Selection and characterization of HER2/neu-binding affibody ligands


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Affibody® (affibody) ligands that are specific for the extracellular domain of human epidermal growth factor receptor 2 (HER2/neu) have been selected by phage display technology from a combinatorial protein library based on the 58 amino acid residue staphylococcal protein A-derived Z domain. The predominant variants from the phage selection were produced in Escherichia coli, purified by affinity chromatography, and characterized by biosensor analyses. Two affibody variants were shown to selectively bind to the extracellular domain of HER2/neu (HER2-ECD), but not to control proteins. One of the variants, denoted His6-ZHER2/neu-4, was demonstrated to bind with nanomolar affinity (~50 nM) to the HER2-ECD molecule at a different site than the monoclonal antibody trastuzumab. Furthermore, radiolabeled His6-ZHER2/neu-4 affibody showed specific binding to native HER2/neu, overexpressed on the SKBR-3 tumor cell line. Such affibody ligands might be considered in tumor targeting applications for radionuclide diagnostics and therapy of adenocarcinomas such as breast and ovarian cancers.

Keywords: affibody/HER2/ligand/phage display/selection

Introduction

Cancer therapy with conventional drugs is often associated with toxicity and limitations in effectiveness. In an attempt to improve cancer treatments, therapy through specific targeting of monoclonal antibodies has been explored since the 1970s. Today, about 15 monoclonal antibodies, humanized to various degrees, are approved for human use. During recent years, the humanized monoclonal antibody trastuzumab (Herceptin®) has been used in breast cancer therapy, since it is directed to the human epidermal growth factor receptor 2 (HER2/neu), overexpressed in ~20–30% of breast cancers (Slamon et al., 1989). Upon interaction with cell-specific markers, antibodies can show direct cytotoxic effects by several mechanisms of action, e.g. blocking of growth factor receptors (Baselga and Mendelsohn, 1994), induction of apoptosis (Trauth et al., 1989), and formation of anti-idiotypic antibodies (Fagerberg et al., 1994), or indirect effects, such as antibody-dependent cellular cytotoxicity (ADCC) (Steplewski et al., 1983) and complement-mediated cytotoxicity (Ballare et al., 1995). As the cytotoxic actions induced by antibodies often are insufficient, they have been coupled to various toxic substances, including radioactive isotopes (Wilder et al., 1996), toxins (Kreitman, 1999) and chemotherapeutic drugs (Garnett et al., 1985), to enhance their potency.

Although monoclonal antibodies offer many attractive features for tumor targeting, they are also correlated with high manufacturing costs, various side effects and limited efficacy. This might be due to their rather large size, causing slow blood clearance, liver uptake and poor tissue penetration. Therefore, smaller antibody fragments, such as F(ab) (Winter et al., 1994), diabody (Holliger et al., 1993), scFv (Bird et al., 1988; Huston et al., 1988), Fv (Davies and Reichmann, 1999) and various single domain antibodies (Desmyter et al., 2002; Holt et al., 2003) have been developed (Nilsson et al., 2000).

Antibody fragment approaches are indeed promising, although some limitations seem to exist. Stability issues, both in terms of proteolysis and shelf-life, are obvious challenges, and antibody fragments do not normally fold inside cells, since they contain disulfide bridges, whose formation is crucial for the correct folding of the molecules. The various antibody approaches have recently been complemented by efforts based on different protein scaffolds (Nygren et al., 2003; Xu et al., 2002; Koide et al., 2002; Gräslund et al., 2002), to engineer adenoviral tropism (Henning et al., 2002) and to inhibit receptor interactions (Sandström et al., 2003). Thus, affibody ligands might exhibit therapeutic potential and merit further investigation.

