Anti-HER3/HER4 antigen binding proteins binding to the beta-hairpin of HER3 and the beta-hairpin of HER4

The invention relates to anti-HER3/HER4 antigen binding proteins, e.g. anti-HER3/HER4 antibodies, that bind to the beta-hairpin of HER3 and the beta-hairpin of HER4, methods for selecting these antigen binding proteins, their preparation and use as medicament.

Background of the Invention

The HER protein family consists of 4 members: HER1, also named epidermal growth factor receptor (EGFR) or ErbB-1, HER2, also named ErbB-2, ErbB-3, also named HER3 and ErbB-4, also named HER4. The ErbB family *proteins* are receptor tyrosine kinases and represent important mediators of cell growth, differentiation and survival. The HER family represent receptors proteins of different ligands like the neuregulin (NRG) family, amphiregulin, EGF and (TGF-a). Heregulin (also called HRG or neuregulin NRG-1) is e.g. a ligand for HER3 and HER4.

Human HER3 (ErbB-3, ERBB3, c-erbB-3,c-erbB3, receptor tyrosine-protein kinase erbB-3, SEQ ID NO: 3) encodes a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases which also includes HER1 (also known as EGFR), HER2, and HER4 (Kraus, M.H. et al, PNAS 86 (1989) 9193-9197; Plowman, G.D. et al, PNAS 87 (1990) 4905-4909; Kraus, M.H. et al, PNAS 90 (1993) 2900-2904). Like the prototypical epidermal growth factor receptor, the transmembrane receptor HER3 consists of an extracellular ligand-binding domain (ECD), a dimerization domain within the ECD, a transmembrane domain, an intracellular protein tyrosine kinase domain (TKD) and a C-terminal phosphorylation domain. This membrane-bound protein has a Heregulin (HRG) binding domain within the extracellular domain but not an active kinase domain. It therefore can bind this ligand but not convey the signal into the cell through protein phosphorylation. However, it does form heterodimers with other HER family members which do have kinase activity. Heterodimerization leads to the activation of the receptor-mediated signaling pathway and transphosphorylation of its intracellular domain. Dimer formation between HER family members expands the signaling potential of HER3 and is a means not only for signal diversification but also signal amplification. For example the HER2/HER3 heterodimer induces one of the most important mitogenic signals via the PI3K and AKT pathway among HER

family members (Sliwkowski M.X., et al, J. Biol. Chem. 269 (1994) 14661-14665; Alimandi M, et al, Oncogene. 10 (1995) 1813-1821; Hellyer, N.J., J. Biol. Chem. 276 (2001) 42153-4261; Singer, E., J. Biol. Chem. 276 (2001) 44266-44274; Schaefer, K.L., Neoplasia 8 (2006) 613-622) For an overview of HER3 and its varoius interactions within the HER receptor family and the NGR ligands family see e.g. G Sithanandam et al Cancer Gene Therapy (2008) 15, 413–448.

Amplification of this gene and/or overexpression of its protein have been reported in numerous cancers, including prostate, bladder, and breast tumors. Alternate transcriptional splice variants encoding different isoforms have been characterized. One isoform lacks the intermembrane region and is secreted outside the cell. This form acts to modulate the activity of the membrane-bound form. Additional splice variants have also been reported, but they have not been thoroughly characterized.

Interestingly in its equilibrium state, the HER3 receptor exists in its "closed confirmation", which does mean, the heterodimerization HER3beta-hairpin motive is tethered via non-covalent interactions to the HER3ECD domain IV (see Figure 1c and 1 d). It is supposed, that the "closed" HER3 conformation can be opened via the binding of the ligand heregulin at a specific HER3 heregulin binding site. This takes place at the HER3 interface formed by the HER3 ECD domains I and domain III. By this interaction it is believed, that the HER3 receptor is activated and transferred into its "open conformation" (see Figure 1e and 1b and e.g. Baselga, J. et al, Nat Rev Cancer 9 (2009). 463-475 and Desbois-Mouthon, C., at al, Gastroenterol Clin Biol 34 (2010) 255-259). In this open conformation heterodimerization and transignal induction with HER2 is possible (see Figure 1b)

WO 2003/013602 relates to inhibitors of HER activity, including HER antibodies. WO 2007/077028 and WO 2008/100624 also relate to HER3 antibodies.

WO 97/35885 and WO2010/127181 relate to HER3 antibodies.

Human HER4 (also known as ErbB-4 ERBB4, v-erb-a erythroblastic leukemia viral oncogene homolog 4, p180erbB4 avian erythroblastic leukemia viral (v-erb-b2) oncogene homolog 4; SEQ ID NO:5) is a single-pass type I transmembrane protein with multiple furin-like cysteine rich domains, a tyrosine kinase domain, a phosphotidylinositol-3 kinase binding site and a PDZ domain binding motif (Plowman G D, wt al, PNAS 90:1746-50(1993); Zimonjic D B, et al, Oncogene 10:1235-7(1995); Culouscou J M, et al, J. Biol. Chem. 268:18407-10(1993)). The protein binds to and is activated by neuregulins-2 and -3, heparin-binding EGF-like

growth factor and betacellulin. Ligand binding induces a variety of cellular responses including mitogenesis and differentiation. Multiple proteolytic events allow for the release of a cytoplasmic fragment and an extracellular fragment. Mutations in this gene have been associated with cancer. Alternatively spliced variants which encode different protein isoforms have been described; however, not all variants have been fully characterized.

Anti-HER4 antibodies for use in anti-cancer therapy are known e.g. from US 5,811,098, US 7,332,579 or Hollmén M, et al, Oncogene. 28 (2009) 1309-19 (anti-ErbB-4 antibody mAb 1479).

So far it was not possible to select antigen binding proteins like e.g. antibodies that specifically bind to the beta-hairpin of HER3 and/or HER4 as these beta-hairpins of HER3 or of HER4 both represent hidden epitopes, which are not accessible in the equilibrium state of these receptors (see Figure 1).

Summary of the Invention

We now have found a method using the beta-hairpins of HER3 and HER4 functionally presented in a 3-dimensional orientation within SlyD scaffolds (see e.g Figure 2, and the polypeptides of SEQ ID NOs. 13, and 17 to 24) to obtain such antigen binding proteins, in particular antibodies.

The invention provides a method for selecting an antigen binding protein, in particular an antibody that binds to human HER3 and binds to human HER4,

wherein the antigen binding protein, in particular the antibody, binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) of human HER3 and binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) of human HER4;

wherein

a) at least one polypeptide selected from the group consisting of:

SEQ ID NO: 13	TtSlyD-FKBP-Her3,
SEQ ID NO: 17	TtSlyDcas-Her3,
SEQ ID NO: 18	TtSlyDcys-Her3,
SEQ ID NO: 19	TgSlyDser-Her3, and
SEQ ID NO: 20	TgSlyDcys-Her3,

which comprises the amino acid sequence of SEQ ID NO:1;

and

b) at least one polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,
SEQ ID NO: 22 TtSlyDcys-Her4,
SEQ ID NO: 23 TgSlyDser-Her4, and
SEQ ID NO: 24 TgSlyDcys-Her4,

which comprises the amino acid sequence of SEQ ID NO:2;

are used to select antigen binding proteins, in particular antibodies, which show binding to both, the at least one polypeptide under a) and the at least one polypeptide under b)

and thereby selecting an antigen binding protein, in particular an antibody, that binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) and within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2).

The invention provides antigen binding protein, in particular an antibody, obtained by such selection method.

The invention provides an isolated antigen binding protein, in particular an antibody, that binds to human HER3 and binds to human HER4, wherein the antigen binding protein, in particular the antibody, binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) of human HER3 and binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) of human HER4.

The invention provides an isolated antigen binding protein that binds to human HER3 and that binds to human HER4,

a) wherein the antigen binding protein binds to a polypeptide of

SEQ ID NO: 18 TtSlyDcys-Her3,

and

b) wherein the antigen binding protein binds to a polypeptide of

SEQ ID NO: 22 TtSlyDcys-Her4.

The invention further provides an isolated antigen binding protein that binds to human HER3 and that binds to human HER4,

a) wherein the antigen binding protein binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide of SEQ ID NO: 18 (TtSlyDcys-Her3), and

b) wherein the antigen binding protein binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide of SEQ ID NO: 22 (TtSlyDcys-Her4).

The invention provides an isolated antibody that binds to human HER3 and that binds to human HER4,

a) wherein the antibody binds to a polypeptide of

SEQ ID NO: 18 TtSlyDcys-Her3,

and

b) wherein the antibody binds to a polypeptide of

SEQ ID NO: 22 TtSlyDcys-Her4.

The invention further provides an isolated antibody that binds to human HER3 and that binds to human HER4,

a) wherein the antibody binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide of SEQ ID NO: 18 (TtSlyDcys-Her3), and

b) wherein the antibody binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide of SEQ ID NO: 22 (TtSlyDcys-Her4).

The invention provides an isolated antibody that binds to human HER3 and that binds to human HER4, wherein the antibody

a) binds to the amino acid sequence of SEQ ID NO:1; and/or

b) binds to the amino acid sequence SEQ ID NO:1 in activated HER3; and/or

c) binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 13	TtSlyD-FKBP-Her3,
SEQ ID NO: 17	TtSlyDcas-Her3,
SEQ ID NO: 18	TtSlyDcys-Her3,
SEQ ID NO: 19	TgSlyDser-Her3, and
SEQ ID NO: 20	TgSlyDcys-Her3;

and/or

d) binds to the ß-hairpin region of HER3; and/or

e) inhibits the heterodimerisation of HER3/HER2 heterodimers; and/or

f) binds to HER3-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 4.0 or higher (Ka (+Heregulin))/(Ka (-Heregulin)); and/or

g) binds to HER3-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 2.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)); and/or

h) specifically binds to the amino acid sequence of SEQ ID NO:2; and/or

i) binds to the amino acid sequence SEQ ID NO:2 in activated HER4; and/or

j) binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 21	TtSlyDcas-Her4,
SEQ ID NO: 22	TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and
SEQ ID NO: 24 TgSlyDcys-Her4;

and/or

k) binds to the ß-hairpin region of HER4; and/or

l) binds to HER4-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 20.0 or higher (Ka (+Heregulin))/(Ka (-Heregulin)); and/or

m) binds to HER4-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 5.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)); and/or

n) shows as monovalent Fab fragment the same or higher biological activity as compared to its bivalent parent full length antibody; and/or

o) inhibits the HER3 phosporylation in MCF-7 cells; and/or

p) does not compete for binding to HER3 with Heregulin/ induces binding of Heregulin to HER3; and/or

q) inhibits the proliferation of MDA-MB-175 cells tumor cells; and/or

r) shows tumor growth inhibitory activity in vivo; and/or

s) binds with an affinity of a KD value $\leq 1 \times 10^{-8}$ M to HER3-ECD (in one embodiment with a KD value of 1×10^{-8} M to 1×10^{-13} M; (in one embodiment with a KD value of 1×10^{-9} M to 1×10^{-13} M); and/or

t) binds with an affinity of a KD value $\leq 1 \times 10^{-8}$ M to HER4-ECD (in one embodiment with a KD value of 1×10^{-8} M to 1×10^{-13} M; (in one embodiment with a KD value of 1×10^{-9} M to 1×10^{-13} M); and/or

u) binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43) and to a polypeptide of consisting of VYNPTTFQLE (SEQ ID NO:44); and/or

v) binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43); and/or

w) binds to a polypeptide consisting of VYNPTTFQLE (SEQ ID NO:44); and/or

x) binds in a FACS assay to HER3 expressing T47D cells; and wherein the antibody induces an at least 25% higher percentage of internalization of HER3 in the presence of Heregulin as compared to the percentage of internalization of HER3 in the presence of Heregulin when measured after 1 h after antibody exposure.

In one embodiment such anti-HER3/HER4 antibody is a monoclonal antibody.

In one embodiment such anti-HER3/HER4 antibody is a human, humanized, or chimeric antibody.

In one embodiment such anti-HER3/HER4 antibody is an antibody fragment that binds human HER3 and that binds human HER4.

In one embodiment such anti-HER3/HER4 antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26, and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

In one embodiment such anti-HER3/HER4 antibody comprises (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In one embodiment such anti-HER3/HER4 antibody comprises

i) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30;

ii) or a humanized variant of the HVRs of the antibody under i) (a), (b), (d) and/or (e).

In one embodiment such anti-HER3/HER4 antibody comprises

i) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30; or

ii) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30; or

iii) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30; or

iv) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In one embodiment such anti-HER3/HER4 antibody comprises (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:33; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:36; or (c) a VH sequence as in (a) and a VL sequence as in (b). In one embodiment such anti-HER3/HER4 antibody comprises a VH

sequence of SEQ ID NO: 33. In one embodiment such anti-HER3/HER4 antibody comprises a VL sequence of SEQ ID NO: 36. In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO:33 and a VL sequence of SEQ ID NO:36.

In one embodiment such anti-HER3/HER4 antibody comprises (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:33; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:37; or (c) a VH sequence as in (a) and a VL sequence as in (b). In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO: 33. In one embodiment such anti-HER3/HER4 antibody comprises a VL sequence of SEQ ID NO: 37. In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO:33 and a VL sequence of SEQ ID NO:37.

In one embodiment such anti-HER3/HER4 antibody comprises (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:34; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:36; or (c) a VH sequence as in (a) and a VL sequence as in (b). In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO: 34. In one embodiment such anti-HER3/HER4 antibody comprises a VL sequence of SEQ ID NO: 36. In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO:34 and a VL sequence of SEQ ID NO:36.

In one embodiment such anti-HER3/HER4 antibody comprises (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:34; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:37; or (c) a VH sequence as in (a) and a VL sequence as in (b). In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO: 34. In one embodiment such anti-HER3/HER4 antibody comprises a VL sequence of SEQ ID NO: 37. In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO:34 and a VL sequence of SEQ ID NO:37.

In one embodiment such anti-HER3/HER4 antibody comprises (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:35; (b) a VL sequence having at least 95% sequence identity to the amino acid

sequence of SEQ ID NO:36; or (c) a VH sequence as in (a) and a VL sequence as in (b). In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO: 35. In one embodiment such anti-HER3/HER4 antibody comprises a VL sequence of SEQ ID NO: 36. In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO:35 and a VL sequence of SEQ ID NO:36.

In one embodiment such anti-HER3/HER4 antibody comprises (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:35; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:37; or (c) a VH sequence as in (a) and a VL sequence as in (b). In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO: 35. In one embodiment such anti-HER3/HER4 antibody comprises a VL sequence of SEQ ID NO: 37. In one embodiment such anti-HER3/HER4 antibody comprises a VH sequence of SEQ ID NO:35 and a VL sequence of SEQ ID NO:37.

In one embodiment such anti-HER3/HER4 antibody is a full length IgG1 antibody or IgG4 antibody.

In one embodiment such anti-HER3/HER4 antibody is a Fab fragment.

The invention further provides an isolated nucleic acid such anti-HER3/HER4 antibody.

The invention further provides a host cell comprising such nucleic acid.

The invention further provides a method of producing an antibody comprising culturing such host cell so that the antibody is produced

In on embodiment such method further comprises recovering the antibody from the host cell.

The invention further provides an immunoconjugate comprising such anti-HER3/HER4 antibody and a cytotoxic agent.

The invention further provides a pharmaceutical formulation comprising such anti-HER3/HER4 antibody and a pharmaceutically acceptable carrier.

The invention further provides the anti-HER3/HER4 antibody described herein for use as a medicament. The invention further provides the anti-HER3/HER4 antibody described herein, or the immunoconjugate comprising the anti-HER3/HER4 antibody and a cytotoxic agent, for use in treating cancer. The invention further provides the anti-HER3/HER4 antibody described herein for use in inhibition of HER3/HER2 dimerization.

Use of such anti-HER3/HER4 antibody, or an immunoconjugate comprising the anti-HER3/HER4 antibody and a cytotoxic agent, in the manufacture of a medicament. Such use wherein the medicament is for treatment of cancer.Such use wherein the medicament is for the inhibition of HER3/HER2 dimerization.

The invention further provides a method of treating an individual having cancer comprising administering to the individual an effective amount of the anti-HER3/HER4 antibody described herein, or an immunoconjugate comprising the anti-HER3/HER4 antibody and a cytotoxic agent.

The invention further provides a method of inducing apoptosis in a cancer cell in an individual sufferning from cancer comprising administering to the individual an effective amount of an immunoconjugate comprising the anti-HER3/HER4 antibody described herein and a cytotoxic agent, thereby inducing apoptosis in a cancer cell in the individual.

One embodiment of the invention is a polypeptide selected from the group consisting of:

i) SEQ ID NO: 13 TtSlyD-FKBP-Her3,
ii) SEQ ID NO: 17 TtSlyDcas-Her3,
iii) SEQ ID NO: 18 TtSlyDcys-Her3,
iv) SEQ ID NO: 19 TgSlyDser-Her3, and
v) SEQ ID NO: 20 TgSlyDcys-Her3,

which polypeptide comprises the amino acid sequence of SEQ ID NO:1

One embodiment of the invention is a polypeptide selected from the group consisting of:

i) SEQ ID NO: 21 TtSlyDcas-Her4,
ii) SEQ ID NO: 22 TtSlyDcys-Her4,

iii) SEQ ID NO: 23 TgSlyDser-Her4,and
iv) SEQ ID NO: 24 TgSlyDcys-Her4,

which polypeptide comprises the amino acid sequence of SEQ ID NO:2.

The invention further provides the use of one of such polypeptides selected from the group consisting of:

i) SEQ ID NO: 13 TtSlyD-FKBP-Her3,
ii) SEQ ID NO: 17 TtSlyDcas-Her3,
iii) SEQ ID NO: 18 TtSlyDcys-Her3,
iv) SEQ ID NO: 19 TgSlyDser-Her3, and
v) SEQ ID NO: 20 TgSlyDcys-Her3,

for eliciting an immune response against SEQ ID NO:1 in an experimental animal.

The invention further provides the use of one of such polypeptides selected from the group consisting of:

i) SEQ ID NO: 21 TtSlyDcas-Her4,
ii) SEQ ID NO: 22 TtSlyDcys-Her4,
iii) SEQ ID NO: 23 TgSlyDser-Her4,and
iv) SEQ ID NO: 24 TgSlyDcys-Her4,

for eliciting an immune response against SEQ ID NO:2 in an experimental animal.

The invention further provides a method for producing an antibody specifically binding to the ß-hairpin of HER3 with the amino acid sequence of SEQ ID NO:1 comprising the following steps:

a) administering to an experimental animal a polypeptide selected from the group consisting of:

i) SEQ ID NO: 13 TtSlyD-FKBP-Her3,

ii) SEQ ID NO: 17 TtSlyDcas-Her3,

iii) SEQ ID NO: 18 TtSlyDcys-Her3,

iv) SEQ ID NO: 19 TgSlyDser-Her3, and

v) SEQ ID NO: 20 TgSlyDcys-Her3,

for at least one time, whereby the polypeptide comprises the ß-hairpin of HER3 with the amino acid sequence of SEQ ID NO:1,

b) recovering from the experimental animal three to ten days after the last administration of the polypeptide B-cells that produce the antibody specifically binding to the ß-hairpin of HER3 with the amino acid sequence of SEQ ID NO:1, and

c) cultivating a cell comprising a nucleic acid encoding the antibody specifically binding to the the ß-hairpin of HER3 with the amino acid sequence of SEQ ID NO:1 and recovering the antibody from the cell or the cultivation medium and thereby producing an antibody specifically binding to a target antigen.

The invention further provides the use of a polypeptide selected from the group consisting of:

i) SEQ ID NO: 13 TtSlyD-FKBP-Her3,

ii) SEQ ID NO: 17 TtSlyDcas-Her3,

iii) SEQ ID NO: 18 TtSlyDcys-Her3,

iv) SEQ ID NO: 19 TgSlyDser-Her3, and

v) SEQ ID NO: 20 TgSlyDcys-Her3,

for epitope mapping, whereby the polypeptide comprises the epitope in the the ß-hairpin of HER3 with the amino acid sequence of SEQ ID NO:1.

A method for producing an antibody specifically binding to the ß-hairpin of HER4 with the amino acid sequence of SEQ ID NO:2 comprising the following steps:

a) administering to an experimental animal a polypeptide selected from the group consisting of:

i) SEQ ID NO: 21 TtSlyDcas-Her4,

ii) SEQ ID NO: 22 TtSlyDcys-Her4,

iii) SEQ ID NO: 23 TgSlyDser-Her4,and

iv) SEQ ID NO: 24 TgSlyDcys-Her4,

for at least one time, whereby the polypeptide comprises the ß-hairpin of HER4 with the amino acid sequence of SEQ ID NO:2,

b) recovering from the experimental animal three to ten days after the last administration of the polypeptide B-cells that produce the antibody specifically binding to the ß-hairpin of HER4 with the amino acid sequence of SEQ ID NO:2, and

c) cultivating a cell comprising a nucleic acid encoding the antibody specifically binding to the the ß-hairpin of HER4 with the amino acid sequence of SEQ ID NO:2 and recovering the antibody from the cell or the cultivation medium and thereby producing an antibody specifically binding to a target antigen.

The invention further provides the use of a polypeptide selected from the group consisting of:

i) SEQ ID NO: 21 TtSlyDcas-Her4,

ii) SEQ ID NO: 22 TtSlyDcys-Her4,

iii) SEQ ID NO: 23 TgSlyDser-Her4,and

iv) SEQ ID NO: 24 TgSlyDcys-Her4,

for epitope mapping, whereby the polypeptide comprises the epitope in the the ß-hairpin of HER4 with the amino acid sequence of SEQ ID NO:2.

Using the beta-hairpins of HER3 and HER4 functionally presented in a 3-dimensional orientation within SlyD scaffolds (see e.g Figure 2, and the polypeptides of SEQ ID NOs. 13, and 17 to 24) the anti-HER3/HER4 antigen binding proteins, in particular antibodies, described herein binding to these beta-hairpins could be selected. It was found that the antigen binding proteins, in particular antibodies,according to the invention have highly valuable properties such as strong growth inhibition of HER3 expressing cancer cells, strong inhibition of HER3 mediated signal transduction (such as e.g. HER3 phoshorylation) which is related to cancer cell proliferation, or very specific pharmacokinetic properties (such as faster association rates and higher Molar Ratios of the binding the

activated HER3 in the presence of Heregulin ("open conformation) when compared to the absence of Heregulin ("closed conformation").

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Schematic overview of "closed" and "open" HER3 conformation and the influence of the Neuregulin family ligands (like e.g. Heregulin abbreviated here as HR) on the conformation change.

Figure 2 3D-structure of the beta-hairpin of HER3 functionally presented in a 3-dimensional orientation within a SlyD scaffold of Thermus thermophiles.

Figure 3 SDS-PAGE analysis of Ni-NTA purification of TtSlyD-FKBP-Her3. E1 and E2 show the purified fractions 12 and 13.SN: *E.coli* lysate supernatant before purification.

Figure 4 SEC elution profile of a Ni-NTA purified fraction of Thermus thermophilus SlyD-FKBP-Her-3.

Figure 5 Testing of specificity and reactivity in IHC of the selected clones. All three clones showed binding to Her3 and cross reactivity against Her4. No cross reactivity against Her1 and Her2 was detectable.

Figure 6 FACS analysis of M-05-74 antibody induced time dependent HER3 internalization in T47D cells.

Figure 7 Biacore sensorgram overlay plot. 1: 100nM M-05-74*Heregulin/Her-3 ECD interaction. 2: 100 nM M-08-11*Heregulin/Her-3 ECD interaction. 3&4: 100nM M-05-74 and 100 nM M-08-11*Her-3 ECD interaction. 5: buffer reference.

Figure 8 Sensorgram overlay of the Biacore epitope-binning experiment. The primary antibody M-05-74 (M-074 in the Figure) presented the Her-3 ECD to the secondary antibodies M-208, GT (=8B8), M-05-74 and M-08-11 (M-011 in the Figure 8) (M-. The noise of the measurement was 5 RU.

Figure 9 Biacore sensorgram overlay plot. 1: 90 nM Heregulin*Her-3 ECD complex on M-05-74. 2: 90 nM Heregulin*Her-3 ECD complex on M-08-11. 3: 90 nM Heregulin*Her-3 ECD complex on 8B8 antibody.

Figure 10 Schematic Mode of Actions identified by Biacore functional assays. 1: M-08-11 binds to the Heregulin activated Her-3 ECD and induces a delayed Heregulin dissociation, whereby M-08-11 stays

in the Her-3 ECD receptor complex. 2: M-05-74 binds to the Heregulin activated Her-3 ECD. Heregulin is trapped in the complex and the antibody stays in the complex 3: 8B8 binds the Heregulin activated Her-3 ECD. The whole complex dissociates from the antibody.

Figure 11 Strategy of the epitope mapping and alanine-scan approach. The peptide hairpin sequences (peptide hairpin) of EGFR, Her-2 ECD, Her-3 ECD and Her-4 ECD including their structural embeddings (structural) were investigated. Cysteins were replaced by serines.

Figure 12 CelluSpots™ Synthesis and Epitope Mapping of epitopes of antibody M-05-74 on HER3 and HER4. Anti-HER3/HER4 antibody M-05-74 binds to HER3 ECD binding epitope VYNKLTFQLEP (SEQ ID NO:43) and to HER4 ECD binding epitope VYNPTTFQLE (SEQ ID NO:44).

Figure 13 Results from the CelluSpots™ Ala-Scan of anti HER3/HER4 antibody M-05-74 (named M-074 in the Figure) and anti-HER3 antibody M-08-11 (named M-011) with no HER4 crossreactivity) - the amino acids which are contributing most to the binding of anti-HER3/HER4 antibody M-05-74 to its HER3 ECD binding epitope VYNKLTFQLEP (SEQ ID NO:43) and to its HER4 ECD binding epitope VYNPTTFQLE (SEQ ID NO:44) are underlined/bold.

Figure 14 Binding of M-05-74 (M-074) induces/promotes binding of HRG to the HER3-ECD.

Figure 15 Inhibition of HER2/HER3 heterodimers/heterodimerization (Imunoprecipitation and Western Blot) in MCF7 cells (HER3-IP = immunoprecipitation with HER3 antibody/ HER2-IP = immunoprecipitation with HER3 antibody).

Figure 16 Treatment of MDA-MB175 cells with M-05-74 resulted in inhibition of cell proliferation.

Figure 17 Treatment with M-05-74 (M-074) (10mg/kg q7d, i.p.) resulted in tumor stasis a FaDu HNSCC transplanted xenografts.

Figure 18 Treatment with M-05-74-Fab-Pseudomonas exotoxin conjugate (M-074-PE) (10mg/kg q7d, i.p.) resulted in stronger inhibition of cell proliferation in the presence (bold line) of HRG than in the absence (thin line) of HRG.

Figure 19 In vivo tumor cell growth inihibiton by M-05-74-Fab-Pseudomonas exotoxin conjugate (M-05-74-PE). Legend: closed

line (vehicle); dotted line (M-05-74-Fab-Pseudomonas exotoxin conjugate (M-05-74-PE)).

Figure 20 Biacore sensorgram overlay plot: binding of the antibody M-05-74 (1) of the present invention to TtSlyDcys-Her3 (SEQ ID NO: 18) in comparison with anti-HER3 antibody MOR09823 (2) described in WO2012/22814. While the antibody of the present M-05-74 (1) shows a clear binding signal to TtSlyDcys-Her3 (SEQ ID NO: 18), the antibody anti-HER3 antibody MOR09823 (2) shows no binding at all to TtSlyDcys-Her3 (SEQ ID NO: 18). Control measurement (3) without antibody at all did not shown any binding to TtSlyDcys-Her3 (SEQ ID NO: 18).

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

I. DEFINITIONS

An "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

An "affinity matured" antibody refers to an antibody with one or more alterations in one or more hypervariable regions (HVRs), compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for antigen.

