Evaluating the therapeutic potential of a dimeric HER3-binding affibody construct in comparison with a monoclonal antibody, seribantumab

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ABSTRACT A number of monoclonal antibodies targeting HER3 are currently under clinical investigation as potential cancer therapeutics. We have earlier generated high affinity (low picomolar) affibody molecules targeting HER3. These are small, 58 amino acid, non-immunoglobulin based scaffold proteins that have proved suitable for tumor targeting applications, previously primarily for molecular imaging purposes. Our high affinity HER3-binding affibody molecule has demonstrated to have anti-proliferative capacity on HER3-positive tumor cells. When formatted as a bivalent construct, in which the two affibody moieties are flanking a small albumin-binding domain (ABD), we have recently demonstrated that tumor growth could be delayed in mice for HER3-positive xenografts. In this study, we have modified the construct further and reduced the size. In a comparative study, we evaluated safety, the capacity to delay tumor growth in mice with BxPC-3 xenografts, and mouse survival. Our novel construct was compared to the HER3-specific monoclonal antibody seribantumab (MM-121), presently in clinical development. They were found to be equally potent in their therapeutic effects and in their safety profile. We conclude that this format of bivalent HER3-binding affibody molecules seems promising for further evaluation as candidate therapeutics for treatment of HER3-overexpressing tumors.

Introduction

The use of monoclonal antibodies or their derivatives to improve survival of patients with disseminated cancer is today established. Several mechanisms of action are utilized, including delivery of cytotoxic payloads (e.g. toxins, drugs or radionuclides) and recruitment of the immune system. An important type of mechanism of action is based on prevention of mitogenic signaling through critical transmembrane receptors. Currently, a major group of cancer-associated antibody targets belong to the tyrosine kinase receptor family 1 (the human epidermal growth factor receptor (HER) family). Clinically successful antibodies, such as cetuximab, panitumumab, trastuzumab, ado-trastuzumab emtansine and pertuzumab target EGFR or HER2, i.e. members of the HER family. Recently, the importance of another member of this family, HER3, as a cancer-associated target has also been recognized [1,2]. Although the tyrosine kinase of HER3 is not fully functional, heterodimers of HER3 with EGFR and especially HER2 provide extremely potent mitogenic signaling [3]. Furthermore, there are accumulated evidences that HER3 expression and its signaling is associated with resistance to anti-cancer therapy [4].

Discoveries in tumor biology have been translated into development of therapeutic anti-HER3 antibodies. Follow-

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Despite clinical success, the size, solubility and stability limitations of monoclonal antibodies have urged researchers to search for complementing strategies. Binding proteins based on small non-immunoglobulin scaffolds are considered as a valuable alternative or complement to antibodies for imaging and therapy of cancer [13].

Affibody molecules are small (6.5 kDa) engineered scaffold proteins [14], which can be selected for high affinity to cancer-associated targets. They possess excellent properties for tumor targeting [15]. First clinical studies with HER2-targeting affibody molecules demonstrated that these proteins are non-toxic, non-immunogenic and capable of target-specific accumulation in tumors [16, 17]. We have earlier reported the selection of anti-HER3 affibody molecules [18] and further affinity maturation to low picomolar affinity [19]. It was demonstrated that the anti-HER3 affibody molecules inhibited heregulin-promoted cancer cell growth in vitro by suppressing heregulin-induced HER3 phosphorylation [20]. In the development of a therapeutic agent, fast renal excretion of the affibody molecule should be prevented. This is a common limitation of scaffold proteins for therapeutic application.
We have earlier demonstrated that a fusion with an albumin binding domain (ABD) prolonged residence of an anti-HER2 affibody molecule in blood [21]. ABD is a scaffold protein (46 amino acid residues, MW = 5.2 kDa) that has been engineered to bind human serum albumin with femtomolar affinity [22,23]. A multimeric construct consisting of two HER3-binding affibody molecules flanking an ABD was previously designed and validated in vitro [24]. Recently, we evaluated the biodistribution and in vivo targeting properties of ZHER3:08699-(S-G)4-ABD035-(S-G)4-ZHER3:08699 (designated 3A3) [25]. It was shown that the growth of a HER3-expressing BxPC-3 pancreatic cell line was inhibited by 3A3 in vitro. Studies in mice using 111In-labeled 3A3 revealed that incorporation of an ABD extended the residence time in blood compared with monomeric HER3-targeting unit. HER3-specific uptake of 111In-labeled 3A3 in HER3-expressing xenografts was further demonstrated [25]. A pilot therapy study demonstrated significantly slower growth of xenografts in mice treated with 3A3 compared with PBS-treated control. However, survival could not be measured as the ethical permit allowed only four weeks of treatment because of the risk for potential unknown side-effects [25].

