

BISPECIFIC TETRAVALENT ANTIBODY TARGETING HER2 AND HER3**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of the filing date of U.S. Provisional Application Ser. No. 63/604,087 filed November 29, 2023, under 35 U.S.C. 119(e), the entire disclosures of which are incorporated by reference herein.

TECHNICAL FIELD

[0002] The present disclosure generally relates to the technical field of antibody therapy for treating cancer, and more particularly relates to bispecific antibodies.

BACKGROUND

[0003] The human epidermal growth factor receptor (EGFR, also known as ErbB1, HER1) family has four members, EGFR, HER2, HER3, and HER4. Deregulation of each member by means of mutation, amplification, and overexpression plays an important role in tumorigenesis and metastasis in a wide variety of tumors. Interruption of EGFR family signaling, either by blocking binding sites on the extracellular domain of the receptor or by inhibiting intracellular tyrosine kinase activity, can prevent the growth of EGFR-family-expressing tumors and improve the patient's condition. For example, HER2 overexpression occurs in 30% of breast cancer patients, indicative of increased disease recurrence and a poor prognosis. HER2 overexpression is also known to occur in stomach, ovarian, and gastric cancer, adenocarcinoma of lung, aggressive forms of uterine cancer, and salivary duct carcinomas. HER2 mutations have been found in non-small-cell lung cancers. The underlying HER2 mutation and amplification produce aberrant growth signals that activate its downstream signaling pathway leading to tumorigenesis. Specifically, HER2 dimerizes with HER3 on the surface of tumor cells, which activates PI3K/AKT signalling that promotes tumor growth and survival.

[0004] Several therapeutic antibodies and small-molecule inhibitors directed against EGFR and HER2 have been approved for use in the treatment of cancer (Arteaga et al. 2012). The examples of therapeutic anti-HER2 antibodies include Trastuzumab and Pertuzumab that are approved for treating several forms of cancers, including breast cancer and gastric cancer (<https://www.herceptin.com/hcp/treating-HER2-cancer.html>). In particular, the monoclonal antibodies against either EGFR or HER2 have demonstrated good clinical responses in colon cancer (Price et al. 2014), squamous cell carcinoma of head and neck (Cohen, 2014), breast and gastric cancers (Arteaga et al. 2012).

[0005] Trastuzumab (Herceptin) and other agents targeting HER2 exerts antitumor efficacy in patients with HER2-expressing breast cancer and stomach cancer. Trastuzumab is a monoclonal antibody that binds to HER2 and the binding increases the activity of p27, a protein that halts cell proliferation. Trastuzumab is effective only in cancers where HER2 is overexpressed. One year of Trastuzumab therapy is recommended for all patients with HER2-positive breast cancer who are also receiving chemotherapy, and there is no additional benefit beyond 12 months.

Pertuzumab is another monoclonal antibody capable of inhibiting dimerization of HER2 with other receptors, such as HER3, and is a FDA-approved therapeutics for use in combination with Trastuzumab and Docetaxel, a chemotherapeutic agent, for the treatment of metastatic HER2-positive breast cancer (Durkee et al. 2016).

[0006] Despite of these success, the long-term benefit seems to be limited in some patients. Many tumors that initially respond to these therapeutic agents eventually progress due to an acquired resistance to the agents. The development of drug resistance reduces the efficacy of these treatments. In the case of HER2-targeted therapies, the resistance can occur via upregulation of HER3 or its ligand HRG. These findings imply that the current therapeutic approaches aiming at inhibiting the activation of HER2/HER3 signalling pathway have failed to provide meaningful clinical benefit (Geuijen et al. 2018; Yu et al. 2019).

[0007] In summary, currently approved antibody therapies targeting HER2 and/or HER3 have disadvantages of either a low response rate to the treatment or the patient developing resistance to the treatment. There is a need of a better treatment for these cancers.

SUMMARY

The present application generally relates to the technical field of antibody therapeutic agents, and more particularly relates to bispecific antibodies against specific epitopes of HER2 and HER3. HER2 and HER3 often form a partnership in promoting cellular transformation that may ultimately leads to tumorigenesis and tumor metastasis. The application provides, among others, bispecific tetravalent antibodies. The application further provides pharmaceutical compositions including the bispecific tetravalent antibodies or their immunoconjugates, the methods of treating diseases such as cancer using the antibodies or their immunoconjugates, the methods of making the antibodies or their immunoconjugates, and the solutions containing the antibodies or their immunoconjugates.

[0008] The application provides bispecific tetravalent antibodies. In one embodiment, the bispecific antibody has a binding affinity to HER2 and HER3. The antibody has a heavy chain (HC) with a heavy chain variable (VH) domain, a light chain (LC) with a light chain variable (VL) domain, and a scFv domain having a scFv light chain variable (VL) domain and a scFv heavy chain variable (VH) domain. In one embodiment, the VL domain and the VH domain form a Fab region. In one embodiment, the heavy chain has a N-terminal and a C-terminal. In one embodiment, each scFv domain is linked to each heavy chain at the N-terminal through a linker.

[0009] In one aspect, the application provides bispecific antibody has the binding specificity to HER2 and HER3, wherein the Fab region has a binding affinity to HER2, and the scFv domain has a binding affinity to HER3.

[0010] In one embodiment, the Fab region has a binding affinity to HER2 with a KD from about 100pM to about 50nM, about 500pM to about 10nM, about 800pM to about 5nM, about 1nM to about 5nM, about 1nM to about 3nM, about 1nM to about 10nM, about 800pM to about 8nM, or about 1nM to about 5nM. In one embodiment, the Fab region has a binding affinity to HER2

with a KD of at least 500pM, 800pM, or 1nM. In one embodiment, the scFv domain has a binding affinity to HER3 with a KD from about 1nM to about 1 mM, 50nM to about 500nM, 80nM to about 300nM, 100nM to about 200nM, or 100nM to about 500nM. In one embodiment, the Fab region has a binding affinity to HER2 with a KD from about 0.1nM to about 10nM, and the scFv domain has a binding affinity to HER3 with a KD from about 80nM to about 200nM. In one embodiment, the antibody has a binding affinity to HER3 that is at least about 50, 75, 80, 90, 100, 120, 150 times to the binding affinity to HER2.

[0011] In one embodiment, the Fab region comprises the VH domain having an amino acid sequence with at least 75%, 80%, 85%, 90%, 95%, 98%, 98% or 100% sequence identity to SEQ ID NO: 10. In one embodiment, the VL domain having an amino acid sequence with at least 75%, 80%, 85%, 90%, 95%, 98%, 98% or 100% sequence identity to SEQ ID NO: 12.

[0012] In one embodiment, the VH domain comprises CDR-H1 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 25, CDR-H2 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 26, CDR-H3 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 27. In one embodiment, the VL domain comprises CDR-L1 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 28, CDR-L2 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 29, CDR-L3 having a sequence identity to SEQ ID NO: 30.

[0013] In one embodiment, the scFv VH domain has an amino acid sequence with at least 75%, 80%, 85%, 90%, 95%, 98%, 98% or 100% sequence identity to SEQ ID NO: 14. In one embodiment, the scFv VL domain has an amino acid sequence with at least 75%, 80%, 85%, 90%, 95%, 98%, 98% or 100% sequence identity to SEQ ID NO: 16.

[0014] In one embodiment, the scFv VH domain comprises CDR-H1 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 31, CDR-H 2 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 32, CDR-H 3 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 33. In one embodiment, the scFv VL domain comprises CDR-L1 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 34, CDR-L 2 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 35, CDR-L 3 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 36.

[0015] In another aspect, the application provides bispecific antibodies having a binding affinity to HER3 and HER2, wherein the Fab region has a binding affinity to HER3, and the scFv domain has a binding affinity to HER2.

[0016] In one embodiment, the Fab region has a binding affinity to HER3 with a KD from about 1pM to about 10pM, 1pM to about 5pM, or 1pM to about 100pM. In one embodiment, the scFv domain has a binding affinity to HER2 with a KD from about 1nM to about 1mM, 50nM to about 300nM, 1pM to about 1mM. In one embodiment, the Fab region has a binding affinity to HER3 with a KD from about 1pM to about 10pM, and the scFv domain has a binding affinity to HER2 with a KD from about 100nM to about 300nM.

[0017] In one embodiment, the Fab region comprises the VH domain having an amino acid sequence with at least 75%, 80%, 85%, 90%, 95%, 98%, 98% or 100% sequence identity to SEQ ID NO: 14. In one embodiment, the Fab region comprises the VL domain having an amino acid sequence with at least 75%, 80%, 85%, 90%, 95%, 98%, 98% or 100% sequence identity to SEQ ID NO: 16.

[0018] In one embodiment, the Fab VH domain comprises CDR-H1 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 31, CDR-H2 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 32, CDR-H3 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 33. In one embodiment, the Fab VL domain comprises CDR-L1 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 34, CDR-L2 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 35, CDR-L3 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 36.

[0019] In one embodiment, the scFv domain comprises the scFv VH domain having an amino acid sequence with at least 75%, 80%, 85%, 90%, 95%, 98%, 98% or 100% sequence identity to SEQ ID NO: 10. In one embodiment, the scFv domain comprises the scFv VL domain having an amino acid sequence with at least 75%, 80%, 85%, 90%, 95%, 98%, 98% or 100% sequence identity to SEQ ID NO: 12.