The term "antigen binding protein" as used herein refers to an antibody as decribed herein or to a scaffold antigen binding protein. In one preferred embodiment the antigen binding protein is an antibody as decribed herein. Scaffold antigen binding proteins are known in the art, for example, fibronectin and designed ankyrin-repeat proteins (DARPins) have been used as alternative scaffolds for antigen-binding

domains, see, e.g., Gebauer and Skerra, Engineered protein scaffolds as next-generation antibody therapeutics. Curr Opin Chem Biol 13:245-255 (2009) and Stumpp et al., Darpins: A new generation of protein therapeutics. Drug Discov Today 13: 695-701 (2008), both of which are incorporated herein by reference in their entirety. B. Criteria for Selecting Parent Variable Domains and Receptors for antigen binding proteins of the invention. In one embodiment a scaffold antigen binding protein is selected from the group consisting of CTLA-4 (Evibody); lipocalin; Protein A derived molecules such as Z-domain of Protein A (Affibody, SpA), A-domain (Avimer/Maxibody); Heat shock proteins such as GroEI and GroES; transferrin (trans-body); ankyrin repeat protein (DARPin); peptide aptamer; C-type lectin domain (Tetranectin); human .gamma.-crystallin and human ubiquitin (affilins); PDZ domains; scorpion toxinkunitz type domains of human protease inhibitors; and fibronectin (adnectin); which has been subjected to protein engineering in order to obtain binding to a ligand other than the natural ligand.

CTLA-4 (Cytotoxic T Lymphocyte-associated Antigen 4) is a CD28-family receptor expressed on mainly CD4+ T-cells. Its extracellular domain has a variable domain- like Ig fold. Loops corresponding to CDRs of antibodies can be substituted with heterologous sequence to confer different binding properties. CTLA-4 molecules engineered to have different binding specificities are also known as Evibodies. For further details see Journal of Immunological Methods 248 (1-2), 31-45 (2001).

Lipocalins are a family of extracellular proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids and lipids. They have a rigid .beta.-sheet secondary structure with a numer of loops at the open end of the conical structure which can be engineered to bind to different target antigens. Anticalins are between 160-180 amino acids in size, and are derived from lipocalins. For further details see Biochim Biophys Acta 1482: 337-350 (2000), US 7,250,297 B1 and US 2007/0224633.

An affibody is a scaffold derived from Protein A of Staphylococcus aureus which can be engineered to bind to antigen. The domain consists of a three-helical bundle of approximately 58 amino acids. Libraries have been generated by randomisation of surface residues. For further details see Protein Eng. Des. Sel. 17, 455-462 (2004) and EP1641818A1Avimers are multidomain proteins derived from the A-domain scaffold family. The native domains of approximately 35 amino acids adopt a defined disulphide bonded structure. Diversity is generated by shuffling of

the natural variation exhibited by the family of A-domains. For further details see Nature Biotechnology 23(12), 1556 - 1561 (2005) and Expert Opinion on Investigational Drugs 16(6), 909-917 (June 2007).

A transferrin is a monomeric serum transport glycoprotein. Transferrins can be engineered to bind different target antigens by insertion of peptide sequences in a permissive surface loop. Examples of engineered transferrin scaffolds include the Trans-body. For further details see J. Biol. Chem 274, 24066-24073 (1999).

Designed Ankyrin Repeat Proteins (DARPins) are derived from Ankyrin which is a family of proteins that mediate attachment of integral membrane proteins to the cytoskeleton. A single ankyrin repeat is a 33 residue motif consisting of two .alpha.-helices and a .beta.-turn. They can be engineered to bind different target antigens by randomising residues in the first .alpha.-helix and a .beta.-turn of each repeat. Their binding interface can be increased by increasing the number of modules (a method of affinity maturation). For further details see J. MoI. Biol. 332, 489-503 (2003), PNAS 100(4), 1700-1705 (2003) and J. MoI. Biol. 369, 1015-1028 (2007) and US20040132028A1.

Fibronectin is a scaffold which can be engineered to bind to antigen. Adnectins consists of a backbone of the natural amino acid sequence of the 10th domain of the 15 repeating units of human fibronectin type III (FN3). Three loops at one end of the .beta.-sandwich can be engineered to enable an Adnectin to specifically recognize a therapeutic target of interest. For further details see Protein Eng. Des. SeI. 18, 435- 444 (2005), US20080139791, WO2005056764 and US6818418B1.

Peptide aptamers are combinatorial recognition molecules that consist of a constant scaffold protein, typically thioredoxin (TrxA) which contains a constrained variable peptide loop inserted at the active site. For further details see Expert Opin. Biol. Ther. 5, 783-797 (2005).

Microbodies are derived from naturally occurring microproteins of 25-50 amino acids in length which contain 3-4 cysteine bridges - examples of microproteins include KalataBI and conotoxin and knottins. The microproteins have a loop which can beengineered to include upto 25 amino acids without affecting the overall fold of the microprotein. For further details of engineered knottin domains, see WO2008098796.

Other antigen binding proteins include proteins which have been used as a scaffold to engineer different target antigen binding properties include human .gamma.-crystallin and human ubiquitin (affilins), kunitz type domains of human protease inhibitors, PDZ- domains of the Ras-binding protein AF-6, scorpion toxins (charybdotoxin), C-type lectin domain (tetranectins) are reviewed in Chapter 7 - Non-Antibody Scaffolds from Handbook of Therapeutic Antibodies (2007, edited by Stefan Dubel) and Protein Science 15:14-27 (2006). Epitope binding domains of the present invention could be derived from any of these alternative protein domains.

The terms "anti-HER3/HER4 antigen binding protein", "an antigen binding protein that binds to (human) HER3 and binds to (human) HER4" and "an antigen binding protein that binds specifically to human HER3 and specifically binds to human HER4" refer to an antigen binding protein that is capable of binding HER3 or HER4 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting HER3 and/or HER4.

The terms "anti-HER3/HER4 antibody", "an antibody that binds to (human) HER3 and binds to (human) HER4" and "an antibody that binds specifically to human HER3 and specifically binds to human HER4" refer to an antibody that is capable of binding HER3 or HER4 with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting HER3 and/or HER4. In one embodiment, the extent of binding of an anti-HER3/HER4 antibody to an unrelated, non-HER3/HER4 protein is less than about 10% of the binding of the antibody to HER3 or HER4 as measured, e.g., by a Surface Plasmon Resonance assay (e.g. BIACORE). In certain embodiments, an antibody that binds to human HER3 has a KD value of the binding affinity for binding to human HER3 of $\leq$ 1 μM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$ 0.001 nM (e.g. 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M, e.g., from 10^{-9} M to 10^{-13} M). In certain embodiments, an antibody that binds to human HER4 has a KD value of the binding affinity for binding to human HER4 of $\leq$ 1 μM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$ 0.001 nM (e.g. 10-8 M or less, e.g. from 10-8 M to 10-13 M, e.g., from 10-9 M to 10-13 M).b In one preferred embodiment the respective KD value of the binding affinities is determined in a Surface Plasmon Resonance assay using the wildtype Extracellular domain (ECD) of human HER3 (HER3-ECD) for the HER3 binding affinity, and wildtype human HER4-ECD for the HER4 binding affinity, respectively.

The term "antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')2; diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

An “antibody that binds to the same epitope” as a reference antibody refers to an antibody that blocks binding of the reference antibody to its antigen in a competition assay by 50% or more, and conversely, the reference antibody blocks binding of the antibody to its antigen in a competition assay by 50% or more. An exemplary competition assay is provided herein.

The term “cancer” as used herein may be, for example, lung cancer, non small cell lung (NSCL) cancer, bronchioloalviolar cell lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, gastric cancer, colon cancer, breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, mesothelioma, hepatocellular cancer, biliary cancer, neoplasms of the central nervous system (CNS), spinal axis tumors, brain stem glioma, glioblastoma multiforme, astrocytomas, schwanomas, ependymonas, medulloblastomas, meningiomas, squamous cell carcinomas, pituitary adenoma, lymphoma, lymphocytic leukemia, including refractory versions of any of the above cancers, or a combination of one or more of the above cancers. In one preferred embodiment such cancer is a breast cancer, ovarian cancer, cervical cancer, lung cancer or prostate cancer. In one preferred embodiment such cancers are further characterized by HER3 and/or HER4 expression or overexpression. One further

embodiment the invention are the anti-HER3/HER4 antibodies of the present invention for use in the simultaneous treatment of primary tumors and new metastases.

The term "chimeric" antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ respectively.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents a cellular function and/or causes cell death or destruction. Cytotoxic agents include, but are not limited to, radioactive isotopes (e.g., At211, I131, I125, Y90, Re186, Re188, Sm153, Bi212, P32, Pb212 and radioactive isotopes of Lu); chemotherapeutic agents or drugs (e.g., methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents); growth inhibitory agents; enzymes and fragments thereof such as nucleolytic enzymes; antibiotics; toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof; and the various antitumor or anticancer agents disclosed below. In one preferred embodiment the "cytotoxic agent" is Pseudomonas exotoxin A or variants thereof. In one preferred embodiment the "cytotoxic agent" is amatoxin or a variants thereof.

"Effector functions" refer to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

An "effective amount" of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

The term "epitope" includes any polypeptide determinant capable of specific binding to an antibody. In certain embodiments, epitope determinant include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl, and, in certain embodiments, may have specific three dimensional structural characteristics, and or specific charge characteristics. An epitope is a region of an antigen that is bound by an antibody.

The term “Fc region” herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991), NIH Publication 91-3242.

"Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

The terms “full length antibody,” “intact antibody,” and “whole antibody” are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be

completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, 5th ed., Bethesda MD (1991), NIH Publication 91-3242, Vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., supra. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al., supra.

A "humanized" antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized variant" of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization. In one preferred embodiment, a murine HVR is grafted into the framework region of a human antibody to prepare the "humanized antibody." See e.g. Riechmann, L., et al., Nature 332 (1988) 323-327; and Neuberger, M.S., et al., Nature 314 (1985) 268-270. The murine variable region amino acid sequence is aligned to a collection of human germline antibody V-genes, and sorted according to sequence identity and homology. The acceptor sequence is selected based on high overall sequence homology and optionally also the presence of the right canonical residues already in the acceptor sequence (see Poul, M-A. and Lefranc, M-P., in "Ingénierie des anticorps banques combinatores" ed. by Lefranc, M-P. and Lefranc, G., Les

Editions INSERM, 1997). The germline V-gene encodes only the region up to the beginning of HVR3 for the heavy chain, and till the middle of HVR3 of the light chain. Therefore, the genes of the germline V-genes are not aligned over the whole V-domain. The humanized construct comprises the human frameworks 1 to 3, the murine HVRs, and the human framework 4 sequence derived from the human JK4, and the JH4 sequences for light and heavy chain, respectively. Before selecting one particular acceptor sequence, the so-called canonical loop structures of the donor antibody can be determined (see Morea, V., et al., Methods, Vol 20, Issue 3 (2000) 267-279). These canonical loop structures are determined by the type of residues present at the so-called canonical positions. These positions lie (partially) outside of the HVR regions, and should be kept functionally equivalent in the final construct in order to retain the HVR conformation of the parental (donor) antibody. In WO 2004/006955 a method for humanizing antibodies is reported that comprises the steps of identifying the canonical HVR structure types of the HVRs in a non-human mature antibody; obtaining a library of peptide sequence for human antibody variable regions; determining the canonical HVR structure types of the variable regions in the library; and selecting the human sequences in which the canonical HVR structure is the same as the non-human antibody canonical HVR structure type at corresponding locations within the non-human and human variable regions. Summarizing, the potential acceptor sequence is selected based on high overall homology and optionally in addition the presence of the right canonical residues already in the acceptor sequence. In some cases simple HVR grafting only result in partial retention of the binding specificity of the non-human antibody. It has been found that at least some specific non-human framework residues are required for reconstituting the binding specificity and have also to be grafted into the human framework, i.e. so called "back mutations" have to be made in addition to the introduction of the non-human HVRs (see e.g. Queen et al., Proc. Natl. Acad. Sci. USA 86 (1989) 10,029-10,033; Co et al., Nature 351 (1991) 501-502). These specific framework amino acid residues participate in FR-HVR interactions and stabilized the conformation (loop) of the HVRs (see e.g. Kabat et al., J. Immunol. 147 (1991) 1709). In some cases also forward-mutations are introduced in order to adopt more closely the human germline sequence. Thus “humanized variant of an antibody according to the invention” (which is e.g. of mouse origin) refers to an antibody, which is based on the mouse antibody sequences in which the VH and VL are humanized by above described standard techniques (including HVR grafting and optionally subsequent mutagenesis of certain amino acids in the

framework region and the HVR-H1, HVR-H2, HVR-L1 or HVR-L2, whereas HVR-H3 and HVR-L3 remain unmodified).

The term "hypervariable region" or "HVR" as used herein refers to each of the regions of an antibody variable domain which are hypervariable in sequence ("complementarity determining regions" or "CDRs") and/or form structurally defined loops ("hypervariable loops") and/or contain the antigen-contacting residues ("antigen contacts"). Generally, antibodies comprise six HVRs: three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). Exemplary HVRs herein include:

(a) hypervariable loops occurring at amino acid residues 26-32 (L1), 50-52 (L2), 91-96 (L3), 26-32 (H1), 53-55 (H2), and 96-101 (H3) (Chothia and Lesk, *J. Mol. Biol.* 196:901-917 (1987));

(b) CDRs occurring at amino acid residues 24-34 (L1), 50-56 (L2), 89-97 (L3), 31-35b (H1), 50-65 (H2), and 95-102 (H3) (Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991));

(c) antigen contacts occurring at amino acid residues 27c-36 (L1), 46-55 (L2), 89-96 (L3), 30-35b (H1), 47-58 (H2), and 93-101 (H3) (MacCallum et al. *J. Mol. Biol.* 262: 732-745 (1996)); and

(d) combinations of (a), (b), and/or (c), including HVR amino acid residues 46-56 (L2), 47-56 (L2), 48-56 (L2), 49-56 (L2), 26-35 (H1), 26-35b (H1), 49-65 (H2), 93-102 (H3), and 94-102 (H3).

Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., *supra*.

An "immunoconjugate" is an antibody conjugated to one or more heterologous molecule(s), including but not limited to a cytotoxic agent.

An "individual" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

An "isolated" antibody is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman, S. et al., J. Chromatogr. B 848 (2007) 79-87.

An "isolated" nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

“Isolated nucleic acid encoding an anti-HER3/HER4 antibody” refers to one or more nucleic acid molecules encoding antibody heavy and light chains (or fragments thereof), including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

The term “Mab” refers to monoclonal antibodies, whereas the term “hMab” refers to humanized variants of such monoclonal antibodies.

A “naked antibody” refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.(Include if Prior art has immunoconjugates).

"Native antibodies" refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

The term “package insert” is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

“Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2

sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

The term "HER3," as used herein, refers to any native HER3 from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice

and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed HER3 as well as any form of HER3 that results from processing in the cell. The term also encompasses naturally occurring variants of HER3, e.g., splice variants or allelic variants. The amino acid sequence of an exemplary human HER3 is shown in SEQ ID NO:3. "Human HER3" (ErbB-3, ERBB3, c-erbB-3,c-erbB3, receptor tyrosine-protein kinase erbB-3, SEQ ID NO: 3) encodes a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases which also includes HER1 (also known as EGFR), HER2, and HER4 (Kraus, M.H. et al, PNAS 86 (1989) 9193-9197; Plowman, G.D. et al, PNAS 87 (1990) 4905-4909; Kraus, M.H. et al, PNAS 90 (1993) 2900-2904). Like the prototypical epidermal growth factor receptor, the transmembrane receptor HER3 consists of an extracellular ligand-binding domain (ECD), a dimerization domain within the ECD, a transmembrane domain, an intracellular protein tyrosine kinase domain (TKD) and a C-terminal phosphorylation domain. This membrane-bound protein has a Heregulin (HRG) binding domain within the extracellular domain but not an active kinase domain. It therefore can bind this ligand but not convey the signal into the cell through protein phosphorylation. However, it does form heterodimers with other HER family members which do have kinase activity. Heterodimerization leads to the activation of the receptor-mediated signaling pathway and transphosphorylation of its intracellular domain. Dimer formation between HER family members expands the signaling potential of HER3 and is a means not only for signal diversification but also signal amplification. For example the HER2/HER3 heterodimer induces one of the most important mitogenic signals via the PI3K and AKT pathway among HER family members (Sliwkowski M.X., et al, J. Biol. Chem. 269 (1994) 14661-14665; Alimandi M, et al, Oncogene. 10 (1995) 1813-1821; Hellyer, N.J., J. Biol. Chem. 276 (2001) 42153-4261; Singer, E., J. Biol. Chem. 276 (2001) 44266-44274; Schaefer, K.L., Neoplasia 8 (2006) 613-622) For an overview of HER3 and its varoius interactions within the HER receptor family and the NGR ligands family see e.g. G Sithanandam et al Cancer Gene Therapy (2008) 15, 413–448.

Interestingly in its equilibrium state, the HER3 receptors exists in its "closed confirmation", which does mean, the heterodimerization HER3 beta-hairpin motive is tethered via non-covalent interactions to the HER3 ECD domain IV (see Figure 1c). It is supposed, that the "closed" HER3 conformation can be opened via the binding of the ligand heregulin at a specific HER3 heregulin binding site. This takes place at the HER3 interface formed by the HER3 ECD domains I and domain

III. By this interaction it is believed, that the HER3 receptor is activated and transferred into its "open conformation" (see Figure 1b and e.g. Baselga, J. et al, Nat Rev Cancer 9 (2009). 463-475 and Desbois-Mouthon, C., at al, Gastroenterol Clin Biol 34 (2010) 255-259). In this open conformation heterodimerization and transignal induction with HER2 is possible (see Figure 1b).

The term "HER4," as used herein, refers to any native HER4 from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice and rats), unless otherwise indicated. The term encompasses "full-length," unprocessed HER4 as well as any form of HER4 that results from processing in the cell. The term also encompasses naturally occurring variants of HER4, e.g., splice variants or allelic variants. The amino acid sequence of an exemplary human HER4 is shown in SEQ ID NO:5. "Human HER4" (also known as ErbB-4 ERBB4, v-erb-a erythroblastic leukemia viral oncogene homolog 4, p180erbB4 avian erythroblastic leukemia viral (v-erb-b2) oncogene homolog 4; SEQ ID NO:5) is a single-pass type I transmembrane protein with multiple furin-like cysteine rich domains, a tyrosine kinase domain, a phosphotidylinositol-3 kinase binding site and a PDZ domain binding motif (Plowman G D, wt al, PNAS 90:1746-50(1993); Zimonjic D B, et al, Oncogene 10:1235-7(1995); Culouscou J M, et al, J. Biol. Chem. 268:18407-10(1993)). The protein binds to and is activated by neuregulins-2 and -3, heparin-binding EGF-like growth factor and betacellulin. Ligand binding induces a variety of cellular responses including mitogenesis and differentiation. Multiple proteolytic events allow for the release of a cytoplasmic fragment and an extracellular fragment. Mutations in this gene have been associated with cancer. Alternatively spliced variants which encode different protein isoforms have been described; however, not all variants have been fully characterized.

As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). (See, e.g., Kindt, T.J. et al. Kuby Immunology, 6th ed., W.H. Freeman and Co., N.Y. (2007), page 91) A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. See, e.g., Portolano, S. et al., J. Immunol. 150 (1993) 880-887; Clackson, T. et al., Nature 352 (1991) 624-628).

The term "vector," as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors".

II. COMPOSITIONS AND METHODS

In one aspect, the invention is based, in part, on the finding that using the beta-hairpins of HER3 and HER4 functionally presented in a 3-dimensional orientation within SlyD scaffolds (see e.g Figure 2, and the polypeptides of SEQ ID NO. 13, and 17 to 24) it was possible to select antibodies which are specific for both, the beta-hairpin of HER3 and HER4.

In certain embodiments, the invention provides an antibody that binds to human HER3 and binds to human HER4, wherein the antibody binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) of human HER3 and binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) of human HER4.

Antibodies of the invention are useful, e.g., for the diagnosis or treatment of cancer.

A. Exemplary anti-HER3/HER4 antigen binding proteins and antibodies

The invention provides an isolated antigen binding protein that binds to human HER3 and that binds to human HER4,

a) wherein the antigen binding protein binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3,

and

b) wherein the antigen binding protein binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4.

The invention further provides an isolated antigen binding protein that binds to human HER3 and that binds to human HER4,

a) wherein the antigen binding protein binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;
and

b) wherein the antigen binding protein binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4.

The invention provides an isolated antigen binding protein that binds to human HER3 and that binds to human HER4,

a) wherein the antigen binding protein binds to a polypeptide of

SEQ ID NO: 18 TtSlyDcys-Her3,

and

b) wherein the antigen binding protein binds to a polypeptide of

SEQ ID NO: 22 TtSlyDcys-Her4.

The invention further provides an isolated antigen binding protein that binds to human HER3 and that binds to human HER4,

a) wherein the antigen binding protein binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide of SEQ ID NO: 18 (TtSlyDcas-Her3), and

b) wherein the antigen binding protein binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide of SEQ ID NO: 22 (TtSlyDcas-Her4).

The invention provides an isolated antibody that binds to human HER3 and that binds to human HER4,

a) wherein the antibody binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3,

and

b) wherein the antibody binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4.

The invention further provides an isolated antibody that binds to human HER3 and that binds to human HER4,

a) wherein the antibody binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;
and

b) wherein the antibody binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4.

The invention provides an isolated antibody that binds to human HER3 and that binds to human HER4,

a) wherein the antibody binds to a polypeptide of

SEQ ID NO: 18 TtSlyDcys-Her3,

and

b) wherein the antibody binds to a polypeptide of

SEQ ID NO: 22 TtSlyDcys-Her4.

The invention further provides an isolated antibody that binds to human HER3 and that binds to human HER4,

a) wherein the antibody binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide of SEQ ID NO: 18 (TtSlyDcas-Her3), and

b) wherein the antibody binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide of SEQ ID NO: 22 (TtSlyDcas-Her4).

In one aspect, The invention provides an isolated antibody that binds to human HER3 and binds to human HER4, wherein the antibody binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) of human HER3 and binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) of human HER4.

In certain embodiments, the invention provides an isolated antibody that binds to human HER3 and that binds to human HER4, wherein the antibody has one or more of the following properties (also each combination of each single property is contemplated herein):

a) the antibody binds to the amino acid sequence of SEQ ID NO:1; and/or

b) the antibody binds to the amino acid sequence SEQ ID NO:1 in activated HER3; and/or

c) the antibody binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;
and/or

d) the antibody binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;
and/or

e) the antibody binds to the ß-hairpin region of HER3; and/or

f) the antibody inhibits the heterodimerisation of HER3/HER2 heterodimers in MCF-7 cells in a HER3/HER2 coprecipitation assay (see Example 7); and/or

g) the antibody binds to HER3-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 4.0 or higher (Ka (+Heregulin))/ (Ka (-Heregulin)) (see Example 3b); and/or

h) the antibody binds to HER3-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 2.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)) (see Example 3b); and/or

i) the antibody binds to the amino acid sequence of SEQ ID NO:2; and/or

j) the antibody binds to the amino acid sequence SEQ ID NO:2 in activated HER4; and/or

k) the antibody binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4;
and/or

l) the antibody binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4;
and/or

m) the antibody binds to the ß-hairpin region of HER4; and/or

n) the antibody binds to HER4-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 20.0 or higher (Ka (+Heregulin))/(Ka (-Heregulin)) (see Example 3b); and/or

o) the antibody binds to HER4-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 5.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)) (see Example 3b); and/or

p) the antibody shows as monovalent Fab fragment the same or higher biological activity as compared to its bivalent parent full length antibody (in a HER3 phosphorylation inhibition assay in MCF-7 cells , when compared in equimolar amounts) (see Example 6); and/or

q) the antibody inhibits the HER3 phosporylation in MCF-7 cells (with at least 90 % at a concentration of 6.66 nM) (see Example 6); and/or

r) the antibody does not compete for binding to HER3 with Heregulin/ induces binding of Heregulin to HER3 (see Example 5); and/or

s) the antibody inhibits the proliferation of MDA-MB-175 tumor cells (with an EC50 of 5 µg/ml or lower); and/or

t) the antibody shows tumor growth inhibitory activity in vivo; and/or

u) the antibody binds with an affinity of a KD value $\leq 1 \times 10^{-8}$ M to HER3-ECD (in one embodiment with a KD value of 1×10^{-8} M to 1×10^{-13} M; (in one embodiment with a KD value of 1×10^{-9} M to 1×10^{-13} M); and/or

v) the antibody binds with an affinity of a KD value ≤ 1 x 10^{-8} M to HER4-ECD (in one embodiment with a KD value of 1 x 10^{-8} M to 1 x 10^{-13} M; (in one embodiment with a KD value of 1 x 10^{-9} M to 1 x 10^{-13} M); and/or

w) the antibody binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43) and to a polypeptide of consisting of VYNPTTFQLE (SEQ ID NO:44); and/or

x) the antibody binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43); and/or

y) the antibody binds to a polypeptide consisting of VYNPTTFQLE (SEQ ID NO:44); and/or

z) the antibody binds in a FACS assay to HER3 expressing T47D cells; and wherein the antibody induces an at least 25% higher percentage of internalization of HER3 in the presence of Heregulin as compared to the percentage of internalization of HER3 in the presence of Heregulin when measured after 1 h after antibody exposure. (see example 2e).

In certain embodiments, the invention provides an isolated antibody that binds to human HER3 and that binds to human HER4, wherein the antibody has one or more of the following properties (also each combination of each single property is contemplated herein):

a) the antibody binds to the amino acid sequence of SEQ ID NO:1; and
the antibody binds to the amino acid sequence of SEQ ID NO:2;

and/or

b) the antibody binds to the amino acid sequence SEQ ID NO:1 in activated HER3; and
the antibody binds to the amino acid sequence SEQ ID NO:2 in activated HER4;

and/or

c) the antibody binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;

and the antibody binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4;

and/or

d) the antibody binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;

the antibody binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4;

and/or

e) the antibody binds to the ß-hairpin region of HER3; and the antibody binds to the ß-hairpin region of HER4;

and/or

f) the antibody binds to HER3-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 4.0 or higher (Ka (+Heregulin))/ (Ka (-Heregulin)) (see Example 3b); and
the antibody binds to HER4-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 20.0 or higher (Ka (+Heregulin))/(Ka (-Heregulin)) (see Example 3b);

and/or

g) the antibody binds to HER3-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 2.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)) (see Example 3b); and
the antibody binds to HER4-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 5.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)) (see Example 3b);

and/or

h) the antibody binds with an affinity of a KD value ≤ 1 x 10^{-8} M to HER3-ECD (in one embodiment with a KD value of 1 x 10^{-8} M to 1 x 10^{-13} M; (in one embodiment with a KD value of 1 x 10^{-9} M to 1 x 10^{-13} M); and

the antibody binds with an affinity of a KD value ≤ 1 x 10^{-8} M to HER4-ECD (in one embodiment with a KD value of 1 x 10^{-8} M to 1 x 10^{-13} M; (in one embodiment with a KD value of 1 x 10^{-9} M to 1 x 10^{-13} M);

and/or

i) the antibody binds to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNKLTFQLEP (SEQ ID NO:43) and to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNPTTFQLE (SEQ ID NO:44); and/or

j) the antibody binds to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNKLTFQLEP (SEQ ID NO:43); and/or

k) the antibody binds to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNPTTFQLE (SEQ ID NO:44).