The aim of this study was to compare in vivo efficacy and safety in targeted therapy treatment of HER3-expressing xenografts using a bivalent anti-HER3 ABD-fused affibody construct and anti-HER3 antibody seribantumab.

The affibody construct was re-designed for this study. The albumin-binding domain ABD035 was replaced by a de-immunized ABD084 variant having retained high affinity for serum albumin of different species [26, 27]. For HER3-targeting, the alternative ZHER3:08698 clone [19] was utilized, which in monomeric form has demonstrated better tumor localization than ZHER3:08699 [28]. To reduce size, which potentially could improve penetration of tumor tissue, the (S-G)4 linkers between the protein domains were replaced by shorter G4S linkers, resulting in molecular weight reduction from 22.5 to 19.7 kDa. The new construct ZHER3:08698-G4S-ABD084-G4S-ZHER3:08698 was designated 3A3s.

To validate the design, we investigated if the length of linkers influenced the capacity of 3A3 to inhibit phosphorylation of HER3 in vitro. Binding kinetics of 3A3s to human HER3 and albumin as well as their murine counterpart were measured. The capacity of 3A3s to inhibit HER3 phosphorylation and growth of HER3 expressing cells in vitro was confirmed, and in vivo xenograft growth inhibition was compared with the inhibition obtained with seribantumab. The influence of treatment of HER3-expressing xenografts with 3A3s and seribantumab on mouse survival was investigated, and the safety of 3A3s treatment was furthermore assessed.

**Materials and Methods**

**Design, production and characterization of the bivalent affibody constructs.** We have previously published the generation and characterization of a bivalent HER3-targeting affibody construct, ZHER3:08699-(S-G)4-ABD035-(S-G)4-ZHER3:08699, (22.5 kDa), denoted 3A3 [25]. In the present study, a very similar but slightly smaller construct

(19.7 kDa) was generated according to the same principle; ZHER3:08698-G4S-ABD094-G4S-ZHER3:08698, hereinafter denoted 3A3s. The novel construct differs in that (1) shorter linkers between the binding moieties were used, that (2) a deimmunized variant of ABD [26,27] was introduced, and that (3) a different but very similar HER3-binding affibody molecule ZHER3:08699 was chosen [19]. The same size-matched non-HER3-binding construct as used previously, denoted TAT [24,25] was included as control.

The bivalent fusion proteins 3A3s and TAT were produced in E. coli BL21*DE3 cells (Novagen, Madison, WI) and purified as described by us previously [25]. Briefly, affinity chromatography on an anti-ABD-Sepharose matrix (Affibody AB, Solna, Sweden) was followed by reverse phase HPLC and size-exclusion chromatography. Finally, the endotoxin content was analyzed (APL AB, Stockholm) and found to sufficiently low for in vivo studies (<0.2 endotoxin units/ml).

