

THERAPEUTIC B7-H4 BINDING MOLECULES

CROSS-REFERENCE TO RELATED APPLICATIONS

- [0001]** This application claims priority benefit of U.S. Provisional Patent Application No. 63/077,207, filed September 11, 2020, which is incorporated by reference herein in its entirety for all purposes.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

- [0002]** Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 50,724 Byte ASCII (Text) file named “ B7H4-100-WO-PCT.txt” created on September 8, 2021.

FIELD OF THE INVENTION

- [0003]** The present invention relates to binding molecules (e.g. antibodies) for the treatment of cancer, and related antibody-drug conjugates.

BACKGROUND

- [0004]** Despite years of research into and development of potential anti-cancer drugs, cancer remains one of the leading diseases globally, with one in three individuals developing some form of cancer in their lifetime. Two of the most common cancers types are breast cancer and lung cancer.
- [0005]** The principal therapies for cancer remain chemotherapy and radiotherapy. However, these therapies are associated with various undesirable side effects, from fatigue through to sickness and hair loss. These issues are exacerbated by the often lengthy courses of chemotherapy used.
- [0006]** Over the last couple of decades, a number of antibody therapies for cancer have been developed and marketed, leading to a reduction in the need for harsh forms of therapy (e.g. surgery and chemotherapy) for a number of cancer types. Although the

availability of methodology for producing antibodies (e.g. monoclonal antibodies) has greatly improved over this time period, there are relatively few clinically available anti-cancer antibodies, and even fewer that may be used to target a broad spectrum of cancer types. Furthermore, there is a need to increase the potency of therapeutic antibodies, which is generally limited by the target antigen and subsequent effects on the cancer cell following antibody binding.

[0007] The present invention solves one or more of the above-mentioned problems.

SUMMARY OF THE INVENTION

[0008] The inventors have surprisingly found that the transmembrane polypeptide, B7-H4, is highly expressed in a multiple cell types (e.g. breast, lung, and pancreatic cancer), which is consistent with a role of a cancer antigen. The inventors have successfully generated antibodies which show high (e.g. better binding compared to commercially available antibodies) binding to B7-H4 expressing cells, both *in vitro* and *in vivo*. Advantageously, the antibodies can target multiple different cancer cell types expressing B7-H4, exemplifying the broad utility of the antibodies as anti-cancer therapies.

[0009] Furthermore, the antibodies can advantageously be linked/ conjugated to suitable drugs/ cytotoxins (e.g. to provide Antibody-drug conjugates (ADC)), thus increasing the potency of the antibodies as a therapy by allowing for targeted toxin delivery to cancer cells.

DETAILED DESCRIPTION

[0010] Thus, in one aspect the invention provides an antibody or antigen binding fragment thereof which binds to B7-H4 (e.g. a B7-H4 epitope), the antibody or antigen binding fragment thereof comprising:

- i. a heavy chain CDR1 (HCDR1), a heavy chain CDR2 (HCDR2), a heavy chain CDR3 (HCDR3), a light chain CDR1 (LCDR1), a light chain CDR2 (LCDR2), and a light chain CDR3 (LCDR3) comprising the amino acid sequence of SEQ ID NO: 1,

SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof;

- ii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof;
- iii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof;
- iv. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof; or
- v. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof.

[0011] An antibody or antigen binding fragment thereof may suitably be comprised within a pharmaceutical composition, for example within a formulation suitable for administration to a patient.

[0012] In another aspect, there is provided a pharmaceutical composition comprising an antibody or antigen binding fragment thereof, the antibody or antigen binding fragment thereof comprising:

- i. a heavy chain CDR1 (HCDR1), a heavy chain CDR2 (HCDR2), a heavy chain CDR3 (HCDR3), a light chain CDR1 (LCDR1), a light chain CDR2 (LCDR2), and a light chain CDR3 (LCDR3) comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof;

- ii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof;
- iii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof;
- iv. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof; or
- v. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof.

[0013] The term “pharmaceutical composition” refers to a preparation that is in such form as to permit the biological activity of the active ingredient to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the composition would be administered. Such composition can be sterile, and can comprise a pharmaceutically acceptable carrier, such as physiological saline. Suitable pharmaceutical compositions can comprise one or more of a buffer (e.g., acetate, phosphate or citrate buffer), a surfactant (e.g., polysorbate), a stabilizing agent (e.g., human albumin), a preservative (e.g., benzyl alcohol), and absorption promoter to enhance bioavailability, and/or other conventional solubilizing or dispersing agents.

[0014] Furthermore, the antibody or antigen binding fragment thereof of the invention has been demonstrated to target and suppress growth of B7-H4 positive tumours *in vivo*. Thus, the invention embraces the above defined antibody or antigen binding fragment thereof and the above defined pharmaceutical composition for use in a method of treating cancer. Preferably, said cancer comprises a cancer cell which expresses B7-H4.

[0015] In one aspect there is provided an antibody or antigen binding fragment thereof for use in treating a cancer (for example, wherein said cancer comprises a cancer cell that expresses B7-H4), wherein the antibody or antigen binding fragment comprises:

- i. a heavy chain CDR1 (HCDR1), a heavy chain CDR2 (HCDR2), a heavy chain CDR3 (HCDR3), a light chain CDR1 (LCDR1), a light chain CDR2 (LCDR2), and a light chain CDR3 (LCDR3) comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof;
- ii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof;
- iii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof;
- iv. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof; or
- v. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof.

[0016] In other words, one aspect of the invention provides a method of treating a cancer (for example, wherein said cancer comprises a cancer cell that expresses B7-H4), the method comprising administering to a subject an effective amount of an antibody or antigen binding fragment comprising:

- i. a heavy chain CDR1 (HCDR1), a heavy chain CDR2 (HCDR2), a heavy chain CDR3 (HCDR3), a light chain CDR1 (LCDR1), a light chain CDR2 (LCDR2), and a

light chain CDR3 (LCDR3) comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof;

- ii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof;
- iii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof;
- iv. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof; or
- v. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof.

[0017] In yet other words, the invention embraces, in another aspect, use of an antibody or antigen binding fragment thereof in the manufacture of a medicament for the treatment of cancer (for example, wherein said cancer comprises a cancer cell that expresses B7-H4), said antibody or antigen binding fragment comprising:

- i. a heavy chain CDR1 (HCDR1), a heavy chain CDR2 (HCDR2), a heavy chain CDR3 (HCDR3), a light chain CDR1 (LCDR1), a light chain CDR2 (LCDR2), and a light chain CDR3 (LCDR3) comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof;
- ii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO:

10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof;

- iii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof;
- iv. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof; or
- v. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof.

[0018] Certain definitions and preferred embodiments will now be outlined. It should be understood that the following definitions and embodiments may pertain to any aspect described herein, e.g. any method, composition, and/or composition for use in therapy described herein.

[0019] The term “epitope” refers to a target protein region (e.g. polypeptide) capable of binding to (e.g. being bound by) an antibody or antigen binding fragment of the invention.

[0020] B7-H4 (also known as V-set domain-containing T-cell activation inhibitor 1, encoded by the *VTCTN1* gene) is a transmembrane polypeptide of the B7 family of co-stimulatory proteins. B7-H4 is understood to be expressed on the surface of antigen-presenting cells for interactions with ligands of immune cells (e.g. T-lymphocytes, with CD28 being a potential ligand). Without wishing to be bound by theory, the present inventors’ observation that B7-H4 is highly expressed on cells of various cancer types suggests that this molecule is a tumour-associated antigen. As such, the ability of the claimed antibody to target (and optionally deliver a cytotoxin to) a B7-H4 expressing renders said antibody particularly suitable for use in cancer therapy. Furthermore, B7-H4

expression is not limited to a particular cancer type, such that it represents a target antigen for treating a broad spectrum of cancer types.

- [0021]** The RNA, DNA, and amino acid sequences of B7-H4 are known to those skilled in the art and can be found in many databases, for example, in the databases of the National Center for Biotechnology Information (NCBI) and UniProt. Examples of these sequences found at UniProt are at Q7Z7D3 (VTCN1_HUMAN) for human B7-H4; and Q7TSP5 (VTCN1_MOUSE) for mouse B7-H4. The nucleotide sequence encoding for human B7-H4 may be SEQ ID NO: 53, more preferably SEQ ID NO: 54. The polypeptide sequence of human B7-H4 is preferably SEQ ID NO: 55.
- [0022]** In one embodiment, the antibody or antigen binding fragment thereof comprises a HCDR1, a HCDR2, a HCDR3, a LCDR1, LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EPQ-E02” or “EPQ-E02” herein.
- [0023]** In one embodiment, the antibody or antigen binding fragment thereof comprises a HCDR1, a HCDR2, a HCDR3, a LCDR1, LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EOB-F05” or “EOB-F05” herein.
- [0024]** In one embodiment, the antibody or antigen binding fragment thereof comprises a HCDR1, a HCDR2, a HCDR3, a LCDR1, LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EO5-E07” or “EO5-E07” herein.
- [0025]** In one embodiment, the antibody or antigen binding fragment thereof comprises a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28,

SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EP0-C07” or “EP0-C07” herein.

[0026] In a particularly preferred embodiment, the antibody or antigen binding fragment thereof comprises a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EQD-E02” or “EQD-E02”.

[0027] In other words, the antibody or antigen binding fragment thereof may preferably comprise:

- a HCDR1 comprising the amino acid sequence of SEQ ID NO: 7, or a functional variant thereof;
- a HCDR2 comprising the amino acid sequence of SEQ ID NO: 8, or a functional variant thereof;
- a HCDR3 comprising the amino acid sequence of SEQ ID NO: 9, or a functional variant thereof;
- a LCDR1 comprising the amino acid sequence of SEQ ID NO: 10, or a functional variant thereof;
- a LCDR2 comprising the amino acid sequence of SEQ ID NO: 11, or a functional variant thereof; and
- a LCDR3 comprising the amino acid sequence of SEQ ID NO: 12, or a functional variant thereof.

[0028] In one embodiment, the antibody or antigen binding fragment thereof comprises:

- i. a HCDR1 comprising the amino acid sequence of SEQ ID NO: 1, or a functional variant thereof;
- ii. a HCDR2 comprising the amino acid sequence of SEQ ID NO: 2, or a functional variant thereof;
- iii. a HCDR3 comprising the amino acid sequence of SEQ ID NO: 3, or a functional variant thereof;

- iv. a LCDR1 comprising the amino acid sequence of SEQ ID NO: 4, or a functional variant thereof;
- v. a LCDR2 comprising the amino acid sequence of SEQ ID NO: 5, or a functional variant thereof; and
- vi. a LCDR3 comprising the amino acid sequence of SEQ ID NO: 6, or a functional variant thereof.

[0029] In one embodiment, the antibody or antigen binding fragment thereof comprises:

- i. a HCDR1 comprising the amino acid sequence of SEQ ID NO: 13, or a functional variant thereof;
- ii. a HCDR2 comprising the amino acid sequence of SEQ ID NO: 14, or a functional variant thereof;
- iii. a HCDR3 comprising the amino acid sequence of SEQ ID NO: 15, or a functional variant thereof;
- iv. a LCDR1 comprising the amino acid sequence of SEQ ID NO: 16, or a functional variant thereof;
- v. a LCDR2 comprising the amino acid sequence of SEQ ID NO: 17, or a functional variant thereof; and
- vi. a LCDR3 comprising the amino acid sequence of SEQ ID NO: 18, or a functional variant thereof.

[0030] In one embodiment, the antibody or antigen binding fragment thereof comprises:

- i. a HCDR1 comprising the amino acid sequence of SEQ ID NO: 19, or a functional variant thereof;
- ii. a HCDR2 comprising the amino acid sequence of SEQ ID NO: 20, or a functional variant thereof;
- iii. a HCDR3 comprising the amino acid sequence of SEQ ID NO: 21, or a functional variant thereof;
- iv. a LCDR1 comprising the amino acid sequence of SEQ ID NO: 22, or a functional variant thereof;
- v. a LCDR2 comprising the amino acid sequence of SEQ ID NO: 23, or a functional variant thereof; and

- vi. a LCDR3 comprising the amino acid sequence of SEQ ID NO: 24, or a functional variant thereof.

[0031] In one embodiment, the antibody or antigen binding fragment thereof comprises:

- i. a HCDR1 comprising the amino acid sequence of SEQ ID NO: 25, or a functional variant thereof;
- ii. a HCDR2 comprising the amino acid sequence of SEQ ID NO: 26, or a functional variant thereof;
- iii. a HCDR3 comprising the amino acid sequence of SEQ ID NO: 27, or a functional variant thereof;
- iv. a LCDR1 comprising the amino acid sequence of SEQ ID NO: 28, or a functional variant thereof;
- v. a LCDR2 comprising the amino acid sequence of SEQ ID NO: 29, or a functional variant thereof; and
- vi. a LCDR3 comprising the amino acid sequence of SEQ ID NO: 30, or a functional variant thereof.

[0032] Additionally or alternatively, an antibody or antigen binding fragment thereof described herein may be described by means of a variable heavy (VH) chain and a variable light (VL) chain thereof.

[0033] Suitable a variable heavy (VH) chain sequences (which the antibody or antigen binding fragment thereof may comprise) are outlined in an individualised manner below:

- SEQ ID NO: 31, or a functional variant thereof;
- SEQ ID NO: 33, or a functional variant thereof
- SEQ ID NO: 43, or a functional variant thereof
- SEQ ID NO: 45, or a functional variant thereof
- SEQ ID NO: 46, or a functional variant thereof
- SEQ ID NO: 47, or a functional variant thereof
- SEQ ID NO: 35, or a functional variant thereof
- SEQ ID NO: 37, or a functional variant thereof
- SEQ ID NO: 39, or a functional variant thereof

[0034] Particularly suitable variable heavy (VH) chain sequences (which the antibody or antigen binding fragment thereof may comprise) are outlined in an individualised manner below:

- SEQ ID NO: 45, or a functional variant thereof
- SEQ ID NO: 33, or a functional variant thereof
- SEQ ID NO: 43, or a functional variant thereof
- SEQ ID NO: 46, or a functional variant thereof
- SEQ ID NO: 47, or a functional variant thereof

[0035] Suitable variable light (VL) chain sequences (which the antibody or antigen binding fragment thereof may comprise) are outlined in an individualised manner below:

- SEQ ID NO: 32, or a functional variant thereof
- SEQ ID NO: 34, or a functional variant thereof
- SEQ ID NO: 36, or a functional variant thereof
- SEQ ID NO: 38, or a functional variant thereof
- SEQ ID NO: 40, or a functional variant thereof

[0036] A preferred variable light (VL) chain sequence (which the antibody or antigen binding fragment thereof may comprise) may comprise an amino acid sequence of SEQ ID NO: 34 (or a functional variant thereof).

[0037] For example, in one embodiment, the antibody or antigen binding fragment thereof comprises:

- i. a variable heavy chain comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to the amino acid sequence of SEQ ID NO: 31, 33, 35, 37, or 39, or a functional variant thereof; and
- ii. a variable light chain comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to the amino acid sequence of SEQ ID NO: 32, 34, 36, 38, or 40, or a functional variant thereof.

[0038] For example, in one embodiment, the antibody or antigen binding fragment thereof comprises:

- i. a variable heavy chain comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to the amino acid sequence of SEQ ID NO: 31, 33, 35, 37, 39, 43, 45, 46, or 47, or a functional variant thereof; and
- ii. a variable light chain comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to the amino acid sequence of SEQ ID NO: 32, 34, 36, 38, or 40, or a functional variant thereof.

[0039] Suitably, the antibody or antigen binding fragment thereof may comprise:

- i. a variable heavy chain comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to the amino acid sequence SEQ ID NO: 33, or a functional variant thereof; and
- ii. a variable light chain comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof.

[0040] More suitably, the antibody or antigen binding fragment thereof may comprise:

- i. a variable heavy chain comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to the amino acid sequence SEQ ID NO: 45, or a functional variant thereof; and
- ii. a variable light chain comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof.

[0041] In one embodiment, the antibody or antigen binding fragment thereof comprises:

- a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 31, and SEQ ID NO: 32, respectively, or a functional variant thereof;
- a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 33 and SEQ ID NO: 34, respectively, or a functional variant thereof;
- a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 43 and SEQ ID NO: 34, respectively, or a functional variant thereof;
- a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 45 and SEQ ID NO: 34, respectively, or a functional variant thereof;

- a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 46 and SEQ ID NO: 34, respectively, or a functional variant thereof;
- a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 47 and SEQ ID NO: 34, respectively, or a functional variant thereof;
- a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 35 and SEQ ID NO: 36, respectively, or a functional variant thereof;
- a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 37 and SEQ ID NO: 38, respectively, or a functional variant thereof; or
- a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 39 and SEQ ID NO: 40, respectively, or a functional variant thereof.

[0042] In a preferable embodiment the antibody or antigen binding fragment thereof comprises: a variable heavy (VH) chain comprising the amino acid sequence of SEQ ID NO: 45, 33, 43, 46 or 47 (or a functional variant thereof); and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 34 (or a functional variant thereof). For example, the VH of SEQ ID NOs: 33, 45, 46 and/ 47 may correspond to “germlined” versions of the VH of SEQ ID NO: 33 (e.g. all having same CDR sequences, but with framework variations). Advantageously, each variant retains equivalent binding properties.

[0043] In one embodiment, the antibody or antigen binding fragment thereof comprises: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 31, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 32, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EPD-E02” or “EPD-E02”.

[0044] In one embodiment, the antibody or antigen binding fragment thereof comprises: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 35, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 36, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EOB-F05” or “EOB-F05”.

- [0045]** In one embodiment, the antibody or antigen binding fragment thereof comprises: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 37, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 38, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EO5-E07” or “EO5-E07”.
- [0046]** In one embodiment, the antibody or antigen binding fragment thereof comprises: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 39, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 40, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EP0-C07” or “EP0-C07”.
- [0047]** In one embodiment, the antibody or antigen binding fragment thereof comprises: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 33, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “ZY0EQD-E02” or “EQD-E02”.
- [0048]** In one embodiment, the antibody or antigen binding fragment thereof comprises: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 43, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof.
- [0049]** In one embodiment, the antibody or antigen binding fragment thereof comprises: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 46, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof.
- [0050]** In one embodiment, the antibody or antigen binding fragment thereof comprises: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 47, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof.

- [0051] In a preferable embodiment, the antibody or antigen binding fragment thereof comprises: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 45, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof. An antibody or antigen binding fragment thereof comprising said sequences may be referred to as “EQD-E02_GL”.
- [0052] In one embodiment, the antibody or antigen binding fragment thereof comprises a variable heavy chain comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to a reference amino acid sequence of SEQ ID NO: 43. In one embodiment, the antibody or antigen binding fragment thereof comprises a variable heavy chain comprising an amino acid sequence of SEQ ID NO: 43. For example, the antibody or antigen binding fragment thereof may comprise a variable heavy chain comprising an amino acid sequence of SEQ ID NO: 43, and a variable light chain comprising an amino acid sequence of SEQ ID NO: 34.
- [0053] Additionally or alternatively, an antibody or antigen binding fragment thereof described herein may be described by means of a heavy chain and/or light chain thereof.
- [0054] In one embodiment, the antibody or antigen binding fragment thereof comprises a light chain (e.g. comprising a VL and constant light chain) comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to the amino acid sequence of SEQ ID NO: 44. In a preferable embodiment, the antibody or antigen binding fragment thereof comprises a light chain (e.g. comprising a VL and constant light chain) comprising the amino acid sequence of SEQ ID NO: 44.
- [0055] In one embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain (e.g. comprising a VH and constant heavy chain) comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to a reference amino acid sequence of SEQ ID NO: 48. For example, the antibody or antigen binding fragment thereof may comprise a heavy chain (e.g. comprising a VH and constant heavy chain) comprising the amino acid sequence of SEQ ID NO: 48. Such heavy chain may be referred to as “E02-GL-Maia-heavy chain”.

- [0056]** In one embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain (e.g. comprising a VH and constant heavy chain) comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to a reference amino acid sequence of SEQ ID NO: 49. For example, the antibody or antigen binding fragment thereof may comprise a heavy chain (e.g. comprising a VH and constant heavy chain) comprising the amino acid sequence of SEQ ID NO: 49. Such heavy chain may be referred to as “E02-GLY-Maia-heavy chain”.
- [0057]** In one embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain (e.g. comprising a VH and constant heavy chain) comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to a reference amino acid sequence of SEQ ID NO: 50. For example, the antibody or antigen binding fragment thereof may comprise a heavy chain (e.g. comprising a VH and constant heavy chain) comprising the amino acid sequence of SEQ ID NO: 50. Such heavy chain may be referred to as “E02-GLQ-Maia-heavy chain”.
- [0058]** In a preferred embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain (e.g. comprising a VH and constant heavy chain) comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to a reference amino acid sequence of SEQ ID NO: 51. In a more preferable embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain (e.g. comprising a VH and constant heavy chain) comprising the amino acid sequence of SEQ ID NO: 51. Such heavy chain may be referred to as “E02-GL-WT-heavy chain”.
- [0059]** In one embodiment, the antibody or antigen binding fragment thereof comprises a light chain constant region comprising an amino acid sequence having at least 70%, 75%, 80%, 90%, 95% or 100% sequence identity to a reference amino acid sequence of SEQ ID NO: 42. In a preferable embodiment, the antibody or antigen binding fragment thereof comprises light chain constant region comprising an amino acid sequence of SEQ ID NO: 42.
- [0060]** In one embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain constant region comprising the amino acid sequence of SEQ ID NO: 41.

More preferably, the antibody or antigen binding fragment thereof comprises a heavy chain constant region comprising the amino acid sequence of SEQ ID NO: 52.

[0061] In a preferable embodiment, the antibody or antigen binding fragment thereof comprises a light chain (e.g. comprising a VL and constant light chain) comprising the amino acid sequence of SEQ ID NO: 44 and a heavy chain (e.g. comprising a VH and constant heavy chain) comprising the amino acid sequence of SEQ ID NO: 51.

[0062] Advantageously, the inventors have demonstrated that an antibody or antigen binding fragment of the claims may target a broader spectrum of B7-H4 expressing cells when compared with existing (commercially) available antibodies reported to target B7-H4. Thus, not only have the inventors provided an antibody (or antigen binding fragment) thereof having affinity and specificity for a clinically relevant target, but have demonstrated a unique advantage (e.g. unexpected technical effect) associated therewith.

[0063] For example, Figure 4 demonstrates that an exemplary antibody of the claims binds to wide spectrum of cancer cell types with affinity not achievable with antibodies: Biosciences 14-5949 anti-Human B7H4 mouse IgG; US biological B0000-35B anti Human B7H4 mouse IgG; R and D systems AF2514 anti Mouse B7H4 goat IgG1; and Sigma SAB2500141 anti B7H4 Goat IgG1.

