COMPOSITION COMPRISING ANTIBODY THAT BINDS TO DOMAIN II OF HER2 AND ACIDIC VARIANTS THEREOF

Related Applications

This application claims the benefit of U.S. Provisional Patent Application No. 61/024825, filed 30 January 2008, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

Field of the Invention

The present invention concerns a composition comprising a main species HER2 antibody that binds to domain II of HER2, and acidic variants thereof. The invention also relates to pharmaceutical formulations comprising the composition, and therapeutic uses for the composition.

Background of the Invention

HER2 Antibodies

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 *erb*B1 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-α), 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-α and EGF, have been evaluated as therapeutic agents in the treatment of such malignancies. See, *e.g.*, Baselga and Mendelsohn., *supra*; Masui *et al. Cancer Research* 44:1002-1007 (1984); and Wu *et al. J. Clin. Invest.* 95:1897-1905 (1995).

The second member of the HER family, $p185^{neu}$, was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The

activated form of the *neu* proto-oncogene results from a point mutation (valine to glutamic acid) in the transmembrane region of the encoded protein. Amplification of the human homolog of *neu* is observed in breast and ovarian cancers and correlates with a poor prognosis (Slamon *et al.*, *Science*, 235:177-182 (1987); Slamon *et al.*, *Science*, 244:707-712 (1989); and US Pat No. 4,968,603). To date, no point mutation analogous to that in the *neu* proto-oncogene has been reported for human tumors. Overexpression of HER2 (frequently but not uniformly due to gene amplification) has also been observed in other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon, thyroid, pancreas and bladder. See, among others, King *et al.*, *Science*, 229:974 (1985); Yokota *et al.*, *Lancet*: 1:765-767 (1986); Fukushige *et al.*, *Mol Cell Biol.*, 6:955-958 (1986); Guerin *et al.*, *Oncogene Res.*, 3:21-31 (1988); Cohen *et al.*, *Oncogene*, 4:81-88 (1989); Yonemura *et al.*, *Cancer Res.*, 51:1034 (1991); Borst *et al.*, *Gynecol. Oncol.*, 38:364 (1990); Weiner *et al.*, *Cancer Res.*, 50:421-425 (1990); Kern *et al.*, *Cancer Res.*, 50:5184 (1990); Park *et al.*, *Cancer Res.*, 49:6605 (1989); Zhau *et al.*, *Mol. Carcinog.*, 3:254-257 (1990); Aasland *et al. Br. J. Cancer* 57:358-363 (1988); Williams *et al. Pathobiology* 59:46-52 (1991); and McCann *et al.*, *Cancer*, 65:88-92 (1990). HER2 may be overexpressed in prostate cancer (Gu *et al. Cancer Lett.* 99:185-9 (1996); Ross *et al. Hum. Pathol.* 28:827-33 (1997); Ross *et al. Cancer* 79:2162-70 (1997); and Sadasivan *et al. J. Urol.* 150:126-31 (1993)).

Antibodies directed against the rat $p185^{neu}$ and human HER2 protein products have been described. Drebin and colleagues have raised antibodies against the rat *neu* gene product, $p185^{neu}$ See, for example, Drebin *et al.*, *Cell* 41:695-706 (1985); Myers *et al.*, *Meth. Enzym.* 198:277-290 (1991); and WO94/22478. Drebin *et al. Oncogene* 2:273-277 (1988) report that mixtures of antibodies reactive with two distinct regions of $p185^{neu}$ result in synergistic anti-tumor effects on *neu*-transformed NIH-3T3 cells implanted into nude mice. See also U.S. Patent 5,824,311 issued October 20, 1998.

Hudziak *et al.*, *Mol. Cell. Biol.* 9(3):1165-1172 (1989) describe the generation of a panel of HER2 antibodies which were characterized using the human breast tumor cell line SK-BR-3. Relative cell proliferation of the SK-BR-3 cells following exposure to the antibodies was determined by crystal violet staining of the monolayers after 72 hours. Using this assay, maximum inhibition was obtained with the antibody called 4D5 which inhibited

cellular proliferation by 56%. Other antibodies in the panel reduced cellular proliferation to a lesser extent in this assay. The antibody 4D5 was further found to sensitize HER2-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See also U.S. Patent No. 5,677,171 issued October 14, 1997. The HER2 antibodies discussed in Hudziak *et al.* are further characterized in Fendly *et al. Cancer Research* 50:1550-1558 (1990); Kotts *et al. In Vitro* 26(3):59A (1990); Sarup *et al. Growth Regulation* 1:72-82 (1991); Shepard *et al. J. Clin. Immunol.* 11(3):117-127 (1991); Kumar *et al. Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis *et al. Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras *et al. Oncogene* 9:1829-1838 (1994); Vitetta *et al. Cancer Research* 54:5301-5309 (1994); Sliwkowski *et al. J. Biol. Chem.* 269(20):14661-14665 (1994); Scott *et al. J. Biol. Chem.* 266:14300-5 (1991); D'souza *et al. Proc. Natl. Acad. Sci.* 91:7202-7206 (1994); Lewis *et al. Cancer Research* 56:1457-1465 (1996); and Schaefer *et al. Oncogene* 15:1385-1394 (1997).

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, Trastuzumab or HERCEPTIN®; U.S. Patent 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 September 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein.

Other HER2 antibodies with various properties have been described in Tagliabue *et al. Int. J. Cancer* 47:933-937 (1991); McKenzie *et al. Oncogene* 4:543-548 (1989); Maier *et al. Cancer Res.* 51:5361-5369 (1991); Bacus *et al. Molecular Carcinogenesis* 3:350-362 (1990); Stancovski *et al. PNAS (USA)* 88:8691-8695 (1991); Bacus *et al. Cancer Research* 52:2580-2589 (1992); Xu *et al. Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk *et al. Cancer Research* 52:2771-2776 (1992);Hancock *et al. Cancer Res.* 51:4575-4580 (1991); Shawver *et al. Cancer Res.* 54:1367-1373 (1994); Arteaga *et al. Cancer Res.* 54:3758-3765 (1994); Harwerth *et al. J. Biol. Chem.* 267:15160-15167 (1992); U.S. Patent No. 5,783,186; and Klapper *et al. Oncogene* 14:2099-2109 (1997).

Homology screening has resulted in the identification of two other HER receptor family members; HER3 (US 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-α), 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. Patent 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 TGFα 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)).

To target the HER signaling pathway, rhuMAb 2C4 (Pertuzumab) was developed as a humanized antibody that inhibits the dimerization of HER2 with other HER receptors, thereby inhibiting ligand-driven phosphorylation and activation, and downstream activation of the RAS and AKT pathways. In a phase I trial of Pertuzumab as a single agent for treating solid tumors, 3 subjects with advanced ovarian cancer were treated with pertuzumab. One had a durable partial response, and an additional subject had stable disease for 15 weeks. Agus *et al. Proc Am Soc Clin Oncol* 22: 192, Abstract 771 (2003).

Antibody Variant Compositions

US Patent No. 6,339,142 describes a HER2 antibody composition comprising a mixture of anti-HER2 antibody and one or more acidic variants thereof, wherein the amount of the acidic variant(s)is less than about 25%. Trastuzumab is the exemplified HER2 antibody.

Reid *et al.* Poster presented at Well Characterized Biotech Pharmaceuticals conference (January, 2003) "Effects of Cell Culture Process Changes on Humanized Antibody Characteristics" describes an unnamed, humanized IgG1antibody composition with N-terminal heterogeneities due to combinations of VHS signal peptide, N-terminal glutamine, and pyroglutamic acid on the heavy chain thereof.

Harris *et al.* "The Ideal Chromatographic Antibody Characterization Method" talk presented at the IBC Antibody Production Conference (February, 2002) reports a VHS extension on the heavy chain of E25, a humanized anti-IgE antibody.

Rouse *et al.* Poster presented at WCBP "'Top Down' Glycoprotein Characterization by High Resolution Mass Spectrometry and Its Application to Biopharmaceutical Development" (January 6-9, 2004) describes a monoclonal antibody composition with N-terminal heterogeneity resulting from $^{-3}$AHS or $^{-2}$HS signal peptide residues on the light chain thereof.

In a presentation at IBC Meeting (September, 2000) "Strategic Use of Comparability Studies and Assays for Well Characterized Biologicals," Jill Porter discussed a late-eluting form of ZENAPAX™ with three extra amino acid residues on the heavy chain thereof.

US2006/0018899 describes a composition comprising a main species pertuzumab antibody and an amino-terminal leader extension variant, as well as other variant forms of the pertuzumab antibody.

Summary

In a first aspect, there is provided a composition comprising a main species HER2 antibody that binds to domain II of HER2, and an acidic variant thereof comprising a disulfide reduced variant, a non-reducible variant or both of the main species antibody.

In a second aspect, there is provided a pharmaceutical formulation comprising the composition as defined above in a pharmaceutically acceptable carrier.

In a third aspect, there is provided the pharmaceutical formulation as defined above for use in treating HER2 positive cancer in a patient.

In a fourth aspect, there is provided a method of making an evaluated pharmaceutical composition, comprising:

(1) preparing a composition comprising a main species HER2 antibody that binds to domain II of HER2, and acidic variant(s) of that main species HER2 antibody and (2) evaluating the acidic variant(s) in the composition, and confirming that the amount thereof is less than about 25% of the total composition, wherein the acidic variant(s) comprise a disulfide reduced variant, a non-reducible variant or both.

In a fifth aspect, there is provided a composition produced by the method as defined above.

According to a first embodiment, the disclosure concerns a composition comprising a main species HER2 antibody that binds to domain II of HER2, and acidic variants thereof wherein the acidic variants include glycated variant, disulfide reduced variant, or non-reducible variant. Preferably, the acidic variants include glycated variant, deamidated variant, disulfide reduced variant, sialylated variant, and non-reducible variant. Desirably, the amount of the acidic variants is less than about 25%.

In another embodiment, the disclosure provides a composition comprising a main species HER2 antibody comprising variable light and variable heavy sequences in SEQ ID Nos. 3 and 4, respectively, and acidic variants of the main species antibody, wherein the acidic variants include glycated variant, deamidated variant, disulfide reduced variant, sialylated variant, and non-reducible variant.

The disclosure also concerns pharmaceutical formulations comprising the compositions in a pharmaceutically acceptable carrier.

Additionally, the disclosure relates to a method of treating HER2 positive cancer in a patient comprising administering the pharmaceutical formulation to the patient in an amount effective to treat the cancer. With respect to such methods, as demonstrated in the Example herein, preferably the main species antibody and acidic variants have essentially the same pharmacokinetics.

In another embodiment, the disclosure concerns a method of making a pharmaceutical composition comprising: (1) preparing a composition comprising a main species HER2 antibody that binds to domain II of HER2, and acidic variants thereof including glycated variant, disulfide reduced variant, or non-reducible variant, and (2) evaluating the acidic variants in the composition, and confirming that the amount thereof is less than about 25%. In one embodiment, the acidic variants are evaluated by a method selected from the group

consisting of ion exchange chromatography wherein the composition is treated with sialidase, reduced capilliary electrophoresis with sodium dodecyl sulfate (CE-SDS), non-reduced CE-SDS, boronate chromatography, and peptide mapping.

Brief Description of the Drawings

Figure 1 provides a schematic of the HER2 protein structure, and amino acid sequences for Domains I-IV (SEQ ID Nos.19-22, respectively) of the extracellular domain thereof.

Figures 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) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 1 and 2, respectively); V_L and V_H domains of humanized 2C4 version 574 (SEQ ID Nos. 3 and 4, respectively), and human V_L and V_H consensus frameworks (hum κ1, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 5 and 6, respectively). Asterisks identify differences between humanized 2C4 version 574 and murine monoclonal antibody 2C4 or between humanized 2C4 version 574 and the human framework. Complementarity Determining Regions (CDRs) are in brackets.

Figures 3A and 3B show the amino acid sequences of Pertuzumab light chain (SEQ ID No. 15) and heavy chain (SEQ ID No. 16). CDRs are shown in bold. The carbohydrate moiety is attached to Asn 299 of the heavy chain.

Figures 4A and 4B show the amino acid sequences of Pertuzumab light chain (SEQ ID No. 17) and heavy chain (SEQ ID No. 18), each including an intact amino terminal signal peptide sequence.

Figure 5 depicts, schematically, binding of 2C4 at the heterodimeric binding site of HER2, thereby preventing heterodimerization with activated EGFR or HER3.

Figure 6 depicts coupling of HER2/HER3 to the MAPK and Akt pathways.

Figure 7 compares activities of Trastuzumab and Pertuzumab.

Figures 8A and 8B show the amino acid sequences of Trastuzumab light chain (SEQ ID No. 13) and heavy chain (SEQ ID No. 14).

Figures 9A and 9B depict a variant Pertuzumab light chain sequence (SEQ ID No. 23) and a variant Pertuzumab heavy chain sequence (SEQ ID No. 24).

Figure 10 shows experimental design for isolation of cation exchange MP (Main Peak) and AV (Acidic Variants), cell culture, recovery, and PK (pharmacokinetics)

evaluation and analytical testing. Fresh media = standard media; spent media = standard media after 12 days of cell culture, cells were removed by centrifugation. Dissolved oxygen, pH, and other parameters were not controlled.

Figure 11 shows a typical DIONEX PROPAC™ cation exchange (CEX) chromatogram from Example 1.