The monoclonal antibody seribantumab was produced in Chinese hamster ovary cells and purified via affinity chromatography (Evitria, Zürich, Switzerland). Its purity was confirmed using analytical size exclusion chromatography (Superdex 200 10/300 GL) on AKTA Explorer (both GE Healthcare, Uppsala, Sweden)

To confirm that the shorter linkers did not impair the function of the 3A3 fusion proteins, two different 3A3 constructs with different linker lengths were compared in their ability to inhibit heregulin-induced signaling in an in vitro assay using MCF-7 cells (ATCC HTB-22). MCF-7 cells were trypsinated and seeded at 25,000 cells per well in an EnSpire-LFC microplate (Perkin Elmer, Waltham, MA, cat no 6055408) and allowed to grow for 20 h at 37°C in complete medium (RPMI 1640 medium with L-glut (Lonza, Basel, Switzerland) supplemented with sodium pyruvate (Lonza), non-essential amino acids (Lonza), penicillin/streptomycin (Lonza) + 10% fetal calf serum (FCS) (Gibco Life Technologies, Carlsbad, CA)). The cells were
washed once with Hanks balanced salt solution (HBSS; Sigma-Aldrich, Darmstadt, Germany, cat no H9269). 100 µl HBSS was added per well and the plates were incubated at ambient temperature for 1.5 h inside the EnSpire Instrument (Perkin Elmer) to be used for assay readout. The ability of the two 3A3 constructs with different linker length to block heregulin-induced signaling was analyzed by mixing the proteins in a 5-fold dilution series with a fixed concentration of heregulin (1 nM) in a final assay volume of 130 µl/well. The redistribution of dynamic mass upon addition of stimuli was recorded by an EnSpire Instrument every 60 s for 1 h. The half maximal inhibitory concentrations (IC50) were determined from the dose response curves.

Retained affinity of the 3A3s to murine ErbB3 and human HER3 (Sino Biological, Beijing, China) was verified on a BiaCore T200 system (GE Healthcare) using a CM5 sensor chip with immobilized murine and human serum albumin (both Sigma-Aldrich) according to a previously published protocol [24]. Both bivalent binders were captured on the surface, 3A3s at 100 nM and TAT at 25 nM for 12 s to allow the ABD to bind to the immobilized surface with both target binding affibody moieties available for interaction. After the capture step dilutions of murine ErbB3 and human HER3 were injected at concentrations between 25 and 250 nM for 150 s for association and 1800 s for dissociation. Separately, 3A3s and TAT were injected at concentrations between 1 and 25 nM to determine binding to albumin. Flow rates were kept at 10 µl/min with PBST running buffer in all experiments and 10 mM hydrochloric acid to regenerate the chip-surface. A 1:1 binding model was used to determine the affinities towards murine albumin. A model allowing for a heterogeneous ligand was used to fit data to determine the affinities towards murine and human ErbB3/HER3.

The 3A3s fusion protein was subjected to circular dichroism at 195-250 nm and 20°C using a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) in a cell with an optical path-length of 1 mm. In addition, the ellipticity at 221 nm was measured for each Affibody molecule while heating the sample from 20 to 90°C (5°C/min).

**Cell proliferation assay.** Cells for in vitro and in vivo experiments were purchased from American Type Tissue Culture Collection (ATCC, Manassas, VA). The cell line BxPC-3 (human pancreatic carcinoma) was cultured in RPMI media (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and maintained at 37°C and 5% CO2. Trypsin-EpTA (0.25% trypsin, 0.02% EDTA in PBS) (Biochrom, Berlin, Germany) was used to detach cells.

The cell proliferation assays were performed as previously described [25]. Cells were incubated for 6 days with 0.05 to 900 nM of either bivalent affibody construct or no construct. BxPC-3 cells are autocrine for heregulin [11], which was therefore not added to the cells. Cell survival was measured by adding 10 µl of cell counting kit 8 (CCK8) solution (Sigma-Aldrich) and further incubated at 37°C for 6 h before measuring the optical density at 450 nm with a Tecan Sunrise microplate reader (Tecan Group, Männedorf, Switzerland). OD measurements were collected and adjusted to the maximal OD reached by wells containing no affibody constructs.

