecular imaging has great potential in this area [8]. One great advantage is that it can be used to
identify and quantify tumor markers, e.g. HER2 (human epidermal growth factor receptor 2) or estrogen receptors in breast cancer, without the invasiveness of a biopsy or surgery [9].

Radionuclide imaging techniques, which include single-photon emission computed tomography (SPECT) and positron emission tomography (PET), are powerful tools for detecting and staging tumors. Today, the most common molecular imaging method in oncology is $^{18}$F-FDG-PET (fluoridedioxoyglucose) [10].

For more than 50 years $^{131}$I has been used for therapy of thyroid cancer, but besides this few methods for targeted radionuclide therapy (TRT) are routinely used at the clinics. Two examples are Bexxar ($^{131}$I-tositumomab) and Zevalin ($^{90}$Y-ibritumomab tiuxetan) which are approved by the FDA (food and drug administration, USA) for clinical use in the treatment of CD20-positive non-Hodgkin’s lymphoma. Both compounds have shown impressive clinical outcomes with overall response rates of 80-100% and in some cases, no evidence of disease for several years [11]. So far TRT has not been very successful in treatment of solid tumors. Two reasons for this are the inability to deliver a sufficient radiation dose to tumor cells and the generally lower radiosensitivity of solid tumors [12]. During the last decade the field of TRT has expanded and the gradual shift towards treating patients with minimal residual disease instead of bulky tumors has given rise to several promising (phase I and II) studies [12,13,14].

TRT is a complex field that has to take many components into consideration. The choice of targeting agent and radionuclide is of course of great importance. Simplified, $\beta$- emitters are often the best choice for solid tumors while $\alpha$-emitters may be better suited for micrometastases due to their shorter range. Also, the radiolabeling method is of importance since it can have large effects on for example binding affinity, biodistribution and retention of the radionuclide [15]. Other important factors to evaluate when choosing radionuclide for therapy is the half-life and the possibility for production [6].

When using tumor targeting, both for imaging and in particular for therapy purposes where a toxic effector might be used, it is of great importance to be aware of the cellular events followed by binding of the target. The major goal in tumor targeting is either to ensure that the effector stays at the target for as long as possible, or that it relocates to the cell nucleus where the main target – the DNA, is situated. The targeting agent itself may also have a biological effect on the cell, which could be both good and bad. A dream scenario would be that the targeting agent delivers the toxic substance to the cell while simultaneously sensitizing the cell to its lethal effects.
2.2 The EGFR/ErbB Family

The epidermal growth factor receptor (EGFR) family consists of four members; EGFR, HER2, HER3 and HER4 (also known as ErbB1-4), from now on denoted as EGFR and HER2-4, while ErbB refers to all four receptors. The most extensively studied receptors are EGFR and HER2. During recent years HER3 has also come into focus, but less is known about HER4.

The ErbB receptors have evolved from a single ligand-receptor combination found in *C. elegans* to a complex signaling network in humans. Upon ligand binding to the receptor a signal is brought from the outside of the cell to the inside, and eventually to the nucleus, where changes in gene expression cause the cell to adjust to the environment. The many combinations in the ErbB-receptor network results in several regulatory levels and signal diversity, making it a robust system [16,17].

The receptors are expressed in most normal tissues, and the absence of one of the Erbb's is fatal during embryonic development. Abnormal expression and signaling of the receptors is associated with the development and progression of several forms of cancer and in addition to enhanced invasiveness and resistance to chemotherapy and radiation (see below for each receptor). Apart from cancer, the ErbB family has also been linked to other diseases like psoriasis, inflammation and Alzheimer’s diseases [18].

2.2.1 Structure

The ErbBs are type I receptor tyrosine kinases and they share the same structural basis. Each receptor contains an extracellular domain (ECD), a transmembrane domain (TM) and an intracellular tyrosine kinase domain flanked by a juxtamembrane region and carboxyl tail. The ECD is composed of four different subdomains (I-IV). Natural ligands bind to domain I and III, and domain II is known as the dimerization domain. The receptors can be posed in a “closed” and an “open” position in which the dimerization arm is exposed (reviewed in [19]). It is generally accepted that binding of a ligand stabilizes the receptor in the open position, which enables dimerization and leads to activation, resulting in downstream signaling and subsequent cellular processes such as proliferation, migration and apoptosis (reviewed in [20]). HER2 and HER3 are special since HER2 always has its dimerization arm exposed [21] and HER3 lacks a fully functional tyrosine kinase [22]. Figure 2 shows a simplified version of the four receptors and two receptors forming a heterodimer.

The receptors are controlled by at least 11 different EGF-related ligands, including EGF and neuregulin (NRG). Some ligands are receptor specific while others can bind more than one receptor. HER2 does not have any known ligand [23,24].
2.2.2 Receptor Dimerization

Receptor dimerization and activation is yet not fully understood. Crystallography studies have focused on the function of the extracellular domain in dimerization and have given a fairly good view of the ECD rearrangement required for ligand-induced dimerization. Unliganded receptors adopt an auto-inhibited configuration with the dimerization arm hidden [25]. Upon ligand binding, the receptor undergoes conformational changes that expose the dimerization arm [26,27]. The dimer interface is formed by domain II and a small part of domain IV [28]. It was earlier believed that one ligand could bind two receptors simultaneously and thus pull the receptors together, but it has been shown that two ligands are present in each EGFR homodimer [26,27]. Dimerization is thus entirely receptor-mediated since binding of a ligand merely stabilizes the receptor in the open position.

The steps between ligand binding and initiation of downstream signaling are more complex than previously believed. Seemingly, receptors can form both active and inactive dimers. Exposure of the dimerization arm is not sufficient for the formation of an active dimer but the intracellular part must also contribute. For the receptors to become active, asymmetric dimers must be formed in which the C-lobe of one kinase domain acts as an activator when binding to the N-lobe of the other receptor, resulting in kinase activation (see Figure 2) [29].
Further complicating the understanding of dimerization is the documentation of a variety of different dimers, with different requisitions for dimerization. Pre-formed dimers, even in the absence of ligand stimulation, have been reported by several groups [30,31], and some reports also speak of a higher order of oligomers, or receptor clusters [32,33,34]. Dimers formed by the kinase domain, and not the ECD, have also been studied. These so-called “quasi-dimers” are autoinhibited by the carboxyl-tail, which needs to unfold to enable dimerization [35,36]. Yet others have reported that for ligand-independent homodimerization, the extracellular domain is not needed and only the intracellular part is sufficient to form homodimers driven by over-expression [37,38].

The biological effect of inactive dimers and oligomers are to date not well understood. One possible explanation is that the inactive dimers are intermediate states to activation with the function of increasing ligand affinity [39,40,41], and/or to prevent ligand-independent activation [36].

2.2.3 Receptor Activation

Kinase activation leads to phosphorylation of tyrosine residues of the C-terminal tail. These residues serve as docking sites for signaling molecules containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains [42], which in turn activates numerous signal transduction pathways. The effect on downstream signaling, and hence the biological outcome, depends on the composition of the receptor pair and the identity of the ligand [43,44,45]. Heterodimers are regarded as more signaling-potent than homodimers [46,47].

Depending on which phosphotyrosine sites are phosphorylated different signaling proteins are engaged. The number of docking sites for certain signaling proteins differs between the receptors, for example EGFR has several shc/grb2 binding sites to activate mitogen-activated protein kinase (MAPK) [48], while HER3 has more phosphatidylinositol-3-OH kinase (PI3K) binding sites to initiate Akt signaling [49].

The ErbB receptors are capable of binding several docking proteins including: growth-factor-receptor-bound-2 (Grb2), Src-homology-2-containing (Shc), PI3K, STAT5 and Cbl, amongst others [48]. The focus of this thesis will be on the signaling pathways MAPK, PI3K/Akt and PLCγ, see the following section “Cell Signaling”. To conclude, activation of the ErbBs is regulated at multiple levels by several auto-inhibitory mechanisms.
2.2.4 EGFR

EGFR was the first receptor within the family to be identified, and it is also the most studied receptor, often used as a model of how the ErbB family works and functions. It was discovered by Stanley Cohen and co-workers in 1980 as the receptor to EGF [50]. Six years later, Cohen was awarded with the Nobel Prize in medicine for his discovery of growth factors.

EGFR can be bound by all the ligands in the family with the exception of the NRGs, and can form heterodimers with all three co-receptors. The ligands are EGF, transforming growth factor \( \alpha \) (TGF-\( \alpha \)), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR), epigen (EPN), ampiregulin (AR) [51].

Ligand binding to EGFR results in rapid internalization [52]. The main internalization pathway is through clathrin-mediated endocytosis, in which the receptor-ligand complexes are clustered in clathrin coated pits and transported to early endosomes [53,54]. Depending on the pH-stability of the ligand, the receptor is then either ubiquitinated and subsequently degraded, or recycled to the cell surface [53]. The other receptors are considered as endocytosis-impaired [55], and are more or less inefficiently degraded [53]. Heterodimerization with HER2 decreases internalization, especially when HER2 is overexpressed [56].

