Title: PREDICTING RESPONSE TO A HER INHIBITOR

Abstract: The present invention concerns methods for predicting response of patients with HER2 expressing tumor to treatment with HER inhibitors. In particular, the present invention concerns the use of low HER2 mRNA levels as a selection criterion for treating HER2 positive cancer patients, such as breast cancer patients, with HER inhibitor, such as pertuzumab.
PREDICTING RESPONSE TO A HER INHIBITOR

Related Application

This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/395,899 filed on May 19, 2010, which application is fully incorporated herein by reference.

Field of the Invention

The present invention concerns methods for predicting response of patients with HER2 expressing tumor to treatment with HER inhibitors. In particular, the present invention concerns the use of low HER2 mRNA levels as a selection criterion for treating HER2 positive cancer patients, such as breast cancer patients, with a HER inhibitor, such as pertuzumab.

Description of Related Art

HER Receptors and Antibodies Thereagainst

The HER family of receptor tyrosine kinases are important mediators of cell growth, differentiation and survival. The receptor family includes four distinct members including epidermal growth factor receptor (EGFR, ErbB1, or HER1), HER2 (ErbB2 or p185 neu), HER3 (ErbB3) and HER4 (ErbB4 or tyro2).

EGFR, encoded by the erbB1 gene, has been causally implicated in human malignancy. In particular, increased expression of EGFR has been observed in breast, bladder, lung, head, neck and stomach cancer as well as glioblastomas. Increased EGFR receptor expression is often associated with increased production of the EGFR ligand, transforming growth factor alpha (TGF-a), by the same tumor cells resulting in receptor activation by an autocrine stimulatory pathway. Baselga and Mendelsohn Pharmac. Ther. 64:127-154 (1994). Monoclonal antibodies directed against the EGFR or its ligands, TGF-a and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, e.g., Baselga and Mendelsohn., supra; Masui et

Antibodies directed against the rat p\textsuperscript{185}neu and human HER2 protein products have been described.

Drebin and colleagues have raised antibodies against the rat neu gene product, p\textsuperscript{185}neu See, for example, Drebin et al, Cell 41:695-706 (1985); Myers et al, Meth. Enzym. 198:277-290 (1991); and W094/22478. Drebin et al. Oncogene 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions of p\textsuperscript{185}neu result in synergistic anti-tumor effects on neu-transformed NIH-3T3 cells implanted into nude mice. See also U.S. Pat. No. 5,824,311 issued Oct. 20, 1998.

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., J. Clin. Oncol. 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein.


Homology screening has resulted in the identification of two other HER receptor family members; HER3 (U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. PNAS (USA) 86:9193-9197 (1989)) and HER4 (EP Pat Appln No 599,274; Plowman et al., Proc. Natl. Acad. Sci. USA, 90:1746-1750 (1993); and Plowman et al., Nature, 366:473-475 (1993)). Both of these receptors display increased expression on at least some breast cancer cell lines.

The HER receptors are generally found in various combinations in cells and heterodimerization is thought to increase the diversity of cellular responses to a variety of HER ligands (Earp et al. Breast Cancer Research and Treatment 35: 115-132 (1995)). EGFR is bound by six different ligands; epidermal growth factor (EGF), transforming growth factor alpha (TGF-a), amphiregulin, heparin binding epidermal growth factor (HB-EGF), betacellulin and epiregulin (Groenen et al. Growth Factors 11:235-257 (1994)). A family of heregulin proteins resulting from alternative splicing of a single gene are ligands for HER3 and HER4. The heregulin family includes alpha, beta and gamma heregulins (Holmes et al., Science, 256:1205-1210 (1992); U.S. Pat. No. 5,641,869; and Schaefer et al. Oncogene 15:1385-1394 (1997)); neu differentiation factors (NDFs), glial growth factors (GGFs); acetylcholine receptor inducing activity (ARIA); and sensory and motor neuron derived factor (SMDF). For a review, see Groenen et al. Growth Factors 11:235-257 (1994); Lemke, G. Molec. & Cell. Neurosci. 7:247-262 (1996) and Lee et al. Pharm. Rev. 47:51-85 (1995).

Recently three additional HER ligands were identified; neuregulin-2 (NRG-2) which is reported to bind either HER3 or HER4 (Chang et al. Nature 387 509-512 (1997); and Carraway et al. Nature 387:512-516 (1997)); neuregulin-3 which binds HER4 (Zhang et al. PNAS (USA) 94(18):9562-7 (1997)); and neuregulin-4 which binds HER4 (Harari et al. Oncogene 18:2681-89 (1999)) HB-EGF, betacellulin and epiregulin also bind to HER4.

While EGF and TGF-a do not bind HER2, EGF stimulates EGFR and HER2 to form a heterodimer, which activates EGFR and results in transphosphorylation of
HER2 in the heterodimer. Dimerization and/or transphosphorylation appears to activate the HER2 tyrosine kinase. See Earp et al., supra. Likewise, when HER3 is co-expressed with HER2, an active signaling complex is formed and antibodies directed against HER2 are capable of disrupting this complex (Sliwkowski et al., J. Biol. Chem., 269(20): 14661-14665 (1994)). Additionally, the affinity of HER3 for heregulin (HRG) is increased to a higher affinity state when co-expressed with HER2. See also, Levi et al, Journal of Neuroscience 15: 1329-1340 (1995); Morrissey et al, Proc. Natl. Acad. Sci. USA 92: 1431-1435 (1995); and Lewis et al, Cancer Res., 56:1457-1465 (1996) with respect to the HER2-HER3 protein complex. HER4, like HER3, forms an active signaling complex with HER2 (Carraway and Cantley, Cell 78:5-8 (1994)).

Diagnostics and Prognostics

Patients treated with the HER2 antibody trastuzumab are selected for therapy based on HER2 overexpression/amplification. See, for example, WO99/31 140 (Paton et al), US2003/0170234A1 (Hellmann, S.), and US2003/0147884 (Paton et al); as well as WO01/89566, US2002/0067485, and US2003/0134434 (Mass et al). See, also, US2003/0152572, Cohen et al., concerning immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) for detecting HER2 overexpression and amplification.

Patients treated with pertuzumab can be selected for therapy based on HER activation or dimerization. Patent publications concerning pertuzumab and selection of patients for therapy therewith include: WO01/00245 (Adams et al.); US2003/0086924 (Sliwkowski, M.); US2004/0013667A1 (Sliwkowski, M.); as well as WO2004/008099A2, and US2004/0106161 (Bossenmaier et al).


Pertuzumab (also known as recombinant human monoclonal antibody 2C4; OMNITARG™, Genentech, Inc, South San Francisco) represents the first in a new class of agents known as HER dimerization inhibitors (HDI) and functions to inhibit the ability of HER2 to form active heterodimers with other HER receptors (such as EGFR/HER1, HER3 and HER4) and is active irrespective of HER2 expression levels. See, for example, Harari and Yarden, Oncogene 19:6102-14 (2000; Yarden and Sliwkowski Nat Rev Mol Cell Biol, 2:127-37 (2001); Sliwkowski Nat Struct Biol 10:158-9 (2003); Cho, et al, Nature 421:756-60 (2003); and Malik, et al. Pro Am Soc Cancer Res 44:176-7 (2003).
Pertuzumab blockade of the formation of HER2-HER3 heterodimers in tumor cells has been demonstrated to inhibit critical cell signaling, which results in reduced tumor proliferation and survival (Agus et al. Cancer Cell 2:127-37 (2002)).

Pertuzumab has undergone testing as a single agent in the clinic with a phase 1a trial in patients with advanced cancers and phase II trials in patients with ovarian cancer and breast cancer as well as lung and prostate cancer. In a Phase I study, patients with incurable, locally advanced, recurrent or metastatic solid tumors that had progressed during or after standard therapy were treated with pertuzumab given intravenously every 3 weeks. Pertuzumab was generally well tolerated. Tumor regression was achieved in 3 of 20 patients evaluable for response. Two patients had confirmed partial responses. Stable disease lasting for more than 2.5 months was observed in 6 of 21 patients (Agus et al. Pro Am Soc Clin Oncol 22:192 (2003)). At doses of 2.0-15 mg/kg, the pharmacokinetics of pertuzumab was linear, and mean clearance ranged from 2.69 to 3.74 mL/day/kg and the mean terminal elimination half-life ranged from 15.3 to 27.6 days. Antibodies to pertuzumab were not detected (Allison et al. Pro Am Soc Clin Oncol 22:197 (2003)).

Sergina et al. report that the biological marker with which to assess the efficacy of HER tyroskine kinase inhibitors (TKIs) should be the transphosphorylation of HER3 rather than the autophosphorylation. Sergina et al. Nature 445(7126): 437-441 (2007).


**Summary of the Invention**

The present invention is based, at least in part, on the unexpected experimental finding that low HER2 mRNA expression levels in a HER2 positive cancer are an indication that a patient with such cancer is likely to respond well to treatment with a
HER inhibitor, especially a HER dimerization inhibitor, such as a HER2 dimerization inhibitor (e.g. pertuzumab), when administered alone or in combination with other anti-cancer agent(s), such as other HER2 antibody or antibodies (e.g. trastuzumab) and/or one or more chemotherapeutic agents. This is true even when the cancer progressed following prior cancer treatment, such as prior treatment with trastuzumab.

In one aspect, the invention concerns a method for treating a patient with a HER2 positive cancer, comprising administering a therapeutically effective amount of a HER dimerization inhibitor to the patient, wherein the patient's cancer expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the same cancer type.

In another aspect, the invention concerns a method for treating a patient with HER2 positive breast cancer comprising administering a therapeutically effective amount of pertuzumab to the patient, wherein the patient's cancer expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in HER2 positive breast cancer.

In yet another aspect, the invention concerns a method for selecting a patient with HER2 expressing cancer for treatment with a HER dimerization inhibitor comprising (i) identifying the patient as HER2 positive by measuring HER2 protein expression or HER2 gene amplification in a cancer sample from the patient, and (ii) measuring HER2 mRNA expression in the cancer sample, wherein the patient is selected for treatment with a HER dimerization inhibitor is the cancer sample expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the same cancer type.

In one embodiment, HER2 protein expression is measured by immunohistochemistry (IHC).

In another embodiment, HER2 gene amplification is measured by fluorescence in situ hybridization (FISH) or chromogenic in situ hybridization (CISH).
In a further embodiment, HER2 protein expression in the tumor is grade 2+ by IHC. In a still further embodiment, HER2 protein expression in the tumor is grade 3+ by IHC.

In a further aspect, the invention concerns an article of manufacture comprising, packaged together, a pharmaceutical composition comprising a HER dimerization inhibitor in a pharmaceutically acceptable carrier and a label stating that the inhibitor or pharmaceutical composition is indicated for treating a patient with a type of cancer which is able to respond to a HER dimerization inhibitor, wherein the patient's cancer is HER2 positive and expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the same cancer type.

In a still further aspect, the invention concerns a method for manufacturing a HER dimerization inhibitor or a pharmaceutical composition thereof comprising combining in a package the inhibitor or pharmaceutical composition and a label stating that the inhibitor or pharmaceutical composition is indicated for treating a patient with a type of cancer which is able to respond to a HER dimerization inhibitor, wherein the patient's cancer is HER2 positive and expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the cancer type.

In another aspect, the invention concerns a method for advertising a HER dimerization inhibitor or a pharmaceutically acceptable composition thereof comprising promoting, to a target audience, the use of the HER dimerization inhibitor or pharmaceutical composition thereof for treating a patient population with a type of cancer, where the patient's cancer is HER2 positive and expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the cancer type.

The invention further concerns an article of manufacture comprising, packaged together, a pharmaceutical composition comprising pertuzumab in a pharmaceutically acceptable carrier and a label stating that the pertuzumab or pharmaceutical composition is indicated for treating a patient with a type of cancer which is able to respond to pertuzumab, wherein the patient's cancer is HER2 positive and expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the cancer type.
The following specific embodiments concern all aspects listed herein or discussed throughout the disclosure:

In one embodiment, the cancer is HER2 positive breast cancer, preferably HER2 positive metastatic breast cancer, more preferably HER2 positive metastatic breast cancer that has progressed during or following other, e.g. standard of care, cancer treatment, such as treatment with trastuzumab or another HER antibody and/or a chemotherapeutic anti-cancer agent.

In another embodiment, the cancer, such as breast cancer, is characterized by overexpression of HER2 at the protein level as determined by immunohistochemistry (IHC) or based on Her2 gene amplification.

In yet another embodiment, the patient's cancer expresses HER2 mRNA at a level which is less than the 33rd percentile for HER2 mRNA expression in the cancer type.

In a further embodiment, the patient's cancer expresses HER2 mRNA at a level which is less than the 25th percentile for HER2 mRNA expression in the cancer type.

In yet another embodiment, HER2 mRNA expression has been determined using polymerase chain reaction (PCR), such as quantitative real time polymerase chain reaction (qRT-PCR).

In a further embodiment, the HER dimerization inhibitor is a HER2 dimerization inhibitor, which may, for example, inhibit HER heterodimerization.

In a particular embodiment, the HER dimerization inhibitor is an antibody, such as an EGFR, HER2, or HER3 antibody.

In another embodiment, the HER2 antibody binds to Domain II of HER2 extracellular domain.

In yet another embodiment, the HER2 antibody binds to a junction between domains I, II and III of HER2 extracellular domain.
In a further embodiment, the HER2 antibody comprises the variable light and variable heavy amino acid sequences in SEQ ID Nos. 11 and 12, respectively.

In a preferred embodiment, the HER2 antibody is pertuzumab.

Antibodies herein include naked, intact antibodies, antibody fragments and antibody conjugates, for example.

In a particular embodiment, the cancer is breast cancer, such as advanced breast cancer which progressed during prior treatment with a chemotherapeutic agents, such as with a HER2 antibody, e.g. trastuzumab.

Efficacy of treatment can be measured using a variety of end points, including objective response (OR), clinical benefit response (CBR), progression free survival (PFS), and overall survival (OS).

The treatment methods according to the present invention may include treatment with one or more additional anti-cancer therapeutic agents, which may, for example, be selected from the group consisting of HER antibody, chemotherapeutic agent, antibody directed against a tumor associated antigen, anti-hormonal compound, cardioprotectant, cytokine, EGFR-targeted drug, anti-angiogenic agent, tyrosine kinase inhibitor, COX inhibitor, non-steroidal anti-inflammatory drug, farnesyl transferase inhibitor, antibody that binds oncofetal protein CA 125, HER2 vaccine, HER targeting therapy, Raf or ras inhibitor, liposomal doxorubicin, topotecan, taxane, dual tyrosine kinase inhibitor, TLK286, EMD-7200, a medicament that treats nausea, a medicament that prevents or treats skin rash or standard acne therapy, a medicament that treats or prevents diarrhea, a body temperature-reducing medicament, and a hematopoietic growth factor.

The additional therapeutic agent may, for example be a HER2 antibody, such as trastuzumab, alone or in combination with one or more anti-cancer agents.
**Brief Description of Drawings**

FIG. 1 provides a schematic of the HER2 protein structure, and amino acid sequences for Domains I-IV (SEQ ID Nos. 1-4, respectively) of the extracellular domain thereof.

FIGS. 2A and 2B depict alignments of the amino acid sequences of the variable light (V_L) (FIG. 2A) and variable heavy (V_H) (FIG. 2B) chain domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 5 and 6, respectively); V_L and V_H domains of variant 574/pertuzumab (SEQ ID Nos. 7 and 8, respectively), and human V_L and V_H consensus frameworks (humKl, light κ subgroup 1; humlll, heavy subgroup III) (SEQ ID Nos. 9 and 10, respectively). Asterisks identify differences between variable domains of pertuzumab and murine monoclonal antibody 2C4 or between variable domains of pertuzumab and the human framework. Complementarity Determining Regions (CDRs) are in brackets.

FIGS. 3A and 3B show the amino acid sequences of pertuzumab light chain (FIG. 3A; SEQ ID No. 11) and heavy chain (FIG. 3B; SEQ ID No. 12). CDRs are shown in bold. Calculated molecular mass of the light chain and heavy chain are 23,526.22 Da and 49,216.56 Da (cysteines in reduced form). The carbohydrate moiety is attached to Asn 299 of the heavy chain.

FIG. 4 depicts, schematically, binding of 2C4 at the heterodimeric binding site of HER2, thereby preventing heterodimerization with activated EGFR or HER3.

FIG. 5 depicts coupling of HER2/HER3 to the MAPK and Akt pathways.

FIGS. 6A and 6B show the amino acid sequences of trastuzumab light chain (FIG. 6A; SEQ ID No. 13) and heavy chain (FIG. 6B; SEQ ID No. 14), respectively.

FIG. 7 shows HER2-CR (qRT-PCR) - Objective Response (OR) and Clinical Benefit Response (CBR), estimated response rates as a function of HER2 biomarker values.

FIG. 8 shows the results of HER2-CR (qRT-PCR) - OR and CBR analysis by HER2 mRNA expression quartiles.
FIG. 9 evaluates progression-free survival (PFS) in breast cancer patients treated with pertuzumab + Herceptin® (trastuzumab) as described in Example 1 based on HER2 mRNA levels determined by qRT-PCR.

FIG. 10 illustrates HER2-CR (qRT-PCR) interactions with hormone receptor status. Kaplan-Meier estimates of PFS in breast cancer patients treated with pertuzumab + Herceptin® (trastuzumab) as described in Example 1 as a function of estrogen receptor and progesterone receptor status, respectively.

FIG. 11 is a scatter plot showing correlations of HER2 vs. HER3 mRNA levels in breast cancer patients treated with pertuzumab + Herceptin® (trastuzumab) as described in Example 1.

**Detailed Description of the Invention**

A. Definitions

A "HER receptor" is a receptor protein tyrosine kinase which belongs to the HER receptor family and includes EGFR, HER2, HER3 and HER4 receptors. The HER receptor will generally comprise an extracellular domain, which may bind an HER ligand and/or dimerize with another HER receptor molecule; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. The HER receptor may be a "native sequence" HER receptor or an "amino acid sequence variant" thereof. Preferably the HER receptor is native sequence human HER receptor.

The terms "ErbBl," "HER1", "epidermal growth factor receptor" and "EGFR" are used interchangeably herein and refer to EGFR as disclosed, for example, in Carpenter et al. Ann. Rev. Biochem. 56:881-914 (1987), including naturally occurring mutant forms thereof (e.g. a deletion mutant EGFR as in Humphrey et al. PNAS (USA) 87:4207-4211 (1990)). erbBl refers to the gene encoding the EGFR protein product.

The expressions "ErbB2" and "HER2" are used interchangeably herein and refer to human HER2 protein described, for example, in Semba et al., PNAS (USA)
The term "erbB2" refers to the gene encoding human ErbB2 and "neu" refers to the gene encoding rat p185.sup.neu. Preferred HER2 is native sequence human HER2.

Herein, "HER2 extracellular domain" or "HER2ECD" refers to a domain of HER2 that is outside of a cell, either anchored to a cell membrane, or in circulation, including fragments thereof. In one embodiment, the extracellular domain of HER2 may comprise four domains: "Domain I" (amino acid residues from about 1-195; SEQ ID NO:1), "Domain II" (amino acid residues from about 196-319; SEQ ID NO:2), "Domain 111" (amino acid residues from about 320-488: SEQ ID NO:3), and "Domain IV" (amino acid residues from about 489-630; SEQ ID NO:4) (residue numbering without signal peptide). See Garrett et al. Mol. Cell. 11:495-505 (2003), Cho et al. Nature 421: 756-760 (2003), Franklin et al. Cancer Cell 5:317-328 (2004), and Plowman et al. Proc. Natl. Acad. Sci. 90:1746-1750 (1993), as well as FIG. 1 herein.

"ErbB3" and "HER3" refer to the receptor polypeptide as disclosed, for example, in U.S. Pat. Nos. 5,183,884 and 5,480,968 as well as Kraus et al. PNAS (USA) 86:9193-9197 (1989).


By "HER ligand" is meant a polypeptide which binds to and/or activates a HER receptor. The HER ligand of particular interest herein is a native sequence human HER ligand such as epidermal growth factor (EGF) (Savage et al., J. Biol. Chem. 247:7612-7621 (1972)); transforming growth factor alpha (TGF-.alpha.) (Marquardt et al, Science 223:1079-1082 (1984)); amphiregulin also known as schwannoma or keratinocyte autocrine growth factor (Shoyab et al. Science 243:1074-1076 (1989); Kimura et al. Nature 348:257-260 (1990); and Cook et al. Mol. Cell. Biol. 11:2547-2557 (1991)); betacellulin (Shing et al, Science 259:1604-1607 (1993);
binding epidermal growth factor (HB-EGF) (Higashiyama et al., Science 251:936-939
(1991)); epieregulin (Toyoda et al., J. Biol. Chem. 270:7495-7500 (1995); and
Komurasaki et al. Oncogene 15:2841-2848 (1997)); a heregulin (see below);
(NRG-4) (Harari et al. Oncogene 18:2681-89 (1999)); and cripto (CR-1) (Kannan et
al. J. Biol. Chem. 272(6):3330-3335 (1997)). HER ligands which bind EGFR include
EGF, TGF-a, amphiregulin, betacellulin, HB-EGF and epieregulin. HER ligands which
bind HER3 include heregulins. HER ligands capable of binding HER4 include

"Heregulin" (HRG) when used herein refers to a polypeptide encoded by the
heregulin gene product as disclosed in U.S. Pat. No. 5,641,869, or Marchionni et al,
Nature, 362:312-318 (1993). Examples of heregulins include heregulin-a, heregulin-
β1, heregulin-p2 and heregulin-p3 (Holmes et al, Science, 256:1205-1210 (1992);
and U.S. Pat. No. 5,641,869); neu differentiation factor (NDF) (Peles et al. Cell 69:
205-216 (1992)); acetylcholine receptor-inducing activity (ARIA) (Falls et al. Cell
72:801-815 (1993)); glial growth factors (GGFs) (Marchionni et al, Nature, 362:312-
318 (1993)); sensory and motor neuron derived factor (SMDF) (Ho et al. J. Biol.
Chem. 270:14523-14532 (1995)); γ- heregulin (Schaefer et al. Oncogene 15:1385-
1394 (1997)).

A "HER dimer" herein is a noncovalently associated dimer comprising at least
two HER receptors. Such complexes may form when a cell expressing two or more
HER receptors is exposed to an HER ligand and can be isolated by
immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski et al.,
J. Biol. Chem., 269(20): 14661-14665 (1994), for example. Other proteins, such as a
cytokine receptor subunit (e.g. gpl30) may be associated with the dimer. Preferably,
the HER dimer comprises HER2.

A "HER heterodimer" herein is a noncovalently associated heterodimer
comprising at least two different HER receptors, such as EGFR-HER2, HER2-HER3
or HER2-HER4 heterodimers.
A "HER inhibitor" is an agent which interferes with HER activation or function. Examples of HER inhibitors include HER antibodies (e.g. EGFR, HER2, HER3, or HER4 antibodies); EGFR-targeted drugs; small molecule HER antagonists; HER tyrosine kinase inhibitors; HER2 and EGFR dual tyrosine kinase inhibitors such as lapatinib/GW572016; antisense molecules (see, for example, WO2004/87207); and/or agents that bind to, or interfere with function of, downstream signaling molecules, such as MAPK or Akt (see FIG. 5). Preferably, the HER inhibitor is an antibody or small molecule which binds to a HER receptor.

