THERAPY OF PLATINUM-RESISTANT CANCER

Related Applications

This is a non-provisional application filed under 37 CFR 1.53(b) claiming claiming the benefit under 35 USC §119(e) of provisional application 60/580,333 filed June 16, 2004, the contents of which are incorporated herein by reference.

Field of the Invention

The present invention concerns a method for treating platinum-resistant, ovarian cancer, primary peritoneal carcinoma or fallopian tube carcinoma, with the combination of a HER2 antibody that effectively inhibits HER dimerization as well as gemcitabine.

Background of the Invention

HER 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)).

Ovarian Cancer

Ovarian cancer is the most common cause of death from malignancy of the female reproductive tract. There are an estimated 24,000 new diagnoses per year in the United States, with approximately 13,000 deaths from the disease. Patients with advanced ovarian cancer are frequently treated with platinum-based chemotherapy, often combined with a taxane. After these agents have failed, there are few therapeutic options. Patients with platinum-sensitive disease are often re-treated with platinum, but a substantial proportion of patients have a short duration of response after re-treatment. For those with platinum-resistant disease outcome is less favorable. Topotecan is approved by the Food and Drug Administration (FDA) for patients who have have failed initial or subsequent chemotherapy; liposomal doxorubicin is approved only for patients with ovarian cancer that is refractory to both platinum- and paclitaxel-based chemotherapy regimens. Topotecan and liposomal doxorubicin

have shown a partial response rate of 6% and 12% respectively in patients with platinum-resistant disease, with a median progression-free survival of 14 –18 weeks. More recently, promising results with gemcitabine have been reported in platinum-resistant ovarian cancer with partial responses at 16%, leading to increasing use of this agent as 2nd line therapy. However, there is a clear need for new and improved therapeutic options for patients with advanced ovarian cancer for whom existing therapies have failed.

The ErbB or human epidermal growth factor receptor (HER) family of receptor tyrosine kinases are implicated in the pathogenesis of ovarian cancer. To target the HER signaling pathway, pertuzumab (rhuMAb 2C4) 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.

Gemcitabine has been used in a variety of tumors and is indicated for use in pancreatic and lung cancer. The most common toxicities with use of single agent gemcitabine include cytopenias, with an incidence of anemia and neutropenia of 68% and 63%, respectively. Another common toxicity is nausea and vomiting, with a combined incidence of 69%, with a 13% grade III and a 1% grade IV incidence. Diarrhea occurs less frequently at 19%. Rash occurs more commonly at 30%, with only a 1% grade III incidence. Gemcitabine has been combined with many other chemotherapeutic agents, such as the taxanes, anthracyclines, and platinums without any significant increases or unexpected toxicities.

Trastuzumab has been combined with gemcitabine in several combinations of different chemotherapies in phase II trials and was also well tolerated with no observed cardiac or unexpected toxicities. Safran *et al. Proc Am. Soc. Clin. Oncol.* 20:130a (2001), Miller *et al. Oncology* 15(2): 38-40 (2001). See, also, Zinner *et al. Proc. Am. Soc. Clin. Oncol.* 20:328a (2001), Nagourney *et al. Breast Cancer Res. Treat.* 57:116, Abstract 475 (1999), Bun *et al. Proc. Am. Assoc. Canc. Res.* 41:719, Abstract #4571 (2000), Konecny *et al. Breast Cancer Res Treat* 57: 114, Abstract 467 (1999), O'Shaugnessy *et al. Sem. Oncol* 2(suppl3):22-26 (2004), Sledge *et al. Sem. Oncol.* 2(suppl3):19-21 (2003), Zinner *et al. Lung Cancer* 44(1):99-110 (2004), Gatzemeier *et al. Ann of Oncol.* 15:19-27 (2004), concerning the combination of Trastuzumab and Gemcitabine.

In a phase I trial of Omnitarg 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).

Summary of the Invention

The present invention concerns, in a first aspect, a method for treating a platinum-resistant cancer selected from the group consisting of ovarian cancer, primary peritoneal carcinoma and fallopian tube carcinoma, comprising administering to a patient a HER2 antibody that inhibits HER dimerization more effectively than Trastuzumab, and an antimetabolite chemotherapeutic agent, each in amounts effective to treat the cancer.

In another aspect, the invention provides a method for treating platinum-resistant cancer selected from the group consisting of ovarian cancer, primary peritoneal carcinoma and fallopian tube carcinoma, comprising administering to a patient a HER2 antibody that binds to a heterodimeric binding site on HER2, and gemcitabine, each in amounts effective to treat the cancer.

In yet a further aspect, the invention concerns a method for treating platinum-resistant cancer selected

from the group consisting of ovarian cancer, primary peritoneal carcinoma and fallopian tube carcinoma, comprising administering to a patient a HER2 antibody that binds to Domain II of HER2, and gemcitabine, each in amounts effective to treat the cancer.

Brief Description of the Drawings

Figures 1A and 1B depict epitope mapping of residues 22-645 within the extracellular domain (ECD) of HER2 (amino acid sequence, including signal sequence, shown in Fig. 1A; SEQ ID NO:13) as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura *et al. J. of Virology* 67(10):6179-6191 (1993); and Renz *et al. J. Cell Biol.* 125(6):1395-1406 (1994)). The various HER2-ECD truncations or point mutations were prepared from cDNA using polymerase chain reaction technology. The HER2 mutants were expressed as gD fusion proteins in a mammalian expression plasmid. This expression plasmid uses the cytomegalovirus promoter/enhancer with SV40 termination and polyadenylation signals located downstream of the inserted cDNA. Plasmid DNA was transfected into 293 cells. One day following transfection, the cells were metabolically labeled overnight in methionine and cysteine-free, low glucose DMEM containing 1% dialyzed fetal bovine serum and 25 μCi each of ^{35}S methionine and ^{35}S cysteine. Supernatants were harvested and either the HER2 monoclonal antibodies or control antibodies were added to the supernatant and incubated 2-4 hours at 4°C. The complexes were precipitated, applied to a 10-20% Tricine SDS gradient gel and electrophoresed at 100 V. The gel was electroblotted onto a membrane and analyzed by autoradiography. As shown in Fig. 1B, the HER2 antibodies 7C2, 7F3, 2C4, 7D3, 3E8, 4D5, 2H11 and 3H4 bind various HER2 ECD epitopes.

Figures 2A and 2B show the effect of HER2 monoclonal antibodies 2C4 and 7F3 on rHRGβ1 activation of MCF7 cells. Fig. 2A shows dose-response curves for 2C4 or 7F3 inhibition of HRG stimulation of tyrosine phosphorylation. Fig. 2B shows dose-response curves for the inhibition of ^{125}I-labeled $rHRG\beta1_{177-244}$ binding to MCF7 cells by 2C4 or 7F3.

Figure 3 depicts inhibition of specific ^{125}I-labeled $rHRG\beta1_{177-244}$ binding to a panel of human tumor cell lines by the HER2 monoclonal antibodies 2C4 or 7F3. Monoclonal antibody-controls are isotype-matched murine monoclonal antibodies that do not block rHRG binding. Nonspecific ^{125}I-labeled $rHRG\beta1_{177-244}$ binding was determined from parallel incubations performed in the presence of 100 nM rHRGβ1. Values for nonspecific ^{125}I-labeled $rHRG\beta1_{177-244}$ binding were less than 1% of the total for all the cell lines tested.

Figures 4A and 4B show the effect of monoclonal antibodies 2C4 and 4D5 on proliferation of MDA-MB-175 (Fig. 4A) and SK-BR-3 (Fig. 4B) cells. MDA-MB-175 and SK-BR-3 cells were seeded in 96 well plates and allowed to adhere for 2 hours. Experiment was carried out in medium containing 1% serum. HER2 antibodies or medium alone were added and the cells were incubated for 2 hours at 37°C. Subsequently rHRGβ1 (1nM) or medium alone were added and the cells were incubated for 4 days. Monolayers were washed and stained/fixed with 0.5% crystal violet. To determine cell proliferation the absorbance was measured at 540 nm.

Figures 5A and 5B show the effect of monoclonal antibody 2C4, Trastuzumab antibody or an anti-EGFR antibody on heregulin (HRG) dependent association of HER2 with HER3 in MCF7 cells expressing low/normal levels of HER2 (Fig. 5A) and SK-BR-3 cells expressing high levels of HER2 (Fig. 5B); see Example 2 below.

Figures 6A and 6B compare the activities of intact murine monoclonal antibody 2C4 (mu 2C4) and a chimeric 2C4 Fab fragment. Fig. 6A shows inhibition of ^{125}I-HRG binding to MCF7 cells by chimeric 2C4 Fab

or intact murine monoclonal antibody 2C4. MCF7 cells were seeded in 24-well plates (1 x 10^5 cells/well) and grown to about 85% confluency for two days. Binding experiments were conducted as described in Lewis *et al. Cancer Research* 56:1457-1465 (1996). Fig. 6B depicts inhibition of rHRGβ1 activation of p180 tyrosine phosphorylation in MCF7 cells performed as described in Lewis *et al. Cancer Research* 56:1457-1465 (1996).

Figures 7A and 7B depict alignments of the amino acid sequences of the variable light (V_L) (Fig. 7A) and variable heavy (V_H) (Fig. 7B) 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 8A to C show binding of chimeric Fab 2C4 (Fab.v1) and several humanized 2C4 variants to HER2 extracellular domain (ECD) as determined by ELISA in Example 3.

Figure 9 is a ribbon diagram of the V_L and V_H domains of monoclonal antibody 2C4 with white CDR backbone labeled (L1, L2, L3, H1, H2, H3). V_H sidechains evaluated by mutagenesis during humanization (see Example 3, Table 2) are also shown.

Figure 10 depicts the effect of monoclonal antibody 2C4 or Trastuzumab on EGF, TGF-α, or HRG-mediated activation of mitogen-activated protein kinase (MAPK).

Figures 11A and 11B show the amino acid sequences of Trastuzumab light chain (SEQ ID NO:14) and Trastuzumab heavy chain (SEQ ID NO:15), respectively.

