

MULTISPECIFIC ANTIGEN-BINDING MOLECULES AND USES THEREOF

FIELD

[0001] The present invention relates to the field of therapeutic proteins, and in particular, to the field of therapeutic proteins that are capable of inactivating, blocking, attenuating, eliminating and/or reducing the concentration of one or more target molecules *in vitro* or *in vivo*.

BACKGROUND

[0002] Therapeutic treatments often require the inactivation or blocking of one or more target molecules that act on or in the vicinity of a cell. For example, antibody-based therapeutics often function by binding to a particular antigen expressed on the surface of a cell, or to a soluble ligand, thereby interfering with the antigen's normal biological activity. Antibodies and other binding constructs directed against various cytokines (e.g., IL-1, IL-4, IL-6, IL-13, IL-22, IL-25, IL-33, etc.), or their respective receptors, for instance, have been shown to be useful in treating a wide array of human ailments and diseases. Therapeutic agents of this type typically function by blocking the interaction between the cytokine and its receptor in order to attenuate or inhibit cellular signaling. In certain contexts, however, it would be therapeutically beneficial to inactivate or inhibit the activity of a target molecule in a manner that does not necessarily involve blocking its physical interaction with another component. One way in which such non-blocking attenuation of a target molecule could be achieved would be to reduce the extracellular or cell surface concentration of the target molecule. Although genetic and nucleic acid-based strategies for reducing the amount or concentration of a given target molecule are known in the art, such strategies are often fraught with substantial technical complications and unintended side effects in therapeutic settings. Accordingly, alternative non-blocking strategies are needed to facilitate the inactivation or attenuation of various target molecules for therapeutic purposes.

BRIEF SUMMARY

[0003] The present invention is based, at least in part, on the concept of attenuating or inactivating a target molecule by facilitating or bringing about a physical linkage between the target molecule and an internalizing effector protein. Through this type of physical intermolecular linkage, the target molecule can be forced to be internalized into the cell along with the internalizing effector protein, and processed by the intracellular degradative machinery, or otherwise attenuated, sequestered, or inactivated. This mechanism represents a novel and inventive strategy for inactivating or attenuating the activity of a target molecule without necessarily blocking the

interaction between the target molecule and its binding partners.

[0004] Accordingly, the present invention provides a multispecific antigen-binding molecule that is capable of simultaneously binding a target molecule (T) and an internalizing effector protein (E). More specifically, the present invention provides a multispecific antigen-binding molecule comprising a first antigen-binding domain (D1), and a second antigen-binding domain (D2), wherein D1 specifically binds T, and D2 specifically binds E, and wherein the simultaneous binding of T and E by the multispecific antigen-binding molecule attenuates the activity of T to a greater extent than the binding of T by D1 alone. The enhanced attenuation of the activity of T may be due to the forced internalization/degradation of T through its physical linkage to E; however, other mechanisms of action are possible and are not excluded from the scope of the present invention.

[0005] In addition, the present invention provides methods of using the multispecific antigen-binding molecule to inactivate or attenuate the activity of a target molecule (T). In particular, the present invention provides a method for inactivating or attenuating the activity of T by contacting T and an internalizing effector protein (E) with a multispecific antigen-binding molecule, wherein the multispecific antigen-binding molecule comprises a first antigen-binding domain (D1) and a second antigen-binding domain (D2), wherein D1 specifically binds T, and wherein D2 specifically binds E; and wherein the simultaneous binding of T and E by the multispecific antigen-binding molecule attenuates the activity of T to a greater extent than the binding of T by D1 alone.

[0006] In certain embodiments of the present invention, D1 and/or D2 comprise(s) at least one antibody variable region. For example, the multispecific antigen-binding molecule can, in some embodiments, be a bispecific antibody, wherein D1 comprises an antibody heavy and light chain variable region (HCVR/LCVR) pair that specifically binds T, and wherein D2 comprises an HCVR/LCVR pair that specifically binds E. Alternatively, D1 and/or D2 may comprise a peptide or polypeptide that specifically interacts with the target molecule (T) and/or the internalizing effector protein (E). For example, if the target molecule is a cell surface receptor, then D1 may comprise a portion of a ligand that specifically binds the cell surface receptor target molecule. Similarly, if the internalizing effector protein is a cell surface internalizing receptor, then D2 may comprise a portion of a ligand that specifically binds the cell surface internalizing receptor. In certain embodiments, D1 comprises an antibody variable region that specifically binds T, and D2 comprises a peptide or polypeptide that specifically binds E. In yet other embodiments, D1 comprises a peptide or polypeptide that specifically binds T, and D2 comprises an antibody variable region that specifically binds E. In any configuration, however, the end result is that T and E are capable of being physically linked, directly or indirectly, via the simultaneous binding of T and E by a multispecific

antigen-binding molecule.

[0007] Other embodiments will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[0008] **Figure 1 (panels A-D)** provides schematic representations of four general exemplary mechanisms of action for the multispecific antigen binding molecules of the present invention. In each illustrated configuration D1 is a first antigen-binding domain; D2 is a second antigen binding domain; T is a target molecule; E is an internalizing effector protein; and R is a receptor which internalizes upon binding E. **Panel A** depicts the situation in which both T and E are membrane-associated. **Panel B** depicts the situation in which T is soluble and E is membrane-associated. **Panel C** depicts the situation in which T is membrane-associated and E is a soluble protein that interacts with, and is internalized into the cell via the interaction of E and R. **Panel D** depicts the situation in which T is soluble and E is a soluble protein that interacts with, and is internalized into the cell via the interaction of E and R.

[0009] **Figure 2** shows the results of an immunoprecipitation experiment performed on two different cells (Cell-1 expressing FcγR1 alone, and Cell-2 expressing Krm2 and FcγR1) following incubation for different amounts of time (0, 15, 30 and 60 minutes) with a DKK1-mFc multispecific antigen-binding molecule.

[0010] **Figure 3** shows the relative IL-4-induced luminescence produced by Stat6-luc reporter HEK293 cells in the presence and absence of an anti-IL-4R/anti-CD63 multispecific antigen binding protein ("ab conjugate") or control constructs ("control 1" and "control 2") at various concentrations of IL-4.

[0011] **Figure 4** shows the results of an experiment carried out in the same manner as the experiment shown in Figure 3, except that CD63 expression was significantly reduced in the reporter cell line by an siRNA directed against CD63.

[0012] **Figure 5** shows the results of an experiment carried out in a similar manner as the experiments shown in Figures 3 and 4, except that the reporter cells were incubated with the multispecific antigen binding protein ("Ab conjugate") or control constructs ("control 1" and "control 2") at time zero (panels A and B), for 2 hours (panels C and D) or overnight (panels E and F) prior to the addition of IL-4 ligand. The top row of bar graphs (panels A, C, and E) represent the results of experiments conducted in cells expressing normal levels of CD63 ("untransfected"), while the bottom row of bar graphs (panels B, D, and F) represents the results of experiments conducted in

cells in which CD63 expression was significantly reduced in the reporter cell line by an siRNA directed against CD63.

[0013] **Figure 6** shows the results of an experiment carried out in a similar manner as the experiments shown in Figures 3 and 4, except that the reporter cells were incubated with the anti-IL-4R/anti-CD63 multispecific antigen binding protein ("Ab conjugate") or control constructs ("control 1" and "control 2") for 15 minutes (panel A), 30 minutes (panel B), 1 hour (panel C) or 2 hours (panel D) prior to the addition of IL-4 ligand.

[0014] **Figure 7** shows the results of an experiment in which Stat6-luc reporter cells were treated with 10 pM IL-4 in the presence of various dilutions of an anti-IL-4R x anti-CD63 bispecific antibody ("bispecific"), or control constructs (anti-IL-4R monospecific, or mock bispecific that only binds IL-4R).

[0015] **Figure 8** shows the results of experiments in which HEK293 cells were treated with a SOST construct labeled with a myc tag and a pH-sensitive label (that produces a fluorescent signal at low pH), along with the various mono-specific and bispecific antibodies as shown. Results are expressed in terms of number of fluorescent spots (*i.e.*, labeled vesicles) per cell. **Panel A** shows the results following incubation on ice for 3 hours, **panel B** shows the results following 1 hour incubation at 37°C, and **panel C** shows the results following 3 hours incubation at 37°C.

[0016] **Figure 9** shows the results of experiments in which HEK293 cells were treated with fluorescently-labeled lipopolysaccharide (LPS) from *E. coli* (**Panel A**) or *S. minnesota* (**Panel B**), along with an anti-CD63 x anti-LPS bispecific antibody, control antibodies, or LPS only, for various times, followed by quenching of non-internalized (*i.e.*, surface bound) fluorophore. Fluorescent signal therefore reflects internalized LPS under the various conditions shown. Results are expressed in terms of number of fluorescent spots (*i.e.*, labeled vesicles) per cell.

[0017] **Figure 10** shows the mean fluorescence in arbitrary units from Alexa488 labeled FeID1-mycv-myc-his. Blue histograms depict cell surface label. Red histograms depict internalized label. Group 1 on the X-axis represents cells treated with anti-HLA-B x anti-FeID1 bispecific antibody; group 2 represents cells treated with anti-HLA-B parental bivalent monospecific antibody; group 3 represents treatment with IgG isotype controls. **Panel A** shows binding and internalization of FeID1-mmh-488 by C1Rneo B-lymphoblastoid cells that do not express MHC1. **Panel B** shows binding and internalization of FeID1-mmh-488 by C1Rneo B-lymphoblastoid cells that express MHC1.

[0018] **Figure 11** depicts internalization of prolactin receptor (PRLR) and HER2 on T47D cells by measuring the amount of PRLR or HER2 remaining on the cell surface at 0 to 60 minutes after

transferring cells from 4°C to 37°C. Percent surface receptor remaining over time is shown. Squares represent PRLR and triangles represent HER2.

[0019] **Figure 12** depicts co-localization of prolactin receptor (PRLR) and HER2 with lysosomes in T47D cells. Percent receptor associated with lysosomes over time is shown. Squares represent PRLR and triangles represent HER2.

[0020] **Figure 13** depicts schematically the PRLR and HER2 truncations and chimeras.

[0021] **Figure 14** depicts fluorescent micrographs of HEK293 cells expressing various PRLR and HER2 constructs and chimeras. The subpanels to the left in each panel depict cells at 4°C before internalization. The subpanels to the right in each panel depict cells after one hour at 37°C. Panel A depicts cells expressing full-length PRLR (PRLR FL). Panel B depicts cells expressing full-length HER2 (PRLR FL). Panel C depicts cells expressing the PRLRectoHER2cytoTM construct. Panel D depicts cells expressing the HER2ectoPRLRcytoTM construct.

[0022] **Figure 15** depicts fluorescent micrographs of HEK293 cells expressing various PRLR constructs and truncations. Panel A depicts cells expressing full length PRLR, stained for PRLR, after 1 hour at 4°C. Panel B depicts cells expressing full length PRLR, stained for PRLR, after 1 hour at 37°C. Panel C depicts cells expressing truncated PRLR with 42 residues of the cytoplasmic domain, stained for PRLR, after 1 hour at 4°C. Panel D depicts cells expressing truncated PRLR with 42 residues of the cytoplasmic domain, stained for PRLR, after 1 hour at 37°C. Panel E depicts cells expressing truncated PRLR with only 21 residues of the cytoplasmic domain, stained for PRLR, after 1 hour at 4°C. Panel F depicts cells expressing truncated PRLR with only 21 residues of the cytoplasmic domain, stained for PRLR, after 1 hour at 37°C.

[0023] **Figure 16** depicts western blots of cell lysates of cells expressing full length PRLR (panel A), cells expressing truncated PRLR with 42 residues of the cytoplasmic domain remaining (panel B), and cells expressing truncated PRLR with 21 residues of the cytoplasmic domain remaining (panel C). The upper subpanels are stained with anti-PRLR antibody. The lower subpanels are stained for beta-actin to control for loading.

[0024] **Figure 17** depicts the percentage of cells in early mitosis versus levels of surface expression of PRLR or HER2 or PRLRectoHER2cytoTM (Panel A), PRLR or HER2ectoPRLRcytoTM (Panel B) after treatment with either PRLR-DM1 or HER2-DM1.

[0025] **Figure 18** depicts a western blot of whole cell lysates expressing various PRLR constructs, truncations, and substitutions at 0 hours and 4 hours post CHX treatment. The upper panel is stained for PRLR, and the lower panel is stained for beta-actin to control for loading.

[0026] **Figure 19** depicts a western blot of whole cell lysates of HEK 293 cells induced to express full length PRLR at 0 hours, 1 hour, 2 hours, and 4 hours post CHX treatment. The upper panel is stained for HER2, and the lower panel is stained for PRLR.

[0027] **Figure 20** depicts a western blot of whole cell lysates of HEK 293 cells induced to express the cytoplasmic truncation form of PRLR at 0 hours, 2 hours, and 4 hours post CHX treatment. The upper panel is stained for HER2, and the lower panel is stained for PRLR.

[0028] **Figure 21** depicts co-localization of HER2 with lysosomes in T47D cells treated with the HER2 x PRLR bispecific antibody. Percent lysosomes associated with the HER2 receptor is shown over time. Squares represent cells treated with the HER2 x PRLR bispecific antibody and triangles represent cells treated with non-binding control antibodies.

[0029] **Figure 22** is a histogram depicting the percentage of cell cycle arrested T47D/HER2 cells (Y-axis) treated with 1 nM, 10 nM, or 30 nM of PRLR-ADC (A), HER2-ADC plus HER2 x PRLR bispecific antibody (B), HER2-ADC alone (C), non-binding control ADC (D), or no treatment (E).

[0030] **Figure 23** is a dot blot depicting percentage of viable T47D cells expressing HER2 *versus* increasing amount of drug: PRLR-ADC (A; squares), HER2-ADC plus HER2 x PRLR bispecific antibody (B; diamonds), HER2-ADC alone (C; up triangles), non-binding control ADC (D; circles), or non-binding control ADC plus HER2 x PRLR bispecific antibody (E; down triangles).

[0031] **Figure 24** shows a western blot panel of mouse serum probed with anti-FcID1 antibody at various times (15 minutes, 6 hours, 1 day, 2 days, 3 days, 4 days and 6 days) post-treatment with an anti-HLAB x anti-FcID1 bispecific antibody, phosphate buffered saline, anti-FcID1 bivalent monospecific antibody, and anti-HLAB bivalent monospecific antibody.

[0032] **Figure 25** shows a histogram depicting the proportion of FcID1-Fc cleared from mouse serum above baseline levels and normalized to the initial value (Y-axis). Item 1 depicts treatment with anti-HLAB x anti-FcID1 bispecific antibody; item 2 depicts treatment with anti-FcID1 bivalent monospecific antibody; and item 3 depicts treatment with anti-HLAB bivalent monospecific antibody (X-axis).

[0033] **Figure 26** is a histogram depicting pHrodo®-hHJV-mmh uptake into HEK293 cells. Y-axis depicts integrated intensity (arbitrary units) of pHrodo® signal. At the X-axis, Anti-Myc::PCSK9 depicts the effect on HJV uptake due to no antibody (open bar), α -Myc::PCSK9FL (solid filled bar), α -Myc::PCSK9LC (horizontal line filled bar), and α -Myc::PCSK9SC (stipple filled bar); Anti-HJV::PCSK9 depicts the effect on HJV uptake due to no antibody (open bar), α -HJV-N::PCSK9FL (solid filled bar), α -HJV-N::PCSK9LC (horizontal line filled bar), and α -HJV-N::PCSK9SC (stipple

filled bar); and no antibody controls for background uptake.