**Phospho-HER3 ELISA.** BxPC-3 cells were seeded in 6-well plates (2 000 000/well) and allowed to grow for 8 h at 37°C in complete medium. The cells were then starved in medium containing 0.5% FBS for 24 h. The ZHER3 affibody molecule, 3A3s (ZHER3-ABD-ZHER3) and TAT (ZTaq-ABD-ZTaq, negative control) were added to the wells (200 nM) and incubated for 10 minutes at 37°C. Further, heregulin (4 nM) was added to the wells and incubated for 10 minutes at 37°C. A well containing cells and heregulin was included as a positive control. All samples were treated in duplicates. The media was removed and the cells were washed twice with 1.4 ml ice-cold PBS, and 0.4 ml 1x lysis buffer (1% NP-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin) was added to each well. The cell plates were kept on ice and the cells were detached with a cell scraper, transferred to Eppendorf tubes on ice, lysed in a sonicator bath for 45 seconds and centrifuged for 10 minutes at 14 000 rpm. The supernatant from each tube was collected and used in the phospho-HER3 ELISA.

DuoSet IC ELISA kit (R&D Systems, Minneapolis, MN) for detection of phosphorylated HER3 was used according to manufacturer's instructions as described below. 96-well plates were coated with anti-HER3 capture antibody at 4 µg/ml in PBS overnight at room temperature. The plate was washed and blocked with blocking buffer (1% BSA, 0.05% NaN3 in PBS, pH 7.2-7.4) for 2 h at room temperature. After washing, a 1:1 mixture of "diluent #12" (1% NP-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM activated sodium orthovanadate) and cell lysate was added to each well in triplicates and incubated for 2 h at room temperature. The plate was further washed and incubated for 2 h at room temperature with anti-phospho-tyrosine-HRP, diluted 1:2000 in "diluent #14" (20 mM Tris, 137 mM NaCl, 0.05% Tween 20, 0.1% BSA, pH 7.2-7.4). The plate was washed and substrate solution (1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine), R&D Systems) was added. The reaction was stopped with stop solution (2 M H2SO4) and the plate was analyzed in an Enmax microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

**In vitro HER3 binding specificity.** In vitro specificity experiment was performed with the aim to confirm binding specificity of 3A3s and to check cross reactivity of 3A3s affibody construct and seribantumab. BxPC-3 cells (106 cells/dish) were seeded the day before experiment in 6 well plates. This experiment was performed in triplicate. Cells were incubated with 0.1 nM of 111In-3A3 in media (preparation was described in detail previously [25]. Additional cell dishes were pre-treated for 15 min with 50 nM of 3A3, seribantumab or bevacizumab (Avastin, Roche, Basel, Switzerland), as a non-specific control. Cells were incubated for 1 h at ambient temperature and harvested using Trypsin-EDTA. Cell associated radioactivity was measured using an automated gamma well counter (3-inch Na(Tl) detector, 2480 Wizard2, PerkinElmer).
**Determination of dosage.** A pharmacokinetics–pharmacodynamics (PK/PD) simulation approach was used to determine the dosage regimen for the affibody molecule. A simulation of the exposure levels of seribantumab and affibody molecule was performed using a one-compartment model in the PK/PD simulation software Maxsim2 (Fraunhofer–Chalmers Research Centre Industrial Mathematics, Gothenburg, Sweden). The dose for seribantumab was based on data published by Schoebel et al [29]. PK-parameters for the 3A3s were adopted from in house data and the dose level was iterated in order to achieve similar levels of exposure for the antibody and affibody construct.

**Mouse xenograft studies.** Three groups (n=10) of female Balb/c nu/nu mice were subcutaneously implanted with 5×106 cells BxPC-3 per animal. Treatment was started 7 days after tumor inoculation with 600 µg either 3A3s or seribantumab in 150 µl PBS. The control group was treated with equal volume of PBS. Injections were done intraperitoneally, 3 times per week, and treatment continued until the mice reached humane end point: tumor’s size exceeded 1 cm3, ulcerated, necrotic or infected tumor, animal weight loss over 15% of initial weight, weight loss exceeded 10% within one week, debilitation reached BS2 in Body Condition Scoring, or animals conceded as sick according to Guidelines for Pain and Distress in Laboratory Animals from US National Institute of Cancer. Mice were controlled twice per week. The tumors were measured using calipers and volumes were calculated using ellipsoid formula.