A "HER dimerization inhibitor" is an agent which inhibits formation of a HER dimer or HER heterodimer. Preferably the HER dimerization inhibitor is a HER2 dimerization inhibitor and/or inhibits HER heterodimerization. Preferably, the HER dimerization inhibitor is an antibody, for example an antibody which binds to HER2 at the heterodimeric binding site thereof. The most preferred HER dimerization inhibitor herein is pertuzumab or MAb 2C4. Binding of 2C4 to the heterodimeric binding site of HER2 is illustrated in FIG. 4. Other examples of HER dimerization inhibitors include antibodies which bind to EGFR and inhibit dimerization thereof with one or more other HER receptors (for example EGFR monoclonal antibody 806, MAb 806, which binds to activated or "untethered" EGFR; see Johns et al, J. Biol. Chem. 279(29):30375-30384 (2004)); antibodies which bind to HER3 and inhibit dimerization thereof with one or more other HER receptors; antibodies which bind to HER4 and inhibit dimerization thereof with one or more other HER receptors; peptide dimerization inhibitors (U.S. Pat. No. 6,417,168); antisense dimerization inhibitors; etc.

A "HER2 dimerization inhibitor" is an agent that inhibits formation of a dimer or heterodimer comprising HER2.

As defined herein, tumors are considered "HER2 positive" if they are 3+ by immunohistochemistry (IHC) or have a positive fluorescence *in situ* hybridization (FISH) or chromogenic *in situ* hybridization (CISH) result.

A "HER antibody" is an antibody that binds to a HER receptor. Optionally, the HER antibody further interferes with HER activation or function. Preferably, the HER antibody binds to the HER2 receptor. A HER2 antibody of particular interest herein is
pertuzumab. Another example of a HER2 antibody is trastuzumab. Examples of EGFR antibodies include cetuximab and ABX0303.

"HER activation" refers to activation, or phosphorylation, of any one or more HER receptors. Generally, HER activation results in signal transduction (e.g. that caused by an intracellular kinase domain of a HER receptor phosphorylating tyrosine residues in the HER receptor or a substrate polypeptide). HER activation may be mediated by HER ligand binding to a HER dimer comprising the HER receptor of interest. HER ligand binding to a HER dimer may activate a kinase domain of one or more of the HER receptors in the dimer and thereby results in phosphorylation of tyrosine residues in one or more of the HER receptors and/or phosphorylation of tyrosine residues in additional substrate polypeptides(s), such as Akt or MAPK intracellular kinases, see, FIG. 5, for example.

"Phosphorylation" refers to the addition of one or more phosphate group(s) to a protein, such as a HER receptor, or substrate thereof.

An antibody which "inhibits HER dimerization" is an antibody which inhibits, or interferes with, formation of a HER dimer. Preferably, such an antibody binds to HER2 at the heterodimeric binding site thereof. The most preferred dimerization inhibiting antibody herein is pertuzumab or MAb 2C4. Binding of 2C4 to the heterodimeric binding site of HER2 is illustrated in FIG. 4. Other examples of antibodies which inhibit HER dimerization include antibodies which bind to EGFR and inhibit dimerization thereof with one or more other HER receptors (for example EGFR monoclonal antibody 806, MAb 806, which binds to activated or "untethered" EGFR; see Johns et al, J. Biol. Chem. 279(29):30375-30384 (2004)); antibodies which bind to HER3 and inhibit dimerization thereof with one or more other HER receptors; and antibodies which bind to HER4 and inhibit dimerization thereof with one or more other HER receptors.

An antibody which "blocks ligand activation of a HER receptor more effectively than trastuzumab" is one which reduces or eliminates HER ligand activation of HER receptor(s) or HER dimer(s) more effectively (for example at least about 2-fold more effectively) than trastuzumab. Preferably, such an antibody blocks HER ligand activation of a HER receptor at least about as effectively as murine
monoclonal antibody 2C4 or a Fab fragment thereof, or as pertuzumab or a Fab fragment thereof. One can evaluate the ability of an antibody to block ligand activation of a HER receptor by studying HER dimers directly, or by evaluating HER activation, or downstream signaling, which results from HER dimerization, and/or by evaluating the antibody-HER2 binding site, etc. Assays for screening for antibodies with the ability to inhibit ligand activation of a HER receptor more effectively than trastuzumab are described in Agus et al. Cancer Cell 2: 127-137 (2002) and WO01/00245 (Adams et al.). By way of example only, one may assay for: inhibition of HER dimer formation (see, e.g., FIG. 1A-B of Agus et al. Cancer Cell 2: 127-137 (2002); and WO01/00245); reduction in HER ligand activation of cells which express HER dimers (WO01/00245 and FIG. 2A-B of Agus et al. Cancer Cell 2: 127-137 (2002), for example); blocking of HER ligand binding to cells which express HER dimers (WO01/00245, and FIG. 2E of Agus et al. Cancer Cell 2: 127-137 (2002), for example); cell growth inhibition of cancer cells (e.g. MCF7, MDA-MD-134, ZR-75-1, MD-MB-175, T-47D cells) which express HER dimers in the presence (or absence) of HER ligand (WO01/00245 and FIGS. 3A-D of Agus et al. Cancer Cell 2:127-137 (2002), for instance); inhibition of downstream signaling (for instance, inhibition of HRG-dependent AKT phosphorylation or inhibition of HRG- or TGF.alpha.-dependent MAPK phosphorylation) (see, WO01/00245, and FIG. 2C-D of Agus et al. Cancer Cell 2: 127-137 (2002), for example). One may also assess whether the antibody inhibits HER dimerization by studying the antibody-HER2 binding site, for instance, by evaluating a structure or model, such as a crystal structure, of the antibody bound to HER2 (See, for example, Franklin et al. Cancer Cell 5:3 17-328 (2004)).

A "heterodimeric binding site" on HER2, refers to a region in the extracellular domain of HER2 that contacts, or interfaces with, a region in the extracellular domain of EGFR, HER3 or HER4 upon formation of a dimer therewith. The region is found in Domain II of HER2. Franklin et al. Cancer Cell 5:317-328 (2004).

The HER2 antibody may "inhibit HRG-dependent AKT phosphorylation" and/or inhibit "HRG- or TGFα-dependent MAPK phosphorylation" more effectively (for instance at least 2-fold more effectively) than trastuzumab (see Agus et al. Cancer Cell 2: 127-137 (2002) and WO01/00245, by way of example).
The HER2 antibody may be one which, like pertuzumab, does "not inhibit HER2 ectodomain cleavage" (Molina et al. Cancer Res. 61:4744-4749 (2001)). Trastuzumab, on the other hand, can inhibit HER2 ectodomain cleavage.

A HER2 antibody that "binds to a heterodimeric binding site" of HER2, binds to residues in domain II (and optionally also binds to residues in other of the domains of the HER2 extracellular domain, such as domains I and III), and can sterically hinder, at least to some extent, formation of a HER2-EGFR, HER2-HER3, or HER2-HER4 heterodimer. Franklin et al. Cancer Cell 5:317-328 (2004) characterize the HER2-pertuzumab crystal structure, deposited with the RCSB Protein Data Bank (ID Code IS78), illustrating an exemplary antibody that binds to the heterodimeric binding site of HER2.

An antibody that "binds to domain II" of HER2 binds to residues in domain II and optionally residues in other domain(s) of HER2, such as domains I and III. Preferably the antibody that binds to domain II binds to the junction between domains I, II and III of HER2.

Protein "expression" refers to conversion of the information encoded in a gene into messenger RNA (mRNA) and then to the protein. Herein, a sample or cell that "expresses" a protein of interest (such as HER2) is one in which mRNA encoding the protein, or the protein, including fragments thereof, is determined to be present in the sample or cell. It is important, however, the distinguish between mRNA and protein expression levels. Patient response to treatment with a HER inhibitor (e.g., a HER2 inhibitor) is predicted herein based on mRNA (and not protein) expression levels.

A sample, cell, tumor or cancer which "expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression" in a type of cancer is one in which the level of HER2 mRNA expression is considered a "low HER2 mRNA level" to a skilled person for that type of cancer. Generally, such level will be in the range from about 0 to less than about 50%, relative to HER2 mRNA levels in a population of samples, cells, tumors, or cancers of the same cancer type. For instance the population which is used to arrive at the median expression level may be cancer samples generally, or subgroupings thereof. The examples herein, demonstrate how
the median expression level can be determined. This may constitute an absolute value of expression.

The technique of "polymerase chain reaction" or "PCR" as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Pat. No. 4,683,195 issued 28 Jul. 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis et al, Cold Spring Harbor Symp. Quant. Biol. 51: 263 (1987); Erlich, ed., PCR Technology, (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample, comprising the use of a known nucleic acid (DNA or RNA) as a primer and utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

"Quantitative real time polymerase chain reaction" or "qRT-PCR" refers to a form of PCR wherein the amount of PCR product is measured at each step in a PCR reaction. This technique has been described in various publications including Cronin et al, Am. J. Pathol. 164(l):35-42 (2004); and Ma et al, Cancer Cell 5:607-616 (2004).

The term "microarray" refers to an ordered arrangement of hybridizable array elements, preferably polynucleotide probes, on a substrate.

The term "polynucleotide," when used in singular or plural, generally refers to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA
including single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. The term "polynucleotide" specifically includes cDNAs. The term includes DNAs (including cDNAs) and RNAs that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritiated bases, are included within the term "polynucleotides" as defined herein. In general, the term "polynucleotide" embraces all chemically, enzymatically and/or metabolically modified forms of unmodified polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

The term "oligonucleotide" refers to a relatively short polynucleotide, including, without limitation, single-stranded deoxyribonucleotides, single- or double-stranded ribonucleotides, RNA:DNA hybrids and double-stranded DNAs. Oligonucleotides, such as single-stranded DNA probe oligonucleotides, are often synthesized by chemical methods, for example using automated oligonucleotide synthesizers that are commercially available. However, oligonucleotides can be made by a variety of other methods, including in vitro recombinant DNA-mediated techniques and by expression of DNAs in cells and organisms.

The phrase "gene amplification" refers to a process by which multiple copies of a gene or gene fragment are formed in a particular cell or cell line. The duplicated region (a stretch of amplified DNA) is often referred to as "amplicon." Usually, the amount of the messenger RNA (mRNA) produced also increases in the proportion of the number of copies made of the particular gene expressed.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon
probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers, (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, typically: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50 °C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42 °C; or (3) employ 50% formamide, 5 X SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 X Denhardt's solution, sonicated salmon sperm DNA (50 µg/g/ml), 0.1% SDS, and 10% dextran sulfate at 42 °C, with washes at 42 °C in 0.2% SDS, SSC (sodium chloride/sodium citrate) and 50% formamide at 55 °C, followed by a high-stringency wash consisting of 0.1 X SSC containing EDTA at 55 °C.

"Moderately stringent conditions" may be identified as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and % SDS) less stringent that those described above. An example of moderately stringent conditions is overnight incubation at 37 °C in a solution comprising: 20% formamide, 5 X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 X Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 X SSC at about 37-50 °C. The skilled artisan will
recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

A "native sequence" polypeptide is one which has the same amino acid sequence as a polypeptide (e.g., HER receptor or HER ligand) derived from nature, including naturally occurring or allelic variants. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring human polypeptide, murine polypeptide, or polypeptide from any other mammalian species.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different
immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "V_H." The variable domain of the light chain may be referred to as "V_L." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three HVRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The HVRs in each chain are held together in close proximity by the FR regions and, with the HVRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, National Institute of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in the binding of an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.
The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these maybe further divided into subclasses (isotypes), e.g., IgGi, IgG2, IgG3, IgG4, IgAi, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al. Cellular and Mol. Immunology, 4th ed. (W.B. Saunders, Co., 2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain an Fc region.

A "naked antibody" for the purposes herein is an antibody that is not conjugated to a cytotoxic moiety or radiolabel.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen binding region thereof. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.
"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three HVRs of each variable domain interact to define an antigen-binding site on the surface of the V\textsubscript{H}-V\textsubscript{L} dimer. Collectively, the six HVRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three HVRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CHI) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CHI domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')\textsubscript{2} antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the sc Fv to form the desired structure for antigen binding. For a review of scFv, see, e.g., Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds., (Springer-Verlag, New York, 1994), pp. 269-315. scFv fragments herein specifically include "small modular immunopharmaceuticals" (SMIPs) such as disclosed in US2005/0180970A1 and US2005/0186216 A1 assigned to Trubion.

The term "diabodies" refers to antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V\textsubscript{H}) connected to a
light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a
linker that is too short to allow pairing between the two domains on the same chain,
the domains are forced to pair with the complementary domains of another chain and
create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies
are described more fully in, for example, EP 404,097; WO 1993/01 161; Hudson et al,
6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al, Nat.

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 except for possible mutations, e.g.,
naturally occurring mutations, that may be present in minor amounts. Thus, the
modifier "monoclonal" indicates the character of the antibody as not being a mixture
of discrete antibodies. In certain embodiments, such a monoclonal antibody typically
includes an antibody comprising a polypeptide sequence that binds a target, wherein
the target-binding polypeptide sequence was obtained by a process that includes the
selection of a single target binding polypeptide sequence from a plurality of
polypeptide sequences. For example, the selection process can be the selection of a
unique clone from a plurality of clones, such as a pool of hybridoma clones, phage
clones, or recombinant DNA clones. It should be understood that a selected target
binding sequence can be further altered, for example, to improve affinity for the
target, to humanize the target binding sequence, to improve its production in cell
culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc.,
and that an antibody comprising the altered target binding sequence is also a
monoclonal antibody of this invention. 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. In addition to their
specificity, monoclonal antibody preparations are advantageous in that they are
typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being
obtained from a substantially homogeneous population of antibodies, and is not to be

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (see, e.g., U.S. Pat. No. 4,816,567; and Morrison et al, Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984)). Chimeric antibodies include PRIMATIZED® antibodies wherein the antigen-binding region of the antibody is derived from an antibody produced by, e.g., immunizing macaque monkeys with the antigen of interest.
"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a HVR of the recipient are replaced by residues from a HVR of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, FR residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, e.g., Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992). See also, e.g., Vaswani and Hamilton, Ann. Allergy, Asthma & Immunol. 1:105-115 (1998); Harris, Biochem. Soc. Transactions 23:1035-1038 (1995); Hurle and Gross, Curr. Op. Biotech. 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409.

Humanized HER2 antibodies include huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7 and huMAb4D5-8 or trastuzumab (HERCEPTIN®) as described in Table 3 of U.S. Pat. No. 5,821,337 expressly incorporated herein by reference; humanized 520C9 (W093/21319); and humanized 2C4 antibodies such as pertuzumab as described herein.

For the purposes herein, "trastuzumab," "HERCEPTIN®," and "huMAb4D5-8" refer to an antibody comprising the light and heavy chain amino acid sequences shown in FIGs 6A and 6B, SEQ ID Nos. 13 and 14, respectively.
Herein, "pertuzumab" and "OMNITARG™" refer to an antibody comprising the light and heavy chain amino acid sequences shown in FIGs 3A and 3B, SEQ ID Nos. 11 and 12, respectively.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries.


"Framework" or "FR" residues are those variable domain residues other than the HVR residues as herein defined.

The term "variable domain residue numbering as in Kabat" or "amino acid position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al, supra. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by
alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

Throughout the present specification and claims, the Kabat numbering system is generally used when referring to a residue in the variable domain (approximately, residues 1-107 of the light chain and residues 1-113 of the heavy chain) (e.g., Kabat et al., Sequences of Immunological Interest. 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)).

The "EU numbering system" or "EU index" is generally used when referring to a residue in an immunoglobulin heavy chain constant region (e.g., the EU index reported in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991) expressly incorporated herein by reference). Unless stated otherwise herein, references to residues numbers in the variable domain of antibodies means residue numbering by the Kabat numbering system. Unless stated otherwise herein, references to residue numbers in the constant domain of antibodies means residue numbering by the EU numbering system (e.g., see U.S. Provisional Application No. 60/640,323, Figures for EU numbering).

An "affinity matured" antibody is one with one or more alterations in one or more HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). In one embodiment, an affinity matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies may be produced using certain procedures known in the art. For example, Marks et al. Bio/Technology 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling.


Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region)
of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: Clq 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.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue.

Unless indicated otherwise herein, the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat et al., supra. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

A "functional Fc region" possesses an "effector function" of a native sequence Fc region. Exemplary "effector functions" include Clq binding; CDC; Fc receptor binding; ADCC; phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g., an antibody variable domain) and can be assessed using various assays as disclosed, for example, in definitions herein.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes);
native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and
native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

A "variant Fc region" comprises an amino acid sequence which differs from
that of a native sequence Fc region by virtue of at least one amino acid modification,
preferably one or more amino acid substitution(s). Preferably, the variant Fc region
has at least one amino acid substitution compared to a native sequence Fc region or to
the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid
substitutions, and preferably from about one to about five amino acid substitutions in
a native sequence Fc region or in the Fc region of the parent polypeptide. The variant
Fc region herein will preferably possess at least about 80% homology with a native
sequence Fc region and/or with an Fc region of a parent polypeptide, and most
preferably at least about 90% homology therewith, more preferably at least about 95%
homology therewith.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an
antibody. In some embodiments, an FcR is a native human FcR. In some
embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and
includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic
variants and alternatively spliced forms of those receptors. FcγRII receptors include
FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which
have similar amino acid sequences that differ primarily in the cytoplasmic domains
thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based
activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB
contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic
domain. (see, e.g., Daeron, Annu. Rev. Immunol. 15:203-234 (1997)). FcRs are
reviewed, for example, in Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991);
126:330-41 (1995). Other FcRs, including those to be identified in the future, are
encompassed by the term "FcR" herein.

The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn,
which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J.
of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are

Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides with a variant Fc region are administered. WO 2000/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. See also, e.g., Shields et al. J. Biol. Chem. 9(2):6591-6604 (2001).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. In certain embodiments, the cells express at least Fc.gamma.RII and perform ADCC effector function(s). Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells, and neutrophils. The effector cells may be isolated from a native source, e.g., from blood.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. NK cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The primary cells for mediating ADCC, NK cells, express FcyRIII only, whereas monocytes express FcyRI, Fc.gamma.RII, and FcyRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or 5,821,337 or U.S. Pat. No. 6,737,056 (Presta), may be performed. Useful effector cells for such assays include PBMC and 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 et al. PNAS (USA) 95:652-656 (1998).

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway
is initiated by the binding of the first component of the complement system (Clq) to antibodies (of the appropriate subclass), which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g., as described in Gazzano-Santoro et al., J. Immunol. Methods 202:163 (1996), maybe performed. Polypeptide variants with altered Fc region amino acid sequences (polypeptides with a variant Fc region) and increased or decreased Clq binding capability are described, e.g., in U.S. Pat. No. 6,194,551 B1 and WO 1999/51642. See also, e.g., Idusogie et al. J. Immunol. 164: 4178-4184 (2000).

The term "Fc region-comprising antibody" refers to an antibody that comprises an Fc region. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during purification of the antibody or by recombinant engineering of the nucleic acid encoding the antibody. Accordingly, a composition comprising an antibody having an Fc region according to this invention can comprise an antibody with K447, with all K447 removed, or a mixture of antibodies with and without the K447 residue.

The term "main species antibody" herein refers to the antibody structure in a composition which is the quantitatively predominant antibody molecule in the composition. In one embodiment, the main species antibody is a HER2 antibody, such as an antibody that binds to Domain II of HER2, antibody that inhibits HER dimerization more effectively than trastuzumab, and/or an antibody which binds to a heterodimeric binding site of HER2. The preferred embodiment herein of the main species antibody is one comprising the variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4, and most preferably comprising the light chain and heavy chain amino acid sequences in SEQ ID Nos. 11 and 12 (pertuzumab).

An "amino acid sequence variant" antibody herein is an antibody with an amino acid sequence which differs from a main species antibody. Ordinarily, amino acid sequence variants will possess at least about 70% homology with the main species antibody, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with the main species antibody. The amino acid sequence variants possess substitutions, deletions, and/or additions at certain positions within or adjacent to the amino acid sequence of the main species antibody. Examples of amino acid sequence variants herein include an acidic variant (e.g. deamidated
antibody variant), a basic variant, an antibody with an amino-terminal leader extension (e.g. VHS-) on one or two light chains thereof, an antibody with a C-terminal lysine residue on one or two heavy chains thereof, etc, and includes combinations of variations to the amino acid sequences of heavy and/or light chains.

The antibody variant of particular interest herein is the antibody comprising an amino-terminal leader extension on one or two light chains thereof, optionally further comprising other amino acid sequence and/or glycosylation differences relative to the main species antibody.

A "glycosylation variant" antibody herein is an antibody with one or more carbohydrate moieties attached thereto which differ from one or more carbohydrate moieties attached to a main species antibody. Examples of glycosylation variants herein include antibody with a Gl or G2 oligosaccharide structure, instead a GO oligosaccharide structure, attached to an Fc region thereof, antibody with one or two carbohydrate moieties attached to one or two light chains thereof, antibody with no carbohydrate attached to one or two heavy chains of the antibody, etc, and combinations of glycosylation alterations.

Where the antibody has an Fc region, an oligosaccharide structure may be attached to one or two heavy chains of the antibody, e.g. at residue 299 (298, Eu numbering of residues). For pertuzumab, GO was the predominant oligosaccharide structure, with other oligosaccharide structures such as GO-F, G-I, Man5, Man6, Gl-1, Gl(I-6), Gl(I-3) and G2 being found in lesser amounts in the pertuzumab composition.

Unless indicated otherwise, a "Gl oligosaccharide structure" herein includes G-I, Gl-1, Gl(I-6) and Gl(I-3) structures.

An "amino-terminal leader extension" herein refers to one or more amino acid residues of the amino-terminal leader sequence that are present at the amino-terminus of any one or more heavy or light chains of an antibody. An exemplary amino-terminal leader extension comprises or consists of three amino acid residues, VHS, present on one or both light chains of an antibody variant.
A "deamidated" antibody is one in which one or more asparagine residues thereof has been derivitized, e.g. to an aspartic acid, a succinimide, or an iso-aspartic acid.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. A "cancer type" herein refers to a particular category or indication of cancer. Examples of such cancer types include, but are not limited to, carcinoma, lymphoma, blastoma (including medulloblastoma and retinoblastoma), sarcoma (including liposarcoma and synovial cell sarcoma), neuroendocrine tumors (including carcinoid tumors, gastrinoma, and islet cell cancer), mesothelioma, schwannoma (including acoustic neuroma), meningioma, adenocarcinoma, melanoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include breast cancer (including metastatic breast cancer), squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, testicular cancer, esophageal cancer, tumors of the biliary tract, as well as head and neck cancer, as well as subtypes of any of such cancers, including, but not limited to chemotherapy-resistant, platinum-resistant, advanced, refractory, and/or recurrent types thereof.

A "cancer type which is able to respond to a HER inhibitor" is one which when treated with a HER inhibitor, such as a HER2 antibody or small molecule inhibitor, shows a therapeutically effective benefit in the patient therewith according to any of the criteria for therapeutic effectiveness known to the skilled oncologist, including those elaborated herein, but particularly in terms of objective response (OR) or clinical benefit response (CBR), including survival, such as progression free survival (PFS) and/or overall survival (OS). Preferably, such cancer is breast cancer, including metastatic breast cancer (MBC), but other types of cancer, such as ovarian
cancer, peritoneal cancer, fallopian tube cancer, non-small cell lung cancer (NSCLC), prostate cancer, and colorectal cancer are also included herein. Most preferably, the cancer is metastatic breast cancer (MBC).