Figures 12A and 12B show the amino acid sequences of Pertuzumab light chain (SEQ ID NO:16) and Pertuzumab heavy chain (SEQ ID NO:17), respectively.

Figure 13 depicts, schematically, binding of 2C4 at the heterodimeric binding site of HER2, thereby preventing heterodimerization with activated EGFR or HER3.

Figure 14 depicts coupling of HER2/HER3 to the MAPK and Akt pathways.

Figure 15 compares activity of Trastuzumab and Pertuzumab.

Figure 16 depicts schematically the various domains of HER2.

Detailed Description of the Preferred Embodiments

I. Definitions

An "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. The HER receptor may be a "native sequence" HER receptor or an "amino acid sequence variant" thereof. Preferably the HER receptor is native sequence human HER receptor.

The extracellular domain of HER2 comprises four domains, Domain I (amino acid residues from about 1-195), Domain II (amino acid residues from about 196-320), Domain III (amino acid residues from about 321-488), and Domain IV (amino acid residues from about 489-632) (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), and Fig. 16 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.

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

The term "amino acid sequence variant" refers to polypeptides having amino acid sequences that differ to some extent from a native sequence polypeptide. Ordinarily, amino acid sequence variants will possess at least about 70% homology with at least one receptor binding domain of a native HER ligand or with at least one ligand binding domain of a native HER receptor, and preferably, they will be at least about 80%, more preferably at least about 90% homologous with such receptor or ligand binding domains. The amino acid sequence variants possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence of the native amino acid sequence.

"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.

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 variants generally being present in minor amounts. 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 variant thereof. Preferably, the intact antibody has one or more effector functions.

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.

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. 14 and 15, respectively.

Herein, "Pertuzumab" and "OMNITARG™" refer to an antibody comprising the light and heavy chain amino acid sequences in SEQ ID NOS. 16 and 17, 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 murine monoclonal antibody 2C4, a Fab fragment of murine monoclonal antibody 2C4, 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 Examples 1-2 and 4 herein. 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 Example 2 herein); reduction in HER ligand activation of cells which express HER dimers (Example 1 herein and Fig. 2A-B of Agus *et al. Cancer Cell* 2: 127-137 (2002), for example); blocking of HER ligand binding to cells which express HER dimers (Example 1 herein, 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 (Example 1 herein and Figs. 3A-D of Agus *et al. Cancer Cell* 2: 127-137 (2002), for instance); inhibition of downstream signaling (for instance, inhibition of HRG-dependent AKT phosphorylation or inhibition of HRG- or TGFα- dependent MAPK phosphorylation) (see, Example 4 herein, 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 Example 4 herein, 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. Preferably the antibody that binds to domain II binds to the junction between domains I, II and III of HER2.

An "ovary" is one of the two small, almond-shaped organs located on either side of the uterus in a female.

A "fallopian tube" or "oviduct" is one of the two fine tubes leading from the ovaries of female mammals into the uterus.

The "peritoneum" is the epithelial lining of a body cavity such as the abdomen.

"Ovarian cancer" is a potentially life-threatening malignancy, that develops in one or both ovaries. By the time symptoms of ovarian cancer appear, the ovarian tumor may have grown large enough to shed cancer cells throughout the abdomen. Ovarian cancer cells that have spread outside the ovaries are referred to as metastatic ovarian cancers. Ovarian tumors tend to spread to the diaphragm, intestine and/or omentum (a fatty layer that covers and pads organs in the abdomen). Cancer cells can also spread to other organs through lymph channels and the bloodstream. Ovarian cancer to be treated herein includes the three primary classes of malignant ovarian tumors, namely, epithelial tumor, germ cell tumor, and stromal tumor.

"Primary peritoneal carcinoma" refers to a cancer that arises in the peritoneum. Primary peritoneal carcinoma may be very similar to epithelial ovarian cancer in terms of microscopic appearance, symptoms, pattern of spread, and prognosis. A woman who has had her ovaries removed can still get primary peritoneal carcinoma.

"Fallopian tube carcinoma" refers to cancer of the fallopian tube and/or broad ligament.

An "epithelial tumor" develops in a layer of cube-shaped cells known as the germinal epithelium, which surrounds the outside of the ovaries. Epithelial tumors account for up to 90% of all ovarian cancers.

A "germ cell tumor" is found in egg-maturation cell(s) of the ovary. Germ cell tumors, which account for about 3% of all ovarian cancers, occur most often in teenagers and young women.

A "stromal tumor" develops from connective tissue cells that hold the ovary together and that produce the female hormones, estrogen and progesterone. Stromal tumors account for 6% of all ovarian cancers.

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

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 (*e.g.* any one or more residues in the region from about residue 22 to about residue 584 of HER2, inclusive; see Figs. 1A-B). Epitope 2C4 comprises residues from domain II in the extracellular domain of HER2. 2C4 and Pertuzumab binds to the extracellular domain of HER2 at the junction of domains I, II and III. Franklin *et al. Cancer Cell* 5:317-328 (2004).

The "epitope 4D5" is the region in the extracellular domain of HER2 to which the antibody 4D5 (ATCC CRL 10463) and Trastuzumab bind. This epitope is close to the transmembrane domain of HER2, and within Domain IV of HER2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such

as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of HER2 (*e.g.* any one or more residues in the region from about residue 529 to about residue 625, inclusive; see Figs. 1A-B).

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; see Figs. 1A-B).

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

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

"Overall survival" refers to the patient remaining alive for a defined period of time, such as 1 year, 5 years, etc, *e.g.*, from the time of diagnosis or treatment.

"Progression free survival" refers to the patient remaining alive, without the cancer getting worse.

An "objective response" refers to a measurable response, including complete response (CR) or partial response (PR).

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

"Partial response" refers to a decrease in the size of one or more tumors or lesions, or in the extent of cancer in the body, in response to treatment.

A "HER-expressing cancer" is one comprising cells which have HER protein present at their cell surface.

A "HER2-expressing cancer" is one which produces sufficient levels of HER2 at the surface of cells thereof, such that a HER2 antibody can bind thereto and have a therapeutic effect with respect to the cancer.

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 CYTOXAN® cyclosphosphamide; 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 gamma1I

and calicheamicin omegaI1 (see, *e.g.*, Agnew, *Chem Intl. Ed. Engl.*, 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; 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, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin 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 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; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; 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; an epothilone; 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; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, *e.g.*, TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids such as retinoic acid; capecitabine; 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 and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON® toremifene; aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR®

vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

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

"Gemcitabine" or " 2'-deoxy-2', 2'-difluorocytidine monohydrochloride (b-isomer)" is a nucleoside analogue that exhibits antitumor activity. The empirical formula for gemcitabine HCl is C9H11F2N3O4 · HCl. Gemcitabine HCl is sold by Eli Lilly under the trademark GEMZAR®.

A "platinum-based chemotherapeutic agent" comprises an organic compound which contains platinum as an integral part of the molecule. Examples of platinum-based chemotherapeutic agents include carboplatin, cisplatin, and oxaliplatinum.

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

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

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.); 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 (see WO98/50433, Abgenix). The anti-EGFR antibody may be conjugated with a cytotoxic agent, thus generating an immunoconjugate (see, *e.g.*, EP659,439A2, Merck Patent GmbH). Examples of small molecules that bind to EGFR include ZD1839 or Gefitinib (IRESSA™; Astra Zeneca), CP-358774 or Erlotinib HCL (TARCEVA™; Genentech/OSI) and AG1478, AG1571 (SU 5271; Sugen).

A "tyrosine kinase inhibitor"is a molecule which inhibits to some extent 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 as well as small molecule HER2 tyrosine kinase inhibitor such as TAK165 available from Takeda, dual-HER inhibitors such as EKB-569 (available from Wyeth) which preferentially binds EGFR but inhibits both HER2 & EGFR-overexpressing cells, GW572016 (available from Glaxo) an oral HER2 and EGFR tyrosine kinase inhibitor, and 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); or as described in any of the following patent publications: US Patent No. 5,804,396; WO99/09016 (American Cyanimid); WO98/43960 (American Cyanamid); WO97/38983 (Warner Lambert); WO99/06378 (Warner Lambert); WO99/06396 (Warner Lambert); WO96/30347 (Pfizer, Inc); WO96/33978 (Zeneca); WO96/3397 (Zeneca); and WO96/33980 (Zeneca).

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. 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) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

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

(ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

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.

(iii) Humanized antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al., Nature*, 321:522-525 (1986); Riechmann *et al., Nature*, 332:323-327 (1988); 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.

Example 3 below 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 murine monoclonal antibody 2C4 (or a Fab fragment thereof) and/or binds HER2 essentially as effectively as murine monoclonal antibody 2C4 (or a Fab fragment thereof). The humanized antibody herein may, for example, comprise nonhuman hypervariable region residues incorporated into a human variable heavy domain and may further comprise a framework region (FR) substitution at a position selected from the group consisting of 69H, 71H and 73H utilizing the variable domain numbering system set forth in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991). In one embodiment, the humanized antibody comprises FR substitutions at two or all of positions 69H, 71H and 73H.

An exemplary humanized antibody of interest herein comprises variable heavy domain complementarity determining residues GFTFTDYTMX, where X is 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 domain amino acid sequence in SEQ ID NO:4.

The humanized antibody may comprise variable light domain 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 domain 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 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 murine 2C4 or 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.

(iv) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_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.

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

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

(v) 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.

(vi) Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the HER2 protein. Other such antibodies may combine a HER2 binding site with binding site(s) for EGFR, HER3 and/or HER4. Alternatively, a HER2 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (*e.g.* CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the HER2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express HER2. These antibodies possess a HER2-binding arm and an arm which binds the cytotoxic agent (*e.g.* saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies).

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).

(vii) 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, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1

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).

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.

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).

To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. 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.

