

ANTI-ERBB-2 ANTIBODIES AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of Singapore application number
5 10201603812X, filed 12 May 2016, the contents of it being hereby incorporated by reference
in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates generally to antibodies. Specifically, the present
10 invention relates to anti-ERBB2 antibodies and their uses thereof.

BACKGROUND OF THE INVENTION

[0003] Antibody-based therapy has in recent years become an important treatment
strategy for cancer. Such therapy functions through mediating alterations in antigen or
15 receptor function, modulating the immune system or delivering a specific drug that is
conjugated to an antibody that targets a specific antigen.

[0004] The fundamental basis of antibody-based cancer therapy is the fact that cancerous
tissues express an array of antigens that may be overexpressed, selectively expressed or
mutated compared to normal, non-cancerous tissue. Antibodies against a specific antigen on a
20 cancerous tissue can be used to target and kill the cancerous tissue.

[0005] However, a key challenge in developing candidate therapeutic antibodies for
cancer is the identification of antigens suitable for antibody-based therapy. Suitability of an
antigen for therapy is dependent on various factors including but not limited to the nature of
the antigen (e.g. accessibility, abundance, location of expression on cancerous cells etc.),
25 therapeutic approach, antibody affinity and other pharmacokinetic properties.

[0006] ERBB2 receptor tyrosine kinase, also known as HER2, HER2/neu and CD340,
belongs to the epidermal growth factor receptor (HER/EGFR/ERBB) family and
overexpression of ERBB2 has been detected in various cancers. Currently, some anti-ERBB2
antibodies such as Herceptin® and KADCYLA® are commercially available. However,
30 these available antibodies are costly and have been shown to bind to normal cells. Binding of
these available antibodies are also not consistent across cancer cell lines. There is therefore a

need to develop novel antibodies against ERBB2 that address the disadvantages of the anti-ERBB2 antibodies that are currently available.

SUMMARY

5 [0007] In one aspect, there is provided an antigen-binding protein, or an antigen-binding fragment thereof, comprising (i) a heavy chain variable domain comprising a VHCDR1 having the amino acid sequence GYTFSNYWIE (SEQ ID NO: 3); a VHCDR2 having the amino acid sequence EILPGSDSTNYNEKFKG (SEQ ID NO: 4), and a VHCDR3 having the amino acid sequence GGSNYGYFDY (SEQ ID NO: 5); and (ii) a light chain variable
10 domain comprising a VLCDR1 having the amino acid sequence KASQDVGTA (SEQ ID NO: 6), a VLCDR2 having the amino acid sequence WASTRHT (SEQ ID NO: 7), and a VLCDR3 having the amino acid sequence QQYSSYRT (SEQ ID NO: 8).

[0008] In one aspect, there is provided an antigen-binding protein or an antigen-binding fragment thereof, that competes with the antigen binding protein as disclosed herein for
15 binding to ErbB2 receptor protein kinase.

[0009] In one aspect, there is provided an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein comprising a radioisotope or a cytotoxin conjugated thereto.

[0010] In one aspect, there is provided a composition comprising a physiologically
20 acceptable carrier and a therapeutically effective amount of the antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein.

[0011] In one aspect, there is provided a use of an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein in the manufacture of a medicament for treating cancer.

25 [0012] In one aspect, there is provided a method for detecting cancer in a subject, the method comprising: contacting a sample obtained from the subject with an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein *in vitro*; detecting the binding of the antigen-binding protein, or antigen-binding fragment thereof in the sample; correlating the binding with a level of binding in a control sample to determine the level of
30 binding in the sample, wherein an increase in the level of binding in the sample relative to the control sample is indicative of cancer.

[0013] In one aspect, there is provided a kit when used in the method as disclosed herein, comprising an antigen-binding protein or an antigen-binding fragment thereof as disclosed herein, together with instructions for use.

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DEFINITIONS

[0014] The terms “ERBB2 tyrosine kinase receptor” and “ERBB2” are used interchangeably, and include variants, isoforms, species homologs of ERBB2 tyrosine kinase receptor and analogs having at least one common epitope with ERBB2 receptor tyrosine kinase. Estrogen Related Receptor Beta (ERBB2) is also known in the art as cluster of differentiation 340 (CD340), human epidermal growth factor receptor 2 (HER2) or Neu.

[0015] The term "immune response" refers to the action of, for example, lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by the above cells or the liver (including antibodies, cytokines, and complements) that results in selective damage to, destruction of, or elimination from the human body of invading pathogens, cells or tissues infected with pathogens, cancerous cells, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

[0016] The term “antigen binding protein” as used herein refers to whole antibodies, antibody fragments (i.e., "antigen-binding portion") and other protein constructs, such as domains, or single chains thereof which are capable of binding to an antigen.

[0017] The term “antibody” is used herein in the broadest sense to refer to molecules with an immunoglobulin-like domain and includes monoclonal, recombinant, polyclonal, chimeric, humanised, bispecific and heteroconjugate antibodies; a single variable domain, a domain antibody, antigen binding fragments, immunologically effective fragments, single chain Fv, diabodies, Tandabs™, etc (for a summary of alternative “antibody” formats see Holliger and Hudson, Nature Biotechnology, 2005, Vol 23, No. 9, 1126-1136).

[0018] An "antibody" also refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen-binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be

further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL 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. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system.

[0019] The term "antigen-binding fragment" of an antibody, as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., ERBB2 tyrosine kinase receptor). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding fragment" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR).

[0020] The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

[0021] The term "humanized antibody" is intended to refer to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences.

[0022] The term "chimeric antibody" is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody.

[0023] The term "high affinity" for an antibody refers to an antibody having a K_D of 10^{-7} or less, 10^{-8} M or less, more preferably 10^{-9} M or less and even more preferably 10^{-10} M or less for a target antigen.

[0024] As used herein, the term "subject" includes any human or nonhuman animal. The
5 term "nonhuman animal" includes all vertebrates, e.g., mammals and non-mammals, such as nonhuman primates, sheep, dogs, cats, horses, cows, chickens, amphibians, reptiles, etc. Except when noted, the terms "patient" or "subject" are used interchangeably.

BRIEF DESCRIPTION OF THE DRAWINGS

10 [0025] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0026] **Fig. 1.** Binding of A19 to various cancer cell lines. Membrane staining of tumor cells and FACS staining on live cells was performed. The cells were incubated with A19 on
15 ice for 45 mins, washed and stained with secondary FITC. The binding of A19 is analysed by flow cytometry and binding is based on the population shift from the negative control.

[0027] **Fig. 2.** A) Isotyping of A19. Isotyping was carried out with Roche isotyping strip. B) Identification of target antigen. IP on SKOV3 membrane preparation was carried out, followed by western blot. The corresponding band was excised from a parallel SDS Page gel
20 and the antigen target identified via MS. A19 is: an IgG1; has 2 distinct bands of different molecular weights; isotyping/immunoprecipitation-Mass Spectrometry (IP/MS) was performed on both bands.