A "cancer type which is able to respond to a HER dimerization inhibitor" is one which when treated with a HER dimerization inhibitor, such as pertuzumab, shows a therapeutically effective benefit in the patient therewith according to any of the criteria for therapeutic effectiveness known to the skilled oncologist, including those elaborated herein, but particularly in terms of objective response (OR) or clinical benefit response (CBR), including survival, such as progression free survival (PFS) and/or overall survival (OS). Preferably, such cancer is breast cancer, including metastatic breast cancer (MBC), but other types of cancer, such as ovarian cancer, peritoneal cancer, fallopian tube cancer, non-small cell lung cancer (NSCLC), prostate cancer, and colorectal cancer are also included herein. Most preferably, the cancer is metastatic breast cancer (MBC).

In particular, a "cancer type which is able to respond to a HER inhibitor" or a "cancer type which is able to respond to a HER dimerization inhibitor" is a cancer identified as HER-positive according to standard procedures, i.e., immunohistochemistry and/or in situ hybridization. In one preferred embodiment the present invention relates to treatment of patients having HER-positive metastatic breast cancer. Preferably the HER-positive cancer is a HER2-positive cancer. Preferably the HER inhibitor is a HER dimerization inhibitor, especially a HER2 dimerization inhibitor.

An "effective response" and similar wording refers to a response to the HER dimerization inhibitor, HER inhibitor or chemotherapeutic agent that is significantly higher than a response from a patient that does not express HER2 mRNA at the designated level.

An "advanced" cancer is one which has spread outside the site or organ of origin, either by local invasion or metastasis.
A "refractory" cancer is one which progresses even though an anti-tumor agent, such as a chemotherapeutic agent, is being administered to the cancer patient. An example of a refractory cancer is one which is platinum refractory.

A "recurrent" cancer is one which has regrown, either at the initial site or at a distant site, after a response to initial therapy.

Herein, a "patient" is a human patient. The patient may be a "cancer patient," i.e. one who is suffering or at risk for suffering from one or more symptoms of cancer.

A "tumor sample" herein is a sample derived from, or comprising tumor cells from, a patient's tumor. Examples of tumor samples herein include, but are not limited to, tumor biopsies, circulating tumor cells, circulating plasma proteins, ascitic fluid, primary cell cultures or cell lines derived from tumors or exhibiting tumor-like properties, as well as preserved tumor samples, such as formalin-fixed, paraffin-embedded tumor samples or frozen tumor samples.

A "fixed" tumor sample is one which has been histologically preserved using a fixative.

A "formalin-fixed" tumor sample is one which has been preserved using formaldehyde as the fixative.

An "embedded" tumor sample is one surrounded by a firm and generally hard medium such as paraffin, wax, celloidin, or a resin. Embedding makes possible the cutting of thin sections for microscopic examination or for generation of tissue microarrays (TMAs).

A "paraffin-embedded" tumor sample is one surrounded by a purified mixture of solid hydrocarbons derived from petroleum.

Herein, a "frozen" tumor sample refers to a tumor sample which is, or has been, frozen.

A cancer or biological sample which "displays HER expression, amplification, or activation" is one which, in a diagnostic test, expresses (including overexpresses) a
HER receptor, has amplified HER gene, and/or otherwise demonstrates activation or phosphorylation of a HER receptor.

A cancer cell with "HER receptor overexpression or amplification" is one which has significantly higher levels of a HER receptor protein or gene compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. HER receptor overexpression may be determined in a diagnostic or prognostic assay by evaluating increased levels of the HER protein present on the surface of a cell (e.g. via an immunohistochemistry assay; IHC). Gene amplification may be measured, for example, by fluorescent in situ hybridization (FISH; see W098/45479 published October, 1998). One may also study HER receptor overexpression or amplification by measuring shed antigen (e.g., HER extracellular domain) in a biological fluid such as serum (see, e.g., U.S. Pat. No. 4,933,294 issued Jun. 12, 1990; WO91/05264 published Apr. 18, 1991; U.S. Pat. No. 5,401,638 issued Mar. 28, 1995; and Sias et al. J. Immunol. Methods 132: 73-80 (1990)). Aside from the above assays, various in vivo assays are available to the skilled practitioner. For example, one may expose cells within the body of the patient to an antibody which is optionally labeled with a detectable label, e.g. a radioactive isotope, and binding of the antibody to cells in the patient can be evaluated, e.g. by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

Conversely, a cancer which "does not overexpress or amplify HER receptor" is one which does not have higher than normal levels of HER receptor protein or gene amplification compared to a noncancerous cell of the same tissue type.

Herein, an "anti-tumor agent" refers to a drug used to treat cancer. Non-limiting examples of anti-tumor agents herein include chemotherapeutic agents, HER inhibitors, HER dimerization inhibitors, HER antibodies, antibodies directed against tumor associated antigens, anti-hormonal compounds, cytokines, EGFR-targeted drugs, anti-angiogenic agents, tyrosine kinase inhibitors, growth inhibitory agents and antibodies, cytotoxic agents, antibodies that induce apoptosis, COX inhibitors, farnesyl transferase inhibitors, antibodies that binds oncofetal protein CA 125, HER2 vaccines, Raf or ras inhibitors, liposomal doxorubicin, topotecan, taxane, dual
tyrosine kinase inhibitors, TLK286, EMD-7200, pertuzumab, trastuzumab, erlotinib, and bevacizumab.

An "approved anti-tumor agent" is a drug used to treat cancer which has been accorded marketing approval by a regulatory authority such as the Food and Drug Administration (FDA) or foreign equivalent thereof.

Where a HER inhibitor or HER dimerization inhibitor is administered as a "single anti-tumor agent" it is the only anti-tumor agent administered to treat the cancer, i.e. it is not administered in combination with another anti-tumor agent, such as chemotherapy.

By "standard of care" herein is intended the anti-tumor agent or agents that are routinely used to treat a particular form of cancer.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially a HER expressing cancer cell either in vitro or in vivo. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of HER expressing cells in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin.

Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazime, mechlorethamine, cisplatin, methotrexate, 5-fluouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13.

Examples of "growth inhibitory" antibodies are those which bind to HER2 and inhibit the growth of cancer cells overexpressing HER2. Preferred growth inhibitory HER2 antibodies inhibit growth of SK-BR-3 breast tumor cells in cell culture by greater than 20%, and preferably greater than 50% (e.g. from about 50% to about 100%) at an antibody concentration of about 0.5 to 30 μg/ml, where the growth
inhibition is determined six days after exposure of the SK-BR-3 cells to the antibody (see U.S. Pat. No. 5,677,171 issued Oct. 14, 1997). The SK-BR-3 cell growth inhibition assay is described in more detail in that patent and hereinbelow. The preferred growth inhibitory antibody is a humanized variant of murine monoclonal antibody 4D5, e.g., trastuzumab.

An antibody which "induces apoptosis" is one which induces programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies). The cell is usually one which overexpresses the HER2 receptor. Preferably the cell is a tumor cell, e.g. a breast, ovarian, stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. In vitro, the cell maybe a SK-BR-3, BT474, Calu 3 cell, MDA-MB-453, MDA-MB-361 or SKOV3 cell. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold, induction of annexin binding relative to untreated cell in an annexin binding assay using BT474 cells (see below). Examples of HER2 antibodies that induce apoptosis are 7C2 and 7F3.

The "epitope 2C4" is the region in the extracellular domain of HER2 to which the antibody 2C4 binds. In order to screen for antibodies which bind to the 2C4 epitope, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Preferably the antibody blocks 2C4's binding to HER2 by about 50% or more. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 2C4 epitope of HER2. Epitope 2C4 comprises residues from Domain II in the extracellular domain of HER2. 2C4 and pertuzumab binds to the extracellular domain of HER2 at the junction of domains I, II and III. Franklin et al. Cancer Cell 5:317-328 (2004).
The "epitope 4D5" is the region in the extracellular domain of HER2 to which the antibody 4D5 (ATCC CRL 10463) and trastuzumab bind. This epitope is close to the transmembrane domain of HER2, and within Domain IV of HER2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of HER2 (e.g. any one or more residues in the region from about residue 529 to about residue 625, inclusive of the HER2 ECD, residue numbering including signal peptide).

The "epitope 7C2/7F3" is the region at the N terminus, within Domain 1, of the extracellular domain of HER2 to which the 7C2 and/or 7F3 antibodies (each deposited with the ATCC, see below) bind. To screen for antibodies which bind to the 7C2/7F3 epitope, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on HER2 (e.g. any one or more of residues in the region from about residue 22 to about residue 53 of the HER2 ECD, residue numbering including signal peptide).

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with cancer as well as those in which cancer is to be prevented. Hence, the patient to be treated herein may have been diagnosed as having cancer or may be predisposed or susceptible to cancer.

The terms "therapeutically effective amount" or "effective amount" refer to an amount of a drug effective to treat cancer in the patient. The effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. The effective amount may extend progression free survival (e.g. as measured by Response
Evaluation Criteria for Solid Tumors, RECIST, or CA-125 changes, result in an objective response (including a partial response, PR, or complete response, CR), results in a clinical benefit response (CBR), improve survival (including overall survival and progression-free survival) and/or improve one or more symptoms of cancer (e.g. as assessed by FOSI). Most preferably, the therapeutically effective amount of the drug is effective to improve progression-free survival (PFS) and/or overall survival (OS).

"Survival" refers to the patient remaining alive, and includes overall survival as well as progression free survival.

By "extending survival" is meant increasing overall or progression free survival in a treated patient relative to an untreated patient (i.e. relative to a patient not treated with a HER inhibitor, HER dimerization inhibitor, such as pertuzumab), or relative to a patient who does not express HER2 mRNA at the designated level, and/or relative to a patient treated with an approved anti-tumor agent (such as topotecan or liposomal doxorubicin, where the cancer is breast cancer, such as metastatic breast cancer).

An "objective response" (OR) refers to a measurable response, including complete response (CR) and partial response (PR). Objective response by tumor measurement according to RECIST criteria has occurred if there is documented and confirmed complete response (CR) or partial response (PR).

A "clinical benefit response" (CBR) includes patients who have met the criteria for objective response at any time and for any duration (at least 4 weeks) and patients whose best response was stable disease (defined according to RECIST criteria) that lasts at least 6 months (or 8 cycles of therapy).

By "complete response" or "CR" is intended to refer to the disappearance of all signs of cancer in response to treatment. This does not always mean the cancer has been cured.

"Partial response" or "PR" refers to a decrease in the size of one or more tumors or lesions, or in the extent of cancer in the body, in response to treatment.
"Duration of response" is defined for patients with a best overall response of CR or PR. Response lasts from the date that either the complete response or partial response (whichever is earlier if the partial response precedes a complete response) was first recorded to the date on which progressive disease was first noted or date of death. Duration of complete response lasts from the date of complete response to the date of the first observation of progressive disease or date of death. Patients with no documented progression after CR or PR or who have not died are censored at the last date at which there is valid documentation of response (i.e. tumor measurement recorded on the eCRF).

"Time to response" is defined for all patients as the time from the date of first dose of study medication to the date of first CD or PR, which will be the date the response is first radiographically documented. Patients who never respond will be censored at the last valid tumor measurement. Time to response may be summarized using Kaplan-Meier methodology.

"Time to progression" is defined as the interval between the day of first dose of medication and the first documentation of progressive disease. In the context of the clinical trial described in Example 1, patients who are withdrawn from the study (or die) without documented progression are censored at the date of the last tumor assessment when the patient was known to be progression-free. Patients without baseline tumor assessments are censored on study day 1. Patients who withdraw due to insufficient therapeutic response without documentation of progression are considered to have met the end-point.

"Progression-free survival" (PFS) refers to the patient remaining alive, without the cancer progressing or getting worse. In the context of the clinical trial described in Example 1, progression-free survival (PFS) is defined as the time from date of first dose of study medication to documented disease progression or death at any time, regardless of the cause. Patients who do not progress or die while on study or during follow-up are censored at the last valid tumor assessment. Patients who withdraw due to insufficient therapeutic response without documentation of progression are considered to have met the end-point. Patients with no post-baseline tumor measurements but known to be alive are censored on study day 1.
"Overall survival" refers to the patient remaining alive for a defined period of time, such as 1 year, 3 years, 5 years, etc from the time of diagnosis or treatment. For assessing the results of the clinical trial described in Example 1, for example, overall survival at 3 years is deemed to provide a clinically relevant and mature result, providing sufficient data. The analysis may be carried out at alternative time points depending on the quality of the data collected.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At$^{211}$, I$^{131}$, I$^{125}$, Y$^{90}$, Re$^{188}$, Re$^{188}$, Sm$^{153}$, Bi$^{22}$, P$^{32}$ and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXAN®); alkyl sulfonates such as busulfan, imposulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne
antibiotics (e.g., calicheamicin, especially calichearnicin gammall and calicheamicin omegall (see, e.g., Nicolaou et al, Angew. Chem. Intl. Ed. Engl, 33: 183-186 (1994)); CDP323, an oral alpha-4 integrin inhibitor; dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection (DOXIL®), liposomal doxorubicin TLC D-99 (MYOCET®), peglylated liposomal doxorubicin (CAELYX®), and deoxydoxorubicin), esorubicin, darubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelarnycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziqoune; elfonithine; elliptinium acetate; etogluclid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; m菩提nanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; P5K® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sизofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotritylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; dacarbazine; mannoucaine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoid, e.g., paclitaxel (TAXOL®), albumin-engineered nanoparticle formulation of paclitaxel (ABRAXANE™), and docetaxel (TAXOTERE®); chloranbucil; 6-thioguanine;
mercaptopurine; methotrexate; platinum agents such as cisplatin, oxaliplatin, and carboplatin; vincas, which prevent tubulin polymerization from forming microtubules, including vinblastine (VELBAN®), vincristine (ONCOVIN®), vindesine (ELDISINE® & FILDESIN®), and vinorelbine (NAVELBINE®); etoposide (VP-16); ifosfamide; mitoxantrone; leucovorin; novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoids such as retinoic acid, including bexarotene (TARGRETIN®); bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), etidronate (DIDROCAL®), NE-58095, zoledronic acid/zoledronate (ZOMETA®), alendronate (FOSAMAX®), pamidronate (AREDIA®), tiludronate (SKELID®), or risedronate (ACTONEL®); troxacinabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVESTIN® vaccine, LEUVESTIN® vaccine, and VAX ID® vaccine; topoisomerase 1 inhibitor (e.g., LURTOTECAN®); rmRH (e.g., ABARELIX®); BAY439006 (sorafenib; Bayer); SU-1 1248 (Pfizer); perifosine, COX-2 inhibitor (e.g. celecoxib or etoricoxib), proteosome inhibitor (e.g. PS341); bortezomib (VELCADE®); CCI-779; tipifamib (RI 1577); orafenib, ABT5 10; Bcl-2 inhibitor such as oblimersen sodium (GENASENSE®); pixantrone; EGFR inhibitors (see definition below); tyrosine kinase inhibitors (see definition below); and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovorin.

Herein, chemotherapeutic agents include "anti-hormonal agents" or "endocrine therapeutics" which act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer. They may be hormones themselves, including, but not limited to: anti-estrogens with mixed agonist/antagonist profile, including, tamoxifen (NOLVADEX®), 4-hydroxytamoxifen, toremifene (FARESTON®), idoxifene, droloxifene, raloxifene (EVISTA®), tricofene, keoxifene, and selective
estrogen receptor modulators (SERMs) such as SERM3; pure anti-estrogens without agonist properties, such as fulvestrant (FASLODEX®), and EM800 (such agents may block estrogen receptor (ER) dimerization, inhibit DNA binding, increase ER turnover, and/or suppress ER levels); aromatase inhibitors, including steroidal aromatase inhibitors such as formestane and exemestane (AROMASIN®), and nonsteroidal aromatase inhibitors such as anastrazole (ARIMIDEX®), letrozole (FEMARA®) and aminoglutethimide, and other aromatase inhibitors include vorozole (RIVISOR®), megestrol acetate (MEGASE®), fadrozole, and 4(5)-imidazoles; lutenizing hormone-releasing hormone agonists, including leuprolide (LUPRON® and ELIGARD®), goserelin, buserelin, and tripterelin; sex steroids, including progestines such as megestrol acetate and medroxyprogesterone acetate, estrogens such as diethylstilbestrol and premarin, and androgens-retinoids such as fluoxymesterone, all transretionic acid and fenretinide; onapristone; anti-progesterones; estrogen receptor down-regulators (ERDs); anti-androgens such as flutamide, nilutamide and bicalutamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above.

An "antimetabolite chemotherapeutic agent" is an agent which is structurally similar to a metabolite, but can not be used by the body in a productive manner. Many antimetabolite chemotherapeutic agents interfere with the production of the nucleic acids, RNA and DNA. Examples of antimetabolite chemotherapeutic agents include gemcitabine (GEMZAR®), 5-fluorouracil (5-FU), capecitabine (XELODA™), 6-mercaptopurine, methotrexate, 6-thioguanine, pemtrexed, raltitrexed, arabinosylcytosine ARA-C cytarabine (CYTOSAR-U®), dacarbazine (DTIC-DOME®), azacytosine, deoxyctosine, pyrimidene, fludarabine (FLUDARA®), cladarabine, 2-deoxy-D-glucose etc. The preferred antimetabolite chemotherapeutic agent is gemcitabine.

"Gemcitabine" or "2'-deoxy-2',2'-difluorocytidine monohydrochloride (b-isomer)" is a nucleoside analogue that exhibits antitumor activity. The empirical formula for gemcitabine HC1 is C₉H₁₁F₂N₃O₄ HCl. Gemcitabine HC1 is sold by Eli Lilly under the trademark GEMZAR.
A "platinum-based chemotherapeutic agent" comprises an organic compound which contains platinum as an integral part of the molecule. Examples of platinum-based chemotherapeutic agents include carboplatin, cisplatin, and oxaliplatinum.

By "platinum-based chemotherapy" is intended therapy with one or more platinum-based chemotherapeutic agents, optionally in combination with one or more other chemotherapeutic agents.

By "chemotherapy-resistant" cancer is meant that the cancer patient has progressed while receiving a chemotherapy regimen (i.e. the patient is "chemotherapy refractory"), or the patient has progressed within 12 months (for instance, within 6 months) after completing a chemotherapy regimen.

By "platinum-resistant" cancer is meant that the cancer patient has progressed while receiving platinum-based chemotherapy (i.e. the patient is "platinum refractory"), or the patient has progressed within 12 months (for instance, within 6 months) after completing a platinum-based chemotherapy regimen.

An "anti-angiogenic agent" refers to a compound which blocks, or interferes with to some degree, the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or antibody that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. The preferred anti-angiogenic factor herein is an antibody that binds to vascular endothelial growth factor (VEGF), such as bevacizumab (AVASTIN®).

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-a and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth
factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-P; platelet-growth factor; transforming growth factors (TGFs) such as TGF-a and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

As used herein, the term "EGFR-targeted drug” refers to a therapeutic agent that binds to EGFR and, optionally, inhibits EGFR activation. Examples of such agents include antibodies and small molecules that bind to EGFR. Examples of antibodies which bind to EGFR include MAb 579 (ATCC CRL HB 8506), MAb 455 (ATCC CRL HB8507), MAb 225 (ATCC CRL 8508), MAb 528 (ATCC CRL 8509) (see, U.S. Pat. No. 4,943,533, Mendelsohn et al.) and variants thereof, such as chimerized 225 (C225 or Cetuximab; ERBUTIX®) and reshaped human 225 (H225) (see, WO 96/40210, Imclone Systems Inc.); IMC-11F8, a fully human, EGFR-targeted antibody (Imclone); antibodies that bind type II mutant EGFR (U.S. Pat. No. 5,212,290); humanized and chimeric antibodies that bind EGFR as described in U.S. Pat. No. 5,891,996; and human antibodies that bind EGFR, such as ABX-EGF (see WO98/50433, Abgenix); EMD 55900 (Stragliotto et al. Eur. J. Cancer 32A:636-640 (1996)); EMD7200 (matuzumab) a humanized EGFR antibody directed against EGFR that competes with both EGF and TGF-alpha for EGFR binding; and mAb 806 or humanized mAb 806 (Johns et al, J. Biol. Chem. 279(29):30375-30384 (2004)). The anti-EGFR antibody may be conjugated with a cytotoxic agent, thus generating an immunoconjugate (see, e.g., EP659,439A2, Merck Patent GmbH). Examples of small molecules that bind to EGFR include ZD1839 or Gefitinib (IRESSA™; Astra Zeneca); CP-358774 or Erlotinib (TARCEVA™; Genentech/OSI); and AG1478, AG1571 (SU 5271; Sugen); EMD-7200.
A "tyrosine kinase inhibitor" is a molecule which inhibits tyrosine kinase activity of a tyrosine kinase such as a HER receptor. Examples of such inhibitors include the EGFR-targeted drugs noted in the preceding paragraph; small molecule HER2 tyrosine kinase inhibitor such as TAK165 available from Takeda; CP-724,714, an oral selective inhibitor of the ErbB2 receptor tyrosine kinase (Pfizer and OSI); dual-HER inhibitors such as EKB-569 (available from Wyeth) which preferentially binds EGFR but inhibits both HER2 and EGFR-overexpressing cells; GW572016 (available from Glaxo) an oral HER2 and EGFR tyrosine kinase inhibitor; PKI-166 (available from Novartis); pan-HER inhibitors such as canertinib (CI-1033; Pharmacia); Raf-1 inhibitors such as antisense agent ISIS-5132 available from ISIS Pharmaceuticals which inhibits Raf-1 signaling; non-HER targeted TK inhibitors such as Imatinib mesylate (Gleevec™) available from Glaxo; MAPK extracellular regulated kinase 1 inhibitor CI-1040 (available from Pharmacia); quinazolines, such as PD 153035, 4-(3-chloroanilino) quinazoline; pyridopyrimidines; pyrimidopyrimidines; pyrrolopyrimidines, such as CGP 59326, CGP 60261 and CGP 62706; pyrazolopyrimidines, 4-(phenylamino)-7H-pyrrolo[2,3-d]pyrimidines; curcumin (diferuloyl methane, 4,5-bis(4-fluoroanilino)phthalimide); tyrphostines containing nitrothiophene moieties; PD-0183805 (Warner-Lamber); antisense molecules (e.g. those that bind to HER-encoding nucleic acid); quinoxalines (U.S. Pat. No. 5,804,396); tyrphostins (U.S. Pat. No. 5,804,396); ZD6474 (Astra Zeneca); PTK-787 (Novartis/Schering AG); pan-HER inhibitors such as CI-1033 (Pfizer); Affmitac (ISIS 3521; Isis/Lilly); Imatinib mesylate (Gleevec; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); CI-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Sugen); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-1C1 1 (Imclone); or as described in any of the following patent publications: U.S. Pat. No. 5,804,396; WO99/09016 (American Cyanimid); WO98/43960 (American Cyanamid); WO97/38983 (Warner Lambert); WO99/06378 (Warner Lambert); WO99/06396 (Warner Lambert); WO96/30347 (Pfizer, Inc); WO96/33978 (Zeneca); WO96/3397 (Zeneca); and WO96/33980 (Zeneca).
A "loading" dose herein generally comprises an initial dose of a therapeutic agent administered to a patient, and is followed by one or more maintenance dose(s) thereof. Generally, a single loading dose is administered, but multiple loading doses are contemplated herein. Usually, the amount of loading dose(s) administered exceeds the amount of the maintenance dose(s) administered and/or the loading dose(s) are administered more frequently than the maintenance dose(s), so as to achieve the desired steady-state concentration of the therapeutic agent earlier than can be achieved with the maintenance dose(s).

A "maintenance" dose herein refers to one or more doses of a therapeutic agent administered to the patient over a treatment period. Usually, the maintenance doses are administered at spaced treatment intervals, such as approximately every week, approximately every 2 weeks, approximately every 3 weeks, or approximately every 4 weeks.