Figure 12 shows analysis of pertuzumab starting material and CEX fractions. AV = acidic variant; MP = main peak; and BV = basic variant.

Figure 13 reveals CEX of main peak (MP) spiked into cell culture media and incubated for 12 days.

Figure 14 describes main peak incubation conditions.

Figure 15 summarizes methods for characterization of acidic variants.

Figure 16 shows pertuzumab concentration versus time in the PK studies in Example 1.

Figure 17 provides the area under the curve (AUC) and geometric mean ratios from the PK study in Example 1.

Detailed Description of the Preferred Embodiments

I. Definitions

The term "main species antibody" herein refers to the antibody amino acid sequence structure in a composition which is the quantitatively predominant antibody molecule in the composition. Preferably, 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. 15 and 16 (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%, and 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.* a deamidated antibody variant), a basic variant, the antibody with an amino-terminal leader extension (*e.g.* VHS-) on one or two light chains thereof, antibody with a C-terminal lysine residue on one or two heavy chains thereof, antibody with one or more oxidized methionine residues, etc. and includes combinations of variations to the amino acid sequences of heavy and/or light chains.

An "acidic variant" is a variant of the main species antibody which is more acidic than the main species antibody. An acidic variant has gained negative charge or lost positive charge relative to the main species antibody. Such acidic variants can be resolved using a separation methodology, such as ion exchange chromatography, that separates proteins according to charge. Acidic variants of a main species antibody elute earlier than the main peak upon separation by cation exchange chromatography.

A "disulfide reduced variant" has one more disulfide-bonded cysteine(s) chemically reduced to the free thiol form. This variant can be monitored by hydrophobic interaction chromatography or by sizing methodology such as Capillary Electrophoresis with Sodium Dodecyl Sulfate (CE-SDS), *e.g.* as described in Example 1. Herein, a "non-reducible variant" is a variant of the main species antibody that cannot be chemically reduced to heavy and light chain by treatment with a reducing agent such as dithiothreitol. Such variants can be assessed by treating the composition with a reducing agent and evaluating the resulting composition using a methodology that evaluates protein size, such as Capillary Electrophoresis with Sodium Dodecyl Sulfate (CE-SDS), for instance using the techniques described in Example 1 below.

A "glycosylation variant" antibody herein is an antibody with one or more carbohydrate moeities attached thereto wh ich differ from one or more carbohydate moieties attached to a main species antibody. Examples of glycosylation variants herein include antibody with a G1 or G2 oligosaccharide structure, instead a G0 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, antibody which is syalidated, etc, as well as combinations of such glycosylation alterations.

Where the antibody has an Fc region, an oligosaccharide structure such as that shown in Fig. 14 herein may be attached to one or two heavy chains of the antibody, *e.g.* at residue 299. For Pertuzumab, G0 was the predominant oligosaccharide structure, with other oligosaccharide structures such as G0-F, G-1, Man5, Man6, G1-1, G1(1-6), G1(1-3) and G2 being found in lesser amounts in the Pertuzumab composition.

Unless indicated otherwise, a "G1 oligosaccharide structure" herein includes G1(1-6) and G1(1-3) structures.

For the purposes herein, "sialylated variant" is a variant of the main species antibody comprising one or more sialylated carbohydrate moieties attached to one or two heavy chains thereof. A sialylated variant can be identified by evaluating a composition (for example by ion exchange chromatography) with or without sialidase treatment, *e.g.* as described in the example.

A "glycated variant" is an antibody to which a sugar, such as glucose, has been covalently attached. This addition can occur by reaction of glucose with a lysine residue on the protein (*e.g.* in cell culture media). A glycated variant can be identified by mass spectrometry analysis of the reduced antibody evaluating the increase in mass of heavy or light chains. A glycated variant can also be quantified by boronate chromatography as explained in Example 1 below. A glycated variant differs from a glycosylation 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. An example of a deamidated antibody is a pertuzumab variant, wherein Asn-386 and/or Asn-391 on one or two heavy chains of pertuzumab are deamidated.

A "amino-terminal leader extension variant" herein refers to a main species antibody with one or more amino acid residues of the amino-terminal leader sequence at the amino-terminus of any one or more heavy or light chains of the main species 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.

"Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the

alignment are well known in the art. One such computer program is "Align 2", authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, DC 20559, on December 10, 1991.

For the purposes herein, "cation exchange analysis" refers to any method by which a composition comprising two or more compounds is separated based on charge differences using a cation exchanger. A cation exchanger generally comprises covalently bound, negatively charged groups. Preferably, the cation exchanger herein is a weak cation-exchanger and/or comprises a carboxylate functional group, such as the PROPAC WCX-10™ cation exchange column sold by Dionex.

A "HER receptor" is a receptor protein tyrosine kinase which belongs to the HER receptor family and includes EGFR, HER2, HER3 and HER4 receptors and other members of this family to be identified in the future. The HER receptor will generally comprise an extracellular domain, which may bind an HER ligand; a lipophilic transmembrane domain; a conserved intracellular tyrosine kinase domain; and a carboxyl-terminal signaling domain harboring several tyrosine residues which can be phosphorylated. Preferably the HER receptor is native sequence human HER receptor.

The extracellular domain of HER2 comprises four domains, Domain I (amino acid residues from about 1-195), Domain II (amino acid residues from about 196-319), Domain III (amino acid residues from about 320-488), and Domain IV (amino acid residues from about 489-630) (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), or Plowman *et al. Proc. Natl. Acad. Sci.* 90:1746-1750 (1993). See, also, Fig. 1 herein.

The terms "ErbB1," "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)). *erb*B1 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)* 82:6497-6501 (1985) and Yamamoto *et al. Nature* 319:230-234 (1986) (Genebank accession number

X03363). The term "*erb*B2" refers to the gene encoding human ErbB2 and "*neu*" refers to the gene encoding rat p185^{neu}. Preferred HER2 is native sequence human HER2.

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

The terms "ErbB4" and "HER4" herein refer to the receptor polypeptide as disclosed, for example, in 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), including isoforms thereof, *e.g.*, as disclosed in WO99/19488, published April 22, 1999.

By "HER ligand" is meant a polypeptide which binds to and/or activates an 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-α) (Marquardt *et al., Science* 223:1079-1082 (1984)); amphiregulin also known as schwanoma 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); and Sasada *et al. Biochem. Biophys. Res. Commun.* 190:1173 (1993)); heparin-binding epidermal growth factor (HB-EGF) (Higashiyama *et al., Science* 251:936-939 (1991)); epiregulin (Toyoda *et al., J. Biol. Chem.* 270:7495-7500 (1995); and Komurasaki *et al. Oncogene* 15:2841-2848 (1997)); a heregulin (see below); neuregulin-2 (NRG-2) (Carraway *et al., Nature* 387:512-516 (1997)); neuregulin-3 (NRG-3) (Zhang *et al., Proc. Natl. Acad. Sci.* 94:9562-9567 (1997)); neuregulin-4 (NRG-4) (Harari *et al. Oncogene* 18:2681-89 (1999)) or cripto (CR-1) (Kannan *et al. J. Biol. Chem.* 272(6):3330-3335 (1997)). HER ligands which bind EGFR include EGF, TGF-α, amphiregulin, betacellulin, HB-EGF and epiregulin. HER ligands which bind HER3 include heregulins. HER ligands capable of binding HER4 include betacellulin, epiregulin, HB-EGF, NRG-2, NRG-3, NRG-4 and heregulins.

"Heregulin" (HRG) when used herein refers to a polypeptide encoded by the heregulin gene product as disclosed in U.S. Patent No. 5,641,869 or Marchionni *et al., Nature*, 362:312-318 (1993). Examples of heregulins include heregulin-α, heregulin-β1, heregulin-β2 and heregulin-β3 (Holmes *et al., Science*, 256:1205-1210 (1992); and U.S.

Patent 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)). The term includes biologically active fragments and/or amino acid sequence variants of a native sequence HRG polypeptide, such as an EGF-like domain fragment thereof (*e.g.* $HRG\beta1_{177-244}$).

A "HER dimer" herein is a noncovalently associated dimer comprising at least two different 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. Examples of such HER dimers include EGFR-HER2, HER2-HER3 and HER3-HER4 heterodimers. Moreover, the HER dimer may comprise two or more HER2 receptors combined with a different HER receptor, such as HER3, HER4 or EGFR. Other proteins, such as a cytokine receptor subunit (*e.g.* gp130) may be associated with the dimer.

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).

"HER activation" or "HER2 activation" refers to activation, or phosphorylation, of any one or more HER receptors, or HER2 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.

The term "antibody" herein is used in the broadest sense and specifically covers intact 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.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variants that may arise during production of the monoclonal antibody, such as those variants described herein. In contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are 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 construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature,* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991), for example.

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 (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA*, 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (*e.g.* Old World Monkey, Ape etc) and human constant region sequences.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen-binding or variable 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 fragment(s).

An "intact antibody" is one which comprises an antigen-binding variable region as well as a light chain constant domain (C_L) and heavy chain constant domains, C_H1, C_H2 and C_H3. The constant domains may be native sequence constant domains (*e.g.* human native sequence constant domains) or amino acid sequence variants thereof. Preferably, the intact antibody has one or more effector functions, and comprises an oligosaccharide structure attached to one or two heavy chains thereof.

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. Examples of antibody effector functions include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor; BCR), etc.

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.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of

Health, Bethesda, MD (1991). The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different "classes". There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into "subclasses" (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (*e.g.* Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is 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 US Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be *assessed in vivo*, *e.g.*, in a animal model such as that disclosed in Clynes *et al. PNAS (USA)* 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, *e.g.* from blood or PBMCs as described herein.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and Fcγ RIII subclasses, including allelic variants and alternatively

spliced forms of these 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 review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al., Immunomethods* 4:25-34 (1994); and de Haas *et al., J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al., J. Immunol.* 117:587 (1976) and Kim *et al., J. Immunol.* 24:249 (1994)).

"Complement dependent cytotoxicity" or "CDC" refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (*e.g.* an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, *e.g.* as described in Gazzano-Santoro *et al., J. Immunol. Methods* 202:163 (1996), may be performed.

"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 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 both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions 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*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region generally comprises amino acid residues from a "complementarity determining region" or "CDR" (*e.g.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*e.g.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework Region" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

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-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions 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 also contains the constant domain of the light chain and the first constant domain (CH1) 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 CH1 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 at least one free thiol group. $F(ab')_2$ 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.

The "light chains" of antibodies 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.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Plückthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994). HER2 antibody scFv fragments are described in WO93/16185; U.S. Patent No. 5,571,894; and U.S. Patent No. 5,587,458.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a variable heavy domain (V_H) connected to a variable light 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 are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993).

"Humanized" forms of non-human (*e.g.*, rodent) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (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 are made to further refine antibody performance. In general, the 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 also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see 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).

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. Patent 5,821,337 expressly incorporated herein by reference; humanized 520C9 (WO93/21319) and humanized 2C4 antibodies 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 in SEQ ID NOS. 13 and 14, respectively.

Herein, "Pertuzumab" and "rhuMAb 2C4," refer to an antibody comprising the variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4, respectfully.

Where Pertuzumab is an intact antibody, it preferably comprises the light chain and heavy chain amino acid sequences in SEQ ID Nos. 15 and 16, respectively.

A "naked antibody" is an antibody (as herein defined) that is not conjugated to a heterologous molecule, such as a cytotoxic moiety or radiolabel.

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 diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more 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 a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, 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.

A HER2 antibody which "inhibits HER dimerization more effectively than Trastuzumab" is one which reduces or eliminates HER dimers more effectively (for example at least about 2-fold more effectively) than Trastuzumab. Preferably, such an antibody inhibits HER2 dimerization at least about as effectively as an antibody selected from the group consisting of intact murine monoclonal antibody 2C4, a Fab fragment of murine monoclonal antibody 2C4, intact Pertuzumab, and a Fab fragment of Pertuzumab. One can evaluate HER dimerization inhibition 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 HER dimerization 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 dimerization by assessing, for example, 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

(WO01/00245and 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 (WO01/00245and 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 TGFα- 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* 5:317-328 (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 does "not inhibit HER2 ectodomain cleavage" (Molina *et al. Cancer Res.* 61:4744-4749(2001)).

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.

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, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, 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. Patent No. 5,677,171 issued October 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 may be 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. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 2C4 epitope of HER2 using 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. Epitope 2C4 comprises residues from domain II in the extracellular domain of HER2. 2C4 and Pertuzumab bind 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, in Fig. 1).

The "epitope 7C2/7F3" is the region at the N terminus, within Domain I, 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 HER2 in Fig. 1).

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disease as well as those

in which the disease is to be prevented. Hence, the patient to be treated herein may have been diagnosed as having the disease or may be predisposed or susceptible to the disease.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer 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 squamous cell cancer (*e.g.* epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, 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, breast cancer, 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, esophagael cancer, tumors of the biliary tract, as well as head and neck cancer.

The term "effective amount" refers to an amount of a drug effective to treat disease in the patient. Where the disease is cancer, 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, result in an objective response (including a partial response, PR, or complete respose, CR), increase overall survival time, and/or improve one or more symptoms of cancer.

A "HER2 positive cancer" is one comprising cells which have HER2 protein present at their cell surface.