[0034] Figure 27 is a dot blot showing serum iron levels in micrograms per deciliter serum (Y-axis) one week after treatment of hHLAB transgenic mice with anti-HJV-blocking bivalent monospecific antibody (1); anti-Myc::PCSK9 full length fusion protein (2); anti-HJV-non-blocking bivalent monospecific antibody (3); and anti-HJV-non-blocker::PCSK9 full length fusion protein (4).

DETAILED DESCRIPTION

[0035] Before the present invention is described, it is to be understood that this invention is not limited to particular methods and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0036] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. As used herein, the term "about," when used in reference to a particular recited numerical value, means that the value may vary from the recited value by no more than 1%. For example, as used herein, the expression "about 100" includes 99 and 101 and all values in between (e.g., 99.1, 99.2, 99.3, 99.4, etc.).

[0037] Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All patents, applications and non-patent publications mentioned in this specification are incorporated herein by reference in their entireties.

MULTISPECIFIC ANTIGEN-BINDING MOLECULES

[0038] The present inventors have surprisingly discovered that a target molecule's activity can be attenuated by linking the target molecule to an internalizing effector protein via a multispecific antigen-binding molecule.

[0039] Accordingly, the present invention provides multispecific antigen binding molecules comprising a first antigen-binding domain (also referred to herein as "D1"), and a second antigen-binding domain (also referred to herein as "D2"). D1 and D2 each bind different molecules. D1 specifically binds a "target molecule". The target molecule is also referred to herein as "T". D2 specifically binds an "internalizing effector protein". The internalizing effector protein is also referred to herein as "E". According to the present invention, the simultaneous binding of T and E by the

multispecific antigen-binding molecule attenuates the activity of T to a greater extent than the binding of T by D1 alone. As used herein, the expression "simultaneous binding," in the context of a multispecific antigen-binding molecule, means that the multispecific antigen-binding molecule is capable of contacting both a target molecule (T) and an internalizing effector protein (E) for at least some period of time under physiologically relevant conditions to facilitate the physical linkage between T and E. Binding of the multispecific antigen-binding molecule to the T and E components may be sequential; e.g., the multispecific antigen-binding molecule may first bind T and then bind E, or it may first bind E first and then bind T. In any event, so long as T and E are both bound by the multispecific antigen-binding molecule for some period of time (regardless of the sequential order of binding), the multispecific antigen-binding molecule will be deemed to "simultaneously bind" T and E for purposes of the present disclosure. Without being bound by theory, the enhanced inactivation of T is believed to be caused by the internalization and degradative rerouting of T within a cell due to its physical linkage to E. The multispecific antigen-binding molecules of the present invention are thus useful for inactivating and/or reducing the activity and/or extracellular concentration of a target molecule without directly blocking or antagonizing the function of the target molecule.

[0040] According to the present invention, a multispecific antigen-binding molecule can be a single multifunctional polypeptide, or it can be a multimeric complex of two or more polypeptides that are covalently or non-covalently associated with one another. As will be made evident by the present disclosure, any antigen binding construct which has the ability to simultaneously bind a T and an E molecule is regarded as a multispecific antigen-binding molecule. Any of the multispecific antigen-binding molecules of the invention, or variants thereof, may be constructed using standard molecular biological techniques (e.g., recombinant DNA and protein expression technology), as will be known to a person of ordinary skill in the art.

ANTIGEN-BINDING DOMAINS

[0041] The multispecific antigen-binding molecules of the present invention comprise at least two separate antigen-binding domains (D1 and D2). As used herein, the expression "antigen-binding domain" means any peptide, polypeptide, nucleic acid molecule, scaffold-type molecule, peptide display molecule, or polypeptide-containing construct that is capable of specifically binding a particular antigen of interest. The term "specifically binds" or the like, as used herein, means that the antigen-binding domain forms a complex with a particular antigen characterized by a dissociation constant (K_D) of 500 pM or less, and does not bind other unrelated antigens under ordinary test conditions. "Unrelated antigens" are proteins, peptides or polypeptides that have less than 95% amino acid identity to one another.

[0042] Exemplary categories of antigen-binding domains that can be used in the context of the present invention include antibodies, antigen-binding portions of antibodies, peptides that specifically interact with a particular antigen (e.g., peptibodies), receptor molecules that specifically interact with a particular antigen, proteins comprising a ligand-binding portion of a receptor that specifically binds a particular antigen, antigen-binding scaffolds (e.g., DARPins, HEAT repeat proteins, ARM repeat proteins, tetratricopeptide repeat proteins, and other scaffolds based on naturally occurring repeat proteins, etc., [see, e.g., Boersma and Pluckthun, 2011, *Curr. Opin. Biotechnol.* 22:849-857, and references cited therein]), and aptamers or portions thereof.

[0043] In certain embodiments in which the target molecule or the internalizing effector protein is a receptor molecule, an "antigen-binding domain," for purposes of the present invention, may comprise or consist of a ligand or portion of a ligand that is specific for the receptor. For example, if the target molecule (T) is IL-4R, the D1 component of the multispecific antigen-binding molecule may comprise the IL-4 ligand or a portion of the IL-4 ligand that is capable of specifically interacting with IL-4R; or if the internalizing effector protein (E) is transferrin receptor, the D2 component of the multispecific antigen-binding molecule may comprise transferrin or a portion of transferrin that is capable of specifically interacting with the transferrin receptor.

[0044] In certain embodiments in which the target molecule or the internalizing effector protein is a ligand that is specifically recognized by a particular receptor (e.g., a soluble target molecule), an "antigen-binding domain," for purposes of the present invention, may comprise or consist of the receptor or a ligand-binding portion of the receptor. For example, if the target molecule (T) is IL-6, the D1 component of the multispecific antigen-binding molecule may comprise the ligand-binding domain of the IL-6 receptor; or if the internalizing effector protein (E) is an indirectly internalized protein (as that term is defined elsewhere herein), the D2 component of the multispecific antigen-binding molecule may comprise a ligand-binding domain of a receptor specific for E.

[0045] Methods for determining whether two molecules specifically bind one another are well known in the art and include, for example, equilibrium dialysis, surface plasmon resonance, and the like. For example, an antigen-binding domain, as used in the context of the present invention, includes polypeptides that bind a particular antigen (e.g., a target molecule [T] or an internalizing effector protein [E]) or a portion thereof with a K_D of less than about 500 pM, less than about 400 pM, less than about 300 pM, less than about 200 pM, less than about 100 pM, less than about 90 pM, less than about 80 pM, less than about 70 pM, less than about 60 pM, less than about 50 pM, less than about 40 pM, less than about 30 pM, less than about 20 pM, less than about 10 pM, less than about 5 pM, less than about 4 pM, less than about 2 pM, less than about 1 pM, less than about

0.5 pM, less than about 0.2 pM, less than about 0.1 pM, or less than about 0.05 pM, as measured in a surface plasmon resonance assay.

[0046] The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore™ system (Biacore Life Sciences division of GE Healthcare, Piscataway, NJ).

[0047] The term " K_D ", as used herein, means the equilibrium dissociation constant of a particular protein-protein interaction (e.g., antibody-antigen interaction). Unless indicated otherwise, the K_D values disclosed herein refer to K_D values determined by surface plasmon resonance assay at 25°C.

ANTIBODIES AND ANTIGEN-BINDING FRAGMENTS OF ANTIBODIES

[0048] As indicated above, an "antigen-binding domain" (D1 and/or D2) can comprise or consist of an antibody or antigen-binding fragment of an antibody. The term "antibody," as used herein, means any antigen-binding molecule or molecular complex comprising at least one complementarity determining region (CDR) that specifically binds to or interacts with a particular antigen (e.g., T or E). The term "antibody" includes immunoglobulin molecules comprising four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, as well as multimers thereof (e.g., IgM). Each heavy chain comprises a heavy chain variable region (abbreviated herein as HCVR or V_H) and a heavy chain constant region. The heavy chain constant region comprises three domains, C_{H1} , C_{H2} and C_{H3} . Each light chain comprises a light chain variable region (abbreviated herein as LCVR or V_L) and a light chain constant region. The light chain constant region comprises one domain (C_{L1}). The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. In different embodiments of the invention, the FRs of the antibodies of the invention (or antigen-binding portion thereof) may be identical to the human germline sequences, or may be naturally or artificially modified. An amino acid consensus sequence may be defined based on a side-by-side analysis of two or more CDRs.

[0049] The D1 and/or D2 components of the multispecific antigen-binding molecules of the present invention may comprise or consist of antigen-binding fragments of full antibody molecules. The terms "antigen-binding portion" of an antibody, "antigen-binding fragment" of an antibody, and

the like, as used herein, include any naturally occurring, enzymatically obtainable, synthetic, or genetically engineered polypeptide or glycoprotein that specifically binds an antigen to form a complex. Antigen-binding fragments of an antibody may be derived, *e.g.*, from full antibody molecules using any suitable standard techniques such as proteolytic digestion or recombinant genetic engineering techniques involving the manipulation and expression of DNA encoding antibody variable and optionally constant domains. Such DNA is known and/or is readily available from, *e.g.*, commercial sources, DNA libraries (including, *e.g.*, phage-antibody libraries), or can be synthesized. The DNA may be sequenced and manipulated chemically or by using molecular biology techniques, for example, to arrange one or more variable and/or constant domains into a suitable configuration, or to introduce codons, create cysteine residues, modify, add or delete amino acids, etc.

[0050] Non-limiting examples of antigen-binding fragments include: (i) Fab fragments; (ii) F(ab')₂ fragments; (iii) Fd fragments; (iv) Fv fragments; (v) single-chain Fv (scFv) molecules; (vi) dAb fragments; and (vii) minimal recognition units consisting of the amino acid residues that mimic the hypervariable region of an antibody (*e.g.*, an isolated complementarity determining region (CDR) such as a CDR3 peptide), or a constrained FR3-CDR3-FR4 peptide. Other engineered molecules, such as domain-specific antibodies, single domain antibodies, domain-deleted antibodies, chimeric antibodies, CDR-grafted antibodies, diabodies, triabodies, tetrabodies, minibodies, nanobodies (*e.g.* monovalent nanobodies, bivalent nanobodies, etc.), small modular immunopharmaceuticals (SMIPs), and shark variable IgNAR domains, are also encompassed within the expression "antigen-binding fragment," as used herein.

[0051] An antigen-binding fragment of an antibody will typically comprise at least one variable domain. The variable domain may be of any size or amino acid composition and will generally comprise at least one CDR which is adjacent to or in frame with one or more framework sequences. In antigen-binding fragments having a V_H domain associated with a V_L domain, the V_H and V_L domains may be situated relative to one another in any suitable arrangement. For example, the variable region may be dimeric and contain V_H-V_H, V_H-V_L or V_L-V_L dimers. Alternatively, the antigen-binding fragment of an antibody may contain a monomeric V_H or V_L domain.

[0052] In certain embodiments, an antigen-binding fragment of an antibody may contain at least one variable domain covalently linked to at least one constant domain. Non-limiting, exemplary configurations of variable and constant domains that may be found within an antigen-binding fragment of an antibody of the present invention include: (i) V_H-C_H1; (ii) V_H-C_H2; (iii) V_H-C_H3; (iv) V_H-C_H1-C_H2; (v) V_H-C_H1-C_H2-C_H3; (vi) V_H-C_H2-C_H3; (vii) V_H-C_L; (viii) V_L-C_H1; (ix) V_L-C_H2; (x) V_L-C_H3; (xi)

V_L-C_H1-C_H2; (xii) V_L-C_H1-C_H2-C_H3; (xiii) V_L-C_H2-C_H3; and (xiv) V_L-C_L. In any configuration of variable and constant domains, including any of the exemplary configurations listed above, the variable and constant domains may be either directly linked to one another or may be linked by a full or partial hinge or linker region. A hinge region may consist of at least 2 (e.g., 5, 10, 15, 20, 40, 60 or more) amino acids which result in a flexible or semi-flexible linkage between adjacent variable and/or constant domains in a single polypeptide molecule. Moreover, an antigen-binding fragment may comprise a homo-dimer or hetero-dimer (or other multimer) of any of the variable and constant domain configurations listed above in non-covalent association with one another and/or with one or more monomeric V_H or V_L domain (e.g., by disulfide bond(s)).

[0053] The multispecific antigen-binding molecules of the present invention may comprise or consist of human antibodies and/or recombinant human antibodies, or fragments thereof. The term "human antibody", as used herein, includes antibodies having variable and constant regions derived from human germline immunoglobulin sequences. Human antibodies may nonetheless include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs and in particular CDR3. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

[0054] The multispecific antigen-binding molecules of the present invention may comprise or consist of recombinant human antibodies or antigen-binding fragments thereof. The term "recombinant human antibody", as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further below), antibodies isolated from a recombinant, combinatorial human antibody library (described further below), antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes (see e.g., Taylor et al. (1992) Nucl. Acids Res. 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L

sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

BISPECIFIC ANTIBODIES

[0055] According to certain embodiments, the multispecific antigen-binding molecules of the invention are bispecific antibodies; *e.g.*, bispecific antibodies comprising an antigen-binding arm that specifically binds a target molecule (T) and an antigen-binding arm that specifically binds an internalizing effector protein (E). Methods for making bispecific antibodies are known in the art and may be used to construct multispecific antigen-binding molecules of the present invention. Exemplary bispecific formats that can be used in the context of the present invention include, without limitation, *e.g.*, scFv-based or diabody bispecific formats, IgG-scFv fusions, dual variable domain (DVD)-Ig, Quadroma, knobs-into-holes, common light chain (*e.g.*, common light chain with knobs-into-holes, etc.), CrossMab, CrossFab, (SEED)body, leucine zipper, Duobody, IgG1/IgG2, dual acting Fab (DAF)-IgG, and Mab² bispecific formats (*see, e.g.*, Klein *et al.* 2012, mAbs 4:6, 1-11, and references cited therein, for a review of the foregoing formats).

MULTIMERIZING COMPONENTS

[0056] The multispecific antigen-binding molecules of the present invention, in certain embodiments, may also comprise one or more multimerizing component(s). The multimerizing components can function to maintain the association between the antigen-binding domains (D1 and D2). As used herein, a "multimerizing component" is any macromolecule, protein, polypeptide, peptide, or amino acid that has the ability to associate with a second multimerizing component of the same or similar structure or constitution. For example, a multimerizing component may be a polypeptide comprising an immunoglobulin C_H3 domain. A non-limiting example of a multimerizing component is an Fc portion of an immunoglobulin, *e.g.*, an Fc domain of an IgG selected from the isotypes IgG1, IgG2, IgG3, and IgG4, as well as any allotype within each isotype group. In certain embodiments, the multimerizing component is an Fc fragment or an amino acid sequence of 1 to about 200 amino acids in length containing at least one cysteine residues. In other embodiments, the multimerizing component is a cysteine residue, or a short cysteine-containing peptide. Other multimerizing domains include peptides or polypeptides comprising or consisting of a leucine zipper, a helix-loop motif, or a coiled-coil motif.

