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Effect of Herceptin® and two HER2-binding affibodies on intracellular signalling pathways

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Title (English)

Effect of Herceptin® and two HER2-binding affibodies on intracellular signalling pathways

Title (Swedish)

Abstract

The aim of the present study was to investigate the effect of the two HER2-binding affibodies, $(Z_{her2:4})_2$ and Z_{00342}, on intracellular signal transduction pathways and to compare to that of the monoclonal antibody trastuzumab (Herceptin®). The results demonstrate that the signal transduction proteins Erk1/2, Akt and PLCγ1, as well as the HER2 receptor itself, are all affected by treatment with both the two affibodies and trastuzumab. Thus, all three substances are biologically active. The results indicate that $(Z_{her2:4})_2$ has abilities to induce proliferation and increase resistance to apoptosis, while Z_{00342} showed a similar inhibitory effect on HER2 expressing cells as trastuzumab. Z_{00342} may thus be better suited for diagnostic and therapeutic purposes than $(Z_{her2:4})_2$.

Keywords

HER2, affibody, trastuzumab, signal transduction, Erk, Akt, PLCγ1

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Effect of Herceptin® and two HER2-binding affibodies on intracellular signalling pathways

Lina Ekerljung

Sammanfattning

HER2 är en receptor med framträdande roll inom cellsignalering då den till exempel sänder ut signaler som påverkar cellernas förmåga att växa, överleva och migrera. Följaktligen är HER2 en viktig måltavla inom cancerforskningen, dels för att blockera signalering och dels för att receptorn kan användas som måltavla vid cancerdiagnostik och tumörspecifik terapi. Vid tumörspecifik terapi fästes ett cellgift till en bindarmolekyl specifik för receptorn och de skadliga effekterna kan på så sätt begränsas till tumörvävnad.

Herceptin® är ett läkemedel som används framförallt mot bröstcancer. Det är en antikropp som binder HER2 och blockerar receptorns signaler. Ett lovande alternativ till antikroppar som bindarmolekyl vid tumörspecifik terapi är affibodymolekyler. De är en typ av bindarmolekyler med mindre storlek än antikroppar och kan därför lättare penetrera tumörer.

I den här studien har effekten på cellsignalering jämförts mellan Herceptin och två HER2-bindande affibodymolekyler, en dubbelbindande form $(Z_{HER2:4})_2$ och en förbättrad enkelbindande form Z_{00342}. Även studier av celltillväxt har gjorts för att undersöka substansernas biologiska effekt på tillväxt och strålkänslighet.

I studien visade sig de tre substanserna alla ha olika effekt på signaleringsvägarna. Den dubbelbindande formen visade kapacitet att stimulera tillväxt hos tumörceller medan den enkelbindande affibodymolekylen hade samma hämmande effekt som Herceptin.

Examensarbete 20 p i Molekylär Bioteknikprogrammet

Uppsala universitet februari 2005

1. Introduction

1.1 Cancer Therapy

1.1.1 ErbB Receptors as Therapeutic Targets

HER2 (ErbB-2 or Neu) is a tyrosine kinase receptor and one of four members in the HER (Human EGF-like Receptor, EGF stands for Epidermal Growth Factor) family (also known as subclass I receptor tyrosine kinases (RTK)). The others are EGFR / ErbB-1, HER3 / ErbB-3 and HER4 / ErbB-4 [1, 5]. In normal cells the functions of these receptors are critical for the signal transduction (ST) network that regulates proliferation, differentiation and migration of cells [1, 12]. Since most human tumours have aberrant signal transduction elements [12] and the ErbB receptors are critical for ST pathways, the receptors have been pursued as therapeutic targets. Given that a large number of cancer cells show excessive EGFR and HER2 expression relative to normal cells, most efforts have focused on these two receptors. Over-expression of HER2 occurs in many human tumours, e.g. ovarian, gastric and particularly breast tumours. The receptor is over-expressed in 25-30% of breast cancers and is associated with a poor patient prognosis and resistance to conventional cancer therapies, like chemotherapy and radiation therapy [7]. Over-expression of HER2 in breast cancer is often a result of amplification of the HER2 gene and leads to a constitutive signal [5, 19, 12].

There are several ways to target the receptors. One strategy is to utilize an antibody that binds to the receptor and inhibits it. A second approach is to use low-molecular-weight tyrosine kinase inhibitors, which inhibit receptor activation. Other strategies are e.g. gene therapy and antibodies conjugated to a toxin or a cytotoxic drug [5, 19, 12].

1.1.2 Therapeutic Agents

A monoclonal antibody directed against HER2 called trastuzumab, Herceptin®, is already in therapeutic use for treatment of breast cancer. Trastuzumab recognizes an epitope on the extra-cellular domain of HER2 and blocks downstream signalling even though it activates the receptor itself [13]. Trastuzumab also down-regulates the amount of HER2 at the cell surface and activates the human complement cascade since it contains IgG1 [1, 13, 19]. Only a fraction of patients treated with Herceptin responds to the therapy, mainly those with the highest levels of HER2 [1, 5].

One problem with using antibodies like trastuzumab as therapeutic agents is that they do not efficiently penetrate solid tumours because of their high molecular weight. Monoclonal antibodies are also correlated with high manufacturing costs and various side effects. A class of affinity ligands denoted affibody molecules, Affibody®, are promising alternatives to antibodies. Their robust structure together with their low molecular weight (7 kDa, to compare with trastuzumab at 150 kDa) make them suitable for many different applications, like detection reagents and inhibitors to receptor interactions [18]. In figure 1, a comparison between an antibody and an affibody molecule is shown to visualise the size difference. Affibodies are based on a three-helix domain derived from one of the IgG-binding domains of staphylococcal protein A and can be selected to target desired molecules through phage display. In the present study, two HER2-binding affibodies have been investigated, a bivalent

affibody and an affinity maturated monovalent affibody, denoted $(Z_{HER2:4})_2$ and Z_{00342} respectively. The affibodies bind to another extracellular region of HER2 than trastuzumab does, although the exact binding site has so far not been determined [18].

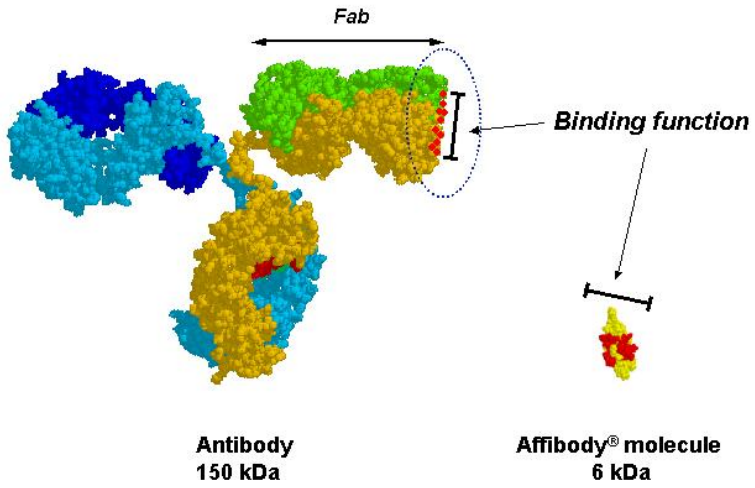

Figure 1. A comparision of an antibody and an Affibody® molecule. The illustration is used with permission from Affibody AB (Bromma, Sweden).