(viii) Screening for antibodies with the desired properties

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

To identify an antibody which blocks ligand activation of a HER receptor, the ability of the antibody to block HER ligand binding to cells expressing the HER receptor (*e.g.* in conjugation with another HER receptor with which the HER receptor of interest forms a HER hetero-oligomer) may be determined. For example, cells naturally expressing, or transfected to express, HER receptors of the HER hetero-oligomer may be incubated with the antibody and then exposed to labeled HER ligand. The ability of the 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 Example 1 below. HER2 monoclonal antibodies may be added to each well and incubated for 30 minutes. ^{125}I-labeled $rHRG\beta1_{177-224}$ (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 Example 1 below. 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 rHRG$\beta 1_{177-244}$ 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 Example 2 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 (see, *e.g.* Figs. 1A and 1B herein) and/or one can study the antibody-HER2 structure (Franklin *et al. Cancer Cell* 5:317-328 (2004)) to see what domain(s) of HER2 is/are bound by the antibody.

(ix) 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).

(x) Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.* a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.*, Massey, *Nature* 328: 457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the HER2 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al.*, *Nature*, 312: 604-608 (1984).

(xi) 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).

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).

III. Pharmaceutical Formulations

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), 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). Preferred lyophilized HER2 antibody formulations are described in WO 97/04801, expressly incorporated herein by reference.

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, 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.

IV. Screening Patients for Therapy

According to a preferred embodiment of the invention herein, the patient selected for therapy has a tumor displaying HER (and preferably HER2) activation. In one embodiment, the extent of HER (or HER2) activation in cancer cells significantly exceeds the level of activation of that receptor in non-cancerous cells of the same tissue type. Such excessive activation may result from overexpression of the HER receptor and/or greater than normal levels of a HER ligand available for activating the HER receptor in the cancer cells. Such excessive activation may cause and/or be caused by the malignant state of a cancer cell. In some embodiments, the cancer will be subjected to a diagnostic or prognostic assay to determine whether amplification and/or overexpression of a HER receptor is occurring which results in such excessive activation of the HER receptor. Alternatively, or additionally, the cancer may be subjected to a diagnostic or prognostic assay to determine whether amplification and/or overexpression a HER ligand is occurring in the cancer which attributes to excessive activation of the receptor. In a subset of such cancers, excessive activation of the receptor may result from an autocrine stimulatory pathway. Various assays for determining HER activation will be described in more detail below.

(i) HER dimers

Tumors samples can be assessed for the presence of HER dimers, as indicating HER or HER2 activation. Any method known in the art may be used to detect HER2 dimers, such as EGFR-HER2, HER2-HER3, in tumors. Several preferred methods are described below. These methods detect noncovalent protein-protein interactions or otherwise indicate proximity between proteins of interest.

Immunoaffinity-based methods, such as immunoprecipitation or ELISA, may be used to detect HER dimers. In one embodiment, HER2 antibodies are used to immunoprecipitate complexes comprising HER2 from tumor cells, and the resulting immunoprecipitant is then probed for the presence of EGFR or HER3 by immunoblotting. In another embodiment, EGFR or HER3 antibodies may be used for the immunoprecipitation step and the immunoprecipitant then probed with HER2 antibodies. In a further embodiment, HER ligands specific to EGFR, HER3, EGFR/HER2 complexes or HER2/HER3 complexes may be used to precipitate complexes, which are then probed for the presence of HER2. For example, ligands may be conjugated to avidin and complexes purified on a biotin column.

In other embodiments, such as ELISA or antibody "sandwich"-type assays, antibodies to HER2 are immobilized on a solid support, contacted with tumor cells or tumor cell lysate, washed, and then exposed to antibody against EGFR or HER3. Binding of the latter antibody, which may be detected directly or by a secondary antibody conjugated to a detectable label, indicates the presence of heterodimers. In certain embodiments, EGFR or HER3 antibody is immobilized, and HER2 antibody is used for the detection step. In other embodiments HER ligands may be used in place of, or in combination with HER antibodies.

Chemical or UV cross-linking may also be used to covalently join dimers on the surface of living cells. Hunter *et al.*, *Biochem. J.*, 320:847-53. Examples of chemical cross-linkers include dithiobis(succinimidyl) propionate (DSP) and 3,3′dithiobis(sulphosuccinimidyl) propionate (DTSSP). In one embodiment, cell extracts

from chemically cross-linked tumor cells are analyzed by SDS-PAGE and immunoblotted with antibodies to EGFR and/or HER3. A supershifted band of the appropriate molecular weight most likely represents EGFR-HER2 or HER2-HER3 dimers, as HER2 is the preferred dimerization partner for EGFR and HER3. This result may be confirmed by subsequent immunoblotting with HER2 antibodies.

Fluorescence resonance energy transfer (FRET) may also be used to detect EGFR-HER2 or HER2-HER3 dimers. FRET detects protein conformational changes and protein-protein interactions in vivo and in vitro based on the transfer of energy from a donor fluorophore to an acceptor fluorophore. Selvin, *Nat. Struct. Biol.*, 7:730-34 (2000). Energy transfer takes place only if the donor fluorophore is in sufficient proximity to the acceptor fluorophore. In a typical FRET experiment, two proteins or two sites on a single protein are labeled with different fluorescent probes. One of the probes, the donor probe, is excited to a higher energy state by incident light of a specified wavelength. The donor probe then transmits its energy to the second probe, the acceptor probe, resulting in a reduction in the donor's fluorescence intensity and an increase in the acceptor's fluorescence emission. To measure the extent of energy transfer, the donor's intensity in a sample labeled with donor and acceptor probes is compared with its intensity in a sample labeled with donor probe only. Optionally, acceptor intensity is compared in donor/acceptor and acceptor only samples. Suitable probes are known in the art and include, for example, membrane permeant dyes, such as fluorescein and rhodamine, organic dyes, such as the cyanine dyes, and lanthanide atoms. Selvin, *supra*. Methods and instrumentation for detecting and measuring energy transfer are also known in the art. Selvin, *supra*.

FRET-based techniques suitable for detecting and measuring protein-protein interactions in individual cells are also known in the art. For example, donor photobleaching fluorescence resonance energy transfer (pbFRET) microscopy and fluorescence lifetime imaging microscopy (FLIM) may be used to detect the dimerization of cell surface receptors. Selvin, supra; Gadella & Jovin, *J. Cell Biol.*, 129:1543-58 (1995). In one embodiment, pbFRET is used on cells either "in suspension" or "*in situ*" to detect and measure the formation of EGFR-HER2 or HER2-HER3 dimers, as described in Nagy *et al.*, *Cytometry*, 32:120-131 (1998). These techniques measure the reduction in a donor's fluorescence lifetime due to energy transfer. In a particular embodiment, a flow cytometric Foerster-type FRET technique (FCET) may be used to investigate EGFR-HER2 and HER2-HER3 dimerization, as described in Nagy et al., supra, and Brockhoff *et al.*, *Cytometry*, 44:338-48 (2001).

FRET is preferably used in conjunction with standard immunohistochemical labeling techniques. Kenworthy, *Methods*, 24:289-96 (2001). For example, antibodies conjugated to suitable fluorescent dyes can be used as probes for labeling two different proteins. If the proteins are within proximity of one another, the fluorescent dyes act as donors and acceptors for FRET. Energy transfer is detected by standard means. Energy transfer may be detected by flow cytometric means or by digital microscopy systems, such as confocal microscopy or wide-field fluorescence microscopy coupled to a charge-coupled device (CCD) camera.

In one embodiment of the present invention, HER2 antibodies and either EGFR or HER3 antibodies are directly labeled with two different fluorophores, for example as described in Nagy *et al, supra*. Tumor cells or tumor cell lysates are contacted with the differentially labeled antibodies, which act as donors and acceptors for FRET in the presence of EGFR-HER2 or HER2-HER3 dimers. Alternatively, unlabeled antibodies against HER2 and either EGFR or HER3 are used along with differentially labeled secondary antibodies that serve as donors and

acceptors. See, for example, Brockhoff et al., supra. Energy transfer is detected and the presence of dimers is determined if the labels are found to be in close proximity.

In other embodiments HER receptor ligands that are specific for HER2 and either HER1 or HER3 are fluorescently labeled and used for FRET studies.

In still other embodiments of the present invention, the presence of dimers on the surface of tumor cells is demonstrated by co-localization of HER2 with either EGFR or HER3 using standard direct or indirect immunofluorescence techniques and confocal laser scanning microscopy. Alternatively, laser scanning imaging (LSI) is used to detect antibody binding and co-localization of HER2 with either EGFR or HER3 in a high-throughput format, such as a microwell plate, as described in Zuck *et al, Proc. Natl. Acad. Sci. USA,* 96:11122-27 (1999).

In further embodiments, the presence of EGFR-HER2 and/or HER2-HER3 dimers is determined by identifying enzymatic activity that is dependent upon the proximity of the dimer components. A HER2 antibody is conjugated with one enzyme and an EGFR or HER3 antibody is conjugated with a second enzyme. A first substrate for the first enzyme is added and the reaction produces a second substrate for the second enzyme. This leads to a reaction with another molecule to produce a detectable compound, such as a dye. The presence of another chemical breaks down the second substrate, so that reaction with the second enzyme is prevented unless the first and second enzymes, and thus the two antibodies, are in close proximity. In a particular embodiment tumor cells or cell lysates are contacted with a HER2 antibody that is conjugated with glucose oxidase and a HER3 or HER1 antibody that is conjugated with horse radish peroxidase. Glucose is added to the reaction, along with a dye precursor, such as DAB, and catalase. The presence of dimers is determined by the development of color upon staining for DAB.

Dimers may also be detected using methods based on the eTag™ assay system (Aclara Bio Sciences, Mountain View, CA), as described, for example, in U.S. Patent Application 2001/0049105, published December 6, 2001, both of which are expressly incorporated by reference in their entirety. An eTag™, or "electrophoretic tag," comprises a detectable reporter moiety, such as a fluorescent group. It may also comprise a "mobility modifier," which consists essentially of a moiety having a unique electrophoretic mobility. These moieties allow for separation and detection of the eTag™ from a complex mixture under defined electrophoretic conditions, such as capillary electrophoresis (CE). The portion of the eTag™ containing the reporter moiety and, optionally, the mobility modifier is linked to a first target binding moiety by a cleavable linking group to produce a first binding compound. The first target binding moiety specifically recognizes a particular first target, such as a nucleic acid or protein. The first target binding moiety is not limited in any way, and may be for example, a polynucleotide or a polypeptide. Preferably, the first target binding moiety is an antibody or antibody fragment. Alternatively, the first target binding moiety may be a HER receptor ligand or binding-competent fragment thereof.