[0020] In one embodiment, the scFv VH domain comprises CDR-H1 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 25, CDR-H2 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 26, CDR-H3 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 27. In one embodiment, the scFv VL domain comprises CDR-L1 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 28, LCDR CDR-L2 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 29, CDR CDR-L3 having at least 98%, 99%, or 100% sequence identity to SEQ ID NO: 30.

[0021] The tetravalent bispecific antibody may include a heavy chain having at least 60%, 70% 80%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO: 2, 6, or 22. In one embodiment, the tetravalent bispecific antibody may include the light chain having at least 60%, 70% 80%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO: 4, 8, or 24.

[0022] In one embodiment, the Fab VH domain comprises an amino acid sequence having at least 60%, 70% 80%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO: 10, or 14. In one embodiment, the Fab VL domain comprises an amino acid sequence having at least 60%, 70% 80%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO: 12, or 16.

[0023] In one embodiment, the scFv domain comprises an amino acid sequence having at least 60%, 70% 80%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO: 18, or 20.

[0024] In one embodiment, the scFv VH comprises an amino acid sequence having at least 60%, 70% 80%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO: 10, or 14. In one embodiment, the scFv VL domain comprises an amino acid sequence having at least 60%, 70% 80%, 90%, 95%, 98%, or 99% sequence identity to SEQ ID NO: 12, or 16.

[0025] The linker may include a flexible GC linker having from about 10 amino acids to about 20 amino acids. In one embodiment, the linker comprises an amino acid sequence (Gly-Gly-Gly-Gly-Ser)_m, and wherein m is an integer of at least 3. In one embodiment, m may be 3, 4, 5, 6, 7, 8, 9, or 10. In one embodiment, m is 4.

[0026] In another aspect, the application provides nucleic acid sequences encoding the antibodies as disclosed herein. In one embodiment, the isolated nucleic acid sequence encodes the bispecific tetravalent antibodies as disclosed herein.

[0027] In a further aspect, the application provides expression vectors. In one embodiment, the expression vector comprising the isolated nucleic acid sequences encoding the bispecific antibodies.

[0028] In a further aspect, the application provides host cells. In one embodiment, the host cell comprises the isolated nucleic acid sequence encoding the bispecific antibodies. The host cells may be prokaryotic cells or eukaryotic cells.

[0029] In a further aspect, the application provides immunoconjugates. In one embodiment, the immunoconjugate includes the bispecific antibody conjugated to a drug moiety or cytotoxic agent. In one embodiment, the bispecific antibody may comprise a Fab region having a binding affinity to HER2 and a scFv domain having a binding affinity to HER3. In one embodiment, the bispecific antibody may comprise a Fab region having a binding affinity to HER3 and a scFv domain having a binding affinity to HER2. In one embodiment, the drug moiety or cytotoxic agent comprises a radioisotope, a radionuclide, a therapeutic agent, a chemotherapeutic agent, or a combination thereof. In one embodiment, the drug moiety or cytotoxic agent may be an imaging agent.

[0030] In a further aspect, the application provides pharmaceutical compositions. In one embodiment, the pharmaceutical composition comprises the bispecific tetravalent antibodies as disclosed herein or their immunoconjugates. In one embodiment, the bispecific antibody may comprise a Fab region having a binding affinity to HER2 and a scFv domain having a binding affinity to HER3. In one embodiment, the application may further include optionally a pharmaceutically acceptable carrier.

[0031] In one embodiment, the pharmaceutical composition may further include a cytotoxic agent. In one embodiment, the cytotoxic agent may be a radioisotope, a radionuclide, a therapeutic agent, a chemotherapeutic agent, or a combination thereof.

[0032] In a further aspect, the application provides methods for treating or preventing diseases. In one embodiment, the disease is a cancer. In one embodiment, the method for treating or preventing cancer in a subject includes the step of administering to the subject a pharmaceutical composition comprising the bispecific antibody, or its immunoconjugate. In one embodiment, the method for treating or preventing cancer in a subject includes the step of administering to the subject an effective amount of the bispecific antibody, or its immunoconjugate. In one

embodiment, the bispecific antibody may comprise a Fab region having a binding affinity to HER2 and a scFv domain having a binding affinity to HER3.

[0033] In one embodiment, the method of treating or preventing the cancer may include co-administering an effective amount of a therapeutic agent. In one embodiment, the therapeutic agent may be an antibody, a chemotherapy agent, an enzyme, or a combination thereof.

[0034] The cancer may be any cancer expressing HER2 or/and HER3. In one embodiment, the cancer comprises cells expressing HER3 or EGFR, and wherein the cancer comprises breast cancer, colorectal cancer, pancreatic cancer, head and neck cancer, melanoma, ovarian cancer, endometrial cancer, epidermal cancer, prostate cancer, non-small lung cell cancer, small cell lung cancer, glioma, esophageal cancer, nasopharyngeal cancer, kidney cancer, gastric cancer, liver cancer, bladder cancer, cervical cancer, brain cancer, lymphoma, leukaemia, or myeloma.

[0035] In a further aspect, the application provides methods for making the bispecific antibodies and their immunoconjugates. In one embodiment, the method for producing the bispecific antibody includes the steps of culturing a host cell such that the DNA sequence encoding the bispecific antibody is expressed, and purifying said bispecific antibody. In one embodiment, the method for producing the immunoconjugate includes the steps of conjugating the bispecific with the cytotoxic agent to provide the immunoconjugate, and purifying said immunoconjugate.

[0036] In a further aspect, the application provides a solution containing the bispecific antibodies or their immunoconjugates. In one embodiment, the solution comprises an effective concentration of the bispecific antibody, or its immunoconjugate. In one embodiment, the solution is blood plasma in a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] The foregoing and other features of this disclosure may become more fully apparent from the following description and appended claims, taken in conjunction with the accompanying drawings. Understanding that these drawings depict only several embodiments arranged in accordance with the disclosure and are, therefore, not to be considered limiting of its scope, the disclosure may be described with additional specificity and detail through use of the accompanying drawings, in which:

[0038] **Figure 1** shows a schematic diagram of bispecific tetravalent antibodies that has two light chains and two heavy chains, wherein the pairing of the heavy and light chains forms a Fab region called the first binding domain D1, and the heavy chain monomer contains a scFv binding domain (D2) linked to the N-terminal Fab (D1)(**1A**) and three anti-HER2XHER3 bispecific tetravalent antibodies and two parental bivalent monospecific antibodies (**1B**);

[0039] **Figure 2** depicts the VH-VL pairing of trastuzumab and MM-111 Fvs, showing the contact between Q39 of VH and Q38 of VL (Kabat numbering) (**2A**), the correct pairing with reversed charge orientation (VH+/VL- versus VH-/VL+) (**2B top**), and the incorrect pairing (**2B bottom**);

[0040] **Figure 3** shows the Octet binding affinity of the anti-HER2XHER3 bispecific tetravalent antibodies, SI-71X24, SI-71X25, and SI-71X33 (**3A**) for HER2 (top) and HER3 (bottom), and the

parental bivalent antibodies, SI-4C12 (trastuzumab) and SI-1C16 (MM-111) (**3B**) for HER2 (top) and HER3 (bottom);

[0041] **Figure 4** shows the anti-proliferation activity of SI-71X24 and SI-71X25 against BT-474 breast cancer cells (**4A**) and SI-71X33 against FaDu head and neck cancer cells (**4B**) in the Alamar-blue proliferation assay, revealing the unexpected pro-proliferation activity of SI-71X24;

[0042] **Figure 5** shows the pro-proliferation effects of SI-71X24 on FaDu cells (head and neck cancer), **5A**) and Oka-C-1 (lung squamous cell carcinoma), **5B**), compared to SI-71X33 in the confluence proliferation assay; and the average gMFI signal of cell binding by SI-71X25 and SI-71X24 (**5C**); and

[0043] **Figure 6** shows the average gMFI signals of antibody (10 nM of SI-71X24, SI-71X25, and the anti-CD20 Rituximab as an antibody control) in 11 HER2/HER3-expressing cancer cell lines and 1 HER2/HER3 negative lung cancer cells (COR-L279); bar graph represents the average gMFI in the anti-Fc AF647 APC channel; the dots represent replicates (n=4); titles indicate tissue of origin, cell line name, anti-HER2 antibody binding capacity, and anti-HER3 antibody binding capacity.

DETAILED DESCRIPTION

[0044] This disclosure provides a protein that has superior therapeutic properties or efficacies than currently known bispecific antibodies or similar antibodies that target one or two members of the EGFR family. In one embodiment, the antibody is configured structurally to efficiently target two members of EGFR family, HER2 and HER3, with the cancer cell proliferative activity as the endpoint. In the anti-HER2xHER3 bispecific tetravalent antibody, the unexpected finding is that the position of the HER2 and HER3 binding domains has been changed to have opposite effects on cancer cell proliferation. Typically, antigen-binding fragments function independently in a multi-specific antibody and contribute to its overall function. In the case of HER2 and HER3, the parental monospecific antibodies are known for their ability of inhibiting cancer cell proliferation, and the anti-HER2xHER3 bispecific antibody is expected to combine the anti-proliferation activity. This application disclosed a novel and surprising structure-function relationship that is pivotal in the development of highly effective antibodies, such as those described herein. These antibodies demonstrate significantly superior therapeutic advantages over existing antibodies in the prior art for the treatment of HER2/HER3-expressing cancers.

[0045] The terms “a”, “an” and “the” as used herein are defined to mean “one or more” and include the plural unless the context is inappropriate.

