PERTUZUMAB VARIANTS AND EVALUATION THEREOF

This non-provisional application filed under 37 CFR §1.53(b), claims the benefit under 35 USC §119(e) of U.S. Provisional Application Serial No. 61/812,603, filed April 16, 2013, which is incorporated by reference in entirety.

Sequence Listing

The instant application contains a Sequence Listing submitted via EFS-Web and hereby incorporated by reference in its entirety. Said ASCII copy, created April 8, 2014, is named P5584R1-WO_SeqList.txt, and is 31,363 bytes in size.

Field of the Invention

The present invention concerns variants of Pertuzumab. In particular, it concerns: an unpaired cysteine variant comprising Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab, an afucosylated variant of Pertuzumab, a low-molecular-weight-species (LMWS) of Pertuzumab, and a high-molecular-weight-species (HMWS) or Pertuzumab. The invention further concerns the isolated variants, compositions, pharmaceutical compositions, and articles of manufacture comprising the variants, as well as methods of making and characterizing the variants and compositions thereof.

Background of the Invention

Pertuzumab (PERJETA®) (also called rhuMAb 2C4) is a monoclonal antibody (MAb) which is the first of its class in a line of agents called "HER dimerization inhibitors." By binding to HER2, it inhibits dimerization of HER2 with other HER receptors and thus inhibits tumor growth. Pertuzumab received United Stated Food and Drug Administration (US FDA) approval for the treatment of HER2-positive metastatic breast cancer on June 8, 2012.

US Patent No. 7,862,817 (Adams et al.) describe a humanized variant of the 2C4 antibody called humanized 2C4 version 574 or recombinant humanized monoclonal antibody 2C4 (rhuMAb 2C4). The antibody bound Subdomain II in the Human Epidermal Growth Factor Receptor 2 (HER2) extracellular domain (ECD). The rhuMAb 2C4 antibody was produced on a laboratory scale and shown to bind to HER2 and inhibit growth of MDA-175 cells (which express HER2 at a 1+ level)

and MCF7 xenografts implanted into mice. See, also, Adams et al. *Cancer Immunol. Immunother.* 55(6):717-727 (2006)

US Patent No. 6,339,142 (Blank and Basey) describes a HER2 antibody composition comprising a mixture of anti-HER2 antibody and one or more acidic variants thereof, wherein the amount of the acidic variant(s) is less than about 25%. Humanized monoclonal antibody 4D5 variant 8 (humMAb4D5-8 or Trastuzumab) is the exemplified HER2 antibody.

US Patent No. 7,560,111, US Patent No. 7,879,325, and US Patent No. 8,241,630 (Kao et al.) describe a variant of Pertuzumab (rhuMAb 2C4) comprising an amino terminal leader extension (VHS-) on one or both light chains of the antibody, the so-called "VHS-variant." When, Reference Material (Phase I), Lot S9802A (Phase II), and 400 L scale Process Development Material were tested for free thiol using the Ellman's analysis at native conditions, free thiol level was below the limit of detection in all materials tested. From 1-2% of the Pertuzumab in the compositions tested were afucosylated (G0-F) as determined by capillary electrophoresis (CE). See Table 5 of US Patent No. 7,560,111 (Kao et al.).

WO 2009/099829 (Harris et al.) describe acidic variants of pertuzumab including: deamidated variant, glycated variant, disulfide reduced variant, non-reducible variant, and sialylated variant. The variants were characterized as disclosed as follows:

Table 1: Acidic Variants in WO 2009/099829 (Harris et al.)

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

CEX = cation exchange. CE-SDS = Capillary Electrophoresis with Sodium Dodecyl Sulfate.

The experimental method used to characterize the disulfide reduced variant in WO 2009/099829 (Harris et al.), non-reduced CE-SDS of intact antibody, evaluated reduced inter-chain disulfide bonds, rather than intra-chain disulfide bonds.

Zhang et al. *Anal. Chem.* 84(16):7112-7123 (2012) report a recombinant antibody (mAb A) having unpaired cysteines (Cys22 and Cys96) in the variable heavy (VH domain) thereof. The

unpaired cysteines were found to have no significant impact on binding of the antibody to CD20, and mAb A with unpaired cysteines was fully active in a potency assay (complement-dependent cytotoxicity, CDC, assay).

WO 2009/009523 (Kao et al.) discloses prevention of inter-chain disulfide bond reduction during recombinant production of the ocreclizumab (rhuMAb 2H7) antibody which binds CD20.

Harris, R. *Dev. Biol.* (Basel, Switzerland) 122: 117-127 (2005) disclosed unpaired cysteines (Cys22 and Cys96) in the variable heavy (VH) doman of omalizumab, a humanized anti-IgE antibody. The unpaired cysteine form had significantly lower potency.

Summary of the Invention

The experimental data herein concerns variant forms of Pertuzumab, including an unpaired cysteine variant, afucosylated variant, low-molecular-weight-species (LMWS), and high-molecular-weight-species (HMWS). Means for identifying, characterizing, and quantifying these variants are valuable in the manufacture and quality control methods for the Pertuzumab drug composition.

Thus, in a first aspect, the invention concerns a composition comprising Pertuzumab and unpaired cysteine variant thereof, wherein the unpaired cysteine variant comprises Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab. The unpaired cysteine variant includes a heterodimer variant (comprising Cys23/Cys88 unpaired cysteines in only one variable light domain of Pertuzumab) and/or a homodimer variant (comprising Cys23/Cys88 unpaired cysteines in both variable light domains of Pertuzumab).

The composition optionally further comprises one or more additional variants of Pertuzumab such as afucosylated variant, low-molecular-weight-species (LMWS) variant, high-molecular-weight-species (HMWS) variant, glycated variant, disulfide reduced variant, non-reducible variant, deamidated variant, sialylated variant,VHS-variant, C-terminal lysine variant, methionine-oxidized variant, G1 glycosylation variant, G2 glycosylation variant, and non-glycosylated heavy chain variant.

The invention also concerns a composition comprising Pertuzumab and an afucosylated variant of Pertuzumab, wherein the amount of the afucosylated variant is from 0.9 to 4.1% of the composition. In one embodiment, the invention concerns a composition comprising Pertuzumab and an afucosylated variant of Pertuzumab, wherein the amount of the afucosylated variant is greater than 2% of the composition. According to this embodiment, the amount of the afucosylated variant is greater than that reported in US Patent No. 7,560,111, US Patent No. 7,879,325, and US Patent No. 8,241,630 (Kao et al.).

In an additional aspect, the invention concerns a composition comprising a mixture of Pertuzumab, low-molecular-weight species (LMWS) of Pertuzumab, and high-molecular-weight-species (HMWS) of Pertuzumab, wherein the amount of LMWS is $\leq 1.6\%$ and the amount of HMWS is $\leq 1.7\%$.

The invention also concerns a composition comprising a mixture of Pertuzumab, Peak 1, and Peak 2, wherein the amount of Peak 1 is $\leq 0.5\%$ and the amount of Peak 2 is $\leq 1.0\%$ as measured by reduced capilliary electrophoresis sodium dodecyl sulphate (R-CE-SDS) assay.

Additional aspects of the invention concern pharmaceutical compositions, articles of manufacture, and methods of treating a cancer patient using or comprising the compositions herein.

In an additional aspect, the invention concerns a method for evaluating a Pertuzumab composition comprising: (1) measuring the amount of unpaired cysteine variant in the composition, wherein the unpaired cysteine variant comprises Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab; and/or (2) measuring the amount of afucosylated Pertuzumab in the composition; and/or (3) measuring the amount of low-molecular-weight-species (LMWS) or high-molecular-weight-species (HMWS) of Pertuzumab in the composition.

In yet an additional aspect, the invention concerns a method for evaluating the biological activity of a Pertuzumab composition comprising measuring the amount of afucosylated Pertuzumab variant in the composition to determine the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of the composition, and confirming the amount of afucosylated Pertuzumab is in the range from about 0.9 to about 4.1%.

In another aspect, the invention concerns a method for making a composition comprising: (1) producing a composition comprising Pertuzumab and one or more variants thereof, and (2) subjecting the composition so-produced to an analytical assay to evaluate the amount of the variant(s) therein, wherein the variant(s) comprise: (i) unpaired cysteine variant comprising Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab; and/or (ii) afucosylated variant of Pertuzumab; and/or (iii) high-molecular-weight-species (HMWS) of Pertuzumab; and/or (iv) low-molecular-weight-species (LMWS) of Pertuzumab, and/or (v) Peak 1 fragment(s) of Pertuzumab, and/or (vi) Peak 2 fragment(s) of Pertuzumab.

In another aspect, the invention concerns an isolated variant of Pertuzumab, wherein the isolated variant comprises: (a) an unpaired cysteine variant of Pertuzumab, wherein the variant is a heterodimer variant comprising Cys23/Cys88 unpaired cysteines in only one variable light domain of Pertuzumab; and/or (b) an unpaired cysteine variant of Pertuzumab, wherein the variant is a homodimer variant comprising Cys23/Cys88 unpaired cysteines in both variable light domains of

Pertuzumab; and/or (c) afucosylated variant of Pertuzumab; and/or (d) high-molecular-weight-species (HMWS) of Pertuzumab; and/or (e) low-molecular-weight-species (LMWS) of Pertuzumab; and/or (f) Peak 1 fragment(s) of Pertuzumab, and/or (g) Peak 2 fragment(s) of Pertuzumab.

In an additional aspect, the invention concerns a method for evaluating fragmentation of a Pertuzumab composition comprising measuring the amount of Peak 1 and Peak 2 in the composition by reduced capilliary electrophoresis sodium dodecyl sulphate (R-CE-SDS) assay and confirming the amount of Peak 1 is $\leq 5\%$ and the amount of Peak 2 is $\leq 1.0\%$.

Brief Description of the Drawings

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

Figures 2A and 2B depict alignments of the amino acid sequences of the variable light (VL) (Fig. 2A) and variable heavy (VH) (Fig. 2B) domains of murine monoclonal antibody 2C4 (SEQ ID Nos. 5 and 6, respectively); VL and VH domains of variant 574/Pertuzumab (SEQ ID Nos. 7 and 8, respectively), and human VL and VH consensus frameworks (hum κ1, light kappa subgroup I; humIII, heavy subgroup III) (SEQ ID Nos. 9 and 10, respectively). Asterisks identify differences between variable domains of Pertuzumab and murine monoclonal antibody 2C4 or between variable domains of Pertuzumab and the human framework. Complementarity Determining Regions (CDRs) are in brackets.

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

Figures 4A and 4B show the amino acid sequences of Trastuzumab light chain (Fig. 4A; SEQ ID NO. 13) and heavy chain (Fig. 4B; SEQ ID NO. 14), respectively. Boundaries of the variable light and variable heavy domains are indicated by arrows.

Figures 5A and 5B depict a variant Pertuzumab light chain sequence (Fig. 5A; SEQ ID NO. 15) and a variant Pertuzumab heavy chain sequence (Fig. 5B; SEQ ID NO. 16), respectively.

Figure 6 depicts the structure of (main species) Pertuzumab including its 4 inter-chain and 12 intra-chain disulfide bonds, including the Cys23/Cys88 intra-chain disulfide bonds in each of the variable light (VL) domains. Domains depicted are: VL = variable light domain; VH = variable heavy domain; CL = light chain constant domain; CH1 = heavy chain constant domain 1; CH2 =

heavy chain constant domain 2; CH3 = heavy chain constant domain 3.

Figure 7 shows non-reduced (native) trypic peptide maps of Pertuzumab.

Figure 8 depicts tryptic peptide maps of reduced and non-reduced Pertuzumab (full scale).

Figure 9 depicts tryptic peptide maps of reduced and non-reduced Pertuzumab (0-120 minutes).

Figure 10 depicts tryptic peptide maps of reduced and non-reduced Pertuzumab (120-204 minutes).

Figure 11 depicts hydrophobic interaction chromatography (HIC) analysis of papain-digested Pertuzumab.

Figure 12 depicts HIC analysis of papain-digested Pertuzumab (expanded view).

Figure 13 depicts HIC analysis of intact Pertuzumab. Peaks comprising: enriched free thiol homodimer (free thiols on both light chains), free thiol heterodimer (free thiol on one light chain), and wild-type homodimer (main species antibody) are shown.

Figure 14 depicts activity of Pertuzumab (Batch anti2C4907-2) in the antibody-dependent cell-mediated cytotoxicity (ADCC) assay.

Figure 15 depicts impact of G0-F level on ADCC activity. Samples tested were phase III Pertuzumab (G0-F = 2.2%) and phase I Pertuzumab (G0-F = 0.8%).

Figure 16 depicts capillary electrophoresis analysis of N-linked oligosaccharides released from Pertuzumab.

Figure 17 depicts capillary electrophoresis analysis of N-linked oligosaccharides released from Pertuzumab (expanded view). Note: The G1 oligosaccharide has two isomeric forms (labeled G1 and G1') wherein the terminal galactose residue is attached to either the α1-6 branch or the α1-3 branch.

Figure 18 depicts Reversed Phase-High Performance Liquid Chromatography (HP-HPLC) for Pertuzumab Fab and Fc (limited Lys-C digestion) separation. Limited Lys-C digested

Pertuzumab and limited Lys-C digested Pertuzumab then treated with N-ethymaleimide (NEM) are shown.

Figure 19 depicts peptide mapping confirming Pertuzumab free thiol Fab contains free Cys23 and Cys88 at the light chain thereof. L2 peptide from the Fab containing free thiols was labeled by NEM and thus shifted in the peptide map analysis.

Figure 20 depicts schematically: main species or wild-type IgG1 (Fig. 20A), Cys23/Cys88 heterodimer variant (Fig. 20B), and Cys23/Cys88 homodimer variant (Fig. 20C).

Figure 21 depicts % G0-F versus ADCC activity for Pertuzumab batches using the assay in Example 4 herein.

Figure 22 depicts schematically binding of Pertuzumab at the heterodimeric binding site of HER2, thereby preventing heterodimerization with activated EGFR or HER3.

Figure 23 compares activities of Trastuzumab (which binds to Subdomain IV near the juxtamembrane domain of HER2 ECD) and Pertuzumab (which binds to Subdomain II of HER2 ECD).

Figures 24A and 24B depict oligosaccharide structures attached to an IgG antibody.

Figure 25 depicts Size Exclusion Chromatography (SEC) analysis of Pertuzumab (full scale).

Figure 26 depicts SEC analysis of Pertuzumab (expanded scale). Peaks include main peak (main species antibody), high molecular weight species (HMWS), and low molecular weight species (LMWS).

Figure 27 depicts Size Exclusion-High Performance Liquid Chromatography (SE-HPLC) analysis of Pertuzumab samples. Sample A is representative Pertuzumab Drug Product batch. Sample B is Pertuzumab batch subjected to light exposure at 1.2 mlux hours. Sample C is a Pertuzumab batch subjected to light exposure at 3.6 mlux hours. Sample D is a Pertuzumab batch subjected to acid treatment at pH 3.2. Sample E is purified basic variants from Ion Exchange-HPLC (IE-HPLC).

Figure 28 depicts concordance plot of analytical ultracentrifugation (AUC) sedimentation velocity and SE-HPLC analysis. The error bars represent two standard deviations from n=3 determination. All other data points denote a single determination. Circles denote samples that have

HMWS levels below the level of detection of the AUC.

Figure 29 depicts Capillary Electrophoresis Sodium Dodecyl Sulfate Analysis (CE-SDS) with Laser-Induced Fluorescence (LIF) detection of non-reduced Pertuzumab.

Figure 30 depicts CE-SDS-LIF of non-reduced (NR) Pertuzumab (expanded view).

Figures 31A and 31B depict SE-HPLC Chromatograms for Example 6: full scale (Fig 31A) and expanded scale (Fig. 31B).

