Engineering and characterization of a bispecific HER2 x EGFR-binding affibody molecule

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HER2 (human epidermal-growth-factor receptor-2; ErbB2) and EGFR (epidermal-growth-factor receptor) are overexpressed in various forms of cancer, and the co-expression of both HER2 and EGFR has been reported in a number of studies. The simultaneous targeting of HER2 and EGFR has been discussed as a strategy with which to potentially increase efficiency and selectivity in molecular imaging and therapy of certain cancers. In an effort to generate a molecule capable of bispecifically targeting HER2 and EGFR, a gene fragment encoding a bivalent HER2-binding affibody molecule was genetically fused in-frame with a bivalent EGFR-binding affibody molecule via a $(G_4S)_3$ [$(Gly_4\text{-}Ser)_3$]-encoding gene fragment. The encoded 30 kDa affibody construct $(Z_{HER2})_2$–$(G_4S)_3$–$(Z_{EGFR})_2$, with potential for bs (bispecific) binding to HER2 and EGFR, was expressed in *Escherichia coli* and characterized in terms of its binding capabilities. The retained ability to bind HER2 and EGFR separately was demonstrated using both biosensor technology and flow-cytometric analysis, the latter using HER2- and EGFR-overexpressing cells. Furthermore, simultaneous binding to HER2 and EGFR was demonstrated in: (i) a sandwich format employing real-time biospecific interaction analysis where the bs affibody molecule bound immobilized EGFR and soluble HER2; (ii) immunofluorescence microscopy, where the bs affibody molecule bound EGFR-overexpressing cells and soluble HER2; and (iii) a cell–cell interaction analysis where the bs affibody molecule bound HER2-overexpressing SKBR-3 cells and EGFR-overexpressing A-431 cells. This is, to our knowledge, the first reported bs affinity protein with potential ability for the simultaneous targeting of HER2 and EGFR. The potential future use of this and similar constructs, capable of bs targeting of receptors to increase the efficacy and selectivity in imaging and therapy, is discussed.

Introduction

An increasing number of clinically approved treatments for cancer have been developed on the basis of knowledge about molecular targets that are specific for, or overexpressed in, the tumour and their molecular function. The use of mAbs (monoclonal antibodies) is a well-established approach for targeting of tumour-associated antigens both in *in vivo* diagnostics and in therapy. Today, nine mAbs are approved by US Food and Drug Administration (FDA) for therapeutic use in oncology and several more are in preclinical and clinical development. Efficient tumour targeting for therapy or molecular imaging is dependent on sufficient tumour uptake and selectivity. Furthermore, fast clearance to achieve good contrast is particularly important in molecular imaging. Advances in protein engineering has led to the generation of a number of antibody derivatives [e.g. scFvs (single-chain variable fragments), Fabs (Fab fragments), diabodies (small recombinant bispecific antibodies), minibodies (recombinant antibodies in which the heavy- and light-chain variable regions are part of the same polypeptide chain, which also includes the heavy-chain hinge region and one heavy-chain constant domain) and dAbs (single-domain antibodies)] and more recently also to other scaffold proteins [e.g. Kunitz domains, knottins, Adnectins™, anticalins, DARPins (designed ankyrin-repeat proteins) and

Key words: bispecific (bs) affibody molecule, human epidermal-growth-factor receptor-3 (ErbB), microwell array, protein engineering, tumour targeting.

Abbreviations used: ABD, albumin-binding domain; BiTE, bispecific T-cell engager; bs, bispecific; DTT, dithiothreitol; EGFR, epidermal-growth-factor receptor; ECD, extracellular domain; FBS, fetal bovine serum; $(G_4S)_3$, $(Gly_4\text{-}Ser)_3$; hEGFR, human EGFR-1; hEGFR-Fc, hEGFR Fc chimaera; HER2/ErbB2, human EGFR-2; HER3/ErbB3, human EGFR-3; hHER2-Fc, HER2 Fc chimaera; His_6, hexahistidine; IMAC, immobilized-metal-ion affinity chromatography; mAb, monoclonal antibody; scFv, single-chain variable fragment.

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affibody molecules], of which several have been investigated for targeting of cancer-associated target molecules.

For certain applications it is desirable to increase the potency or selectivity of the affinity proteins. One way to do this is to engineer bs (bispecific) binding proteins for dual targeting. The bs binding proteins can be of several different formats, such as bs mAbs, diabodies and tandem scFvs [1,2]. The primary use of bs mAbs has been to redirect cytotoxic immune effector cells for enhanced killing of tumour cells. In such approaches, one arm of the bs mAb targets surface markers on tumour cells and the other arm binds proteins expressed on effector cells, such as CD3, CD16 or CD64, which are expressed on for example T lymphocytes or natural killer (NK) cells. Several bs mAbs targeting CD3 and tumour antigens have been generated, and their efficacy in T-cell retargeting toward these antigens have been documented in tumour models *in vitro* and *in vivo* [3–5]. BiTEs (bs T-cell engagers) are based on single-chain antibodies and, to date, various mouse models have demonstrated high levels of activity of BiTE antibodies [3,6,7]. Recently, a study on the BiTE antibody blinatumomab, with dual specificity for CD19 and CD3, also showed clinical activity in non-Hodgkin's B-cell lymphoma patients at doses well below those reported for the monoclonal antibody rituximab [8]. In other approaches, one arm of the bs mAb targets surface markers on tumour cells and the other arm can be directed to radionuclides, drugs and toxins. Pretargeting with these bs mAbs allows for loading of a sufficient amount of antibody on the tumour and is followed by clearance from normal tissue before administration of the cytotoxic molecule, thus decreasing unwanted toxicity to normal organs [9,10].

A possible way to improve selectivity and thereby possibly enhance the imaging contrast, and potentially also therapeutic efficacy, is by simultaneous targeting of two different receptors. By using a bs binding protein, the targeting of tumour cells expressing both receptors should be favoured compared with the 'background' targeting of normal tissues that express only one receptor. Some initial reports can be found on the development of antibody-based molecules for simultaneous targeting of two tumour-associated targets [11–13]. An anti-EGFR (epidermal-growth-factor receptor) × anti-(insulin-like-growth-factor receptor) di-diabody showed effective blocking of EGF and insulin-like-growth-factor-stimulated cell activation and, when tested *in vivo*, antitumour activity could be demonstrated [11]. In another study, the bs targeting of carcinoembryonic antigen and HER2 (human EGFR-2; ErbB2) with an anti-(carcinoembryonic antigen) × anti-HER2 bs mAb showed comparable or improved tumour uptake in cells positive for both receptors [12]. A recent study of an anti-HER2 × HER3 (human EGFR-3; ErbB3) bs scFv demonstrated successful tumour targeting and tumour growth inhibition [13]. Furthermore, targeting of HER2 and HER3 with the bs scFv exhibited significantly greater *in vivo* targeting of HER2/HER3-expressing tumours than did molecules with only one functional arm for targeting of HER2 or HER3 [13].