[0046] The terms “polypeptide”, “peptide”, and “protein”, as used herein, are interchangeable and are defined to mean a biomolecule composed of amino acids linked by a peptide bond.

[0047] The term “antigen” refers to an entity or fragment thereof which can induce an immune response in an organism, particularly an animal, more particularly a mammal including a human. The term includes immunogens and regions thereof responsible for antigenicity or antigenic determinants.

[0048] The terms “antigen- or epitope-binding portion or fragment”, “variable domain”, “variable region”, “variable region sequence”, or “binding domain” refer to fragments of an antibody that are capable of binding to an antigen (such as HER2 and HER3 in this application). These fragments may be capable of the antigen-binding function and additional functions of the intact antibody.

[0049] The terms “Fv” or “scFv” refers to the minimum antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) can recognize and bind antigen, although at a lower affinity than the entire binding site. Examples of binding fragments include, but are not limited to, a single-chain Fv fragment (scFv) consisting of variable light chain (VL) and variable heavy chain (VH) domains of a single arm of an antibody connected in a single polypeptide chain by a synthetic linker, or a Fab fragment which is a monovalent fragment consisting of VL, constant light (CL), VH and constant heavy 1 (CH1) domain. Antibody fragments can be even smaller sub-fragments and can consist of domains as small as a single CDR domain, in particular the CDR3 regions from either the VL and/or VH domains.

[0050] Antibody fragments are produced using conventional methods known to those skilled in the art. The antibody fragments can be screened for utility using the same techniques employed with intact antibodies. Purified monoclonal antibodies can be cleaved with an enzyme, such as pepsin, and subjected to HPLC gel filtration. Papain digestion of antibodies produces two identical antigen binding fragments, called “Fab” fragments, each with a single antigen binding site, and a residual “Fc” fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen. The appropriate fraction containing Fab fragments can then be collected and concentrated by membrane filtration and the like. For further description of general techniques for the isolation of active fragments of antibodies.

[0051] The term “antibody” is used in the broadest sense and specifically covers single monoclonal antibodies and/or recombinant antibodies (including agonist and antagonist antibodies), antibody compositions with polypeptopic specificity, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity. In some embodiments, the antibody may be monoclonal, polyclonal, chimeric, single chain, multi-specific or multi-effective, human and humanized antibodies, as well as active fragments thereof. Examples of active fragments of molecules that bind to known antigens include Fab, $F(ab')_2$, scFv and Fv fragments, including the products of a Fab immunoglobulin expression library and epitope-binding fragments of any of the antibodies and fragments mentioned above.

[0052] In some embodiments, antibody may include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e. molecules that contain a binding site and that immunospecifically bind an antigen. A typical antibody refers to heterotetrameric protein comprising typically of two heavy (H) chains and two light (L) chains. Each heavy chain is comprised of a heavy chain variable domain (abbreviated as VH) and a heavy chain constant domain. Each light chain is comprised of a light chain variable domain (abbreviated as VL) and a light chain constant domain. The light chains of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains. The VH and VL regions can be further subdivided into domains of hypervariable complementarity determining regions (CDR), and more conserved regions called framework regions (FR). Each variable domain (either VH or VL) is typically composed of three CDRs and four FRs, arranged in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 from amino-terminal to carboxy-terminal. Within the variable regions of the light and heavy chains there are binding regions that interacts with the antigen.

[0053] Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, and IgG4; IgA1 and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

[0054] The term “valency” refers to valency of antibody referring the number of antigenic determinants that an individual antibody molecule can bind. The valency of all antibodies is at least two, whereas “antibody affinity” refers to the tendency of an antibody to bind to a specific epitope at the surface of an antigen, i.e., to the strength of the interaction.

[0055] The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier “monoclonal” indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with

the present disclosure may be made by the hybridoma method first described by Kohler & Milstein or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). "Recombinant" means the antibodies are generated using recombinant nucleic acid techniques in exogeneous host cells. Monoclonal antibodies can be produced using various methods, including without limitation, mouse hybridoma, phage display, recombinant DNA, molecular cloning of antibodies directly from primary B cells, and antibody discovery methods. Monoclonal antibodies may include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity.

[0056] The term "humanized antibody" refers to a type of engineered antibody having its CDRs derived from a non-human donor immunoglobulin, the remaining immunoglobulin-derived parts of the molecule being derived from one (or more) human immunoglobulin(s). In addition, framework support residues may be altered to preserve binding affinity. Methods to obtain "humanized antibodies" are well known to those skilled in the art.

[0057] The terms "isolated" or "purified" refer to a biological molecule free from at least some of the components with which it naturally occurs. Either "isolated" or "purified," when used to describe the various polypeptides disclosed herein, means a polypeptide that has been identified and separated and/or recovered from a cell or cell culture from which it was expressed. Ordinarily, a purified polypeptide will be prepared by at least one purification step. An "isolated" or a "purified" antibody refers to an antibody which is substantially free of other antibodies having different antigenic a binding specificity.

[0058] The term "immunogenic" refers to substances which elicit or enhance the production of antibodies, T-cells or other reactive immune cells directed against an immunogenic agent and contribute to an immune response in humans or animals. An immune response occurs when an individual produces sufficient antibodies, T-cells and other reactive immune cells against administered immunogenic compositions of the present disclosure to moderate or alleviate the disorder to be treated. While the immunogenic response generally includes both cellular (T cell) and humoral (antibody) arms of the immune response, antibodies directed against therapeutic proteins (anti-drug antibodies, ADA) may consist of IgM, IgG, IgE, and/or IgA isotypes.

[0059] The terms "specific binding", "specifically binds to", or "is specific for a particular antigen or an epitope" means that the binding is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target.

[0060] The term “affinity” refers to a measure of the attraction between two polypeptides, such as antibody/antigen, receptor/ligand, etc. The intrinsic attraction between two polypeptides can be expressed as the binding affinity equilibrium dissociation constant (KD) of a particular interaction. A KD binding affinity constant can be measured, e.g., by Bio-Layer Interferometry, where KD is the ratio of kdis (the dissociation rate constant) to kon (the association rate constant), as $KD = kdis/kon$.

[0061] Specific binding for a particular antigen or an epitope can be exhibited, for example, by an antibody having a KD for an antigen or epitope of at least about 10^{-4} M, at least about 10^{-5} M, at least about 10^{-6} M, at least about 10^{-7} M, at least about 10^{-8} M, at least about 10^{-9} M, alternatively at least about 10^{-10} M, at least about 10^{-11} M, at least about 10^{-12} M, or greater, where KD refers to the equilibrium dissociation constant of a particular antibody-antigen interaction. Typically, an antibody that specifically binds an antigen will have a KD that is 20-, 50-, 100-, 500-, 1000-, 5,000-, 10,000- or more times greater for a control molecule relative to the antigen or epitope.

[0062] Also, specific binding for a particular antigen or an epitope can be exhibited, for example, by an antibody having a KA or Ka for an antigen or epitope of at least 20-, 50-, 100-, 500-, 1000-, 5,000-, 10,000- or more times greater for the epitope relative to a control, where KA or Ka refers to an association rate of a particular antibody-antigen interaction.

[0063] It is considered by the application that the bispecific antibody potentially has the advantage over any combination therapy, which often has greater toxicity than a single agent treatment. Bispecific agents, such as bispecific antibody as disclosed in the application, may act as a single agent targeting the same antigens as the combination therapy does but with the increased efficacy and response rate and reduced toxicity when compared to the combination therapy. In comparison to the combination therapy using two monoclonal antibodies, a bispecific antibody therapeutics can be less toxic to patients and/or more potent due to the increased binding specificity.

[0064] In one aspect, the application provides a bispecific tetravalent antibody having a N terminal and a C terminal, comprising at least two binding domains, wherein the binding domain comprises a Fab region and a scFv domain. The scFv domain may be attached to either the N terminal or the C terminal of the antibody. The Fab region and the scFv domain each independently have a binding specificity to different proteins in the EGFR family.

[0065] In some embodiments, scFv molecules described herein contain a linker of $(G_mS)_n$ that operably links the VH and VL, regardless of the V-region orientation (LH or HL). The remaining positions in the bispecific antibody may consist of a human IgG Fc or IgG null Fc heavy chain, VH-CH1-Hinge-CH2-CH3, and its corresponding kappa or lambda light chain, VL-CL. Those scFv domains were genetically linked through a linker of $(G_mS)_n$ to either N-terminal or C-terminal of IgG heavy chain, resulting in a contiguous ~ 75 kDa heavy chain monomer peptide. When co-

transfected with the appropriate light chain, the final symmetric bispecific molecule may be purified through the human IgG Fc (Protein A) and assayed to assess functional activity.

[0066] In one embodiment, the binding domain having the binding specificity to HER2 comprises Trastuzumab, which is given by intravenous infusion as a HER2 inhibitor medication used for the treatment of HER2+ breast cancer.

[0067] In one embodiment, the binding domain having the binding specificity to HER3 comprises MM-111, a bispecific HER2 and HER3 binding protein. MM-111 is a human serum albumin protein (HSA)-backed bispecific antibody fragment comprises one therapeutic binding to HER3, but its binding to HER2 alone is not sufficient to be considered as a therapeutic binding. In contrast, Trastuzumab comprises one single therapeutic binding to HER2.

[0068] The bispecific tetravalent antibodies may include an immunoglobulin G (IgG) moiety with two heavy chains and two light chains and two scFv moieties being covalently connected to either C or N terminals of the heavy or light chains via a linker, such as (Gly-Gly-Gly-Gly-Ser)_n linkers or a (Gly-Gly-Gly-Ser)_n linkers or (G_mS)_n or (SG_m)_n linkers.