Figures 32A and 32B depict non-reduced CE-SDS (NR-CE-SDS) electropherograms for Example 6: full scale (Fig. 32A) and expanded scale (Fig. 32B).

Figures 33A and 33B depict reduced CE-SDS (R-CE-SDS) electropherograms for Example 6: full scale (Fig. 33A), and expanded scale (Fig. 33B).

Figure 34 provides a comparison of NR-CE-SDS and R-CE-SDS electropherograms for an acid treated sample (expanded scale).

Figure 35 depicts correlation of Fab quantitation between NR-CE-SDS and SE-HPLC.

Detailed Description of the Preferred Embodiments

I. Definitions

"Paired cysteines" herein refers to two cysteine residues that form a disulfide bond in a protein, such as an antibody. Such disulfide bond can be an inter-chain disulfide bond (e.g. disulfide bond between heavy and light chains of an antibody, or between two heavy chains of an antibody), or intra-chain disulfide bond (e.g. within a light chain of an antibody or within a heavy chain of an antibody). Most IgG1 antibodies comprise four inter-chain disulfide bonds and twelve intra-chain disulfide bonds. See Fig. 6.

An "unpaired cysteine variant" is a variant of a protein (e.g. an antibody such as Pertuzumab) in which one or more paired cysteines are not in the disulfide bonded state. Such unpaired cysteines may not have been paired to form a disulfide bond (e.g. when the protein originally folded into its tertiary structure) or may have formed a disulfide bond but which has later broken (e.g. during manufacture or upon storage). The unpaired cysteines are are often referred to as free thiols or free sulfhydryls. In one embodiment, the unpaired cysteines are from an intra-chain disulfide bond. In one embodiment, the unpaired cysteines are in a light chain, e.g. a variable light domain of the antibody.

In one embodiment, the unpaired cysteine variant is a Cys23/Cys88 variant.

A "Cys23/Cys88" unpaired cysteine variant lacks an intra-molecular disulfide bond at cysteine residues 23 and 88 in one or both variable light domains of the antibody. See Fig. 20(b) and (c) herein.

A "homodimer variant" lacks Cys23/Cys88 disulfide bonds in both variable light domains of the antibody. See Fig. 20(c) herein.

A "heterodimer variant" lacks only one Cys23/Cys88 disulfide bond in one variable light domain of an antibody. See Fig. 20(b) herein.

An "afucosylated variant" is a glycosylation variant of an antibody in which one or both of the oligosaccharide structures attached to residue Asn299 of one or both heavy chains lacks fucose, e.g. lacks Fucα(1->6), in the core oligosaccharide structure.

A "low-molecular-weight-species" or "LMWS" of Pertuzumab comprises a fragment of Pertuzumab that has a molecular weight less than that of main species or intact Pertuzumab (e.g. where the intact Pertuzumab has a molecular weight of about 145,197 Da measuring its peptide chains only). The LMWS can be detected by size exclusion high performance liquid chromatography (SE-HPLC) and/or non-reduced Capillary Electrophoresis with Sodium Dodecyl Sulfate (CE-SDS) for example as in Example 5. In one embodiment, the LMWS comprises or consists of "Peak 6" as obtained by CE-SDS (see, e.g., Example 5).

A "high-molecular-weight-species" or "HMWS" comprises a preparation of Pertuzumab having a molecular weight that is greater than the main species or intact Pertuzumab (e.g. where the intact Pertuzumab has a molecular weight of about 145,197 Da measuring its peptide chains only). The HMWS can be detected by size exclusion high performance liquid chromatography (SE-HPLC) and/or non-reduced Capillary Electrophoresis with Sodium Dodecyl Sulfate (CE-SDS) assay for example as in Example 5.

"Peak 1" herein refers to Pertuzumab fragment(s) which are of a size smaller than Pertuzumab light chain (LC). Peak 1 fragment(s) can be separated from main species Pertuzumab by CE-SDS assay, preferably by reduced CE-SDS (R-CE-SDS) assay. See, for example, Figure 33B, Table 16, and Table 18 herein. Preferably, the amount of peak 1 in a Pertuzumab composition is $\leq$ 0.5%. Optionally, the R-CE-SDS assay is carried out as described in Example 6 and the corrected peak area (CPA) provides the % peak 1 in a composition.

"Peak 2" herein refers to Pertuzumab fragment(s) which are of a size larger than Pertuzumab light chain (LC) and smaller than Pertuzumab non-glycosylated heavy chain (NGHC). Peak 2 can be separated from main species Pertuzumab by CE-SDS, preferably by reduced (R-CE-SDS) assay. Peak 2 excludes peak 3 that can appear during R-CE-SDS assay as explained in Example 6 herein. See, for example, Figure 33B, Table 16, and Table 18 herein. Preferably, the amount of peak 2 in a Pertuzumab composition is $\leq$ 1.0%. Optionally, the R-CE-SDS assay is carried out as described in Example 6 and the corrected peak area (CPA) provides the % peak 2 in a composition.

"Fragmentation" refers to polypeptide chain cleavage, e.g. cleavage of Pertuzumab light chain and/or heavy chain. It does not include the dissociation of non-covalently associated polypeptide chains during NR-CE-SDS analysis, for example.

An "analytical assay" is an assay which qualitatively assesses and/or quantitatively measures the presence or amount of an analyte (e.g. an antibody variant) in a composition. The composition subjected to the assay can be a purified composition, including a pharmaceutical composition.

A "Fab hydrophobic interaction chromatograpy assay" or "Fab HIC assay" comprises generating fragments (e.g. Fab fragments) of the antibodies in a composition (e.g. using papain enzyme) and subjecting the antibody fragments thus generated to HIC in order to separate unpaired cysteine variants from main species Pertuzumab. An exemplarly such assay is disclosed in Example 1 herein.

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

The expression "HER2" refers 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).

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

A "HER dimer" herein is a noncovalently associated dimer comprising at least two HER receptors. Such complexes may form when a cell expressing two or more HER receptors is exposed to an HER ligand and can be isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Sliwkowski *et al.*, *J. Biol. Chem.*, 269(20):14661-14665 (1994), for example. Other proteins, such as a cytokine receptor subunit (*e.g.* gp130) may be associated with the dimer. Preferably, the HER dimer comprises HER2.

A "HER heterodimer" herein is a noncovalently associated heterodimer comprising at least two different HER receptors, such as EGFR-HER2, HER2-HER3 or HER2-HER4 heterodimers.

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

"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 specifically include Trastuzumab and humanized 2C4 antibodies such as Pertuzumab as described and defined herein.

An "intact antibody" herein is one which comprises two antigen binding regions, and an Fc region. Preferably, the intact antibody has a functional Fc region. In one embodiment, "intact Pertuzumab" has a molecular weight of about 145,197 Da measuring its peptide chains only.

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.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 449 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K449 residues removed, antibody populations with no K449 residues removed, and antibody populations having a mixture of antibodies with and without the K449 residue.

Unless indicated otherwise, herein the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat *et al., Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991), expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

A "functional Fc region" possesses an "effector function" of a native sequence Fc region. Exemplary "effector functions" include 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. Such effector functions generally require the Fc region to be combined with a binding domain (*e.g.* an antibody variable domain) and can be assessed using various assays.

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

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

The term "main species antibody" or "wild type antibody" herein refers to the antibody amino acid sequence structure in a composition which is the quantitatively predominant antibody molecule in the composition. Preferably, the main species antibody is a HER2 antibody, such as an antibody that binds to Subdomain II of HER2, antibody that inhibits HER dimerization more effectively than Trastuzumab, and/or binds to a heterodimeric binding site on HER2. In one

embodiment, the main species antibody is one comprising CDR-H1 (SEQ ID NO: 17 or 23), CDR-H2 (SEQ ID NO: 18), and CDR-H3 (SEQ ID NO: 19), CDR-L1 (SEQ ID NO: 20), CDR-L2 (SEQ ID NO: 21 or 24) and CDR-L3 (SEQ ID NO: 22), the VL and VH amino acid sequences in SEQ ID NOs. 7 and 8, respectively (see Figs. 2A-2B), and optionally, the light chain amino acid sequences in SEQ ID NOs. 11 or 15 and heavy chain amino acid sequences in SEQ ID NOs. 12 or 16 (see Figs. 3A-3B and 5A-5B). In one embodiment, the main species antibody is Pertuzumab.

An antibody which "inhibits HER dimerization" is an antibody which inhibits, or interferes with, formation of a HER dimer or heterodimer. In one embodiment, such an antibody binds to HER2 at the heterodimeric binding site thereof. The most preferred dimerization inhibiting antibody herein is Pertuzumab.

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 Subdomain II of HER2 (SEQ ID NO: 2). Franklin *et al. Cancer Cell* 5:317-328 (2004).

A HER2 antibody that "binds to a heterodimeric binding site" of HER2, binds to residues in Subdomain II (SEQ ID NO: 2) and optionally also binds to residues in other of the domains of the HER2 extracellular domain, such as Subdomains I and III (SEQ ID NOs: 1 and 3), 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 Subdomain II" of HER2 binds to residues in Subdomain II (SEQ ID NO: 2) and optionally residues in other Subdomain(s) of HER2, such as Subdomains I and III (SEQ ID NOs: 1 and 3, respectively).

For the purposes herein, "Pertuzumab" and "rhuMAb 2C4", which are used interchangeably, refer to an antibody comprising the variable light (VL) and variable heavy (VH) amino acid sequences in SEQ ID NOs: 7 and 8, respectively. Figs. 22 and 23 herein illustrate exemplary biological functions of Pertuzumab. Where Pertuzumab is an intact antibody, it preferably comprises an IgG1 antibody; in one embodiment comprising the light chain amino acid sequence in SEQ ID NO: 11 or 15, and heavy chain amino acid sequence in SEQ ID NO: 12 or 16. The antibody is optionally produced by recombinant Chinese Hamster Ovary (CHO) cells. The terms "Pertuzumab" and "rhuMAb 2C4" herein cover biosimilar or intended copies of the drug with the United States Adopted Name (USAN) or International Nonproprietary Name (INN): Pertuzumab.

For the purposes herein, "Trastuzumab" and rhuMAb4D5", which are used interchangeably, refer to an antibody comprising the variable light (VL) and variable heavy (VH) amino acid sequences from within SEQ ID Nos: 13 and 14, respectively (see Figs. 4A-4B). Where Trastuzumab is an intact antibody, it preferably comprises an IgG1 antibody; in one embodiment comprising the

light chain amino acid sequence of SEQ ID NO: 13 and the heavy chain amino acid sequence of SEQ ID NO: 14. The antibody is optionally produced by Chinese Hamster Ovary (CHO) cells. The terms "Trastuzumab" and "rhuMAb4D5" herein cover biosimilar or intended copies of the drug with the United States Adopted Name (USAN) or International Nonproprietary Name (INN): Trastuzumab.

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

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

A Adisulfide reduced variant@ has one or more inter-chain disulfide-bonded cysteine(s) chemically reduced to the free thiol form. This variant can be monitored by non-reduced Capillary Electrophoresis with Sodium Dodecyl Sulfate (CE-SDS), *e.g.* as described in WO 2009/099829 (Harris et al.).

Herein, a Anon-reducible variant@ or "incompletely reduced variant" is a variant of the main species antibody that cannot be chemically reduced to heavy chain and light chain by treatment with a reducing agent such as dithiothreitol. Such variants can be assessed by treating the composition with a reducing agent and evaluating the resulting composition using a methodology that evaluates protein size, such as Capillary Electrophoresis with Sodium Dodecyl Sulfate (CE-SDS), for instance using the techniques described in WO 2009/099829 (Harris et al.).

A Aglycosylation variant@ antibody herein is an antibody with one or more carbohydrate moeities attached thereto which differ from one or more carbohydate moieties attached to a main species antibody. In one embodiment, the glycosylation variant has oligosaccharide structures attached to one or both heavy chains of an antibody, e.g. at residue 299 of the heavy chain. In one embodiment, the main species antibody (e.g. Pertuzumab) comprises G0 oligosaccharide as the predominant oligosaccharide attached to its Fc region. Exemplary oligosaccharide structures attached

to IgG1 are depicted in Figs. 24A-24B. Examples of glycosylation variants herein include afucosylated variant, antibody with a G1 or G2 oligosaccharide structure, instead a G0 oligosaccharide structure, attached to an Fc region thereof ("G1 glycosylation variant" or "G2 glycosylation variant"), antibody with no carbohydrate attached to one or two heavy chains of the antibody ("non-glycosylated heavy chain variant"), sialylated variant, etc, as well as combinations of such glycosylation alterations. See, e.g. US Patent 7,560,111 (Kao et al.).

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

Unless indicated otherwise, a AG1 oligosaccharide structure@ herein includes G1(1-6) and G1(1-3) structures.

For the purposes herein, Asialylated variant@ is a variant of the main species antibody comprising one or more sialylated carbohydrate moieties attached to one or two heavy chains thereof. A sialylated variant can be identified by evaluating a composition (for example by ion exchange chromatography) with or without sialidase treatment, *e.g.* as described in WO 2009/099829.

A Aglycated variant@ is an antibody to which a sugar, such as glucose, has been covalently attached, e.g. to one or both light chains thereof. This addition can occur by reaction of glucose with a lysine residue on the protein (*e.g.* in cell culture media). A glycated variant can be identified by mass spectrometry analysis of the reduced antibody evaluating the increase in mass of heavy or light chains. A glycated variant can also be quantified by boronate chromatography as explained in WO 2009/099829 (Harris et al.).

A Adeamidated@antibody is one in which one or more asparagine residues thereof has been derivitized, *e.g.* to an aspartic acid, a succinimide, or an iso-aspartic acid. An example of a deamidated antibody is a pertuzumab variant, wherein Asn-386 and/or Asn-391 on one or two heavy chains of pertuzumab are deamidated. See WO 2009/099829 (Harris et al.), for example.

An Aamino-terminal leader extension variant@ herein refers to a main species antibody with one or more amino acid residues of the amino-terminal leader sequence at the amino-terminus of any one or more heavy or light chains of the main species antibody. An exemplary amino-terminal leader extension comprises or consists of three amino acid residues, VHS-, present on one or both light chains of an antibody variant, designated a "VHS-variant" herein. See, US Patent 7,560,111 (Kao et al.).

A "C-terminal lysine variant" refers to a variant comprising a lysine (K) residue at the C-terminus of the heavy chain thereof. See, US Patent 7,560,111 (Kao et al.).

A "methionine-oxidized variant" refers to a variant comprising one or more oxidized methionine residues therein, e.g. oxidized Met-254. See, US Patent 7,560,111 (Kao et al.).

The term "cancer" refers to the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer herein include breast cancer (e.g. metastatic breast cancer), gastric (or stomach) cancer, ovarian cancer, primary peritoneal cancer, and fallopian tube cancer. Examples of cancer herein include HER2-positive cancer and low HER3 cancer.

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

A "HER2-positive" cancer comprises cancer cells which have higher than normal levels of HER2. Examples of HER2-positive cancer include HER2-positive breast cancer and HER2-positive gastric cancer. Methods for identifying HER2-positive cancer include: assays that measure HER2 protein such as immunohistochemistry assay (IHC), assays that measure HER2-encoding nucleic acid such as *in situ* hybridization (ISH), including fluorescent *in situ* hybridization (FISH; see WO98/45479 published October, 1998) and chromogenic *in situ* hybridization (CISH; see, e.g. Tanner et al., *Am. J. Pathol.* 157(5): 1467–1472 (2000); Bella et al., *J. Clin. Oncol.* 26: (May 20 suppl; abstr 22147) (2008)), southern blotting, or polymerase chain reaction (PCR) techniques, such as quantitative real time PCR (qRT-PCR); shed antigen (e.g. HER2 ECD) assays (see, *e.g.*, U.S. Patent No. 4,933,294 issued June 12, 1990; and U.S. Patent 5,401,638 issued March 28, 1995); and *in vivo* assays. Optionally, HER2-positive cancer has an immunohistochemistry (IHC) score of 2+ or 3+ and/or an *in situ* hybridization (ISH) amplification ratio ≥2.0.