In certain embodiments, the invention provides an isolated antibody that binds to human HER3 and that binds to human HER4, wherein:

the antibody binds to the amino acid sequence of SEQ ID NO:1; and
the antibody binds to the amino acid sequence of SEQ ID NO:2.

In certain embodiments, the invention provides an isolated antibody that binds to human HER3 and that binds to human HER4, wherein:

the antibody binds to the amino acid sequence SEQ ID NO:1 in activated HER3; and
the antibody binds to the amino acid sequence SEQ ID NO:2 in activated HER4;

In certain embodiments, the invention provides an isolated antibody that binds to human HER3 and that binds human HER4, wherein the antibody binds to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNKLTFQLEP (SEQ ID NO:43) (of human HER3) and to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNPTTFQLE (SEQ ID NO:44) (of human HER4).

In one aspect, the invention provides an anti-HER3/HER4 antibody comprising at least one, two, three, four, five, or six HVRs selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VH HVR sequences selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In one embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In another embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27 and HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In a further embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27, HVR-L3 comprising the amino acid sequence of SEQ ID NO:30, and HVR-H1 comprising the amino acid sequence of SEQ ID NO:25. In a further embodiment, the antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

In another aspect, the invention provides an antibody comprising at least one, at least two, or all three VL HVR sequences selected from (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In one embodiment, the antibody comprises (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28; (b) HVR-L2

comprising the amino acid sequence of SEQ ID NO:29; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, an antibody of the invention comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences selected from (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26, and (iii) HVR-H3 comprising an amino acid sequence selected from SEQ ID NO:27; and (b) a VL domain comprising at least one, at least two, or all three VL HVR sequences selected from (i) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28, (ii) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29, and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, the invention provides an antibody comprising (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and (f) HVR-L3 comprising an amino acid sequence selected from SEQ ID NO:30.

In one aspect, the invention provides an anti-HER3/HER4 antibody comprising at least one, two, three, four, five, or six HVRs selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VH HVR sequences selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In one embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In another embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27 and HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In a further embodiment, the antibody comprises HVR-H3 comprising the amino acid

sequence of SEQ ID NO:27, HVR-L3 comprising the amino acid sequence of SEQ ID NO:30, and HVR-H2 comprising the amino acid sequence of SEQ ID NO:39. In a further embodiment, the antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

In another aspect, the invention provides an antibody comprising at least one, at least two, or all three VL HVR sequences selected from (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In one embodiment, the antibody comprises (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, an antibody of the invention comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences selected from (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39, and (iii) HVR-H3 comprising an amino acid sequence selected from SEQ ID NO:27; and (b) a VL domain comprising at least one, at least two, or all three VL HVR sequences selected from (i) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40, (ii) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41, and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, the invention provides an antibody comprising (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (f) HVR-L3 comprising an amino acid sequence selected from SEQ ID NO:30.

In one aspect, the invention provides an anti-HER3/HER4 antibody comprising at least one, two, three, four, five, or six HVRs selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence

of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VH HVR sequences selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In one embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In another embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27 and HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In a further embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27, HVR-L3 comprising the amino acid sequence of SEQ ID NO:30, and HVR-H2 comprising the amino acid sequence of SEQ ID NO:39. In a further embodiment, the antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

In another aspect, the invention provides an antibody comprising at least one, at least two, or all three VL HVR sequences selected from (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In one embodiment, the antibody comprises (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, an antibody of the invention comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences selected from (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39, and (iii) HVR-H3 comprising an amino acid sequence selected from SEQ ID NO:27; and (b) a VL domain comprising at least one, at least two, or all three VL HVR sequences selected from (i) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40, (ii) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42, and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, the invention provides an antibody comprising (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (f) HVR-L3 comprising an amino acid sequence selected from SEQ ID NO:30.

In one aspect, the invention provides an anti-HER3/HER4 antibody comprising at least one, two, three, four, five, or six HVRs selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VH HVR sequences selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In one embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In another embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27 and HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In a further embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27, HVR-L3 comprising the amino acid sequence of SEQ ID NO:30, and HVR-H2 comprising the amino acid sequence of SEQ ID NO:39. In a further embodiment, the antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

In another aspect, the invention provides an antibody comprising at least one, at least two, or all three VL HVR sequences selected from (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In one embodiment, the antibody comprises (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2

comprising the amino acid sequence of SEQ ID NO:41; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, an antibody of the invention comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences selected from (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39, and (iii) HVR-H3 comprising an amino acid sequence selected from SEQ ID NO:27; and (b) a VL domain comprising at least one, at least two, or all three VL HVR sequences selected from (i) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40, (ii) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41, and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, the invention provides an antibody comprising (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (f) HVR-L3 comprising an amino acid sequence selected from SEQ ID NO:30.

In one aspect, the invention provides an anti-HER3/HER4 antibody comprising at least one, two, three, four, five, or six HVRs selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three VH HVR sequences selected from (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In one embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27. In another embodiment, the antibody comprises HVR-H3 comprising the amino acid sequence of SEQ ID NO:27 and HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In a further embodiment, the antibody comprises HVR-H3 comprising the amino acid

sequence of SEQ ID NO:27, HVR-L3 comprising the amino acid sequence of SEQ ID NO:30, and HVR-H2 comprising the amino acid sequence of SEQ ID NO:39. In a further embodiment, the antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

In another aspect, the invention provides an antibody comprising at least one, at least two, or all three VL HVR sequences selected from (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30. In one embodiment, the antibody comprises (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, an antibody of the invention comprises (a) a VH domain comprising at least one, at least two, or all three VH HVR sequences selected from (i) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25, (ii) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39, and (iii) HVR-H3 comprising an amino acid sequence selected from SEQ ID NO:27; and (b) a VL domain comprising at least one, at least two, or all three VL HVR sequences selected from (i) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40, (ii) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42, and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, the invention provides an antibody comprising (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39; (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27; (d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (f) HVR-L3 comprising an amino acid sequence selected from SEQ ID NO:30.

In another aspect, the invention provides an anti-HER3/HER4 antibody comprising

i) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25;
(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26;
(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28; (e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and (f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30;

ii) or a humanized variant of the HVRs of the antibody under i) (a), (b), (d) and/or (e).

In another aspect, the invention provides an anti-HER3/HER4 antibody comprising

(a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38;
(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;
(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;
(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;
(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and
(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30:

In another aspect, the invention provides an anti-HER3/HER4 antibody comprising

(a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38;
(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;
(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;
(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;
(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and
(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, the invention provides an anti-HER3/HER4 antibody comprising

(a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25;
(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;
(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;
(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;
(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and
(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, the invention provides an anti-HER3/HER4 antibody comprising

(a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25;
(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;
(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;
(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and
(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In any of the above embodiments, an anti-HER3/HER4 antibody is humanized. In one embodiment, an anti-HER3/HER4 antibody comprises HVRs as in any of the above embodiments, and further comprises an acceptor human framework, e.g. a human immunoglobulin framework or a human consensus framework. In another embodiment, an anti-HER3/HER4 antibody comprises HVRs as in any of the above embodiments, and further comprises a VH comprising a framework region of human germline IGHV1-46-01 or IMGT_hVH_1_46 and a VL comprising a framework region of human germline IGKV3-11-01 or IMGT_hVK_1_39. Frameork regions and sequences of human germlines are described in Poul, M-A. and Lefranc, M-P., in "Ingénierie des anticorps banques combinatores" ed. by Lefranc, M-P. and Lefranc, G., Les Editions INSERM, 1997. Human heavy and light chain variable framework regions of all human germlines are listed e.g. in Lefranc, M.P., Current Protocols in Immunology (2000) - Appendix 1P A.1P.1-A.1P.37 and are accessible via IMGT, the international ImMunoGeneTics information system® (http://imgt.cines.fr) or via http://vbase.mrc-cpe.cam.ac.uk.

In another aspect, an anti-HER3/HER4 antibody comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:33. In certain embodiments, a VH sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-HER3/HER4 antibody comprising that sequence retains the ability to bind to HER3, and HER4, respectively. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO:33. In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-HER3/HER4 antibody comprises the VH sequence in SEQ ID NO:33, including post-translational modifications of that sequence. In a particular embodiment, the VH comprises one, two or three HVRs selected from: (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38, (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39, and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

In another aspect, an anti-HER3/HER4 antibody comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:34. In certain embodiments, a VH sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-HER3/HER4 antibody comprising that sequence retains the ability to bind to HER3, and HER4, respectively. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO:34. In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-HER3/HER4 antibody comprises the VH sequence in SEQ ID NO:34, including post-translational modifications of that sequence. In a particular embodiment, the VH comprises one, two or three HVRs selected from: (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25, (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39, and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

In another aspect, an anti-HER3/HER4 antibody comprises a heavy chain variable domain (VH) sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:35. In certain embodiments, a VH sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-HER3/HER4 antibody comprising that sequence retains the ability to bind to HER3, and HER4, respectively. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO:35. In certain embodiments, substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-HER3/HER4 antibody comprises the VH sequence in SEQ ID NO:35, including post-translational modifications of that sequence. In a particular embodiment, the VH comprises one, two or three HVRs selected from: (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25, (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39, and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

In another aspect, an anti-HER3/HER4 antibody is provided, wherein the antibody comprises a light chain variable domain (VL) having at least 90%, 91%, 92%, 93%,

94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:36. In certain embodiments, a VL sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-HER3/HER4 antibody comprising that sequence retains the ability to bind to HER3, and HER4, respectively. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO:36. In certain embodiments, the substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-HER3/HER4 antibody comprises the VL sequence in SEQ ID NO:36, including post-translational modifications of that sequence. In a particular embodiment, the VL comprises one, two or three HVRs selected from (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, an anti-HER3/HER4 antibody is provided, wherein the antibody comprises a light chain variable domain (VL) having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the amino acid sequence of SEQ ID NO:37. In certain embodiments, a VL sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity contains substitutions (e.g., conservative substitutions), insertions, or deletions relative to the reference sequence, but an anti-HER3/HER4 antibody comprising that sequence retains the ability to bind to HER3, and HER4, respectively. In certain embodiments, a total of 1 to 10 amino acids have been substituted, inserted and/or deleted in SEQ ID NO:37. In certain embodiments, the substitutions, insertions, or deletions occur in regions outside the HVRs (i.e., in the FRs). Optionally, the anti-HER3/HER4 antibody comprises the VL sequence in SEQ ID NO:37, including post-translational modifications of that sequence. In a particular embodiment, the VL comprises one, two or three HVRs selected from (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

In another aspect, an anti-HER3/Her4 antibody is provided, wherein the antibody comprises a VH as in any of the embodiments provided above, and a VL as in any of the embodiments provided above. In one embodiment, the antibody comprises the VH and VL sequences in SEQ ID NO:33 and SEQ ID NO:36,

the VH and VL sequences in SEQ ID NO:33 and SEQ ID NO:37
the VH and VL sequences in SEQ ID NO:34 and SEQ ID NO:36,
the VH and VL sequences in SEQ ID NO:34 and SEQ ID NO:37,
the VH and VL sequences in SEQ ID NO:35 and SEQ ID NO:36, or
the VH and VL sequences in SEQ ID NO:33 and SEQ ID NO:37,
respectively, including post-translational modifications of those sequences.

In another aspect, an anti-HER3/Her4 antibody is provided, wherein the antibody comprises a heavy chain variable domain (VH) sequence having at least 95%, sequence identity and a light chain variable domain (VL) having at least 95%, or 100% sequence identity to antibody comprising

a) the VH and VL sequences in SEQ ID NO:33 and SEQ ID NO:36,
b) the VH and VL sequences in SEQ ID NO:33 and SEQ ID NO:37
c) the VH and VL sequences in SEQ ID NO:34 and SEQ ID NO:36,
d) the VH and VL sequences in SEQ ID NO:34 and SEQ ID NO:37,
e) the VH and VL sequences in SEQ ID NO:35 and SEQ ID NO:36, or
f) the VH and VL sequences in SEQ ID NO:33 and SEQ ID NO:37,
respectively,
wherein the antibody has one or more of the following properties:

In certain embodiments, the invention provides an isolated antibody that binds to human HER3 and that binds to human HER4, wherein the antibody has one or more of the following properties: the antibody

a) binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;

b) binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;

c) inhibits the heterodimerisation of HER3/HER2 heterodimers in MCF-7 cells in a HER3/HER2 coprecipitation assay;

d) binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4;

e) binds to a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4;

f) shows as monovalent Fab fragment the same or higher biological activity as compared to its bivalent parent full length antibody (when compared in equimolar amounts in a HER3 phosphorylation inhibition assay in MCF-7 cells);

g) shows tumor growth inhibitory activity in vivo;

h) binds with an affinity of a KD value ≤ 1 x 10-8 M to HER3-ECD (in one embodiment with a KD value of 1 x 10-8 M to 1 x 10-13 M; (in one embodiment with a KD value of 1 x 10-9 M to 1 x 10-13 M);

i) binds with an affinity of a KD value ≤ 1 x 10-8 M to HER4-ECD (in one embodiment with a KD value of 1 x 10-8 M to 1 x 10-13 M; (in one embodiment with a KD value of 1 x 10-9 M to 1 x 10-13 M);

j) wherein the antibody binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43) or to a polypeptide of consisting of VYNPTTFQLE (SEQ ID NO:44);

k) wherein the antibody binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43); and/or

l) wherein the antibody binds to a polypeptide consisting of VYNPTTFQLE (SEQ ID NO:44).

In a further aspect, the invention provides an antibody that binds to the same epitope as an anti-HER3/HER4 antibody provided herein. For example, in certain embodiments, an antibody is provided that binds to the same epitope as an anti-HER3/HER4 antibody comprising a VH sequence of SEQ ID NO:31 and a VL sequence of SEQ ID NO:32 . In certain embodiments, an antibody is provided that binds to an epitope within a fragment of human HER3 consisting of amino acids VYNKLTFQLEP (SEQ ID NO:43) and/or that binds to an epitope within a fragment of human HER4 consisting of amino acids VYNPTTFQLE (SEQ ID NO:44).

1. Antibody Affinity

In certain embodiments, an antibody provided herein has a dissociation constant KD of ≤ 1 μM, ≤ 100 nM, ≤ 10 nM, ≤ 1 nM, ≤ 0.1 nM, ≤ 0.01 nM, or ≤ 0.001 nM (e.g. 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M, e.g., from 10^{-9} M to 10^{-13} M).

In one preffered embodiment, KD is measured using surface plasmon resonance assays using a BIACORE®) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with *N*-ethyl-*N'*- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8,

to 5 μg/ml (~0.2 μM) before injection at a flow rate of 5 μl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20™) surfactant (PBST) at 25°C at a flow rate of approximately 25 μl/min. Association rates (k_{on} or ka) and dissociation rates (k_{off} or kd) are calculated using a simple one-to-one Langmuir binding model (BIACORE® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant KD is calculated as the ratio kd/ka (k_{off}/k_{on}.) See, e.g., Chen, Y. et al., J. Mol. Biol. 293 (1999) 865-881. If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophotometer (Aviv Instruments) or a 8000-series SLM-AMINCO ™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

2. Antibody Fragments

In certain embodiments, an antibody provided herein is an antibody fragment. Antibody fragments include, but are not limited to, Fab, Fab', Fab'-SH, $F(ab')_2$, Fv, and scFv fragments, and other fragments described below. For a review of certain antibody fragments, see Hudson, P.J. et al., Nat. Med. 9 (2003) 129-134. For a review of scFv fragments, see, e.g., Plueckthun, A., In; The Pharmacology of Monoclonal Antibodies, Vol. 113, Rosenburg and Moore (eds.), Springer-Verlag, New York (1994), pp. 269-315; see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and $F(ab')_2$ fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No. 5,869,046.

Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific. See, for example, EP 0 404 097; WO 1993/01161; Hudson, P.J. et al., Nat. Med. 9 (2003) 129-134; and Holliger, P. et al., Proc. Natl. Acad. Sci. USA 90 (1993) 6444-6448. Triabodies and tetrabodies are also described in Hudson, P.J. et al., Nat. Med. 9 (20039 129-134).

Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; *see,* e.g., U.S. Patent No. 6,248,516 B1).

Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. *E. coli* or phage), as described herein.

3. Chimeric and Humanized Antibodies

In certain embodiments, an antibody provided herein is a chimeric antibody. Certain chimeric antibodies are described, e.g., in U.S. Patent No. 4,816,567; and Morrison, S.L. et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855). In one example, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a "class switched" antibody in which the class or subclass has been changed from that of the parent antibody. Chimeric antibodies include antigen-binding fragments thereof.

In certain embodiments, a chimeric antibody is a humanized antibody. Typically, a non-human antibody is humanized to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. Generally, a humanized antibody comprises one or more variable domains in which HVRs, e.g., CDRs, (or portions thereof) are derived from a non-human antibody, and FRs (or portions thereof) are derived from human antibody sequences. A humanized antibody optionally will also comprise at least a portion of a human constant region. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the HVR residues are derived), e.g., to restore or improve antibody specificity or affinity.

Humanized antibodies and methods of making them are reviewed, e.g., in Almagro, J.C. and Fransson, J., Front. Biosci. 13 (2008) 1619-1633, and are further described, e.g., in Riechmann, I. et al., Nature 332 (1988) 323-329; Queen, C. et al., Proc. Natl. Acad. Sci. USA 86 (1989) 10029-10033; US Patent Nos. 5, 821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri, S.V. et al., Methods 36 (2005) 25-

34 (describing SDR (a-CDR) grafting); Padlan, E.A., Mol. Immunol. 28 (1991) 489-498 (describing "resurfacing"); Dall'Acqua, W.F. et al., Methods 36 (2005) 43-60 (describing "FR shuffling"); and Osbourn, J. et al., Methods 36 (2005) 61-68 and Klimka, A. et al., Br. J. Cancer 83 (2000) 252-260 (describing the "guided selection" approach to FR shuffling). Morea, V., et al., Methods, Vol 20, Issue 3 (2000) 267-279) and WO2004/006955 (approach via canonical structures).

4. Human Antibodies

In certain embodiments, an antibody provided herein is a human antibody. Human antibodies can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk, M.A. and van de Winkel, J.G., Curr. Opin. Pharmacol. 5 (2001) 368-374 and Lonberg, N., Curr. Opin. Immunol. 20 (2008) 450-459.

Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic mice, the endogenous immunoglobulin loci have generally been inactivated. For review of methods for obtaining human antibodies from transgenic animals, see Lonberg, N., Nat. Biotech. 23 (2005) 1117-1125. See also, e.g., U.S. Patent Nos. 6,075,181 and 6,150,584 describing XENOMOUSE™ technology; U.S. Patent No. 5,770,429 describing HuMab® technology; U.S. Patent No. 7,041,870 describing K-M MOUSE® technology, and U.S. Patent Application Publication No. US 2007/0061900, describing VelociMouse® technology). Human variable regions from intact antibodies generated by such animals may be further modified, e.g., by combining with a different human constant region.

Human antibodies can also be made by hybridoma-based methods. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described. (See, e.g., Kozbor, D., J. Immunol. 133 (1984) 3001-3005; Brodeur, B.R. et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York (1987), pp. 51-63; and Boerner, P. et al., J. Immunol. 147 (1991) 86-95) Human antibodies generated via human B-cell hybridoma technology are also described in Li, J. et al., Proc.

Natl. Acad. Sci. USA 103 (2006) 3557-3562. Additional methods include those described, for example, in U.S. Patent No. 7,189,826 (describing production of monoclonal human IgM antibodies from hybridoma cell lines) and Ni, J., Xiandai Mianyixue 26 (2006) 265-268 (describing human-human hybridomas). Human hybridoma technology (Trioma technology) is also described in Vollmers, H.P. and Brandlein, S., Histology and Histopathology 20 (2005) 927-937 and Vollmers, H.P. and Brandlein, S., Methods and Findings in Experimental and Clinical Pharmacology 27 (2005) 185-191.

Human antibodies may also be generated by isolating Fv clone variable domain sequences selected from human-derived phage display libraries. Such variable domain sequences may then be combined with a desired human constant domain. Techniques for selecting human antibodies from antibody libraries are described below.

5. Library-Derived Antibodies

Antibodies of the invention may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics. Such methods are reviewed, e.g., in Hoogenboom, H.R. et al., Methods in Molecular Biology 178 (2001) 1-37 and further described, e.g., in the McCafferty, J. et al., Nature 348 (1990) 552-554; Clackson, T. et al., Nature 352 (1991) 624-628; Marks, J.D. et al., J. Mol. Biol. 222 (1992) 581-597; Marks, J.D. and Bradbury, A., Methods in Molecular Biology 248 (2003) 161-175; Sidhu, S.S. et al., J. Mol. Biol. 338 (2004) 299-310; Lee, C.V. et al., J. Mol. Biol. 340 (2004) 1073-1093; Fellouse, F.A., Proc. Natl. Acad. Sci. USA 101 (2004) 12467-12472; and Lee, C.V. et al., J. Immunol. Methods 284 (2004) 119-132.

In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter, G. et al., Ann. Rev. Immunol. 12 (1994) 433-455. Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of

antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths, A.D. et al., EMBO J. 12 (1993) 725-734. Finally, naive libraries can also be made synthetically by cloning non-rearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom, H.R. and Winter, G., J. Mol. Biol. 227 (1992) 381-388. Patent publications describing human antibody phage libraries include, for example: US Patent No. 5,750,373, and US Patent Publication Nos. 2005/0079574, 2005/0119455, 2005/0266000, 2007/0117126, 2007/0160598, 2007/0237764, 2007/0292936, and 2009/0002360.

Antibodies or antibody fragments isolated from human antibody libraries are considered human antibodies or human antibody fragments herein.

6. Multispecific Antibodies

In certain embodiments, an antibody provided herein is a multispecific antibody, e.g. a bispecific antibody. Multispecific antibodies are monoclonal antibodies that have binding specificities for at least two different sites. In certain embodiments, one of the binding specificities is for HER3/HER4 and the other is for any other antigen. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express HER3 or HER4. Bispecific antibodies can be prepared as full length antibodies or antibody fragments.

Techniques for making multispecific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein, C. and Cuello, A.C., Nature 305 (1983) 537-540, WO 93/08829, and Traunecker, A. et al., EMBO J. 10 (1991) 3655-3659), and "knob-in-hole" engineering (see, e.g., U.S. Patent No. 5,731,168). Multi-specific antibodies may also be made by engineering electrostatic steering effects for making antibody Fc-heterodimeric molecules (WO 2009/089004); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan, M. et al., Science 229 (1985) 81-83); using leucine zippers to produce bi-specific antibodies (see, e.g., Kostelny, S.A. et al., J. Immunol. 148 (1992) 1547-1553; using "diabody" technology for making bispecific antibody fragments (see, e.g., Holliger, P. et al., Proc. Natl. Acad. Sci. USA 90 (1993) 6444-6448); and using single-chain Fv (sFv) dimers (see, e.g. Gruber, M et al., J. Immunol. 152

(1994) 5368-5374); and preparing trispecific antibodies as described, e.g., in Tutt, A. et al., J. Immunol. 147 (1991) 60-69).

Engineered antibodies with three or more functional antigen binding sites, including "Octopus antibodies," are also included herein (see, e.g. US 2006/0025576).

The antibody or fragment herein also includes a "Dual Acting Fab" or "DAF" comprising an antigen binding site that binds to HER3/HER4 as well as another, different antigen (see, US 2008/0069820, for example).

The antibody or fragment herein also includes multispecific antibodies described in WO 2009/080251, WO 2009/080252, WO 2009/080253, WO 2009/080254, WO 2010/112193, WO 2010/115589, WO 2010/136172, WO 2010/145792, and WO 2010/145793.

7. Antibody Variants

In certain embodiments, amino acid sequence variants of the antibodies provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding.

a) Substitution, Insertion, and Deletion Variants

In certain embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitutional mutagenesis include the HVRs and FRs. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". More substantial changes are provided in Table 1 under the heading of "exemplary substitutions," and as further described below in reference to amino acid side chain classes. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired

activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

(1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;

(2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

(5) residues that influence chain orientation: Gly, Pro;

(6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antibody and/or will have substantially retained certain biological properties of the parent antibody. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more HVR residues are mutated and the variant antibodies displayed on phage and screened for a particular biological activity (e.g. binding affinity).

Alterations (e.g., substitutions) may be made in HVRs, e.g., to improve antibody affinity. Such alterations may be made in HVR "hotspots," i.e., residues encoded by codons that undergo mutation at high frequency during the somatic maturation process (see, e.g., Chowdhury, P.S., Methods Mol. Biol. 207 (2008) 179-196), and/or SDRs (a-CDRs), with the resulting variant VH or VL being tested for binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom, H.R. et al. in Methods in Molecular Biology 178 (2002) 1-37. In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. CDR-H3 and CDR-L3 in particular are often targeted.

In certain embodiments, substitutions, insertions, or deletions may occur within one or more HVRs so long as such alterations do not substantially reduce the ability of the antibody to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in HVRs. Such alterations may be outside of HVR "hotspots" or SDRs. In certain embodiments of the variant VH and VL sequences provided above, each HVR either is unaltered, or contains no more than one, two or three amino acid substitutions.

A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham, B.C. and Wells, J.A., Science 244 (1989) 1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as arg, asp, his, lys, and glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g. for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

b) Glycosylation variants

In certain embodiments, an antibody provided herein is altered to increase or decrease the extent to which the antibody is glycosylated. Addition or deletion of glycosylation sites to an antibody may be conveniently accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed.

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, e.g., Wright, A. and Morrison, S.L., TIBTECH 15 (1997) 26-32. The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an antibody of the invention may be made in order to create antibody variants with certain improved properties.

In one embodiment, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e. g. complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues); however, Asn297 may also be located about ± 3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. See, e.g., US 2003/0157108; US 2004/0093621. Examples of publications related to "defucosylated" or "fucose-deficient" antibody variants include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO 2005/053742; WO 2002/031140; Okazaki, A. et al., J. Mol. Biol. 336 (2004) 1239-1249; Yamane-Ohnuki, N. et al., Biotech. Bioeng. 87 (2004) 614-622. Examples of cell lines capable of producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka, J. et al., Arch. Biochem. Biophys. 249 (1986) 533-545; US 2003/0157108; and WO 2004/056312, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8,* knockout CHO cells (see, e.g., Yamane-Ohnuki, N. et al., Biotech. Bioeng. 87 (2004) 614-622; Kanda, Y. et al., Biotechnol. Bioeng. 94 (2006) 680-688; and WO 2003/085107).

Antibodies variants are further provided with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc region of the antibody is bisected by GlcNAc. Such antibody variants may have reduced fucosylation and/or improved ADCC function. Examples of such antibody variants are described, e.g., in WO 2003/011878; US Patent No. 6,602,684; and US 2005/0123546. Antibody variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function. Such antibody variants are described, e.g., in WO 1997/30087; WO 1998/58964; and WO 1999/22764.

c) Fc region variants

In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (*e.g.* a substitution) at one or more amino acid positions.

In certain embodiments, the invention contemplates an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half life of the antibody *in vivo* is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express Fc(RIII only, whereas monocytes express FcgammaRI, FcgammaRII and FcgammaRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch, J.V. and Kinet, J.P., Annu. Rev. Immunol. 9 (1991) 457-492. Non-limiting examples of *in vitro* assays to assess ADCC activity of a molecule of interest is described in U.S. Patent No. 5,500,362 (see, e.g. Hellstrom, I. et al., Proc. Natl. Acad. Sci. USA 83 (1986) 7059-7063; and Hellstrom, I. et al., Proc. Natl. Acad. Sci. USA 82 (1985) 1499-1502); U.S. Patent No. 5,821,337 (see Bruggemann, M. et al., J. Exp. Med. 166 (1987) 1351-1361). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA; and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for

such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, *e.g.*, in an animal model such as that disclosed in Clynes, R. et al., Proc. Natl. Acad. Sci. USA 95 (1998) 652-656. C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. See, e.g., C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro, H. et al., J. Immunol. Methods 202 (1996) 163-171; Cragg, M.S. et al., Blood 101 (2003) 1045-1052; and Cragg, M.S. and M.J. Glennie, Blood 103 (2004) 2738-2743). FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art (see, e.g., Petkova, S.B. et al., Int. Immunol. 18 (2006: 1759-1769).