A cancer which "overexpresses" a HER receptor is one which has significantly higher levels of a HER receptor, such as HER2, at the cell surface thereof, 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). Alternatively, or additionally, one may measure levels of HER-encoding nucleic acid in the cell, *e.g.* via fluorescent *in situ* hybridization (FISH; see WO98/45479 published October, 1998), southern blotting, or polymerase chain reaction (PCR) techniques, such as real time quantitative PCR (RT-PCR). One may also study HER receptor overexpression by measuring shed antigen (*e.g.*, HER extracellular domain) in a biological fluid such as serum (see, *e.g.*, U.S. Patent No. 4,933,294 issued June 12, 1990; WO91/05264 published April 18, 1991; U.S. Patent 5,401,638 issued March 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 HER2 receptor" is one which does not express higher than normal levels of HER2 receptor compared to a noncancerous cell of the same tissue type.

A cancer which "overexpresses" a HER ligand is one which produces significantly higher levels of that ligand compared to a noncancerous cell of the same tissue type. Such overexpression may be caused by gene amplification or by increased transcription or translation. Overexpression of the HER ligand may be determined diagnostically by evaluating levels of the ligand (or nucleic acid encoding it) in the patient, *e.g.* in a tumor biopsy or by various diagnostic assays such as the IHC, FISH, southern blotting, PCR or *in vivo* assays described above.

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^{186}, Re^{188}, Sm^{153}, Bi^{212}, 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 cyclosphosphamide (CYTOXAN®); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide 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 ranimnustine; antibiotics such as the enediyne antibiotics (*e. g.*, calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII (see, *e.g.*, Agnew, *Chem Intl. Ed. Engl.*, 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, 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), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, 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; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; dacarbazine; mannomustine; 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; leucovovin; novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (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®); troxacitabine (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, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; topoisomerase 1 inhibitor (*e.g.*, LURTOTECAN®); rmRH (*e.g.*, ABARELIX®); BAY439006 (sorafenib; Bayer); SU-11248 (Pfizer); perifosine, COX-2 inhibitor (*e.g.* celecoxib or etoricoxib), proteosome inhibitor (*e.g.* PS341); bortezomib (VELCADE®); CCI-779; tipifarnib (R11577); orafenib, ABT510; 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 leucovovin.

Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens with mixed agonist/antagonist profile, including, tamoxifen (NOLVADEX®), 4-hydroxytamoxifen, toremifene (FARESTON®), idoxifene, droloxifene, raloxifene (EVISTA®), trioxifene, 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 including vorozole (RIVISOR®), megestrol acetate (MEGASE®), fadrozole, imidazole; lutenizing hormone-releaseing 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; testolactone; and pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above.

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, US Patent 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 (US Patent No. 5,212,290); humanized and chimeric antibodies that bind EGFR as described in US Patent No. 5,891,996; and human antibodies that bind EGFR, such as ABX-EGF or Panitumumab (see WO98/50433, Abgenix/Amgen); 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 (EMD/Merck); human EGFR antibody, HuMax-EGFR (GenMab); fully human antibodies known as E1.1, E2.4, E2.5, E6.2, E6.4, E2.11, E6. 3 and E7.6. 3 and described in US 6,235,883; MDX-447 (Medarex Inc); 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). EGFR antagonists include small molecules such as compounds described in US Patent Nos: 5,616,582, 5,457,105, 5,475,001, 5,654,307, 5,679,683, 6,084,095, 6,265,410, 6,455,534, 6,521,620, 6,596,726, 6,713,484, 5,770,599, 6,140,332, 5,866,572, 6,399,602, 6,344,459, 6,602,863, 6,391,874, 6,344,455, 5,760,041, 6,002,008, and 5,747,498, as well as the following PCT publications: WO98/14451, WO98/50038, WO99/09016, and WO99/24037. Particular small molecule EGFR antagonists include OSI-774 (CP-358774, erlotinib, TARCEVA® Genentech/OSI

Pharmaceuticals); PD 183805 (CI 1033, 2-propenamide, N-[4-[(3-chloro-4-fluorophenyl)amino]-7-[3-(4-morpholinyl)propoxy]-6-quinazolinyl]-, dihydrochloride, Pfizer Inc.); ZD1839, gefitinib (IRESSA®) 4-(3'-Chloro-4'-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy)quinazoline, AstraZeneca); ZM 105180 ((6-amino-4-(3-methylphenyl-amino)-quinazoline, Zeneca); BIBX-1382 (N8-(3-chloro-4-fluoro-phenyl)-N2-(1-methyl-piperidin-4-yl)-pyrimido[5,4-d]pyrimidine-2,8-diamine, Boehringer Ingelheim); PKI-166 ((R)-4-[4-[(1-phenylethyl)amino]-1H-pyrrolo[2,3-d]pyrimidin-6-yl]-phenol); (R)-6-(4-hydroxyphenyl)-4-[(1-phenylethyl)amino]-7H-pyrrolo[2,3-d]pyrimidine); CL-387785 (N-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butynamide); EKB-569 (N-[4-[(3-chloro-4-fluorophenyl)amino]-3-cyano-7-ethoxy-6-quinolinyl]-4-(dimethylamino)-2-butenamide) (Wyeth); AG1478 (Sugen); AG1571 (SU 5271; Sugen); dual EGFR/HER2 tyrosine kinase inhibitors such as lapatinib (GW 572016 or N-[3-chloro-4-[(3 fluorophenyl)methoxy]phenyl]6[5[[[2methylsulfonyl)ethyl]amino]methyl]-2-furanyl]-4-quinazolinamine; Glaxo-SmithKline) or cyanoguanidine quinazoline and cyanoamidine quinazolamine derivatives.

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; lapatinib (GW572016; available from Glaxo-SmithKline) 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 (GLEEVAC©) available from Glaxo; MAPK extracellular regulated kinase I 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 (US Patent No. 5,804,396); tryphostins (US Patent No. 5,804,396); ZD6474 (Astra Zeneca); PTK-787 (Novartis/Schering AG); pan-HER inhibitors such as CI-1033 (Pfizer); Affinitac (ISIS 3521; Isis/Lilly); Imatinib mesylate (Gleevac; Novartis); PKI 166 (Novartis); GW2016 (Glaxo SmithKline); CI-1033 (Pfizer); EKB-569 (Wyeth); Semaxinib (Sugen); ZD6474 (AstraZeneca); PTK-787 (Novartis/Schering AG); INC-1C11 (Imclone); cyanoguanidine quinazoline and cyanoamidine quinazolamine derivatives; or as described in any of the following patent publications: US Patent No. 5,804,396; WO99/09016 (American Cyanamid); 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); WO96/33980 (Zeneca); and US2005/0101617.

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-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α 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.

II. HER2 Antibody Variant Compositions

The present invention concerns, at least in part, certain HER2 antibody compositions. Preferably, the HER2 antibody (either or both of the main species HER2 antibody and antibody variant thereof) is one which binds to Domain II of HER2, inhibits HER dimerization more effectively than Trastuzumab, and/or 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. 15 and 16 (Pertuzumab).

The composition herein comprises a main species HER2 antibody that binds to domain II of HER2, and acidic variants thereof wherein the acidic variants include one, two, or three of glycated variant, disulfide reduced variant, and non-reducible variant. The acidic variants in the composition may include one, two, three, four, or five of glycated variant, deamidated variant, disulfide reduced variant, sialylated variant, and non-reducible variant. Preferably, the total amount of all acidic variants in the composition is less than about 25%. In one embodiment, the glycated variant, deamidated variant, disulfide reduced variant, sialylated variant, and non-reducible variant constitute at least about 75-80% of the acidic variants in the composition.

The invention additionally concerns a composition comprising a main species HER2 antibody comprising variable light and variable heavy sequences in SEQ ID Nos. 3 and 4, respectively, and acidic variants of the main species antibody, wherein the acidic variants include one, two, three, four, or five of glycated variant, deamidated variant, disulfide reduced variant, sialylated variant, and non-reducible variant.

The invention provides a method of making a pharmaceutical composition comprising: (1) preparing a composition comprising a main species HER2 antibody that binds to domain II of HER2, and acidic variants thereof including glycated variant, disulfide reduced variant, or non-reducible variant, and (2) evaluating the acidic variants in the composition, and confirming that the amount thereof is less than about 25%. The method contemplates combining the composition before, during, or after after step (2) with a pharmaceutically acceptable carrier. In one embodiment, the composition evaluated in step (2) is in a pharmaceutically acceptable carrier.

In one embodiment, at least about 75-80% of the acidic variants (constituting the less than about 25% of the composition) are selected from: glycated variant, deamidated variant, disulfide reduced variant, sialylated variant, and non-reducible variant.

The acidic variants may be evaluated by a variety of methods, but preferably such methods include one, two, three, four, or five of: ion exchange chromatography (IEC) wherein the composition is treated with sialidase before, after, and/or during the IEC (*e.g.* to evaluate sialylated variant), reduced CE-SDS (*e.g.* to evaluate disulfide reduced variant), non-reduced CE-SDS (*e.g* to evaluate non-reducible variant), boronate chromatography (*e.g.* to evaluate glycated variant), and peptide mapping (*e.g.* to evaluate deamidated variant).

In one embodiment, the overall acidic variants are evaluated by ion exchange chromatography, for example using a weak cation exchanger and/or cation exchanger with carboxylate functional group (for example, using a DIONEX PROPAC™ WCX-10 chromatography column). In one embodiment of such chromatography the conditions for the chromatography involve Buffer A of 20mM BisTris, pH 6.0; Buffer B of 20mM BisTris, 200mM NaCl, pH 6.0; and a gradient of 0.5% Buffer B at 1.0mL/min.

The composition optionally includes an amino-terminal leader extension variant. 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 intact antibody or antibody fragment (*e.g.* Fab of $F(ab')_2$ fragments), but preferably both are intact 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 lower detection limit of any assay (preferably cation exchange 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%, and preferably from about 8% to about 12 %) of the antibody molecules in the composition comprise an amino-terminal leader extension. Such percentage amounts are preferably determined using cation exchange analysis.

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 (such an antibody variant may be present in an amount from about 1% to about 20%), antibody with one or more oxidized methionine residues (for example, Pertuzumab comprising oxidized met-254)etc.

Moreover, aside from the sialylated variant discussed above, the main species antibody or variant may comprise additional 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, etc.

Optionally, the antibody comprising one or two light chains, wherein either or both of the light chains comprise the amino acid sequence in SEQ ID No. 23 (including variants thereof such as those disclosed herein). The antibody further comprises one or two heavy chains, wherein either or both of the heavy chains comprise the amino acid sequence in SEQ ID NO. 16 or SEQ ID NO. 24 (including variants thereof such as those disclosed herein).

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.

III. Production of HER2 Antibodies

A description follows as to exemplary techniques for the production of the antibodies used in accordance with the present invention. The HER2 antigen to be used for production of antibodies may be, *e.g.*, a soluble form of the extracellular domain of HER2 or a portion thereof, containing the desired epitope. Alternatively, cells expressing HER2 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 HER2 useful for generating antibodies will be apparent to those skilled in the art.

(i) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variants that may arise during production of the monoclonal antibody, such as those variants described herein. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent 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, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland 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 recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al., Curr. Opinion in Immunol.*, 5:256-262 (1993) and Plückthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, monoclonal antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature*, 348:552-554 (1990). Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al., Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al., Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

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. Patent 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.

(ii) 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); Verhoeyen *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. Patent 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.

US Patent No. 6,949,245 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-α and/or HRG mediated activation of MAPK essentially as effectively as intact murine monoclonal antibody 2C4 (or a Fab fragment thereof) and/or binds HER2 essentially as effectively as intact 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 complementarity determining residues GFTFTDYTMX, where X is preferrably D or S (SEQ ID NO:7); DVNPNSGGSIYNQRFKG (SEQ ID NO:8); and/or NLGPSFYFDY (SEQ ID NO:9), 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 amino acid sequence in SEQ ID NO:4.

The humanized antibody may comprise variable light complementarity determining residues KASQDVSIGVA (SEQ ID NO:10); SASYX^{1}X^{2}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:11); and/or QQYYIYPYT (SEQ ID NO:12), *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 amino acid sequence in SEQ ID NO:3.

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. 3 and 4, respectively (*i.e.* variant 574). The affinity matured antibody preferably binds to HER2 receptor with an affinity superior to that of intact murine 2C4 or intact variant 574 (*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 IgG1 antibody.

(iii) 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_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. Patent 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 described by Marks *et al., J. Mol. Biol.* 222:581-597 (1991), or Griffith *et al., EMBO J.* 12:725-734 (1993). See, also, U.S. Patent Nos. 5,565,332 and 5,573,905.

Human antibodies may also be generated by *in vitro* activated B cells (see U.S. Patents 5,567,610 and 5,229,275).

Human HER2 antibodies are described in U.S. Patent No. 5,772,997 issued June 30, 1998 and WO 97/00271 published January 3, 1997.

(iv) Antibody fragments

Various techniques have been developed for the production of antibody fragments. 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. Patent No. 5,571,894; and U.S. Patent No. 5,587,458. The antibody fragment may also be a "linear antibody", *e.g.*, as described in U.S. Patent 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

(v) 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).

WO 96/16673 describes a bispecific HER2/FcγRIII antibody and U.S. Patent No. 5,837,234 discloses a bispecific HER2/FcγRI antibody IDM1 (Osidem). A bispecific HER2/Fcα antibody is shown in WO98/02463. U.S. Patent No. 5,821,337 teaches a bispecific HER2/CD3 antibody. MDX-210 is a bispecific HER2-FcγRIII Ab.