[0064] Preferably, antibody or antigen binding fragment thereof described herein is capable of binding to B7-H4 as an integral component of a cancer cell (for example, B7-H4 as an integral component of a cell membrane of a cancer cell).

[0065] An antibody or antigen binding fragment thereof described herein may bind to an OVCAR4 cell line and/or a CHO cell line (e.g. which may lack an exogenous nucleic acid encoding B7-H4). For example, the antibody or antigen binding fragment thereof binds to a B7-H4 (e.g. a B7-H4 epitope) of an OVCAR4 cell line and/or a CHO cell line (e.g. which may lack an exogenous nucleic acid encoding B7-H4). Suitably, the antibody or antigen binding fragment thereof described herein may bind to an OVCAR4 cell line and a CHO cell line (e.g. which may lack an exogenous nucleic acid encoding B7-H4).

[0066] In one embodiment, the antibody or antigen binding fragment thereof binds to an OVCAR4 cell line and/or CHO cell line (e.g. which may lack an exogenous nucleic acid encoding B7-H4) with higher affinity when compared to one or more antibody selected

from E Biosciences 14-5949 anti-Human B7H4 mouse IgG, US biological B0000-35B anti Human B7H4 mouse IgG, R and D systems AF2514 anti-Mouse B7H4 goat IgG1, Sigma SAB2500141 anti B7H4 Goat IgG1, Isotype 1 CAT004 SP06-003, Isotype 2 R and D Normal goat IgG control (AB-108C), AdD serotec MCA2632, Epitomics 2516-1, eBiosciences, 145972-82, eBioscience 145970-85, or a combination thereof. For example, the antibody or antigen binding fragment thereof may bind to an OVCAR4 cell line and/or CHO cell line (e.g. which may lack an exogenous nucleic acid encoding B7-H4) with higher affinity when compared to one or more antibody selected from E Biosciences 14-5949 anti-Human B7H4 mouse IgG, US biological B0000-35B anti Human B7H4 mouse IgG, R and D systems AF2514 anti-Mouse B7H4 goat IgG1, and Sigma SAB2500141 anti B7H4 Goat IgG1, or a combination thereof.

[0067] In a preferable embodiment, the antibody or antigen binding fragment thereof binds to an OVCAR4 cell line with higher affinity when compared to E Biosciences 14-5949 anti-Human B7H4 mouse IgG.

[0068] Reference to “E Biosciences 14-5949 anti-Human B7H4 mouse IgG” may be used interchangeably with the term “B7-H4 Monoclonal Antibody (H74), eBioscience” herein. Said antibody is available from ThermoFisher Scientific (Catalog # 14-5949-82).

[0069] In another preferable embodiment, the antibody or antigen binding fragment thereof binds to an OVCAR4 cell line with higher affinity when compared to US biological B0000-35B anti Human B7H4 mouse IgG.

[0070] Said affinity (e.g. binding affinity) can be measured by any suitable method of measuring binding affinity described herein.

[0071] The OVCAR4 cell line is a human ovary carcinoma cell line. The OVCAR4 cell line is obtainable from the National Cancer Institute for the transfer of cell lines from the Division of Cancer Treatment and Diagnosis Tumor Repository. The Chinese hamster ovary (CHO) cell line is an epithelial cell line derived from the ovary of the Chinese hamster, and is widely obtainable.

[0072] As described above, an antibody or antigen binding fragment thereof of the invention may be comprised within a pharmaceutical composition. The pharmaceutical composition may comprise one or more pharmaceutically acceptable excipient(s). In one

embodiment, a pharmaceutical composition of the invention can comprise a pharmaceutically acceptable, non-toxic, sterile carrier such as physiological saline, non-toxic buffers, preservatives and the like. Suitable formulations for use in the therapeutic methods disclosed herein are described in Remington's Pharmaceutical Sciences, 22nd ed., Ed. Lloyd V. Allen, Jr. (2012).

- [0073] In one embodiment, a pharmaceutical composition of the invention may be comprised within one or more formulation selected from a capsule, a tablet, an aqueous suspension, a solution, a nasal aerosol, or a combination thereof.
- [0074] In one embodiment, the pharmaceutical composition comprises more than one type of antibody or antigen binding fragment of the invention. For example, a pharmaceutical composition may comprise two or more selected from an antibody, an antigen-binding fragment, an antibody or antigen binding fragment thereof conjugated to a cytotoxin, or a combination thereof.
- [0075] The term “a pharmaceutically effective amount” of an antibody or antigen-binding fragment means an amount sufficient to achieve effective binding to a target and to achieve a benefit, e.g., to ameliorate symptoms of a disease or condition or to detect a substance or a cell.
- [0076] In one embodiment, a pharmaceutical composition may comprise a buffer (e.g., acetate, phosphate or citrate buffer), a surfactant (e.g., polysorbate), optionally a stabilizer agent (e.g., human albumin), etc.
- [0077] Suitably, the antibody or antigen binding fragment of the invention binds to B7-H4 molecule with sufficient affinity such that the antibody is useful as a therapeutic agent or a diagnostic reagent in targeting B7-H4.
- [0078] In one embodiment, the antibody or antigen binding fragment thereof binds to a B7-H4 (preferably a human B7-H4) with a dissociation constant (KD) of $\leq 1 \mu\text{M}$, $\leq 100 \text{ nM}$, $\leq 10 \text{ nM}$, $\leq 1 \text{ nM}$, $\leq 0.1 \text{ nM}$, $\leq 10 \text{ pM}$, $\leq 1 \text{ pM}$, or $\leq 0.1 \text{ pM}$. In one embodiment, the antibody or antigen binding fragment thereof binds to a B7-H4 (preferably a human B7-H4) with a KD of between about 0.1nM to about 40nM, between about 0.5nM to about 30nM, between about 1nM to about 20nM, or between about 1.5nM to about 20nM.

- [0079] In a preferable embodiment, the antibody or antigen binding fragment thereof binds to a B7-H4 (preferably a human B7-H4) with a KD of between about 23nM to about 27nM. In a more preferable embodiment, the antibody or antigen binding fragment thereof binds to a B7-H4 (preferably a human B7-H4) with a KD of between about 1nM to about 1.5nM.
- [0080] The KD measurements (binding affinity) may be carried out by any suitable assay known in the art. Suitable assays include an affinity assay performable via a KinExA system (e.g., KinExA 3100, KinExA 3200, or KinExA 4000) (Sapidyne Instruments, Idaho), or ForteBio Octet system.
- [0081] In one embodiment, the extent of binding of an antibody or antigen binding fragment thereof of the invention to an unrelated, non-B7-H4 protein is less than about 10%, 5%, 2% or 1 % (preferably less than about 10%) of the binding of the antibody (or antigen binding fragment thereof) to B7-H4 (preferably human B7-H4). Said binding may be measured, e.g., by a radioimmunoassay (RIA), BIACORE® (using recombinant B7-H4 as the analyte and antibody as the ligand, or vice versa), KINEXA®, ForteBio Octet system, or other binding assays known in the art.
- [0082] In one embodiment, the antibody or antigen binding fragment thereof does not bind to one or more selected from a human B7-H1 molecule, a human B7-H2 molecule, a human B7-H3 molecule, a human BTN1A1 molecule, a human HHLA2 molecule, a human BTN3A2 molecule, or a combination thereof. In a preferable embodiment, the antibody or antigen binding fragment thereof does not bind to one or more selected from a human B7-H1 molecule, a human B7-H2 molecule, a human B7-H3 molecule, or a combination thereof.
- [0083] The term “does not bind” means that the antibody or antigen binding fragment thereof described herein does not substantially bind to one of more of said molecules (e.g. human B7-H1 molecule, a human B7-H2 molecule, a human B7-H3 molecule, a human BTN1A1 molecule, a human HHLA2 molecule, a human BTN3A2 molecule, or a combination thereof). The term “substantially no” when used in the context of binding herein may mean less than 5%, 2%, 1%, 0.5% or 0.1% of cells expressing one or more of said molecules in a cell culture become bound by the antibody or antigen binding

fragment thereof described herein (upon contact therewith). Suitably, the term “substantially no” when used in the context of binding herein may mean no such cells become bound.

[0084] In one embodiment, the antibody or antigen binding fragment thereof does not bind to a human B7-H1 molecule, a human B7-H2 molecule, a human B7-H3 molecule, a human BTN1A1 molecule, a human HHLA2 molecule, or a human BTN3A2 molecule. In a preferable embodiment, the antibody or antigen binding fragment thereof does not bind to a human B7-H1 molecule, a human B7-H2 molecule, or a human B7-H3 molecule.

[0085] In one embodiment, the B7-H4 polypeptide is comprised within a B7-H4 polypeptide sequence, or a fragment thereof.

[0086] A “B7-H4 polypeptide” may comprise the full length polypeptide sequence of B7-H4 (e.g. SEQ ID NO.: 55), or may comprise a fragment of B7-H4 of any length of the full length polypeptide sequence of B7-H4 (e.g. comprising a polypeptide sequence of 5%, 15%, 25%, 35%, 45%, 55%, 65%, 75%, 85% or 95% of the full length polypeptide sequence of B7-H4) which comprises an epitope which can bind (e.g. be bound by) an antibody or antigen binding fragment of the invention. The B7-H4 polypeptide may comprise a sequence having 75%, 80%, 85%, 90% or 90% sequence identity to the sequence of SEQ ID NO.: 55. Preferably, the B7-H4 polypeptide comprises the sequence of SEQ ID NO.: 55.

[0087] The antibody or antigen binding fragment has high affinity for B7-H4 both *in vitro* and *in vivo*, and thus may advantageously be used in methods for detecting a B7-H4 epitope, and associated methods of diagnosis.

[0088] To “treat” refers to therapeutic measures that cure, slow down, alleviate symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder. Thus, those in need of treatment include those already with the disorder. In one embodiment, a subject is successfully “treated” for a disease or disorder (preferably cancer), according to the methods provided herein if the patient shows, e.g., total, partial, or transient alleviation or elimination of symptoms associated with the disease or disorder (preferably cancer).

[0089] In one embodiment, a method of the invention may be used to prevent the onset of a cancer comprising a cancer cell which expresses B7-H4. To “prevent” refers to prophylactic or preventative measures that prevent and/or slow the development of a targeted pathologic condition or disorder. Thus, those in need of prevention include those prone to have or susceptible to the disorder. In one embodiment, a disease or disorder (preferably cancer) is successfully prevented according to the methods provided herein if the patient develops, transiently or permanently, e.g., fewer or less severe symptoms associated with the disease or disorder, or a later onset of symptoms associated with the disease or disorder, than a patient who has not been subject to the methods of the invention.

[0090] The terms “subject”, “individual” and “patient” are used interchangeably herein to refer to a mammalian subject. In one embodiment the “subject” is a human, domestic animals, farm animals, sports animals, and zoo animals, e.g., humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, etc. In one embodiment, the subject is a cynomolgus monkey (*Macaca fascicularis*). In a preferable embodiment, the subject is a human. In methods of the invention, the subject may not have been previously diagnosed as having cancer. Alternatively, the subject may have been previously diagnosed as having cancer. The subject may also be one who exhibits disease risk factors, or one who is asymptomatic for cancer. The subject may also be one who is suffering from or is at risk of developing cancer. Thus, in one embodiment, a method of the invention may be used to confirm the presence of cancer in a subject. For example, the subject may previously have been diagnosed with cancer by alternative means. In one embodiment, the subject has been previously administered a cancer therapy.

[0091] In one embodiment, methods of treatment of the invention comprise one or more administration step selected from oral, intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal, or vaginal, inhalation, topical, or a combination thereof. In a preferable embodiment, the administration is intravenous or intraarterial (e.g. by injection or drip), or a combination thereof.

- [0092] In one embodiment, the antibody or antigen binding fragment thereof is delivered directly to the site of the adverse cellular population (e.g. thereby increasing the exposure of the diseased tissue to the therapeutic agent). In one embodiment, the administration is directly to the airway, e.g., by inhalation or intranasal administration.
- [0093] In a preferable embodiment, a cancer referred to herein is a cancer characterised by the expression (preferably overexpression) of a B7-H4 molecule. In other words, a cancer referred to herein may comprise a cancerous cell that expresses B7-H4. Said cancerous cell may be comprised within a tumor.
- [0094] In one embodiment, the cancer is one or more selected from breast cancer, ovarian cancer, endometrial cancer, cholangiocarcinoma, NSCLC (squamous and adenocarcinoma), pancreatic cancer, and gastric cancer.
- [0095] In one embodiment, the cancer is one or more selected from colorectal cancer, HNSCC, prostate cancer, lung cancer (e.g. NSCLC or SCLC), breast cancer, ovarian cancer, pancreatic cancer, gastric cancer, cholangiocarcinoma, melanoma, endometrial cancer, hematological cancer (AML, MM, DLBCL), and cancers comprising CSCs.
- [0096] In a preferable embodiment, the cancer is lung cancer, breast cancer, or a combination thereof. For example, the cancer may be lung cancer. The cancer may be breast cancer. The cancer may be ovarian cancer.
- [0097] In one embodiment, the cancer is one or more breast cancer selected from hormone receptor (HR)-positive (HR+) breast cancer, human epidermal growth factor receptor 2 positive (HER2+) breast cancer, triple negative breast cancer (TNBC). A subject may be Herceptin eligible. A subject may have received treatment with Herceptin.
- [0098] In one embodiment, the cancer is one or more non-small-cell lung carcinoma (NSCLC) preferably selected from squamous NSCLC, adenocarcinoma NSCLC, or a combination thereof.
- [0099] An antibody or antigen binding fragment thereof also finds utility in detecting a cancer cell, for example as part of a diagnostic method.
- [0100] In a further aspect, there is provided a method for detecting the presence or absence of a B7-H4 polypeptide (e.g. a B7-H4 polypeptide epitope) in a sample, comprising:

- a. contacting a sample with an antibody or antigen binding fragment thereof, or a pharmaceutical composition comprising an antibody or antigen binding fragment thereof, to provide an antibody-antigen complex; wherein said antibody or antigen binding fragment thereof comprises:
 - i. a heavy chain CDR1 (HCDR1), a heavy chain CDR2 (HCDR2), a heavy chain CDR3 (HCDR3), a light chain CDR1 (LCDR1), a light chain CDR2 (LCDR2), and a light chain CDR3 (LCDR3) comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof;
 - ii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof;
 - iii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof;
 - iv. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof; or
 - v. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof.
- b. detecting the presence or absence of said antibody-antigen complex; and
- c. wherein the presence of the antibody-antigen complex confirms the presence of a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope); or
- d. wherein the absence of the antibody-antigen complex confirms the absence of B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope).

[0101] In a related aspect, there is provided a method for detecting the presence or absence of a cancer cell expressing a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope) in a sample, comprising:

- a. contacting a sample with an antibody or antigen binding fragment thereof, or a pharmaceutical composition comprising an antibody or antigen binding fragment thereof, to provide an antibody-antigen complex; wherein said antibody or antigen binding fragment thereof comprises:
 - i. a heavy chain CDR1 (HCDR1), a heavy chain CDR2 (HCDR2), a heavy chain CDR3 (HCDR3), a light chain CDR1 (LCDR1), a light chain CDR2 (LCDR2), and a light chain CDR3 (LCDR3) comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof;
 - ii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof;
 - iii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof;
 - iv. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof; or
 - v. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof;
- b. detecting the presence or absence of said antibody-antigen complex; and

- c. wherein the presence of the antibody-antigen complex confirms the presence of a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope); or
- d. wherein the absence of the antibody-antigen complex confirms the absence of B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope).

[0102] The invention embraces a corresponding use of the antibody or antigen binding fragment thereof of the invention for detecting a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope).

[0103] In one embodiment, the presence of antibody-antigen complex is indicative of the presence of a cancer cell, and the absence of the antibody-antigen complex is indicative of the absence of a cancer cell. For example, the method may comprise confirming the presence of cancer where an antibody-antigen complex is detected, or not confirming the presence of cancer where an antibody-antigen complex is not detected.

[0104] In a preferable embodiment, the cancer cell is a cancer cell expressing a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope).

[0105] Thus, the present invention embraces corresponding use of the method steps described herein in methods of diagnosing a subject with a cancer, preferably wherein said cancer comprises a B7-H4 expressing cancer cell.

[0106] In one embodiment, a method of detection or method of diagnosis may comprise measuring the expression level of B7-H4 on a cell (or tissue) obtainable from a subject, and comparing the measured expression level with a standard B7-H4 expression in a control cell (or tissue), wherein an increase in the expression level compared to the control is indicative of the presence of cancer. Preferably, said control sample comprises a non-cancer (e.g. normal) cell.

[0107] An “antibody-antigen complex” means a complex (e.g. macromolecular complex) comprising a B7-H4 antigen which has become bound to an antibody. The term “antibody-antigen complex” may be used synonymously with the terms “bound B7-H4-antibody complex” and “antibody bound to a B7-H4”.

[0108] An antibody-antigen complex may be detected by any means known to the skilled person. In one embodiment, the antibody (or antigen binding fragment thereof) is labelled

with a detectable label. Said label may be an epi-fluorescent label. In a preferable embodiment, the antibody is labelled with 800CW.

[0109] In one embodiment, an antibody-antigen complex is detected by means of a secondary (e.g. detection) antibody which binds the antibody and/or antibody-antigen complex.

[0110] Suitably, said secondary antibody comprises a detection means, such as a tag/label to aid detection. Said detection means is preferably conjugated to the secondary antibody. Examples of suitable labels include detectable labels such as radiolabels or fluorescent or coloured molecules, enzymatic markers or chromogenic markers – e.g. dyes that provide a visible colour change upon binding of the detection antibody to an antigen. By way of example, the label may be fluorescein-isothiocyanate (FITC), R-phycoerythrin, Alexa 532, CY3 or digoxigenin. The label may be a reporter molecule, which is detected directly, such as by detecting its fluorescent signal, or by exposure of the label to photographic or X-ray film. Alternatively, the label is not directly detectable, but may be detected, for example, in a two-phase system. An example of indirect label detection is binding of an antibody to the label.

[0111] In a preferable embodiment, said secondary antibody comprises a fluorescent tag, and an antibody-antigen complex is detected by the fluorescence emitted from a, antibody-antigen-secondary antibody complex. An “antibody-antigen-secondary antibody complex” means a complex comprising an antigen (e.g. B7-H4) which has become bound to an antibody, wherein said complex has further become bound by a secondary antibody which binds said antibody and/or antibody-antigen complex.

[0112] Suitably, an antibody-antigen complex is detected when the signal (preferably fluorescence) emitted from the detection label is greater than the signal detected in a control comprising no antibody (e.g. no antibody which binds a B7-H4). Said control may alternatively comprise a B7-H4, but the sample is not applied to said control.

[0113] Suitably, a “sample” is a sample obtained from a subject (e.g. biopsy), cell line, tissue culture, or other source of cells potentially expressing B7-H4. In preferable embodiment, a sample is a biopsy from a subject. Said biopsy may be taken from a tumour, or a site at risk of developing a tumour.

- [0114] In a preferable embodiment, the sample is an isolated sample obtainable (e.g. obtained) from a subject.
- [0115] In a preferable embodiment, the B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope) is an integral component of a cancer cell, more preferably an integral component of the cell membrane of a cancer cell.
- [0116] The present invention encompasses the antibodies (e.g. the antibody or antigen binding fragment) defined herein having the recited CDR sequences or variable heavy and variable light chain sequences (reference antibodies), as well as functional variants thereof. A functional variant binds to the same target antigen as the reference antibody, and preferably exhibits the same antigen cross-reactivity as the reference antibody. The functional variants may have a different affinity for the target antigen when compared to the reference antibody, but substantially the same affinity is preferred.
- [0117] The term “reference antibody” is used to conveniently refer, in comparison, to an antibody or antigen thereof of the invention. Thus, the term “reference antibody” refers to an antibody or antigen thereof of the invention. For example, the reference antibody may mean an antibody or antigen binding fragment thereof comprising a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively. More particularly, the reference antibody may mean an antibody or antigen binding fragment thereof comprising a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 33; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34. Preferably, the reference antibody may mean an antibody or antigen binding fragment thereof comprising a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 45; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34.
- [0118] In one embodiment functional variants of a reference antibody show sequence variation at one or more CDRs when compared to corresponding reference CDR sequences. Thus, a functional antibody variant may comprise a functional variant of a CDR. Where the term “functional variant” is used in the context of a CDR sequence, this means that the CDR has at most 2, preferably at most 1 amino acid differences when

compared to a corresponding reference CDR sequence, and when combined with the remaining 5 CDRs (or variants thereof) enables the variant antibody to bind to the same target antigen as the reference antibody, and preferably to exhibit the same antigen cross-reactivity as the reference antibody. A functional variant may be referred to as a “variant antibody”.

[0119] In one embodiment a variant antibody (or antigen binding fragment thereof) comprises:

- a light chain CDR1 having at most 2 amino acid difference when compared to a corresponding reference CDR sequence;

- a light chain CDR2 having at most 2 amino acid difference when compared to a corresponding reference CDR sequence;

- a light chain CDR3 having at most 2 amino acid difference when compared to a corresponding reference CDR sequence;

- a heavy chain CDR1 having at most 2 amino acid difference when compared to a corresponding reference CDR sequence;

- a heavy chain CDR2 having at most 2 amino acid difference when compared to a corresponding reference CDR sequence; and

- a heavy chain CDR3 having at most 2 amino acid difference when compared to a corresponding reference CDR sequence;

wherein the variant antibody binds to the same target antigen as the reference antibody, and preferably exhibits the same antigen cross-reactivity (or lack thereof) as the reference antibody.

[0120] Preferably a variant antibody (or antigen binding fragment thereof) comprises:

- a light chain CDR1 having at most 1 amino acid difference when compared to a corresponding reference CDR sequence;

- a light chain CDR2 having at most 1 amino acid difference when compared to a corresponding reference CDR sequence;

- a light chain CDR3 having at most 1 amino acid difference when compared to a corresponding reference CDR sequence;

a heavy chain CDR1 having at most 1 amino acid difference when compared to a corresponding reference CDR sequence;

a heavy chain CDR2 having at most 1 amino acid difference when compared to a corresponding reference CDR sequence; and

a heavy chain CDR3 having at most 1 amino acid difference when compared to a corresponding reference CDR sequence;

wherein the variant antibody binds to the same target antigen as the reference antibody, and preferably exhibits the same antigen cross-reactivity (or lack thereof) as the reference antibody.