The linking group preferably comprises a cleavable moiety, such as an enzyme substrate, or any chemical bond that may be cleaved under defined conditions. When the first target binding moiety binds to its target, the cleaving agent is introduced and/or activated, and the linking group is cleaved, thus releasing the portion of the eTag™ containing the reporter moiety and mobility modifier. Thus, the presence of a "free" eTag™ indicates the binding of the target binding moiety to its target.

Preferably, a second binding compound comprises the cleaving agent and a second target binding moiety that specifically recognizes a second target. The second target binding moiety is also not limited in any way and

may be, for example, an antibody or antibody fragment or a HER receptor ligand or binding competent ligand fragment. The cleaving agent is such that it will only cleave the linking group in the first binding compound if the first binding compound and the second binding compound are in close proximity.

In an embodiment of the present invention, a first binding compound comprises an eTag™ in which an antibody to HER2 serves as the first target binding moiety. A second binding compound comprises an antibody to EGFR or HER3 joined to a cleaving agent capable of cleaving the linking group of the eTag™. Preferably the cleaving agent must be activated in order to be able to cleave the linking group. Tumor cells or tumor cell lysates are contacted with the eTag™, which binds to HER2, and with the modified EGFR or HER3 antibody, which binds to EGFR or HER3 on the cell surface. Unbound binding compound is preferable removed, and the cleaving agent is activated, if necessary. If EGFR-HER2 or HER2-HER3 dimers are present, the cleaving agent will cleave the linking group and release the eTag™ due to the proximity of the cleaving agent to the linking group. Free eTag™ may then be detected by any method known in the art, such as capillary electrophoresis.

In one embodiment, the cleaving agent is an activatable chemical species that acts on the linking group. For example, the cleaving agent may be activated by exposing the sample to light.

In another embodiment, the eTag™ is constructed using an antibody to EGFR or HER3 as the first target binding moiety, and the second binding compound is constructed from an antibody to HER2.

In yet another embodiment, the HER dimer is detected using an antibody or other reagent which specifically or preferentially binds to the dimer as compared to binding thereof to either HER receptor in the dimer.

(ii) HER2 phosphorylation

Immunoprecipitation with EGFR, HER2, or HER3 antibody as discussed in the previous section may optionally be followed by a functional assay for dimers, as an alternative or supplement to immunoblotting. In one embodiment, immunoprecipitation with HER3 antibody is followed by an assay for receptor tyrosine kinase activity in the immunoprecipitant. Because HER3 does not have intrinsic tyrosine kinase activity, the presence of tyrosine kinase activity in the immunoprecipitant indicates that HER3 is most likely associated with HER2. Graus-Porta *et al.*, *EMBO J.*, 16:1647-55 (1997); Klapper *et al.*, *Proc. Natl. Acad. Sci. USA*, 96:4995-5000 (1999). This result may be confirmed by immunoblotting with HER2 antibodies. In another embodiment, immunoprecipitation with HER2 antibody is followed by an assay for EGFR receptor tyrosine kinase activity. In this assay, the immunoprecipitant is contacted with radioactive ATP and a peptide substrate that mimics the in vivo site of transphosphorylation of HER2 by EGFR. Phosphorylation of the peptide indicates co-immunoprecipitation and thus dimerization of EGFR with HER2. Receptor tyrosine kinase activity assays are well known in the art and include assays that detect phosphorylation of target substrates, for example, by phosphotyrosine antibody, and activation of cognate signal transduction pathways, such as the MAPK pathway.

Phosphorylation of HER receptor may be assessed by immunoprecipitation of one or more HER receptors, such as HER2 (HER2) receptor, and Western blot analysis. For example, positivity is determined by the presence of a phospho-HER2 band on the gel, using an anti-phosphotyrosine antibody to detect phosphorylated tyrosine residue(s) in the immunoprecipitated HER receptor(s). Anti-phosphotyrosine antibodies are commercially available from PanVera (Madison, WI), a subsidiary of Invitrogen, Chemicon International Inc. (Temecula, CA), or Upstate Biotechnology (Lake Placid, NY). Negativity is determined by the absence of the band.

In another embodiment, phosphorylation of HER2 (HER2) receptor is assessed by immunohistochemistry using a phospho-specific HER2 antibody (clone PN2A; Thor *et al.*, *J. Clin. Oncol.*, 18(18):3230-3239 (2000)).

Other methods for detecting phosphorylation of HER receptor(s) include, but are not limited to, KIRA ELISA (U.S. Patent Nos. 5,766,863; 5,891,650; 5,914,237; 6,025,145; and 6,287,784), mass spectrometry (comparing size of phosphorylated and non-phosphorylated HER2), and e-tag proximity assay with both a HER (*e.g.* HER2) antibody and phospho-specific or phospho-tyrosine specific antibody (*e.g.*, using the eTag™assay kit available from Aclara BioSciences (Mountain View, CA). Details of the eTag assay are described hereinabove.

One may also use phospho-specific antibodies in cellular array to detect phosphorylation status in a cellular sample of signal transduction protein (US 2003/0190689).

(iii) HER2 ligands

Levels of a HER ligand, such as TGF-α, in or associated with the tumor may be determined according to known procedures. Such assays may detect protein and/or nucleic acid encoding it in the sample to be tested. In one embodiment, HER ligand levels in the tumor may be determined using immunohistochemistry (IHC); see, for example, Scher *et al. Clin. Cancer Research* 1:545-550 (1995). Alternatively, or additionally, one may evaluate levels of HER ligand-encoding nucleic acid in the sample to be tested; *e.g.* via FISH, southern blotting, or PCR techniques.

(iv) Non-HER2 overexpressing cancer

While the cancer may be characterized by overexpression of the HER2 receptor, the present application further provides a method for treating cancer which is not considered to be a HER2-overexpressing.

To determine HER2 expression in the cancer, various diagnostic/prognostic assays are available. In one embodiment, HER2 overexpression may be analyzed by IHC, *e.g.* using the HERCEPTEST® (Dako). Parrafin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a HER2 protein staining intensity criteria as follows:

Score 0 no staining is observed or membrane staining is observed in less than 10% of tumor cells.

Score 1+ a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane.

Score 2+ a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells.

Score 3+ a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells.

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

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

0 = 0-10,000 copies/cell,

1+ = at least about 200,000 copies/cell,

2+ = at least about 500,000 copies/cell,

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

Overexpression of HER2 at the 3+ level, which leads to ligand-independent activation of the tyrosine kinase (Hudziak *et al.*, *Proc. Natl. Acad. Sci. USA,* 84:7159-7163 (1987)), occurs in approximately 30% of breast cancers, and in these patients, relapse-free survival and overall survival are diminished (Slamon *et al.*, *Science,* 244:707-712 (1989); Slamon *et al.*, *Science,* 235:177-182 (1987)).

Alternatively, or additionally, FISH assays such as the INFORM™ (sold by Ventana, Arizona) or PATHVISION™ (Vysis, Illinois) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of HER2 overexpression in the tumor.

In one embodiment, the cancer will be one which expresses (and may overexpress) EGFR, such expression may be evaluated as for the methods for evaluating HER2 expression as noted above.

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

V. Treatment with the HER2 Antibodies

It is contemplated that, according to the present invention, the HER2 antibodies may be used to treat chemotherapy-resistant cancer or platinum-resisant cancer, such as platinum-resistant cancer. The cancer will generally comprise HER2-expressing cells, such that the HER2 antibody herein is able to bind to the cancer cells.

The patient is treated with a combination of the HER2 antibody, preferably Pertuzumab, and an antimetabolite chemotherapeutic agent, preferably gemcitabine. The combined administration includes coadministration or concurrent administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Thus, the antimetabolite chemotherapeutic 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 antimetabolite chemotherapeutic 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 antimetabolite chemotherapeutic agent and the HER2 antibody are administered concurrently to the patient, in a single formulation or separate formulations.

Treatment with the combination will result in an improvement in the signs or symptoms of cancer. For instance, such therapy may result in an improvement in survival (overall survival and/or progression free survival) relative to a patient treated with the antimetabolite chemotherapeutic agent only, and/or may result in an objective clinical response (partial or complete). Moreover, treatment with the combination of the antimetabolite chemotherapeutic agent (*e.g.* gemcitabine) and the HER2 antibody (*e.g.* Pertuzumab) may result in a synergistic, or greater than additive, therapeutic benefit to the patient.

Preferably, the HER2 antibody 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 (or HER2 antibody immunoconjugate) and antimetabolite chemotherapeutic agent are administered to a human patient in accord with known methods, such as intravenous administration, *e.g.*, as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The HER2 antibody and

antimetabolite chemotherapeutic agent may, but need not be, administered by the same administration route. Intravenous administration of both the antibody and antimetabolite chemotherapeutic agent is preferred.

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 approximately every 3 weeks. In another embodiment, the HER2 antibody is administered as a dose of approximately 1050mg administered approximately every 3 weeks.

The antimetabolite chemotherapeutic agent is usually administered at dosages known therefor, or optionally lowered due to combined action of the drugs or negative side effects attributable to administration of the antimetabolite chemotherapeutic agent. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). Where the antimetabolite chemotherapeutic agent is gemcitabine, preferably, it is administered at a dose between about 600mg/m^2 to 1250mg/m^2 (for example approximately 1000mg/m^2), for instance, on days 1 and 8 of a 3-week cycle.