[0028] **Fig. 3.** MS peptide coverage. Peptide sequences from MS were mapped against the protein sequences of Isoform 4 of Receptor Tyrosine Kinase (Her2, Erbb-2) (top sequence)
25 and Receptor tyrosine-protein kinase Erbb-2 (bottom sequence).

[0029] **Fig. 4.** Validation of identity via cross probing. Immunoprecipitation (IP) was carried out with A19 and immunoblotted with Herceptin. Similarly, IP was carried out with Herceptin and immunoblotted with A19. In both experiments, the same antigen was detected confirming that the antigen target of A19 is Erbb-2. The same antigens were detected in both
30 sets of IP. The cell line used was SKOV3.

[0030] **Fig. 5.** Validation of identity via knock down with siRNA. Knocked down of Erbb-2 was carried out using siRNA. Via western blot, all three antibodies to Erbb-2 showed

diminished binding, confirming that the antigen target is Erbb-2. The cell line used was SKOV3.

[0031] **Fig. 6.** Glycan analysis. Upon treatment with periodate, the binding of A19 is abolished. The figure shows actin bands at 39 kDa (protein-binding control) and monoclonal antibody (mAb) A4 as the positive control for periodate treatment. Fig. 6 shows A19 and Herceptin binds to glycans. The cell line used was SKOV3.

[0032] **Fig. 7.** N-linked glycan analysis. Upon enzymatic removal of N-linked glycans by PNGase, the binding of A19 was abolished. Fig. 7 shows that A19 and Herceptin bind to N-linked glycans. The cell line used was SKOV3.

10 [0033] **Fig. 8.** O-linked glycan analysis. O-linked glycan synthesis was inhibited in culture with Benzyl-alpha-GalNAc. The loss of O-linked glycans was confirmed by the loss of binding of TRA-1-60. Binding of A19 was similar to the control, confirming that the binding of A19 is not to O-linked glycans. The cell line used was HES-3. Fig. 8 shows that A19 and Herceptin do not bind to O-linked glycans.

15 [0034] **Fig. 9.** *In vitro* ADCC activity of A19. A19 does not exhibit ADCC activity when cultured with either ovarian and breast cancer cells. ADCC activity was measured as fold induction of the NFAT pathway using an ADCC reporter bio-assay (Promega). Fig. 9 shows that chimeric A19 does not elicit ADCC.

[0035] **Fig. 10.** Bioactivity of naked A19. A19 was spiked into SKOV3 at various doses and viability measured through Cell Titre Glow (CTG) at Day 2 (T2) and Day 5 (T5). The viability of the cells at various doses of A19 was comparable to the control at both T2 and T5. The cell line used was SKOV3. Fig. 10 shows that naked A19 has no effect on cell growth *in vitro*.

25 [0036] **Fig. 11.** Bioactivity of naked A19. A19 was spiked into SKOV3 at various doses ranging from 0 to 5 mg/ml and the growth monitored real-time. There is no difference in growth for all doses of A19. The cell line used was SKOV3. Fig. 11 shows that naked A19 has no effect on cell growth *in vitro*.

[0037] **Fig. 12.** A19 internalizes into cells. A19 was conjugated to a dye (pHRodo, ThermoFisher Scientific) which fluoresces brightly when the antibody complex enters the cells to an acidic environment. The dotted histogram represents cells not treated with antibodies while the shaded histogram represents cells treated with antibodies. The negative control consist of the pHRodo dye (not conjugated to any antibody), while mAb2448 is a

known mAb that internalizes into the cells (positive control). The cell line used was SKOV3. Fig. 12 shows that chimeric A19 internalizes into cells.

[0038] **Fig. 13.** A19 internalizes into cells. Immuno-staining of A19 at T=5min shows that the antibody forms a bright ring around the cell. After 1hr, the antibody internalizes into the cell, as observed by the punctuated and scattered staining of A19 inside the cell. The cell line used was SKOV3.

[0039] **Fig. 14.** A19 can be used as an antibody-drug conjugate (ADC). A19 is conjugated to the toxin saporin via a secondary mAb or via streptavidin-biotin affinity. As an ADC, A19 kills cells *in vitro*. The cell line used was SKOV3. Fig. 14 shows that chimeric A19 can be used as ADC.

[0040] **Fig. 15.** A19 as an ADC kills cells that highly express Erbb-2. Out of the various cancer cells, SKOV3 and SKBR3 express high levels of Erbb2. A19-ADC has effect only on SKOV3 and SKBR3 *in vitro*. Fig. 15 shows that ADC is observed on other cancers and that a significant ADC effect is observed on cell lines that highly express Erbb-2 whilst there is a slight effect on COLO205.

[0041] **Fig. 16.** A19-ADC kills cells in a dosage dependent manner. As an ADC, A19 kills SKOV3 *in vitro*, in a dose-dependent manner. It is noted that the dosage used is 1000x lower compared to that of naked A19. The cell line used was SKOV3. Fig. 16 shows that A19 works as an ADC. Naked A19 (5mg/ml) has no effect on cell proliferation.

[0042] **Fig. 17.** A19-ADC suppresses tumour growth *in vivo*. Three doses of A19-ADC (37.5ug per dose) was administered 1 week apart, intraperitoneally. Tumour size was tracked over 2 months. Cells were injected into the right flank of mice (subcutaneous). Fig. 17 shows that A19 works as an ADC *in vivo* although conjugation is sub-optimal. The cell line used was SKOV3. There was no animal model for MCF7.

[0043] **Fig. 18.** Competitive binding between A19 and Herceptin. A19 and Herceptin were individually conjugated to Alexafluor 488 and APC respectively. Individual stains showed that the antibodies bind to the cells (Q1 for A19, Q3 for Herceptin). When the antibodies were incubated together, they do not compete with each other (Q2). Fig. 18 shows that chimeric A19 does not bind to the same epitope as Herceptin. The cell line used was SKOV3.

[0044] **Fig. 19.** Isoforms that the monoclonal antibodies (mAbs) detect. All 3 mAbs detect various isoforms of Erbb-2. The isoform that is probably common to the 3 mAbs is the upper band or isoform 4. Fig. 19 shows that A19's antigen target is Erbb-2/Her2.

[0045] **Fig. 20.** Variable gene sequence and protein sequence of A19. Variable gene sequences of the A19 heavy (SEQ ID NO: 9) and light chains (SEQ ID NO: 10) and the protein sequence of the A19 heavy (SEQ ID NO: 1) and light chains (SEQ ID NO: 2) are shown.