A "medicament" is an active drug to treat cancer, such as a HER inhibitor, a HER dimerization inhibitor (such as pertuzumab) or a chemotherapeutic agent (such as gemcitabine).

A "target audience" is a group of people or an institution to whom or to which a particular medicament is being promoted or intended to be promoted, as by marketing or advertising, especially for particular uses, treatments, or indications, such as individual patients, patient populations, readers of newspapers, medical literature, and magazines, television or internet viewers, radio or internet listeners, physicians, drug companies, etc.

A "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, contraindications, other therapeutic products to be combined with the packaged product, and/or warnings concerning the use of such therapeutic products, etc.
B. General Description of the Invention

I. Production of Antibodies

Since, in the preferred embodiment, the HER inhibitor is an antibody, a description follows as to exemplary techniques for the production of HER antibodies used in accordance with the present invention. The HER antigen to be used for production of antibodies may be, e.g., a soluble form of the extracellular domain of a HER receptor or a portion thereof, containing the desired epitope. Alternatively, cells expressing HER at their cell surface (e.g. NIH-3T3 cells transformed to overexpress HER2; or a carcinoma cell line such as SK-BR-3 cells, see Stancovski et al. PNAS (USA) 88:8691-8695 (1991)) can be used to generate antibodies. Other forms of HER receptor useful for generating antibodies will be apparent to those skilled in the art.

(i) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl₂, or RiN=C=NR, where R and Ri are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the
same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal Antibodies

Various methods for making monoclonal antibodies herein are available in the art. For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al, Nature, 256:495 (1975), by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human
heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); and Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., Anal. Biochem., 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies: Principles and Practice, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional antibody purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is 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 murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as E. coli cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce antibody protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on


The DNA also may be modified, for example, by substituting the coding sequence for human heavy chain and light chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; and Morrison, et al, Proc. Natl. Acad. Sci. USA, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) **Humanized Antibodies**

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import"
variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeven et al., Science, 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., J. Immunol, 151:2296 (1993); Chothia et al, J. Mol. Biol, 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al, J. Immunol, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e.,
the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

WOI/00245 describes production of exemplary humanized HER2 antibodies which bind HER2 and block ligand activation of a HER receptor. The humanized antibody of particular interest herein blocks EGF, TGF-a and/or HRG mediated activation of MAPK essentially as effectively as murine monoclonal antibody 2C4 (or a Fab fragment thereof) and/or binds HER2 essentially as effectively as murine monoclonal antibody 2C4 (or a Fab fragment thereof). The humanized antibody herein may, for example, comprise nonhuman hypervariable region residues incorporated into a human variable heavy domain and may further comprise a framework region (FR) substitution at a position selected from the group consisting of 69H, 71H and 73H utilizing the variable domain numbering system set forth in Kabat et al. Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). In one embodiment, the humanized antibody comprises FR substitutions at two or all of positions 69H, 71H and 73H.

An exemplary humanized antibody of interest herein comprises variable heavy domain complementarity determining residues GFTFTDYTMX, where X is preferably D or S (SEQ ID NO: 15); DVNPNSGSIYNQR FKG (SEQ ID NO: 16); and/or NLGPSFYFDY (SEQ ID NO: 17), optionally comprising amino acid modifications of those CDR residues, e.g. where the modifications essentially maintain or improve affinity of the antibody. For example, the antibody variant of interest may have from about one to about seven or about five amino acid substitutions in the above variable heavy CDR sequences. Such antibody variants may be prepared by affinity maturation, e.g., as described below. The most preferred humanized antibody comprises the variable heavy chain amino acid sequence in SEQ ID NO: 12, or one or more of CDR1, CDR2 and CDR3 from that sequence.
The humanized antibody may comprise variable light domain complementarity determining residues KASQDV SIGVA (SEQ ID NO: 18); SASYX^X \_3, where X^1 is preferably R or L, X^2 is preferably Y or E, and X^3 is preferably T or S (SEQ ID NO: 19); and/or QQYYIYPYT (SEQ ID NO:20), e.g. in addition to those variable heavy domain CDR residues in the preceding paragraph. Such humanized antibodies optionally comprise amino acid modifications of the above CDR residues, e.g. where the modifications essentially maintain or improve affinity of the antibody. For example, the antibody variant of interest may have from about one to about seven or about five amino acid substitutions in the above variable light CDR sequences. Such antibody variants may be prepared by affinity maturation, e.g., as described below. The most preferred humanized antibody comprises the variable light domain amino acid sequence in SEQ ID NO:1 1, or one or more of CDR1, CDR2 and CDR3 from that sequence.

The present application also contemplates affinity matured antibodies which bind HER2 and block ligand activation of a HER receptor. The parent antibody may be a human antibody or a humanized antibody, e.g., one comprising the variable light and/or variable heavy sequences of SEQ ID Nos. 11 and 12, respectively (i.e. comprising the VL and/or VH of pertuzumab). The affinity matured antibody preferably binds to HER2 receptor with an affinity superior to that of murine 2C4 or pertuzumab (e.g. from about two or about four fold, to about 100 fold or about 1000 fold improved affinity, e.g. as assessed using a HER2-extracellular domain (ECD) ELISA). Exemplary variable heavy CDR residues for substitution include H28, H30, H34, H35, H64, H96, H99, or combinations of two or more (e.g. two, three, four, five, six, or seven of these residues). Examples of variable light CDR residues for alteration include L28, L50, L53, L56, L91, L92, L93, L94, L96, L97 or combinations of two or more (e.g. two to three, four, five or up to about ten of these residues).

Various forms of the humanized antibody or affinity matured antibody are contemplated. For example, the humanized antibody or affinity matured antibody may be an antibody fragment, such as a Fab, which is optionally conjugated with one or more cytotoxic agent(s) in order to generate an immunoconjugate. Alternatively, the humanized antibody or affinity matured antibody may be an intact antibody, such as
an intact IgGl antibody. The preferred intact IgGl antibody comprises the light chain sequence in SEQ ID NO: 11 and the heavy chain sequence in SEQ ID NO: 12.

(iv) Human Antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J.sub.H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al, Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al, Nature, 362:255-258 (1993); Bruggermann et al, Year in Immuno., 7:33 (1993); and U.S. Pat. Nos. 5,591,669, 5,589,369 and 5,545,807. Alternatively, phage display technology (McCafferty et al, Nature 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology 3:564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al, Nature, 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques

As discussed above, human antibodies may also be generated by in vitro activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).


(v) Antibody Fragments

Various techniques have been developed for the production of antibody fragments comprising one or more antigen binding regions. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992); and Brennan et al, Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form F(ab')\(_2\) fragments (Carter et al, Bio/Technology 10:163-167 (1992)). According to another approach, F(ab')\(_2\) fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific Antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the HER2 protein. Other such antibodies may combine a HER2 binding site with binding site(s) for EGFR, HER3 and/or HER4. Alternatively, a HER2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte.
such as a T-cell receptor molecule (e.g. CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the HER2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express HER2. These antibodies possess a HER2-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab′)₂ bispecific antibodies).


Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al, Nature, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al, EMBO J., 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CHI) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments
when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

According to another approach described in U.S. Pat. No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C.sub.H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking
agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage.

Brennan et al., Science, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')₂ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from E. coli, which can be chemically coupled to form bispecific antibodies. Shalaby et al., J. Exp. Med., 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')₂ molecule. Each Fab' fragment was separately secreted from E. coli and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al, J. Immunol, 148(5): 1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al, Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993) has
provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (\(V_H\)) connected to a light-chain variable domain (\(V_L\)) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the \(V_H\) and \(V_L\) domains of one fragment are forced to pair with the complementary \(V_L\) and \(V_H\) domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., J. Immunol, 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. J. Immunol. 147: 60 (1991).

(vii) Other Amino Acid Sequence Modifications

Amino acid sequence modification(s) of the antibodies described 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 the antibody are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, 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 is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells Science, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be
predetermined. For example, to analyze the performance of a mutation at a given site, 
ala scanning or random mutagenesis is conducted at the target codon or region and the 
expressed antibody variants are screened for the desired activity.

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 antibody with an N-terminal 
methionyl residue or the antibody fused to a cytotoxic polypeptide. 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.

Another type of variant is an amino acid substitution variant. These variants 
have at least one amino acid residue in the antibody molecule replaced by a different 
residue. The sites of greatest interest for substitutional mutagenesis include the 
hypervariable regions, but FR alterations are also contemplated. Conservative 
substitutions are shown in Table 1 under the heading of "preferred substitutions". If 
such substitutions result in a change in biological activity, then more substantial 
changes, denominated "exemplary substitutions" in Table 1, or as further described 
below in reference to amino acid classes, may be introduced and the products 
screened.
TABLE 1

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

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Amino acids may be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)):
(1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M)

(2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gin (Q)

(3) acidic: Asp (D), Glu (E)

(4) basic: Lys (K), Arg (R), His (H)

Alternatively, naturally occurring residues may be divided into groups based on common side-chain properties:

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

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

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

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

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(6) aromatic: Trp, Tyr, Phe.

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

Any cysteine residue not involved in maintaining the proper conformation of the antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

A particularly preferred 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 development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within
each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and human HER2. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antibody alters the original glycosylation pattern of the antibody. By altering is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that
lacks fucose attached to a Fc region of the antibody are described in US Pat Appl No US 2003/0157108 Al, Presta, L. See also US 2004/0093621 Al (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetyl glucosamine (GlcNAc) in the carbohydrate attached to an Fc region of the antibody are referenced in WO03/01 1878, Jean-Mairet et al. and U.S. Pat. No. 6,602,684, Umana et al.

Antibodies with at least one galactose residue in the oligosaccharide attached to an Fc region of the antibody are reported in WO97/30087, Patel et al. See, also, WO98/58964 (Raju, S.) and W099/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof.

It may be desirable to modify the antibody of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron et al, J. Exp Med. 176:1 191-1 195 (1992) and Shopes, B. J. Immunol. 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional crosslinkers as described in Wolff et al. Cancer Research 53:2560-2565 (1993).

Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson et al. Anti-Cancer Drug Design 3:219-230 (1989).

WO00/42072 (Presta, L.) describes antibodies with improved ADCC function in the presence of human effector cells, where the antibodies comprise amino acid substitutions in the Fc region thereof. Preferably, the antibody with improved ADCC comprises substitutions at positions 298, 333, and/or 334 of the Fc region (Eu numbering of residues). Preferably the altered Fc region is a human IgG1 Fc region comprising or consisting of substitutions at one, two or three of these positions. Such substitutions are optionally combined with substitution(s) which increase Clq binding and/or CDC.
Antibodies with altered Clq binding and/or complement dependent cytotoxicity (CDC) are described in WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.). The antibodies comprise an amino acid substitution at one or more of amino acid positions 270, 322, 326, 327, 329, 313, 333 and/or 334 of the Fc region thereof (Eu numbering of residues).

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Pat. No. 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG1, IgG2, IgG3, or IgG4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

Antibodies with improved binding to the neonatal Fc receptor (FcRn), and increased half-lives, are described in WO00/42072 (Presta, L.) and US2005/0014934A1 (Hinton et al.). These antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. For example, the Fc region may have substitutions at one or more of positions 238, 250, 256, 265, 272, 286, 303, 305, 307, 311, 312, 314, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424, 428 or 434 (Eu numbering of residues). The preferred Fc region-comprising antibody variant with improved FcRn binding comprises amino acid substitutions at one, two or three of positions 307, 380 and 434 of the Fc region thereof (Eu numbering of residues).

Engineered antibodies with three or more (preferably four) functional antigen binding sites are also contemplated (US Appln No. US2002/0004587 Al, Miller et al).

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.
(viii) Screening for Antibodies with the Desired Properties

Techniques for generating antibodies have been described above. One may further select antibodies with certain biological characteristics, as desired.

To identify an antibody which blocks ligand activation of a HER receptor, the ability of the antibody to block HER ligand binding to cells expressing the HER receptor (e.g. in conjugation with another HER receptor with which the HER receptor of interest forms a HER hetero-oligomer) may be determined. For example, cells naturally expressing, or transfected to express, HER receptors of the HER hetero-oligomer may be incubated with the antibody and then exposed to labeled HER ligand. The ability of the antibody to block ligand binding to the HER receptor in the HER hetero-oligomer may then be evaluated.

For example, inhibition of HRG binding to MCF7 breast tumor cell lines by HER2 antibodies may be performed using monolayer MCF7 cultures on ice in a 24-well-plate format essentially as described in WO 1/00245. HER2 monoclonal antibodies may be added to each well and incubated for 30 minutes. 125I-labeled rHRGpi 177-224 (25 pm) may then be added, and the incubation may be continued for 4 to 16 hours. Dose response curves may be prepared and an IC50 value may be calculated for the antibody of interest. In one embodiment, the antibody which blocks ligand activation of a HER receptor will have an IC50 for inhibiting HRG binding to MCF7 cells in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC50 for inhibiting HRG binding to MCF7 cells in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

Alternatively, or additionally, the ability of an antibody to block HER ligand-stimulated tyrosine phosphorylation of a HER receptor present in a HER hetero-oligomer may be assessed. For example, cells endogenously expressing the HER receptors or transfected to expressed them may be incubated with the antibody and then assayed for HER ligand-dependent tyrosine phosphorylation activity using an anti-phosphotyrosine monoclonal (which is optionally conjugated with a detectable label). The kinase receptor activation assay described in U.S. Pat. No. 5,766,863 is
also available for determining HER receptor activation and blocking of that activity by an antibody.

In one embodiment, one may screen for an antibody which inhibits HRG stimulation of p\(\text{i80}\) tyrosine phosphorylation in MCF7 cells essentially as described in WO 1/00245. For example, the MCF7 cells may be plated in 24-well plates and monoclonal antibodies to HER2 may be added to each well and incubated for 30 minutes at room temperature; then HRG\(\beta\). sub.177-244 may be added to each well to a final concentration of 0.2 nM, and the incubation may be continued for 8 minutes. Media may be aspirated from each well, and reactions may be stopped by the addition of 100 \(\mu\)l of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 \(\mu\)l) may be electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (at 1 \(\mu\)g/ml) immunoblots may be developed, and the intensity of the predominant reactive band at M\(_r\). about. 180,000 may be quantified by reflectance densitometry. The antibody selected will preferably significantly inhibit HRG stimulation of p\(\text{i80}\) tyrosine phosphorylation to about 0-35% of control in this assay. A dose-response curve for inhibition of HRG stimulation of p\(\text{i80}\) tyrosine phosphorylation as determined by reflectance densitometry may be prepared and an IC\(_{50}\) for the antibody of interest may be calculated. In one embodiment, the antibody which blocks ligand activation of a HER receptor will have an IC\(_{50}\) for inhibiting HRG stimulation of p\(\text{i80}\) tyrosine phosphorylation in this assay of about 50 nM or less, more preferably 10 nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC\(_{50}\) for inhibiting HRG stimulation of p\(\text{i80}\) tyrosine phosphorylation in this assay may, for example, be about 100 nM or less, more preferably 50 nM or less.

One may also assess the growth inhibitory effects of the antibody on MDA-MB-175 cells, e.g., essentially as described in Schaefer et al. Oncogene 15:1385-1394 (1997). According to this assay, MDA-MB-175 cells may be treated with a HER2 monoclonal antibody (10 \(\mu\)g/mL) for 4 days and stained with crystal violet. Incubation with a HER2 antibody may show a growth inhibitory effect on this cell line similar to that displayed by monoclonal antibody 2C4. In a further embodiment, exogenous HRG will not significantly reverse this inhibition. Preferably, the antibody
will be able to inhibit cell proliferation of MDA-MB-175 cells to a greater extent than monoclonal antibody 4D5 (and optionally to a greater extent than monoclonal antibody 7F3), both in the presence and absence of exogenous HRG.

In one embodiment, the HER2 antibody of interest may block heregulin dependent association of HER2 with HER3 in both MCF7 and SK-BR-3 cells as determined in a co-immunoprecipitation experiment such as that described in WOO I/00245 substantially more effectively than monoclonal antibody 4D5, and preferably substantially more effectively than monoclonal antibody 7F3.

To identify growth inhibitory HER2 antibodies, one may screen for antibodies which inhibit the growth of cancer cells which overexpress HER2. In one embodiment, the growth inhibitory antibody of choice is able to inhibit growth of SK-BR-3 cells in cell culture by about 20-100% and preferably by about 50-100% at an antibody concentration of about 0.5 to 30 µg/ml. To identify such antibodies, the SK-BR-3 assay described in U.S. Pat. No. 5,677,171 can be performed. According to this assay, SK-BR-3 cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillin streptomycin. The SK-BR-3 cells are plated at 20,000 cells in a 35 mm cell culture dish (2 ml/35 mm dish). 0.5 to 30 µg/ml of the HER2 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the SK-BR-3 cells by about 20-100% or about 50-100% may be selected as growth inhibitory antibodies. See U.S. Pat. No. 5,677,171 for assays for screening for growth inhibitory antibodies, such as 4D5 and 3E8.

In order to select for antibodies which induce apoptosis, an annexin binding assay using BT474 cells is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10 µg/ml of the monoclonal antibody. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca²⁺ binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1 µg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVER™. CellQuest
software (Becton Dickinson). Those antibodies which induce statistically significant
levels of annexin binding relative to control are selected as apoptosis-inducing
antibodies. In addition to the annexin binding assay, a DNA staining assay using
BT474 cells is available. In order to perform this assay, BT474 cells which have been
treated with the antibody of interest as described in the preceding two paragraphs are
incubated with 9 μg/ml HOECHST 33342™ for 2 hr at 37 °C, then analyzed on an
EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™
software (Verity Software House). Antibodies which induce a change in the
percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or
greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-
apoptotic antibodies using this assay. See W098/17797 for assays for screening for
antibodies which induce apoptosis, such as 7C2 and 7F3.

To screen for antibodies which bind to an epitope on HER2 bound by an
antibody of interest, a routine cross-blocking assay such as that described in
Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and
David Lane (1988), can be performed to assess whether the antibody cross-blocks
binding of an antibody, such as 2C4 or pertuzumab, to HER2. Alternatively, or
additionally, epitope mapping can be performed by methods known in the art and/or
one can study the antibody-HER2 structure (Franklin et al. Cancer Cell 5:317-328
(2004)) to see what domain(s) of HER2 is/are bound by the antibody.

(ix) Pertuzumab Compositions

In one embodiment of a HER2 antibody composition, the composition
comprises a mixture of a main species pertuzumab antibody and one or more variants
thereof. The preferred embodiment herein of a pertuzumab main species antibody is
one comprising the variable light and variable heavy amino acid sequences in SEQ ID
Nos. 11 and 12 or one or more heavy and/or light chain CDRs from those sequences
(including deamidated and/or oxidized variants of those sequences). In one
embodiment, the composition comprises a mixture of the main species pertuzumab
antibody and an amino acid sequence variant thereof comprising an amino-terminal
leader extension. Preferably, the amino-terminal leader extension is on a light chain of
the antibody variant (e.g. on one or two light chains of the antibody variant). The
main species HER2 antibody or the antibody variant may be an full length antibody or
antibody fragment (e.g. Fab or F(ab')2 fragments), but preferably both are full length antibodies. The antibody variant herein may comprise an amino-terminal leader extension on any one or more of the heavy or light chains thereof. Preferably, the amino-terminal leader extension is on one or two light chains of the antibody. The amino-terminal leader extension preferably comprises or consists of VHS-. Presence of the amino-terminal leader extension in the composition can be detected by various analytical techniques including, but not limited to, N-terminal sequence analysis, assay for charge heterogeneity (for instance, cation exchange chromatography or capillary zone electrophoresis), mass spectrometry, etc. The amount of the antibody variant in the composition generally ranges from an amount that constitutes the detection limit of any assay (preferably N-terminal sequence analysis) used to detect the variant to an amount less than the amount of the main species antibody. Generally, about 20% or less (e.g. from about 1% to about 15%, for instance from 5% to about 15%) of the antibody molecules in the composition comprise an amino-terminal leader extension. Such percentage amounts are preferably determined using quantitative N-terminal sequence analysis or cation exchange analysis (preferably using a high-resolution, weak cation-exchange column, such as a PROPAC WCX-10™ cation exchange column). Aside from the amino-terminal leader extension variant, further amino acid sequence alterations of the main species antibody and/or variant are contemplated, including but not limited to an antibody comprising a C-terminal lysine residue on one or both heavy chains thereof, a deamidated antibody variant, etc.

Moreover, the main species antibody or variant may further comprise glycosylation variations, non-limiting examples of which include antibody comprising a G1 or G2 oligosaccharide structure attached to the Fc region thereof, antibody comprising a carbohydrate moiety attached to a light chain thereof (e.g. one or two carbohydrate moieties, such as glucose or galactose, attached to one or two light chains of the antibody, for instance attached to one or more lysine residues), antibody comprising one or two non-glycosylated heavy chains, or antibody comprising a sialidated oligosaccharide attached to one or two heavy chains thereof etc.
The composition may be recovered from a genetically engineered cell line, e.g. a Chinese Hamster Ovary (CHO) cell line expressing the HER2 antibody, or may be prepared by peptide synthesis.

(x) Immunoconjugates

The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g. a small molecule toxin or an enzymatically active toxin of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof), or a radioactive isotope (i.e., a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above. Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, a maytansine (U.S. Pat. No. 5,208,020), a trichothene, and CC1065 are also contemplated herein.

In one preferred embodiment of the invention, the antibody is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine may, for example, be converted to May-SS-Me which may be reduced to May-SH3 and reacted with modified antibody (Chari et al. Cancer Research 52: 127-131 (1992)) to generate a maytansinoid-antibody immunoconjugate.

Another immunoconjugate of interest comprises an antibody conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to,\( \text{Yi}^1, a_2^1, a_3^1, N\text{-acetyl-} \gamma t^1, \) PSAG and \( \tau^1 \) (Hinman et al. Cancer Research 53: 3336-3342 (1993) and Lode et al. Cancer Research 58: 2925-2928 (1998)). See, also, U.S. Pat. Nos. 5,714,586; 5,712,374; 5,264,586; and 5,773,001 expressly incorporated herein by reference.

Enzymatically active toxins and fragments thereof which can be used include 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. See, for example, WO 93/21232 published Oct. 28, 1993.

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g. a ribonuclease or a DNA endonuclease).

A variety of radioactive isotopes are available for the production of radioconjugated HER2 antibodies. Examples include At$^{211}$, I$^{131}$, I$^{125}$, Y$^{90}$, Re$^{186}$, Re$^{188}$, Sm$^{153}$, Bi$^{212}$, P$^{32}$ and radioactive isotopes of Lu.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipiminate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareddehyde), bis-azo compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazenium derivatives (such as bis-(p-diazeniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as l,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al. Science 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methylidene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/1 1026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al. Cancer Research 52: 127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, e.g. by recombinant techniques or peptide synthesis.

The auristatin peptides, auristatin E (AE) and monomethylauristatin (MMAE), synthetic analogs of dolastatin (WO 02/088172), have been conjugated as drug moieties to: (i) chimeric monoclonal antibodies cBR96 (specific to Lewis Y on carcinomas); (ii) cACIO which is specific to CD30 on hematological malignancies.