**Immunohistochemistry.** At the time of euthanasia, tumors (together with eventual metastases), kidneys and livers were excised and preserved in 37% formaldehyde until paraffinized. The tissues were subsequently embedded in paraffin. Haematoxylin and Eosin (HE) and immunohistochemical (IHC) staining and slide scanning were performed at the Swedish SciLifeLab facilities. HE staining (Haemotox- lin (Mayers’ Histolab, Stockholm, Sweden), Eosin (0.2%, Histolab)) of 4-µm sections was carried out followed by dehydration and mounting in Pertex (Histolab). IHC staining was performed in accordance with protocols described elsewhere [30]. In brief, 4-µm sections collected on SuperFrost Plus slides were deparaffinized in xylene, re-hydrated in graded alcohols, blocked for endogenous peroxidase, and subjected to heat-induced antigen retrieval using PT module buffer 1 (pH 6, Thermo Fisher Scientific) in a Decloaking Chamber (Biocare Medical, Pacheco, CA) at 125°C for 4 minutes. Automated IHC was performed using a LabVision Autostainer 480S (Thermo Fisher Scientific). Primary antibody monoclonal mouse anti-human HER3 (Clone DAK-H3-IC, 0.2 mL, DAKO, Carpinteria, CA) was diluted in UltraAb Diluent (Thermo Fisher Scientific), and applied to the slides for 30 min at room temperature. The slides were further incubated with the secondary reagent (anti-rabbit/mouse horse redish peroxidase–conjugated UltraVision; Thermo Fisher Scientific) for 30 min at room temperature. Following the washing steps, the slides were developed for 5 min using the avidin–biotin peroxidase staining technique (Vector Elite; Vector Laboratories, Burlingame, CA) using 3,3-diaminobenzidine as the substrate. Slides were counterstained in Mayers hematoxylin (01820, Histolab) for 5 minutes using the Autostainer XL. (Leica, Wetzlar, Germany), and then rinsed in lithium carbonate water (diluted 1:5 from saturated solution) for 1 minute. The slides were dehydrated in graded ethanol and lastly cover-slipped (PERTEX, Histolab) using an automated glass cover slipper (CV5030, Leica). The slides were scanned using the automated scanning system Aperio XT (Aperio Technologies, Vista CA). Slides were examined by a pathologist.

**Statistical analysis.** Obtained values are presented as average with standard deviation if not stated otherwise. Data were assessed either by an unpaired, two-tailed t-test for comparison of two groups or by one-way ANOVA with Bonferroni correction for multiple comparisons. Log-rank test for trend was used for multiple comparison of survival in treated and control groups. GraphPad Prism (version 6 for Windows GraphPad Software) was used in order to determine significant differences (p<0.05).

**Results and discussion**

**Design, production and in vitro characterization of the HER3-binding affibody protein.** The aim of this study was to compare two different HER3-targeting affinity proteins, the 20 kDa affibody-based 3A3s fusion protein and a monoclonal antibody seribantumab [8] currently in clinical development (Fig. 1), for their ability to reduce tumor cell growth. The 3A3s fusion protein [ZHER1:08698-G4S-ABD094-G4S-ZHER1:08698] comprises two HER3-binding affibody units ZHER1:08698 [19], flanking a 46 amino acid albumin binding domain, ABD094 [26, 27].

In order to confirm that the shorter G4S linkers of 3A3s did not negatively influence the performance as compared to the previously described 3A3 construct [25] having four SiG linkers flanking ABD, the constructs ability to inhibit heregulin-induced signaling was analyzed in an in vitro cell assay (Fig 1S). Two 3A3s variants, differing only in the length of the linkers, with either short (G4S) or longer (G8S) linkers were compared. The results showed that the two constructs had a very similar inhibitory capacity. The obtained IC50 values for 3A3 with one or four G4S linkers were 0.7 nM and 0.6 nM, respectively. The effect of linker length between the affibody- and ABD moieties was indeed marginal, and the variant with shorter linker was thus chosen for further studies since a reduced size may have advantages in terms of penetration of tumor tissue. The same size-matched fusion protein TAT, binding the non-mammalian target Taq polymerase, that was included in our previous study [25] was used as a control protein for cell proliferation studies.

