

HER2-BINDING POLYPEPTIDE

Field of the invention

The present disclosure concerns a new polypeptide and fusion proteins which comprise said new polypeptide which bind to Human Epidermal Growth Factor Receptor 2 (in the following referred to as HER2). The present disclosure also
5 relates to use of such a HER2-binding polypeptide or fusion proteins as a diagnostic agent, prognostic agent, theranostic agent and/or medicament, more particularly use thereof in the diagnosis, prognosis and/or treatment of forms of cancer characterized by over-expression of HER2.

10 Background

Human epidermal growth factor receptor 2 (HER2, HER2/neu, CD340) is a 185 kDa transmembrane protein belonging to the HER tyrosine kinase family. It consists of an extracellular domain, a transmembrane/juxtamembrane domain, and an intracellular tyrosine kinase domain that can interact with multiple signaling
15 molecules. The ligand to HER2 is unknown. Under physiological conditions HER2 signaling is activated by heterodimerization with other receptors of the HER family, with HER2 being the preferred dimerization partner of the other family members EGFR/HER1, HER3, HER4.

HER2 is overexpressed in a substantial fraction of breast, ovarian,
20 gastroesophageal and gastric cancers. Its elevated expression leads to constitutive and uncontrolled HER2 signaling that promotes cancer growth associated with poor prognosis. Other ways of HER2 signaling activation are proteolytic cleavage of its extracellular domain (ECD) or activating mutations. HER2 mutations occur in several cancer types with varying frequencies, e.g., in around 4% of lung, breast, and
25 gastroesophageal cancers, in 9-12% of bladder cancers, and 4-6% of colorectal cancers (Bose *et al.*, 2021, N Engl J Med 385(13):1241-1243; Cocco *et al.*, 2021, Pharmacol Ther. 199:188–196).

In the last two decades, HER2-targeted therapy has dramatically changed the prognosis for patients with advanced HER2-positive breast cancer, and notable
30 progress has been made in the treatment of other HER2-positive solid tumors such as gastroesophageal and gastric cancers, as well as NSCLC. Enhertu[®], an antibody-drug conjugate consisting of the humanized monoclonal antibody trastuzumab

(Herceptin®) covalently linked to the topoisomerase I inhibitor deruxtecan, is the first HER2-targeting large molecule with an approval to treat HER2-mutated cancer, namely NSCLC with activating HER2 mutations (Li *et al.*, 2022, N Engl J Med 386:241-51).

5 Despite the significant improvement in the treatment of advanced HER2-positive solid tumors, almost all patients treated with currently available HER2-targeted therapies such as trastuzumab and pertuzumab (Perjeta®), or ADCs (e.g., trastuzumab-emtansin (Kadcyla® and trastuzumab-deruxtecan)) eventually experience disease progression (Kelly and Janjigian, 2016, J Gastrointest Oncol. 7(5):750-62; Siravegna *et al.*, 2018, Cancer Cell. 34(1):148-62; Zeng *et al.*, 2021, Journal of the National Cancer Center. 1:58-73). Over time, most of these patients advance to the point where no approved HER2-targeted therapy controls the progression of the disease, creating a high unmet medical need in this patient population. This population also includes those who develop brain metastases, 10 which are a common cause of death (Loibl S. *et al.*, 2017, The Lancet 389:2415-2429).

 HER2 targeted polypeptides based on the Z scaffold have been developed for both diagnostic and therapeutic purposes. WO2009080810 disclosed an engineered HER2 binding Z variant which in clinical studies has demonstrated excellent 20 sensitivity and specificity in the detection of HER2 expressing metastases in patients diagnosed with breast cancer (Sörensen *et al.*, 2014, The Journal of Nuclear Medicine 55(5):730-735; Sörensen *et al.*, 2016, Theranostics 6(2): 262-271; Alhuseinalkhudhur *et al.*, 2023, J Nucl Med 64(9):1364-1370). Clinical evaluation was made with ¹¹¹In and ⁶⁸Ga radiolabeled conjugates suitable for SPECT- and PET based 25 imaging, respectively. WO2022171892 disclosed a further modified version of this Z variant comprising a C-terminal -GGGC chelate suitable for radiolabeling optionally without the need of an intermediate conjugation step. A first-in-human study with this Z variant labelled with ^{99m}Tc demonstrated the molecule to be safe and well tolerated and with the capacity to discriminate between breast cancer tumors with 30 high and low HER2 expression levels (Bragina *et al.*, 2023, Theranostics 13(14):4858-4871). To date, clinical use of both these referenced HER2 targeted Z variants has been limited to imaging studies using a monomeric Z moiety. In a preclinical setting, the same Z variant in fusion with ABD and labeled with ¹⁷⁷Lu was administered to

HER2-expressing human xenograft mice, resulting in an extended survival compared to mice treated with vehicle. However, a noticeable accumulation of the molecule, was detected in the liver (Liu *et al.*, 2023, *Cancers* 15(9):2409). Attempts to improve the biodistribution of Z-ABD fusion polypeptides for radionuclide therapy by e.g.

5 positioning a radionuclide-chelator complex at the N-terminus (Liu *et al.*, 2022, *Pharmaceutics* 14(11):2519) or on the middle of helix 1 of ABD to increase the distance between albumin and chelator (Liu *et al.*, 2021, *Pharmaceutics* 13(6):839) resulted in substantially elevated renal uptake, making these constructs unsuitable for radionuclide therapy.

10 Thus, there is still a need to develop therapies for HER2 positive cancer for first line treatment and for providing recurrent/refractory cancer patients with a targeted radiotherapy option to treat patients in relapse on current treatments.

Furthermore, because the tissue penetration rate of a molecule is negatively
15 associated with its size, a relatively large antibody molecule inherently has poor tissue distribution and penetration capacity. As such, it may not be an optimal targeting moiety for delivery of radioisotopes to solid tumors in a therapeutic setting. Moreover, although antibodies are widely used in a variety of routine contexts owing to high affinity and specificity to a multitude of possible antigens,
20 such as for analytical, purification, diagnostic and therapeutic purposes, they still suffer from drawbacks. Such drawbacks include aggregation tendencies and long systemic half-lives which, in the case of targeted radiotherapy, increase the risk of damaging healthy tissue. When used for diagnostic (e.g. imaging) purposes, monoclonal antibodies again have drawbacks due to their long half-lives that result
25 in poor contrast unless sufficient time is allowed for levels of unbound antibody to decline. Thus, monoclonal antibodies are not always optimal for therapeutic and diagnostic uses.

From the above, it is clear that there is continued need for provision of agents with a high affinity and specificity for HER2 to be used as a targeting probe
30 for therapeutic, diagnostic, and theranostic purposes, as well as agents with low immunogenicity to ensure safe and well tolerated therapeutics.

Description

It is an object of the present disclosure to satisfy the requirements of a continued provision of agents with an affinity for HER2, through the provision of a polypeptide that is characterized by specific binding to HER2.

It is a further object of the disclosure to provide a HER2-binding polypeptide that can readily be used as a moiety in a fusion protein.

It is a further object of the disclosure to provide a HER2-binding polypeptide or fusion protein comprising a chelating environment provided by a chelator, wherein a radionuclide may be coordinated, chelated or complexed to the polypeptide or fusion protein.

A further object of the disclosure is to provide a composition comprising said HER2-binding polypeptide, said fusion protein, or said radiolabeled polypeptide or fusion protein, and at least one pharmaceutically acceptable excipient or carrier.

A further object of the disclosure is to provide methods which utilize said HER2-binding polypeptide, fusion protein, radiolabeled HER2-binding polypeptide or fusion protein, or composition comprising any of the above, which are amenable to use in prognostic and/or diagnostic applications.

A further object of the disclosure is to provide methods which utilize said HER2-binding polypeptide, fusion protein, radiolabeled HER2-binding polypeptide or fusion protein, or composition comprising any of the above, which are amenable to use in therapeutic, including theranostic, applications.

A further object of the disclosure is to provide a HER2-binding polypeptide which can be produced using recombinant protein expression or synthesized by chemical peptide synthesis.

These and other objects are met by the different aspects of the present disclosure. Thus, in a first aspect, the disclosure provides a HER2-binding polypeptide, comprising the amino acid sequence [XYZ]:

EMRNAYWEIALLPNLTNQKRAFIRKLYDDPSQSSELLSEAKKLND SQAPEPE

(SEQ ID NO:1).

In accordance herewith, the inventors have found that a HER2-binding polypeptide, comprising the amino acid sequence SEQ ID NO:1 exhibits surprising advantages, for example in comparison with other HER2-directed therapies. The herein disclosed polypeptide binds to different epitopes compared to other HER2-
5 directed therapies such as the monoclonal antibodies trastuzumab and pertuzumab (Eigenbrot *et al.*, 2010, PNAS 107(34):15039–15044) and the small-molecule drugs lapatinib (Tykerb®) and tucatinib (Tukysa®), that exert their therapeutic effects through antibody-mediated immunomodulatory activities, as well as disruption of
10 polypeptide holds potential in advanced HER2-positive breast cancer and other solid tumors resistant to these agents, and can also be used simultaneously due to the difference in binding site (Rexer and Arteaga, 2012, Crit Rev Oncog. 17(1):1-16; Kulukian *et al.*, 2020, Mol Cancer Ther 19(4):976-987).

The use of the disclosed polypeptide as a targeting probe offers several
15 advantages compared to monoclonal antibodies, including smaller size, lower production costs, possibility to conjugate a payload site-specifically resulting in well-defined construct with reproducible biodistribution profile and easy engineering.

Furthermore, previous studies with other Z-variants have demonstrated that incorporation of negatively charged amino acids is associated with lower uptake in
20 liver and that substitution of amino acids with hydrophobic side chains by amino acids with hydrophilic ones can result in rapider excretion from blood as well as reduced hepatic uptake (Oroujeni *et al.*, 2018, Amino Acids, 50:981-994; Hofström *et al.*, 2013, J. Med. Chem. 56:4966–4974). Therefore, in the inventive polypeptide (SEQ ID NO:12), lysine (K) in position 58 has been replaced with a glutamic acid (E)
25 and an additional negatively charged glutamic acid (E) has been added within the linker, in position 60. Furthermore, proline (P) has been introduced in position 59 to provide a more rigid linker which may favor in directing the HER2 binding and the albumin binding paratopes away from each other. Proline is also less hydrophobic than valine (V) present in the disclosed comparator molecules introduced in the
30 Examples, the comparator molecules being H2ABPP2 (SEQ ID NO:13), H2ABPP3 (SEQ ID NO:14) and H2ABPP4 (SEQ ID NO:15).

A polypeptide according to this aspect of the disclosure can be used as an alternative to HER2-binding antibodies and to known HER2-binding Z variants in diverse applications. As non-limiting examples, it is useful in the treatment of cancers characterized by HER2 overexpression, in the diagnosis and/or prognosis of
5 cancers characterized by HER2 overexpression both *in vivo* and *in vitro*, in targeting of other therapeutic, diagnostic or prognostic agents to cells overexpressing HER2, in histochemical methods for the detection of HER2, in methods of separation and other applications. The polypeptide according to this aspect may prove useful in any method which relies on affinity for HER2 of a reagent. Thus, the polypeptide may be
10 used as a detection reagent, a capture reagent or a separation reagent in such methods, as a diagnostic and/or prognostic agent for diagnostics and/or prognostics *in vivo* or *in vitro*, or as a therapeutic agent in its own right or as a means for targeting other therapeutic or diagnostic agents to the HER2 protein. Methods that employ the polypeptide according to this aspect of the disclosure *in vitro* may be
15 performed in different formats, such as in microtiter plates, in protein arrays, on biosensor surfaces, on tissue sections, and so on.

The terms "HER2-binding" and "binding affinity for HER2" as used in this specification refer to a property of a polypeptide which may be tested for example
20 by ELISA by Kinetic Exclusion Assay (KinExA®) and/or by various sensor-based technologies, such as surface plasmon resonance (SPR) technology, biolayer interferometry (BLI; e.g. Octet®) and quartz crystal microbalance (QCM) technology.

For example, HER2-binding affinity may be tested in an experiment in which samples of the polypeptide are captured on antibody-coated ELISA plates and
25 biotinylated HER2 is added followed by streptavidin-conjugated HRP. TMB substrate is added and the absorbance at 450 nm is measured using a multi-well plate reader. The skilled person may then interpret the results obtained by such experiments to establish at least a qualitative measure of the binding affinity of the polypeptide for HER2. If a quantitative measure is desired, for example to determine the EC50 value
30 (the half maximal effective concentration) for the interaction, ELISA may also be used. The response of the polypeptide against a dilution series of biotinylated HER2 is measured using ELISA as described above. The skilled person may then interpret

the results obtained by such experiments, and EC50 values may be calculated from the results using for example GraphPad Prism 5 and nonlinear regression.

The affinity of a binding interaction, e.g. defined as the equilibrium dissociation constant (K_D), may also be determined by different sensor-based methods as mentioned above. Importantly, as these technologies measure binding in real-time, the kinetics properties of the interaction can also be assessed, including the rate of association (k_a) and rate of dissociation (k_d). One such technology is based on surface plasmon resonance (SPR). Here, HER2, or a fragment thereof, is immobilized on a sensor chip and samples of the polypeptide whose affinity is to be determined are prepared by serial dilution and injected over the chip. Alternatively, the polypeptide to be tested is immobilized on a sensor chip of the instrument, and a sample containing HER2, or a fragment thereof, is passed over the chip. Binding values may for example be defined in a Biacore (Cytiva), Sierra (Bruker), Catterra (Catterra) or ProteOn XPR 36 (Bio-Rad) instrument. K_D values and/or kinetic constants may then be calculated from the results using for example the 1:1 Langmuir binding model of the Biacore Insight Evaluation software, or other suitable model and software, provided by the instrument manufacturer. Analogously, HER2-binding kinetics and affinity may also be assessed by BLI, an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer. Measurements by BLI technology may for example be performed in an Octet® HTX system (Sartorius) and data monitored and analyzed by appropriate model and software provided by the instrument manufacturer.

A measure of HER2-binding affinity may also be obtained using a continuous-flow system based on QCM technology. To monitor binding interactions, one of the interacting molecules, or fragment thereof, is immobilized on the sensor surface and the sample containing the other one is injected over the sensor surface. The signal output is given in frequency (Hz) and is directly related to changes in mass on the sensor surface. Kinetic measurements by QCM technology may for example be performed in an Attana A200® (Attana) instrument. Data is collected by Attester Software and K_D values may then be calculated from the results using for example a 1:1 Langmuir binding model and subsequently processed in the Evaluation Software, or other suitable software, provided by the instrument manufacturer.