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DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0046] In a first aspect the present invention refers to an antigen-binding protein, or an antigen-binding fragment thereof, comprising (i) a heavy chain variable domain comprising a VHCDR1 having the amino acid sequence GYTFSNYWIE (SEQ ID NO: 3); a VHCDR2
10 having the amino acid sequence EILPGSDSTNYNEKFKG (SEQ ID NO: 4), and a VHCDR3 having the amino acid sequence GGSNYGYFDY (SEQ ID NO: 5); and (ii) a light chain variable domain comprising a VLCDR1 having the amino acid sequence KASQDVGTA
VA (SEQ ID NO: 6), a VLCDR2 having the amino acid sequence WASTRHT (SEQ ID NO: 7), and a VLCDR3 having the amino acid sequence QQYSSYRT (SEQ ID NO: 8).

15 [0047] In one embodiment, the antigen-binding protein, or antigen-binding fragment thereof comprises heavy and light chain CDR regions that are about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99% identical to SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8.

[0048] In a preferred embodiment, the heavy chain variable region of the antigen-binding
20 protein, or antigen-binding fragment thereof as disclosed herein may comprise the amino acid sequence set forth in SEQ ID NO:1.

[0049] In one embodiment, the antigen-binding protein, or antigen-binding fragment thereof as disclosed herein may comprise a heavy chain variable region which comprises an amino acid sequence having about 80%, about 85%, about 90%, about 95%, about 96%,
25 about 97%, about 98% or about 99% identity to the amino acid sequence set forth in SEQ ID NO:1.

[0050] In another preferred embodiment, the light chain variable region of the antigen-binding protein, or antigen-binding fragment thereof as disclosed herein may comprise the amino acid sequence set forth in SEQ ID NO:2.

30 [0051] In another embodiment, the antigen-binding protein, or antigen-binding fragment thereof as disclosed herein may comprise a light chain variable region which comprises an amino acid sequence having about 80%, about 85%, about 90%, about 95%, about 96%,

about 97%, about 98% or about 99% identity to the amino acid sequence set forth in SEQ ID NO:2

[0052] As used herein, the percent homology between two amino acid sequences is equivalent to the percent identity between the two sequences. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology=# of identical positions/total # of positions x100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[0053] In one embodiment, the antigen-binding protein, or antigen-binding fragment thereof as disclosed herein is selected from the group consisting of monoclonal, recombinant, polyclonal, chimeric, humanised, bispecific and heteroconjugate antibodies; a single variable domain, a domain antibody, antigen binding fragments, immunologically effective fragments, single chain Fv, a single chain antibody, a univalent antibody lacking a hinge region, a minibody, diabodies, and Tandabs™.

[0054] In a further embodiment, the antigen-binding protein, or antigen-binding fragment thereof is a polyclonal or monoclonal antibody. In a preferred embodiment, the antigen-binding protein, or antigen-binding fragment thereof is a monoclonal antibody.

[0055] In a further preferred embodiment, the monoclonal antibody is A19. The monoclonal antibody may be humanized.

[0056] The antigen-binding protein, or antigen-binding fragment thereof of the present invention may bind to ERBB2 receptor protein kinase. In some embodiments, the antigen-binding protein, or antigen-binding fragment thereof may bind to one or more isoforms of ERBB2 receptor protein kinase. Examples of isoforms of ERBB2 receptor protein kinase include isoforms 1, 2, 3, 4, 5 and 6. In some embodiments, the isoforms may be truncated. In other embodiments, the antigen-binding protein, or antigen-binding fragment thereof may bind to a truncated isoform 4 of ERBB2 receptor protein kinase. In a further embodiment, the truncated isoform 4 does not comprise any hydrophobic regions.

[0057] In some embodiments, the antigen-binding protein, or antigen-binding fragment thereof binds to a glycan on the ERBB2 receptor protein kinase. In some embodiments, the antigen-binding protein, or antigen-binding fragment thereof binds to an extracellular glycan on the ERBB2 receptor protein kinase.

[0058] As used herein, glycan refers to a polysaccharide that may be homo- or hetero polymers of monosaccharides. Glycans include N-linked glycans and O-linked glycans. N-linked glycans are glycans whose monosaccharides are linked to the nitrogen in the side chain of asparagine. O-linked glycans are glycans whose monosaccharides are linked on a serine or threonine amino acid residue.

[0059] In a preferred embodiment, the antigen-binding protein, or antigen-binding fragment thereof may bind to an N-linked glycan on the ERBB2 receptor protein kinase. In a further preferred embodiment, the N-linked glycan may be located at one or more of amino acid positions 66, 124, 187, 259, 530, 571 and 629 of ERBB2 receptor protein kinase.

[0060] In another aspect, the present invention relates to an antigen-binding protein or an antigen-binding fragment thereof that competes with the antigen binding protein as disclosed herein for binding to the same epitope of ERBB2 receptor protein kinase. Competition with respect to binding may refer to binding affinity or to binding mechanism. For example, an antigen-binding protein, or an antigen-binding fragment thereof that competes with the antigen binding protein as disclosed herein for binding to ERBB2 receptor protein kinase may compete by binding to the same ERBB2 epitope with at least the same affinity or with higher affinity. Competitive binding may also be achieved by reducing avidity of binding.

[0061] In another aspect, the present invention relates to an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein conjugated to a therapeutic moiety, such as a cytotoxin, a drug (e.g., an immunosuppressant), a radiotoxin or a radioisotope.

[0062] Such conjugates are referred to herein as "immunoconjugates" or "antibody drug conjugates (ADCs)". Immunoconjugates that include one or more cytotoxins are referred to as "immunotoxins." A cytotoxin or cytotoxic agent includes any agent that is detrimental to (e.g., kills) cells. Examples include monomethyl auristatin E (MMEA-1), mertansine (DM-1) and saporin, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents also include, for example, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan,

dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine). Other
5 examples of therapeutic cytotoxins that can be conjugated to an antibody of the invention include duocarmycins, calicheamicins, maytansines and auristatins, and derivatives thereof.

[0063] In a preferred embodiment, the cytotoxin is selected from the group consisting of monomethyl auristatin E (MMEA-1), mertansine (DM-1) and saporin.

[0064] Cytotoxins can be conjugated to antibodies of the invention using linker
10 technology available in the art. Examples of linker types that have been used to conjugate a cytotoxin to an antibody include, but are not limited to, hydrazones, thioethers, esters, disulfides and peptide-containing linkers.

[0065] Antibodies of the present invention also can be conjugated to a radioactive isotope to generate cytotoxic radiopharmaceuticals, also referred to as radioimmunoconjugates.
15 Examples of radioactive isotopes that can be conjugated to antibodies for use diagnostically or therapeutically include, but are not limited to, iodine¹³¹, indium¹¹¹, yttrium⁹⁰ and lutetium¹⁷⁷. Methods for preparing radioimmunconjugates are established in the art. Examples of radioimmunoconjugates are commercially available, including Zevalin.TM. (IDEC Pharmaceuticals) and Bexxar.TM. (Corixa Pharmaceuticals), and similar methods can
20 be used to prepare radioimmunoconjugates using the antibodies of the invention.