One potential cancer target is HER2/neu (also called ErbB2), a transmembrane protein belonging to the human epidermal growth factor tyrosine kinase receptor family. Increased...
HER2/neu activity is associated with increased proliferation and decreased apoptotic capacity. HER2/neu is often overexpressed in different cancers, including breast and ovarian (Wang and Hung, 2001), but is expressed only to a small extent or not at all in many normal adult tissues (Natali et al., 1990; Press et al., 1990). In patients, overexpression of HER2/neu is associated with short disease-free time and decreased overall survival and it has also been shown that the overexpression is often preserved in metastases (Gancberg et al., 2002). Together, these factors provide strong arguments for the development of targeting strategies directed against HER2/neu.

In this study, we present the selection and characterization of affibody variants binding to a recombinant extracellular domain of HER2/neu (HER2-ECD), which was used as target during biopanning. The novel selected affibody ligands were characterized for their binding to HER2-ECD in biosensor studies. Furthermore, it was investigated whether the selected affibody molecules bound to the same HER2/neu site as the trastuzumab monoclonal antibody. In addition, the specific binding to native HER2/neu, overexpressed in the tumor cell line SKBR-3, was evaluated.

Materials and methods

Strains, vectors, and phagemid library

The amber suppressor Escherichia coli strain RRIΔM15 (Rüther, 1982) was used as bacterial host for phage production and cloning procedure. The phagemid vector pAffi1 and the construction of the phagemid library used in this study, a portion (~8.7x10⁹) of Zlib2002 (3x10⁹ members), will be described elsewhere (E.Gunneriusson, unpublished data). Phagemid inserts of selected clones were subcloned into the expression vector pAY81, containing a T7 promoter (Studier et al., 1990), a DNA fragment encoding a hexahistidyl (His6) tag and a multiple cloning site, together with a gene conferring resistance to kanamycin. The E.coli strain BL21(DE3) (Novagen, Madison, WI) was used for protein production from the pAY81 plasmid.

Preparation of phage stocks

Preparation of phage stocks from the library (a portion of Zlib2002) and between selections was performed according to previously described procedures (Nord et al., 1997; Hansson et al., 1999) using the helper phage M13K07 (New England Biolabs, Beverly, MA). PEG/NaCl precipitation yielded phage titers of about 10¹³ p.f.u./ml.

Phage selections

A 100 kDa recombinant extracellular domain of HER2/neu (HER2-ECD) comprising 624 amino acids, corresponding to nucleotides 238–2109 (Horak et al., 2001), was used as target protein during selections. The protein was biotinylated in vitro using EZ-Link™ Sulfo-NHS-LC-Biotin ( Pierce, Rockford, IL). A 30-fold molar excess of biotin was added to HER2-ECD in phosphate-buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, pH 7.2.), and the mixture was incubated at room temperature for 1 h followed by extensive dialysis against PBS at 4°C to remove the surplus of biotin. The biotinylated target protein was immobilized on streptavidin-coated paramagnetic beads (Dynabeads<sup>®</sup> M-280 Streptavidin; Dynal A.S., Oslo, Norway). For each round of selection, beads were washed twice with PBS supplemented with 0.1% Tween-20 (PBST), followed by incubation with biotinylated HER2-ECD in at least 250 µl of PBS, pH 7.2, for 30 min at room temperature under continuous rotation (end over end). For round 1, 40 µg of target protein were incubated with 5 mg of beads, for rounds 2 and 3, 20 µg of target protein were incubated with 2.5 mg of beads, and for round 4, 5 µg of target protein were incubated with 0.63 mg of beads. The beads with the immobilized HER2-ECD were thereafter washed once with PBST. This procedure resulted in an immobilization of ~5 µg of the target protein per mg of beads, as determined by SDS–PAGE analysis.