Another method for determining binding affinity for HER2 is the Kinetic Exclusion Assay (KinExA®; Sapidyne Instruments Inc; Darling and Brault, 2004, Assay and Drug Dev Tech 2(6):647-657) for measurements of the equilibrium binding affinity and kinetics between unmodified molecules in solution. For affinity analysis, 5 the equilibrium dissociation constant, K_D , and the rate of association, k_a , are experimentally determined, while the rate of dissociation, k_d , may be calculated based on the equation $k_d = K_D * k_a$.

A KinExA® K_D analysis requires immobilization of one interaction partner (e.g. the titrated binding partner) to a solid phase, which is then used as a probe to 10 capture the other interaction partner (e.g. the constant binding partner) free in solution once an equilibrium is reached. For each experiment, a series of solutions with a constant concentration of one binding partner and a titration of the other binding partner are equilibrated. The solutions are then briefly exposed to the solid phase and a portion of free constant binding partner is captured and labeled with a 15 fluorescent secondary molecule. The short contact time with the solid phase is less than the time needed for dissociation of the pre-formed complex in solution, meaning that competition between the solution and the solid phase titrated binding partner is “kinetically excluded”. Because the solid phase is only used as a probe for the free constant binding partner in each sample, the solution equilibrium is not 20 altered during measurements. A K_D value is calculated from signals generated from captured free constant binding partner, which are directly proportional to the concentration of free constant binding partner in the equilibrated sample. The data may be analyzed using the KinExA® Pro software and least squares analysis to fit the optimal solutions for the K_D and the Active Binding site Concentration (ABC) to a 25 curve representative of a stoichiometric relevant model, for instance a 1:1 reversible bi-molecular interaction.

Determination of binding kinetics via KinExA® may be done in a similar format as the equilibrium analysis, except measurements are collected “pre 30 equilibrium” and the binding signals are a function of time and total concentration of the titrated binding partner. There are two methods that can be used to determine the k_a . The “direct method” holds the concentrations of titrated and constant binding partners fixed, and the solution is probed over time. The amount of the free constant binding partner in the solution will decrease as the sample moves

toward equilibrium. The “inject method” holds incubation time and one partner’s concentration fixed, while titrating concentrations of the other partner. As the concentration of the titrated binding partner increases, the amount of free constant binding partner will decrease as more complexes are formed.

5 For measuring the affinity and kinetics of labeled polypeptides to receptor molecules on living cells a LigandTracer® instrument may be used. This real-time cell-binding assay technology is based on the continuous detection of a fluorescently or radioactively labeled ligand in a target area and in a reference area on a dish mounted on a rotating support.

10 As the skilled person will realize, the function of any polypeptide, such as the HER2-binding capacity of the HER2-binding polypeptide of the present disclosure, is dependent on the tertiary structure of the polypeptide. It is therefore possible to make minor changes to the sequence of amino acids in a polypeptide, such as adding additional N- and/or C-terminal amino acids without affecting the function
15 thereof. Thus, the disclosure encompasses modified variants of the HER2-binding polypeptide (SEQ ID NO:1), which have retained HER2-binding characteristics.

 As the skilled person will realize, minor changes can be made to the sequence of amino acids in a polypeptide without affecting the basic structure of the polypeptide and affecting the function thereof. That is, the overall folding of the Cα
20 backbone of the polypeptide according to this embodiment of the invention is substantially the same as related HER2-binding polypeptides of the present invention, e.g. having the same elements of secondary structure in the same order etc. Thus, a HER2-binding polypeptide can comprise the amino acid sequence [XYZ] (SEQ ID NO:1) according to the present disclosure and can in turn comprise one or
25 more additional N- and/or C-terminal amino acid(s), thereof forming a longer amino acid sequence, but having essentially the same fold as the original HER2-binding polypeptide. Suitably, said one or more additional N- and/or C-terminal amino acid(s) comprises at least one cysteine residue. This implies that basic structural properties are shared, those properties e.g. resulting in similar CD spectra. The
30 skilled person is aware of other parameters that are relevant.

 Different modifications of, and/or additions to, the polypeptide according to this aspect of the disclosure may be performed in order to tailor the polypeptide to the specific use intended, without departing from the scope of the present teaching.

Such modifications and additions are described in more detail below, and may comprise additional amino acids comprised in the same polypeptide chain, or labels and/or therapeutic agents that are chemically conjugated or otherwise bound to the polypeptide according to this aspect of the disclosure.

5 Thus, an aspect of the disclosure also encompasses polypeptides in which the HER2-binding polypeptide described above is present as a HER2-binding domain, to which additional amino acid residues have been added at either terminal. In some embodiments, the HER2-binding polypeptide as described herein comprises an amino acid sequence selected from:

10 K-[XYZ] (SEQ ID NO:2); VDAKYAK-[XYZ] (SEQ ID NO:3); AEAKFAK-[XYZ] (SEQ ID NO:4); AEAKYAK-[XYZ] (SEQ ID NO:5); ADNNTFNK-[XYZ] (SEQ ID NO:6); ADAQQNNFNK-[XYZ] (SEQ ID NO:7); AQHDE-[XYZ] (SEQ ID NO:8); and VDNKFNK-[XYZ] (SEQ ID NO:9); wherein [XYZ] is as defined herein.

15 In a further embodiment, there is provided a HER2-binding polypeptide as described herein, which comprises an amino acid sequence selected from:

 K-[XYZ] (SEQ ID NO:2); VDAKYAK-[XYZ] (SEQ ID NO:3); AEAKFAK-[XYZ] (SEQ ID NO:4); and AEAKYAK-[XYZ] (SEQ ID NO:5); wherein [XYZ] is as defined herein.

20 In another further embodiment, there is provided a HER2-binding polypeptide as described herein, comprising the amino acid sequence AEAKYAK-[XYZ] (SEQ ID NO:5); wherein [XYZ] is as defined herein.

 Additional amino acid residues may play a role in the binding of HER2 by the polypeptide, but may also serve other purposes, related for example to one or more of the production, purification, stabilization, coupling or detection of the polypeptide. Additional amino acid residues may comprise one or more amino acid residues added for purposes of chemical coupling, such as addition of a cysteine amino acid. Additional amino acid residues may also comprise a "tag" for
25 purification or detection of the polypeptide, such as a hexahistidine (H₆) tag, or a "myc" tag or a "FLAG" tag. Such a tag may for example enable interaction with antibodies specific to the tag or immobilized metal affinity chromatography (IMAC) as the case may be. The skilled person is aware of other alternatives.

Following this line of thought, it may be beneficial that the HER2-binding polypeptide as defined herein is part of a fusion protein comprising a second or further moieties. Second and further moiety/moieties of the fusion protein in such a protein may suitably have a desired biological activity.

Thus, in a further aspect, the disclosure provides a fusion protein comprising:

- a first moiety consisting of a HER2-binding polypeptide as described herein; and
- a second moiety consisting of a polypeptide having a desired biological activity.

In further embodiments, there is provided a fusion protein as described herein, wherein said desired biological activity is a binding activity.

In another further embodiment, there is provided a fusion protein as described herein, wherein said binding activity increases the *in vivo* half-life and/or changes the biodistribution properties of the fusion protein or conjugate. In one embodiment, there is provided a fusion protein as described herein, wherein said binding activity increases the *in vivo* half-life of the fusion protein or conjugate. In another embodiment, there is provided a fusion protein as described herein, wherein said binding activity changes the biodistribution properties of the fusion protein or conjugate.

The disclosure also encompasses fusion proteins comprising multimers of the HER2-binding polypeptide comprising SEQ ID NO:1. It may be of interest, e.g. when using the polypeptide according to the invention for diagnosis or treatment of cancer or in a method of purification of HER2, to obtain even stronger binding of HER2 than is possible with one polypeptide according to the invention. In this case, the provision of a multimer, such as a dimer, trimer or tetramer, of the polypeptide may provide the necessary avidity effects. The multimer may consist of a suitable number of polypeptides according to the invention. The linked polypeptide "units" in a multimer according to the invention may be connected by covalent coupling using known organic chemistry methods, or expressed as one or more fusion polypeptides in a system for recombinant expression of polypeptides, or joined in any other fashion, either directly or via a linker, for example an amino acid linker.

Additionally, "heterogenic" fusion polypeptides, in which the polypeptide comprising SEQ ID NO:1 constitutes a first domain, or first moiety, and the second and further moieties have other functions than binding HER2, are also contemplated and fall within the ambit of the present disclosure. The second and further

5 moiety/moieties of such a fusion polypeptide may comprise a binding domain with affinity for another target than HER2. Non-limiting examples of targets for such second and further moiety/moieties may be selected from the group consisting of CD3, CD16, CD137 (4-1BB), CTLA-4, EGFR, FGFR1, HER3, VEGF, PD1, PD-L1 and cMet. The result is then a fusion polypeptide having at least one HER2-binding domain and

10 at least one domain with affinity for said other target molecule. This makes possible the creation of multispecific agents that may be used in several applications, such as used as therapeutic and/or diagnostic and/or prognostic agents, or as reagents for capture, detection or separation. The preparation of such multispecific multimers of polypeptides, in which at least one polypeptide domain comprises SEQ ID NO:1, may

15 be effected as described above for the multimer of several HER2-binding "units".

The second or further moiety or moieties may be a variant of a domain derived from protein A of *Staphylococcus aureus*, such as a Z variant, or comprise an unrelated, naturally occurring or recombinant, protein having a binding affinity for a target (or a fragment thereof retaining the binding capability of the naturally

20 occurring or recombinant protein). Another example of such a binding protein, which has an affinity for human serum albumin and may be used as fusion partner with the polypeptide according to the invention, is one of the albumin binding domains from protein G of *Streptococcus* strain G148 (Nygren P-Å *et al.*, 1988 Mol Recogn 1:69-74), such as the GA1, GA2 or GA3 domain. The GA3 domain of protein

25 G is also denoted ABD, i.e. albumin binding domain. Derivatives of ABD with improved properties vis-à-vis the wild type GA3 domain of protein G have been disclosed, for example in WO2009/016043, WO2012/004384 and WO2014/048977. A fusion polypeptide between the HER2-binding polypeptide comprising SEQ ID NO:1 and an albumin binding domain of streptococcal protein G thus also falls

30 within the scope of the present disclosure. When the disclosed polypeptide is administered to a human subject as a diagnostic, prognostic or therapeutic agent, or as a targeting agent, the fusion thereof to a moiety which binds serum albumin may prove beneficial, in that the half-life *in vivo* of such a fusion protein is likely to be

extended compared with the half-life of the HER2-binding moiety in isolation (this principle has been described e.g. in WO1991/01743). By binding to albumin *in vivo*, the biodistribution properties of the polypeptide may change. In particular, albumin binding may reduce glomerular filtration, facilitate reabsorption back to the circulation and thereby reduce renal uptake of the polypeptide which may be critical when a cytotoxic payload or a radionuclide is conjugated to the polypeptide. In addition, albumin is known to accumulate in tumor tissue and incorporation of an albumin binding domain may thus enhance the tumor accumulation of such fusion protein.

Thus, in another embodiment, there is provided a fusion protein as described herein, wherein said second moiety comprises an albumin binding domain of streptococcal protein G or a derivative thereof. In a further embodiment, there is provided a fusion protein as described herein, wherein said albumin binding domain of streptococcal protein G or a derivative thereof is the GA3 domain or a derivative thereof. In yet another further embodiment, there is provided a fusion protein as described herein, wherein said albumin binding domain comprises an amino acid sequence selected from:

i) LAX₃AKX₆X₇ANX₁₀ELDX₁₄YGVSDFYKRLIX₂₆KAKTVEGVEALKX₃₉X₄₀ILX₄₃X₄₄LP
(SEQ ID NO:10)
wherein independently of each other
X₃ is selected from E, S, Q and C;
X₆ is selected from E, S and C;
X₇ is selected from A and S;
X₁₀ is selected from A, S and R;
X₁₄ is selected from A, S, C and K;
X₂₆ is selected from D and E;
X₃₉ is selected from D and E;
X₄₀ is selected from A and E;
X₄₃ is selected from A and K;
X₄₄ is selected from A, S and E;
L in position 45 is present or absent; and
P in position 46 is present or absent; and

ii) an amino acid sequence which has at least 95% identity to the sequence defined in i). In one embodiment, said amino acid sequence has at least 97% identity to the sequence defined in i).

5 The term "% identity", as used throughout the specification, may for example be calculated as follows. The query sequence is aligned to the target sequence using the CLUSTAL W algorithm (Thompson *et al.*, 1994, Nucleic Acids Research, 22: 4673-4680). A comparison is made over the window corresponding to the shortest of the aligned sequences. The shortest of the aligned sequences may in some instances be
10 the target sequence. In other instances, the shortest of the aligned sequences may be the query sequence. The amino acid residues at each position are compared and the percentage of positions in the query sequence that have identical correspondences in the target sequence is reported as % identity.

15 In a further embodiment, there is provided a fusion protein as described herein, wherein said albumin binding domain comprises an amino acid sequence as defined in SEQ ID NO:11.

 As contemplated for the other aspects as described herein, the fusion protein
20 of a further aspect may also comprise an additional amino acid residue or residues. These amino acid residues may play another role or roles than in the binding of HER2 by the polypeptide. These other purposes may be related for example to one or more of the production, purification, stabilization, coupling or detection of the polypeptide. Different modifications of, and/or additions to, the fusion protein
25 according to this aspect of the disclosure may be performed in order to tailor the fusion protein to the specific use intended, without departing from the scope of the present teaching. Such modifications and additions are described in more detail below, and may comprise additional amino acids comprised in the first, second, or further moieties, or labels and/or therapeutic agents that are chemically conjugated
30 or otherwise bound to the fusion protein according to this aspect of the disclosure. Such additional amino acid residues may comprise one or more amino acid residues added for purposes of chemical coupling, such as addition of a cysteine amino acid. Such additional amino acid residues may also comprise a "tag" for purification or

detection of the polypeptide, such as a hexahistidine (H₆) tag, or a “myc” tag or a “FLAG” tag. Such a tag may for example enable interaction with antibodies specific to the tag or immobilized metal affinity chromatography (IMAC) as the case may be. The skilled person is aware of other alternatives.

5 Thereof, the disclosure provides a fusion protein as described herein, wherein said amino acid sequence comprises a cysteine residue, either already present in the sequence or as an additional amino acid residue. In a further embodiment, there is provided a fusion protein as described herein, wherein said cysteine residue is situated at the C-terminal end of the fusion protein. In one
10 embodiment, there is provided a fusion protein as described herein, wherein said amino acid sequence is as defined in SEQ ID NO:12.