Both bivalent fusion proteins were expressed in E. coli, and recovered by affinity chromatography and reversed phase HPLC to a purity exceeding 98%, (Fig. 2S A). The molecular masses as measured by mass spectrometry (Fig. 2S B), 19,709 Da for 3A3s, and 22,126 Da for TAT, were in a very good agreement with the expected theoretical values, 19,709 Da and 22,124 Da, respectively. The monoclonal antibody seribantumab was produced in CHO cells and recovered by affinity chromatography. The purity was assessed using analytical size exclusion chromatography and was found to be 96% (Fig. 2S C).

For investigation of the thermal stability, the 3A3s affibody...
A fusion protein was subjected to circular dichroism spectroscopy. The melting temperature of 3A3s was determined by measuring the ellipticity at 221 nm while heating the proteins from 20 to 90°C. The obtained melting curve corresponded to a Tm value of 65°C (Figure S3A), which is in the same range as the original HER3-specific affibody molecules [24]. Additionally, spectra at wavelengths ranging from 195-250 nm were obtained before and after the variable temperature measurement in order to assess the reversibility of the unfolding after heat treatment (Figure S3B). The results showed that the 3A3s fusion protein maintained the alpha-helical content and completely refolded after heating to 90°C, as shown by the overlap between spectra generated before and after heating.

Binding to HER3, mErbB3 and serum albumin was verified by surface plasmon resonance (SPR) and the best fit for the HER3 and mErbB3 interactions was observed using a heterogeneous ligand-binding model (Fig. 2). This is likely due to that the affinity for HER3 is influenced by the orientation of the affibody molecules, i.e. the N- or C-terminal fusion affecting the binding differently. Hence 3A3s has a distinct KD value towards HER3 for both its N- and C-terminal HER3 binding moiety, 4.8±3.3 nM and 0.4±0.5 nM (Fig. 2). The affinities for mErbB3 for 3A3s are 35±26 nM and 146±114 nM. The affinity for HSA was determined to 3.4±0.5 nM and for MSA 2.4±0.4 nM (Fig. 2). The affinities for the serum albumins are likely influenced by the fusion protein context and were lower than previously reported [22].

111In-labeled 3A3s demonstrated target-specific binding to HER3 expressing BxPC-3 cells. Cells pre-treated with non-labeled 3A3s had significantly lower (p < 0.0001) radioactivity uptake than control cells (Fig. 3A). It was also demonstrated that pre-treatment of cells with molar equivalent concentration of seribantumab significantly (p < 0.001) decreased cellular uptake of radioactivity, demonstrating that both therapeutic anti-HER3 proteins compete for the same epitope of HER3. Pre-treatment of cells with non-targeting mAb (bevasizumab) did not influence cellular uptake of radioactivity (Fig. 3A).

The effect of 3A3s on HER3-signaling was demonstrated in a phosphorylation assay. The level of HER3 phosphorylation was analyzed using phosphor-tyrosine-specific detection antibodies in a sandwich ELISA (Fig. 3B). The pancreatic cancer cell line, BxPC-3 with the estimated HER3 expression of 12±2 x 103 [31], was used in this assay. The cells were stimulated by addition of heregulin, which resulted in significant increase in HER3 tyrosine phosphorylation levels (p < 0.005), as compared to cells without addition of heregulin. Cells that were treated with both heregulin and anti-HER3 monomeric or dimeric affibody (ZHER3 or 3A3s) demonstrated HER3 phosphorylation levels that were similar as for heregulin-unstimulated cells (p < 0.001). Similar mechanism of action was reported for seribantumab [8]. TAT was included as a negative control, and as expected, it did not affect the HER3 phosphorylation, which remained at the same level as heregulin stimulated cells.