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 (CH1) 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. Patent 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_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. Patent 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. Patent 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).

(vi) Other amino acid sequence modifications

Amino acid sequence modification(s) of the HER2 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 HER2 antibody are prepared by introducing appropriate nucleotide changes into the HER2 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 HER2 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 HER2 antibody, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the HER2 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 HER2 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 HER2 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 a HER2 antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the HER2 antibody

molecule include the fusion to the N- or C-terminus of the HER2 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 HER2 antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions or CDRs, but FR or Fc region 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

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

Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

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), Gln (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:

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

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

(3) acidic: Asp, Glu;

(4) basic: His, Lys, Arg;

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

(6) aromatic: Trp, Tyr, Phe.

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

Any cysteine residue not involved in maintaining the proper conformation of the HER2 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 huma 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-aceylgalactosamine, 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, any oligosaccharide structure attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that lacks fucose attached to an Fc region of the antibody are described in US Pat Appl No US 2003/0157108 A1, Presta, L. See also US 2004/0093621 A1 (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetylglucosamine (GlcNAc) in the oligosaccharide structure attached to an Fc region of the antibody are referenced in WO03/011878, Jean-Mairet *et al.* and US Patent No. 6,602,684, Umana *et al.* Antibodies with at least one galactose residue in an oligosaccharide structure attached to an Fc region of the antibody are reported in WO97/30087, Patel *et al.* See, also, WO98/58964 (Raju, S.) and WO99/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof. Antibody compositions comprising main species antibody with such carbohydrate structures attached to one or two heavy chains of the Fc region are contemplated herein.

Nucleic acid molecules encoding amino acid sequence variants of the HER2 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 HER2 antibody.

(vii) 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 HER2 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 WO01/00245. HER2 monoclonal antibodies may be added to each well and incubated for 30 minutes. ^{125}I-labeled rHRGβ1$_{177-224}$ (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 IC_{50} value may be calculated for the antibody of interest. In one embodiment, the antibody which blocks ligand activation of an HER receptor will have an IC_{50} for inhibiting HRG binding to MCF7 cells in this assay of about 50nM or less, more preferably 10nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG binding to MCF7 cells in this assay may, for example, be about 100nM or less, more preferably 50nM or less.

Alternatively, or additionally, the ability of the HER2 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. Patent 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 p180 tyrosine phosphorylation in MCF7 cells essentially as described in WO01/00245. 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 rHRGβ1$_{177-244}$ 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 µl of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 µl) may be electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (at 1 µg/ml) immunoblots may be developed, and the intensity of the predominant reactive band at M_r ~180,000 may be quantified by reflectance densitometry. The antibody selected will preferably significantly inhibit HRG stimulation of p180 tyrosine phosphorylation to about 0-35% of control in this assay. A dose-response curve for inhibition of HRG stimulation of p180 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 p180 tyrosine phosphorylation in this assay of about 50nM or less, more preferably 10nM or less. Where the antibody is an antibody fragment such as a Fab fragment, the IC_{50} for inhibiting HRG stimulation of p180 tyrosine phosphorylation in this assay may, for example, be about 100nM or less, more preferably 50nM 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 treated with a HER2 monoclonal antibody (10µg/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 WO01/00245 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. Patent 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 35mm cell culture dish (2mls/35mm 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 US 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 10μg/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 Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (*e.g.* annexin V-FTIC) (1μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ 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 9μg/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

WO98/17797 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.

(viii) 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. Patent 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 a HER2 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, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ^I_1 (Hinman *et al. Cancer Research* 53: 3336-3342 (1993) and Lode *et al. Cancer Research* 58: 2925-2928 (1998)). See, also, US Patent 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 October 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 such as a deoxyribonuclease; DNase).

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 adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al. Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. 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 HER2 antibody and cytotoxic agent may be made, *e.g.* by recombinant techniques or peptide synthesis.

In yet another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.* avidin) which is conjugated to a cytotoxic agent (*e.g.* a radionucleotide).

(ix) Other antibody modifications

Other modifications of the antibody 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-(methylmethacylate) 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).

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 cyotoxicity (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.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers 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. Preferably the altered Fc region is a human IgG1 Fc region comprising or consisting of substitutions at one, two or three of these positions.

Antibodies with altered C1q binding and/or complement dependent cytotoxicity (CDC) are described in WO99/51642, US Patent No. 6,194,551B1, US Patent No. 6,242,195B1, US Patent No. 6,528,624B1 and US Patent 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.

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 US Patent 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.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule. Antibodies with substitutions in an Fc region thereof and increased serum half-lives are also described in WO00/42072 (Presta, L.).

Engineered antibodies with three or more (preferably four) functional antigen binding sites are also contemplated (US Appln No. US2002/0004587 A1, Miller *et al.*).

The HER2 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 WO97/38731 published October 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Patent 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).

IV. Pharmaceutical Formulations

Therapeutic formulations of the compositions of the present invention are prepared for storage by mixing the composition with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. 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 HER2 antibody formulations are described in WO 97/04801. Particularly preferred formulations for the present composition are described in US20006/088523.

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. For example, it may be desirable to further provide antibodies which bind to EGFR, HER2 (*e.g.* an antibody which binds a different epitope on HER2), HER3, HER4, or vascular endothelial factor (VEGF) in the one formulation. Alternatively, or additionally, the composition may further comprise a

chemotherapeutic agent, cytotoxic agent, cytokine, growth inhibitory agent, anti-hormonal agent, EGFR-targeted drug, anti-angiogenic agent, tyrosine kinase inhibitor, and/or cardioprotectant. 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-(methylmethacylate) 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 γ 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.

V. Treatment with the HER2 Antibody Composition

It is contemplated that, according to the present invention, the HER2 antibody may be used to treat cancer. The cancer will generally be HER2 positive, such that the HER2 antibody herein is able to bind to the cancer cells. In one embodiment, the cancer expresses low HER3 (*e.g.* ovarian cancer) or has elevated HER2:HER3 ratio (*e.g.* ovarian cancer). Various cancers that can be treated with the composition are listed in the definitions section above.

Preferred cancers to be treated herein include: breast cancer, including HER2 positive

breast cancer, optionally in combination with trastuzumab and a taxoid such as docetaxel, and including neoadjuvant therapy of breast cancer; ovarian cancer (including both platinum-resistant and platinum-sensitive ovarian cancer) (see US2006/0013819, for example); lung cancer (including non small cell lung cancer, NSCLC), optionally in combination with an EGFR inhibitor (see US 2007/0020261 for example); colorectal cancer, etc.

It is also contemplated that the HER2 antibody may be used to treat various non-malignant diseases or disorders, such as autoimmune disease (*e.g.* psoriasis); endometriosis; scleroderma; restenosis; polyps such as colon polyps, nasal polyps or gastrointestinal polyps; fibroadenoma; respiratory disease; cholecystitis; neurofibromatosis; polycystic kidney disease; inflammatory diseases; skin disorders including psoriasis and dermatitis; vascular disease; conditions involving abnormal proliferation of vascular epithelial cells; gastrointestinal ulcers; Menetrier's disease, secreting adenomas or protein loss syndrome; renal disorders; angiogenic disorders; ocular disease such as age related macular degeneration, presumed ocular histoplasmosis syndrome, retinal neovascularization from proliferative diabetic retinopathy, retinal vascularization, diabetic retinopathy, or age related macular degeneration; bone associated pathologies such as osteoarthritis, rickets and osteoporosis; damage following a cerebral ischemic event; fibrotic or edemia diseases such as hepatic cirrhosis, lung fibrosis, carcoidosis, throiditis, hyperviscosity syndrome systemic, Osler Weber-Rendu disease, chronic occlusive pulmonary disease, or edema following burns, trauma, radiation, stroke, hypoxia or ischemia; hypersensitivity reaction of the skin; diabetic retinopathy and diabetic nephropathy; Guillain-Barre syndrome; graft versus host disease or transplant rejection; Paget's disease; bone or joint inflammation; photoaging (*e.g.* caused by UV radiation of human skin); benign prostatic hypertrophy; certain microbial infections including microbial pathogens selected from adenovirus, hantaviruses, Borrelia burgdorferi, Yersinia spp. and Bordetella pertussis; thrombus caused by platelet aggregation; reproductive conditions such as endometriosis, ovarian hyperstimulation syndrome, preeclampsia, dysfunctional uterine bleeding, or menometrorrhagia; synovitis; atheroma; acute and chronic nephropathies (including proliferative glomerulonephritis and diabetes-induced renal disease); eczema; hypertrophic scar formation; endotoxic shock and fungal infection; familial adenomatosis polyposis; neurodedenerative diseases (*e.g.* Alzheimer's disease, AIDS-related dementia, Parkinson's disease, amyotrophic lateral sclerosis, retinitis

pigmentosa, spinal muscular atrophy and cerebellar degeneration); myelodysplastic syndromes; aplastic anemia; ischemic injury; fibrosis of the lung, kidney or liver; T-cell mediated hypersensitivity disease; infantile hypertrophic pyloric stenosis; urinary obstructive syndrome; psoriatic arthritis; and Hasimoto's thyroiditis. Preferred non-malignant indications for therapy herein include psoriasis, endometriosis, scleroderma, vascular disease (*e.g.* restenosis, artherosclerosis, coronary artery disease, or hypertension), colon polyps, fibroadenoma or respiratory disease (*e.g.* asthma, chronic bronchitis, bronchieactasis or cystic fibrosis).

Treatment with the HER2 antibody will result in an improvement in the signs or symptoms of disease. For instance, where the disease being treated is cancer, such therapy may result in an improvement in survival (overall survival and/or progression free survival) and/or may result in an objective clinical response (partial or complete).

Preferably, the HER2 antibody in the composition administered is a naked antibody. However, the HER2 antibody administered may be conjugated with a cytotoxic agent. Preferably, the immunoconjugate and/or HER2 protein to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate 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 HER2 antibody is 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, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. Intravenous administration of antibody composition is prefered.

For the prevention or treatment of disease, the appropriate dosage of HER2 antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the HER2 antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the HER2 antibody, and the discretion of the attending physician. The HER2 antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 50 mg/kg (*e.g.* 0.1-20mg/kg) of HER2 antibody is an initial

candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. In one embodiment, the initial infusion time for the HER2 antibody may be longer than subsequent infusion times, for instance approximately 90 minutes for the initial infusion, and approximately 30 minutes for subsequent infusions (if the initial infusion is well tolerated). The preferred dosage of the HER2 antibody will be in the range from about 0.05mg/kg to about 10mg/kg. Thus, one or more doses of about 0.5mg/kg, 2.0mg/kg, 4.0mg/kg or 10mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, *e.g.* every week or every three weeks (*e.g.* such that the patient receives from about two to about twenty, *e.g.* about six doses of the HER2 antibody). An initial higher loading dose, followed by one or more lower doses may be administered. In one embodiment, the HER2 antibody is administered as a loading dose of approximately 840 mg followed by approximately 420 mg administered approximately every 3 weeks. In another embodiment, the HER2 antibody is administered as a loading dose of approximately 1050mg followed by approximately 525mg administered approximately every 3 weeks.

Other therapeutic agents may be combined with the HER2 antibody. Such 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 other therapeutic agent may be administered prior to, or following, administration of the HER2 antibody. In this embodiment, the timing between at least one administration of the other therapeutic agent and at least one administration of the HER2 antibody is preferably approximately 1 month or less, and most preferably approximately 2 weeks or less. Alternatively, the other therapeutic agent and the HER2 antibody are administered concurrently to the patient, in a single formulation or separate formulations.

Examples of other therapeutic agents that can be combined with the HER2 antibody include any one or more of: a chemotherapeutic agent, such as an anti-metabolite, *e.g.* gemcitabine; a second, different HER2 antibody (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); a second antibody

directed against another 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 Erlitonib, Gefitinib, 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 HER2 AutoVac vaccine from Pharmexia, or APC8024 protein vaccine from Dendreon, or HER2 peptide vaccine from GSK/Corixa); another HER targeting therapy (*e.g.* trastuzumab, cetuximab, gefitinib, erlotinib, CI1033, GW2016 etc); Raf and/or ras inhibitor (see, for example, WO 2003/86467); Doxil; Topetecan; taxane; GW572016; TLK286; 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 body temperature-reducing medicament such as acetaminophen, diphenhydramine, or meperidine; hematopoietic growth factor, etc.

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 HER2 antibody. Treatment with the combination of the HER2 antibody composition and other therapeutic agent may result in a synergistic, or greater than additive, therapeutic benefit to the patient.

If a chemotherapeutic agent is administered, it 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 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. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992).

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

VII. Deposit of Materials

The following hybridoma cell lines have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, USA (ATCC):

Antibody Designation	ATCC No.	Deposit Date
7C2	ATCC HB-12215	October 17, 1996
7F3	ATCC HB-12216	October 17, 1996
4D5	ATCC CRL 10463	May 24, 1990
2C4	ATCC HB-12697	April 8, 1999

Further details of the invention are illustrated by the following non-limiting Example. The disclosures of all citations in the specification are expressly incorporated herein by reference.

EXAMPLE 1

This example describes the characterization of a composition comprising a main species HER2 antibody that binds to domain II of HER2 (pertuzumab) and acidic variants thereof.