**EGFR in Cancer**

Deregulation of EGFR has been observed and is associated with poor prognosis in several cancers, for example head and neck, ovarian, breast and colorectal cancers [57]. But perhaps the best known example of EGFR overexpression is found in brain tumors where 40% of glioblastomas have gene amplifications [58]. Many cell types normally express EGFR, predominantly in squamous and glandular epithelium [59].

There are three main mechanisms by which EGFR can mediate tumor initiation and progression: overexpression, mutation and autocrine signaling of ligands that results in constitutive activation [60,61]. The most common mutation, EGFRvIII, has for example been found in gliomas, lung- and breast cancers. This mutation results in a receptor that cannot bind any ligand but is constitutively active [62].

2.2.5 HER2

HER2 is sometimes denoted as p185\(^{\text{ErbB2/neu}}\) or neu. Unlike the other receptors in the family, HER2 does not have a natural ligand. Instead it has a constitutively open dimerization arm, similar to the configuration of the other receptors open state. This structure makes the receptor unable to bind EGF-related peptides because domains I and III are too close for a ligand to fit [24]. Instead HER2 functions as a co-receptor for the other members of the
family. It is actually considered as the preferred dimerization partner for the other receptors [63,64]. Heterodimerization with HER2 leads to more effective signaling, for example by decreasing the rate of ligand dissociation and receptor down-regulation [56,65,66]. In addition, HER2 is capable of recruiting more signaling proteins than the other ErbBs, especially when overexpressed [67].

HER2 is considered as an oncogene that can cause cell transformation even in the absence of a ligand [68]. It is generally thought that when HER2 is overexpressed (approximately \( >10^6 \) receptors/ cell) it can induce ligand-independent dimerization, both with itself and with other receptors of the ErbB family, resulting in constitutive activation [37,46,69,70]. Even though it is likely that HER2 homodimers exist, crystallography studies of the ECD have failed to detect such [24].

Interestingly, recent studies have proposed the existence of a membrane-bound ligand for HER2, more precisely the cell surface mucin Muc4 [71,72].

**HER2 in Cancer**

The second member of the ErbB family was first found as an amplified gene (HER2) in breast cancer [73], and as a product of the neu oncogene in rat neuroblastomas [74]. Since then it has held its position as one of the most studied areas in human cancer and is a pursued target for therapy.

Increased expression and activity of HER2 induces cell transformation and tumorigenesis in several tumor types and model systems (reviewed in [75]). Overexpression due to gene amplification or transcriptional deregulation is seen in approximately 20-30% of breast cancer [76,77]. The HER2 status is often maintained during progression to invasive disease and breast cancer metastases generally overexpress HER2 to the same extent as the corresponding primary tumors [75,78]. Overexpression is also associated with poor patient prognosis, enhanced invasiveness and resistance to conventional therapies, e.g. chemotherapy and radiation [79,80]. Even though HER2 overexpression is not as common as for EGFR, it has been found in several other cancers, for example ovarian- [81], colorectal- [82] and gastric cancer [83]. Mutated HER2 has also been found in a subset of lung cancer [77]. The expression of HER2 is low to medium in normal tissue and generally lower than the expression of EGFR [59].

2.2.6 HER3

HER3 lacks a fully functional kinase domain but is capable of activating other ErbB receptors when forming heterodimers [84]. In fact, HER2-HER3 heterodimers are considered as the most potent receptor couple for mitogenic signaling [67,85]. Moreover, HER3 has 6 binding sites for PI3K and is thus a powerful activator of the PI3K/Akt pathway which affects cell survival. EGFR and HER2 cannot bind PI3K directly [49].
The receptor has two known ligands; NRG 1 (also known as heregulin HRG and neuregulating factor NDF) and NRG2, which both have several splicing variants [51].

**HER3 in Cancer**

High expression of HER3 has been reported in melanomas, colorectal and some lung cancers. Yet it is not considered as transforming when overexpressed and no oncogenic mutations have so far been identified [86]. However, HER3 is crucial for cell growth in tumors driven by HER2 overexpression [87,88]. Increased HER3 signaling is also an escape route for resistance to ErbB-targeted tyrosine kinase inhibitors (TKI) [89]. This makes HER3 interesting for combinatorial therapies that target several ErbB receptors.

**2.2.7 HER4**

HER4 is the least studied member of the ErbB family. It has several ligands of which some are shared by EGFR (HB-EGF, BTC, EPR), some by HER3 (NRG1 and 2) and NRG3 and 4 that are exclusive for HER4 [51].

HER4 has four different isoforms caused by alternative splicing of mRNA. In addition, the JM-a isoform can be cleaved at two different sites. Cleavage results in a soluble intracellular domain (4ICD) that can translocate to the nucleus, where it affects transcription [90]. The other ErbB receptors have also been found in the nucleus [91].

The significance of HER4 in cancer, and especially breast cancer, is unclear. Some reports describe HER4 as a tumor suppressor with pro-apoptotic effects, while others report that it can promote cell growth [92]. The contradicting evidence may be due to the different functions of the four isoforms [93].

**2.3 Cell Signaling**

Important signaling pathways activated by the EGFR family are the mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3 kinase (PI3K)/Akt cascade and the phospholipase Cγ (PLCγ). Signaling through these pathways are important for proliferation, survival and migration [94,95,96]. These pathways are outlined in Figure 3 and will be further discussed below.
Figure 3. Three major signaling pathways downstream of the ErbB receptors; PI3K/Akt, PLCγ and MAPK/Erk1/2 pathways. The PI3K/Akt pathway is associated with increased survival, PLCγ with migration, and MAPK signals are linked with an increase in proliferation.

Figure 3, which illustrates the pathways as more or less separate linear cascades, is a very simplified version of reality. Cellular signaling is a complex system with various crosstalks between a multitude of different players, both within the ErbB family, and to other signaling proteins in the surroundings. If one ErbB is blocked, signaling from other family members will be increased [89,97]. Co-activation of other receptor tyrosine kinases is believed to play an important role in tumor response to targeted therapies [98].

The strength and duration of signaling is regulated by several regulatory loops which give negative or positive feedback [17]. As in the case of MAPK signaling, the duration of the signal can affect the cellular outcome [99]. In general, endocytosis of receptors is considered as a way of down-regulating receptor-activated signaling. However, signaling can continue after internalization. It can even be initiated in the endosomes [54].

There are also several other signaling players affected by the ErbB receptors, for example STAT, catenin and Src [18]. Additionally, all ErbB receptors have also been reported in the nucleus where they may act as transcription factors [100].
2.3.1 MAPK

There are at least four distinct MAPK cascades; extracellular signal-regulated kinases (Erk)1 and 2, Jun amino-terminal kinases (Jnk)1/2/3, p38-MAPK and Erk5. Even though all are regulated by growth factors, only Erk1/2 is regarded as growth factor controlled while the others are considered more or less as stress-induced cascades [99]. All functional combinations of receptors and ligands are able to activate the Erk1/2 MAPK pathway, either directly through the SH2-binding domain or indirectly through binding of the Shc adaptor to the PTB-binding domain [20].

The MAPK signaling pathway is a three layer signaling cascade in which one MAPkinase phosphorylates the other (MAPKKK-MAPKK-MAPK). The Erk1/2 MAPK pathway starts with recruitment of Grb-2 to the phosphorylated receptor (or the adaptor protein Shc). This leads to a linear kinase cascade that culminates in activation of Erk1/2 (see Figure 3). One important regulator upstream of Erk1/2 is Ras. Erk1/2 phosphorylates several cytoplasmic proteins and is also translocated to the nucleus where it activates multiple transcription factors, for example regulators of cell cycle progression [85,95].

The Erk1/2 cascade was initially identified as a growth-promoting pathway, but has also been found to contribute to cell migration, tumor invasion and cell survival [99]. A constitutively active form of MAPK-kinases can transform cells in culture [101]. Approximately 30% of human tumors contain oncogenic mutations in the Ras genes, causing constitutive activation of downstream effectors, e.g. Erk1/2 [102,103].

2.3.2 PI3K / Akt

Activation of PI3K occurs through binding of its regulatory p85 subunit to a phosphotyrosine site on the receptor. This results in the recruitment of several signaling effectors, e.g. Akt (also known as protein kinase B (PKB)), which is recruited to the plasma membrane and activated by phosphorylation (see Figure 3 for further details). Cytoplasmatic Akt, and Akt translocated to the nucleus, target regulators of apoptosis and cell survival, e.g. Bad and caspase 9 [104]. PI3K can also be activated by Ras in the MAPK pathway [95,105]. The Akt cascade is negatively regulated by PTEN (phosphatase and tensin homologue deleted on chromosome ten). Several cancers have mutations in PTEN, resulting in constitutive activation of Akt [106]. Signaling through the PI3K/Akt pathway has come forth as an important player in cancer tumorigenesis, especially with regards to resistance to ErbB-targeted therapy [107,108].
2.3.3 PLCγ

PLCγ (PLCγ 1) is recruited to the membrane by activated EGFR and HER2, but can also be activated by PI3K products [20]. Activated PLCγ hydrolyzes lipids that generate second messengers. This results in calcium release and thereby activation of calcium/calcmodulin-dependent kinases (see Figure 3) [109]. PKC can also activate Raf, thus implicating crosstalk to the MAPK pathways [95]. PLCγ promotes tumor invasion and is required for cell motility induced by growth factors, e.g. through EGFR signaling [109]. The activity of PLCγ has also been related to radiation and chemotherapy resistance [110].