[0121] For example, a variant of the antibody or antigen binding fragment may comprise:

a heavy chain CDR1 having at most 2 amino acid difference when compared to SEQ ID NO: 7;

a heavy chain CDR2 having at most 2 amino acid difference when compared to SEQ ID NO: 8; and

a heavy chain CDR3 having at most 2 amino acid difference when compared to SEQ ID NO: 9;

a light chain CDR1 having at most 2 amino acid difference when compared to SEQ ID NO: 10;

a light chain CDR2 having at most 2 amino acid difference when compared to SEQ ID NO: 11;

a light chain CDR3 having at most 2 amino acid difference when compared to SEQ ID NO: 12;

wherein the variant antibody binds to a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope), and preferably exhibits the same antigen cross-reactivity (or lack thereof) as the reference antibody or antigen binding fragment.

[0122] For example, a variant of the antibody or antigen binding fragment may (preferably) comprise:

a heavy chain CDR1 having at most 1 amino acid difference when compared to SEQ ID NO: 7;

a heavy chain CDR2 having at most 1 amino acid difference when compared to SEQ ID NO: 8; and

a heavy chain CDR3 having at most 1 amino acid difference when compared to SEQ ID NO: 9;

a light chain CDR1 having at most 1 amino acid difference when compared to SEQ ID NO: 10;

a light chain CDR2 having at most 1 amino acid difference when compared to SEQ ID NO: 11;

a light chain CDR3 having at most 1 amino acid difference when compared to SEQ ID NO: 12;

wherein the variant antibody binds to a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope), and preferably exhibits the same antigen cross-reactivity (or lack thereof) as the reference antibody or antigen binding fragment.

[0123] The foregoing can be applied analogously to variants of the other antibodies described herein, wherein the amino acid differences are defined relative to the CDR sequences thereof, and wherein the variant antibody binds to the same target antigen as said antibodies, and preferably exhibits the same antigen cross-reactivity.

[0124] In one embodiment, a variant antibody may have at most 5, 4 or 3 amino acid differences total in the CDRs thereof when compared to a corresponding reference antibody, with the proviso that there is at most 2 (preferably at most 1) amino acid differences per CDR. Preferably a variant antibody has at most 2 (more preferably at most 1) amino acid differences total in the CDRs thereof when compared to a corresponding reference antibody, with the proviso that there is at most 2 amino acid differences per CDR. More preferably a variant antibody has at most 2 (more preferably at most 1) amino acid differences total in the CDRs thereof when compared to a corresponding reference antibody, with the proviso that there is at most 1 amino acid difference per CDR.

[0125] The amino acid difference may be an amino acid substitution, insertion or deletion. In one embodiment the amino acid difference is a conservative amino acid substitution as described herein.

[0126] In one embodiment a variant antibody has the same framework sequences as the exemplary antibodies described herein. In another embodiment the variant antibody may comprise a framework region having at most 2, preferably at most 1 amino acid difference (when compared to a corresponding reference framework sequence). Thus, each framework region may have at most 2, preferably at most 1 amino acid difference (when compared to a corresponding reference framework sequence).

[0127] In one embodiment a variant antibody may have at most 5, 4 or 3 amino acid differences total in the framework regions thereof when compared to a corresponding reference antibody, with the proviso that there is at most 2 (preferably at most 1) amino acid differences per framework region. Preferably a variant antibody has at most 2 (more preferably at most 1) amino acid differences total in the framework regions thereof when compared to a corresponding reference antibody, with the proviso that there is at most 2 amino acid differences per framework region. More preferably a variant antibody has at most 2 (more preferably at most 1) amino acid differences total in the framework regions thereof when compared to a corresponding reference antibody, with the proviso that there is at most 1 amino acid difference per framework region.

[0128] Thus, a variant antibody may comprise a variable heavy chain and a variable light chain as described herein, wherein:

the heavy chain has at most 14 amino acid differences (at most 2 amino acid differences in each CDR and at most 2 amino acid differences in each framework region) when compared to a heavy chain sequence herein; and

the light chain has at most 14 amino acid differences (at most 2 amino acid differences in each CDR and at most 2 amino acid differences in each framework region) when compared to a light chain sequence herein;

wherein the variant antibody binds to the same target antigen as the reference antibody, and preferably exhibits the same antigen cross-reactivity (or lack thereof) as the reference antibody.

[0129] Said variant heavy or light chains may be referred to as “functional equivalents” of the reference heavy or light chains.

[0130] In one embodiment a variant antibody may comprise a variable heavy chain and a variable light chain as described herein, wherein:

the heavy chain has at most 7 amino acid differences (at most 1 amino acid difference in each CDR and at most 1 amino acid difference in each framework region) when compared to a heavy chain sequence herein; and

the light chain has at most 7 amino acid differences (at most 1 amino acid difference in each CDR and at most 1 amino acid difference in each framework region) when compared to a light chain sequence herein;

wherein the variant antibody binds to the same target antigen as the reference antibody, and preferably exhibits the same antigen cross-reactivity (or lack thereof) as the reference antibody.

Antibody-drug conjugates (ADCs)

[0131] Advantageously, an antibody or antigen binding fragment thereof of the invention may comprise a heterologous agent. In one embodiment, an antibody or antigen binding fragment of the invention is linked to a heterologous agent. In a preferable embodiment, the antibody or antigen binding fragment is conjugated to a heterologous agent. Suitably, “conjugated” means linked via a covalent or ionic bond. Preferably, said heterologous agent is a cytotoxin.

[0132] The heterologous agent may simply be referred to as an “agent” or “active agent”. For example, in alternative language, an antibody or antigen binding fragment thereof of the invention may comprise an active agent. In one embodiment, an antibody or antigen binding fragment of the invention is linked to an active agent. In a preferable embodiment, the antibody or antigen binding fragment is conjugated to an active agent.

[0133] The heterologous/ active agent can be a drug. Preferably, the heterologous/ active is a cytotoxin.

[0134] It is particularly preferred that an antibody or antigen binding fragment thereof of the invention is linked (e.g. conjugated) to a heterologous/ active agent in methods of treatment, as described below.

- [0135] An agent and/or cytotoxin of the invention may be conjugated to the antibody or antigen binding fragment thereof by means of a spacer (e.g. at least one spacer). In one embodiment, the spacer is a peptide spacer. In one embodiment, the spacer is a non-peptide (e.g. chemical) spacer.
- [0136] The cytotoxic agent or cytotoxin can be any molecule known in the art that inhibits or prevents the function of cells and/or causes destruction of cells (cell death), and/or exerts anti-neoplastic/anti-proliferative effects. A number of classes of cytotoxic agents are known to have potential utility in ADC molecules. These include, but are not limited to, topoisomerase I inhibitors, amanitins, auristatins, daunomycins, doxorubicins, duocarmycins, dolastatins, enediynes, lexitropsins, taxanes, puromycins, maytansinoids, vinca alkaloids, tubulysins and pyrrolobenzodiazepines (PBDs). Examples of such cytotoxic agents are AFP, MMAF, MMAE, AEB, AEVB, auristatin E, paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretastatin, chaliceamicin, maytansine, DM-1, vinblastine, methotrexate, and netropsin, and derivatives and analogs thereof. Additional disclosure regarding cytotoxins suitable for use in ADCs can be found, for example, in International Patent Application Publication Nos. WO 2015/155345 and WO 2015/157592, incorporated by reference herein in their entirety.
- [0137] For example, the antibody or antigen binding fragment may be conjugated to such heterologous agent to provide an “antibody-drug conjugate” (ADC).
- [0138] The agent is typically linked to, or “loaded onto” the antibody or antigen-binding fragment. The agent loading (p) is the average number of agent(s) per antibody or antigen-binding fragment (e.g. the Ligand unit).
- [0139] The average number of agents per antibody (or antigen-binding fragment) in preparations of ADCs from conjugation reactions may be characterized by conventional means such as UV, reverse phase HPLC, HIC, mass spectroscopy, ELISA assay, and electrophoresis. The quantitative distribution of ADC in terms of p may also be determined. By ELISA, the averaged value of p in a particular preparation of ADC may be determined (Hamblett et al (2004) Clin. Cancer Res. 10:7063-7070; Sanderson et al

(2005) Clin. Cancer Res. 11:843-852). In some instances, separation, purification, and characterization of homogeneous ADC, where p is a certain value from ADC with other drug loadings, may be achieved by means such as reverse phase HPLC or electrophoresis. Such techniques are also applicable to other types of conjugates.

[0140] Cysteine amino acids may be engineered at reactive sites in an antibody (or antigen-binding fragment thereof) and which preferably do not form intrachain or intermolecular disulfide linkages (Junutula, et al., 2008b Nature Biotech., 26(8):925-932; Dornan et al (2009) Blood 114(13):2721-2729; US 7521541; US 7723485; WO2009/052249). The engineered cysteine thiols may react with a linker within an agent (e.g. of formula I below) which may have thiol-reactive, electrophilic groups such as maleimide or alpha-halo amides to form ADC with cysteine engineered antibodies. The location of the drug unit can thus be designed, controlled, and known. The drug loading can be controlled since the engineered cysteine thiol groups typically react with drug-linker reagents in high yield. Engineering an IgG antibody to introduce a cysteine amino acid by substitution at a single site on the heavy or light chain gives two new cysteines on the symmetrical antibody. A drug loading near 2 can be achieved with near homogeneity of the conjugation product ADC.

[0141] Where more than one nucleophilic or electrophilic group of the antibody or antigen binding fragment thereof reacts with an agent, then the resulting product may be a mixture of ADC compounds with a distribution of agent units attached to an antibody, e.g. 1, 2, 3, etc. Liquid chromatography methods such as polymeric reverse phase (PLRP) and hydrophobic interaction (HIC) may separate compounds in the mixture by agent loading value. Preparations of ADC with a single agent loading value (p) may be isolated.

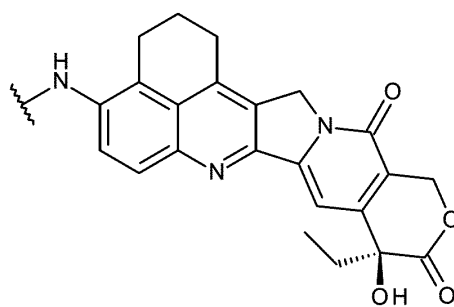
[0142] Thus, the antibody-drug conjugate compositions of the invention may include mixtures of antibody-drug conjugates where the antibody or antigen binding fragment thereof has one or more agent moieties and where the agent moieties may be attached to the antibody or antigen binding fragment thereof at various amino acid residues.

[0143] In one embodiment, the average number of agents per antibody (or antigen-binding fragment thereof) is in the range 1 to 20. In some embodiments the range is

selected from 1 to 10, 2 to 10, 2 to 8, 2 to 6, and 4 to 10. In some embodiments, there is one agent per antibody (or antigen-binding fragment thereof). In some embodiments, the number of agents per antibody (or antigen-binding fragment thereof) can be expressed as a ratio of agent (i.e., drug) to antibody. This ratio is referred to as the Drug to Antibody Ratio (DAR).” The DAR is the average number of drugs (i.e., agents) linked to each antibody. In one embodiment of the present invention, the DAR is in the range 1 to 20. In some embodiments the range of DAR is selected from 1 to 10, 2 to 10, 2 to 8, 2 to 6, and 4 to 10. In a particular embodiment of the present invention, the DAR is about 8. In a particular embodiment of the present invention, the DAR is 8.

- [0144]** In one embodiment, the antibody or antigen-binding fragment is conjugated to one or more heterologous agent selected from the group consisting of a topoisomerase I inhibitor, a tubulysin derivative, a pyrrolobenzodiazepine, an antimicrobial agent, a therapeutic agent, a prodrug, a peptide, a protein, an enzyme, a lipid, a biological response modifier, a pharmaceutical agent, a lymphokine, a heterologous antibody, a fragment of a heterologous antibody, a detectable label, a polyethylene glycol (PEG), a radioisotope, or a combination thereof.
- [0145]** In one embodiment, the antibody antigen binding fragment is conjugated to one or more cytotoxin selected from a topoisomerase I inhibitor, tubulysin derivative, a pyrrolobenzodiazepine, or a combination thereof. For example, the antibody or antigen binding fragment thereof is conjugated to one or more cytotoxin selected from the group consisting of topoisomerase I inhibitor SG3932, SG4010, SG4057 or SG4052 (the structures of which are provided below); tubulysin AZ1508, pyrrolobenzodiazepine SG3315, pyrrolobenzodiazepine SG3249, or a combination thereof.
- [0146]** It is preferred that the antibody or antigen binding fragment thereof may be conjugated to a topoisomerase I inhibitor. Topoisomerase inhibitors are chemical compounds that block the action of topoisomerase (topoisomerase I and II), which is a type of enzyme that controls the changes in DNA structure by catalyzing the breaking and rejoining of the phosphodiester backbone of DNA strands during the normal cell cycle.

[0147] A general example of a suitable topoisomerase I inhibitor is represented by the following compound:

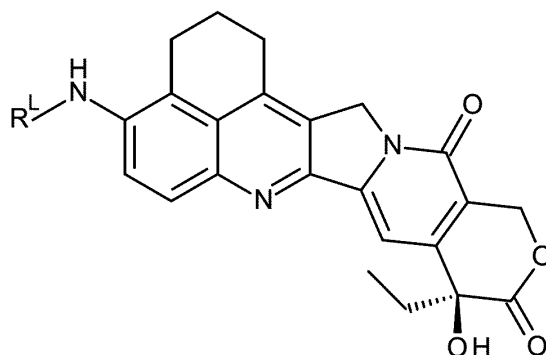


A*

Said compound is denoted as A*, and may be referred to as a “Drug Unit” herein.

[0148] The compound (e.g. A*) is preferably provided with a linker for connecting (preferably conjugating) to an antibody or antigen binding fragment described herein (which may be referred to as a “Ligand Unit”). Suitably, the linker is attached (e.g. conjugated) in a cleavable manner to an amino residue, for example, an amino acid of an antibody or antigen binding fragment described herein.

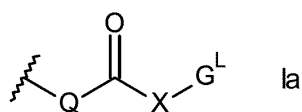
[0149] More particularly, an example of a suitable topoisomerase I inhibitor is represented by the following compound, with the formula “I”:



I

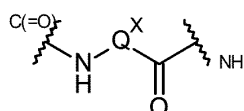
and salts and solvates thereof, wherein R^L is a linker for connection to an antibody or antigen binding fragment thereof described herein (e.g. the Ligand Unit), wherein said linker is preferably selected from:

(ia):



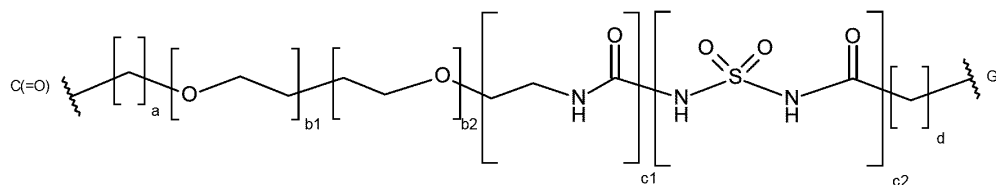
wherein

Q is:



, where Q^X is such that Q is an amino-acid residue, a dipeptide residue, a tripeptide residue or a tetrapeptide residue;

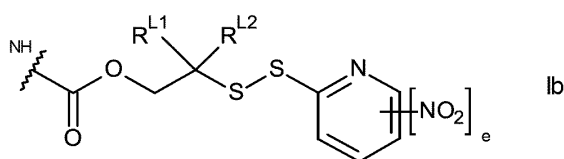
X is:



where $a = 0$ to 5 , $b1 = 0$ to 16 , $b2 = 0$ to 16 , $c1 = 0$ or 1 , $c2 = 0$ or 1 , $d = 0$ to 5 , wherein at least $b1$ or $b2 = 0$ (i.e. only one of $b1$ and $b2$ may not be 0) and at least $c1$ or $c2 = 0$ (i.e. only one of $c1$ and $c2$ may not be 0);

G^L is a linker for connecting to an antibody or antigen binding fragment thereof described herein (e.g. the Ligand Unit); or

(ib):



where R^{L1} and R^{L2} are independently selected from H and methyl, or together with the carbon atom to which they are bound form a cyclopropylene or cyclobutylene group; and

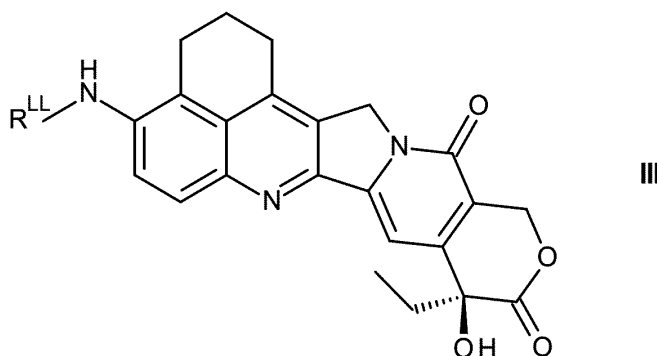
e is 0 or 1 .

[0150] It will be understood by the person skilled in the art that more than one of said agent(s) (e.g. topoisomerase I inhibitor) may be conjugated to the antibody or antigen binding fragment thereof.

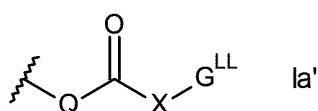
[0151] For example, a conjugate (e.g. antibody-drug conjugate) of the invention may be of the general formula IV:



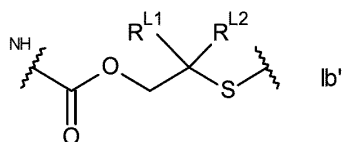
or a pharmaceutically acceptable salt or solvate thereof, wherein L is an antibody or antigen binding fragment thereof described herein (e.g. the Ligand Unit), D^L is a topoisomerase I inhibitor having a linker (e.g. Drug Linker unit) that is of formula III:



R^{LL} is a linker connected to an antibody or antigen binding fragment thereof described herein (e.g. the Ligand Unit), wherein the linker is preferably selected from (ia'):



where Q and X are as defined above and G^{LL} is a linker connected to an antibody or antigen binding fragment thereof described herein (e.g. the Ligand Unit); and (ib'):



where R^{L1} and R^{L2} are as defined above; and

p is an integer of from 1 to 20.

[0152] The drug loading is represented by p, the number of topoisomerase I inhibitor(s) (e.g. Drug units) per antibody or antigen binding fragment thereof (e.g. Ligand Unit). Drug loading may range from 1 to 20 Drug units (D) per Ligand unit. For compositions, p represents the average drug loading of the conjugates in the composition, and p ranges from 1 to 20.

[0153] Accordingly, the inventor embraces a conjugate comprising an antibody or antigen binding fragment thereof described herein (e.g. the Ligand Unit) covalently linked to at least one topoisomerase I inhibitor (e.g. Drug unit, such as A* illustrated above). Said inhibitor is preferably linked to the antibody or antigen binding fragment thereof by a linker (e.g. Linker unit), such as a linker described above as R^L and/or R^{LL} . In other words, the invention embraces an antibody or antigen binding fragment thereof described herein (e.g. the Ligand Unit) with one or more topoisomerase I inhibitors attached, preferably via a linker (e.g. Drug-Linker units). The antibody or antigen binding fragment thereof (representing a Ligand unit), described more fully above, is a targeting agent that binds to a target moiety. More particularly, this Ligand unit can, for example, specifically bind to a B7-H4 on a target cell, to which the Drug unit is thus delivered. Accordingly, the present invention also provides methods for the treatment of, for example, various cancers and other disorders with an ADC (e.g. cancers/ disorders which are associated with the presence of cells, preferably cancerous cells, which express B7-H4).

Further preferences

[0154] Certain features of the topoisomerase I inhibitors described above are particularly preferred and may be defined in more detail as set out below. By way of example, a preferred embodiment of feature Q^X (e.g. within the linker of 1a described above) will be outlined.

[0155] The following preferences may apply to all aspects of the invention as described above, or may relate to a single aspect. The preferences may be combined together in any combination.

[0156] Various definitions which pertain to certain terms in this section are provided under the heading “Definitions” provided below.

Q^x

[0157] In one embodiment, Q is an amino acid residue. The amino acid may be a natural amino acid or a non-natural amino acid. For example, Q may be selected from: Phe, Lys, Val, Ala, Cit, Leu, Ile, Arg, and Trp, where Cit is citrulline.

[0158] In one embodiment, Q comprises a dipeptide residue. The amino acids in the dipeptide may be any combination of natural amino acids and non-natural amino acids. In some embodiments, the dipeptide comprises natural amino acids. Where the linker is a cathepsin labile linker, the dipeptide is the site of action for cathepsin-mediated cleavage. The dipeptide then is a recognition site for cathepsin.

[0159] In one embodiment, Q is selected from:

NH-Phe-Lys-C=O ,
 NH-Val-Ala-C=O ,
 NH-Val-Lys-C=O ,
 NH-Ala-Lys-C=O ,
 NH-Val-Cit-C=O ,
 NH-Phe-Cit-C=O ,
 NH-Leu-Cit-C=O ,
 NH-Ile-Cit-C=O ,
 NH-Phe-Arg-C=O ,
 NH-Trp-Cit-C=O , and
 NH-Gly-Val-C=O ;
 where Cit is citrulline.

[0160] Preferably, Q is selected from:

NH-Phe-Lys-C=O ,
 NH-Val-Ala-C=O ,
 NH-Val-Lys-C=O ,

NH-Ala-Lys-C=O , and

NH-Val-Cit-C=O .

[0161] More preferably, Q is selected from NH-Phe-Lys-C=O , NH-Val-Cit-C=O or NH-Val-Ala-C=O .

[0162] Other suitable dipeptide combinations include:

NH-Gly-Gly-C=O ,

NH-Gly-Val-C=O

NH-Pro-Pro-C=O , and

NH-Val-Glu-C=O .

[0163] Other dipeptide combinations may be used, including those described by Dubowchik et al., *Bioconjugate Chemistry*, 2002, 13,855-869, which is incorporated herein by reference.