Aside from the HER2 antibody and antimetabolite chemotherapeutic agent, other therapeutic regimens may be combined therewith. For example, a second (third, fourth, etc) chemotherapeutic agent(s) may be administered, wherein the second chemotherapeutic agent is either another, different antimetabolite chemotherapeutic agent, or a chemotherapeutic agent that is not an antimetabolite. For example, the second chemotherapeutic agent may be a taxane (such as Paclitaxel or Docetaxel), capecitabine, or platinum-based chemotherapeutic agent (such as Carboplatin, Cisplatin, or Oxaliplatin), anthracycline (such as doxorubicin, including, liposomal doxorubicin), topotecan, pemetrexed, vinca alkaloid (such as vinorelbine), and TLK 286. "Cocktails" of different chemotherapeutic agents may be administered.

Other therapeutic agents that may be combined with the HER2 antibody and antimetabolite chemotherapeutic agent include any one or more of: 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 TARCEVA®, IRESSA® or Cetuximab); an anti-angiogenic agent (especially Bevacizumab sold by Genentech under the trademark AVASTIN™); a tyrosine kinase inhibitor; a COX inhibitor (for instance a COX-1 or COX-2 inhibitor); non-steroidal anti-inflammatory drug, Celecoxib (CELEBREX®); farnesyl transferase inhibitor (for example, Tipifarnib/ZARNESTRA® R115777 available from Johnson and Johnson or Lonafarnib SCH66336 available from Schering-Plough); antibody that binds oncofetal protein CA 125 such as Oregovomab (MoAb B43.13); HER2 vaccine (such as 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.

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

Aside from administration of the antibody protein to the patient, the present application contemplates administration of the antibody by gene therapy. Such administration of nucleic acid encoding the antibody is encompassed by the expression "administering an antibody". See, for example, WO96/07321 published March 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells; *in vivo* and *ex vivo*. For *in vivo* delivery the nucleic acid is injected directly into the patient, usually at the site where the antibody is required. For *ex vivo* treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, *e.g.* U.S. Patent Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for *ex vivo* delivery of the gene is a retrovirus.

The currently preferred *in vivo* nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.* capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular

localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu *et al.*, *J. Biol. Chem.* 262:4429-4432 (1987); and Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson *et al.*, *Science* 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

VI. 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 Examples. The disclosures of all citations in the specification are expressly incorporated herein by reference.

Example 1

Production and Characterization of Monoclonal Antibody 2C4

The murine monoclonal antibodies 2C4, 7F3 and 4D5 which specifically bind the extracellular domain of HER2 were produced as described in Fendly *et al., Cancer Research* 50:1550-1558 (1990). Briefly, NIH 3T3/HER2-3_{400} cells (expressing approximately 1 x 10^5 HER2 molecules/cell) produced as described in Hudziak *et al Proc. Natl. Acad. Sci. (USA)* 84:7159-7163 (1987) were harvested with phosphate buffered saline (PBS) containing 25mM EDTA and used to immunize BALB/c mice. The mice were given injections i.p. of 10^7 cells in 0.5ml PBS on weeks 0, 2, 5 and 7. The mice with antisera that immunoprecipitated ^{32}P-labeled HER2 were given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified HER2 membrane extract on weeks 9 and 13. This was followed by an i.v. injection of 0.1 ml of the HER2 preparation and the splenocytes were fused with mouse myeloma line X63-Ag8.653.

Hybridoma supernatants were screened for HER2-binding by ELISA and radioimmunoprecipitation.

The HER2 epitopes bound by monoclonal antibodies 4D5, 7F3 and 2C4 were determined by competitive binding analysis (Fendly *et al. Cancer Research* 50:1550-1558 (1990)). Cross-blocking studies were done on antibodies by direct fluorescence on intact cells using the PANDEX™ Screen Machine to quantitate fluorescence. Each monoclonal antibody was conjugated with fluorescein isothiocyanate (FITC), using established procedures (Wofsy *et al. Selected Methods in Cellular Immunology*, p. 287, Mishel and Schiigi (eds.) San Francisco: W.J. Freeman Co. (1980)). Confluent monolayers of NIH 3T3/HER2-3_{400} cells were trypsinized, washed once, and resuspended at 1.75 x 10^6 cell/ml in cold PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3. A final concentration of 1% latex particles (IDC, Portland, OR) was added to reduce clogging of the PANDEX™ plate membranes. Cells in suspension, 20 µl, and 20 µl of purified monoclonal antibodies (100µg/ml to 0.1 µg/ml) were added to the PANDEX™ plate wells and incubated on ice for 30 minutes. A predetermined dilution of FITC-labeled monoclonal antibodies in 20 µl was added to each well, incubated for 30 minutes, washed, and the fluorescence was quantitated by the PANDEX™. Monoclonal antibodies were considered to share an epitope if

each blocked binding of the other by 50% or greater in comparison to an irrelevant monoclonal antibody control. In this experiment, monoclonal antibodies 4D5, 7F3 and 2C4 were assigned epitopes I, G/F and F, respectively.

The growth inhibitory characteristics of monoclonal antibodies 2C4, 7F3 and 4D5 were evaluated using the breast tumor cell line, SK-BR-3 (see Hudziak *et al. Molec. Cell. Biol.* 9(3):1165-1172 (1989)). Briefly, SK-BR-3 cells were detached by using 0.25% (vol/vol) trypsin and suspended in complete medium at a density of 4×10^5 cells per ml. Aliquots of 100 µl (4×10^4 cells) were plated into 96-well microdilution plates, the cells were allowed to adhere, and 100 µl of media alone or media containing monoclonal antibody (final concentration 5 µg/ml) was then added. After 72 hours, plates were washed twice with PBS (pH 7.5), stained with crystal violet (0.5% in methanol), and analyzed for relative cell proliferation as described in Sugarman *et al. Science* 230:943-945 (1985). Monoclonal antibodies 2C4 and 7F3 inhibited SK-BR-3 relative cell proliferation by about 20% and about 38%, respectively, compared to about 56% inhibition achieved with monoclonal antibody 4D5.

Monoclonal antibodies 2C4, 4D5 and 7F3 were evaluated for their ability to inhibit HRG-stimulated tyrosine phosphorylation of proteins in the M_r 180,000 range from whole-cell lysates of MCF7 cells (Lewis *et al. Cancer Research* 56:1457-1465 (1996)). MCF7 cells are reported to express all known HER receptors, but at relatively low levels. Since HER2, HER3, and HER4 have nearly identical molecular sizes, it is not possible to discern which protein is becoming tyrosine phosphorylated when whole-cell lysates are evaluated by Western blot analysis.

However, these cells are ideal for HRG tyrosine phosphorylation assays because under the assay conditions used, in the absence of exogenously added HRG, they exhibit low to undetectable levels of tyrosine phosphorylation proteins in the M_r 180,000 range.

MCF7 cells were plated in 24-well plates and monoclonal antibodies to HER2 were added to each well and incubated for 30 minutes at room temperature; then $rHRG\beta1_{177-244}$ was added to each well to a final concentration of 0.2 nM, and the incubation was continued for 8 minutes. Media was carefully aspirated from each well, and reactions were 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) was electrophoresed on a 4-12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (4G10, from UBI, used at 1 µg/ml) immunoblots were developed, and the intensity of the predominant reactive band at M_r~180,000 was quantified by reflectance densitometry, as described previously (Holmes *et al. Science* 256:1205-1210 (1992); Sliwkowski *et al. J. Biol. Chem.* 269:14661-14665 (1994)).

Monoclonal antibodies 2C4, 7F3, and 4D5, significantly inhibited the generation of a HRG-induced tyrosine phosphorylation signal at M_r 180,000. In the absence of HRG, none of these antibodies were able to stimulate tyrosine phosphorylation of proteins in the M_r 180,000 range. Also, these antibodies do not cross-react with EGFR (Fendly *et al. Cancer Research* 50:1550-1558 (1990)), HER3, or HER4. Antibodies 2C4 and 7F3 significantly inhibited HRG stimulation of p180 tyrosine phosphorylation to <25% of control. Monoclonal antibody 4D5 was able to block HRG stimulation of tyrosine phosphorylation by ~50%. Fig. 2A shows dose-response curves for 2C4 or 7F3 inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry. Evaluation of these inhibition curves using a 4-parameter fit yielded an IC_{50} of 2.8 ± 0.7 nM and 29.0 ± 4.1 nM for 2C4 and 7F3, respectively.

Inhibition of HRG binding to MCF7 breast tumor cell lines by HER2 antibodies was performed with monolayer cultures on ice in a 24-well-plate format (Lewis *et al. Cancer Research* 56:1457-1465 (1996)). HER2

monoclonal antibodies were added to each well and incubated for 30 minutes. ^{125}I-labeled rHRGβ1$_{177-224}$ (25 pm) was added, and the incubation was continued for 4 to 16 hours. Fig. 2B provides dose-response curves for 2C4 or 7F3 inhibition of HRG binding to MCF7 cells. Varying concentrations of 2C4 or 7F3 were incubated with MCF7 cells in the presence of ^{125}I-labeled rHRGβ1, and the inhibition curves are shown in Fig. 2B. Analysis of these data yielded an IC_{50} of 2.4 ± 0.3 nM and 19.0 ± 7.3 nM for 2C4 and 7F3, respectively. A maximum inhibition of ~74% for 2C4 and 7F3 were in agreement with the tyrosine phosphorylation data.

To determine whether the effect of the HER2 antibodies observed on MCF7 cells was a general phenomenon, human tumor cell lines were incubated with 2C4 or 7F3 and the degree of specific ^{125}I-labeled rHRGβ1 binding was determined (Lewis *et al. Cancer Research* 56:1457-1465 (1996)). The results from this study are shown in Fig. 3. Binding of ^{121}I-labeled rHRGβ1 could be significantly inhibited by either 2C4 or 7F3 in all cell lines, with the exception of the breast cancer cell line MDA-MB-468, which has been reported to express little or no HER2. The remaining cell lines are reported to express HER2, with the level of HER2 expression varying widely among these cell lines. In fact, the range of HER2 expression in the cell lines tested varies by more than 2 orders of magnitude. For example, BT-20, MCF7, and Caov3 express ~10^4 HER2 receptors/cell, whereas BT-474 and SK-BR-3 express ~10^6 HER2 receptors/cell. Given the wide range of HER2 expression in these cells and the data above, it was concluded that the interaction between HER2 and HER3 or HER4, was itself a high-affinity interaction that takes place on the surface of the plasma membrane.