The four rounds of biopanning were performed as follows. To avoid unspecific binders, all tubes used in this procedure were pretreated with PBST supplemented with 0.1% gelatin. To further avoid binders against the streptavidin present on the paramagnetic beads, ~1 ml of the phage stock supplemented with 0.1% gelatin was for round 1 and 2 preincubated with 0.5 mg of the beads, previously washed twice with PBST. The unbound phage stock was after that subjected to biopanning against the HER2-ECD-coated beads for 2 h at room temperature under continuous rotation. The beads were washed once with PBST in round 1, three times in round 2, six times in round 3 and 12 times in round 4. The bound phages were subsequently eluted with 500 µl of 0.1 M glycine–HCl, pH 2.2, for 10 min at room temperature, followed by immediate neutralization with 50 µl of 1 M Tris–HCl, pH 8.0. The eluted phages were used to infect log phase RRIΔM15 cells for 20 min at 37°C. The infected cell suspensions were spread on TYE agar plates (15 g/l agar, 8 g/l NaCl, 10 g/l tryptone and 6 g/l yeast extract), supplemented with 2% glucose and 100 mg/l ampicillin, followed by overnight incubation at 37°C. The grown colonies were collected by resuspension in tryptic soy broth (TSB, 30 g/l; Merck, Darmstadt, Germany), supplemented with 2% glucose and 100 mg/l ampicillin, and a fraction (100 times excess of cells compared to the phage titer after elution) was used for inoculation, leading to the next generation of phage stock. The selection process was monitored by titrating the phage stocks before selection and after elution. A serial dilution of phages was allowed to infect log phase RRIΔM15 cells for 20 min at 37°C, followed by plating on TYE agar plates, supplemented with 2% glucose and 100 mg/l ampicillin, and overnight incubation at 37°C.

DNA sequencing

After four rounds of biopanning, DNA sequencing of phagemid (pAffi1) inserts was performed on 49 randomly picked colonies using specific sequencing primers and Big Dye terminators (Amersham Biosciences, Upsala, Sweden). The Sanger fragments were analyzed on a DNA sequencer ABI Prism<sup>®</sup> 3700 Analyzer (Applied Biosystems, Foster City, CA). Subcloned DNA fragments were verified by the same procedure.

DNA constructions

DNA fragments encoding different variants of Z<sub>HER2/neu</sub> were subcloned into the expression vector pAY81. The fragments were digested from the pAffi1 vector with XhoI (ER0691; Fermentas, Vilnius, Lithuania) and SalI (ER0641; Fermentas), and ligated into the pAY81 vector, previously restricted with the same enzymes and dephosphorylated using calf intestine alkaline phosphatase (CIAP; Fermentas). The cleaved DNA fragments were purified on low-melt agarose gel (Pronadisa, Madrid, Spain) prior to ligation with T4 DNA Ligase (Fermentas). The ligations resulted in expression vectors denoted...
pAY81-ZHER2/neu:4, -ZHER2/neu:7 encoding the different affibody ligands fused to an N-terminal His6 tag, allowing purification by immobilized metal ion affinity chromatography (IMAC). All plasmid preparations were, after cultivation of transformed E. coli cells overnight, performed using QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

Protein production and purification
Selected affibody variants were expressed as His6-tagged fusion proteins from the pAY81 plasmids in E. coli strain BL21(DE3). Cells were inoculated in 15 ml of TSB medium, containing 50 mg/l kanamycin, and grown in shake flasks overnight at 37°C. Fresh TSB (100 ml), supplemented with 5 g/l yeast extract and 50 mg/l kanamycin, was inoculated with 1 ml of the overnight cultures and the cells were grown at 37°C until A600nm ~0.6–1.0, when gene expression was induced by addition of isopropyl β-d-thiogalactoside (IPTG; Apollo Scientific, Derbyshire, UK) to a final concentration of 1 mM. After 3–4 h of cultivation at 37°C, the cell cultures were harvested by centrifugation (4000 g, 15 min, 4°C). The cell pellets were subsequently resuspended in 25 ml of denaturing buffer (6 M Gua–HCl, 47.4 mM NaH2PO4, 2.65 mM Na2HPO4, 10 mM Tris–HCl, 0.1 M NaCl, pH 8.0), and disrupted by sonication. β-Mercaptoethanol was added to the cell suspensions to a final concentration of 10 mM and left with magnetic stirring for 1 h at room temperature. Centrifugation at 15 000 g for 10 min at 4°C resulted in pelleted insoluble material and denatured proteins in the supernatant.