[0066] The antibody conjugates of the invention can be used to modify a given biological response, and the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, an enzymatically active
25 toxin, or active fragment thereof, such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor or interferon-gamma.; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

[0067] In some embodiments, the antigen-binding protein, or an antigen-binding fragment comprising a radioisotope or cytotoxin conjugated thereto is internalized into a cell upon
30 binding to ERBB2 receptor tyrosine kinase. Internalization of the antigen-binding protein or

antigen-binding fragment comprising a radioisotope or cytotoxin conjugated thereto releases the radioisotope or cytotoxin and may trigger cell death.

[0068] In some embodiments, the antigen-binding protein, or an antigen-binding fragment thereof may trigger cell death by antibody-dependent cell-mediated cytotoxicity (ADCC) or
5 complement dependent cytotoxicity (CDC).

[0069] In another aspect, the present invention provides a composition comprising a physiologically acceptable carrier and a therapeutically effective amount of the antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein.

[0070] Compositions may include one or a combination of (e.g., two or more different)
10 antigen-binding protein, antigen-binding fragment thereof, antibodies, or immunoconjugates or bispecific molecules of the invention. For example, a pharmaceutical composition of the invention can comprise a combination of antibodies (or immunoconjugates or bispecifics) that bind to different epitopes on the target antigen or that have complementary activities.

[0071] Pharmaceutical compositions of the invention also can be administered in
15 combination therapy, i.e., combined with other agents. In some embodiments, the compositions of the present invention may comprise a further active pharmaceutical ingredient selected from the group consisting of bevacizumab, carboplatin, paclitaxel or gefitinib. In other embodiments, the compositions of the present invention may be administered with chemotherapy.

[0072] As used herein, "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g., by injection or infusion).
25 Depending on the route of administration, the active compound, i.e., antibody, immunoconjugate, or bispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

[0073] Pharmaceutically acceptable carriers include sterile aqueous solutions or
30 dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is

incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[0074] The pharmaceutical compounds of the invention may include one or more
5 pharmaceutically acceptable salts. A "pharmaceutically acceptable salt" or "physiologically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects. Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric,
10 hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanolic acids, hydroxy alkanolic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline metals or alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic
15 amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

[0075] A pharmaceutical composition of the invention also may include a pharmaceutically acceptable anti-oxidant. Examples of pharmaceutically acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine
20 hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

[0076] Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by
30 the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

[0077] These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

[0078] A composition of the present invention can be administered via one or more routes of administration using one or more of a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. Preferred routes of administration for antibodies of the invention include intravenous, intramuscular, intradermal, intraperitoneal, subcutaneous, spinal or other parenteral routes of administration, for example by injection or infusion. The phrase "parenteral administration" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

[0079] Alternatively, an antibody of the invention can be administered via a non-parenteral route, such as a topical, epidermal or mucosal route of administration, for example, intranasally, orally, vaginally, rectally, sublingually or topically.

[0080] In one aspect, the present invention relates to a use of an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein in the manufacture of a medicament for treating cancer.

[0081] It will be generally understood that cancer treatment includes one or more of inhibiting growth of cancer cells, suppressing proliferation of cancer cells, triggering cell death, and activating host immune response to cancer cells.

[0082] Preferred cancers which may be treated using the antigen-binding protein, or antigen-binding fragment thereof as disclosed herein include cancers typically responsive to immunotherapy. Non-limiting examples of preferred cancers for treatment include melanoma (e.g., metastatic malignant melanoma), renal cancer (e.g. clear cell carcinoma), prostate

cancer (e.g. hormone refractory prostate adenocarcinoma), breast cancer, colon cancer and lung cancer (e.g. non-small cell lung cancer). Additionally, the invention includes refractory or recurrent malignancies whose growth may be inhibited using the antibodies of the invention.

- 5 [0083] Examples of other cancers that may be treated using the methods of the invention include bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina,
- 10 carcinoma of the vulva, Hodgkin's Disease, non-Hodgkin's lymphoma, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemias including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic
- 15 leukemia, solid tumors of childhood, lymphocytic lymphoma, cancer of the bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, environmentally induced cancers including those induced by asbestos, and
- 20 combinations of said cancers.

[0084] In some embodiments, the cancer may be selected from a cancer that expresses an epitope that the antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein binds.

- [0085] In a preferred embodiment, the cancer is selected from breast, colorectal, ovarian,
- 25 lung, retinoblastoma, gastric, cervical and pancreatic cancer.

[0086] In other embodiments, the medicament disclosed herein may be administered with a further active pharmaceutical ingredient.

[0087] In yet other embodiments, the medicament disclosed herein may be administered with chemotherapy.

- 30 [0088] The further active pharmaceutical agent or chemotherapy may be administered separately, simultaneously or sequentially with the medicament, composition, antigen-binding protein, or antigen-binding fragment thereof as disclosed herein. Sequentially as used

herein, refers to administration of the further active pharmaceutical agent or chemotherapy before or after administration of the medicament, composition, antigen-binding protein, or antigen-binding fragment thereof. Administration of the further active pharmaceutical agent or chemotherapy may take place immediately, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days or 7 days before and/or after administration of the medicament, composition, antigen-binding protein, or antigen-binding fragment thereof.

[0089] In another aspect, there is provided a method for detecting cancer in a subject, the method comprising: contacting a sample obtained from the subject with an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein *in vitro*; detecting the binding of the antigen-binding protein, or antigen-binding fragment thereof in the sample; correlating the binding with a level of binding in a control sample to determine the level of binding in the sample, wherein an increase in the level of binding in the sample relative to the control sample is indicative of cancer.

[0090] In some embodiments, the control sample is from the same subject. In some embodiments, the control sample is from a different subject.

[0091] In some embodiments, in the method disclosed herein, the antigen-binding protein, or antigen-binding fragment thereof may bind to ERBB2 receptor protein kinase. In some embodiments, the antigen-binding protein, or antigen-binding fragment thereof may comprise a detectable label.

[0092] As used herein, a detectable label includes fluorescent, chemiluminescent, phosphorescent and chromogenic labels. The label may be constitutively detectable, or may be detectable upon binding with a cell or substrate. Examples of detectable labels include but are not limited to Alexa Fluor® dyes, FITC, TRITC, PE, Texas Red, Cy® dyes, GFP, YFP, RFP, CFP, APC, R-PE, Qdot® probes, SYTOX Green, propidium iodide, biotin, horseradish peroxidase, alkaline phosphatase. In a preferred embodiment, the detectable label is selected from biotin, alkaline phosphatase, horseradish peroxidase, FITC, PE or Cy® Dyes.