Several studies have suggested that simultaneous targeting of the epidermal-growth-factor receptors EGFR and HER2 could be valuable. Variable expression levels, but rather frequent expression, of both HER2 and EGFR has been reported in a number of studies in disseminated bladder [14,15], colorectal [16,17] and prostate cancer [18,19]. The prognostic value of the co-expression of EGFR and HER2 in breast-cancer patients has also been studied and a more severe prognosis for the co-expression than for expression of either EGFR or HER2 was demonstrated [20,21]. These data suggest that simultaneous targeting of EGFR and HER2 would be of value for molecular-imaging efforts and could improve the selectivity for targeted therapy.

Affibody molecules are affinity proteins that have been developed by combinatorial protein engineering of the 58-amino-acid residue Z domain scaffold, which is derived from staphylococcal Protein A [22]. Affibody molecules have shown potential in a wide variety of applications, e.g. as detection reagents [23–25], in bioseparation [26–28], for depletion in proteomics applications [29] and for tumour-targeting applications [30–34]. The small size (~6.5 kDa), generally high stability, efficient production in *Eschercihia coli* or by chemical synthesis and straightforward thiol-based site-specific coupling or labelling are favourable properties for affinity proteins [35]. The single protein chain also allows for straightforward construction of different fusion proteins. Affibody molecules have been genetically fused to other proteins, e.g. ABD (albumin-binding domain) [36] or Fc fragment [37] or to another identical affibody molecule to increase the functional affinity (avidity) [38–40]. bs targeting has been achieved with an affibody–ABD fusion molecule, to allow binding to both the target protein and human serum albumin in order to obtain increased *in vivo* half-life [36] or to Fc to take advantage of Fc functions. The small size of the affibody molecule could be an advantage in comparison with other affinity proteins for the generation of bs molecules. Obstacles in the development of bs mAbs have been: (i) the difficulty in producing material with enough quality and yield, e.g. undesired products might be formed because of undesired paring between the polypeptide chains [41], (ii) the high costs related to mammalian cell production, which sometimes is needed, since not all antibody derivatives give functional products upon *E. coli* expression, and (iii) the stability *in vivo* of bs proteins that are dependent on stable pairing of polypeptide chains [11]. Affibody molecules could hold a potential here because of their efficient production in *E. coli* (gram amounts per litre from bacterial cultures are not uncommon) and the straightforward genetic fusion of different affibody molecules to form single

polypeptide proteins. Affibody molecules have also shown high serum stability (F. Y. Frejd, unpublished work).

Affibody molecules targeting receptors within the receptor tyrosine kinase family have previously been successfully generated. A high-affinity HER2-specific affibody molecule, generated by phage-display selection, has been investigated in several preclinical studies [30,42–44] and also in a pilot clinical study [32]. An EGFR-binding affibody molecule has demonstrated high affinity and selectivity and good contrast in molecular imaging in xenografted mice models [31].

In the present paper we describe the generation of a HER2 × EGFR bs affibody molecule with potential for dual binding to the target proteins HER2 and EGFR. Binding was investigated to immobilized proteins by biosensor analysis and to HER2 and EGFR-overexpressing cells in flow-cytometric analysis. Simultaneous binding of both targets was evaluated by immunofluorescence microscopy using a microwell array. In addition, the ability of the bs affibody molecule to interact with both targets in their native form on the cell surface was also investigated using LigandTracer® (a semi-automated instrument for the analysis of protein–cell interactions manufactured by Ridgeview Instruments). The potential of a HER2 × EGFR bs affibody molecule as a tool for molecular imaging and therapy is discussed.

Materials and methods

DNA constructions

The gene encoding the bs affibody protein was constructed by cloning of vectors encoding the HER2- and EGFR-binding affibody proteins. The amber-suppressor *Escherichia coli* strain RR1ΔM15 [45] was used for subcloning procedures. Expression vectors pAY442 and pAY430, containing a T7 promoter [46], a DNA fragment encoding a His_6 (hexahistidine) tag and a multiple cloning site, together with a gene conferring resistance to kanamycin, were used. Plasmid pAY430 codes in addition for a unique C-terminal cysteine residue for direct labelling. The gene encoding the HER2-binding affibody molecule was recovered from a vector containing a $(Z_{HER2:342})_2$ construct previously described by Orlova et al. [30]. The vector was first restricted with endonucleases AccI and NotI, followed by ligation of a gene fragment (containing an SfiI restriction site) encoding the $(GGGGS)_3$ linker. The gene encoding the EGFR-binding affibody molecule was recovered from a vector containing a $Z_{EGFR:1907}$ construct previously described by Friedman et al. [31]. A gene fragment (containing an SfiI restriction site) encoding the $(GGGGS)_3$ linker was introduced to the vector by amplifying the $Z_{EGFR:1907}$ fragment with specific primers introducing the linker, followed by PstI/AccI and SfiI/AccI restriction and ligation. A second affibody gene construct was introduced head-to-tail, giving a vector encoding the $(Z_{EGFR:1907})_2$ with a N-terminal $(GGGGS)_3$ linker fragment. The two vectors encoding the dimeric HER2- and EGFR-binding entities were subsequently PstI/SfiI-restricted and the fragment encoding the HER2-binding affibody linker moiety was ligated into the $(Z_{EGFR:1907})_2$-containing vector. The ligation resulted in expression vectors encoding, under the control of the T7 promoter, two versions of the bs affibody molecule fused to an N-terminal His_6 tag, and differing only in that the latter vector encodes a variant with a C-terminal cysteine residue.