2.4 Targeting Agents for the ErbB-Family

Due to their overexpression and involvement in development of many cancers, the ErbB receptors (particularly EGFR and HER2) have been pursued as therapeutic targets for several decades. So far, there are two types of drugs in clinical use that target the ErbB receptors: monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs). Table 1 summarizes EGFR and HER2 targeting drugs.

Table 1. Examples of ErbB targeting agents.

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Type</th>
<th>Status</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Cetuximab/Erbitux®</td>
<td>mAb</td>
<td>appr. 2004</td>
<td>Colorectal, head and neck</td>
</tr>
<tr>
<td>EGFR</td>
<td>Panitumumab/Vectibix®</td>
<td>mAb</td>
<td>appr. 2006</td>
<td>Colorectal</td>
</tr>
<tr>
<td>HER2</td>
<td>Trastuzumab/Herceptin®</td>
<td>mAb</td>
<td>appr. 1998</td>
<td>Breast</td>
</tr>
<tr>
<td>HER2</td>
<td>Pertuzumab/Omnitarg</td>
<td>mAb</td>
<td>ICT</td>
<td>Prostate, breast, ovarian</td>
</tr>
<tr>
<td>EGFR</td>
<td>Gefitinib/Imressa®</td>
<td>TKI</td>
<td>appr. 2004</td>
<td>NSCLC</td>
</tr>
<tr>
<td>EGFR</td>
<td>Erlotinib/Tarceva®</td>
<td>TKI</td>
<td>appr. 2005</td>
<td>NSCLC, pancreatic</td>
</tr>
<tr>
<td>EGFR/</td>
<td>Lapatinib/Tykerb®</td>
<td>TKI</td>
<td>appr. 2007</td>
<td>Breast</td>
</tr>
</tbody>
</table>

Abbreviations: (mAb) monoclonal antibody, (TKI) tyrosine kinase inhibitor, (appr.) approved for clinical use by the FDA (Food and Drug Administration, USA), (ICT) in clinical trials, (NSCLC) non-small cell lung carcinoma.

2.4.1 Monoclonal Antibodies

There are two monoclonal antibodies that target EGFR, cetuximab and panitumumab. Both bind to the subdomain III of EGFR and prevent ligand binding [111]. Cetuximab has been shown to sensitize head and neck patients to irradiation [112], but neither of the drugs seem to have an effect on patients with mutations in the ras genes [111]. The most common side effect of EGFR-targeted drugs is skin toxicity [113].
Trastuzumab is a monoclonal antibody that targets HER2. It binds to the extracellular domain IV [21] and effects downstream signaling. Trastuzumab has been reported to down-regulate the amount of HER2 at the cell surface and activate the human complement cascade [114]. Some of its effects are thought to be mediated by inhibition of the PI3K/Akt pathway, and this signaling cascade is also believed to play an important role in trastuzumab resistance [108]. Today, trastuzumab is used for treatment of HER2-positive metastatic breast cancer. Trastuzumab in combination with chemotherapy has given the best results and significantly improved overall survival [115]. Pertuzumab is another antibody that binds HER2. By binding domain II it prevents receptor dimerization [116]. Currently, pertuzumab is undergoing clinical trials.

2.4.2 Tyrosine Kinase Inhibitors

TKIs target the kinase activity of the receptors. They are small molecule inhibitors that bind to the intracellular domain and prevent signal transduction. Two TKIs that target EGFR have been approved for clinical use, gefitinib and erlotinib. However, only patients with mutated EGFR kinase domain, resulting in constitutively active receptor, respond to gefitinib treatment [117]. Lapatinib is a dual EGFR and HER2 tyrosine kinase inhibitor that reversibly inhibits the ATP-binding site of their kinase domains. It has been proposed that lapatinib may be able to overcome trastuzumab resistance [118].

2.5 Affibody Molecules

The affibody molecules are originally derived from domain B of the staphylococcal protein A. This so-called Z domain consists of 58 amino acids in a three-helix bundle. By randomizing 13 surface-exposed amino acids, libraries containing up to $10^{11}$ molecules with the potential to bind to as many different targets have been created [119,120], see Figure 4. Selection of new binders is most often done by phage display but other methods such as ribosome display [120] and cell surface display [121] have also been used. If the affinity for the target is insufficient, affinity maturation can improve binding. One way to do this is by creating a new library where only some of the 13 amino acids are randomized [122]. Affibody molecules binding to a wide range of targets have been generated, for example insulin, amyloid beta peptide, CD28, TNF-α, HER3 and IgE [123].

Even though the affibody molecules are small (about 6 kDa for monomers and 15 kDa for dimers), they have a binding area similar in size to that of an antibody. Multimeric constructs can be generated by head-to-tail genetic
fusion, which can be used to increase affinity [124] or, as shown in this thesis, to generate a binder with different biological properties. The affibody molecules are stable at high temperature and in a wide pH-range [125]. By introduction of a terminal cysteine residue to the otherwise cysteine free scaffold, site specific labeling or immobilization on a solid surface can be performed. Another advantage is the possibility of synthetic production that gives the opportunity of adding conjugates like fluorophores or chelating groups for radiometal labeling [126,127].

![Image of Ig-binding domains of Protein A](image)

*Figure 4.* The affibody molecules are based on the Z-domain scaffold derived from domain B of the Ig-binding region of protein A. Large libraries of potential binders can be generated by randomizing 13 amino acids on helices 1 and 2 (indicated by yellow). Printed with permission from Affibody AB.

Affibody molecules have been used for a wide variety of applications ranging from biotechnological applications (e.g. immunoprecipitation and affinity chromatography) to clinically intended tumor targeting (e.g. imaging and therapy) [123]. Ongoing clinical studies at Akademiska hospital in Uppsala are using $^{111}$In-labeled ABY-025 for SPECT/CT imaging in breast cancer patients. ABY-025 is a DOTA-conjugated variant of Z$_{HER2:2891}$, which is a more stable variant of Z$_{HER2:342}$ due to reengineering of the scaffold [128]. So far the results from the study are promising. HER2-expressing metastases in brain, axilla and adrenal gland were visible 24 hours post-injection, and the opportunity to visualize the HER2 status in metastases has been used to modify further treatment of patients (personal communication with Jörgen Carlsson).
2.5.1 Affibody Molecules Used in the Present Study

In this thesis, several different affibody molecules have been used. The HER2-targeting $(Z_{HER2:4})_2$ is a dimeric version of the first HER2-targeting affibody generated by Wikman et al. This first binder had an affinity for HER2 of 50 nM, but a fast off-rate [129]. The binding properties were improved by the dimeric $(Z_{HER2:4})_2$ which has a $K_D$ of 3 nM [124]. In the affinity matured version $Z_{HER2:342}$, binding has been further enhanced and it has an apparent affinity for HER2 of 22 pM [122]. Schematic figures of the three HER2-binding ligands are presented in Figure 5.

The EGFR-targeting affibody molecule $Z_{EGFR:955}$ has been generated and described by Friedman et al. [130]. In the present study, the dimeric $(Z_{EGFR:955})_2$ and the affinity matured $Z_{EGFR:1907}$ [131] have been used. According to Biacore analysis they bind EGFR with a $K_D$ of 50 nM and 5 nM, respectively [130,131].

A bispecific affibody molecule that simultaneously targets both EGFR and HER2 has previously been described by us and a group at KTH [132]. Paper V describes the generation of six new bispecific-targeting affibody molecules based on monomeric ligands.
3 The Present Study

3.1 Aim

The aim of my Ph.D. work was to evaluate new ErbB-targeting affibody molecules regarding their cellular effects \textit{in vitro} upon receptor binding. The affibody molecules are intended for tumor targeting with the prospect of clinical use in imaging or therapy. Ligand binding to an ErbB receptor may have an impact on the cell. When used \textit{in vivo}, it is important to be aware of these effects as they may have consequences for the continued growth of the tumor.

Three types of affibody molecules have been studied;
- HER2-targeting (paper I-III).
- EGFR-targeting (paper IV).
- Simultaneous targeting of both EGFR and HER2 (paper V).
3.2 Summary of Included Papers

Studying the cellular effects of ligand binding has been achieved by studies of intracellular signaling (paper I-V), cell proliferation (paper I-II and V), migration and apoptosis (paper I), binding kinetics (paper II, III and V), survival and receptor dimerization (III and V). Additionally, the generation of six bispecific affibody molecules is described in paper V.

Human cancer cells in cultivation were used as the model in which the effects were studied. The cell lines used all overexpress at least one of the receptors in the ErbB family. Table 2 summarizes the cell lines and their expression levels of EGFR and HER2.