[0093] The detectable labels may be detected in an assay selected from flow cytometry, tissue section or immunohistochemistry.

[0094] In some embodiments, in the method as disclosed herein, the cancer detected may be selected from a cancer that expresses an epitope that the antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein binds.

5 [0095] In preferred embodiments, in the method as disclosed herein, the cancer detected may be selected from breast, colorectal, ovarian, lung, retinoblastoma, gastric, cervical and pancreatic cancer.

[0096] In another aspect, there is provided a kit when used in the method as disclosed herein, comprising an antigen-binding protein, or an antigen-binding fragment thereof as disclosed herein, together with instructions for use.

10 [0097] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention
15 in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may
20 be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0098] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or
25 negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0099] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of
30 any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

[00100] Non-limiting examples of the invention and comparative examples will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

5 [00101] **Materials and Methods**

[00102] *Flow Cytometry Analysis*

[00103] Cells were harvested using trypsin (Invitrogen, USA) to obtain a single cell suspension of 2×10^5 cells in 10 μ L of ice-cold 1% bovine serum albumin (BSA) (Sigma-Aldrich, USA) in phosphate buffered saline (PBS, Invitrogen, USA). Cells were incubated for
10 45 minutes at 4°C in 100 μ L of hybridoma culture supernatant containing monoclonal antibody A19 or 5 μ g of purified mAbs. Cells were then washed with ice-cold 1% BSA/PBS, and incubated with polyclonal goat anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (FITC) at a dilution of 1:500 (DAKO, Denmark) for 15min in the dark. Cells were then washed with ice-cold 1% BSA/PBS and resuspended in 200 μ L of 1% BSA/PBS
15 for analysis on FACScalibur flow cytometer (BD Biosciences, USA).

[00104] *Isotyping*

[00105] Isotyping was performed with Mouse Monoclonal Antibody Isotyping kit from Roche (Roche, #11493027001). The protocol was carried out according to manufacturer's instructions. Briefly, the pellet in the tube was reconstituted with 150 μ L of hybridoma culture
20 supernatant. The solution was thoroughly mixed by vortexing before adding the isostrip. The results were analyzed after 10min of incubation.

[00106] *Membrane Protein Extraction*

[00107] Adherent cells were scraped in PBS (Invitrogen, USA) and centrifuged at 3000rpm for 5 minutes. The cells were washed in ice-cold PBS (Invitrogen, USA) and centrifuged at
25 3000rpm for 5min. The resulting pellet was resuspended in Homogenize Buffer Mix (BioVision, USA) and sonicated using Misonix Sonicator 3000 under the following conditions: a total process time of 5min consisting of repeated cycles of 5s pulse on and 10 seconds pulse off. The resulting homogenate was transferred to a 1.5mL microcentrifuge tube and centrifuged at 700g for 10min at 4°C to remove debris. The supernatant was then
30 collected and centrifuged at 10000g for 30min at 4°C. The resulting supernatant was discarded and the pellet containing membrane protein extract was collected for subsequent analysis.

[00108] *Immunoprecipitation (IP)*

[00109] The membrane protein was solubilized with 2% Triton in PBS. Immunoprecipitation was carried out using the Phynexus instrument (Phynexus Inc, California, USA), loaded with Protein G tips (Phynexus Inc, #PTR 92-05-02). The automated
5 program allowed sequential incubation with A19 or Herceptin, solubilized protein samples and washing buffers. Low pH elution was performed at the final step and the eluted sample was neutralized before use.

[00110] *SDS PAGE Gel and Western Blot*

[00111] The samples were boiled at 95oC after adding 5X sample loading dye and
10 subjected to SDS-PAGE using 4-12% gradient NuPAGE Bis-Tris gel (#NP0335 Box) with 1X MOPS buffer (#NP001) (all from Life Technologies). The proteins were separated at 100-120V for 1-2hr. The samples were prepared in duplicates, one set used for Western blot transfer onto PVDF membrane and the other for silver staining. The membrane blot was blocked with 5% low fat milk for 30min before incubating overnight at 4oC with diluted
15 culture supernatant from the primary antibody (1:3) with blocking buffer. Blots were washed with 0.1% Tween in PBS, and incubated with horseradish peroxidase (HRP) conjugated anti-mouse or anti-human Ig (1:10000, DAKO) at room temperature for 1hr. Finally, the blots were developed using chemiluminescence, ECL prime Western blotting detection reagent (GE Healthcare, #RPN2232). The protein band on the silver stained gel that corresponded to
20 the Western blot was excised and digested with trypsin prior to antigen target identification using mass spectrometry (LC/MS-MS). For target validation, A19 and commercial antibodies against Erbb-2 (Herceptin/Abcam) were diluted 1:100 for IP and 1:1000 for Western blotting.

[00112] *siRNA Knockdown*

[00113] To validate the identity of target antigen, knockdown of target antigen was carried
25 out with siRNA against Erbb-2 (Ambion, #103546) using Lipofectamine® RNAiMAX according to the transfection protocol provided by the manufacturer. siRNA negative control was used as the scrambled control. Briefly, 1 x 10⁵ SKOV3 cells were seeded into 6-well plate and allowed to adhere overnight. Master mixes of Lipofectamine was mixed with master mixes of scrambled and Erbb-2 siRNA (1:1) and allowed to stand for 20min at room
30 temperature. Culture media was aspirated from the 6-well plate and replaced with 3ml/well of fresh media. 200μL of the mix was added into the respective wells dropwise and evenly distributed throughout the wells. The cells were left to incubate at 37°C for 48hr. The cells

were harvested by scrapping and lysed with 2% Triton in PBS. Total protein concentration was quantified with DC Protein Assay (Bio-Rad Laboratories) and Western blot carried out as previously described.

[00114] *PerIodate*

- 5 [00115] Proteins were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were rinsed twice with 100mM sodium acetate (Merck, Germany), pH 4.5 and subsequently incubated in the dark twice with 100mM sodium meta-perIodate (Sigma-Aldrich, USA) for 15min each. Sodium acetate was added into the control instead of sodium meta-perIodate. After incubation, membranes were rinsed 4 times with 100mM sodium acetate, followed by PBS. The membranes were then incubated with 0.5M sodium borohydride (Sigma Aldrich, USA) for 30min at room temperature. After incubation, the membranes were rinsed once with PBS and blocked in 5% milk in PBS-Tween for 30min at room temperature. Thereafter, the blots were probed with primary antibodies and detection via chemiluminescence.