1.1.3 Radiation

The most important effect of radiation, from a cancer therapeutic point of view, is the arrest of cell proliferation. This is mediated through severe damage in DNA, which is the critical target for radiation. There are different types of DNA damage: single strand breaks, base damage, double strand breaks and cross-links. Single strand breaks and base damage can effectively be repaired by the cell repair system. Also double strand breaks can be repaired, but with lower accuracy and this damage can thus give rise to rearrangements of genes and ultimately lead to mitotic cell death or apoptosis. The probability of reaching a lethal effect of radiation on a cell increases with the radiation dose delivered. The main part of the damage caused by radiation occurs through ionisation of cellular water, which renders free radicals that have the capacity to damage biomolecules. Only a small part of the damage is caused by direct ionisation of biomolecules [16].

1.2 HER2

All members in the HER family have an extracellular ligand-binding region with two cystein-rich domains, a transmembrane segment and an intracellular (cytoplasmic) protein tyrosine kinase containing domain [1, 4, 5]. The receptors are inactive when present in a monomeric state but when a ligand binds the extracellular domain of a receptor formation of hetero- and homodimers is induces. The formation of dimers leads to autophosphorylation on the tyrosine residues in the intracellular domain. These phosphorylated residues serve as docking sites for a variety of different

signalling molecules and consequently lead to a variety of intracellular signalling pathways [4, 5, 6].

The HER ligands can be classified into two groups: EGF-like ligands that bind EGFR (for example epidermal growth factor, EGF) and neuregulins that binds to HER3 and HER4. HER2 is not able to bind EGF-like ligands because of a major difference in the structure of its extracellular region in comparison to EGFR and HER3. In fact, HER2 does not have any cognate ligand [5]. In the absence of a cognate ligand, HER2 can be activated by two different mechanisms. One mechanism is through the formation of ligand-dependent HER2-based heterodimers, which relies on the binding of a ligand to EGFR, HER3 or HER4 [1, 4]. Activation of HER2 is also thought to occur through over-expression of HER2, which leads to spontaneously formed homodimers. This process is dependent upon extremely high HER2 concentration on the cell surface, in the order of thousands of times higher than in normal cells, but independent on receptor ligands [1, 19].

HER2 is the preferred heterodimerisation partner for all the ErbB receptors. It decreases the rate of internalisation and degradation as well as increases recycling of the other receptor partner. The fact that HER2 exists in a conformation favourable to dimerisation is also thought to improve its capability to form heterodimers [19, 10]. Depending of the dimerisation partner, a given receptor can acquire different signalling properties [4]. Activation of HER2-containing dimers leads to an increase in cell proliferation, migration and resistance to apoptosis [12].

1.3 Signal Transduction

Downstream of the receptor, signalling often occurs via phosphorylation cascades. Depending on the identities of the receptors in the dimer and also the identity of the ligand, different pathways can be activated [19, 10]. All ErbB receptor dimers and ligands can activate the MAPK (mitogen-activated protein kinase, also known as extra-cellular signal-regulated kinase, ERK1 / ERK2) pathway. Activation of the PI(3)K (phosphatidylinosithol-3-kinase) pathway is induced with different amplitudes by different ErbB receptors depending on the receptors ability to bind the p85 regulatory subunit. PLCγ (phospholipas Cγ) is recruited to the membrane by activated EGFR and HER2, but can also be activated by PI(3)K products [5, 10]. Simplified, MAPK is the key component in the machinery that leads to induction of proliferation (by inducing cell-cycle progression through the G1 state), PLCγ has a major role in migration and PI(3)K in anti-apoptosis [1, 17]. In reality, the signalling pathways are much more complex and the ways of interactions are numerous. The three signalling pathways and their active components are shown in figure 2.

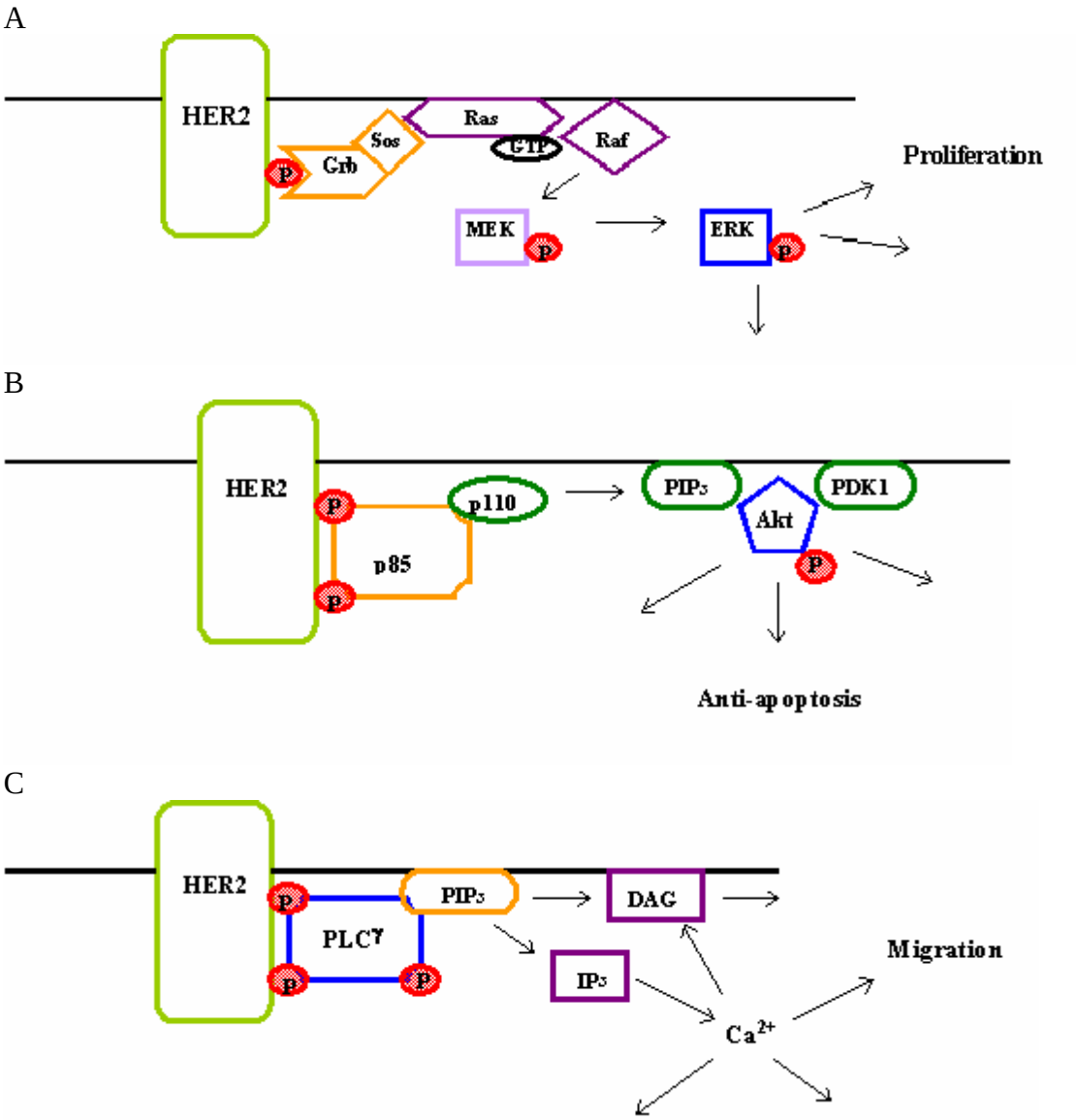

Figure 2. The three different signalling pathways are described with their components, a) MAPK, b) PI(3)K and c) PLCγ. The red encircled P:s indicate phosphorylation and the arrows indicate further activation.