Table 2. Cell lines used in the present study. Additionally, HER3 is expressed in MCF-7 (~10⁴) and SKBR-3 (~10⁵).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR*</th>
<th>HER2*</th>
<th>Tumor type origin</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKBR-3</td>
<td>10⁴-10⁵</td>
<td>2-6x10⁶</td>
<td>Adenocarcinoma (breast)</td>
<td>I-III, V</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>2x10⁵</td>
<td>10⁷</td>
<td>Adenocarcinoma (ovary)</td>
<td>I-II</td>
</tr>
<tr>
<td>MCF-7</td>
<td>5x10³</td>
<td>2x10⁴</td>
<td>Adenocarcinoma (breast)</td>
<td>III</td>
</tr>
<tr>
<td>A-431</td>
<td>2x10⁶</td>
<td>2x10⁵</td>
<td>Squamous-cell carcinoma (epithelial vulva)</td>
<td>IV-V</td>
</tr>
<tr>
<td>U-343</td>
<td>6x10⁵</td>
<td>3x10⁴</td>
<td>Glioblastoma (brain)</td>
<td>IV</td>
</tr>
</tbody>
</table>

*Approximate number of receptors per cell according to the literature [41,70,133].
3.2.1 HER2-Targeting - Paper I-III

Three different HER2-binding affibody molecules have been studied regarding their cellular effects. In paper I, \((Z_{HER2:4})_2\) and \(Z_{HER2:342}\) were studied, while the focus of paper II and III is mainly on \((Z_{HER2:342})_2\). The ligands are sometimes denoted as \((Z4)_2\), \(Z342\) and \((Z342)_2\) to improve readability. The clinically used antibody trastuzumab was used as a reference substance. Three HER2-expressing cell lines have been used: SKBR-3, SKOV-3 and MCF-7.

Intracellular Signaling

Activation of the downstream signaling pathways was studied by examining the phosphorylation status of Erk1/2, Akt, PLC\(\gamma\) and the receptor itself (p-HER2). This was done either by SDS-PAGE and western blot or by ELISA. To quantify the results from the western blot experiments, the AIDA or Image J softwares were used to measure the intensity of the immunoblotting bands. Figure 6 shows the phosphorylation levels of HER2, Erk1/2 and Akt in SKBR-3 cells, and Figure 7 shows the p-HER2 status in SKOV-3 and MCF-7 cells.

![Figure 6](image_url)

*Figure 6. Phosphorylation and total levels of HER2 (A), Erk1/2 (B) and Akt (C) in SKBR-3 cells. A) Immunoprecipitation (IP) by HER2-specific antibody (Ab) was followed by SDS-PAGE and immunoblotting (Ib) with Abs specific for phosphotyrosine (pTyr) or total HER2. B, C) Immunoblotting was done with Abs specific for phosphorylated Erk1/2 (pErk), total Erk (Erk2), phosphorylated Akt (pAkt) or total Akt. Results from one representative experiment of three are shown.*

Generally, the effects of the monomeric \(Z_{HER2:342}\) were modest in both SKBR-3 and SKOV-3 cells. The dimeric affibody molecules, on the other hand, had more pronounced effects. Both \((Z_{HER2:4})_2\) and \((Z_{HER2:342})_2\) activated the receptor, increased phosphorylation of Erk1/2 (although not in SKOV-3, since Erk1/2 is constitutively active in this cell line) and resulted in a tendency of increased phosphorylation levels of Akt.
It should be noted that trastuzumab induced phosphorylation of HER2 but decreased phosphorylation of Akt. This was seen in both cell lines. The literature on phosphorylation status of HER2 upon trastuzumab treatment is somewhat contradicting. Most groups report that the antibody does not have any effect on the activation status [108,134], yet others confirm our results showing increased phosphorylation [135].

Figure 7. Phosphorylation levels of HER2 in (A) SKOV-3 cells, (B) MCF-7 cells. A) Immunoprecipitation was done with HER2-specific antibody prior to SDS-PAGE, followed by immunoblotting by antibodies specific for phosphotyrosine (pTyr) or total HER2. Phosphorylation of HER2 is plotted as pTyr divided by total HER2. Error bars represent standard deviation (n=3). B) Total cell lysates subjected to ELISA and detected by p-HER2-specific antibodies. Numbers indicates minutes of stimulation with \((Z_{HER2:342})_2\).

**Cell Growth**

The effects on cell growth of the three different affibody molecules were tested in SKBR-3 and SKOV-3 cells. Cells were continuously treated with approximately 10 nM of each substance for a long period of time (33-56 days). The cell culture medium and treatment substances were changed three times a week. Once a week the cells were counted and a suitable amount of cells were reseeded to allow further proliferation. In the experiment described in paper II, the treatments were terminated after 56 days and the cells were further cultivated in complete cell culture medium without supplementing with the substances. Cell growth was monitored for an additional 4-6 weeks. Control experiments that were performed in the HER2-negative cell line MDA-231 showed that \((Z_{HER2:342})_2\) had no effect compared to untreated cells (data not shown).
Figure 8. Cell growth of SKBR-3 (A & B) and SKOV-3 (C & D) cells. The cells were continuously treated with the substances for 56 days after which the treatments were ended, indicated by interrupted lines in A and C. Cell growth after cessation of treatment is plotted in B and D. The number of cells is normalized to the starting value within each group, which in figure B and D is the total number of cells at day 56. The lines in A, C and D represent curve fits performed by non-linear regression of exponential growth. Error bars represent standard deviations (n=3). The experiment was repeated with similar results.

As can be seen in Figure 8, the substances have different effects and the outcomes of treatment are also different in the two cell lines. Generally, the effects of ZHER2:342 were minor. In SKOV-3 cells, both dimeric affibody molecules had a small but significant growth-promoting effect. In SKBR-3 the consequences of treatment with the substances differed. Both (ZHER2:4)2 and (ZHER2:342)2 had a similarly suppressive effect on cell growth as the therapeutic drug trastuzumab. Interestingly, the cells treated by dimeric affibody molecules were further growth-suppressed when the treatments were ceased (Figure 8B). This phenomenon was again seen when the experiment was repeated (data not shown). Trastuzumab treated SKBR-3 cells immediately resumed to the same growth rate as untreated cells.

The differences between the cell lines could be explained by the fact that SKOV-3 is a cell line with low expression of PTEN [108] and lacks ARHI [136], which is a tumor suppressor protein sharing 60 % amino acid homo-
logy with Ras, leading to constitutively active Erk [108]. The low expression of PTEN is also a reason to why trastuzumab has no effect on SKOV-3, as the antibody is believed to recruit PTEN and thereby inhibit Akt signaling [134]. That SKOV-3 cells are resistant to trastuzumab treatment has been shown before [108].

The phosphorylation levels of Erk1/2 and Akt in SKBR-3 cells after prolonged treatment were studied, in an attempt to learn more about the molecular mechanism behind the growth inhibitory effects of the affibody molecules and trastuzumab seen in the proliferation study (Figure 8). This was done by reseeding some of the cells after counting, and after sufficient time for attachment the cells were lysed and subjected to SDS-PAGE and western blot. Figure 9 shows the levels of phosphorylated Erk1/2 and Akt in SKBR-3 cells at week 3, 8, 9 and 12. No deviations from the untreated control could be detected in SKOV-3 cells (data not shown).

Figure 9. Phosphorylation levels of Erk1/2 (A) and Akt (B) in SKBR-3 cells from the proliferation study described in Figure 8. The phosphorylation levels are the intensity of the western blot bands quantified by the Image J software and normalized to the untreated control, which is indicated as a dotted line. Mean values and standard deviations from three measurements are shown.

Surprisingly considering the decrease in cell growth, both \((Z_{\text{HER2}:4})_2\) and \((Z_{\text{HER2}:342})_2\) strongly increased the levels of phosphorylated Erk1/2 after eight weeks of treatment (Figure 9A). It has been reported that prolonged hyperactivation of the Erk-pathway promotes cell cycle arrest and cell senescence [137,138]. A drastic drop in p-Erk1/2 levels were seen the week after, when the treatment had ended. Apart from this, the effects of all substances on phosphorylated Erk1/2 and Akt were modest. Nevertheless, it can be noted that trastuzumab decreased phosphorylation of Akt during treatment, which was also noted in the short time study (Figure 6). This further supports the general theory that some of trastuzumabs effects are mediated through attenuation of the PI3K/Akt pathway [108,134]. The phosphorylation level of Akt immediately resumed to untreated levels, as did the growth rate, upon the cessation of treatment. In contrast, cells treated by \((Z_{\text{HER2}:4})_2\) or \((Z_{\text{HER2}:342})_2\) had decreased levels of p-Akt at weeks nine and twelve (Figure 9B). These
differences in signaling patterns indicate that even though trastuzumab and the dimeric affibody molecules target the same receptor and induce similar growth inhibitory effects on SKBR-3 cells, the effects are mediated through different molecular mechanisms.

Migration
A so-called wound-healing assay was used to study the effects on migration, as previously described [139]. In this experiment “wounds” were introduced into a confluent layer of SKOV-3 cells, which were treated with \((Z_{\text{HER2:4}})_2\), \(Z_{\text{HER2:342}}\), trastuzumab or left untreated. Cell migration to close the wounds was followed for 72h by measuring the width of the wound and taking phase-contrast microscopic pictures. The experiment was performed in serum-free cell culture medium to minimize proliferation. The result is presented by photographs in Figure 10 and as a graph in Figure 11.