The His6-ZHER2/neu fusion proteins were recovered by IMAC purification on Talon Metal Affinity Resin (BD Biosciences, CA) columns under denaturing conditions. The IMAC columns were pulsed twice with 10 ml of denaturing buffer and 10 ml of elution buffer (8 M urea, 0.1 M NaCl, 29.6 mM HAc, 70.4 mM NaAc, 50 mM NaH2PO4, pH 5.0), and equilibrated with 25 ml of denaturing buffer. Supernatants containing the dissolved His6-ZHER2/neu proteins were diluted with 25 ml of denaturing buffer and filtered (0.45 μm; Sartorius, Göttingen, Germany), before the samples were applied to the columns. After washing with 30 ml of denaturing buffer, the bound proteins were released with elution buffer. Renaturation of the purified fusion proteins was performed by extensive dialysis against PBS (10 mM phosphate, 154 mM NaCl, pH 7.2) at 4°C. Protein concentration was calculated from absorbance measurements at 280 nm, using the appropriate extinction coefficient for each protein, and also determined by amino acid analysis (Amino-syraanalyscentralen, Uppsala, Sweden). The purified proteins were further analyzed by SDS–PAGE on Tris–glycine 16% homogeneous gels, using a Novex system (Invitrogen, Carlsbad, CA).

Biosensor analyses
A BIAcore® 2000 instrument (Biacore AB, Uppsala, Sweden) was used for real-time biospecific interaction analysis (BIA) between selected affibody molecules and the target protein. HER2-ECD (diluted in 10 mM NaAc, pH 4.5) was immobilized (~2200 RU) on the carboxylated dextran layer of one flow-cell surface of a CM5 sensor chip (research grade) (Biacore) by amine coupling, according to the manufacturer’s instructions. Another flow-cell surface was activated and deactivated to be used as a reference surface, and human IgG (Amersham Biosciences), HIV-1 gp120 (Protein Sciences Corp., Meriden, CT), and an albumin binding region of streptococcal protein G (BB; Ståhl et al., 1999) were immobilized on separate flow-cell surfaces on CM5 sensor chips, to serve as negative controls. For all affibody samples, the buffer was changed to HBS-EP (5 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) by gel filtration using NAPFm-10 columns, according to the manufacturer’s protocol (Amersham Biosciences), and the samples were there after filtered (0.45 μm; Millipore, Billerica, MA). Binding analyses were performed at 25°C, and HBS-EP was used as running buffer. In a first experiment, 5 μM of each affibody (diluted in HBS-EP) was injected over all surfaces with a flow rate of 5 μl/min. As a negative control, an unrelated affibody was also injected. In a second experiment, the His6-ZHER2/neu:4 and His6-ZHER2/neu:7 affibody variants were subjected to a kinetic analysis, in which the proteins were injected over a HER2-ECD surface at different concentrations (0–5 μM, with 0.0098 μM as the lowest concentration for His6-ZHER2/neu:4 and 0.0196 μM for His6-ZHER2/neu:7) diluted in HBS-EP) with a flow rate of 30 μl/min. The dissociation equilibrium constant (KD), the association rate constant (ka), and the dissociation rate constant (kd) were calculated using BIAevaluation 3.2 software (Biacore), assuming a one-to-one binding. For the first two Biacore analyses, the samples were run in duplicates in random order, and after each injection the flow cells were regenerated by injection of 10 mM HCl. In a third experiment, the His6-ZHER2/neu:4 variant was evaluated for binding competition with the monoclonal antibody trastuzumab. To obtain a reference curve, His6-ZHER2/neu:4 was injected over the HER2-ECD surface at a concentration of 5 μM and the surface was regenerated with 10 mM HCl. The HER2-ECD surface was subsequently saturated by repeated injections with trastuzumab at a concentration of 0.2 μM until no significant additional response was observed. Directly following the trastuzumab injections, the same amount of the His6-ZHER2/neu:4 affibody as in the reference experiment was injected.