Protein expression and purification

The HER2 × EGFR bs affibody molecule was expressed as a His_6-tagged protein from pAY442 and pAY430 plasmid in *E. coli* strain BL21(DE3) (Novagen). A preculture of cells was inoculated in fresh TSB + YE medium (30 g/l Tryptic Soy Broth supplemented with 5 g/l yeast extract) containing 50 mg/l kanamycin and grown in shake flasks at 37 °C until an attenuance (D_{600}) of ~ 0.8 was reached, at which time protein expression was induced by addition of isopropyl L-thio-β-D-galactopyranoside (Apollo Scientific Ltd) to a final concentration of 1 mM. After 4 h culturing at 37 °C, cells were harvested by centrifugation at 6000 ***g*** for 10 min at 4 °C. The cell pellet was frozen overnight, thawed, then resuspended in denaturing buffer (7 M urea, 100 mM NaH_2PO_4 and 10 mM Tris/HCl, pH 8.0) containing 100 units/ml benzonase (a promiscuous endonuclease). After incubation at 10 °C for 30 min, the cells were disrupted by sonication (Sonics VibraCell, Sonics & Materials, Inc.; pulse 5.0 s/5.0 s for 2 min, 50 % amplitude). After centrifugation at 16 000 ***g*** for 20 min at 4 °C, the supernatant was passed through 0.45-μm-pore-size filter and the denatured protein was loaded on to a His GraviTrap column (GE 11-0033-99; GE Healthcare) and washed with binding buffer (20 mM sodium phosphate, 0.5 M NaCl and 20 mM imidazole, pH 7.4), followed by washing buffer (20 mM sodium phosphate, 0.5 M NaCl and 60 mM imidazole, pH 7.4). The bound protein was eluted with imidazole buffer (20 mM sodium phosphate, 0.5 M NaCl and 500 mM imidazole, pH 7.4) and then applied to a PD-10 column (GE Healthcare) equilibrated with PBS (pH 7.4), and eluted with PBS. The proteins were denoted $(Z_{HER2:342})_2$–$(G_4S)_3$ [$(Gly_4\text{-}Ser)_3$]–$(Z_{EGFR:1907})_2$ and $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$–Cys. Protein concentrations were estimated from A_{280} using the appropriate absorption coefficient for each protein. To confirm the purity and correct molecular mass of the proteins, they were analysed by SDS/PAGE (NuPAGE 4–12 % BisTris gel; Invitrogen) and stained with SimplyBlue™ Safe Stain (Invitrogen).

Biosensor analysis

A Biacore® 2000 instrument (GE Healthcare) was used for real-time biospecific interaction analysis of the bs $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$ affibody molecule and its interaction with

the target proteins HER2 and EGFR. hHER2-Fc (HER2 Fc chimaera), hEGFR (human EGFR-1) and hEGFR-Fc (hEGFR Fc chimaera) (diluted in 10 mM sodium acetate, pH 4.5; R&D Systems) were immobilized (~2700 response units) by amine coupling on the carboxylated dextran layer of three flow-cell surfaces of a CM5 sensor chip (GE Healthcare), according to the manufacturer's instructions. Another flow-cell surface was activated and de-activated as a reference surface. The bs affibody molecule was diluted in a running buffer, HBS (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA and 0.005% surfactant P20, pH 7.4), before binding analysis was performed at 25°C. In a first experiment, the bs $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$ affibody molecule was injected at concentrations ranging from 125 nM to 2 μM over all surfaces with a flow rate of 30 μl/min. Dimeric EGFR-binding molecule $(Z_{EGFR:1907})_2$ [31] and HER2-binding molecule $(Z_{HER2:342})_2$ [30] were also injected as controls. After each injection the flow cells were regenerated by the injection of 10 ml of 10 mM HCl.

In a second experiment, 5 μM $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$ affibody protein was injected over all flow-cell surfaces at a flow rate of 30 μl/min. Following a short dissociation time of 1 min, 115 nM hHER2-Fc or 100 nM hEGFR-Fc respectively (diluted in the running buffer) was injected. After each injection the flow cells were regenerated by the injection of 10 ml of 10 mM HCl.

Fluorophore labelling

The $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$-Cys affibody molecule was labelled directly at the unique C-terminal cysteine residue with Alexa Fluor® 488 (Invitrogen). Approx. 1 mg of affibody molecule in 0.5 ml of PBS, pH 8, was reduced with 20 mM DTT (dithiothreitol) for 30 min, at 40°C and gentle shaking. Excess of DTT was removed on a NAP-5 size-exclusion column (GE Healthcare) equilibrated with ammonium acetate (5 mM, pH 5.5). A 10 mM solution of Alexa Fluor® 488 was added to a 5-fold excess and the protein was incubated for 2 h at 40°C in the dark with gentle shaking. Extensive dialysis was performed to remove unreacted fluorophore. The protein concentration was estimated by SDS/PAGE (NuPAGE gel) and preserved binding performance was verified by Biacore® biosensor analysis.

Cell culture

The EGFR-rich human squamous carcinoma cell line A-431 [DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen)] was grown at 37°C in a humidified 5% CO_2 environment in Dulbecco's-modified Eagle's medium supplemented with 10% (v/v) FBS (fetal bovine serum) and 1% antibiotic/antimycotic solution (all from Invitrogen). The HER2-expressing human ovary adenocarcinoma cell line SK-OV-3 [ECACC (European Collection of Cell Cultures)] was cultured in McCoy's 5a medium (Invitrogen) supplemented with serum and antibiotic as described above. For real-time cell–cell interaction analysis, the HER2-expressing human adenocarcinoma cell line SKBR-3 (A.T.C.C.) was cultured in RPMI medium and the A-431 cell line (A.T.C.C.) was cultured in Ham's F10 medium supplemented with 10% FBS, glutamine (2 mM), streptomycin (100 μg/ml) and penicillin (100 IU/ml) (all from Biochrom).

Microwell array for imaging

The microwell array used for cell analysis in immunofluorescence microscopy has the outer format of a standard microscopic glass slide (26 mm × 76 mm) holding 672 microwells [47]. It consists of a microwell-etched silicon grid (500 μm thickness) that has been anodically bonded to a glass slide (500 μm thickness), resulting in a total thickness of 1 mm. Each well has the bottom area of 650 μm × 650 μm and holds a volume of 0.5 μl. A thin gas-permeable poly(dimethylsiloxane) membrane was used to seal the wells in cell culturing, thereby preventing evaporation and contamination. Membrane fabrication is described in detail elsewhere [47].