- 15 [00116] *PNGase Digestion*

- [00117] PNGase digestion was carried out according to manufacturer's protocol (New England Biolabs). Briefly, 10-20µg of glycoprotein was first denatured in 1x glycoprotein Denaturing Buffer at 95°C for 10 minutes. Denatured proteins were then incubated with 1µl sialidase at 37°C. Subsequently, 1x G7 Reaction Buffer and 10% NP-40 were added and incubated with 2µl of PNGase F at 37°C for 1hr. Digested proteins were subsequently resolved on SDS-PAGE and transferred to Western blot.

[00118] *Inhibition of O-linked Glycosylation in hESC*

- [00119] Human embryonic stem cell line, HES-3 was obtained from ES Cell International (ESI, Singapore, <http://escellinternational>). The cells were cultured at 37°C, 5% CO₂ on matrigel-coated culture dishes supplemented daily with conditioned media (CM) from immortalized mouse feeders, ΔE-MEF. The media used for culturing hESC was KNOCKOUT (KO) media which contained 85% KO-DMEM (DMEM, Dulbecco's modified Eagle's medium) supplemented with 15% KO serum replacer, 1 mM L-glutamine, 25 U/ml penicillin, 25 U/ml streptomycin, 1% non-essential amino acids (NEAA), 0.1 mM 2-mercaptoethanol, and 5 ng/mL of recombinant human fibroblast growth factor-2 (FGF-2) (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>). To passage hESC, briefly, once the culture reached confluency, the cells were mechanically cut with cell cutter (Invitrogen) into

small square cell sheets, scraped from the culture dish using cell scraper and transferred to a fresh matrigel-coated culture dish. Culture dishes were pre-incubated with matrigel (Becton Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>) at 4°C overnight or at room temperature for at least 4hr. Cells are passaged at a ratio of 1:6 or 1:8 depending on the confluency.

[00120] Four days after passaging, hESC in culture were spiked with optimized amount of Benzyl- α -GalNac in CM and incubated for 24hr. For the negative control, hESC were fed with CM or CM with the same volume of DMSO as the inhibitor. Cells were trypsinised and resuspended as single cell suspension in 1% BSA/PBS. Flow cytometry analysis was carried out as described previously.

[00121] *Antibody-dependent cell-mediated cytotoxicity assay*

[00122] ADCC activity was measured using a reporter bioassay (Promega; ADCC Reporter Bioassay, #G7010). The ADCC bioassay was carried out according to the manufacturer's protocol. Briefly, cells were seeded at 5,000 cells per well in a 96-well clear bottom black tissue culture plates (Corning; #3904) in low 4% IgG-serum (Promega; #G711A) media. Serial dilutions of primary antibody were incubated in triplicate wells for approximately 15 min at 37°C, 5% CO₂. Following incubation, engineered effector cells were added to the wells at approximately 150,000 cells per well. After more than 5 h (or as indicated in results), Bio-Glo™ Luciferase Assay Substrate (Promega; #G719A and #G720A) was added to the wells and luminescence was measured using the Infinite® 200 microplate reader (Tecan).

[00123] *CellTiter-Glo® (CTG) Luminescent Cell Viability Assay*

[00124] Cells were seeded (1000 cells/90μL/well) to 96-well plates (black, clear flat bottom) in culture media and incubated overnight at 37°C, 5% CO₂. Stocks of mAbs with varying concentrations or mAb conjugated with toxins were prepared accordingly and added in volumes of 10μL to the cultures. The cultures were incubated for another 72hr at 37°C, 5% CO₂. Metabolically active cells were measured based on the presence of ATP, using the CellTiter-Glo® (CTG) Luminescent Cell Viability Assay kit (Promega). CTG substrate was added in volumes of 100μL to each well and incubated for 15min in the dark at RT on a shaker. Luminescence was measured using TECAN M2000.

[00125] *Real-time Monitoring of Cell Proliferation*

[00126] Cell growth was continuously monitored over time using the xCelligence® real-time analyzer (Roche). Adherence of cells was monitored based on cell impedance. Cell

culture media (40µL) was first loaded onto the 96-well E-plate to measure background impedance. Cells were then plated at 1,000 cells/50µL per well and allowed to grow overnight in normal cell culture conditions. Stocks of mAbs with varying concentrations or mAb conjugated with toxins were prepared accordingly and spiked in volumes of 10µL to the cultures. All experiments were done in at least 3 wells per treatment condition.

[00127] *Antibody Drug Conjugates (ADCs)*

[00128] Primary mAbs were complexed with appropriate secondary antibody conjugates: mAb-ZAP or HUM-ZAP (Advanced Targeting Systems), at 1:1 molar ratio for 15 min at room temperature before spiking into the cultures. Alternatively, mAbs were biotinylated using the EZ-Link™ Sulfono-NHS-Biotin kit (Thermo Fisher Scientific) prior to incubating with Streptavidin Saporin (1:1 molar ratio).

[00129] *Biotinylation of mAbs*

[00130] mAbs were biotinylated using the EZ-Link™ Sulfono-NHS-Biotin kit (Thermo Fisher Scientific). Briefly, 50µL of Biotin Regent was added to 1ml of mAb (2mg/ml, in PBS) and incubated at room temperature for 30min. Non-reacted biotin was removed by dialysis.

[00131] *Internalization Studies*

[00132] Biotinylated mAbs was incubated with equimolar of pHRedo® Red Avidin (Thermo Fisher Scientific, #P35362) in the dark and on ice for 5min prior to use. 5µg of conjugated mAbs was added to the cells and incubated in the dark and at room temperature for 2hrs before analysis on the FACS Calibur via the FL2-H channel. Real time video capture of the internalization was carried out on the DeltaVision (GE Healthcare Life Sciences).

[00133] *Immuno-Fluorescence*

[00134] Cells were trypsinised, seeded at 2000 cells/well on two 24-well plates (Plate 1 and Plate 2) and left overnight in the incubator at 37°C, 5% CO₂. Both plates of cells were pre-chilled by washing twice with fresh cold media and topped up with 1ml of cold media. Primary antibody was then added into the wells (final concentration of 4µg/ml). For the 1st plate, incubation was carried out on ice for 5 mins. For the 2nd plate, incubation was carried out at 37°C for an hour to facilitate internalization. After the primary mAb incubation, both plates were washed twice with cold PBS and subsequently fixed with 4% Paraformaldehyde /PBS for 15min. The cells were washed twice with cold PBS and permeabilized with 0.5% Triton-X/PBS for 10min. The cells were again washed twice and blocked with 10% Fetal

Bovine Serum/PBS for 10min. The cells were washed twice again with PBS and incubated with anti-mouse Alexafluor® 488 and DAPI (Thermo Fisher Scientific) for 30min in the dark. Excess dyes were washed off with PBS and 500µl 1% BSA/PBS was added to each well before imaging.