Cell culture
Human breast cancer cell line SKBR-3, known to express ~2×10⁶ HER2/neu per cell, was purchased from ATCC (American Type Culture Collection, Manassas, VA). The cells were cultured in complemented medium, containing RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and PEST (100 IU/ml penicillin and 100 μg/ml streptomycin), all from Biochrom KG (Berlin, Germany). The cells were cultured at 37°C in humified air containing 5% CO₂, and seeded in 3 cm Petri dishes 3 days before the experiment.

Radiolabeling
Labeling precursor, N-succinimidyl-p-(trimethylstannyl) benzoate (SPMB), was prepared according to Orlova et al. (2000), and 5 μg of SPMB were added to 5 MBq of 125I in a 5% solution of acetic acid. To start the reaction, 40 μg of chloramine-T (Sigma, St Louis, MO) in aqueous solution were added. During 5 min, the reaction mixture was agitated, and 80 μg of sodium meta-bisulfate (Aldrich, Steinheim, Germany) in aqueous solution were added to stop the reaction. The radiolabeled precursor was added to 40 μg of His6-ZHER2/neu:4 or His6-ZHER2/neu:7 in 0.07 M borate buffer, pH 9.2. The coupling reaction, in which the labeled precursor is conjugated to amine groups on the protein, was performed at
room temperature for 45 min with continuous shaking. Labeled affibody molecules were separated from low molecular weight products using a NAP\textsuperscript{TM}-5 size exclusion column (Amersham Biosciences) equilibrated with PBS. The radiolabeled affibody molecules were then analyzed using BIAcore technology to verify that the labeling procedure had not affected the binding affinity to HER2-ECD.

The monoclonal anti-HER2 antibody trastuzumab, Herceptin\textsuperscript{®} (purchased from Apoteket AB, Sweden) desalted on a PD-10 column (Amersham Biosciences), was radiolabeled with \textsuperscript{125}I using the chloramine-T method previously described (Sundberg et al., 2003).

Cellular tests
To each dish of \(\sim750\) 000 SKBR-3 cells, 28 ng of labeled His\textsubscript{6}Z\textsubscript{HER2/neu}:4, His\textsubscript{6}Z\textsubscript{HER2/neu}:7 or 470 ng trastuzumab in 1 ml of complemented medium, described above, were added. This amount corresponds to a theoretical 1:1 ratio between the targeting agent and HER2 molecules available on the cells. Three dishes without cells were treated in the same way, in order to determine unspecific and not cell-dependent binding. The resulting radioactivity value was subtracted from all others. To analyze the specificity of cell binding and epitope competition between the targeting agents, three dishes were treated not only with labeled affibody or antibody, but also with a 500-fold excess of unlabeled affibody or antibody. After 3 h of incubation at 37\(^\circ\)C, the radioactive medium was removed and the dishes were washed rapidly three times with ice-cold serum-free medium. Cells were trypsinated with 0.5 ml trypsin/EDTA solution (0.25%/0.02% in PBS; Biochrom KG) for 15 min at 37\(^\circ\)C. The cells were then resuspended in 1 ml of complemented medium, and 0.5 ml of the cell suspension was used for cell counting and the remaining 1 ml was used for radioactivity measurement in an automated gamma counter.

Results and discussion

Library and selection of affibody variants
Phage display in vitro selection technology was used to isolate novel affibody ligands, binding to the extracellular domain of HER2/neu (HER2-ECD), from a portion of the combinatorial protein library Zlib2002. The library is based on the 58 residue staphylococcal protein A-derived Z domain, and has been constructed by combinatorial substitution mutagenesis of 13 positions located at the surface responsible for Fc binding activity, essentially as described earlier (Nord et al., 1995). The different members (\(\sim3\times10^9\)) of the library are monovalently displayed on protein III of M13 bacteriophage particles using a phagemid vector system (Nord et al., 1995, 1997).