 With regard to the description above of fusion proteins incorporating the HER2-binding polypeptide according to the invention, it is to also be noted that the
15 albumin binding domain moiety in the inventive fusion protein, H2ABPP1 (SEQ ID NO:12), and H2ABPP2 (SEQ ID NO:13) and H2ABPP3 (SEQ ID NO:14) presented in the Examples section as further comparator molecules, are overall both more negatively charged and less hydrophobic when compared to the main comparator H2ABPP4 (SEQ ID NO:15), which comprises a different albumin binding domain moiety.
20 H2ABPP4 has been published previously (Orlova *et al.*, 2013, J Nucl Med, 54(6):961-8, Liu *et al.*, 2023, *supra*) and is regarded as the main comparator for H2ABPP1. In total (over the whole Z-ABD polypeptide), the mutations present in H2ABPP1 involve a net decrease of three basic amino acids, and a net increase of four acidic amino acids compared to H2ABPP4. Modifications at the C-terminal end of the Z-moiety
25 and the linker involve a net decrease of one basic amino acid, and a net increase of one acidic amino acid. Such sequence manipulations (increasing the number of acidic amino acid residues and decreasing the number of basic amino acid residues, or the opposite), are effective at changing the isoelectric point. In addition, H2ABPP1 (SEQ ID NO:12) contains a deimmunized version of the albumin binding domain
30 moiety compared to the albumin binding domain moiety in H2ABPP4 (SEQ ID NO:15), which may be critical for clinical uses.

 With regard to the description above of fusion proteins incorporating the HER2-binding polypeptide according to the invention, it is to be noted that the

designation of first, second and further moieties is made for clarity reasons to distinguish between the HER2-binding moiety or moieties on the one hand, and moieties exhibiting other functions on the other hand. These designations are not intended to refer to the actual order of the different domains in the polypeptide chain of the fusion protein. Thus, for example, said first moiety may without restriction appear at the N-terminal end, in the middle, or at the C-terminal end of the fusion protein.

The disclosure further encompasses embodiments in which the polypeptide of one aspect or the fusion protein of another aspect has been provided with a label group, such as at least one fluorophore, biotin or a radioactive isotope, for example for purposes of detection of the polypeptide or fusion protein. A majority of radionuclides have a metallic nature and metals are typically incapable of forming stable covalent bonds with elements presented in proteins and peptides. For this reason, labeling of proteins and peptides with radioactive metals is performed with the use of chelators, i.e. multidentate ligands, which form non-covalent compounds, called chelates, with the metal ions. Thereof, in a further aspect of the disclosure, there is provided a HER2-binding polypeptide as described herein or fusion protein as described herein, comprising a chelating environment provided by a chelator, wherein a radionuclide may be coordinated, chelated or complexed to the polypeptide or fusion protein.

One example of a chelator is the polyaminopolycarboxylate type of chelator. Two classes of such polyaminopolycarboxylate chelators can be distinguished: macrocyclic and acyclic chelators. In one embodiment, there is provided a HER2-binding polypeptide or fusion protein as described herein, comprising a chelating environment provided by a polyaminopolycarboxylate chelator coupled to the HER2-binding polypeptide or fusion protein via a thiol group. In a further embodiment, there is provided a HER2-binding polypeptide or fusion protein as described herein, wherein the polyaminopolycarboxylate chelator is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), diethylenetriaminepentaacetic acid or a derivative thereof.

The most commonly used macrocyclic chelators for radioisotopes of indium, gallium, lutetium, yttrium, bismuth, radioactinides and radiolanthanides are

different derivatives of DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid). Thereof, in another embodiment, there is provided a HER2 binding polypeptide or fusion protein as described herein, wherein the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid derivative is 1,4,7,10-
5 tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide. In yet a further embodiment, there is provided a fusion protein consisting of SEQ ID NO:12 coupled to 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide.

The molecular design of polypeptides has a strong impact on their
10 biodistribution influencing interaction with their molecular target in tumors and in normal tissue as well as off-target interactions, i.e. unspecific binding to blood proteins. For example, position and chemical nature of a label might impact predominant excretion pathway as well as uptake and retention in excretory organs (Tolmachev *et al.*; 2020, Cancers 12(3):651). One problem is that there is a variety
15 off-target interaction, which are difficult to model. Thus, the search for optimal biodistribution requires careful experimental assessment of the constructs. Variables to consider, and where some examples can be taken from the development of Z-ABD fusion conjugates are:

- overall architecture of the complex, including valency, i.e. number of
20 targeting Z moieties in a Z-ABD construct, and positions of the Z moiety in relation to ABD and to each other (Xu *et al.*; 2020, Cancers 13(1):85; Yin *et al.*, 2021, Pharmaceutics 13(11):1974);
- amino acid composition of a linker between the Z moiety and ABD (Xu *et al.*, 2022, Pharmaceutics 14(3):522)
- 25 • charge and hydrophilicity of a spacer between the targeting protein and cytotoxic payload (Ding *et al.*, 2019, Cancers 11(8):1168);
- number of drugs conjugated to a targeting protein (Ding *et al.*, 2021, Pharmaceutics 13(3):430);
- the physico-chemical properties of a cytotoxic payload (Yin *et al.*, 2023, J
30 Control Release 355:515-527) and
- positioning of a payload (Liu 2021 *supra*, Liu 2022 *supra*).

In one embodiment, the inventive fusion protein comprises a HER2-targeted polypeptide, a linker, and an albumin-binding domain (ABD), conjugated to a maleimide-DOTA via a unique C-terminal cysteine. The molecular weight of the polypeptide is approximately 12 kDa, but when the molecule binds to human albumin after administration, the molecular weight increases to approximately 80 kDa. For radionuclide therapy one of the main issues is high renal uptake. The association to the albumin binding domain is expected to reduce glomerular filtration, facilitate reabsorption back to circulation, and promote a favorable biodistribution suitable to radiotherapy. The inventive polypeptide or fusion protein as disclosed herein surprisingly provides several technical improvements over the prior art, such as an improved biodistribution profile, wherein there is a reduced uptake of the H2ABPP1 in liver and spleen as well as a favorable tumor uptake of H2ABPP1. The utilization of the albumin binding domain as introduced in SEQ ID NO:11 and 12, is also expected to reduce immunogenicity when compared to other molecules, such as ABDwt or ABD035.

In embodiments in which the polypeptide, or fusion protein is labeled with an imaging agent (e.g. radioactive agent), measuring the amount of labeled polypeptide present in a tissue, such as in a tumor tissue, may be done using imaging equipment, such as through acquiring radioactivity counts or images of radiation density, or derivatives thereof such as radiation concentration. In one embodiment, there is provided a radiolabeled HER2-binding polypeptide or fusion protein consisting of a radiochelate of the HER2-binding polypeptide or fusion protein as described herein and a radionuclide. In a specific embodiment, said radionuclide is suitable for medical imaging. In this specific embodiment, said radionuclide is selected from the group consisting of ^{72}As , ^{76}Br , ^{55}Co , ^{61}Cu , ^{64}Cu , ^{18}F , $^{18}\text{F}[\text{AlF}]$, ^{19}F , ^{66}Ga , ^{67}Ga , ^{68}Ga , $^{110\text{m}}\text{In}$, ^{111}In , ^{123}I , ^{124}I , ^{131}I , ^{177}Lu , ^{51}Mn , $^{52\text{m}}\text{Mn}$, ^{52}Mn , ^{186}Re , ^{188}Re , ^{44}Sc , ^{149}Tb , ^{152}Tb , ^{155}Tb , ^{161}Tb , $^{99\text{m}}\text{Tc}$, ^{45}Ti , ^{86}Y , and ^{89}Zr , such as from ^{68}Ga , ^{111}In , ^{177}Lu , ^{152}Tb and ^{155}Tb . Thus, in another specific embodiment, said radionuclide is selected from the group consisting of ^{68}Ga , ^{111}In , ^{177}Lu , ^{152}Tb and ^{155}Tb . In a particular embodiment, the radionuclide suitable for medical imaging is ^{177}Lu . In another particular embodiment, the radionuclide suitable for medical imaging is ^{111}In . In another particular embodiment, the radionuclide suitable for

medical imaging is ^{68}Ga . In another specific embodiment, said radionuclide is suitable for therapy. In this specific embodiment, said radionuclide is selected from the group consisting of ^{225}Ac , ^{212}Bi , ^{213}Bi , ^{67}Cu , ^{166}Ho , ^{177}Lu , ^{212}Pb , ^{149}Pm , ^{186}Re , ^{188}Re , ^{153}Sm , ^{149}Tb , ^{161}Tb , ^{227}Th and ^{90}Y , such as ^{225}Ac , ^{227}Th , ^{149}Tb , ^{161}Tb and ^{177}Lu . Thus, in
5 another specific embodiment, said radionuclide is selected from the group consisting of ^{225}Ac , ^{227}Th , ^{149}Tb , ^{161}Tb and ^{177}Lu . In a particular embodiment, the radionuclide suitable for therapy is ^{177}Lu .

When reference is made to a labeled polypeptide, this should be understood
10 as a reference to all aspects of polypeptides as described herein, including HER2-binding polypeptides and fusion proteins comprising a HER2-binding polypeptide. Thus, a labeled polypeptide may contain only the HER2-binding polypeptide and e.g. a radionuclide, which may be chelated or covalently coupled to the HER2-binding polypeptide, or contain the HER2-binding polypeptide, a radionuclide and a second
15 moiety such as a small molecule having a desired biological activity, for example a therapeutic efficacy, or a second moiety having a desired binding activity, for example a half-life extending property. A labeled polypeptide may contain a HER2-binding polypeptide in heterodimeric form and e.g. a radionuclide, which may be chelated or covalently coupled to the HER2-binding polypeptide, or contain the
20 HER2-binding polypeptide in heterodimeric form, a therapeutic radionuclide and a second moiety such as a small molecule having a desired biological activity, for example a therapeutic efficacy. Further, the radionuclide as described herein may serve multiple purposes, i.e. said radionuclide may fulfill both the capacity of a radionuclide suitable for therapy and the capacity of a radionuclide suitable for
25 medical imaging. Thus, the radionuclide labeled molecule is said to be suitable as a theranostic, which is a molecule suitable for both therapy and medical imaging. The term "theranostic" as used herein can be used interchangeably with the term "theragnostic". Furthermore, the skilled person will understand that a theranostic or theragnostic molecule could also be understood as 1) the same molecule, such as a
30 polypeptide or fusion protein, labeled with the same radionuclide for both imaging and therapy as described previously, 2) use of the same molecule, such as a polypeptide or fusion protein, but labeled with different radionuclides for imaging and therapy, and/or 3) use of different molecules, such as different polypeptides or

different fusion proteins, each labeled with different radionuclides for imaging and therapy. This way, the qualities of a theranostic could also be fulfilled, wherein one radionuclide could be suitable for therapy while the other radionuclide could be suitable for medical imaging. The skilled person will be able to create many other possible variants based on the teaching herein. The labeled polypeptide or fusion protein may be suitable for both therapy, and for medical imaging for the purpose of detection and for monitoring of the treatment response. It can be further contemplated, that payloads other than radionuclides could be conjugated via a cysteine residue, such as cytotoxic payloads. Some examples of cytotoxic payloads that can be conjugate to cysteine residues are: maytansinoids, such as DM1 and DM4; auristatins, such as monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF); DNA-damaging agents such as duocarmycins, pyrrolobenzodiazepines (PBDs), and calicheamicins; camptothecins, such as SN-38; taxanes; tubulysins; STING-agonists; TLR-agonists, Topoisomerase II inhibitors; small interfering RNA; antisense oligonucleotides; and protein degrader payloads.

A further aspect of the disclosure provides a composition comprising a HER2-binding polypeptide of an aspect or a fusion protein of another aspect or a radiolabeled polypeptide or fusion protein of a further aspect, and at least one pharmaceutically acceptable excipient or carrier. Said composition may suitably be formulated for a purpose selected from storage, transport, lyophilization, concentration, or any combination thereof. Suitably, said at least one pharmaceutically acceptable excipient or carrier is selected to enable or enhance the administration of radiolabeled polypeptide to a subject to be treated or imaged. In a particular embodiment, the composition according to this aspect is suitable for use as a medicament, a diagnostic agent *in vivo* or a prognostic agent *in vivo* without further manipulation. In another embodiment, said composition is suitable for lyophilization. Lyophilization of a composition according to this embodiment yields a lyophilized powder of said polypeptide. Said lyophilized powder of the polypeptide constitutes a further aspect of the disclosure, and may offer advantages in terms of transport, storage, handling and radiolabeling of the polypeptide when it is intended for use as a medicament or as a diagnostic or prognostic agent *in vivo*. The person of skill in the art of pharmaceutical compositions and formulation is able to select the

proper excipients or carriers for a given application of the HER2-binding polypeptide, fusion protein, or radiolabeled polypeptide or fusion protein to create a composition according to this further aspect without undue experimentation.

5 The present disclosure also encompasses different aspects of using the above-described HER2-binding polypeptide, whether radiolabeled or not, and compositions. It also encompasses various methods for treatment, diagnosis and prognosis in which the polypeptide is useful due to its binding characteristics. When referring to the "HER2-binding polypeptide" in the following description of these uses and methods, this term is intended to encompass the HER2-binding
10 polypeptide alone, but also all those molecules based on this polypeptide described above that e.g. incorporate the HER2-binding polypeptide as a moiety in a fusion protein and/or are conjugated to a label, a chelator, a therapeutic and/or diagnostic agent and/or are provided with additional amino acid residues as a tag or for other purposes. As explained above, such fusion proteins, derivatives etc. form a part of
15 the present disclosure. The "HER2-binding polypeptide" is also intended to encompass the compositions of the polypeptide, the fusion protein or the radiolabeled polypeptide or fusion protein according to the above-mentioned aspect.

20 Thus, in a further aspect, there is provided a method for *in vivo* imaging of the body of a mammalian, including human, subject having or suspected of having a cancer characterized by overexpression of HER2, comprising the steps:

- administering a radiolabeled polypeptide or fusion protein as described herein, optionally comprised in a composition as described herein, into the
25 body of the mammalian subject; and
- obtaining one or more images of at least a part of the subject's body using a medical imaging instrument, said image(s) indicating the presence of the radionuclide inside the body.

In one embodiment, there is provided a method as described herein, comprising,
30 before the administration step, a step of preparing a radiolabeled polypeptide or fusion protein as described herein, which step comprises mixing the polypeptide or fusion protein as described herein with the radionuclide suitable for medical imaging. In a further embodiment, there is provided a method as described herein,

wherein said mixing is carried out at a pH value in the range of pH 6.0 – 7.0, such as carried out at a pH value selected from the group consisting of pH 6.0, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH 6.8, pH 6.9 and pH 7.0. In a further embodiment, said mixing is carried out at a pH value in the range of 6.3 – 6.7, such as carried out at a pH value selected from the group consisting of pH 6.3, pH 6.4, pH 6.5, pH 6.6 and pH 6.7. In a more specific embodiment, said mixing is carried out at a pH value of 6.5.