The growth inhibitory effects of monoclonal antibodies 2C4 and 4D5 on MDA-MB-175 and SK-BR-3 cells in the presence or absence of exogenous rHRGβ1 was assessed (Schaefer *et al. Oncogene* 15:1385-1394 (1997)). HER2 levels in MDA-MB-175 cells are 4-6 times higher than the level found in normal breast epithelial cells and the HER2-HER4 receptor is constitutively tyrosine phosphorylated in MDA-MB-175 cells. MDA-MB-175 cells were treated with a HER2 monoclonal antibodies 2C4 and 4D5 (10μg/mL) for 4 days. In a crystal violet staining assay, incubation with 2C4 showed a strong growth inhibitory effect on this cell line (Fig. 4A). Exogenous HRG did not significantly reverse this inhibition. On the other hand 2C4 revealed no inhibitory effect on the HER2 overexpressing cell line SK-BR-3 (Fig. 4B). Monoclonal antibody 2C4 was able to inhibit cell proliferation of MDA-MB-175 cells to a greater extent than monoclonal antibody 4D5, both in the presence and absence of exogenous HRG. Inhibition of cell proliferation by 4D5 is dependent on the HER2 expression level (Lewis *et al. Cancer Immunol. Immunother.* 37:255-263 (1993)). A maximum inhibition of 66% in SK-BR-3 cells could be detected (Fig.4B). However this effect could be overcome by exogenous HRG.

Example 2

HRG Dependent Association of HER2 with HER3 is Blocked by Monoclonal Antibody 2C4

The ability of HER3 to associate with HER2 was tested in a co-immunoprecipitation experiment. 1.0 x 10^6 MCF7 or SK-BR-3 cells were seeded in six well tissue culture plates in 50:50 DMEM/Ham's F12 medium containing 10% fetal bovine serum (FBS) and 10 mM HEPES, pH 7.2 (growth medium), and allowed to attach overnight. The cells were starved for two hours in growth medium without serum prior to beginning the experiment

The cells were washed briefly with phosphate buffered saline (PBS) and then incubated with either 100 nM of the indicated antibody diluted in 0.2% w/v bovine serum albumin (BSA), RPMI medium, with 10 mM HEPES, pH 7.2 (binding buffer), or with binding buffer alone (control). After one hour at room temperature, HRG

was added to a final concentration of 5 nM to half the wells (+). A similar volume of binding buffer was added to the other wells (-). The incubation was continued for approximately 10 minutes.

Supernatants were removed by aspiration and the cells were lysed in RPMI, 10 mM HEPES, pH 7.2, 1.0% v/v TRITON X-100™, 1.0% w/v CHAPS (lysis buffer), containing 0.2 mM PMSF, 10 μg/ml leupeptin, and 10 TU/ml aprotinin. The lysates were cleared of insoluble material by centrifugation.

HER2 was immunoprecipitated using a monoclonal antibody covalently coupled to an affinity gel (Affi-Prep 10, Bio-Rad). This antibody (Ab-3, Oncogene Sciences) recognizes a cytoplasmic domain epitope. Immunoprecipitation was performed by adding 10 μl of gel slurry containing approximately 8.5 μg of immobilized antibody to each lysate, and the samples were allowed to mix at room temperature for two hours. The gels were then collected by centrifugation. The gels were washed batchwise three times with lysis buffer to remove unbound material. SDS sample buffer was then added and the samples were heated briefly in a boiling water bath.

Supernatants were run on 4-12% polyacrylamide gels and electroblotted onto nitrocellulose membranes. The presence of HER3 was assessed by probing the blots with a polyclonal antibody against a cytoplasmic domain epitope thereof (c-17, Santa Cruz Biotech). The blots were visualized using a chemiluminescent substrate (ECL, Amersham).

As shown in the control lanes of Figs. 5A and 5B, for MCF7 and SK-BR-3 cells, respectively, HER3 was present in a HER2 immunoprecipitate only when the cells were stimulated with HRG. If the cells were first incubated with monoclonal antibody 2C4, the HER3 signal was abolished in MCF7 cells (Fig. 5A, lane 2C4 +) or substantially reduced in SK-BR-3 cells (Fig. 5B, lane 2C4+). As shown in Figs 5A-B, monoclonal antibody 2C4 blocks heregulin dependent association of HER3 with HER2 in both MCF7 and SK-BR-3 cells substantially more effectively than Trastuzumab. Preincubation with Trastuzumab decreased the HER3 signal in MCF7 lysates but had little or no effect on the amount of HER3 co-precipitated from SK-BR-3 lysates. Preincubation with an antibody against the EGF receptor (Ab-1, Oncogene Sciences) had no effect on the ability of HER3 to co-immunoprecipitate with HER2 in either cell line.

Example 3

Humanized 2C4 Antibodies

The variable domains of murine monoclonal antibody 2C4 were first cloned into a vector which allows production of a mouse/human chimeric Fab fragment. Total RNA was isolated from the hybridoma cells using a Stratagene RNA extraction kit following manufacturer's protocols. The variable domains were amplified by RT-PCR, gel purified, and inserted into a derivative of a pUC119-based plasmid containing a human kappa constant domain and human C_H1 domain as previously described (Carter *et al. PNAS (USA)* 89:4285 (1992); and U.S. Patent No. 5,821,337). The resultant plasmid was transformed into *E. coli* strain 16C9 for expression of the Fab fragment. Growth of cultures, induction of protein expression, and purification of Fab fragment were as previously described (Werther *et al. J. Immunol.* 157:4986-4995 (1996); Presta *et al. Cancer Research* 57: 4593-4599 (1997)).

Purified chimeric 2C4 Fab fragment was compared to the murine parent antibody 2C4 with respect to its ability to inhibit ^{125}I-HRG binding to MCF7 cells and inhibit rHRG activation of p180 tyrosine phosphorylation in MCF7 cells. As shown in Fig. 6A, the chimeric 2C4 Fab fragment is very effective in disrupting the formation of the high affinity HER2-HER3 binding site on the human breast cancer cell line, MCF7. The relative IC_{50} value calculated for intact murine 2C4 is 4.0 ± 0.4nM, whereas the value for the Fab fragment is 7.7 ± 1.1nM. As

illustrated in Fig. 6B, the monovalent chimeric 2C4 Fab fragment is very effective in disrupting HRG-dependent HER2-HER3 activation. The IC_{50} value calculated for intact murine monoclonal antibody 2C4 is 6.0 ± 2nM, whereas the value for the Fab fragment is 15.0 ± 2nM.

DNA sequencing of the chimeric clone allowed identification of the CDR residues (Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991)) (Figs. 7A and B). Using oligonucleotide site-directed mutagenesis, all six of these CDR regions were introduced into a complete human framework (V_L kappa subgroup I and V_H subgroup III) contained on plasmid VX4 as previously described (Presta *et al.*, *Cancer Research* 57: 4593-4599 (1997)). Protein from the resultant "CDR-swap" was expressed and purified as above. Binding studies were performed to compare the two versions. Briefly, a NUNC MAXISORP™ plate was coated with 1 microgram per ml of HER2 extracellular domain (ECD; produced as described in WO 90/14357) in 50 mM carbonate buffer, pH 9.6, overnight at 4°C, and then blocked with ELISA diluent (0.5% BSA, 0.05% polysorbate 20, PBS) at room temperature for 1 hour. Serial dilutions of samples in ELISA diluent were incubated on the plates for 2 hours. After washing, bound Fab fragment was detected with biotinylated murine anti-human kappa antibody (ICN 634771) followed by streptavidin-conjugated horseradish peroxidase (Sigma) and using 3,3',5,5'-tetramethyl benzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as substrate. Absorbance was read at 450 nm. As shown in Fig. 8A, all binding was lost on construction of the CDR-swap human Fab fragment.

To restore binding of the humanized Fab, mutants were constructed using DNA from the CDR-swap as template. Using a computer generated model (Fig. 9), these mutations were designed to change human framework region residues to their murine counterparts at positions where the change might affect CDR conformations or the antibody-antigen interface. Mutants are shown in Table 2.

Table 2

Designation of Humanized 2C4 FR Mutations

Mutant no.	Framework region (FR) substitutions
560	ArgH71Val
561	AspH73Arg
562	ArgH71Val, AspH73Arg
568	ArgH71Val, AspH73Arg, AlaH49Gly
569	ArgH71Val, AspH73Arg, PheH67Ala
570	ArgH71Val, AspH73Arg, AsnH76Arg
571	ArgH71Val, AspH73Arg, LeuH78Val
574	ArgH71Val, AspH73Arg, IleH69Leu
56869	ArgH71Val, AspH73Arg, AlaH49Gly, PheH67Ala

Binding curves for the various mutants are shown in Figs. 8A-C. Humanized Fab version 574, with the changes ArgH71Val, AspH73Arg and IleH69Leu, appears to have binding restored to that of the original chimeric 2C4 Fab fragment. Additional FR and/or CDR residues, such as L2, L54, L55, L56, H35 and/or H48, may be modified (*e.g.* substituted as follows - IleL2Thr; ArgL54Leu; TyrL55Glu; ThrL56Ser; AspH35Ser; and ValH48Ile)

in order to further refine or enhance binding of the humanized antibody. Alternatively, or additionally, the humanized antibody may be affinity matured (see above) in order to further improve or refine its affinity and/or other biological activities.

Humanized 2C4 version 574 was affinity matured using a phage-display method. Briefly, humanized 2C4.574 Fab was cloned into a phage display vector as a geneIII fusion. When phage particles are induced by infection with M13KO7 helper phage, this fusion allows the Fab to be displayed on the N-terminus of the phage tail-fiber protein, geneIII (Baca *et al. J Biol Chem.* 272:10678 (1997)).