Antibodies with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

Certain antibody variants with improved or diminished binding to FcRs are described. (See, e.g., U.S. Patent No. 6,737,056; WO 2004/056312, and Shields, R.L. et al., J. Biol. Chem. 276 (2001) 6591-6604)

In certain embodiments, an antibody variant comprises an Fc region with one or more amino acid substitutions which improve ADCC, e.g., substitutions at positions 298, 333, and/or 334 of the Fc region (EU numbering of residues).

In some embodiments, alterations are made in the Fc region that result in altered (*i.e.*, either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in US Patent No. 6,194,551, WO 99/51642, and Idusogie, E.E. et al., J. Immunol. 164 (2000) 4178-4184.

Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer, R.L. et al., J. Immunol. 117 (1976) 587-593, and Kim, J.K. et al., J. Immunol. 24 (1994) 2429-2434), are described in US 2005/0014934. Those antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. Such Fc variants include those with

substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc region residue 434 (US Patent No. 7,371,826).

See also Duncan, A.R. and Winter, G., Nature 322 (1988) 738-740; US 5,648,260; US 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

d) Cysteine engineered antibody variants

In certain embodiments, it may be desirable to create cysteine engineered antibodies, e.g., "thioMAbs," in which one or more residues of an antibody are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the antibody. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Cysteine engineered antibodies may be generated as described, e.g., in U.S. Patent No. 7,521,541.

e) Antibody Derivatives

In certain embodiments, an antibody provided herein may be further modified to contain additional non-proteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer is attached, they can be the

same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

In another embodiment, conjugates of an antibody and non-proteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the non-proteinaceous moiety is a carbon nanotube (Kam, N.W. et al., Proc. Natl. Acad. Sci. USA 102 (2005) 11600-11605). The radiation may be of any wavelength, and includes, but is not limited to, wavelengths that do not harm ordinary cells, but which heat the non-proteinaceous moiety to a temperature at which cells proximal to the antibody-non-proteinaceous moiety are killed.

B. Recombinant Methods and Compositions

Antibodies may be produced using recombinant methods and compositions, e.g., as described in U.S. Patent No. 4,816,567. In one embodiment, isolated nucleic acid encoding an anti-HER3/HER4 antibody described herein is provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (e.g., the light and/or heavy chains of the antibody). In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (e.g., has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and an amino acid sequence comprising the VH of the antibody, or (2) a first vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the antibody. In one embodiment, the host cell is eukaryotic, e.g. a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., Y0, NS0, Sp20 cell). In one embodiment, a method of making an anti-HER3/HER4 antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antibody, as provided above, under conditions suitable for expression of the antibody, and optionally recovering the antibody from the host cell (or host cell culture medium).

For recombinant production of an anti-HER3/HER4 antibody, nucleic acid encoding an antibody, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such nucleic acid may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody).

Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., US 5,648,237, US 5,789,199, and US 5,840,523. (See also Charlton, K.A., In: Methods in Molecular Biology, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2003), pp. 245-254, describing expression of antibody fragments in *E. coli*.) After expression, the antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized," resulting in the production of an antibody with a partially or fully human glycosylation pattern. See Gerngross, T.U., Nat. Biotech. 22 (2004) 1409-1414; and Li, H. et al., Nat. Biotech. 24 (2006) 210-215.

Suitable host cells for the expression of glycosylated antibody are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures can also be utilized as hosts. *See,* e.g., US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants).

Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells as described, e.g., in Graham, F.L. et al., J. Gen Virol. 36 (1977) 59-74); baby hamster kidney cells

(BHK); mouse sertoli cells (TM4 cells as described, e.g., in Mather, J.P., Biol. Reprod. 23 (1980) 243-252); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK; buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TRI cells, as described, e.g., in Mather, J.P. et al., Annals N.Y. Acad. Sci. 383 (1982) 44-68; MRC 5 cells; and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including DHFR$^-$ CHO cells (Urlaub, G. et al., Proc. Natl. Acad. Sci. USA 77 (1980) 4216-4220); and myeloma cell lines such as Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody production, see, e.g., Yazaki, P. and Wu, A.M., Methods in Molecular Biology, Vol. 248, Lo, B.K.C. (ed.), Humana Press, Totowa, NJ (2004), pp. 255-268.

C. Assays and Antibody selection methods

Anti-HER3/HER4 antibodies (or antigen binding proteins) provided herein may be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art.

One aspect of the invention is a method for selecting an antibody (or antigen binding protein) that binds to human HER3 and binds to human HER4, wherein the antibody (or antigen binding protein) binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) of human HER3 and binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) of human HER4; wherein

a) at least one polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3,

which comprises the amino acid sequence of SEQ ID NO:1;

and

b) at least one polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4,

which comprises the amino acid sequence of SEQ ID NO:2;

are used (in a binding assay) to select antibodies (or antigen binding proteins), which show binding to both, the at least one polypeptide under a) and the at least one polypeptide under b)

and thereby selecting an antibody (or antigen binding protein) that binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) (within human HER3) and within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) (within human HER4).

In one embodiment the selection method further comprises a step wherein the selected antibodies (or antigen binding proteins) are counterscreened with the polypeptides (tested for binding to the polypeptides) selected from the group consisiting of:

SEQ ID NO: 14 TtSlyD-Wildtype

SEQ ID NO: 15 TtSlyDcas

SEQ ID NO: 16 TgSlyDΔIF

to confirm that the selected antibodies (or antigen binding proteins) do not bind to the polypetide scaffolds which are not comprising amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) or the amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2).

The invention provides an antibody (or antigen binding protein) obtained by such selection method.

A method for selecting an antibody (or antigen binding protein) specifically binding to a human HER3 and human HER4 comprising the following steps:

a) determining the binding affinity of a plurality of antibodies (or antigen binding proteins) to the ß-hairpin of HER3 with the amino acid sequence of SEQ ID NO:1, whereby ß-hairpin of HER3 is presented as polypeptide selected from the group consisting of:

i) SEQ ID NO: 13 TtSlyD-FKBP-Her3,

ii) SEQ ID NO: 17 TtSlyDcas-Her3,

iii)SEQ ID NO: 18 TtSlyDcys-Her3,

iv)SEQ ID NO: 19 TgSlyDser-Her3, and

v) SEQ ID NO: 20 TgSlyDcys-Her3,

which comprise the ß-hairpin of HER3 with the amino acid sequence of SEQ ID NO:1,

b) selecting the antibody having an apparent complex stability above a pre-defined threshold level;

c) determining the binding affinity of the selected antibodies (or antigen binding proteins) under step b) to the ß-hairpin of HER4 with the amino acid sequence of SEQ ID NO:2, whereby ß-hairpin of HER4 is presented as polypeptide selected from the group consisting of:

i) SEQ ID NO: 21 TtSlyDcas-Her4,

ii) SEQ ID NO: 22 TtSlyDcys-Her4,

iii)SEQ ID NO: 23 TgSlyDser-Her4,and

iv)SEQ ID NO: 24 TgSlyDcys-Her4,

which comprise the ß-hairpin of HER4 with the amino acid sequence of SEQ ID NO:2,

d) selecting the antibody (or antigen binding protein) having an apparent complex stability above a pre-defined threshold level.

1. Binding assays and other assays

In one aspect, an antibody (or antigen binding protein) of the invention is tested for its antigen binding activity, e.g., by known methods such as ELISA, Western blot, including surface plasmon resonance (e.g. BIACORE) , etc.

In another aspect, competition assays may be used to identify an antibody that competes with M-05-74 for binding to HER3 and/or to HER4. In certain embodiments, such a competing antibody binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by M-05-74. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris, G.E. (ed.), Epitope Mapping Protocols, In: Methods in Molecular Biology, Vol. 66, Humana Press, Totowa, NJ (1996). Further methods are described in detail in Example 4 using the CelluSpot™ technology.

In an exemplary competition assay, immobilized HER3 or HER4 is incubated in a solution comprising a first labeled antibody that binds to HER3 or HER4, respectively (e.g., M-05-74) and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to HER3 or HER4. The second antibody may be present in a hybridoma supernatant. As a control, immobilized HER3 or HER4 is incubated in a solution comprising the first labeled antibody but not the second unlabeled antibody. After incubation under conditions permissive for binding of the first antibody to HER3 or HER4, excess unbound antibody is removed, and the amount of label associated with immobilized HER3 or HER4 is measured. If the amount of label associated with immobilized HER3 or HER4 is substantially reduced in the test sample relative to the control sample, then that indicates that the second antibody is competing with the first antibody for binding to HER3 or HER4. See Harlow, E. and Lane, D., Antibodies: A Laboratory Manual, Chapter 14, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988).

2. Activity assays

In one aspect, assays are provided for identifying anti-HER3/HER4 antibodies (or antigen binding proteins) thereof having biological activity. Biological activity may include, e.g., inibition of HER3 (or HER4) phosphorylation, inibition of cancer cell proliferation of HER3 and/or HER4 expressing or overexpressing cancer cells, inihibition of HER3/HER2 heterodimerization, (time-dependant) internalization via FACS assay, in vivo tumor growth inhibition in xenograft animal (e.g. mouse or

rat) models with xenografted HER3 and/or HER4 expressing or overexpressing cancer cells. Antibodies having such biological activity in vivo and/or in vitro are also provided.

In certain embodiments, an antibody of the invention is tested for such biological activity. Exemplary vitro or in vivo assays for specified biological activities are described in Example 2e, 3, 5 to 9, and 11.

D. Immunoconjugates

The invention also provides immunoconjugates comprising an anti-HER3/HER4 antibody (or antigen binding protein) described herein conjugated to one or more cytotoxic agents, such as chemotherapeutic agents or drugs, growth inhibitory agents, toxins (e.g., protein toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes.

In one embodiment, an immunoconjugate is an antibody-drug conjugate (ADC) in which an antibody is conjugated to one or more drugs, including but not limited to a maytansinoid (see US 5,208,020, US 5,416,064 and EP 0 425 235 B1); an auristatin such as monomethyl auristatin drug moieties DE and DF (MMAE and MMAF) (see US 5,635,483, US 5,780,588, and US 7,498,298); a dolastatin; a calicheamicin or derivative thereof (see US 5,712,374, US 5,714,586, US 5,739,116, US 5,767,285, US 5,770,701, US 5,770,710, US 5,773,001, and US 5,877,296; Hinman, L.M. et al., Cancer Res. 53 (1993) 3336-3342; and Lode, H.N. et al., Cancer Res. 58 (1998) 2925-2928); an anthracycline such as daunomycin or doxorubicin (see Kratz, F. et al., Curr. Med. Chem. 13 (2006) 477-523; Jeffrey, S.C. et al., Bioorg. Med. Chem. Lett. 16 (2006) 358-362; Torgov, M.Y. et al., Bioconjug. Chem. 16 (2005) 717-721; Nagy, A. et al., Proc. Natl. Acad. Sci. USA 97 (2000) 829-834; Dubowchik, G.M. et al., Bioorg. & Med. Chem. Letters 12 (2002) 1529-1532; King, H.D. et al., J. Med. Chem. 45 (20029 4336-4343; and U.S. Patent No. 6,630,579); methotrexate; vindesine; a taxane such as docetaxel, paclitaxel, larotaxel, tesetaxel, and ortataxel; a trichothecene; and CC1065.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins,

Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to a Pseudomonas exotoxin A or variants thereof. Pseudomonas exotoxin A or variants thereof are described e.g in WO2011/32022, WO2009/32954, WO2007/031741, WO2007/016150, WO2005/052006 and Liu W, et al, PNAS 109 (2012) 11782-11787.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example TC^{99m} or I^{123}, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, MRI), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

Conjugates of an antibody and cytotoxic agent may be made a) either using recombination expression techniques (e.g for the expression of amino acid sequence based toxines fused to a Fab or Fv antibody fragment e.g. in E.coli) or b) using polypeptide coupling techniques (like sortase enzyme based coupling of amino acid sequence based toxines to a Fab or Fv antibody fragment) or c) using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta, E.S. et al., Science 238 (1987) 1098-1104. Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triamine pentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO 94/11026. The linker may be a “cleavable linker” facilitating release of a

cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari, R.V. et al., Cancer Res. 52 (1992) 127-131; U.S. Patent No. 5,208,020) may be used.

The immunuoconjugates or ADCs herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL., U.S.A).

E. Methods and Compositions for Diagnostics and Detection

In certain embodiments, any of the anti-HER3/HER4 antibodies (or antigen binding proteins) provided herein is useful for detecting the presence of HER3 and/or HER4, respectively in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue, such as tumor tissues.

In one embodiment, an anti-HER3/HER4 antibody for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of HER3 or HER4, respectively, in a biological sample is provided. In certain embodiments, the method comprises contacting the biological sample with an anti-HER3/HER4 antibody as described herein under conditions permissive for binding of the anti-HER3/HER4 antibody to HER3 or HER4, respectively, and detecting whether a complex is formed between the anti-HER3/HER4 antibody and HER3 or HER4, respectively. Such method may be an *in vitro* or *in vivo* method. In one embodiment, an anti-HER3/HER4 antibody is used to select subjects eligible for therapy with an the anti-HER3/HER4 antibodies antibody, e.g. where HER3 and HER4, respectively are both biomarkers for selection of patients.

Exemplary disorders that may be diagnosed using an antibody of the invention include cancer.

In certain embodiments, labeled anti-HER3/HER4 antibodies are provided. Labels include, but are not limited to, labels or moieties that are detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly,

e.g., through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

F. Pharmaceutical Formulations

Pharmaceutical formulations of an anti-HER3/HER4 antibody (or antigen binding protein) as described herein are prepared by mixing such antibody having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed.) (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyl dimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as poly(vinylpyrrolidone); amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers herein further include interstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rhuPH20 (HYLENEX®, Baxter International,

Inc.). Certain exemplary sHASEGPs and methods of use, including rhuPH20, are described in US Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

Exemplary lyophilized antibody formulations are described in US Patent No. 6,267,958. Aqueous antibody formulations include those described in US Patent No. 6,171,586 and WO 2006/044908, the latter formulations including a histidine-acetate buffer.

The formulation herein may also contain more than one active ingredients as necessary for the particular indication being treated.

Active ingredients may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methyl methacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed.) (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules.

The formulations to be used for *in vivo* administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

G. Therapeutic Methods and Compositions

Any of the anti-HER3/HER4 antibodies (or antigen binding proteins) or immunoconjugates of the anti-HER3/HER4 antibodies (or antigen binding proteins) conjugated to a cytotoxic agent, provided herein may be used in therapeutic methods.

In one aspect, an anti-HER3/HER4 antibody or immunoconjugate of the anti-HER3/HER4 antibody conjugated to a cytotoxic agent for use as a medicament is provided. In further aspects, an anti-HER3/HER4 antibody or immunoconjugate of

the anti-HER3/HER4 antibody conjugated to a cytotoxic agent for use in treating cancer is provided. In certain embodiments, an anti-HER3/HER4 antibody or immunoconjugates of the anti-HER3/HER4 antibody conjugated to a cytotoxic agent for use in a method of treatment is provided. In certain embodiments, the invention provides an anti-HER3/HER4 antibody or immunoconjugate of the anti-HER3/HER4 antibody conjugated to a cytotoxic agent for use in a method of treating an individual having cancer comprising administering to the individual an effective amount of the anti-HER3/HER4 antibody or the immunoconjugate of the anti-HER3/HER4 antibody conjugated to a cytotoxic agent. In further embodiments, the invention provides an anti-HER3/HER4 antibody or immunoconjugate of the anti-HER3/HER4 antibody conjugated to a cytotoxic agent for use in inducing apoptosis in a cancer cell/ or inhibiting cancer cell proliferation. In certain embodiments, the invention provides an anti-HER3/HER4 antibody or immunoconjugate of the anti-HER3/HER4 antibody conjugated to a cytotoxic agent for use in a method of inducing apoptosis in a cancer cell/ or inhibiting cancer cell proliferation in an individual comprising administering to the individual an effective of the the anti-HER3/HER4 antibody or immunoconjugate of the anti-HER3/HER4 antibodies conjugated to a cytotoxic agent to induce apoptosis in a cancer cell/ or to inhibit cancer cell proliferation. An "individual" according to any of the above embodiments is preferably a human.

In a further aspect, the invention provides for the use of an anti-HER3/HER4 antibody or an immunoconjugate of the anti-HER3/HER4 antibody conjugated to a cytotoxic agent in the manufacture or preparation of a medicament. In one embodiment, the medicament is for treatment of cancer. In a further embodiment, the medicament is for use in a method of treating cancer comprising administering to an individual having cancer an effective amount of the medicament. In a further embodiment, the medicament is for for inducing apoptosis in a cancer cell/ or inhibiting cancer cell proliferation. In a further embodiment, the medicament is for use in a method of inducing apoptosis in a cancer cell/ or inhibiting cancer cell proliferation in an individual suffering from cancer comprising administering to the individual an amount effective of the medicament to induce apoptosis in a cancer cell/ or to inhibit cancer cell proliferation. An "individual" according to any of the above embodiments may be a human.

In a further aspect, the invention provides a method for treating cancer. In one embodiment, the method comprises administering to an individual having cancer

an effective amount of an anti-HER3/HER4 antibody. An "individual" according to any of the above embodiments may be a human.

In a further aspect, the invention provides a method for inducing apoptosis in a cancer cell/ or inhibiting cancer cell proliferation in an individual suffering from cancer. In one embodiment, the method comprises administering to the individual an effective amount of an anti-HER3/HER4 antibody or an immunoconjugate of the anti-HER3/HER4 antibody conjugated to a cytotoxic compound to induce apoptosis in a cancer cell/ or to inhibit cancer cell proliferation in the individual suffering from cancer. In one embodiment, an "individual" is a human.

In a further aspect, the invention provides pharmaceutical formulations comprising any of the anti-HER3/HER4 antibodies provided herein, e.g., for use in any of the above therapeutic methods. In one embodiment, a pharmaceutical formulation comprises any of the anti-HER3/HER4 antibodies provided herein and a pharmaceutically acceptable carrier.

An antibody of the invention (and any additional therapeutic agent) can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Various dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

Antibodies of the invention would be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as described herein, or about from 1

to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

For the prevention or treatment of disease, the appropriate dosage of an antibody of the invention (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (e.g. 0.5mg/kg - 10 mg/kg) of antibody can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.05 mg/kg to about 10 mg/kg. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 4.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (*e.g.* such that the patient receives from about two to about twenty, or *e.g.* about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the antibody. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

It is understood that any of the above formulations or therapeutic methods may be carried out using an immunoconjugate of the invention in place of or in addition to an anti-HER3/HER4 antibody.

III. Articles of Manufacture

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described

above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody of the invention. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

It is understood that any of the above articles of manufacture may include an immunoconjugate of the invention in place of or in addition to an anti-HER3/HER4 antibody.

Description of the amino acid sequences

SEQ ID NO: 1	ß-Hairpin of human HER3
SEQ ID NO: 2	ß-Hairpin of human HER4
SEQ ID NO: 3	human HER3
SEQ ID NO: 4	human HER3 Extracellular Domain (ECD)
SEQ ID NO: 5	human HER4
SEQ ID NO: 6	human HER4 Extracellular Domain (ECD)
SEQ ID NO: 7	human HER1
SEQ ID NO: 8	human HER1 Extracellular Domain (ECD)
SEQ ID NO: 9	human HER2

SEQ ID NO: 10	human HER2 Extracellular Domain (ECD)
SEQ ID NO: 11	Human Heregulin fragment (HRG)
SEQ ID NO: 12	Human Heregulin β-1 fragment (as provided from Preprotech)
SEQ ID NO: 13	TtSlyD-FKBP-Her3
SEQ ID NO: 14	TtSlyD-Wildtype
SEQ ID NO: 15	TtSlyDcas
SEQ ID NO: 16	TgSlyDΔIF
SEQ ID NO: 17	TtSlyDcas-Her3
SEQ ID NO: 18	TtSlyDcys-Her3
SEQ ID NO: 19	TgSlyDser-Her3
SEQ ID NO: 20	TgSlyDcys-Her3
SEQ ID NO: 21	TtSlyDcas-Her4
SEQ ID NO: 22	TtSlyDcys-Her4
SEQ ID NO: 23	TgSlyDser-Her4
SEQ ID NO: 24	TgSlyDcys-Her4
SEQ ID NO: 25	heavy chain HVR-H1, M-05-74
SEQ ID NO: 26	heavy chain HVR-H2, M-05-74
SEQ ID NO: 27	heavy chain HVR-H3, M-05-74
SEQ ID NO: 28	light chain HVR-L1, M-05-74
SEQ ID NO: 29	light chain HVR-L2, M-05-74
SEQ ID NO: 30	light chain HVR-L3, M-05-74
SEQ ID NO: 31	heavy chain variable domain VH, M-05-74
SEQ ID NO: 32	light chain variable domain VL, M-05-74
SEQ ID NO: 33	humanized variant 1 of heavy chain variable domain VH, M-05-74_VH1
SEQ ID NO: 34	humanized variant 2 of heavy chain variable domain VH, M-05-74_VH2
SEQ ID NO: 35	humanized variant 3 of heavy chain variable domain VH, M-05-74_VH3
SEQ ID NO: 36	humanized variant 1 of light chain variable domain VL, M-05-74_VL1
SEQ ID NO: 37	humanized variant 2 of light chain variable domain VL, M-05-74_VL2
SEQ ID NO:38	humanized variant 1 of HVR-H1, M-05-74_ HVR-H1_V1
SEQ ID NO:39	humanized variant 1 of HVR-H2, M-05-74_ HVR-H2_V1
SEQ ID NO:40	humanized variant 1 of HVR-L1, M-05-74_ HVR-L1_V1
SEQ ID NO: 41	humanized variant 1 of HVR-L2, M-05-74_ HVR-L2_V1

SEQ ID NO:42	humanized variant 2 of HVR-L2, M-05-74_ HVR-L2_V2
SEQ ID NO:43	binding epitope within ß-hairpin of human HER3
SEQ ID NO:44	binding epitope within ß-hairpin of human HER4
SEQ ID NO:45	Pseudomonas exotoxin variant PE24LR8M_3G (including a GGG linker)
SEQ ID NO:46	Light chain of M-05-74 (M-05-74_LC)
SEQ ID NO:47	Heavy chain of M-05-74 HC with sortase tag (M-05-74_HC)
SEQ ID NO:48	Heavy chain of M-05-74 HC conjugated to Pseudomonas exotoxin variant PE24LR8M (Fab-074-PE heavy chain 1)
SEQ ID NO:49	Heavy chain of M-05-74 HC conjugated to Pseudomonas exotoxin variant PE24LR8M (Fab-074-PE heavy chain 2) as direct PE24LR8M fusion
SEQ ID NO: 50	soluble S.aureus sortase A
SEQ ID NO: 51	heavy chain variable domain VH, <Her3> M-08-11
SEQ ID NO: 52	light chain variable domain VL, <Her3> M-08-11
SEQ ID NO: 53	human kappa light chain constant region
SEQ ID NO: 54	human lambda light chain constant region
SEQ ID NO: 55	human heavy chain constant region derived from IgG1
SEQ ID NO: 56	human heavy chain constant region derived from IgG1 mutated on L234A and L235A
SEQ ID NO: 57	human heavy chain constant region derived from IgG1 mutated on L234A, L235A and P329G
SEQ ID NO: 58	human heavy chain constant region derived from IgG4

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

In the following several embodiments of the invention are listed:

1. A method for selecting an antigen binding protein that binds to human HER3 and binds to human HER4, wherein the antigen binding protein binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) of human HER3 and binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) of human HER4;

   wherein

a) at least one polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3,

which comprises the amino acid sequence of SEQ ID NO:1;

and

b) at least one polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4,

which comprises the amino acid sequence of SEQ ID NO:2;

are used to select antigen binding proteins, which show binding to both, the at least one polypeptide under a) and the at least one polypeptide under b)

and thereby selecting an antigen binding protein that binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) and within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2).

2. An antigen binding protein obtained by the selection method of embodiment 1.

3. The method of embodiment 1, or the antigen binding protein of embodiment 2, wherein the antigen binding protein is an antibody.

4. An isolated antigen binding protein that binds to human HER3 and binds to human HER4, wherein the antigen binding protein binds within an amino

acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) of human HER3 and binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) of human HER4.

5. The antigen binding protein of embodiment 3

a) wherein the antigen binding protein binds to a polypeptide of

SEQ ID NO: 18 TtSlyDcys-Her3,

and

b) wherein the antigen binding protein binds to a polypeptide of

SEQ ID NO: 22 TtSlyDcys-Her4.

6. The antigen binding proteinof embodiments 4 or 5 wherein the antigen binding protein is an antibody.

7. An isolated antibody that binds to human HER3 and that binds to human HER4, wherein the antibody has one or more of the following properties:

a) the antibody binds to the amino acid sequence of SEQ ID NO:1; and/or

b) the antibody binds to the amino acid sequence SEQ ID NO:1 in activated HER3; and/or

c) the antibody binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;
and/or

d) the antibody binds to the ß-hairpin region of HER3; and/or

e) the antibody inhibits the heterodimerisation of HER3/HER2 heterodimers; and/or

f) the antibody binds to HER3-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 4.0 or higher (Ka (+Heregulin))/(Ka (-Heregulin)); and/or

g) the antibody binds to HER3-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 2.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)); and/or

h) the antibody binds to the amino acid sequence of SEQ ID NO:2; and/or

i) the antibody binds to the amino acid sequence SEQ ID NO:2 in activated HER4; and/or

j) the antibody binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4;

and/or

k) the antibody binds to the ß-hairpin region of HER4; and/or

l) the antibody binds to HER4-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 20.0 or higher (Ka (+Heregulin))/(Ka (-Heregulin)); and/or

m) the antibody binds to HER4-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 5.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)); and/or

n) the antibody shows as monovalent Fab fragment the same or higher biological activity as compared to its bivalent parent full length antibody; and/or

o) the antibody inhibits the HER3 phosporylation in MCF-7 cells; and/or

p) the antibody does not compete for binding to HER3 with Heregulin/ induces binding of Heregulin to HER3; and/or

q) the antibody inhibits the proliferation of MDA-MB-175 tumor cells; and/or

r) the antibody shows tumor growth inhibitory activity in vivo; and/or

s) the antibody binds with an affinity of a KD value $\leq 1 \times 10^{-8}$ M to HER3-ECD; and/or

t) the antibody binds with an affinity of a KD value $\leq 1 \times 10^{-8}$ M to HER4-ECD; and/or

u) the antibody binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43) and to a polypeptide consisting of VYNPTTFQLE (SEQ ID NO:44); and/or

v) the antibody binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43); and/or

w) the antibody binds to a polypeptide consisting of VYNPTTFQLE (SEQ ID NO:44); and/or

x) the antibody binds in a FACS assay to HER3 expressing T47D cells; and wherein the antibody shows an at least 25% higher percentage of internalization in the presence of Heregulin as compared to the percentage of internalization in the presence of Heregulin when measured after 1 h after antibody exposure.

8. An antibody that binds to human HER3 and that binds human HER4, wherein the antibody binds to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNKLTFQLEP (SEQ ID NO:43) (of human HER3) and to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNPTTFQLE (SEQ ID NO:44) (of human HER4).

9. The antibody of any one of embodiments 6 to 8, which is a human, humanized, or chimeric antibody.

10. The antibody of any one of embodiments 6 to 8, which is an antibody fragment that binds human HER3 and that binds human HER4.