[0057] In certain embodiments, the multispecific antigen-binding molecules of the present invention comprise two multimerizing domains, M1 and M2, wherein D1 is attached to M1 and D2 is attached to M2, and wherein the association of M1 with M2 facilitates the physical linkage of D1 and D2 to one another in a single multispecific antigen-binding molecule. In certain embodiments, M1

and M2 are identical to one another. For example, M1 can be an Fc domain having a particular amino acid sequence, and M2 is an Fc domain with the same amino acid sequence as M1. Alternatively, M1 and M2 may differ from one another at one or more amino acid position. For example, M1 may comprise a first immunoglobulin (Ig) C_H3 domain and M2 may comprise a second Ig C_H3 domain, wherein the first and second Ig C_H3 domains differ from one another by at least one amino acid, and wherein at least one amino acid difference reduces binding of the targeting construct to Protein A as compared to a reference construct having identical M1 and M2 sequences. In one embodiment, the Ig C_H3 domain of M1 binds Protein A and the Ig C_H3 domain of M2 contains a mutation that reduces or abolishes Protein A binding such as an H95R modification (by IMGT exon numbering; H435R by EU numbering). The C_H3 of M2 may further comprise a Y96F modification (by IMGT; Y436F by EU). Further modifications that may be found within the C_H3 of M2 include: D16E, L18M, N44S, K52N, V57M, and V82I (by IMGT; D356E, L358M, N384S, K392N, V397M, and V422I by EU) in the case of an IgG1 Fc domain; N44S, K52N, and V82I (IMGT; N384S, K392N, and V422I by EU) in the case of an IgG2 Fc domain; and Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (by IMGT; Q355R, N384S, K392N, V397M, R409K, E419Q, and V422I by EU) in the case of an IgG4 Fc domain.

INTERNALIZING EFFECTOR PROTEINS (E)

[0058] In the context of the present invention, the D2 component of the multispecific antigen-binding molecule specifically binds an internalizing effector protein ("E"). An internalizing effector protein is a protein that is capable of being internalized into a cell or that otherwise participates in or contributes to retrograde membrane trafficking. In some instances, the internalizing effector protein is a protein that undergoes transcytosis; that is, the protein is internalized on one side of a cell and transported to the other side of the cell (e.g., apical-to-basal). In many embodiments, the internalizing effector protein is a cell surface-expressed protein or a soluble extracellular protein. However, the present invention also contemplates embodiments in which the internalizing effector protein is expressed within an intracellular compartment such as the endosome, endoplasmic reticulum, Golgi, lysosome, etc. For example, proteins involved in retrograde membrane trafficking (e.g., pathways from early/recycling endosomes to the trans-Golgi network) may serve as internalizing effector proteins in various embodiments of the present invention. In any event, the binding of D2 to an internalizing effector protein causes the entire multispecific antigen-binding molecule, and any molecules associated therewith (e.g., a target molecule bound by D1), to also become internalized into the cell. As explained below, internalizing effector proteins include proteins that are directly internalized into a cell, as well as proteins that are indirectly internalized into a cell.

[0059] Internalizing effector proteins that are directly internalized into a cell include membrane-associated molecules with at least one extracellular domain (e.g., transmembrane proteins, GPI-anchored proteins, etc.), which undergo cellular internalization, and are preferably processed via an intracellular degradative and/or recycling pathway. Specific non-limiting examples of internalizing effector proteins that are directly internalized into a cell include, e.g., CD63, MHC-I (e.g., HLA-B27), Kremen-1, Kremen-2, LRP5, LRP6, LRP8, transferrin receptor, LDL-receptor, LDL-related protein 1 receptor, ASGR1, ASGR2, amyloid precursor protein-like protein-2 (APLP2), apelin receptor (APLNR), MAL (Myelin And Lymphocyte protein, a.k.a. VIP17), IGF2R, vacuolar-type H⁺ ATPase, diphtheria toxin receptor, folate receptor, glutamate receptors, glutathione receptor, leptin receptors, scavenger receptors (e.g., SCARA1-5, SCARB1-3, CD36), etc.

[0060] In certain embodiments, the internalizing effector protein is prolactin receptor (PRLR). It was discovered that PRLR is, not only a target for certain therapeutic applications, but is also an effective internalizing effector protein on the basis of its high rate of internalization and turn-over. The potential for PRLR as an internalizing effector protein, for example, is illustrated in WO2015/026907, where it is demonstrated, *inter alia*, that anti-PRLR antibodies are effectively internalized by PRLR-expressing cells in vitro.

[0061] In embodiments in which E is a directly internalized effector protein, the D2 component of the multispecific antigen-binding molecule can be, e.g., an antibody or antigen-binding fragment of an antibody that specifically binds E, or a ligand or portion of a ligand that specifically interacts with the effector protein. For example, if E is Kremen-1 or Kremen-2, the D2 component can comprise or consist of a Kremen ligand (e.g., DKK1) or Kremen-binding portion thereof. As another example, if E is a receptor molecule such as ASGR1, the D2 component can comprise or consist of a ligand specific for the receptor (e.g., asialoorosomucoid [ASOR] or Beta-GalNAc) or a receptor-binding portion thereof.

[0062] Internalizing effector proteins that are indirectly internalized into a cell include proteins and polypeptides that do not internalize on their own, but become internalized into a cell after binding to or otherwise associating with a second protein or polypeptide that is directly internalized into the cell. Proteins that are indirectly internalized into a cell include, e.g., soluble ligands that are capable of binding to an internalizing cell surface-expressed receptor molecule. A non-limiting example of a soluble ligand that is (indirectly) internalized into a cell via its interaction with an internalizing cell surface-expressed receptor molecule is transferrin. In embodiments wherein E is transferrin (or another indirectly internalized protein), the binding of D2 to E, and the interaction of E with transferrin receptor (or another internalizing cell-surface expressed receptor molecule), causes the

entire multispecific antigen-binding molecule, and any molecules associated therewith (e.g., a target molecule bound by D1), to become internalized into the cell concurrent with the internalization of E and its binding partner.

[0063] In embodiments in which E is an indirectly internalized effector protein such as a soluble ligand, the D2 component of the multispecific antigen-binding molecule can be, e.g., an antibody or antigen-binding fragment of an antibody that specifically binds E, or a receptor or portion of a receptor that specifically interacts with the soluble effector protein. For example, if E is a cytokine, the D2 component can comprise or consist of the corresponding cytokine receptor or ligand-binding portion thereof.

TARGET MOLECULES (T)

[0064] In the context of the present invention, the D1 component of the multispecific antigen-binding molecule specifically binds a target molecule ("T"). A target molecule is any protein, polypeptide, or other macromolecule whose activity or extracellular concentration is desired to be attenuated, reduced or eliminated. In many instances, the target molecule to which D1 binds is a protein or polypeptide [*i.e.*, a "target protein"]; however, the present invention also includes embodiments wherein the target molecule ("T") is a carbohydrate, glycoprotein, lipid, lipoprotein, lipopolysaccharide, or other non-protein polymer or molecule to which D1 binds. According to the present invention, T can be a cell surface-expressed target protein or a soluble target protein. Target binding by the multispecific antigen-binding molecule may take place in an extracellular or cell surface context. In certain embodiments, however, the multispecific antigen-binding molecule binds a target molecule inside the cell, for example within an intracellular component such as the endoplasmic reticulum, Golgi, endosome, lysosome, etc.

[0065] Examples of cell surface-expressed target molecules include cell surface-expressed receptors, membrane-bound ligands, ion channels, and any other monomeric or multimeric polypeptide component with an extracellular portion that is attached to or associated with a cell membrane. Non-limiting, exemplary cell surface-expressed target molecules that may be targeted by the multispecific antigen-binding molecule of the present invention include, e.g., cytokine receptors (e.g., receptors for IL-1, IL-4, IL-6, IL-13, IL-22, IL-25, IL-33, etc.), as well as cell surface targets including other type 1 transmembrane receptors such as PRLR, G-protein coupled receptors such as GCGR, ion channels such as Nav1.7, ASIC1 or ASIC2, non-receptor surface proteins such as MHC-I (e.g., HLA-B*27), etc.

[0066] In embodiments in which T is a cell surface-expressed target protein, the D1 component of

the multispecific antigen-binding molecule can be, e.g., an antibody or antigen-binding fragment of an antibody that specifically binds T, or a ligand or portion of a ligand that specifically interacts with the cell surface-expressed target protein. For example, if T is IL-4R, the D1 component can comprise or consist of IL-4 or a receptor-binding portion thereof.

[0067] Examples of soluble target molecules include cytokines, growth factors, and other ligands and signaling proteins. Non-limiting exemplary soluble target protein that may be targeted by the multispecific antigen-binding molecule of the present invention include, e.g., IL-1, IL-4, IL-6, IL-13, IL-22, IL-25, IL-33, SOST, DKK1, etc. Soluble targets molecules also include, e.g., non-human target molecules such as allergens (e.g., Fel D1, Betv1, CryJ1), pathogens (e.g., *Candida albicans*, *S. aureus*, etc.), and pathogenic molecules (e.g., lipopolysaccharide [LPS], lipotechoic acid [LTA], Protein A., toxins, etc.). In embodiments in which T is a soluble target molecule, the D1 component of the multispecific antigen-binding molecule can be, e.g., an antibody or antigen-binding fragment of an antibody that specifically binds T, or a receptor or portion of a receptor that specifically interacts with the soluble target molecule. For example, if T is IL-4, the D1 component can comprise or consist of IL-4R or a ligand-binding portion thereof.

[0068] Target molecules also include tumor-associated antigens, as described elsewhere herein.

pH-DEPENDENT BINDING

[0069] The present invention provides multispecific antigen-binding molecules comprising a first antigen-binding domain (D1) and a second antigen-binding domain (D2), wherein one or both of the antigen-binding domains (D1 and/or D2) binds its antigen (T or E) in a pH-dependent manner. For example, an antigen-binding domain (D1 and/or D2) may exhibit reduced binding to its antigen at acidic pH as compared to neutral pH. Alternatively, an antigen-binding domain (D1 and/or D2) may exhibit enhanced binding to its antigen at acidic pH as compared to neutral pH. Antigen-binding domains with pH-dependent binding characteristics may be obtained, e.g., by screening a population of antibodies for reduced (or enhanced) binding to a particular antigen at acidic pH as compared to neutral pH. Additionally, modifications of the antigen-binding domain at the amino acid level may yield antigen-binding domains with pH-dependent characteristics. For example, by substituting one or more amino acid of an antigen-binding domain (e.g., within a CDR) with a histidine residue, an antigen-binding domain with reduced antigen-binding at acidic pH relative to neutral pH may be obtained.

[0070] In certain embodiments, the present invention includes multispecific antigen-binding molecules comprising a D1 and/or D2 component that binds its respective antigen (T or E) at acidic pH with a K_D that is at least about 3, 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45,

50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more times greater than the K_D of the D1 and/or D2 component for binding to its respective antigen at neutral pH. pH dependent binding may also be expressed in terms of the $t_{1/2}$ of the antigen-binding domain for its antigen at acidic pH compared to neutral pH. For example, the present invention includes multispecific antigen-binding molecules comprising a D1 and/or D2 component that binds its respective antigen (T or E) at acidic pH with a $t_{1/2}$ that is at least about 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, or more times shorter than the $t_{1/2}$ of the D1 and/or D2 component for binding to its respective antigen at neutral pH.

[0071] Multispecific antigen-binding molecules of the present invention that comprise a D1 and/or D2 component with reduced antigen binding at acidic pH as compared to neutral pH, when administered to animal subjects, may in certain embodiments exhibit slower clearance from circulation as compared to comparable molecules that do not exhibit pH-dependent binding characteristics. According to this aspect of the invention, multispecific antigen-binding molecules with reduced antigen binding to either T and/or E at acidic pH as compared to neutral pH are provided which exhibit at least 2 times slower clearance from circulation relative to comparable antigen-binding molecules that do not possess reduced antigen binding at acidic pH as compared to neutral pH. Clearance rate can be expressed in terms of the half-life of the antibody, wherein a slower clearance correlates with a longer half-life.

[0072] As used herein, the expression "acidic pH" means a pH of 6.0 or less. The expression "acidic pH" includes pH values of about 6.0, 5.95, 5.8, 5.75, 5.7, 5.65, 5.6, 5.55, 5.5, 5.45, 5.4, 5.35, 5.3, 5.25, 5.2, 5.15, 5.1, 5.05, 5.0, or less. As used herein, the expression "neutral pH" means a pH of about 7.0 to about 7.4. The expression "neutral pH" includes pH values of about 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3, 7.35, and 7.4.

ATTENUATION OF TARGET MOLECULE ACTIVITY

[0073] As noted elsewhere herein, and as demonstrated by the working Examples herein below, the present inventors have discovered that the simultaneous binding of a target molecule (T) and an internalizing effector protein (E) by a multispecific antigen-binding molecule attenuates the activity of T to a greater extent than the binding of T by the first antigen-binding domain (D1) component of the multispecific antigen-binding molecule alone. As used herein, the expression "attenuates the activity of T to a greater extent than the binding of T by D1 alone" means that, in an assay in which the activity of T can be measured using cells that express E, the level of T activity measured in the presence of a multispecific antigen-binding molecule is at least 10% lower than the level of T activity measured in the presence of a control construct containing D1 by itself (*i.e.*, not physically linked to the second antigen-binding domain (D2)). For instance, the level of T activity measured in

the presence of the multispecific antigen-binding molecule may be about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% lower than the level of T activity measured in the presence of a control construct containing D1 by itself.

[0074] A non-limiting, illustrative assay format for determining whether a multispecific antigen-binding molecule attenuates the activity of a target molecule to a greater extent than the binding of the target molecule by the D1 domain alone is shown in working Examples 1 and 2, herein below. In Example 1, for instance, "T" is the interleukin-4 receptor (IL-4R), and "E" is CD63. The multispecific antigen-binding molecule of Example 1 is a 2-antibody conjugate comprising an anti-IL-4R mAb linked to an anti-CD63 mAb via a streptavidin/biotin linker. Thus, "D1" in this exemplary construct is the antigen-binding domain (HCVR/LCVR pair) of the anti-IL-4R antibody, and "D2" is the antigen-binding domain (HCVR/LCVR pair) of the anti-CD63 antibody. For the experiments of Examples 1 and 2, a cell-based assay format was used that produces a reporter signal when IL-4R activity is stimulated by the addition of exogenous IL-4 ligand. The amount of IL-4-induced reporter activity detected in the presence of the multispecific antigen-binding molecule was compared to the amount of IL-4-induced reporter activity detected in the presence of control constructs containing the anti-IL-4R antibody either connected to an irrelevant control immunoglobulin (control 1), or combined with, but not physically connected to, the anti-CD63 antibody (control 2). The control constructs thus produce the condition in which T is bound by D1 alone (*i.e.*, wherein D1 is not a part of the multispecific antigen-binding molecule *per se*). If the extent of target molecule activity (represented by the reporter signal) observed in the presence of the multispecific antigen-binding molecule is at least 10% less than the amount of target molecule activity observed in the presence of a control construct comprising the D1 component not physically linked to the D2 component (*e.g.*, control 1 or control 2), then for purposes of the present disclosure, it is concluded that "the simultaneous binding of T and E by the multispecific antigen-binding molecule attenuates the activity of T to a greater extent than the binding of T by D1 alone."