[0164] In some embodiments, Q is a tripeptide residue. The amino acids in the tripeptide may be any combination of natural amino acids and non-natural amino acids. In some embodiments, the tripeptide comprises natural amino acids. Where the linker is a cathepsin labile linker, the tripeptide is the site of action for cathepsin-mediated cleavage. The tripeptide then is a recognition site for cathepsin. Tripeptide linkers of particular interest are:

$\text{NH-Glu-Val-Ala-C=O}$

$\text{NH-Glu-Val-Cit-C=O}$

$\text{NH-}\alpha\text{Glu-Val-Ala-C=O}$

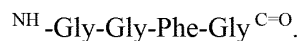
$\text{NH-}\alpha\text{Glu-Val-Cit-C=O}$

[0165] In some embodiments, Q is a tetrapeptide residue. The amino acids in the tetrapeptide may be any combination of natural amino acids and non-natural amino acids. In some embodiments, the tetrapeptide comprises natural amino acids. Where the linker is a cathepsin labile linker, the tetrapeptide is the site of action for cathepsin-mediated cleavage. The tetrapeptide then is a recognition site for cathepsin. Tetrapeptide linkers of particular interest are:

$\text{NH-Gly-Gly-Phe-Gly-C=O}$; and

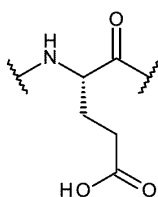
$\text{NH-Gly-Phe-Gly-Gly-C=O}$.

[0166] In some embodiments, the tetrapeptide is:

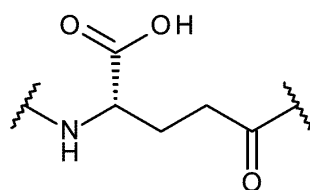


[0167] In the above representations of peptide residues, $^{\text{NH}}$ - represents the N-terminus, and $^{\text{C=O}}$ represents the C-terminus of the residue. The C-terminus binds to the NH of A*.

[0168] Glu represents the residue of glutamic acid, i.e.:



α Glu represents the residue of glutamic acid when bound via the α -chain, i.e.:



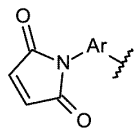
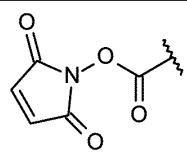
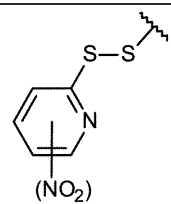
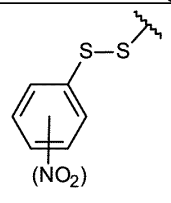
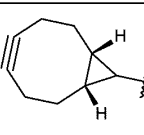
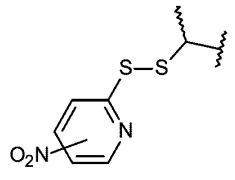
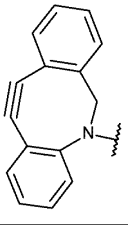
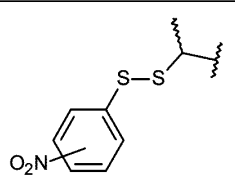
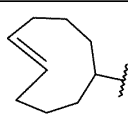
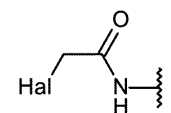
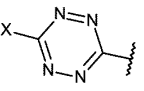
[0169] In one embodiment, the amino acid side chain is chemically protected, where appropriate. The side chain protecting group may be a group as discussed above. Protected amino acid sequences are cleavable by enzymes. For example, a dipeptide sequence comprising a Boc side chain-protected Lys residue is cleavable by cathepsin.

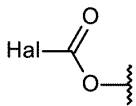
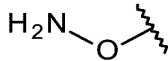
[0170] Protecting groups for the side chains of amino acids are well known in the art and are described in the Novabiochem Catalog, and as described above.

G^L

[0171] G^L may be selected from:

(G ^{L1-1})		(G ^{L6})	
----------------------	--	--------------------	--

(G ^{L1-2})		(G ^{L7})	
(G ^{L2})		(G ^{L8})	
(G ^{L3-1})	 where the NO ₂ group is optional	(G ^{L9})	
(G ^{L3-2})	 where the NO ₂ group is optional	(G ^{L10})	
(G ^{L3-3})	 where the NO ₂ group is optional	(G ^{L11})	
(G ^{L3-4})	 where the NO ₂ group is optional	(G ^{L12})	
(G ^{L4})		(G ^{L13})	

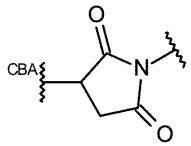
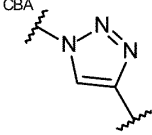
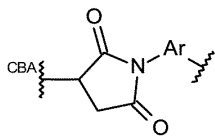
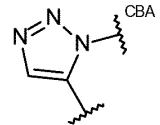
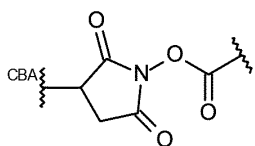
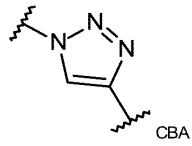
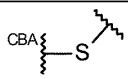
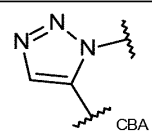
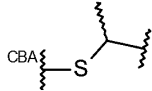
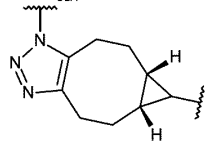
	Where Hal = I, Br, Cl		
(G ^{L5})		(G ^{L14})	

where Ar represents a C₅₋₆ arylene group, e.g. phenylene, and X represents C₁₋₄ alkyl.

[0172] In some embodiments, G^L is selected from G^{L1-1} and G^{L1-2}. In some of these embodiments, G^L is G^{L1-1}.

G^{LL}

[0173] G^{LL} may be selected from:

(G ^{LL1-1})		(G ^{LL8-1})	
(G ^{LL1-2})		(G ^{LL8-2})	
(G ^{LL2})		(G ^{LL9-1})	
(G ^{LL3-1})		(G ^{LL9-2})	
(G ^{LL3-2})		(G ^{LL10})	

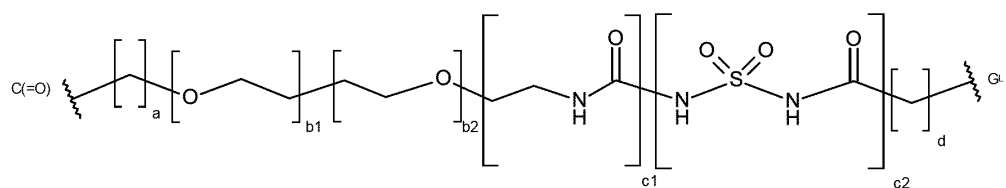
(G ^{LL-4})		(G ^{LL11})	
(G ^{LL5})		(G ^{LL12})	
(G ^{LL6})		(G ^{LL13})	
(G ^{LL7})		(G ^{LL14})	

where Ar represents a C₅₋₆ arylene group, e.g. phenylene and X represents C₁₋₄ alkyl.

[0174] In some embodiments, G^{LL} is selected from G^{LL1-1} and G^{LL1-2}. In some of these embodiments, G^{LL} is G^{LL1-1}.

X

[0175] X is preferably:



where a = 0 to 5, b₁ = 0 to 16, b₂ = 0 to 16, c = 0 or 1, d = 0 to 5, wherein at least b₁ or b₂ = 0 and at least c₁ or c₂ = 0.

a may be 0, 1, 2, 3, 4 or 5. In some embodiments, a is 0 to 3. In some of these embodiments, a is 0 or 1. In further embodiments, a is 0.

b1 may be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16. In some embodiments, b1 is 0 to 12. In some of these embodiments, b1 is 0 to 8, and may be 0, 2, 3, 4, 5 or 8.

b2 may be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16. In some embodiments, b2 is 0 to 12. In some of these embodiments, b2 is 0 to 8, and may be 0, 2, 3, 4, 5 or 8. Preferably, only one of b1 and b2 may not be 0.

c1 may be 0 or 1. c2 may be 0 or 1. Preferably, only one of c1 and c2 may not be 0.

d may be 0, 1, 2, 3, 4 or 5. In some embodiments, d is 0 to 3. In some of these embodiments, d is 1 or 2. In further embodiments, d is 2. In further embodiments, d is 5.

[0176] In some embodiments of X, a is 0, b1 is 0, c1 is 1, c2 is 0 and d is 2, and b2 may be from 0 to 8. In some of these embodiments, b2 is 0, 2, 3, 4, 5 or 8. In some embodiments of X, a is 1, b2 is 0, c1 is 0, c2 is 0 and d is 0, and b1 may be from 0 to 8. In some of these embodiments, b1 is 0, 2, 3, 4, 5 or 8. In some embodiments of X, a is 0, b1 is 0, c1 is 0, c2 is 0 and d is 1, and b2 may be from 0 to 8. In some of these embodiments, b2 is 0, 2, 3, 4, 5 or 8. In some embodiments of X, b1 is 0, b2 is 0, c1 is 0, c2 is 0 and one of a and d is 0. The other of a and d is from 1 to 5. In some of these embodiments, the other of a and d is 1. In other of these embodiments, the other of a and d is 5. In some embodiments of X, a is 1, b2 is 0, c1 is 0, c2 is 1, d is 2, and b1 may be from 0 to 8. In some of these embodiments, b2 is 0, 2, 3, 4, 5 or 8.

[0177] In some embodiments, R^L is of formula Ib. In some embodiments, R^{LL} is formula Ib'.

[0178] R^{L1} and R^{L2} may be independently selected from H and methyl, or together with the carbon atom to which they are bound form a cyclopropylene or cyclobutylene group.

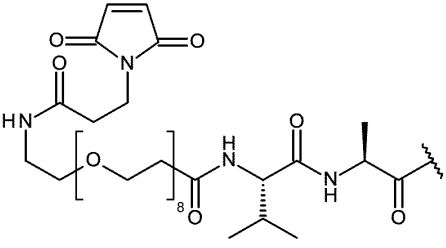
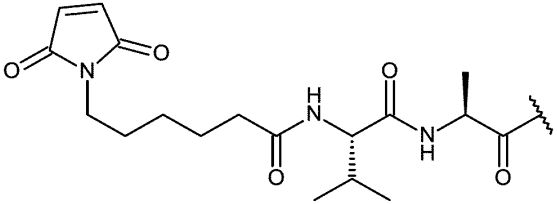
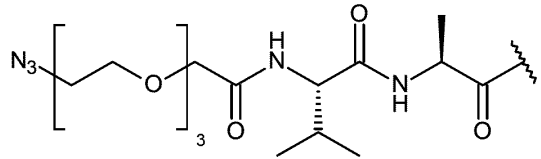
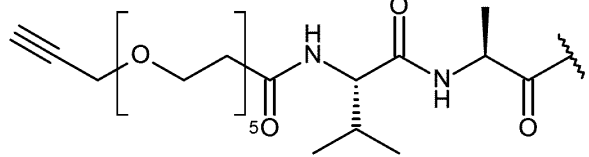
[0179] In some embodiments, both R^{L1} and R^{L2} are H. In some embodiments, R^{L1} is H and R^{L2} is methyl. In some embodiments, both R^{L1} and R^{L2} are methyl.

[0180] In some embodiments, R^{L1} and R^{L2} together with the carbon atom to which they are bound form a cyclopropylene group. In some embodiments, R^{L1} and R^{L2} together with the carbon atom to which they are bound form a cyclobutylene group.

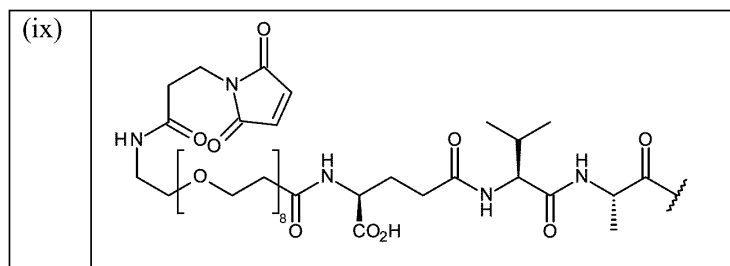
[0181] In the group Ib, in some embodiments, e is 0. In other embodiments, e is 1 and the nitro group may be in any available position of the ring. In some of these embodiments, it is in the ortho position. In others of these embodiments, it is in the para position.

[0182] In some embodiments where compounds described herein are provided in a single enantiomer or in an enantiomerically enriched form, the enantiomerically enriched form has an enantiomeric ratio greater than 60:40, 70:30, 80:20 or 90:10. In further embodiments, the enantiomeric ratio is greater than 95:5, 97:3 or 99:1.

[0183] In some embodiments, R^L is selected from:

(i)	
(ii)	
(iii)	
(iv)	

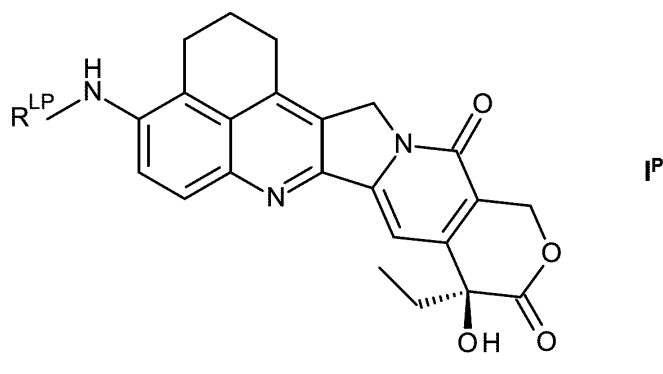
(v)	
(vi)	
(vii)	
(viii)	



[0184] In some embodiments, R^{LL} is a group derived from the R^L groups above.

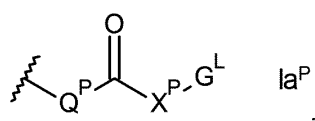
[0185] Having outlined said preferences above, certain preferred topoisomerase I-linker (e.g. Drug Linker unit) formulas are now described.

[0186] In some embodiments, the compound of formula **I** is of the formula **I^P**:



and salts and solvates thereof, wherein R^{LP} is a linker for connection to an antibody or antigen binding fragment thereof described herein, wherein said linker is selected from:

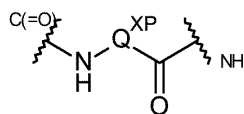
(ia):



wherein

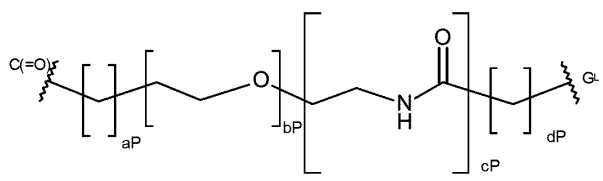
Q^P is:

52



, where Q^{XP} is such that Q^P is an amino-acid residue, a dipeptide residue or a tripeptide residue;

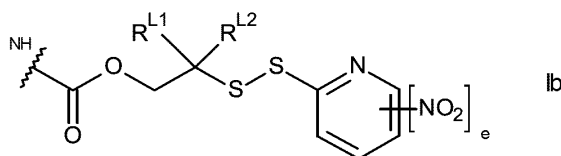
X^P is:



where $aP = 0$ to 5 , $bP = 0$ to 16 , $cP = 0$ or 1 , $dP = 0$ to 5 ;

G^L is a linker for connecting to an antibody or antigen binding fragment thereof described herein (e.g. Ligand Unit);

(ib):



where R^{L1} and R^{L2} are independently selected from H and methyl, or together with the carbon atom to which they are bound form a cyclopropylene or cyclobutylene group; and

e is 0 or 1.

aP may be 0, 1, 2, 3, 4 or 5. In some embodiments, aP is 0 to 3. In some of these embodiments, aP is 0 or 1. In further embodiments, aP is 0.

bP may be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16. In some embodiments, b is 0 to 12. In some of these embodiments, bP is 0 to 8, and may be 0, 2, 4 or 8.

cP may be 0 or 1.

dP may be 0, 1, 2, 3, 4 or 5. In some embodiments, dP is 0 to 3. In some of these embodiments, dP is 1 or 2. In further embodiments, dP is 2.

[0187] In some embodiments of X^P , aP is 0, cP is 1 and dP is 2, and bP may be from 0 to 8. In some of these embodiments, bP is 0, 4 or 8.

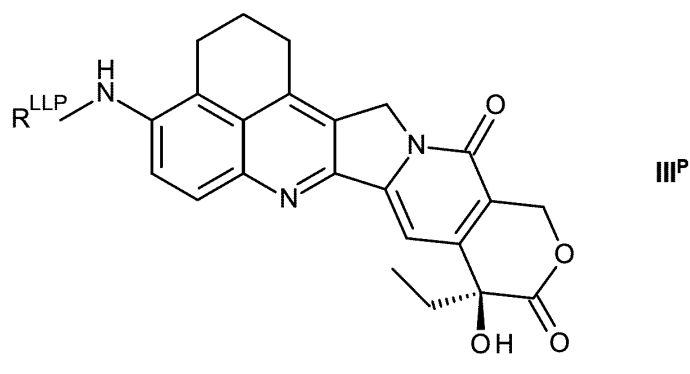
[0188] The preferences for Q^X above for compounds of Formula **I** may apply to Q^{XP} (for example, where appropriate).

[0189] The preferences for G^L , R^{L1} , R^{L2} and e above for compounds of Formula **I** may apply to compounds of Formula **I^P**.

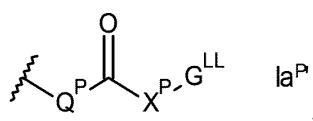
[0190] In some embodiments, the conjugate of formula **IV** is of the formula **IV^P**:



or a pharmaceutically acceptable salt or solvate thereof, wherein L is an antibody or antigen binding fragment thereof described herein (e.g. Ligand Unit), D^{LP} is a topoisomerase I inhibitor (e.g. Drug Linker unit) that is of formula **III^P**:

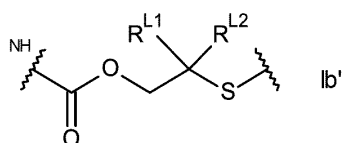


R^{LLP} is a linker connected to the antibody or antigen binding fragment thereof (e.g. Ligand unit), wherein said linker is selected from (ia'):



where Q^P and X^P are as defined above and G^{LL} is a linker connected to an antibody or antigen binding fragment thereof described herein (e.g. Ligand Unit); and (ib'):

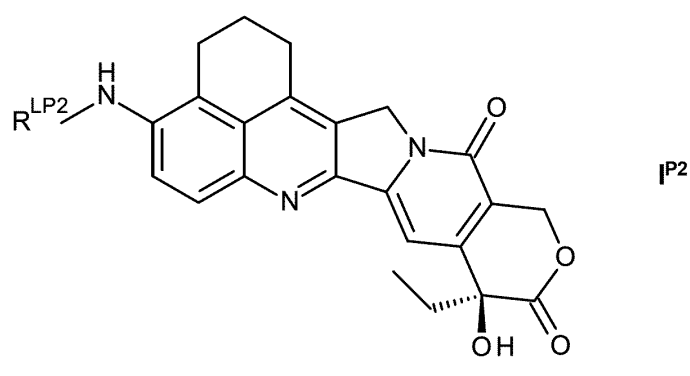
54



where R^{L1} and R^{L2} are as defined above; and

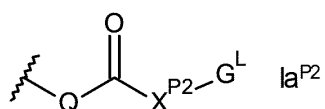
p is an integer of from 1 to 20.

[0191] In some embodiments, the compound of formula **I** is of the formula **I^{P2}**:



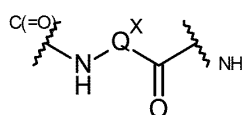
and salts and solvates thereof, wherein R^{LP2} is a linker for connection to an antibody or antigen binding fragment thereof described herein, wherein said linker is selected from:

(ia):



wherein

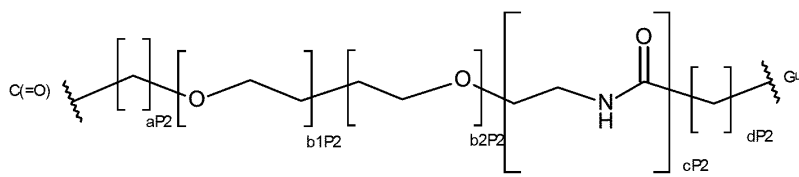
Q is:



, where Q^X is such that Q is an amino-acid residue, a dipeptide residue, a tripeptide residue or a tetrapeptide residue;

X^{P2} is:

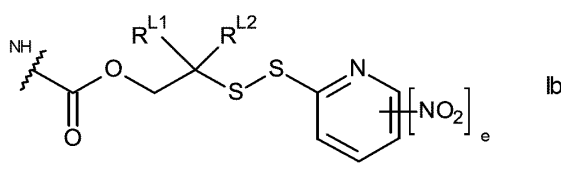
55



where aP2 = 0 to 5, b1P2 = 0 to 16, b2P2 = 0 to 16, cP2 = 0 or 1, dP2 = 0 to 5, wherein at least b1P2 or b2P2 = 0 (i.e. only one of b1 and b2 may not be 0);

G^L is a linker for connecting to an antibody or antigen binding fragment thereof described herein (e.g. Ligand Unit);

(ib):



where R^{L1} and R^{L2} are independently selected from H and methyl, or together with the carbon atom to which they are bound form a cyclopropylene or cyclobutylene group; and

e is 0 or 1.

[0192] aP2 may be 0, 1, 2, 3, 4 or 5. In some embodiments, aP2 is 0 to 3. In some of these embodiments, aP2 is 0 or 1. In further embodiments, aP2 is 0.

[0193] b1P2 may be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16. In some embodiments, b1P2 is 0 to 12. In some of these embodiments, b1P2 is 0 to 8, and may be 0, 2, 3, 4, 5 or 8.

[0194] b2P2 may be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16. In some embodiments, b2P2 is 0 to 12. In some of these embodiments, b2P2 is 0 to 8, and may be 0, 2, 3, 4, 5 or 8.

[0195] Preferably, only one of b1P2 and b2P2 may not be 0.

[0196] cP2 may be 0 or 1.

[0197] dP2 may be 0, 1, 2, 3, 4 or 5. In some embodiments, dP2 is 0 to 3. In some of these embodiments, dP2 is 1 or 2. In further embodiments, dP2 is 2. In further embodiments, dP2 is 5.

[0198] In some embodiments of X^{P2} , aP2 is 0, b1P2 is 0, cP2 is 1 and dP2 is 2, and b2P2 may be from 0 to 8. In some of these embodiments, b2P2 is 0, 2, 3, 4, 5 or 8. In some embodiments of X^{P2} , aP2 is 1, b2P2 is 0, cP2 is 0 and dP2 is 0, and b1P2 may be from 0 to 8. In some of these embodiments, b1P2 is 0, 2, 3, 4, 5 or 8. In some embodiments of X^{P2} , aP2 is 0, b1P2 is 0, cP2 is 0 and dP2 is 1, and b2P2 may be from 0 to 8. In some of these embodiments, b2P2 is 0, 2, 3, 4, 5 or 8. In some embodiments of X^{P2} , b1P2 is 0, b2P2 is 0, cP2 is 0 and one of aP2 and dP2 is 0. The other of aP2 and d is from 1 to 5. In some of these embodiments, the other of aP2 and d is 1. In other of these embodiments, the other of aP2 and dP2 is 5.