The MAPK signalling pathway starts with recruitment of Grb-2 to the phosphorylated receptor. This leads to a linear kinase cascade that culminates in activation of Erk/MAPK. Erk phosphorylates several cytoplasmic and membranal proteins, and is rapidly translocated to the nucleus, where it activates a variety of transcription factors. Activation of PI(3)K occurs through binding of the regulatory p85 subunit to a phosphotyrosine site on the receptor. This results in the recruitment of several signalling effectors, e.g. Akt that is recruited to the plasma membrane and phosphorylated. Phosphorylation of Akt causes its activation and translocation to the nucleus where it targets regulators of apoptosis and cell growth. Phospholipase Cγ (PLCγ) is recruited to the membrane through binding to the activated receptor. Activated PLCγ hydrolyses lipids and that generates second messengers. This results in calcium release and thereby activation of calcium/calmodulin-dependent kinases [3, 10].

In many cases, phosphorylation correlates with enzymatic activity and can therefore be used as a way to measure protein activity, albeit indirectly. The activation of a certain signalling pathway is therefore possible to detect by the use of antibodies specific for the active and inactive form of a molecule in that pathway.

1.4 Aim of Study

The aim of this project was to examine the effect of the HER2-binding affibodies, $(Z_{HER2:4})_2$ and Z_{00342} (i.e. the bivalent affibody and the affinity maturated monovalent affibody) on intracellular signal transduction. These effects were compared to those of the therapeutic agent Herceptin® (trastuzumab) that is already in clinical use. The main approach to examine this was by analysing selected signal transduction pathways. The intracellular signalling proteins chosen were Erk, PLCγ and Akt. Possible activation and repression of the HER2 receptor itself was also investigated. Finally, the effect of these substances had on living cells, cell growth and growth inhibition was also studied.

2. Materials and Methods

2.1 Treatment Substances

The monoclonal HER2-directed antibody trastuzumab, Herceptin®, was purchased from Apoteket AB (Sweden), and desalted on a PD-10-column (Amersham Biosciences, Uppsala, Sweden) to remove low molecular weight compounds from the formulation. The EGF used was a human recombinant EGF from Chemicon International (Temecula, USA). The affibodies were developed and supplied by Affibody AB (Bromma, Sweden). The bivalent, $(Z_{HER2:4})_2$, was made as previously described [12], and the affinity maturated monovalent affibody, Z_{00342}, according to a manuscript in progress.

2.2 Cell Cultivation

Human ovarian carcinoma cells (SKOV-3), that over-express HER2 (2×10^6 HER2 per cell), were cultivated in McCoy's 5A medium complemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS). All chemicals were from Biochrom KG (Berlin, Germany).

2.2.1 Signal Activity Assay

For the signal activation experiment, 2 million cells were seeded in 75 cm^2 bottles (Nunc A/S, Denmark) and cultivated for approximately 45 hours after which the cells were starved overnight in medium only containing 0.1 % FBS. The amount of cells was chosen to give as high protein concentration in the lysates as possible without the number of cells in one bottle being so large that it inhibited cell growth. Addiation of the different substances was done at different time points. For the affibodies and trastuzumab, four different exposure times were used; 15 minutes, 2 hours, 4 hours and 8 hours. EGF was used as a positive control to ensure that stimulation of the cells was possible. The incubation time for EGF was solely one hour. The cells were treated with 16.6 nM trastuzumab, 16.6 nM $(Z_{HER2:4})_2$, 16.4 nM Z_{00342} or 1.67 nM EGF. These concentrations correspond to a theoretical ratio of about 7.5:1 between substance and receptor. This ratio was chosen to make sure that each receptor had the opportunity to encounter the substances, considering diffusion and amount, without being too affected by the physical nature of the substances. During treatment, 6 ml medium with 0.1% FBS in each bottle was used.

2.2.2 Growth Study

For the growth experiments, 24 bottles (25 cm^2, Nunc A/S, Denmark) were cultivated with 10 ml medium.

Twelve bottles were used for studying proliferation, out of which 3 bottles were treated with trastuzumab (16.6 nM), 3 with the $(Z_{HER2:4})_2$ (16.6 nM) and 3 with Z_{00342} (16.4 nM). The remaining three were used as untreated controls. The media were changed three times a week and the three substances added at the same times. At the starting point, 50 000 cells were seeded in each bottle. Cell counting was done once a

week in an automated cell counter and after each cell counting 45 000 cells were seeded to new bottles for continuous cultivation. The remaining cells were discharged.

Twelve bottles were used to study growth inhibition by measuring the surviving fraction after irradiation. Also here, 3 bottles were used for each substance and the remaining 3 were irradiated but chemically non-treated controls. In each bottle, 300 000 cells were seeded 48 hours before irradiation. The substances were added 4 hours prior to irradiation (at the same concentrations as before). The cells were irradiated with 4 Gy of γ-radiation using a ^{137}Cs source, and then incubated in the same medium for additionally 20 hours. After that, the media were changed to normal medium (complete McCoy's 5A). The cells were then cultivated as above and counted once a week to follow cell growth.

2.3 Cell Lysate Preparation

After the media was removed, the cells were washed once with ice-cold PBS (phosphate buffered saline) followed by 1 ml lysis buffer (pH 7.4, 1% Triton X-100, 150 mM NaCl, 20 mM Tris, 5 mM EDTA, 10% Glycerol) containing protease and phosphatase inhibitors (1% trasylol, 1mM Na_3VO_4 and 1mM PMSF (phenyl methyl sulfonyl fluoride)). After 10 minutes of incubation on ice while shaking, the lysates were centrifuged at 20 800 g for ten minutes and 4°C. The pellets were discarded and the supernatant kept on ice, or frozen at –20°C.

2.4 Analysis of Signal Activation

2.4.1 Antibodies

Activation of MAPK can be detected by using the antibody pErk that binds to phosphorylated Erk1 and Erk2, and an antiserum, EET, that binds independently of phosphorylation to Erk2. PI(3)K activation is detected by analysing the phosphorylation status of the downstream effector protein Akt using the antibody pAkt which binds phosphorylated Akt, and the antibody Akt that binds the effector protein independently of phosphorylation. The SFE antibody binds directly to lipase PLCγ1, but its activation is measured indirectly by analysing its phosphorylation (phosphorylation of PLCγ leads to activation). The antibody used is an anti-phosphotyrosine antibody, pTyr, which binds phosporylated proteins. This antibody was also used for measuring phosphorylated HER2. For detecting HER2 independently of activation, a HER2 specific antibody was used.

In the present study the following antibodies were used: Antibodies specific for Akt and mouse anti-phosphotyrosine antibody, py99, were purchased from Santa Cruz Biotechnology (Santa Cruz, USA); rabbit p-Akt (Ser 473), rabbit phospho-p44/42 Erk (Thr 202/ Tyr 204) and the HER2 antibody from cell Signaling Technology (Beverly, USA). Rabbit antisera against PLCγ1 (SFE) and Erk-2 (EET) were produced at the Ludwig Institute for Cancer Research (Uppsala, Sweden) as previously described [11, 9].

2.4.2 Immunoprecipitation and SDS-PAGE

The protein concentrations in the cell lysates were determined using Pierce BCA™ protein assay kit (Rockford, USA). Since equal protein loading in each lane on a gel is of importance and not the actual protein concentration in each cell lysate, only relative concentrations were determined. The lysate with the lowest protein concentration was loaded with the largest volume and the amount taken from the other lysates were set relatively their protein concentration.