A cell growth inhibition experiment was performed to assess the effect of treatment with the 3A3s affibody con-
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Figure 3. In vitro characterization of 3A3s. A. In vitro specificity test. BxPC-3 cells were incubated with 1 nM of 111In-3A3 with pre-incubation with 50 nM of 3A3s, MM-121 or non-HER3-specific mAb (bevacizumab). Cell associated uptake was normalized to uptake of cells without pre-treatment. Data are presented as an average for 3 dishes ± SD. B. Inhibition efficacy. HER3 phosphorylation was quantified in BxPC-3 cells treated with 4 nM heregulin and compared with phosphorylation in cells in presence of 200 nM 3A3s, ZHER3, or ZTaq. Phosphorylation in non-treated cells was used as a control. The signal was normalized to the signal in 4 nM heregulin treated cells. Data are presented as an average for 9 dishes ± SD. Un-paired t-test was used to determine significant difference (** p < 0.01, *** p < 0.001).

Figure 4. Cell proliferation of the BxPC-3 pancreatic cancer cell line analyzed by measuring cell growth inhibitory effects of the bivalent fusion protein 3A3s (filled triangles) and TAT control construct (filled squares) incubated at concentrations ranging from 0.05 to 900 nM for 144 h. Cell viability was measured using a CCK8-kit. Maximum inhibition was set to the lowest absorbance signal of 3A3s treated cells.

Tumor growth inhibition in xenografted mice. The dosage for seribantumab was based on data published by Schoeberl et al [29]. To determine the dosage regimen for the affibody construct, a pharmacokinetics-pharmacodynamics simulation approach was used using a one-compartment model. A simulation of the exposure levels of seribantumab and a body molecule was performed, which suggested that 600 µg/dose of the affibody construct and 600 µg/dose for seribantumab, 3 times/week for 12 weeks was sufficient to achieve a similar level of exposure (Fig. S4).

An in vivo therapy experiment was performed in female mice bearing HER3 expressing BxPC-3 xenografts. Therapy started at day 7 after xenograft implantation and the experiment reached a humane end point at day 68. Mice were visually controlled and weighed twice per week. Exterior, general conditions, behavior, and body-scoring were similar in both treated groups to that in the control group. Body weight did not differ between the three groups (Fig. S5A). All animals were euthanized when xenograft volume reached 1 cm³. Pathological examination of liver and kidney tissue of euthanized animals did not show any differences between control and treated groups (Fig. S5B). Xenograft growth was slower in both treated groups than control, with doubling times of 13±1 days for 3A3s, 12±1 for seribantumab and 10±1 for control group (difference between 3A3s group and control group was significant, p < 0.05), respectively. Xenograft volumes were significantly smaller (p < 0.05) in both treated groups than in control group but there was no difference between treated groups (Fig. 5A). Median survival was also significantly longer for treated groups than for control group: control 41 days, seribantumab-treated 54 days and 3A3s-treated 60 days (p < 0.0001). There was no significant difference in median survival between treated groups (p = 0.45).

Histological analysis of the excised xenografts showed a poorly differentiated tumor with solid growth pattern and presence of variable areas of necrosis. Between the three groups no relevant differences were observed neither in growth pattern nor areas of necrosis (Fig. S6).

The immunohistochemical analysis of the HER3 receptor expression in the xenografts demonstrated a distinct membranous staining in most tumor cells of all three groups, independent of treatment (Fig. 5C). There was a tendency that the central areas of the solid tumor islands showed a more intense expression than cells located in the periphery near the stroma. This phenomenon was more pronounced in treated xenografts. No obvious difference in staining pattern was observed in kidney and liver tissue between treated and untreated mice.