A "low HER3" cancer comprises cancer cells which have lower than normal levels of HER3. Examples of low HER3 cancers include ovarian, primary peritoneal, and fallopian tube carcinoma. See, for example, US Patent No. 7,981,418 (Amler et al.). In one embodiment, low HER3 is determined based on HER3 mRNA expression levels (concentration ratio equal or lower than 2.81, as assessed by qRT-PCR on a COBAS z480® instrument).

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 essentially to the 2C4 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Preferably the antibody blocks 2C4's binding to HER2 by about 50% or more. Alternatively, epitope mapping can be performed to assess whether the antibody binds essentially to the 2C4 epitope of HER2. Epitope 2C4 comprises residues from Subdomain II (SEQ ID NO: 2) in the extracellular domain of HER2. 2C4 and Pertuzumab binds to the extracellular domain of HER2 at the junction of Subdomains I, II and III (SEQ ID NOs: 1, 2, and 3, respectively). Franklin *et al. Cancer Cell* 5:317-328 (2004).

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

be predisposed or susceptible to cancer.

The 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 response, CR), increase overall survival time, and/or improve one or more symptoms of cancer (*e.g.* as assessed by FOSI).

A "fixed" or "flat" dose of a therapeutic agent herein refers to a dose that is administered to a human patient without regard for the weight (WT) or body surface area (BSA) of the patient. The fixed or flat dose is therefore not provided as a mg/kg dose or a mg/m^2 dose, but rather as an absolute amount of the therapeutic agent.

A "container" refers to an object that can be used to hold or contain a pharmaceutical composition or composition. Examples of containers herein include a vial, syringe, intravenous bag, etc.

An "intravenous bag" or "IV bag" is a bag that can hold a solution which can be administered via the vein of a patient. In one embodiment, the solution is a saline solution (e.g. about 0.9% or about 0.45% NaCl). Optionally, the IV bag is formed from polyolefin or polyvinal chloride.

A "vial" is a container suitable for holding a liquid or lyophilized preparation. In one embodiment, the vial is a single-use vial, e.g. a 20-cc single-use vial with a stopper.

A "package insert" is a leaflet that, by order of the Food and Drug Administration (FDA) or other regulatory authority, must be placed inside the package of every prescription drug. The leaflet generally includes the trademark for the drug, its generic name, and its mechanism of action; states its indications, contraindications, warnings, precautions, adverse effects, and dosage forms; and includes instructions for the recommended dose, time, and route of administration.

A "pharmaceutical composition" is a composition comprising a pharmaceutically active drug (e.g. Pertuzumab and variant forms such as those disclosed herein) and one or more "pharmaceutically active excipients" (e.g. buffer, stabilizer, tonicity modifier, preservative, surfactant, etc) that can be safely administered to a human patient. Such compositions may be liquid or lyophilized, for example.

A "recombinant" protein is one which has been produced by a genetically modified host cell, such as a Chinese Hamster Ovary (CHO) host cell.

"Manufacturing scale" refers to production of a protein drug (e.g. antibody) at a commercial scale, e.g. at 12,000 liter (L) or more, using a commercial process approved by the FDA or other

regulatory authority.

"Purifying" refers to one or more purification steps, such as Protein A chromatography, ion exchange chromatography, etc.

"Isolated" variant refers to the variant which has been separated from the main species or wild-type antibody by one or more purification or analytical procedures. Such isolated variant can be evaluated for its biological activity and/or potency.

II. Antibody Compositions

(i) Main Species Antibody

The antibody compositions herein comprise an antibody that binds HER2 (a HER2 antibody), optionally a humanized HER2 antibody. The humanized antibodies 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 VH CDR residues:

- GFTFTDYTMX (SEQ ID NO: 17), where X is preferably D or S, e.g. GFTFTDYTMD (SEQ ID NO: 23) for CDR-H1;
- DVNPNSGGSIYNQRFKG (SEQ ID NO: 18) for CDR-H2; and/or
- NLGPSFYFDY (SEQ ID NO: 19) for CDR-H3, optionally comprising amino acid modifications of those CDR residues, *e.g.* where the modifications essentially maintain or improve affinity of the antibody. For example, an antibody variant for use in the methods of the present invention 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 humanized antibody may comprise VL CDR residues:

- KASQDVSIGVA (SEQ ID NO: 20) for CDR-L1;
- 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: 21), e.g. SASYRYT (SEQ ID NO: 24) for CDR-L2; and/or
- QQYYIYPYT (SEQ ID NO: 22) for CDR-L3,

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.

The present application also contemplates affinity matured antibodies which bind HER2. The parent antibody may be a human antibody or a humanized antibody, *e.g.*, one comprising the variable light and/or variable heavy sequences of SEQ ID NOs. 7 and 8, respectively (*i.e.* comprising the VL and/or VH of Pertuzumab). An affinity matured variant of Pertuzumab preferably binds to HER2 receptor with an affinity superior to that of murine 2C4 or Pertuzumab (*e.g.* from about two or about four fold, to about 100 fold or about 1000 fold improved affinity, *e.g.* as assessed using a HER2 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. Alternatively, the humanized antibody or affinity matured antibody may be an intact antibody, such as an intact IgG1 antibody.

Preferably, the HER2 antibody (either or both of the main species HER2 antibody and antibody variant thereof) is one which binds to Subdomain II of HER2, inhibits HER dimerization more effectively than Trastuzumab, and/or binds to a heterodimeric binding site of HER2. The preferred embodiment herein of the main species antibody is one comprising the variable light and variable heavy amino acid sequences in SEQ ID Nos. 3 and 4, and most preferably comprising the light chain amino acid sequences in SEQ ID No. 11 or 15 and heavy chain amino acid sequence in SEQ ID No. 12 or 16.

(ii) Unpaired Cysteine Variants

Examples 1 and 3 herein describe unpaired cysteine variants of Pertuzumab. Analytical assays for isolating, characterizing, and quantifying such variants include assays which specifically evaluate intra-chain disulfide bonds (as distinct from inter-chain disulfide bonds), for example, Hydrophobic Interaction Chromatography (HIC) analysis of antibody fragments (e.g. of Fab fragment) as in Example 1, HIC of an intact antibody as in Example 1, peptide mapping analysis of differentially tagged antibodies as in Example 3, and/or Reversed Phase High Performance Liquid Chromatography (RP-HPLC) as in Example 3 herein and in Zhang et al. *Anal. Chem.* 84(16):7112-7123 (2012).

Generally, the predominant form of Pertuzumab comprises a disulfide bond between Cys23 and Cys88 in both of the VL domains of its two Fab domains. See Figure 6.

One unpaired cysteine variant herein, a heterodimer variant, lacks the Cys23/Cys88 disulide bond in the variable light (VL) domain of only one of it two Fab regions. See Fig. 20(b). This was determined to be the predominant unpaired cysteine variant.

A further unpaired cysteine variant herein, a homodimer variant, lacks the Cys23/Cys88 disulfide bonds in both of its Fab regions. See Fig. 20(c).

In one embodiment, the amount of the unpaired cysteine variant in the composition (including homodimer and heterodimer variant) is ≤ about 25%, for example, as determined by Fab hydrophobic interaction chromatography (HIC).

In one embodiment, the amount of the homodimer variant in the composition is ≤ 4.9% as determined by HIC of intact antibody.

In one embodiment, the amount of heterodimer variant in the composition is from about 13% to about 18%, for example, as determined by HIC of intact antibody.

The composition optionally further comprises one or more additional variants as described below.

The invention also concerns an isolated unpaired cysteine variant of Pertuzumab, wherein the unpaired cysteine variant comprises Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab. Such isolated unpaied cysteine variant may comprise or consist of a heterodimer variant and/or a homodimer variant. Such variants can be isolated using HIC or other purification methods, and may be subjected to a biological assay such as the potency assay (using HER2-positive breast cancer cells) as in Example 1 below.

(iii) Afucosylated Variant

Examples 2 and 4 herein describe afucosylated variants of Pertuzumab and demonstrate how to determine ADCC activity based on the percentage of afucosylated Pertuzumab in a composition.

In one embodiment, the invention concerns a composition comprising Pertuzumab and an afucosylated variant of Pertuzumab, wherein the amount of the afucosylated variant is greater than 2% of the composition. See, for example, anti2C4907-2, and Run 1 in Table 9 below.

In an alternative embodiment, the invention concerns a composition comprising Pertuzumab and an afucosylated variant of Pertuzumab, wherein the amount of the afucosylated variant is from 0.9 to 4.1% of the composition. This amount of afucosylated variant may, for example, be quantified using the validated CE-LIF assay in Example 4.

Optionally, the composition further comprises the unpaired cysteine variants (heterodimer and/or homodimer as described in the previous section) and/or additional variants to be described below.

(iv) LMWS and HMWS

The invention further concerns a low-molecular-weight species (LMWS) of Pertuzumab and/or high-molecular-weight-species (HMWS) of Pertuzumab in either isolated form or in compositions comprising the variant(s) and the main species antibody. The LMWS and HMWS can be isolated, characterized, and quantified using various techniques, including, without limitation, size exclusion high performance liquid chromatography (SE-HPLC), and/or Capillary Electrophoresis Sodium Dodecyl Sulfate (CE-SDS).

Using SE-HPLC assay (e.g. as in Example 5), the amount of main species Pertuzumab and HMWS or LMWS in a composition may be:

Main Peak: ≥ about 96%, e.g., ≥ about 96.7%, ≥ about 97.3%, e.g., ≥ about 97.4%.
HMWS: ≤ about 2%, e.g., ≤ about 1.7%, e.g., ≤ about 1.5%, e.g. ≤ about 1.4%, e.g. ≤ about 0.8%.
LMWS: ≤ about 2%, e.g., ≤ about 1.6%, e.g., ≤ about 1.2%, e.g. ≤ about 0.6%.

Using NR-CE-SDS assay (e.g. as in Example 5), the amount of main species Pertuzumab and HMWS or LMWS in a composition may be:

Main Peak: ≥ about 95%, e.g., ≥ about 96.0%, e.g., ≥ about 97.8%

HMWS: ≤ about 1%, e.g. ≤ about 0.6%.

LMWS: ≤ about 4%, e.g. ≤ about 3.4%.

For example, the amount of Main Peak or main species Pertuzumab (excluding LMWS and HMWS) as determined by CE-SDS may be about 95% to about 99%, e.g., from about 96.0% to about 97.8%, e.g. from about 95.3% to about 97.3% Main Peak.

Optionally, the LMWS comprises or consists of "Peak 6" as obtained by NR-CE-SDS (see, e.g. Example 5). Such Peak 6 may be determined to be about 0.9% to about 2.3%, e.g. about 2% to about 2.3% of the composition.

(v) Peak 1 and Peak 2 Fragments of Pertuzumab

The invention further concerns a Peak 1 fragment(s) and/or Peak 2 fragment(s) of Pertuzumab in either separated or isolated form or in compositions comprising the fragment(s) and the main species antibody. Peak 1 and Peak 2 can be isolated, characterized, and quantified using various techniques, including, without limitation, size exclusion high performance liquid chromatography (SE-HPLC), and/or Capillary Electrophoresis Sodium Dodecyl Sulfate (CE-SDS), including R-CE-SDS and NR-CE-SDS. In one embodiment, Peak 1 and Peak 2 are separated and/or analyzed by R-CE-SDS, e.g. as described in Examples 5 and 6 and the corrected peak area (CPA) provides the % Peak 1 or Peak 2 in the composition.

Using R-CE-SDS assay (e.g. as in Examples 5 and 6), the amount of Peak 1 in a composition is is $\leq$ 5% (e.g. from 0.13% to 0.41% CPA) and the amount of Peak 2 in a composition is $\leq$ 1.0% (e.g. from 0.47% to 0.74% CPA).

(vi) Additional Variants

The compositions herein optionally comprise additional variants of Pertuzumab such as those described in US Patent 7,560,111 (Kao et al.) and/or in WO 2009/099829 (Harris et al.).

Examples of such additional variants include, without limitation, any one or more of: glycated variant, disulfide reduced variant, non-reducible variant, deamidated variant, sialylated variant, VHS-variant, C-terminal lysine variant, methionine-oxidized variant, afucosylated variant, G1 glycosylation variant, G2 glycosylation variant, and non-glycosylated heavy chain variant.

For example, the composition may comprise acidic variants (see WO 2009/099829, Harris et al.), wherein the acidic variants in the composition may include one, two, three, four, or five of glycated variant, deamidated variant, disulfide reduced variant, sialylated variant, and non-reducible variant. Preferably, the total amount of all acidic variants in the composition is less than about 25%. In one embodiment, the glycated variant, deamidated variant, disulfide reduced variant, sialylated variant, and non-reducible variant constitute at least about 75-80% of the acidic variants in the composition.

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

The composition optionally includes an amino-terminal leader extension variant. Preferably, the amino-terminal leader extension is on a light chain of the antibody variant (*e.g.* on one or two light chains of the antibody variant). The antibody variant herein may comprise an amino-terminal leader extension on any one or more of the heavy or light chains thereof. Preferably, the amino-terminal leader extension is on one or two light chains of the antibody. The amino-terminal leader extension preferably comprises or consists of VHS- (i.e. VHS-variant). Presence of the amino-terminal leader extension in the composition can be detected by various analytical techniques including, but not limited to, N-terminal sequence analysis, assay for charge heterogeneity (for instance, cation exchange chromatography or capillary zone electrophoresis), mass spectrometry, etc. The amount of the antibody variant in the composition generally ranges from an amount that constitutes the lower detection limit of any assay (preferably cation exchange analysis) used to detect the variant to an amount less than the amount of the main species antibody. Generally, about 20% or less (*e.g.* from about 1% to about 15%, for instance from 5% to about 15%, and preferably from about 8% to about 12 %) of the antibody molecules in the composition comprise an amino-terminal leader extension. Such percentage amounts are preferably determined using cation exchange analysis.

Further amino acid sequence alterations of the main species antibody and/or variant are contemplated, including but not limited to an antibody comprising a C-terminal lysine residue on one or both heavy chains thereof (such an antibody variant may be present in an amount from about 1% to about 20%), antibody with one or more oxidized methionine residues (for example, Pertuzumab comprising oxidized Met-254) etc.

Moreover, aside from the afucosylated variant and sialylated variant discussed above, the main species antibody or variant may comprise additional glycosylation variations, non-limiting examples of which include antibody comprising a G1 or G2 oligosaccharide structure attached to the Fc region thereof, antibody comprising one or two non-glycosylated heavy chains, etc.

III. Manufacturing and Analytical Methods

According to one embodiment of the invention, a method for evaluating a Pertuzumab composition is provided which comprises one, two, three, or four of: (1) measuring the amount of unpaired cysteine variant in the composition, wherein the unpaired cysteine variant comprises Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab, and/or (2) measuring the amount of afucosylated Pertuzumab in the composition, and/or (3) measuring the amount of low-molecular-weight-species (LMWS) of Pertuzumab in the composition, and/or (4) measuring the amount of high-molecular-weight-species (HMWS) of Pertuzumab in the composition. Optionally, all four analytical assays are performed on a composition comprising Pertuzumab and variants thereof.