The proteins in the cell lysates were separated by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). As running gel, an 8% (for one gel: 2.5 ml 40% acrylamide, 2 ml 2 M Tris-Cl pH 8.8, 5.6 ml dH_2O, 10 µl TEMED, 40 µl 10% APS) or 10% (for one gel: 2.5 ml 40% acrylamide, 2 ml 2 M Tris-Cl pH 8.8, 5.6 ml dH_2O, 10 µl TEMED, 40 µl 10% APS) was used and as stacking gel a 4% gel (0.6 ml 40% acrylamide, 0.8 ml 0.5 M Tris-Cl pH 6.8, 4 ml dH_2O, 6 µl TEMED, 30 µl 10% APS). The 8% running gel was used for analysing PLCγ- and HER2-phosphorylation while the 10% running gel was used for analysing Erk- and Akt-phosphorylation. Equal amounts of protein (approximately 20 µl lysate) with 20 µl reducing sample buffer (280 µl 2×sample buffer, 200 µl 21% SDS, 20 µl 2-mercaptoetanol) were heated for 5 minutes at 95°C before loaded on the gels. The gels were run at 150 V for approximately one hour in 1×electrophoresis buffer (0.025 M Tris, 0.192 M Glycin, 0.1% SDS). As protein size marker, a broad range prestained protein marker from New England Biolabs (Beverly, USA) was used.

Since pTyr binds all phosphorylated proteins, PLCγ and HER2 must be selected before immunoblotting. By immunoprecepitation, an antigen can be selected from a solution before electrophoresis and the probed protein will, optimally, be the only protein loaded on the gel. A protein-specific antibody is incubated with the lysate, whereafter sepharose conjugated protein A is added. Protein A binds the antibodies, and after centrifugation, the sepharose beads form a pellet that allows for separation of the antibody-complexes from the rest of the proteins. For immunoprecipitation, an equal amount of protein (approximately 300 – 500 µl lysate) was incubated on ice with the antibody of interest (SFE or HER2 antibody) over night. The antibody dilutions were: 1:100 for HER2 and 1:50 for SFE. To each sample, 40 µl of protein-A sepharose (protein A sepharose 4B conjugated, Zymed laboratories inc. (San Francisco, USA)) was added and the samples were incubated rotating at 4°C for at least 30 minutes. The samples were then centrifuged and washed three times with ice-cold lysis buffer. After elution of the bound protein by boiling for 5 minutes in 40 µl reducing sample buffer, the samples were ready for SDS-PAGE.

2.4.3 Transfer and Western Blotting

After SDS-PAGE the proteins are transferred from the gel to a membrane. By using immunoblotting, where the membrane is probed with an antibody specific to the protein, the protein can be selected. This antibody is then probed by a second antibody, which has horseradish peroxidase conjugated. When developing the membrane, a luminol reagent is used and the peroxidase on the second antibody catalyses the luminol to emit light. The emission, and thereby the amount of protein, can then be detected by a cooled charge-coupled device (CCD) camera or x-ray film.

By stripping the membrane of antibodies, the membrane can be re-probed by another set of antibodies, e.g. antibodies independent of phosphorylation.

The proteins were transferred to a PVDF membrane (immobilon-P Transfer Membrane, pore size 0.45 um, from Millipore, USA) by semi-dry transfer. For each gel three filters (Gel Blotting Papier GB005 from Schlicher & Schnell, Germany) and semi-dry buffer (5.82 g Tris, 2.93 g Glycin, 1.875 ml 20% SDS, 200 ml MeOH, 800 ml dH_2O) was used. The transfers were run at 15 V in room temperature for 60 or 75 minutes depending on the number of gels transferred at the same time. After transfer, the membrane were blocked in 5% BSA (bovine serum albumin) in PBS-T (PBS with 0.05% Tween-20) or TBS-T (Tris buffer saline with 0.1% Tween-20) for one hour in room temperature to prevent unspecific binding of the antibodies to the membrane itself. This was followed by incubation with their respective primary antibody for two hours at room temperature or over night at 4°C. The antibodies were diluted with PBS-T, 1% BSA and 0.1% NaN_3. The antibody dilutions were: 1:1000 for p-Erk, p-Akt and HER2-antibody, 1:300 for SFE and EET and 1 μg/ml for py99 and Akt. These mixtures were reused several times. Before incubation with a secondary antibody the membranes were washed 3×5 minutes in PBS-T or TBS-T. The secondary antibodies were diluted in PBS-T, anti-mouse 1:10 000 and anti-rabbit 1:30 000. The incubation time was 1 hour in room temperature. The species specificity of the secondary antibody was chosen to match the primary antibody used. This was followed by another 3×5 minutes wash and an additional 5 minute wash in PBS. The membranes were developed using a CCD-camera together with a luminol reagent (western blotting luminol reagent from Santa Cruz biotechnology (Santa Cruz, USA) or Lumi-light PLUS western blotting from Roche Diagnostics (Indianapolis, USA)) according to the manufacturer's instructions. After developing, the membranes were stripped from antibodies using 0.4 M NaOH for 10 minutes at room temperature and then washed 3×5 minutes in PBS-T or TBS-T. After this, the procedure (from blocking in BSA) could be re-done with other antibodies of interest, i.e. antibodies independent of phosphorylation. To analyse the pictures, the AIDA (Advanced Image Data Analyzer from Fuji film (Minami-Ashigara, Japan)) software, version 3.10, was used.

3. Results

3.1 Signal Activity Assay

Each membrane was developed twice; once probed for phosphorylation and once to give the total amount of each protein. In figure 3, blots of cells treated with the bivalent affibody can be seen as an example.

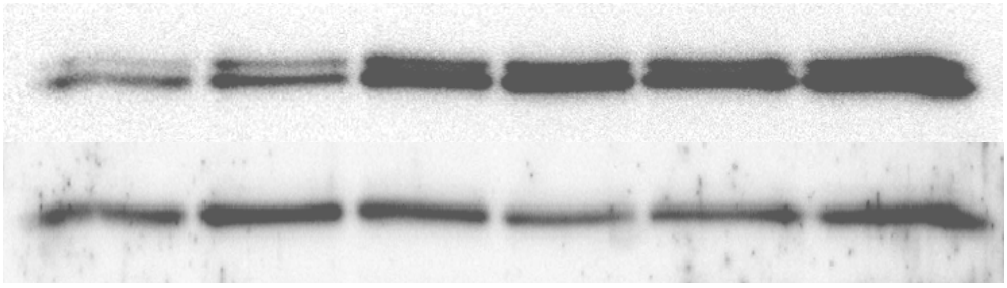

Figure 3. Two blots of bivalent affibody treated cells. The upper panel shows a blot probed by pErk, which target phosphorylated Erk1 and Erk2 (thereof the two parallel bands). In the lower panel, the proteins are probed by EET that target Erk2 independently of phosphorylation. The six parallel bands represent (from the left) untreated, treated with EGF, and treated with the bivalent affibody (at four different time points).

The emission of the bands of interest in each blot was quantified by the AIDA software, and the resulting values were used to calculate the quotients between phosphorylated and total amount of protein. To make the comparison between different results of the same experiment feasible, each of the individual measurements was normalised against the measurement of the corresponding untreated cells. In this way, the mean values of the standardised quotients from all experiments (three or four) could be plotted with error bars showing the standard error of the mean. The resulting diagrams are showed in figures 4-7.

3.1.1 Effects on MAP-kinase

As seen in figure 4, the exposure by trastuzumab gave a slight decrease in phosphorylation of Erk at longer exposure times. A small decrease of phosphorylation is also seen for the affinity maturated monovalent affibody, while the bivalent affibody shows a tendency to increase phosphorylation of Erk. The one-hour exposure to EGF resulted in a small decrease in phosphorylation. Thus, the MAP-kinases were robustly activated in the absence of any factor.

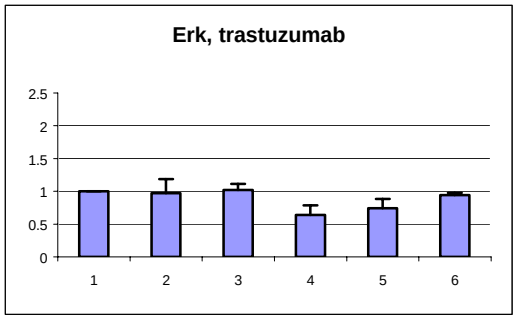

Figure 4a.