Conventional means of attaching, i.e. linking through covalent bonds, a drug moiety to an antibody generally leads to a heterogeneous mixture of molecules where the drug moieties are attached at a number of sites on the antibody. For example, cytotoxic drugs have typically been conjugated to antibodies through the often-numerous lysine residues of an antibody, generating a heterogeneous antibody-drug conjugate mixture. Depending on reaction conditions, the heterogeneous mixture typically contains a distribution of antibodies with from 0 to about 8, or more, attached drug moieties. In addition, within each subgroup of conjugates with a particular integer ratio of drug moieties to antibody, is a potentially heterogeneous mixture where the drug moiety is attached at various sites on the antibody. Analytical and preparative methods may be inadequate to separate and characterize the antibody-drug conjugate species molecules within the heterogeneous mixture resulting from a conjugation reaction. Antibodies are large, complex and structurally diverse biomolecules, often with many reactive functional groups. Their reactivities with linker reagents and drug-linker intermediates are dependent on factors such as pH, concentration, salt concentration, and co-solvents. Furthermore, the multistep conjugation process may be nonreproducible due to difficulties in controlling the reaction conditions and characterizing reactants and intermediates.

Cysteine thiols are reactive at neutral pH, unlike most amines which are protonated and less nucleophilic near pH 7. Since free thiol (RSH, sulfhydryl) groups
are relatively reactive, proteins with cysteine residues often exist in their oxidized form as disulfide-linked oligomers or have internally bridged disulfide groups. Extracellular proteins generally do not have free thiols (Garman, 1997, Non-Radioactive Labelling: A Practical Approach, Academic Press, London, at page 55).

Antibody cysteine thiol groups are generally more reactive, i.e. more nucleophilic, towards electrophilic conjugation reagents than antibody amine or hydroxyl groups. Cysteine residues have been introduced into proteins by genetic engineering techniques to form covalent attachments to ligands or to form new intramolecular disulfide bonds (Better et al (1994) J. Biol. Chem. 13:9644-9650; Bernhard et al (1994) Bioconjugate Chem. 5:126-132; Greenwood et al (1994) Therapeutic Immunology 1:247-255; Tu et al (1999) Proc. Natl. Acad. Sci. USA 96:4862-4867; Kanno et al (2000) J. of Biotechnology, 76:207-214; Chmura et al (2001) Proc. Natl. Acad. Sci. USA 98(15):8480-8484; U.S. Pat. No. 6,248,564). However, engineering in cysteine thiol groups by the mutation of various amino acid residues of a protein to cysteine amino acids is potentially problematic, particularly in the case of unpaired (free Cys) residues or those which are relatively accessible for reaction or oxidation. In concentrated solutions of the protein, whether in the periplasm of E. coli, culture supernatants, or partially or completely purified protein, unpaired Cys residues on the surface of the protein can pair and oxidize to form intermolecular disulfides, and hence protein dimers or multimers. Disulfide dimer formation renders the new Cys unreactive for conjugation to a drug, ligand, or other label. Furthermore, if the protein oxidatively forms an intramolecular disulfide bond between the newly engineered Cys and an existing Cys residue, both Cys thiol groups are unavailable for active site participation and interactions. Furthermore, the protein may be rendered inactive or non-specific, by misfolding or loss of tertiary structure (Zhang et al (2002) Anal. Biochem. 311:1-9).

Cysteine-engineered antibodies have been designed as FAB antibody fragments (thioFab) and expressed as full-length, IgG monoclonal (thioMab) antibodies (Junutula, J. R. et al. (2008) J Immunol Methods 332:41-52; US 2007/0092940, the contents of which are incorporated by reference). ThioFab and ThioMab antibodies have been conjugated through linkers at the newly introduced cysteine thiols with thiol-reactive linker reagents and drug-linker reagents to prepare
antibody drug conjugates (Thio ADC). For further information see, also U.S. Publication Nos. 2007/0092940; 2009/0068202; 2010/0003766.

Other immunoconjugates are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylinethacrylate) 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, Oslo, A., Ed., (1980).

The antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al, Proc. Natl. Acad. Sci. USA, 82:3688 (1985); Hwang et al, Proc. Natl. Acad. Sci. USA, 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and W097/38731 published Oct. 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al. J. Biol. Chem. 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon et al. J. National Cancer Inst. 81(19)1484 (1989)

II. Diagnostic Methods

In one aspect, the invention herein provides a method for selecting a therapy for a patient with a type of HER2 positive cancer (e.g. breast cancer) which is able to respond to a HER inhibitor or HER dimerization inhibitor (e.g. pertuzumab)
comprising determining HER2 mRNA expression in a cancer sample from the patient and selecting a HER inhibitor or HER dimerization inhibitor as the therapy if the cancer sample expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the cancer type.

The patients selected for treatment with a HER inhibitor, such as a HER dimerization inhibitor, in accordance with the present invention are with a cancer characterized by (i) HER expression, preferably HER2 expression, more preferably HER2 overexpression at the protein level or as determined by a gene amplification (e.g. FISH) assay, and (ii) low HER2 mRNA levels, as determined, for example, by RT-PCR. In particular, a patient diagnosed with a HER expressing, e.g. HER2 expressing or overexpressing cancer is identified as a good candidate for treatment with a HER inhibitor (or HER dimerization inhibitor), e.g. HER inhibitor (or HER2 dimerization inhibitor) in accordance with the present invention if the HER2 mRNA level in the patient's cancer is lower than the median of the HER2 mRNA level in the same cancer type. The median or percentile mRNA expression level can be determined essentially contemporaneously with measuring HER2 mRNA expression in the patient's cancer, or may have been determined previously.

Prior to the therapeutic methods described below, HER2 mRNA expression in the patient's cancer is/are assessed. Generally, a biological sample is obtained from the patient in need of therapy, which sample is subjected to one or more diagnostic assay(s), usually at least one in vitro diagnostic (IVD) assay. However, other forms of evaluating HER2 expression, such as in vivo diagnosis, are expressly contemplated herein. The biological sample is usually a tumor sample, preferably from breast cancer, such as metastatic breast cancer (MBC) tumor sample.

The biological sample herein may be a fixed sample, e.g. a formalin fixed, paraffin-embedded (FFPE) sample, or a frozen sample.

Various methods for determining expression of mRNA include, but are not limited to, gene expression profiling, polymerase chain reaction (PCR) including quantitative real time PCR (qRT-PCR), microarray analysis, serial analysis of gene expression (SAGE), MassARRAY, and Gene Expression Analysis by Massively Parallel Signature Sequencing (MPSS) etc. Such mRNA analysis is preferably
performed using the technique of polymerase chain reaction (PCR), or by microarray analysis. Where PCR is employed, a preferred form of PCR is quantitative real time PCR (qRT-PCR). The preferred qRT-PCR assay is that as described in Example 2 below.

The steps of a representative protocol for profiling gene expression using fixed, paraffin-embedded tissues as the RNA source, including mRNA isolation, purification, primer extension and amplification are given in various published journal articles (for example: Godfrey et al. J. Molec. Diagnostics 2: 84-91 (2000); Specht et al., Am. J. Pathol. 158: 419-29 (2001)). Briefly, a representative process starts with cutting about 10 microgram thick sections of paraffin-embedded tumor tissue samples. The RNA is then extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be included, if necessary, and RNA is reverse transcribed using gene specific promoters followed by PCR. Finally, the data are analyzed to identify the best treatment option(s) available to the patient on the basis of the characteristic gene expression pattern identified in the tumor sample examined.

Various exemplary methods for determining gene expression will now be described in more detail.

(i) Gene Expression Profiling

In general, methods of gene expression profiling can be divided into two large groups: methods based on hybridization analysis of polynucleotides, and methods based on sequencing of polynucleotides. The most commonly used methods known in the art for the quantification of mRNA expression in a sample include northern blotting and in situ hybridization (Parker & Barnes, Methods in Molecular Biology 106:247-283 (1999)); RNAse protection assays (Hod, Biotechniques 13:852-854 (1992)); and polymerase chain reaction (PCR) (Weis et al, Trends in Genetics 8:263-264 (1992)). Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. Representative methods for sequencing-based gene expression analysis include Serial Analysis of Gene Expression (SAGE), and gene expression analysis by massively parallel signature sequencing (MPSS).
(ii) Polymerase Chain Reaction (PCR)

Of the techniques listed above, a sensitive and flexible quantitative method is PCR, which can be used to compare mRNA levels in different sample populations, in normal and tumor tissues, with or without drug treatment, to characterize patterns of gene expression, to discriminate between closely related mRNAs, and to analyze RNA structure.

The first step is the isolation of mRNA from a target sample. The starting material is typically total RNA isolated from human tumors or tumor cell lines, and corresponding normal tissues or cell lines, respectively. Thus RNA can be isolated from a variety of primary tumors, including breast, lung, colon, prostate, brain, liver, kidney, pancreas, spleen, thymus, testis, ovary, uterus, etc., tumor, or tumor cell lines, with pooled DNA from healthy donors. If the source of mRNA is a primary tumor, mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin-fixed) tissue samples. General methods for mRNA extraction are well known in the art and are disclosed in standard textbooks of molecular biology, including Ausubel et al., Current Protocols of Molecular Biology, John Wiley and Sons (1997). Methods for RNA extraction from paraffin embedded tissues are disclosed, for example, in Rupp and Locker, Lab Invest. 56:A67 (1987), and De Andres et al., BioTechniques 18:42044 (1995). In particular, RNA isolation can be performed using purification kit, buffer set and protease from commercial manufacturers, such as Qiagen, according to the manufacturer's instructions. For example, total RNA from cells in culture can be isolated using Qiagen RNeasy mini-columns. Other commercially available RNA isolation kits include MASTERPURE® Complete DNA and RNA Purification Kit (EPICENTRE®, Madison, Wis.), and Paraffin Block RNA Isolation Kit (Ambion, Inc.). Total RNA from tissue samples can be isolated using RNA Stat-60 (Tel-Test). RNA prepared from tumor can be isolated, for example, by cesium chloride density gradient centrifugation.

As RNA cannot serve as a template for PCR, the first step in gene expression profiling by PCR is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. The two most commonly used reverse transcriptases are avilo myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT). The
reverse transcription step is typically primed using specific primers, random hexamers, or oligo-dT primers, depending on the circumstances and the goal of expression profiling. For example, extracted RNA can be reverse-transcribed using a GENEAMP™ RNA PCR kit (Perkin Elmer, Calif., USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction. Although the PCR step can use a variety of thermostable DNA-dependent DNA polymerases, it typically employs the Taq DNA polymerase, which has a 5'-3' nuclease activity but lacks a 3'-5' proofreading endonuclease activity. Thus, TAQMAN® PCR typically utilizes the 5'-nuclease activity of Taq or Tth polymerase to hydrolyze a hybridization probe bound to its target amplicon, but any enzyme with equivalent 5' nuclease activity can be used. Two oligonucleotide primers are used to generate an amplicon typical of a PCR reaction. A third oligonucleotide, or probe, is designed to detect nucleotide sequence located between the two PCR primers. The probe is non-extendible by Taq DNA polymerase enzyme, and is labeled with a reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative interpretation of the data.

TAQMAN® PCR can be performed using commercially available equipment, such as, for example, ABI PRISM 7700® Sequence Detection System® (Perkin-Elmer-Applied Biosystems, Foster City, Calif., USA), or Lightcycler (Roche Molecular Biochemicals, Mannheim, Germany). In a preferred embodiment, the 5' nuclease procedure is run on a real-time quantitative PCR device such as the ABI PRISM 7700® Sequence Detection System. The system consists of a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system amplifies samples in a 96-well format on a thermocycler. During amplification, laser-induced fluorescent signal is collected in real-time through fiber optics cables for all 96 wells,
and detected at the CCD. The system includes software for running the instrument and for analyzing the data.

5'-Nuclease assay data are initially expressed as Ct, or the threshold cycle. As discussed above, fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The point when the fluorescent signal is first recorded as statistically significant is the threshold cycle (Ct).

To minimize errors and the effect of sample-to-sample variation, PCR is usually performed using an internal standard. The ideal internal standard is expressed at a constant level among different tissues, and is unaffected by the experimental treatment. RNAs most frequently used to normalize patterns of gene expression are mRNAs for the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and P-actin.

A more recent variation of the PCR technique is quantitative real time PCR (qRT-PCR), which measures PCR product accumulation through a dual-labeled fluorogenic probe (i.e., TAQMAN® probe). Real time PCR is compatible both with quantitative competitive PCR, where internal competitor for each target sequence is used for normalization, and with quantitative comparative PCR using a normalization gene contained within the sample, or a housekeeping gene for PCR. For further details see, e.g. Held et al, Genome Research 6:986-994 (1996).

The steps of a representative protocol for profiling gene expression using fixed, paraffin-embedded tissues as the RNA source, including mRNA isolation, purification, primer extension and amplification are given in various published journal articles (for example: Godfrey et al, J. Molec. Diagnostics 2: 84-91 (2000); Specht et al., Am. J. Pathol. 158: 419-29 (2001)). Briefly, a representative process starts with cutting about 10 microgram thick sections of paraffin-embedded tumor tissue samples. The RNA is then extracted, and protein and DNA are removed. After analysis of the RNA concentration, RNA repair and/or amplification steps may be included, if necessary, and RNA is reverse transcribed using gene specific promoters followed by PCR.
According to one aspect of the present invention, PCR primers and probes are designed based upon intron sequences present in the gene to be amplified. In this embodiment, the first step in the primer/probe design is the delineation of intron sequences within the genes. This can be done by publicly available software, such as the DNA BLAT software developed by Kent, W., Genome Res. 12(4):656-64 (2002), or by the BLAST software including its variations. Subsequent steps follow well established methods of PCR primer and probe design.

In order to avoid non-specific signals, it is important to mask repetitive sequences within the introns when designing the primers and probes. This can be easily accomplished by using the Repeat Masker program available on-line through the Baylor College of Medicine, which screens DNA sequences against a library of repetitive elements and returns a query sequence in which the repetitive elements are masked. The masked intron sequences can then be used to design primer and probe sequences using any commercially or otherwise publicly available primer/probe design packages, such as Primer Express (Applied Biosystems); MGB assay-by-design (Applied Biosystems); Primer3 (Rozen and Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, N.J., pp 365-386).

Factors considered in PCR primer design include primer length, melting temperature (Tm), and G/C content, specificity, complementary primer sequences, and 3'-end sequence. In general, optimal PCR primers are generally 17-30 bases in length, and contain about 20-80%, such as, for example, about 50-60% G+C bases. Tm's between 50 and 80 °C, e.g. about 50 to 70 °C. are typically preferred.

The preferred conditions, primers, probes, and internal reference (G6PDH) are as described in Example 1 below.

(iii) **Microarrays**

Differential gene expression can also be identified, or confirmed using the microarray technique. Thus, the expression profile of breast cancer-associated genes can be measured in either fresh or paraffin-embedded tumor tissue, using microarray technology. In this method, polynucleotide sequences of interest (including cDNAs and oligonucleotides) are plated, or arrayed, on a microchip substrate. The arrayed sequences are then hybridized with specific DNA probes from cells or tissues of interest. Just as in the PCR method, the source of mRNA typically is total RNA isolated from human tumors or tumor cell lines, and corresponding normal tissues or cell lines. Thus RNA can be isolated from a variety of primary tumors or tumor cell lines. If the source of mRNA is a primary tumor, mRNA can be extracted, for example, from frozen or archived paraffin-embedded and fixed (e.g. formalin-fixed) tissue samples, which are routinely prepared and preserved in everyday clinical practice.

In a specific embodiment of the microarray technique, PCR amplified inserts of cDNA clones are applied to a substrate in a dense array. Preferably at least 10,000 nucleotide sequences are applied to the substrate. The microarrayed genes, immobilized on the microchip at 10,000 elements each, are suitable for hybridization under stringent conditions. Fluorescently labeled cDNA probes may be generated through incorporation of fluorescent nucleotides by reverse transcription of RNA extracted from tissues of interest. Labeled cDNA probes applied to the chip hybridize with specificity to each spot of DNA on the array. After stringent washing to remove non-specifically bound probes, the chip is scanned by confocal laser microscopy or by another detection method, such as a CCD camera. Quantitation of hybridization of each arrayed element allows for assessment of corresponding mRNA abundance. With dual color fluorescence, separately labeled cDNA probes generated from two sources of RNA are hybridized pairwise to the array. The relative abundance of the transcripts from the two sources corresponding to each specified gene is thus
determined simultaneously. The miniaturized scale of the hybridization affords a
convenient and rapid evaluation of the expression pattern for large numbers of genes.
Such methods have been shown to have the sensitivity required to detect rare
transcripts, which are expressed at a few copies per cell, and to reproducibly detect at
least approximately two-fold differences in the expression levels (Schena et al., Proc.
Natl. Acad. Sci. USA 93(2): 106-149 (1996)). Microarray analysis can be performed
by commercially available equipment, following manufacturer's protocols, such as by
using the Affymetrix GENCHIP™ technology, or Incyte's microarray technology.

The development of microarray methods for large-scale analysis of gene
expression makes it possible to search systematically for molecular markers of cancer
classification and outcome prediction in a variety of tumor types.

(iv) Serial Analysis of Gene Expression (SAGE)

Serial analysis of gene expression (SAGE) is a method that allows the
simultaneous and quantitative analysis of a large number of gene transcripts, without
the need of providing an individual hybridization probe for each transcript. First, a
short sequence tag (about 10-14 bp) is generated that contains sufficient information
to uniquely identify a transcript, provided that the tag is obtained from a unique
position within each transcript. Then, many transcripts are linked together to form
long serial molecules, that can be sequenced, revealing the identity of the multiple
tags simultaneously. The expression pattern of any population of transcripts can be
quantitatively evaluated by determining the abundance of individual tags, and
identifying the gene corresponding to each tag. For more details see, e.g. Velculescu

(v) MassARRAY Technology

The MassARRAY (Sequenom, San Diego, Calif.) technology is an automated,
high-throughput method of gene expression analysis using mass spectrometry (MS)
for detection. According to this method, following the isolation of RNA, reverse
transcription and PCR amplification, the cDNAs are subjected to primer extension.
The cDNA-derived primer extension products are purified, and dipensed on a chip
array that is pre-loaded with the components needed for MALTI-TOF MS sample
preparation. The various cDNAs present in the reaction are quantitated by analyzing the peak areas in the mass spectrum obtained.

(vi) *Gene Expression Analysis by Massively Parallel Signature Sequencing (MPSS)*

This method, described by Brenner et al, Nature Biotechnology 18:630-634 (2000), is a sequencing approach that combines non-gel-based signature sequencing with in vitro cloning of millions of templates on separate 5 microgram diameter microbeads. First, a microbead library of DNA templates is constructed by in vitro cloning. This is followed by the assembly of a planar array of the template—containing microbeads in a flow cell at a high density (typically greater than 3 times, 106 microbeads/cm2). The free ends of the cloned templates on each microbead are analyzed simultaneously, using a fluorescence-based signature sequencing method that does not require DNA fragment separation. This method has been shown to simultaneously and accurately provide, in a single operation, hundreds of thousands of gene signature sequences from a yeast cDNA library.

While, in accordance with the present invention, patients are identified for treatment with a HER inhibitor, such as a HER (HER2) dimerization inhibitor, based on low HER2 mRNA levels in the patients' cancer, the cancer of eligible patients is further characterized by HER2 expression, preferably HER2 overexpression at the protein level and/or by gene amplification as determined, for example, by FISH.

(vii) *Immunohistochemistry*

Immunohistochemistry methods are suitable for detecting HER2 expression at the protein level. Thus, antibodies or antisera, preferably polyclonal antisera, and most preferably monoclonal antibodies specific for each marker are used to detect expression. The antibodies can be detected by direct labeling of the antibodies themselves, for example, with radioactive labels, fluorescent labels, hapten labels such as, biotin, or an enzyme such as horse radish peroxidase or alkaline phosphatase. Alternatively, unlabeled primary antibody is used in conjunction with a labeled secondary antibody, comprising antisera, polyclonal antisera or a monoclonal
antibody specific for the primary antibody. Immunohistochemistry protocols and kits are well known in the art and are commercially available.

(viii) **Proteomics**

HER2 protein expression levels in a tumor (cancer) sample can also be determined by proteomics methods. The term "proteome" is defined as the totality of the proteins present in a sample (e.g. tissue, organism, or cell culture) at a certain point of time. Proteomics includes, among other things, study of the global changes of protein expression in a sample (also referred to as "expression proteomics"). Proteomics typically includes the following steps: (1) separation of individual proteins in a sample by 2-D gel electrophoresis (2-D PAGE); (2) identification of the individual proteins recovered from the gel, e.g. my mass spectrometry or N-terminal sequencing, and (3) analysis of the data using bioinformatics.

In one embodiment, HER2 protein expression may be analyzed by IHC, e.g. using the HERCEPTEST® (Dako). Paraffm embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a HER2 protein staining intensity criteria as follows:

Score 0 no staining is observed or membrane staining is observed in less than 10% of tumor cells. Score 1+ a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane. Score 2+ a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells. Score 3+ a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

Those tumors with 0 or 1+ scores for HER2 overexpression assessment may be characterized as not overexpressing HER2, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing HER2.

Tumors overexpressing HER2 may be rated by immunohistochemical scores corresponding to the number of copies of HER2 molecules expressed per cell, and can been determined biochemically:

0=0-10,000 copies/cell,

1+=at least about 200,000 copies/cell,
2+= at least about 500,000 copies/cell,
3+= at least about 2,000,000 copies/cell.

Overexpression of HER2 at the 3+ level, which leads to ligand-independent activation of the tyrosine kinase (Hudziak et al., Proc. Natl. Acad. Sci. USA, 84:7159-7163 (1987)), occurs in approximately 30% of breast cancers, and in these patients, relapse-free survival and overall survival are diminished (Slamon et al., Science, 244:707-712 (1989); Slamon et al, Science, 235:177-182 (1987)). Alternatively, or additionally, FISH assays such as the INFORM™ (sold by Ventana, Ariz.) or PATHVISION™ (Vysis, 111.) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of HER2 amplification in the tumor.

HER2 expression may also be evaluated using an in vivo diagnostic assay, e.g. by administering a molecule (such as an antibody) which binds the molecule to be detected and is tagged with a detectable label (e.g. a radioactive isotope) and externally scanning the patient for localization of the label.

The tumors selected for treatment in accordance with the present invention are characterized by HER2 protein expression, preferably HER2 protein overexpression, more preferably HER2 protein overexpression at +2 or +3 level, as determined by IHC, and/or by HER2 amplification as determined by FISH, and, additionally by low HER2 mRNA expression levels, as determined, for example, by RT-PCR. Preferably, the cancer is breast cancer, more preferably metastatic breast cancer, including breast cancer that progressed during a prior cancer treatment, such as prior treatment with a chemotherapeutic anti-cancer agent and/or antibody, such as a different HER2 antibody, e.g., trastuzumab.

III. Pharmaceutical Formulations

Therapeutic formulations of the HER inhibitor, HER dimerization inhibitor, or chemotherapeutic agent used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), generally in the form of
lyophilized formulations or aqueous solutions. Antibody crystals are also contemplated (see US Pat Appln 2002/0136719). Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl 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 polyvinylpyrrolidone; 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 TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Lyophilized antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

The preferred pertuzumab formulation for therapeutic use comprises 30 mg/mL pertuzumab in 20 mM histidine acetate, 120 mM sucrose, 0.02% polysorbate 20, at pH 6.0. An alternate pertuzumab formulation comprises 25 mg/mL pertuzumab, 10 mM histidine-HCl buffer, 240 mM sucrose, 0.02% polysorbate 20, pH 6.0.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Various drugs which can be combined with the HER inhibitor, or HER dimerization inhibitor are described in the Treatment Section below. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-methylmethacrylate) 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 semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and .gamma. ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D(-)-3-hydroxybutyric acid.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Accordingly, a method for manufacturing a HER inhibitor, or HER dimerization inhibitor (such as pertuzumab), or a pharmaceutical composition thereof is provided, which method comprises combining in a package the inhibitor or pharmaceutical composition and a label stating that the inhibitor or pharmaceutical composition is indicated for treating a patient with a type of cancer (for example, breast cancer) which is able to respond to the inhibitor, wherein the patient's cancer expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the cancer type.