In one embodiment, there is provided a method of diagnosing a cancer characterized by overexpression of HER2, comprising

- performing the method of *in vivo* imaging as described herein, and
- establishing said diagnosis on the basis of the obtained images.

In another related embodiment, there is provided a method of establishing prognosis for a cancer characterized by overexpression of HER2, comprising

- performing the method of *in vivo* imaging as described herein, and
- establishing said prognosis on the basis of the obtained images.

In another related embodiment, there is provided a method of monitoring response to treatment of a cancer characterized by overexpression of HER2, comprising

- performing the method of *in vivo* imaging as described herein, and
- establishing said treatment response on the basis of the obtained images.

In a further embodiment, there is provided a method of diagnosing a cancer, a method of establishing prognosis for a cancer or a method of monitoring treatment response as described herein, wherein the cancer is selected from breast cancer, ovarian cancer, gastric cancer, colorectal cancer, prostate cancer, bladder cancer, salivary cancer, lung cancer and cancer in the esophagus. In a more specific embodiment, there is provided a method of diagnosing a cancer, a method of establishing prognosis for a cancer or a method of monitoring treatment response as described herein, wherein said cancer is breast cancer.

In a related aspect, there is provided a method of treatment of a mammalian, including human, subject having a cancer characterized by overexpression of HER2, comprising the step of administering a therapeutically effective amount of a radiolabeled polypeptide or fusion protein as described herein, optionally comprised
5 in a composition as described herein, to said subject. In one embodiment, there is provided a method of treatment as described herein, comprising, before the administration step, a step of preparing a radiolabeled polypeptide or fusion protein as described herein, which step comprises mixing the polypeptide or fusion protein as described herein with the radionuclide suitable for therapy. In a further
10 embodiment, there is provided a method of treatment as described herein, wherein said mixing is carried out at a pH value in the range of pH 6.0 – 7.0, such as carried out at a pH value selected from the group consisting of pH 6.0, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH 6.8, pH 6.9 and pH 7.0. In a further embodiment, said mixing is carried out at a pH value in the range of 6.3 – 6.7, such
15 as carried out at a pH value selected from the group consisting of pH 6.3, pH 6.4, pH 6.5, pH 6.6 and pH 6.7. In a more specific embodiment, said mixing is carried out at a pH value of 6.5. In a further embodiment, there is provided a method of treatment as described herein, wherein the cancer is selected from breast cancer, ovarian cancer, gastric cancer, colorectal cancer, prostate cancer, bladder cancer, salivary
20 cancer, lung cancer and cancer in the esophagus. In a more specific embodiment, there is provided a method of treatment wherein said cancer is breast cancer.

In a related aspect, there is provided a HER2-binding polypeptide or fusion protein as described herein or composition as described herein, for use as a medicament. In
25 one embodiment, there is provided a HER2-binding polypeptide or fusion protein or composition for use as a medicament, wherein said use is in the method of treatment as described herein.

In another related aspect, there is provided a HER2-binding polypeptide or fusion
30 protein or composition as described herein for use as a diagnostic agent *in vivo*. In one embodiment, there is provided a HER2-binding polypeptide or fusion protein or composition for use as a diagnostic agent *in vivo*, wherein said use is in the method of diagnosing a cancer characterized by overexpression of HER2 as described herein.

In a closely related aspect, there is provided a HER2-binding polypeptide or fusion protein or composition as described herein for use as a prognostic agent *in vivo*. In one embodiment, there is provided a HER2-binding polypeptide or fusion protein or
5 composition for use as a prognostic agent *in vivo*, wherein said use is in the method of establishing a prognosis for a cancer characterized by overexpression of HER2 as described herein.

In a further closely related aspect, there is provided a HER2-binding polypeptide or
10 fusion protein or composition as described herein for use as a monitoring agent *in vivo*. In one embodiment, there is provided a HER2-binding polypeptide or fusion protein or composition for use as a monitoring agent *in vivo*, wherein said use is in the method of monitoring treatment response for a cancer characterized by overexpression of HER2 as described herein.

15 In another related aspect, there is provided a radiolabeled polypeptide or fusion protein as described herein for use as a diagnostic agent *in vivo*. In one embodiment, there is provided a radiolabeled polypeptide or fusion protein as described herein for use as a diagnostic agent *in vivo*, wherein said use is in the method of diagnosing
20 a cancer characterized by overexpression of HER2 as described herein.

In another closely related aspect, there is provided a radiolabeled polypeptide or fusion protein as described herein for use as a prognostic agent *in vivo*. In one embodiment, there is provided a radiolabeled polypeptide or fusion protein as
25 described herein for use as a prognostic agent *in vivo*, wherein said use is in the method of establishing a prognosis for a cancer characterized by overexpression of HER2 as described herein.

In another closely related aspect, there is provided a radiolabeled polypeptide or
30 fusion protein as described herein for use as a monitoring agent *in vivo*. In one embodiment, there is provided a radiolabeled polypeptide or fusion protein as described herein for use as a monitoring agent *in vivo*, wherein said use is in the

method of monitoring treatment response for a cancer characterized by overexpression of HER2 as described herein.

5 In another related aspect, there is provided a radiolabeled polypeptide or fusion protein as described herein for use as a medicament. In one embodiment, there is provided a radiolabeled polypeptide or fusion protein as described herein for use as a medicament, wherein said use is in a method of treatment as described herein.

10 In a related aspect, there is provided a composition as described herein for use as a diagnostic agent *in vivo*. In one embodiment, there is provided a composition as described herein for use as a diagnostic agent *in vivo*, wherein said use is in the method of diagnosing a cancer characterized by overexpression of HER2 as described herein.

15 In a closely related aspect, there is provided a composition as described herein for use as a prognostic agent *in vivo*. In one embodiment, there is provided a composition as described herein for use as a prognostic agent *in vivo*, wherein said use is in the method of establishing a prognosis for a cancer characterized by overexpression of HER2 as described herein.

20 In another closely related aspect, there is provided a composition as described herein for use as a monitoring agent *in vivo*. In one embodiment, there is provided a composition as described herein for use as a monitoring agent *in vivo*, wherein said use is in the method of monitoring treatment response for a cancer characterized by overexpression of HER2 as described herein.

25 In another related aspect, there is provided a composition as described herein for use as a medicament. In one embodiment, there is provided a composition as described herein for use as a medicament, wherein said use is in a method of treatment as described herein.

Another aspect of the disclosure relates to a nucleic acid encoding the polypeptide or fusion protein as described herein.

A further aspect of the disclosure relates to an expression vector comprising the nucleic acid of the previous aspect.

Yet another aspect of the disclosure relates to a host cell comprising the expression vector of the previous aspect.

5

The latter three aspects of the invention are tools for the production of a polypeptide according to the invention, and the skilled person will be able to obtain them and put them into practical use without undue burden, given the information herein concerning the polypeptide that is to be expressed and given the current
10 level of skill in the art of recombinant expression of proteins. The polypeptide according to the disclosure may also be produced by other known means, including chemical synthesis or expression in different prokaryotic or eukaryotic hosts, including plants and transgenic animals. Thus, in a further aspect, the disclosure provides a method of producing a HER2-binding polypeptide or fusion protein as
15 described herein, comprising:

- culturing a host cell as described herein under conditions permissive of expression of said polypeptide from said expression vector, and
- isolating said polypeptide or fusion protein.

20 In another aspect, the disclosure further provides a method of producing a HER2-binding polypeptide or fusion protein as described herein by non-biological peptide synthesis using amino acids and/or amino acid derivatives having protected reactive side-chains, the non-biological peptide synthesis comprising:

- step-wise coupling of the amino acids and/or the amino acid derivatives to
25 form a polypeptide or fusion protein as described herein having protected reactive side-chains,
- removal of the protecting groups from the reactive side-chains of the polypeptide or fusion protein, and
- folding of the polypeptide or fusion protein in aqueous solution.

30

In a further aspect, the disclosure further provides a method of producing a radiolabeled polypeptide or fusion protein as described herein, which step comprises mixing the polypeptide or fusion protein as described herein or the

polypeptide or fusion protein produced according to either a method comprising culturing a host cell as described herein, or a method comprising non-biological peptide synthesis as described herein, with a radionuclide. In another further aspect, the disclosure further provides a method of producing a radiolabeled polypeptide or fusion protein as described herein, which step comprises mixing the polypeptide or fusion protein as described herein with a radionuclide. In one embodiment, said mixing is carried out at a pH value in the range of pH 6.0 – 7.0, such as carried out at a pH value selected from the group consisting of pH 6.0, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH 6.8, pH 6.9 and pH 7.0. In a further embodiment, said mixing is carried out at a pH value in the range of 6.3 – 6.7, such as carried out at a pH value selected from the group consisting of pH 6.3, pH 6.4, pH 6.5, pH 6.6 and pH 6.7. In a specific embodiment, said mixing is carried out at a pH value of 6.5.

While the invention has been described with reference to various exemplary aspects and embodiments, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the scope of the invention. In addition, many modifications may be made to adapt a particular situation or molecule to the teachings of the invention without departing from the essential scope thereof. Therefore, it is intended that the invention not be limited to any particular embodiment contemplated, but that the invention will include all embodiments falling within the scope of the appended claims.

The different aspects of the disclosure will now be illustrated in detail through the description of experiments conducted in accordance therewith. The examples to follow are not to be interpreted as limiting. In the examples, reference is made to the appended figures.

Brief description of the figures

Figure 1 shows the result of SDS-PAGE analysis. Purified H2ABPP1 is seen in lane 3 (non-reduced sample) and lane 7 (reduced sample). (Lane 2 and Lane 6 are irrelevant for the invention described herein).

5 **Figure 2** shows the RP-UPLC integrated 220 nm chromatogram from injection of purified H2ABPP1. Unconjugated Cys-Cys dimer is indicated as peak B and constituted approximately 4% of the main peak A.

10 **Figure 3** is an image capture of the IEF gel characterizing the isoelectric point (pI) of DOTA conjugated H2ABPP1. Lane 1: IEF marker (Serva, cat. no. 39212); Lane 3: H2ABPP1.

Figure 4 shows circular dichroism (CD) spectra. (A) CD spectra collected before (broken line) and after (solid line) heat-induced denaturation of H2ABPP1 and (B) melting curve of H2ABPP1.

15 **Figure 5** shows SE-HPLC analyses. (A) Overlay of 220 nm chromatogram from injections of the GF-standard mixture (670 and 158 kDa, co-elution at 2.129 min; 44 kDa, elution at 2.755 min; 17 kDa elution at 3.469 min; and 1.35 kDa elution at 4.946) and the 0.2 M NH₄Ac pH 6.0 buffer. (B) 220 nm chromatogram from the injection of H2ABPP1, which elutes at 3.263 min (indicated with an arrow), i.e. slightly earlier than the 17 kDa standard shown in Figure 5A.

20 **Figure 6** shows sensorgrams depicting the interaction of H2ABPP1 with (A) human HER2 in an MCK analysis and with (B) HSA and (C) MSA in SCK analyses. Injected concentrations of H2ABPP1 were 10, 3.3, 1.1, 0.4 and 0.1 nM and in (B) and (C) also 0.04 nM. Gray lines represent measured responses and black lines shows fitted curves using a 1:1 Langmuir interaction model.

25 **Figure 7** shows sensorgrams depicting the interaction of H2ABPP1 with human HER2 in (A) absence or (B) presence of 100 nM HSA and (C) 900 nM MSA. Injected concentrations of H2ABPP1 were 10, 3.3, 1.1, 0.4 and 0.1 nM in (A) and (B) and 90, 30, 10, 3.3 and 1.1 nM in (C). Gray lines represent measured responses and black lines shows fitted curves using a 1:1 Langmuir interaction model.

30 **Figure 8** shows the result of *in vitro* specificity test for ¹⁷⁷Lu-H2ABPP1 (A and B) and reference fusion proteins ¹⁷⁷Lu-H2ABPP2 (C and D) and ¹⁷⁷Lu-H2ABPP3 (E and F) on SKOV3 and BT474 cells in the presence (A, C, E) and in the absence (B, D, F) of HSA. The data are presented as an average value from 3 samples ± SD.

Figure 9 shows LigandTracer sensorgrams of (A) ^{177}Lu -H2ABPP1, and reference fusion proteins (C) ^{177}Lu -H2ABPP2 and (E) ^{177}Lu -H2ABPP3 binding to HER2-expressing SKOV3 cells in the presence of HSA, and corresponding InteractionMaps for (B) ^{177}Lu -H2ABPP1, (D) ^{177}Lu -H2ABPP2 and (F) ^{177}Lu -H2ABPP3. Binding was measured at three concentrations of radiolabeled fusion protein 0.25 nM, 0.75 nM and 2.25 nM. Data are representatives from duplicates.

Figure 10 shows binding and cellular processing of ^{177}Lu -H2ABPP1 (A and B), and reference fusion proteins ^{177}Lu -H2ABPP2 (C and D) and ^{177}Lu -H2ABPP3 (E and F) HER2-expressing SKOV3 (A, C and E) and BT474 (B, D, F) cells in the presence of HSA. Data are presented as an average ($n = 3$) value $\pm$ SD.

Figure 11 shows the biodistribution of ^{177}Lu -H2ABPP1, and reference fusion proteins ^{177}Lu -H2ABPP2, ^{177}Lu -H2ABPP3 and ^{177}Lu -H2ABPP4 in Balb/c nu/nu mice bearing SKOV3 xenografts at (A) 48 h and (B) 264 h post injection. Data for GI tract are presented as %ID per whole sample. Data are presented as an average ($n = 4$) value $\pm$ SD.

Figure 12 shows P-values of pairwise comparison of uptake of the tested fusion proteins in tumor and some critical organs: (A) tumor (B) blood, (C) bone, (D) kidneys, (E) liver and (F) spleen, 48 h after administration to Balb/c nu/nu mice bearing SKOV3 xenografts. Data are presented as an average ($n = 4$) value $\pm$ SD. P-values are calculated using one-way ANOVA. Significant differences are highlighted in gray.

Figure 13 shows P-values of pairwise comparison of uptake of the tested fusion proteins in tumor and some critical organs: (A) tumor (B) blood, (C) bone, (D) kidneys, (E) liver and (F) spleen, 264 h after administration to Balb/c nu/nu mice bearing SKOV3 xenografts. Data are presented as an average ($n = 4$) value $\pm$ SD. P-values are calculated using one-way ANOVA. Significant differences are highlighted in gray.

Figure 14 shows the results from the *in vivo* specificity test of ^{177}Lu -H2ABPP1 evaluated by comparing the biodistribution in HER2-positive SKOV3 and HER2-negative Ramos xenografts in Balb/c nu/nu mice 48 h after injection. Results of *ex vivo* measurements are presented as % ID/g $\pm$ SD ($n = 4$).