[0075] The binding of T by D1 alone may, in some embodiments, result in partial attenuation of the activity of T (as in the case of Example 1 where the treatment of reporter cells with an anti-IL-4R antibody alone [*i.e.*, controls 1 and 2] caused a small level of attenuation of IL-4 signaling relative to untreated cells). In other embodiments, the binding of T by D1 alone will result in no detectable attenuation of the activity of T; that is, the biological activity of T may be unaffected by the binding of T by D1 alone. In any event, however, the simultaneous binding of T and E by a multispecific antigen-binding molecule of the invention will attenuate the activity of T to a greater extent than the binding of T by D1 alone.

[0076] Alternative assay formats and variations on the assay format(s) exemplified herein will be apparent to persons of ordinary skill in the art, taking into account the nature of the specific target molecule and effector proteins to which any given multispecific antigen-binding molecule may be directed. Any such format can be used in the context of the present invention to determine whether the simultaneous binding of T and E by a multispecific antigen-binding molecule attenuates the activity of T to a greater extent than the binding of T by D1 alone.

TUMOR TARGETING

[0077] In another aspect of the invention, the multispecific antigen-binding molecules are useful for targeting tumor cells. According to this aspect of the invention, the target molecule "T" to which D1 binds is a tumor-associated antigen. In certain instances, the tumor-associated antigen is an antigen that is not ordinarily internalized. The internalizing effector protein "E" to which D2 binds may be tumor specific, or it may be expressed on both tumor and non-tumor cells of an individual. Any of the internalizing effector proteins mentioned elsewhere herein may be targeted for anti-tumor applications of the invention.

[0078] As used herein, the term "tumor-associated antigen" includes proteins or polypeptides that are preferentially expressed on the surface of a tumor cell. The expression "preferentially expressed," as used in this context, means that the antigen is expressed on a tumor cell at a level that is at least 10% greater (*e.g.*, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 150%, 200%, 400%, or more) than the expression level of the antigen on non-tumor cells. In certain embodiments, the target molecule is an antigen that is preferentially expressed on the surface of a tumor cell selected from the group consisting of a renal tumor cell, a colon tumor cell, a breast tumor cell, an ovarian tumor cell, a skin tumor cell, a lung tumor cell, a prostate tumor cell, a pancreatic tumor cell, a glioblastoma cell, a head and neck tumor cell and a melanoma cell. Non-limiting examples of specific tumor-associated antigens include, *e.g.*, AFP, ALK, BAGE proteins, β -catenin, bcr-abl, BRCA1, BORIS, CA9, carbonic anhydrase IX, caspase-8, CD40, CDK4, CEA, CTLA4, cyclin-B1, CYP1B1, EGFR, EGFRvIII, ErbB2/Her2, ErbB3, ErbB4, ETV6-AML, EphA2, Fra-1, FOLR1, GAGE proteins (*e.g.*, GAGE-1, -2), GD2, GD3, GloboH, glypican-3, GM3, gp100, Her2, HLA/B-raf, HLA/k-ras, HLA/MAGE-A3, hTERT, LMP2, MAGE proteins (*e.g.*, MAGE-1, -2, -3, -4, -6, and -12), MART-1, mesothelin, ML-IAP, Muc1, Muc16 (CA-125), MUM1, NA17, NY-BR1, NY-BR62, NY-BR85, NY-ESO1, OX40, p15, p53, PAP, PAX3, PAX5, PCTA-1, PLAC1, PRLR, PRAME, PSMA (FOLH1), RAGE proteins, Ras, RGS5, Rho, SART-1, SART-3, Steap-1, Steap-2, survivin, TAG-72, TGF- β , TMPRSS2, Tn, TRP-1, TRP-2, tyrosinase, and uroplakin-3.

[0079] The multispecific antigen-binding molecule, according to this aspect of the invention, may

be conjugated to a drug, toxin, radioisotope, or other substance which is detrimental to the viability of a cell. Alternatively, the drug or toxin may be a substance which does not directly kill a cell, but renders a cell more susceptible to killing by other external agents. In yet other embodiments involving tumor targeting, the multispecific antigen-binding molecule of the invention is not itself conjugated to a drug, toxin or radioisotope, but instead is administered in combination with a second antigen-binding molecule specific for the target (T) (herein referred to as an "accomplice molecule"), wherein the accomplice molecule is conjugated to a drug, toxin or radioisotope. In such embodiments, the multispecific antigen binding molecule will preferably bind to an epitope on the target molecule (T) that is distinct from and/or non-overlapping with the epitope recognized by the accomplice molecule (*i.e.*, to allow for simultaneous binding of the multispecific antigen-binding molecule and the accomplice molecule to the target).

[0080] In a related embodiment, the present invention also includes anti-tumor combinations, and therapeutic methods, comprising: (a) a toxin- or drug-conjugated antigen-binding molecule that specifically binds a tumor-associated antigen; and (b) a multispecific antigen-binding molecule comprising (i) a first binding domain that specifically binds an internalizing effector protein (*e.g.*, with low affinity) and (ii) a second binding domain that specifically binds the toxin- or drug-conjugated antigen-binding molecule. In this embodiment, the multispecific antigen-binding molecule functions to link the toxin- or drug-conjugated antigen-binding molecule to the internalizing effector protein, which thereby functions to physically link the tumor associated antigen to the internalizing effector protein. Internalization of the toxin-labeled anti-tumor-associated antigen antibody via its connection to the internalizing effector protein would consequently result in targeted tumor cell killing.

[0081] According to certain embodiments of the tumor-targeting aspects of the invention, the multispecific antigen-binding molecule (or accomplice antibody) may be conjugated to one or more cytotoxic drugs selected from the group consisting of: calicheamicin, esperamicin, methotrexate, doxorubicin, melphalan, chlorambucil, ARA-C, vindesine, mitomycin C, cis-platinum, etoposide, bleomycin, 5-fluorouracil, estramustine, vincristine, etoposide, doxorubicin, paclitaxel, larotaxel, tesetaxel, orataxel, docetaxel, dolastatin 10, auristatin E, auristatin PHE and maytansine-based compounds (*e.g.*, DM1, DM4, etc.). The multispecific antigen-binding molecule (or accomplice antibody) may also, or alternatively, be conjugated to a toxin such as diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolacca americana* proteins, etc. The multispecific antigen-binding molecule (or accomplice antibody) may also, or alternatively, be conjugated to one or more radioisotope selected from the group consisting of ²²⁵Ac, ²¹¹At, ²¹²Bi, ²¹³Bi, ¹⁸⁶Rh, ¹⁸⁸Rh,

¹⁷⁷Lu, ⁹⁰Y, ¹³¹I, ⁶⁷Cu, ¹²⁵I, ¹²³I, ⁷⁷Br, ¹⁵³Sm, ¹⁶⁶Ho, ⁶⁴Cu, ¹²¹Pb, ²²⁴Ra and ²²³Ra. Thus, this aspect of the invention includes multispecific antigen-binding molecules that are antibody-drug conjugates (ADCs) or antibody-radioisotope conjugates (ARCs).

[0082] In the context of tumor killing applications, the D2 component may, in certain circumstances, bind with low affinity to the internalizing effector protein "E". Thus, the multispecific antigen-binding molecule will preferentially target tumor cells that express the tumor-associated antigen. As used herein, "low affinity" binding means that the binding affinity of the D2 component for the internalizing effector protein (E) is at least 10% weaker (*e.g.*, 15% weaker, 25% weaker, 50% weaker, 75% weaker, 90% weaker, etc.) than the binding affinity of the D1 component for the target molecule (T). In certain embodiments, "low affinity" binding means that the D2 component interacts with the internalizing effector protein (E) with a K_D of greater than about 10 nM to about 1 μ M, as measured in a surface plasmon resonance assay at about 25°C.

[0083] The simultaneous binding of a multispecific antigen-binding molecule to an internalizing effector protein and a tumor-associated antigen will result in preferential internalization of the multispecific antigen-binding molecule into tumor cells. If, for example, the multispecific antigen-binding molecule is conjugated to a drug, toxin or radioisotope (or if the multispecific antigen-binding molecule is administered in combination with an accomplice antibody that is conjugated to a drug, toxin or radioisotope), the targeted internalization of the tumor-associated antigen into the tumor cell via its linkage to the multispecific antigen-binding molecule, will result in extremely specific tumor cell killing.

PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION METHODS

[0084] The present invention includes pharmaceutical compositions comprising a multispecific antigen-binding molecule. The pharmaceutical compositions of the invention can be formulated with suitable carriers, excipients, and other agents that provide improved transfer, delivery, tolerance, and the like.

[0085] The present invention also includes methods for inactivating or attenuating the activity of a target molecule (T). The methods of the present invention comprise contacting a target molecule with a multispecific antigen-binding molecule as described herein. In certain embodiments, the methods according to this aspect of the invention comprise administering a pharmaceutical composition comprising a multispecific antigen-binding molecule to a patient for whom it is desirable and/or beneficial to inactivate, attenuate, or otherwise decrease the extracellular concentration of a target molecule.

[0086] Various delivery systems are known in the art and can be used to administer the pharmaceutical compositions of the present invention to a patient. Methods of administration that can be used in the context of the present invention include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The pharmaceutical compositions of the invention may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.), and may be administered together with other biologically active agents. Administration can be systemic or local. For example, a pharmaceutical composition of the present invention can be delivered subcutaneously or intravenously with a standard needle and syringe. In addition, with respect to subcutaneous delivery, a pen delivery device can be used to administer a pharmaceutical composition of the present invention to a patient.

EXAMPLES

[0087] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1. Use of a Multispecific Antigen-Binding Molecule to Induce Degradation of a Cell Surface Receptor via Linkage with an Internalizing Effector Protein

[0088] As an initial proof-of-concept experiment, a multispecific antigen-binding molecule was created which is capable of binding (a) an internalizing effector molecule and (b) a cell surface receptor target molecule. In this Example, the internalizing effector protein is Kremen-2 (Krm2), and the cell surface receptor target molecule is an Fc receptor (FcγR1 [Fc-gamma-R1]).

[0089] Kremen molecules (Krm1 and Krm2) are cell-surface proteins known to mediate WNT signaling by directing the internalization and degradation of the WNT pathway signaling molecules LRP5 and LRP6. Internalization of LRP5/6 is accomplished via the soluble interacting protein DKK1. In particular, DKK1 links Kremen to LRP5/6 on the cell surface, and because of this linkage, the internalization of Kremen drives the internalization and degradation of LRP5 and LRP6. (See Li *et al.*, PLoS One 5(6):e11014).

[0090] The present inventors sought to exploit the Kremen-binding properties of DKK1 and the

internalization properties of Kremen to induce the internalization of FcγR1. To facilitate Kremen-mediated internalization/degradation of FcγR1, a multispecific antigen-binding molecule was constructed consisting of DKK1 fused to a mouse Fc (DKK1-mFc, having the amino acid sequence of SEQ ID NO:1). As explained elsewhere herein, a multispecific antigen-binding molecule is defined as a molecule comprising a first antigen-binding domain (D1) which specifically binds a target molecule, and a second antigen-binding domain (D2) which specifically binds an internalizing effector protein. In this proof-of-concept Example, the "first antigen-binding domain" is the mFc component which specifically binds the target molecule FcγR1, and the "second antigen-binding domain" is the DKK1 component which specifically binds the internalizing effector protein Kremen.

[0091] An experiment was first conducted to determine whether DKK1-mFc can be endocytosed into cells in a Kremen-dependent manner. For this experiment, two cell lines were used: Cell-1, an HEK293 cell line engineered to express FcγR1 but not Kremen-2, and Cell-2, an HEK293 cell line engineered to express both FcγR1 and Kremen-2. A 1:10 dilution of DKK1-mFc conditioned medium was added to the respective cell lines and allowed to incubate at 37°C for 90 minutes. After the 90 minute incubation, cells were stained with Alexa-488-labeled anti-mouse IgG antibody to detect the DKK1-mFc molecule. Using fluorescence microscopy, it was observed that virtually no DKK1-mFc was localized inside Cell-1 (lacking Kremen); however, substantial amounts of DKK1-mFc were detected within Cell-2 which expresses Kremen-2. Thus, these results show that the multispecific antigen-binding molecule DKK1-mFc can be internalized into cells in a Kremen-dependent manner.

[0092] Next, a time-course experiment was conducted to determine whether DKK1-mFc can induce FcγR1 degradation in a Kremen-dependent manner. A brief description of the experimental protocol is as follows: Cell-1 (expressing only FcγR1) and Cell-2 (expressing Kremen-2 and FcγR1) were treated with 2 mg/ml NHS-Sulfo-Biotin for 15 minutes on ice to label all cell surface expressed proteins. Cells were then washed and resuspended in 400 μl of medium and divided into four 100 μl aliquots which were treated with DKK1-mFc for varying amounts of time (0 min, 15 min, 30 min and 60 min) at 37°C. Following DKK1-mFc incubation, cells were pelleted and treated with protease inhibitors. Lysates of the cells from the different incubation time points were subjected to FcγR1 immunoprecipitation. For the FcγR1 immunoprecipitation, mouse anti-FcγR1 antibody was added to cell lysates and incubated for 1 hour at 4°C. Then Protein-G beads were added and the mixture was incubated for 1 hour at 4°C. The beads were then washed and the proteins eluted and subjected to SDS-PAGE. Proteins were transferred to membrane and probed with HRP-labeled streptavidin to reveal relative amounts of remaining surface-exposed FcγR1 protein in each sample. Results are shown in Figure 2.

[0093] As illustrated in Figure 2, the amount of surface-exposed FcγR1 protein in Cell-1 samples (expressing FcγR1 but not Kremen-2) remained relatively constant regardless of the amount of time the cells were exposed to DKK1-mFc. By contrast, the amount of surface-exposed FcγR1 protein in Cell-2 samples (expressing both Kremen-2 and FcγR1) decreased substantially with increasing incubation times with DKK1-mFc. Thus, this experiment demonstrates that DKK1-mFc induces degradation of cell surface expressed FcγR1 in a Kremen-2-dependent manner.

[0094] Taken together, the foregoing results show that a multispecific antigen-binding molecule that simultaneously binds a cell surface target molecule (FcγR1) and an internalizing effector protein (Kremen-2), can induce degradation of the target molecule in an effector protein-dependent manner.

Example 2. IL-4R Activity is Attenuated Using a Multispecific Antigen-Binding Molecule with Specificity for IL-4R and CD63

[0095] In a further set of proof-of-concept experiments, a multispecific antigen-binding molecule was constructed which is capable of simultaneously binding a cell surface-expressed target molecule (*i.e.*, IL-4R) and a cell surface-expressed internalizing effector protein (*i.e.*, CD63). The purpose of these experiments was to determine whether IL-4R activity on a cell can be attenuated to a greater extent by physically linking IL-4R to an effector molecule that is internalized and targeted for degradation within the lysosome (in this case, CD63). In other words, this Example was designed to test whether the normal internalization and degradation of CD63 could be used to force the internalization and degradative rerouting of IL-4R within a cell.