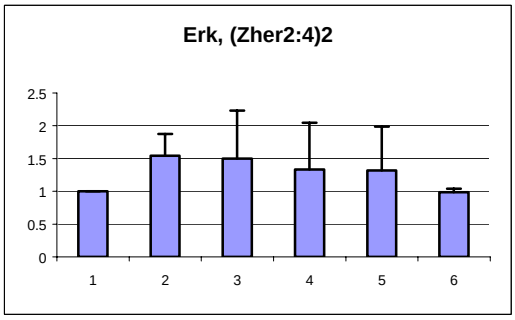

Figure 4b.

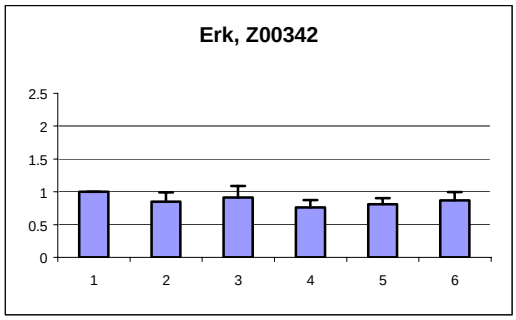

Figure 4c.

Figure 4. Phosphorylation of the MAPK-signalling pathway after stimulation with a) trastuzumab, b) $(Z_{her2:4})_2$ and c) Z_{00342}.
1. Untreated
2. 15 min. exposure of each substance.
3. 2 h exposure of each substance.
4. 4 h exposure of each substance.
5. 8 h exposure of each substance.
6. 1 h exposure of EGF.

3.1.2 Effects on PI(3)K – Akt Pathway

Figure 5 shows that the phosphorylation of the PI(3)K signalling pathway was clearly amplified by exposure to the bivalent affibody while trastuzumab on the other hand slightly decreased the phosporylation with time. For the affinity maturated monovalent affibody, a small increase is seen. It is also clear that exposure to EGF stimulated phosphorylation of Akt. Thus, in regard to Akt phosphorylation, the two affibody substances clearly distinguishes themselves from trastuzumab.

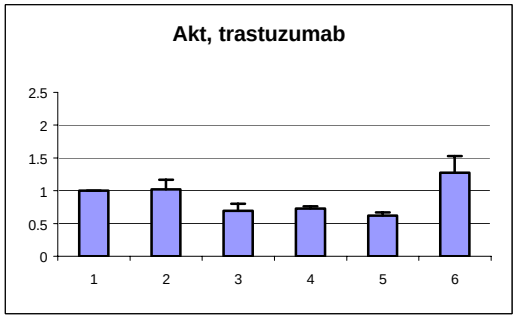

Figure 5a.

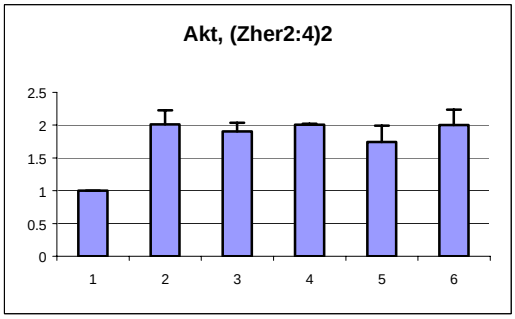

Figure 5b.

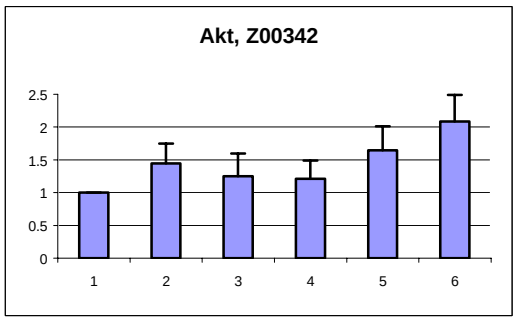

Figure 5c.

Figure 5. Phosphorylation of the PI(3)K signalling pathway after stimulation with a) trastuzumab, b) $(Z_{her2:4})_2$ and c) Z_{00342}.
1. Untreated
2. 15 min. exposure of each substance.
3. 2 h exposure of each substance.
4. 4 h exposure of each substance.
5. 8 h exposure of each substance.
6. 1 h exposure of EGF.

3.1.3 Effects on PLCγ Phosphorylation.

For the result of PLCγ phosphorylation by trastuzumab, the 15 minutes exposure was chosen to normalise with instead of the untreated, as for the others. This was due to the fact that in one individual experiment the untreated showed no phosphorylation at all and this value was hence was impossible to divide with. From figure 6 it is apparent that trastuzumab treatment resulted in strong phosphorylation of PLCγ. The bivalent affibody seemed to increase phosphorylation with time while exposure to the monovalent affibody showed a small decrease. EGF treatment gave a small increase

in phosphorylation. It is of note that the monovalent and the bivalent forms of affibodies affected PLCγ phosphorylation in different ways.

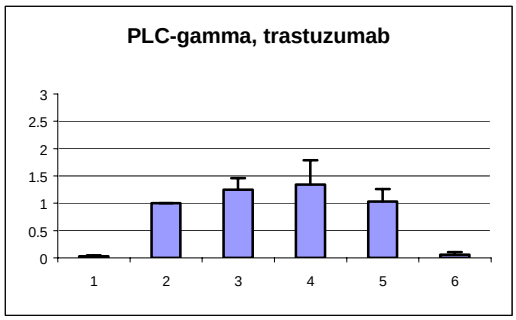

Figure 6a.

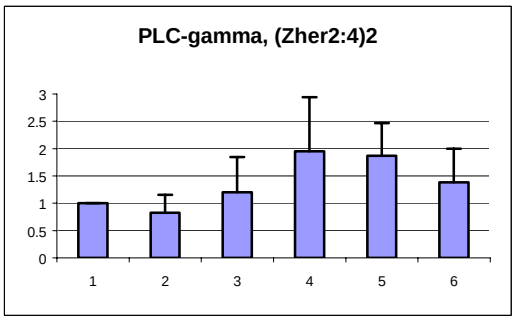

Figure 6b.

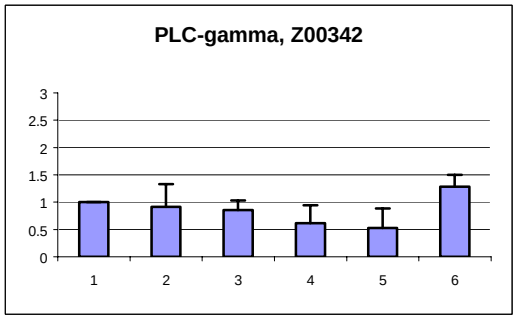

Figure 6c.

Figure 6. Phosphorylation of the PLCγ signalling pathway after stimulation with a) trastuzumab, b) $(Z_{her2:4})_2$ and c) Z_{00342}. For trastuzumab the 15 minutes exposure was used to normalise with.
1. Untreated
2. 15 min. exposure of each substance.
3. 2 h exposure of each substance.
4. 4 h exposure of each substance.
5. 8 h exposure of each substance.
6. 1 h exposure of EGF.

3.1.4 Effects on HER2 Phosphorylation

As can be seen in figure 7, the HER2 receptor itself was robustly phosphorylated by both trastuzumab and the bivalent affibody. For the bivalent affibody, the stimulation increased with time, while trastuzumab rather gave a decrease in phosphorylation with time. The affinity maturated monovalent affibody decreased phosphorylation of HER2.