[0069] It is known that having a single therapeutic agent poses significant challenges due to the selection of binding moieties and the backbone structure that may affect the binding efficiency *in vivo* and the therapeutic efficacy in patients. For example, ALM is a bispecific antibody targeting HER2/HER3, which has antiproliferative activity to tumor cells *in vitro*. But a short circulating half-life makes it an unlikely candidate drug due to rapid renal clearance. These bispecific antibodies may inhibit different receptor-mediated oncogenic signaling simultaneously therefore overcome resistance in EGFR family inhibitor or monoclonal antibody treatment.

[0070] In one embodiment, the binding domain having the binding specificity to HER2 comprises Trastuzumab. Trastuzumab inhibits HER2 phosphorylation and consequently, its downstream signaling pathways (Gijzen et al. 2010; Goel et al. 2015). But not all patients with HER2 overexpression in cancer cells can benefit from trastuzumab therapy due to the initial or acquired resistance (Luque-Cabal et al. 2016). The precise mechanism of the resistance to Trastuzumab remains unclear. Both the HER2/HER3 heterodimer and its downstream signaling play crucial roles in the tumor resistance and metastasis of HER2-positive cancer (Wang et al. 2016; Yang et al. 2017). Trastuzumab may inhibit ligand-independent HER3/HER2 interactions rather than blocking HER2 signaling. In fact, many forms of HER2-amplified metastatic cancer, which do not respond to or are eventually resistant to trastuzumab, often recover the phospho-HER3 and PI3K-Akt-mTOR downstream signals (Diaz-Serrano, et al. 2018).

[0071] HER2-targeted bispecific antibodies exhibit significant efficiency in preclinical studies for treating drug-resistant HER2-expressing malignant tumors. HER2-targeted bispecific antibodies include MM-111, ALM, PB4188, and MCLA-128 (McDonagh et al. 2012; Robinson et al. 2008; Geuijen et al. 2018; and Schram et al., 2022). MM-111 targets the HER2/HER3 heterodimer, blocks heregulin binding, and inhibits downstream signaling pathways. In contrast, Trastuzumab alone had no effect on heregulin-induced paclitaxel resistance. MM-111 has a higher-affinity

HER2 arm to target HER2-amplified tumors as compared with its HER3 arm (McDonagh et al. 2012). Upon its binding to HER2-positive cells with high avidity, MM-111 can effectively block submaximal ligand-driven proliferation but not supramaximal activation. This is because the HER2 binding moiety of MM-111 does not have any sustained effect on tumor cell growth when compared with that of Trastuzumab, which binds to a different epitope in the region IV of HER2 (Neve et al. 2001). Indeed, the combination therapy of Trastuzumab and MM-111 shows significantly greater activity than either antibody used alone (McDonagh et al. 2012).

[0072] MCLA-128 (Zenocutuzumab, Zeno) is a bispecific humanized immunoglobulin G1 (IgG1) containing two different Fab arms targeting the extracellular domains of HER2 and HER3. A recent clinical study (Schram et al., 2022) reveals that Zeno mediates durable clinical responses in a small group of patients with *NRG1* fusion-positive cancers. Having a single therapeutic agent poses significant challenges due to the selection of binding moieties and the backbone structure that may affect the binding efficiency *in vivo* and the therapeutic efficacy in patients.

[0073] The present application disclosed anti-HER2xHER3 bispecific tetravalent antibodies characterized by their symmetric configuration and tetravalent composition of binding domains.

EXAMPLES

Example 1. Anti-HER2xHER3 Bispecific Tetravalent Antibodies

[0074] A bispecific tetravalent antibody that simultaneously targets the HER2 domain IV (referred to as HER2 in this application) and HER3 was constructed by fusing a single chain Fv (scFv) binding fragment to the N-terminal of a human IgG1 heavy chain (**Figure 1A**). This anti-HER2xHER3 antibody has two heavy chains and two light chains, forming two typical bivalent Fab regions, and a pair of scFvs linked to the N-terminal of the heavy chain monomer. SI-71X25 and SI-71X24 are typical anti-HER2xHER3 tetravalent antibodies, which have the same structure as shown in **Figure 1B**. SI-71X25 and SI-71X24 differ in the binding specificity of the Fab and scFv, which are characterized by the reciprocal switch as either a Fab region or a scFv moiety. SI-71X25 features two anti-HER2 Fab regions and two anti-HER3 scFv domains, while SI-71X24 features two anti-HER3 Fab regions and two anti-HER2 scFv domains. It is worth noting that the HER2-binding variable domain was from Trastuzumab (targeting domain IV), while the HER3-binding variable domain was derived from MM-111. The two bivalent monospecific antibodies, SI-4C12 and SI-1C16, were generated in house and were the same as the two parental antibodies, Trastuzumab and MM111, respectively (**Figure 1C**).

Salt bridge engineering for antibody stabilization

[0075] The anti-HER2xHER3 bispecific tetravalent antibodies may be more highly aggregated following initial protein A purification than bispecific antibodies in some other structural formula. It was postulated that the aggregation could be due to improper pairing of VH and VL domains during expression. The examination of Trastuzumab/MM-111 Fvs reveals that most VH/VL interface residues are bulky hydrophobics. Both Trastuzumab and MM-111 Fvs contain contacts between Q39 of the VH and Q38 of the VL (Kabat numbering, **Figure 2A**). Mutating these polar

but uncharged Gln residues to charged residues could form salt bridge. Importantly, reversing the charge orientation (VH+/VL- versus VH-/VL+) could favor the correct pairing while disfavoring the incorrect chain pairing (**Figure 2B**). Used Discovery Studio to predict stability of all charge mutants to determine which are most likely to stabilize the correct pairings and destabilize the incorrect pairings. For this purpose, SI-71X33 was an optimized version of SI-71X25 (**Figure 1B**).

Generation of Anti-HER2xHER3 Antibodies

[0076] SI-71X25, SI-71X24, and SI71X33 were designed and cloned to comprise a classical IgG1 antibody fused to an scFv at the N-terminal of the heavy chain, thus forming a bispecific, bivalent-Fab antibody (**Figure 1A, Table 1**). They are characterized by the fact that both binding domains are bivalent. SI-71X25 and SI-71X33 are characterized by having two anti-HER2 Fab regions and two anti-HER3 scFv domains, while SI-71X24 is characterized by having two anti-HER3 Fab regions and two anti-HER2 scFv domains (**Figure 1B**).

[0077] The antibodies were expressed by transiently transfecting the expression plasmids for heavy and light chains in the ExpiCHO system (Thermo Fisher). Briefly, 5.6µg of each expression plasmid was brought to 2.4ml with OptiPRO SFM medium containing 33.8µg sheared herring sperm carrier DNA. 2.2ml of OptiPRO SFM medium containing 192µl Expifectamine CHO reagent was added to the DNA and incubated at room temperature for 5 minutes. The resulting mixture was then added to 60ml ExpiCHO cells at 6×10^6 cells/ml in a 250ml Erlenmeyer flask and incubated at 37°C, 5% CO₂, 150rpm. Cells were fed with 21ml ExpiCHO feed and 360 µl of CHO enhancer at 24 hours post-transfection and shifted to 32°C, 5% CO₂, 150rpm. Cells were fed again at 48 hours post-transfection with 21ml ExpiCHO feed. Culture supernatant was harvested 9 days post-transfection, spun for 20min at 7500rpm to pellet the cells and then passed through a 0.2mm filter. Expression titer was quantitated using biolayer interferometry on an Octet384 system with protein A sensors and a standard curve prepared with purified antibody.

[0078] Proteins were purified from the harvested supernatant using a 5ml MabSelect Prisma protein-A column (Cytiva). The column was equilibrated with phosphate-buffered saline. The supernatant was then passed through the column at a flow rate of 5 ml/min. The column was washed with 25ml PBS. Protein was then eluted by passing 15ml of 50 mM sodium acetate, pH 3.5 through the column. The eluted protein was immediately neutralized by addition of 1/10th volume of 1M sodium acetate, pH7.0.

[0079] Immediately after first-step protein-A or His tag purification, proteins were analyzed by analytical SEC using Waters Acquity UPLC H-Class with ACQUITY UPLC® Protein BEH SEC 200Å, 4.6mm x 150mm, 1.7 µm column. PBS (125 mM sodium phosphate, 137 mM sodium chloride, pH 6.8) was used as mobile phase for 10-minute runs at 0.3 ml/min, injecting 15 µg protein. Proteins were further purified by preparative SEC using Superdex Increase 10/300 GL column in mobile phase of 25 mM sodium acetate, 125 mM NaCl, pH 5.5, ultimately to be buffer-exchanged into 25 mM sodium acetate, 125 mM NaCl, 10% sucrose, pH 5.5. Final samples contained >95% protein of interest as assessed by analytical SEC and were used for subsequent assays.

Example 2. Characterization of Anti-HER2xHER3 Antibodies

[0080] Biolayer interferometry (Octet) binding assays were performed on an Octet384 instrument to quantify binding kinetics of antibodies to HER2 and HER3. For affinity experiments, antibody was captured to anti-human Fc (AHC) sensor tips by loading for 150 seconds at 75nM. After a 60-second baseline step, a 180-second association phase with serial dilutions (0-100 nM for HER2; 0-400 nM for HER3; 1:2 dilution factor) of His-tagged HER2 or HER3 (expressed and purified in-house) in assay buffer (phosphate-buffered saline containing 1% BSA, 0.05% Tween20) was performed, followed by a 300-second dissociation phase in assay buffer. Regeneration was achieved using 10 mM glycine, pH 1.5. Binding curves were globally fit to a 1:1 model to extract the dissociation constants, K_D , and kinetic association and dissociation rates.