Four rounds of phage display selection were performed using biotinylated recombinant HER2-ECD as target protein immobilized on streptavidin-coated paramagnetic beads. To increase the selection stringency, decreasing amounts of target protein together with increasing number of washing steps were employed for each round. DNA sequencing performed on 49 randomly picked colonies from the fourth round of biopanning revealed seven different clones, among which one clone was represented 33 times, indicating significant convergence (Figure 1). Such convergence typically indicates better binding affibody molecules (Nord et al., 1997), but all sequences represented more than once were selected for further characterization, i.e. clones 2, 4, 7 and 8. An alignment of the 13 randomized amino acid residues of the seven unique variants

![Alignment of affibody variants](attachment:alignment.png)

* Amber stop

Fig. 1. Amino acid sequence corresponding to the wild-type Z domain aligned to deduced amino acid sequences of different affibody variants selected against HER2-ECD. The 13 randomized amino acid residues (Q9, Q10, N11, F13, Y14, L17, H18, E24, E25, R27, N28, Q32, K35) are presented. Amino acid residues that occur at the same position in more than one of the variants are presented in bold. Note, an amber stop codon is included in the Z\textsubscript{HER2/neu:25} variant. Horizontal bars indicate amino acid identities. Figures to the right represent the number of times each variant revealed upon DNA sequencing of 49 colonies. The three \(\alpha\)-helices in the wild-type Z domain are boxed.
Production of selected affibody molecules

DNA fragments encoding four different affibody variants (ZHER2/neu:2, ZHER2/neu:4, ZHER2/neu:7, and ZHER2/neu:8) (Figure 1) were subcloned into the pAY81 vector, resulting in expression vectors encoding, under the control of the T7 promoter, the different affibody molecules fused to an N-terminal His6 peptide tag. The His6-ZHER2/neu fusion proteins were expressed intracellularly in E. coli cells, and thereafter purified on IMAC columns under denaturing conditions. Upon SDS–PAGE analysis, the proteins were observed as specific bands of expected molecular weight (8.7 kDa), indicating pure and stable affibody molecules (Figure 2 and data not shown). Interestingly, the His6-ZHER2/neu:7 variant was migrating as a somewhat larger protein than the His6-ZHER2/neu:4 variant (Figure 2), although they differ only in eight amino acid positions (Figure 1). Such deviation in migration has been observed earlier for similar proteins (Gräslund et al., 2000). Estimations from absorbance measurements at 280 nm demonstrated high expression levels (100–200 mg/l of cell culture) for all four proteins.

Biosensor binding analyses of selected affibody ligands

Purified affibody ligands were analyzed for HER2-ECD binding by real-time biospecific interaction analysis using a BIAcore biosensor instrument. Prior to sample injection, all affibody proteins were subjected to renaturation and the buffer was changed to the running buffer. The different affibody molecules were subsequently injected over separate sensor chip flow-cell surfaces containing the immobilized target protein HER2-ECD and the control proteins IgG, gp120, and BB, respectively. The His6-ZHER2/neu:2, His6-ZHER2/neu:4, and His6-ZHER2/neu:7 affibody proteins were all shown to bind to HER2-ECD, although the binding of His6-ZHER2/neu:4 and His6-ZHER2/neu:7 (Figure 3A) was considerably stronger than the binding of His6-ZHER2/neu:2 (data not shown). For His6-ZHER2/neu:8 and the unrelated control affibody, no obvious

Fig. 2. SDS–PAGE analysis (Tris–glycine 16% homogeneous gel, reducing conditions) of the expressed and IMAC-purified affibody fusion proteins His6-ZHER2/neu:4 (lane 1) and His6-ZHER2/neu:7 (lane 2). Lane M, marker proteins with molecular masses in kilodaltons. Protein bands were visualized with Coomassie Brilliant Blue staining.