5 [00135] *In Vivo Model*

[00136] The antibody drug conjugate was prepared by conjugating biotinylated A19 to Streptavidin Saporin (Advanced Targeting Systems) as described earlier. For the animal model, the pre-emptive model was adopted. Each nude mouse was injected in the right flank, subcutaneously, with 5 x 10⁶ SKOV3 cells in 100µL volume PBS/matrigel (1:1 volume; BD
10 Matrigel™ Matrix, #354234). The drug (37.5µg per dose) was administered intra-peritoneal at Day 0, 7 and 14. Tumour size was monitored over 70 days.

[00137] *Conjugation of mAbs to Fluorophores*

[00138] Antibodies A19 and Herceptin were conjugated to Alexafluor 488 and Allophycocyanin (APC) respectively using the LYNX Rapid Conjugation Kit® (AbD
15 Serotec) according to manufacturer's protocol. Briefly, 100-150µg of antibody is used for every 100µg of fluorophore. To the antibody sample, 1µl of the Modifier reagent is added to every 10µl of antibody and gently mixed. The mixed antibody-modifier sample is added directly onto the LYNX lyophilized is resuspended by gentle pipetting the solution up and down twice. The sample is incubated at room temperature for 3hr. After the incubation, 1µl
20 of Quencher is added to every 10µl antibody used. The final sample is left to stand for 30min before use.

[00139] *Competitive Assay*

[00140] 5µg of conjugated mAbs were incubated individually or dually with single cells suspension of SKOV3 (0.5 x 10⁶ cells per 100µl 1% BSA/PBS) on ice for 30min. The cells
25 were washed and resuspended in 200µl buffer. Bindings of A19 and Herceptin were analysed on the FACS Calibur via the FL1-H and FL4-H channels respectively.

[00141] **Results**

[00142] The experimental data demonstrates that A19 binds to various cancer cell lines and that the isotype of A19 is IgG1. From immunoprecipitation/mass spectrometry, the antigen
30 target of A19 is shown to be Erbb-2. A19 binds to two isoforms. The upper band in Fig. 3 is identified as Isoform 4 of Receptor Tyrosine Kinase while the lower band is identified as Receptor tyrosine-protein kinase Erbb2. The peptide coverage (from MS) compared to the

protein sequence for A19 is: (1) 39% for Isoform 4 of Receptor Tyrosine Kinase. (2) 14% for Receptor tyrosine-protein kinase Erbb-2.

[00143] Immunoprecipitation (IP) was carried out with A19 and immunoblotted with Herceptin. Similarly, IP was carried out with Herceptin and immunoblotted with A19. In both
5 experiments, the same antigen was detected confirming that the antigen target of A19 is Erbb-2.

[00144] Knock down of Erbb-2 was carried out using siRNA. Protein load across all conditions (LP, SC and KD) were normalized as evident by the actin bands. Binding of anti-Erbb-2 and Herceptin was diminished in the KD lane but not in the other 2 controls,
10 confirming the knock down of Erbb-2. Diminished binding of A19 was also observed, confirming that the antigen target of A19 is indeed Erbb-2.

[00145] For the perIodate assay, sodium metaperiodate was used to oxidize the carbohydrate moiety of the glycoproteins by opening saccharide rings of vicinal diols, producing 2 aldehyde groups which are reduced to hydroxyls by the reducing agent sodium
15 borohydride. Upon treatment with perIodate, the binding of Herceptin and A19 was abolished. This shows that A19 is binding to glycans on Erbb-2.

[00146] Upon enzymatic removal of N-linked glycans by PNGase, the binding of Herceptin and A19 was abolished. This shows that A19 binds to N-linked glycans on Erbb-2.

[00147] O-linked glycan synthesis was inhibited in culture with Benzyl-alpha-GalNAc. The
20 loss of O-linked glycans was confirmed by the loss of binding of TRA-1-60 post-inhibition. The binding of Herceptin and A19 to Benzyl-alpha-GalNAc treated cells was similar to the controls, confirming that the binding of A19 is not to O-linked glycans.

[00148] The variable genes from A19 mouse hybridoma were isolated and cloned into multi-promoter single expression vectors containing human IgG₁ constant genes. These
25 vectors were then transfected into DG44 Chinese hamster ovary (CHO) mammalian cell lines for production. The chimeric A19 was tested for ADCC activity using an ADCC reporter bioassay (Promega). Herceptin exhibited ADCC activity when cultured with either ovarian and breast cancer cells. However, A19 does not exhibit ADCC activity when cultured with either ovarian and breast cancer cells. As a naked antibody, A19 has no effect on the proliferation
30 of SKOV3 and has no effect on the proliferation of SKOV3.

[00149] When the cells were incubated with pHRodo dye (negative control), there was no shift in fluorescence. When the cells were incubated with the positive control mAb 2448

(conjugated to pHRedo), an increase in fluorescence was observed. Similarly, cells incubated with A19 conjugated with pHRedo showed an increase in fluorescence, indicating that the mAb internalizes into the cells.

[00150] The immunofluorescence staining confirms that A19 internalizes into cells.

5 Viabilities of the cells were comparable when incubated with A19 or saporin-toxin alone. As an ADC, A19 kills cells *in vitro* and kills cells that highly express Erbb-2.

[00151] Based on the binding of A19 and through flow cytometry analysis, SKOV3 and SKBR3 express high levels of Erbb-2. The other 4 cell lines express moderate or low levels of Erbb-2 (compared to SKOV3 and SKBR3). The viabilities of COLO205, MCF-7, CAMA-

10 1 and BT474 remained high when incubated with A19-ADC. On the other hand, the viabilities of SKOV3 and SKBR3 were significantly reduced when incubated with A19-ADC, indicating that as an ADC, A19 kills cells that highly express Erbb-2.

[00152] Through real-time monitoring, A19-ADC kills cells in a dosage dependent manner. It is noted that the dosage used is 1000x lower compared to that of naked A19.

15 [00153] A19-ADC was generated via biotinylation of the mAb and conjugated to streptavidin-Saporin. Although this conjugation was sub-optimal, the A19-ADC is able to suppress tumour growth *in vivo*. On the contrary, in the other 2 control groups (Saporin or A19 only), tumour suppression was not observed.

[00154] When A19 and Herceptin were incubated together with the cells, they do not
20 compete with each other. This shows that A19 and Herceptin bind to different epitopes. All 3 mAbs (A19, Herceptin and commercial antibody) detect various sets of Erbb-2 isoforms. The isoform that is probably common to the 3 mAbs is the upper band or isoform 4.