The invention also concerns a method for making a composition comprising: (1) producing a composition comprising Pertuzumab and one or more variants thereof, and (2) subjecting the composition so-produced to one or more analytical assay(s) to evaluate the amount of the variant(s) therein. The analytical assay(s) can evaluate and quantify the amount of any one or more of: (i) unpaired cysteine variant comprising Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab and/or (ii) a heterodimer variant comprising Cys23/Cys88 unpaired cysteines in only one variable light domain of Pertuzumab and/or (iii) a homodimer variant comprising Cys23/Cys88 unpaired cysteines in both variable light domains of Pertuzumab and/or (iv) afucosylated variant of Pertuzumab and/or (v) high-molecular-weight-species (HMWS) of Pertuzumab and/or (vi) low-molecular-weight-species (LMWS) of Pertuzumab, and/or (vii) Peak 1 fragment(s) of Pertuzumab and/or (viii) Peak 2 fragment(s) of Pertuzumab. Thus, one, two, three, four, five, six, seven or eight of these variants can be analyzed.

Optionally, the analytical assay evaluates, quantifies, or isolates unpaired cysteine variant, including heterodimer and/or homodimer variants. For example, the analytical assay may comprises Hydrophobic Interaction Chromatography (HIC) of an antibody fragment (e.g. Fab fragment) or of an intact antibody (see, e.g. Example 1), peptide mapping analysis (see, e.g. Example 3), or Reversed Phase High Performance Liquid Chromatography (HPLC) (see, e.g., Example 3).

In one embodiment, the amount of the unpaired cysteine variant (heterodimer and/or homodimer variant) in the composition is $\leq$ about 25% as determined by Fab hydrophobic interaction chromatography (HIC).

In one embodiment, the amount of the homodimer variant in the composition is $\leq 4.9\%$ as determined by hydrophobic interaction chromatography (HIC) of intact antibody.

In one embodiment, the amount of the heterodimer variant in the composition is from about 13% to about 18% as determined by hydrophobic interaction chromatography (HIC) of intact antibody.

Optionally, the analytical assay evaluates, quantifies, or isolates afucosyled variant. The amount of afucosylation can be used to determine or quantify biological activity, e.g. ADCC, of the composition.

In addition, the method comprises evaluating the biological activity of a Pertuzumab composition comprising measuring the amount of afucosylated Pertuzumab variant in the composition to determine the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of the composition, and confirming the amount of afucosylated Pertuzumab is in the range from about 0.9%

to about 4.1%. For instance, the method comprises measuring the amount of afucosylated Pertuzumab using capillary electrophoresis-laser-induced fluorescence (CE-LIF).

Optionally, the analytical assay for evaluating afucosylation is capillary electrophoresis (CE), including capillary electrophoresis-laser-induced fluorescence (CE-LIF), see, Examples 2 and 4 below. The amount of afucosylated variant is optionally from about 0.9 to about 4.1% of the composition (e.g. as measured by CE-LIF in Example 4). In one embodiment, the amount of afucosylated variant is greater than 2% of the composition (e.g. as measured by CE-LIF in Example 4).

Optionally, the analytical assay evaluates, quantifies, or isolates low-molecular-weight species (LMWS) and/or high-molecular-weight-species (HMWS) of Pertuzumab. Exemplary assays include SE-HPLC and/or CE-SDS (see, for example, Example 5 below).

In one embodiment, the analytical assay comprises SE-HPLC (e.g. as in Example 5), and the amount of main species Pertuzumab, HMWS or LMWS in a composition thus analyzed is determined to be:

Main Peak: ≥ about 96%, e.g., ≥ about 96.7%, ≥ about 97.3%, e.g., ≥ about 97.4%.
HMWS: ≤ about 2%, e.g., ≤ about 1.7%, e.g., ≤ about 1.5%, e.g. ≤ about 1.4%, e.g. ≤ about 0.8%.
LMWS: ≤ about 2%, e.g., ≤ about 1.6%, e.g., ≤ about 1.2%, e.g. ≤ about 0.6%.

In one embodiment, the analytical assay comprises CE-SDS (e.g. as in Example 5), and the amount of main species Pertuzumab and HMWS or LMWS Pertuzumab in a composition thus analyzed is determined to be:

Main Peak: ≥ about 95%, e.g., ≥ about 96.0%, e.g., ≥ about 97.8%

HMWS: ≤ about 1%, e.g. ≤ about 0.6%.

LMWS: ≤ about 4%, e.g. ≤ about 3.4%.

In one embodiment, a composition is evaluated by NR-CE-SDS, and the amount of Main Peak or main species Pertuzumab (excluding LMWS and HMWS) is found to be from about 95% to about 99%, e.g., from about 96.0% to about 97.8%, e.g. from about 95.3% to about 97.3% of the composition thus analyzed.

In one embodiment, the amount of "Peak 6" in a composition is evaluated by CE-SDS (see, e.g. Example 5), and the amount of Peak 6 LMWS is determined to be about 0.9% to about 2.3%, e.g. about 2% to about 2.3% of a composition thus analyzed.

In one embodiment, the amount of Peak 1 and/or Peak 2 in a composition is evaluated by R-CE-SDS (see, e.g. Examples 5 and 6), and the amount of Peak 1 is determined to be ≤ 5% (e.g. from 0.13% to 0.41% CPA) and the amount of Peak 2 is determined to be ≤ 1.0% (e.g. from 0.47% to 0.74% CPA).

The methods optionally further comprise combining the purified composition with one or more pharmaceutically acceptable excipients to make a pharmaceutical composition. In addition, the pharmaceutical composition can be put into a container which is packaged together with a package insert (e.g. with prescribing information instructing the user thereof to use the pharmaceutical composition to treat cancer) so as to make an article of manufacture.

IV. Pharmaceutical Compositions

Pharmaceutical compositions comprising Pertuzumab and variants thereof are prepared for storage by mixing the composition having the desired degree of purity with optional pharmaceutically acceptable excipients (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), generally in the form of lyophilized formulations or aqueous solutions. Antibody crystals are also contemplated (see US Pat Appln 2002/0136719). Pharmaceutically acceptable excipients are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as histidine acetate; antioxidants including ascorbic acid and methionine; 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 polysorbates (e.g. polysorbate 20 or 80), PLURONICS™ or polyethylene glycol (PEG).

Lyophilized antibody formulations are described in U.S. Pat. No. 6,267,958, US Patent No. 6,685,940 and US Patent No. 6,821,515, expressly incorporated herein by reference. An exemplary Trastuzumab pharmaceutical composition is a sterile, white to pale yellow preservative-free lyophilized powder for intravenous (IV) administration, comprising 440 mg Trastuzumab, 400 mg α,α-trehalose dehydrate, 9.9 mg L-histidine-HCl, 6.4 mg L-histidine, and 1.8 mg polysorbate 20. Reconsitution of 20 mL of bacteriostatic water for injection (BWFI), containing 1.1% benzyl alcohol as a preservative, yields a multi-dose solution containing 21 mg/mL Trastuzumab, at pH of approximately 6.0.

An exemplary Pertuzumab pharmaceutical composition for therapeutic use comprises 30mg/mL Pertuzumab in 20mM histidine acetate, 120mM sucrose, 0.02% polysorbate 20, at pH 6.0. An alternate Pertuzumab formulation comprises 25 mg/mL Pertuzumab, 10 mM histidine-HCl buffer,

240 mM sucrose, 0.02% polysorbate 20, pH 6.0.

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

V. Therapeutic Applications and Uses

The compositions herein can be used to treat cancer, such as HER2-positive breast cancer, e.g, metastatic or locally recurrent, unresectable breast cancer, or *de novo* Stage IV disease, is defined as immunohistochemistry (IHC) 3+ and/or fluorescence *in situ* hybridization (FISH) amplification ratio ≥2.0. Optionally, the patients in the population have not received previous treatment or have relapsed after adjuvant therapy, have a left ventricular ejection fraction (LVEF) of ≥50% at baseline, and/or have an Eastern Cooperative Oncology Group performance status (ECOG PS) of 0 or 1.

In an alternative embodiment, the composition can be used to treat early-stage HER2-positive breast cancer, e.g., in combination with Trastuzumab, and chemotherapy, wherein the chemotherapy comprises anthracycline-based chemotherapy, or carboplatin-based chemotherapy. In one embodiment, the chemotherapy comprises anthracycline-based chemotherapy, e.g. comprising 5-FU, epirubicin, and cyclophosphamide (FEC). In an alternative embodiment, the chemotherapy comprises carboplatin-based chemotherapy, e.g. comprising taxane (e.g. Docetaxel), Carboplatin in addition to HERCEPTIN®/Trastuzumab (e.g. TCH regimen). In one embodiment, the composition is administered concurrently with the anthracycline-based chemotherapy or with the carboplatin-based chemotherapy, e.g. wherein the Pertuzumab, Trastuzumab and chemotherapy are administered in 3-week cycles with Pertuzumab, Trastuzumab and the chemotherapy being administered on day-1 of each cycle. The early-stage HER2-positive breast cancer therapy contemplated herein includes neoadjuvant and adjuvant therapy.

In yet another embodiment, the composition can be used to treat HER2-positive gastric cancer, optionally in combination with Trastuzumab and a chemotherapy, such as a platin (e.g. cisplatin) and/or a fluoropurimidine (e.g. capecitabine and/or 5-fluorouracil (5-FU)).

In an alternative embodiment, the composition may be used to treat HER2-positive breast cancer optionally in combination with Trastuzumab and vinorelbine. The breast cancer according to this embodiment is optionally metastatic or locally advanced. Optionally, the patient has not previously received systemic non-hormonal anticancer therapy in the metastatic setting.

In another aspect, the compostion is used to treat HER2-positive breast cancer in a patient comprising administering the composition, Trastuzumab, and aromatase inhibitor (e.g. anastrazole or letrozole) to the patient. According to this embodiment, the breast cancer is advanced breast cancer, including hormone receptor-positive breast cancer such as estrogen receptor (ER)-positive and/or progesterone receptor (PgR)-positive breast cancer. Optionally, the patient has not previously received systemic nonhormonal anticancer therapy in the metastatic setting. This treatment method

optionally further comprises administering induction chemotherapy (e.g. comprising taxane) to the patient.

In an additional aspect, the composition is used to treat low HER3 cancer, such as ovarian cancer, primary peritoneal, or fallopian tube cancer. See, for example, US Patent 7,981,418 (Amler et al.) and U.S. Patent Publication US-2006-0013819-A1 (Kelsey, S.).

The antibodies and chemotherapeutic treatments are administered to a human patient in accord with known methods. Specific administration schedules and formulations are described in the examples herein.

According to one particular embodiment of the invention, approximately 840mg (loading dose) of Pertuzumab is administered, followed by one or more doses of approximately 420mg (maintenance dose(s)) of Pertuzumab. The maintenance doses are preferably administered about every 3 weeks, for a total of at least two doses, until clinical progressive disease, or unmanageable toxicity, e.g from 6 to 20 doses. Longer treatment periods, including more treatment cycles, are also contemplated.

According to another particular embodiment where the cancer is gastric cancer, Pertuzumab is administered at a dose of 840 mg for all treatment cycles.

VI. Articles of Manufacture

One embodiment of an article of manufacture herein comprises a container, such as a vial, syringe, or intravenous (IV) bag containing the composition or pharmaceutical composition herein. Optionally, the article of manufacture further comprises a package insert with prescribing information describing how to use the composition according to the previous section herein.

VII. Deposit of Biological 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**
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

Cys23/Cys88 Unpaired Cysteine Variant of Pertuzumab and Characterization Thereof

Pertuzumab is a humanized monoclonal antibody (MAb) based on a human IgG1(κ) framework. The recombinant antibody is produced by Chinese Hamster Ovary (CHO) cells and comprises two heavy chains (449 amino acid residues each) and two light chains (214 amino acid residues each) with inter-chain and intra-chain disulfide bonds. The light-chain and heavy-chain sequences of pertuzumab are shown in Figs. 3A and 3B, respectively. The calculated molecular mass of intact pertuzumab is 145,197 Da (peptide chains only, without heavy-chain C-terminal lysine residue).

The CH2 domain of each heavy chain also has a single conserved glycosylation site at Asn299.

Pertuzumab differs from Trastuzumab (HERCEPTIN®) in the complementarity determining regions (CDRs) of the light chain (12 amino acid differences) and the heavy chain (29 amino acid differences), and the fact that it binds to a different epitope on the human epidermal growth factor receptor 2 ($p185^{HER2}$). Binding of pertuzumab to the HER2 receptor on human epithelial cells prevents HER2 from forming complexes with other members of the HER receptor family (including EGFR, HER3, HER4) and forming HER2 homodimers. By blocking complex formation, pertuzumab inhibits ligand-initiated intracellular signaling through two major signal pathways, mitogen-activated protein (MAP) kinase and phosphoinositide 3-kinase (PI3K), resulting in inhibition of cell proliferation and survival, respectively.

This example concerns the identification and characterization of an unpaired cysteine variant of Pertuzumab: the Cys23/Cys88 unpaired cysteine variant comprising unpaired cysteines in one or both light chains of the antibody.

Free sulfhydryls were measured using Ellman's reagent, and showed a reactive free sulfhydryl content of 0.1–0.3 moles per mole protein. Hydrophobic interaction chromatography (HIC) analysis and peptide map analysis revealed unpaired cysteine residues at Cys23 and Cys88 on one or both light chains. Using papain HIC, levels of the Fab variant containing free sulfhydryls at these sites were found to be 12.7%–13.5% in Pertuzumab materials produced using the commercial manufacturing process. HIC analysis of the intact antibody indicated that the two major forms are 78%–85% wild-type Pertuzumab and 13.4%–18.4% Pertuzumab heterodimer (unpaired cysteine pair on one arm).

MATERIALS AND METHODS

Compositions Tested: This example describes the characterization of the current Pertuzumab Reference Standard Batch anti2C4907-2 and Run 1, representing Phase III clinical material, and five Phase III/commercial batches (Runs 3-7), all produced at 12,000 liter (L) scale using the commercial process. Comparison is also made to the previous Reference Standard Batch anti2C4-900-1, which is representative of the Phase I/II clinical material.

The compositions tested were drug substance batches formulated in the commercial formulation at 30 mg/mL in 20 mM L-histidine acetate, 120 mM sucrose, and 0.02% (w/v) polysorbate 20 at pH 6.0. Batch anti2C4-900-1 was formulated earlier in clinical development at 25 mg/mL in 10 mM L-histidine chloride, 240 mM sucrose, and 0.02% (w/v) polysorbate 20 at pH 6.0.

Disulfide Bond Analysis by Non-Reduced Peptide Map Analysis and Mass Spectrometry: To denature pertuzumab under non-reducing conditions and alkylate any buried free sulfhydryl groups, approximately 0.5 mg of pertuzumab in formulation buffer was mixed with denaturing buffer (consisting of 8 M GdHCl, 10 mM N-ethylmaleimide (NEM), 0.1 M sodium acetate, pH 5.0) and then incubated at 37°C for 3 hours. The solution was buffer exchanged into 600 µL of 0.1 M Tris, 1 mM $CaCl_2$, pH 7.0 using NAP-5 columns. Acetonitrile (ACN) was added to each sample to achieve a concentration of 10%. The trypsin digestion was carried out at an enzyme to substrate ratio of 1:10 (w/w) at 37°C for 16 hours. The resulting peptides were separated by RP-HPLC using the methods described below for sulfitolysis tryptic maps.