Figure 15 shows imaging of Balb/c nu/nu mice bearing SKOV3 and Ramos xenografts 48 h after injection of ^{177}Lu -H2ABPP1. The scale is linear showing arbitrary units normalized to a maximum count rate.

Figure 16 shows biodistribution of ^{177}Lu -H2ABPP1 in Balb/c nu/nu mice bearing HER2-expressing SKOV3 xenografts 4-336 h post administration. Data are presented as an average ($n = 4$) value $\pm$ SD.

Figure 17 shows an overview of timing of interventions in the experimental therapeutic study performed in mice.

Figure 18 shows tumor growth curves for individual mice from five groups receiving: (A) vehicle (1% BSA in PBS), (B) trastuzumab, (C) a single injection of ^{177}Lu -H2ABPP1 (10 μg , 21 MBq), (D) two injections of ^{177}Lu -H2ABPP1 (10 μg , 21 MBq each, four weeks apart), and (E) a single injection of ^{177}Lu -H2ABPP1 (10 μg , 21 MBq) in combination with trastuzumab. The doses and frequency of trastuzumab injections were 4 mg/kg the first week and 2 mg/kg for the next five consecutive weeks.

Figure 19 shows the survival of mice treated with vehicle (1% BSA in PBS), trastuzumab, a single injection ^{177}Lu -H2ABPP1, double injections of ^{177}Lu -H2ABPP1, and a combination of ^{177}Lu -H2ABPP1 and trastuzumab. The doses were as described for Figure 18.

20 Examples

The following examples disclose the generation and characterization of a novel HER2 targeted fusion protein, H2ABPP1 (SEQ ID NO:12), as well as H2ABPP1 coupled to mal-DOTA, both comprising the amino acid sequence of a novel HER2 targeted polypeptide as defined in SEQ ID NO:1. H2ABPP1 (SEQ ID NO:12), a fusion protein, was engineered with a cysteine residue situated at the C-terminal end of the fusion protein and coupled to the chelator mal-DOTA. The *in vitro* and *in vivo* properties of radiolabeled H2ABPP1 (SEQ ID NO: 12) were compared with the properties of three radiolabeled reference HER2-binding fusion proteins, H2ABPP2 (SEQ ID NO:13), H2ABPP3 (SEQ ID NO:14) and H2ABPP4 (SEQ ID NO:15). All four fusion proteins are identical with regard to the amino acid residues comprising the HER2 targeted polypeptide as defined by the amino acids 1-50 in SEQ ID NO:1, and thus identical in amino acid residues interacting with HER2, but the novel fusion protein H2ABPP1 (SEQ ID NO:12) differs in linker and/or scaffold residues compared to H2ABPP2 (SEQ

ID NO:13), H2ABPP3 (SEQ ID NO:14), and H2ABPP4 (SEQ ID NO:15). Because changes in the linker and/or scaffold might be associated with undesirable changes in properties, for example affect radiolabeling as well as changes in biodistribution and imaging properties, a clinical translation of a new radiolabeled fusion protein requires a new preclinical evaluation. For the purpose of such evaluation, radiolabeling was here made with ^{177}Lu . Importantly, the Examples surprisingly showed that the hepatic uptake of H2ABPP1, was considerably reduced compared to the hepatic uptake of H2ABPP4, as was the uptake in spleen. Furthermore, an increased tumor uptake of H2ABPP1 was observed in comparison to H2ABPP4. The Examples also surprisingly showed that the survival time of mice treated with a single dose of H2ABPP1 labeled with ^{177}Lu was longer than for mice treated with vehicle or the HER2 targeted monoclonal antibody trastuzumab. To conclude, favorable *in vitro* and *in vivo* properties, such as HER2-binding, biodistribution and tumor-targeting, were demonstrated for H2ABPP1, making this novel fusion protein suitable for use in diagnostic, prognostic, therapeutic, theranostic, and monitoring applications.

Example 1

Production of H2ABPP1

20 *Materials and methods*

The gene encoding H2ABPP1 (SEQ ID NO:12) was cloned in a T7 promoter driven expression vector and transformed into *E. coli* T7E2 electrocompetent cells. H2ABPP1 was expressed in autoinducing medium (Overnight Express TB, Novagen, supplemented with 50 mg/L neomycin) inoculated with precultures. The culture was incubated at 37°C and 150 rpm for 16 h before the cells were harvested by centrifugation. The cells were disrupted by sonication. Purification was performed by affinity chromatography using a resin comprising an immobilized anti-ABD ligand (*in-house* developed) followed by Reverse Phase Chromatography (RPC) using a 3 mL Resource RPC column (Cytiva). The RPC eluate was buffer exchanged to endotoxin free 1 × DPBS (Corning) supplemented with 10 mM DTT, using PD-10 columns (Cytiva). Concentration of the sample was performed by centrifugation using Amicon Ultra-15 Ultracel 3K Centrifugal filter (Merck Millipore). For conjugation experiments, the sample was buffer exchanged to Chelex® treated buffer, 0.2 M

NH₄Ac, pH 6.0, using PD-10 columns. Conjugation with mal-DOTA was performed by addition of ~3.4 × molar excess of maleimido-mono-amide-DOTA (mal-DOTA; Macrocyclics, cat. no. B-272) and incubation at 22 °C for 1 h and 600 rpm. Excess mal-DOTA was removed by buffer exchange to Chelex® treated buffer, 0.2 M NH₄Ac, pH 6.0, using PD-10 columns.

Protein concentrations were determined by absorbance measurements at 280 nm. The purity of mal-DOTA conjugated H2ABPP1 (SEQ ID NO:12) was analyzed by SDS-PAGE stained with Coomassie Blue and RP-UPLC-MS analysis.

10 *Results*

H2ABPP1 (SEQ ID NO:12) was successfully produced and purified. The expression level was estimated to 20 mg product per gram pellet with the product expressed in a soluble form. SDS-PAGE analysis of mal-DOTA conjugated H2ABPP1 (SEQ ID NO:12) showed that the sample contained a small fraction of unconjugated peptide in dimeric form when non-reduced sample was analyzed, but in monomeric form when the sample was reduced (Figure 1). Thus, the dimer formation is reversible. No other product related impurities were observed on the SDS-PAGE gel. The fraction of unconjugated H2ABPP1 was determined to 4% by RP-UPLC and the purity of the batch was determined to 95% (Figure 2). Unless otherwise stated, all subsequent analyses were performed using mal-DOTA conjugated H2ABPP1 (SEQ ID NO:12).

Example 2

Characterization of H2ABPP1

Materials and methods

25 Isoelectric Focusing (IEF): The pI of H2ABPP1 was determined by IEF using Novex™ pH 3-10 IEF Protein Gels (Invitrogen, cat. no. EC6655BOX).

Circular Dichroism (CD) measurement: H2ABPP1 was prepared in 1 × DPBS at a concentration of 0.5 mg/mL. A CD spectrum at 250–195 nm was obtained at 20°C. In addition, a variable temperature measurement (VTM) was performed. In the VTM, the absorbance was measured at 221 nm while the temperature was raised from 20°C to 90°C, with a temperature slope of 5°C/min. A new CD spectrum was obtained at 20°C after the heating procedure, in order to study the refolding ability of H2ABPP1. The CD measurements were performed on a Jasco J-810

spectropolarimeter (Jasco Scandinavia AB) using a cell with an optical path length of 1 mm.

Size exclusion high performance liquid chromatography (SE-HPLC): H2ABPP1 at a concentration of 0.5 mg/mL was analyzed on a Superdex 75 Increase, 5/150 GL column (Cytiva). 1 × DPBS was used as running buffer and the flow rate was 0.5 mL/min. The 220 and 280 nm signals were monitored. A blank (20 µL 0.2 M NH₄Ac pH 6.0) and GF-standard mixture (670, 158, 44, 17 and 1.35 kDa; Bio-Rad) were also analyzed.

10 *Results*

IEF: The IEF analysis determined the pI of H2ABPP1 to 5.2, and charge variants in proximity to the isoelectric point of H2ABPP1 were observed (Figure 3).

CD measurement: The reversible folding ability of H2ABPP1 after heating to 90 °C was analyzed by circular dichroism spectroscopy. The superimposed spectra measured before and after the variable temperature measurement show that H2ABPP1 refolds reversible after heating to 90 °C, i.e., the alpha helical structure is retained when cooling down to 20 °C after the heating (Figure 4A). The melting curve from the variable temperature measurement at 221 nm is shown in Figure 4B. However, since H2ABPP1 is a fusion protein comprising one Z moiety and one ABD moiety having different melting temperatures, no clear transition is obtained and the melting temperature (T_m) of H2ABPP1 can therefore not be determined.

SE-HPLC: Chromatograms recorded at 220 nm of reference standard and H2ABPP1 are shown in Figure 5A and 5B, respectively. H2ABPP1 elutes at 3.262 min, slightly earlier than the retention time for the 17 kDa standard. Thus, H2ABPP1 with a molecular weight of 12,60 kDa appears to eluate as a dimer rather than as a monomer in this SE-HPLC analysis. No peaks were observed before the elution of the H2ABPP1 main peak, i.e. no higher aggregates of H2ABPP1 were detected.

Example 3

Binding of H2ABPP1 to HER2 and albumin

Materials and methods

Kinetic binding analyses were performed by surface plasmon resonance (SPR) measurements using a Biacore 8K instrument (Cytiva). The Biacore Insight Evaluation

software and use of the Langmuir 1:1 binding model was applied for determination of kinetic constants; the associate rate constant (k_a), dissociation rate constant (k_d) and dissociation constant (K_D).

- 5 Binding of H2ABPP1 to HER2: A multicycle kinetics (MCK) assay was used for analysis. H2ABPP1 was diluted in HBS-EP+ (Cytiva) to 10, 3.3, 1.1, 0.4 and 0.1 nM, and injected at a flowrate of 100 μ L/min for 120 s over a chip surface with immobilized HER2 (Sino Biological, cat. no. 10004-H08H), followed by a dissociation for 900 s. A 30 s injection of 25 mM HCl buffer at 30 μ L/min was performed for surface regeneration between each analyte injection.
- 10 Binding of H2ABPP1 to HSA and MSA: A single cycle kinetics (SCK) assay was used for analysis. H2ABPP1 was diluted in HBS-EP+ to 10, 3.3, 1.1, 0.4, 0.1 and 0.04 nM, and injected at a flowrate of 50 μ L/min for 240 s over a chip surface with immobilized HSA (Albumedix, cat. no. 205-005) and MSA (Sigma Aldrich, cat. no. A3559), respectively, followed by a dissociation for 7200 s. Two 30 s injections of
- 15 regeneration buffer were performed for surface regeneration between analyte injections.

- Binding of H2ABPP1 to HER2 +/- HSA and MSA: An MCK assay was used for analysis. H2ABPP1 was assessed for binding to immobilized HER2 in the absence or presence of HSA or MSA, respectively. H2ABPP1 was diluted to 10, 3.3, 1.1, 0.4 and 0.1 nM in
- 20 HBS-EP+ with or without of 100 nM HSA (Recombunin Prime, cat. no. Albumedix, 200-010) or diluted to 90, 30, 10, 3.3 and 1.1 nM with or without 900 nM MSA. A pre-analyte contact time of 60 s, a contact time of 250 s and a post-analyte contact time of 450 s were used at a flow rate of 30 μ L/min. A 30 s injection of 25 mM HCl buffer at 30 μ L/min was performed for surface regeneration between each analyte
- 25 injection.

Results

- Obtained sensorgrams for binding of H2ABPP1 to HER2, HSA and MSA, respectively are displayed in Figure 6A-C and the corresponding kinetic values presented in Table
- 30 1. The results of binding of H2ABPP1 to HER2 assessed in absence or presence of HSA and MSA, respectively are shown in Figure 7A-C and the corresponding kinetic values presented in Table 2. The H2ABPP1 binding affinities (K_D values) were determined to 340 pM, 25 pM and 360 pM for binding to HER2, HSA and MSA,

respectively. Pre-incubation of H2ABPP1 with HSA or MSA showed retained binding to HER2, but with an effect on the binding affinity. The calculated affinities in the presence of HSA and MSA were determined to 1 and 10 nM, respectively, approximately 5- and 50-times weaker, than in the absence of albumin. This is
5 mainly due to a slower on-rate.

Table 1. Kinetic data of H2ABPP1 binding to human HER2, HSA and MSA, respectively.

Immobilized target	k_a (1/Ms)	k_d (1/s)	K_D (M)
HER2	1.5×10^6	5.1×10^{-4}	3.4×10^{-10}
HSA	1.1×10^6	2.8×10^{-5}	2.5×10^{-11}
MSA	2.2×10^6	8.0×10^{-4}	3.6×10^{-10}

10 Table 2. Kinetic data of H2ABPP1 binding to human HER2 in the absence or presence of HSA.

Analyte	k_a (1/Ms)	k_d (1/s)	K_D (M)
H2ABPP1	3×10^6	5×10^{-4}	2×10^{-10}
H2ABPP1 + HSA	5×10^5	7×10^{-4}	1×10^{-9}
H2ABPP1 + MSA	6×10^4	6×10^{-4}	1×10^{-8}

Example 4

Radiolabeling of H2ABPP1 and reference fusion proteins

15 This Experiment describes the radiolabeling of H2ABPP1 and the reference fusion proteins H2ABPP2 (SEQ ID NO:13), H2ABPP3 (SEQ ID NO:14) and H2ABPP4 (SEQ ID NO:15) with ¹⁷⁷Lu. H2ABPP2 and H2ABPP3 was produced essentially as described in Example 1 for H2ABPP1, and H2ABPP4 was produced as described previously (Orlova
et al. 2013, Nucl Med. 54:961-968).

20

Materials and methods

Labeling with ¹⁷⁷Lu: 30 µL of 0.2 M ammonium acetate (pH 6.0) was added to 20 µg of mal-DOTA conjugated polypeptide. The mixture was incubated with [¹⁷⁷Lu]LuCl₃ (Curium Pharma; 30-35 MBq) at 65°C for 30 min. Purification was performed using
25 NAP-5 columns, pre-equilibrated and eluted with 1% BSA in PBS. The radiochemical

yield and purity were analyzed by instant thin layer chromatography (ITLC-SG) (Agilent Technologies) and validated by radio-HPLC. To evaluate label stability, aliquots of fresh radiolabeled fusion protein were incubated with a 500-fold molar excess of EDTA at 37°C for 60 min. Incubation was also performed in PBS as a control. Samples were evaluated in triplicate.