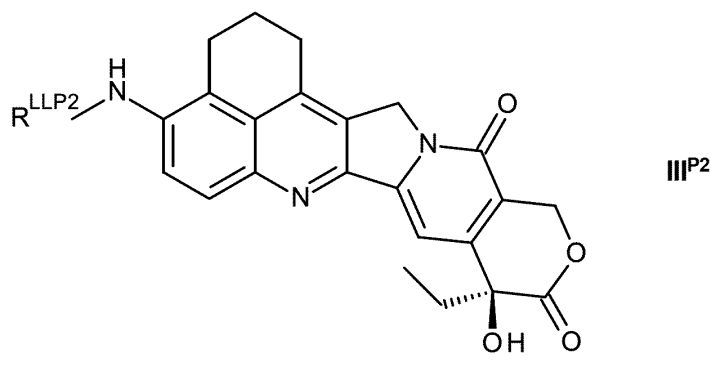
[0199] The preferences for Q^X above for compounds of Formula **I** may apply to Q^X in Formula Ia^{P2} (e.g. where appropriate).

[0200] The preferences for G^L , R^{L1} , R^{L2} and e above for compounds of Formula **I** may apply to compounds of Formula **I**^{P2}.

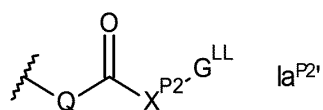
[0201] In some embodiments, the conjugate of formula **IV** is of the formula **IV**^{P2}:



or a pharmaceutically acceptable salt or solvate thereof, wherein L is an antibody or antigen binding fragment thereof described herein (e.g. Ligand unit), D^{LP2} is a topoisomerase I inhibitor (e.g. Drug Linker unit) that is of formula **III**^{P2}:

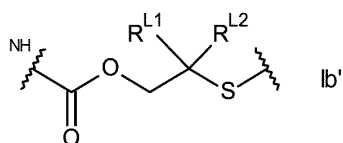


R^{LLP2} is a linker connected to the antibody or antigen binding fragment thereof (e.g. Ligand unit), wherein said linker is selected from (ia'):



where Q and X^{P2} are as defined above and G^{LL} is a linker connected to the antibody or antigen binding fragment thereof; and

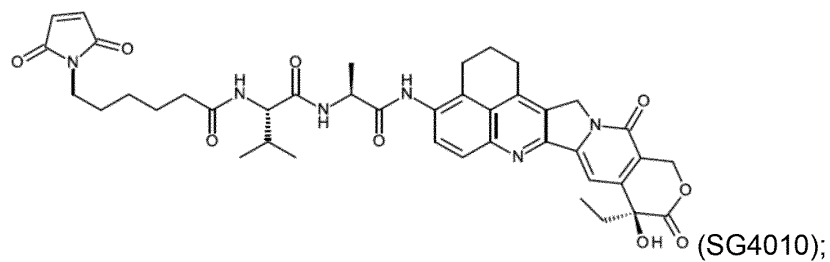
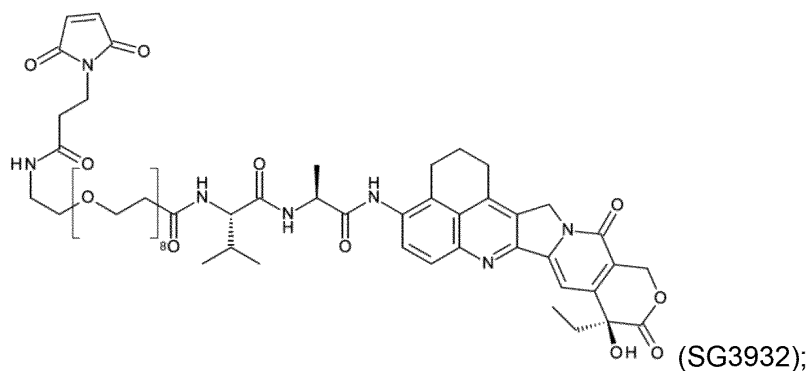
(ib'):



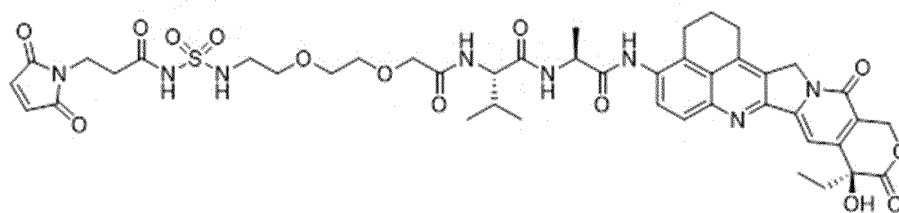
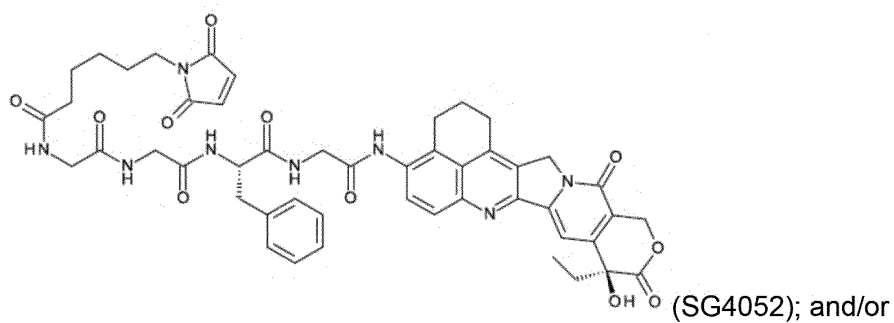
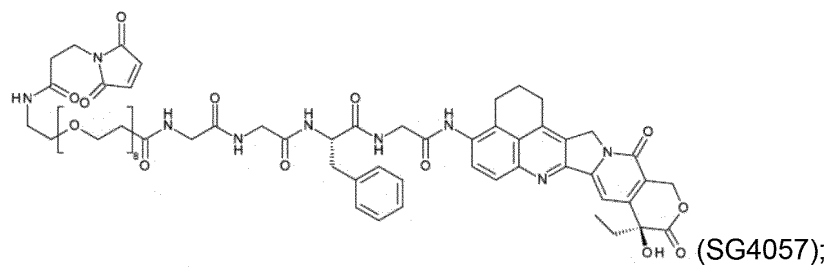
where R^{L1} and R^{L2} are as defined above; and

p is an integer of from 1 to 20.

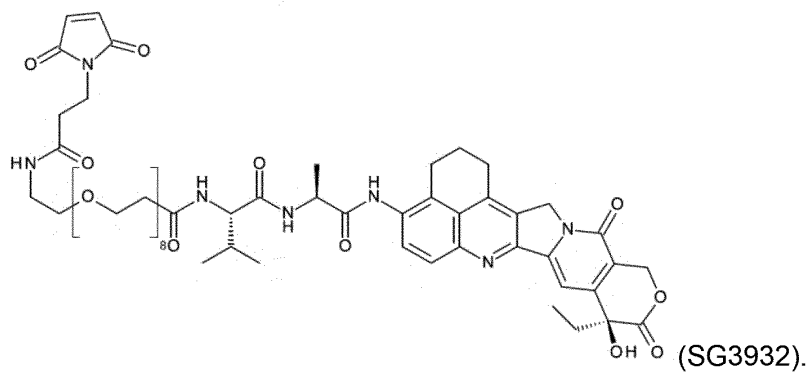
[0202] Particularly suitable topoisomerase I inhibitors include those having the following formulas:



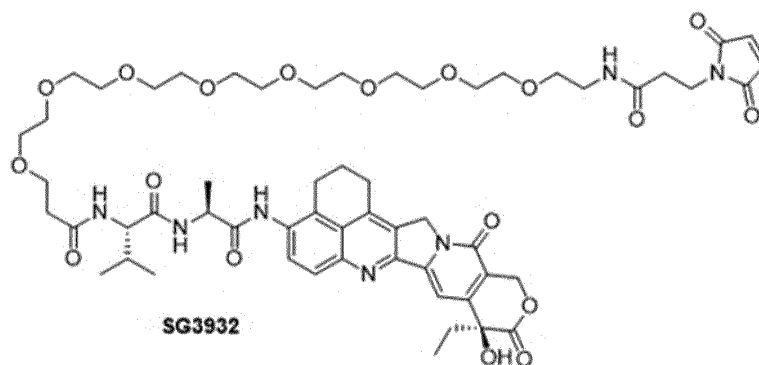
58



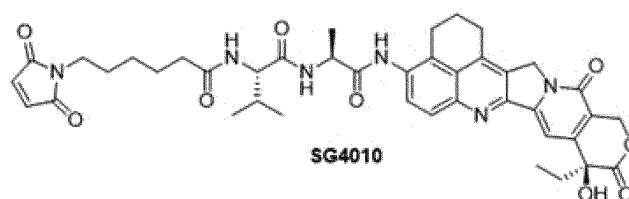
[0203] SG3932 is particularly preferred. Thus, in preferable embodiment, an antibody or antigen binding fragment thereof described herein is conjugated to a topoisomerase I inhibitor having the following formula (e.g. SG3932):



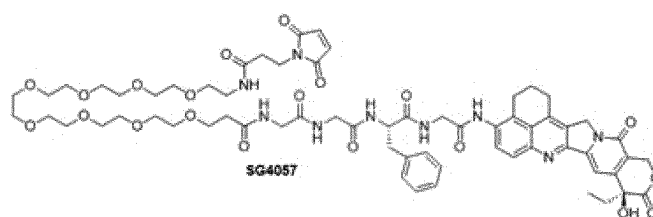
[0204] For the avoidance of doubt, the numeral '8' specifies that the structure within boxed parentheses is repeated eight times. Thus, another representation of SG3932 is:



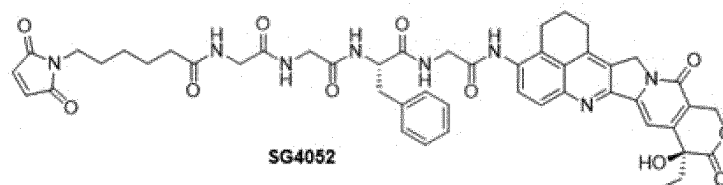
[0205] Another representation of SG4010 is:



[0206] Another representation of SG4057 is:



[0207] Another representation of SG4052 is:

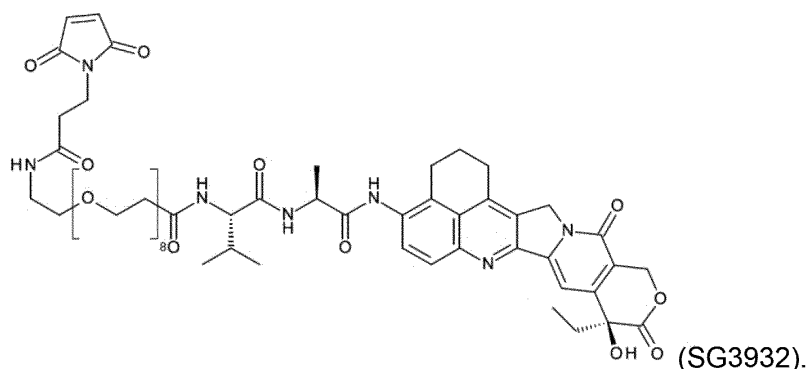


[0208] Any antibody or antigen binding fragment thereof described herein may be conjugated to one or more of said topoisomerase I inhibitor(s).

[0209] In a preferred aspect, there is provided an antibody or antigen binding fragment thereof which binds to a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope), comprising:

- i. a HCDR1 comprising the amino acid sequence of SEQ ID NO: 7, or a functional variant thereof;
- ii. a HCDR2 comprising the amino acid sequence of SEQ ID NO: 8, or a functional variant thereof;
- iii. a HCDR3 comprising the amino acid sequence of SEQ ID NO: 9, or a functional variant thereof;
- iv. a LCDR1 comprising the amino acid sequence of SEQ ID NO: 10, or a functional variant thereof;
- v. a LCDR2 comprising the amino acid sequence of SEQ ID NO: 11, or a functional variant thereof; and
- vi. a LCDR3 comprising the amino acid sequence of SEQ ID NO: 12, or a functional variant thereof;

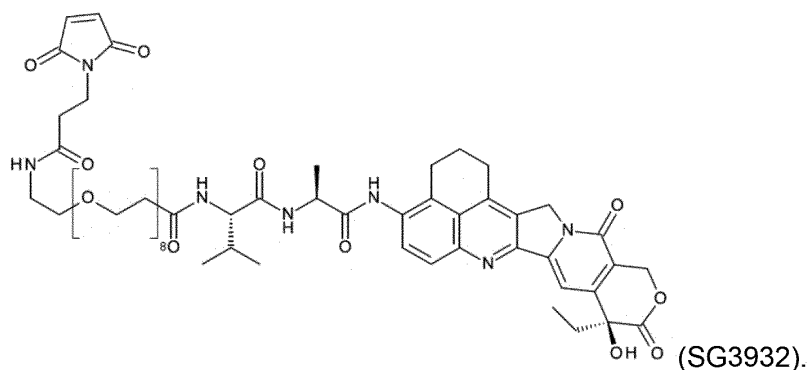
wherein the antibody or antigen binding fragment thereof is conjugated to a SG3932:



[0210] Another preferred aspect provides an antibody or antigen binding fragment thereof comprising: a variable heavy chain comprising the amino acid sequence of SEQ

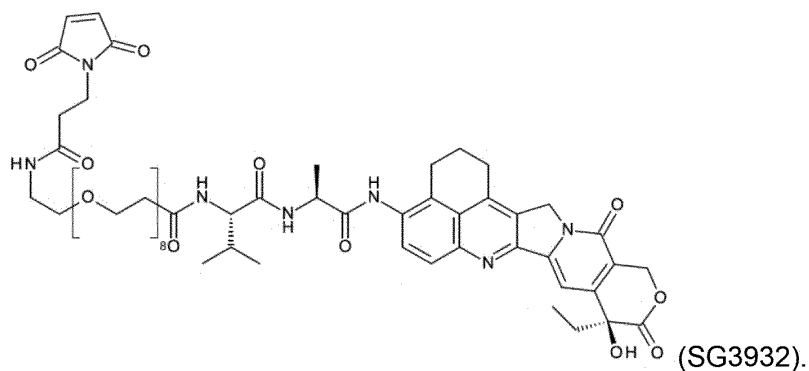
ID NO: 33, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof;

wherein the antibody or antigen binding fragment thereof is conjugated to a SG3932:



[0211] A particularly preferred aspect provides an antibody or antigen binding fragment thereof comprising: a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 45, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof;

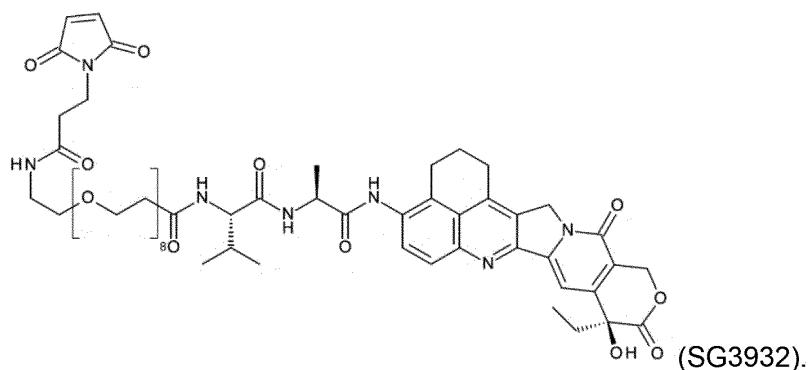
wherein the antibody or antigen binding fragment thereof is conjugated to a SG3932:



[0212] Another preferred aspect provides an antibody or antigen binding fragment thereof comprising: a heavy chain comprising the amino acid sequence of SEQ ID NO:

51, or a functional variant thereof; and a light chain comprising the amino acid sequence of SEQ ID NO: 44, or a functional variant thereof;

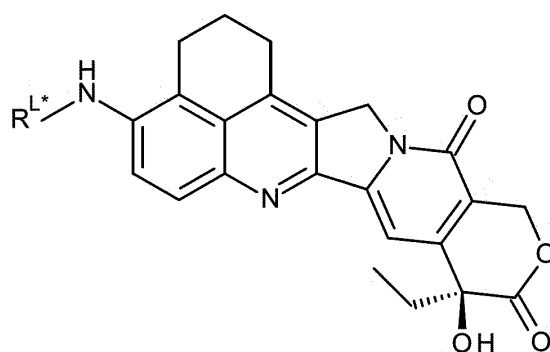
wherein the antibody or antigen binding fragment thereof is conjugated to a SG3932:



Synthesis of Topoisomerase I inhibitors

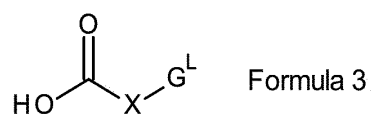
[0213] For completion, certain general synthetic routes for the preparation of preferred topoisomerase I inhibitor(s) will now be described. Further details may be found in the Examples section.

[0214] Compounds of formula I where R^L is of formula Ia may be synthesised from a compound of Formula 2:



Formula 2

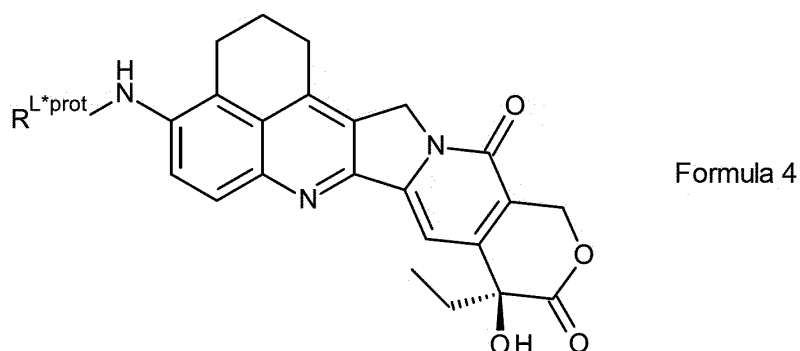
where R^{L*} is -QH by linking a compound of Formula 3:



or an activated version thereof.

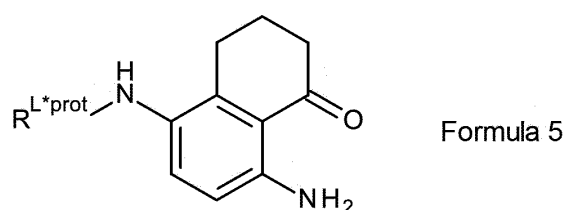
[0215] Such a reaction may be carried out under amide coupling conditions.

[0216] Compounds of Formula 2 may be synthesised by the deprotection of a compound of Formula 4:



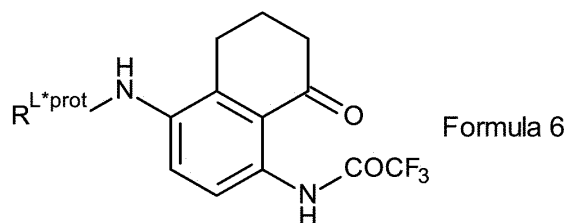
where R^{L*prot} is $-Q-Prot^N$, where $Prot^N$ is an amine protecting group.

[0217] Compounds of Formula 4 may be synthesised by the coupling of a compound of Formula 5:



with the compound A3 using the Friedlander reaction.

[0218] Compounds of Formula 5 may be synthesised from compounds of Formula 6:



by removal of the trifluoroacetamide protecting group.

[0219] Compounds of Formula 6 may be synthesised by coupling: $R^{L*prot}\text{-OH}$ to the compound I7.

[0220] Compounds of formula I where R^L is of formula Ia or Ib may be synthesised from the compound I11 by coupling of the compound $R^L\text{-OH}$, or an activated form thereof.

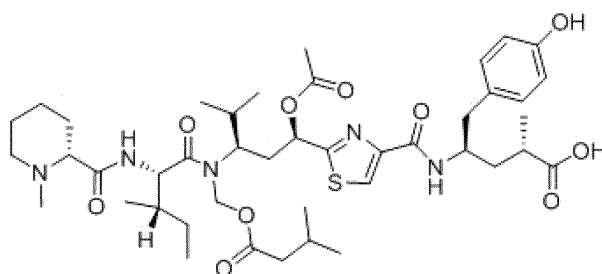
Amine protecting groups:

[0221] Amine protecting groups are well-known to those skilled in the art. Particular reference is made to the disclosure of suitable protecting groups in Greene's Protecting Groups in Organic Synthesis, Fourth Edition, John Wiley & Sons, 2007 (ISBN 978-0-471-69754-1), pages 696-871.

Further ADCs

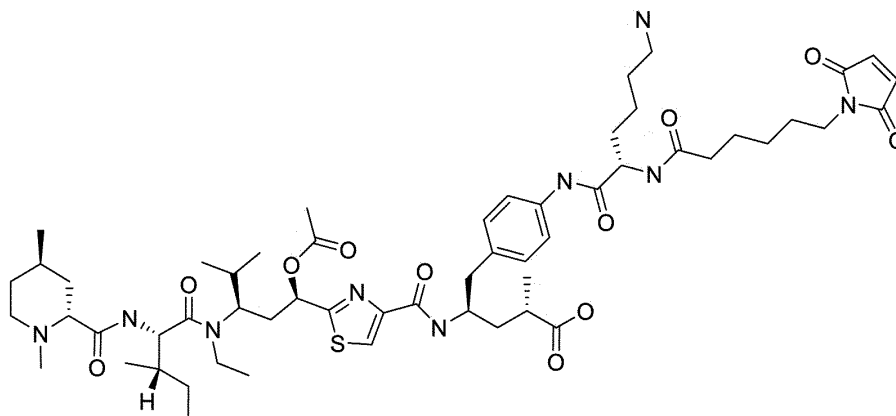
[0222] Although topoisomerase I inhibitors are preferred as outlined above, it should be noted that any suitable agent (e.g. drug/ cytotoxin) may be linked to an antibody or antigen binding fragment thereof of the invention. Examples of other suitable agents are outlined below.

[0223] In one embodiment, the cytotoxin is a tubulysin or tubulysin derivative. In one embodiment, the cytotoxin is Tubulysin A, having the following chemical structure:

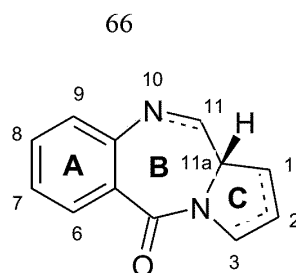


[0224] Tubulysins are members of a class of natural products isolated from myxobacterial species. As cytoskeleton-interacting agents, tubulysins are mitotic poisons that inhibit tubulin polymerization and lead to cell cycle arrest and apoptosis. As used herein, the term “tubulysin” refers both collectively and individually to the naturally occurring tubulysins and analogs and derivatives of tubulysins. Illustrative examples of tubulysins are disclosed, for example, in WO2004005326A2, WO2012019123A1, WO2009134279A1, WO2009055562A1, WO2004005327A1, US7776841, US7754885, US20100240701, US7816377, US20110021568, and US20110263650, incorporated herein by reference. It is to be understood that such derivatives include, for example, tubulysin prodrugs or tubulysins that include one or more protection or protecting groups, one or more linking moieties.