Claims

1. An antigen-binding protein, or an antigen-binding fragment thereof, comprising (i) a heavy chain variable domain comprising a VHCDR1 having the amino acid sequence
5 GYTFSNYWIE (SEQ ID NO: 3); a VHCDR2 having the amino acid sequence
EILPGSDSTNYNEKFKG (SEQ ID NO: 4), and a VHCDR3 having the amino acid
sequence GGSNYGYFDY (SEQ ID NO: 5); and (ii) a light chain variable domain
comprising a VLCDR1 having the amino acid sequence KASQDVGTAVA (SEQ ID
NO: 6), a VLCDR2 having the amino acid sequence WASTRHT (SEQ ID NO: 7), and a
10 VLCDR3 having the amino acid sequence QQYSSYRT (SEQ ID NO: 8).
2. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim
1, comprising heavy and light chain CDR regions that are about 80%, about 85%,
about 90%, about 95%, about 96%, about 97%, about 98% or about 99% identical to
15 the heavy and light chain CDR regions of (i) and (ii).
3. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim
1, wherein the heavy chain variable region comprises the amino acid sequence set
forth in SEQ ID NO:1.
20
4. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim
3, comprising a heavy chain variable region which comprises an amino acid sequence
having about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about
98% or about 99% identity to the amino acid sequence set forth in SEQ ID NO:1.
25
5. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim
1, wherein the light chain variable region comprises the amino acid sequence set forth
in SEQ ID NO:2.
- 30 6. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim
5, comprising a light chain variable region which comprises an amino acid sequence

having about 80%, about 85%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99% identity to the amino acid sequence set forth in SEQ ID NO:2.

- 5 7. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in any one of claims 1 to 6, wherein the antigen binding protein is selected from the group consisting of monoclonal, recombinant, polyclonal, chimeric, humanised, bispecific and heteroconjugate antibodies; a single variable domain, a domain antibody, antigen binding fragments, immunologically effective fragments, single chain Fv, a single chain antibody, a univalent antibody lacking a hinge region, a minibody, diabodies, and Tandabs™.
- 10
8. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim 7, wherein the binding protein is a monoclonal antibody.
- 15 9. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim 8, wherein the monoclonal antibody is A19.
10. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim 8 or claim 9, wherein the monoclonal antibody is humanised.
- 20 11. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in any one of claims 1 to 10, wherein the antigen-binding protein, or antigen-binding fragment thereof, binds to ERBB2 receptor protein kinase.
- 25 12. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim 11, wherein the antigen-binding protein, or an antigen-binding fragment thereof binds to isoform 4 of ERBB2 receptor protein kinase.
- 30 13. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim 12, wherein the antigen-binding protein, or antigen-binding fragment thereof binds to a truncated isoform 4 of ERBB2 receptor protein kinase.

14. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim 13, wherein the truncated isoform 4 does not comprise any hydrophobic regions.
- 5 15. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in any one of claims 11 to 14, wherein the antigen-binding protein, or antigen-binding fragment thereof binds to a glycan on the ERBB2 receptor protein kinase.
- 10 16. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim 15, wherein the antigen-binding protein, or antigen-binding fragment thereof binds to an N-linked glycan on the ERBB2 receptor protein kinase.
- 15 17. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim 16, wherein the antigen-binding protein, or antigen-binding fragment thereof binds to an N-linked glycan located at one or more of amino acid positions 66, 124, 187, 259, 530, 571 and 629 of ERBB2 receptor protein kinase.
- 20 18. An antigen-binding protein or an antigen-binding fragment thereof, that competes with the antigen binding protein as claimed in any one of claims 1 to 17 for binding to ERBB2 receptor protein kinase.
- 25 19. An antigen-binding protein, or an antigen-binding fragment thereof, as claimed in any one of claims 1 to 18, comprising a radioisotope or a cytotoxin conjugated thereto.
20. The antigen-binding protein, or antigen-binding fragment thereof, as claimed in claim 19, wherein the antibody is conjugated with a cytotoxin selected from the group consisting of monomethyl auristatin E (MMEA-1), mertansine (DM-1) and saporin.
- 30 21. The antigen-binding protein, or antigen-binding fragment thereof as claimed in claim 19 or 20, wherein the antigen-binding protein, or an antigen-binding fragment comprising a radioisotope or cytotoxin conjugated thereto is internalized into a cell upon binding to ERBB2 receptor tyrosine kinase.

22. A composition comprising a physiologically acceptable carrier and a therapeutically effective amount of the antigen-binding protein, or an antigen-binding fragment thereof, as claimed in any one of claims 1 to 21.
- 5 23. The composition as claimed in claim 22, wherein the composition comprises a further active pharmaceutical ingredient selected from bevacizumab, carboplatin, herceptin or paclitaxel.
- 10 24. Use of an antigen-binding protein, or an antigen-binding fragment thereof, as claimed in any one of claims 1 to 21, in the manufacture of a medicament for treating cancer.
25. Use of claim 24, wherein the cancer is selected from breast, colorectal, ovarian, lung, retinoblastoma, gastric, cervical and pancreatic cancer.
- 15 26. Use of claims 24 or 25, wherein the medicament is to be administered with a further active pharmaceutical ingredient.
- 20 27. Use of claim 24 or 25, wherein the medicament is to be administered with chemotherapy.
- 25 28. Use of claim 26 or 27, wherein the further pharmaceutical agent or chemotherapy is to be administered separately, simultaneously or sequentially.
- 30 29. A method for detecting cancer in a subject, the method comprising: contacting a sample obtained from the subject with an antigen-binding protein, or an antigen-binding fragment thereof as claimed in any one of claims 1 to 21 *in vitro*; detecting the binding of the antigen-binding protein, or antigen-binding fragment thereof in the sample; correlating the binding with a level of binding in a control sample to determine the level of binding in the sample, wherein an increase in the level of binding in the sample relative to the control sample is indicative of cancer.
- 30 30. The method according to claim 29, wherein the control sample is from the same subject.

31. The method according to claim 29, wherein the control sample is from a different subject.
- 5 32. The method according to any one of claims 29 to 31, wherein the antigen-binding protein, or antigen-binding fragment thereof, binds ERBB2 receptor protein kinase.
33. The method according to any one of claims 29 to 32, wherein the antigen-binding protein, or antigen-binding fragment thereof, comprises a detectable label.
- 10 34. The method according to claim 33, wherein the detectable label is selected from biotin, alkaline phosphatase, horseradish peroxidase, FITC, PE or Cy Dyes.
35. The method according to claims 33 or 34, wherein the detectable label is detected in an assay selected from flow cytometry, tissue section or immunohistochemistry.
- 15 36. The method as claimed in any one of claims 29 to 35, wherein the cancer is selected from breast, colorectal, ovarian, lung, retinoblastoma, gastric, cervical and pancreatic cancer.
- 20 37. A kit when used in the method of any one of claims 29 to 36, comprising an antigen-binding protein, or an antigen-binding fragment thereof as claimed in any one of claims 1 to 21, together with instructions for use.