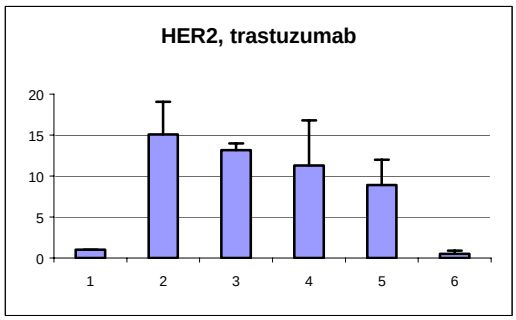

Figure 7a.

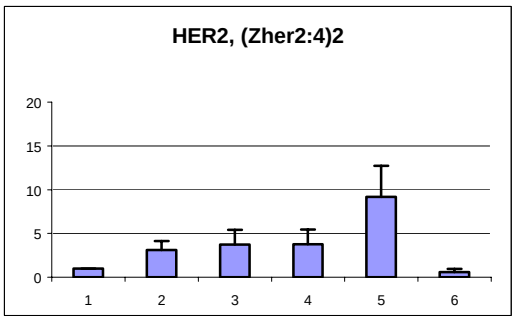

Figure 7b.

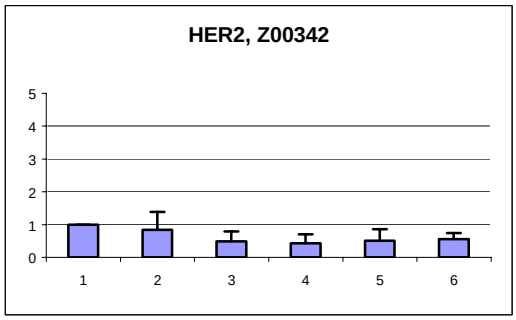

Figure 7c.

Figure 7. Phosphorylation of the HER2 receptor after stimulation with a) trastuzumab, b) $(Z_{her2:4})_2$ and c) Z_{00342}.
1. Untreated
2. 15 min. exposure of each substance.
3. 2 h exposure of each substance.
4. 4 h exposure of each substance.
5. 8 h exposure of each substance.
6. 1 h exposure of EGF.

Since HER2 showed little or no phosphorylation when treated with EGF at the time used in the experiment, an extra treatment period was used in one set of experiment to make sure activation was possible. This time, a second induction time of 15 minutes for EGF was used. From the diagram in figure 8, it is apparent that the 15-minutes induction time phosphorylated the HER2 receptor far more than the one-hour stimulation.

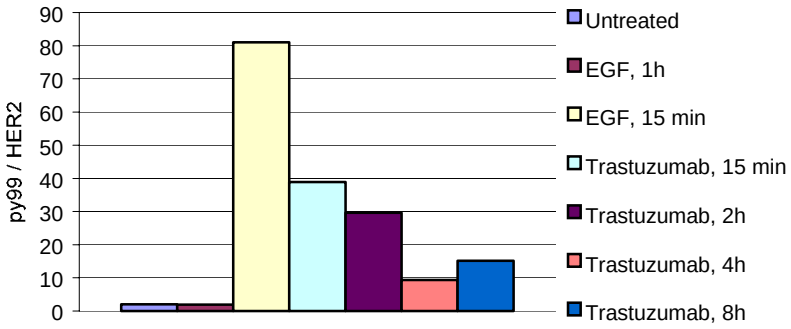

Figure 8. Phosphorylation of HER2 after exposure to EGF and trastuzumab for various times.

3.1.5 Summary of Individual ST Results

In figure 9, the results of each individual experiment are illustrated by arrows showing the change in phosphorylation of each signalling protein compared to untreated cells. As seen in figure 9, some of the results differ between individual experiments while other results are stable. The results from cells treated with trastuzumab were stable, while treatment by the affinity maturated monovalent affibody gave ambiguous results. Phosphorylation of HER2 and Akt induced by the bivalent affibody are both explicit, but analysis of phosphorylation of Erk and PLCγ showed unstable results.

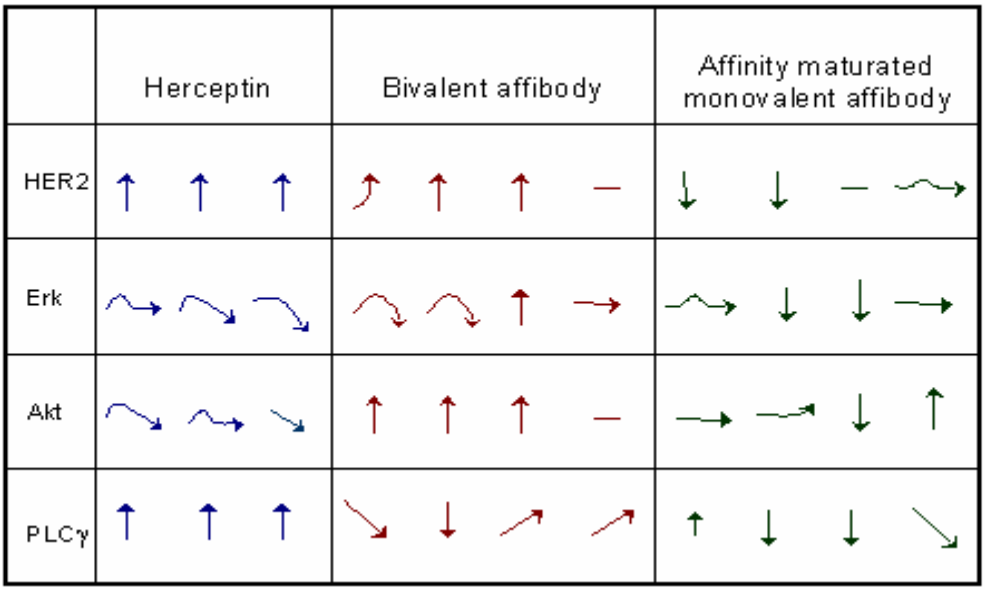

Figure 9. The arrows show the change in phosphorylation / activation compared to the control. Upward pointing arrows correspond to an increased activation and consequently, downward pointing arrows correspond to decreased activation. The horizontal movement of an arrow shows a change in time, i.e. the four different induction times give different activation levels, with right as the direction of increased time. A short arrow indicates that the change is of a small magnitude.

3.2 Growth Study

Possibly, the addition of the substances could alter the cells ability to adhere on the bottle surface and thereby influence the growth time. To see if this was the case, an experiment was made to ensure that the differences in growth rate depended on the assumed altered signal cascade and not on altered adherence. A number of 45 000 cells were seeded in each bottle, three bottles for each substance and three bottles with untreated cells. The same concentrations of the substances as in the growth experiment were used. After two hours, the media were removed and the cells were counted in the automated cell counter. The results presented in table 1, indicate no deviation in adherence between the cells treated with the different substances and those left untreated.

	Number of cells	Adhered fraction
Untreated	39 853	89% ± 2%
Trastuzumab	40 653	90% ± 5%
$(Z_{HER2:4})_2$	38 627	86% ± 6%
Z_{00342}	38 587	86% ± 2%

Table 1. Number of cells is the average number of cells from each group of three bottles. The adhered fraction is the percentage of cells that had adhered after two hours, i.e. the quotient between average and the number of cells seeded in each bottle (45 000), with standard deviation.

For the proliferation study, where the different substances were continuously added, the cells were cultivated for 33 days. During this time, the cells were counted six times, and after each time only a small fraction from each bottle, 45 000 cells, were seeded for further cultivation. To make up for this fractionation each individual cell

number value was multiplied by its corresponding split factor (if for example, only 0.5 ml of 10 ml cell suspension was seeded for continuous cultivation, the next value for number of cells was multiplied by 10 / 0.5 = 20). The average numbers of cells from each series are plotted in the diagram in figure 10.