Sulfitolysis Tryptic Peptide Map: To generate the pertuzumab peptide maps, the protein was digested with trypsin after reduction and sulfitolysis of the cysteine residues. Aliquots (1 mg) of pertuzumab were added to 360 mM Tris-HCl pH 8.6, 6 M guanidine hydrochloride (GdHCl), 2 mM ethylenediaminetetraacetic acid (EDTA), 13 mM sodium sulfite, and 38 mM sodium tetrathionate for reduction and sulfitolysis of the cysteine residues. Samples were incubated at 37°C for 20 minutes. Sulfitolyzed samples were loaded onto PD-10 columns and eluted with 10 mM Tris, 0.1 mM $CaCl_2$, pH 8.3. Following buffer exchange, 20 µL of a 10% octyl-B-glucoside solution and 20 µL of 1 mg/mL trypsin were added. Samples were incubated at 37°C for 5 hours. The digestion reaction was quenched with 25 µL of 10% trifluoroacetic acid (TFA). The resulting peptides were separated by RP-HPLC using a Zorbax 300SB-C8 column (4.6 mm × 150 mm). The peptides were separated after a 5 minute hold at initial conditions with a linear gradient from 0% to 17% solvent B in 57 minutes, to 32% solvent B at 149 minutes, to 45% solvent B at 162 minutes, and to 95% solvent B at 173 minutes. At 179 minutes, the column was reconditioned at 100% solvent A for

25 minutes, for a total run time of 204 minutes. Solvent A consisted of 0.1% TFA in water and solvent B consisted of 0.08% TFA in acetonitrile. The column was maintained at 37°C and eluted at a flow rate of 0.5 mL/min. The elution profile was monitored at 214 nm and 280 nm. Masses of the tryptic peptides were determined by liquid chromatography-mass spectrometric (LC-MS) analysis of the separated digest mixture using an LTQ ORBITRAP™ mass spectrometer.

Free Sylfydryl Content by Ellman's Analysis: The Pertuzumab samples were buffer exchanged into reaction buffer (100 mM potassium phosphate, 1 mM EDTA, 8 M urea, pH 8) and adjusted to a concentration that resulted in free thiol concentrations within the standard curve range. A solution of dithionitrobenzene (DTNB) (10 mM) and a cysteine standard curve (eight points between 0 and 100 μM) were prepared in reaction buffer. On a 96-well plate, 165 μL of sample or standard were added to triplicate wells. The reaction was initiated by the addition of 10 μL of DTNB and then incubated for 30 minutes. After incubation, absorbance was measured at 412 nm using a SPECTRAMAX M^2® plate reader. The concentration of free thiol was calculated using the linear equation obtained from the standard curve. The concentration of the protein was determined using the absorbance at 280 nm obtained from a spectrophotometer. The free thiol is reported as moles of free thiol per mole of Pertuzumab.

Papain HIC: For papain-digested Pertuzumab samples, the samples were digested with papain after removing the C-terminal lysine with carboxypeptidase B (CpB). The Fab and Fc domains were separated by HIC using a PolyPropyl Aspartamide column (4.6 mm × 100 mm, 1500 Å, 3 μm). Solvent A consisted of 1.6 M ammonium sulfate, 20 mM potassium phosphate, pH 6.05 and solvent B consisted of 20 mM potassium phosphate pH 6.05. The analytes were separated with a gradient from 0% to 18% solvent B from 3 to 6 minutes, to 24% solvent B at 21 minutes. The column was maintained at 25°C with a flow rate of 0.8 mL/min. The elution profile was monitored at 280 nm.

HIC of Intact Antibody: Intact Pertuzumab samples were separated by HIC using a PolyPropyl Aspartamide column (9.4 mm × 100 mm, 1500 Å, 3 μm). Solvent A consisted of 1.0 M ammonium sulfate, 20 mM potassium phosphate, pH 6.05 and solvent B consisted of 20 mM potassium phosphate, pH 6.05. The analytes were separated isocratically with 12% solvent B for 25 minutes. The column was maintained at 30°C with a flow rate of 2 mL/min. The elution profile was monitored at 280 nm.

SDS-PAGE with Peptide Mass Fingerprinting: Both reduced and non-reduced Pertuzumab samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples (5 μg) were denatured by heating in the presence of SDS-PAGE sample buffer for 5–

10 minutes at 60 ± 2°C with iodoacetamide for non-reduced samples. Samples were reduced for 15–20 minutes at 60°C ± 2°C in the presence of 80 mM dithiothreitol (DTT). The denatured samples were separated in 4%–20% polyacrylamide gradient gels and stained with SYPRO™ Ruby dye to obtain the protein banding pattern. Along with the pertuzumab samples, molecular weight standards and SYPRO™ Ruby–stain sensitivity standards (2 ng/lane and 8 ng/lane bovine serum albumin (BSA)) were included on the gels.

Peptide mass fingerprinting is an analytical technique for protein identification. The gels were loaded with 10 μg of commercial Reference Standard Batch anti2C4907-2 and Run 5. All bands separated by SDS-PAGE were cleaved into peptides by trypsin. The absolute masses of the peptides are accurately measured with BRUKER™ matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI–TOF MS). The peptide mass lists were used to identify proteins by searching protein sequences. All observed bands in both the non-reduced and reduced Pertuzumab were identified by peptide mass fingerprinting.

Potency by Bioassay: The Pertuzumab potency method assesses the potency of Pertuzumab by measuring its ability to inhibit proliferation of a human HER2-expressing breast cancer cell line. In a typical assay, 96-well microtiter plate(s) were seeded with the breast cancer cells and incubated in a humidified incubator. After incubation, the media was removed and varying concentrations of Pertuzumab Reference Standard, assay control, and sample(s) were added to the plate(s). The plate(s) were then incubated, and the relative number of viable cells was quantitated indirectly using a redox dye, ALAMARBLUE®. The fluorescence was measured using excitation at 530 nm and emission at 590 nm. ALAMARBLUE® is blue and nonfluorescent in its oxidized state, but is reduced by the cell's intracellular environment to a pink form that is highly fluorescent (Page et al. *Int. J. Oncol.* 3: 473-476 (1993)). The changes in color and fluorescence are proportional to the number of viable cells. The results, expressed in relative fluorescence units (RFU), were plotted against the Pertuzumab concentrations and a parallel line program was used to estimate the anti-proliferative activity of Pertuzumab samples relative to the Reference Standard.

RESULTS

Assignment of Disulfide Bonds: There are 32 cysteines in Pertuzumab, forming 16 disulfide bonds, of which four are inter-chain and 12 are intra-chain linkages. However, because of the multimeric nature of the molecule, there are only nine distinct disulfide bonds. The native protein was digested with trypsin to achieve release of all disulfide-linked peptides. The chromatographic profiles for the Pertuzumab batches are shown in Fig. 7. Reversed-phase LC-MS analysis of the digest of commercial Reference Standard Batch anti2C4907-2 yielded all of the expected disulfide-linked peptide-pairs (Table 2).

Table 2: Disulfide-Linked Peptide-Pairs Identified by LC-MS

Expected[a]	Disulfide Linkage	Found	Expected Mass (Da)[b]	Observed Mass (Da)[b]
T2H=T10H	Cys22=Cys96	T2H=T10H	3429.48	3429.48
T13H=T14H	Cys146=Cys202	T13H=T14H	7917.92	7917.92
T19H=T19H	Cys228=Cys228 Cys231=Cys231[c]	T19H=T19H	5455.78	5455.79
T21H=T27H	Cys263=Cys323	T21H=T27H	2329.10	2329.10
T35H=T40H	Cys369=Cys427	T35H=T40H	3845.82	3845.82
T18H=T20L	Cys222=Cys214	T18H=T20L	757.24	757.24
T18H=T20L	Cys222=Cys214	T18H=T19L–T20L[d]	1261.49	1261.49
T2L=T7L	Cys23=Cys88	T2L=T7L	5393.48	5393.48
T11L=T18L	Cys134=Cys194	T11L=T18L	3556.75	3556.75

Note: An equal sign (=) represents a disulfide bond.
H = heavy chain; L = light chain; LC-MS = high-performance liquid chromatography mass spectrometry; T = tryptic peptide.

[a] Refer to Figs. 9 and 10.

[b] Monoisotopic masses (MH^+).

[c] Disulfides are inferred. The T19H dimer assignment did not include verification of Cys228=Cys228 and Cys231=Cys231 disulfides.

[d] The presence of this disulfide-linked pair has been confirmed through the use of an alternate enzyme, Lys-C, that does not cleave T19L and T20L.

Identified peptides were further confirmed by the identification of expected peptides from the peptide-pairs upon reduction of the disulfides (Fig. 8 with expanded views in Figs. 9 and 10). A dimer of heavy-chain peptide T19H (T19H=T19H) was identified as containing two disulfide bonds; identification of the Cys228=Cys228 and Cys231=Cys231 pairs is inferred. One disulfide pair, T18H=T20L, was detected by LC-MS but eluted close to the void volume and was not identifiable as a distinct peak on ultraviolet (UV) chromatograms. The presence of this disulfide pair was further confirmed by LC-MS analysis of a Lys-C digest, wherein the peptide T18H=T19L–T20L was observed. No unexpected linkages were found. One disulfide bond is partially unpaired, as discussed below.

Free Sulfhydryl Analysis: All cysteine residues in properly folded pertuzumab should be involved in disulfide bonds. Ellman's assay (Ellman, G. *Arch. Biochem. Biophys.* 82: 70-77 (1959)), a method for measuring the free sulfhydryl content of peptides and proteins, was used to determine if reactive unmodified (free) thiols are present in pertuzumab. All materials were evaluated for free thiol (unpaired cysteine residue) content and results are summarized in Table 3.

Table 3: Free Thiol Content by Ellman's Assay

Batch Name	Moles of Free Thiols per Mole of Pertuzumab
anti2C4-900-1	0.06
anti2C4907-2	0.15
Run 1	0.28
Run 3	0.16
Run 4	0.17
Run 5	0.16
Run 6	0.16
Run 7	0.14

Note: Free thiol levels were determined by Ellman's assay in the presence of 8 M urea.

Approximately 0.1–0.3 moles of free thiols per mole of pertuzumab were observed in all batches analyzed. In the absence of 8 M urea, free thiol levels were below the quantitation limit (QL; approximately 0.1 mole free thiol per mole protein) in all materials tested, indicating that the free thiols (i.e., unpaired cysteines) present in pertuzumab molecules were buried and inaccessible to Ellman's reagent under non-denaturing condition.

Analysis of pertuzumab materials by HIC after CpB and papain digestion revealed an additional peak between the Fc and Fab peaks which was identified as a Fab variant containing unpaired cysteine residues at Cys23 and Cys88 (Figs. 11 and 12, labeled as free-thiol Fab). This identification was confirmed by LC-MS tryptic peptide mapping, wherein the sample was subjected to denaturation in the presence of NEM prior to reduction and tryptic digestion. The extent of the free thiol Fab variant using the papain HIC method was measured across pertuzumab batches and found to be consistent using the current process (Table 4).

Table 4: Relative Amount of Cys23/Cys88 Unpaired Cysteine Fab Variant as Determined by Papain HIC or Calculated Intact Antibody Variant

Batch Name	Percent Unpaired Cysteine Fab Variant	Percent Intact Antibody Variant*
anti2C4-900-1	9.4	17.9
anti2C4907-2	12.7	23.8
Run 1	13.2	24.6
Run 3	13.3	24.9
Run 4	13.5	25.2
Run 5	13.3	24.9
Run 6	12.9	24.2
Run 7	13.2	24.6

Note: The percent unpaired cysteine Fab peak was obtained by dividing the unpaired cysteine Fab peak area by the peak areas of unpaired cysteine Fab + Fab.

* Calculated as described below.

By the papain HIC assay, the values for material produced using the commercial process ranged from 12.7% to 13.5%, while the value for Reference Standard Batch 2C4-900-1 (Phase I/II) was slightly lower at 9.4%.

Converting % Fab Variant from Papain HIC to Estimated % Intact Antibody Variant: The relative amount of Fab fragments containing unpaired cysteines can be used to calculate the relative distribution of heterodimer or homodimer forms of unpaired cycteine varints. If papain HIC assay shows that 10% (or x%) of Fab fragments from pertuzumab contain unpair cycteines at Cys23/Cys88, then there should be 10 Fab fragments containing unpaired cysteines released from every 50 pertuzumab molecules because the digestion of 50 antibodies by papain should yield 100 Fab fragments. Assuming that these 10 Fab fragments are from 10 different pertuzumab molecules, the relative amount pertuzumab containing one Fab with Cys23/Cys88 unpaired cysteines is approximately 20%, i.e. 10 out 50 pertuzumab molecules, (or 2x%). More precisely, if the probability of a pertuzumab with two Fabs containing Cys23/Cys88 unparied cysteines is taken into account, then the relative amount of pertuzumab heterodimer unpaired cysteine variants should be 2 × 10% × 90% = 18% (or 2 × x% × [100-x] %). In addition, the relative amount of pertuzumab homodimer unpaired cysteine variants should be at 10% × 10% = 1% (or x% × x%). In this case, the relative among of pertuzumab containing 2 Fabs with no unpaired cysteines on either Fab should be at 90% × 90% = 81% (or [100-x]% × [100-x]%).

In addition wild-type homodimer (without unpaired cysteines) and heterodimer (with unpaired cysteines on one Fab) can be quantified directly by HIC. HIC of intact antibody separates pertuzumab into two major peaks (Fig. 13), which were identified as wild-type homodimer (without free thiols) and heterodimer (with free thiol pair on one Fab) by LC-MS tryptic peptide mapping. The minor front shoulder peak was also collected and characterized by papain HIC as predominantly homodimer (free thiol pair on both Fabs, approximately 40%) and pertuzumab with Fc oxidation. Using the HIC of intact antibody, pertuzumab was estimated to contain approximately 17%–18% heterodimer for materials produced using the current process, and 13% using the Phase I/II process (Table 5). Without being bound by any one theory, it is possible that the increased amount of the unpaired cysteine variant produced by the commercial process (relative to the phase I/II process) may result from protein (i.e. VL domain) folding rate accuring faster than thiol oxidation (disulfide formation) rate, thus trapping free cysteines in the variant.

Table 5: Relative Amount of Intact Unpaired Cysteine Variants as Determined by HIC of Intact Pertuzumab

	Peak		
Batch Name	Wild-Type Homodimer (%)	Unpaired cysteine Heterodimer (%)	Partially Enriched Unpaired cysteine Homodimer (%)
anti2C4-900-1	84.7	13.4	1.9
anti2C4907-2	78.9	18.2	2.9
Run 1	78.4	18.4	3.2
Run 3	79.1	17.6	3.2
Run 4	79.3	17.3	3.4
Run 5	79.1	17.4	3.5
Run 6	79.7	17.2	3.1
Run 7	79.3	17.3	3.4

Note: The percent relative peak was obtained by dividing the individual peak area by the total peak area of all three peaks.

Since an unpaired cysteine pair at Cys23/Cys88 on the light chain of pertuzumab was observed by HIC, purified fractions of each of the unpaired cysteine variants were tested in the anti-proliferation assay. The unpaired cysteine containing Fab was purified and estimated to have reduced potency (estimated potency ~50% relative to the native Fab) (Table 6).

Table 6: Anti-Proliferation of Unpaired Cysteine Fab Variant

Pertuzumab Samples and Conditions	Mean % Activity (n=2)	% Difference
Native Fab	100	N/A
Unpaired Cysteine-Fab	50[a]	67

Note: Percent activity reported relative to Native Fab.
[a] Estimated potency value. Dose response curves are not parallel, and the lower plateau does not converge.

In addition, three intact forms (wild-type homodimer, free-thiol containing heterodimer, and unpaired cysteine containing homodimer) were isolated by HIC and tested with the anti-proliferation potency assay. See Table 7.

Table 7: Anti-Proliferation Activities of Full Length Pertuzumab Unpaired Cysteine Variants Fractions

Pertuzumab Samples and Conditions	Anti-proliferation	
	Mean % Activity (n = 3)	CV (%)
Starting Material	110	11
Heterodimer	112	7
Wild-Type Homodimer	104	15
Unpaired cysteine-Containing Homodimer[a]	90	15

Note: Percent activity reported relative to pertuzumab Reference Standard (Batch anti2C4907-2).
[a] The fraction contains approximately 40% unpaired cysteine-containing homodimer and 60% heterodimer or wild-type homodimer mixture.