Figure 10. Photographs of the wound-healing assay taken in a phase-contrast microscope. Cell migration to close the wounds in confluent SKOV-3 cells was followed for 72h. Representative photographs from three experiments are shown.
Figure 11. Quantification of the migration assay. “Wounds” were introduced in confluent SKOV-3 cells that were treated with $(Z_\text{HER2:4})_2$, $Z_\text{HER2:342}$, trastuzumab or left untreated. Three wounds were introduced in each group. The widths of the wounds were measured in a microscope using a scale bar. The width of the wounds at time zero were set as 0% wound healing. Serum-free cell culture medium was used to minimize proliferation.

The cells treated with $(Z_\text{HER2:4})_2$ closed the wound faster than the untreated cells. Trastuzumab decreased the rate of healing while $Z_\text{HER2:342}$ had no effect. However, proliferation may also have some effect on the outcome of this assay, even though serum-free cell culture medium was used. Increased migration has the risk of causing enhanced metastasizing in a clinical setting.

Survival
The radiosensitizing effect of the affibody molecules, in particular $(Z_\text{HER2:342})_2$, were evaluated in SKBR-3, MCF-7 and SKOV-3 cells. As shown in Figure 12A, treatment with $(Z_\text{HER2:342})_2$ decreased SKBR-3 survival 4-fold after irradiation with 8 Gy compared to an irradiated control ($S_{8\text{Gy}}$ 0.006 and 0.023, respectively, $P<0.01$). After 4 Gy of radiation, the two groups did not significantly differ. For MCF-7 cells, treatment with $(Z_\text{HER2:342})_2$ did not alter the survival, either at 4 or 8 Gy (Figure 12B).
Figure 12. Survival measured by clonogenic survival after γ-irradiation in SKBR-3 (A) and MCF-7 cells (B). Cells were treated with 10 nM \((Z_{HER2:342})_2\) for 2 hours prior to irradiation and allowed to repair for 16 hours before reseeding. After 3 weeks of cultivation, colonies containing more than 50 cells were counted and the survival fraction was calculated. Mean values and standard deviations are calculated on at least 7 replicates.

To verify the results, a different survival model was also used. Proliferation of SKBR-3 and SKOV-3 cells exposed to external γ-irradiation were followed until exponential growth was reached. This time, treatment with monomeric ZHER2:342 was also included. Figure 13A shows the growth of SKBR-3 cells treated by \((Z_{HER2:342})_2\). Extrapolation of the curve fit results in an estimate of the surviving fraction of cells that is responsible for the regrowth [140]. The surviving fraction calculated by this method was similar to the result from the clonogenic survival, rendering a significant decrease in survival compared to the control (\(S_{6\text{Gy}} 0.015\) for \((Z_{HER2:342})_2\) compared to \(S_{6\text{Gy}} 0.05\) for the control \((P<0.01)\)). Figure 13B shows the number of SKBR-3 cells after 28 days of cultivation after irradiation with 2 and 6 Gy. Notably, the monomeric \(Z_{HER2:342}\) did not sensitize for radiation, on the contrary it seems to have a protective effect at the lower dose. No deviations from the untreated control could be detected in SKOV-3 cells (data not shown).
Figure 13. Cell growth of SKBR-3 cells after exposure to external γ-irradiation. A) Cells treated by 17 nM (Z_{HER2:342})_2 for 2 hours prior to irradiation. Curve fits are based on the data points where exponential growth has been reached. B) Number of cells after 4 weeks of cultivation after irradiation. Mean values and standard deviations of 3 values are shown.

It should be noted that MCF-7 cells are more radiosensitive than SKBR-3 cells, mean survival after 8 Gy (S_{8Gy}) was 6% compared to 23%, respectively. SKOV-3 was the least sensitive cell line and had a calculated S_{8Gy} of 3% according to the growth extrapolation method. It has been reported that increased levels of HER2 is corresponding to increased radioresistance [141], which is in agreement with this study. However, also other differences between the cell lines can have an effect. In the case of SKOV-3 it is likely that the low expression of PTEN is contributing to its radioresistance.

**Dimerization**

The membrane impermeable crosslinker bis[sulfosuccinimidyl] suberate (BS^3) was used to examine receptor dimerization. After stimulation by the affibody molecules and natural ligands, the cells were treated with BS^3 prior to cell lysis. This 11 Å long reagent crosslinks extracellular proteins through the amino groups. Monomeric and dimeric receptors were then separated by SDS-PAGE and detected by western blot.
Figure 14. Dimerization assay. SKBR-3 cells were treated with 10 nM \((Z_{\text{HER2:342}})^2\) for 5, 15, 60 and 120 minutes. \(Z_{\text{HER2:342}}\) was used at 100 nM for 1h. Untreated cells (-), and cells treated with EGF and nrg1-\(\beta1\) for 15 minutes were used as controls. Cells were cross-linked by 5 nM BS\(^3\) for 30 minutes before cell lysis. Total cell lysates were then subjected to SDS-PAGE and western blot with (A) HER2- and (B) EGFR-specific antibodies. An antibody recognizing \(\beta\)-actin was used to ensure equal loading.

As can be seen in Figure 14, \((Z_{\text{HER2:342}})^2\) strongly increased the levels of dimeric HER2 (represented by the upper band). The monomeric \(Z_{\text{HER2:342}}\) on the other hand did not induce any receptor dimerization. As expected, no changes in the levels of dimeric EGFR (Figure 14B) or HER3 (data not shown) could be detected upon treatment by HER2-specific affibody molecules. A small tendency for increased levels of dimeric HER2 and EGFR could be detected after treatment by nrg1-\(\beta1\) and EGF, respectively. Since EGFR is known to rapidly internalize upon stimulation by EGF [52], it is possible that a large part of the EGF induced EGFR-dimers had already internalized after 15 minutes and that a shorter stimulation time for EGF would have been more suitable.

The dimerization assay indicates that \((Z_{\text{HER2:342}})^2\) are capable of inducing receptor dimerization. However, the assay cannot distinguish between fully functional receptor dimers and receptors that are merely present at the cell membrane in such close proximity to each other that they can be cross-linked by BS\(^3\). Since HER2 has a dimerization arm that is always exposed, it is possible that \((Z_{\text{HER2:342}})^2\) can span the dimer interface and induce homodimerization by pulling two receptors together.

Conclusions from Paper I-III

One of the most striking aspects of the results is the differences in effect between monomeric and dimeric ligands. Even though \(Z_{\text{HER2:342}}\) and \((Z_{\text{HER2:342}})^2\) are based on the same peptide and should bind to the same epitope of HER2, their effects on intracellular signaling and biological outcome are not the same. Generally, the effects of the monomeric \(Z_{\text{HER2:342}}\) were small, while the dimeric binders \((Z_{\text{HER2:342}})^2\) and \((Z_{\text{HER2:4}})^2\) were capable
of promoting phosphorylation of HER2 and inducing changes in signal transduction and biological outcome. For example, treatment with \((Z\text{HER2:342})_2\) decreased cell growth and increased radiosensitivity in SKBR-3 cells. A possible explanation for the observed differences was found in the results of the dimerization study. The dimeric \((Z\text{HER2:342})_2\) is capable of inducing HER2 dimerization, while the monomeric \(Z\text{HER2:342}\) is not. However, the assay cannot distinguish between functional receptor dimers and receptors that simply are close enough to be cross-linked. Taken together with all the results from paper I-III it is likely that the dimeric affibody molecules are capable of inducing effective HER2 dimerization.

It is intriguing that increased HER2-dimerization, which is generally considered as mediating oncogenic effects, would result in decreased cell growth and increased radiosensitivity. High expression of HER2, as in the SKBR-3 cell line, is believed to result in ligand-independent dimerization, both with itself and with other receptors of the ErbB family \([37,142]\). It is possible that the \((Z\text{HER2:342})_2\)-induced dimers differ from these “naturally” induced receptor dimers. A possible way to examine this would be to study the phosphorylation of specific tyrosine residues in HER2. Another potential explanation is that \((Z\text{HER2:342})_2\) might prevent ligand-induced formation of the more signaling potent heterodimers by locking HER2 in homodimers. However, this must be studied in a cell line with a more balanced expression level of the different receptors than in SKBR-3. Additionally, also other bivalent binders that target the ErbB-family have been shown to sensitize to irradiation, for example trastuzumab and cetuximab \([112,141]\).

The effects of the ligands are also dependent on the cell line. While \((Z\text{HER2:342})_2\) has a slightly growth promoting effect in SKOV-3 cells, it decreases growth of SKBR-3 cells. This finding could provide clues as to how the ligand induces its biological effects. Since SKOV-3 has constitutively active Erk1/2 and Akt, it is likely that at least part of the effects induced by \((Z\text{HER2:342})_2\) are mediated by these pathways.