Fig. 3. Biosensor binding studies. (A) Sensograms obtained after injection of the purified His6-ZHER2/neu:4 (squares) and His6-ZHER2/neu:7 (circles) affibody variants over sensor chip flow-cell surfaces containing amine-coupled HER2-ECD (filled squares/circles) or human IgG (open squares/circles). (B) Sensograms obtained after injection of the His6-ZHER2/neu:4 affibody variant over a HER2-ECD flow-cell surface at selected concentrations: 312.5 nM (filled diamonds), 156.3 nM (filled circles), 78.2 nM (filled triangles), 39.1 nM (open squares), 19.6 nM (open diamonds), and 9.8 nM (open circles). (C) Sensograms obtained after injection of the His6-ZHER2/neu:7 affibody variant over a HER2-ECD flow-cell surface at selected concentrations: 625 nM (filled squares), 312.5 nM (filled diamonds), 156.3 nM (filled circles), 78.2 nM (filled triangles), 39.1 nM (open squares), and 19.6 nM (open diamonds). All samples were run at 25 °C in duplicates in random order, and the response obtained from an activated and deactivated reference surface has been subtracted from all curves.
binding to HER2-ECD was observed (data not shown). The reason why the His6-ZHER2/neu:8 affibody molecule did not bind its target HER2-ECD in the BIAcore experiment, although having certain sequence similarities to the binding clones (Figure 1), is not obvious and cannot be fully explained from the present data. However, this clone was the least similar of those characterized, and could potentially bind to a slightly different site that might not be optimally presented on the HER2-ECD immobilized on the biosensor chip. Furthermore, as expected, no significant binding could be seen to the control proteins; IgG (Figure 3A), HIV-1 gp120 (data not shown), and streptococcal protein BB (data not shown). These results suggest that the His6-ZHER2/neu:4 and His6-ZHER2/neu:7 affibody variants bind selectively to the target protein HER2-ECD.

The His6-ZHER2/neu:4 and His6-ZHER2/neu:7 affibody ligands were subjected to a kinetic analysis in order to determine the kinetic binding constants. Prior to the kinetic analysis, the protein concentration was determined by amino acid analysis. The affibody molecules were then injected over a HER2-ECD flow-cell surface at different concentrations, ranging from 0 to 5 μM with 9.8 nM as the lowest concentration for His6-ZHER2/neu:4 and 19.6 nM for His6-ZHER2/neu:7. Upon evaluation of the binding curves (Figure 3B and C), the dissociation equilibrium constant (K_D) was determined to be ~50 nM for His6-ZHER2/neu:4 and ~140 nM for His6-ZHER2/neu:7, the latter estimated by steady-state determination. The reason for the difference in K_D is most likely due to the marked difference in dissociation rate, as can be seen in Figure 3A. For His6-ZHER2/neu:4, the association rate constant (k_a) was calculated to be ~1.8×10^10 M^-1 s^-1 and the dissociation rate constant (k_d) ~9.9×10^-3 s^-1, while for His6-ZHER2/neu:7, the k_a and k_d values were difficult to estimate due to the fast association and dissociation kinetics (K_D determined through estimation at steady state). Thus, the His6-ZHER2/neu:4 affibody variant showing stronger binding to its target was selected for further characterization.

To investigate if the His6-ZHER2/neu:4 affibody binds to the same HER2-ECD site as the monoclonal antibody trastuzumab, the affibody was injected over a HER2-ECD flow-cell surface, before and after saturation with trastuzumab (Figure 4). First, as a reference, the His6-ZHER2/neu:4 affibody molecule was, as in Figure 3A, allowed to bind to, and dissociate from, the immobilized extracellular domain of HER2/neu (data not shown). Secondly, the trastuzumab monoclonal antibody was allowed to bind to HER2-ECD (Figure 4A), and a typical association curve was obtained. The HER2-ECD surface was subsequently saturated by repeated injections with trastuzumab until no significant additional response was observed (data not shown). Directly following the trastuzumab injections, the same amount of the His6-ZHER2/neu:4 affibody as in the reference experiment was injected (Figure 4B). Both binding curves of His6-ZHER2/neu:4 had a similar appearance and approximately the same amount of affibody could be bound to the surface irrespective of preceding trastuzumab saturation, indicating that the affibody binds to a different site on the HER2-ECD molecule.