[0225] In one embodiment, the cytotoxin is tubulysin 1508, also referred to herein as “AZ1508” and described in more detail in WO 2015157594, incorporated herein by reference, having the following structure:



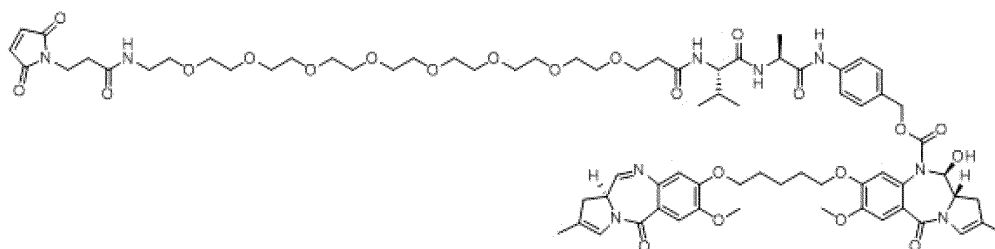
[0226] In another embodiment, the cytotoxin may be a pyrrolobenzodiazepine (PBD) or a PBD derivative. PBD translocates to the nucleus where it crosslinks DNA, preventing replication during mitosis, damaging DNA by inducing single strand breaks, and subsequently leading to apoptosis. Some PBDs have the ability to recognize and bond to specific sequences of DNA; the preferred sequence is PuGPu. PBDs are of the general structure:



[0227] PBDs differ in the number, type and position of substituents, in both their aromatic A rings and pyrrolo C rings, and in the degree of saturation of the C ring. In the B-ring there is either an imine (N=C), a carbinolamine (NH-CH(OH)), or a carbinolamine methyl ether (NH-CH(OMe)) at the N10-C11 position which is the electrophilic centre responsible for alkylating DNA. All of the known natural products have an (S)-configuration at the chiral C11a position which provides them with a right-handed twist when viewed from the C ring towards the A ring. This gives them the appropriate three-dimensional shape for isohelicity with the minor groove of B-form DNA, leading to a snug fit at the binding site. Their ability to form an adduct in the minor groove enables them to interfere with DNA processing, hence their use as anti-tumour agents.

[0228] The first PBD anti-tumor antibiotic, anthramycin, was discovered in 1965. Since then, a number of naturally occurring PBDs have been reported, and over 10 synthetic routes have been developed to a variety of analogues. Family members include abbeymycin, chicamycin, DC-81, mazethramycin, neothramycins A and B, porothramycin, prothracarcin, sibanomicin (DC-102), sibiromycin and tomamycin. PBDs and ADCs comprising them are also described in WO 2015/155345 and WO 2015/157592, incorporated in their entirety herein by reference.

[0229] In one embodiment, the cytotoxin is PBD 3249, also referred to herein as “SG3249” and described in more detail in WO 2014/057074, incorporated herein by reference, having the following structure:

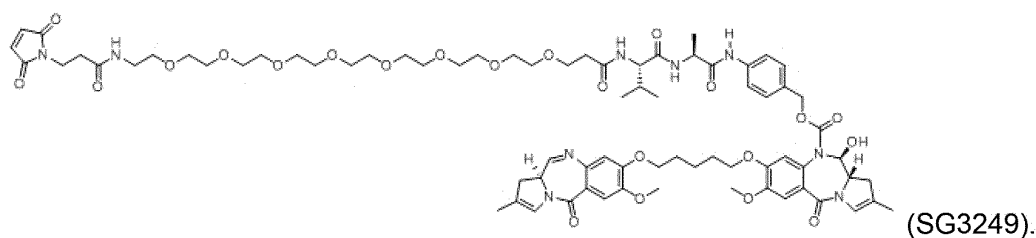


[0230] Thus, the antibody or antigen binding fragment thereof is conjugated to a pyrrolobenzodiazepine SG3249 cytotoxin.

[0231] For example, in one aspect, there is provided an antibody or antigen binding fragment thereof which binds to a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope), comprising:

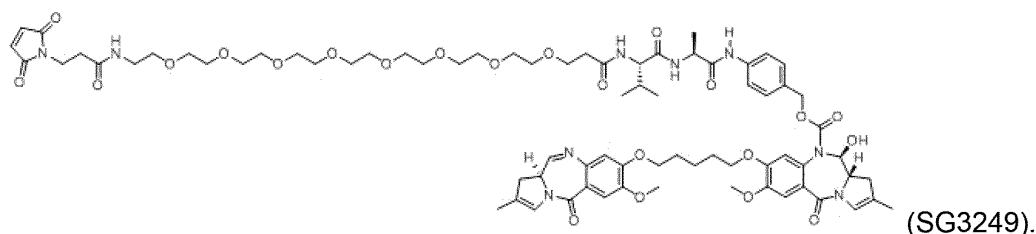
- i. a HCDR1 comprising the amino acid sequence of SEQ ID NO: 7, or a functional variant thereof;
- ii. a HCDR2 comprising the amino acid sequence of SEQ ID NO: 8, or a functional variant thereof;
- iii. a HCDR3 comprising the amino acid sequence of SEQ ID NO: 9, or a functional variant thereof;
- iv. a LCDR1 comprising the amino acid sequence of SEQ ID NO: 10, or a functional variant thereof;
- v. a LCDR2 comprising the amino acid sequence of SEQ ID NO: 11, or a functional variant thereof; and
- vi. a LCDR3 comprising the amino acid sequence of SEQ ID NO: 12, or a functional variant thereof;

wherein the antibody or antigen binding fragment thereof is conjugated to a pyrrolobenzodiazepine SG3249 cytotoxin:

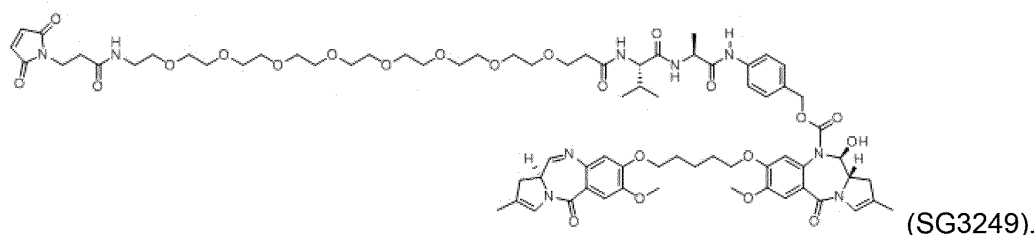


[0232] In another aspect, there is provided an antibody or antigen binding fragment thereof which binds to a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope), comprising: antibody or antigen binding fragment thereof comprising a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 33, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO:

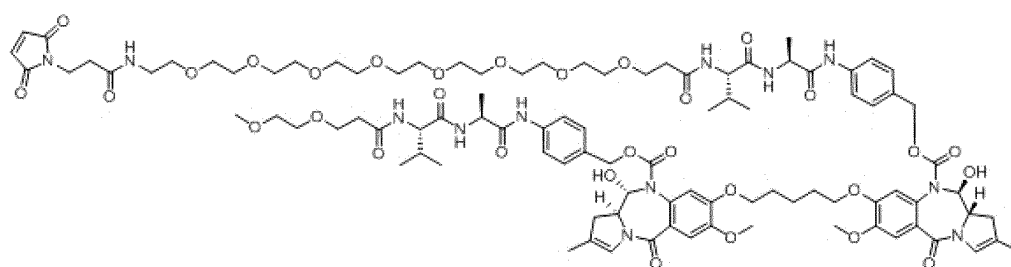
34, or a functional variant thereof; wherein the antibody or antigen binding fragment thereof is conjugated to a pyrrolobenzodiazepine SG3249 cytotoxin:



[0233] In another aspect, there is provided an antibody or antigen binding fragment thereof which binds to a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope), comprising: antibody or antigen binding fragment thereof comprising a variable heavy chain comprising the amino acid sequence of SEQ ID NO: 45, or a functional variant thereof; and a variable light chain comprising the amino acid sequence of SEQ ID NO: 34, or a functional variant thereof; wherein the antibody or antigen binding fragment thereof is conjugated to a pyrrolobenzodiazepine SG3249 cytotoxin:



[0234] In one embodiment, the cytotoxin is PBD 3315, also referred to herein as "SG3315" and described in more detail in WO 2015/052322, incorporated herein by reference, having the following structure:



[0235] The antibody or antigen binding fragment thereof is preferably conjugated to a pyrrolobenzodiazepine SG3249 cytotoxin through a cysteine residue.

- [0236] The antibody or antigen fragment thereof of the invention may be conjugated to heterologous agents (preferably a cytotoxin) using site-specific or non-site specific methods of conjugation. In one embodiment, the antibodies and antigen fragment thereof comprise one, two, three, four or more therapeutic moieties. In one embodiment, all therapeutic moieties are the same.
- [0237] Conventional conjugation strategies for antibodies or antigen-binding fragments thereof rely on randomly conjugating the payload to the antibody or fragment through lysines or cysteines. In one embodiment, the antibody or antigen-binding fragment thereof is randomly conjugated to a heterologous agent (preferably a cytotoxin), for example, by partial reduction of the antibody or fragment, followed by reaction with a desired agent, with or without a linker moiety attached. The antibody or fragment may be reduced using DTT or similar reducing agent. The agent with or without a linker moiety attached can then be added at a molar excess to the reduced antibody or fragment in the presence of DMSO. After conjugation, excess free cysteine may be added to quench unreacted agent. The reaction mixture may then be purified and buffer-exchanged into PBS.
- [0238] In one embodiment, an agent (e.g. cytotoxin) is conjugated to an antibody or antigen binding fragment thereof by site-specific conjugation. In one embodiment, site-specific conjugation of therapeutic moieties to antibodies using reactive amino acid residues at specific positions yields homogeneous ADC preparations with uniform stoichiometry.
- [0239] The site specific conjugation can be through a cysteine, residue or a non-natural amino acid. In a preferable embodiment, the heterologous agent (preferably cytotoxin) is conjugated to the antibody or antigen binding fragment thereof through at least one cysteine residue.
- [0240] In one embodiment, the heterologous agent (preferably cytotoxin) is chemically conjugated to the side chain of an amino acid (preferably at a specific Kabat position in the Fc region). In one embodiment, the agent (e.g. the cytotoxic or imaging agent) is conjugated to the antibody or antigen binding fragment thereof through a cysteine substitution of at least one of positions 239, 248, 254, 273, 279, 282, 284, 286, 287, 289,

297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 440, 441, 442, 443 and 446, wherein the numbering corresponds to the EU index in Kabat. In one embodiment, the specific Kabat positions are 239, 442, or both. In one embodiment, the specific positions are Kabat position 442, an amino acid insertion between Kabat positions 239 and 240, or both. In one embodiment, the heterologous agent (preferably cytotoxin) is conjugated to the antibody or antigen binding fragment thereof through a thiol-maleimide linkage. In some aspects, the amino acid side chain is a sulfhydryl side chain.

[0241] In one embodiment, the antibody or antigen binding fragment thereof comprises a light chain (e.g. preferably comprising a VL and constant light chain) comprising the amino acid sequence of SEQ ID NO: 44 and a heavy chain (e.g. comprising a VH and constant heavy chain) comprising the amino acid sequence of SEQ ID NO: 48; wherein the antibody or antigen binding fragment thereof is conjugated to a pyrrolobenzodiazepine SG3249 cytotoxin; preferably wherein the pyrrolobenzodiazepine SG3249 cytotoxin is conjugated to the cysteine residue at amino acid position 240 of said heavy chain.

[0242] In one embodiment, the antibody or antigen binding fragment thereof comprises a light chain (e.g. preferably comprising a VL and constant light chain) comprising the amino acid sequence of SEQ ID NO: 44 and a heavy chain (e.g. comprising a VH and constant heavy chain) comprising the amino acid sequence of SEQ ID NO: 51; wherein the antibody or antigen binding fragment thereof is conjugated to a pyrrolobenzodiazepine SG3249 cytotoxin.

[0243] Reference herein to an antibody or antigen-binding fragment conjugated to a cytotoxin is synonymous with the term “antibody drug conjugate (ADC)”, or “anti-B7-H4 ADC”.

[0244] In one embodiment, the antibody or antigen binding fragment thereof (e.g., anti-B7-H4 ADC) delivers a cytotoxic payload to a cell (preferably a B7-H4-expressing cell) and inhibits or suppresses proliferation (e.g. of a tumour) by at least 10%, or at least 20%, or at least 30%, or at least 40%, or at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90% or about 100% (preferably at least 40%) relative to a level of

inhibition or suppression in the absence of the antibody or antigen binding fragment thereof (e.g., anti-B7-H4 ADC). Cellular proliferation can be assayed using art recognized techniques which measure rate of cell division, and/or the fraction of cells within a cell population undergoing cell division, and/or rate of cell loss from a cell population due to terminal differentiation or cell death (e.g., thymidine incorporation).

[0245] In one embodiment, the antibody or antigen fragment thereof (e.g., anti-B7-H4 ADC) of the invention binds to B7-H4 on the surface of a cell, and is internalised into the cell. In one embodiment, the antigen or antibody fragment thereof is internalised into a cell (preferably a B7-H4-expressing cell) with an IC₅₀ at 10 minutes of about 100 ng/ml to about 1 µg/ml, about 100 ng/ml to about 500 ng/ml, about 100 ng/ml to about 250 ng/ml, about 250 ng/ml to about 500 ng/ml, about 350 ng/ml to about 450 ng/ml, about 500 ng/ml to about 1 µg/ml, about 500 ng/ml to about 750 ng/ml, about 750 ng/ml to about 850 ng/ml, or about 900 ng/ml to about 1 µg/ml.

[0246] In one embodiment, the antibody or antigen fragment thereof (e.g., anti-B7-H4 ADC) is internalised into a cell (preferably a B7-H4-expressing cell) with an IC₅₀ at 30 minutes of about 100 ng/ml to about 1 µg/ml, about 100 ng/ml to about 500 ng/ml, about 100 ng/ml to about 250 ng/ml, about 250 ng/ml to about 500 ng/ml, about 250 ng/ml to about 350 ng/ml, about 350 ng/ml to about 450 ng/ml, about 500 ng/ml to about 1 µg/ml, about 500 ng/ml to about 750 ng/ml, about 750 ng/ml to about 850 ng/ml, or about 900 ng/ml to about 1 µg/ml.

[0247] In one embodiment, the antibody or antigen fragment thereof (e.g., anti-B7-H4 ADC) is internalised into a cell (preferably a B7-H4-expressing cell) with an IC₅₀ at 120 minutes of about 50 ng/ml to about 500 ng/ml, about 50 ng/ml to about 100 ng/ml, about 100 ng/ml to about 200 ng/ml, about 200 ng/ml to about 300 ng/ml, about 300 ng/ml to about 400 ng/ml, or about 400 ng/ml to about 500 ng/ml.

[0248] In one embodiment, the antibody or antigen fragment thereof (e.g., anti-B7-H4 ADC) is internalised into a cell (preferably a B7-H4-expressing cell) with an IC₅₀ at 8 hours of about 5 ng/ml to about 250 ng/ml, about 10 ng/ml to about 25 ng/ml, about 25 ng/ml to about 50 ng/ml, about 50 ng/ml to about 100 ng/ml, about 100 ng/ml to about 150 ng/ml, about 150 ng/ml to about 200 ng/ml, or about 200 ng/ml to about 250 ng/ml.

- [0249] For the avoidance of doubt, reference to a “conjugate” herein means an antibody or antigen binding fragment conjugated to a heterologous agent (preferably a cytotoxin) including any such agent described above.
- [0250] In addition to the therapeutic applications of an antibody or antigen binding fragment of the invention described above, the “conjugates” of the present invention may be also used in a method of therapy. Thus, also provided is a method of treatment, comprising administering to a subject in need of treatment a therapeutically-effective amount of a conjugate described herein (e.g. conjugate of formula IV). The term “therapeutically effective amount” is an amount sufficient to show benefit to a patient. Such benefit may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage, is within the responsibility of general practitioners and other medical doctors.
- [0251] A conjugate may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated. Examples of treatments and therapies include, but are not limited to, chemotherapy (the administration of active agents, including, e.g. drugs); surgery; and radiation therapy.
- [0252] Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to the active ingredient, i.e. a conjugate/ ADC of the invention (e.g. formula IV), a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous, or intravenous.
- [0253] Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol,

propylene glycol or polyethylene glycol may be included. A capsule may comprise a solid carrier such as a gelatin.

[0254] For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

[0255] Preferably, the conjugates can be used to treat proliferative disease. The term "proliferative disease" pertains to an unwanted or uncontrolled cellular proliferation of excessive or abnormal cells which is undesired, such as, neoplastic or hyperplastic growth, whether *in vitro* or *in vivo*. The term "proliferative disease" may alternatively be referred to as "cancer".

[0256] A suitable proliferative disease (e.g. cancer) will preferably be characterised by the presence cancerous cells that express B7-H4.

[0257] Examples of proliferative conditions include, but are not limited to, benign, pre-malignant, and malignant cellular proliferation, including but not limited to, neoplasms and tumours (e.g., histiocytoma, glioma, astrocyoma, osteoma), cancers (e.g. lung cancer, small cell lung cancer, gastrointestinal cancer, bowel cancer, colon cancer, breast carcinoma, ovarian carcinoma, prostate cancer, testicular cancer, liver cancer, kidney cancer, bladder cancer, pancreatic cancer, brain cancer, sarcoma, osteosarcoma, Kaposi's sarcoma, melanoma), leukemias, psoriasis, bone diseases, fibroproliferative disorders (e.g. of connective tissues), and atherosclerosis. Other cancers of interest include, but are not limited to, haematological; malignancies such as leukemias and lymphomas, such as non-Hodgkin lymphoma, and subtypes such as DLBCL, marginal zone, mantle zone, and follicular, Hodgkin lymphoma, AML, and other cancers of B or T cell origin. Any type of cell may be treated, including but not limited to, lung, gastrointestinal (including, e.g. bowel, colon), breast (mammary), ovarian, prostate, liver (hepatic), kidney (renal), bladder, pancreas, brain, and skin.

- [0258] The antibody-drug conjugate may be labelled, for example to aid detection of cell binding (*in vitro* or *in vivo*). The label may be a biotin label. In another embodiment, the label may be a radioisotope.
- [0259] In another aspect, there is provided a polynucleotide comprising a nucleic acid sequence encoding an antibody or antigen binding fragment thereof of the invention.
- [0260] In one embodiment, the polynucleotide may be an isolated polynucleotide.
- [0261] The sequence(s) (e.g. polynucleotide sequence(s)) of the present invention include sequences that have been removed from their naturally occurring environment, recombinant or cloned (e.g. DNA) isolates, and chemically synthesized analogues or analogues biologically synthesized by heterologous systems.
- [0262] The sequence(s) (e.g. polynucleotide sequence(s)) of the present invention may be prepared by any means known in the art. For example, large amounts of the sequence(s) may be produced by replication and/or expression in a suitable host cell. The natural or synthetic DNA fragments coding for a desired fragment will typically be incorporated into recombinant nucleic acid constructs, typically DNA constructs, capable of introduction into and replication in a prokaryotic or eukaryotic cell. Usually the DNA constructs will be suitable for autonomous replication in a unicellular host, such as yeast or bacteria, but may also be intended for introduction to and integration within the genome of a cultured bacterial, insect, mammalian, plant or other eukaryotic cell lines.
- [0263] The sequence(s) (e.g. polynucleotide sequence(s)) of the present invention may also be produced by chemical synthesis, e.g. a polynucleotide by the phosphoramidite method or the tri-ester method and may be performed on commercial automated oligonucleotide synthesizers. A double-stranded (e.g. DNA) fragment may be obtained from the single stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strand together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.
- [0264] When applied to a sequence (e.g. polynucleotide sequence) of the invention, the term “isolated” preferably denotes that the sequence has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences (but

may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators), and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment.

[0265] Another aspect provided herein is a host cell comprising a polynucleotide, said polynucleotide comprising a nucleic acid sequence encoding an antibody or antigen binding fragment thereof of the invention.

[0266] In one embodiment, the polynucleotide encodes a VH chain of an antibody or antigen binding fragment thereof. In one embodiment, a polynucleotide of the invention may encode a VL chain of an antibody or antigen binding fragment thereof. In one embodiment, the polynucleotide may encode a VH and a VL chain of an antibody or antigen binding fragment thereof. In one embodiment, the polynucleotide may further encode a leader sequence (e.g. which functions as a secretory sequence for controlling transport of a polypeptide from the cell).

[0267] In another aspect there is provided a vector (e.g. plasmid) comprising the polynucleotide of the invention.

[0268] Variants of a polynucleotide described above are embraced by the invention. Polynucleotide variants can contain alterations in the coding regions, non-coding regions, or both. In one embodiment, a polynucleotide variant comprises an alteration that produces silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. In one embodiment, a polynucleotide variant is produced by a silent substitution due to the degeneracy of the genetic code. A polynucleotide variant can be produced for a variety of reasons, e.g., to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*). Vectors and cells comprising said polynucleotide variant are also provided.

[0269] The present invention embraces methods for producing an antibody or antigen binding fragment thereof that binds to a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope), comprising expressing a polynucleotide in a host cell, said polynucleotide

comprising a nucleic acid sequence encoding an antibody or antigen binding fragment thereof of the invention.

[0270] The present invention further embraces an antibody or antigen binding fragment thereof obtainable by said methods for producing an antibody or antigen binding fragment thereof that binds to a B7-H4 polypeptide (e.g. B7-H4 polypeptide epitope).

[0271] In a preferable embodiment, the method for producing an antibody or antigen binding fragment thereof comprises (a) culturing the host cell and (b) isolating the antibody or antigen binding fragment thereof expressed from the cell.

[0272] Suitable host cells for expression of an antibody or antigen binding fragment thereof of the invention include a prokaryote, yeast, insect, or higher eukaryotic cells (preferably wherein the polynucleotide is under the control of appropriate promoters). Prokaryotes include gram negative or gram-positive organisms, for example *E. coli* or *bacilli*. Higher eukaryotic cells include established cell lines of mammalian origin as described herein. Cell-free translation systems can also be employed.