The dimeric \((Z\text{HER2:342})_2\), and perhaps also \((Z\text{HER2:4})_2\), show promising characteristics. It sensitizes SKBR-3 cells to irradiation and decreases cell growth to the same extent as the clinically approved mAb trastuzumab. This makes \((Z\text{HER2:342})_2\) interesting for therapy purposes, while \(Z\text{HER2:342}\) might be better suited for imaging since it does not seem to induce any large effects on cells.
3.2.2 EGFR-Targeting - Paper IV

In this article, the intracellular signaling effects of the EGFR-binding affibody molecule \( Z_{\text{EGFR,955}} \)\(^2 \) are examined. The clinically approved antibody cetuximab (Erbitux\textsuperscript{®}) and the natural ligand EGF were included as reference molecules. The studies were performed in the EGFR-expressing cell lines A-431 and U-343.

**Intracellular Signaling**

The effect on the downstream signaling pathways MAPK/Erk and PI3K/Akt were analyzed by studying the phosphorylation levels of Erk1/2 and Akt, see Figure 15. All three substances were able to induce phosphorylation of Erk1/2 in both cell lines. The low background levels of activated Erk1/2 were rapidly increased upon stimulation. While the phosphorylation caused by EGF was still visible after 2 hours, signals caused by \( Z_{\text{EGFR,955}} \)\(^2 \) and cetuximab were more transient. The substances were also able to increase phosphorylation of Akt in A-431 cells, but not in the U-343 cells. This was due to the high background level of activated Akt since the U-343 cell line has mutations in PTEN which result in constitutive activation of Akt [143].

**Figure 15.** Phosphorylated Erk1/2 (A) and Akt (B) in A-431 (upper panel) and U-343 (lower panel) cells. Treatment time is indicated in minutes. Untreated cells were used as negative controls (-), and cells incubated with EGF for 15 minutes as positive control (+).

**Receptor Phosphorylation**

The EGFR receptor was phosphorylated by EGF and cetuximab in both cell lines. Treatment by EGF resulted in strong phosphorylation as early as 5 minutes after stimulation and lasted for at least 2 hours in A-431 cells while it was more transient in U-343 cells. Phosphorylation induced by cetuximab was weaker. \( Z_{\text{EGFR,955}} \)\(^2 \), on the other hand, did not induce phosphorylation of EGFR. This was very surprising since the affibody molecule can activate downstream signaling. Phosphorylated EGFR is shown in Figure 16.
Phosphorylation levels are shown to the left and total receptor amount to the right. Treatment time is indicated in minutes. Untreated cells were used as negative controls (-), and cells incubated with EGF for 15 minutes as positive control (+).

Figure 16. Phosphorylated EGFR in A-431 (A) and U-343 (B) cells. To see if the downstream signaling was caused by phosphorylation of one of the other receptors in the family, HER2, HER3 and HER4 were also studied. However, no phosphorylation after treatment by (ZEGFR:955)2 could be detected in either of these receptors, see Figure 17. Cetuximab, on the other hand, phosphorylated all receptors. Treatment with EGF resulted in activation of HER2 and HER4 in A-431 but not HER3.

Figure 17. Phosphorylated receptors in A-431 (A) and U-343 cells (B). Phosphorylation levels are shown to the left and total receptor amount to the right. Cells were treated with approximately 5-10 nM of substances for 15 minutes. Untreated cells were used as controls (-).

To see if the downstream signaling was caused by phosphorylation of one of the other receptors in the family, HER2, HER3 and HER4 were also studied. However, no phosphorylation after treatment by (ZEGFR:955)2 could be detected in either of these receptors, see Figure 17. Cetuximab, on the other hand, phosphorylated all receptors. Treatment with EGF resulted in activation of HER2 and HER4 in A-431 but not HER3.

One explanation for the lack of detectable receptor phosphorylation despite activation of Erk1/2 and Akt by (ZEGFR:955)2 could be due to the limitations of the western blot method and the antibodies used. The levels of phosphorylated receptor could be so low that we are not able to detect them, yet still
sufficient for activation of the signaling cascades. The receptors are probably present at the cell membrane in the “closed” configuration in equilibrium with the “open” configuration (further discussed in section 2.2). It is thus possible that the affibody ligand can bind to a receptor in the open state with its dimerization arm exposed. If one ligand happens to catch two open receptors, dimerization might occur and the signaling cascades are activated. Since the probability for this should be low, it could explain the very low levels of phosphorylated receptor (which are too low for detection). Phosphorylation of Erk1/2 without notable activation of HER2 was observed in a concentration study of the HER2-binding affibody \((Z_{\text{HER2:4}})_2\) on SKBR-3 cells (data not shown). Phosphorylation of Erk1/2 was noted after stimulation by such low amounts as 0.2 nM of \((Z_{\text{HER2:4}})_2\) while phosphorylation of the receptor was visible first at 10 nM.

Another explanation could be that \((Z_{\text{EGFR:955}})_2\) binds to EGFR in such a way that EGFR interacts with other protein tyrosine kinases. EGFR is for example known to be activated by G protein-coupled receptor (GPCR) and growth hormone activation of Jak2 (Janus kinase) [100].

**Conclusions from Paper IV**

A surprising observation from this study was that \((Z_{\text{EGFR:955}})_2\) could induce activation of the downstream signaling proteins Erk1/2 and Akt without any obvious phosphorylation of EGFR. The most likely explanation for this is that the level of phosphorylated receptors (EGFR or other members of the ErbB family) is too low to be detected by our method.

The fact that the induced signaling pattern of \((Z_{\text{EGFR:955}})_2\) is similar to that induced by cetuximab makes the affibody molecule a potentially interesting candidate for EGFR-targeting therapy, especially since Cetuximab has been shown to sensitize to irradiation [112]. Cetuxumab and \((Z_{\text{EGFR:955}})_2\) have also been shown to compete for the same binding site and are internalized in a similar manner in A-431 cells [144].
A bispecific EGFR- and HER2-targeting affibody molecule has previously been generated and described by us and collaborators at KTH [132]. A problem with this ligand was that it could not simultaneously bind both EGFR and HER2 if the HER2-binding part was allowed to bind first. Only if EGFR was targeted first, binding could occur to both receptors, see Figure 18. This ligand was denoted \((Z_{\text{HER2:342}})_2-(\text{G}_4\text{S})_3-(Z_{\text{EGFR:1907}})_2\), and was thus constructed of a dimeric HER2-binder, \((Z_{\text{HER2:342}})_2\), and a dimeric EGFR-binder, \((Z_{\text{EGFR:1907}})_2\). A probable explanation for the domination of the HER2-binding part could be the differences in affinity to the receptors. \((Z_{\text{HER2:342}})_2\) has a higher association rate and a more than 100-fold better affinity for HER2 than \((Z_{\text{EGFR:1907}})_2\) has for EGFR [122,131]. It is therefore likely that binding to HER2 takes place first. The lack of subsequent detectable binding to EGFR could be caused by inaccessibility of the EGFR-binding epitope in the bispecific affibody molecule due to steric interference or rigidity. Other possible explanations are methodological limitations and/or insufficient concentrations to allow detection of the binding with lower affinity.

![Figure 18](image-url)

**Figure 18.** A) The binding of \(^{14}\text{C}\)-labelled A-431 cells to SKBR-3 cells was monitored in real-time by LigandTracer White. B) The opposite set-up, \(^{14}\text{C}\)-labelled SKBR-3 cells were allowed to bind A-431 cells. The \(^{14}\text{C}\)-labelled cell suspension was pre-incubated with bispecific affibody molecule, and then added to a cell dish with SKBR-3 or A-431 cells and the uptake of \(^{14}\text{C}\) was measured. \(^{13}\text{C}\)-labelled cells without bispecific affibody molecule were used as negative controls. (cps) counts/s.

**Production**

The second generation of bispecific affibody molecules was based on monomeric ligands. For the EGFR-binding part \(Z_{\text{EGFR:1907}}\), was used. This is an affinity matured version of \(Z_{\text{EGFR:955}}\) which is evaluated in its dimeric form in paper IV. \(Z_{\text{EGFR:1907}}\) is reported to bind to EGFR with an affinity of 5 nM [131]. Two different HER2-binding ligands were used, \(Z_{\text{HER2:4}}\) which binds...
to HER2 with an affinity of 50 nM [129], and the affinity maturated binder \( Z_{\text{HER2:342}} \), which has an affinity of 22 pM to HER2 [122]. The affinities towards the receptors are thus more equal in some of these constructs than in the former bispecific ligand [132].

The two binding domains, directed towards EGFR and HER2, were assembled head-to-tail and separated by a spacer sequence to facilitate flexibility and separation of the two binding arms. This so-called linker was a 20 amino acid long glycine or serine based sequence, \( (G_4S)_3 \) and \( (S_4G)_3 \). Furthermore, the influence of the order of the affibody molecules constituting the bispecific constructs was investigated by alternating the placement of the EGFR- and HER2-binding domains either at the N- or the C-terminus. The six different constructs are listed in Table 3. The constructs are herewith denoted by their roman numerals (I-VI).

Table 3. Constructs of six bispecific binders. To improve readability, the constructs are herewith named by roman numerals.