Flow-cytometric analysis

Binding of the Alexa Fluor® 488-labelled bs affibody molecule to A-431 cells and SK-OV-3 cells was analysed using a FACS Vantage SE (BD Biosciences) flow cytometer. Subconfluent A-431 cells and SK-OV-3 cells were detached with a trypsin/0.53 mM EDTA solution (Invitrogen), centrifuged (200 ***g*** for 3 min) and resuspended in ice-cold PBSB (PBS supplemented with 1% BSA). Single cell suspensions were prepared whereby 150 000 cells/well were added to a V-shaped 96-well microtitre plate (Greiner Bio-one). The A-431 and SK-OV-3 cells were incubated for 1 h at 4°C with gentle shaking with one of the following: (i) PBSB; (ii) a more-than-1000-fold molar excess of dimeric EGFR-binding affibody molecule $(Z_{EGFR:1907})_2$; or (iii) a more-than-1000-fold molar excess of HER2-binding affibody molecule, $(Z_{HER2:342})_2$ (molar excesses are relative to the cell receptor number), followed by the addition of $(Z_{HER2:342})_2$–$(G_4S)_3$-$(Z_{EGFR:1907})_2$–Alexa Fluor® 488 affibody molecule to a final concentration of 2 μg/ml and incubated for 1 h at 4°C in the dark with gentle shaking. All dilutions were made in PBSB and the incubations were performed in a total volume of 150 μl. Subsequently, cells were washed with 150 μl of ice-cold PBSB, followed by resuspension in 300 μl of PBSB and kept in the dark on ice until flow-cytometric analysis. As controls, the anti-EGFR mAb, Ab30 (2 μg/ml; Abcam), and the anti-HER2 mAb, Trastuzumab (2 μg/ml; Roche), were used with the secondary antibodies [goat-anti-mouse–Alexa Fluor 647 and goat-anti-human-Alexa Fluor® 488 (5 μg/ml; from Molecular Probes and Invitrogen) respectively)].

Immunofluorescence microscopy

Immunofluorescence microscopy was used to analyse the binding of the bs affibody molecule to A-431 and SK-OV-3 cells in two separate experiments. In a first experiment, specific cell binding of the bs $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$–Alexa Fluor® 488 affibody molecule to SK-OV-3 cells was studied with a laser scanning confocal microscope (LSM 510 META; Carl Zeiss) using a 40 ×/0.8 NA (numerical aperture) objective. The fluorescent dye Alexa Fluor® 488 was detected using 488 nm excitation and a 505 nm longpass filter. At 2 days before the experiment, 50 000 SK-OV-3 cells were seeded in 35 mm-diameter cell-culture dishes (BD Falcon) and incubated at 37 °C in 5% CO_2. At the time for analysis, the cells were washed with 1 ml of PBSB before incubation with one of the following: (i) PBSB; (ii) an approx. 1000-fold molar excess of the two dimeric affibody molecules $(Z_{EGFR:1907})_2$ (50 nM) and $(Z_{HER2:342})_2$ (165 nM); (iii) an approx. 1000-fold molar excess of the $(Z_{HER2:342})_2$ (165 nM); and (iv) an approx. 1000-fold molar excess of the dimeric affibody molecule $(Z_{EGFR:1907})_2$ (50 nM) (molar excesses are relative to the cell receptor number) for 20 min at 20 °C. Subsequently, the $(Z_{HER2:342})_2$–$(G_4S)_3$-$(Z_{EGFR:1907})_2$–Alexa Fluor® 488 affibody molecule was added to a final concentration of 1 μg/ml and incubated for 10 min at 20 °C in the dark. The cells were washed once with 1 ml of PBSB and kept in 5 ml of PBSB during analysis.

In a second experiment, the simultaneous binding of the bs affibody molecule to the native receptor on the cell and to soluble target protein was studied using a laser scanning confocal microscope (LSM 5 PASCAL; Carl Zeiss) equipped with a 20 × -magnification 0.5-numerical-aperture objective. The fluorescent dyes Alexa Fluor® 488 and Nile Red were detected using 488 and 543 nm excitation respectively, together with a 505–530 nm bandpass filter and a 560 nm longpass filter. Microwell arrays were seeded with A-431 cells at a cell concentration of 200 cells/microwell, and a gas-permeable top membrane was used to seal the wells, followed by incubation overnight at 37 °C in 5% CO_2. Non-adherent cells were removed by washing with McCoy + 4% FBS (McCoy's medium supplemented with 4% FBS). Washing was performed by dipping the microwell arrays in 10 ml of washing buffer. Absorption on tissues (Precision Wipes; Kimtech Science) was used to empty the microwells, and 30 areas (of five wells each) per microwell array were incubated with the $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$ affibody molecule (660 nM), together with the soluble target protein HER2-ECD (extracellular domain) [48] (660 nM), in McCoy + 4% FBS in a total volume of 5 μl/five wells for 30 min at 20 °C. As a negative control, an irrelevant affibody molecule $(Z_{taq})_2$ was used in a concentration of 660 nM. The microwell arrays were washed twice with McCoy + 4% FBS, fixed with ice-cold 4% paraformaldehyde (Sigma–Aldrich) for 15 min at 20 °C, washed once with PBS and incubated with 2 μg/ml Trastuzumab in PBSB for 20 min at 20 °C. The arrays were then washed twice with PBSB, followed by incubation with the secondary goat anti-human–Alexa Fluor® 488 antibody (5 μg/ml; 5 μl/five wells; 20 min at 20 °C in the dark). Two rounds of washing were performed before the addition of 1 ml of the lipophilic stain Nile Red (0.1 μM; Molecular Probes) to the entire microwell arrays. Mounting of the microwell arrays in PBSB with a coverglass was followed by immunofluorescence analysis.

Real-time cell–cell interaction analysis

A-431 cells were cultured with 80 kBq of [^{14}C]thymidine (PerkinElmer) for three cell cycles. Approx. 10^6 ^{14}C-A-431 cells in suspension were incubated with 16.6 nM $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$ for 45 min in 4 ml of cell culture medium. To remove unbound bs affibody molecules, the cell suspension was centrifuged (175 ***g***) and washed once, and the pellet was resuspended in 1 ml of cell culture medium. SKBR-3 cells were seeded on a local part of a 100 mm cell culture dish by tilting the dish during cultivation as described previously [49] and cultured until the cell number was approx. 10^6. The part of the cell culture dish where no cells were cultured was used as a reference surface in the analysis. The ^{14}C-A-431 cell suspension was added to the SKBR-3 cell dish and real-time cell–cell interaction was monitored in 20 °C with LigandTracer® White by measurement of ^{14}C. As a negative control ^{14}C-A-431 without pre-incubation with bs $Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$ affibody molecule was used.