[0081] Protein stability is a key parameter defined by the difference in free energy between the folded and unfolded states. For protein therapeutics, stability may impact immunogenicity, pharmacokinetics, and even efficacy, and reduction of aggregation can help to develop therapeutics that are easier to manufacture and safer for patients. In addition, expression efficiency and protein yield directly determine the cost of protein therapeutics. If proteins can be more efficiently expressed to reach higher titers and increased yield of purified protein, manufacturing costs can be reduced significantly.

[0082] After transient expression in ExpiCHO cells, titer of antibodies was quantitated using biolayer interferometry. All proteins were expressed in the ExpiCHO expression system, indicating they are stable enough to be efficiently produced. Immediately following first-step protein A purification, antibodies were evaluated for stability and aggregation by analytical size-exclusion chromatography on a Waters UPLC system (**Table 2**). SI-71X24 had a larger percentage of the protein of interest (POI) after first step purification than SI-71X25. Interestingly SI-71X33 had a lower percentage of high molecular weight aggregate than its non-salt bridge variant (SI-71X25). The data suggests that the salt bridge mutations are indeed stabilizing the correct pairing of V domains during expression and initial purification.

Example 3. Octet Binding Affinity for Anti-HER2xHER3 Antibodies

[0083] The affinities of the anti-HER2xHER antibodies to HER2 (**Table 3**) and HER3 (**Table 4**) were assessed using Biolayer interferometry. As shown in **Figure 3A**, SI-71X24 shows impaired binding to both HER2 and HER3, relative to that of SI-71X25 and SI-71X33. In addition, the K_D calculation may not be entirely accurate due to the very slow k_{off} at $1.00E-07$ in SI-71X24 (**Table 3**). It is worth noting that the HER2 and HER3 binding response of SI-71X33 is higher than that of its non-salt bridge variant (SI-71X25). These data support the notion that the salt bridge stabilizes the correct pairing of the V regions, thereby increasing the proportion of molecules with functional antigen binding domains.

Example 4. The Anti-Proliferation Effect of Anti-HER2xHER3 Antibodies

Breast cancer cells (BT-474)

[0084] To test the effect of anti-HER2xHER3 bispecific antibodies on the growth of breast cancer cells, HER2/HER3-expressing BT-474 cells were incubated with varying concentrations of test antibodies and proliferation was measured using Alamar blue.

[0085] The breast ductal carcinoma cell line BT-474 was purchased from ATCC (cat #HTB-20) and was maintained in Hybri-Care medium supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. BT-474 cells were detached from flasks with trypsin and diluted to 1.2x10⁵ cells/ml in medium + 1% FBS. 50µl of cell suspension (6000 cells) was seeded to interior 60 wells of 96-well tissue culture plates. Outer wells were filled with 300µl sterile H₂O to minimize evaporation in interior wells. Cells were allowed to adhere for 4 hours at 37°C, 5% CO₂. Antibodies to be tested were diluted to 2X final concentration in Hybri-Care medium + 1% FBS. 50µl of test antibodies were added to each well for a total volume of 100µl per well. Each antibody was tested in triplicate at the following final concentrations: 100nM, 25nM, 6.25nM, 1.563nM, 0.391nM, 0.098nM, 0.024nM, 0.006nM, and 0.0015nM. Each plate contained two antibodies at those concentrations tested in triplicate. Six control wells per plate contained cells with medium only. Immediately following addition of test compounds, 10µl alamar blue (Thermo Fisher cat# DAL1100) was added to three of the medium only control wells on each plate. Cells were incubated for 2 hours at 37°C, 5% CO₂. Following the two-hour incubation, 110µl sample from each of the control wells was removed and placed in a black, opaque 96-well plate. This plate was centrifuged for 5 minutes at 2000RPM to remove any bubbles. Fluorescence was then measured (excitation = 535nm, emission = 595nm) on a Molecular Devices FilterMax F5 microplate reader. Measured control fluorescence values (C_{start}) serve as the baseline to measure assay endpoint proliferation. Plates were returned to 37°C, 5% CO₂ for 7 days (168 hours). Following incubation, 10µl alamar blue was added to each test well as well as the other three control (medium only) wells. Following 2 hours incubation at 37°C, 5% CO₂, fluorescence was measured as described above. Endpoint control fluorescence values (C_{end}) and test well fluorescence values (T_{end}) were used to calculate the % of control proliferation using the following formula:

[0086] % of control proliferation = $((T_{end}-C_{start})/(C_{end}-C_{start}))*100$

[0087] The result shows that SI-71X25 exerts anti-proliferative effect while SI-71X24 exerted pro-proliferative activity (**Figure 4A**). This finding is unexpected because their parental antibodies, Trastuzumab and MM-111, are known inhibitors to cancer cell proliferation and tumor growth. To confirm the anti-proliferative activity of SI-71X25, SI-71X33 was tested together with its parental antibodies, Trastuzumab and MM-111 (**Figure 4B**, see details below).

Head and neck cancer cells (FaDu)

[0088] Using the protocol that measures percent confluence, SI-71X33 along with its parental antibodies, SI-4C12 (anti-HER2 mAb) and SI-1C16 (anti-HER3 mAb), was analyzed for their proliferation activity. The result showed that SI-71X33 exerts the anti-proliferative activity to FaDu head and neck cancer cells (**Figure 4B**). Data points were analysed by GraphPad Prism and

the inhibition curve was fitted by nonlinear regression [log(inhibitor) vs. response, 4 parameters] and its IC₅₀ value was calculated (**Table 5**). If SI-71X25 is considered as a potent inhibitor for treating BT-474 breast cancer cells, SI-71X33 is an optimized potent inhibitor for FaDu cells.

Implication to other cancers

[0089] To evaluate the binding activity of SI-71X25, SI-71X33, and SI-71X24 to HER2/HER3 positive cancer, a panel of cancer cell lines were used for indications including breast cancer (SK-BR-3 and BT-474), pancreatic cancer (CFPAC-1), gastric cancer (NUGC-4), lung cancer (NCI-H358), endometrial cancer (Ishikawa), liver cancer (HepG2), epidermal cancer (A-431), head and neck cancer (FaDu), another form of lung cancer (Oka-C-1), and colon cancer (Colo-320). The gMFI APC signal supposedly correlates with the density of antibody expression per cell. HER2/HER3 negative lung cancer cells, COR-L279, were used to demonstrate no detectable non-specific binding by the same assay. The results showed that the optimized SI-71X33 antibody could at least increase the binding to SK-BR-3, BT-474, CFPAC-1 and NUGC-4 cells (**Figure 6**).

[0090] Both SI-71X25 (and SI-71X33) and SI-71X24 are anti-HER2xHER3 bispecific tetravalent antibodies, characterized by the attachment of an scFv domain linked to the N-terminal of each Fab domain. The results of the study showed that swapping the positions of HER2 and HER3 binding domains did not affect the binding specificity, but the effect on the proliferation activity was diametrically opposed, with anti-HER2/D1 having proliferative activity. SI-71X25 is characterized by the pairing of anti-HER2/D1 with anti-HER3/D2, whereas SI-71x24 is characterized by the pairing of anti-HER3/D1 with anti-HER2/D2. With HER2 binding comes from the two scFv domain, the HER2-specific KD is 0.001nM. Compared with the KD values of SI-71X25, SI-71X33, and SI-4C12, the HER2 binding affinity of SI-71X24 is significantly enhanced (**Table 3**). This significant increase in HER2 binding affinity may be an important reason for the shift in the SI-71X24 from anti-proliferative activity to pro-proliferative activity. The high affinity binding level may be sufficient to activate the HER2 signaling pathway, just as ligand binding to the HER2 receptor. HER2 is a tumor-associated antigen that has been used to develop targeted therapies for the treatment of various solid tumors, such as breast cancer, ovarian cancer, uterine cancer, gastric cancer, and lung cancer. The discovery of this structure-function relationship may help in the design and implementation of antibody therapies for the treatment of HER2-expressing cancer.

TABLES

Table 1. The anti-HER2xHER3 bispecific tetravalent antibodies are homodimers of antibody monomers comprising a Fab region and a scFv domain at the C-terminal of the heavy chain.

Protein	Heavy chain	Light chain	D1 (Fab)	D2 (scFv)
SI-71X24	N-D2-linker-D1-C	VL-C λ	HER3	HER2
SI-71X25	N-D2-linker-D1-C	VL-C κ	HER2	HER3
SI-77X33	N-D2-linker-D1-C	VL-C κ	HER2 VH-Q39E/VL-Q38R	HER3 VH-Q39R/VL-Q38E

Table 2. Expression titer and stability assessment of anti-HER2xHER3 bispecific tetravalent antibodies by using analytical size-exclusion chromatography after first step protein A purification.

Protein	Titer ($\mu\text{g/ml}$)	POI %	HMW %	LMW %
SI-71X24	56.5	77.8	22.2	0.0
SI-71X25	188.7	51.9	48.1	0.0
SI-77X33	267.3	97.0	3.0	0.0

Table 3. The binding kinetics (affinity) of anti-HER2xHER3 bispecific tetravalent antibodies to HER2 in solution.