[0096] First, a multispecific antigen-binding molecule was constructed that is able to bind both IL-4R and CD63. Specifically, a streptavidin-conjugated anti-IL-4R antibody and a biotinylated anti-CD63 antibody were combined in a 1:1 ratio to produce an anti-IL-4R:anti-CD63 conjugate (*i.e.*, a multispecific antigen-binding molecule that specifically binds both IL-4R and CD63). The anti-IL-4R antibody used in this Example is a fully human mAb raised against the IL-4R extracellular domain. (The anti-IL-4R antibody comprised a heavy chain variable region having SEQ ID NO:3 and a light chain variable region having SEQ ID NO:4). The anti-CD63 antibody used in this Example is the mouse anti-human CD63 mAb clone MEM-259, obtained from Biolegend (San Diego, CA), catalog. No. 312002.

[0097] Two control constructs were also created: Control-1 = streptavidin-conjugated anti-IL-4R antibody combined in a 1:1 ratio with biotinylated control mouse IgG1kappa antibody; and Control-2 = streptavidin-conjugated anti-IL-4R antibody combined in a 1:1 ratio with non-biotinylated anti-CD63 antibody. The anti-IL-4R antibody used in the experimental and control constructs for this

Example is an antibody that is known to specifically bind IL-4R and only partially block IL-4-mediated signaling.

[0098] The experimental cell line used in this Example is an HEK293 cell line containing a STAT6-luciferase reporter construct and additional STAT6 ("HEK293/STAT6-luc cells"). The cells used in this experiment express both IL-4R and CD63 on their surface. When treated with IL-4 in the absence of any inhibitors, this cell line produces a dose-dependent detectable chemiluminescence signal which reflects the extent of IL-4-mediated signaling.

[0099] In an initial experiment, the experimental anti-IL-4R/anti-CD63 multispecific molecule, or the control constructs, were added to the HEK293/STAT6-luc cells so that the final concentration of anti-IL-4R antibody in the media was 12.5 nM. Reporter signal was measured at increasing concentrations of IL-4 in the presence and absence of the experimental and control constructs (Figure 3). As seen in Figure 3, The anti-IL-4R/anti-CD63 multispecific molecule ("ab conjugate") inhibited IL-4-mediated signaling to a significantly greater extent than either control construct.

[0100] To confirm that the effect observed in Figure 3 was dependent on CD63, the same experiment described above was carried out, except that CD63 expression was significantly reduced in the reporter cell line using an siRNA directed against CD63. With CD63 expression significantly reduced, the enhanced inhibitory activity of the anti-IL-4R/anti-CD63 multispecific molecule was no longer observed (Figure 4). This result suggests that the ability of the anti-IL-4R/anti-CD63 multispecific molecule to attenuate IL-4-mediated signaling is due to the simultaneous binding of the multispecific molecule to IL-4R and CD63 and the consequent internalization and degradation of the entire antibody-IL-4R-CD63 complex.

[0101] Similar experiments were next carried out in which the anti-IL-4R/anti-CD63 multispecific molecule, or the control constructs, were allowed to incubate with the HEK293/STAT6-luc reporter cell line for various amounts of time prior to the addition of IL-4. In a first set of such experiments, the molecules were allowed to incubate with the reporter cell line for 0 hours (*i.e.*, added concurrently with IL-4), 2 hours, or overnight prior to the addition of 50 pM IL-4. Luciferase activity was measured six hours after the addition of IL-4. Results are shown in Figure 5, top panel ("untransfected"). In a further set of experiments, a similar protocol was carried out, except that the experimental or control molecules were allowed to incubate with the reporter cell line for 15 minutes, 30 minutes, 1 hour or 2 hours prior to the addition of 50 pM IL-4. Results are shown in Figure 6.

[0102] The results summarized in Figures 5 and 6 show that the anti-IL-4R/anti-CD63

multispecific molecule is able to inhibit IL-4-mediated signaling, and that this inhibitory effect is enhanced with longer incubation times. As with the initial set of experiments, it was confirmed using CD63 siRNA that the inhibitory effect of the anti-IL-4R/anti-CD63 multispecific molecule was dependent on CD63 expression (Figure 5 bottom panel ["CD63 siRNA"]).

[0103] In summary, this Example provides further proof-of-concept for the inhibition of a target molecule activity through the use of a multispecific antigen-binding molecule that is capable of simultaneously binding both the target molecule (in this case IL-4R) and an internalizing effector protein (in this case CD63) to thereby cause the internalization and degradative rerouting of the target molecule within a cell. Stated differently, the simultaneous binding of IL-4R and CD63 by the exemplary multispecific antigen-binding molecule attenuated the activity of IL-4R to a substantially greater extent (*i.e.*, > 10%) than the binding of IL-4R by the control constructs alone.

Example 3. An Anti-IL-4R x Anti-CD63 Bispecific Antibody Attenuates IL-4R Activity in a CD63-Dependent Manner

[0104] The experiments of Example 2, herein, show that an anti-IL-4R/anti-CD63 multispecific molecule inhibits IL-4-mediated signaling in a CD63-dependent manner. In those experiments, the multispecific antigen-binding molecule consisted of two separate monoclonal antibodies (anti-IL-4R and anti-CD63) that were connected via a biotin-streptavidin linkage. To confirm that the results observed with that proof-of-concept multispecific antigen-binding molecule are generalizable to other multispecific antigen-binding molecule formats, a true bispecific antibody was constructed.

[0105] Standard bispecific antibody technology was used to construct a bispecific antibody consisting of a first arm specific for IL-4R and a second arm specific for CD63. The IL-4R-specific arm contained an anti-IL-4R heavy chain paired with a CD63-specific light chain. The CD63-specific light chain was paired with the IL-4R specific heavy chain solely for purposes of convenience of construction; nevertheless, the pairing of the anti-IL-4R heavy chain with the anti-CD63 light chain retained full specificity for IL-4R and did not exhibit binding to CD63. The CD63-specific arm contained an anti-CD63 heavy chain paired with an anti-CD63 light chain (the same light chain as used in the IL-4R arm). The anti-IL-4R heavy chain (comprising SEQ ID NO:3) was derived from the full anti-IL-4R antibody as used in Example 2; However, the anti-CD63 heavy and light chains were derived from the anti-CD63 antibody designated H5C6, obtained from the Developmental Studies Hybridoma Bank (University of Iowa Department of Biology, Iowa City, IA). As with the full anti-IL-4R antibody used in Example 2, the anti-IL-4R component of the bispecific antibody used in this Example exhibited only moderate IL-4R blocking activity on its own.

[0106] An IL-4 luciferase assay was carried out to assess the blocking activity of the anti-IL-4R x anti-CD63 bispecific antibody. Briefly, serial dilutions of anti-IL-4R x anti-CD63 bispecific antibody or control molecules were added to HEK293/STAT6-luc reporter cells (see Example 2). Under normal conditions, these cells produce a detectable luciferase signal when treated with IL-4. For this experiment, 10 pM IL-4 was then added to the cells, and luciferase activity was quantified for each dilution of antibody used. The controls used in this assay were: (a) mock bispecific antibody that binds IL-4R with one arm and has a non-functional anti-CD63 arm (*i.e.*, containing one anti-IL-4R heavy chain and one anti-CD63 heavy chain, both paired with the anti-IL-4R light chain); (b) anti-IL-4R monospecific antibody; and (c) buffer (PBS) only (without antibody). Results are shown in Figure 7. As shown in Figure 7, for the control samples used, luciferase activity remained relatively high even at the highest antibody concentrations, whereas for the bispecific antibody, luciferase activity declined significantly as antibody concentration increased. These results confirm that simultaneous binding of IL-4R and CD63 by a bispecific antibody causes substantial inhibition of IL-4R activity.

Example 4. Internalization of SOST Using a Multispecific Antigen-Binding Molecule That Simultaneously Binds SOST and CD63

[0107] In this Example, the ability of multispecific antigen-binding molecules to promote the internalization of the soluble target molecule SOST (sclerostin) was assessed. For these experiments, the target molecule was a fusion protein consisting of a human SOST protein tagged with a pHrodo™ moiety (Life Technologies, Carlsbad, CA) and a myc tag. The pHrodo™ moiety is a pH-sensitive dye that is virtually non-fluorescent at neutral pH and brightly fluorescent in an acidic environment such as the endosome. The fluorescent signal, therefore, can be used as an indicator of cellular internalization of the SOST fusion protein. The multispecific antigen-binding molecules for these experiments were bispecific antibodies with binding specificity for both CD63 (an internalizing effector protein) and the SOST fusion protein (a soluble target molecule), as described in more detail below.

[0108] The experiments were conducted as follows: Briefly, HEK293 cells were plated at 10,000 cells/well in poly-D-lysine coated 96 well plates (Greiner Bio-One, Monroe, NC). After allowing the cells to settle overnight, the media was replaced with media containing antibody (5 µg/mL, as described below), pHrodo™-myc-tagged-SOST (5 µg/mL), heparin (10 µg/mL), and Hoechst 33342. The cells were then incubated for either 3 hours on ice or 3 hours at 37°C. All cells were washed twice prior to imaging in PBS, and the number of fluorescent spots per cell, as well as the corresponding fluorescence intensity, was counted to establish the extent of pHrodo-myc-tagged-SOST cellular internalization in the presence of the various antibody constructs.

[0109] The antibodies used in this Example were as follows: (1) anti-CD63 monospecific antibody (clone H5C6, Developmental Studies Hybridoma Bank, University of Iowa Department of Biology, Iowa City, IA); (2) anti-myc antibody (clone 9E10, Schiweck *et al.*, 1997, FEBS Lett. 414(1):33-38); (3) anti-SOST antibody (an antibody having the heavy and light chain variable regions of the antibody designated "Ab-B" in US Patent No. 7,592,429); (4) anti-CD63 x anti-myc bispecific antibody (*i.e.*, a multispecific antigen-binding molecule comprising an anti-CD63 arm derived from the antibody H5C6 and an anti-myc arm derived from 9E10); (5) anti-CD63 x anti-SOST bispecific antibody #1 (*i.e.*, a multispecific antigen-binding molecule comprising an anti-CD63 arm derived from the antibody H5C6 and an anti-SOST arm derived from "Ab-B"); and (6) anti-CD63 x anti-SOST bispecific antibody #2 (*i.e.*, a multispecific antigen-binding molecule comprising an anti-CD63 arm derived from the antibody H5C6 and an anti-SOST arm derived from the antibody designated "Ab-20" in US Patent No. 7,592,429). The bispecific antibodies used in these experiments were assembled using the so-called "knobs-into-holes" methodology (see, *e.g.*, Ridgway *et al.*, 1996, Protein Eng. 9(7):617-621).

[0110] Results of the internalization experiments are shown in Figure 8. Figure 8 shows the number of spots (labeled vesicles) per cell, under the various treatment conditions tested. Taken together, the results of these experiments demonstrate that the bispecific constructs, which simultaneously bind CD63 and SOST (either directly or via the myc tag), caused the greatest amount of SOST internalization as reflected by the fluorescence intensity and number of fluorescent spots per cell over time at 37°C. Thus, the multispecific antigen-binding molecules used in this Example are able to effectively direct the internalization of a soluble target molecule.

Example 5. Changes in Bone Mineral Density in Mice Treated with A Multispecific Antigen-Binding Molecule that Binds CD63 and SOST

[0111] An anti-CD63 x anti-SOST multispecific antigen-binding molecule, as described in Example 4, is next tested for its ability to increase bone mineral density in mice. Five groups of mice (about 6 mice per group) are used in these experiments. The treatment groups are as follows: (I) untreated negative control mice; (II) mice treated with a blocking anti-SOST monospecific antibody that is known to increase bone mineral density on its own (positive control); (III) mice treated with a bispecific antibody that specifically binds CD63 and SOST but does not inhibit SOST activity on its own or only slightly inhibits SOST activity on its own; (IV) mice treated with an anti-CD63 parental antibody (*i.e.*, a monospecific antibody containing the same anti-CD63 antigen-binding domain as in the bispecific antibody); and (V) mice treated with an anti-SOST parental antibody (*i.e.*, a monospecific antibody containing the same anti-SOST antigen-binding domain as in the bispecific antibody). The amount of antibody administered to the mice in each group is about

10 to 25 mg/kg.

[0112] It is expected that mice in group III (treated with an anti-SOST x anti-CD63 bispecific antibody) will exhibit an increase in bone mineral density that is at least comparable to that which is observed in the mice of group II (treated with a known blocking anti-SOST antibody), even though the anti-SOST component of the bispecific antibody does not inhibit SOST activity on its own (as confirmed by the mice in Group V which are expected to *not* exhibit an increase in bone mineral density). The increase in bone mineral density that is expected in the mice of group III is believed to be driven by CD63-mediated internalization of SOST, as observed in the cellular experiments of Example 4, above.

Example 6. Cellular Internalization of Lipopolysaccharide (LPS) Mediated by a Multispecific Antigen-Binding Molecule That Simultaneously Binds LPS and CD63

[0113] This Example illustrates the use of a multispecific antigen-binding molecule of the invention to direct the internalization of a non-protein target molecule, namely lipopolysaccharide (LPS). LPS is a component of the outer membrane of Gram-negative bacteria and is known to contribute to septic shock. Anti-LPS antibodies have been investigated as possible treatment agents for sepsis. The experiments of the present Example were designed to assess the ability of a multispecific antigen-binding molecule to promote the internalization of LPS.

[0114] The multispecific antigen-binding molecule used in this Example was a bispecific antibody with one arm directed to LPS (target) and the other arm directed to CD63 (internalizing effector protein). The anti-LPS arm was derived from the antibody known as WN1 222-5. (DiPadova *et al.*, 1993, *Infection and Immunity* 61(9):3863-3872; Muller-Loennies *et al.*, 2003, *J. Biol. Chem.* 278(28):25618-25627; Gomery *et al.*, 2012, *Proc. Natl. Acad. Sci USA* 109(51):20877-20882; US 5,858,728). The anti-CD63 arm was derived from the H5C6 antibody (see Example 4). The anti-LPS x anti-CD63 bispecific antibody (*i.e.*, multispecific antigen-binding molecule) was assembled using the so-called "knobs-into-holes" methodology (see, *e.g.*, Ridgway *et al.*, 1996, *Protein Eng.* 9(7):617-621).

[0115] Two LPS species were used in these experiments: *E. coli* LPS and *Salmonella minnesota* LPS. Both versions were obtained as fluorescent-labeled molecules (ALEXA-FLUOR®-488-labeled LPS, Life Technologies, Carlsbad, CA).

[0116] Experiments were conducted as follows: HEK293 cells were plated in 96-well PDL-coated imaging plates. After overnight rest, media was replaced with fresh medium. Fluorescently labeled LPS (either *E. coli*- or *S. minnesota*-derived) was added in regular medium. Next, the anti-LPS x anti-CD63 bispecific antibody, or control half-antibodies paired with dummy Fc, were added to the

samples. Following various incubation times at 37°C (1 hour and 3 hours) or on ice (3 hours), cells from the LPS-treated samples were processed as follows: washed – quenched with anti-ALEXA-FLUOR®-488 antibody – washed & fixed. The anti-ALEXA-FLUOR®-488 antibody quenches fluorescence from non-internalized (i.e., surface bound) fluorophore. Thus, any fluorescence observed in the quenching antibody-treated samples is due to internalized LPS. The level of fluorescence from each sample at the various time points was measured.