In addition, a method for manufacturing a chemotherapeutic agent (such as gemcitabine or paclitaxel or docetaxel) or a pharmaceutical composition thereof is provided, wherein the method comprises combining in a package the chemotherapeutic agent or pharmaceutical composition and a label stating that the chemotherapeutic agent or pharmaceutical composition is indicated for treating a patient with a type of cancer (exemplified by breast cancer), wherein the patient's cancer expresses HER2 mRNA at a level lower than the median level for HER2 mRNA expression in the cancer type.
IV. Treatment with HER Inhibitors

The invention herein provides a method for treating a patient with a type cancer which is able to respond to a HER inhibitor or HER dimerization inhibitor, comprising administering a therapeutically effective amount of the inhibitor to the patient, wherein the patient's cancer expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the cancer type. Preferably the patient's cancer expresses both HER2 and HER3 at a level which is less than the 33rd percentile, or the 25th percentile for HER2 mRNA expression in the cancer type.

In a particularly preferred embodiment, the invention provides a method for treating a patient with breast cancer comprising administering a therapeutically effective amount of pertuzumab to the patient, as a single agent or in combination with trastuzumab, wherein the patient's cancer expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in breast cancer. In this embodiment, preferably the patient's cancer expresses HER2 mRNA at a level which is less than the 33rd percentile, or the 25th percentile for HER2 mRNA expression in breast cancer. The breast cancer preferably is advanced HER2 positive breast cancer, more preferably HER2 positive breast cancer that progressed during prior therapy, such as prior trastuzumab therapy.

Examples of various cancer types that can be treated with a HER inhibitor or HER dimerization inhibitor are listed in the definition section above. Preferred cancer types include breast cancer, including metastatic breast cancer (MBC); ovarian cancer; peritoneal cancer; fallopian tube cancer; lung cancer, including non-small cell lung cancer (NSCLC); prostate cancer; and colorectal cancer. In one embodiment, the cancer which is treated is advanced, refractory, recurrent, chemotherapy-resistant, and/or platinum-resistant cancer. In another embodiment, the cancer is breast cancer that progressed during prior therapy, such as during prior trastuzumab therapy.

Therapy with the HER inhibitor, HER dimerization inhibitor and/or chemotherapeutic agent preferably extends survival, including progression free survival (PFS) and/or overall survival (OS). In one embodiment, therapy with the HER inhibitor or HER dimerization inhibitor extends survival at least about 20%
more than survival achieved by administering an approved anti-tumor agent, or standard of care, for the cancer being treated.

In the preferred embodiment, the method involves treating a patient with breast cancer, specially breast cancer that progressed during prior therapy, such as prior trastuzumab therapy. Administration of pertuzumab to the patient, either alone or in combination with trastuzumab, may, for example, extend survival at least about 20% more than survival achieved by trastuzumab without the administration of pertuzumab.

The HER inhibitor, or HER dimerization inhibitor, other HER antibodies, such as anti-HER2 antibodies, e.g. trastuzumab, and/or chemotherapeutic agent are administered to a human patient in accord with known methods, such as intravenous administration, e.g., as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of the antibody is preferred.

For the prevention or treatment of cancer, the dose will depend on the type of cancer to be treated, as defined above, the severity and course of the cancer, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the drug, and the discretion of the attending physician.

In one embodiment, a fixed dose of inhibitor is administered. The fixed dose may suitably be administered to the patient at one time or over a series of treatments. Where a fixed dose is administered, preferably it is in the range from about 20 mg to about 2000 mg of the inhibitor. For example, the fixed dose may be approximately 420 mg, approximately 525 mg, approximately 840 mg, or approximately 1050 mg of the inhibitor, such as pertuzumab.

Where a series of doses are administered, these may, for example, be administered approximately every week, approximately every 2 weeks, approximately every 3 weeks, or approximately every 4 weeks, but preferably approximately every 2 weeks or every 3 weeks. The fixed doses may, for example, continue to be
administered until disease progression, adverse event, or other time as determined by the physician. For example, from about two, three, or four, up to about 17 or more fixed doses may be administered.

In one embodiment, one or more loading dose(s) of the antibody are administered, followed by one or more maintenance dose(s) of the antibody. In another embodiment, a plurality of the same dose are administered to the patient.

According to one preferred embodiment of the invention, a fixed dose of HER dimerization inhibitor (e.g. pertuzumab) of approximately 840 mg (loading dose) is administered, followed by one or more doses of approximately 420 mg (maintenance dose(s)) of the antibody. The maintenance doses are preferably administered about every 3 weeks, for a total of at least two doses, up to 17 or more doses.

According to another preferred embodiment of the invention, one or more fixed dose(s) of approximately 1050 mg of the HER dimerization inhibitor (e.g. pertuzumab) are administered, for example every 3 weeks. According to this embodiment, one, two or more of the fixed doses are administered, e.g. for up to one year (17 cycles), and longer as desired.

In another embodiment, a fixed dose of approximately 1050 mg of the HER dimerization inhibitor (e.g. pertuzumab) is administered as a loading dose, followed by one or more maintenance dose(s) of approximately 525 mg. About one, two or more maintenance doses may be administered to the patient every 3 weeks according to this embodiment.

While the HER inhibitor, HER dimerization inhibitor or chemotherapeutic agent may be administered as a single anti-tumor agent, the patient is optionally treated with a combination of the inhibitor (or chemotherapeutic agent), and one or more (additional) chemotherapeutic agent(s). Exemplary chemotherapeutic agents herein include: gemcitabine, carboplatin, paclitaxel, docetaxel, topotecan, and/or liposomal doxorubicin. Preferably at least one of the chemotherapeutic agents is a mitotic inhibitor, such as paclitaxel or docetaxel, or an antimetabolite chemotherapeutic agent such as gemcitabine. The combined administration includes coadministration or concurrent administration, using separate formulations or a single
pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Thus, the mitotic inhibitor, such as a taxene, or the antimetabolite chemotherapeutic agent may be administered prior to, or following, administration of the HER inhibitor, such as a HER dimerization inhibitor. In this embodiment, the timing between at least one administration of the antimetabolite chemotherapeutic agent and at least one administration of the inhibitor is preferably approximately 1 month or less, and most preferably approximately 2 weeks or less. Alternatively, the antimetabolite chemotherapeutic agent and the inhibitor are administered concurrently to the patient, in a single formulation or separate formulations. Treatment with the combination of the chemotherapeutic agent (e.g. antimetabolite chemotherapeutic agent such as gemcitabine) and the inhibitor (e.g. pertuzumab) may result in a synergistic, or greater than additive, therapeutic benefit to the patient.

In a preferred embodiment, the HER dimerization inhibitor, such as pertuzumab, is administered in combination with one or more of: a second, different HER inhibitor, HER dimerization inhibitor (for example, a growth inhibitory HER2 antibody such as trastuzumab, or a HER2 antibody which induces apoptosis of a HER2-overexpressing cell, such as 7C2, 7F3 or humanized variants thereof); an antibody directed against a different tumor associated antigen, such as EGFR, HER3, HER4; anti-hormonal compound, e.g., an anti-estrogen compound such as tamoxifen, or an aromatase inhibitor; a cardioprotectant (to prevent or reduce any myocardial dysfunction associated with the therapy); a cytokine; an EGFR-targeted drug (such as TARCEVA®, IRESSA® or cetuximab); an anti-angiogenic agent (especially bevacizumab sold by Genentech under the trademark AVASTIN™); a tyrosine kinase inhibitor; a COX inhibitor (for instance a COX-1 or COX-2 inhibitor); non-steroidal anti-inflammatory drug, celecoxib (CELEBREX®); farnesyl transferase inhibitor (for example, Tipifarnib/ZARNESTRA® R115777 available from Johnson and Johnson or Lonafarnib SCH66336 available from Schering-Plough); antibody that binds oncofetal protein CA 125 such as Oregovomab (MoAb B43.13); HER2 vaccine (such as HER2AutoVac vaccine from Pharmexia, or APC8024 protein vaccine from Dendreon, or HER2 peptide vaccine from GSK/Corixa); another HER targeting therapy (e.g. trastuzumab, cetuximab, ABX-EGF, EMD7200, gefitinib, erlotinib,
CP724714, CI1033, GW572016, IMC-1 1F8, TAK165, etc); Raf and/or ras inhibitor
(see, for example, WO 2003/86467); topoisomerase 1 inhibitor such as topotecan; taxane; HER2 and EGFR dual tyrosine
kinase inhibitor such as lapatinib/GW572016; TLK286 (TELCYTA®); EMD-7200; a
medicament that treats nausea such as a serotonin antagonist, steroid, or
benzodiazepine; a medicament that prevents or treats skin rash or standard acne
therapies, including topical or oral antibiotic; a medicament that treats or prevents
diarrhea; a body temperature-reducing medicament such as acetaminophen,
diphenhydramine, or meperidine; hematopoietic growth factor, etc.

Particularly desired chemotherapeutic agents for combining with the inhibitor,
e.g. for therapy of breast cancer, include: a taxoid such as paclitaxel or docetaxel;
topotecan; or liposomal doxorubicin; an antimetabolite chemotherapeutic agent such
as gemcitabine; a platinum compound such as carboplatin;

An antimetabolite chemotherapeutic agent, if administered, is usually
administered at dosages known therefor, or optionally lowered due to combined
action of the drugs or negative side effects attributable to administration of the
antimetabolite chemotherapeutic agent. Preparation and dosing schedules for such
chemotherapeutic agents may be used according to manufacturers' instructions or as
determined empirically by the skilled practitioner. Where the antimetabolite
chemotherapeutic agent is gemcitabine, preferably, it is administered at a dose
between about 600 mg/m² to 1250 mg/m.sup.2 (for example approximately 1000
mg/m²), for instance, on days 1 and 8 of a 3-week cycle.

Aside from the inhibitor and mitotic inhibitor and/or antimetabolite
chemotherapeutic agent, other therapeutic regimens may be combined therewith. For
example, a second (third, fourth, etc) chemotherapeutic agent(s) may be administered,
wherein the second chemotherapeutic agent is either another, different antimetabolite
chemotherapeutic agent, or a chemotherapeutic agent that is not an antimetabolite. For
example, the second chemotherapeutic agent may be a taxane (such as paclitaxel or
docetaxel), capecitabine, or platinum-based chemotherapeutic agent (such as
carboplatin, cisplatin, or oxaliplatin), anthracycline (such as doxorubicin, including,
liposomal doxorubicin), topotecan, pemetrexed, vinca alkaloid (such as vinorelbine),
and TLK 286. "Cocktails" of different chemotherapeutic agents may be administered.
Suitable dosages for any of the above coadministered agents are those presently used and may be lowered due to the combined action (synergy) of the agent and inhibitor.

In addition to the above therapeutic regimes, the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

Where the inhibitor is an antibody, preferably the administered antibody is a naked antibody. However, the inhibitor administered may be conjugated with a cytotoxic agent. Preferably, the conjugated inhibitor and/or antigen to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the conjugate in killing the cancer cell to which it binds. In a preferred embodiment, the cytotoxic agent targets or interferes with nucleic acid in the cancer cell. Examples of such cytotoxic agents include maytansinoids, calicheamicins, ribonucleases and DNA endonucleases.

The present application contemplates administration of the inhibitor by gene therapy. See, for example, WO96/07321 published Mar. 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells; in vivo and ex vivo. For in vivo delivery the nucleic acid is injected directly into the patient, usually at the site where the antibody is required. For ex vivo treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, e.g. U.S. Pat. Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for ex vivo delivery of the gene is a retrovirus.
The currently preferred in vivo nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Choi, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al, J. Biol. Chem. 262:44294432 (1987); and Wagner et al, Proc. Natl. Acad. Sci. USA 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson et al, Science 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

V. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the diseases or conditions 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, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds or contains a composition which is effective for treating the disease or condition of choice 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 the HER dimerization inhibitor, such as pertuzumab, or chemotherapeutic agent, such as gemcitabine.

The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable diluent buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. The article of manufacture may further include other materials desirable
from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

The kits and articles of manufacture of the present invention also include information, for example in the form of a package insert or label, indicating that the composition is used for treating cancer where the patient's cancer expresses HER2 and HER3 at a defined level depending on the drug. The insert or label may take any form, such as paper or on electronic media such as a magnetically recorded medium (e.g., floppy disk) or a CD-ROM. The label or insert may also include other information concerning the pharmaceutical compositions and dosage forms in the kit or article of manufacture.

Generally, such information aids patients and physicians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following information regarding the HER dimerization inhibitor or chemotherapeutic agent may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdosage, proper dosage and administration, how supplied, proper storage conditions, references and patent information.

In a specific embodiment of the invention, an article of manufacture is provided comprising, packaged together, a pharmaceutical composition comprising a HER inhibitor, or HER dimerization inhibitor in a pharmaceutically acceptable carrier and a label stating that the inhibitor or pharmaceutical composition is indicated for treating a patient with a type of cancer which is able to respond to a HER inhibitor, or HER dimerization inhibitor, wherein the patient's cancer expresses HER2 and HER3 at a level less than the median level for HER2 and HER3 expression, respectively in the cancer type.

In an optional embodiment of this inventive aspect, the article of manufacture herein further comprises a container comprising a second medicament, wherein the HER inhibitor or HER dimerization inhibitor is a first medicament, and which article further comprises instructions on the package insert for treating the patient with the second medicament, in an effective amount. The second medicament may be any of
those set forth above, with an exemplary second medicament being another HER2 antibody or a chemotherapeutic agent.

The package insert is on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds or contains a composition that is effective for treating cancer type 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 the HER inhibitor, HER dimerization inhibitor, or chemotherapeutic agent. The label or package insert indicates that the composition is used for treating cancer in a subject eligible for treatment with specific guidance regarding dosing amounts and intervals of inhibitor and any other medicament being provided. The article of manufacture may further comprise an additional container comprising a pharmaceutically acceptable diluent buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution, and/or dextrose solution. The article of manufacture may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.


VI. Methods of Advertising

The invention herein also encompasses a method for advertising a HER inhibitor, HER dimerization inhibitor (for instance pertuzumab) or a pharmaceutically acceptable composition thereof comprising promoting, to a target audience, the use of the inhibitor or pharmaceutical composition thereof for treating a patient population with a type of cancer (such as ovarian cancer), where the patient's cancer expresses HER2 and HER3 at a level less than the median level for HER2 and HER3 expression, respectively in the cancer type.

Advertising is generally paid communication through a non-personal medium in which the sponsor is identified and the message is controlled. Advertising for purposes herein includes publicity, public relations, product placement, sponsorship, underwriting, and sales promotion. This term also includes sponsored informational public notices appearing in any of the print communications media designed to appeal to a mass audience to persuade, inform, promote, motivate, or otherwise modify behavior toward a favorable pattern of purchasing, supporting, or approving the invention herein.

The advertising and promotion of the diagnostic method herein may be accomplished by any means. Examples of advertising media used to deliver these messages include television, radio, movies, magazines, newspapers, the internet, and billboards, including commercials, which are messages appearing in the broadcast media. Advertisements also include those on the seats of grocery carts, on the walls of an airport walkway, and on the sides of buses, or heard in telephone hold messages or in-store PA systems, or anywhere a visual or audible communication can be placed.

More specific examples of promotion or advertising means include television, radio, movies, the internet such as webcasts and webinars, interactive computer networks intended to reach simultaneous users, fixed or electronic billboards and other public signs, posters, traditional or electronic literature such as magazines and
newspapers, other media outlets, presentations or individual contacts by, e.g., e-mail, phone, instant message, postal, courier, mass, or carrier mail, in-person visits, etc.

The type of advertising used will depend on many factors, for example, on the nature of the target audience to be reached, e.g., hospitals, insurance companies, clinics, doctors, nurses, and patients, as well as cost considerations and the relevant jurisdictional laws and regulations governing advertising of medicaments and diagnostics. The advertising may be individualized or customized based on user characterizations defined by service interaction and/or other data such as user demographics and geographical location.

VII. Kits of the invention

The materials for use in the methods of the present invention are suited for preparation of kits produced in accordance with well known procedures. The invention thus provides kits comprising agents, which may include gene-specific or gene-selective probes and/or primers, for quantitating the expression of HER2 mRNA or mRNA of any other marker that might be used in combination with HER2 in the diagnostic assays herein, such as, for example, HER3 mRNA. Such kits may optionally contain reagents for the extraction of RNA from samples, in particular fixed paraffin-embedded tissue samples and/or reagents for RNA amplification. In addition, the kits may optionally comprise the reagent(s) with an identifying description or label or instructions relating to their use in the methods of the present invention. The kits may comprise containers (including microtiter plates suitable for use in an automated implementation of the method), each with one or more of the various reagents (typically in concentrated form) utilized in the methods, including, for example, pre-fabricated microarrays, buffers, the appropriate nucleotide triphosphates (e.g., dATP, dCTP, dGTP and dTTP; or rATP, rCTP, rGTP and UTP), reverse transcriptase, DNA polymerase, RNA polymerase, and one or more probes and primers of the present invention (e.g., appropriate length poly(T) or random primers linked to a promoter reactive with the RNA polymerase).

All aspects of the present invention may also be practiced such that a limited number of additional genes that are co-expressed with the disclosed genes, for
example as evidenced by high Pearson correlation coefficients, are included in a prognostic or predictive test in addition to and/or in place of disclosed genes.

Having described the invention, the same will be more readily understood through reference to the following Examples, which is provided by way of illustration, and is not intended to limit the invention in any way.

All references cited throughout this disclosure are expressly incorporated by reference herein.

**Example 1**

Study design of Phase II clinical trial to evaluate the clinical efficacy and safety of pertuzumab administered in combination with Herceptin® in breast cancer patients who progressed on Herceptin®-based therapy.

**Glossary of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Anthracyclines and cyclophosphamide</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AE</td>
<td>Adverse Event</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT (SGPT)</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ANC</td>
<td>Absolute neutrophil count</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AST (SGOT)</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the plasma concentration-time curve</td>
</tr>
<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CISH</td>
<td>Chromogenic in situ hybridization</td>
</tr>
<tr>
<td>Cmax</td>
<td>Maximum plasma concentration</td>
</tr>
<tr>
<td>CR</td>
<td>Complete Response</td>
</tr>
<tr>
<td>CT</td>
<td>Computer Tomography</td>
</tr>
<tr>
<td>eCRF</td>
<td>Electronic Case Report Form(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>eForm</td>
<td>Electronic form</td>
</tr>
<tr>
<td>EC50</td>
<td>Plasma concentration associated with half-maximal effect</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>ECOG</td>
<td>Eastern Cooperative Oncology Group</td>
</tr>
<tr>
<td>EDC</td>
<td>Electronic data capture</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ESF</td>
<td>Eligibility screening form</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedde</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>G-SCF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>H0</td>
<td>Null hypothesis</td>
</tr>
<tr>
<td>H1</td>
<td>Alternative hypothesis</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HER1</td>
<td>Human Epidermal growth factor Receptor 1</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal growth factor Receptor 2</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonization</td>
</tr>
<tr>
<td>ID SMB</td>
<td>Independent Data Safety Monitoring Board</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IRB/IEC</td>
<td>Institutional Review Board/Independent Ethics Committee</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridization</td>
</tr>
<tr>
<td>ITT</td>
<td>Intent to treat</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>keo</td>
<td>Equilibration rate constant</td>
</tr>
<tr>
<td>LVEF</td>
<td>Left ventricular ejection fraction</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum Tolerated Dose</td>
</tr>
<tr>
<td>NCI-CTCAE</td>
<td>National Cancer Institute-Common Toxicity Criteria for Adverse Events</td>
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Background

Pertuzumab (P), a fully humanized monoclonal antibody, targets the dimerization epitope of human epidermal growth factor receptor 2 (HER2), preventing dimerization of HER2 with other HER family members like HER3 and inducing antibody-dependent cell-mediated cytotoxicity. As a single agent it has activity against HER2-positive breast cancer, but in combination with trastuzumab (Herceptin®), the level of activity is greatly enhanced, with an overall clinical benefit rate of 50% in 66 patients who had been treated in 2 cohorts in a Phase II study (Gelmon et al, 2008 ASCO Annual Meeting; J Clin Oncol 26: 2008 (May 20 suppl; abstr 1026).

This is an exploratory, two-stage phase II, single arm study of the efficacy and safety of pertuzumab and Herceptin® in HER2 positive metastatic breast cancer patients who have progressed following Herceptin® based chemotherapy.

Study Design

The first 27 patients are recruited and treated with pertuzumab and Herceptin® to form the first cohort of patients, and are assessed to determine whether the study can move into the second stage at an interim analysis.

The second cohort contains the patients recruited following a decision to proceed into the second stage and the patients recruited before the interim analysis. These patients receive pertuzumab and Herceptin®.

Once the first two stages have been completed, a third cohort of 27 patients (to ensure that 24 are evaluable) is recruited to evaluate the activity and safety associated with single agent pertuzumab. Efficacy is assessed after all patients have completed eight cycles of single agent pertuzumab (approximately 24 weeks), or withdrawn consent, or are lost to follow-up, whichever is earlier. Cohort 3 patients are
treated with pertuzumab as a single agent. If they fail to respond or respond and then relapse, then Herceptin® may be administered at the investigator's discretion.

Additionally, efficacy in the patients in cohort 3 receiving the combined study treatment (pertuzumab and Herceptin®) is of secondary interest and is assessed. This can occur at any point after the last patient has completed at least eight cycles of study treatment (approximately 24 weeks), or withdrawn consent, or are lost to follow-up, whichever is earlier.

To participate in the trial, a patient must fulfill all inclusion/exclusion criteria and must consent to allow storage of serum and tumor tissue samples for biomarker research.

Once enrolled, a patient receive treatment until disease progression or withdrawal from the trial for any reason. If a patient withdraws from the trial for reasons other than disease progression, she is followed-up until disease progression. All patients are followed up for survival until the last patient has died, or withdrawn consent, or is lost to follow-up, or has reached 3 years after the last study dose, whichever is earlier.

In case of withdrawal from the trial due to cardiac toxicity the patient must be followed-up for cardiac outcome whenever possible.

Approximately 28 days after the last dose of study medication, the patient will be asked to perform a final safety assessment (called Final Visit).

The primary clinical cut-off of this study is when all patients enrolled into stages one and two have completed approximately 24 weeks of pertuzumab and Herceptin® treatment or withdrawn due to any reason prior to that, whichever is earlier. The main clinical study report is prepared following the analysis of these data. All data collected after the clinical cut-off, including data on the 3rd cohort, are added to the study report as an addendum.

The combination of pertuzumab and Herceptin® is a two stage study design which allows an early termination of the trial in case of lack of efficacy or safety concerns.
Twenty-four evaluable patients are recruited in the first stage of the trial, cohort 1. After all 24 evaluable patients have completed at least 2 cycles of therapy and one efficacy evaluation after the 2nd cycle of therapy, or withdrawn prematurely due to death or progressive disease before completing 2 cycles of therapy, recruitment is stopped and the interim analysis is conducted. If, before the planned interim analysis time-point, there is clear evidence of efficacy, recruitment is not halted.

At the interim analysis, efficacy and safety parameters are evaluated in cohort 1.

Depending on the outcome of the interim analysis, recruitment may continue into the second stage, in which case 34 additional evaluable patients will be needed to complete cohort 2. Otherwise, the study is terminated.

**Target population**

Female patients with HER2-positive metastatic breast cancer who have progressed on Herceptin®-based therapy administered as last treatment for metastatic disease. A maximum of 3 prior chemotherapy regimens (including neo-adjuvant, adjuvant and metastatic disease) are allowed.