Results

Generation of a bs affibody molecule

A gene construct encoding a bs affibody molecule, targeting both HER2 and EGFR, was constructed by introducing a 20-amino-acid $(G_4S)_3$ linker between the dimeric versions of HER2- and EGFR-binding affibody molecules, resulting in the $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$ affibody molecule (Figures 1A and 1B). The $(Z_{HER2:342})_2$–$(G_4S)_3$–$(Z_{EGFR:1907})_2$ affibody molecule is hereafter referred to as 'bs affibody molecule'. Dimeric affibody molecules were used in order to allow for additional flexibility in the potential dual binding of HER2 and EGFR receptors on cells. The His_6-tagged bs affibody molecule, also available in a version having a C-terminal cysteine residue suitable for directed labelling, was produced in *E. coli* and the IMAC (immobilized-metal-ion affinity chromatography)-purified fusion protein was observed as a single band of expected molecular size (~30 kDa) on SDS/PAGE, indicating a pure and stable protein (Figure 1C).

Single and dual binding biosensor analysis

In order to study whether the bs affibody molecule was capable of binding to the target proteins HER2 and EGFR,

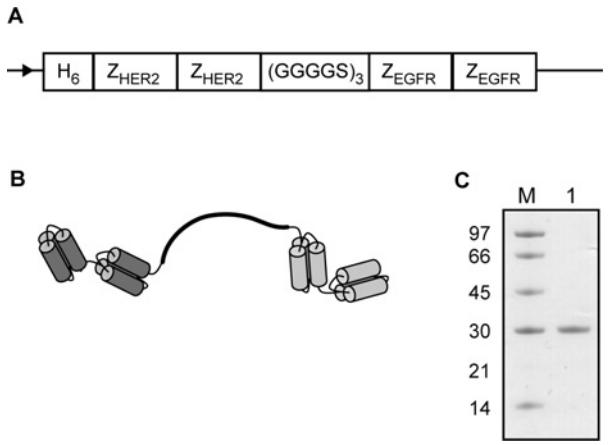

Figure 1 Schematic representation of the bs affibody construct and SDS/PAGE of the purified bs affibody protein

(**A**) The N-terminal His_6 tag and the $(G_4S)_3$ linker are indicated. (**B**) Schematic illustration of the three-dimensional structure of the bs affibody molecule in which each affibody moiety consists of a triple α-helical bundle. (**C**) Analysis of the expressed and purified bs affibody protein by SDS/PAGE under reducing conditions. Lane M, molecular-mass markers (values on the left are in kDa); lane 2, HER2 × EGFR bs affibody. The gel was stained with SimplyBlue™ Safe Stain.

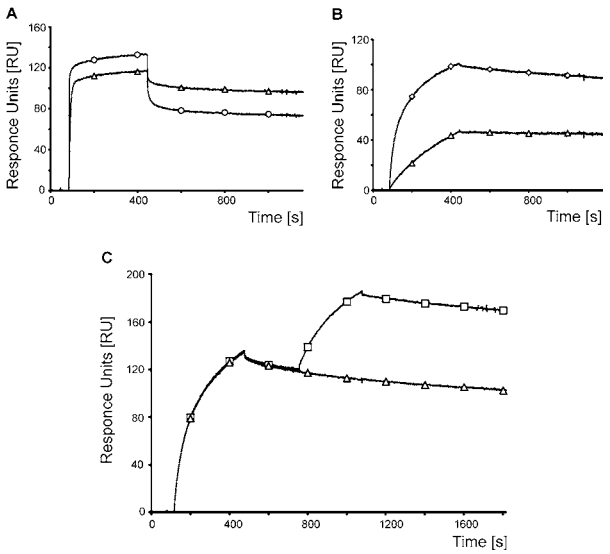

Figure 2 Biacore® biosensor analysis

Binding of the bs affibody molecule (△) was compared with the binding of the parental dimeric proteins; (**A**) $(Z_{HER2:342})_2$ (○) on the HER2 surface and (**B**) $(Z_{EGFR:1907})_2$ (◇) on the EGFR surface. (**C**) Sensorgram of the bs affibody molecule injected over an EGFR-immobilized surface at a concentration of 5 μM was followed by the injection of 115 nM soluble hHER2-Fc (□) to visualize subsequent binding to both target proteins. As a control, a sensorgram of bs affibody molecule (△) at a concentration of 5 μM is shown.

a real-time biospecific interaction analysis using a Biacore® instrument was performed. The bs affibody molecule was injected at different concentrations over the flow-cell surfaces of a sensor chip containing immobilized HER2 and EGFR respectively. The affinities (equilibrium dissociation constant, K_D) of the monomeric affibody molecules were previously determined to be 22 pM for $Z_{HER2:342}$ [30] and 5.4 nM for $Z_{EGFR:1907}$ [31]. The dimer format of each affibody molecule in the bs affibody protein will most likely give an additional increase in functional affinity (avidity), as demonstrated previously [38,39]. Furthermore, high selectivity of the $Z_{HER2:342}$ and $Z_{EGFR:1907}$ affibody molecules to their respective target protein has been shown in previous studies [30,39]. In the present study, binding to HER2 and EGFR was confirmed for the bs affibody protein (Figure 2). When comparing the binding of the bs affibody molecule to the binding of the parental dimeric $(Z_{HER2:342})_2$ and $(Z_{EGFR:1907})_2$ proteins used as controls (Figures 2A and 2B), the bs affibody protein showed a similar binding profile as the $(Z_{HER2:342})_2$ when injected over the HER2 surface (Figure 2A). A retained high association rate and a low dissociation rate was demonstrated, in accordance with previous data for $Z_{HER2:342}$ [30]. The decrease in binding to HER2 during the first seconds of the dissociation phase is likely to be due to differences in the buffer, but it does not influence the low dissociation rate. When injecting the bs affibody protein over the EGFR-immobilized surface, a lower association rate and reduced binding capacity was shown as compared with $(Z_{EGFR:1907})_2$ (Figure 2B). The low dissociation rate was, however, similar for both the bs affibody protein and $(Z_{EGFR:1907})_2$ (Figure 2B).