[0117] Figure 9 expresses the results of these experiments in terms of the number of labeled vesicles per cell. As shown in Figure 9, only cells treated with the anti-CD63 x anti-LPS bispecific antibody demonstrated significant numbers of labeled vesicles that increased over time. Cells treated with labeled LPS and the control antibodies did not exhibit appreciable numbers of fluorescent vesicles, indicating that LPS was not internalized under those treatment conditions.

[0118] This Example therefore demonstrates that an anti-LPS x anti-CD63 bispecific antibody causes internalization of LPS into cells in a manner that requires simultaneous binding of LPS and CD63. Accordingly, these results support the use of multispecific antigen-binding molecules of the invention to promote cellular internalization of target molecules such as LPS for the treatment of diseases and disorders such as sepsis.

Example 7. Cellular Internalization and Degradation of Her2 Mediated by a Multispecific Antigen-Binding Molecule That Simultaneously Binds Her2 and PRLR

[0119] This Example includes three separate experiments which illustrate the use of a multispecific antigen-binding molecule of the invention to direct the internalization and degradation of a tumor-associated antigen (Her2). The elimination of Her2 from the surface of tumor cells would be a desirable approach for the treatment of certain types of cancers characterized by high Her2 expression (e.g., breast cancer, gastric cancer, gastro-esophageal cancers, etc.).

[0120] The multispecific antigen-binding molecules used in the experiments of this Example were bispecific antibodies with one arm directed to Her2 (target) and the other arm directed to PRLR (internalizing effector protein).

Experiment 1

[0121] As an initial experiment, the ability of an anti-Her2 x anti-PRLR bispecific antibody (referred to herein as "Her2xPRLR bsAb1") to degrade endogenous Her2 in T47D breast cancer cells was evaluated. T47D breast cancer cells express low levels of HER2 and are generally recalcitrant to anti-HER2 therapy (see Horwitz *et al.*, "Variant T47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance," *Cell*. 1982 Mar;28(3):633-42).

[0122] The anti-Her2 arm was derived from the antibody known as trastuzumab. The anti-PRLR arm was derived from a fully human anti-PRLR antibody. The anti-Her2 x anti-PRLR bispecific antibody (*i.e.*, multispecific antigen-binding molecule) was assembled using the so-called "knobs-into-holes" methodology (see, *e.g.*, Ridgway *et al.*, 1996, Protein Eng. 9(7):617-621). Other constructs used in this Experiment were trastuzumab (mono-specific antibody), anti-PRLR Ab (mono-specific antibody, referred to as H1H6958N2, as set forth in US Patent Application Publication No. 2015/0056221, the disclosure of which is incorporated by reference herein in its entirety), and a mono-specific Control antibody (directed against an irrelevant non-human antigen).

[0123] For this experiment, T47D cells expressing endogenous levels of Her2 and PRLR receptors were grown in RPMI (Irvine Scientific, 9160) supplemented with 10% FBS (ATCC, 30-2020), 10mM Hepes, 1mM Sodium Pyruvate, and 10ug/ml Insulin. Cycloheximide (CHX) at 50ug/ml was used to stop protein synthesis. T47D cells were treated with either Cycloheximide and Trastuzumab (CHX/Trastuzumab), Cycloheximide and Her2xPRLR bsAb1 (CHX/bsAb1), Cycloheximide and PRLR Ab (CHX/PRLR Ab), Cycloheximide and Control Ab (CHX/Control Ab), or with Cycloheximide, PRLR Ab and Trastuzumab for either 0, 1, 2, or 4 hours.

[0124] Cells were lysed on ice in RIPA buffer (100 mM Tris-HCl, 300 mM NaCl, 2% NP-40, 1% Sodium deoxycholate, and 0.2% SDS) (Boston BioProducts BP-116X) supplemented with protease and phosphatase inhibitors (Thermo Fisher, 1861280), followed by sonication (Qsonica Model Q55, three pulses). The sonicated lysates were diluted with 2X SDS Sample Buffer 1:1, heated at 95°C for 10min, followed by centrifugation at 13,000 rpm for 10 min at room temperature. Supernatants were resolved on 4-20% Novex Tris-Glycine gels and blotted to PVDF membranes.

[0125] Her2 antibodies (Dako, A0485) at 1:300 or PRLR antibodies (Life Technologies, 35-9200) at 1:250 were used for primary labeling of membranes followed by HRP-conjugated secondary antibody at 1:5000, and chemiluminescence detection with ECL (Amersham, RPN2106). Quantitation of the Western blots was performed by calculating net intensity of bands with CareStream software (Kodak). Results are shown in Table 1. Values are expressed in terms of relative units compared to background.

[0126] As shown in Table 1, upon inhibition of protein synthesis with Cycloheximide, PRLR was shown to undergo rapid and complete degradation with all treatments used; the process was not affected by any of the antibodies tested. By contrast, in the presence of Cycloheximide, Her2 was degraded in cells treated with Her2xPRLR bsAb1, but not with the mixture of Trastuzumab and anti-PRLR Ab, individual PRLR Abs, Trastuzumab, or Control Antibodies. This result suggests that Her2xPRLR bsAb1 serves to bridge Her2 to PRLR on the cell surface which is followed by

degradation of the Her2-bsAb1-PRLR complex.

Table 1

Treatment	Time (hr)	Her2	PRLR
Cycloheximide + Trastuzumab	0	5.87	6.67
	1	2.51	4.28
	2	5.38	2.89
	4	5.66	0.18
Cycloheximide + Her2xPRLR bsAb1	0	3.99	6.48
	1	5.79	3.68
	2	2.36	1.62
	4	0.70	0.04
Cycloheximide + anti-PRLR Ab	0	5.36	5.75
	1	4.48	1.24
	2	4.24	0.69
	4	6.32	0
Cycloheximide + Control Antibody	0	3.36	4.66
	1	3.09	3.10
	2	4.98	3.30
	4	4.74	0.23
Cycloheximide + Trastuzumab + anti-PRLR Ab	0	5.37	6.12
	1	4.22	2.16
	2	4.48	0.44
	4	5.22	0

Experiment 2

[0127] In a second experiment, the dose-dependent effects of Her2 x PRLR multispecific antigen-binding proteins on Her2 degradation *in vitro*, as well as the potential effects on PRLR levels, were investigated. Two different Her2 x PRLR bispecific antibodies ("Her2xPRLR bsAb1" and "Her2xPRLR bsAb2") were used in this experiment. Both bispecific antibodies were constructed using the knobs-into-holes methodology. The anti-Her2 arm was identical for both bispecific antibodies and was derived from the antibody known as trastuzumab. The anti-PRLR arms of Her2xPRLR bsAb1 and Her2xPRLR bsAb2 were derived from two different fully human anti-PRLR antibodies.

[0128] For this experiment, T47D cells expressing endogenous levels of Her2 and PRLR

receptors were grown in RPMI (Irvine Scientific, 9160) supplemented with 10% FBS (ATCC, 30-2020), 10mM Hepes, 1mM Sodium Pyruvate and 10ug/ml Insulin. T47D cells were treated for 6 hours with either bsAb1 or bsAb2 at 10ug/ml, 3ug/ml, 1 µg/ml, 0.3 µg/ml, 0.03 µg/ml, or 0 µg/ml.

[0129] Cells were lysed on ice in RIPA buffer (100 mM Tris-HCl, 300 mM NaCl, 2% NP-40, 1% Sodium deoxycholate, and 0.2% SDS) (Boston BioProducts BP-116X) supplemented with protease and phosphatase inhibitors (Thermo Fisher, 1861280), followed by sonication (Qsonica Model Q55, three pulses). The sonicated lysates were diluted with 2X SDS Sample Buffer 1:1, heated at 95°C for 10 min, followed by centrifugation at 13,000 rpm for 10 min at room temperature. Supernatants were resolved on 4-20% Novex Tris-Glycine gels and blotted to PVDF membranes.

[0130] Her2 antibodies (Dako, A0485) at 1:300, PRLR antibodies (Life Technologies, 35-9200) at 1:250, or beta-Actin antibodies (Genetex, GTX100313) at 1:10,000 were used for primary labeling of membranes followed by HRP-conjugated secondary antibody at 1:5000, and chemiluminescence detection with ECL (Amersham, RPN2106).

[0131] Quantitation of the Western blots was performed by calculating net intensity of bands with CareStream software (Kodak). To control for loading, normalization to actin was used as follows: the sample with highest actin net intensity was used as normalization control. Actin net intensity of each sample was divided by normalization control value to get a relative value of the sample. Net intensities of each target protein (Her2 or PRLR) were divided by the calculated relative actin value for the sample to get a normalized Her2 or PRLR value (arbitrary units). Results are shown in Table 2.

[0132] As shown in Table 2, both Her2xPRLR bsAb1 and Her2xPRLR bsAb2 induced Her2 degradation in a dose dependent manner in T47D cells. In contrast, the levels of PRLR were not affected by bispecific antibody treatment. This result is consistent with PRLR undergoing constitutive surface turnover on the surface of these cells.

Table 2

Treatment	Concentration (µg/ml)	Her2	PRLR
Her2xPRLR bsAb1	0	6.47	2.49
	0.03	3.44	0.95
	0.1	1.46	1.10
	0.3	1.47	1.33
	1.0	1.10	1.63
	3.0	1.27	1.64

	10.0	0.98	1.30
Her2xPRLR bsAb2	0	6.16	3.48
	0.03	4.37	2.71
	0.1	3.12	3.64
	0.3	2.43	3.42
	1.0	1.43	2.85
	3.0	1.55	2.52
	10.0	1.59	2.70

Experiment 3

[0133] The foregoing experiments suggest that Her2 degradation by a Her2xPRLR bispecific antibody requires that the bispecific antibody simultaneously bind PRLR (an internalizing effector protein) and Her2 (a target protein). To confirm this principle, a third experiment was conducted to assess the effect of blocking either the PRLR- or Her2-binding arms of the Her2xPRLR bsAb1 on Her2 internalizing activity.

[0134] For this experiment T47D cells expressing endogenous levels of Her2 and PRLR receptors were grown in RPMI (Irvine Scientific, 9160) supplemented with 10% FBS (ATCC, 30-2020), 10 mM Hepes, 1 mM Sodium Pyruvate and 10 µg/ml Insulin. Her2xPRLR bsAb1 (10 µg/ml) was left untreated (i.e., both arms unblocked), or incubated with either a soluble PRLR protein construct ("PRLR.mmh" to block the PRLR-binding arm) or a soluble Her2 protein construct ("Her2.mmh" to block the Her2-binding arm) at 37°C for 1h (1:2 molar ratio) before adding to T47D cells for either 0, 2, 4, or 6 hours.

[0135] At the end of the incubation period, cells were lysed on ice in RIPA buffer (100 mM Tris-HCl, 300 mM NaCl, 2% NP-40, 1% Sodium deoxycholate, and 0.2% SDS) (Boston BioProducts BP-116X) supplemented with protease and phosphatase inhibitors (Thermo Fisher, 1861280), followed by sonication (Qsonica Model Q55, three pulse). The sonicated lysates were diluted with 2X SDS Sample Buffer 1:1, and heated at 95°C for 10min, followed by centrifugation at 13,000 rpm for 10 min at room temperature. Supernatants were resolved on 4-20% Novex Tris-Glycine gels and blotted to PVDF membranes.

[0136] Her2 antibodies (Dako, A0485) at 1:300 or beta-Actin antibodies (Genetex, GTX100313) at 1:10,000 were used for primary labeling of membranes followed by HRP-conjugated secondary antibody at 1:5000, and chemiluminescence detection with ECL (Amersham, RPN2106).

[0137] Quantitation of the Western blots was performed by calculating net intensity of bands with CareStream software (Kodak). To control for loading, normalization to actin was used as follows:

the sample with highest actin net intensity was used as normalization control. Actin net intensity of each sample was divided by normalization control value to get a relative value of the sample. Net intensity of Her2 band was divided by the calculated relative actin value for the sample to get a normalized Her2 value (arbitrary units). Results are shown in Table 3.

[0138] As shown in Table 3, Her2xPRLR bsAb1-mediated Her2 degradation was completely prevented in T47D cells by blocking either Her2 or PRLR arms of Her2xPRLR bsAb1, indicating that Her2 degradation occurred via its physical connection to PRLR which was mediated by simultaneous Her2 and PRLR binding by the Her2xPRLR bsAb1 multispecific antigen binding protein.

Example 8. Increased Potency of Trastuzumab Emtansine Mediated by a Multispecific Antigen-Binding Molecule That Simultaneously Binds Her2 and PRLR

[0139] This Example illustrates the use of multispecific antigen-binding molecules of the invention to increase the potency of an antibody-drug conjugate ("ADC"). More specifically, this Example demonstrates the use of a multispecific antigen-binding molecule comprising a first binding domain directed to an internalizing effector protein and a second binding domain directed to a tumor-associated target antigen, in combination with a second antigen-binding molecule specific for the tumor-associated antigen target (also referred to herein as an "accomplice molecule"), wherein the accomplice molecule is an ADC. In the experiments set forth herein below, the internalizing effector protein is prolactin receptor (PRLR), the tumor-associated antigen target is Her2, and the accomplice molecule is an ADC specific for Her2 (*i.e.*, trastuzumab emtansine (T-DM1)).

[0140] Trastuzumab is a recombinant humanized monoclonal antibody that binds to the extracellular domain of Her2. Trastuzumab and its corresponding ADC, Trastuzumab-emtansine, or T-DM1, have been successfully used in patients with strong Her2 overexpression, as assessed by immunohistochemistry (IHC 3+). However, there is currently no effective therapy available for patients with Her2 IHC2+ and Her2 IHC1+ tumors.

[0141]

Table 3

Treatment	Time (hr)	Her2
Her2xPRLR bsAb1	0	2.84
	2	2.39
	4	1.31
	6	0.42

Her2xPRLR bsAb1 + PRLR.mmh	0	3.06
	2	1.94
	4	2.65
	6	2.30
Her2xPRLR bsAb1 + Her2.mmh	0	2.16
	2	2.30
	4	2.56
	6	1.47

[0142] In this Example, the ability of four different anti-Her2 x anti-PRLR bispecific antibodies (referred to herein as "Her2xPRLR bsAb36," "Her2xPRLR bsAb37," "Her2xPRLR bsAb42" and "Her2xPRLR bsAb45") to increase the cell-killing potency of T-DM1 on Her2-expressing cells was evaluated. The bispecific antibodies used in this Example were constructed from four different anti-Her2 arms and two different anti-PRLR arms, as summarized in Table 4.

Table 4

Bispecific Antibody	Anti-Her2 Arm	Anti-PRLR Arm
Her2xPRLR bsAb36	anti-Her2 Ab-1	anti-PRLR Ab-1
Her2xPRLR bsAb37	anti-Her2 Ab-2	anti-PRLR Ab-1
Her2xPRLR bsAb42	anti-Her2 Ab-3	anti-PRLR Ab-2
Her2xPRLR bsAb45	anti-Her2 Ab-4	anti-PRLR Ab-2

[0143] Standard methods were used to construct the anti-Her2 x anti-PRLR bispecific antibodies (*i.e.*, multispecific antigen-binding molecules). Control experiments were also conducted using a control ADC (*i.e.*, an antibody-drug conjugate comprising an antibody directed against an irrelevant non-human protein conjugated to DM1), or an anti-PRLR-DM1 ADC (the antibody-drug conjugate referred to as H1H6958N2 in US 2015/0056221, the disclosure of which is incorporated by reference herein in its entirety).