Pertuzumab is a recombinant humanized monoclonal antibody, generated based on human IgG1(κ) framework. It comprises two heavy chains (448 residues) and two light chains (214 residues). The two heavy chains are linked by two interchain disulfides and each light chain is attached to a heavy chain through one interchain disulfide. There is an N-linked glycosylation site in the Fc region of pertuzumab at Asn-299 of the two heavy chains. Pertuzumab differs from HERCEPTIN® (Trastuzumab) in the epitope binding regions of the light chain (12 amino acid differences) and the heavy chain (30 amino acid differences). As a result of these differences, pertuzumab binds to a completely different epitope on the HER2 receptor. Binding of pertuzumab to the HER2 receptor on human epithelial cells prevents it from forming complexes with other members of the HER receptor family (Agus *et al., Cancer Cell* 2:127-137 (2002)). By blocking complex formation, pertuzumab prevents the growth-stimulatory effects of ligands for the complexes (*e.g.*, EGF and heregulin). *In vitro* experiments demonstrated that both pertuzumab and pertuzumab-Fab inhibit the binding of heregulin (HRG) to MCF7 cells, and that the HRG-stimulated phosphorylation of the HER2-HER3 complex can be inhibited by both pertuzumab and pertuzumab-Fab (Agus *et al., Cancer Cell* 2:127-137 (2002)). Furthermore, *in vivo* inhibition of tumor growth by pertuzumab and a polyethylene glycol

derivatized Fab of pertuzumab were found to be comparable in a murine prostate cancer xenograft model (Agus *et al., Cancer Cell* 2:127-137 (2002)). These data suggest that the Fc region of the antibody is not necessary for the inhibition of tumor growth, and moreover, bivalency and Fc-mediated effector functions are not required for *in vivo* or *in vitro* biological activity.

In this example, the main peak of pertuzumab was collected from a cation exchange column and incubated in cell culture media or processed using standard antibody purification operations. Acidic variants formed upon incubation of main peak with cell culture media components. Acidic variants of monoclonal antibodies are modified forms of the desired product that elute earlier than the main peak upon separation by cation exchange chromatography. Subtle differences in the amount and/or distribution of acidic variants are often observed pre- and post-process changes and pose a challenge to demonstrating product comparability. Purification operations had little effect on formation of acidic variants. Variants identified in the acidic variant fraction included glycated variant, deamidated variant, disulfide-reduced variant, sialylated variant, and non-reducible variant. Collectively, the acidic variants were fully potent.

Amongst other things, the purpose of this study was to: better understand the impact of cell culture and recovery processes on pertuzumab acidic variant formation, characterize the predominant acidic variants of pertuzumab, and evaluate the impact of acidic variants on pharmacokinetics (PK).

Rat pharmacokinetic results show that the area under the curve for the acidic variant fraction and main peak fraction were equivalent to pertuzumab starting material (geometric mean ratios 0.96 and 0.95, respectively). These results demonstrate that although acidic variants are chemically different from the main peak they have equivalent pharmacokinetics.

Methods and Results

Figure 10 depicts the experimental design for isolation of cation exchange MP (Main Peak) and AV (Acidic Variants), cell culture, recovery, and PK (pharmacokinetics) evaluation and analytical testing. Fresh media = standard media; spent media = standard media after 12 days of cell culture, cells were removed by centrifugation. Dissolved oxygen, pH, and other parameters were not controlled.

A. Isolation of Main Peak and Acidic Variants

Charge variants of pertuzumab were separated on a 4.0 x 250 mm DIONEX PROPAC WCX-10™ cation exchange (CEX) column using the following conditions:

Buffer A: 20mM BisTris, pH 6.0

Buffer B: 20mM BisTris, 200mM NaCl, pH 6.0

Gradient: 0.5% Buffer B delivered at 1.0 mL/min

Column Temperature: 35°C

Detection: 280 nm

A typical chromatogram is shown in Figure 11. AV (acidic variant) and MP (main peak) fractions were collected.

Potency and monomer content were similar among the pertuzmab starting material, main peak, and acidic variants (Figure 12). Purity of the main peak and acidic variant CEX fractions was acceptable for pharmacokinetics studies based on the criteria of 90% purity by CEX (Figure 12).

B. Main Peak Spiking Experiments

Pertuzumab main peak isolated by CEX was spiked into either fresh or spent cell culture media (no cells) and incubated for 12 days at 37°C as outlined in Figure 10. Samples at various time points were directly analyzed by CEX or after isolation by protein A. Main peak was also spiked into media +/- various media components such as glucose and peptone. In addition, main peak was processed through standard recovery operations such as protein A chromatorgraphy (ProA), low pH treatment, and SP Sepharose Fast Flow (SPFF) for multiple cycles and analyzed by CEX.

CEX profiles of main peak incubated for 12 days in fresh or spent media were similar (Figures 13 and 14). Main peak decreased more after incubation in fresh media than in spent media. Removal of various media components did affect the decrease of main peak. Percent main peak in incubated samples was the same with and without protein A isolation. Incubation in media buffer alone caused a loss of main peak.

Protein A isolation of main peak from media did not affect the CEX profile demonstrating that modifications during incubation do not affect protein A binding or elution. Recovery operations had little or no effect on the percent CEX main peak.

C. Characterization of Acidic Variants

Pertuzumab acidic variants were isolated by CEX from pertuzumab starting material or main peak incubated in cell culture media. The isolated acidic variants were analyzed by the methods listed in Figure 15. Acidic variants comprise 21% of total peak area, therefore about 80% (17% of 21%) of acidic variants were identified. Deamidated forms could not be quantified.

Forms identified in acidic variants generated by main peak incubated with media were the same as those identified in pertuzumab starting material. The following forms were detected: sialylated variant, disulfide reduced variant, glycated variant, non-reducible variant, and deamidated variant. Higher order glycated forms were identified by electrospray ionization-mass spectrometry (ESI-MS) after reduction and PNGase treatment.

D. Pharmacokinetics (PK) Study

A single intravenous (IV) dose of 10 mg/kg, 12 rats per arm, 3 arms (acidic variants, main peak, pertuzumab starting material). Extensive PK sampling was conducted for 35 days. Geometric Mean Ratio of AUC (Day 0-14) between acidic, main peak, and pertuzumab starting material. Geometric Mean Ratio = GM Sample/GM IgG1 Starting Material. Pertuzumab concentration versus time curve were similar for the pertuzumab starting material, acidic variant, and main peak (Figures 16 and 17). No significant difference in exposure was observed between acidic variants, main peak, and pertuzumab starting material. The GMR was ~1.0 with 90% CI between 0.80 – 1.25.

Conclusions

Multiple cell culture factors contribute to acidic variant formation, but recovery was not shown to effect acidic variant formation. Disulfide reduced, non-reducible, sialylated, glycated, and deamidated variants were identified in the acidic fraction. Acidic fraction isolated from pertuzumab starting material and those generated by incubation of CEX main peak contained the same forms. Acidic variants, main peak, and pertuzumab starting material had the same pharmacokinetics.

CLAIMS

Claim 1. A composition comprising a main species HER2 antibody that comprises light chain and heavy chain amino acid sequences in SEQ ID NOs. 15 and 16 respectively and binds to domain II of HER2, and acidic variants of that main species antibody, wherein the main species HER2 antibody is the antibody amino acid sequence structure in the composition which is the quantitatively predominant antibody molecule in the composition and the main species HER2 antibody and the acidic variants are all intact antibodies, wherein the acidic variants include a disulfide reduced variant, a non-reducible variant or both variants of the main species antibody.

Claim 2. The composition of claim 1, wherein the acidic variant comprises a disulfide reduced variant of the main species HER2 antibody.

Claim 3. The composition of claim 1 or claim 2, wherein the acidic variant comprises a non-reducible variant of the main species HER2 antibody.

Claim 4. The composition of claim 1 or claim 2, wherein the composition further comprises a glycated variant, a deamidated variant or a sialylated variant.

Claim 5. The composition of claim 1 or claim 2, wherein the composition comprises a glycated variant, a deamidated variant, a disulfide reduced variant, a sialylated variant, and a non-reducible variant.

Claim 6. The composition of claim 1 or claim 2, wherein the amount of the acidic variant(s) is less than about 25% of the total composition.

Claim 7. The composition of claim 1 or claim 2, comprising an amino-terminal leader extension variant of the main species HER2 antibody.

Claim 8. The composition of claim 7, wherein the amino-terminal leader extension comprises VHS-.

Claim 9. The composition of claim 8, wherein the amino-terminal leader extension consists of VHS-.

Claim 10. The composition of claim 1 or claim 2, further comprising an amino acid sequence variant of the main species HER2 antibody selected from the group consisting of an antibody comprising a C-terminal lysine residue on one or both heavy chains thereof, and an antibody with one or more oxidized methionine residues.

Claim 11. A pharmaceutical formulation comprising the composition of claim 1 or claim 2 in a pharmaceutically acceptable carrier.

Claim 12. The pharmaceutical formulation of claim 11 for use in treating HER2 positive cancer in a patient.

Claim 13. The pharmaceutical formulation of claim 12, wherein the cancer is selected from the group consisting of breast cancer, ovarian cancer, lung cancer, and colorectal cancer.

Claim 14. The pharmaceutical formulation of claim 11 or 12, wherein the pharmaceutical formulation is to be administered in combination with Trastuzumab.

Claim 15. The pharmaceutical formulation of claim 14, for use in treating breast cancer.

Claim 16. The pharmaceutical formulation of claim 11, wherein the main species HER2 antibody and acidic variant(s) have essentially the same pharmacokinetics.

Claim 17. A method of making an evaluated pharmaceutical composition, comprising:
(1) preparing a composition comprising a main species HER2 antibody that comprises light chain and heavy chain amino acid sequences in SEQ ID NOs. 15 and 16 respectively and binds to domain II of HER2, and acidic variants of that main species HER2 antibody including a disulfide reduced variant, a non-reducible variant or both variants of the main species antibody, wherein the main species HER2 antibody is the antibody amino acid sequence structure in the composition which is the quantitatively predominant antibody molecule in the composition and the main species HER2 antibody and the acidic variants are all intact antibodies, and (2) evaluating the acidic variants in the composition, and confirming that the

amount thereof is less than about 25% of the total composition.

Claim 18. The method of claim 17, wherein the composition comprises a glycated variant, a deamidated variant, a disulfide reduced variant, a sialylated variant, and a non-reducible variant.

Claim 19. The method of claim 17 or claim 18, wherein step (2) comprises evaluating the acidic variants by a method selected from the group consisting of ion exchange chromatography wherein the composition is treated with sialidase, reduced capillary electrophoresis with sodium dodecyl sulfate (CE-SDS), non-reduced CE-SDS, boronate chromatography, and peptide mapping.

Claim 20. The method of claim 17 or claim 18, wherein step (2) comprises evaluating the acidic variants by ion exchange chromatography.

Claim 21. The method of claim 20 which comprises cation exchange chromatography using a cation exchanger with a carboxylate functional group.

Claim 22. The method of claim 21, wherein the conditions for the chromatography involve: Buffer A of 20 mM BisTris, pH 6.0; Buffer B of 20 mM BisTris, 200 mM NaCl, pH 6.0; and a gradient of 0.5% Buffer B at 1.0 mL/min.

Claim 23. The method of claim 17 or claim 18, comprising combining the composition after step (2) with a pharmaceutically acceptable carrier.

Claim 24. The method of claim 17 or claim 18, wherein the composition evaluated in step (2) is in a pharmaceutically acceptable carrier.

Claim 25. A composition produced by the method of claim 17 or claim 18.

P4169R1WOSEQLIST
Sequence Listing

<110> GENENTECH, INC.
HARRIS, REED J.
MOTCHNIK, PAUL A.