<table>
<thead>
<tr>
<th>Name</th>
<th>Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>( Z_{\text{HER2:4}} \cdot (S_4G)<em>3 \cdot Z</em>{\text{EGFR:1907}} )</td>
</tr>
<tr>
<td>II</td>
<td>( Z_{\text{EGFR:1907}} \cdot (S_4G)<em>3 \cdot Z</em>{\text{HER2:4}} )</td>
</tr>
<tr>
<td>III</td>
<td>( Z_{\text{HER2:342}} \cdot (S_4G)<em>3 \cdot Z</em>{\text{EGFR:1907}} )</td>
</tr>
<tr>
<td>IV</td>
<td>( Z_{\text{EGFR:1907}} \cdot (S_4G)<em>3 \cdot Z</em>{\text{HER2:342}} )</td>
</tr>
<tr>
<td>V</td>
<td>( Z_{\text{HER2:4}} \cdot (G_4S)<em>3 \cdot Z</em>{\text{EGFR:1907}} )</td>
</tr>
<tr>
<td>VI</td>
<td>( Z_{\text{EGFR:1907}} \cdot (G_4S)<em>3 \cdot Z</em>{\text{HER2:4}} )</td>
</tr>
</tbody>
</table>

**Binding Properties**

The binding properties of the six constructs were examined both in Biacore and in a cellular setting in LigandTracer. The kinetic data are presented in Table 4 and Table 5, respectively. Both methods give similar results, showing that all constructs are able to bind both receptors. It is also evident that binding of all three receptor targeting ligands are benefited by placement at the N-terminus of the construct. This is especially notable in the Biacore setup, while it is most evident for constructs V and VI in the LigandTracer experiment. In SKBR-3 cells, in which the binding predominately originates from the HER2-binding part, construct V has higher affinity than VI. The opposite is shown in A-431 cells where binding is predominantly towards EGFR, here construct VI has higher affinity than V (see Table 5). According to the Biacore analysis, constructs III and IV are the most promising candidates. Especially construct IV shows avidity when allowed to bind to both targets (Table 4).
Table 4. Kinetic data from the Biacore assay. Bispecific affibody molecules (I-VI) were injected over immobilized EGFR, HER2 and a mixed surface containing both EGFR and HER2. Kinetic parameters were estimated using TraceDrawer, using a 1:1-binding model.

<table>
<thead>
<tr>
<th></th>
<th>EGFR</th>
<th>HER2</th>
<th>EGFRxHER2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_a$ (M$^{-1}$s$^{-1}$)</td>
<td>$k_d$ (s$^{-1}$)</td>
<td>$K_D$ (nM)</td>
</tr>
<tr>
<td>I</td>
<td>1.1 x 10^4</td>
<td>5.3 x 10^-4</td>
<td>47</td>
</tr>
<tr>
<td>II</td>
<td>1.7 x 10^4</td>
<td>5.0 x 10^-4</td>
<td>29</td>
</tr>
<tr>
<td>III</td>
<td>6.8 x 10^3</td>
<td>5.9 x 10^-4</td>
<td>86</td>
</tr>
<tr>
<td>IV</td>
<td>1.1 x 10^4</td>
<td>6.1 x 10^-4</td>
<td>55</td>
</tr>
<tr>
<td>V</td>
<td>1.3 x 10^4</td>
<td>5.6 x 10^-4</td>
<td>43</td>
</tr>
<tr>
<td>VI</td>
<td>1.5 x 10^4</td>
<td>5.2 x 10^-4</td>
<td>35</td>
</tr>
</tbody>
</table>

NA = not available.

Interestingly, bispecific constructs containing the novel (S$_4$G)$_3$ linker (I and II) displayed a higher affinity in cell binding, as compared to constructs containing the (G$_4$S)$_3$ linker (V and VI), see Table 5. An avidity effect of constructs I and II were seen in SKBR-3 cells in the LigandTracer experiment. Blocking only one of the receptors resulted in weak but visible binding, which was notable during the association phase but had a very quick off-rate. Without any pre-blocking a clear binding rate could be seen both during the binding- and retention phase, clearly indicating avidity. In A-431 cells, constructs II-V showed increased binding when both receptors were present compared to when only EGFR was available. When EGFR was blocked, no binding could be detected in this cell line (Table 5).

Table 5. Kinetic data from binding assays in LigandTracer. Kinetic parameters were estimated using TraceDrawer, using a 1:1-binding model.

<table>
<thead>
<tr>
<th></th>
<th>Binding to SKBR-3 cells</th>
<th>Binding to A-431 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_D$ (nM)</td>
<td>$K_D$ (nM)</td>
</tr>
<tr>
<td>I</td>
<td>No block</td>
<td>Pre-block $Z_{HER2:4}/Z_{HER2:342}$</td>
</tr>
<tr>
<td></td>
<td>ZHER2:4</td>
<td>ZHER2:342</td>
</tr>
<tr>
<td>I</td>
<td>14</td>
<td>W</td>
</tr>
<tr>
<td>II</td>
<td>24</td>
<td>W</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>V</td>
<td>370</td>
<td>ND</td>
</tr>
<tr>
<td>VI</td>
<td>1000</td>
<td>ND</td>
</tr>
</tbody>
</table>

W = weak binding, no kinetic parameters could be calculated.

ND = not detectable (no signal of binding was obtained).
A possible explanation for the discrepancy between the two methods (as between the two cell lines) is the variances in receptor numbers. In the Biacore set-up, the EGFR and the HER2 receptor are present in equal numbers, while in LigandTracer it depends on the cell line. SKBR-3 cells have approximately $6 \times 10^6$ HER2 receptors but only $4 \times 10^5$ EGFR receptors [41]. Furthermore, in Biacore the binding is measured to recombinant proteins in an artificial environment, while the binding in LigandTracer is measured to functional receptors in living cells.

Apparently, the balance in receptor number as well as the differences in affinity for the two targets have large impact on the behavior of a bispecific binder. This can be exemplified by construct IV: In SKBR-3 cells, binding is predominantly originating from the high affinity HER2-binder $Z_{\text{HER2:}342}$, and no apparent contribution is made by the EGFR-binding part. On the contrary, in A-431 cells and to the mixed receptor surface in the Biacore assay, the construct shows avidity.

**Cellular Effects**

The dimerization study presented in Figure 19 clearly shows that all six bispecific affibody molecules induce dimeric EGFR in SKBR-3 cells, but no dimeric receptors could be detected in A-431 cells that mostly express high numbers of EGFR. However, the assay cannot distinguish between EGFR-containing heterodimers and homodimers. Immunoblotting with an antibody specific for HER2 rendered ambiguous results. This was probably due to the high background level of dimerized HER2 which conceals the small changes induced by the bispecific affibody molecules (data not shown).

![Figure 19. Dimerization assay of the bispecific binders. Monomeric affibody molecules and EGF were used as controls. After stimulation by 100 nM of each substance for 1 h at 37°C, the chemical crosslinker BS3 was added. EGF-treatment lasted only 5 minutes to minimize internalization. After cell lysis, the samples were subjected to SDS-PAGE, followed by transfer and western blot. A) SKBR-3 cells. B) A-431 cells.](image)

The phosphorylation studies, which were performed both by ELISA and western blot, show a small but significant increase in phosphorylated EGFR after treatment with bispecific binders. Figure 20 shows the phosphorylation levels of EGFR measured by ELISA.
Figure 20. Phosphorylation study of the EGFR receptor by ELISA. SKBR-3 cells (A) or A-431 cells (B) were stimulated with 100 nM of each substance for 1 h at 37°C. EGF-treatment was only 5 minutes in order to minimize internalization. To enable comparison between different experiments, the obtained values were normalized to their respective untreated control. The graphs show mean values from 4 experiments with the standard deviations. In A) construct I-VI and EGF deviate significantly from the untreated control (unpaired t-test, P<0.05).

Both the dimerization and the phosphorylation studies indicate that the bispecific affibody molecules are able to dimerize the receptors when both HER2 and EGFR are present. However, the assay cannot distinguish between fully functional receptor dimers and receptors that are merely present at the cell membrane in such proximity of each other that they can be cross-linked by BS3. The increase in EGFR phosphorylation indicates activation of the receptor and not only a close proximity of the receptors. It is also difficult to say if we are detecting hetero- or homodimers, but heterodimers are more likely as the monomeric ligands do not seem to be able to induce dimerization and activation of the receptors (Figure 19 and Figure 20). Since the configuration of EGFR needs to change for the receptors to dimerize, and binding of the monomeric ligand does not seem capable of inducing this change, a possibility is that the Z_{EGFR,1907} part of the bispecific ligand catches an EGFR in its open state. The HER2-binding part binds to HER2 and the receptors are thus pulled together by the bispecific ligand. If the EGFR that Z_{EGFR,1907} binds to is in the “closed” configuration, no dimerization and phosphorylation of the receptor is possible. This could explain the low levels of phosphorylated EGFR observed.

Why no significant HER2 dimerization and phosphorylation could be measured in SKBR-3 cells might be explained by the high background level of activated HER2, together with the known differences in receptor number of EGFR and HER2. There are more than ten times as many HER2 as EGFR on SKBR-3 cells [41,70]. Probably the small increase of heterodimeric
HER2 is concealed by the large amount of HER2 dimers already existing and can therefore not be quantified by our methods.