11. The antibody of any one of embodiments 6 to 8, wherein the antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26, and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

12. The antibodyof any one of embodiments 6 to 8, or 11, comprising (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

13. The antibody of any one of embodiments 6 to 8, wherein the antibody comprises

i) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30;

ii) or a humanized variant of the HVRs of the antibody under i) (a), (b), (d) and/or (e).

14. The antibody of any one of embodiments 6 to 8 or 13, wherein the antibody comprises

i) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30; or

ii) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30; or

iii) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30; or

iv) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

15. The antibody of any one of embodiments 6 to 8, comprising (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:33; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:36; or (c) a VH sequence as in (a) and a VL sequence as in (b).

16. The antibody of embodiment 15, comprising a VH sequence of SEQ ID NO: 33.

17. The antibody of embodiment 135, comprising a VL sequence of SEQ ID NO: 36.

18. An antibody comprising a VH sequence of SEQ ID NO:33 and a VL sequence of SEQ ID NO:36.

19. The antibody of any one of embodiments 6 to8, comprising (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:33; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:37; or (c) a VH sequence as in (a) and a VL sequence as in (b).

20. The antibody of embodiment 19, comprising a VH sequence of SEQ ID NO: 33.

21. The antibody of embodiment 19, comprising a VL sequence of SEQ ID NO: 37.

22. An antibody comprising a VH sequence of SEQ ID NO:33 and a VL sequence of SEQ ID NO:37.

23. The antibody of any one of embodiments 6 to 8, comprising (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:34; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:36; or (c) a VH sequence as in (a) and a VL sequence as in (b).

24. The antibody of embodiment 23, comprising a VH sequence of SEQ ID NO: 34.

25. The antibody of embodiment 23, comprising a VL sequence of SEQ ID NO: 36.

26. An antibody comprising a VH sequence of SEQ ID NO:34 and a VL sequence of SEQ ID NO:36.

27. The antibody of any one of embodiments 6 to 8, comprising (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:34; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:37; or (c) a VH sequence as in (a) and a VL sequence as in (b).

28. The antibody of embodiment 27, comprising a VH sequence of SEQ ID NO: 34.

29. The antibody of embodiment 27, comprising a VL sequence of SEQ ID NO: 37.

30. An antibody comprising a VH sequence of SEQ ID NO:34 and a VL sequence of SEQ ID NO:37.

31. The antibody of any one of embodiments 6 to 8, comprising (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:35; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:36; or (c) a VH sequence as in (a) and a VL sequence as in (b).

32. The antibody of embodiment 31, comprising a VH sequence of SEQ ID NO: 35.

33. The antibody of embodiment 31, comprising a VL sequence of SEQ ID NO: 36.

34. An antibody comprising a VH sequence of SEQ ID NO:35 and a VL sequence of SEQ ID NO:36.

35. The antibody of any one of embodiments 6 to 8, comprising (a) a VH sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:35; (b) a VL sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:37; or (c) a VH sequence as in (a) and a VL sequence as in (b).

36. The antibody of embodiment 35, comprising a VH sequence of SEQ ID NO: 35.

37. The antibody of embodiment 35, comprising a VL sequence of SEQ ID NO: 37.

38. An antibody comprising a VH sequence of SEQ ID NO:35 and a VL sequence of SEQ ID NO:37.

39. The antibody of any one of embodiments6 to 38, which is a full length IgG1 antibody or IgG4 antibody.

40. The antibody of any one of embodiments 6 to 38, which is a Fab fragment.

41. Isolated nucleic acid encoding the antibody of any one of embodiments 6 to 38.

42. A host cell comprising the nucleic acid of embodiment41.

43. A method of producing an antibody comprising culturing the host cell of embodiment 42 so that the antibody is produced, and recovering said antibody from said cell culture or the cell culture supernatant.

44. An immunoconjugate comprising the antibody of any one of embodiments 6 to 38 and a cytotoxic agent.

45. A pharmaceutical formulation comprising the antibody of any one of embodiments 6 to 38, or the immunoconjugate of embodiment 44, and a pharmaceutically acceptable carrier.

46. The antibody of any one of embodiments 6 to 38, or the immunoconjugate of embodiment 44, for use as a medicament.

47. The antibody of any one of embodiments 6 to 38, or the immunoconjugate of embodiment 44, for use in treating cancer.

48. The antibody of any one of embodiments 6 to 38 for use in inhibition of HER3/HER2 dimerization.

49. Use of the antibody of any one of embodiments 6 to 38, or an immunoconjugate of embodiment 44, in the manufacture of a medicament.

50. The use of embodiment 49, wherein the medicament is for treatment of cancer.

51. The use of embodiment 49, wherein the medicament is for the inhibition of HER3/HER2 dimerization.

52. A method of treating an individual having cancer comprising administering to the individual an effective amount of the antibody of any one of the preceding embodiments, or an immunoconjugate comprising the antibody of any one of the preceding embodiments and a cytotoxic agent.

53. A method of inducing apoptosis in a cancer cell in an individual sufferning from cancer comprising administering to the individual an effective amount of an immunoconjugate comprising the antibody of any one of the preceding embodiments and a cytotoxic agent, thereby inducing apoptosis in a cancer cell in the individual.

54. A polypeptide selected from the group consisting of:

i) SEQ ID NO: 13 TtSlyD-FKBP-Her3,

ii) SEQ ID NO: 17 TtSlyDcas-Her3,

iii) SEQ ID NO: 18 TtSlyDcys-Her3,

iv) SEQ ID NO: 19 TgSlyDser-Her3, and

v) SEQ ID NO: 20 TgSlyDcys-Her3,

which polypeptide comprises the amino acid sequence of SEQ ID NO:1

55. A polypeptide selected from the group consisting of:

i) SEQ ID NO: 21 TtSlyDcas-Her4,

ii) SEQ ID NO: 22 TtSlyDcys-Her4,

iii) SEQ ID NO: 23 TgSlyDser-Her4,and

iv) SEQ ID NO: 24 TgSlyDcys-Her4,

which polypeptide comprises the amino acid sequence of SEQ ID NO:2

Examples:

Materials & general methods

Recombinant DNA techniques

Standard methods were used to manipulate DNA as described in Sambrook, J. *et al.*, Molecular Cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions.

Gene synthesis

Desired gene segments were prepared from oligonucleotides made by chemical synthesis. The 400 - 1600 bp long gene segments, which were flanked by singular restriction endonuclease cleavage sites, were assembled by annealing and ligating oligonucleotides including PCR amplification and subsequently cloned via the indicated restriction sites e.g. EcoRI/ BlpI or BsmI/XhoI into the expression vectors described below. The DNA sequences of the subcloned gene fragments were confirmed by DNA sequencing. Gene synthesis fragments were ordered according to given specifications at Geneart (Regensburg, Germany).

DNA sequence determination

DNA sequences were determined by double strand sequencing performed at Sequiserve GmbH (Vaterstetten, Germany).

DNA and protein sequence analysis and sequence data management

Infomax's Vector NT1 Advance suite version 11.5.0 was used for sequence creation, mapping, analysis, annotation and illustration.

Example 1

Preparation of antigen and screening proteins - Generation of functional ß-hairpin HER3 and ß-hairpin HER4 constructs for selecting antibodies binding to the ß-hairpin of HER3 and the ß-hairpin of HER4

To generate functional ß-Hairpin HER3 and HER4 constructs, the amino acid sequences of the ß-Hairpins of HER3 (SEQ ID NO: 1) and HER4 (SEQ ID NO: 2), were grafted into a SlyD polypeptide framework comprising a FKBP domain. In such constructs the grafted ß-Hairpins are freely accessible in contrast to the hidden structure in the native unactivated conformation of HER3 or HER4 (in the absence of ligand as e.g. HRG) (see Figure 1c and 1 d where the ß-Hairpin of HER3 is hidden).

All fused SlyD polypeptides can be purified and refolded by using almost identical protocols. *E. coli* BL21 (DE3) cells transformed with the particular expression plasmid were grown at 37°C in LB medium containing the respective antibiotic for selective growth (Kanamycin 30 µg/ml, or Ampicillin (100 µg/ml)) to an OD600 of 1.5, and cytosolic overexpression was induced by adding 1 mM isopropyl-ß-D-thiogalactoside (IPTG). Three hours after induction, cells were harvested by centrifugation (20 min at 5,000 g), frozen and stored at -20 °C. For cell lysis, the frozen pellet was resuspended in chilled 50 mM sodium phosphate buffer (pH 8.0) supplemented with 7 M GdmCl and 5 mM imidazole. Thereafter the suspension was stirred for 2-10 hours on ice to complete cell lysis. After centrifugation (25,000 g, 1 h) and filtration (cellulose nitrate membrane, 8.0 µm, 1.2 µm, 0.2 µm), the lysate was applied onto a Ni-NTA column equilibrated with the lysis buffer. In the subsequent washing step the imidazole concentration was raised to 10 mM (in 50 mM sodium phosphate buffer (pH 8.0) comprising 7 M GdmCl) and 5 mM TCEP was added in order to keep the thiol moieties in a reduced form and to prevent premature disulfide bridging. At least 15 to 20 volumes of the reducing washing

buffer were applied. Thereafter, the GdmCl solution was replaced by 50 mM sodium phosphate buffer (pH 8.0) comprising 100 mM NaCl, 10 mM imidazole, and 5 mM TCEP to induce conformational refolding of the matrix-bound SlyD fusion polypeptide. In order to avoid reactivation of co-purifying proteases, a protease inhibitor cocktail (Complete® EDTA-free, Roche) was added to the refolding buffer. A total of 15 to 20 column volumes of refolding buffer were applied in an overnight procedure. Thereafter, both TCEP and the Complete® EDTA-free inhibitor cocktail were removed by washing with 10 column volumes 50 mM sodium phosphate buffer (pH 8.0) comprising 100 mM NaCl and 10 mM imidazole. In the last washing step, the imidazole concentration was raised to 30 mM (10 column volumes) in order to remove tenacious contaminants. The refolded polypeptide was then eluted by applying 250 mM imidazole in the same buffer. Protein-containing fractions were assessed for purity by Tricine-SDS-PAGE (Schaegger, H. and von Jagow, G., Anal. Biochem. 166 (1987) 368-379). Subsequently, the protein was subjected to size-exclusion-chromatography (SuperdexTM HiLoad, Amersham Pharmacia) using potassium phosphate as the buffer system (50 mM potassium phosphate buffer (pH 7.0), 100 mM KCl, 0.5 mM EDTA). Finally, the protein-containing fractions were pooled and concentrated in an Amicon cell (YM10) to a concentration of ~ 5 mg/ml. Exemplarily SDS-PAGE analysis of Ni-NTA purification of TtSlyD-FKBP-Her3 is shown in Figure 3 and SEC elution profile of a Ni-NTA purified fraction of Thermus thermophilus SlyD-FKBP-Her-3 is shown in Figure 4. The *Thermus thermophilus* SlyD (TtSlyD)-Her-3 fusion polypeptide could be purified successfully as a soluble and stable polypeptide in its monomeric form. The final yield was quantified at 16.4 mg purified protein from fraction 12 and 13.

Table 2: Summary of the amino acid sequences of the developed SlyD-based epitope scaffolds (which carry the HER3 dimerization domain fragment (ß-Hairpin of HER3 (SEQ ID NO: 1)) as insert or the HER4 dimerization domain fragment (ß-Hairpin of HER4 (SEQ ID NO: 2)) as insert).

TtSlyD-FKBP-Her3, TtSlyDcas-Her3, TtSlyDcys-Her3, Thermococcus gammatolerans TgSlyDser-Her3 and TgSlyDcys-Her3 carry the Her3 dimerization domain fragment (ß-Hairpin of HER3 (SEQ ID NO: 1)) as insert and were used as immunogens and as positive controls in ELISA screening.

TtSlyD-Wildtype, TtSlyDcas, TgSlyDΔIF were used as negative controls in the ELISA screening (without the Her3 dimerization domain fragment (ß-Hairpin of

HER3 (SEQ ID NO: 1)) or the Her4 dimerization domain fragment (ß-Hairpin of HER4 (SEQ ID NO: 2)) as insert).

TtSlyDcas-Her4, TtSlyDcys-Her4, TgSlyDser-Her4 and TgSlyDcys-Her4 (which carry the Her4 dimerization domain fragment (ß-Hairpin of HER4 (SEQ ID NO: 2)) as insert) were used in the ELISA screening to check the developed clones for HER4 crossreactivity.

As the epitope scaffolds are expressed in E.coli the N-terminal methionine residue can be present or not. (Nt = N-terminal; Ct = C-terminal)

Table 2

TtSlyD-FKBP-Her3	Nt-MRSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEGEAFQAHVPAEKAYGAGSPQPLVYNKLTFQLEPNPHTKGSSGKDLDFQVEVVKVREATPEELLHGHAHGGGSRKHHHHH HHH-Ct
TtSlyD-Wildtype	Nt-MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEGEAFQAHVPAEKAYGPHDPEGVQVVPLSAFPEDAEVVPGAQFYAQDMEGNPMPLTVVAVEGEEVTVDFNHPLAGKDLDFQVEVVKVREATPEELLHGHAHGGGSRKHHHHHHHH-Ct
TtSlyDcas	Nt-MRSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEGEAFQAHVPAEKAYGAGSGSSGKDLDFQVEVVKVREATPEELLHGHAHGGGSRKHHHHHHHH-Ct
TgSlyDΔIF	Nt-MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVGAGEIIPGIEEALLGMELGEKKEVVVPPEKGYGATGHPGIIPPHATAIFEIEVVEIKKAGEALEHHHHHHLEHHHHHH-Ct
TtSlyDcas-Her3	Nt-MRSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEGEAFQAHVPAEKAYGAGSPQPLVYNKLTFQLEPNPHTKGSSGKDLDFQVEVVKVREATPEELLHGHAHGGGSRKHHHHHHHH-Ct
TtSlyDcys-Her3	Nt-MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEGEAFQAHVPAEKAYGPCGPQPLVYNKLTFQLEPNPHTGCGKDLDFQVEVVKVREATPEELLHGHAHGGGSHHHHHHHH-Ct

TgSlyDser-Her3	Nt-MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVGAGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMPSGPQPLVYNKLTFQLEPNPHTGSAGKTAIFEIEVVEIKKAGEAGGGSRKHHHHHHHH-Ct
TgSlyDcys-Her3	Nt-MRGSKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVGAGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMPCGPQPLVYNKLTFQLEPNPHTGCAGKTAIFEIEVVEIKKAGEAGGGSHHHHHHHH-Ct
TtSlyDcas-Her4	Nt-MRSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEGEAFQAHVPAEKAYGAGSPQTFVYNPTTFQLEHNFNAKGSSGKDLDFQVEVVKVREATPEELLHGHAHGGGSRKHHHHHHHH-Ct
TtSlyDcys-Her4	Nt-MRGSKVGQDKVVTIRYTLQVEGEVLDQGELSYLHGHRNLIPGLEEALEGREEGEAFQAHVPAEKAYGPCGPQTFVYNPTTFQLEHNFNAGCGKDLDFQVEVVKVREATPEELLHGHAHGGGSHHHHHHHH-Ct
TgSlyDser-Her4	Nt-MKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVGAGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMPSGPQTFVYNPTTFQLEHNFNAGSAGKTAIFEIEVVEIKKAGEAGGGSRKHHHHHHHH-Ct
TgSlyDcys-Her4	Nt-MRGSKVERGDFVLFNYVGRYENGEVFDTSYESVAREQGIFVEEREYSPIGVTVGAGEIIPGIEEALLGMELGEKKEVVVPPEKGYGMPCGPQTFVYNPTTFQLEHNFNAGCAGKTAIFEIEVVEIKKAGEAGGGSHHHHHHHH-Ct

Example 2

a) Immunisation and Selection of HER3 antibodies

For the generation of antibodies against the ß-hairpin of HER3 and HER4, Balb/C, NMRI or SJL mice were immunized with different antigens. As antigens the following proteins were used: full length Her3 ECD, or the epitope scaffold proteins TtSlyD-FKBP12-Her3, TtSlyDcys-Her3, TtSlyDcas-Her3, TgSlyDcys-Her3 and TgSlyDser-Her3. The TtSlyD-FKBP12-Her3 variant represents the first

generation epitope scaffold, used for generation of Her3 dimerization domain specific antibodies. Although the general principal of using SlyD variants as epitope scaffolds could already be demonstrated using the first generation SlyD-FKBP12 scaffold, improved variants of the scaffold with higher stability were developed. These SlyD variants are derived from Thermos thermophilus and Thermococcus gammatolerans.

All mice were subjected to 3 immunizations at the time points 0, 6 and 10 weeks after start of the immunization campaign. At each time point each mouse was immunized with 100 µg endotoxin free immunogen dissolved in 100 µl PBS. For the first immunization the immunogen was mixed with 100 µl CFA. For the second and third immunization the immunogen was mixed with IFA. The first and the third immunization were applied via the intraperitoneal route, the second immunization was applied subcutaneously. 2 and 3 days prior to the preparation of spleenocyte for antibody development using hybridoma technology, the mice were subjected to intravenous booster immunizations with 12.5 µg immunogen in 100 µl PBS and without adjuvant.

Titer analysis

For the determination of serum titers against the respective immunogen and against the screening proteins a small amount of serum of each mouse was collected in week 11 after start of the immunization campaign. For the ELISA the immunogen or the screening scaffold proteins were immobilized on the plate surface. Her3 ECD was immobilized at a concentration of 1 µg/ml and the scaffold proteins TtSlyD-FKBP12-Her3, TtSlyD-FKBP12, TtSlyDcys-Her3, TtSlyDcas-Her3, TtSlyDcas, TgSlyDcys-Her3, TgSlyDser-Her3 and TgSlyDΔIF were used at a concentration of 0.5 µg/ml. The scaffold proteins TtSlyDcas and TgSlyDΔIF were used as negative controls. The sera from each mouse were diluted in PBS with 1% BSA and the dilutions were added to the plates. The sera were tested at dilutions 1:300, 1:900, 1:2700, 1:8100, 1:24300, 1:72900, 1:218700 and 1:656100. Bound antibody was detected with a HRP-labeled $F(ab`)_2$ goat anti-mouse Fcγ (Dianova) and ABTS (Roche) as a substrate.

Even on the level of serum titration it was already obvious that immunized mice developed antibodies against the Her3 ß-hairpin domain. In mice immunized with Her3 ECD this can be shown by titration against one of the scaffold proteins containing the dimerization ß-hairpin loop. The strongly reduced signal can be

explained by the fact, that the majority of antibodies raised by immunization with Her3 ECD are targeting other parts within the ECD and only a small fraction is binding to the dimerization ß-hairpin domain. In mice immunized with Her3 dimerization loop containing scaffolds the fraction of antibodies targeting the loop can be shown by titration against Her3 ECD (positive control) and titration against an control scaffold without Her3 insertion (negative control).

b) Antibody Development and ELISA Screening/Selection

The use of the here described epitope scaffold technology offers in principal two strategies for the development of antibodies targeting the Her3 dimerization domain (ß-Hairpins of HER3 (SEQ ID NO: 1)). One strategy is to immunize with the full length Her3 ECD and to use the scaffolds to screen for the dimerization domain specific antibodies. The other strategy is the direct use of the scaffold for immunization and to use the Her3 ECD, a scaffold with another backbone or a scaffold without insertion for counter screening. Antibodies were developed with hybridoma technology by fusing primary B-cells with P3X63Ag8.653 myeloma cells. 2 days after the final booster immunization, immunized mice were sacrificed and spleen cell populations were prepared. The spleenocytes were fused with P3X63Ag8.653 by using the PEG fusion technology. The cellular batch culture from the fusion was incubated overnight at 37°C under 5% CO_2. The following day the cellular batch containing fused cells was centrifuged for 10 min at 400 g. Thereafter, the cells were suspended in hybridoma selection media supplemented with 0.1x azaserine-hypoxanthine (Sigma) and were seeded at a concentration of $2.5x10^4$ cells per well in 96well plates. The plates were cultured for at least 1 week at 37°C under 5% CO_2. 3 days prior to ELISA analysis the selection media was changed.

Primary culture supernatants were tested in ELISA against Her3 ECD and various scaffold proteins. The testing against the scaffold proteins was done to demonstrate that the selected clones are binding to the dimerization domain ß-hairpin of native Her3 ECD. The testing against the control scaffolds TtSlyDcas and TgSlyDΔIF was done to show that the selected clones are binding the inserted Her3 derived sequence and not the scaffold backbone. To check for cross reactivity the resulting clones were tested against the full length ECDs of the other members of the Her family namely, Her1, Her2 und Her4. As shown all selected clones are highly specific for Her3 and a highly specific cross reactivity to HER4 could be detected, while no cross reactivity to other members of the Her family were detected. For the

ELISA the screening an antigen down format was used. Her3 ECD was immobilized at a concentration of 1 µg/ml and the scaffold proteins TtSlyD-FKBP12-Her3, TtSlyD-FKBP12, TtSlyDcys-Her3, TtSlyDcas-Her3, TtSlyDcas, TgSlyDcys-Her3, TgSlyDser-Her3 and TgSlyDΔIF were immobilized at a concentration of 0.5 µg/ml. Hybridoma Supernatant was added to the plates and incubated for 1 h at room temperature. Bound antibody was detected with a HRP-labeled F(ab`)$_2$ goat anti-mouse Fcγ (Dianova) and ABTS (Roche) was used as a HRP-substrate.

Table 3: Evaluation of the selected clones by ELISA. The clones were tested against the scaffold proteins TtSlyDcas-Her3, TtSlyDcys-Her3, TgSlyDser-Her3 and TgSlyDcys-Her3 and the full length Her3 ECD to verify their Her3 dimerization domain insert (ß-Hairpin of HER3 (SEQ ID NO: 1)) specificity. As negative controls the scaffold proteins TtSlyDcas and TgSlyDΔIF were used. Additionally, clones were tested against full length ECDs of Her1, Her2, Her3 and Her4 to verify potential cross reactivity. Clones show binding to full length Her3 ECD and are cross reactive against full length Her4 ECD.

Klon	TtSlyD-			TgSlyD-						
	cas	cas-Her3	cys-Her3	ΔIF	ser-Her3	cys-Her3	Her1 ECD	Her2 ECD	Her3 ECD	Her4 ECD
M-05-74	0.023	3.133	3.150	0.020	3.159	3.159	0.018	0.020	3.152	3.170
M-15-02	0.040	1.763	1.522	0.040	1.980	1.785	0.024	0.025	3.153	3.192
M-15-03	0.045	1.772	1.850	0.039	1.628	1.461	0.020	0.024	3.171	3.234
M-15-04	0.040	1.847	1.457	0.033	1.833	1.500	0.067	0.064	3.175	3.186
M-15-05	0.041	1.443	1.482	0.046	1.886	1.485	0.020	0.021	3.156	3.216
M-15-08	0.041	1.569	1.707	0.040	1.746	1.532	0.019	0.023	3.195	3.181
M-15-09	0.057	1.870	1.929	0.076	1.799	1.640	0.024	0.037	3.234	3.200
M-15-11	0.044	1.714	1.636	0.056	2.005	1.693	0.029	0.031	3.103	3.218
M-16-01	0.039	1.653	1.793	0.037	1.860	1.637	0.024	0.032	3.184	3.212

c) **Immunohistochemistry**

All selected clones were tested for reactivity and specificity in IHC. Therefore HEK293 cells were transiently transfected with plasmids coding for full length HER1, HER2, HER3 or HER4, respectively. 2 days after transfection the different cell lines now expressing HER1, HER2, HER3 or HER4were harvested, subsequently fixed in formalin and embedded in Agarose for generation of IHC controls. After an additional fixation in formalin overnight the Agarose blocks were embedded in paraffin. Untransfected HEK293 cells were used as negative controls and treated accordingly to the transfected cells. After paraffin embedding 3 µm thin sections were prepared using a microtome. The sections were mounted on glass microscopy slides and dried for 2 h. All further steps of the immunohistochemical staining procedure were carried out using a Ventana Benchmark XT. The slides were dewaxed and antigen retrieval was performed by applying heat for 1 hour. For antigen retrieval the Ventana buffer CC1 was used. The antibodies were used at a concentration of 1 µg/ml. For the detection of bound antibody the Ventana UltraView detection kit was used. Results are shown in Figure 5. All three clones showed binding to HER3 and cross reactivity against HER4. No cross reactivity against HER1 and HER2 was detectable.

d) **DNA Sequencing of selected anti-Her3 Hybridoma**

To obtain the DNA sequences of the selected hybridoma clones a 5` Race PCR was conducted. For the RT-PCR total RNA was prepared from $5x10^6$ cells by using a total RNA purification kit (Qiagen). The reverse transcription and the PCR were conducted using a 5`prime RACE PCR kit (Roche). The resulting PCR fragments from heavy and light chain were purified by gel electrophoresis and subsequent gel purification. The PCR fragments were cloned using the Topo Zero-Blunt cloning kit (Invitrogen) and transformed into competent cells. Several clones from each hybridoma were submitted for sequencing to obtain a consensus sequences for the selected clones. M-05-74 M-15-02 M-15-04 were submitted for sequencing which resulted in identical VH and VL sequences for all 3 clones. M-15-03, M-15-05, M-15-08, M-15-09, M-15-11, M-16-01 were sequenced analogously and also resulted in identical VH and VL sequences fo all clones.

e) **Time dependent internalization analyses of M-05-74 via FACS**

Binding and internalization of HER3 by the selected clone M-05-74 to HER3 was analyzed in FACS using the HER3 expressing tumor cell line T47D. $5x10^5$ cells were treated with 50 ng Recombinant Human Heregulin fragment (HRG) (SEQ ID NO: 11). The fragment including amino acid of SEQ ID NO: 11 was cloned in pCDNA.1 vector (Invitrogen). The HRG fragment was expressed in FreeStyle™ 293-F cells according to the protocol described by Invitrogen. (FreeStyle™ 293 Expression system Catalog no. K9000-01). Purified HRG fragment was solved in 20mM Histidin,140mM NaCl; pH6.0 and stored by -80C.

Untreated (-) cells were used as negative controls. Shortly after Heregulin induced activation, 1 µg of M-05-74 was added to the cells. The cells were incubated for 0, 5, 15, 30, 45, 60, 75, 90, 105, 120, 180 or 240 min at 37°C. After incubation the cells were immediately put on ice. The cells were washed with 3 ml FACS buffer once and then stained for 30 minutes with 1 µg of a R-Phycoerythrin Goat Anti-Mouse IgG (H+L) secondary antibody. Flow cytometry was carried out using a FACSCantoTM flow cytometer (BD Biosciences). Results are FACS analysis of M-05-74 induced, time dependent HER3 receptor internalization in T47D cells. M-05-74 shows binding to the expressed HER3 ECD, with or without supplemented recombinant human Heregulin fragment (HRG). M-05-74 leads to Her3 receptor internalization over a 4 h time period. Results are shown in Figure 6. The isotype control is indicated as a constant horizontal black bar. M-05-74 shows binding to the expressed Her3 ECD, with or without Human Heregulin fragment (-) and (+HRG). M-05-74 leads to Her3 receptor internalization over a 4 h time period. The isotype control is indicated as a constant horizontal black bar. In the presence of HRG the antibody induced internalization of HER3 was faster (e.g after 1h , at least 25 % more HER3 were internalized in the presence of HRG (+ HRG) when compared to the value in the absence of HRG (-).