These data demonstrate that an unpaired cysteine variant of pertuzumab is present in the composition manufactured at commercial scale. The HIC methods (evaluating Fab fragment or intact antibody) in this Example or peptide mapping in Example 3 below are assays that can be used to evaluate the presence and quantity of the unpaired cysteine variant in a pertuzumab composition.

EXAMPLE 2

Afucosylated Pertuzumab Composition and Characterization Thereof

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) is an aspect of cell-mediated immunity by which an effector cell actively lyses a target cell that has bound antigen-specific antibodies. Pertuzumab exhibited ADCC activity when tested with HER2 3+ cells but very little activity was observed with HER2 1+ cells (Fig. 14).

The levels of afucosylation in the pertuzumab Phase I and Phase III Reference Standards were measured using capillary electrophoresis. The higher level of afucosylated material (G0-F = 2.2%) in the pertuzumab Phase III Reference Standard correlates with the higher ADCC activity observed compared to Phase I Reference Standard (Fig. 15), which had lower G0-F (0.8%). An enzymatically deglycosylated pertuzumab was also prepared and tested and showed no binding to FcγRIIIa and no ADCC activity (Table 8).

Table 8: Biological Activities of Deglycosylated Pertuzumab

Pertuzumab Samples and Conditions	Mean % Activity (n = 3)				
	Anti-Proliferation	HER2 Binding	FcγRIIIa Binding	ADCC	FcRn Binding
Control	90	105	106	101	85
Deglycosylated	87	94	No Activity	No Activity	72

Note: Percent activity reported relative to pertuzumab Reference Standard (Batch anti2C4907-2). ADCC = antibody-dependent cell-mediated cytotoxicity.

These data show that measuring G0-F (afucosylated) pertuzumab is an effective means for quantifying pertuzumab's ADCC activity. Experiments to quantify afucosylation are as follows.

Oligosaccharide Analysis by Capillary Electrophoresis (CE): Pertuzumab samples (250–500 μg) were purified using Protein A solid phase extraction affinity tips (PHYTIPS™) and an automated liquid handling system. Pertuzumab samples were eluted from the protein A resin using 12 mM hydrochloric acid, pH 2.0 and neutralized using 10 μL of 50 mM sodium succinate. The resulting sample was incubated with 2.5 U/mL of PNGase F for 15 hours at 37°C. The protein was precipitated by heating the solution at 95°C for 5 minutes and was removed by centrifugation. The supernatant solutions containing released oligosaccharides were vacuum dried. The released glycans were derivatized with 8-aminopyrene-1,2,6-trisulfonic acid (APTS) in a 15% acetic acid solution containing sodium cyanoborohydride at 55°C for two hours. Analyses of derivatized glycans were performed with a capillary electrophoresis (CE) system equipped with a fluorescence detection module using an argon-ion laser (488 nm excitation, 520 nm emission) and an N-CHO coated capillary (50 μm × 50 cm). The running buffer was 40 mM ε-amino-n-caproic acid/acetic acid, pH 4.5, 0.2% hydroxypropyl methylcellulose (HPMC). Samples were injected into the capillary by pressure at 0.5 psi. The separation was performed at 20 kV, and the capillary temperature was maintained at 20°C.

Pertuzumab contains an N-linked oligosaccharide site in the C_H2 domain of the Fc portion of the molecule at Asn299. The relative distribution of the neutral oligosaccharides found at this site for each batch was determined using CE after treatment with PNGase F and labeling with APTS.

The electropherograms from CE analysis of the released, derivatized oligosaccharides are shown in Fig. 16 with expanded-view profiles in Fig. 17. Relative amounts of oligosaccharides in pertuzumab for the materials analyzed are summarized in Table 9.

Table 9: Distribution of Oligosaccharide Structures in Pertuzumab (Percent Peak Area)

Batch Name	G0-F	G0-GlcNAc	Man5	G0	G1[a]	G2
anti2C4-900-1	0.8	2.7	1.2	72.1	20.4	2.0
anti2C4907-2	2.2	0.8	0.3	63.6	27.6	3.0
Run 1	2.5	1.6	0.2	62.4	29.1	3.4
Run 3	1.7	1.2	0.3	70.3	23.4	2.2
Run 4	1.8	1.8	0.3	75.4	18.3	1.4
Run 5	1.4	1.0	0.4	71.8	22.4	2.1
Run 6	1.1	1.0	0.2	73.3	21.3	1.9
Run 7	1.2	0.8	0.2	69.7	24.5	2.5

Note: The total % may not add exactly to 100% due to rounding. In addition, minor species (< 0.5%) may have been included in the total percent peak area but not reported in this table.
[a] Sum of the two G1 isomers (refer to Fig. 17).

The oligosaccharide with G0 structure is the predominant species in all materials (62%–75%). The G0 glycoform was slightly more abundant in Runs 3-7 and the previous Reference Standard Batch 2C4-900-1 (70%–75%) compared to the current Reference Standard Batch anti2C4907-2 and Run 1 (62%–64%). The G1 glycoform was observed as two peaks corresponding to the two isomers with the terminal galactose on either branch of the biantennary structure. The areas of these two peaks were combined in order to determine the relative amount of G1 glycoform. The G1 and G2 glycoforms account for approximately 18%–29% and 1%–3%, respectively of the released oligosaccharides for all the materials. Peaks arising from other oligosaccharide structures were also observed in the electropherograms (all present at 3% or less). These structures include G0-F (G0 lacking core fucose), G0-GlcNAc (G0 lacking one GlcNAc), Man5, and other minor glycoforms (Ma and Nashabeh *Anal Chem* 71:5185–92 (1999)). Oligosaccaride structures on pertuzumab were consistent with those found CHO–derived MAbs (Ma and Nashabeh, *supra*) and naturally occurring human immunoglobulins (Flynn et al. *Mol Immunol* 47:2074–82 (2010)).

EXAMPLE 3

Peptide Mapping and RP-HPLC for Evaluating Unpaired Cysteine Variant

Materials: Materials and devices used in the experiments include: 3-[N-Morpholino]propanesulfonic Acid (MOPS; Sigma-Aldrich), N-ethylmaleimide (d0-NEM; Thermo

Scientific, Rockford, Il), N-ethylmaleimide (d5-NEM; Cambridge Isotope Laboratories, Andover, MA), L-Cysteine (Sigma-Aldrich), Trypsin (Promega, Madison, WI), Trifluoroacetic acid (TFA; Fisher, Fair Lawn, NJ), Acetonitrile (ACN, Burdick & Jackson, Muskegon, MI). All chemicals and reagents were used as received with no further purification.

Differential N-Ethylmaleimide (NEM) Labeling of Antibodies: Differential NEM tagging method allowed free thiols already present in the antibodies to be tagged with d0-NEM and remaining disulfide bridges to be reduced and tagged with d5-NEM. For initial d0-NEM tagging, 100 µL antibody (3 mg/mL) was gently mixed with 400 µL Denaturing Buffer (7.5 M GdnHCl, pH 5) containing 6.25 mM d0-NEM and incubated at 37°C for 2 h. 20 µL Cysteine (125 mM) was added to the sample and incubated at 37°C for 15 minutes to inactivate remaining d0-NEM. To reduce remaining disulfide bridges in the antibody, 10 µL TCEP (0.5 M) was added to the sample and incubated at 37°C for 30 minutes. 70 µL d5-NEM (171 mM) was then added to the sample and incubated at 37°C for 2 h to tag the free thiols created by the reduced disulfide bridges. 0.5 mL of the differential NEM tagged sample was buffer exchanged using NAP-5 columns and eluted with 0.6 mL MOPS buffer (20 mM MOPS, 0.5 mM TCEP, pH 7).

Peptide map analysis of Antibodies: Differential NEM tagged samples were digested with trypsin at a 1:50 (w/w) trypsin:antibody ratio at 37°C for 2 h. Digestions were quenched with 10 % TFA. The trypsin digested differential NEM tagged samples were separated using an Agilent 1200 HPLC system (Agilent, Palo Alto, CA). A Jupiter C18 column (250 x 2 mm, 5 µm) (Phenomenex, Torrance, CA) with 300 Å pore size was employed for chromatographic separation of samples. The injection volume was 95 µL, and the column temperature was 55°C. The mobile phase A was 0.1% TFA in water and mobile phase B was 0.08% TFA in 90% ACN (v/v). Initial conditions were set at 100% mobile phase A and kept for the first 3 minutes after sample injection. Mobile phase B was increased to 10% over the next 20 minutes and then further increased to 40% until 160 minutes and 100% until 162 minutes all over linear gradients. Mobile phase B was held at 100% until 170 minutes. The column was the re-equilibrated at 100% mobile phase A until 195 minutes. The flow rate was kept at 0.28 mL/min.

The effluent from the HPLC was directly connected to the electrospray ionization source of LTQ ORBITRAP™ mass spectrometer operating in a positive ion mode. The spray voltage was 4.5kV, and the capillary temperature was 300°C. The mass spectrometer was operated in the data dependent fashion to switch automatically between MS and MS/MS modes. Survey full scan MS spectra were acquired from m/z 300 to m/z 2000 in the FT-Orbitrap with a resolution set for R =60,000 at m/z 400. The five most intense ions were fragmented in the linear ion trap using collision induced dissociation (CID) at normalized collisional energy of 35% with an activation time of 30 ms and isolation width of 2.5 m/z units. The dynamic exclusion (DE) function was enabled to reduce data redundancy and allow low-intensity ions to be selected for data dependent MS/MS scans. The

dynamic exclusion parameters were as follows: a repeat duration of 30 seconds, an exclusion list size of 500, an exclusion duration of 90 seconds, a low exclusion mass width 0.76, a high exclusion mass width of 1.56, and a repeat count of 2. The data analyses were performed using XCALIBUR™ software.

The current Pertuzumab Reference Standard Batch anti2C4907-2 was analyzed using the method described above. It was found that 10.9% of the T2L peptides (produced by trpsin digestion and containing Cys23) and 8.3% of the T7L peptides (produced by trpsin digestion and containing Cys88) were tagged with d0-NEM. Because only unpaired cysteines were tagged with d0-NEM in this experiment, these results suggest that approximately 10% of the Cys23 and Cys88 in anti2C4907-2 are not linked by a disulfide bond (i.e. 10% unpaired cysteins variants). Using a calculation method smilar to that described above to convert the percent unpaired cysteine Fab variant into percent unpaired cysteine intact variant, it was estimated that 18% of the pertuzumab molecules in anti2C4907-2 are heterodimer unpaired cysteine variants, 1% of the pertuzumab molecules are homodimer unpaired cysteine variants, and 81% are the wild-type homodimer form (without unpaired cysteines). These results are in a general agreement with the results from HIC analysis of either the Fab fragments or the intact pertuzumab.

Limited Endoproteinase Lys-C Digestion to Generate the Fab: The Fab fragment of MAb A was generated through limited Lys-C digestion procedure. Briefly, MAb A (1 mg/ml) was mixed with Lys-C enzyme at 1:400 ratio in 100 mM Tris, pH 7.6, and then the mixture was incubated at 37 °C for 30 minutes. The reaction mixture was tagged with NEMin pH 5.5, 350 mM sodium acetate and 8M Guanidine HCl. The digests were analyzed with an RP-HPLC method described below.

RP-HPLC Conditions: RP-HPLC analysis was performed on an AGILENT 1200™ HPLC system (Palo Alto, CA, USA) equipped with a binary gradient pump, autosampler, temperature-controlled column compartment, and a diode array detector. The system included a Pursuit 3 diphenyl reversed phase column (150 x 4.6 mm, 3 μm, Varian, Lake Forest, CA, USA) that was run at 75 °C and 1ml/min. The separation was monitored using absorbance at 280 nm. The mobile phase consisted of 0.1 % TFA in water (mobile phase A) and 0.09% TFA in ACN (mobile phase B). The 38-minute method began with a three minute gradient from 32% to 36 % mobile phase B, followed by an 18 minute linear gradient to 42% mobile phase B. The column was washed at 95 % mobile phase B for 5 minute and equilibrated at 32 % mobile phase B for 10 minutes.

RP-HPLC analysis of free thiol Fab generated by limited Lys-C digestion (Fig. 18) indicated the free thiol Fab is around 13%, consistent with the HIC in Example 1. The NEM tagged free thiol Fab becomes more hydrophobic, thus eluted later compared with free thiol Fab (Fig. 18) and further confirmed the presence of free thiol. See also Fig. 19 in which peptide mapping confirms free thiol Fab.

EXAMPLE 4

Afucosylation Quantification by CE-LIF

This example describes a fully validated capillary electrophoresis-laser-induced fluorescence (CE-LIF) assay for quantifying afucosylated Pertuzumab variant. Modifications to the methods disclosed in Example 2 above include: no robotic sample preparation, no Protein A purification step ensuring consistent protein concentrations among samples, and changes to the electrophoretic parameters (buffer excipient concentration).

In the assay, Pertuzumab samples are diluted to 10 mg/mL using formulation buffer, and buffer exchanged into Peptide-N-Glycanase F (PNGase F) digest buffer. The asparagine-linked oligosaccharides are then released enzymatically with PNGase F. The released glycans are subsequently derivatized with 8-aminopyrene-1,3,6-trisulfonic acid (APTS), a negatively charged fluorophore. APTS provides all glycans with three negative charges, which allow their rapid electrophoretic analysis. The mixture containing the excess derivatizing agent and the APTS-glycan conjugates is analyzed by CE using a coated capillary that reduces the electroosmotic flow. The separation is monitored with a laser-induced fluorescence system using an argon-ion laser with an excitation wavelength of 488 nm and an emission band pass filter of 520 nm.

Using the assay, a correlation plot is shown in Fig. 21. Using the correlation plot (%ADCC = 30.133 + 12.439x, where x = %G0-F) and an ADCC range of 40 – 135%, the final specification for Pertuzumab corresponds to 0.9– 4.1% G0-F.

Thus, using this validated CE-LIF assay, it is possible to evaluate Pertuzumab compositions to confirm the biological activity in terms of ADCC is within the desired range (40-135% ADCC activity = 0.9-4.1% G0-F).

EXAMPLE 5

Pertuzumab High-Molecular-Weight-Species (HMWS), Low-Molecular-Weight-Species (LMWS) and Characterization Thereof

Pertuzumab was analyzed by SE-HPLC and CE-SDS to determine the amount of high-molecular-weight species (HMWS), generally dimer, and low-molecular-weight species (LMWS). There was no difference in HMWS upon dilution, suggesting that the aggregates are non-dissociable. There was good agreement between analytical ultracentrifugation (AUC) and SE-HPLC results in terms of HMWS quantitation, showing no evidence of size-exclusion chromatography missing or underestimating major HMWS.

MATERIALS AND METHODS

Pertuzumab Compositions Tested: This example describes the characterization of the current Pertuzumab Reference Standard Batch anti2C4907-2 and Run 1, representing Phase III clinical material, and five Phase III/commercial batches (Runs 3-7), all produced at 12,000 liter (L) scale using the commercial process.

Isolated HMWS: To prepare representative HMWS used for biological characterization, a pertuzumab batch from Run 3 was injected onto a preparative HPLC system using a preparative SE-HPLC column (TSK G3000SW, 21.5 mm × 600 mm) and the same isocratic mobile phase as described above at 4.5 mL/min. High-molecular-weight species were fraction collected and subsequently buffer exchanged into formulation buffer. HMWS were shown to be 70% pure by subsequent SE-HPLC analysis, with the remainder predominantly main peak. The main peak was also collected and shown to be 100% pure.

Isolated LMWS: To prepare isolated LMWS, a batch Number from Run 3 was digested with papain and subjected to fraction collection using preparative HPLC, as above. The predominant forms were verified to be Fc and Fab by intact ESI-MS analysis. LMWS were shown to be 99% pure by subsequent analytical SE-HPLC. Isolated Fab variants were also prepared using papain treatment and collected by preparative IE-HPLC. The Fab variant was shown to be 100% pure by subsequent analytical SE-HPLC.