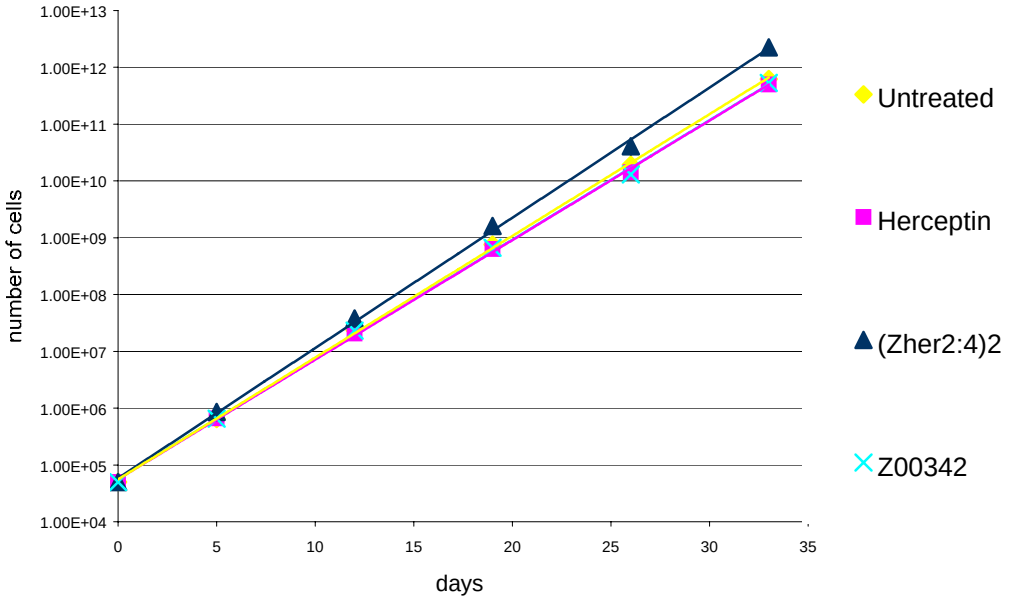

Figure 10. Cell growth of the proliferation study with continuous addition of substances. The y-axis has a logarithmic scale and the trendlines are exponentially fitted.

From figure 10, it is obvious that the bivalent affibody stimulated proliferation. After 33 days of cultivation the cells treated with the bivalent affibody were 3.6 times as many as the untreated cells. A cell doubling time of 32.6 hours corresponds to the trendline added to the series of the bivalent affibody. The curve of the untreated cells corresponds to a doubling time of 33.8 hours, while trastuzumab and the affinity maturated monovalent affibody resulted in the doubling times of 34.3 and 34.5 hours respectively. Thus, exposure to trastuzumab or the monovalent affibody resulted in a small decrease of proliferation. The data from diagram 10 is presented in table 2.

	Cell doubling time	Normalised number of cells after 33 days
Untreated	33.8 h ± 0.36 h	1
Trastuzumab	34.3 h ± 0.34 h	0.81
$(Z_{HER2:4})_2$	32.6 h ± 0.58 h	3.61
Z_{00342}	34.5 h ± 0.45 h	0.85

Table 2. Data from the proliferation study. The cell doubling times with standard deviations are from the fitted curves in figure 10.

For the growth inhibition study, the cells were cultivated for 30 days after which the experiment was interrupted. The numbers of cells are plotted in figure 11 in the same way as the cell numbers in figure 10. Normalised values from the untreated group from the proliferation study are also added to the diagram as untreated, non-irradiated.

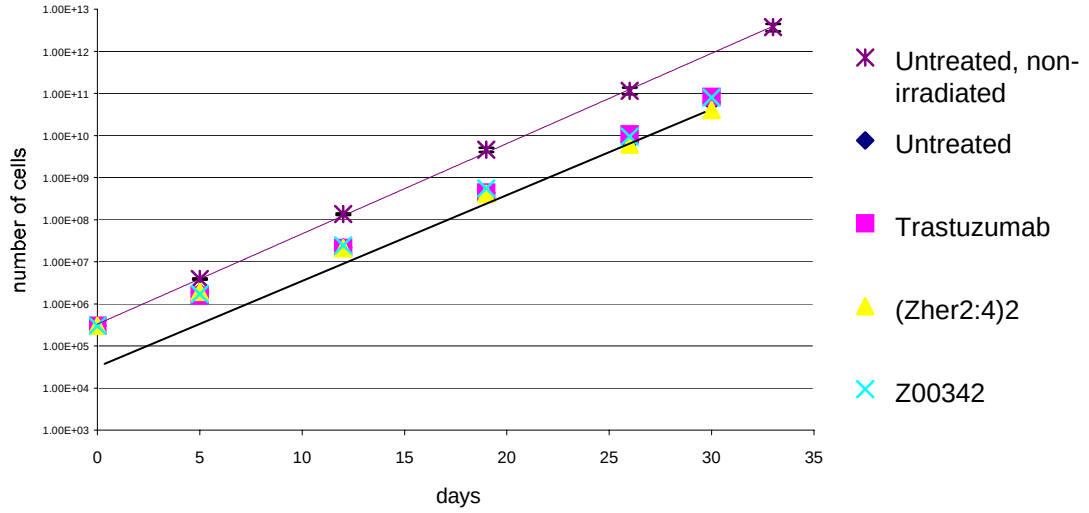

Figure 11. Cell growth of the growth inhibition study after radiation of 4 Gy and an initial 24 h treatment with substances. The y-axis has a logarithmic scale and the trendlines are exponentially fitted. The lower trendline is fitted in regard of the last three values (at day 19, 26 and 30) to give the surviving fraction.

Cell number values from the last three time points in figure 11, where the growth has reached exponential phase, were plotted separately. The trendlines were extrapolated back to day zero (as visualised in figure 11) and the values from where the trendlines cross the y-axis were used to calculate the surviving fraction. The quotients between the values of the non-irradiated, untreated cells and the values of the different irradiated cells determined the surviving fractions. The results are presented in table 3. The surviving fraction of the cells treated with the affibodies was higher than those untreated, while trastuzumab treatment gave a lower percentage of survival. Especially exposure to the bivalent affibody seems to have increased the amount of cells that have survived after the irradiation.

	Surviving fraction	Stdv
Untreated	26.9%	12.9%
Trastuzumab	15.7%	5.0%
$(Z_{HER2:4})_2$	53.7%	30.5%
Z_{00342}	33.9%	15.8%

Table 3. The surviving fractions with standard deviations after irradiation of the growth inhibition study.

4. Discussion

The results from the signal activity assays demonstrate that both affibodies affect the HER2 receptor and its downstream signalling. The effects of the two affibodies differ both from those of trastuzumab and between themselves. The reason for why they differ compared to trastuzumab probably relates to the fact that they do not share the same epitope on HER2 as trastuzumab [18]. According to previous studies, antibodies that share the same epitope on an ErbB receptor often have the same range of activity [20]. Alternatively, it can be a result of the difference in size, as has been reported before [6]. As seen in figure 9, cells treated with trastuzumab resulted in more stable results than cells treated with any of the affibodies. Especially exposure to the monovalent form gave unstable results. One can speculate that a bivalent molecule may be able to stabilise and even induce HER2 dimers through binding to one receptor with each arm, and thus lead to more efficient activation of signal transduction compared to treatment with a monovalent molecule. This could explain the differences in results between the bivalent and the monovalent affibody.