Example 3

a) Kinetic screening/ binding properties of HER3 antibodies

The kinetic screening was performed according to Schraeml et al. (Schraml, M. and M. Biehl, Methods Mol Biol 901 (2012) 171-181) on a BIAcore 4000 instrument, mounted with a Biacore CM5 sensor. In all assay the test antibodies were captured. The system was under the control of the software version V1.1. The instrument buffer was HBS-EP (10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA,

0.05 % (w/v) P20). The system operated at 25 °C. 30 µg/ml Rabbit polyclonal antibody (RAM IgG,(Rabbit anti Mouse IgG with Fc gamma specificity) GE Healthcare) in 10 mM sodium acetate buffer (pH 4.5) was immobilized using EDC/NHS chemistry according to the manufacturer's instructions on the spots 1, 2, 4 and 5 in the flow cells 1, 2, 3 and 4. The sensor was saturated using 1M ethanolamine. In each flow cell, referenced signals were calculated using spots 1-2 and spots 5-4, spot 3 served as a blanc control. The antigen (human recombinant Her-3 ECD (68 kDa), and recombinant Thermus thermophilus SlyD FKBP-Her3 (15 kDa) comprising the ß-hairpin peptide of HER3 (SEQ ID NO:1)) was diluted at 150 nM in instrument buffer supplemented with 1mg/ml CMD(Carboxymethyldextran, Sigma). to suppress unspecific binding. Prior to their application the hybridoma culture supernatants were diluted 1:5 in instrument buffer. The diluted mixtures were injected at a flow rate of 30 µl/min for 2 min. The antibody capture level (CL) in response units was monitored. Immediately thereafter the respective antigen was injected at a flow rate of 30 µl/min for 3 min association time. Thereafter, the antibody-antigen complex dissociation signal was recorded for 5 min. The sensor was regenerated by injecting a 10 mM glycine-HCl solution (pH 1.7) for 2 min at a flow rate of 30 µl/min. The recorded signal shortly before the end of the injection of the antigen was denoted as binding late (BL) in response units. The recorded signal shortly before the end of the recording of the dissociation is denoted as stability late (SL) in response units. The dissociation rate constants were determined calculated The antibody-antigen complex stability in minutes was calculated with the following formula: ln(2)/60*kd . The Molar Ratio was calculated with the formula: MW (antibody) / MW(antigen) *BL (antigen)/ CL (antibody).

Binding Late (BL) represents the response units at the end of the analyte injection. The amount of antibody captured as a ligand on the sensor surface is measured as Capture Level (CL) in response units. Together with the information of the molecular weights of the tested analytes, the antibody and the analyte in solution, the Molar Ratio can be calculated. In case the sensor was configurated with a suitable amount of antibody ligand capture level, each antibody should be able to functionally bind at least to one analyte in solution, which is represented by a Molar Ratio of MR = 1.0. Then, the Molar Ratio is also an indicator for the valence mode of analyte binding. The maximum valence can be MR = 2 for an antibody binding two analytes, one with each Fab valence. In case of steric limitations or a dysfunctional analyte binding, the Molar Ratio can indicate understoichiometric

binding, like it is the case when the Her-3 ECD is being bound in its "closed" conformation. The maximum assay deviation in the determination of the Molar Ratio is MR = 0.2.

Screening/Selection of anti-HER3/HER4 antibody M-05-74:

In one experiment, the kinetic screening was driven with hybridoma primary cultures from different fusions, which were obtained from an immunization of mice with human recombinant Her-3 ECD. The aim was to select cultures with binding specificity for the Her-3 heterodimerization domain ß-hairpin peptide (SEQ ID NO:1). As antigens in solution human recombinant Her-3 ECD (68 kDa), and recombinant Thermus thermophilus SlyD FKBP-Her3 (15 kDa) comprising the ß-hairpin peptide of HER3 (SEQ ID NO:1) were used. A positive hit was classified as a primary culture supernatant with binding activity versus both antigens.

The Table 4 exemplarily shows primary culture supernatants, from which M-05-74 fulfills these requirements, indicating epitope specificity for the ß-hairpin of HER3. Therefore this is a suitable method of screening of anti-HER3 antibodies which bind to the Her-3 hairpin of SEQ ID NO:1.

Table 4: Exemplary results obtained from a kinetic screening experiment with a set of hybridoma primary cultures from fusions, wherein antibody M-05-74 was identified as binding to both HER3 ECD and the ß-hairpin of HER3 (SEQ ID NO:1) within the thermo SlyD-Her3 construct.

Ligand	Analyte	binding late BL [RU]	stability late SL [RU]	kd [1/s]	t/2 diss [min]	T [°C]	CL [RU]	MR [-]
M-04-06	human-Her3-ECD	17	16	4.13E-04	28	25	134	0.3
M-04-06	thermo SlyD-Her3	-4	-4	n.d.	n.d.	25	134	-0.3
M-04-140	human-Her3-ECD	-1	1	n.d.	n.d.	25	110	0.0

Ligand	Analyte	binding late BL [RU]	stability late SL [RU]	kd [1/s]	t/2 diss [min]	T [°C]	CL [RU]	MR [-]
M-04-140	thermo SlyD-Her3	-6	-5	n.d.	n.d.	25	112	-0.5
M-05-20	human-Her3-ECD	32	33	4.98E-05	232	25	623	0.1
M-05-20	thermo SlyD-Her3	-9	-6	n.d.	n.d.	25	625	-0.1
M-05-30	human-Her3-ECD	122	123	3.74E-05	309	25	521	0.5
M-05-30	thermo SlyD-Her3	-3	-2	n.d.	n.d.	25	525	-0.1
M-05-44	human-Her3-ECD	55	55	3.42E-05	337	25	373	0.3
M-05-44	thermo SlyD-Her3	-7	-6	n.d.	n.d.	25	369	-0.2
M-05-74	**human-Her3-ECD**	**75**	**79**	**<1.00E-05**	**>1155**	**25**	**318**	**0.5**
M-05-74	**thermo SlyD-Her3**	**33**	**32**	**1.20E-04**	**96**	**25**	**315**	**1.1**
M-05-82	human-Her3-ECD	0	1	n.d.	n.d.	25	205	0.0
M-05-82	thermo SlyD-Her3	-4	-5	n.d.	n.d.	25	204	-0.2

It has been found that M-05-74 shows a reduced Molar Ratio in its binding to the human Her-3 ECD analyte (MR = 0.5), whereas in its binding to analyte Thermus

thermophilus SlyD FKBP-Her3 comprising the ß-hairpin HER3 (SEQ ID NO:1) M-05-74 shows an improved Molar Ratio (MR = 1.1), indicating a functional, stoichiometric 1:1 binding with improved epitope accessibility (compared to human Her-3 ECD).

b) Kinetics of HER3 antibodies M-05-74, M-205 and M-208 kinetics to investigate the mode of action of M-05-74 in the absence and presence of Heregulin (HRG)

In its equilibrium state, the Her-3 ECD is in its "closed confirmation", which does mean, the heterodimerization Her-3 beta-hairpin motive is tethered via non-covalent interactions to the Her-3 ECD domain IV (see Figure 1c and d) . It is supposed, that the "closed" Her-3 conformation can be opened via the binding of the ligand heregulin at a specific Her-3 heregulin binding site. This takes place at the Her-3 interface formed by the Her-3 ECD domains I and domain III. By this interaction it is believed, that the Her-3 receptor is activated and transferred into its "open conformation" (see Figure 1b and e). When this occurs, the Her-3 beta-hairpin is accessible for the described antibodies. This mode of action can be simulated in vitro by a Biacore experiment.

A Biacore T100 instrument (GE Healthcare) was used to kinetically assess the monoclonal antibodies for their behavior to the heregulin-activated Her-3 Extracellular Domain (Her3_ECD). A CM5 series sensor was mounted into the system and was normalized in HBS-ET buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% w/v Tween 20) according to the manufacturer's instructions. The sample buffer was the system buffer supplemented with 1 mg/ml CMD (Carboxymethyldextran, Sigma #86524). The system operated at 25 °C. 6500 RU RAM-Fcγ (relative units of Fcγ-fragment RamIgG, GE Healthcare) were immobilized according to the manufacturer's instructions using EDC/NHS chemistry on all four flow cells. The sensor was deactivated using 1M ethanolamine.

The binding activity of the respective antibody against the analytes was kinetically tested. Antibodies were captured at 35 nM concentration by a 1 min injection at 5 µl/min. The flow rate was set to 100 µl/min.

The analytes in solution tested were human Heregulin fragment (HRG) (SEQ ID NO:11), a 44 kDa homodimeric protein (prepared according to Example 2e), human recombinant HER2 ECD (SEQ ID NO:10) (69.6 kDa), human recombinant

HER3 ECD (SEQ ID NO:4) (68 kDa), human recombinant HER4 ECD (SEQ ID NO:6), and 100 nM of the Her-3 ECD and the Her-4 ECD each incubated with a 5-fold molar excess of Heregulin for 60 min at room temperature resulting in HER3 ECD-HRG complex and HER4 ECD-HRG complex (Addition of MWs for complexes).

Analytes in solution were injected at different concentration steps of 0 nM, 1.1 nM, 3.7 nM, 11.1 nM , 33.1 nM and 90 nM for 3.5 min. The dissociation was monitored for 15 min. Where possible, kinetic signatures were evaluated according to a Langmuir fit.

Table 5a: SPR-resolved kinetic data of M-05-74 (=M-074), M-205 and M-208

Antibody	CL RU	Analyte in solution	T °C	k_a 1/Ms	k_d 1/s	K_D M	K_D nM	BL RU	MR	Chi² RU²
M-074	535	HRG	25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M-074	530	HER2_ECD	25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M-074	648	HER3-ECD	25	1.3E+04	2.8E-05	2.2E-09	2	70	0.2	0.1
M-074	712	HER4-ECD	25	6.7E+03	1.0E-03	1.5E-07	150	27	0.1	0.1
M-074	546	HER3-ECD-HRG	25	6.3E+04	2.7E-04	4.2E-09	4	160	0.6	2.3
M-074	719	HER4-ECD-HRG	25	1.6E+05	8.3E-04	5.2E-09	5	349	0.6	0.0
M-205	591	HRG	25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M-205	588	HER2_ECD	25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M-205	605	HER3-ECD	25	4.9E+04	1.0E-04	2.0E-09	2	235	1.0	1.3
M-205	597	HER3-ECD-HRG	25	3.7E+04	1.2E-04	3.2E-09	3	164	0.4	0.3
M-208	777	HRG	25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M-208	771	HER2_ECD	25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M-208	822	HER3-ECD	25	5.8E+04	5.3E-05	9.1E-10	1	367	1.0	9.4
M-208	795	HER3-ECD-HRG	25	5.0E+04	1.4E-04	2.8E-09	3	390	1.1	17.6

MR = Molar Ratio, BL = Binding Late, CL = Capture Level ; n.d. = not detectable = no bindingThe Molar Ratio was calculated with the formula: MW (antibody) / MW(antigen) *BL (antigen)/ CL (antibody).

The antibody M-205 is a murine monoclonal antibody with binding activity versus an epitope nearby the Her-3 ECD Heregulin binding site (described as Mab205.10.2 in WO2011/076683). M-205 competes with Heregulin around its binding site on the Her-3 ECD.

The antibody M-208 is a murine monoclonal antibody with binding activity versus the Her-3 ECD domain IV. M-208 binds to the Her-3 ECD independently of the Her-3 ECD conformational state.

M-05-74 (=M-074 in Table 5) binds to the Her-3 ECD in its active "open" conformation (on the presence of ligand (e.g. heregulin HRG) with improved kinetics, due to a better accessibility of the Her-3 hairpin in its "open" conformation. The MR is at least two fold higher.

No antibody binding (n.d.) was observed versus the negative control analytes Heregulin beta (HRG) and the extracellular HER-2 domain (HER2_ECD). The tested antibodies showed all binding to the Her3-ECD (HER3_ECD), but with strongly differing BL values.

M-05-74 binds to the Her-3 ECD in its "closed" conformation with slower association rate constant k_a = 1.3E+04 1/Ms and smaller BL (70 RU) than when compared to the clones M-205 with faster k_a = 4.9 E+04 1/Ms and high signal amplitude at BL (235 RU) and M-208 with faster k_a = 5.8E+04 1/Ms and also high signal amplitude at BL (367RU). This implicates on the stoichiometry of the binding (MR), where M-205 (MR = 1.0) and M-208 (MR = 1.0) both show a functional 1:1 binding for the HER3-ECD, whereas M-05-74 shows a non-functional binding (MR = 0.2). Here it is supposed, that this interaction of M-05-74 versus the Her-3 ECD is residual binding of a portion of structurally handicapped Her-3 ECD analyte. This is also supposed for the interaction of M-05-74 versus the Her-4 ECD, which also shows a non-functional binding with BL (27 RU) and (MR = 0.1).

A surprising result is the more than 4-fold increase (nearly 5 fold) of the M-05-74 association rate constant k_a from the "closed" Her-3 ECD to the "open" Her-3 ECD/Heregulin complex; from k_a = 1.3E+04 1/Ms (Her3_ECD) to k_a = 6.3E+04 1/Ms (Her3-ECD-HRG). So M-05-74 binds to HER3-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and absence of Heregulin (Ka (-Heregulin)) of 4.0 or higher (Ka (+Heregulin))/ (Ka (-Heregulin) = ka (Her3-ECD-HRG)/ ka (Her3-ECD) = 6.3E+04 [1/Ms]/1.3E+04

[1/Ms]) = 4.85)) .Thereby the Molar Ratio improves 3-fold, indicating now a 1:1 interaction of M-05-74 with the Her-3 ECD Heregulin complex. Thus binds M-05-74 to HER3-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 3.0 (MR (+Heregulin))/ (MR (-Heregulin) = 0.6/0.2 = 3).

This is also valid for the Her-4 ECD/Heregulin complex, where the Molar Ratio improves 6-fold, indicating a 1:1 interaction of M-05-74 with the Her-4 ECD Heregulin complex. Thus binds M-05-74 to HER4-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 3.0 (MR (+Heregulin))/ (MR (-Heregulin) = 0.6/0.1 = 6). And furthermore surprisingly the M-05-74 association rate constant ka increases from the "closed" Her-4 ECD to the "open" Her-4 ECD/Heregulin complex from ka = 6.7E+03 1/Ms (Her3_ECD) to ka = 1.6E+05 more than 20-fold. So M-05-74 binds to HER4-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and absence of Heregulin (Ka (-Heregulin)) of 20.0 or higher (Ka (+Heregulin))/ (Ka (-Heregulin) = ka (Her4-ECD-HRG)/ ka (Her4-ECD) = 6.7E+04 [1/Ms]/1.6E+05 [1/Ms]) = 23.88)).

As expected, the Heregulin displacer M-205, reduces its BLvalue and the Molar Ratio. The Molar Ratio is decreased 2.5-fold, from a fully functional 1:1 interaction with MR = 1.0 (Her3-ECD) with 235 RU at BL into a less functional MR = 0.4 (Her3-ECD-HRG) with 164 RU at BL. This indicates the loss in functionality due to the competing presence of excess Heregulin.

The antibody M-208, which binds to the Her-3 ECD domain IV remains completely unaffected by the presence of Heregulin. No significiant change of the Molar Ratios MR could be detected.

The Figure 7 shows the mode of binding of the anti-HER3/HER4 ß-hairpin antibody M-05-74 to the Heregulin-activated Her-3 ECD complex. M-05-74 (see plot 1) captures and prevents the Heregulin dissociation from the complex. M-05-74 is a trap for Heregulin ("Heregulin-sink"). M-05-74 does not compete with Heregulin for a binding site on the Her-3 ECD. For comparison M-08-11 (plot 2) is shown; M-08-11 (VH and VL see SEQ ID NO: 51 and 52) is another HER3 ß-Hairpin binder with no HER4 ECD and HER4 ß-hairpin crossreactivity, which binds to a different epitope than M-05-74.

Ia further experiment also HER1 ECD, T.T.SlyD-cysHer3 and T.T.SlyD-cas without the HER3 ß-hairpin were included in the measurement– results are shown in Table 5b , which substantially reveals the same binding properties of M-05-74.

A Biacore T200 instrument (GE Healthcare) was mounted with a CM5 series sensor. The sensor was normalized in HBS-ET buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% w/v Tween 20) according to the manufacturer's instructions. The sample buffer was the system buffer supplemented with 1 mg/ml CMD (Carboxymethyldextran, Sigma #86524). The system operated at 25 °C. 6500 RU RAM-Fcγ (relative units of Fcγ-fragment RamIgG, GE Healthcare) were immobilized according to the manufacturer's instructions using amine coupling EDC/NHS chemistry on all four flow cells. The sensor was deactivated using 1M ethanolamine. Monoclonal antibodies were captured (CL, Capture Level) on the sensor surface by a 1 min injection at 10 µl/min. Concentration dependent kinetics were measured.A concentration series of the analytes HER-1-ECD, HER-2-ECD, HER-3-ECD, HER-4-ECD, T.T.SlyD-cysHer3 and T.T.SlyD-cas were injected each at 0 nM, 1.1 nM, 3.3 nM, 2x 10 nM, 30 nM and 90 nM. Heregulin beta (HRG) was injected at 0 nM, 17 nM, 2 x 50 nM, 150 nM and 450 nM, 90 nM HER-3 ECD and 90 nM HER-4 ECD were preincubated for 2 hrs with a five-fold molar excess of HRG beta and were injected at HER concentrations steps of 0 nM, 1.1 nM, 3.3 nM, 2x 10 nM, 30 nM and 90 nM. All analytes were injected for 5 min association time and 10 min dissociation time at 100 µl/min flow rate. The sensor capture system was regenerated by a 3 min injection at 10 µl/min of 10 mM glycine pH 1.7. Where possible kinetic data was evaluated using the Biacore T200 evaluation software. HER-3-ECD, HER-4-ECD and T.T.SlyD-cysHer3 kinetics were evaluated using a Langmuir fitting model. HER-3-ECD-HRG and HER-4-ECD-HRG kinetics of M-5-74, were evaluated according to a Langmuir fitting model.

Table 5b: SPR-resolved kinetic data of M-05-74

	Analyte	**CL (Ab)**	**k_a**	**k_d**	**K_D**	**RMax**	**MR**	**Chi²**	**T**
Antibody	**in solution**	RU	1/Ms	1/s	M	RU		RU²	°C
M-5-74	HER1-ECD	288	n.d.	n.d.	n.d.	1	n.d.	0	25
	HER2-ECD	287	n.d.	n.d.	n.d.	1	n.d.	0	
	HER3-ECD	289	9,6E+04	1,1E-04	1,1E-09	19	**0,1**	0,05	
	HER4-ECD	285	1,6E+04	8,2E-04	5,1E-08	13	**0,1**	0,01	

	Analyte	CL (Ab)	k_a	k_d	K_D	RMax	MR	Chi²	T
Antibody	**in solution**	RU	1/Ms	1/s	M	RU		RU²	°C
	HER3-ECD-HRG	312	1,0E+05	2,9E-04	2,8E-09	195	**0,8**	2,2	
	HER4-ECD-HRG	301	9,9E+04	8,1E-04	8,1E-09	179	**0,8**	1,8	
	HRG	301	n.d.	n.d.	n.d.	0	n.d.	0,0	
	T.T.SlyD-cysHer3	486	3,0E+04	2,4E-04	7,8E-09	88	**1,9**	0,02	
	T.T.SlyD-cas	490	n.d.	n.d.	n.d.	0,5	0,0	0,06	

MR = Molar Ratio, BL = Binding Late, CL = Capture Level ; n.d. = not detectable = no bindingM-05-74 binds HER-3-ECD-HRG and HER-4-ECD-HRG with 1:1 stoichiometry and inactive HER-3-ECD and HER-4-ECD with 10:1 stoichiometry. M-05-74 binds HER-3-ECD and HER-3-ECD-HRG with higher affinity than HER-4-ECD and HER-4-ECD-HRG. M-05-74 does not interact with HER-1, HER-2 and HRG. M-05-74 binds T.T.SlyD-cysHer3 with 1:2 stoichiometry and does not interact with T.T.SlyD-cas.

Example 4

Epitope mapping of anti-HER3 antibody M-05-74 and mode of action analysis

M-05-74 with a unique epitope (ß-hairpin of HER3 and HER4)

A Biacore 2000 (GE Healthcare) instrument was used to assess the accessible epitopes clone culture supernatants for their binding specificity. A CM5 sensor was mounted into the system and was normalized in HBS-ET buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% w/v Tween 20) according to the manufacturer's instructions. The sample buffer was the system buffer supplemented with 1 mg/ml CMD (Carboxymethyldextran, Sigma). The system operated at 37 °C. 10000 RU RAM-Fcγ (relative units of Fcγ-fragment Rabbit Anti-Mouse IgG/ Jackson Laboratories) were immobilized according to the manufacturer's instructions using EDC/NHS chemistry on all four flow cells. The sensor was deactivated using 1M ethanolamine.

At a flow rate of 10 µl/min the primary antibody 50 nM anti-HER3 M-05-74 was captured for 1 min on all flow cells. The flow rate was set to 30 µl/min and an IgG blocking solution (50 µg/ml IgG (20:2:1 IgG1-Fcγ, IgG2a-Fcγ, IgG2b), Roche)

was injected for 5 minutes. The antigen Her-3 ECD was injected at 1.5 µM for 3 min.

Afterwards, 100 nM of each anti-HER3 secondary antibodies (a) M-05-74 b) 8B8 from WO97/35885 (named GT in the Figure) c) M-208 which binds to domainIV of HER3, and d) M-08-11; another HER3 ß-Hairpin binder with no HER4 ECD and HER4 ß-hairpin crossreactivity) was injected for 3 minutes at 30 µl/min. Acidic regeneration of the sensor surface was achieved using three consecutive injections of 10 mM Glycine pH 1.7 at 30µl/min for 60 sec.

The noise of the measurement is defined by the rebinding of the secondary M-05-74 injection, which re-saturates the already dissociated primary M-05-74. The experiment showed (see Figure 8), that M-208 and M-05-74 occupy distinct epitopes on the Her-3 ECD, because the secondary M-208 signal completely saturates the Her-3 ECD in the presence of M-05-74. M-08-11 binding is completely blocked by the presence of M-05-74. The M-08-11 secondary signal is even below noise. Nevertheless M-08-11 binds to a different epitope than M-05-74 as M-08-11 does not bind to human HER4 ECD and HER4 ß-hairpin. (see also below the exact epitope mapping data with the ß-hairpins of HER3 and HER4). The 8B8 (=GT) secondary antibody produces a significant signal in the presence of M-05-74, which is above noise. Therefore the 8B8 (=GT) antibody binds another epitope than M-05-74 and M-08-11.

M-05-74 with unique epitope and mode of actions

A Biacore B3000 instrument (GE Healthcare) was used to kinetically assess the clone culture M-05-74 and the antibody 8B8 (from WO 97/35885, named GT in the Figures) to the "closed" conformation of Her-3 ECD and the "open", Heregulin-activated Her-3 ECD. A CM5 series sensor was mounted into the system and was normalized in HBS-ET buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% w/v Tween 20) according to the manufacturer's instructions. The sample buffer was the system buffer supplemented with 1 mg/ml CMD (Carboxymethyldextran). The system operated at 25 °C. 10000 RU RAM-Fcγ (relative units of Fcγ-fragment Rabbit Anti-Mouse IgG / Jackson Laboratories) were immobilized according to the manufacturer's instructions using EDC/NHS chemistry on all flow cells. The sensor was deactivated using 1M ethanolamine. Analytes in solution were injected at 100 µl/min at different concentration steps of 0 nM, 1.1 nM, 3.7 nM, 11.1 nM , 33.1 nM and 90 nM for 2 min. The dissociation

was monitored for 5 min. Acidic regeneration of the sensor surface was achieved using three consecutive injections of 10 mM Glycine pH 1.7 at 30 µl/min for 60 sec. Kinetic data were evaluated according to a Langmuir fit.

Table 6: Langmuir kinetics of M-05-74 in comparison to 8B8 (GT). 8B8 with lower antigen complex stability (t/2diss) and less functionality (MR).

Antibody	CL (RU)	Analyte in solution	T (°C)	ka (1/Ms)	t/2-diss (min)	BL (RU)	MR	Chi² (RU²)
8B8	339.3	ECD-HRG	25	3.21E+05	0.8	90	**0.4**	2.57
M-074	314.7	ECD-HRG	25	6.6E+04	18	199	**0.8**	0.773
8B8	347.3	Her-3 ECD	25	1.02E+05	5.3	13.1	**0.1**	0.12
M-074	318.2	Her-3 ECD	25	2.04E+04	28	36	**0.2**	0.122
8B8	476.1	ttSlyD-Her3	25	n.d.	n.d.	n.d.	n.d.	n.d.
M-074	468	ttSlyD-Her3	25	8.75E+04	4.9	68.1	**1.5**	0.174

MR = Molar Ratio, BL = Binding Late, CL = Capture Level

In the table above kinetic data of the antibody clone M-05-74 and the antibody 8B8 are listed. M-05-74 binds to the Heregulin-activated Her-3 ECD with high functionality MR = 0.8. M-05-74 and acts as Heregulin trap. (see also Figure Biacore sensogram Example 3b and Figure 7).

The complex stability of the 8B8 antibody with t1/2 diss = 0.8 min is weak. 8B8 binds with an , MR = 0.4 .No separated dissociation phases of the 8B8 antibody and the Heregulin dissociation can be identified. Heregulin completely dissociates off in the same timeframe and with the same velocity, like 8B8. 8B8 antibody does not delay the heregulin dissociation.

M-05-74 functionally binds (MR = 1.5) to the Thermus thermophilus SlyD FKBP-Her3 comrpising th HER3 ß-Hairpin of SEQ ID NO:1 with KD = 27 nM. Since the antibody 8B8 does not bind to the HER3 ß-Hairpin comprising *Thermus thermophilus* SlyD FKBP-Her-3 fusion polypeptide this antibody targets another epitope than M-05-74.

Figure 9 is an overlay plot of the biacore sensogramms of anti-HER3/HER4 antibody M-05-74, anti-HER3 antibody M-08-11 and anti-HER3 antibody 8B8 (from WO97/35885) showing the different binding modes of actions. Anti-HER3/HER4 antibody M-05-74 traps the Heregulin-activated Her-3 ECD (1) with t1/2 diss = 18min and acts Heregulin-sink. Anti-HER3 antibody M-08-11 HER3 (ß-Hairpin binder with no HER4 ECD and HER4 ß-hairpin crossreactivity) delays

the Heregulin dissociation (2) and produces a complex two-state kinetic. 8B8 antibody (3) is does not trap Heregulin and also not delays the Heregulin dissociation from the Her-3 ECD/Heregulin complex. Since it is a perfect Langmuir interaction, the Heregulin/Her-3 ECD complex quickly and completely dissociates as intact complex from the 8B8 antibody.

In Figure 10 a scheme of these binding modes of action is shown: **1**: M-08-11 binds to the Heregulin activated Her-3 ECD and induces a delayed Heregulin dissociation, whereby M-08-11 stays in the Her-3 ECD receptor complex. **2**: M-05-74 binds to the Heregulin activated Her-3 ECD. Heregulin is trapped in the complex and the antibody stays in the complex. **3**: 8B8 binds the Heregulin activated Her-3 ECD. The whole complex dissociates from the antibody.

Peptide-based 2D Epitope Mapping

In another embodiment a peptide-based epitope mapping experiment was done to characterize the Her-3 ECD epitopes by using the CelluSpots™ Synthesis and Epitope Mapping technology. Epitope mappings were carried out by means of a library of overlapping, immobilized peptide fragments (length: 15 amino acids) corresponding to the sequences of human Her-1 ECD, Her-2 ECD,Her-3 ECD and Her-4 ECD peptide hairpins. In Figure 11, the strategy of the epitope mapping and alanine-scan approach is shown. The peptide hairpin sequences (ß-hairpin) of HER1(EGFR) ECD, HER2 ECD,HER3 ECD and HER4 ECDincluding their structural embeddings (structural) were investigated. Cysteins were replaced by serines. For antibody selection of the antibodies via binding to such ß-hairpins, the ß-hairpins of HER3 and HER4 are defined by SEQ ID NO:1 and SEQ ID NO:2.