SE-HPLC: Aliquots of pertuzumab were diluted to 10 mg/mL with mobile phase (0.2 M potassium phosphate, pH 6.2, 0.25 M potassium chloride). Samples were separated on a TSK G3000SW$_{XL}$ column (7.8 mm × 300 mm) that was eluted isocratically. The flow rate was at 0.5 mL/min, and column temperature was at ambient temperature. The elution profile was monitored at 280 nm. For detection by multi-angle light scattering (MALS), pertuzumab samples were separated using two columns in sequence connected inline to a WYATT DAWN HELEO™ MALS detector (using 658 nm laser, 17 detectors) and a WYATT OPTILAB™ rex refractive index detector.

CE-SDS: Each Pertuzumab batch was derivatized with 5 carboxytetramethylrhodamine succinimidyl ester, a fluorescent dye. After removing the free dye using NAP-5 columns, non-reduced samples were prepared by adding 40 mM iodoacetamide and heating at 70°C for 5 minutes. For the analysis of reduced samples, the derivatized pertuzumab was mixed with sodium dodecyl sulfate (SDS) and 1 M DTT to a final concentration of 1% SDS (v/v). Samples were then heated at 70°C for 20 minutes. The prepared samples were analyzed on a CE system using a 50 m inner diameter × 31.2 cm fused silica capillary maintained at 20°C throughout the analysis. Samples were introduced into the capillary by electrokinetic injection at 10 kV for 40 seconds. The separation was conducted at a constant voltage of 15 kV in the reversed polarity (negative to positive)

mode using CE-SDS running buffer as the sieving medium. An argon ion laser operating at 488 nm was used for fluorescence excitation with the resulting emission signal monitored at 560 nm.

RESULTS AND DISCUSSION

SE-HPLC provides quantitative information about the molecular size distribution of a native protein. The SE-HPLC profiles for the pertuzumab batches are shown in Fig. 25, and an expanded view of the profiles is shown in Fig. 26. The relative peak area distribution of size-exclusion peaks is listed in Table 10.

Table 10: Relative Size Distribution of Pertuzumab by Size-Exclusion Chromatography

	Peak		
Batch Name	HMWS (%)	Main Peak (%)	LMWS (%)
anti2C4-900-1	0.1	99.8	0.1
anti2C4907-2[a]	0.2	99.8	0.0
Run 1	0.2	99.8	0.0
Run 3	0.2	99.8	0.0
Run 4	0.1	99.8	0.0
Run 5	0.2	99.8	0.0
Run 6	0.2	99.8	0.0
Run 7	0.2	99.8	0.0

Note: The total percent may not add exactly to 100% due to rounding.
HMWS = high-molecular-weight species; LMWS = low-molecular-weight species.
[a] Values obtained from Reference Standard anti2C4907-2.

The proportion of pertuzumab eluting in the main peak was more than 99% for all materials. The amount of high-molecular-weight species (HMWS) ranged from 0.1% to 0.2%, and the low-molecular-weight species (LMWS) was $\leq$ 0.1%. All batches displayed similar chromatographic profiles. A purified HMWS fraction, including dimer and higher aggregates, was shown to have 46% potency relative to Reference Standard Batch anti2C4907-2.

SE-HPLC was performed on both neat and diluted samples held at 30°C to examine pertuzumab HMWS for both fast and slow-dissociating aggregates that could result from dilution and/or prolonged exposure to elevated room temperature. No decrease was seen in the HMWS content of diluted and/or heated Reference Standard Batch anti2C4907-2 as compared to the control.

SE-HPLC separation combined with MALS performed on Reference Standard Batch anti2C4907-2 confirmed the SE-HPLC main peak to be monomer, with a molecular weight of approximately 150 kDa.

AUC in sedimentation velocity mode was used to characterize the HMWS present in pertuzumab samples. Sedimentation velocity is a technique independent from size exclusion chromatography that measures the levels of HMWS in a sample in the absence of a solid column matrix. AUC was performed on pertuzumab samples with increasing levels of HMWS to determine if SE-HPLC is able to detect all major pertuzumab HMWS consistently by comparing the levels and species of aggregates determined by sedimentation velocity to those determined by SE-HPLC. Five samples ranging from 0.2% to 7.2% total HMWS (determined by SE-HPLC) were characterized by sedimentation velocity and labeled A–E in Table 11and Fig. 27.

These samples consisted of a representative pertuzumab Drug Product batch (labeled A) and four samples with enriched HMWS. The samples with enriched HMWS were chosen to be representative of a wide range of degradation mechanisms (exposure to light, exposure to acidic pH, and purified IE-HPLC basic variants).

SE-HPLC shows one major HMWS peak for samples A, B, C and E and two HMWS peaks for sample D (Fig. 27). For samples A, B, C and E, AUC showed only one HMWS peak with a sedimentation coefficient at about 9.1S. In sample D, AUC showed two HMWS peaks with sedimentation coefficients of about 9.1S and 10.8S. The HMWS detected by AUC are consistent with the SE-HPLC results; both methods show one main degradation product, with minor levels of a larger HMWS in sample D.

A comparison of the quantitative results of these samples from the two methods is presented in Table 11.

Table 11: Comparison of AUC and SE-HPLC Results

	% HMWS (total)	
Sample	AUC	SE-HPLC
A	1.2 [31.4% RSD][a]	0.2
B	1.9	1.3
C	4.8	5.5
D	6.4	6.6
E	7.6 [7.9% RSD]	7.2

Note 1: Sample A consists of a representative pertuzumab Drug Product batch, Sample B consists of a pertuzumab batch subjected to light exposure at 1.2 mlux hours, Sample C consists of a pertuzumab batch subjected to light exposure at 3.6 mlux hours, Sample D consists of a pertuzumab batch subjected to acid treatment at pH 3.2, and Sample E consists of purified basic variants from IE-HPLC.

Note 2: Refer to Fig. 27 for corresponding SE-HPLC chromatograms.

AUC = analytical ultracentrifugation; HMWS = high-molecular-weight species; RSD = relative standard deviation; SE-HPLC = size-exclusion high-performance liquid chromatography.

[a] Samples A and B have HMWS levels that are below the limit of quantitation of the AUC technique.

For samples C, D, and E there is good agreement in the percent HMWS measured by both techniques. The low level of HMWS present in samples A and B prevents an accurate quantitation of the species by AUC, which has an estimated Limit of Quantitation of 3.7% (Gabrielson and Arthur, *Methods* 54:83-91 (2011)). This is reflected by an apparent discrepancy in percent HMWS between SE-HPLC and AUC (Table 11). A correlation across a range of HMWS levels was evaluated. The correlation coefficient (Lin, L, *Biometrics* 45:255–68 (1989)) was calculated to be 0.97 (n=5) indicating good agreement between AUC and SE-HPLC for the quantitation of HMWS (Fig. 28).

These results confirm that SE-HPLC is robust in measuring HMWS for pertuzumab. SE-HPLC is able to detect and accurately quantitate all HMWS species observed by AUC.

Size-based heterogeneity, analyzed by SE-HPLC, SDS-PAGE, and CE-SDS, was consistent among the batches. The SE-HPLC assay showed similar levels of HMWS (0.1%–0.2%) and LMWS (0.0%–0.1%) for all batches tested. The banding patterns developed by SDS-PAGE analysis for reduced and non-reduced samples were consistent, as were the electrophoretic profiles generated by CE-SDS.

In one embodiment, the amounts of the main species Pertuzumab and HMWS variant and LMWS variant as evaluated by SE-HPLC is as follows:

≥ 96% Main Peak e.g., ≥ 96.7% Main Peak, e.g., ≥ 97.3% Main Peak e.g., ≥ 97.4% Main Peak ≤ 2% HMWS, e.g., ≤ 1.7% HMWS, e.g., ≤ 1.5% HMWS, e.g., ≤ 1.4% HMWS, e.g. ≤ 0.8% HMWS.

≤ 2% LMWS, e.g., ≤ 1.6% LMWS, e.g., ≤ 1.2% LMWS, e.g. ≤ 0.6% LMWS.

Both the pertuzumab HMWS and LMWS fractions purified by SE-HPLC exhibited a decreased anti-proliferation activity compared to the main peak and control, which was fully potent. All size variants showed comparable HER2 binding activity and FcRn binding activity compared to the control, except for the LMWS, which showed lower FcRn binding. Since the LMWS sample contains 2/3 Fab fragments and 1/3 Fc fragments, the lower anti proliferation and FcRn binding activity are as expected. The HMWS showed higher Fc RIIIa (CD16) V158 binding activity, but lower ADCC activity. The LMWS showed lower Fc RIIIa (CD16) V158 binding activity, and no ADCC activity was observed for this variant (Table 12).

Table 12: Biological Activities of Pertuzumab Main Peak, HMWS, and LMWS

Pertuzumab Samples and Conditions	Mean % Activity (n = 3)				
	Anti-Proliferation	HER2 Binding	FcγRIIIa Binding	ADCC	FcRn Binding
Control	103	108	91[b]	80[b]	80
Main Peak	104	96	96	79	87
HMWS	46	82	522	38	73
LMWS	12[a]	73[a]	23[a]	No Activity	7[a]

Note: Percent activity reported relative to pertuzumab Reference Standard (Batch anti2C4907-2).
ADCC = antibody-dependent cell-mediated cytotoxicity; HMWS = high-molecular-weight species; LMWS = low-molecular-weight species.

[a] The LMWS sample consist of 2/3 Fab and 1/3 Fc fragments. The value shown reflects nM/nM adjustment based on molecular weight (Fab = 47644 Da, Fc = 52800 Da, and the full length antibody = 148088 Da.

[b] The pertuzumab Reference Standard (Batch anti2C4907-2) has a G0-F level of 2.2%, while the control sample had a G0-F of 1.7%. Results have not been corrected for difference in afucosylated material level.

Capillary Electrophoresis Sodium Dodecyl Sulfate (CE-SDS): CE-SDS with laser-induced fluorescence (LIF) detection analysis is a high-sensitivity assay that provides a means of quantitatively assessing the molecular size distribution of proteins under denaturing conditions. In the CE-SDS analysis of non-reduced samples (Fig. 29), pertuzumab migrated as a prominent peak consisting of 96%–98% of the total peak area with minor peaks representing LMWS and HMWS. The amount of HMWS determined by this technique was 0.6% for all materials tested. The remaining species migrated as LMWS as shown in Fig. 30 (expanded view). The sample heating-induced fragmentation is minimized with alkylation (Salas-Solano et al. *Anal Chem* 78:6583–6594 (2006)). The relative distribution of the species separated by CE-SDS is listed in Table 13.

Table 13: Relative Distribution of Non-Reduced Pertuzumab by CE-SDS (Percent Peak Area)

	Peak							
Batch Name	1	2	3	4	5	6	Main	HMWS
anti2C4-900-1	0.1	0.2	0.1	0.2	0.2	0.9	97.8	0.6
anti2C4907-2	0.1	0.3	0.1	0.3	0.2	1.9	96.5	0.6
Run 1	0.1	0.3	0.1	0.4	0.2	1.7	96.7	0.6
Run 3	0.1	0.4	0.1	0.3	0.2	2.3	96.0	0.6
Run 4	0.1	0.4	0.1	0.3	0.2	2.3	96.1	0.6
Run 5	0.1	0.3	0.1	0.2	0.2	2.0	96.4	0.6
Run 6	0.1	0.4	0.1	0.3	0.2	2.2	96.2	0.6
Run 7	0.1	0.4	0.0	0.2	0.2	2.1	96.3	0.6

Note: The total % may not add exactly to 100% due to rounding.
CE-SDS = capillary electrophoresis sodium dodecyl sulfate; HMWS = high-molecular-weight species.

A minor difference was observed wherein Peak 6 increased from 0.9% in the Reference Standard Batch anti2C4-900-1 (Phase I/II process) to 1.7%–2.3% for Reference Standard Batch anti2C4907-2, Run 1, and Runs 3–7.

In one embodiment, the Pertuzumab main peak (excluding LMWS and HMWS) as separated or isolated by NR-CE-SDS is from about 95% to about 99%, e.g., from about 96.0% to about 97.8%. Optionally, the amount of HMWS is $\leq$ 1%, e.g. $\leq$ 0.6% and the amount of LMWS is $\leq$ 4%, e.g. $\leq$ 3.4% as separated or isolated by CE-SDS.

EXAMPLE 6

Detection and Quantification of Pertuzumab Fragmentation

The purpose of this example was to evaluate size exclusion chromatography (SE-HPLC), reduced capillary electrophoresis sodium dodecyl sulfate (R-CE-SDS), and non-reduced CE-SDS (NR-CE-SDS) methods for the detection of pertuzumab fragments.

MATERIALS AND METHODS

Samples evaluated in this study are summarized below. These include pertuzumab samples that have been subjected to various stressed conditions which might result in increased fragmentation.

- Reference Standard (2C4907-2)
- Thermally stressed (42 days, 40°C)
- Acid treated (pH 3.2, 1 day, 40°C)
- Accelerated stability (30 days at 40°C then stored at approximately 5°C)
- Real time Drug Product (DP) stability (T=0 and T=548 days and stored at approximately 5°C) and corresponding Drug Substance (DS)

SE-HPLC was carried out as described in Example 5 above, with the following reportable values: LMWS, Main Peak, HMWS, and all other significant peaks above limit of quantification (LOQ).

Reduced CE-SDS (R-CE-SDS) was carried out according to Example 5 above, with reportable values: Peak 1, LC, Peak 2, Peak 3, NGHC, HC, Peak 5, Inc. Red., and other significant peaks above LOQ.

Non-reduced CE-SDS (NR-CE-CDS) was carried out as in Example 5 above, with sample preparation excluding the antibody reduction step to allow non-reduced analysis by eliminated dithiothreitol (DTT) from the SDS complexation step.

Qualitative results obtained by SE-HPLC, NR-CE-SDS, and R-CE-SDS are presented in Figs. 31A-B, Figs. 32A-B, and Figs. 33A-B, respectively, as well as Tables 14, 15, and 16, respectively.

Peak identifications are based on Hunt & Nashabeh *Analytical Chemistry* 71: 2390-2397 (1999), and Ma & Nashabeh *Chromatographia Supplement* 53: S75-S89 (2001). For NR-CE-SDS analysis, a small peak after Peak 2 is typically included as part of Peak 2 during data reporting. For this study, this small peak is reported separately as Peak 2a to differentiate the fragment from light chain (LC).

Table 14: SE-HPLC Quantitative Data (% Peak Area)

	HMWS (%)	Main Peak (%)	LMWS (%)
2C4907-2	0.18	99.77	0.04
Thermal	0.40	98.96	0.64
Acid Treated	7.09	92.65	0.26
Accelerated Stability	0.26	99.23	0.51
DP Stability	0.19	99.68	0.13

Table 15: NR-CE-SDS Quantitative Data (% CPA)

	Peak 1	Peak 2 (LC)	Peak 2a	Peak 3 (Fab)	Peak 4 (HL)	Peak 5 (HH/des Fab)	Peak 6 (HHL)	Main Peak	HMWS
2C4907-2	0.12	0.29	0.05	0.07	0.35	0.18	1.90	96.32	0.73
Thermal	0.42	0.70	0.09	0.61	0.52	1.36	3.62	91.99	0.71
Acid Treated	0.15	0.37	0.17	0.35	0.45	0.66	3.19	93.42	1.25
Accel. Stability	0.35	0.59	0.09	0.48	0.44	1.07	3.25	92.90	0.84
DP Stability	0.22	0.37	0.06	0.20	0.33	0.43	2.53	95.27	0.60

LC = Light Chain, HC = Heavy Chain, L = Light, H= Heavy

Table 16: R-CE-SDS Quantitative Data (% CPA)

	Peak 1	LC	Peak 2	Peak 3	NGHC	HC	Peak 5	Inc. Red.
2C4907-2	0.31	25.30	0.92	2.44	2.81	66.82	0.48	0.91
Thermal	0.54	26.16	1.12	3.13	2.81	64.54	0.27	1.43
Acid Treated	0.38	25.04	5.58[c]	2.77	2.41	62.91	0.19	0.71
Accel. Stability	0.51	25.01	0.98	2.99	3.16	66.10	0.25	1.03
DP Stability	0.19	26.34	0.69	3.21	2.83	65.93	0.24	0.57

NGHC = Non-Glycosylated Heavy Chain, HC = Heavy Chain, Inc. Red. = Incompletely Reduced

Data Evaluation: The percent peak area (or percent corrected peak area, %CPA, for CE-SDS) of relevant fragments was compared to determine if either of the CE-SDS methods provide non-redundant information as compared to SE-HPLC. Relevant fragments include peaks with

unknown structure, or those that are known to contain products derived from cleavage of polypeptide chain(s). These fragments are distinct from the dissociable non-disulfide bonded heavy and/or light chain fragments that are present in antibody products and are commonly observed by CE-SDS. Fragment peaks must be resolved from other peaks to enable sensitive detection and accurate quantitation.