[0273] In one aspect, there is provided a kit comprising an antigen or antibody binding fragment described herein. There is further embraced use of said kit in the methods of the present invention.

[0274] In one embodiment, a kit comprises an isolated (e.g. purified) antigen or antibody binding fragment of the invention. In one embodiment, a kit comprises an isolated (e.g. purified) antigen or antibody binding fragment of the invention, wherein the antigen or antibody binding fragment comprises an agent (e.g. conjugated cytotoxin) described herein. In one embodiment, the kit comprises one or more container. The kit may provide the antigen or antibody binding fragment and the agent individually (e.g. the agent is not conjugated to the antigen or antibody binding fragment, but is in a form suitable for conjugation thereto); optionally wherein the kit is further provided with instructions and/or reagents for conjugating the agent to the antigen or antibody binding fragment. In one embodiment, the kit comprises all of the components necessary and/or sufficient to perform a detection assay, including all controls, directions for performing assays, and any necessary software for analysis and presentation of results.

[0275] An antibody or antigen binding fragment thereof of the invention can be used in assays for immunospecific binding by any method known in the art. The immunoassays that can be used include, but are not limited to, competitive and non-competitive assay systems using techniques such as Western blot, RIA, ELISA, ELISPOT, "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, and protein A immunoassays.

[0276] An antibody or antigen binding fragment thereof of the invention can be employed histologically, as in immunofluorescence, immunoelectron microscopy, or non-immunological assays, for example, for *in situ* detection of B7-H4 or conserved variants or peptide fragments thereof. *In situ* detection can be accomplished by removing a histological specimen from a patient, and applying thereto a labelled an antibody or antigen binding fragment thereof of the invention, e.g., applied by overlaying the labelled antibody or antigen binding fragment thereof onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of B7-H4, or conserved variants or peptide fragments, but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Antibodies

[0277] The term "antibody" covers monoclonal antibodies and fragments thereof (e.g. exhibiting the desired biological activity). In a preferable embodiment, an antibody of the present invention is a monoclonal antibody. In a more preferable embodiment, the antibody is a fully human monoclonal antibody. In one embodiment, methods of the invention may employ polyclonal antibodies.

[0278] In particular, an antibody is a protein including at least one or two, heavy (H) chain variable regions (abbreviated herein as VHC), and at least one or two light (L) chain variable regions (abbreviated herein as VLC). The VHC and VLC 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). The extent of the framework region and CDRs has been precisely defined (see, Kabat, E.A., et al. Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, 1991, and Chothia, C. et al, J. Mol. Biol. 196:901-917, 1987). Preferably, each VHC and VLC is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, DR2, FR3, CDR3, FR4. The VHC or VLC chain of the antibody can further include all or part of a heavy or light chain constant region. In one embodiment, the antibody is a tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains, wherein the heavy and light immunoglobulin chains are interconnected by, e.g., disulfide bonds. The heavy chain constant region includes three domains, CH1, CH2 and CH3. The light chain constant region is comprised of one domain, CL. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The term "antibody" includes intact immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof), wherein the light chains of the immunoglobulin may be of types kappa or lambda. The term antibody, as used herein, also refers to a portion of an antibody that binds to one of the above-mentioned markers, e.g., a molecule in which one or more immunoglobulin chains is not full length, but which binds to a marker. Examples of binding portions encompassed within the term antibody include (i) a Fab fragment, a monovalent fragment consisting of the VLC, VHC, 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 Fc fragment consisting of the VHC and CH1 domains; (iv) a Fv fragment consisting of the VLC and VHC domains of a single arm of an antibody, (v) a dAb fragment (Ward et al, Nature 341:544-546, 1989), which consists of a VHC domain; and (vi) an isolated complementarity determining region (CDR) having sufficient framework to bind, e.g. an antigen binding portion of a variable region. An antigen binding portion of a light chain variable region and an antigen binding portion of a heavy chain variable region, e.g., the two domains of the Fv fragment, VLC and VHC, can be joined, using recombinant methods, by a synthetic linker that enables them to be made as

a single protein chain in which the VLC and VHC regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 1A1-ATi-Al β ; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also encompassed within the term antibody. These may be obtained using conventional techniques known to those skilled in the art, and the portions are screened for utility in the same manner as are intact antibodies.

- [0279] In one embodiment, the antibody or antigen binding fragment is one or more selected from a murine antibody, a humanized antibody, a chimeric antibody, a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a multispecific antibody, or a combination thereof.
- [0280] In one embodiment, the antigen-binding fragment is one or more selected from a Fv fragment, an Fab fragment, an F(ab')₂ fragment, an Fab' fragment, a dsFv fragment, an scFv fragment, an sc(Fv)₂ fragment, or a combination thereof.
- [0281] In a preferable embodiment, the antibody or antigen binding fragment thereof is a monoclonal antibody (mAb).
- [0282] In one embodiment, the antibody or antigen binding fragment thereof (e.g. mAb) of the invention is a scFV.
- [0283] In one embodiment, the antibody or antigen binding fragment thereof can bind to B7-H4 molecules across species, e.g., the antibody or fragment can bind to mouse B7-H4, rat B7-H4, rabbit, human B7-H4 and/or cynomolgus monkey B7-H4. In one embodiment, the antibody or fragment can bind to human B7-H4 and cynomolgus monkey B7-H4. In one embodiment, the antibody or antigen binding fragment can also bind to mouse B7-H4.
- [0284] In one embodiment, the antibody or antigen binding fragment thereof can specifically bind to B7-H4, e.g., human B7-H4 and cynomolgus monkey B7-H4, but does not specifically bind to human B7-H1, B7-H2, and/or B7-H3.
- [0285] In one embodiment, the antibody or antigen-binding fragment thereof can include, in addition to a VH and a VL, a heavy chain constant region or fragment thereof. In one embodiment, the heavy chain constant region is a human heavy chain constant region, e.g., a human IgG constant region, e.g., a human IgG1 constant region. In one

embodiment (preferably where the antibody or antigen-binding fragment thereof is conjugated to an agent, such as a cytotoxic agent), a cysteine residue is inserted between amino acid S239 and V240 in the CH2 region of IgG1. This cysteine is referred to as “a 239 insertion” or “239i.”

[0286] In one embodiment, the antibody or antigen binding fragment thereof may comprise a heavy chain constant region comprising the amino acid sequence of SEQ ID NO: 41. Preferably, the antibody or antigen binding fragment thereof may comprise a heavy chain constant region comprising the amino acid sequence of SEQ ID NO: 52.

[0287] In one embodiment, a heavy chain constant region or fragment thereof, e.g., a human IgG constant region or fragment thereof, can include one or more amino acid substitutions relative to a wild-type IgG constant domain wherein the modified IgG has an increased half-life compared to the half-life of an IgG having the wild-type IgG constant domain. For example, the IgG constant domain can contain one or more amino acid substitutions of amino acid residues at positions 251-257, 285-290, 308-314, 385-389, and 428-436, wherein the amino acid position numbering is according to the EU index as set forth in Kabat. In one embodiment the IgG constant domain can contain one or more of a substitution of the amino acid at Kabat position 252 with Tyrosine (Y), Phenylalanine (F), Tryptophan (W), or Threonine (T), a substitution of the amino acid at Kabat position 254 with Threonine (T), a substitution of the amino acid at Kabat position 256 with Serine (S), Arginine (R), Glutamine (Q), Glutamic acid (E), Aspartic acid (D), or Threonine (T), a substitution of the amino acid at Kabat position 257 with Leucine (L), a substitution of the amino acid at Kabat position 309 with Proline (P), a substitution of the amino acid at Kabat position 311 with Serine (S), a substitution of the amino acid at Kabat position 428 with Threonine (T), Leucine (L), Phenylalanine (F), or Serine (S), a substitution of the amino acid at Kabat position 433 with Arginine (R), Serine (S), Isoleucine (I), Proline (P), or Glutamine (Q), or a substitution of the amino acid at Kabat position 434 with Tryptophan (W), Methionine (M), Serine (S), Histidine (H), Phenylalanine (F), or Tyrosine. In a preferable embodiment, the IgG constant domain can contain amino acid substitutions relative to a wild-type human IgG constant domain including as substitution of the amino acid at Kabat position 252 with Tyrosine (Y), a

substitution of the amino acid at Kabat position 254 with Threonine (T), and a substitution of the amino acid at Kabat position 256 with Glutamic acid (E). In one embodiment, the antibody or antigen-binding fragment thereof comprises a heavy chain, wherein the heavy chain is a human IgG1 YTE mutant.

[0288] In one embodiment, the antibody or antigen-binding fragment thereof can include, in addition to a VH and a VL, and optionally a heavy chain constant region or fragment thereof, a light chain constant region or fragment thereof. In one embodiment, the light chain constant region is a kappa lambda light chain constant region, e.g., a human kappa constant region or a human lambda constant region.

[0289] In one embodiment, the antibody or antigen binding fragment thereof comprises a light chain constant region comprising the amino acid sequence of SEQ ID NO: 42.

[0290] In one embodiment, a VH and/or VL amino acid sequence can have 85%, 90%, 95%, 96%, 97%, 98% or 99% similarity to a sequence set forth herein. In one embodiment, a VH and/or VL amino acid sequence may comprise 1, 2, 3, 4, 5 or more substitutions, e.g., conservative substitutions relative to a sequence set forth herein. A B7-H4 antibody having VH and VL regions having a certain percent similarity to a VH region or VL region, or having one or more substitutions, e.g., conservative substitutions can be obtained by mutagenesis (e.g., site-directed or PCR-mediated mutagenesis) of nucleic acid molecules encoding VH and/or VL regions described herein, followed by testing of the encoded altered antibody for binding to B7-H4 and optionally testing for retained function using the functional assays described herein.

[0291] The affinity or avidity of an antibody or antigen binding fragment thereof for an antigen can be determined experimentally using any suitable method well known in the art, e.g., flow cytometry, enzyme-linked immunosorbent assay (ELISA), or radioimmunoassay (RIA), or kinetics (e.g., KINEXA® or BIACORE™ analysis). Direct binding assays as well as competitive binding assay formats can be readily employed. (See, e.g., Berzofsky et al., *Antibody-Antigen Interactions*, In *Fundamental Immunology*, Paul, W. E., Ed., Raven Press: New York, N.Y. (1984); Kuby, *Immunology*, W. H. Freeman and Company: New York, N.Y. (1992); and methods described herein.) The measured affinity of a particular antibody-antigen interaction can vary if measured under

different conditions (e.g., salt concentration, pH, temperature). Thus, measurements of affinity and other antigen-binding parameters (e.g., K_D or K_d , K_{on} , K_{off}) are made with standardized solutions of antibody and antigen, and a standardized buffer, as known in the art.

[0292] In one embodiment, the antibody or antigen-binding fragment thereof, can bind to B7-H4-expressing cells with an IC_{50} lower than about 500 nM, lower than about 350 nM, lower than about 250 nM, lower than about 150 nM, lower than about 100 nM, lower than about 75 nM, lower than about 60 nM, lower than about 50 nM, lower than about 40 nM, lower than about 30 nM, lower than about 20 nM, lower than about 15 nM, lower than about 10 nM, lower than about 5 nM, lower than about 1 nM, lower than about 500 pM, lower than about 350 pM, lower than about 250 pM, lower than about 150 pM, lower than about 100 pM, lower than about 75 pM, lower than about 60 pM, lower than about 50 pM, lower than about 40 pM, lower than about 30 pM, lower than about 20 pM, lower than about 15 pM, lower than about 10 pM, or lower than about 5 pM. Preferably, said IC_{50} is measured by flow cytometry.

[0293] A “monoclonal antibody” (mAb) refers to a homogeneous antibody population involved in the highly specific recognition and binding of a single antigenic determinant, or epitope. This is in contrast to polyclonal antibodies that typically include different antibodies directed against different antigenic determinants. The term “monoclonal antibody” encompasses both intact and full-length monoclonal antibodies as well as antibody fragments (such as Fab, Fab', F(ab')₂, Fv), single chain (scFv) mutants, fusion proteins comprising an antibody portion, and any other modified immunoglobulin molecule comprising an antigen recognition site. Furthermore, “monoclonal antibody” refers to such antibodies made in any number of ways including, but not limited to, hybridoma, phage selection, recombinant expression, and transgenic animals.

[0294] In a preferable embodiment, the antibody or antigen binding fragment thereof (e.g. mAb) of the invention is a humanised antibody or antigen binding fragment thereof. Suitably, said humanised the antibody or antigen binding fragment thereof is an IgG.

[0295] The term “humanised antibody” refers to an antibody derived from a non-human (e.g., murine) immunoglobulin, which has been engineered to contain minimal non-

human (e.g., murine) sequences. Typically, humanized antibodies are human immunoglobulins in which residues from the complementary determining region (CDR) are replaced by residues from the CDR of a non-human species (e.g., mouse, rat, rabbit, or hamster) that have the desired specificity, affinity, and capability (Jones et al., 1986, *Nature*, 321:522-525; Riechmann et al., 1988, *Nature*, 332:323-327; Verhoeyen et al., 1988, *Science*, 239:1534-1536). In some instances, the Fv framework region (FW) residues of a human immunoglobulin are replaced with the corresponding residues in an antibody from a non-human species that has the desired specificity, affinity, and capability.

[0296] Humanised antibodies can be further modified by the substitution of additional residues either in the Fv framework region and/or within the replaced non-human residues to refine and optimize antibody specificity, affinity, and/or capability. In general, humanized antibodies will comprise substantially all of at least one, and typically two or three, variable domains containing all or substantially all of the CDR regions that correspond to the non-human immunoglobulin whereas all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. Humanized antibody can also comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Examples of methods used to generate humanized antibodies are described in U.S. Pat. Nos. 5,225,539 or 5,639,641.

[0297] A “variable region” of an antibody refers to the variable region of the antibody light chain or the variable region of the antibody heavy chain, either alone or in combination. The variable regions of the heavy and light chain each consist of four framework regions (FW) connected by three complementarity-determining regions (CDRs), also known as hypervariable regions. The CDRs in each chain are held together in close proximity by the FW regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (i.e., Kabat et al. *Sequences of Proteins of Immunological Interest*, (5th ed., 1991, National Institutes of Health, Bethesda Md.)); and (2) an approach based on crystallographic studies of antigen-antibody complexes (Al-lazikani et al. (1997) J.

Molec. Biol. 273:927-948)). In addition, combinations of these two approaches are sometimes used in the art to determine CDRs.

[0298] The “Kabat numbering system” is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (e.g., Kabat et al., Sequences of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)).

[0299] The amino acid position numbering as in Kabat, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Using this numbering system, the actual linear amino acid sequence can contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FW or CDR of the variable domain. For example, a heavy chain variable domain can include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g., residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FW residue 82.

[0300] The Kabat numbering of residues can be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a “standard” Kabat numbered sequence. Chothia refers instead to the location of the structural loops (Chothia and Lesk, J. Mol. Biol. 196:901-917 (1987)). The end of the Chothia CDR-H1 loop, when numbered using the Kabat numbering convention, varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The table below lists the positions of the amino acids comprising the variable regions of the antibodies in each system.

Region	Kabat	AbM	Chothia
LCDR1	L24-L34	L24-L34	L24-L34
LCDR2	L50-L56	L50-L56	L50-L56

LCDR3	L89-L97	L89-L97	L89-L97
HCDR1 ¹	H31-H35B	H26-H35B	H26-H32...34
HCDR1 ²	H31-H35	H26-H35	H26-H32
HCDR2	H50-H65	H50-H58	H52-H56
HCDR3	H95-H102	H95-H102	H95-H102

¹Kabat Numbering²Chothia Numbering

[0301] ImMunoGeneTics (IMGT) also provides a numbering system for the immunoglobulin variable regions, including the CDRs. See, e.g., Lefranc, M.P. et al., Dev. Comp. Immunol. 27: 55-77(2003). The IMGT numbering system is based on an alignment of more than 5,000 sequences, structural data, and characterization of hypervariable loops and allows for easy comparison of the variable and CDR regions for all species. According to the IMGT numbering schema, VH-CDR1 is at positions 26 to 35, VH-CDR2 is at positions 51 to 57, VH-CDR3 is at positions 93 to 102, VL-CDR1 is at positions 27 to 32, VL-CDR2 is at positions 50 to 52, and VL-CDR3 is at positions 89 to 97.

[0302] As used throughout the specification the VH CDRs sequences described correspond to the classical Kabat numbering locations, namely Kabat VH-CDR1 is at positions 31-35, VH-CDR2 is a positions 50-65, and VH-CDR3 is at positions 95-102. VL-CDR1, VL-CDR2 and VL-CDR3 also correspond to classical Kabat numbering locations, namely positions 24-34, 50-56 and 89-97, respectively.

[0303] In one embodiment, an antibody of the invention a human antibody.

[0304] The term “human antibody” means an antibody produced in a human or an antibody having an amino acid sequence corresponding to an antibody produced in a human made using any technique known in the art. This definition of a human antibody includes intact or full-length antibodies, fragments thereof, and/or antibodies comprising at least one human heavy and/or light chain polypeptide such as, for example, an antibody comprising murine light chain and human heavy chain polypeptides.

[0305] In one embodiment, an antibody of the invention a chimeric antibody.

[0306] The term “chimeric antibodies” refers to antibodies in which the amino acid sequence of the immunoglobulin molecule is derived from two or more species. Typically, the variable region of both light and heavy chains corresponds to the variable

region of antibodies derived from one species of mammals (e.g., mouse, rat, rabbit, etc.) with the desired specificity, affinity, and capability while the constant regions are homologous to the sequences in antibodies derived from another (usually human) to avoid eliciting an immune response in that species.

[0307] The terms “YTE” or “YTE mutant” refer to a mutation in IgG1 Fc that results in an increase in the binding to human FcRn and improves the serum half-life of the antibody having the mutation. A YTE mutant comprises a combination of three mutations, M252Y/S254T/T256E (EU numbering Kabat et al. (1991) Sequences of Proteins of Immunological Interest, U.S. Public Health Service, National Institutes of Health, Washington, D.C.), introduced into the heavy chain of an IgG1. See U.S. Patent No. 7,658,921, which is incorporated by reference herein. The YTE mutant has been shown to increase the serum half-life of antibodies approximately four-times as compared to wild-type versions of the same antibody (Dall'Acqua et al., J. Biol. Chem. 281:23514-24 (2006); Robbie et al., (2013) Antimicrob. Agents Chemother. 57, 6147-6153). See also U.S. Patent No. 7,083,784, which is hereby incorporated by reference in its entirety.

[0308] “Binding affinity” generally refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, “binding affinity” refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (KD). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention.

[0309] Potency of the antibody or antigen binding fragment thereof is normally expressed as an IC50 value, in ng/ml unless otherwise stated. IC50 is the median inhibitory concentration of an antibody molecule. In functional assays, IC50 is the concentration that reduces a biological response by 50% of its maximum. In ligand-

binding studies, IC₅₀ is the concentration that reduces receptor binding by 50% of maximal specific binding level. IC₅₀ can be calculated by any number of means known in the art.

[0310] The fold improvement in potency for the antibody or antigen binding fragment thereof of the invention as compared to a reference antibody can be at least about 2-fold, at least about 4-fold, at least about 6-fold, at least about 8-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 60-fold, at least about 70-fold, at least about 80-fold, at least about 90-fold, at least about 100-fold, at least about 110-fold, at least about 120-fold, at least about 130-fold, at least about 140-fold, at least about 150-fold, at least about 160-fold, at least about 170-fold, or at least about 180-fold or more.

[0311] Binding potency of an antibody is normally expressed as an EC₅₀ value, in nM or pM unless otherwise stated. EC₅₀ is the concentration of a drug that induces a median response between baseline and maximum after a specified exposure time. EC₅₀ can be calculated by any number of means known in the art.

Antibody preparation

[0312] The antibodies of the present invention can be obtained using conventional techniques known to persons skilled in the art and their utility confirmed by conventional binding studies – an exemplary method is described in Example 2. By way of example, a simple binding assay is to incubate the cell expressing an antigen with the antibody. If the antibody is tagged with a fluorophore, the binding of the antibody to the antigen can be detected by FACS analysis.

[0313] Antibodies of the present invention can be raised in various animals including mice, rats, rabbits, goats, sheep, monkeys or horses. Antibodies may be raised following immunisation with individual capsular polysaccharides, or with a plurality of capsular polysaccharides. Blood isolated from these animals contains polyclonal antibodies – multiple antibodies that bind to the same antigen. Antigens may also be injected into chickens for generation of polyclonal antibodies in egg yolk. To obtain a monoclonal antibody that is specific for a single epitope of an antigen, antibody-secreting

lymphocytes are isolated from an animal and immortalized by fusing them with a cancer cell line. The fused cells are called hybridomas, and will continually grow and secrete antibody in culture. Single hybridoma cells are isolated by dilution cloning to generate cell clones that all produce the same antibody; these antibodies are called monoclonal antibodies. Methods for producing monoclonal antibodies are conventional techniques known to those skilled in the art (see e.g. *Making and Using Antibodies: A Practical Handbook*. GC Howard. CRC Books. 2006. ISBN 0849335280). Polyclonal and monoclonal antibodies are often purified using Protein A/G or antigen-affinity chromatography.

[0314] The antibody or antigen binding fragment thereof of the invention may be prepared as a monoclonal anti-B7-H4 antibody, which can be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature* 256:495 (1975). Using the hybridoma method, a mouse, hamster, or other appropriate host animal, is immunized as described above to elicit the production by lymphocytes of antibodies that will specifically bind to an immunizing antigen. Lymphocytes can also be immunized in vitro. Following immunization, the lymphocytes are isolated and fused with a suitable myeloma cell line using, for example, polyethylene glycol, to form hybridoma cells that can then be selected away from unfused lymphocytes and myeloma cells. Hybridomas that produce monoclonal antibodies directed specifically against a chosen antigen as determined by immunoprecipitation, immunoblotting, or an in vitro binding assay, e.g., radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA), can then be propagated either in in vitro culture using standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, 1986) or in vivo as ascites tumors in an animal. The monoclonal antibodies can then be purified from the culture medium or ascites fluid using known methods.

[0315] Alternatively, the antibody or antigen binding fragment thereof (e.g. as monoclonal antibodies) can also be made using recombinant DNA methods as described in U.S. Patent No. 4,816,567. The polynucleotides encoding a monoclonal antibody are isolated from mature B-cells or hybridoma cell, such as by RT-PCR using oligonucleotide primers that specifically amplify the genes encoding the heavy and light

chains of the antibody, and their sequence is determined using conventional procedures. The isolated polynucleotides encoding the heavy and light chains are then cloned into suitable expression vectors, which when transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, monoclonal antibodies are generated by the host cells. Also, recombinant monoclonal antibodies or antigen-binding fragments thereof of the desired species can be isolated from phage display libraries expressing CDRs of the desired species as described in McCafferty et al., *Nature* 348:552-554 (1990); Clackson et al., *Nature*, 352:624-628 (1991); and Marks et al., *J. Mol. Biol.* 222:581-597 (1991).