To determine whether the bs affibody molecule could bind HER2 and EGFR simultaneously, the bs affibody protein was injected over an EGFR-immobilized flow-cell surface, followed by injection of soluble HER2 protein (Figure 2C). The injection of soluble HER2 was performed with a short delay in order to facilitate the read-out of the two binding events. Owing to the low dissociation rate of the bs affibody protein, the delay in injection did not significantly affect the binding of soluble HER2 protein to the bs affibody molecule. The injection of the bs affibody molecule over the EGFR-immobilized surface followed by injection of soluble HER2 showed successful simultaneous binding of both moieties of the bs affibody molecule to their respective targets (Figure 2C). As a control, soluble EGFR protein was injected after the bs affibody protein and, as expected, no second binding event could be seen (results not shown). In the reverse set-up, the bs affibody molecule was injected over a HER2-immobilized flow-cell surface followed by injection of soluble EGFR. Binding of the bs affibody molecule to immobilized HER2 could be seen, but, surprisingly, no binding to soluble EGFR was detected (results not shown). The reason for this lack of EGFR binding is not evident, but was further investigated in cellular assays described below.

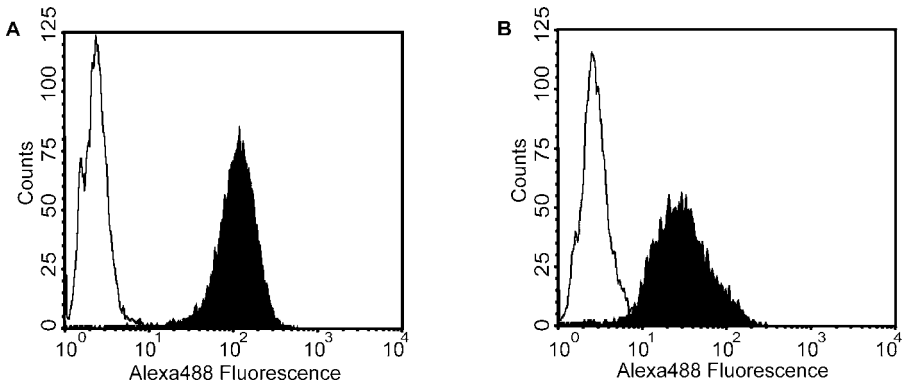

Figure 3 Flow-cytometric analysis of the binding of fluorescently labelled bs affibody molecule to (A) A-431 and (B) SK-OV-3 cells

Graphs show cells only (white area) and the binding of bs affibody molecule to EGFR or HER2 on cells (black area). The bs affibody molecule was labelled directly on a unique C-terminal cysteine residue with Alexa Fluor® 488.

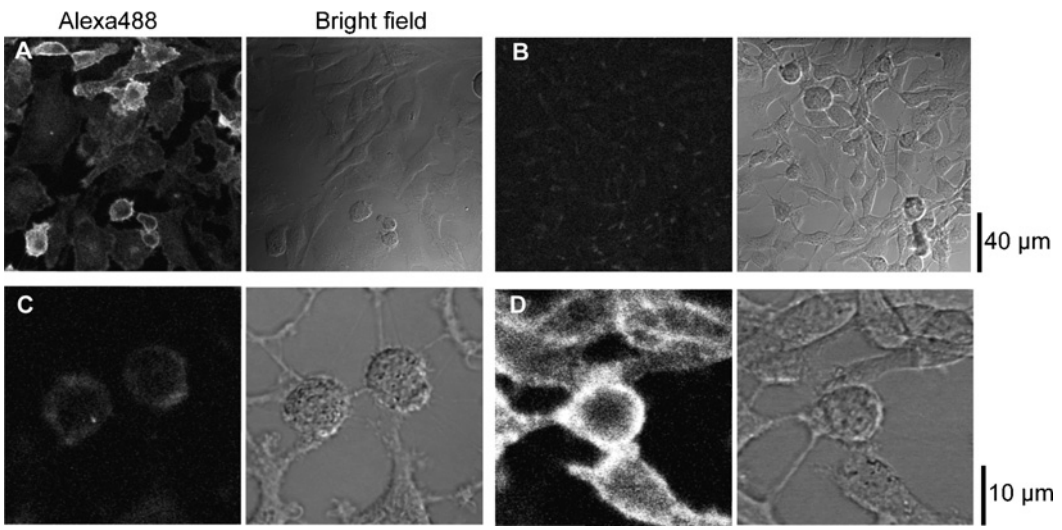

Figure 4 Cell binding and selectivity of the bs affibody molecule

SK-OV-3 cells were exposed to fluorescently labelled bs affibody molecule and visualized by immunofluorescence microscopy (left panels) and bright field images (right panels). (**A**) The Alexa Fluor® 488-labelled bs affibody molecule incubated with SK-OV-3 cells. Thereafter, in three separate blocking experiments (**B**–**D**), the Alexa Fluor® 488-labelled bs affibody molecule was incubated with SK-OV-3 cells after (**B**) pre-incubation with an excess of both $(Z_{EGFR:1907})_2$ and $(Z_{HER2:342})_2$, (**C**) pre-incubation with an excess of $(Z_{HER2:342})_2$ and (**D**) pre-incubation with an excess of $(Z_{EGFR:1907})_2$.

Analysis of the binding and selectivity of the bs affibody molecule to cells expressing EGFR and HER2

The bs affibody molecule was subjected to flow-cytometric analysis in order to confirm binding and selectivity to native HER2 and EGFR on human cell lines with high HER2 and EGFR expression. Expression of HER2 and EGFR was confirmed using commercial anti-EGFR and anti-HER2 mAbs (results not shown). Before flow-cytometric analysis, the bs affibody molecule was fluorescently labelled with Alexa Fluor® 488 utilizing the unique C-terminal cysteine residue. In the first experiment, binding of the bs affibody molecule to EGFR-expressing A-431 cells (Figure 3A) and to HER2-expressing SK-OV-3 cells (Figure 3B) was demonstrated. In the second experiment, flow cytometry was used to confirm selectivity in the binding of the bs affibody molecule to native HER2 and EGFR on cells by incubation of SK-OV-3 and A-431 cells with an excess of either $(Z_{HER2:342})_2$ or $(Z_{EGFR:1907})_2$, followed by incubation with the bs affibody molecule. In accordance with the high receptor expression of HER2 on SK-OV-3 and high receptor expression of EGFR on A-431 cells, an excess of $(Z_{EGFR:1907})_2$ resulted in an efficient inhibition of binding of the bs affibody molecule to A-431 cells, but no apparent decrease in the binding to SK-OV-3 cells (results not shown). The opposite result was seen with an excess of $(Z_{HER2:342})_2$, where a decrease in of bs affibody molecule binding to SK-OV-3 cells was observed, but, again, no noticeable decrease in binding

to A-431 cells (results not shown). Results from flow-cytometric analysis confirmed the selective binding of the bs affibody molecule to the native receptors on both cell lines.