Results

The labeling of H2ABPP1 (SEQ ID NO:12), H2ABPP2 (SEQ ID NO:13), H2ABPP3 (SEQ ID NO:14) and H2ABPP4 (SEQ ID NO:15) with ^{177}Lu resulted in a yield of 85.2%, 74.7%, 90.0% and 98%, respectively, after 30 min of incubation. Purification of H2ABPP1, H2ABPP2 and H2ABPP3 using NAP-5 columns provided a radiochemical purity of 99% for each radiolabeled compound (H2ABPP4 was used without further purification). After incubation with a 500-fold molar excess of EDTA for 1 h, the radiochemical purity for H2ABPP1, H2ABPP2 and H2ABPP3 was $99 \pm 0\%$, $97 \pm 2\%$, and $98 \pm 1\%$, respectively. Same result, within error, was obtained for samples incubated in PBS.

Example 5

In vitro studies of radiolabeled H2ABPP1 and reference fusion proteins

This experiment describes *in vitro* binding specificity and binding affinity studies of radiolabeled H2ABPP1 (SEQ ID NO:12) and radiolabeled reference fusion proteins H2ABPP2 (SEQ ID NO:13) and H2ABPP3 (SEQ ID NO:14), radiolabeled as described in Example 4, to ovarian carcinoma SKOV3 and breast carcinoma BT474 cells.

Materials and methods

In vitro binding specificity: The HER2 binding specificity of radiolabeled compounds was evaluated using ovarian carcinoma SKOV3 and breast carcinoma BT474 cells (both cell lines were obtained from the American Type Culture Collection, ATCC). Experiments were performed in triplicate. Approximately 1×10^6 cells were seeded per well in 6-well plates the day before the experiment and maintained in 2 mL complete RPMI 1640 medium at 5% CO_2 and 37°C. In three control dishes each, SKOV-3 and BT-474 cells were pre-saturated with a 1000-fold excess of each non-labeled polypeptide for 30 min before addition of labeled conjugate. The cells in

- both pre-saturated and non-saturated dishes were incubated with a labeled conjugate (0.5 nM) for 1 h at 37°C. The medium was collected, and the cells were washed with PBS followed by incubation with trypsin-EDTA solution. The radioactivity in detached cells and media was measured using an automated
- 5 gamma-spectrometer equipped with an NaI (TI) well detector (2480 Wizard, Wallac). To also evaluate the impact of HSA on binding specificity, HSA was added to the complete RPMI 1640 medium to obtain a concentration of 100 nM, and HSA-containing medium was used in the experiment, performed as described above. Data was analyzed by unpaired 2-tailed t-test.
- 10 *In vitro* binding affinity: The binding kinetics of the ^{177}Lu -labeled fusion proteins binding to SKOV3 cells were measured using a LigandTracer Yellow instrument (Ridgeview Instruments) as previously described (Björke et al., 2006 Appl. Radiat. Isot., 64:901–905). SKOV-3 cells were seeded on a local area of a cell culture dish (Nunclon™, Size 100620, NUNC A/S). The measurements were performed in
- 15 duplicates at three concentrations, 0.25 nM, 0.75 nM and 2.25 nM, of each radiolabeled fusion protein. To investigate the influence of HSA on cell binding affinity, the experiment was performed in the presence of 100 nM HSA in the cell medium. The data was analyzed using the Interaction Map software (Ridgeview Diagnostics) to calculate the dissociation constant at equilibrium (K_D).
- 20 Cellular processing: Cellular processing of radiolabeled fusion proteins by HER2-expressing cells were studied in the presence of HSA in SKOV3 and BT474 cell lines. Approximately 1×10^6 cells were seeded per dish (3 cm petri dishes). A set of three dishes was used for each data point. Cells were incubated with a 5 nM solution of radiolabeled fusion proteins at 37°C. At pre-determined time points (1, 2, 4, 6 and 24
- 25 h), a group of three dishes was removed from the incubator, and the medium was collected. The cells were washed with PBS and treated with 0.2 M glycine buffer containing 4 M urea, pH 2.0, for 5 min on ice. The cells were washed with an additional 1 mL glycine buffer, whereafter the acidic fractions were collected, and their radioactivity was measured. This activity was considered as membrane bound.
- 30 After that, cells were incubated with 1 mL of 1 M NaOH at 37°C for 20 min and collected following an additional rinse with 1 mL 1M NaOH. The measured radioactivity in alkaline fractions was considered as an internalized activity.

Results

In vitro binding specificity: The results of the *in vitro* binding specificity of ^{177}Lu -H2ABPP1 and the reference fusion proteins ^{177}Lu -H2ABPP2 and ^{177}Lu -H2ABPP3 are shown in Figure 8. Binding of ^{177}Lu -labeled conjugates to HER2-expressing SKOV3 and BT474 cells was significantly lower ($p < 0.001$) for samples pre-saturated with an excess amount of non-labeled fusion protein, both in the presence and absence of HSA. This result demonstrates that the binding is HER2 mediated.

In vitro binding affinity: The binding of ^{177}Lu -H2ABPP1, and the reference fusion proteins ^{177}Lu -H2ABPP2 and ^{177}Lu -H2ABPP3 to HER2-expressing SKOV-3 cells in the presence of HSA as evaluated by LigandTracer measurements are shown in Figure 9. The InteractionMap analysis for all labeled fusion proteins showed a one-to-two binding with high association and low dissociation rates, resulting in predominant affinity in sub-nanomolar range, Table 3. ^{177}Lu -H2ABPP1 showed the highest affinity (lowest apparent K_D) of the strongest and dominant interaction.

Table 3. Apparent K_D s of ^{177}Lu labeled fusion proteins interacting with HER2 expressing SKOV-3 cells in the presence of HSA.

Analyte	K_{D1} (pM)	Weight (%)	K_{D2} (nM)	Weight (%)
H2ABPP1	400 ± 19	57 ± 1	14.2 ± 0.6	26 ± 0
H2ABPP2	529 ± 23	51 ± 2	10.8 ± 0.1	34 ± 1
H2ABPP3	554 ± 50	53 ± 0	13.6 ± 0.1	31 ± 1

Cellular processing: The results of cellular processing experiment of ^{177}Lu -H2ABPP1, and the reference fusion proteins ^{177}Lu -H2ABPP2 and ^{177}Lu -H2ABPP3 in SKOV3 and BT474 cells in the presence of HSA are shown in Figure 10. All variants demonstrated a rapid binding to HER2-expressing cells during the first 2 hours of incubation. All variants showed slow internalization in SKOV3 and BT474 cells, less than 20% in 24 h.

Example 6

In vivo biodistribution study of radiolabeled H2ABPP1 and reference fusion proteins

This experiment describes *in vivo* biodistribution studies of radiolabeled H2ABPP1 and radiolabeled reference fusion proteins H2ABPP2, H2ABPP3, and H2ABPP4,

labeled as described in Example 4, in mice bearing HER2-positive or HER2-negative xenografts.

Materials and methods

5 Animal handling: Animal experiments were performed in accordance with national legislation for work with laboratory animals. Approval was granted by the Ethical Committee for Animal Research in Uppsala. Four mice per data point were used in biodistribution experiments. HER2-positive and HER2-negative xenografts were established by subcutaneous injection of approximately 10^7 SKOV3 cells or Ramos
10 cells, respectively, in the hind legs of female BALB/C nu/nu mice. Mice were euthanized at pre-determined time points by an intraperitoneal injection of anesthesia, Ketalar-Rompun solution (Ketalar: 10 mg/mL, Rompun: 1 mg/mL). Organs of interest were excised, weighed and their activity was measured. The tissue uptake values were calculated as the percentage of injected dose per gram of
15 the sample (%ID/g).

Biodistribution in tumor-bearing mice: The biodistribution of the labeled inventive fusion protein ^{177}Lu -H2ABPP1, and the labeled reference fusion proteins ^{177}Lu -H2ABPP2, ^{177}Lu -H2ABPP3 and ^{177}Lu -H2ABPP4, respectively, was evaluated in mice bearing SKOV3 xenografts. Eight groups of mice were injected intravenously with
20 ^{177}Lu -labeled fusion proteins in 100 μL of 1% BSA in PBS. The injected activity was 270 kBq/mouse, and the injected amount of protein was adjusted to 10 μg /mouse using corresponding non-labeled compound. The average tumor weight was 0.67 ± 0.31 g. Mice were euthanized at 48 or 264 h post injection (p.i.). The blood, lung, liver, spleen, small intestine wall, kidney, tumor, muscle, bone, gastro-intestine, and
25 the remaining carcass were collected. Organs and tissue samples were weighed, and their radioactivity was measured. Organs and tumor uptake were calculated as the percentage of the injected dose per gram of the sample (% ID/g), while for gastro-intestines and carcass uptake, the percentage of injected dose (% ID) per whole sample was calculated.

30 Dosimetry evaluation: To rank the fusion proteins, a simplified assessment of dosimetry was performed by calculating areas under curves (AUC) by trapezoid method using decay uncorrected uptake values for potentially critical organs, i.e. kidneys, liver and bones, as well as for tumors. This is based on that the absorbed

doses are proportional to residence in a tissue, which is determined by AUC. ^{177}Lu -H2ABPP1 was selected for further studies due to having the best targeting properties (see Tables 4 and 5 in the Result section).

Biodistribution of ^{177}Lu -H2ABPP1 in tumor bearing mice was measured in mice bearing SKOV3 xenografts to obtain the data for dosimetry evaluation. Biodistribution in mice bearing HER2-negative Ramos xenografts was measured 48 h p.i. to check if the accumulation of ^{177}Lu -H2ABPP1 in tumors was HER2-specific. The average animal weight was 18.1 ± 1.6 g at the time of the experiment. The average SKOV-3 tumor weight was 0.4 ± 0.2 g. The average Ramos tumor weight was 0.8 ± 0.4 g. Mice were injected intravenously with $10 \mu\text{g}$ (270 kBq) ^{177}Lu -H2ABPP1 per mouse in $100 \mu\text{L}$ of 1% BSA in PBS. At 4, 24, 48, 72, 168 and 336 h p.i., one group of mice with SKOV3 was euthanized and the biodistribution was measured. For assessment of dosimetry in humans, the murine biodistribution data for ^{177}Lu -H2ABPP1 were upscaled using the “percent kg/g method” according to the following equation:

$$(\%IA/\text{organ})_{\text{human}} = [(\%IA/\text{g})_{\text{animal}} \times (\text{kg}_{\text{TBweight}})_{\text{animal}} \times (\text{g}_{\text{organ}}/(\text{kg}_{\text{TBweight}})_{\text{human}})]$$

as described in Stabin, 2008, Springer, pp. 83–86 and Kirschner *et al.*, 1975, J Nucl Med 16:248–249.

The organ weight from reference adult female (ICRP publication 23) phantom were used for upscaling. The uptake value was fitted by an exponential function and areas under curves were calculated to determine residence times. OLINDA/EXM 1.0 software was used to estimate absorbed doses.

In vivo imaging: *In vivo* imaging was performed to confirm the biodistribution data. One mouse with SKOV3 xenografts and one mouse with Ramos xenografts were injected with 10.5 MBq ($8 \mu\text{g}$) of ^{177}Lu -H2ABPP1. The imaging was performed 48 h p.i. using nanoScan SPECT/CT (Mediso Medical Imaging Systems). The data were reconstructed using Tera-Tomo™ 3D SPECT Software.

Results

Biodistribution in tumor-bearing mice: Results of the comparative biodistribution of the labeled inventive fusion protein ^{177}Lu -H2ABPP1 and the labeled reference fusion

proteins ^{177}Lu -H2ABPP2, ^{177}Lu -H2ABPP3 and ^{177}Lu -H2ABPP4 in Balb/c nu/nu mice bearing SKOV3 xenografts at 48 and 264 h p.i. are shown in Figure 11A and 11B, respectively, with a more detailed comparison in Figure 12 and 13.

At 48 h p.i., there was no significant difference between uptake of the labeled fusion proteins in tumor and in some normal critical organs, like blood and bone (Figure 12A-C). The kidney uptake of ^{177}Lu -H2ABPP1 and the reference fusion proteins ^{177}Lu -H2ABPP2 and ^{177}Lu -H2ABPP3 was significantly (ca. 1.5-fold, $p < 0.05$) higher than the uptake of ^{177}Lu -H2ABPP4 (Figure 12D). However, the uptake of ^{177}Lu -H2ABPP1, ^{177}Lu -H2ABPP2 and ^{177}Lu -H2ABPP3 in liver and spleen was significantly ($p < 0.05$) lower than the uptake of ^{177}Lu -H2ABPP4 (Figure 12E-F).

At 264 h p.i., the tumor uptake of ^{177}Lu -H2ABPP1 was significantly (ca. 1.5-fold, $p < 0.05$) higher than the uptake of ^{177}Lu -H2ABPP4. No significant difference of the tumor uptake was observed between the reference fusion proteins ^{177}Lu -H2ABPP2, ^{177}Lu -H2ABPP3 and ^{177}Lu -H2ABPP4 (Figure 13A). Uptake of all labeled compounds in blood and bone showed no significant difference (Figure 13B-C). In line with the results at 48 h, the kidney uptake of ^{177}Lu -H2ABPP1, ^{177}Lu -H2ABPP2 and ^{177}Lu -H2ABPP3 was significantly (ca. 1.5-fold, $p < 0.05$) higher, and the uptake in liver and spleen was significantly ($p < 0.05$) lower, than the uptake of ^{177}Lu -H2ABPP4 (Figure 13D-F).

Although the kidney uptake of ^{177}Lu -H2ABPP1, ^{177}Lu -H2ABPP2 and ^{177}Lu -H2ABPP3 was higher than of ^{177}Lu -H2ABPP4 it was lower than for Z-ABD fusion variants as well as for corresponding monomeric Z variant as published previously in Liu *et al.* 2021 (*supra*) and Liu *et al.* 2022 (*supra*). More precisely, the renal uptake of ^{177}Lu -ABY-271 was 1.7-fold higher compared to uptake of ^{177}Lu -H2ABPP1 (12.5 ± 1.0 vs 7.5 ± 1.0 %ID/g, p -value = 0.00503 in unpaired t-test) 48 h after injection; the renal uptake of ^{177}Lu -PEP40233 was 3-fold higher (23.2 ± 1.9 vs 7.5 ± 1.0 %ID/g, p -value = 5.8×10^{-6} in unpaired t-test) 48 h after injection.

In vivo specificity and imaging: Results from the *in vivo* specificity test showed that the uptake of ^{177}Lu -H2ABPP1 in HER2-expressing SKOV-3 xenografts in BALB/C nu/nu mice was 9.5-folds ($p < 0.0005$) higher than the uptake in HER2-negative Ramos xenografts, while the accumulation of radioactivity in other organs was the same (Figure 14). This indicates that the *in vivo* accumulation of ^{177}Lu -H2ABPP1 is highly

HER2-specific. The results were confirmed by the imaging performed 48 h after injection of ¹⁷⁷Lu-H2ABPP1 both in SKOV-3 and Ramos xenografts (Figure 15).
Dosimetry evaluation: The results of the simplified assessment of dosimetry are presented in Tables 4 and 5. The data show that ¹⁷⁷Lu-H2ABPP1 provides the largest
5 AUC for tumor and the smallest AUC for liver and bone (which is supposed to give an estimate for a dose to the bone marrow) Table 4. ¹⁷⁷Lu-H2ABPP1 clearly showed the best dose ratios between tumor and liver and tumor and bone and the next best dose ratio between tumor and kidney (Table 5).