Protein	Response (nm)	KD (nM)	kon ($\text{M}^{-1}\text{s}^{-1}$)	koff (s^{-1})
SI-71X24	0.191	0.001	2.40E+05	1.00E-07
SI-71X25	0.281	1.26	1.98E+05	2.48E-04
SI-77X33	0.481	1.52	163E+05	2.48E-04
SI-4C12	0.578	1.34	2.75E+05	3.04E-04
SI-1C16	No binding	No binding	No binding	No binding

Table 4. The binding kinetics (affinity) of anti-HER2xHER3 bispecific tetravalent antibodies to HER3 in solution.

Protein	Response (nm)	KD (nM)	kon ($M^{-1}s^{-1}$)	koff (s^{-1})
SI-71X24	0.130	256	2.38E+05	6.11E-02
SI-71X25	0.237	118	3.33E+05	3.94E-02
SI-77X33	0.365	151	2.87E+05	4.34E-02
SI-4C12	No binding	No binding	No binding	No binding
SI-1C16	0.351	168	3.24E+05	5.43E-02

Table 5. Potency and efficacy parameters for BT-474 proliferation assay using anti-HER2/HER3 tetravalent antibodies.

Protein	IC50 (nM)	Efficacy (%)
SI-71X24	0.35*	-16.4
SI-71X25	4.4	30.7

* SI-71X24 is pro-proliferative - value is EC50

SEQUENCE LISTING

SEQ ID	Description
1	SI-71X24 heavy chain nucleotide sequence
2	SI-71X24 heavy chain amino acid sequence
3	SI-71X24 light chain nucleotide sequence
4	SI-71X24 light chain amino acid sequence
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6	SI-71X25 heavy chain amino acid sequence
7	SI-71X25 light chain nucleotide sequence
8	SI-71X25 light chain amino acid sequence
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10	anti-HER2 trastuzumab variable heavy (VH) domain amino acid sequence
11	anti-HER2 trastuzumab variable light (VL) domain nucleotide sequence
12	anti-HER2 trastuzumab variable light (VL) domain amino acid sequence
13	anti-HER3 MM-111 variable heavy (VH) domain nucleotide sequence
14	anti-HER3 MM-111 variable heavy (VH) domain amino acid sequence
15	anti-HER3 MM-111 variable light (VL) domain nucleotide sequence
16	anti-HER3 MM-111 variable light (VL) domain amino acid sequence
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18	anti-HER2 trastuzumab scFv amino acid sequence

19	anti-HER3 MM-111 scFv nucleotide sequence
20	anti-HER3 MM-111 scFv amino acid sequence
21	SI-71X33 heavy chain nucleotide sequence
22	SI-71X33 heavy chain amino acid sequence
23	SI-71X33 light chain nucleotide sequence
24	SI-71X33 light chain amino acid sequence
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26	anti-HER2 trastuzumab CDR-H2 amino acid sequence
27	anti-HER2 trastuzumab CDR-H3 amino acid sequence
28	anti-HER2 trastuzumab CDR-L1 amino acid sequence
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30	anti-HER2 trastuzumab CDR-L3 amino acid sequence
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SIBA077 - HER2 x HER3 symmetric sequence listing

CDR's underlined in amino acid sequences

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>SEQ ID 04 SI-71X24 light chain amino acid sequence

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SGLQADDEADYYCSSYGSSTHVI FGGGTKVTVLGLQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWK
ADSSPVKAGVETTTPSKQSNKYAASSYLSLTPEQWKSRSYSQVTHEGSTVEKTVAPTECS

>SEQ ID 05 SI-71X25 heavy chain nucleotide sequence

CAGGTGCAGCTGCAGGAGTCGGGGGAGGCCTGGTCAAGCCTGGAGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGG
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ACCGCGATGGAAGTGCAGTTACTATGTGACTCTGTGAAGGGCCGATTACCATCTCCAGAGACGACGCCAAGAAC
TCACTGTATCTGCAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACTGTGCGAGAGATCGTGGGGTGGG
CTACTTCGATCTCTGGGGCCGTGGCACCCCTGGTCACCGTGTCTAGCGGTGGAGGCGGTTCAGGCGGAGGTGGTTCCG
GCGGTGGCGGCTCCAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATCTCC
TGCACTGGAACCAGCAGTGACGTTGGTGGTTATAACTTTGTCTCCTGGTACCAACAACACCCAGGCAAAGCCCCAA
ACTCATGATCTATGATGTCACTGATCGGCCCTCAGGGGTGTCTGATCGCTTCTCCGGCTCCAAGTCTGGCAACACGG
CCTCCCTGATCATCTCTGGCCTCCAGGCTGACGACGAGGCTGATTATTACTGCAGCTCATATGGGAGCAGCAGCACT
CATGTGATTTTCGGCGGAGGGACCAAGGTGACCGTCTTAGGCGGTGGAGGGTCCGGCGGTGGTGGATCCGAGGTTCA
GCTGGTGGAGTCTGGCGGTGGCTGGTGCAGCCAGGGGGCTCACTCCGTTTGTCTGTGCAGCTTCTGGCTTCAACA
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CCTGCAGATGAACAGCCTGCGTGTCTGAGGACACTGCCCTATATTATTGTTCTAGATGGGGGACGGCTTCTATG
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GCACCCCTCCTCAAAGACACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGGT
GACGGTGTCTGGAACTCAGCGCCCTGACCAGCGCGGTGCACACCTTCCCGGCTGTCTACAGTCTCAGGACTCT
ACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAG
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GAGGTGCATAATGCCAAGACAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCTCACCGT

CCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCATCGAGA
AAACCATCTCCAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCATCCGGGAGGAGATGACC
AAGAACCAGGTGAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGG
GCAGCCGGAGAACAACATAAGACCACGCCTCCCGTGTGGACTCCGACGGCTCCTTCTTCTCTATAGCAAGCTCA
CCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTAC
ACGCAGAAGAGCCTCTCCCTGTCTCCGGT

>SEQ ID 06 SI-71X25 heavy chain amino acid sequence

QVQLQESGGGLVPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANINRDGSASYVDSVKGRFTISRDDAKN
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CTGTSSDVGGYNFVSWYQQHPGKAPKLMYDVSDRPSGVSDRFSGSKSGNTASLIISGLQADDEADYYCSSYSSST
HVIFFGGTQKVTVLGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPT
NGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGLVTVSSASTKGPSVFPL
APSSKSTSGGTAALGLVKDYFPEPVTVSWNSGALTSKVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHK
PSNTKVDKKVEPKSCDKHTHTCPPCPAPELGGPSVFLFPPPKPDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMT
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHY
TQKSLSLSPG

>SEQ ID 07 SI-71X25 light chain nucleotide sequence

GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCCTCTGTGGGCGATAGGGTCACCATCACCTGCCGTGCCAG
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CCTTCCTCTACTCTGGAGTCCCTTCTCGCTTCTCTGGCTCCAGATCTGGGACGGATTCACTCTGACCATCAGCAGT
CTGCAGCCGGAAGACTTCGCAACTTATTACTGTACAGCAACATTATACTACTCCTCCCACGTTCCGACAGGGTACCAA
GGTGGAGATCAAACGTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA
CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTC
CAATCGGGTAAC'TCCCAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCTGAC
GCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCA
CAAAGAGCTTCAACAGGGGAGAGTGT

>SEQ ID 08 SI-71X25 light chain amino acid sequence

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSRSGTDFTLTISS
LQPEDFATYYCQQHYTTPPTFGQGTKEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNAL
QSGNSQESVTEQDSKSTYLSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

>SEQ ID 09 anti-HER2 trastuzumab variable heavy (VH) domain nucleotide sequence

GAGGTTACAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGGGCTCACTCCGTTTGTCTGTGCAGCTTCTGG
CTTCAACATTAAGACACCTATATACTGGGTGCGTCAGGCCCCGGTAAGGGCCTGGAATGGGTTGCAAGGATTT
ATCCTACGAATGGTTATACTAGATATGCCGATAGCGTCAAGGGCCGTTTCACTATAAGCGCAGACACATCCAAAAAC
ACAGCCTACCTGCAGATGAACAGCCTGCGTGTGAGGACACTGCCGTCTATTATTGTTCTAGATGGGAGGGGACGG
CTTCTATGCTATGGACTACTGGGGTCAAGGAACCTGGTCACCGTCTCCTCG

>SEQ ID 10 anti-HER2 trastuzumab variable heavy (VH) domain amino acid sequence

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKN
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>SEQ ID 11 anti-HER2 trastuzumab variable light (VL) domain nucleotide sequence

GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCCTCTGTGGGCGATAGGGTCACCATCACCTGCCGTGCCAG
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CCTTCCTCTACTCTGGAGTCCCTTCTCGCTTCTCTGGCTCCAGATCTGGGACGGATTTCCTCTGACCATCAGCAGT
CTGCAGCCGGAAGACTTCGCAACTTATTACTGTACGAACATTATACTACTCCTCCCACGTTCCGACAGGGTACCAA
GGTGGAGATCAAA

>SEQ ID 12 anti-HER2 trastuzumab variable light (VL) domain nucleotide sequence
DIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSSGSRSGTDFTLTIS
LQPEDFATYYCQQHYYTTPPTFGQGTKVEIK