In a third experiment, immunofluorescence microscopy was used to investigate the binding and selectivity of the bs affibody molecule to SK-OV-3 cells in more detail. A similar blocking approach as for the previous experiment was utilized. Fluorescently labelled bs affibody showed significant binding to SK-OV-3 cells (Figure 4A). The binding of fluorescently labelled bs affibody protein could be completely blocked by the pre-incubation of an excess of non-labelled $(Z_{EGFR:1907})_2$ and $(Z_{HER2:342})_2$ (Figure 4B). Furthermore, when the SK-OV-3 cells were pre-incubated with an excess of only non-labelled $(Z_{HER2:342})_2$ prior to incubation with the fluorescently labelled bs affibody, the fluorescence was reduced to background levels (Figure 4C), whereas the same excess of $(Z_{EGFR:1907})_2$ could not inhibit the fluorescence signals (Figure 4D). Results from immunofluorescence microscopy demonstrate specific binding of the bs affibody molecule to the HER2 receptor on the SK-OV-3 cells. Taken together, these results confirm the selective binding of the bs affibody molecule to the native receptors on both cell lines, visualized in detail for SK-OV-3 cells.

Dual binding of bs affibody molecule using microwell immunofluorescence imaging

The next step was to confirm that the bs affibody molecule was capable of binding both target molecules simultaneously in a cellular assay as a continuation of the biosensor analysis data described above. An experiment was set up to study whether a cell-bound bs affibody could simultaneously bind the second target protein in solution. Owing to the high reagent cost of soluble target proteins, a microwell array (holding 0.5 μl/well and 672 wells) was used (Figure 5A) [47]. The microwell array, conveniently treated as a standard microscopic glass slide in terms of washing and staining, was found to be a suitable tool for parallel imaging on low-volume samples. The binding events were analysed using immunofluorescence microscopy (the detection set-up is schematically shown in Figure 5B). EGFR-expressing A-431 cells, cultured on the glass bottom of the microwells, were incubated with bs affibody protein and soluble HER2–ECD target protein and subsequently detected with an anti-HER2 antibody and fluorophore-conjugated secondary antibody. Successful simultaneous binding of the bs affibody molecule to A-431 cells and soluble target protein could be demonstrated (Figure 5C). No binding was observed for the negative control affibody molecule $(Z_{taq})_2$ (Figure 5D). In agreement with the biosensor analysis data, the reverse set-up, with adherent HER2-expressing SK-OV-3 cells in the microwell followed by incubation with bs affibody molecule and soluble EGFR protein, did not result in simultaneous binding (result not shown). This unexpected lack of detectable binding to adherent HER2-expressing cells and soluble EGFR was thus further investigated in a cell-to-cell interaction study that is described below.

Dual cell–cell binding of bs affibody molecule using real-time cell–cell interaction analysis

We next evaluated whether the bs affibody molecule was capable of simultaneous binding of the two target proteins on two different cell lines. Simultaneous binding of the bs affibody molecule to both HER2-overexpressing SKBR-3 cells and to EGFR-overexpressing A-431 cells was investigated in a real-time cell–cell interaction analysis using a LigandTracer® White device and ^{14}C-labelled cells. bs affibody molecule was pre-incubated with ^{14}C-A-431 cells and added to adherent SKBR-3 cells, demonstrating simultaneous binding of the bs affibody molecule to both cell types (Figure 6). The binding of ^{14}C-A-431 cells incubated with the bs affibody molecule was approx. 3-fold higher to the SKBR-3 cell area compared to the reference area and was seen as early as 2 h. As expected, no binding was seen in the negative control (i.e. pre-incubation without the bs affibody molecule) and low binding similar to that seen in the reference area was detected (Figure 6). In accordance with experiments described above, the reverse set-up, where a cell suspension of pre-incubated bs affibody molecule and ^{14}C-SKBR-3 cells were incubated with adherent A-431 cells, did not show any cell–cell interactions (results not shown). To summarize, this cell–cell binding assay demonstrated dual binding of the bs affibody molecule to native receptors. This particular bs affibody molecule can therefore be proposed as a potential molecule for dual targeting, and future studies on different biological effects will be motivated by these findings.

Discussion

The receptor tyrosine kinases HER2 and EGFR are overexpressed in various malignancies and are associated with a poor patient prognosis. They are therefore interesting targets for cancer imaging and therapy and have been extensively studied. Approved therapeutic strategies include blocking of ligand binding or blocking of receptor dimerization. One possible application for bs molecules would be to bind surface receptors on tumour cells in such a way that active dimerization, and thus cell signalling, is sterically inhibited. bs molecules could also be pictured as binding two tumour-associated receptors on the same cell to increase the selectivity by preferably directing the bs molecule to cells expressing both receptors instead of binding to cells expressing only one of these receptors.

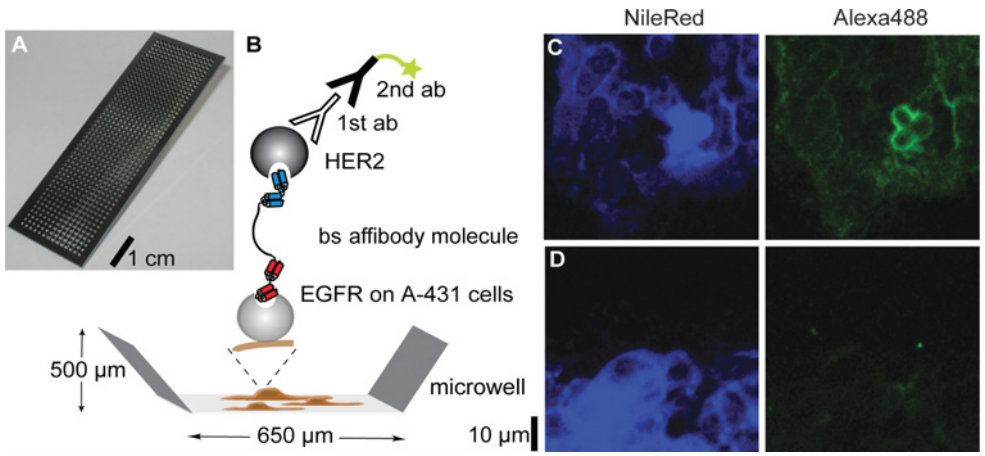

Figure 5 Dual binding of bs affibody molecule

A-431 cells were exposed to bs affibody molecule and soluble HER2 in a microwell array and visualized by immunofluorescent microscopy. For detection, the anti-HER2 antibody (Trastuzumab) and Alexa Fluor® 488-conjugated goat anti-human antibody ('Alexa488'; right column, green) was used together with the cell lipophilic stain Nile Red ('NileRed'; left column, blue). (**A**) Microwell array with 672 wells, holding 0.5 μl/well. (**B**) Schematic illustration of the experimental set-up, showing the dimeric binding entities of the bs affibody molecule in red and blue. Adherent A-431 cells incubated with (**C**) bs affibody molecule and soluble HER2 or (**D**) an irrelevant affibody molecule, $(Z_{Taq})_2$, and soluble HER2. 1st ab, first antibody; 2nd ab, second antibody.