10 *Table 4. AUC for ¹⁷⁷Lu-labeled fusion proteins in Balb/c nu/nu mice bearing SKOV3 xenografts.*

Polypeptide	Tumor	Kidney	Liver	Bone
¹⁷⁷ Lu-H2ABPP1	3784	854.5	684.3	178.4
¹⁷⁷ Lu-H2ABPP2	3077	819.7	869.8	198.7
¹⁷⁷ Lu-H2ABPP3	3025	852.7	788.6	182.8
¹⁷⁷ Lu-H2ABPP4	2967	580.2	1378	188.6

Table 5. Ratios between AUC for tumors and critical organs for ¹⁷⁷Lu-labeled fusion proteins in Balb/c nu/nu mice bearing SKOV3 xenografts.

Polypeptide	Tumor-to-organ AUC ratio		
	Kidney	Liver	Bone
¹⁷⁷ Lu-H2ABPP1	4.43	5.53	21.21
¹⁷⁷ Lu-H2ABPP2	3.75	3.54	15.49
¹⁷⁷ Lu-H2ABPP3	3.55	3.84	16.55
¹⁷⁷ Lu-H2ABPP4	5.11	2.15	15.73

15 Additional collected biodistribution data of ¹⁷⁷Lu-H2ABPP1 in Balb/c nu/nu mice bearing HER2-expressing SKOV3 xenografts are presented in Figure 16. A slow clearance of ¹⁷⁷Lu-H2ABPP1 from the blood was observed, with a biological half-life of 23 h. Clearance of ¹⁷⁷Lu-H2ABPP1 from normal organs and tissues followed
20 clearance from the blood. The tumor uptake of ¹⁷⁷Lu-H2ABPP1 was 15 ± 2%ID/g at 24 h p.i.. By this time point, the tumor uptake was already higher than the radioactivity concentration in blood and all other organs and tissues. The uptake of

^{177}Lu -H2ABPP1 in the tumor peaked around 48-72 h after injection, followed by slow washout. At this time point, tumor uptake of ^{177}Lu -H2ABPP1 was 4-fold higher than uptake in kidney.

- For assessment of dosimetry in humans, the murine biodistribution data for ^{177}Lu -H2ABPP1 were upscaled. Estimated absorbed doses after injection of ^{177}Lu -H2ABPP1 in humans are provided in Table 6. According to calculations using OLINDA/EXM 1.0, the highest absorbed doses are expected in heart wall, osteogenic cells, small intestine wall, spleen, liver, and kidneys. The expected effective dose is 0.128 mSv/MBq.

10

Table 6. Calculated absorbed doses of ^{177}Lu -H2ABPP1 in humans using OLINDA/EXM 1.0.

Target	^{177}Lu -H2ABPP1 mGy/MBq	Target	^{177}Lu -H2ABPP1 mGy/MBq
Adrenalin glands	0.065	Skin	0.055
Brain	0.011	Spleen	0.228
Breasts	0.060	Thymus	0.070
Gallbladder wall	0.065	Thyroid	0.058
Lower large intestine wall	0.327	Urinary bladder wall	0.060
Small intestine wall	0.252	Uterus	0.062
Stomach wall	0.074	Total body	0.099
Upper large intestine wall	0.064	Effective dose equivalent (mSv/MBq)	0.228
Heart wall	1.410	Effective dose (mSv/mBq)	0.128
Kidneys	0.209		
Liver	0.251		
Lungs	0.163		
Muscle	0.046		
Ovaries	0.063		
Pancreas	0.058		
Red marrow	0.121		
Osteogenic cells	0.643		

Example 7

In vivo experimental radionuclide therapy study of ^{177}Lu -H2ABPP1

This Example describes an experimental therapeutic study in mice investigating the effect on tumor growth and survival time of animals treated with ^{177}Lu -H2ABPP1, and co-treatment by ^{177}Lu -H2ABPP1 and the HER2-targeting antibody trastuzumab. The H2ABPP1 used in this therapy experiment was radiolabeled essentially as described in Example 4, but at pH 6.5 instead of pH 6.0.

Materials and methods

Female Balb/c nu/nu mice were subcutaneously implanted with SKOV3 cells (approximately 10^7 cells per animal) on the abdomen area. The mice bearing SKOV3 xenografts were randomized into 5 groups of 7-9 mice and the treatment of animals was initiated six days after implantation. By the time of injection, the average tumor volume was $0.16 \pm 0.04 \text{ cm}^3$ and the average mouse weight was $18.2 \pm 0.8 \text{ g}$. Mice with a body weight less than 17 g were excluded from the study.

An overview of timing of interventions in the experimental therapeutic study is presented in Figure 17. In one group, mice were injected i.v. with $10 \mu\text{g}$ (21 MBq) of ^{177}Lu -H2ABPP1. In another group, mice received two injections of ^{177}Lu -H2ABPP1, the first at day 1 and the second at day 29. Animals in the control group received vehicle, 1% BSA in PBS, only. To evaluate the effect of trastuzumab, one group of mice was weekly injected s.c. with trastuzumab (Roche GmbH) with a loading dose of 4 mg/kg for the first week on day 0 and then with 2 mg/kg for five consecutive weeks. To evaluate the effect of combination therapy of trastuzumab and ^{177}Lu -H2ABPP1, one group of mice was injected i.v. with $10 \mu\text{g}$ (21 MBq) of ^{177}Lu -H2ABPP1 on day 1 and with trastuzumab at the same doses and time points as the group injected with trastuzumab alone. Mice were weighted twice a week and tumors were measured using a caliper. The tumor volume (V) was calculated using the formula $V = 1/2 \times (\text{length} \times \text{width}^2)$. Mice were euthanized if their tumor volume exceeded 1000 mm^3 or became ulcerated, or if a mouse lost over 10% of their weight within one week, or more than 15% since the study began. According to ethical permit, all animals had to be euthanized by day 90 after the treatment start. After euthanasia, kidneys, livers and tumors were collected and examined by an

animal pathologist (at BioVet AB veterinary medicine lab, Sollentuna, Sweden) to detect any histopathologic changes.

Results

5 The result of the experimental therapy is presented in Figures 18 and 19. The growth of tumor for individual animals is shown in Figure 18. The last animal in the group receiving vehicle was sacrificed at day 42. Tumor growth in the group of mice treated with trastuzumab followed a similar pattern as in the group receiving vehicle, but with some delay of tumor growth. The last animal in the trastuzumab-
10 treated group was euthanized at day 70, and the median survival time of this group was 45 days, which was significantly longer than that of the group receiving vehicle (38 days, $p < 0.0005$, Mantel-Cox test). Different tumor growth patterns were observed in groups treated with a single or double injection of ^{177}Lu -H2ABPP1 or its combination with trastuzumab, compared with the pattern in the group receiving
15 vehicle. From day 14, the average tumor volume was significantly lower ($p < 0.05$, one-way ANOVA with Bonferroni correction for multiple comparisons) in groups treated with a single or double injection of ^{177}Lu -H2ABPP1, or its combination with trastuzumab, compared to in the group receiving vehicle. The survival time of mice treated with a single injection of ^{177}Lu -H2ABPP1, or a combination treatment with
20 trastuzumab, were both significantly ($p < 0.0005$) longer than the survival time of mice treated with trastuzumab alone. At the time of study termination (day 90), six mice out of seven in the group treated with a single injection of ^{177}Lu -H2ABPP1 and all eight mice in the group treated with ^{177}Lu -H2ABPP1/trastuzumab combination were alive. Complete tumor remission was seen in five mice in the group treated
25 with the combination therapy and in one mouse treated with a single injection of ^{177}Lu -H2ABPP1. In the group treated with double injections of ^{177}Lu -H2ABPP1, two mice were alive at the study termination, whereas the other five mice had to be sacrificed after the second administration due to decline in body weight. This resulted in a 45-days median survival of mice in this group, which is significantly
30 shorter ($p < 0.05$) compared with mice treated with a single injection of ^{177}Lu -H2ABPP1 and with its combination with trastuzumab.

The mice tolerated the experimental therapies well, except for the group of mice treated with double injections of ^{177}Lu -H2ABPP1. No significant difference was observed in the average mice weight between control group and the groups treated with single radionuclide therapy and/or with trastuzumab. The pathology examination concluded the renal and hepatic toxicity of ^{177}Lu -H2ABPP1 to be minimal to mild. More precisely, only minimal to mild changes in the liver and a trend to mildly increased scores for proximal tubule single-cell apoptosis were seen in animals treated with ^{177}Lu -H2ABPP1.

10

Example 8
Radiolabeling of H2ABPP1 at a higher pH

In Example 2, the isoelectric point of mal-DOTA conjugated H2ABPP1 was determined to 5.2, and charge variants in proximity to the isoelectric point of H2ABPP1 were observed. A reduced solubility may be encountered for proteins at pHs close to or at their pI, causing formation of aggregates and in worst case precipitations. On the other hand, radiolabeling is usually performed at a lower pH for several reasons such as increased labeling efficiency, to minimize side reactions (e.g. to prevent hydroxide formation), and due to that many radioisotopes are more stable at low pH (Breeman *et al* 2003, Eur J Nucl Med Mol Imaging 30(6):917-20). To minimize the risk of solubility issues with H2ABPP1, which becomes especially important for large scale production where the yield should be maximized for a lower cost of goods, radiolabeling at a pH of 6.5 instead of 6.0 (as used in Example 4) was assessed. The labeling buffer was also switched from ammonium acetate to sodium acetate, as ammonium acetate is not available in GMP quality. The aim was to obtain a product that is stable for at least two days at a yield sufficient for at least one patient dose.

Materials and methods

Labeling with ^{177}Lu : H2ABPP1, at a final concentration of 2 mg/ml in 0.2 M NaAc, pH 6.5 containing 2 mg/mL sodium ascorbate, was labeled with ^{177}Lu LuCl₃ (Curium Pharma) at an activity concentration of about 1000 MBq/mL. The mixture was

5 incubated at 65°C for 30 min at an agitation of 450 rpm. Purification was performed by solid phase extraction using Oasis HLB Plus Light cartridges (Waters) eluted with 1 mL of a buffer containing 50% ethanol and 50% 0.4 M NaAc, pH 6.5. The sample was further diluted in dilution buffer containing 0.9% NaCl, 10-20 mg/mL sodium ascorbate at target pH 6.5 (6.3-6.6) to an activity concentration of 300 MBq/mL. The

10 labelling efficiency and stability were analyzed by ITLC and RP-HPLC on day 0, 1 and 3. Samples were stored at 2-8°C.

Results

The radiolabeling was surprisingly successful also at a pH of 6.5 and showed

15 acceptable stability up to 3 days (stored at 2-8°C). The results from the ITLC and HPLC analyses are summarized in Table 7.

Table 7. Result of stability analysis of different formulations

	Parameter	Crude product 2mg/mL sodium ascorbate ~ 872 MBq/mL No purification No ethanol Protein content 0.4 mg/mL	Formulation A 10 mg/mL sodium ascorbate ~ 304 MBq/mL 7% ethanol Protein content 0.17 mg/mL	Formulation B 20mg/mL sodium ascorbate ~ 304 MBq/mL 7% ethanol Protein content 0.17 mg/mL
Day 0	RCP by ITLC	97.8 % ROI	99.0 % ROI	99.2 % ROI
	RCP by HPLC, RD	94.6 %	98.6 %	98.7 %
	RCP by HPLC, UV ₂₃₀	99.2 %	97.6 %	96.4 %
Day 1	RCP by ITLC	93.4 % ROI	98.1 % ROI	97.9 % ROI
	RCP by HPLC, RD	n.a.	98.0 %	98.6 %
	RCP by HPLC, UV ₂₃₀	n.a.	99.4 %	93.3 %

Day 2	RCP by ITLC	89.4 % ROI	96.6 % ROI	97.9 %
	RCP by HPLC, RD	n.a.	96.9 %	97.8 %
	RCP by HPLC, UV ₂₃₀	n.a.	96.4%	98.5 %
Day 3	RCP by ITLC	83.2 % ROI	96.2 % ROI	97.5 % ROI
	RCP by HPLC, RD	n.a.	95.8 %	97.0%
	RCP by HPLC, UV ₂₃₀	n.a.	98.4%	97.1 %

RCP = Radiochemical purity

RD = Radio detector

ROI = Region of Interest

UV₂₃₀ = UV absorbance at 230 nm

5 n.a. = not analyzed

ITEMIZED LISTING OF EMBODIMENTS

1. A HER2-binding polypeptide, comprising the amino acid sequence [XYZ]:
10 EMRNAYWEIALLPNLTNQQKRAFIRKLYDDPSQSSELLSEAKKLNDSQAPEPE
 (SEQ ID NO:1).
2. A HER2-binding polypeptide according to item 1, which comprises an amino acid
sequence selected from:
K-[XYZ] (SEQ ID NO:2); VDAKYAK-[XYZ] (SEQ ID NO:3); AEAKFAK-[XYZ] (SEQ ID NO:4);
15 AEAKYAK-[XYZ] (SEQ ID NO:5); ADN NFNK-[XYZ] (SEQ ID NO:6); ADAQQNNFNK-[XYZ]
 (SEQ ID NO:7); AQHDE-[XYZ] (SEQ ID NO:8); and VDNKFNK-[XYZ] (SEQ ID NO:9);
 wherein [XYZ] is as defined in item 1.
3. A HER2-binding polypeptide according to item 2, which comprises an amino acid
sequence selected from:
20 K-[XYZ] (SEQ ID NO:2); VDAKYAK-[XYZ] (SEQ ID NO:3); AEAKFAK-[XYZ] (SEQ ID NO:4);
 and AEAKYAK-[XYZ] (SEQ ID NO:5); wherein [XYZ] is as defined in item 1.
4. A HER2-binding polypeptide according to item 3, comprising the amino acid
sequence AEAKYAK-[XYZ] (SEQ ID NO:5); wherein [XYZ] is as defined in item 1.
5. Fusion protein comprising:
25 - a first moiety consisting of a HER2-binding polypeptide according to any preceding
 item; and

- a second moiety consisting of a polypeptide having a desired biological activity.
- 6. A fusion protein according to item 5, wherein said desired biological activity is a binding activity.
- 7. A fusion protein according to item 6, wherein said binding activity increases the *in vivo* half-life and/or changes the biodistribution properties of the fusion protein.
- 8. A fusion protein according to item 7, wherein said second moiety comprises an albumin binding domain of streptococcal protein G or a derivative thereof.
- 9. A fusion protein according to item 8, wherein said albumin binding domain of streptococcal protein G or a derivative thereof is the GA3 domain or a derivative thereof.
- 10. A fusion protein according to item 9, wherein said albumin binding domain comprises an amino acid sequence selected from:
 - i) LAX₃AKX₆X₇ANX₁₀ ELDX₁₄YGVSDY YKRLIX₂₆KAKT VEGVEALKX₃₉X₄₀ ILX₄₃X₄₄LP (SEQ ID NO:10)
- 15 wherein independently of each other
 - X₃ is selected from E, S, Q and C;
 - X₆ is selected from E, S and C;
 - X₇ is selected from A and S;
 - X₁₀ is selected from A, S and R;
 - 20 X₁₄ is selected from A, S, C and K;
 - X₂₆ is selected from D and E;
 - X₃₉ is selected from D and E;
 - X₄₀ is selected from A and E;
 - X₄₃ is selected from A and K;
 - 25 X₄₄ is selected from A, S and E;
 - L in position 45 is present or absent; and
 - P in position 46 is present or absent; and
 - ii) an amino acid sequence which has at least 95% identity to the sequence defined in i).
- 30 11. A fusion protein according to item 10, wherein said albumin binding domain comprises an amino acid sequence as defined in SEQ ID NO:11.