Each peptide synthesized was shifted by one amino acid, *i.e.* it had 14 amino acids overlap with the previous and the following peptide, respectively. For preparation of the peptide arrays the Intavis CelluSpots™ technology was employed. In this approach, peptides are synthesized with an automated synthesizer (Intavis MultiPep RS) on modified cellulose disks which are dissolved after synthesis. The solutions of individual peptides covalently linked to macromolecular cellulose are then spotted onto coated microscope slides. The CelluSpots™ synthesis was carried out stepwise utilizing 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on amino-modified cellulose disks in a 384-well synthesis plate. In each coupling cycle, the corresponding amino acids were activated with a solution of DIC/HOBt in DMF. Between coupling steps un-reacted amino groups were capped with a

mixture of acetic anhydride, diisopropylethyl amine and 1-hydroxybenzotriazole. Upon completion of the synthesis, the cellulose disks were transferred to a 96-well plate and treated with a mixture of trifluoroacetic acid (TFA), dichloromethane, triisoproylsilane (TIS) and water for side chain deprotection. After removal of the cleavage solution, the cellulose bound peptides are dissolved with a mixture of TFA, TFMSA, TIS and water, precipitated with diisopropyl ether and re-suspended in DMSO. The peptide solutions were subsequently spotted onto Intavis CelluSpots™ slides using an Intavis slide spotting robot.

For epitope analysis, the slides prepared as described above were washed with ethanol and then with Tris-buffered saline (TBS; 50 mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 8) before blocking for 16 h at 4°C with 5 mL 10x Western Blocking Reagent (Roche Applied Science), 2.5 g sucrose in TBS, 0.1% Tween 20. The slide was washed with TBS and 0.1% Tween 20 and incubated afterward with 1 µg/mL of the corresponding IGF1 antibodies in TBS and 0.1% Tween 20 at ambient temperature for 2 h and subsequently washed with TBS + 0.1% Tween 20. For detection, the slide was incubated with anti-rabbit / anti-mouse secondary HRP-antibody (1:20000 in TBS-T) followed by incubation with chemiluminescence substrate luminol and visualized with a LumiImager (Roche Applied Science). ELISA-positive SPOTs were quantified and through assignment of the corresponding peptide sequences the antibody binding epitopes were identified.

As depicted in Figure 12, M-05-74 shows a HER3 ECD epitope with the amino acid sequence VYNKLTFQLEP (SEQ ID NO:43) and a crossreactivity to a HER4 ECD epitope with the amino acid sequence VYNPTTFQLE (SEQ ID NO:44) with no detectable signals versus the hairpin motives in EGFR and the HER2 ECD. No signals at all were detectable with the 8B8 antibody, therefore the 8B8 antibody targets epitopes, different from the hairpin peptide motives. M-08-11 shows a HER3 ECD specific epitope with the amino acid sequence PLVYNKLTFQLE with no crossreactivity detectable to the other hairpin sequences of the Her-family.

In Figure 13, the amino acids identified by Ala-Scan which are contributing most to the binding of antiHER3/HER4 antibody M-05-74 to its HER3 ECD binding epitope VYNKLTFQLEP (SEQ ID NO:43) and to its HER4 ECD binding epitope VYNPTTFQLE (SEQ ID NO:44) are underlined/bold.

Example 5

Binding of HRG to HER3-ECD in the presence of HER3 antibody (ELISA)

A Streptavidin-coated 96-well plate was incubated at 4°C with cell culture supernatant containing SBP-tagged HER3-ECD. On the next day the wells were washed three times with washng buffer (PBS + 0.05% Tween-20) and blocked with PBS containing 1% BSA for one hour. After another three washes with washing buffer, 40µl antibody solution (in Delfia Binding Buffer) was added to each well as a 2x stock of the desired final concentrations (10^{-3} to 10^{3}nM, alternatively 10^{-4} to 10^{2}nM). Immediately 40µl of 20nM Europium-labeled Heregulin-beta (PeproTech, Cat. #100-03) was added to achieve a final concentration of 10nM. The plates were incubated on a shaker at room temperature for two hours. Following three washes with Delfia Wash Buffer, Delfia Enhancement Solution was added and incubated on a shaker for 15 minutes (light protected). Finally, the plates were measured in a Tecan Infinite F200 reader using a time-resolved fluorescence measurement protocol. The binding of M-05-74 (named M-074 in Figure 14) can promote binding of HRG to HER3-ECD until a plateau is reached at a signal of 650. Results are shown in Figure 14.

Example 6

a) Inhibition of HER3 phosphorylation in ZR-75-1 cells

Assays were performed in ZR-75-1 cells according to the following protocol: Seed cells with 500,000 cells/well into Poly-D-Lysine coated 6-well plate in RPMI1640 medium with 10% FCS. Incubate for 24h. Remove medium by aspirating, incubate overnight with 500µl/well RPMI 1640 with 0.5% FCS. Add antibodies in 500 µl RPMI 1640 with 0.5% FCS. Incubate for 1h. Add Heregulin-beta (PeproTech, Cat. #100-03)) (final concentration 500ng/ml) for 10 min. To lyse the cells remove medium and add 80 µl ice cold Triton-X-100 cell lysis buffer and incubate for 5 minutes on ice. After transferring the lysate into 1.5 ml reaction tube and centrifugation at 14000 rpm for 15 min at 4°C, transfer supernatant into fresh reaction tubes. Samepes containing equal amounts of protein in SDS loading buffer were separated on SDS PAGE and blotted by using a semi-dry Western Blot to nitrocellulose membranes. Membrans were blocked by 1xNET-buffer + 0.25% gelatine for 1h hour and pHER3 is detected by the antibody αPhospho-HER3/ErbB3 (Tyr1289) (21D3), Cell Signaling, #4791and HER3 by the antibody αErbB3 (C-17), Santa Cruz, #sc-285 respectively. After washing und detection of the signals by an POD coupled secondary antibody, bands were densometricaly

scanned. Percent (%) inhibition of anti-HER3 antibodies M-05-74 on receptor phosphorylation in zr-75-1 cells is shown below in Table 7.

Table 7: % Inhibition of HER3 phosphorylation in ZR-75-1 cells

antibody	pHER3 % inhibiton [10 µg/ml]
Ctrl	0
M-05-74	49

b) Inhibition of HER3 phosphorylation of the bivalent parent M-05-74 and the Fab fragment of M-05-74 (Fab-74)

MCF-7 cells were seeded into 24-Well-plates (1ml RPMI, 10% FCS, 3x105 cells per well) and were incubated at 37°C /5%C02 overnight. After 24 hours the media was replaced with1ml media containing 0.5% FCS. After 48hours the antibodies were added to a final concentration of 10µg/ml, 1µg/ml and 0.1µg/ml (M-05-74) and 6.66µg/ml, 0.66µg/ml and 0.066µg/ml (Fab-074). The plates were incubated at 37°C for 50 minutes and then Heregulin-beta (PeproTech, Cat. #100-03) was added to a final concentration of 500ng/ml. The plates were incubated for a further 10 minutes at 37°C/5%C02. The cells were washed with PBS and lysed in 40µl Triton Lysis Buffer (1% Triton) containing Aprotinin (10µg/ml), Orthovanadate (0.4 mM), Phenylmethylsulfonyl fluoride (1mM). 26µl of the collected lysates were transferred to reaction tubes and 14µl Sample Buffer (NuPAGE LDS Sample Buffer 4x, NuPAGE Sample Reducing Agent 10x) was added. The samples were incubated for 10 minutes at 70°C and then analysed by SDS-PAGE (NuPAGE, 4-12 % Bis-Tris-Mini-Gel). Electroblotting was performed using the iBlot Dry Blotting System (Invitrogen). The nitrocellulose membrane was incubated with phosphoHER3 antibody (α Phospho Her3, Cellsignaling # 4791, Rabbit 1:1000) followed by incubation with HRP-conjugated secondary antibody (goat anti rabbit 1:5000, BioRad cat: 170-6515). Signal was developed using ECL Detection Reagents (Amersham RPN2209) on X-Ray film (Roche Lumi-Film Chemiluminescent Detection Film 11666657001). The anti-HER3 antibody M-05-74 (full length purified from hybridoma) and the Fab fragment of the antibody Fab-74 (obtained py papain cleavage from full length M-05-74) were investigated in eqimolar amounts. Fab fragments were generated by papain digestion of the antibody. Briefly, 1ml of app. 2 mg/ml antibody containing solution was supplemented with 25 mM Cystein and 70 µg papain (Roche). After incubation at

37°C for 1.5 h, the digestion reaction was stopped by addition of iodoacetamide and the reaction mixture was purified by MabSelect Sure (GE Healthcare). The Fab containing flowthrough fraction was further purified by size exclusion chromatography (Superdex 200; GE Healthcare).

Percent (%) inhibition of anti-HER3 antibodies on receptor phosphorylation in MCF7 cells is summarised below and in Table 8. The antibody M-05-74 (full length from hybridoma) and the Fab fragment of this antibody Fab-74 can inhibit HER3 phosporylation in equimolar concentrations to an comparable extent.

Table 8: % Inhibition of HER3 phosphorylation in MCF-7 cells

Antibody	pHER3 % inhibition **[6.66 nM]**	pHER3 % inhibition **[0.66 nM]**
control	0	0
M-05-74 (full length from hybridoma)	94	13
Fab fragment of M-05-74 (Fab-74)	96	14

Example 7

Inhibition of HER2/HER3 heterodimers (Imunoprecipitation and Western Blot) in MCF7 cells

MCF-7 cells were seeded into 6-Well-plates (2ml RPMI, 10% FCS, 8x105 cells per well) and were grown overnight. On the next day the media was exchanged by 2ml starving media containing 0.5% FCS. On day three the antibodies were added to a final concentration of 10µg/ml and the plates were incubated at 37°C. After 50 minutes Heregulin-beta (PeproTech, Cat.#100-03) was added to a final concentration of 500ng/ml and the plates were incubated for another 10 minutes at 37°C. The cells were washed with PBS and lysed in 250µl Triton Lysis Buffer containing 1% Digitonin. 60µl of the collected lysates were transferred to reaction tubes and incubated with 40µl antibody-coupled Sepharose (either Herceptin or HER3-antibody #208) and 500µl Buffer containing 0.3% Digitonin. The reaction

mixes were incubated on a wheel rotator overnight at 4°C. On the next day the reaction mixes were washed three times with 500µl Buffer containing 0.3% Digitonin. After the last wash the supernatant was discarded and 10µl 4x Loading Buffer was added. The tubes were incubated for 10 minutes at 70°C and the supernatants were consequently loaded onto a gel for SDS-PAGE. After the following Semi-Dry Western Blot the membranes containing the samples immunoprecipitated with HER2 antibody were incubated with anti-HER3/HER4 antibody M-05-74 (M-074 in Figure 15), and vice versa. The membranes were then incubated with HRP-conjugated secondary antibody and the ECL signal was transferred onto X-Ray film. Results are shown in Figure 15, showing a strong inhibition of the HER2/HER heterodimer formation (HER2/HER heterodimerization) by the M-05-74.

Example 8

Inhibition of tumor cell proliferation of M-05-74 in MDA-MB-175 cells.

The anti-tumor efficacy of HER3 antibodies M-05-74 in a cell proliferation assay, using MDA-MB-175 cells (VII Human Breast Carcinoma Cells, ATCC catalog no. HTB-25), was assessed. 20,000 cells per well were seeded into sterile 96 well tissue culture plates with DMEM/F12 cell culture medium, containing 10% FCS and incubated at 37°C±1°C with 5 % ±1% CO_2 for one day. The cells are slow growing cells with a doubling time of ca. 3 days. Anti-HER3 antibodies were added in dilution series and further incubated for 6 days. Cell viability was then assessed using the alamarBlue® readout. EC50 values were calculated.

Table 9: EC50 of the Inhibition of tumor cell proliferation of M-05-74 in MDA-MB-175 cells

antibody	EC_{50} [µg/ml]
M-05-74	5,8

Example 9

In vivo antitumor efficacy of anti-HER3 antibody M-05-74

The in vivo antitumor efficacy of the anti-HER3 antibody M-05-74 (M-074) could be detected in cell based models of various tumor origin (e.g. SCCHN and pancreatic cancer) transplanted on SCID beige. As example data are shown for the SCCHN xenograft model FaDu (cell line based).

Test agents

M-05-74 was provided as stock solution from Roche, Penzberg, Germany expressed and purified from hybridoma cells. Antibody buffer included histidine. Antibody solution was diluted appropriately in buffer from stock prior injections.

Cell lines and culture conditions

FaDu human HNSCC cells were originally obtained from ATCC. The tumor cell line was routinely cultured in MEM Eagle medium supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM NEAA at 37 °C in a water-saturated atmosphere at 5 % CO2. Culture passage was performed with trypsin / EDTA 1x splitting every third day.

Animals

Female SCID beige or nude mice were purchased from breeder (e.g. Charles River, Sulzfeld, Germany) and maintained under specific-pathogen-free condition with daily cycles of 12 h light /12 h darkness according to committed guidelines (GV-Solas; Felasa; TierschG). Experimental study protocol was reviewed and approved by local government. After arrival animals were maintained in the quarantine part of the animal facility for one week to get accustomed to new environment and for observation. Continuous health monitoring was carried out on regular basis. Diet food (Provimi Kliba 3337) and water (acidified pH 2.5-3) were provided ad libitum.

Animals were controlled daily for clinical symptoms and detection of adverse effects. For monitoring throughout the experiment body weight of animals was documented.

Animal treatment started after animal randomisation after cell transplantation when median tumor size was about 100–150mm3. Antibody was administered as single agent at 10 mg/kg i.p. q7d once weekly for several weeks depending of the model. The corresponding vehicle was administered on the same days.

FaDu HNSCC xenograft bearing mice were treated with antibody M-05-74 from study day 10 to 24. As a result, treatment with H-74 antibody showed significant anti-tumor efficacy with nearly tumors stasis of s.c. FaDu xenografts. The Tumor Growth Inhibition (TGI) was calculated at 89%.

Treatment with M-05-74 (10mg/kg q7dx3, i.p.) resulted in nearly tumor stasis of FaDu . Results are shown in Figure 17 , wherein M-05-74 is named M-074.

Example 10

Generation of M-05-74-Fab-Pseudomonas exotoxin conjugate (M-05-74-PE)

Expression, purification and renaturation of Fab fragment of M-05-74, PE24 variant, and Fab fragment of M-05-74 conjugated to Pseudomonas exotoxin variant PE24LR8M based on the Sequences of SEQ ID NO:45, 46, 47, 48 (or 49).

Expression of Fab (e.g. for sortase coupling) -Expression vectors

For the expression of the described Fab fragments, variants of expression plasmids for transient expression (e.g. HEK293-F) cells based either on a cDNA organization with or without a CMV-Intron A promoter or on a genomic organization with a CMV promoter were applied.

Beside the antibody expression cassette the vectors contained:
- an origin of replication which allows replication of this plasmid in *E. coli*, and
- a ß-lactamase gene which confers ampicillin resistance in *E. coli*.

The transcription unit of the antibody gene was composed of the following elements:
- unique restriction site(s) at the 5' end
- the immediate early enhancer and promoter from the human cytomegalovirus,
- followed by the Intron A sequence in the case of the cDNA organization,
- a 5'-untranslated region of a human antibody gene,
- an immunoglobulin heavy chain signal sequence,
- the human antibody chain either as cDNA or as genomic organization with the immunoglobulin exon-intron organization
- a 3' untranslated region with a polyadenylation signal sequence, and
- unique restriction site(s) at the 3' end.

The fusion genes comprising the antibody chains as described below were generated by PCR and/or gene synthesis and assembled by known recombinant methods and techniques by connection of the according nucleic acid segments *e.g.* using unique restriction sites in the respective vectors. The subcloned nucleic acid sequences were verified by DNA sequencing. For transient transfections larger

quantities of the plasmids were prepared by plasmid preparation from transformed *E. coli* cultures (Nucleobond AX, Macherey-Nagel).

Cell culture techniques

Standard cell culture techniques were used as described in Current Protocols in Cell Biology (2000), Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J. and Yamada, K.M. (eds.), John Wiley & Sons, Inc.

The Fab fragments were expressed by transient co-transfection of the expression plasmids of the heavy and the light chain in HEK29-F cells growing in suspension as described below.

Transient transfections in HEK293-F system

The Fab fragments were generated by transient transfection with the respective plasmids (e.g. encoding the heavy and modified heavy chain, as well as the corresponding light and modified light chain) using the HEK293-F system (Invitrogen) according to the manufacturer's instruction. Briefly, HEK293-F cells (Invitrogen) growing in suspension either in a shake flask or in a stirred fermenter in serum-free FreeStyle™ 293 expression medium (Invitrogen) were transfected with a mix of the four expression plasmids and 293-Free™ (Novagen) or Fectin (Invitrogen). For 2 L shake flask (Corning) HEK293-F cells were seeded at a density of 1.0E*6 cells/mL in 600 mL and incubated at 120 rpm, 8% CO2. The day after the cells were transfected at a cell density of ca. 1.5E*6 cells/mL with ca. 42 mL mix of A) 20 mL Opti-MEM (Invitrogen) with 600 µg total plasmid DNA (1 µg/mL) encoding the heavy or modified heavy chain, respectively and the corresponding light chain in an equimolar ratio and B) 20 ml Opti-MEM + 1.2 mL 293-Free (Novagen) or Fectin (2 µl/mL). According to the glucose consumption glucose solution was added during the course of the fermentation. The supernatant containing the secreted antibody was harvested after 5-10 days and antibodies were either directly purified from the supernatant or the supernatant was frozen and stored.

Expression of Pseudomonas exotoxin variant PE24-LR8M for sortase coupling- Expression vector

For the expression of PE24-LR8M an E. coli expression plasmid was used.

Beside the expression cassette for the pseudomonas exotoxin A domain III the vector contained:

- an origin of replication from the vector pBR322 for replication in *E. coli* (according to Sutcliffe, G., et al., Quant. Biol. 43 (1979) 77-90),
- the lacI repressor gene from E. coli (Farabaugh, P.J., Nature 274 (1978) 765-769),
- the URA3 gene of Saccharomyces cerevisiae coding for orotidine 5'-phosphate decarboxylase (Rose, M. et al. Gene 29 (1984) 113-124) which allows plasmid selection by complementation of E.coli pyrF deletion strains (uracil auxotrophy).

The transcription unit of the toxin gene was composed of the following elements:

- unique restriction site(s) at the 5' end,
- the T5 hybrid promoter (T5-PN25/03/04 hybrid promoter according to Bujard, H., et al. Methods. Enzymol. 155 (1987) 416-433 and Stueber, D., et al., Immunol. Methods IV (1990) 121-152) including a synthetic ribosomal binding site according to Stueber, D., et al. (see before),
- the pseudomonas exotoxin A domainIII with an N-terminal coupling tag followed by a furin site (SEQ ID NO:45 Pseudomonas exotoxin variant PE24LR8M_3G, including a GGG linker for sortase coupling),
- two bacteriophage-derived transcription terminators, the λ-T0 terminator (Schwarz, E., et al., Nature 272 (1978) 410-414) and the fd-terminator (Beck E. and Zink, B. Gene 1-3 (1981) 35-58),
- unique restriction site(s) at the 3' end.

Cultivation and expression of the Pseudomonas Exotoxin A construct variant PE24-LR8M_3G in an E. coli fed-batch process on chemical defined medium

For the expression of PE24-LR8M_3G_Ecoli (25kDa) the E.coli host/vector system which enables an antibiotic-free plasmid selection by complementation of an E.coli auxotrophy (PyrF) was employed (EP 0 972 838 and US 6,291,245).

An E.coli K12 strain was transformed by electroporation with the expression plasmid. The transformed E.coli cells were first grown at 37 °C on agar plates. A colony picked from this plate was transferred to a 3mL roller culture and grown at 37°C to an optical density of 1-2 (measured at 578nm). Then 1000 µl culture where mixed with 1000 µl sterile 86%-glycerol and immediately frozen at -80°C for long time storage. The correct product expression of this clone was first verified in small

scale shake flask experiments and analyzed with SDS-Page prior to the transfer to the 10L fermenter.

Pre cultivation:

For pre-fermentation a chemical defined medium has been used. For pre-fermentation 220 ml of medium in a 1000 ml Erlenmeyer-flask with four baffles was inoculated with 1.0 ml out of a primary seed bank ampoule. The cultivation was performed on a rotary shaker for 8 hours at 32 °C and 170 rpm until an optical density (578 nm) of 2.9 was obtained. 100 ml of the pre cultivation was used to inoculate the batch medium of the 10L bioreactor.

Fermentation:

For fermentation in a 10l Biostat C, DCU3 fermenter (Sartorius, Melsungen, Germany) a chemical defined batch medium was used. All components were dissolved in deionized water. The alkaline solution for pH regulation was an aqueous 12.5 % (w/v) NH_3 solution supplemented with 11.25 g/l L-methionine.

Starting with 4.2 l sterile batch medium plus 100 ml inoculum from the pre cultivation the batch fermentation was performed at 31 °C, pH 6.9 ± 0.2, 800 mbar back pressureand an initial aeration rate of 10 l/min. The relative value of dissolved oxygen (pO2) was kept at 50 % throughout the fermentation by increasing the stirrer speed up to 1500 rpm. After the initially supplemented glucose was depleted, indicated by a steep increase in dissolved oxygen values, the temperature was shifted to 25 °C and 15 minutes later the fermentation entered the fed-batch mode with the start of both feeds (60 and 14 g/h respectively). The rate of feed 2 is kept constant, while the rate of feed 1 is increased stepwise with a predefined feeding profile from 60 to finally 160 g/h within 7 hours. When carbon dioxide off gas concentration leveled above 2% the aeration rate was constantly increased from 10 to 20 l/min within 5 hours. The expression of recombinant PE24-LR8M_3G_Ecoli protein was induced by the addition of 2.4 g IPTG at an optical density of approx. 120. The target protein is expressed soluble within the cytoplasm.

After 24 hours of cultivation an optical density of 209 is achieved and the whole broth is cooled down to 4-8°C. The bacteria are harvested via centrifugation with a flow-through cntrifuge (13,000 rpm, 13 l/h) and the obtained biomass is stored at -

20 °C until further processing (cell disruption). The yield is 67.5 g dry cells per liter.

Analysis of product formation:

Samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_{Target} = 10) are suspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 µL of each suspension are centrifuged (15,000 rpm, 5 minutes) and each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (= soluble protein fraction) 100 µL and to each pellet (= insoluble protein fraction) 200 µL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 minutes at 95°C under intense mixing to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 µL of each sample are transferred to a 4-20 % TGX Criterion Stain Free polyacrylamide gel (Bio-Rad). Additionally 5 µl molecular weight standard (Precision Plus Protein Standard, Bio-Rad) were applied.

The electrophoresis was run for 60 Minutes at 200 V and thereafter the gel was transferred the GelDOC EZ Imager (Bio-Rad) and processed for 5 minutes with UV radiation. Gel images were analyzed using Image Lab analysis software (Bio-Rad). Relative quantification of protein expression was done by comparing the volume of the product bands to the volume of the 25kDa band of the molecular weight standard.

Cultivation and expression of an antibody fragment light chain construct (VL) and an antibody fragment heavy chain Pseudomonas Exotoxin A variant fusion (Fab-PE24) in an E. coli fed-batch process on chemical defined medium

For the expression of a Fab-light chain (23.4kDa) and a Fab-heavy chain PE24 fusion (48.7 kDa) the E.coli host/vector system which enables an antibiotic-free plasmid selection by complementation of an E.coli auxotrophy (PyrF) was employed (EP 0 972 838 and US 6,291,245).

An E.coli K12 strain was transformed by electroporation with the respective expression plasmids. The transformed E.coli cells were first grown at 37 °C on agar plates. For each transformation a colony picked from this plate was transferred to a

3mL roller culture and grown at 37°C to an optical density of 1-2 (measured at 578nm). Then 1000 µl culture where mixed with 1000 µl sterile 86%-glycerol and immediately frozen at -80°C for long time storage. The correct product expression of these clones was first verified in small scale shake flask experiments and analyzed with SDS-Page prior to the transfer to the 10L fermenter.

Pre-cultivation:

For pre-fermentation a chemical defined medium has been used. For pre-fermentation 220 ml of medium in a 1000 ml Erlenmeyer-flask with four baffles was inoculated with 1.0 ml out of a primary seed bank ampoule. The cultivation was performed on a rotary shaker for 9 hours at 37 °C and 170 rpm until an optical density (578 nm) of 7 to 8 was obtained. 100 ml of the pre cultivation was used to inoculate the batch medium of the 10L bioreactor.

Fermentation (RC52#003):

For fermentation in a 10l Biostat C, DCU3 fermenter (Sartorius, Melsungen, Germany) a chemical defined batch medium was used. The alkaline solution for pH regulation was an aqueous 12.5 % (w/v) NH_3 solution supplemented with 11.25 g/l L-methionine.

Starting with 4.2 l sterile batch medium plus 100 ml inoculum from the pre cultivation the batch fermentation was performed at 31 °C, pH 6.9 ± 0.2, 800 mbar back pressure and an initial aeration rate of 10 l/min. The relative value of dissolved oxygen (pO2) was kept at 50 % throughout the fermentation by increasing the stirrer speed up to 1500 rpm. After the initially supplemented glucose was depleted, indicated by a steep increase in dissolved oxygen values, the temperature was shifted to 37 °C and 15 minutes later the fermentation entered the fed-batch mode with the start of both feeds (60 and 14 g/h respectively). The rate of feed 2 is kept constant, while the rate of feed 1 is increased stepwise with a predefined feeding profile from 60 to finally 160 g/h within 7 hours. When carbon dioxide off gas concentration leveled above 2% the aeration rate was constantly increased from 10 to 20 l/min within 5 hours. The expression of recombinant target proteins as insoluble inclusion bodies located in the cytoplasm was induced by the addition of 2.4 g IPTG at an optical density of approx. 40.

After 24 hours of cultivation an optical density of 185 is achieved and the whole broth is cooled down to 4-8°C. The bacteria are harvested via centrifugation with a

flow-through centrifuge (13,000 rpm, 13 l/h) and the obtained biomass is stored at -20 °C until further processing (cell disruption). The yield is between 40 and 60 g dry cells per liter.

Analysis of product formation:

Samples drawn from the fermenter, one prior to induction and the others at dedicated time points after induction of protein expression are analyzed with SDS-Polyacrylamide gel electrophoresis. From every sample the same amount of cells (OD_{Target} = 10) are suspended in 5 mL PBS buffer and disrupted via sonication on ice. Then 100 µL of each suspension are centrifuged (15,000 rpm, 5 minutes) and each supernatant is withdrawn and transferred to a separate vial. This is to discriminate between soluble and insoluble expressed target protein. To each supernatant (= soluble protein fraction) 100 µL and to each pellet (= insoluble protein fraction) 200 µL of SDS sample buffer (Laemmli, U.K., Nature 227 (1970) 680-685) are added. Samples are heated for 15 minutes at 95°C under intense mixing to solubilize and reduce all proteins in the samples. After cooling to room temperature 5 µL of each sample are transferred to a 4-20 % TGX Criterion Stain Free polyacrylamide gel (Bio-Rad). Additionally 5 µl molecular weight standard (Precision Plus Protein Standard, Bio-Rad) and 3 amounts (0.3 µl, 0.6 µl and 0.9 µl) quantification standard with known target protein concentration (0.1 µg/µl) were applied.

The electrophoresis was run for 60 Minutes at 200 V and thereafter the gel was transferred the GelDOC EZ Imager (Bio-Rad) and processed for 5 minutes with UV radiation. Gel images were analyzed using Image Lab analysis software (Bio-Rad). With the three standards a linear regression curve was calculated with a coefficient of >0.99 and thereof the concentrations of target protein in the original sample was calculated.