<120> COMPOSITION COMPRISING ANTIBODY THAT BINDS TO DOMAIN II OF HER2 AND ACIDIC VARIANTS THEREOF

<130> P4169R1 WO

<141> 2009-01-28

<150> US 61/024,825
<151> 2008-01-30

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50 55 60

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35 40 45

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35 40 45

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35 40 45

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Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
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65 70 75

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95 100 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
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Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr

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Asn Pro Gln Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile
140 145 150

Phe His Lys Asn Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn
155 160 165

Arg Ser Arg Ala Cys His Pro Cys Ser Pro Met Cys Lys Gly Ser
170 175 180

Arg Cys Trp Gly Glu Ser Ser Glu Asp Cys Gln Ser Leu Thr Arg
185 190 195

<210> 20

<211> 124
<212> PRT
<213> Homo sapiens

<400> 20

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 Thr Val Cys Ala Gly Gly Cys Ala Arg Cys Lys Gly Pro Leu Pro
   1               5                  10                  15

 Thr Asp Cys Cys His Glu Gln Cys Ala Ala Gly Cys Thr Gly Pro
              20                  25                  30

 Lys His Ser Asp Cys Leu Ala Cys Leu His Phe Asn His Ser Gly
              35                  40                  45

 Ile Cys Glu Leu His Cys Pro Ala Leu Val Thr Tyr Asn Thr Asp
              50                  55                  60

 Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg Tyr Thr Phe Gly
              65                  70                  75

 Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu Ser Thr Asp
              80                  85                  90

 Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln Glu Val
              95                 100                 105

 Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys Pro
             110                 115                 120

 Cys Ala Arg Val
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<210> 21
<211> 169
<212> PRT
<213> Homo sapiens

<400> 21

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 Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu Val Arg Ala Val
   1               5                  10                  15

 Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys Lys Ile Phe
              20                  25                  30

 Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp Pro Ala
              35                  40                  45

 Ser Asn Thr Ala Pro Leu Gln Pro Glu Gln Leu Gln Val Phe Glu
              50                  55                  60

 Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
              65                  70                  75

 Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile
              80                  85                  90

 Arg Gly Arg Ile Leu His Asn Gly Ala Tyr Ser Leu Thr Leu Gln
              95                 100                 105

 Gly Leu Gly Ile Ser Trp Leu Gly Leu Arg Ser Leu Arg Glu Leu
             110                 115                 120

 Gly Ser Gly Leu Ala Leu Ile His His Asn Thr His Leu Cys Phe
```

125 130 135

Val His Thr Val Pro Trp Asp Gln Leu Phe Arg Asn Pro His Gln
140 145 150

Ala Leu Leu His Thr Ala Asn Arg Pro Glu Asp Glu Cys Val Gly
155 160 165

Glu Gly Leu Ala

<210> 22
<211> 142
<212> PRT
<213> Homo sapiens

<400> 22
Cys His Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro
1 5 10 15

Thr Gln Cys Val Asn Cys Ser Gln Phe Leu Arg Gly Gln Glu Cys
20 25 30

Val Glu Glu Cys Arg Val Leu Gln Gly Leu Pro Arg Glu Tyr Val
35 40 45

Asn Ala Arg His Cys Leu Pro Cys His Pro Glu Cys Gln Pro Gln
50 55 60

Asn Gly Ser Val Thr Cys Phe Gly Pro Glu Ala Asp Gln Cys Val
65 70 75

Ala Cys Ala His Tyr Lys Asp Pro Pro Phe Cys Val Ala Arg Cys
80 85 90

Pro Ser Gly Val Lys Pro Asp Leu Ser Tyr Met Pro Ile Trp Lys
95 100 105

Phe Pro Asp Glu Glu Gly Ala Cys Gln Pro Cys Pro Ile Asn Cys
110 115 120

Thr His Ser Cys Val Asp Leu Asp Asp Lys Gly Cys Pro Ala Glu
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Gln Arg Ala Ser Pro Leu Thr
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<210> 23
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<213> Artificial sequence

<220>
<223> Sequence is synthesized.

<400> 23
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1 5 10 15

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20 25 30

Asp Val Ser Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys

35 40 45

Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly
50 55 60

Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr
65 70 75

Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr
80 85 90

Cys Gln Gln Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr
95 100 105

Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile
110 115 120

Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val
125 130 135

Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln
140 145 150

Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser
155 160 165

Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
170 175 180

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
185 190 195

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys
200 205 210

Ser Phe Asn Arg Gly Glu Cys
215

<210> 24
<211> 449
<212> PRT
<213> Artificial sequence

<220>
<223> Sequence is synthesized.

<400> 24
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
1 5 10 15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr
20 25 30

Asp Tyr Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
35 40 45

Glu Trp Val Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr
50 55 60

Asn Gln Arg Phe Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser
65 70 75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp

80 85 90

Thr Ala Val Tyr Tyr Cys Ala Arg Asn Leu Gly Pro Ser Phe Tyr
95 100 105

Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala
110 115 120

Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
125 130 135

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp
140 145 150

Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu
155 160 165

Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly
170 175 180

Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
185 190 195

Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn
200 205 210

Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr
215 220 225

His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro
230 235 240

Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
260 265 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
275 280 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
290 295 300

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp
305 310 315

Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
320 325 330

Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro
335 340 345

Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met
350 355 360

Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
365 370 375

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
380 385 390

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser

395 400 405

Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
410 415 420

Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
425 430 435

Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
440 445

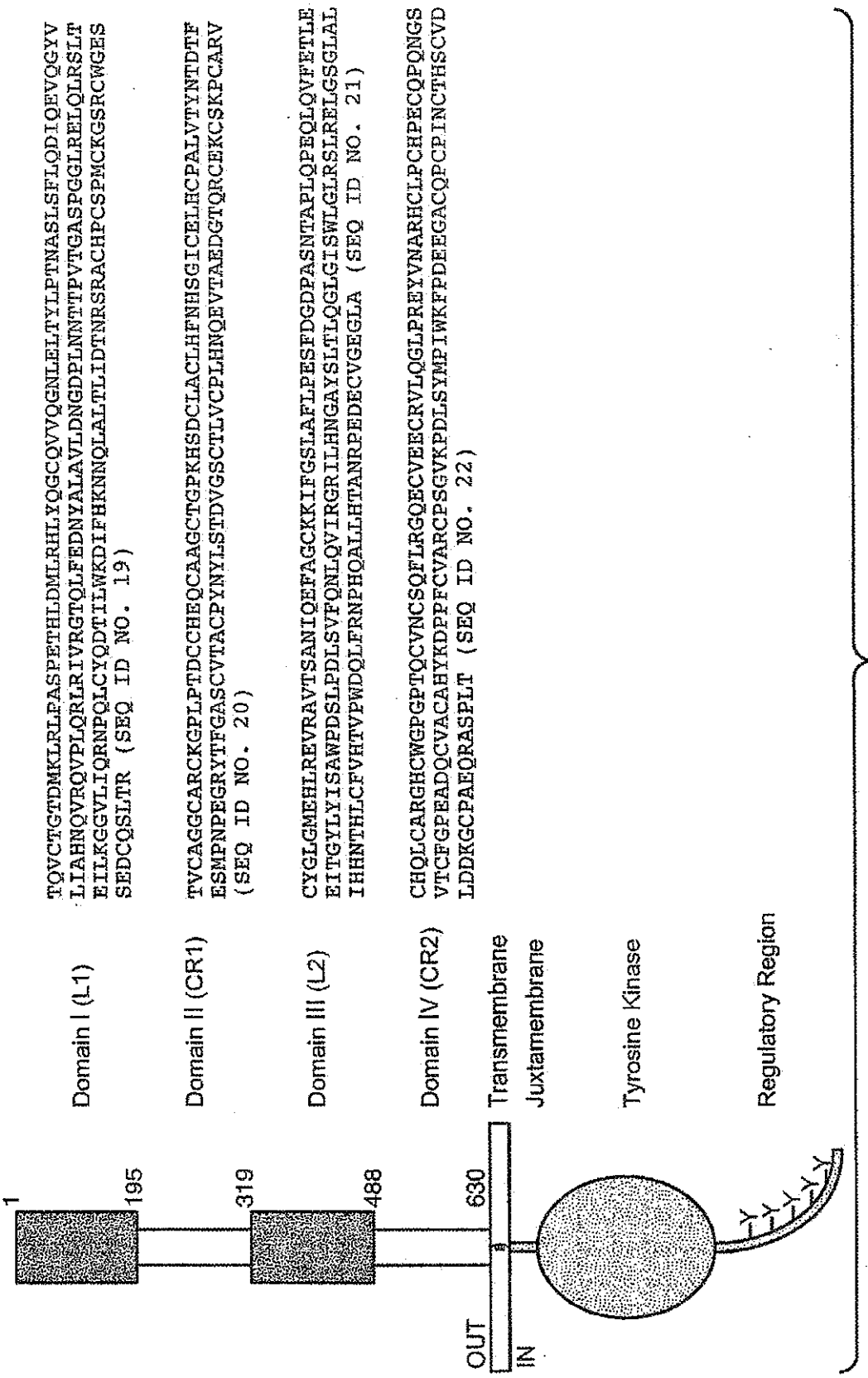

1
195
319
488
630
Domain I (L1)
Domain II (CR1)
Domain III (L2)
Domain IV (CR2)
Transmembrane
Juxtamembrane
Tyrosine Kinase
Regulatory Region
OUT
IN
Y
Y
Y
Y
Y
TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQGYV
LIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLT
EILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGES
SEDCQSLTR (SEQ ID NO. 19)
TVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTF
ESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARV
(SEQ ID NO. 20)
CYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLE
EITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLAL
IHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLA (SEQ ID NO. 21)
CHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGS
VTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVD
LDDKGCPAEQRASPLT (SEQ ID NO. 22)

FIG. 1

Variable Light

```
                10          20          30          40
2C4     DTVMTQSHKIMSTSVGDRVSITC [KASQDVSIGVA] WYQQRP
         **    **** *      *                    *
574     DIQMTQSPSSLSASVGDRVTITC [KASQDVSIGVA] WYQQKP
                                  *   ** ***
hum κI  DIQMTQSPSSLSASVGDRVTITC [RASQSISNYLA] WYQQKP

                50          60          70          80
2C4     GQSPKLLIY [SASYRYT] GVPDRFTGSGSGTDFTFTISSVQA
          **                  * *         *    * *
574     GKAPKLLIY [SASYRYT] GVPSRFSGSGSGTDFTLTISSLQP
                   * *****
hum κI  GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP

                90         100
2C4     EDLAVYYC [QQYYIYPYT] FGGGTKLEIK (SEQ ID NO:1)
           * *                 *   *
574     EDFATYYC [QQYYIYPYT] FGQGTKVEIK (SEQ ID NO:3)
                    *** *
hum κI  EDFATYYC [QQYNSLPWT] FGQGTKVEIK (SEQ ID NO:5)
```

FIG. 2A

Variable Heavy

```
                10          20          30          40
2C4     EVQLQQSGPELVKPGTSVKISCKAS [GFTFTDYTMD] WVKQS
           **  **  *  * *** *                    * *
574     EVQLVESGGGLVQPGGSLRLSCAAS [GFTFTDYTMD] WVRQA
                                    ** * *
hum III EVQLVESGGGLVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

                50 a        60          70          80
2C4     HGKSLEWIG [DVNPNSGGSIYNQRFKG] KASLTVDRSSRIVYM
          *  *   **                    *** *    **** *
574     PGKGLEWVA [DVNPNSGGSIYNQRFKG] RFTLSVDRSKNTLYL
                   ****** *** ****      * * *
hum III PGKGLEWVA [VISGDGGSTYYADSVKG] RFTISRDNSKNTLYL

                abc 90         100ab        110
2C4     ELRSLTFEDTAVYYCAR [NLGPSFYFDY] WGQGTTLTVSS (SEQ ID NO:2)
        ***  **                          **
574     QMNSLRAEDTAVYYCAR [NLGPSFYFDY] WGQGTLVTVSS (SEQ ID NO:4)
                           ********
hum III QMNSLRAEDTAVYYCAR [GRVGYSLYDY] WGQGTLVTVSS (SEQ ID NO:6)
```

FIG. 2B

Amino Acid Sequence for Pertuzumab Light Chain

```
1       10        20        30        40        50        60
|       |         |         |         |         |         |
DIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKAPKLLIYSASYRYTGVPS

        70        80        90        100       110       120
        |         |         |         |         |         |
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYYIYPYTFGQGTKVEIKRTVAAPSVFIFPP

        130       140       150       160       170       180
        |         |         |         |         |         |
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLT

        190       200       210
        |         |         |
LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
```

FIG. 3A

Amino Acid Sequence for Pertuzumab Heavy Chain

```
1       10        20        30        40        50        60
|       |         |         |         |         |         |
EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKGLEWVADVNPNSGGSIY

        70        80        90        100       110       120
        |         |         |         |         |         |
NQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPSFYFDYWGQGTLVTVSSA

        130       140       150       160       170       180
        |         |         |         |         |         |
STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG

        190       200       210       220       230       240
        |         |         |         |         |         |
LYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGP

        250       260       270       280       290       300
        |         |         |         |         |         |  *
SVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS

        310       320       330       340       350       360
        |         |         |         |         |         |
TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM

        370       380       390       400       410       420
        |         |         |         |         |         |
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ

        430       440       448
        |         |         |
QGNVFSCSVMHEALHNHYTQKSLSLSPG
```

FIG. 3B

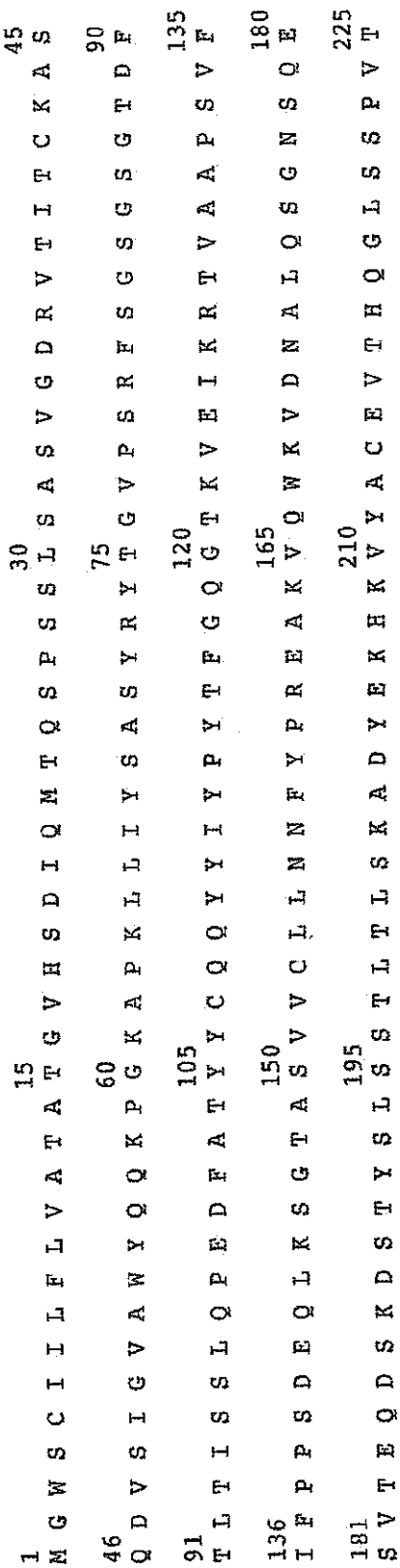

1 M G W S C I I L F L V A T A T G V H S D I Q M T Q S P S S L S A S V G D R V T I T C K A S
46 Q D V S I G V A W Y Q Q K P G K A P K L L I Y S A S Y R Y T G V P S R F S G S G S G T D F
91 T L T I S S L Q P E D F A T Y Y C Q Q Y Y I Y P Y T F G Q G T K V E I K R T V A A P S V F
136 I F P P S D E Q L K S G T A S V V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E
181 S V T E Q D S K D S T Y S L S S T L T L S K A D Y E K H K V Y A C E V T H Q G L S S P V T
226 K S F N R G E C
15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 233
(SEQ ID NO. 17)

FIG. 4A