**Inclusion criteria**

1. Histologically confirmed breast cancer.

2. HER2-positive tumors. HER2 status must be re-confirmed in a central lab before study entry.

3. Availability of a FFPE tumor tissue sample from primary tumor for eligibility (HER2-status) testing and biomarker assessment.

4. Patients with metastatic breast cancer, who have progressed on Herceptin®-based therapy as last treatment for metastatic disease.

5. Patients with ≤3 chemotherapy regimens prior to study entry.

6. The last dose of Herceptin® must have been given ≤9 weeks prior to study day 1 for patients in cohorts 1 and 2 and ≥4 weeks before study day 1 for patients
in cohort 3.

7. At least one measurable lesion according to RECIST. A measurable lesion in a previously irradiated area has to reveal clear signs of progression (increase in size of ≥ 50% or new lesions)

8. At least 4 weeks since prior radiotherapy, with full recovery.

9. At least 4 weeks since major surgery, with full recovery

10. LVEF of ≥ 55% or local parameter for ≥ LLN by echocardiography or MUGA.

11. Performance status ECOG ≤ 2

12. Age ≥ 18 years

13. Signed informed consent

**Exclusion criteria**

1. Prior treatment with any targeted therapy other than Herceptin®, such as cetuximab, bevacizumab, gefitinib or with anticancer vaccine.

2. Prior exposure to the following cumulative doses of anthracyclines: doxorubicin or liposomal doxorubicine > 360 mg/m², epirubicin > 720 mg/m², mitoxantrone > 120 mg/m², idarubicin > 90 mg/m².

3. Known asymptomatic decreases in LVEF to below 50% absolute value during Herceptin® treatment.

4. Patients with known history of any cardiac adverse event which according to the criteria of the investigator was related to Herceptin® therapy.

5. History of congestive heart failure (any NYHA grading), unstable angina, evidence of transmural infarction on ECG, poorly controlled hypertension (systolic > 180 mm Hg and/or diastolic > 100 mm Hg), or hemodynamically significant valvular disease.

6. Other malignancy within the last 5 years, except for carcinoma in situ of the cervix or basal cell carcinoma
7. Absolute Neutrophil Count (ANC) < $1.5 \times 10^9$/L, Platelet count < $100 \times 10^9$/L or Hb < 10 g/dL.

8. Impaired liver function: serum [total] bilirubin > 1.5 x ULN, AST, ALT > 2.5 x ULN, (> 5 x ULN in patients with liver metastases).

9. Serum creatinine > 2 x ULN

10. History or clinical evidence of brain metastases.

11. Severe uncontrolled systemic disease (e.g. hypertension, clinically significant cardiovascular, pulmonary, metabolic, wound-healing, ulcer, or bone fracture).

12. Patients with severe dyspnea at rest requiring supplementary oxygen therapy due to complications of advanced malignancy.

13. Positive pregnancy test in women of childbearing potential

14. Patients with reproductive potential not willing to use effective method of contraception.

15. Pregnant or lactating women.

16. Patients with known infection with HIV, HBV, HCV.

17. Known hypersensitivity to Herceptin®, murine proteins, or to any of its excipients.

18. Treatment with any investigational drug within 28 days prior to the start of the study.

Patients assessed by the investigator to be unable or unwilling to comply with the requirements of the protocol.

**Allowed therapies**

In general, all medications taken by the patient for concomitant diseases should continue during the study and should be recorded on the eCRF.
The following list of allowed medications is provided as guidance, treatments prescribed to the patients should be adapted according to the local standard practice.

- HI and H2 antagonist (e.g. diphenhydramine, cimetidine)
- Analgesics (e.g. paracetamol, meperidine, acetaminophen, opioids)
- Corticosteroids to treat or prevent allergic or infusion reactions
- Antiemetics (approved prophylactic serotonin-antagonists, benzodiazepines, ondansetron etc)
- Medications to treat diarrhea (e.g. loperamide)

**Excluded therapies**

- Any anti-cancer therapies other than those administered in this study
- Any targeted therapy
- Treatment with steroids except for thyroid hormone replacement therapy and short term corticosteroids, in order to treat or prevent allergic or infusion reactions
- High doses of systemic corticosteroids. High dose is considered as > 20 mg of dexamethasone a day (or equivalent) for > 7 consecutive days.
- Any investigational agent, except for those used for this study

Initiation of herbal remedies. Herbal remedies initiated prior to study entry and continuing during the study are permitted and must be reported on the appropriate eCRF form. They should have been initiated a minimum of 2 weeks before study entry.

**Dose/route/regimen**

Cohorts 1 and 2: Herceptin® (trastuzumab): Herceptin® is continued to be administered at the same schedule the patient was following before entry into this study. This means that Herceptin® is administered weekly at the dose of 2 mg/kg during 30 min infusion or every 3 weeks at the dose of 6 mg/kg during a 90 min infusion.
Cohorts 1 and 2: pertuzumab is administered at an initial dose of 840 mg (loading dose) as an i.v. infusion on day 2. Starting on day 22 and every 3 weeks thereafter pertuzumab is administered as an i.v. infusion at a dose of 420 mg. The initial pertuzumab dose is administered over 60 (± 10) minutes and patients are observed for another 60 minutes. If the infusion is well tolerated, subsequent doses are administered over 30 (± 10) minutes and patients are observed for another 60 minutes for fever, chills and other infusion-associated reactions.

Cohorts 1 and 2: At cycle 1, Herceptin® is administered on Day 1 and pertuzumab on Day 2. Starting at Cycle 2 and thereafter, Herceptin® and pertuzumab are administered on the same day. Herceptin® is administered first followed by pertuzumab.

Cohort 3: pertuzumab is administered at an initial dose of 840 mg (loading dose) as an i.v. infusion on day 1. Starting on Day 22 and every 3 weeks thereafter pertuzumab is administered as an i.v. infusion at a dose of 420 mg. The initial pertuzumab dose is administered over 60 (± 10) minutes and patients are observed for another 60 minutes. If the infusion is well tolerated, subsequent doses are administered over 30 (± 10) minutes and patients are observed for another 60 minutes for fever, chills and other infusion-associated reactions.

If the patient fails to respond to treatment or responds and then progresses, at the investigator's discretion, Herceptin® may be added to the treatment regimen. Weekly or three weekly scheduling is acceptable. Herceptin® is administered weekly at the dose of 2 mg/kg during 30 min infusion or every 3 weeks at the dose of 6 mg/kg during a 90 min infusion following loading doses of 4 mg/kg and 8 mg/kg, respectively.

Assessment of efficacy


Secondary: Time to response, duration of response, time to progression, progression-free survival and overall survival.
Exploratory: To evaluate the predictive effect of expression and activation of HER family receptors, HER-ligands and HER downstream signaling components for clinical outcome of treatment with Herceptin® and pertuzumab in HER2-positive breast cancer patients.

Statistical analyses

This is a phase II trial using a two-stage design, with an interim analysis. The purpose of this trial is to provide estimates of both objective response rate (ORR) and clinical benefit response rate (patients with CR, PR or stable disease lasting for at least 8 cycles of therapy or > 6 months), as well as safety.

Sample Size:

At the interim analysis, if there > 2 responders, or 1 responder and > 12 patients in stable disease (SD), or 0 responders and > 13 patients in SD, recruitment will continue. With these cut-off values, the probability of early termination, given an ineffective treatment, is 0.127. At the end of the 2nd stage, with the critical values set at > 8 responders or > 14 patients with clinical benefit response (CBR) out of 58 evaluable patients, the trial will have power of approximately 67% to reject the null hypothesis when the ORR is > 13% or CBR rate is > 25%, at a one-sided alpha level of < 0.10.

To account for a possible 5% inexvaluable rate, an additional 4 patients are recruited, for a total of 62 patients.

Cohort 3 only:

A preliminary assessment of efficacy in the pertuzumab alone patients in cohort 3 is performed when all patients have reached the 8 cycle endpoint. Hence, a further 27 patients (for 24 evaluable) will be recruited.

Any cohort 3 patients that continue on with dual agent study treatment (pertuzumab/Herceptin®) post progression on single agent pertuzumab, will be assessed for tumor response for exploratory purposes only.
Analysis Plan:

Hypothesis Test

An objective response rate of no clinical interest is defined to be $< 7\%$ and a clinical benefit response rate of no clinical interest to be $< 15\%$. So in a multinomial setting, the progressive disease rate is assumed to be $> 85\%$. The null hypothesis is defined as:

$$H_0: p_{o1} > 0.85 \text{ and } p_{o2} < 0.08 \text{ and } p_{o3} < 0.07,$$

where $p_{o1}$ is the probability of having progressive disease as the best response at the end of the trial, $p_{o2}$ is the probability of having stable disease lasting at least for 8 cycles of therapy or $> 6$ months as the best response, and $p_{o3}$ is the probability of having objective response as the best response.

An objective response rate that is clinically meaningful is defined to be at least $13\%$ and a clinical benefit response rate of clinical interest to be at least $25\%$. The progressive disease rate is therefore assumed to be $< 75\%$. The alternative hypothesis is defined to be:

$$H_a: p_{o1} < 0.85 \text{ and } p_{o2} > 0.08 \text{ and } p_{o3} > 0.07,$$

where $p_{o1}, p_{o2}, p_{o3}$ are as defined above.

Since the null hypothesis may be rejected at points that do not satisfy the critical values defined under Sample Size, the trial will only be considered to have met the primary end-point if the ORR or the CBR rate fall within the region bounded by the critical values.

Interim Analysis

An interim analysis is conducted once 24 evaluable patients have completed at least 2 cycles of therapy and had one efficacy assessment after the 2nd cycle (or dropped out due to progressive disease or died before completing 2 cycles of therapy). If there are $\geq 2$ responders, or 1 responder and $\geq 12$ patients in stable disease (SD), or 0 responders and $\geq 13$ patients in SD, recruitment will continue into the 2nd stage. The trial will only be stopped for lack of efficacy or safety concerns. Only patient listings related to the primary efficacy parameter will be
provided. At the interim analysis, an independent DSMB will review selected safety listings and summaries and will recommend to the sponsor to continue or stop the trial, based on safety only.

**Primary Efficacy:**

The objective response rate and clinical benefit response rate will be calculated at the 2nd stage only. If one or more of the critical values are exceeded and the p-value is \( \leq 0.10 \), the null hypothesis may be rejected. Two-sided 80% confidence intervals will be calculated around the ORR and the CBR rate. Additionally, a p-value will be calculated using a single multinomial distribution for \( p_{01}, p_{02}, e^{p_{03}} \).

**Secondary Efficacy:**

**All cohorts:**

Time to response, time to progression, progression-free survival and overall survival are analyzed for all patients. Estimates for the survivor function for the time to event using the Kaplan-Meier approach are displayed graphically. Estimates for the median time to event (if reached) is given and the corresponding two-sided 80% confidence interval around the median is calculated. Duration of response for responding patients only is also analyzed using the Kaplan-Meier approach. For graphical purposes, the start of the response is set to study day 1 and the time to event (the duration) will be the number of days from the start to the end of response.

**Cohort 3 only:**

Following the unexpectedly high rate of activity observed in the first part of the study (cohorts 1 and 2) associated with combined pertuzumab/Herceptin® therapy, a final cohort of 24 evaluable patients (27 to be enrolled) is recruited in order to evaluate the activity of single agent pertuzumab in this group of patients. Patients continuing with dual agent study treatment (pertuzumab/Herceptin®) post progression on single agent pertuzumab, are assessed for tumor response for exploratory purposes only.
Exploratory Biomarker Analysis:

Biomarkers are evaluated on a univariate level regarding their potential for prediction (e.g. search or adaptation of cut-offs) of the clinical endpoints. Further multivariate techniques (e.g. Linear Discriminant Analysis, Multiple Logistic Regression, Principal Component Analysis with Rotation, Cluster Analysis, CART methodology) are employed in order to study combinations of markers.

Biomarker and Response correlations with clinical covariates are investigated. It is checked whether covariates can improve the prediction and whether there is an interaction with the biomarkers. Relevant covariates could become a part of the statistical prediction model.

Safety Analysis:

Adverse event data are presented in frequency tables (overall, by intensity, or by relationship) by body system. Changes in laboratory values are presented in shift tables and summary tables. The total number of cycles with reported abnormal laboratory values are summarized by CTCAE grade for selected laboratory tests. ECOG is summarized by descriptive statistics and change in ECOG status over time is summarized by shift tables. Vital signs are reported in patient listings and information on study drug are summarized by duration and cumulative dose using descriptive statistics.

Rationale for assessment of biomarkers for prediction of response to treatment with pertuzumab and Herceptin®

Breast cancer patients with tumors that display over expression of the HER2 protein or amplification of the HER2 gene are eligible for treatment with Herceptin®, as it has been established that patients with HER2-positive tumors benefit from treatment with Herceptin®.

In a number of xenograft models, including breast cancer, it has been observed that response to pertuzumab is independent of the HER2 expression levels. Rather, the results of xenograft experiments suggest that HER2 activation may be indicative of response to pertuzumab. Activation of HER2 may be assessed
directly by determination of the levels of HER2 (hetero) dimers or phosphorylated
HER2. As the formation of HER2 heterodimers requires the binding of a HER
ligand to EGFR/HER1 or HER3 - which induces a conformational change in these
receptors resulting in the exposition of their dimerization domain, now ready to bind
to the dimerization domain of HER2 - the levels of expression of HER ligands may
represent an indirect means to assess HER2 activation. HER2 activation leads to
activation of multiple signal-transduction pathways, therefore the activated forms of
HER downstream signaling components may also be indicative of HER2
activation. Indeed, a number of factors derived from the HER downstream
signaling pathways and alternative signaling pathways have been suggested to
predict responsiveness to HER-targeting agents, including PTEN (Nagata
She QB, et al., Clin Cancer Res 2003; 9:4340-6), PIP/Akt (Clark AS, et al.,
Molecular Cancer Therapeutics 2002; 1: 707-717), various growth factors and their
receptors such as IGF-1 (Yuhong Lu et al., Journal of the National Cancer Institute
303).

Data derived from the phase II study TOC2689g of pertuzumab in recurrent
ovarian cancer suggest that HER2 activation in tumor cells determined by HER2
phosphorylation and/or HER2 heterodimerization indeed may be predictive for
response to pertuzumab. Quantitative RT-PCR data from TOC2689g suggest that
expression levels of a set of markers may be linked to pHER2 activation, and that
expression levels, as determined in formalin-fixed paraffin embedded tumor tissue,
may predict outcome of pertuzumab treatment. A similar observation was made
in the pertuzumab trial BO16934 in HER2-negative expressing metastatic breast
cancer patients.

Assessment of serum levels of HER2-ECD and the HER-ligands in the phase
II study BO16934 of pertuzumab in HER2 negative metastatic breast cancer and the
phase II study TOC2689g of pertuzumab in recurrent ovarian cancer suggests that
a panel of these markers may identify a subset of patients that may have
better response characteristics. The present study mandates the collection of
baseline serum to further evaluate the relationship of clinical outcome and levels of HER2-ECD and selected HERligands.

**HER2 status testing and biomarker assessment**

Archival tumor samples from the primary tumor are collected for eligibility testing (assessment of HER2 status) and the assessment of tumor tissue biomarkers for pertuzumab response prediction in a central pathology laboratory. The preferred tumor tissue specimen is a tumor block.

**HER2 Status Testing**

The tumor specimen is used for eligibility (HER2 status) testing first. HER2 testing is performed in the following order: (i) IHC HER2 testing - if testing result according to the DAKO scoring is HER2 3+, the patient is eligible. If result is HER2 2+, a (ii) HER2 FISH or a HER2 CISH test is performed - HER2 2+/FISH or CISH positive patients are eligible for this study. All other patients (e.g. HER2 2+/HER2 FISH or CISH negative) are excluded from the study. In particular cases, one may perform FISH or CISH test only or regardless of the IHC result, i.e. if there is a discrepancy between the IHC result performed at the investigational site and the IHC result performed at the central laboratory. In case of discrepancy, all patients who test FISH or CISH positive at the central laboratory are included in the trial.

In case a patient is not eligible for the study based on the central lab HER2 testing result, the tumor sample (block or remainder of the slides) will be returned to the site without performing further biomarker assessments on.

**Biomarker Assessments**

For eligible patients assessments of biomarkers that may indicate response to pertuzumab is performed, as follows:

The following assessments is performed on the tumor tissue:

- Expression of the HER-family receptors
- Expression of HER ligands
- Expression profiles (genes and proteins) associated with HER2 activation
- Expression of markers of the HER signal transduction, alternative signaling pathways and markers for response and resistance to HER inhibitors

- Activation of HER family receptors (e.g. phosphorylation or dimerization forms) The following measurements may be performed on the tumor tissue sample:

  - Determination of tumor cell coding sequence mutations of HER family receptors and other components of the HER signaling pathway.

For assessment of baseline blood serum biomarkers that may indicate response to pertuzumab, a baseline blood serum sample will be collected during screening. Assessments will be performed in a central laboratory, as follows:

The following measurements are performed in baseline serum samples:

- Levels of ECD of HER2
- Levels of HER ligands, and/or other proteins thought to be important for HER family signaling

The following measurements may be performed in baseline serum samples:

- Markers of response to HER inhibitors and HER activation and/or markers that are potentially important for HER family signaling

From the tumor blocks, a maximum of 10 slides are cut and 2 cores of 1.5 mm each will be removed in order to construct TMAs. All tumor blocks are returned to the sites within 16 weeks of being received at the central lab.

Since uncontrolled oxidation processes on the slides may affect the assessment of markers for response to pertuzumab, we strongly recommend sending tumor blocks.
In case that slides are sent to the central lab, a minimum of 25 unstained, freshly cut slides of 4 µη each are required. The slides must be sent to the central lab within 2 days of being cut.

Blood serum samples and tumor tissue are stored at F Hoffmann-La Roche or representative for a maximum of 15 years and then they are destroyed

**Example 2**

**Biomarker correlations with clinical benefit of pertuzumab and trastuzumab treatment of patients with metastatic HER2-positive breast cancer**

**Methods:**

Patients with metastatic HER2-positive breast cancer who had progressed on Herceptin® (H)-based therapy as their last treatment for metastatic disease and who had measurable disease at baseline were enrolled, as described in Example 1. Tumour samples were collected at the time of primary surgery. Treatment with H was restarted and pertuzumab (P) was introduced concurrently. Response rate and stabilisation of disease lasting 6 months (SD) as well as progression-free survival were evaluated. Proteins and mRNA of potential prognostic or predictive significance (HER2, shed HER2, HER3, epidermal growth factor receptor, amphiregulin, betacellulin, c-Myc, p-Akt, PIK3CA, PTEN, transforming growth factor-a) were measured using immunohistochemistry and/or real-time reverse transcriptase quantitative polymerase chain reaction, immunoassay or fluorescence *in situ* hybridization.

*RNS isolation from formalin-fixed paraffin-embedded (FFPE) tissue samples for subsequent RT-PCR*

Starting material for were two tubes per patient containing dry material from FFPET scraped off the slide. RNA was isolated from each tube separately. RNA isolation was performed essentially according to the package insert of High Pure RNA Paraffin Kit (Id. No. 3270289).
After RNA isolation, the two eluates from one patient were combined resulting in 100 µl eluate per patient. One µl of eluate was used for OD260 and 280 measurement using a Nanodrop instrument. RNA concentration as well as purity were calculated. The pooled eluate was divided into two equal aliquots of about 50 µl (one for use on the next day in cDNA synthesis reactions, the other for later use) and frozen at ≤ -65°C.

**cDNA synthesis and qRT-PCR for biomarkers Her1-3, amphiregulin and β-cellulin**

Starting material: purified RNA prepared as described above. cDNA synthesis and RT-PCR were performed according to the package insert of the corresponding LightCycler kits (LabLot 1 for transfer study, pHER2-surrogate-BrCa study, and TOC3258 study, LabLot2 for B017931 and B017929).

The cDNA synthesis of the five targets and the reference gene has to be performed on one day. After cDNA synthesis material has to be stored at -15 to -25°C.

The five target PCRs and the reference PCR have to be run sequentially on the same instrument for each sample. It is not required to run all assays on one day, as there is cDNA for each target that is stored frozen.

The growth curves of Her2 and Her3 RT-PCRs have to be checked visually for correct cp-calculation (maximum of second derivative of first maximum is to be used for cp-calculation). Signal height for the Calibrator to be documented is also taken from the first maximum.

**Results**: 66 pts were recruited, among whom there were 4 complete responses, 12 partial responses and a further 17 pts experiencing SD 6 months, giving an overall clinical benefit rate of 50%.

The results are represented in FIGs 7-11.
FIG. 7 shows HER2-CR (qRT-PCR) - Objective Response (OR) and Clinical Benefit Response (CBR), estimated response rates as a function of HER2 biomarker values.

FIG. 8 shows the results of HER2-CR (qRT-PCR) - OR and CBR analysis by HER2 expression quartiles.

FIG. 9 evaluates progression-free survival (PFS) in breast cancer patients treated with pertuzumab + Herceptin® as described in Example 1 based on HER2 mRNA levels determined by qRT-PCR.

FIG. 10 illustrates HER2-CR (qRT-PCR) interactions with hormone receptor status. Kaplan-Meier estimates of PFS in breast cancer patients treated with pertuzumab + Herceptin® as described in Example 1 as a function of estrogen receptor and progesterone receptor status, respectively.

FIG. 11 is a scatter plot showing correlations of HER2 vs. HER3 mRNA levels in breast cancer patients treated with pertuzumab + Herceptin® as described in Example 1.

As illustrated by the results shown, assessment of the biomarkers suggests that low HER2 mRNA correlates with favourable clinical outcomes in HER2 positive breast cancer. Thus, HER2 mRNA expression has predictive and prognostic value for treatment with HER2 inhibitors, in particular pertuzumab, and with a combination of pertuzumab and Herceptin® (trastuzumab) in cancer patients. Since patients to this study have been included on the basis of high level of HER2 expression on the protein level or based on HER2 gene amplification (HER2 positive patients), the finding that low HER2 mRNA expression levels (as determined by qRT-PCR in the present case) in the same cancer respond better to the described treatment was highly surprising.
Claims:

1. A method for treating a patient with a HER2 positive cancer, comprising administering a therapeutically effective amount of a HER dimerization inhibitor to the patient, wherein the patient's cancer expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the same cancer type.

2. The method of claim 1 wherein the patient's cancer expresses HER2 mRNA at a level which is less than the 33rd percentile for HER2 mRNA expression in the same cancer type.

3. The method of claim 1 wherein the patient's cancer expresses HER2 mRNA at a level which is less than the 25th percentile for HER2 mRNA expression in the same cancer type.

4. The method of any one of claims 1-3, wherein HER2 mRNA expression has been determined using polymerase chain reaction (PCR).

5. The method of claim 4 wherein the PCR is quantitative real time polymerase chain reaction (qRT-PCR).

6. The method of any one of the preceding claims wherein the HER dimerization inhibitor is a HER2 dimerization inhibitor.

7. The method of any one of the preceding claims wherein the HER dimerization inhibitor inhibits HER heterodimerization.

8. The method of any one of the preceding claims wherein the HER dimerization inhibitor is an antibody.

9. The method of claim 8 wherein the antibody binds to a HER receptor selected from the group consisting of EGFR, HER2, and HER3.

10. The method of claim 9 wherein the antibody binds to HER2.

11. The method of claim 10 wherein the HER2 antibody binds to Domain II of HER2 extracellular domain.
12. The method of claim 11 wherein the antibody binds to a junction between domains I, II and III of HER2 extracellular domain.