Purification, Sortase coupling and renaturation (of Fab fragment of M-05-74, PE24 variant, and Fab fragment of M-05-74 conjugated to Pseudomonas exotoxin variant PE24LR8M)

Fab fragment

The Fab fragment was purified by affinity chromatography (Ni Sepharose™ High Perfomance HisTrap™) according to the manufacture´s description. In brief, the supernatant was loaded onto the column equilibrated in 50 mM sodium phosphate

pH 8.0, 300 mM NaCl. Protein elution was performed with the same buffer at pH 7.0 with a washing step containing 4 mM imidazole followed by a gradient up to 100 mM imidazole. Fractions containing the desired Fab fragment were pooled and dialyzed against 20 mM His, 140 mM NaCl, pH 6.0.

PE24 for Sortase coupling

E. coli cells expressing PE24 were lysed by high pressure homogenization (if details are required: Christian Schantz) in 20 mM Tris, 2 mM EDTA, pH 8.0 + Complete protease inhibitor cocktail tablets (Roche). The lysate was filtrated and loaded onto a Q sepharose FF (GE Healthcare) equilibrated in 20 mM Tris, pH 7.4. Protein was eluted with a gradient up to 500 mM NaCl in the same buffer. PE24 containing fractions were identified by SDS PAGE. The combined pool was concentrated and applied to a HiLoad™ Superdex™ 75 (GE Healthcare)equilibrated in 20 mM Tris, 150 mM NaCl, pH 7.4. Fractions containing PE24 were pooled according to SDS PAGE and frozen at -80°C.

Sortase coupling of Fab fragment to PE24

Fab fragment and PE24 were diafiltrated separately into 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$ pH7.5 using Amicon® Ultra 4 centrifugal filter devices (Merck Millipore) and concentrated to 5 – 10 mg/ml. Both proteins and sortase were combined in a 1:1:0.8 molar ratio. After one hour incubation at 37°C the mixture was loaded onto a Ni Sepharose™ High Perfomance HisTrap™) equilibrated in 50 mM sodium phosphate, pH 8.0, 300 mM NaCl. Elution was performed with a gradient up to 100 mM imidazole in the same buffer pH 7.0. The flow through fractions containing the final product Fab-PE24 was concentrated and loaded onto a HiLoad™ Superdex™ 200 (GE Healthcare) in 20 mM Tris, 150 mM NaCl, pH 7.4. Fractions containing the desired coupled protein were pooled and stored at -80°C. As sortase soluble S.aureus sortase A was used (SEQ ID NO: 50). Soluble S.aureus sortase A was expressed and purified using the following expression plasmid: The sortase gene encodes an N-terminally truncated Staphylococcus aureus sortase A (60-206) molecule. The expression plasmid for the transient expression of soluble sortase in HEK293 cells comprised besides the soluble sortase expression cassette an origin of replication from the vector pUC18, which allows replication of this plasmid in E. coli, and a beta-lactamase gene which confers ampicillin resistance in E. coli. The transcription unit of the soluble sortase comprises the following functional elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (P-CMV) including intron A,
- a human heavy chain immunoglobulin 5'-untranslated region (5'UTR),
- a murine immunoglobulin heavy chain signal sequence,
- an N-terminally truncated S.aureus sortase A encoding nucleic acid, and
- the bovine growth hormone polyadenylation sequence (BGH pA).

Renaturation of Fab-PE24 derived from E. coli inclusion bodies

Inclusion bodies of VH-PE24 and VL-C_{kappa} were solubilized separately in 8 M guanidinium hydrochloride, 100 mM Tris-HCl, 1 mM EDTA, pH 8.0 + 100 mM dithiothreitol (DTT). After 12 – 16 hours at RT the pH of the solubilisates was adjusted to 3.0, the centrifuged solutions were dialyzed against 8 M guanidinium hydrochloride, 10 mM EDTA, pH 3.0. The protein concentration was determined by Biuret reaction, the purity of inclusion body preparations was estimated by SDS PAGE. Equimolar amounts of both chains were diluted in two steps into 0.5 M arginine, 2 mM EDTA, pH 10 + 1 mM GSH/1 mM GSSG, to a final concentration of 0.2 – 0.3 mg/ml. After 12 – 16 h at 4 – 10 °C the renaturated protein was diluted with H_2O to < 3 mS/cm and loaded onto a Q sepharose FF (GE healthcare) equilibrated in 20 mM Tris/HCl, pH 7.4. Elution was performed with a gradient up to 400 mM NaCl in the same buffer. Fractions containing the correct product were identified by SDS-PAGE and analytical size exclusion chromatography (SEC). Pooled fractions were concentrated and loaded onto a HiLoad™ Superdex™ 200 (GE Healthcare) in 20 mM Tris, 150 mM NaCl, pH 7.4 or alternatively in 20 mM histidine, 140 mM NaCl, pH 6.0. Fractions were analyzed and pooled according to analytical SEC and stored at -80°C.

Based on SEQ ID NO:46 and 49 the immunoconjugate of Fab fragment of M-05-74with Pseudomonas exotoxin variant PE24LR8M (M-05-74-PE) can be expressed recombinately, purified and renturated also as direct PE24LR8M fusion.

Example 11

Cell killing of different tumor cell lines by M-05-74-Fab-Pseudomonas exotoxin conjugate (M-05-74-PE)

HER3 overexpressing A549 cells were seeded into a white 96-well-plate (flat, transparent bottom, $1x10^4$ cells per well) and were grown in RPMI (10% FCS) overnight. On the next day, the media was exchanged by 50µl starving media (RPMI, 0.5% FCS). After at least 4 hours, 5µl Heregulin-beta (PeproTech,

Cat.#100-03) (HRG beta) was added to a final concentration of 500ng/ml. 50µl Fab-74-PE solution was added to final concentrations of 10, 3.3, 1.1, 0.37, 0.12, 0.04, 0.014, 0.005 and 0.002µg/ml. Plates were incubated for 72h. After 24h and 48h, 5µl Heregulin-beta was added again to a final concentration of 500ng/ml. After 72h the luminescence was measured in a Tecan Infinite F200 Reader using the CellTiter-Glo Luminescent Cell Viability Assay by Promega (Cat.#G7571). The EC50 value for M-05-74-Fab-Pseudomonas exotoxin conjugate (M-05-74-PE) in the abscence of HRG beta was: 1,93 µg/ml and in the presence 0,13 µg/ml.

Table 10: EC50 of Cell killing of A549 cells by M-05-74-Fab-Pseudomonas

presence (+)/ absence (-) of ligand Heregulin-beta (HRG)	EC50 of (M-05-74-PE) (µg/ml)	half max. inhibition
+HRG beta	0,13	30,3
-HRG beta	1,93	19,85

Example 12

Humanized variants of the antibodies according to the invention

The binding specificity of the murine antibody is transferred onto a human acceptor framework to eliminate potential immunogenicity issues arising from sequence stretches that the human body will recognize as foreign. This is done by engrafting the entire HVRs of the murine (donor) antibody onto a human (acceptor) antibody framework, and is called HVR (or CDR)-grafting or antibody humanization.

The murine variable region amino acid sequence is aligned to a collection of human germline antibody V-genes, and sorted according to sequence identity and homology. The acceptor sequence is selected based on high overall sequence homology and optionally also the presence of the right canonical residues already in the acceptor sequence (see Poul, M-A. and Lefranc, M-P., in "Ingénierie des anticorps banques combinatores" ed. by Lefranc, M-P. and Lefranc, G., Les Editions INSERM, 1997).

The germline V-gene encodes only the region up to the beginning of HVR3 for the heavy chain, and till the middle of HVR3 of the light chain. Therefore, the genes of the germline V-genes are not aligned over the whole V-domain. The humanized construct comprises the human frameworks 1 to 3, the murine HVRs, and the

human framework 4 sequence derived from the human JK4, and the JH4 sequences for light and heavy chain, respectively.

Before selecting one particular acceptor sequence, the so-called canonical loop structures of the donor antibody can be determined (see Morea, V., et al., Methods, Vol 20, Issue 3 (2000) 267-279). These canonical loop structures are determined by the type of residues present at the so-called canonical positions. These positions lie (partially) outside of the HVR regions, and should be kept functionally equivalent in the final construct in order to retain the HVR conformation of the parental (donor) antibody.

In WO 2004/006955 a method for humanizing antibodies is reported that comprises the steps of identifying the canonical HVR structure types of the HVRs in a non-human mature antibody; obtaining a library of peptide sequence for human antibody variable regions; determining the canonical HVR structure types of the variable regions in the library; and selecting the human sequences in which the canonical HVR structure is the same as the non-human antibody canonical HVR structure type at corresponding locations within the non-human and human variable regions.

Summarizing, the potential acceptor sequence is selected based on high overall homology and optionally in addition the presence of the right canonical residues already in the acceptor sequence.

In some cases simple HVR grafting only result in partial retention of the binding specificity of the non-human antibody. It has been found that at least some specific non-human framework residues are required for reconstituting the binding specificity and have also to be grafted into the human framework, i.e. so called "back mutations" have to be made in addition to the introduction of the non-human HVRs (see e.g. Queen et al., Proc. Natl. Acad. Sci. USA 86 (1989) 10,029-10,033; Co et al., Nature 351 (1991) 501-502). These specific framework amino acid residues participate in FR-HVR interactions and stabilized the conformation (loop) of the HVRs (see e.g. Kabat et al., J. Immunol. 147 (1991) 1709).

In some cases also forward-mutations are introduced in order to adopt more closely the human germline sequence.

The genes for those designed antibody sequences are generated by conventional PCR techniques. The heavy chain variable region is fused to either the human IgG1

heavy chain constant region (if effector function is required) or to a human IgG1 / IgG4 heavy chain constant region variant (if no effector function is required; IgG1 L234A L235A P329G; IgG4 S228P L235E P329G). The light chain variable domain is fused to either the light chain kappa or lambda constant domain for the construction of the expression plasmids.

Accordingly the mouse anti-HER3/HER4 antibody M-05-74 was humanized to give the following humanized variants of M-05-74:

Table 11: VH and VL sequences of humanized variant antibodies of M-05-74

Humanized antibodies of M-05-74	**humanized variant of VH/SEQ ID NO:**	**humanized variant of light chain variable domain VL//SEQ ID NO:**
huMab M-05-74_1	<Her3> M-05-74_VH1 SEQ ID NO: 33	<Her3> M-05-74_VL1 SEQ ID NO: 36
huMab M-05-74_2	<Her3> M-05-74_VH1 SEQ ID NO: 33	<Her3> M-05-74_VL2 SEQ ID NO: 37
huMab M-05-74_3	<Her3> M-05-74_VH2 SEQ ID NO: 34	<Her3> M-05-74_VL1 SEQ ID NO: 36
huMab M-05-74_4	<Her3> M-05-74_VH2 SEQ ID NO: 34	<Her3> M-05-74_VL2 SEQ ID NO: 37
huMab M-05-74_5	<Her3> M-05-74_VH3 SEQ ID NO: 35	<Her3> M-05-74_VL1 SEQ ID NO: 36
huMab M-05-74_6	<Her3> M-05-74_VH3 SEQ ID NO: 35	<Her3> M-05-74_VL2 SEQ ID NO: 37

Table 12: HVR sequences of humanized variant antibodies of M-05-74

Humanized antibodies of M-05-74	**HVR-H1, HVR-H2, and HVR-H3 of humanized variant/SEQ ID NO:**	**HVR-L1, HVR-L2, and HVR-L3 of humanized variant/SEQ ID NO:**
huMab M-05-74_1	-humanized variant 1 HVR-H1_V1 (SEQ ID NO:38), -humanized variant 1 HVR-H2_V1 (SEQ ID NO:39), -HVR-H3 (SEQ ID NO: 27)	-humanized variant 1 HVR-L1_V1 (SEQ ID NO:40), -humanized variant 1 HVR-L2_V1 (SEQ ID NO:41), -HVR-L3 (SEQ ID NO: 30)

Humanized antibodies of M-05-74	**HVR-H1, HVR-H2, and HVR-H3 of humanized variant/SEQ ID NO:**	**HVR-L1, HVR-L2, and HVR-L3 of humanized variant/SEQ ID NO:**
huMab M-05-74_2	-humanized variant 1 HVR-H1_V1 (SEQ ID NO:38), -humanized variant 1 HVR-H2_V1 (SEQ ID NO:39), -HVR-H3 (SEQ ID NO: 27)	-humanized variant 1 HVR-L1_V1 (SEQ ID NO:40), -humanized variant 1 HVR-L2_V1 (SEQ ID NO:42), -HVR-L3 (SEQ ID NO: 30)
huMab M-05-74_3	-HVR-H1 (SEQ ID NO: 25), -humanized variant 1 HVR-H2_V1 (SEQ ID NO:39), -HVR-H3 (SEQ ID NO: 27)	-humanized variant 1 HVR-L1_V1 (SEQ ID NO:40), -humanized variant 1 HVR-L2_V1 (SEQ ID NO:41), -HVR-L3 (SEQ ID NO: 30)
huMab M-05-74_4	-HVR-H1 (SEQ ID NO: 25), -humanized variant 1 HVR-H2_V1 (SEQ ID NO:39), -HVR-H3 (SEQ ID NO: 27)	-humanized variant 1 HVR-L1_V1 (SEQ ID NO:40), -humanized variant 1 HVR-L2_V1 (SEQ ID NO:42), -HVR-L3 (SEQ ID NO: 30)
huMab M-05-74_5	-HVR-H1 (SEQ ID NO: 25), -humanized variant 1 HVR-H2_V1 (SEQ ID NO:39), -HVR-H3 (SEQ ID NO: 27)	-humanized variant 1 HVR-L1_V1 (SEQ ID NO:40), -humanized variant 1 HVR-L2_V1 (SEQ ID NO:41), -HVR-L3 (SEQ ID NO: 30)
huMab M-05-74_6	-HVR-H1 (SEQ ID NO: 25), -humanized variant 1 HVR-H2_V1 (SEQ ID NO:39), -HVR-H3 (SEQ ID NO: 27)	-humanized variant 1 HVR-L1_V1 (SEQ ID NO:40), -humanized variant 1 HVR-L2_V1 (SEQ ID NO:42), -HVR-L3 (SEQ ID NO: 30)

Antibodies were expressed in mammalian cell culture systems like HEK or CHO, and purified via protein A and size exclusion chromatography. Humanized antibodies were either expressed full-length antibodies or antibody fragments or are included in immunotoxin conjugates (see Example 10). Humanized variants were evaluated with respect to their binding and biological properties as described above.

Antibody	Binding to HER3-ECD KD (binding affinity)	Binding to HER3-ECD-HRG KD (binding affinity)
huMab M-05-74_5	3.2 nM	4.1 nM
huMab M-05-74_6	8.3 nM	8.6 nM

Example 13

In vivo tumor cell growth inihibiton by M-05-74-Fab-Pseudomonas exotoxin conjugate (M-05-74-PE)

The human A431-B34 non-small cell lung cancer cell line cell line, which was stably transfected with an expression vector encoding human HER3, was subcutaneously inoculated into the right flank of female SCID *beige* mice (1×10^7 cells per animal).

On day 21 after tumor inoculation, the animals were randomized and allocated into the treatment group and one vehicle group, resulting in a median tumor volume of ~110 mm^3 per group. On the same day, animals were treated intravenously for 2 cycles, each cycle consisting of 3q7d (every other day), with M-05-74-Fab-Pseudomonas exotoxin conjugate (M-05-74-PE) (1.0 mg/kg). Controls received vehicle (Tris buffer). The two cycles were separated by a one week off-treatment.

Primary tumor volume (TV) was calculated according to the NCI protocol (TV = (length x $width^2$) / 2), where "length" and "width" are long and short diameters of tumor mass in mm (Corbett et al., 1997). Calculation was executed from staging (day 21 after tumor inoculation) until day 42 after tumor inoculation, and values were documented as medians and inter-quartile ranges (IQR) defined as differences of the third and first quartile.

For calculation of percentage tumor growth inhibition (TGI) during the treatment period, every treated group was compared with its respective vehicle control. TV_{day}

$_z$ represents the tumor volume of an individual animal at a defined study day (day z) and $TV_{day\,x}$ represents the tumor volume of an individual animal at the staging day (day x).

The following formula was applied:

$$TGI\,[\%] = 100 - \frac{median(TV(treated)_{day\,z} - TV(treated)_{day\,x})}{median(TV(resp.\,control)_{day\,z} - TV(resp.\,control)_{day\,x})} \times 100$$

Calculations of treatment to control ratio (TCR) with confidence interval (CI) were applied using non-parametric methods. Results of median tumor volumes with inter-quartile ranges are shown in Figure 19. Tumor growth inhibition was 66% of M-05-74-Fab-Pseudomonas exotoxin conjugate (M-05-74-PE) with a TCR of 0.509 (CI:0.33 – 0.734).

Example 14

Binding of the antibody M-05-74 (1) to TtSlyDcys-Her3 (SEQ ID NO: 18) in comparison with anti-HER3 antibody MOR09823 (2) described in WO2012/22814.

A Biacore T200 instrument (GE Healthcare) was mounted with CM5 series sensor and was normalized in HBS-ET+ buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% w/v Tween 20) according to the manufacturer's instructions. The sample buffer was the system buffer supplemented with 1 mg/ml CMD (Carboxymethyldextran). The system operated at 37 °C. A double antibody capture system was established on the sensor surface. 6500 RU mAb<M-IgG>R was immobilized according to the manufacturer's instructions using EDC/NHS chemistry on all flow cells. The sensor was deactivated using 1M ethanolamine. Flow cell 1 served as a reference and was captured for 1 min at 10µl/min with anti-TSH IgG1 antibody. On flow cell 2 M-5-74 was captured for 1 min at 10µl/min. On flow cell 3 a murine anti-human FC pan antibody was captured 1 min at 10 µl/min followed by the injection of the anti-HER3 antibody M-05-74 (1) or of anti-HER3 antibody MOR09823 antibody for 1 min at 10 µl/min. The flow rate was set to 60 µl/min. The analyte in solution TtSlyDcys-HER3 (SEQ ID NO: 18) was injected at concentrations of 0 nM and 150 nM for 5 min and the dissociation was monitored for 600 sec. The sensor was fully regenerated by one injection at 10 µl/min for 3 min with 10 mM glycine pH 1.7 buffer.

Fig.20 depicts a sensorgram overlay plot showing binding signals at 150 nM of, TtSlyDcys-Her3 and buffer . The overlay plot above shows the antibody M-5-74 binding at 150 nM TtSlyDcys-Her3 (1). MOR09823 antibody does not bind TtSlyDcas-Her3 (2). (3) shows the backround binding signal of the TtSlyDcas-HER3 versus the mAb<M-IgG>R capture surface. The anti-HER3 antibody MOR09823 (2) described in WO2012/22814 does not show any interaction at 150 nM TtSlyDcys-Her3 . The positive control antibody M-05-74 (1) shows significiant binding versus TtSlyDcas-Her3. No interaction could be determined with both antibodies when injecting 150 nM TtSlyDcys (no HER-3 insertion) (data not shown).

Patent Claims

1. A method for selecting an antigen binding protein that binds to human HER3 and binds to human HER4, wherein the antigen binding protein binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) of human HER3 and binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) of human HER4;

wherein

a) at least one polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3,

which comprises the amino acid sequence of SEQ ID NO:1;

and

b) at least one polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4,

which comprises the amino acid sequence of SEQ ID NO:2;

are used to select antigen binding proteins, which show binding to both, the at least one polypeptide under a) and the at least one polypeptide under b)

and thereby selecting an antigen binding protein that binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) and within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2).

2. An antigen binding protein obtained by the selection method of claim 1.

3. The method of claim 1, or the antigen binding protein of claim 2 wherein the antigen binding protein is an antibody.

4. An isolated antigen binding protein that binds to human HER3 and binds to human HER4, wherein the antigen binding protein binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) of human HER3 and binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) of human HER4.

5. The antigen binding protein of claim 4

a) wherein the antigen binding protein binds to a polypeptide of

SEQ ID NO: 18 TtSlyDcys-Her3,

and

b) wherein the antigen binding protein binds to a polypeptide of

SEQ ID NO: 22 TtSlyDcys-Her4.

6. The antigen binding protein of claims 4 or 5 wherein the antigen binding protein is an antibody.

7. An isolated antibody that binds to human HER3 and that binds to human HER4, wherein the antibody has one or more of the following properties:

a) the antibody binds to the amino acid sequence of SEQ ID NO:1; and/or

b) the antibody binds to the amino acid sequence SEQ ID NO:1 in activated HER3; and/or

c) the antibody binds within an amino acid sequence of PQPLVYNKLTFQLEPNPHT (SEQ ID NO:1) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 13 TtSlyD-FKBP-Her3,

SEQ ID NO: 17 TtSlyDcas-Her3,

SEQ ID NO: 18 TtSlyDcys-Her3,

SEQ ID NO: 19 TgSlyDser-Her3, and

SEQ ID NO: 20 TgSlyDcys-Her3;
and/or

d) the antibody binds to the ß-hairpin region of HER3; and/or

e) the antibody inhibits the heterodimerisation of HER3/HER2 heterodimers; and/or

f) the antibody binds to HER3-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 4.0 or higher (Ka (+Heregulin))/(Ka (-Heregulin)); and/or

g) the antibody binds to HER3-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 2.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)); and/or

h) the antibody binds to the amino acid sequence of SEQ ID NO:2; and/or

i) the antibody binds to the amino acid sequence SEQ ID NO:2 in activated HER4; and/or

j) the antibody binds within an amino acid sequence of PQTFVYNPTTFQLEHNFNA (SEQ ID NO:2) which is comprised in a polypeptide selected from the group consisting of:

SEQ ID NO: 21 TtSlyDcas-Her4,

SEQ ID NO: 22 TtSlyDcys-Her4,

SEQ ID NO: 23 TgSlyDser-Her4, and

SEQ ID NO: 24 TgSlyDcys-Her4;

and/or

k) the antibody binds to the ß-hairpin region of HER4; and/or

l) the antibody binds to HER4-ECD with a ratio of the association constant (Ka) in presence of Heregulin (Ka (+Heregulin)) and in absence of Heregulin (Ka (-Heregulin)) of 20.0 or higher (Ka (+Heregulin))/(Ka (-Heregulin)); and/or

m) the antibody binds to HER4-ECD with a ratio of the Molar Ratio MR of binding in presence of Heregulin (MR (+Heregulin)) and in absence of Heregulin (MR (-Heregulin)) of 5.0 or higher (MR (+Heregulin))/ (MR (-Heregulin)); and/or

n) the antibody shows as monovalent Fab fragment the same or higher biological activity as compared to its bivalent parent full length antibody; and/or

o) the antibody inhibits the HER3 phosporylation in MCF-7 cells; and/or

p) the antibody does not compete for binding to HER3 with Heregulin/ induces binding of Heregulin to HER3; and/or

q) the antibody inhibits the proliferation of MDA-MB-175 tumor cells; and/or

r) the antibody shows tumor growth inhibitory activity in vivo; and/or

s) the antibody binds with an affinity of a KD value $\leq 1 \times 10^{-8}$ M to HER3-ECD; and/or

t) the antibody binds with an affinity of a KD value $\leq 1 \times 10^{-8}$ M to HER4-ECD; and/or

u) the antibody binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43) and to a polypeptide consisting of VYNPTTFQLE (SEQ ID NO:44); and/or

v) the antibody binds to a polypeptide consisting of VYNKLTFQLEP (SEQ ID NO:43); and/or

w) the antibody binds to a polypeptide consisting of VYNPTTFQLE (SEQ ID NO:44); and/or

x) the antibody binds in a FACS assay to HER3 expressing T47D cells; and wherein the antibody shows an at least 25% higher percentage of internalization in the presence of Heregulin as compared to the percentage of internalization in the presence of Heregulin when measured after 1 h after antibody exposure.

8. An antibody that binds to human HER3 and that binds human HER4, wherein the antibody binds to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNKLTFQLEP (SEQ ID NO:43) and to a polypeptide with a length of 15 amino acids comprising the amino acid sequence VYNPTTFQLE (SEQ ID NO:44).

9. The antibody of any one of claims 6 to 8, wherein the antibody comprises (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25; (b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26, and (c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27.

10. The antibody of claims any one of claims 6 to 9, comprising (a) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28; (b) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and (c) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

11. The antibody of any one of claims 6 to 8, wherein the antibody comprises

i) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:26;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:28;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:29; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30;

ii) or a humanized variant of the HVRs of the antibody under i) (a), (b), (d) and/or (e).

12. The antibody of claims any one of claims 6 to 8, or 11, wherein the antibody comprises

i) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30; or

ii) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:38;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30; or

iii) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:41; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30; or

iv) (a) HVR-H1 comprising the amino acid sequence of SEQ ID NO:25;

(b) HVR-H2 comprising the amino acid sequence of SEQ ID NO:39;

(c) HVR-H3 comprising the amino acid sequence of SEQ ID NO:27;

(d) HVR-L1 comprising the amino acid sequence of SEQ ID NO:40;

(e) HVR-L2 comprising the amino acid sequence of SEQ ID NO:42; and

(f) HVR-L3 comprising the amino acid sequence of SEQ ID NO:30.

13. An antibody comprising :
a) a VH sequence of SEQ ID NO:33 and a VL sequence of SEQ ID NO:36; or
b) a VH sequence of SEQ ID NO:33 and a VL sequence of SEQ ID NO:37; or
c) a VH sequence of SEQ ID NO:34 and a VL sequence of SEQ ID NO:36; or
d) a VH sequence of SEQ ID NO:34 and a VL sequence of SEQ ID NO:37; or
e) a VH sequence of SEQ ID NO:35 and a VL sequence of SEQ ID NO:36; or
f) a VH sequence of SEQ ID NO:35 and a VL sequence of SEQ ID NO:37.

14. The antibody of any one of claims 6to 13, which is a full length IgG1 antibody or IgG4 antibody.

15. The antibody of any one of claims 6 to 13, which is a Fab fragment.

16. An immunoconjugate comprising the antibody of any one of claims 6 to 15 and a cytotoxic agent.

17. The antibody of any one of claims 6 to 15, or the immunoconjugate of claim 16, for use in treating cancer.

18. The antibody of any one of claims 6 to 15 for use in inhibition of HER3/HER2 dimerization.

19. A pharmaceutical formulation comprising the antibody of any one of claims 6 to 15, or the immunoconjugate of claim 16, and a pharmaceutically acceptable carrier.

20. The antibody of any one of claims 6 to 15, or the immunoconjugate of claim 16, for use as a medicament.

21. Use of the antibody of any one of claims 6 to 15, or the immunoconjugate of claim 16, in the manufacture of a medicament.

22. The use of claim 21, wherein the medicament is for treatment of cancer.

23. Isolated nucleic acid encoding the antibody of any one of claims 6 to 15.

24. A host cell comprising the nucleic acid of claim 23.

25. A method of producing an antibody comprising culturing the host cell of claim 24 so that the antibody is produced, and recovering said antibody from said cell culture or the cell culture supernatant.

26. A polypeptide selected from the group consisting of:

i) SEQ ID NO: 13 TtSlyD-FKBP-Her3,

ii) SEQ ID NO: 17 TtSlyDcas-Her3,

iii) SEQ ID NO: 18 TtSlyDcys-Her3,

iv) SEQ ID NO: 19 TgSlyDser-Her3, and

v) SEQ ID NO: 20 TgSlyDcys-Her3,

which polypeptide comprises the amino acid sequence of SEQ ID NO:1

27. A polypeptide selected from the group consisting of:

i) SEQ ID NO: 21 TtSlyDcas-Her4,

ii) SEQ ID NO: 22 TtSlyDcys-Her4,

iii) SEQ ID NO: 23 TgSlyDser-Her4,and

iv) SEQ ID NO: 24 TgSlyDcys-Her4,

which polypeptide comprises the amino acid sequence of SEQ ID NO:2