Figure 21. Clonogenic survival of SKBR-3 cells after irradiation by 8 Gy. Cells were treated with construct IV, Z\textsubscript{HER2:342}, (Z\textsubscript{HER2:342})\textsubscript{2}-(G\textsubscript{4}S)\textsubscript{3}-(Z\textsubscript{EGFR:1907})\textsubscript{2} (denoted as X) or (Z\textsubscript{HER2:342})\textsubscript{2} for 2 hours prior to irradiation and allowed to repair for 16 hours before reseeding. After 3 weeks of cultivation, colonies containing more than 50 cells were counted and the survival fraction was calculated. (Z\textsubscript{HER2:342})\textsubscript{2} and the bispecific (Z\textsubscript{HER2:342})\textsubscript{2}-(G\textsubscript{4}S)\textsubscript{3}-(Z\textsubscript{EGFR:1907})\textsubscript{2} significantly decreased survival (P<0.001, un-paired t-test), while treatment with Z\textsubscript{HER2:342} or construct IV did not differ from the irradiated control. Mean values and standard deviations are calculated on at least 3 replicates.

Clonogenic survival after exposure to external γ-irradiation was performed in SKBR-3 cells. Construct IV was compared to the previously generated bispecific binder, (Z\textsubscript{HER2:342})\textsubscript{2}-(G\textsubscript{4}S)\textsubscript{3}-(Z\textsubscript{EGFR:1907})\textsubscript{2} [132], and their respective HER2-binding ligands, Z\textsubscript{HER2:342} and (Z\textsubscript{HER2:342})\textsubscript{2}. As can be seen in Figure 21, construct IV had no effect on survival, similar to that of its HER2-binding part Z\textsubscript{HER2:342}. (Z\textsubscript{HER2:342})\textsubscript{2}-(G\textsubscript{4}S)\textsubscript{3}-(Z\textsubscript{EGFR:1907})\textsubscript{2} on the other hand (denoted as X in the figure) had a similar radiosensitizing effect as (Z\textsubscript{HER2:342})\textsubscript{2}. These results suggest that (Z\textsubscript{HER2:342})\textsubscript{2}-(G\textsubscript{4}S)\textsubscript{3}-(Z\textsubscript{EGFR:1907})\textsubscript{2} primarily binds to cells by its HER2-binding part.

Furthermore, cell proliferation in SKBR-3 and NCI-N87 cells was also studied. For this assay, construct II was chosen since it was the most promising candidate according to the result from the binding studies in living cells. No differences compared to untreated cells were observed (data not shown).
Conclusions from Paper V

All six bispecific ligands were able to simultaneously bind both EGFR and HER2. The use of monomeric EGFR and HER2 binders, rather than their dimeric equivalents, is more successful in terms of making functional bispecific affinity proteins. It can also be concluded that an N-terminal positioning was favorable for the affinity to the respective targets. Furthermore, the serine linker (S₅G)₃ renders higher affinity of the bispecific binders compared to (G₅S)₃. None of the six bispecific constructs were able to induce any large effects on cellular outcome. Seemingly, they show more resemblance to the monomeric than the dimeric affibody molecules (evaluated in paper I-IV). The observation that the balance in receptor number as well as the differences in affinity for the two targets have large impact on the binding characteristics of a bispecific binder can be used when generating other new bispecific binders. Tuned bispecific binders can potentially be used to implicitly target receptors that are expressed also in normal tissue. By making a bispecific binder that binds moderately good to two different receptors, of which one is expressed also in normal tissue, the avidity effect will keep the bispecific binder bound mainly to cells expressing both receptors. The more a receptor is abundantly expressed in normal tissue, the weaker the respective binder should be selected for the bispecific construct. This opens up the possibility to target EGFR positive tumors without excessive uptake in EGFR-expressing healthy tissues.

The expanding knowledge of the ErbB family suggests that heterodimers might be particularly oncogenic. For many years it has been known that heterodimers are more signaling-potent than homodimers [46,47]. It has also been shown that suppressing one receptor may result in upregulation and increased signaling of another ErbB receptor [89,145]. Additionally, tumors that co-express several ErbB receptors are often more aggressive and associated with a worse prognosis [146,147,148]. This suggests that for cancer prognosis and prediction of response to targeted therapies, the total ErbB expression pattern might be more important than the expression level of one single receptor. Bispecific ligands could thus be used as imaging agents with prognostic value. Another aspect of dual targeting is the possibility of increased tumor specificity since tumors are more likely than healthy tissue to express high amounts of two receptors.
Three types of targeting agents have been studied in this thesis, HER2-specific, EGFR-specific and dual EGFR- and HER2-specific affibody molecules. Studies in this thesis have shown that when these ligands bind to the receptors it can mediate intracellular signals and influence biological outcome. The affibody molecules are primarily intended as targeting agents for imaging and/or therapy of ErbB-expressing human cancers. Knowledge of how they interact and effect cells is a key step towards this goal.

Studies of the HER2-binding ligands showed that the valence of the ligand is of importance and can influence the effects. Treating SKBR-3 cells with the dimeric \((Z_{\text{HER2:342}})_{2}\) induced large effects on intracellular signaling, *in vitro* proliferation and survival, while its monomeric equivalent \(Z_{\text{HER2:342}}\) did not. Dimerization studies showed that \((Z_{\text{HER2:342}})_{2}\) was able to induce western blot bands corresponding to dimeric HER2. Thus, it is possible that \((Z_{\text{HER2:342}})_{2}\) induces HER2 homodimerization. Why HER2-dimerization, which is generally considered as mediating oncogenic effects, would result in decreased proliferation and increased radiosensitivity is somewhat of a mystery. Possible ways to examine this is to see if the \((Z_{\text{HER2:342}})_{2}\)-induced dimers are different from the homodimers induced by overexpression, and if \((Z_{\text{HER2:342}})_{2}\) can prevent ligand-induced activation of HER2 by heterodimerization. The HER2-targeting \((Z_{\text{HER2:342}})_{2}\) sensitizes SKBR-3 cells to γ-irradiation, which makes it interesting for therapy purposes. By labeling \((Z_{\text{HER2:342}})_{2}\) with a suitable radionuclide it could be possible to deliver the toxic effect to the tumors while at the same time sensitizing the tumor cells to irradiation. Since the monomeric \(Z_{\text{HER2:342}}\) did not have any large effects on neither of the cell lines, it could be better suited for imaging.

The EGFR-binding \((Z_{\text{EGFR:955}})_{2}\) was found to activate the downstream signaling pathways MAPK/Erk1/2 and PI3K/Akt, without any detectable phosphorylation of EGFR. This is probably due to the detection limitations of the western blot method and the antibody used. It would be interesting to see if the increased signaling could induce changes in terms of biological outcome. Since \(Z_{\text{EGFR:955}}\) has been shown to partially block the binding of EGF to the receptor [144], it would also be interesting to study if the affibody molecule could inhibit ligand-induced activation of EGFR.
It should be noted that the effects of the same affibody molecule differed in different cell lines. Ideally, similar studies should be performed on additional cell lines to verify that effects that are not cell-line dependent. It could also be of interest to perform studies in a more controlled system, e.g. with transfected receptors and without signaling alterations in the cells.

The bispecific ligands generated and evaluated in paper V were all shown to be able to simultaneously bind to both EGFR and HER2. Their effects on cultured cells were minor. Furthermore, it was shown that N-terminal positioning of the monomeric affibody molecules was favorable to promote the binding to respective target and that the linker (S₄G)₃ renders higher affinity of the bispecific binders compared to (G₄S)₃. Dual targeting of ErbB receptors is interesting from several points of view. First, heterodimerization is considered as more signaling-potent than homodimerization [20]. Second, suppression of one receptor may result in upregulation and increased signaling of another ErbB receptor [89]. Clinically, it has been implicated that coexpression of EGFR and HER2 is correlated with poor patient prognosis for breast cancer patients [146,149]. Bispecific ligands could thus be used as imaging agents with prognostic value. Another aspect of dual targeting is the possibility of increased tumor specificity, since tumors are more likely than healthy tissue to express high amounts of two receptors. It would also be interesting to see if the properties of a bispecific ligand can be changed by varying the length and flexibility of the linker.

Furthermore, HER2-overexpressing tumor cells are dependent on HER3 for proliferation [87], and HER3 has been found as an escape route for cancer cells treated by ErbB-targeting TKIs [89]. A bispecific ligand that targets both these receptors has the opportunity to abrogate the potent signaling of HER2-HER3 dimers. This makes dual targeting of HER2 and HER3 a highly interesting research area.
This work has mainly been carried out at the Division of Biomedical Radiation Sciences (BMS) at Uppsala University with financial support from the Swedish Cancer Society and VINNOVA. Some of the experiments have also been performed at the Ludwig Institute for Cancer Research in Uppsala. Many people have contributed to my work on this thesis and I would like to express my gratitude to them. Especially, I would like to thank:

Mina handledare Lars Gedda och Johan Lennartsson som under olika perioder delat på handledarsetet. Tack för att ni alltid tagit er tid att lära mig nya experiment, förklara komplicerade saker och läsa igenom det jag skrivit. Ni har lärt mig ”allt” jag kan!

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