[0144] To evaluate the ability of Her2xPRLR bispecific antibodies to enhance the cell killing effect of T-DM1, T47D cells expressing endogenous levels of PRLR and overexpressing Her2 to intermediate levels (T47D/Her2) were first grown in RPMI (Irvine Scientific, 9160) supplemented with 10% FBS (ATCC, 30-2020), 10mM Hepes, 1mM Sodium Pyruvate and 10ug/ml Insulin. Cells were then seeded on 96-well plates at 3000 cells/well. The next day cells were left untreated, or treated with a range of concentrations of either T-DM1, or Control ADC, or anti-PRLR-DM1, or a

combination of T-DM1 with 10ug/ml of either Her2xPRLR bsAb42, Her2xPRLR bsAb36, Her2xPRLR bsAb37, or Her2xPRLR bsAb45 for 3 days in triplicates.

[0145] Cell viability was assessed as follows: 3 days post-treatment cells were fixed with 0.25% PFA, 0.1% Saponin, 2ug/ml Hoechst for 20 min, whole well images were acquired on automatic microscope ImageXpress^{MICRO} at 10x and analyzed using Cell Proliferation^{HT} MetzxPressTM Module (nuclear count). Results are summarized in Table 5.

Table 5

Treatment	IC50 (nM)
T-DM1	30.0
Control ADC	200.0
T-DM1 + Her2xPRLR bsAB42	2.0
Control ADC + Her2xPRLR bsAB42	100.0
Anti-PRLR-ADC	0.9
T-DM1 + Her2xPRLR bsAB37	2.0
T-DM1 + Her2xPRLR bsAB36	2.5
T-DM1 + Her2xPRLR bsAB45	1.0

[0146] As shown in Table 5, the cell killing potency of T-DM1 was significantly enhanced by the presence of the Her2xPRLR bispecific antibodies of the present invention. Thus, this Example demonstrates the ability of the multispecific antigen binding proteins of the invention to enhance the potency and efficacy of immunoconjugate molecules directed to target proteins that are not normally internalized by cells in a rapid manner.

[0147] T-DM1 is known to be active against tumors expressing high levels of HER2, while those cells expressing low and intermediate levels of HER2 remain resistant to T-DM1 treatment. In contrast, an anti-PRLR-ADC is capable of inducing robust killing of breast tumor cells expressing low levels of PRLR. The difference in killing efficiency between anti-PRLR-ADC and T-DM1 is likely due to the difference in internalization rates and lysosomal trafficking of their targets respective targets. The intracellular trafficking of PRLR and HER2 were compared.

[0148] In those cells expressing low surface levels of PRLR and HER2, the PRLR was observed to undergo rapid constitutive lysosomal trafficking and degradation that was largely independent of prolactin ligand. HER2, however, did not undergo rapid constitutive lysosomal trafficking and degradation. For example, T47D cells, which express low levels of PRLR and HER2, internalized

80% of the PRLR within 60 minutes, whereas HER was not internalized (Fig. 11). In these experiments, T47D cells were incubated on ice with either CFTM594 -labeled anti-PRLR primary antibody or CFTM594 -labeled anti-HER2 primary antibody. The internalization process was initiated by adding pre-warmed (37°C) media to the cells. At indicated times cells, the cells were fixed with 4% paraformaldehyde to stop internalization, and stained with secondary Alexa Fluor® 488-conjugated goat anti-Human Fab to detect non-internalized antibodies remaining on the cell surface (yellow). Co-localization was quantified in a pixel-by-pixel basis for all sections of the confocal stack. The results are depicted in Figure 11)

[0149] Likewise, PRLR, but not HER2 was observed to be rapidly internalized into the lysosomal compartment of T47D cells. CFTM594-labeled anti-PRLR antibodies or CFTM594-labeled anti-HER2 antibodies were added to T47D cells pre-labeled with fluorescein-3 kDa dextrans. The antibody-receptor complexes were allowed to internalize at 37°C for indicated times (X-axis of Fig. 12), then fixed with 4% paraformaldehyde. Co-localization of internalized antibodies with dextrans-labeled lysosomes was determined. The results are depicted in Fig. 12 and essentially agree with the internalization data of Fig. 11.

[0150] The sequence motifs governing the constitutive turnover of PRLR were mapped to a 21 amino acid region in receptor cytoplasmic domain; PRLR turnover enabled efficient ADC-mediated cell cycle arrest and cell killing. Fig. 13 depicts the various PRLR and HER2 constructs used in the following experiments, including: (1) full length PRLR (PRLR FL), (2) full length HER2 (HER2 FL), (3) the extracellular (Ecto) domain of PRLR fused to the transmembrane (TM) and cytoplasmic (Cyto) portions of HER2 (PRLRectoHER2cytoTM), (4) the extracellular (Ecto) domain of HER2 fused to the transmembrane (TM) and cytoplasmic (Cyto) portions of PRLR (HER2ectoPRLRcytoTM), (5) PRLR with amino acids 301-622 (as measured by unprocessed protein with signal peptide remaining) deleted (PRLR Cyto42), and (6) PRLR with amino acids 280-622 (as measured by unprocessed protein with signal peptide remaining) deleted (PRLR Cyto21).

[0151] To map the internalization effector sequences of PRLR to one of its domains (extracellular, transmembrane, cytoplasmic) HEK293 cells were grown in 24-well optical plates and transiently transfected with a mammalian expression vector encoding PRLR, HER2, HER2ectoPRLRcytoTM, or PRLRectoHER2cytoTM for 24 hours. The transfected cells were then incubated on ice in the presence of CFTM594 -labeled primary antibody, and then warmed to 37°C and incubated for an additional hour. Figure 14 depicts decorated cells at 4°C, when construct is not expected to be internalized), and after 37°C, when those constructs capable of being rapidly internalized would be internalized. Panel A depicts internalized PRLR FL construct at 37°C. Panel B depicts the HER2 FL

construct, which essentially failed to internalize within one hour at 37°C. Panel C depicts the PRLRecto-HER2cyto/TM construct, which also essentially failed to internalize within one hour at 37°C. Panel D depicts the internalized HER2ectoPRLRcyto/TM construct at 37°C. These results suggest that the cytoplasmic domain of PRLR provides the internalization effector sequence.

[0152] The internalization effector sequence was further mapped to the PRLR amino acid residues of about 280 to about 300 (e.g., residues 280 to 300 of SEQ ID NO:11; residues DAHLLLEKKGKSEELLSALGCQD [SEQ ID NO:12]) (a.k.a. PRLR Cyto21-42). Labeled antibody-Ice-to-37°C experiments were performed on HEK293 cells as described above. Figure 15 depicts the cellular uptake of those constructs containing the entire cytoplasmic domain of PRLR (PRLR FL) (panels A and B), the membrane proximal 42 amino acids (PRLR Cyto42) (panels C and D), and the membrane proximal 21 amino acids (PRLR Cyto21) (panels D and E). While the PRLR Cyto42 construct was rapidly internalized, the PRLR Cyto21 construct was not. This demonstrates that the PRLR cytoplasmic domain region between 21 and 42 amino acid proximal to plasma membrane (Cyto21-42) contains information essential for internalization and degradation of PRLR.

[0153] To determine whether internalized PRLR constructs were degraded (*i.e.*, targeted to the lysosome), the transfected HEK293 cells were treated with cycloheximide (CHX) at various times post-warming (0 hours, 2 hours, 4 hours, 6 hours) to arrest protein production. As demonstrated by the western blot of total cell lysates (Figure 16), PRLR FL and PRLR Cyto42, degraded rapidly, while almost no PRLR Cyto21 degradation was detected.

[0154] The effectiveness of the PRLR transmembrane and cytoplasmic domain as an internalization effector to deliver an anti-cell proliferation drug as an antibody drug conjugate (ADC) was tested on transfected HEK293 cells. HEK293 cells were engineered to express PRLR, HER2, or PRLRectoHER2cyto/TM in a tetracycline-controlled fashion (e.g., using the Lenti-X™ Tet-On® System, Clontech, Mountain View, CA). The transfected cells were induced for 24 hours with 0.01 ng/ml, or 0.003 µg/ml, or 0.7 µg/ml of doxycycline to achieve different expression levels of the receptors, which were determined by flow cytometry. Either PRLR conjugated with mertansine (a.k.a. DM1, a.k.a. emtansine) (1 nM) (panel A, Figure 17) or HER2-DM1 (1 nM) (panel B, Figure 17) was added to the respectively transfected cells, which were incubated for another 24h. Cell cycle analysis was performed using phospho-Histone H3 (Ser10) antibody to detect early mitotic cells. The results are depicted in Figure 17, which demonstrates that the transmembrane/cytoplasmic domain of PRLR effectively mediates delivery of drug into the cells.

[0155] Alanine mutagenesis of the cytoplasmic domain, specifically the Cyto21-24 domain, was performed to help map the residues necessary for internalization. The site directed mutagenesis of

two dileucines contained in the Cyto21-42 sequence inhibited the turnover of PRLR Cyto42 on transfected HEK293 cells. T-REx™ HEK293 cells (*i.e.*, stable Tet-On expressing cells, ThermoFisher Scientific, Waltham MA) were engineered to express PRLR Cyto42, PRLR Cyto21, PRLR Cyto42 L283A L284A, PRLR Cyto42 L292A L293A, or PRLR Cyto42 L283A L284A L292A L293A (a.k.a. 4LA) in a tetracycline-controlled fashion (using *e.g.*, the Jump-In™ Cell Engineering Platform, ThermoFisher Scientific, Waltham MA) were induced by Doxycyclin (0.7 µg/ml) for 24 hours and treated with CHX (50 µg/ml) for 0 or 4 hours. The results are depicted in the western blot of Figure 18, which shows a reduction in protein degradation for those constructs lacking Cyto21-42, or the di-leucine repeats. The results support the conclusion that these residues are important for lysosomal targeting.

[0156] Additional experiments demonstrated that PRLR turnover, which is mediated by its cytoplasmic domain, is the driving force directing HER2 to lysosomal degradation, when both receptors are bridged using HER2(T)xPRLR bispecific antibodies. (HER2(T) represents a trastuzumab arm.) The kinetic binding parameters of the bispecific antibody and its parent monospecific antibodies were first assessed. The bispecific antibody showed similar binding kinetics to the extracellular domains of both HER2 and PRLR as its parental antibodies (Table 6).

[0157] To demonstrate that the cytoplasmic domain of PRLR is required for HER2(T)xPRLR bsAb-mediated degradation of endogenous HER2, T-REx™ HEK293 cells were engineered to express either a full length PRLR, or a truncated PRLR lacking the entire cytoplasmic domain (lower panel). The proteins were expressed in a tetracycline-controlled fashion using the Jump-In™ Cell Engineering Platform (ThermoFisher Scientific, Waltham MA). Cells were induced for 24 hours with 0.7 µg/ml doxycycline followed by incubation with CHX combined with the indicated antibodies (Table 6) for different times, lysed and processed for Western Blot.

Table 6

Antibody	Protein	Kinetic Binding Parameters			
		k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	K_D (M)	$T_{1/2}$ (h)
HER2(T) antibody	hHER2.mmh	2.54×10^5	1.01×10^{-3}	3.98×10^{-9}	11
PRLR antibody	hPRLR.mmh	1.20×10^6	4.15×10^{-3}	3.45×10^{-9}	3
HER2(T) x PRLR bispecific	hHER2.mmh	1.70×10^6	2.86×10^{-4}	1.69×10^{-9}	40
	hPRLR.mmh	5.51×10^6	3.36×10^{-4}	6.10×10^{-10}	34

[0158] Figure 19 depicts the western blot of lysates from cells expressing the full length PRLR

construct. Note that the HER2(T) x PRLR bispecific antibody effectively targets HER2 to the lysosome, whereas the anti-HER2(T) antibody does not, demonstrating the effectiveness of PRLR as an internalization effector for low expressing HER2. Figure 20 depicts the western blot of lysates from cells expressing the cytoplasmic truncated PRLR construct. Here, no degradation of PRLR or HER2 was noted under any treatment, thus demonstrating the necessity of the cytoplasmic domain of PRLR to target that receptor and its molecular cargo to the lysosome.

[0159] The HER2xPRLR bispecific antibody induced lysosomal trafficking of HER2, enhanced HER2-ADC-induced cell cycle arrest, and promoted cell killing in T47D cells. To determine lysosome targeting, the T47D cells were treated with either non-binding control antibodies or the HER2 x PRLR bispecific antibody and subjected to the Ice-to-37°C endocytosis assay. Lysosomes and HER2 were stained and the percentage of lysosomal marker pixels occupied by HER2 pixels was determined. The results are depicted in Figure 21, which shows the more than 50% to 60% of the HER2 was targeted to the lysosome by the HER2 x PRLR bispecific antibody within 60 minutes of warming, compared to fewer than 25% in the controls.

[0160] To determine that the HER2 x PRLR bispecific antibody enhanced HER2-DM1-mediated cell cycle arrest, T47D cells expressing HER2 were treated with 1 nM, 10 nM, or 30 nM of PRLR-ADC (A), HER2-ADC plus HER2 x PRLR bispecific antibody (B), HER2-ADC alone (C), non-binding control ADC (D), or no treatment (E). The percentage of early mitotic cells was determined by staining with anti-phospho-Histone (Ser10) antibody, an indicator of cell cycle arrest. The results are depicted in Figure 22, which shows HER2-ADC plus HER2 x PRLR bispecific antibody (B) arresting cells more effectively than HER2-ADC alone (C).

[0161] To determine that the HER2 x PRLR bispecific antibody enhanced HER2-DM1-mediated cell killing, T47D cells expressing HER2 were treated with drug as indicated in Figure 23: PRLR-ADC (A), HER2-ADC plus HER2 x PRLR bispecific antibody (B), HER2-ADC alone (C), non-binding control ADC (D), or non-binding control ADC plus HER2 x PRLR bispecific antibody (E). Figure 23 depicts the results and shows that the HER2 x PRLR bispecific antibody significantly reduced the IC50 for HER2-ADC.

Example 9. High Turnover Rate and Degradation of Target Using hMHC-1 Internalizer

[0162] High turnover proteins were used to degrade soluble and transmembrane targets. Multispecific antigen-binding proteins (e.g., bispecific antibodies) were designed to link the target molecule with “destroyer” proteins (i) known to be rapidly turned over, (ii) demonstrating high target mediated clearance of bivalent antibodies, and (iii) known to traffic to or from the lysosome. For ease of design and manufacturing, bispecific antibodies were made containing a common light

chain, a heavy chain directed to the “destroyer” protein, and a heavy chain directed to the target. One of the heavy chains contained the H95R modification.