12. A fusion protein according to any one of items 5-11, wherein said amino acid sequence comprises a cysteine residue, either already present in the sequence or as an additional amino acid residue.
13. A fusion protein according to item 12, wherein said cysteine residue is situated
- 5 at the C-terminal end of the fusion protein.
14. A fusion protein according to item 13, wherein said amino acid sequence is as defined in SEQ ID NO:12.
15. A HER2-binding polypeptide or fusion protein according to any one of preceding item, comprising a chelating environment provided by a polyaminopolycarboxylate
- 10 chelator coupled to the HER2-binding polypeptide or fusion protein via a thiol group.
16. A HER2-binding polypeptide or fusion protein according to item 15, wherein the polyaminopolycarboxylate chelator is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, 1,4,7-triazacyclononane-1,4,7-triacetic acid, diethylenetriaminepentaacetic acid or a derivative thereof.
- 15 17. A HER2 binding polypeptide or fusion protein according to item 16, wherein the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid derivative is 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide.
18. A fusion protein consisting of SEQ ID NO:12 coupled to 1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide.
- 20 19. A radiolabeled HER2-binding polypeptide or fusion protein consisting of a radiochelate of the HER2-binding polypeptide or fusion protein according to any one of items 15-18 and a radionuclide.
20. A radiolabeled HER2-binding polypeptide or fusion protein according to item 19, wherein said radionuclide is suitable for medical imaging.
- 25 21. A radiolabeled HER2-binding polypeptide or fusion protein according to item 20, wherein said radionuclide is selected from the group consisting of ^{72}As , ^{76}Br , ^{55}Co , ^{61}Cu , ^{64}Cu , ^{18}F , $^{[18\text{F}]\text{AlF}}$, ^{19}F , ^{66}Ga , ^{67}Ga , ^{68}Ga , $^{110\text{m}}\text{In}$, ^{111}In , ^{123}I , ^{124}I , ^{131}I , ^{177}Lu , ^{51}Mn , $^{52\text{m}}\text{Mn}$, ^{52}Mn , ^{186}Re , ^{188}Re , ^{44}Sc , ^{149}Tb , ^{152}Tb , ^{155}Tb , ^{161}Tb , $^{99\text{m}}\text{Tc}$, ^{45}Ti , ^{86}Y , and ^{89}Zr .
22. A radiolabeled HER2-binding polypeptide or fusion protein according to item 19,
- 30 wherein said radionuclide is suitable for therapy.

23. A radiolabeled HER2-binding polypeptide or fusion protein according to item 22, wherein said radionuclide is selected from the group consisting of ^{225}Ac , ^{212}Bi , ^{213}Bi , ^{67}Cu , ^{166}Ho , ^{177}Lu , ^{212}Pb , ^{149}Pm , ^{186}Re , ^{188}Re , ^{153}Sm , ^{149}Tb , ^{161}Tb , ^{227}Th and ^{90}Y .
24. A composition comprising a HER2-binding polypeptide or a fusion protein
- 5 according to any one of items 1-18, or a radiolabeled polypeptide or fusion protein according to any one of items 19-23, and at least one pharmaceutically acceptable excipient or carrier.
25. A method of *in vivo* imaging of the body of a mammalian, including human, subject having or suspected of having a cancer characterized by overexpression of
- 10 HER2, comprising the steps:
- administering a radiolabeled polypeptide or fusion protein according to any one of items 19-21, optionally comprised in a composition according to item 24, into the body of the mammalian subject; and
 - obtaining one or more images of at least a part of the subject's body using a
- 15 medical imaging instrument, said image(s) indicating the presence of the radionuclide inside the body.
26. A method according to item 25, comprising, before the administration step, a step of preparing a radiolabeled polypeptide or fusion protein according to any one of items 19-21, which step comprises mixing the polypeptide or fusion protein
- 20 according to any one of items 1-18 with the radionuclide suitable for medical imaging.
27. A method according to item 26, wherein said mixing is carried out at a pH value in the range of pH 6.0 – 7.0, such as carried out at a pH value selected from the group consisting of pH 6.0, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH
- 25 6.8, pH 6.9 and pH 7.0.
28. A method according to item 27, wherein said mixing is carried out at a pH value in the range of 6.3 – 6.7, such as carried out at a pH value selected from the group consisting of pH 6.3, pH 6.4, pH 6.5, pH 6.6 and pH 6.7.
29. A method according to item 28, wherein said mixing is carried out at a pH value
- 30 of 6.5.
30. A method of diagnosing a cancer characterized by overexpression of HER2, comprising

- performing the method of *in vivo* imaging according to any one of items 25-29, and
 - establishing said diagnosis on the basis of the obtained images.
31. A method of establishing a prognosis for a cancer characterized by overexpression of HER2, comprising
- 5 - performing the method of *in vivo* imaging according to any one of items 25-29, and
- establishing said prognosis on the basis of the obtained images.
32. A method according to any one of items 25-31, wherein the cancer is selected from breast cancer, ovarian cancer, gastric cancer, colorectal cancer, prostate cancer, bladder cancer, salivary cancer, lung cancer and cancer in the esophagus.
- 10 33. A method according item 32, wherein the cancer is breast cancer.
34. A method of treatment of a mammalian, including human, subject having a cancer characterized by overexpression of HER2, comprising the step of administering a therapeutically effective amount of a radiolabeled polypeptide or fusion protein according to any one of items 19 and 22-23, optionally comprised in a
- 15 composition according to item 24, to said subject.
35. A method according to item 34, comprising, before the administration step, a step of preparing a radiolabeled polypeptide or fusion protein according to any one of items 19 and 22-23, which step comprises mixing the polypeptide or fusion protein according to any one of items 1-18 with the radionuclide suitable for
- 20 therapy.
36. A method according to item 35, wherein said mixing is carried out at a pH value in the range of pH 6.0 – 7.0, such as carried out at a pH value selected from the group consisting of pH 6.0, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH 6.8, pH 6.9 and pH 7.0.
- 25 37. A method according to item 36, wherein said mixing is carried out at a pH value in the range of 6.3 – 6.7, such as carried out at a pH value selected from the group consisting of pH 6.3, pH 6.4, pH 6.5, pH 6.6 and pH 6.7.
38. A method according to item 37, wherein said mixing is carried out at a pH value of 6.5.
- 30 39. A method according to any one of items 34-38, wherein the cancer is selected from breast cancer, ovarian cancer, gastric cancer, colorectal cancer, prostate cancer, bladder cancer, salivary cancer, lung cancer and cancer in the esophagus.

40. A method according to item 39, wherein the cancer is breast cancer.
41. A HER2-binding polypeptide or fusion protein according to any one of items 1-18 or composition according to item 24, for use as a medicament.
42. A HER2-binding polypeptide or fusion protein or composition for use according
5 to item 41, wherein said use is in the method according to any one of items 34-40.
43. A HER2-binding polypeptide or fusion protein according to any one of items 1-18 or composition according to item 24 for use as a diagnostic agent *in vivo*.
44. A HER2-binding polypeptide or fusion protein or composition for use according
10 to item 43, wherein said use is in the method according to any one of items 30 and 32-33.
45. A HER2-binding polypeptide or fusion protein according to any one of items 1-18 or composition according to item 24 for use as a prognostic agent *in vivo*.
46. A HER2-binding polypeptide or fusion protein or composition for use according
15 to item 45, wherein said use is in the method according to any one of items 31-33.
47. A radiolabeled polypeptide or fusion protein according to any one of items 19-21
for use as a diagnostic agent *in vivo*.
48. A radiolabeled polypeptide or fusion protein for use according to item 47,
wherein said use is in the method according to any one of items 30 and 32-33.
49. A radiolabeled polypeptide or fusion protein according to any one of items 19-21
20 for use as a prognostic agent *in vivo*.
50. The radiolabeled polypeptide or fusion protein for use according to item 49,
wherein said use is in the method according to any one of items 31-33.
51. The radiolabeled polypeptide or fusion protein according to any one of items 19
and 22-23 for use as a medicament.
- 25 52. The radiolabeled polypeptide or fusion protein for use according to item 51,
wherein said use is in a method according to any one of items 34-40.
53. The composition according to item 24 for use as a diagnostic agent *in vivo*.
54. The composition for use according to item 53, wherein said use is in the method
according to any one of items 30 and 32-33.
- 30 55. The composition according to item 24 for use as a prognostic agent *in vivo*.
56. The composition for use according to item 55, wherein said use is in the method
according to any one of items 31-33.

57. The composition according to item 24 for use as a medicament
58. The composition for use according to item 57, wherein said use is in a method according to any one of items 34-40.
59. A nucleic acid encoding the polypeptide or fusion protein according to any one of
- 5 items 1-14.
60. An expression vector comprising the nucleic acid according to item 59.
61. A host cell comprising the expression vector according to item 60.
62. Method of producing a HER2-binding polypeptide or fusion protein according to any one of items 1-14, comprising
- 10 - culturing a host cell according to item 61 under conditions permissive of expression of said polypeptide from said expression vector, and
- isolating said polypeptide or fusion protein.
63. Method of producing a HER2-binding polypeptide or fusion protein according to any one of items 1-14 by non-biological peptide synthesis using amino acids and/or
- 15 amino acid derivatives having protected reactive side-chains, the non-biological peptide synthesis comprising
- step-wise coupling of the amino acids and/or the amino acid derivatives to form a polypeptide or fusion protein as described herein having protected reactive side-chains,
- 20 - removal of the protecting groups from the reactive side-chains of the polypeptide or fusion protein, and
- folding of the polypeptide or fusion protein in aqueous solution.
64. A method of producing a radiolabeled polypeptide or fusion protein according to any one of items 19-23, which step comprises mixing the polypeptide or fusion
- 25 protein according to any one of items 1-18 or the polypeptide or fusion protein produced according to any one of items 62-63 with a radionuclide.
65. Method according to item 64, wherein said mixing is carried out at a pH value in the range of pH 6.0 – 7.0, such as carried out at a pH value selected from the group consisting of pH 6.0, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH 6.8,
- 30 pH 6.9 and pH 7.0.

66. A method according to item 65, wherein said mixing is carried out at a pH value in the range of 6.3 – 6.7, such as carried out at a pH value selected from the group consisting of pH 6.3, pH 6.4, pH 6.5, pH 6.6 and pH 6.7.

67. A method according to item 66, wherein said mixing is carried out at a pH value
5 of 6.5.

CLAIMS

1. A HER2-binding polypeptide, comprising the amino acid sequence [XYZ]:
EMRNAYWEIALLPNLTNQKRAFIRKLYDDPSQSSELLSEAKKLNDSQAPEPE
5 (SEQ ID NO:1).
2. The HER2-binding polypeptide according to claim 1, comprising the amino acid sequence [XYZ] (SEQ ID NO:1) and one or more additional N- and/or C-terminal amino acid(s).
10
3. Fusion protein comprising:
- a first moiety consisting of a HER2-binding polypeptide according to any one of claims 1-2; and
- a second moiety consisting of a polypeptide having a desired biological activity.
15
4. The fusion protein according to claim 3, wherein said desired biological activity is a binding activity.
5. The fusion protein according to claim 4, wherein said binding activity increases
20 the *in vivo* half-life and/or changes the biodistribution properties of the fusion protein.
6. The HER2-binding polypeptide according to claim 2 or fusion protein according to any one of claims 3-5, wherein said amino acid sequence comprises a cysteine
25 residue, either already present in the sequence or as an additional amino acid residue.
7. The HER2-binding polypeptide or fusion protein according to any preceding claim, comprising a chelating environment provided by a polyaminopolycarboxylate
30 chelator coupled to the HER2-binding polypeptide or fusion protein via a thiol group.

8. A radiolabeled HER2-binding polypeptide or fusion protein consisting of a radiochelate of the HER2-binding polypeptide or fusion protein according to claim 7 and a radionuclide.
- 5 9. The radiolabeled HER2-binding polypeptide or fusion protein according to claim 8, wherein said radionuclide is suitable for therapy.
10. The radiolabeled HER2-binding polypeptide or fusion protein according to claim 9, wherein said radionuclide is selected from the group consisting of ^{225}Ac , ^{212}Bi ,
10 ^{213}Bi , ^{67}Cu , ^{166}Ho , ^{177}Lu , ^{212}Pb , ^{149}Pm , ^{186}Re , ^{188}Re , ^{153}Sm , ^{149}Tb , ^{161}Tb , ^{227}Th and ^{90}Y .
11. A composition comprising a HER2-binding polypeptide or a fusion protein according to any one of claims 1-7, or a radiolabeled polypeptide or fusion protein according to any one of claims 8-10, and at least one pharmaceutically acceptable
15 excipient or carrier.
12. A HER2-binding polypeptide or fusion protein according to any one of claims 1-7 or composition according to claim 11, for use as a medicament, a diagnostic agent *in vivo*, or a prognostic agent *in vivo*.
20
13. A nucleic acid encoding the HER2-binding polypeptide or fusion protein according to any one of claims 1-6.
14. A method of producing a radiolabeled HER2-binding polypeptide or fusion
25 protein according to any one of claims 8-10, which step comprises mixing the polypeptide or fusion protein according to any one of claims 1-7 with a radionuclide.
15. The method according to claim 14, wherein said mixing is carried out at a pH value in the range of pH 6.0 – 7.0, such as carried out at a pH value selected from
30 the group consisting of pH 6.0, pH 6.1, pH 6.2, pH 6.3, pH 6.4, pH 6.5, pH 6.6, pH 6.7, pH 6.8, pH 6.9 and pH 7.0.