From the signal activity assay it is noticeable that both the affibodies induced phosphorylation of Akt. Especially stimulation by the bivalent affibody is evident. Because of Akts major role in anti-apoptosis, this indicates that cells treated with the bivalent affibody have enhanced protection from apoptosis compared to untreated cells. This is also confirmed by the growth inhibition study where cells treated with the bivalent affibody showed a surviving fraction of 54%, compared with a 27% survival of the untreated cells. The corresponding value for cells treated with the affinity maturated monovalent affibody was 34%. Also for trastuzumab, the results of these experiments coincide and both showed a decrease in surviving fraction / phosphorylation of Akt. This deactivation of the PI(3)K-pathway by trastuzumab is in agreement with previous experiments, and is thought to be an important way for Herceptin to induce cell cycle arrest [1, 8].

It is noteworthy that the proliferation study did not indicate that trastuzumab inhibits growth largely. Only a very small decrease in growth compared to the untreated cells was observed. The signal activity assay also indicated a small decrease in phosphorylation of Erk after stimulation with trastuzumab, which agrees with previous studies [1]. Moreover, trastuzumab strongly induced PLCγ1 phosphorylation, which in other model systems has been shown to increase cell motility [15]. However, no studies have indicated problems with increased cell migration after Herceptin treatment. The immuno-response (caused by the human IgG_1 region in trastuzumab) is thought to be an important component of trastuzumab's anti-tumour activity *in vivo*. Additionally, studies have shown that Herceptin only inhibits tumour growth for less than 50 % of the patients with tumours that over-express HER2 [2]. If the SKOV-3 cell line is representative of this group that, together with the lack of immuno-response in the *in vitro* experiment, could also explain why no large decrease in proliferation is visible in this study.

The proliferation study clearly showed an increase in cell growth for cells treated with the bivalent affibody compared to those untreated. After 33 days of cultivation, the cells treated with the bivalent affibody were more than three times as many than those untreated (2.2×10^{12} cells compared to 6.2×10^{11} cells). Treatment with the affinity maturated monovalent affibody caused the same decrease in proliferation as

trastuzumab (5.3×10^{11} cells). Since both increased proliferation and decreased apoptosis could lead to an increased amount of cells, and the bivalent affibody induced phosphorylation in both MAP-kinases and Akt, the results from the growth studies support the signal activity assay. Thus, the bivalent affibody seems to have tumour stimulatory capacities, which other ErbB recptor-binding molecules also have been found to hold [20]. For the affinity maturated monovalent affibody, a small decrease in phosphorylation was visible for both MAP-kinases and PLCγ1.

The fact that both trastuzumab and the bivalent affibody induced phosphorylation of HER2 while the affinity maturated monovalent affibody did not, might depend on a supposed ability for bivalent molecules to induce formation of receptor dimers. One can speculate that the monovalent form even might be able to interrupt dimer-formations, either through competitive inhibition or by inducing conformational changes of the receptor. However, further studies that focus on dimerisation must be done before anything can be said.

One possible explanation to the fact that HER2 did not seem to be activated by the one-hour treatment of EGF (which is a ligand to EGFR and therefore is able to activate HER2 through heterodimerisation) could be a result of HER2 over-expression. A massive over-expression of HER2 could lead to formation of stable homodimers, which are not dependent on EGF. An alternative explanation involves internalisation. This hypothesis means that the receptors are activated, but by the time of cell lysis, one hour after stimulation, the majority of the receptors have already been removed from the cell surface through endocytosis. This down-regulation of the receptors by prolonged exposure to ligand is a well-established occurrence for many receptor-ligand pairs. The internalisation hypothesis was investigated by an experiment with an extra induction time for EGF. As visible in figure 8, HER2 was exceedingly phosphorylated by the shorter induction time, but showed little activation for the longer treatment with EGF. Thus, the result seems to be in agreement with the internalisation hypothesis. The decreasing phosphorylation of HER2 at long exposure to trastuzumab (visible in figure 7a) is possibly also caused by internalisation of the receptor.

From a tumour-inhibiting point of view, the results from the present study of the affibodies might not seem promising, however, studies have shown that there are no simple correlation between *in vitro* growth assays and *in vivo* effects [20]. There is also a need to perform these experiments on other cell lines to obtain more representative results since the results can be cell-line-dependent. It might be advantageous to combine affibody treatment with other therapeutic uses for tumour inhibition since it is a well known fact that combined treatment of agents often gives better results compared with either agent given alone [1, 13]. One alternative that might be useful is to combine affibody treatment with drugs that inhibit Akt activity. However, the main potential use for the HER2 binding affibodies is not direct inhibition of tumour growth but tumour targeting, which can be used both for therapy and diagnostics. In tumour-targeted therapy, a cytotoxic agent, e.g. a radionuclide or a cytostatic drug, is coupled to a targeting agent specific for the tumour(s), and the toxic effects can thereby mostly be spared normal tissues. Radionuclide tumour targeting can for example be used for diagnostics and imaging of HER2-expression in tumours. In breast cancer for instance, it is necessary to detect the level of HER2 to know which medical approach might be the most beneficial. Since no natural ligand has

been found for HER2, it is of great importance to find a good targeting agent for HER2. A previous study has shown the bivalent HER2 binding affibody to be a promising targeting agent for HER2 detection, much because of its small size and high affinity for the receptor [14]. The present study suggests that the affinity maturated monovalent form might be even better suited as a targeting agent. It is smaller than the bivalent form and does not show the same capacity to stimulate proliferation. In fact, the affinity maturated monovalent affibody showed abilities to decrease phosphorylation of HER2 and resulted in a similar decrease of proliferation like trastuzumab. It might therefore be of potential use for inhibition of the receptor and cell growth. However, the results from treatment with the monovalent affibody were in general unstable and more studies to see its *in vivo* effects need to be performed.

5. Conclusions

The aim of the present study was to investigate the effect of the two HER2-binding affibodies, $(Z_{HER2:4})_2$ and Z_{00342} (i.e. the bivalent affibody and the affinity maturated monovalent affibody), on intracellular signal transduction pathways and to compare to that of trastuzumab (Herceptin®). The results demonstrated that the signal transduction proteins Erk1/2, Akt and PLCγ1 as well as the HER2 receptor itself, were all affected by treatment with both the two affibodies and trastuzumab. Thus, all three substances are biologically active. Interestingly, HER2 phosphorylation was induced by trastuzumab and the bivalent affibody but not by the affinity maturated monovalent affibody. In contrast, the monovalent affibody rather decreased phosphorylation of the receptor. It is notable that the effects on the signalling pathways differed between treatment of trastuzumab and the bivalent affibody, even though they both induced phosphorylation of HER2. Both of them induced an increase in PLCγ1 phosphorylation but gave different results for MAP-kinase and Akt. While trastuzumab decreased phosphorylation of them both, the bivalent affibody induced an increase in their phosphorylation. Generally, the affinity maturated monovalent affibody had less effects on signal transduction compared to trastuzumab and the bivalent affibody. The monovalent form had no obvious effect on MAP-kinase, induced a small increase in phosphorylation of Akt and a small decrease in phosphorylation of PLCγ1.

In general, the results from the growth experiments were well in agreement with the results from the signal activity assay. Especially the bivalent affibody's capacity to induce increases in phosphorylation of MAP-kinases and Akt was confirmed by both the growth studies. In the growth study, treatment with the bivalent affibody strongly induced proliferation, and in the growth inhibition study it resulted in a larger surviving fraction, thus indicating increased ability to resist apoptosis. Since the results suggest that the affinity maturated monovalent affibody has a similar inhibitory effect on HER2 expressing cells as trastuzumab, the affinity maturated monovalent form may thus be better suited for diagnostic and therapeutic purposes than the bivalent affibody.

6. Acknowledgements

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