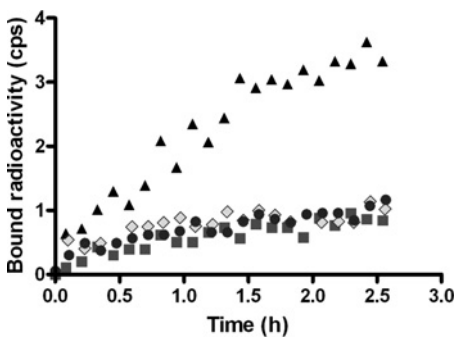

Figure 6 Real-time cell-cell interaction analysis

The binding of ^{14}C-labelled A-431 cells to SKBR-3 cells was monitored in real-time using a LigandTracer® White instrument. The ^{14}C-A-431 cell suspension was pre-incubated with bs affibody molecule. The ^{14}C-A-431 cell suspension was added to the SKBR-3 cell dish and the uptake of ^{14}C to the cell area (▲) and the reference area (■) was measured. ^{14}C-A-431 cells without bs affibody molecule were used as negative controls: ◇, cell area; ●, reference area. cps, counts/s.

In a first effort to generate an affibody molecule capable of binding two tumour-associated receptors, a HER2 × EGFR bs affibody molecule (bs affibody molecule) was constructed by genetic fusion of a dimeric HER2-binding affibody molecule [30] to a dimeric EGFR-binding affibody molecule [31] via a 20-amino-acid $(G_4S)_3$ linker. The bs affibody molecule demonstrated binding both to HER2 and EGFR in biosensor analysis (Figures 2A and 2B). In both cases the low dissociation rate, previously reported for the $Z_{HER2:342}$ and $Z_{EGFR:1907}$, was retained. The somewhat decreased association rate for the bs affibody molecule compared with the $(Z_{EGFR:1907})_2$ when binding to the EGFR-immobilized surface could potentially be explained by steric interference of the linker and HER2-binding moiety, making the EGFR-binding moiety less accessible for binding.

Dual binding of HER2 and EGFR was demonstrated *in vitro* using biosensor analysis, and the bs affibody molecule was found capable of simultaneously binding immobilized EGFR and soluble HER2 protein (Figure 2C). Surprisingly, the reverse set-up of the target proteins could not reproduce dual binding in the biosensor analysis; in this case the bs affibody molecule bound immobilized HER2 as expected, but did not bind soluble EGFR. The lack of dual binding in this case might be due to the EGFR-binding moiety of the bs affibody molecule being less accessible because of a steric interference when the HER2-binding moiety had already been bound to HER2. This was also demonstrated by: (i) immunofluorescence microscopy, which showed that native EGFR on A-431 cells and soluble HER2 protein were bound in a similar manner (Figure 5); and (ii) real-time cell–cell interaction analysis, which showed that the receptors HER2 and EGFR on SKBR-3 and A-431 cells were bound (Figure 6). In both cases, the cell–cell interaction analysis showed simultaneous binding only when the EGFR-binding moiety of the bs affibody molecule was allowed to bind native EGFR on A-431 cells as a first event. In the immunofluorescence analysis the bs affibody molecule and the soluble target protein were added simultaneously to the adherent cells in the microwells. The more-than-100-fold better affinity and higher association rate of the HER2-binding affibody molecule as compared with the EGFR-binding affibody molecule will most likely allow for the binding of the HER2-binding moiety (of the bs affibody molecule) to take place first. When comparing the two set-ups in the immunofluorescence microwell experiment,

simultaneous binding was shown for bs affibody molecule to adherent A-431 cells and soluble HER2 protein, whereas no simultaneous binding was seen for bs affibody molecule to adherent SK-OV-3 cells and soluble EGFR protein. The different binding results are not fully understood, but it seems that the binding of the bs affibody molecule to soluble HER2 will not affect the already existing interaction with native EGFR on cells, but the binding of native HER2 on cells will affect the binding ability of soluble EGFR. The non-accessible EGFR-binding epitope could be due to steric interference or rigidity after binding to HER2 on cells.

In future work with bs affibody molecules it would be interesting to reverse the orientation of the HER2- and EGFR-binding moieties to evaluate whether the decrease in binding performance of the EGFR-binding moiety is because of its C-terminal positioning. Future studies could include different affinities of the affibody binders and the evaluation of different linker lengths to find the optimal bs affibody molecule for HER2 and EGFR targeting. Methods for studying dual binding could include: (i) FRAP (fluorescent recovery after photobleaching), which measures the movement of proteins in the membrane as a correlation of their size; (ii) flow-cytometric analysis to study improvements in dissociation rate; and (iii) cell proliferation (and also *in vivo* tumour growth) as an effect of the bs affibody molecule.

In the present study the successful binding of a bs affibody molecule to two receptors on separate cells could successfully be demonstrated with radioisotope-mediated cell–cell interaction analysis using a LigandTracer® device. Such targeting of two cells with a bs affibody molecule could, for example, be used in the recruitment of cytotoxic T-cells to enhance the killing of tumour cells by genetically fusing, for example, a CD3-binding affibody molecule to a HER2- or EGFR-binding affibody molecule. This strategy has been studied with other bs molecules [3,4,8]. The bs affibody molecule should also have the potential to target two tumour-associated target proteins on the same cell. For certain tumour types, variable expression levels, but rather frequent expression, of both HER2 and EGFR have been reported [14–19], and bs targeting could thus be a valuable strategy to increase the efficiency in cancer imaging or therapy.

The present results show that a bs affibody molecule targeting HER2 and EGFR could be generated with dual HER2 and EGFR binding on cells. We anticipate that such bs affinity proteins for dual targeting of any two target proteins will be increasingly important in future efforts towards molecular imaging and cancer therapy.

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