**Radiolabeling**

The His6-ZHER2/neu:4 and His6-ZHER2/neu:7 affibody proteins were indirectly radiolabeled with ^125_I by amine coupling. Upon BIAcore analysis, to determine if the labeling procedure had affected the binding affinity to HER2-ECD, both affibody variants showed retained affinity (data not shown).

**Cellular tests**

As presented in Figure 5, the His6-ZHER2/neu:4 affibody showed specific binding to SKBR-3 cells, described to express ~2×10^5 HER2/neu molecules per cell. The binding of radiolabeled His6-ZHER2/neu:4 affibody could be efficiently blocked by the addition of an excess of non-labeled affibody, indicating a specific binding to native HER2/neu (Figure 5A). Furthermore, the His6-ZHER2/neu:4 affibody and the monoclonal antibody trastuzumab was found to bind HER2/neu in a non-competitive manner, since the other ligand when added in excess was unable to block binding (Figure 5A and B). This cellular binding assay might in fact suggest that the presence of an excess of trastuzumab monoclonal antibody would improve binding of the His6-ZHER2/neu:4 affibody molecule to HER2/neu (Figure 5A). This effect could potentially be obtained if the trastuzumab binding stabilized the extracellular domain of HER2/neu, but more experimental evidence would need to be available before stating this. These data corroborate the BIAcore results presented in Figure 4, and suggest separate binding sites on HER2/neu for the His6-ZHER2/neu:4 affibody molecule and trastuzumab. The binding of the

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Fig. 4. Binding competition with the monoclonal antibody trastuzumab by biosensor analysis. (A) Sensorgram obtained after one injection of 0.2 μM trastuzumab over a HER2-ECD surface. (B) Sensorgram obtained after injection of 5 μM of the His6-ZHER2/neu:4 affibody over a trastuzumab-saturated HER2-ECD surface. The response obtained from an activated and deactivated reference surface has been subtracted from both curves. Note the significant difference in response between His6-ZHER2/neu:4 and trastuzumab due to their different molecular weights (8.7 and 150 kDa, respectively).
monoclonal antibody trastuzumab. Thus, a HER2/neu binding affibody may have the potential for future use in in vivo diagnostics and therapy of breast, ovarian and other HER2/neu overexpressing tumors. It would in future studies be interesting to evaluate dimerization and affinity maturation strategies (Gunneriusson et al., 1999; Nord et al., 2001) to increase the binding strength of the affibody for improved performance. Successful preparation of HER2/neu-binding affibody ligands may have important implications for radiolabeled tumor targeting, for in vivo imaging purposes or potentially even radiotherapeutic applications. Since affibody molecules are significantly smaller (20 times) than full-sized monoclonal antibodies, they might prove better in penetration of solid tumors. Comparative studies between antibodies and their fragments have demonstrated that smaller targeting molecules provided faster blood clearance and better tumor penetration (Yokota et al., 1992). These factors contribute to better imaging contrast in diagnostics (Behr et al., 1995), and possibly also to better radiotherapeutic efficacy (Ugur et al., 1996).

The half-life of several unrelated affibody molecules seems to be in a rather suitable range for imaging purposes, being in the range of 15 min in mice (data not shown). Radiolabeled receptor ligands, which are even smaller than antibody fragments, also seem to be very promising targeting agents. They typically exhibit rapid clearance from both blood and receptor-negative tissues and have good tumor penetration properties (Froidevaux et al., 2000). A number of other peptide ligands, are under investigation for clinical use (Heppeler et al., 2000). Certain tumor-associated antigens, such as HER2/neu, Ep-CAM, CEA and PSMA, are either not receptors or have no identified natural ligands. In these cases where natural ligands cannot be used as low molecular weight targeting agents, affibody ligands may prove to be promising alternatives for tumor targeting. The efficiency of in vivo targeting for imaging purposes, as well as potential therapeutic efficacy, remain to be investigated in future studies.

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