>SEQ ID 13 anti-HER3 MM-111 variable heavy (VH) domain nucleotide sequence
CAGGTGCAATTGCAGGAGTCGGGGGAGGCCTGGTCAAGCCTGGAGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGG
ATTCACCTTTAGTAGTTATTGGATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTGGCCAACATAA
ACCGCGATGGAAGTGCGAGTTACTATGTGGACTCTGTGAAGGGCCGATTACCATCTCCAGAGACGACGCCAAGAAC
TCACTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACTGTGCGAGAGATCGTGGGGTGGG
CTACTTCGATCTCTGGGGCCGTGGCACCCCTGGTCACCGTCTCGAGC

>SEQ ID 14 anti-HER3 MM-111 variable heavy (VH) domain amino acid sequence
QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYWMSWRQAPGKLEWVANINRDGSASYYVDSVKGRFTISRDDAKN
SLYLQMNSLRADTAVYYCARDRGVGYFDLWGRGTLVTVSS

>SEQ ID 15 anti-HER3 MM-111 variable light (VL) domain nucleotide sequence
CAGTCTGCCCTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATCTCCTGCACTGGAACCCAG
CAGTGACGTTGGTGGTTATAACTTTGTCTCCTGGTACCAACAACACCCAGGCAAAGCCCCAACTCATGATCTATG
ATGTCAGTGATCGGCCCTCAGGGGTGTCTGATCGCTTCTCCGGCTCCAAGTCTGGCAACACGGCTCCTGATCATC
TCTGGCTCCAGGCTGACGACGAGGCTGATTATTACTGCAGCTCATATGGGAGCAGCAGCACTCATGTGATTTTCGG
CGGAGGGACCAAGGTGACCGTCCTA

>SEQ ID 16 anti-HER3 MM-111 variable light (VL) domain amino acid sequence
QSALTQPASVSGSPGQSITISCTGTSSDVGGYNFVSWYQQHPGKAPKLMIDVSDRPSGVSDRFSGSKSGNTASLII
SGLQADDEADYYCSSYGSSSTHVIFGGGTKVTVL

>SEQ ID 17 anti-HER2 trastuzumab scFv nucleotide sequence
GATATCCAGATGACCCAGTCCCCGAGCTCCCTGTCCGCCTCTGTGGGCGATAGGGTCACCATCACCTGCCGTGCCAG
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CCTTCCCTACTCTGGAGTCCCTTCTCGCTTCTCTGGCTCCAGATCTGGGACGGATTCACTCTGACCATCAGCAGT
CTGCAGCCGGAAGACTTCGCAACTTATTACTGTCAGCAACATTATACTACTCCTCCCACGTTCCGGACAGGGTACCAA
GGTGGAGATCAAAGCGGTGGCGGTAGTGGGGGAGCGGTTCTGGCGGTGGAGGGTCCGGCGGTGGAGGATCAGAGG
TTCAGCTGGTGGAGTCTGGCGGTGGCCTGGTGCAGCCAGGGGGCTCACTCCGTTTGTCTGTGCAGCTTCTGGCTTC
AACATTAAAGACACCTATATACACTGGGTGCGTCAGGCCCGGGTAAGGGCCTGGAATGGGTGCAAGGATTTATCC
TACGAATGGTTATACTAGATATGCCGATAGCGTCAAGGGCCGTTTCACTATAAGCGCAGACACATCCAAAAACACAG
CCTACCTGCAGATGAACAGCCTGCGTGTGAGGACACTGCCGTCTATTATTGTTCTAGATGGGGAGGGGACGGCTTC
TATGCTATGGACTACTGGGGTCAAGGAACCCTGGTCACCGTCTCCTCG

>SEQ ID 18 anti-HER2 trastuzumab scFv amino acid sequence
DIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSSGSRSGTDFTLTIS
LQPEDFATYYCQQHYYTTPPTFGQGTKVEIKGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGF
NIKDTYIHWVRQAPGKLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRADTAVYYCSRWGGDGF
YAMDYWGQGTTLTVSS

>SEQ ID 19 anti-HER3 MM-111 scFv nucleotide sequence
CAGGTGCAGCTGCAGGAGTCGGGGGAGGCCTGGTCAAGCCTGGAGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGG
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ACCGCGATGGAAGTGCGAGTTACTATGTGGACTCTGTGAAGGGCCGATTACCATCTCCAGAGACGACGCCAAGAAC
TCACTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACTGTGCGAGAGATCGTGGGGTGGG
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CCTCCCTGATCATCTCTGGCCTCCAGGCTGACGACGAGGCTGATTATTACTGCAGCTCATATGGGAGCAGCAGCACT
CATGTGATTTTCGGCGGAGGGACCAAGGTGACCGTCCTA

>SEQ ID 20 anti-HER3 MM-111 scFv amino acid sequence

QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANINRDGSASYYVDSVKGRFTISRDDAKN
SLYLQMNLSLRAEDTAVYYCARDRGVGYFDLWGRGTLVTVSSGGGGSGGGSGGGGSQSALTQPASVSGSPGQSITIS
CTGTSSDVGGYNFVSWYQHHPGKAPKLMYDVSDRPSGVSDRFSGSKSGNTASLIISGLQADDEADYYCSSYSSST
HVIFGGGTKVTVL

>SEQ ID 21 SI-71X33 heavy chain nucleotide sequence

CAGGTGCAGCTGCAGGAGTCGGGGGGAGGCCTGGTCAAGCCTGGAGGGTCCCTGAGACTCTCCTGTGCAG
CCTCTGGATTACACCTTTAGTAGTTATTGGATGAGCTGGGTCCGCAGAGCTCCAGGGAAGGGGCTGGAGTG
GGTGGCCAACATAAACC CGGATGGAAGTGCAGTTACTATGTGGACTCTGTGAAGGGCCGATTACCATC
TCCAGAGACGACGCCAAGAACTCACTGTATCTGCAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGT
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TAGCGGTGGAGGCGGTTTCAGGCGGAGGTGGTTCGGCGGTGGCGGCTCCAGTCTGCCCTGACTCAGCCT
GCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATCTCCTGCACTGGAACCAGCAGTGACGTTGGTG
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TGATCGGCCCTCAGGGGTCTCTGATCGCTTCTCCGGCTCCAAGTCTGGCAACACGGCCTCCCTGATCATC
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TTCAACATTAAAGACACCTATATACACTGGGTGCGTGAGGCCCCGGGTAAAGGCTGGAATGGGTTGCAA
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CACATCCAAAAACACAGCCTACCTGCAGATGAACAGCCTGCGTGCTGAGGACACTGCCGTCTATTATTGT
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CCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAGCAACACCAA
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CCTGCCCCCATCCCGGATGAGCTGACCAAGAACCAGGTGACCTGACCTGCTGGTCAAAGGCTTCTAT
CCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACCTACAAGACCACGCCTCCCG
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TCTCCGGGT

>SEQ ID 22 SI-71X33 heavy chain amino acid sequence

QVQLQESGGGLVKPGGSLRLSCAASGFTFSSYWMSWVRAPGKGLEWVANINRDGSASYYVDSVKGRFTISR
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ASVSGSPGQSITISCTGTSSDVGGYNFVSWYQHHPGKAPKLMYDVSDRPSGVSDRFSGSKSGNTASLIIS
SGLQADDEADYYCSSYSSSTHVIFGGGTKVTVLGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCAASG
FNIKDTYIHWVREAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNLSLRAEDTAVYYC
SRWGGDGFYAMDYWGQTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVKPKSCDKHTCTPPCPAPE
LLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV
SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY
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SPG

>SEQ ID 23 SI-71X33 light chain nucleotide sequence
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GATTTACTCGGCATCCTTCCTCTACTCTGGAGTCCCTTCTCGCTTCTCTGGCTCCAGATCTGGGACGGAT
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CTTCCCCGCATCTGATGAGCAGTTGAAATCTGGAACGCTCTGTTGTGTGCCTGCTGAATAACTTCTAT
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CAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGA
GAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAAC
AGGGGAGAGTGT

>SEQ ID 24 SI-71X33 light chain amino acid sequence
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQRKPGKAPKLLIYSASFLYSGVPSRFSGRSGTD
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PREAKVQWKVDNALQSGNSQESVTEQDSKSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN
RGEK

>SEQ ID 25 anti-HER2 trastuzumab CDR-H1 amino acid sequence
DTYIH

>SEQ ID 26 anti-HER2 trastuzumab CDR-H2 amino acid sequence
RIYPTNGYTRYADSVKG

>SEQ ID 27 anti-HER2 trastuzumab CDR-H3 amino acid sequence
WGGDGFYAMDY

>SEQ ID 28 anti-HER2 trastuzumab CDR-L1 amino acid sequence
RASQDVNTAVA

>SEQ ID 29 anti-HER2 trastuzumab CDR-L2 amino acid sequence
SASFLYS

>SEQ ID 30 anti-HER2 trastuzumab CDR-L3 amino acid sequence
QQHYTTPPT

>SEQ ID 31 anti-HER3 MM-111 CDR-H1 amino acid sequence
SYWMS

>SEQ ID 32 anti-HER3 MM-111 CDR-H2 amino acid sequence
NINRDGSASYVDSVKG

>SEQ ID 33 anti-HER3 MM-111 CDR-H3 amino acid sequence
DRGVGYFDL

>SEQ ID 34 anti-HER3 MM-111 CDR-L1 amino acid sequence
TGTSSDVGGYNFVS

>SEQ ID 35 anti-HER3 MM-111 CDR-L2 amino acid sequence
DVSDRPS

>SEQ ID 36 anti-HER3 MM-111 CDR-L3 amino acid sequence
SSYGSSSTHVI

Reference

Each of the references cited herein is incorporated by reference in its entirety. The incorporation of these references is not to be construed as an admission that any of the material constitutes prior art to the present application.