Ability to Detect Small Fragments: Small fragments can be observed in both R-CE-SDS and NR-CE-SDS analysis, and are named as Peak 1 in both assays. These peaks retain the same general shape and migration time, and increase similarly under stressed conditions in both assays. Therefore, Peak 1 is presumed to contain the same species in both assays. Both CE-SDS assays are capable of detecting small fragments, as noted in Table 17.

Ability to Detect Fragments Generated by Acid Hydrolysis (Acid Clips): Prolonged exposure to acidic conditions can generate fragments, particularly at the Asp-Pro sequence (pertuzumab heavy chain residues 272-273), as supported by mass spectrometric analysis of the acid-treated sample showing masses at 29039 Da (HC 1-272) and 21513 Da (HC 273-448 with G0 glycan). The theoretical masses for these forms are 29031 Da and 21510 Da, respectively. Based on the expected migration time of these forms, a corresponding peak can be seen clearly in the reduced CE-SDS analysis of the acid-treated sample (Peak 2, Fig. 34), but is detected at a much lower level (lower signal) in the non-reduced assay. It can be postulated that in the NR-CE-SDS assay the Peak 2 fragment is presumably disulfide linked, and therefore not detected. Since the level of Peak 2 detected by R-CE-SDS (5.58%) in the acid-treated sample exceeds the total LMWS as detected by SE-HPLC (0.26%) for this sample, it can be concluded that SE-HPLC is also insufficient for detection of these forms. Therefore, the reduced CE-SDS assay is the only assay presented herein capable of detecting fragmentation generated as a result of acid hydrolysis, as noted in Table 17.

Ability to Detect Fab/DesFab Fragments: There is a good linear correlation ($r^2 = 0.97$) between the LMWS as detected by SE-HPLC, and Peak 3 from the non-reduced CE-SDS assay (Fig. 35). The LMWS was identified to contain the Fab fragment through co-elution studies with enzymatically generated Fab. Similarly, Peak 3 and Peak 5 in the NR-CE-SDS assay were identified through co-migration studies with enzymatically generated Fab and DesFab, respectively (Ma & Nashabeh, *supra*). The desFab peak arises from the heavy chain cleavage that produced the Fab form, so it is presumed to be in a an equivalent molar quantity (corresponds to a 2:1 mass ratio) relative to the Fab fragment, and thus, information on this form can also be indirectly obtained by SE-HPLC as noted in Table 17.

Table 17: Detection Capability for Fragments by SE-HPLC, NR-CE-SDS, and R-CE-SDS

Fragment	SE-HPLC	NR-CE-SDS	R-CE-SDS
Small Fragments	Unknown	Yes	Yes

CE-SDS Peak 1			
Acidic Clips (R-CE-SDS Peak 2)	No	No	Yes
Fab / DesFab	Yes / (indirectly)	Yes	No

Reduced CE-SDS Peak 3: R-CE-SDS Peak 3 is unique for pertuzumab and has not been observed in the CE-SDS analysis of other antibodies. Extended characterization results support the conclusion that Peak 3 is not a product variant or impurity, but rather a method-induced artifact specific to pertuzumab consisting of a dissociable form of LC-LC dimer. Multiple techniques were employed to characterize Peak 3.

Peak 3 is observed by R-CE-SDS with UV and LIF detection, suggesting it is not a dye-labeling or sample preparation artifact.

Upon analysis by R-CE-SDS, purified pertuzumab light chain fractions produce Peak 3 having an apparent MW approximately 2-times the theoretical size of LC.

Peak 3 is not observed when the electrophoretic conditions include a higher capillary temperature, and no other co-migrating fragments are observed under these conditions.

Studies involving single amino acid mutations have identified three amino acid residues in LC CDR1 and CDR2 correlated with LC-LC dimer formation. When any of these three residues is replaced by another amino acid, Peak 3 completely dissociates and is no longer observed by reduced CE-SDS.

SDS-PAGE analysis coupled with MALDI-TOF Protein Mass Fingerprinting (PMF) confirmed no host cell proteins were present in pertuzumab, nor was an analogous band detected at levels observed by CE-SDS.

Taken collectively, these results support the identification of Peak 3 as a method-induced, LC-LC dimer specific to pertuzumab.

DISCUSSION

Evaluation of data obtained from this study indicates that:

(1) NR-CE-SDS does provide non-redundant information as compared to SE-HPLC for the detection of fragments

(2) The non-redundant fragmentation information obtained by the NR-CE-SDS method (as compared to SE-HPLC) can also be obtained using the R-CE-SDS method

As shown in Table 16, the reduced assay detected cleavage products resulting from low pH

exposure, which may occur during Drug Substance manufacture. Table 18 contains the values obtained for reduced CE-SDS Peak 1 and Peak 2 for pertuzumab reference standard, phase III material (n=3), and batches produced using the commercial manufacturing process (n=39). The 95/99 tolerance intervals (TIs) have been calculated for Peak 1 and Peak 2 using a k value of 3.2, and are presented in Table 18. The 95/99 tolerance interval for Peak 1 is 0.0 to 0.4 %CPA. The 95/99 tolerance interval for Peak 2 is 0.3 to 0.9 %CPA.

Table 18: R-CE-SDS Quantitative Data (% CPA) on 43 Batches Tested

n	Batch	Peak 1 (% CPA)	Peak 2 (% CPA)
1	anti2C4907-2	0.31	0.92
2	SSF0001	0.25	0.63
3	SSF0002	0.20	0.62
4	SSF0003	0.27	0.60
5	VV0002	0.27	0.47
6	VV0003	0.28	0.49
7	VV0004	0.25	0.51
8	VV0005	0.41	0.55
9	VV0006	0.27	0.50
10	VV0007	0.29	0.50
11	VV0008	0.30	0.53
12	VV0009	0.26	0.54
13	VV0013	0.19	0.54
14	VV0018	0.17	0.56
15	VV0020	0.17	0.58
16	VV0021	0.18	0.62
17	VV0023	0.14	0.62
18	VV0024	0.13	0.64
19	VV0025	0.18	0.69
20	VV0026	0.13	0.58
21	VV0028	0.17	0.60
22	VV0029	0.18	0.61
23	VV0031	0.18	0.65
24	VV0032	0.16	0.69
25	VV0033	0.18	0.69
26	VV0034	0.18	0.67
27	VV0035	0.17	0.59
28	VV0036	0.17	0.67
29	VV0037	0.19	0.74
30	VV0038	0.19	0.71
31	VV0039	0.19	0.73
32	VV0040	0.19	0.70
33	VV0041	0.18	0.72

34	VV0042	0.19	0.73
35	VV0043	0.19	0.61
36	VV0044	0.20	0.67
37	VV0046	0.20	0.62
38	VV0047	0.22	0.66
39	VV0048	0.22	0.63
40	VV0049	0.20	0.69
41	VV0050	0.18	0.49
42	VV0051	0.16	0.63
43	VV0052	0.18	0.60
	Mean	0.21	0.62
	Standard Deviation	0.06	0.09
	Minimum	0.13	0.47
	Maximum	0.41	0.92
	N	43	43
	K	3.2	3.2
	Lower TI	0.02	0.33
	Upper TI	0.40	0.91

A final acceptance criteria of Peak 1 $\leq$ 0.5% and Peak 2 $\leq$ 1.0% on Drug Substance release is selected herein.

As the pertuzumab Drug Substance is stored frozen, there would be no expected changes on DS stability. In addition, based on R-CE-SDS data obtained for Drug Product at both T=0 and T548d (Table 19), no significant change is observed for any of the named species.

Table 19: R-CE-SDS Quantitative Data (% CPA) for a DP Stability Sample

	Peak 1	LC	Peak 2	Peak 3	NGHC	HC	Peak 5	Inc. Red.
DS Release	0.25	26.72	0.51	2.27	2.76	66.66	0.27	0.56
DP Stability T=0	0.13	26.45	0.54	2.78	2.78	66.53	0.30	0.49
DP Stability T=548d	0.19	26.34	0.69	3.21	2.83	65.93	0.24	0.57

NGHC = Non-Glycosylated Heavy Chain, HC = Heavy Chain, Inc. Red. = Incompletely Reduced

WHAT IS CLAIMED IS:

1. A composition comprising Pertuzumab and unpaired cysteine variant thereof, wherein the unpaired cysteine variant comprises Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab.

2. The composition of claim 1, wherein the unpaired cysteine variant is a heterodimer variant comprising Cys23/Cys88 unpaired cysteines in only one variable light domain of Pertuzumab.

3. The composition of claim 1, wherein the unpaired cysteine variant is a homodimer variant comprising Cys23/Cys88 unpaired cysteines in both variable light domains of Pertuzumab.

4. The composition of any one of the preceding claims, wherein the Pertuzumab and the unpaired cysteine variant each comprise the variable light and variable heavy amino acid sequences in SEQ ID NOs. 7 and 8, respectively.

5. The composition of any one of the preceding claims, wherein the Pertuzumab and the unpaired cysteine variant each comprise the light chain amino acid sequence in SEQ ID No. 11 or 15 and the heavy chain amino acid sequence in SEQ ID No. 12 or 16.

6. The composition of any one of the preceding claims, further comprising one or more additional variants of Pertuzumab, wherein the additional variants are selected from the group consisting of: afucosylated variant, low-molecular-weight-species (LMWS), high-molecular-weight-species (HMWS), glycated variant, disulfide reduced variant, non-reducible variant, deamidated variant, sialylated variant,VHS-variant, C-terminal lysine variant, methionine-oxidized variant, G1 glycosylation variant, G2 glycosylation variant, and non-glycosylated heavy chain variant.

7. The composition of any one of the preceding claims, wherein the amount of the unpaired cysteine variant in the composition is $\leq$ about 25% as determined by Fab hydrophobic interaction chromatography (HIC).

8. The composition of claim 3, wherein the amount of the homodimer variant in the composition is $\leq$ 4.9% as determined by hydrophobic interaction chromatography (HIC) of intact antibody.

9. The composition of claim 2, wherein the amount of the heterodimer variant in the composition is from about 13% to about 18% as determined by hydrophobic interaction chromatography (HIC) of intact antibody.

10. The composition of any one of claims 1 to 9, which has been subjected to an analytical assay to confirm that the amount of the unpaired cysteine variant in the composition is ≤ about 25% as determined by Fab hydrophobic interaction chromatography (HIC).

11. A composition comprising Pertuzumab and an afucosylated variant of Pertuzumab, wherein the amount of the afucosylated variant is from 0.9 to 4.1% of the composition.

12. The composition of claim 11, wherein the amount of the afucosylated variant is greater than 2% of the composition.

13. A composition comprising a mixture of Pertuzumab, low-molecular-weight species (LMWS) of Pertuzumab, and high-molecular-weight-species (HMWS) of Pertuzumab, wherein the amount of LMWS is ≤ 1.6% and the amount of HMWS is ≤ 1.7% as determined by size exclusion high performance liquid chromatography (SE-HPLC).

14. A composition comprising a mixture of Pertuzumab, Peak 1, and Peak 2, wherein the amount of Peak 1 is ≤ 0.5% and the amount of Peak 2 is ≤ 1.0% as measured by reduced capilliary electrophoresis sodium dodecyl sulphate (R-CE-SDS) assay.

15. A pharmaceutical composition comprising the composition of any one of claims 1 to 14 and one or more pharmaceutically acceptable excipients.

16. An article of manufacture comprising a container with the pharmaceutical composition of claim 15 therein, and a package insert with prescribing information instructing the user thereof to use the pharmaceutical composition to treat a cancer patient.

17. A method of treating a patient with cancer comprising administering the composition of any one of claims 1 to 14 or the pharmaceutical composition of claim 15 to a cancer patient.

18. The method of claim 17, wherein the cancer is breast cancer, gastric cancer, ovarian cancer, HER2-positive cancer, or low HER3 cancer.

19. A method for evaluating a Pertuzumab composition comprising: (1) measuring the amount of unpaired cysteine variant in the composition, wherein the unpaired cysteine variant comprises Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab; (2) measuring the amount of afucosylated Pertuzumab in the composition; or (3)

measuring the amount of low-molecular-weight-species (LMWS) or high-molecular-weight-species (HMWS) of Pertuzumab in the composition.

20. The method of claim 19, comprising two or three of (1), (2), and (3).

21. A method for evaluating the biological activity of a Pertuzumab composition comprising measuring the amount of afucosylated Pertuzumab variant in the composition to determine the antibody-dependent cell-mediated cytotoxicity (ADCC) activity of the composition, and confirming the amount of afucosylated Pertuzumab is in the range from about 0.9% to about 4.1%.

22. The method of claim 21, comprising measuring the amount of afucosylated Pertuzumab using capillary electrophoresis-laser-induced fluorescence (CE-LIF).

23. A method for making a composition comprising: (1) producing a composition comprising Pertuzumab and one or more variants thereof, and (2) subjecting the composition so-produced to an analytical assay to evaluate the amount of the variant(s) therein, wherein the variant(s) comprise: (i) unpaired cysteine variant comprising Cys23/Cys88 unpaired cysteines in one or both variable light domains of Pertuzumab; (ii) afucosylated variant of Pertuzumab; (iii) high-molecular-weight-species (HMWS) of Pertuzumab; or (iv) low-molecular-weight-species (LMWS) of Pertuzumab.

24. The method of claim 23, wherein (1) comprises expressing Pertuzumab and the variant(s) from a recombinant Chinese Hamster Ovary (CHO) at manufacturing scale and purifying the composition.

25. An isolated variant of Pertuzumab, wherein the isolated variant comprises: (a) an unpaired cysteine variant of Pertuzumab, wherein the variant is a heterodimer variant comprising Cys23/Cys88 unpaired cysteines in only one variable light domain of Pertuzumab; (b) an unpaired cysteine variant of Pertuzumab, wherein the variant is a homodimer variant comprising Cys23/Cys88 unpaired cysteines in both variable light domains of Pertuzumab; (c) afucosylated variant of Pertuzumab; (d) high-molecular-weight-species (HMWS) of Pertuzumab; or (e) low-molecular-weight-species (LMWS) of Pertuzumab.

26. A method for evaluating fragmentation of a Pertuzumab composition comprising measuring the amount of Peak 1 and Peak 2 in the composition by reduced capilliary electrophoresis sodium dodecyl sulphate (R-CE-SDS) assay and confirming the amount of Peak 1 is $\leq 5\%$ and the amount of Peak 2 is $\leq 1.0\%$.

27. A method for making a composition comprising: (1) producing a composition comprising Pertuzumab and one or more fragments thereof, and (2) subjecting the composition so-produced to an analytical assay to evaluate the amount of Peak 1 and Peak 2 in the composition.