13. The method of claim 11 wherein the HER2 antibody comprises the variable light and variable heavy amino acid sequences in SEQ ID Nos. 11 and 12, respectively.

14. The method of claim 13 wherein the HER2 antibody is pertuzumab.

15. The method of any one of claims 7 to 14 wherein the HER antibody is a naked antibody.

16. The method of any one of claims 7 to 15 wherein the HER antibody is an intact antibody.

17. The method of any one of claims 7 to 15 wherein the HER antibody is an antibody fragment comprising an antigen binding region.

18. The method of any one of claims 7 to 15 wherein the HER antibody is conjugated to a cytotoxic compound.

19. The method of claim 18 wherein the cytotoxic compound is a maytansinoid.

20. The method of claim 18 or claim 19 wherein the antibody comprises engineered cysteine residues.

21. The method of claim 20 wherein the antibody and the cytotoxic compound are linked by a cleavable or non-cleavable covalent linker.

22. The method of claim 21 wherein the covalent linker is selected from the group consisting of N-succinimidy1-3-(2-pyridylthio)propionate (SPDP), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP), iminothiolane (IT), dimethyl adipimidate HCL, disuccinimidyl suberate, glutaraldehyde, bis (p-azidobenzoyl)hexanediamine, bis-(p-diazoniumbenzoyl)-ethylenediamine, toluene 2,6-diisocyanate, and 1,5-difluoro-2,4-dinitrobenzene.
23. The method of any one of the preceding claims wherein the cancer is breast cancer.

24. The method of claim 23 wherein the cancer is HER2 positive advanced breast cancer that progressed during prior treatment with an anti-cancer agent.

25. The method of claim 24 wherein the anti-cancer agent is a different HER antibody.

26. The method of claim 25 wherein the anti-cancer agent is a different HER2 antibody.

27. The method of claim 26 wherein the anti-cancer agent is trastuzumab.

28. The method of any one of the preceding claims which results in objective response (OR) or clinical benefit response (CBR) in the patient.

29. The method of claim 28, which extends progression free survival (PFS) in the patient.

30. The method of claim 28 which extends overall survival (OS) in the patient.

31. The method of any one of the preceding claims wherein the patient progressed on prior treatment with a different HER antagonist.

32. The method of claim 31 wherein the different HER antagonist is trastuzumab.

33. The method of any one of the preceding claims wherein the HER dimerization inhibitor is administered as a single anti-tumor agent.

34. The method of any one of claims 1 to 32 comprising administering a second therapeutic agent to the patient.

35. The method claim 34 wherein the second therapeutic agent is selected from the group consisting of HER antibody, chemotherapeutic agent, antibody directed against a tumor associated antigen, anti-hormonal compound,
cardioprotectant, cytokine, EGFR-targeted drug, anti-angiogenic agent, tyrosine kinase inhibitor, COX inhibitor, non-steroidal anti-inflammatory drug, farnesyl transferase inhibitor, antibody that binds oncofetal protein CA 125, HER2 vaccine, HER targeting therapy, Raf or ras inhibitor, liposomal doxorubicin, topotecan, taxane, dual tyrosine kinase inhibitor, TLK286, EMD-7200, a medicament that treats nausea, a medicament that prevents or treats skin rash or standard acne therapy, a medicament that treats or prevents diarrhea, a body temperature-reducing medicament, and a hematopoietic growth factor.

36. The method of claim 35 wherein the second therapeutic agent is a HER2 antibody.

37. The method of claim 36 wherein the HER2 antibody is trastuzumab.

38. The method of claim 35 wherein the chemotherapeutic agent is selected from the group consisting of gemcitabine, carboplatin, paclitaxel, docetaxel, topotecan, and liposomal doxorubicin.

39. A method for treating a patient with HER2 positive breast cancer comprising administering a therapeutically effective amount of pertuzumab to the patient, wherein the patient's cancer expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in HER2 positive breast cancer.

40. A method for selecting a patient with HER2 expressing cancer for treatment with a HER dimerization inhibitor comprising (i) identifying the patient as HER2 positive by measuring HER2 protein expression or HER2 gene amplification in a cancer sample from the patient and (ii) measuring HER2 mRNA expression in said cancer sample, wherein the patient is selected for treatment with a HER dimerization inhibitor if the cancer sample expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the same cancer type.

41. An article of manufacture comprising, packaged together, a pharmaceutical composition comprising a HER dimerization inhibitor in a pharmaceutically acceptable carrier and a label stating that the inhibitor or pharmaceutical composition is indicated for treating a patient with a type of cancer which is able to respond to a HER dimerization inhibitor, wherein the patient's cancer
is HER2 positive and expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the same cancer type.

42. A method for manufacturing a HER dimerization inhibitor or a pharmaceutical composition thereof comprising combining in a package the inhibitor or pharmaceutical composition and a label stating that the inhibitor or pharmaceutical composition is indicated for treating a patient with a type of cancer which is able to respond to a HER dimerization inhibitor, wherein the patient's cancer is HER2 positive and expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the same cancer type.

43. A method for advertising a HER dimerization inhibitor or a pharmaceutically acceptable composition thereof comprising promoting, to a target audience, the use of the HER dimerization inhibitor or pharmaceutical composition thereof for treating a patient population with a type of cancer, where the patient's cancer is HER2 positive and expresses HER2 mRNA at a level less than the median level for HER2 expression in the same cancer type.

44. An article of manufacture comprising, packaged together, a pharmaceutical composition comprising pertuzumab in a pharmaceutically acceptable carrier and a label stating that the pertuzumab or pharmaceutical composition is indicated for treating a patient with a type of cancer which is able to respond to pertuzumab, wherein the patient's cancer expresses HER2 mRNA at a level less than the median level for HER2 mRNA expression in the cancer type.

45. The article of manufacture of claim 44 further comprising instructions to administer the pertuzumab in combination with trastuzumab.

46. The article of manufacture of claim 44 further comprising instructions to administer the pertuzumab, optionally in combination with trastuzumab, to a patient whose cancer progressed during prior treatment with trastuzumab.

47. The article of manufacture of any of claims 44-46, wherein the cancer is breast cancer.
Domain I (L1)  TQVCTGTDMLKRLPLSPEETHLDMRLHYGQGVQVQGNLELTYLPTNASLSFQLQDIQEVQGVY
LIAHNNQVRQVPLRGLIVRVGTLQFEDNYALAVLDNGPDPLNTTPFVTGASPGGLRLQRLRSTLT
EILGKGGVLIQRNFQLYCVDILWKLDPHFKNQLAALTLLIDTNRSRACHPSCPMSCKGSRCWGES
SEDCQSLTR (SEQ ID NO. 1)

Domain II (CR1)  TVCAAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHLFNHSCIGCELHCAPALVTYNTDTF
ESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPPLHNQEVTAEDGTQRCERKCSKPCARV
(SEQ ID NO. 2)

Domain III (L2)  CYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLALPESFDGPSNTAPLQPEQLFQVETLE
EITGYLYISAWPSLDLPSVFQNLQMRGRILHNGAYSLLQLGQISWGLRSLRELGSGLA
IHHNHTECFVHTVPWQDLFRPQANLLHTANKPEDECVGEGLA (SEQ ID NO. 3)

Domain IV (CR2)  CHQLCARGHCGWPQPTQCVNCSQFLRGQECVYECCRVLQGLPREYNARRHCLPCHPECQPOQGNS
VTCCFGPEADQCVCACAYKDPFCVACRPSVGKPDLSYMIPWKFDEEGACQCPINCTHSYCV
LDDKGCPCAEQRASPLT (SEQ ID NO. 4)

FIG. 1
Variable Light

2C4  DTVMTSQSHKMSTSVGDRSVITC [KASQDVSIQVA] WYQQRP
** **** * * *  
574  DIQMTQSPSSLASAVGDRVTITC [KASQDVSIQVA] WYQQKP
* ** ***

hum kI  DIQMTQSPSSLASAVGDRVTITC [RASQISNYLA] WYQQKP

50  60  70  80

2C4  GQSPKLLIY [SASYRT] GVPQRTFGSGSTGTDFTLTTISVSQA
** * * * * * *  
574  GKPQKLLIY [SASYRT] GVPQRFSGSGSTGTDFTLTILLQP
* ****

hum kI  GKPQKLLIY [AACSLPES] GVPQRFSGSGSTGTDFTLTILLQP

90  100

2C4  EDLAVYYC [QQYIYYPY] FGGTKEIK (SEQ ID NO. 5)
* * * * *  
574  EDFATYYC [QQYIYYPY] FGGTKEIK (SEQ ID NO. 7)
*** *  

hum kI  EDFATYYC [QQYNLFWT] FGGTKEIK (SEQ ID NO. 9)

FIG. 2A

Variable Heavy

2C4  EVQLQSSGPELVKPGTSVKISCKAS [GFTFDTYMD] WVKQS
** ** * * *** *  
574  EVQLVESGGLVQPGGSLRLSCAAS [GFTFDTYMD] WVRQA
** * *  

hum III  EVQLVESGGLVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

50 a  60  70  80

2C4  HGKSKLEWIG [DVNPNSGGSIVQRFKQ] KASLTVDRSSRIYVM
* * * ** * *** * ** *  
574  PGKQLEWVA [DVNPNSGGSIVQRFKQ] RPTLVSQDSSKLTLYL
****** *** **** * * *  

hum III  PGKQLEWVA [VSGDGGSTYADSVKQ] RFTISRDNSKNTLYL

abc  90  100ab  110

2C4  ELRLSTFEDTAVYYCAR [NLGPSFYFDY] WGGTTLTVSS (SEQ ID NO. 6)
*** **  
574  QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGGTTLTVSS (SEQ ID NO. 8)
******

hum III  QMNSLRAEDTAVYYCAR [GRVGYSLDY] WGGTTLTVSS (SEQ ID NO. 10)

FIG. 2B

SUBSTITUTE SHEET (RULE 26)
Amino Acid Sequence for Pertuzumab Light Chain

1  10  20  30  40  50  60
DIQMTQSPSLSASVQGRVTITCKASQDVSGVAWYQQKKPGLKLIALYSASYRTGVPS

70  80  90  100  110  120
RFSKGSGSTDPTTLTISSLQPEDFATYYCQQYIYPFGQGTVKVEIKRTVAAPSVIFPP

130  140  150  160  170  180
SEOLKSGTASSVCLPILNFFPGRAKSQVQWVQVDNAWQSNQSEVTEQDSKDYSTSLT

190  200  210
LSKADYEKHKVYACEVTHQQGLSSPVTSFNRGEC (SEQ ID NO. 11)

FIG. 3A

Amino Acid Sequence for Pertuzumab Heavy Chain

1  10  20  30  40  50  60
EVQLVESGGGLVQPSGLSSLRALSGVFRCAASGFTFTDYGMDVRQAPGGKVLEWVAADVNPNSGSIGY

70  80  90  100  110  120
NQRFKGRFTLSVDRSKNTLYDQMNNSSLRAEDTAVYACARNLGSFYFDYWQGTVLSVTSSA

130  140  150  160  170  180
STKPSVPFPALAPSSKTSSGTAALGCLVRLYFPVEPVTISWNSGALTSGVHTFVPAQLSSG

190  200  210  220  230  240
LYSLSSVVTSPSSGLGTQYVICNQNHKPSMTKVKVEKSCPDCDSKTHQCRCAPELLGGP

250  260  270  280  290  300
SVFLPPKPPTMISRTPETCVVSVDHEDPVEKFNWTVGDGEVHNAKTPREEQYN

310  320  330  340  350  360
TYRVSVLTVLHQQWLNGKEYKCKVSNKALPAIEKTISAKAGQPREPVYTLPPSREEM

370  380  390  400  410  420
TKNQVSLSCTLKVKGYPVPDIAVEWESNGQQPENNYKTTPPVLDSDGSGFLLYSKLVDTKSRWQ

430  440  448
QGNVFCSVMHEALHNHYTQKSLSLSPG (SEQ ID NO. 12)

FIG. 3B

SUBSTITUTE SHEET (RULE 26)
Coupling of HER2/3 to the MAPK and Akt Pathways

Proliferation

MAPK

MEK

Raf

c-Myc

Elk1

p90RSK

MSK1

Ras

GRB2 SOS

SOS

HER3

HRG

GSK

PI3K (p85)

Akt

PDK

IKK

Caspase 9

Bad

FKHR

Survival
Light Chain

1  D I Q M T Q S P S S L S A S V G D R V T I T C R A S Q D V N T A V A W Y Q Q K P G K A P K

46  L L I Y S A S F L Y S G V P S R F S G S R S G T D F T L T I S S L Q P E D F A T Y Y C Q Q

91  H Y T T P P T F G Q G T K V E I K R T V A A P S V F I F P P S D E Q L K S G T A S V V C L


FIG. 6A
Heavy Chain

1  EVQLVESGGGLVQPGSSSLC
15  ASGFNIKDITYIHWRQRPA
30  GKL
46  EWVARIYPNTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAED
75  TAVYVCSSRGGGDGFRYAMDYGQGLTVTVSSASTKGPSVFPLAPSS
90  S
105  KSTSGGTAALGCLVKDYFPEPVPTVSVNSGALTSGVHTFPAVLS
135  QS
150  S
165  GLYSLSSVVTVPSSSLGTQTICNVPNHKPSNTKVDKKEPKSCDK
180  S
195  THTCPCCPAELEGSGPSVFLLFPPKPDKTLQMSRTEPVTCVVDVS
225  S
240  HEDPEVKFNQWYGVHEVKHNAKTKPREEQYNSTYRVSVLTVLHQD
270  DS
285  WLNKEYKCKVSNKALPAPIEKTIASKAGQPREPQVYTLPPSREE
315  E
330  MTKNQVSLTCLVKGFYPSDIAVHWESNGQPPENNYKTTPPVLDSDG
360  S
375  SFLLYSKLTVDKSRWQQGNVFSACSVMHHEALHNHYTQKSLSSPSG
405  SEQ ID NO. 14

FIG. 6B
Study BO17929: Pertuzumab + Herceptin in Breast Cancer

Objective Response: Estimated Response Rate as Function of Biomarker Values

HER2-CR (qRT-PCR) 

No Resp.

High Response

Medium Response

Low Response

Response Rate (%)
HER2-CR (qRT PCR) - OR and CBR (mRNA)

Clinical Benefit Response (CBR)

Study BO17929: Pertuzmab + Herceptin in Breast Cancer
Clinical Benefit Response - Estimated Response Rate as Function of Biomarker Values
HER2-CR (qRT-PCR) - # Resp./Non-Resp.: 29/29 - (Half-Sided Bandwidth=12.5%)

**FIG. 7B**
**HER2-CR (qRT PCR) - OR and CBR**

**Objective Response**
**HER2-CR (qRT-PCR)**

<table>
<thead>
<tr>
<th>Quartile</th>
<th>N</th>
<th># of Responses</th>
<th>Response Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - &lt;25th Pctl</td>
<td>14</td>
<td>5</td>
<td>36%</td>
</tr>
<tr>
<td>25 - &lt;50th Pctl</td>
<td>15</td>
<td>7</td>
<td>47%</td>
</tr>
<tr>
<td>50 - &lt;75th Pctl</td>
<td>14</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>75 - 100th Pctl</td>
<td>15</td>
<td>2</td>
<td>13%</td>
</tr>
</tbody>
</table>

**Clinical Benefit Response**
**HER2-CR (qRT-PCR)**

<table>
<thead>
<tr>
<th>Quartile</th>
<th>N</th>
<th># of Responses</th>
<th>Response Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - &lt;25th Pctl</td>
<td>14</td>
<td>8</td>
<td>57%</td>
</tr>
<tr>
<td>25 - &lt;50th Pctl</td>
<td>15</td>
<td>13</td>
<td>87%</td>
</tr>
<tr>
<td>50 - &lt;75th Pctl</td>
<td>14</td>
<td>2</td>
<td>14%</td>
</tr>
<tr>
<td>75 - 100th Pctl</td>
<td>15</td>
<td>6</td>
<td>40%</td>
</tr>
</tbody>
</table>

**HER2 ≥ 13.5 vs. HER2 < 13.5 (Cutpoint is Median)**

<table>
<thead>
<tr>
<th>Biomarker Subgroup</th>
<th>Response</th>
<th>Count</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2 &lt; 13.5</td>
<td>No</td>
<td>17</td>
<td>59%</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>12</td>
<td>41%</td>
</tr>
<tr>
<td>HER2 ≥ 13.5</td>
<td>No</td>
<td>27</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2</td>
<td>7%</td>
</tr>
</tbody>
</table>

Fisher's Exact Test: Two-sided P-value = 0.0046
Odds Ratio (80% CI): 0.105 [0.036, 0.302]

**HER2 ≥ 13.5 vs. HER2 < 13.5 (Cutpoint is Median)**

<table>
<thead>
<tr>
<th>Biomarker Subgroup</th>
<th>Response</th>
<th>Count</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2 &lt; 13.5</td>
<td>No</td>
<td>8</td>
<td>27.6%</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>21</td>
<td>72.4%</td>
</tr>
<tr>
<td>HER2 ≥ 13.5</td>
<td>No</td>
<td>21</td>
<td>72.4%</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>8</td>
<td>27.6%</td>
</tr>
</tbody>
</table>

Fisher's Exact Test: Two-sided P-value = 0.0014
Odds Ratio (80% CI): 0.145 [0.068, 0.308]
Study BO17929: Pertuzumab + Herceptin in Breast Cancer
Expected Residual Lifetime for PFS (Half-Sided Bandwidth=12.5%)
HER2-CR (qRT-PCR) - # Patients = 58

FIG. 9A
Study BO17929: Pertuzumab + Herceptin in Breast Cancer
Kaplan-Meier Plot for PFS - Variable: HER2-CR (qRT-PCR)
Unstratified Analysis - HER2_CR > 13.5 vs. HER2_CR < 13.5 (Cutpoint is Median) - Total N = 58

FIG. 9B
FIG. 9C

<table>
<thead>
<tr>
<th>Quartile</th>
<th>N</th>
<th>Residual Lifetime (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - &lt;25th Pctl</td>
<td>14</td>
<td>200</td>
</tr>
<tr>
<td>25 - &lt;50th Pctl</td>
<td>15</td>
<td>635</td>
</tr>
<tr>
<td>50 - &lt;75th Pctl</td>
<td>14</td>
<td>154</td>
</tr>
<tr>
<td>75 - 100th Pctl</td>
<td>15</td>
<td>239</td>
</tr>
</tbody>
</table>

HER2-CR (qRT-PCR)

<table>
<thead>
<tr>
<th>% Tile</th>
<th>Abs. Value</th>
<th>N Event (Low)</th>
<th>N Event (High)</th>
<th>Hazard Ratio</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>9.92</td>
<td>20 18</td>
<td>38 35</td>
<td>1.31 (0.896, 1.90)</td>
<td>0.3633</td>
</tr>
<tr>
<td>40</td>
<td>11.2</td>
<td>23 20</td>
<td>35 33</td>
<td>1.57 (1.09, 2.28)</td>
<td>0.1163</td>
</tr>
<tr>
<td>45</td>
<td>12.6</td>
<td>26 23</td>
<td>32 30</td>
<td>1.80 (1.25, 2.59)</td>
<td>0.0399</td>
</tr>
<tr>
<td>50</td>
<td>13.5</td>
<td>29 25</td>
<td>29 28</td>
<td>2.11 (1.46, 3.05)</td>
<td>0.0096</td>
</tr>
<tr>
<td>55</td>
<td>18.0</td>
<td>31 27</td>
<td>27 26</td>
<td>1.96 (1.36, 2.83)</td>
<td>0.0185</td>
</tr>
<tr>
<td>60</td>
<td>21.1</td>
<td>34 30</td>
<td>24 23</td>
<td>2.14 (1.46, 3.12)</td>
<td>0.0104</td>
</tr>
<tr>
<td>65</td>
<td>27.1</td>
<td>37 33</td>
<td>21 20</td>
<td>1.79 (1.22, 2.62)</td>
<td>0.0517</td>
</tr>
</tbody>
</table>
HER2-CR (qRT PCR)

**Interactions with Hormone Receptor Status**

**Estrogen Receptor**

Study BO17929: Pertuzmab + Herceptin in Breast Cancer

KM Plot for PFS - Estrog-/Estrog+ & HER2_CR<13.5/HER2_CR>=13.5 (Cutpoint is Median)

N=58, #Events=53 - Interaction: HR=0.927, p=0.896 - Unstratified Analysis for HER2-CR (qRT-PCR) and Estrogen Rec. Status

<table>
<thead>
<tr>
<th>Condition</th>
<th>Sample Size</th>
<th>HR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrog- / HER2_CR&lt;13.5</td>
<td>11</td>
<td>1.988</td>
<td>0.090</td>
</tr>
<tr>
<td>Estrog+ / HER2_CR&lt;13.5</td>
<td>18</td>
<td>0.671</td>
<td>0.333</td>
</tr>
<tr>
<td>Estrog- / HER2_CR&gt;13.5</td>
<td>19</td>
<td>1.843</td>
<td>0.154</td>
</tr>
<tr>
<td>Estrog+ / HER2_CR&gt;13.5</td>
<td>10</td>
<td>0.622</td>
<td>0.249</td>
</tr>
</tbody>
</table>

**FIG. 10A**

Kaplan - Meier Estimate (%) vs. PFS (Days)
**HER2-CR (qRT PCR)**  
*Interactions with Hormone Receptor Status*

**Progesterone Receptor**

Study BO17929: Pertuzmab + Herceptin in Breast Cancer  
KM Plot for PFS - Prog-/Prog+ & HER2_CR<13.5/HER2_CR>=13.5 (Cutpoint is Median)  
N=52, #Events=47 - Interaction: HR=2.235, p=0.238 - Unstratified Analysis for HER2-CR (qRT-PCR) and Progest. Rec. Status

- **Prog-/HER2_CR<13.5 (n=17)**  
  - HR=1.371  
  - (p=0.367)

- **Prog-/HER2_CR>=13.5 (n=20)**  
  - HR=0.384 (p=0.047)

- **Prog+/HER2_CR<13.5 (n=9)**  
  - HR=3.063 (p=0.057)

- **Prog+/HER2_CR>=13.5 (n=6)**  
  - HR=0.859 (p=0.750)

**FIG. 10B**
Correlations with HER2 mRNA Levels

**HER2 vs. HER3**

Study BO17929: Pertuzmab + Herceptin in Breast Cancer
Scatterplot - HER2-CR (qRT-PCR) vs. HER3-CR (qRT-PCR) (Grey Lines for Quartiles)
n=58, Spearman Correlation = 0.737 (P-Value: <.0001)

**FIG. 11**
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/395 G01N33/574 C07K16/32

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

Further documents are listed in the continuation of Box C.

See patent family annex.

Date of the actual completion of the international search: 18 August 2011

Date of mailing of the international search report: 29/08/2011

Name and mailing address of the ISA:
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer: Kal snor, Inge
INTERNATIONAL SEARCH REPORT

Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.: 43
   because they relate to subject matter not required to be searched by this Authority, namely:

   Claim 43 is directed to a method of doing business and is therefore excluded from search (Art. 17(2)(a)(i) and Rule 39.1(iii) PCT).

2. [ ] Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A,P</td>
<td>AMLER LUKAS C: &quot;HER3 mRNA as a predictive biomarker in anti-cancer therapy&quot;. EXPERT OPINION ON BIOLOGICAL THERAPY, INFORMA HEALTHCARE, UK, vol. 10, no. 9, 1 September 2010 (2010-09-01), pages 1343-1355, XP0090145447, ISSN: 1744-7682, the whole document</td>
<td>1-47</td>
</tr>
</tbody>
</table>