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7. R M Neve ¹, U B Nielsen, D B Kirpotin, M A Poul, J D Marks, C C Benz. Biological effects of anti-ErbB2 single chain antibodies selected for internalizing function *Biochem Biophys Res Commun*. 2001, 280(1):274-9.
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11. Cetuximab: https://www.ema.europa.eu/en/documents/scientific-discussion/erbitux-epar-scientific-discussion_en.pdf
12. Panitumumab: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6763619/#:~:text=Panitumumab%20binds%20EGFR%20with%20an,whether%20this%20characteristic%20is%20favorable>
13. Nimotuzumab: <https://www.nature.com/articles/s41598-019-57279-w/tables/1>
14. Trastuzumab: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6244757/>
15. Pertuzumab: <https://www.tga.gov.au/sites/default/files/auspar-pertuzumab-131001.pdf>

16. Patritumab: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5058629/>
17. MM-121: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3478453/>
18. MM-111: <https://pubmed.ncbi.nlm.nih.gov/22248472/>
19. 2in1: <https://ars.els-cdn.com/content/image/1-s2.0-S1535610811003515-mmc1.pdf>
20. SI-1X6.3(C3): US15/119,694.
21. https://cancerres.aacrjournals.org/content/64/7_Supplement/163.4.short
22. <https://aacrjournals.org/cancerdiscovery/article/12/5/1233/694554/Zenocutuzumab-a-HER2/HER3-Bispecific-Antibody-Is>

CLAIMS

What is claimed is:

1. A bispecific antibody having a binding affinity to HER2 and HER3, comprising,
a heavy chain (HC) having a heavy chain variable (VH) domain, wherein the heavy chain has a N-terminal and a C-terminal,
a light chain (LC) having a light chain variable (VL) domain,
wherein the VL domain and the VH domain form a Fab region, and
a scFv domain having a scFv light chain variable (VL) domain and a scFv heavy chain variable (VH) domain, wherein each scFv domain is linked to each heavy chain at the N-terminal through a linker.
2. The bispecific antibody of Claim 1, wherein the Fab region has a binding affinity to HER2, and the scFv domain has a binding affinity to HER3.
3. The bispecific antibody of Claim 2, wherein the Fab region has a binding affinity to HER2 with a KD from about 0.5nM to about 10nM, and wherein the scFv domain has a binding affinity to HER3 with a KD from about 100nM to about 200nM.
4. The bispecific antibody of Claim 2, wherein the Fab region comprises the VH domain having an amino acid sequence with at least 98% sequence identity to SEQ ID NO: 10 and the VL domain having an amino acid sequence with at least 98% sequence identity to SEQ ID NO: 12.
5. The bispecific antibody of Claim 2, wherein the VH domain comprises CDR-H1 having a sequence identity to SEQ ID NO: 25, CDR-H2 having a sequence identity to SEQ ID NO: 26, CDR-H3 having a sequence identity to SEQ ID NO: 27, and wherein the VL domain comprises CDR-L1 having a sequence identity to SEQ ID NO: 28, CDR-L2 having a sequence identity to SEQ ID NO: 29, CDR-L3 having a sequence identity to SEQ ID NO: 30.
6. The bispecific antibody of Claim 2, wherein the scFv domain comprises the scFv VH domain having an amino acid sequence with at least 98% sequence identity to SEQ ID NO: 14 and the scFv VL domain having an amino acid sequence with at least 98% sequence identity to SEQ ID NO: 16.
7. The bispecific antibody of Claim 2, wherein the scFv VH domain comprises CDR-H1 having a sequence identity to SEQ ID NO: 31, CDR-H 2 having a sequence identity to SEQ ID NO: 32, CDR-H 3 having a sequence identity to SEQ ID NO: 33, and wherein the scFv VL domain comprises CDR-L1 having a sequence identity to SEQ ID NO: 34, CDR-L 2 having a sequence identity to SEQ ID NO: 35, CDR-L 3 having a sequence identity to SEQ ID NO: 36.
8. The bispecific antibody of Claim 1, wherein the Fab region has a binding affinity to HER3, and the scFv domain has a binding affinity to HER2.

9. The bispecific antibody of Claim 8, wherein the Fab region has a binding affinity to HER3 with a KD from about 1pM to about 10pM, and wherein the scFv domain has a binding affinity to HER2 with a KD from about 100nM to about 300nM.

10. The bispecific antibody of Claim 8, wherein the Fab region comprises the VH domain having an amino acid sequence with at least 98% sequence identity to SEQ ID NO: 14 and the VL domain having an amino acid sequence with at least 98% sequence identity to SEQ ID NO: 16.

11. The bispecific antibody of Claim 8, wherein the VH domain comprises CDR-H1 having a sequence identity to SEQ ID NO: 31, CDR-H2 having a sequence identity to SEQ ID NO: 32, CDR-H3 having a sequence identity to SEQ ID NO: 33, and wherein the VL domain comprises CDR-L1 having a sequence identity to SEQ ID NO: 34, CDR-L2 having a sequence identity to SEQ ID NO: 35, CDR-L3 having a sequence identity to SEQ ID NO: 36.

12. The bispecific antibody of Claim 8, wherein the scFv domain comprises the scFv VH domain having an amino acid sequence with at least 98% sequence identity to SEQ ID NO: 10 and the scFv VL domain having an amino acid sequence with at least 98% sequence identity to SEQ ID NO: 12.

13. The bispecific antibody of Claim 8, wherein the scFv VH domain comprises CDR-H1 having a sequence identity to SEQ ID NO: 25, CDR-H2 having a sequence identity to SEQ ID NO: 26, CDR-H3 having a sequence identity to SEQ ID NO: 27, and wherein the scFv VL domain comprises CDR-L1 having a sequence identity to SEQ ID NO: 28, LCDR CDR-L2 having a sequence identity to SEQ ID NO: 29, CDR CDR-L3 having a sequence identity to SEQ ID NO: 30.

14. The bispecific antibody of Claim 1, wherein the heavy chain comprises an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 2, 6, or 22.

15. The bispecific antibody of Claim 1, wherein the light chain comprises an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 4, 8, or 24.

16. The bispecific antibody of Claim 1, wherein the VH domain comprises an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 10, or 14, and wherein the VL domain comprises an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 12, or 16.

17. The bispecific antibody of Claim 1, wherein the scFv domain comprises an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 18, or 20.

18. The bispecific antibody of Claim 1, wherein the scFv VH comprises an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 10, or 14, and wherein the scFv VL domain comprises an amino acid sequence having at least 98% sequence identity to SEQ ID NO: 12, or 16.

19. The bispecific antibody of Claim 1, wherein the linker comprises an amino acid sequence (Gly-Gly-Gly-Gly-Ser)_m, and wherein m is an integer of at least 3.

20. An isolated nucleic acid sequence encoding the bispecific antibody of Claim 1.

21. An expression vector comprising the isolated nucleic acid sequences of Claim 20.

22. A host cell comprising the isolated nucleic acid sequence of Claim 20.

23. A pharmaceutical composition, comprising the bispecific antibody of Claim 2 and optionally a pharmaceutically acceptable carrier.

24. The pharmaceutical composition of Claim 23, further comprising a cytotoxic agent, wherein the cytotoxic agent comprises a radioisotope, a radionuclide, a therapeutic agent, a chemotherapeutic agent, or a combination thereof.

25. An immunoconjugate comprising the bispecific antibody of Claim 2 conjugated to a cytotoxic agent, wherein the cytotoxic agent comprises a radioisotope, a radionuclide, a therapeutic agent, a chemotherapeutic agent, or a combination thereof.

26. A pharmaceutical composition, comprising the immunoconjugate of Claim 25 and optionally a pharmaceutically acceptable carrier.

27. A method for treating or preventing cancer in a subject, said method comprising administering to the subject an effective amount of the bispecific antibody of Claim 2, or the immunoconjugate of Claim 25.

28. The method of Claim 27 further comprises co-administering an effective amount of a therapeutic agent, wherein the therapeutic agent comprises an antibody, a chemotherapy agent, an enzyme, or a combination thereof.

29. The method of Claim 27, wherein the cancer comprises cells expressing HER3 or EGFR, and wherein the cancer comprises breast cancer, colorectal cancer, pancreatic cancer, head and neck cancer, melanoma, ovarian cancer, endometrial cancer, epidermal cancer, prostate cancer, non-small lung cell cancer, small cell lung cancer, glioma, esophageal cancer, nasopharyngeal cancer, kidney cancer, gastric cancer, liver cancer, bladder cancer, cervical cancer, brain cancer, lymphoma, leukaemia, or myeloma.

30. A method for producing the bispecific antibody of Claim 1, comprising
culturing a host cell such that the DNA sequence encoding the bispecific antibody of Claim 1 is expressed, and
purifying said bispecific antibody.

31. A method for producing the immunoconjugate of Claim 25, comprising,
conjugating the bispecific antibody of Claim 2 with the cytotoxic agent to provide the immunoconjugate, and
purifying said immunoconjugate.

32. A solution comprising an effective concentration of the bispecific antibody of Claim 1, or the immunoconjugate of Claim 25, wherein the solution is blood plasma in a subject.