To conclude, our bivalent HER3-targeting 3A3s affibody construct was equally potent in terms of decreased tumor growth and improved survival in xenografted mice, to the monoclonal antibody seribantumab, presently undergoing clinical investigations as a HER3-targeting therapeutic. These results are indeed encouraging, and although the mode of action for 3A3s is not completely investigated, the biological activity is probably the result of several distinct mechanisms. In addition to blocking heregulin-induced signaling, it has previously been reported that bi- and trivalent HER3-specific affibody constructs downregulate HER3 surface expression in different cancer cell types [32], which the immunohistochemistry results obtained in this study supports. If speculating, the bivalent 3A3s might also trap two kinase-deficient HER3 receptors in a non-signaling dimer configuration, preventing them to form signaling heterodimers with for example HER2 or HER3.
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Figure 5. Results of the in vivo HER3-targeting therapy in Balb/c nu/nu female mice bearing BxPC-3 xenografts. Mice were implanted at day 0 and treatment was started at day 7 and continued to humane end point (xenograft's volume over 1 cm3). Mice were injected i.p. with 600 µg of 3A3s or MM121 in 100 µl of PBS 3 times per week, animals in control group were injected with neat PBS. Curves stopped when 3 mice in a group were euthanised (30-33%). Data are presented as an average for 10 animals ± SD. A. Measured xenograft’s volume. From day 20, tumor volumes in the control group were significantly bigger than in the treated groups (p < 0.05). B. Survival until euthanasia criteria were reached. Median survival in treated groups was significantly longer than in control group. C. Immunohistochemical staining of HER3 expression of 4 µm sections of paraffinized tissue samples of BxPC-3 xenografts, livers and kidneys. Tissues were excised from animals euthanized at the humane end point. No relevant differences were observed between treated and control groups in liver and kidneys. The central areas (yellow arrows) of the solid tumor islands showed a more intense expression than the cells that were located in the periphery near the stroma (black arrows).

EGFR. However, further investigations are needed to determine in detail the exact mechanisms. The results also encourage generation of new bivalent affibody constructs targeting other tyrosine kinase receptors for studies on their therapeutic potential as receptor trapping agents.

Acknowledgements

This work was supported by the Swedish Cancer Society (grants CAN2016-463 (SS), CAN2014-474 (AO) and CAN2015/350 (VT)), the Swedish Research Council (grants 621-2012-5236 (SS), 2015-02509 (AO) and 2015-02353 (VT)), the Wallenberg Center for Protein Technology and the ESCAPE Cancer grant from VINNOVA (AO). Dr. Anders Olsson is acknowledged for technical assistance in protein purification and Patrick Micke for assistance with the IHC analysis.

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Supplementary information

Figure S1. Influence of linker length. The two different 3A3 constructs primarily differing in their linker lengths were compared in their ability to inhibit heregulin-induced signaling in an in vitro assay using MCF-7 cells. The assay was performed utilizing a label free system and the results are plotted as recorded response, picomolar (pM) response shift versus concentration. The half maximal inhibitory concentrations (IC50) were determined from the dose response curves.

Figure S2. Purity of the compounds. Characterization of bivalent affibody construct 3A3s and monoclonal antibody MM-121. A. The purity of the 3A3s fusion protein was determined using an analytical column Zorbax 300B-C18 (Agilent Technologies) on a 1200 series RP-HPLC (Agilent Technologies)). B. The molecular mass of 3A3s was confirmed using a 6520 Accurate-Mass Q-TOF LC/MS (Agilent Technologies). C. The purity of the MM121 monoclonal antibody (Evitra AG) was analyzed using analytical SEC (Superdex 200 10/300 GL, GE Healthcare).
Evaluating the therapeutic potential of a dimeric HER3-binding affibody construct in comparison with a monoclonal antibody, seribantumab

Figure S3. Analysis of heat stability and refolding using circular dichroism (CD) spectroscopy. A. Variable temperature measurement (VTM) spectrum obtained at 221 nm while heating the sample from 20 to 91°C. B. Overlay of CD spectra before and after VTM.

Figure S4. Determination of dosage using a one-compartment level. Simulation of antibody and affibody exposure levels performed using a one-compartment model in a PK/PD simulation software. The graphs display the exposure levels plotted as µmol/L versus time.
Figure S5. Monitoring of mice weights in the HER3-targeting therapy experiment. Mice were injected i.p. with 600 µg of 3A3s or MM-121 in 100 µl of PBS 3 times per week, animals in control group were injected with PBS alone. Data are presented as an average for 10 animals ± SD.

Figure S6. Haemotoxylin and Eosin (HE) staining of 4 µm sections of paraffinated tissue samples of BxPC-3 xenografts, livers and kidneys. Tissues were excised from animals euthanized at the humane end point. No relevant differences were observed between treated and control groups.