Individual libraries were constructed for each of the 6 CDRs identified above. In these libraries, the amino acids in the CDRs which were identified using a computer generated model (Fig. 9) as being potentially significant in binding to HER2 were randomized using oligos containing "NNS" as their codons. The libraries were then panned against HER2 ECD coated on NUNC MAXISORP™ plates with 3% dry milk in PBS with 0.2% TWEEN 20® (MPBST) used in place of all blocking solutions. In order to select for phage with affinities higher than that of 2C4.574, in panning rounds 3, 4, and 5, soluble HER2 ECD or soluble Fab 2C4.574 was added during the wash steps as competitor. Wash times were extended to 1 hour at room temperature.

After 5 rounds of panning, individual clones were again analyzed by phage-ELISA. Individual clones were grown in Costar 96-well U-bottomed tissue culture plates, and phage were induced by addition of helper phage. After overnight growth, *E. coli* cells were pelleted, and the phage-containing supernates were transfered to 96-well plates where the phage were blocked with MPBST for 1 hr at room temperature. NUNC MAXISORP™ plates coated with HER2 ECD were also blocked with MPBST as above. Blocked phage were incubated on the plates for 2 hours. After washing, bound phage were detected using horseradish-peroxidase-conjugated anti-M13 monoclonal antibody (Amersham Pharmacia Biotech, Inc. 27-9421-01) diluted 1:5000 in MPBST, followed by 3,3',5,5',-tetramethyl benzidine as substrate. Absorbance was read at 450 nm.

The 48 clones from each library which gave the highest signals were DNA sequenced. Those clones whose sequences occurred the most frequently were subcloned into the vector described above which allows expression of soluble Fabs. These Fabs were induced, proteins purified and the purified Fabs were analyzed for binding by ELISA as described above and the binding was compared to that of the starting humanized 2C4.574 version.

After interesting mutations in individual CDRs were identified, additional mutants which were various combinations of these were constructed and tested as above. Mutants which gave improved binding relative to 574 are described in Table 3.

Table 3

Designation of mutants derived from affinity maturation of 2C4.574

Mutant Name	Change from 574	Mutant/574*
H3.A1	serH99trp, metH34leu	0.380
L2.F5	serL50trp, tyrL53gly, metH34leu	0.087
H1.3.B3	thrH28gln,thrH30ser, metH34leu	0.572
L3.G6	tyrL92pro, ileL93lys, metH34leu	0.569
L3.G11	tyrL92ser, ileL93arg, tyrL94gly, metH34leu	0.561
L3.29	tyrL92phe, tyrL96asn, metH34leu	0.552
L3.36	tyrL92phe, tyrL94leu, tyrL96pro, metH34leu	0.215
654	serL50trp, metH34leu	0.176

655	metH34ser	0.542
659	serL50trp, metH34ser	0.076
L2.F5.H3.A1	serL50trp, tyrL53gly, metH34leu, serH99trp	0.175
L3G6.H3.A1	tyrL92pro, ileL93lys, metH34leu, serH99trp	0.218
H1.3.B3.H3.A1	thrH28gln, thrH30ser, metH34leu, serH99trp	0.306
L3.G11.H3.A1	tyrL92ser, ileL93arg, tyrL94gly, metH34leu, serH99trp	0.248
654.H3.A1	serL50trp, metH34leu, serH99trp	0.133
654.L3.G6	serL50trp, metH34leu, tyrL92pro, ileL93lys	0.213
654.L3.29	serL50trp, metH34leu, tyrL92phe, tyrL96asn	0.236
654.L3.36	serL50trp, metH35leu, tyrL92phe, tyrL94leu, tyrL96pro	0.141

*Ratio of the amount of mutant needed to give the mid-OD of the standard curve to the amount of 574 needed to give the mid-OD of the standard curve in an Erb2-ECD ELISA. A number less than 1.0 indicates that the mutant binds Erb2 better than 574 binds.

The following mutants have also been constructed, and are currently under evaluation:

659.L3.G6	serL50trp, metH34ser, tyrL92pro, ileL93lys
659.L3.G11	serL50trp, metH34ser, tyrL92ser, ileL93arg, tyrL94gly
659.L3.29	serL50trp, metH34ser, tyrL92phe, tyrL96asn
659.L3.36	serL50trp, metH34ser, tyrL92phe, tyrL94leu, tyrL96pro
L2F5.L3G6	serL50trp, tyrL53gly, metH34leu, tyrL92pro, ileL93lys
L2F5.L3G11	serL50trp, tyrL53gly, metH34leu, tyrL92ser, ileL93arg, tyrL94gly
L2F5.L29	serL50trp, tyrL53gly, metH34leu, tyrL92phe, tyrL96asn
L2F5.L36	serL50trp, tyrL53gly, metH34leu, tyrL92phe, tyrL94leu, tyrL96pro
L2F5.L3G6.655	serL50trp, tyrL53gly, metH35ser, tyrL92pro, ileL93lys
L2F5.L3G11.655	serL50trp, tyrL53gly, metH34ser, tyrL92ser, ileL93arg, tyrL94gly
L2F5.L29.655	serL50trp, tyrL53gly, metH34ser, tyrL92phe, tyrL96asn
L2F5.L36.655	serL50trp, tyrL53gly, metH34ser, tyrL92phe, tyrL94leu, tyrL96pro

The following mutants, suggested by a homology scan, are currently being constructed:

678	thrH30ala
679	thrH30ser
680	lysH64arg
681	leuH96val
682	thrL97ala
683	thrL97ser
684	tyrL96phe
685	tyrL96ala
686	tyrL91phe
687	thrL56ala
688	glnL28ala
689	glnL28glu

The preferred amino acid at H34 would be methionine. A change to leucine might be made if there were found to be oxidation at this position.

AsnH52 and asnH53 were found to be strongly preferred for binding. Changing these residues to alanine or aspartic acid dramatically decreased binding. An intact antibody comprising the variable light and heavy domains of humanized version 574 with a human IgG1 heavy chain constant region has been prepared (see U.S. Patent No. 5,821,337). The intact antibody is produced by Chinese Hamster Ovary (CHO) cells. That molecule is designated Pertuzumab herein.

Example 4

Monoclonal Antibody 2C4 Blocks EGF, TGF-α or HRG Mediated Activation of MAPK and Akt kinase

Many growth factor receptors signal through the mitogen-activated protein kinase (MAPK) pathway. These dual specificity kinases are one of the key endpoints in signal transduction pathways that ultimately triggers cancer cells to divide. The ability of monoclonal antibody 2C4 or Trastuzumab to inhibit EGF, TGF-α or HRG activation of MAPK was assessed in the following way.

MCF7 cells (10^5 cells/well) were plated in serum containing media in 12-well cell culture plates. The next day, the cell media was removed and fresh media containing 0.1% serum was added to each well. This procedure was then repeated the following day and prior to assay the media was replaced with serum-free binding buffer (Jones *et al. J. Biol. Chem.* 273:11667-74 (1998); and Schaefer *et al. J. Biol. Chem.* 274:859-66 (1999)). Cells were allowed to equilibrate to room temperature and then incubated for 30 minutes with 0.5 mL of 200 nM Trastuzumab or monoclonal antibody 2C4. Cells were then treated with 1 nM EGF, 1 nM TGF-α or 0.2 nM HRG for 15 minutes. The reaction was stopped by aspirating the cell medium and then adding 0.2 mL SDS-PAGE sample buffer containing 1% DTT. MAPK activation was assessed by Western blotting using an anti-active MAPK antibody (Promega) as described previously (Jones *et al. J. Biol. Chem.* 273:11667-74 (1998)).

As shown in Fig. 10, monoclonal antibody 2C4 significantly blocks EGF, TGF-α and HRG mediated activation of MAPK to a greater extent than Trastuzumab. These data suggest that monoclonal antibody 2C4 binds to a surface of HER2 that is used for its association with either EGFR or HER3 and thus prevents the formation of the signaling receptor complex.

Monoclonal antibody 2C4 was also shown to inhibit heregulin (HRG)-dependent Akt activation.. Activation of the PI3 kinase signal transduction pathway is important for cell survival (Carraway *et al. J. Biol. Chem. 270:* 7111-6 (1995)). In tumor cells, PI3 kinase activation may play a role in the invasive phenotype (Tan *et al. Cancer Reearch. 59:* 1620-1625, (1999)). The survival pathway is primarily mediated by the serine/threonine kinase AKT (Bos *et al. Trends Biochem Sci. 20:* 441-442 (1995). Complexes formed between HER2 and either HER3 or EGFR can initiate these pathways in response to heregulin or EGF, respectively (Olayioye *et al. Mol. & Cell. Biol. 18:* 5042-51 (1998); Karunagaran *et al., EMBO Journal. 15:* 254-264 (1996); and Krymskaya *et al. Am. J. Physiol. 276:* L246-55 (1999)). Incubation of MCF7 breast cancer cells with 2C4 inhibits heregulin-mediated Akt activation. Agus *et al. Cancer Cell* 2: 127-137 (2002). Moreover, the basal level of Akt activation present in the absence of heregulin addition is further reduced by the addition of 2C4.

Example 5

Therapy of Platinum-Resistant Cancer

This example demonstrates the safety, tolerability, and efficacy of pertuzumab in combination with gemcitabine in patients with platinum-resistant ovarian cancer, primary peritoneal carcinoma, or fallopian tube carcinoma. The effect of pertuzumab and gemcitabine on progression free survival, is evaluated in all patients, and in the subset of patients whose tumors contain markers that indicate activation of HER2.

Patients who have progressed while receiving, or within 6 months of receiving, a platinum-based chemotherapy regimen will be eligible for this study. Patients will be randomized to receive either gemcitabine in combination with pertuzumab, or gemcitabine in combination with placebo. Patients treated herein include those who have not received a previous salvage regimen treatment for platinum-resistant disease prior to study entry, and those who have had one prior regimen for platinum-resistant disease.