```
1                                15                                30                                45
M G W S C I I L F L V A T A T G V H S E V Q L V E S G G G L V Q P G G S L R L S C A A S G

46                               60                                75                                90
F T F T D Y T M D W V R Q A P G K G L E W V A D V N P N S G G S I Y N Q R F K G R F T L S

91                               105                               120                               135
V D R S K N T L Y L Q M N S L R A E D T A V Y Y C A R N L G P S F Y F D Y W G Q G T L V T

136                              150                               165                               180
V S S A S T K G P S V F P L A P S S K S T S G G T A A L G C L V K D Y F P E P V T V S W N

181                              195                               210                               225
S G A L T S G V H T F P A V L Q S S G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H

226                              240                               255                               270
K P S N T K V D K K V E P K S C D K T H T C P P C P A P E L L G G P S V F L F P P K P K D

271                              285                               300                               315
T L M I S R T P E V T C V V V D V S H E D P E V K F N W Y V D G V E V H N A K T K P R E E

316                              330                               345                               360
Q Y N S T Y R V V S V L T V L H Q D W L N G K E Y K C K V S N K A L P A P I E K T I S K A

361                              375                               390                               405
K G Q P R E P Q V Y T L P P S R E E M T K N Q V S L T C L V K G F Y P S D I A V E W E S N

406                              420                               435                               450
G Q P E N N Y K T T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H

451                              465
E A L H N H Y T Q K S L S L S P G    (SEQ ID NO. 18)
```

FIG. 4B

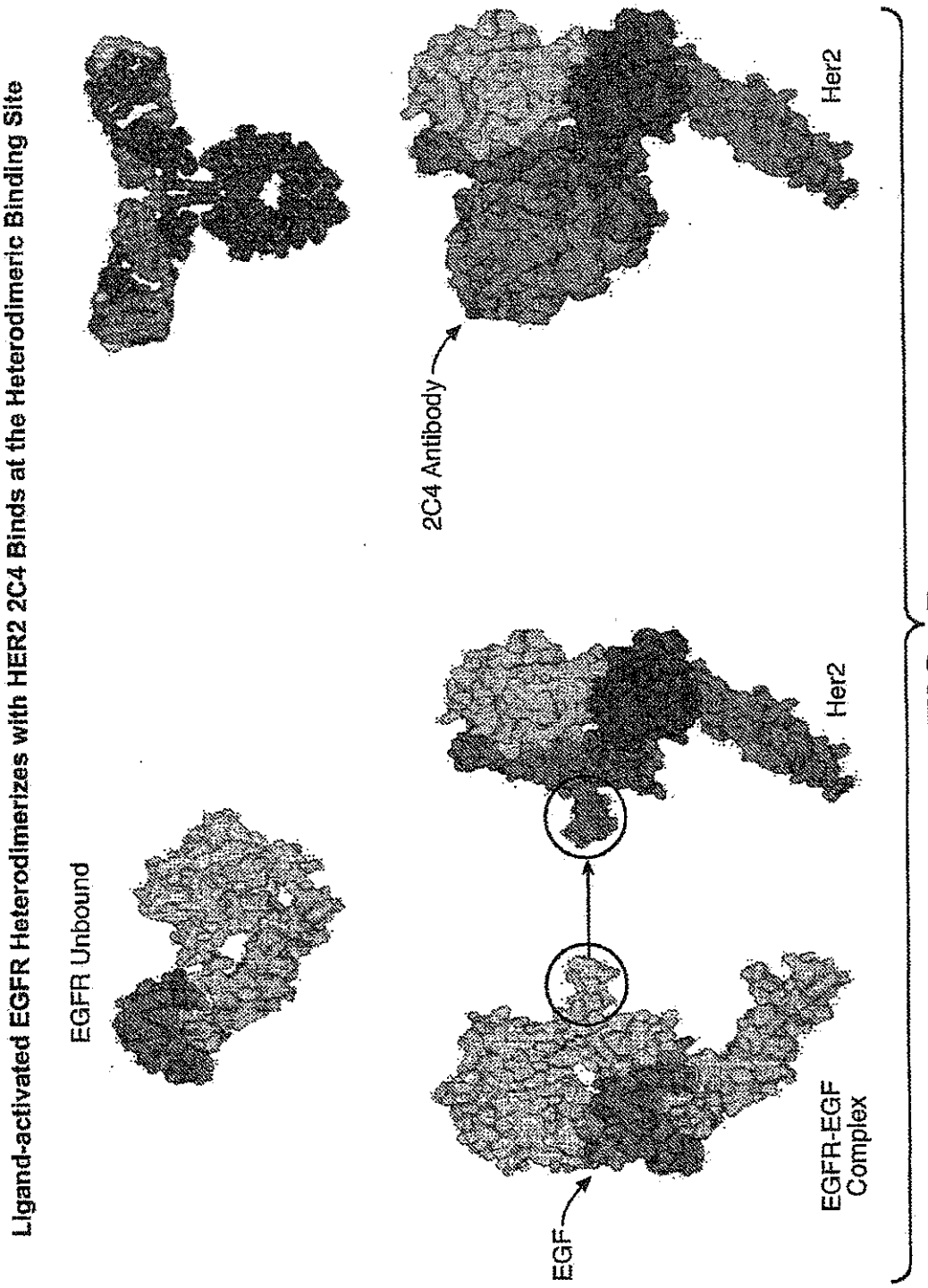

Ligand-activated EGFR Heterodimerizes with HER2 2C4 Binds at the Heterodimeric Binding Site
EGFR Unbound
2C4 Antibody
Her2
Her2
EGF
EGFR-EGF Complex

FIG. 5

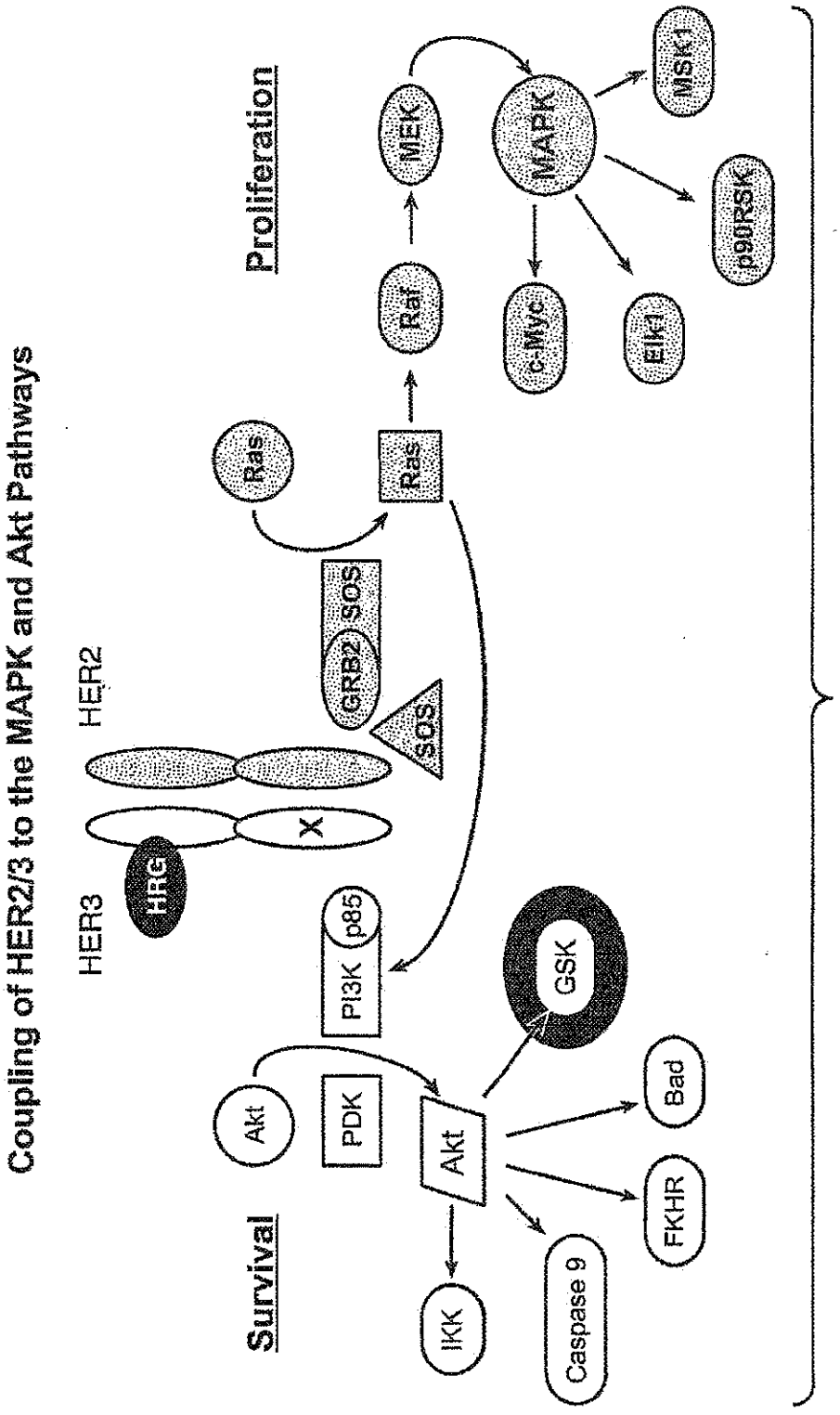
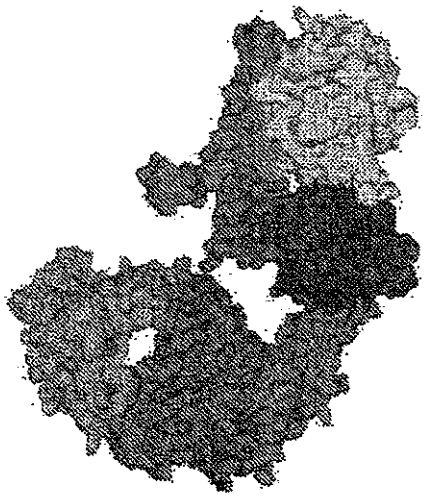

Coupling of HER2/3 to the MAPK and Akt Pathways
HER3
HER2
HRG
Ras
GRB2
SOS
SOS
Ras
Raf
MEK
MAPK
MSK1
p90RSK
c-Myc
Elk-1
Proliferation
PI3K
p85
Akt
PDK
Akt
GSK
Bad
FKHR
IKK
Caspase 9
Survival

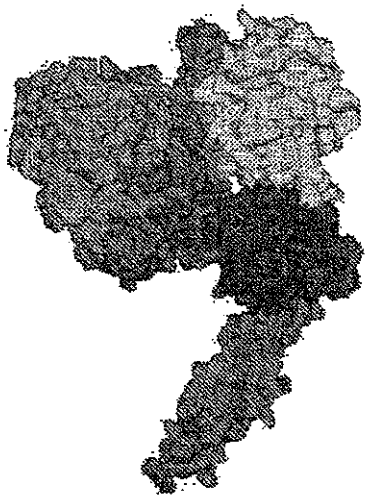
FIG. 6

Trastuzumab
Herceptin



- Binds in IV near JM.
- Protects against receptor shedding
- Moderately affects receptor down-modulation
- Slight effect on HER2's role as a coreceptor

Pertuzumab
Omnitarg



- Binds in II at dimerization interface
- Does not prevent receptor shedding
- Moderately affects receptor down-modulation
- Major effect on HER2's role as a coreceptor

FIG. 7

Light Chain

```
                15                  30                  45
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
                60                  75                  90
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
                105                 120                 135
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
                150                 165                 180
136 L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S T L T
                195                 210     214
181 L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C
```

FIG. 8A

Heavy Chain

```
1                               15                              30                              45
E V Q L V E S G G G L V Q P G G S L R L S C A A S G F N I K D T Y I H W V R Q A P G K G L

46                              60                              75                              90
E W V A R I Y P T N G Y T R Y A D S V K G R F T I S A D T S K N T A Y L Q M N S L R A E D

91                              105                             120                             135
T A V Y Y C S R W G G D G F Y A M D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S

136                             150                             165                             180
K S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S

181                             195                             210                             225
G L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K

226                             240                             255                             270
T H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S

271                             285                             300                             315
H E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D

316                             330                             345                             360
W L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E

361                             375                             390                             405
M T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G