[0163] In this example, the destroyer molecule selected was major histocompatibility complex I MHC-1, more specifically the class I, B isoform (a.k.a. HLA-B). Tight binding monoclonal antibodies to HLA are rapidly cleared, which is a hallmark of a “destroyer” molecule. As an exemplar soluble target molecule, the allergen FelD1 was selected as the target molecule. FelD1 is a tetrameric glycoprotein comprising two disulfide linked heterodimers. Two forms of soluble FelD1 were constructed and tested, a FelD1-myc-myc-his fusion protein (FelD1-mmh) and a FelD1-Fc fusion protein.

[0164] In one set of experiments, FelD1-mmh was labeled with Alexa 488 to aid in tracking the internalization of the target. A bispecific antibody comprising an HLA-B-specific arm (binds destroyer) and a FelD1-specific arm (binds target) (α -HLAB27 $\cdot\alpha$ -FelD1) was used as the multispecific antigen-binding protein. C1Rneo B-lymphoblastoid cells expressing HLA-B were incubated with 10 μ g/ml FelD1-mmh-Alexa 488, and 10 μ g/ml α -HLAB27 $\cdot\alpha$ -FelD1. The cells were incubated overnight to allow time for internalization of the FelD1 target protein. The cells were then stained with anti-Alexa488 antibody (Alexa-Fluor-488-Antibody-Polyclonal/A-11094, Thermo Fisher Scientific, Waltham, MA)), which quenches the fluorescence of Alexa488. The anti-Alexa488 antibody will not quench labeled target that has been internalized, therefore internalized target can be distinguished from target that is associated with the surface of the cell. Here, fluorescence was quantified by FACs.

[0165] Figure 10 depicts the mean fluorescence (arbitrary units quantified by FACS) of surface-bound target and internalized target, for both MHC1 negative cells, which serve as controls, and MHC1 positive cells. The bispecific antibody used was α -HLAB27 $\cdot\alpha$ -FelD1. The parent α -HLAB27 $\cdot\alpha$ -HLAB27 bivalent antibody and non-specific IgG isotype were used as controls. For those cells expressing the MHC1 (destroyer), the cells contacted with α -HLAB27 $\cdot\alpha$ -FelD1 showed an approximately four-fold increase in internalized target molecule relative to surface-associate target molecule. Essentially no effect was detected for the controls. The results are depicted in Figure 10 and in Table 7 below.

[0166] The α -HLAB27 $\cdot\alpha$ -FelD1-mediated clearance of FelD1-Fc from the sera of mice expressing a human HLA-B allele was assessed. Bispecific antibody α -HLAB27 $\cdot\alpha$ -FelD1 and controls (PBS, anti-FelD1 bivalent monospecific, and anti-HLAB27 bivalent monospecific) were administered to mice expressing a human HLA-B allele by subcutaneous injection (10 mg/kg). The following day, 1.6 mg/kg of FelD1-Fc was administered by tail vein injection. (A 3:1 antibody:target ratio was

used.) Serum samples were obtained from tail bleeds taken at 15 minutes, 6 hours, 1 day, 2 days, 3 days, 4 days, 6 days, and 8 days. FeID1 levels were detected and quantified by Western blotting using anti-FeID1 antibodies. The α -HLAB27• α -FeID1 bispecific treatment demonstrated fast FeID1 clearance with a $t_{1/2}$ of < 30 hours, similar to the clearance rate of anti-HLAB27 in the absence of FeID1 (i.e., $t_{1/2}$ of 33 hours) and more than twice the clearance rate of α -HLAB27• α -FeID1 in the absence of FeID1 (i.e., $t_{1/2}$ of 65 hours). The administration of anti-FeID1 did not effect MHC1-mediated clearance, but some moderate clearance was observed, which is attributed to Fc receptors. The results are depicted in Figures 11 and 12.

Table 7: Mean Fluorescence Units (FeID1-mmh-Alexa288)

Antibody	C1R <i>neo</i> B-Lymphoblastoid Cells			
	MHC <i>minus</i> cells		MHC <i>plus</i> cells	
	Surface	Internalized	Surface	Internalized
α -HLAB27• α -FeID1	< 500	< 500	~ 825	~ 3,350
α -HLAB27• α -FeID1	< 500	< 500	< 500	< 500
Isotype control	< 500	< 500	< 500	< 500

Example 10. Degradation of Target Using APLP2/PCSK9 System

[0167] To assess whether proprotein convertase subtilisin/kexin type 9 (PCSK9) and its cell surface partners (such as LDLR and APLP2; see DeVay et al., “Characterization of proprotein convertase subtilisin/kexin type 9 (PCSK9) trafficking reveals a novel lysosomal targeting mechanism via amyloid precursor-like protein 2 (APLP2),” 288(15) J. Biol. Chem. 10805-18 (Apr 12, 2013)) can be used as effectors for antibody-mediated target destruction, anti-target-PCSK9 fusion proteins were made. The fusion protein may be regarded as a model for a bispecific antibody that incorporates an anti-PCSK9 or anti-APLP2 binding arm in lieu of the PCSK9 fusion.

[0168] For these experiments, hemojuvelin (HJV) was used as a model target in part because the in vivo readout of efficacy, i.e., measuring an increase in serum iron one week after the start of treatment, is easy and reliable. HJV is a co-receptor for bone morphogenic protein 6 (BMP6). Blocking HJV inhibits BMP6 signaling and decreases hepcidin levels, which in turn inhibits the iron transporter ferroportin. Ultimately, blocking HJV increases serum iron. (See Core et al., “Hemojuvelin and bone morphogenetic protein (BMP) signaling in iron homeostasis,” 5 Front. Pharmacol. 104 (1-9), May 13, 2014.)

[0169] Six antibody::PCSK9 fusion molecules were generated, each comprising either an anti-HJV non-blocking antibody (α -HJV-n) or anti-Myc antibody (α -Myc) in an hIgG1 backbone fused to

one of three different PCSK9 (e.g., SEQ ID NO:5) variants via a 3x GGGS linker (SEQ ID NO:6). The first variant is full length PCSK9 without the signal sequence, but including the pro domain (Full Length ["PCSK9FL"]; SEQ ID NO:7). The second is the C-terminal domain only, including some internal linker sequence between the catalytic and c-term domains of PCSK9 (Long C-term ["PCSK9LC"]; SEQ ID NO:8). The third is a short variant of the C-terminal domain that does not include this internal linker sequence (Short C-term ["PCSK9SC"]; SEQ ID NO:9). The C-terminus variants are expected to bind only to APLP2 and not to the LDL receptor.

[0170] The antibody::PCSK9 fusions were expressed in and secreted by CHO cells. Plasmids containing both the heavy chain PCSK9 fusion and cognate light chain of the relevant antibody were co-transfected into CHO-K1 cells in 10 cm or 15 cm dishes. The cells were then incubated for 4-5 days to allow production and secretion of antibody::PCSK9, after which the supernatant was harvested and sterilized by filtration through 0.2 micron filters. The CHO cell supernatants containing the fusion proteins were subsequently tested for their ability to internalize soluble HJV protein in vitro.

[0171] Soluble HJV tagged with the fluorophore pHrodo was prepared as follows. The HJV ecto-domain (SEQ ID NO:10) fused to the a myc-myc-6xhis (mmh) tag via a GPG linker was expressed in CHO sups and purified. The purified protein was subsequently labeled with pHrodo® (Thermo Fisher Scientific, Waltham) using N-hydroxysulfosuccinimide (NHS) chemistry.

[0172] HepG2 cells were incubated with a solution containing 50% antibody::PCSK9-containing CHO supernatant, 50% HepG2 media with 5% Lipoprotein deficient serum and 1 µg/ml pHrodo®-labeled hHJV-mmh. HepG2 cells are a convenient model since they express both LDLR and APLP2, key proteins known to internalize PCSK9. However, for assessing the PCSK9-fusion multispecific antigen-binding proteins, the skilled artisan will appreciate that any cell line (natural, induced, ectopically transformed, and the like) in which PCSK9 is internalized, including through binding partners other than LDLR and APLP2 could potentially be used in this assay. Cells were plated at 1.5x10⁵ cells per mL the day before the assay and incubated at 37°C. Internalization was monitored for 24 hours via imaging on an ImageXpress® high content imager (Molecular Devices, Sunnyvale, CA), with the 24 hour time point showing the greatest difference between the constructs and the controls most likely due to pHrodo®-HJV-mmh accumulation in cells throughout the assay. Internalized fluorescence was quantified using MetaXpress® Software (Molecular Devices).

[0173] Incubation with α-HJV-N::PCSK9FL and α-Myc::PCSK9FL increased internalization of the pHrodo®-tagged HJV-mmh protein relative to the no antibody control. Since the pHrodo-tagged HJV-mmh contains two myc-tags, α-Myc::PCSK9FL was expected to act similarly to α-HJV-

N::PCSK9FL in this assay. Interestingly, the fusion proteins containing the PCSK9 C-terminal domains (i.e., α -HJV-N::PCSK9LC and α -HJV-N::PCSK9SC), failed to internalize the labeled HJV protein, possibly due to attenuated LDLR binding. Results are depicted in Figure 26.

Example 11. Degradation of Target *in vivo* Using APLP2/PCSK9 System

[0174] The parent α -HJV-N bivalent monospecific antibody (which does not block BMP binding to HJV), an α -HJV-B bivalent monospecific antibody (which blocks BMP binding to HJV), the α -HJV-N::PCSK9FL fusion protein, and the α -Myc::PCSK9FL fusion protein were tested for their ability to block HJV signaling *in vivo*. The molecules were administered to mice via hydrodynamic delivery (HDD). Serum samples were taken one week later. Since endogenous HJV does not have a myc-myc-his tag, the α -Myc::PCSK9FL fusion protein functions as a negative control *in vivo*. As expected, HDD of the α -HJV-N bivalent multispecific antibody (the positive control) led to a significant increase in serum iron over the negative controls. HDD of α -HJV-N::PCSK9FL (the test molecule), also led to a significant increase in serum iron, of similar magnitude as the blocking antibody, suggesting that this molecule is effective at internalizing and either sequestering or destroying HJV *in vivo* (Figure 27).

[0175] The present invention is not to be limited in scope by the specific embodiments describe herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. Use of a bispecific antibody in the manufacture of a medicament for treatment of a patient with breast cancer, wherein the treatment comprises inhibiting the growth of a breast cancer cell or promoting the killing of a breast cancer cell by contacting the breast cancer cell with the bispecific antibody, wherein:

the bispecific antibody comprises a first antigen-binding domain that specifically binds a tumor target (T), and a second antigen-binding domain that specifically binds an internalizing effector protein (E), wherein T is HER2 and E is a prolactin receptor (PRLR); wherein the breast cancer cell expresses HER2; and wherein HER2 undergoes rapid lysosomal targeting and degradation in the presence of the bispecific antibody.

2. The use according to claim 1, the treatment further comprising contacting the tumor cell with a cytotoxic agent.

3. The use according to claim 2, wherein the cytotoxic agent is an antibody drug conjugate (ADC) comprising a drug, toxin, or radioisotope conjugated to an antibody that specifically binds HER2, preferably wherein the antigen-binding portion of the ADC binds an epitope on HER2 that does not overlap with the epitope on HER2 recognized by the first antigen-binding domain of the bispecific antibody.

4. The use according to claim 2, wherein the cytotoxic agent is a radioisotope, a toxin, or a drug.

5. The use according to claim 3 or 4, wherein the drug is selected from the group consisting of a calicheamicin, an auristatin, or a maytansine-based drug.

6. The use according to any one of claims 1-5, wherein the binding affinity of the bispecific antibody for PRLR is less than the binding affinity of the bispecific antibody for HER2.

7. The use according to any one of claims 2-6, wherein the cytotoxic agent is an antibody drug conjugate (ADC) and the ADC comprises a maytansine-based drug conjugated to an anti-HER2 bivalent monospecific antibody.

8. The use according to claim 2 or claim 4, wherein the cytotoxic agent is an antibody drug conjugate (ADC) and the ADC comprises a maytansine-based drug conjugated to an anti-PRLR bivalent monospecific antibody.

9. The use according to any one of the preceding claims, wherein the breast cancer cell also expresses PRLR.

10. The use according to any one of the preceding claims, wherein the breast cancer cell is characterized as an IHC2+ breast cancer cell.

11. The use according to claim 9 or claim 10, wherein the breast cancer cell is a T47D cell.

12. The use according to any one of the preceding claims, wherein HER2 undergoes rapid lysosomal targeting and degradation within 4 hours of contact.

13. The use according to any one of the preceding claims, wherein the bispecific antibody does not change the expression levels or surface turnover rates of PRLR by the breast cancer cell.

14. A pharmaceutical composition comprising an anti-HER2xPRLR bispecific antibody that binds to (i) PRLR, which is a rapidly internalized cell surface receptor and (ii) HER2, which is a slowly internalized cell surface receptor, wherein the rapidly internalized cell surface receptor and the slowly internalized cell surface receptor are expressed on the same cell.

15. The pharmaceutical composition of claim 14, further comprising a cytotoxic agent.
16. The pharmaceutical composition of claim 15, wherein the cytotoxic agent is an antibody drug conjugate (ADC) comprising a drug, toxin, or radioisotope conjugated to an antibody that specifically binds HER2, preferably wherein the antigen-binding portion of the ADC binds an epitope on HER2 that does not overlap with the epitope on HER2 recognized by the anti-HER2xPRLR bispecific antibody.
17. The pharmaceutical composition of claim 15, wherein the cytotoxic agent is a radioisotope, a toxin, or a drug.
18. The pharmaceutical composition of claim 16 or claim 17, wherein the drug is selected from the group consisting of a calicheamicin, an auristatin, or a maytansine-based drug.
19. The pharmaceutical composition of any one of claims 15-18, wherein the cytotoxic agent comprises a maytansinoid-based drug conjugated to an anti-HER2 bivalent monospecific antibody.
20. The pharmaceutical composition of claim 15 or claim 17, wherein the cytotoxic agent comprises a maytansinoid-based drug conjugated to an anti-PRLR bivalent monospecific antibody.
21. Use of a pharmaceutical composition in the manufacture of a medicament for the treatment of breast cancer, wherein the pharmaceutical composition comprises an anti-HER2xPRLR bispecific antibody that binds to (i) PRLR, which is a rapidly internalized cell surface receptor and (ii) HER2, which is a slowly internalized cell surface receptor, and wherein the rapidly internalized cell surface receptor and the slowly internalized cell surface receptor are expressed on the same cell.

22. The use according to claim 21, further comprising a cytotoxic agent.
23. The use according to claim 22, wherein the cytotoxic agent is an antibody drug conjugate (ADC) comprising a drug, toxin, or radioisotope conjugated to an antibody that specifically binds HER2, preferably wherein the antigen-binding portion of the ADC binds an epitope on HER2 that does not overlap with the epitope on HER2 recognized by the anti-HER2xPRLR bispecific antibody.
24. The use according to claim 22, wherein the cytotoxic agent is a radioisotope, a toxin, or a drug.
25. The use according to claim 23 or claim 24, wherein the drug is selected from the group consisting of a calicheamicin, an auristatin, or a maytansine-based drug.
26. The use according to any one of claims 22-25, wherein the cytotoxic agent comprises a maytansinoid-based drug conjugated to an anti-HER2 bivalent monospecific antibody.
27. The use according to claim 22 or claim 24, wherein the cytotoxic agent comprises a maytansinoid-based drug conjugated to an anti-PRLR bivalent monospecific antibody.