Gemcitabine will be administered at 1000mg/m^2 on days 1 and 8 of each 21 day cycle. Gemcitabine will be infused first over 30 minutes. Dose reductions will be permitted for toxicity. Placebo or pertuzumab will be administered on day 1 of the 21 day cycle. Subjects randomized to receive pertuzumab will be administered an initial loading dose of 840 mg (Cycle 1) followed by 420 mg in Cycles 2 and beyond. Subjects randomized to receive placebo will be administered placebo in the same volume as administered with pertuzumab arm for Cycle 1, Cycles 2 and beyond. Subjects without progressive disease may receive treatment for up to 17 cycles, or 1 year.

Patients will have standard gemcitabine dose reduction and held doses as a result of cytopenias. Pertuzumab will also be held for any held Day 1 gemcitabine doses. Subsequent doses will be at the reduced doses and will not be increased. If dose reduction or holding a dose is required in more than 4 occasions, or if doses are held for more than 3 weeks, then gemcitabine will be discontinued and with the approval of the treating physician and medical monitor, blinded drug may be continued until disease progression. If Day 8 gemcitabine doses are held, then the Day 8 dose will be omitted and the subsequent treatment will commence with the next cycle (Day 22 of the previous cycle).

Gemcitabine will be held and dose reduced as recommended by the following table:

Absolute Granulocyte Count (x10^6/L)		Platelet Count (x10^6/L)	% full dose
>1000	and	>100,000	100
500-999	or	50,000-99,000	75
<500	or	<50,000	Hold

Subsequent doses for any patient requiring dose reduction will be at the reduced dose. If doses are held for more than 3 weeks as a result of cytopenias, patients will be assume to have unacceptable toxicity and will discontinue gemcitabine. If there are no other additional grade III or IV toxicities, continuation of blinded drug will be at the discretion of the physician and medical monitor. Hematological toxicity of gemcitabine has been related to rate of dose administration. Gemcitabine will be given over 30 minutes regardless of total dose. The use of colony-stimulating agents for NCI-CTC Grade 2 cytopenias may be used at the discretion of the treating physician.

The option for crossover to single agent pertuzumab will be offered. A loading dose of 840mg will be administered at the next cycle due with continuation of 420mg with subsequent cycles every 21 days. A paraffin-embedded tumor specimen or unstained paraffin slides of representative tumor containing the cancer will be

obtained and assessed for HER2 phosphorylation status. Approximately 20-40% of ovarian cancer patients may have detectable HER2 phosphorylation.

Response will be assessed at the end of Cycles 2, 4, 6, 8, 12 and 17. Measurable disease will be assessed using the Response Evaluation Criteria for Solid Tumors (RECIST), by clinical evaluation and CT scan or equivalent. Response for subjects with evaluable disease will be assessed according to changes to CA-125 and clinical and radiologic evidence of disease. Responses should be confirmed 4-8 weeks after the initial documentation of response. Evaluable subjects will consist of subjects that have had at least one response assessment and have a determination of HER2 phosphorylation status in their tumor specimen.

The following outcome measures will be assessed.

Primary Efficacy Endpoint

Progression free survival, as determined by investigator assessment using RECIST or CA-125 changes, following initiation of assigned study treatment of all subjects in each arm.

Progression free survival, as determined by investigator assessment using RECIST or CA-125 changes following initiation of assigned study treatment in each arm in the following subgroups:

Subjects with detectable markers of HER2 activation.

Subjects with no detectable markers of HER2 activation.

Secondary Efficacy Endpoints

Objective response (PR or CR)

Duration of response

Survival time

Freedom from progression at 4 months

These endpoints will be assessed in all subjects in each arm and in the following subgroups:

Subjects with detectable markers of HER2 activation.

Subjects with no detectable markers of HER2 activation.

To prevent or treat possible nausea and vomiting, the patient may be premedicated with serotonin antagonists, steroids, and/or benzodiazepines. To prevent or treat possible rash, standard acne therapies, including topical and/or oral antibiotics may be used. Other possible concomitant medications are any prescription medications or over-the-counter preparations used by a subject in the interval beginning 7 days prior to Day 1 and continuing through the last day of the follow-up period. Subjects who experience infusion-associated temperature elevations to >38.5°C or other infusion-associated symptoms may be treated symptomatically with acetaminophen, diphenhydramine, or meperidine. Non-experimental hematopoietic growth factors may be administered for NCI-CTC Grade 2 cytopenias.

The patient treated as described above will show improvement in the signs or symptoms of ovarian cancer, primary peritoneal carcinoma or fallopian tube carcinoma as evaluated by any one or more of the primary or secondary efficacy endpoints. Moreover, the platinum-resistant patient treated as described above with the combination of Pertuzumab and Gemcitabine will show greater improvement in any of the signs or symptoms of the cancer, compared to the platinum-resistant patient treated with Gemcitabine only.

CLAIMS

1. Use of a HER2 antibody that binds to a heterodimeric binding site on HER2 in the manufacture of a medicament for the treatment of platinum-resistant cancer selected from the group consisting of ovarian cancer, primary peritoneal carcinoma and fallopian tube carcinoma, wherein the medicament is to be used in combination with an antimetabolite chemotherapeutic agent.

2. The use of claim 1 wherein the antibody blocks heterodimerization of HER2 with EGFR or HER3.

3. The use of claim 1 or 2 wherein the HER2 antibody binds to Domain II of HER2.

4. The use of claim 1 or 2 wherein said HER2 antibody binds to the junction between domains I, II and III of HER2.

5. The use of claim 3 wherein the HER2 antibody is Pertuzumab.

6. The use of any one of the preceding claims wherein the antimetabolite chemotherapeutic agent is gemcitabine.

7. The use of any one of the preceding claims wherein a sample from said cancer displays HER activation.

8. The use of claim 7 wherein a sample from said cancer displays HER2 activation.

9. The use of any one of the preceding claims wherein administration of said medicament results in an improvement in survival relative to a patient treated with the antimetabolite chemotherapeutic agent only.

10. The use of claim 9 wherein administration of said medicament results in an improvement in progression free survival relative to a patient treated with the antimetabolite chemotherapeutic agent only.

11. The use of claim 9 wherein administration of said medicament results in an improvement in overall survival relative to a patient treated with the antimetabolite chemotherapeutic agent only.

12. The use of any one of the preceding claims wherein administration of said medicament results in an objective response.

13. The use of claim 12 wherein administration of said medicament results in a complete response.

14. The use of claim 12 wherein administration of said medicament results in a partial response.

15. The use of claim 1 wherein the cancer does not overexpress HER2.

16. The use of any one of the preceding claims wherein the HER2 antibody is an intact antibody.

17. The use of any one of claims 1 to 16 wherein the HER2 antibody is an antibody fragment comprising an antigen binding region.

18. The use of any one of the preceding claims wherein the cancer is ovarian cancer.

19. The use of any one of claims 1 to 17 wherein the cancer is primary peritoneal carcinoma.

20. The use of any one of claims 1 to 17 wherein the cancer is fallopian tube carcinoma.

21. Use of a HER2 antibody that binds to a heterodimeric binding site on HER2 in the manufacture of a medicament in a package or two separate packages with instructions for use in the treatment of platinum-resistant cancer selected from the group consisting of ovarian cancer, primary peritoneal carcinoma and fallopian tube carcinoma, wherein said medicament is for use in combination with an antimetabolite chemotherapeutic agent.

22. The use of claim 21 wherein, said instructions provide that the HER2 antibody is administered as a loading dose of approximately 840 mg followed by approximately 420 mg about every 3 weeks.

23. The use of claim 21, wherein said instructions provide that the HER2 antibody is administered as a dose of approximately 1050 mg administered about every 3 weeks.

24. The use of claim 21 wherein the antimetabolite chemotherapeutic agent is gemcitabine.

25. The use of claim 24 wherein said instructions provide that the gemcitabine is administered at a dose between about 600mg/m^2 to 1250mg/m^2 on days 1 and 8 of a 3-week cycle.

26. The use of any one of claims 21-25 wherein said instructions provide that the antimetabolite chemotherapeutic agent is administered prior to, or following, administration of the HER2 antibody.

27. The use of claim 26 wherein said instructions provide that the timing between at least one administration of the antimetabolite chemotherapeutic agent and at least one administration of the HER2 antibody is approximately 1 month or less.

28. The use of claim 27 wherein said instruction provide that the timing between at least one administration of the antimetabolite chemotherapeutic agent and at least one

administration of the HER2 antibody is approximately 2 weeks or less.

29. The use of any one of claims 21 to 25, wherein said instructions provide that the antimetabolite chemotherapeutic agent and the HER2 antibody are administered concurrently to the patient, in a single formulation or separate formulations.

30. The use of any one of claims 21 to 29 wherein said instructions provide for administering a second chemotherapeutic agent to the patient.

31. The use of claim 30 wherein the second chemotherapeutic agent is selected from the group consisting of a taxane, capecitabine, platinum-based chemotherapeutic agent, anthracycline, liposomal doxorubicin, topotecan, pemetrexed, vinca alkaloid, and TLK 286.

32. The use of any one of claims 21 to 31 wherein treatment with the combination of the antimetabolite chemotherapeutic agent and the HER2 antibody results in a synergistic, or greater than additive, therapeutic benefit to the patient.

33. The use of claim 24 wherein treatment with the combination of the gemcitabine and the HER2 antibody results in a synergistic, or greater than additive, benefit to the patient.

34. The use of any one of the preceding claims wherein the HER2 antibody is a naked antibody.

Sequence Listing

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

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

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

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

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

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

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

Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
                395                 400                 405

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

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

His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly
                440                 445
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<210> 16
<211> 214
<212> PRT
<213> Artificial sequence

<220>
<223> Sequence is synthesized.

<400> 16

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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
  1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser
                20                  25                  30

Ile Gly Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

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

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
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65 70 75

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

Tyr Tyr Ile Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
95 100 105

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

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

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

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

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

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

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
200 205 210

Arg Gly Glu Cys

<210> 17
<211> 449
<212> PRT
<213> Artificial sequence

<220>
<223> Sequence is synthesized.

<400> 17
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
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