406                             420                             435                             449
S F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G
```

FIG. 8B

```
1                                           15                                          30                                          45
V H S D I Q M T Q S P S S L S A S V G D R V T I T C K A S Q D V S I G V A W Y Q Q K P G K

46                                          60                                          75                                          90
A P K L L I Y S A S Y R Y T G V P S R F S G S G S G T D F T L T I S S L Q P E D F A T Y Y

91                                          105                                         120                                         135
C Q Q Y Y I Y P Y 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

136                                         150                                         165                                         180
V C L L N N F Y P R E A K V Q W K V D N A L Q S G N S Q E S V T E Q D S K D S T Y S L S S

181                                         195                                         210                 217
T L T L S K A D Y E K H K V Y A C E V T H Q G L S S P V T K S F N R G E C   (SEQ ID NO. 23)
```

FIG. 9A

```
1                                 15                                30                                45
E V Q L V E S G G G L V Q P G G S L R L S C A A S G F T F T D Y T M D W V R Q A P G K G L

46                                60                                75                                90
E W V A D V N P N S G G S I Y N Q R F K G R F T L S V D R S K N T L Y L Q M N S L R A E D

91                                105                               120                               135
T A V Y Y C A R N L G P S F Y F D Y W G Q G T L V T V S S A S T K G P S V F P L A P S S K

136                               150                               165                               180
S T S G G T A A L G C L V K D Y F P E P V T V S W N S G A L T S G V H T F P A V L Q S S G

181                               195                               210                               225
L Y S L S S V V T V P S S S L G T Q T Y I C N V N H K P S N T K V D K K V E P K S C D K T

226                               240                               255                               270
H T C P P C P A P E L L G G P S V F L F P P K P K D T L M I S R T P E V T C V V V D V S H

271                               285                               300                               315
E D P E V K F N W Y V D G V E V H N A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W

316                               330                               345                               360
L N G K E Y K C K V S N K A L P A P I E K T I S K A K G Q P R E P Q V Y T L P P S R E E M

361                               375                               390                               405
T K N Q V S L T C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P V L D S D G S

406                               420                               435                               449
F F L Y S K L T V D K S R W Q Q G N V F S C S V M H E A L H N H Y T Q K S L S L S P G K
```

(SEQ ID NO. 24)

FIG. 9B

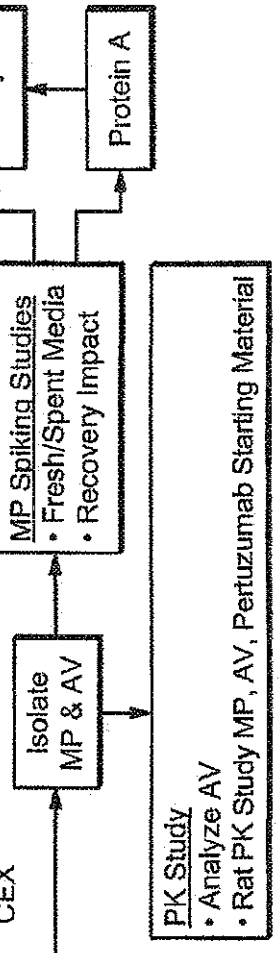

Pertuzumab Starting Material
CEX
Isolate MP & AV
MP Spiking Studies
• Fresh/Spent Media
• Recovery Impact
Analyze
Protein A
PK Study
• Analyze AV
• Rat PK Study MP, AV, Pertuzumab Starting Material

FIG. 10

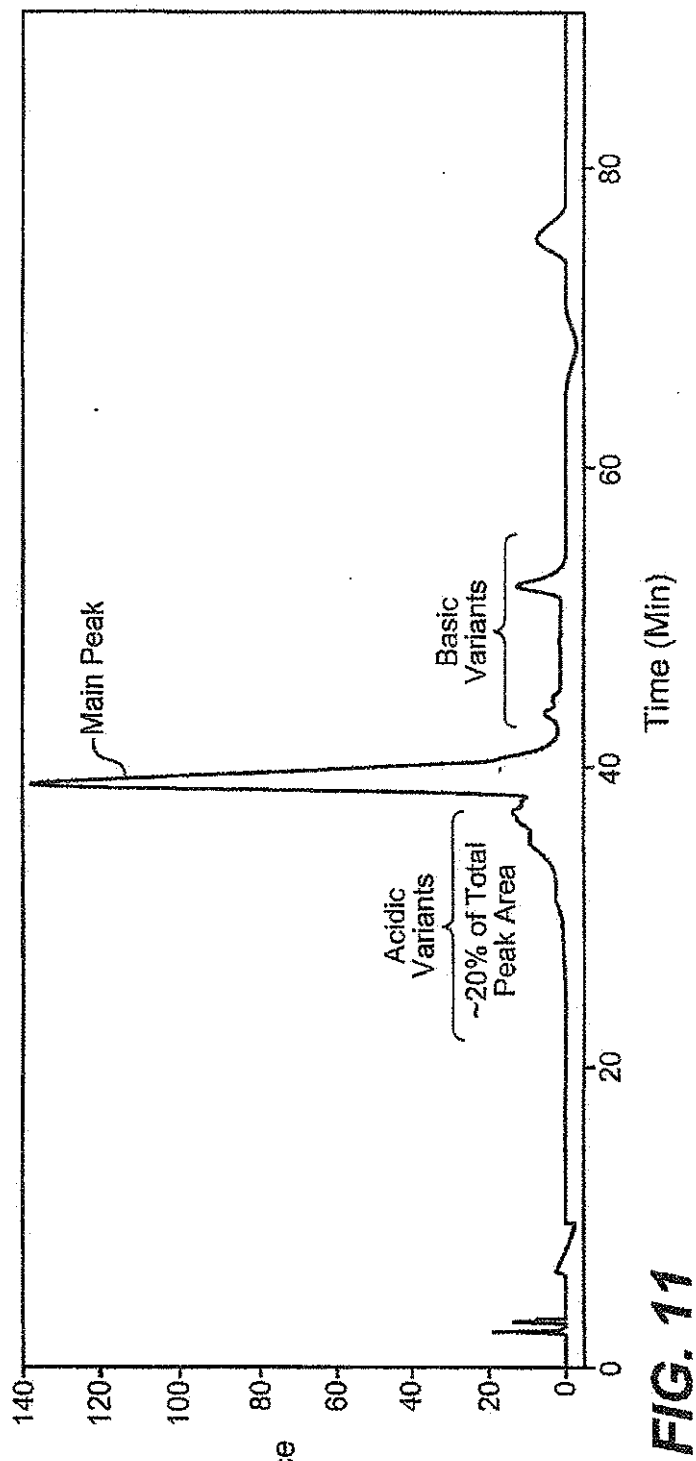

Absorbance (280 nm)
140
120
100
80
60
40
20
0
0
20
40
60
80
Time (Min)
Main Peak
Basic Variants
Acidic Variants
~20% of Total Peak Area

FIG. 11

Analysis of Pertuzumab Starting Material and CEX Fractions			
Analysis	**Starting Material**	**Main Peak**	**Acidic Variants**
CEX (%AV, %MP, %BV)	21, 68, 12	5, 93, 2	95, 4, 2
Potency	94 (Ave. n=2)	109	92
SEC (% Monomer)	99.9	99.9	100

FIG. 12

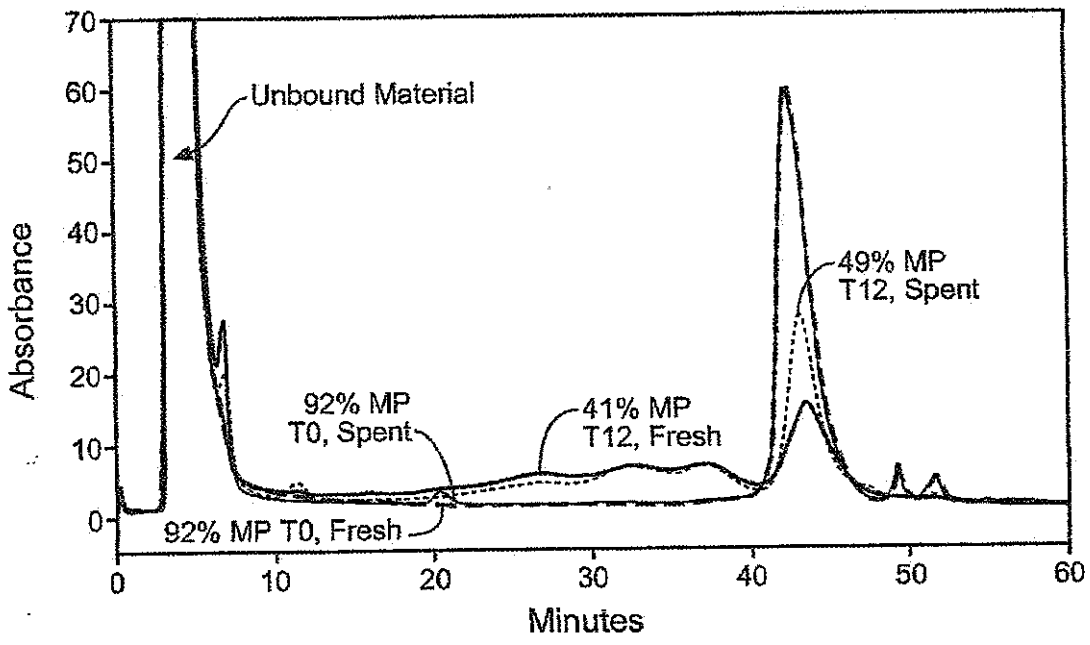


FIG. 13

Main Peak Incubation Conditions	
12d Incubation Sample Condition	**% CEX Main Peak**
Time Zero	90-92
Fresh Media	41%
Spent Media	49%
-Glucose	43%
-Glucose, -Peptone, -Pluronic	41%
Fresh Media + ProA Isolation	37%
Media Buffer	56%

FIG. 14

Methods for Characterization of Acidic Variants	
Method	**Variants Detected***
CEX +/- Sialidase Treatment	6% Sialylated
Reduced CE-SDS	1.5% Incompletely Reduced
Non-reduced CE-SDS	6% Reduced Disulfide
Boronate Chromatography	3.5% Glycated (Higher Order)
Peptide Map	Deamidated

* Percent of Total CEX Peak Area.

FIG. 15

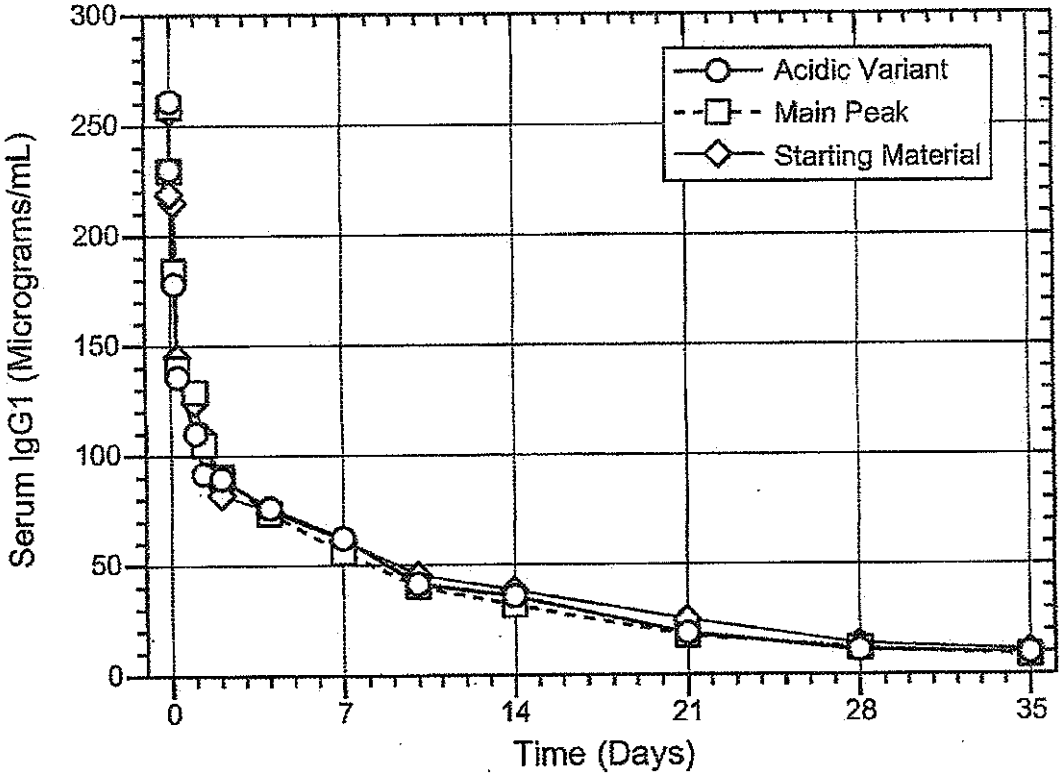


FIG. 16

AUC and Geometric Mean Ratios from PK Study			
Test Material (n=12 for Each Group)	AUC_{0-14} (Day * µg/mL)		Geometric Mean Ratio (CI)
	Mean ± SD	Geometric Mean	
Acidic Variant	910 ± 73.1	907	0.963 (0.905, 1.03)
Main Peak	895 ± 85.5	891	0.946 (0.884, 1.01)
Pertuzumab Starting Material	946 ± 94.6	942	NA

FIG. 17