[0316] The polynucleotide(s) encoding an antibody or an antigen-binding fragment thereof of the invention can further be modified in a number of different manners using recombinant DNA technology to generate alternative antibodies. In some embodiments, the constant domains of the light and heavy chains of, for example, a mouse monoclonal antibody can be substituted (1) for those regions of, for example, a human antibody to generate a chimeric antibody or (2) for a non-immunoglobulin polypeptide to generate a fusion antibody. In some embodiments, the constant regions are truncated or removed to generate the desired antibody fragment of a monoclonal antibody. Site-directed or high-density mutagenesis of the variable region can be used to optimize specificity, affinity, etc. of a monoclonal antibody.

[0317] In one embodiment, the antibody or antigen-binding fragment thereof is a human antibody or antigen-binding fragment thereof. Human antibodies can be directly prepared using various techniques known in the art. Immortalized human B lymphocytes immunized in vitro or isolated from an immunized individual that produce an antibody directed against a target antigen can be generated. See, e.g., Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boemer et al., *J. Immunol.* 147 (1):86-95 (1991); U.S. Patent 5,750,373.

[0318] In one embodiment, the antibody or antigen-binding fragment thereof can be selected from a phage library, where that phage library expresses human antibodies, as described, for example, in Vaughan et al., *Nat. Biotech.* 14:309-314 (1996); Sheets et al.,

Proc. Natl. Acad. Sci. USA, 95:6157-6162 (1998); Hoogenboom and Winter, J. Mol. Biol. 227:381 (1991); and Marks et al., J. Mol. Biol. 222:581 (1991). Techniques for the generation and use of antibody phage libraries are also described in U.S. Patent Nos. 5,969,108; 6,172,197; 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915; 6,593,081; 6,300,064; 6,653,068; 6,706,484; and 7,264,963; and Rothe et al., J. Molec. Biol. 376:1182-1200 (2008), each of which is incorporated by reference in its entirety.

[0319] Affinity maturation strategies and chain shuffling strategies are known in the art and can be employed to generate high affinity human antibodies or antigen-binding fragments thereof. See Marks et al., BioTechnology 10:779-783 (1992), incorporated by reference in its entirety.

[0320] In one embodiment, the antibody or antigen binding fragment thereof (e.g. an monoclonal antibody) can be a humanized antibody. Methods for engineering, humanizing or resurfacing non-human or human antibodies can also be used and are well known in the art. A humanized, resurfaced or similarly engineered antibody can have one or more amino acid residues from a source that is non-human, e.g., but not limited to, mouse, rat, rabbit, non-human primate, or other mammal. These non-human amino acid residues are replaced by residues that are often referred to as “import” residues, which are typically taken from an “import” variable, constant or other domain of a known human sequence. Such imported sequences can be used to reduce immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable characteristic, as known in the art. Suitably, the CDR residues may be directly and most substantially involved in influencing B7-H4 binding. Accordingly, part or all of the non-human or human CDR sequences are preferably maintained while the non-human sequences of the variable and constant regions can be replaced with human or other amino acids.

[0321] Antibodies can also optionally be humanized, resurfaced, engineered or human antibodies engineered with retention of high affinity for the antigen B7-H4 and other favourable biological properties. To achieve this goal, humanized (or human) or engineered anti-B7-H4 antibodies and resurfaced antibodies can be optionally prepared by a process of analysis of the parental sequences and various conceptual humanized and

engineered products using three-dimensional models of the parental, engineered, and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen, such as B7-H4. In this way, FW residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved.

[0322] Humanization, resurfacing or engineering of anti-B7-H4 antibodies or antigen-binding fragments thereof of the present invention can be performed using any known method, such as but not limited to those described in, Jones et al., *Nature* 321:522 (1986); Riechmann et al., *Nature* 332:323 (1988); Verhoeyen et al., *Science* 239:1534 (1988); Sims et al., *J. Immunol.* 151: 2296 (1993); Chothia and Lesk, *J. Mol. Biol.* 196:901 (1987); Carter et al., *Proc. Natl. Acad. Sci. USA* 89:4285 (1992); Presta et al., *J. Immunol.* 151:2623 (1993); U.S. Pat. Nos. 5,639,641, 5,723,323; 5,976,862; 5,824,514; 5,817,483; 5,814,476; 5,763,192; 5,723,323; 5,766,886; 5,714,352; 6,204,023; 6,180,370; 5,693,762; 5,530,101; 5,585,089; 5,225,539; 4,816,567, 7,557,189; 7,538,195; and 7,342,110; International Application Nos. PCT/US98/16280; PCT/US96/18978; PCT/US91/09630; PCT/US91/05939; PCT/US94/01234; PCT/GB89/01334; PCT/GB91/01134; PCT/GB92/01755; International Patent Application Publication Nos. WO90/14443; WO90/14424; WO90/14430; and European Patent Publication No. EP 229246; each of which is entirely incorporated herein by reference, including the references cited therein.

[0323] Anti-B7-H4 humanized antibodies and antigen-binding fragments thereof can also be made in transgenic mice containing human immunoglobulin loci that are capable upon immunization of producing the full repertoire of human antibodies in the absence of

endogenous immunoglobulin production. This approach is described in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016.

[0324] In one embodiment, a fragment (e.g. antibody fragment) of the antibody (e.g. anti-B7-H4 antibody) is provided. Various techniques are known for the production of antibody fragments. Traditionally, these fragments are derived via proteolytic digestion of intact antibodies, as described, for example, by Morimoto et al., J. Biochem. Biophys. Meth. 24:107-117 (1993) and Brennan et al., Science 229:81 (1985). In one embodiment, anti-B7-H4 antibody fragments are produced recombinantly. Fab, Fv, and scFv antibody fragments can all be expressed in and secreted from *E. coli* or other host cells, thus allowing the production of large amounts of these fragments. Such anti-B7-H4 antibody fragments can also be isolated from the antibody phage libraries discussed above. The anti-B7-H4 antibody fragments can also be linear antibodies as described in U.S. Patent No. 5,641,870. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

[0325] According to the present invention, techniques can be adapted for the production of single-chain antibodies specific to B7-H4. See, e.g., U.S. Pat. No. 4,946,778). In addition, methods can be adapted for the construction of Fab expression libraries to allow rapid and effective identification of monoclonal Fab fragments with the desired specificity for B7-H4, or derivatives, fragments, analogs or homologs thereof. See, e.g., Huse et al., Science 246:1275-1281 (1989). Antibody fragments can be produced by techniques known in the art including, but not limited to: F(ab')₂ fragment produced by pepsin digestion of an antibody molecule; Fab fragment generated by reducing the disulfide bridges of an F(ab')₂ fragment; Fab fragment generated by the treatment of the antibody molecule with papain and a reducing agent; or Fv fragments.

[0326] In one embodiment, an antibody or antigen-binding fragment thereof of the invention can be modified in order to increase its serum half-life. This can be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody or antibody fragment, by mutation of the appropriate region in the antibody or antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody or antibody fragment at either end or in the middle (e.g., by DNA or peptide

synthesis), or by YTE mutation. Other methods to increase the serum half-life of an antibody or antigen-binding fragment thereof, e.g., conjugation to a heterologous molecule, such as PEG, are known in the art.

[0327] A modified antibody or antigen-binding fragment thereof as provided herein can comprise any type of variable region that provides for the association of the antibody or polypeptide with B7-H4. In this regard, the variable region can comprise or be derived from any type of mammal that can be induced to mount a humoral response and generate immunoglobulins against the desired antigen. As such, the variable region of an anti-B7-H4 antibody or antigen-binding fragment thereof can be, for example, of human, murine, non-human primate (e.g., cynomolgus monkeys, macaques, etc.) or lupine origin. In one embodiment, both the variable and constant regions of the modified antibody or antigen-binding fragment thereof are human. In one embodiment, the variable regions of a compatible antibody (usually derived from a non-human source) can be engineered or specifically tailored to improve the binding properties or reduce the immunogenicity of the molecule. In this respect, variable regions useful in the present invention can be humanized or otherwise altered through the inclusion of imported amino acid sequences.

[0328] In one embodiment, the variable domains in both the heavy and light chains of an antibody or antigen-binding fragment thereof are altered by at least partial replacement of one or more CDRs and/or by partial framework region replacement and sequence changing. Although the CDRs can be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of different class and in certain embodiments from an antibody from a different species. It is not necessary to replace all of the CDRs with the complete CDRs from the donor variable region to transfer the antigen-binding capacity of one variable domain to another. Rather, it is only necessary to transfer those residues that are necessary to maintain the activity of the antigen-binding site. Given the explanations set forth in U.S. Pat. Nos. 5,585,089, 5,693,761 and 5,693,762, it will be well within the competence of those skilled in the art to carry out routine experimentation to obtain a functional antibody with reduced immunogenicity.

[0329] Alterations to the variable region notwithstanding, those skilled in the art will appreciate that a modified antibody or antigen-binding fragment thereof of this invention will comprise an antibody (e.g., full-length antibody or antigen-binding fragment thereof) in which at least a fraction of one or more of the constant region domains has been deleted or otherwise altered so as to provide desired biochemical characteristics such as increased tumour localization or reduced serum half-life when compared with an antibody of approximately the same immunogenicity comprising a native or unaltered constant region. In one embodiment, the constant region of the modified antibody will comprise a human constant region. Modifications to the constant region compatible with this invention comprise additions, deletions or substitutions of one or more amino acids in one or more domains. That is, the modified antibody disclosed herein can comprise alterations or modifications to one or more of the three heavy chain constant domains (CH1, CH2 or CH3) and/or to the light chain constant domain (CL). In one embodiment, a modified constant region wherein one or more domains are partially or entirely deleted are contemplated. In one embodiment, a modified antibody will comprise domain deleted constructs or variants wherein the entire CH2 domain has been removed (Δ CH2 constructs). In one embodiment, the omitted constant region domain can be replaced by a short amino acid spacer (e.g., 10 residues) that provides some of the molecular flexibility typically imparted by the absent constant region.

[0330] Besides their configuration, it is known in the art that the constant region mediates several effector functions. For example, antibodies bind to cells via the Fc region, with an Fc receptor site on the antibody Fc region binding to an Fc receptor (FcR) on a cell. There are a number of Fc receptors that are specific for different classes of antibody, including IgG (gamma receptors), IgE (eta receptors), IgA (alpha receptors) and IgM (mu receptors). Binding of antibody to Fc receptors on cell surfaces triggers a number of important and diverse biological responses including engulfment and destruction of antibody-coated particles, clearance of immune complexes, lysis of antibody-coated target cells by killer cells (called antibody-dependent cell-mediated cytotoxicity, or ADCC), release of inflammatory mediators, placental transfer and control of immunoglobulin production.

[0331] In one embodiment, an antibody or an antigen-binding fragment thereof provides for altered effector functions that, in turn, affect the biological profile of the administered antibody or antigen-binding fragment thereof. For example, the deletion or inactivation (through point mutations or other means) of a constant region domain can reduce Fc receptor binding of the circulating modified antibody. In other cases it can be that constant region modifications, consistent with this invention, moderate complement binding and thus reduce the serum half-life and nonspecific association of a conjugated cytotoxin. Yet other modifications of the constant region can be used to eliminate disulfide linkages or oligosaccharide moieties that allow for enhanced localization due to increased antigen specificity or antibody flexibility. Similarly, modifications to the constant region in accordance with this invention can easily be made using well-known biochemical or molecular engineering techniques well within the purview of the skilled artisan.

[0332] In one embodiment, the antibody or antigen-binding fragment thereof does not have one or more effector functions. For instance, in one embodiment, the antibody or antigen-binding fragment thereof has no antibody-dependent cellular cytotoxicity (ADCC) activity and/or no complement-dependent cytotoxicity (CDC) activity. In one embodiment, the antibody or antigen-binding fragment thereof does not bind to an Fc receptor and/or complement factors. In one embodiment, the antibody or antigen-binding fragment thereof has no effector function.

[0333] In one embodiment, the antibody or antigen-binding fragment thereof can be engineered to fuse the CH3 domain directly to the hinge region of the respective modified antibodies or fragments thereof. In other constructs a peptide spacer can be inserted between the hinge region and the modified CH2 and/or CH3 domains. For example, compatible constructs can be expressed in which the CH2 domain has been deleted and the remaining CH3 domain (modified or unmodified) is joined to the hinge region with a 5-20 amino acid spacer. Such a spacer can be added, for instance, to ensure that the regulatory elements of the constant domain remain free and accessible or that the hinge region remains flexible. Amino acid spacers can, in some cases, prove to be immunogenic and elicit an unwanted immune response against the construct. In one

embodiment, any spacer added to the construct can be relatively non-immunogenic, or even omitted altogether, so as to maintain the desired biochemical qualities of the modified antibodies.

[0334] Besides the deletion of whole constant region domains, an antibody or antigen-binding fragment thereof provided herein can be modified by the partial deletion or substitution of a few or even a single amino acid in a constant region. For example, the mutation of a single amino acid in selected areas of the CH2 domain can be enough to substantially reduce Fc binding and thereby increase tumor localization. Similarly one or more constant region domains that control the effector function (e.g., complement C1q binding) can be fully or partially deleted. Such partial deletions of the constant regions can improve selected characteristics of the antibody or antigen-binding fragment thereof (e.g., serum half-life) while leaving other desirable functions associated with the subject constant region domain intact. Moreover, the constant regions of the antibody and antigen-binding fragment thereof can be modified through the mutation or substitution of one or more amino acids that enhances the profile of the resulting construct. In this respect it is possible to disrupt the activity provided by a conserved binding site (e.g., Fc binding) while substantially maintaining the configuration and immunogenic profile of the modified antibody or antigen-binding fragment thereof. In one embodiment, there may be an addition of one or more amino acids to the constant region to enhance desirable characteristics such as decreasing or increasing effector function or provide for more cytotoxin or carbohydrate attachment. In one embodiment, it can be desirable to insert or replicate specific sequences derived from selected constant region domains.

[0335] The present invention further embraces variants and equivalents that are substantially homologous an antibody or antigen binding fragment of the invention (e.g. murine, chimeric, humanized or human antibody, or antigen-binding fragments thereof). These can contain, for example, conservative substitution mutations, i.e., the substitution of one or more amino acids by similar amino acids. For example, conservative substitution refers to the substitution of an amino acid with another within the same general class such as, for example, one acidic amino acid with another acidic amino acid, one basic amino acid with another basic amino acid or one neutral amino acid by another

neutral amino acid. What is intended by a conservative amino acid substitution is well known in the art.

- [0336] In one embodiment, the antibody or antigen-binding fragment thereof can be further modified to contain additional chemical moieties not normally part of the protein. Those derivatized moieties can improve the solubility, the biological half-life or absorption of the protein. The moieties can also reduce or eliminate any desirable side effects of the proteins and the like. An overview for those moieties can be found in Remington's Pharmaceutical Sciences, 22nd ed., Ed. Lloyd V. Allen, Jr. (2012).

Definitions

- [0337] The following definitions pertain, in particular, to the description of topoisomerase I inhibitors above, and may even more particularly pertain to the section entitled "further preferences".
- [0338] C₅₋₆ arylene: The term "C₅₋₆ arylene", as used herein, pertains to a divalent moiety obtained by removing two hydrogen atoms from an aromatic ring atom of an aromatic compound.
- [0339] In this context, the prefixes (e.g. C₅₋₆) denote the number of ring atoms, or range of number of ring atoms, whether carbon atoms or heteroatoms.
- [0340] The ring atoms may be all carbon atoms, as in "carboarylene groups", in which case the group is phenylene (C₆).
- [0341] Alternatively, the ring atoms may include one or more heteroatoms, as in "heteroarylene groups". Examples of heteroarylene groups include, but are not limited to, those derived from:
- N₁: pyrrole (azole) (C₅), pyridine (azine) (C₆);
 - O₁: furan (oxole) (C₅);
 - S₁: thiophene (thiole) (C₅);
 - N₁O₁: oxazole (C₅), isoxazole (C₅), isoxazine (C₆);
 - N₂O₁: oxadiazole (furazan) (C₅);
 - N₃O₁: oxatriazole (C₅);
 - N₁S₁: thiazole (C₅), isothiazole (C₅);

N₂: imidazole (1,3-diazole) (C₅), pyrazole (1,2-diazole) (C₅), pyridazine (1,2-diazine) (C₆), pyrimidine (1,3-diazine) (C₆) (e.g., cytosine, thymine, uracil), pyrazine (1,4-diazine) (C₆); and

N₃: triazole (C₅), triazine (C₆).

[0342] C₁₋₄ alkyl: The term “C₁₋₄ alkyl” as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from a carbon atom of a hydrocarbon compound having from 1 to 4 carbon atoms, which may be aliphatic or alicyclic, and which may be saturated or unsaturated (e.g. partially unsaturated, fully unsaturated). The term “C_{1-n} alkyl” as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from a carbon atom of a hydrocarbon compound having from 1 to n carbon atoms, which may be aliphatic or alicyclic, and which may be saturated or unsaturated (e.g. partially unsaturated, fully unsaturated). Thus, the term “alkyl” includes the sub-classes alkenyl, alkynyl, cycloalkyl, etc., discussed below.

[0343] Examples of saturated alkyl groups include, but are not limited to, methyl (C₁), ethyl (C₂), propyl (C₃) and butyl (C₄).

[0344] Examples of saturated linear alkyl groups include, but are not limited to, methyl (C₁), ethyl (C₂), n-propyl (C₃) and n-butyl (C₄).

[0345] Examples of saturated branched alkyl groups include iso-propyl (C₃), iso-butyl (C₄), sec-butyl (C₄) and tert-butyl (C₄).

[0346] C₂₋₄ Alkenyl: The term “C₂₋₄ alkenyl” as used herein, pertains to an alkyl group having one or more carbon-carbon double bonds.

[0347] Examples of unsaturated alkenyl groups include, but are not limited to, ethenyl (vinyl, -CH=CH₂), 1-propenyl (-CH=CH-CH₃), 2-propenyl (allyl, -CH=CH₂), isopropenyl (1-methylvinyl, -C(CH₃)=CH₂) and butenyl (C₄).

[0348] C₂₋₄ alkynyl: The term “C₂₋₄ alkynyl” as used herein, pertains to an alkyl group having one or more carbon-carbon triple bonds.

[0349] Examples of unsaturated alkynyl groups include, but are not limited to, ethynyl (-C≡CH) and 2-propynyl (propargyl, -CH₂-C≡CH).

[0350] C₃₋₄ cycloalkyl: The term “C₃₋₄ cycloalkyl” as used herein, pertains to an alkyl group which is also a cyclyl group; that is, a monovalent moiety obtained by removing a

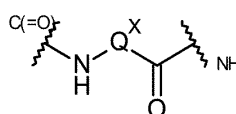
hydrogen atom from an alicyclic ring atom of a cyclic hydrocarbon (carbocyclic) compound, which moiety has from 3 to 7 carbon atoms, including from 3 to 7 ring atoms.

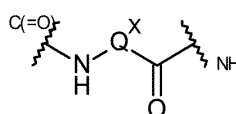
[0351] Examples of cycloalkyl groups include, but are not limited to, those derived from: saturated monocyclic hydrocarbon compounds:

cyclopropane (C₃) and cyclobutane (C₄); and

unsaturated monocyclic hydrocarbon compounds:

cyclopropene (C₃) and cyclobutene (C₄).



[0352] Connection labels: In the formula , the superscripted labels C(=O) and NH indicate the group to which the atoms are bound. For example, the NH group is shown as being bound to a carbonyl (which is not part of the moiety illustrated), and the carbonyl is shown as being bound to a NH group (which is not part of the moiety illustrated).

Salts

[0353] It may be convenient or desirable to prepare, purify, and/or handle a corresponding salt of the active compound/ agent, for example, a pharmaceutically-acceptable salt. Examples of pharmaceutically acceptable salts are discussed in Berge, *et al.*, *J. Pharm. Sci.*, **66**, 1-19 (1977).

[0354] For example, if the compound is anionic, or has a functional group which may be anionic (e.g. -COOH may be -COO⁻), then a salt may be formed with a suitable cation. Examples of suitable inorganic cations include, but are not limited to, alkali metal ions such as Na⁺ and K⁺, alkaline earth cations such as Ca²⁺ and Mg²⁺, and other cations such as Al³⁺. Examples of suitable organic cations include, but are not limited to, ammonium ion (i.e. NH₄⁺) and substituted ammonium ions (e.g. NH₃R⁺, NH₂R₂⁺, NHR₃⁺, NR₄⁺). Examples of some suitable substituted ammonium ions are those derived from: ethylamine, diethylamine, dicyclohexylamine, triethylamine, butylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine, benzylamine,

phenylbenzylamine, choline, meglumine, and tromethamine, as well as amino acids, such as lysine and arginine. An example of a common quaternary ammonium ion is $N(CH_3)_4^+$.

[0355] If the compound is cationic, or has a functional group which may be cationic (e.g. $-NH_2$ may be $-NH_3^+$), then a salt may be formed with a suitable anion. Examples of suitable inorganic anions include, but are not limited to, those derived from the following inorganic acids: hydrochloric, hydrobromic, hydroiodic, sulfuric, sulfurous, nitric, nitrous, phosphoric, and phosphorous.

[0356] Examples of suitable organic anions include, but are not limited to, those derived from the following organic acids: 2-acetoxybenzoic, acetic, ascorbic, aspartic, benzoic, camphorsulfonic, cinnamic, citric, edetic, ethanedithionic, ethanesulfonic, fumaric, glucelonic, gluconic, glutamic, glycolic, hydroxymaleic, hydroxynaphthalene carboxylic, isethionic, lactic, lactobionic, lauric, maleic, malic, methanesulfonic, mucic, oleic, oxalic, palmitic, pantoic, pantothenic, phenylacetic, phenylsulfonic, propionic, pyruvic, salicylic, stearic, succinic, sulfanilic, tartaric, toluenesulfonic, trifluoroacetic acid and valeric. Examples of suitable polymeric organic anions include, but are not limited to, those derived from the following polymeric acids: tannic acid, carboxymethyl cellulose.

Solvates

[0357] It may be convenient or desirable to prepare, purify, and/or handle a corresponding solvate of the active compound. The term “solvate” is used herein in the conventional sense to refer to a complex of solute (e.g. active compound, salt of active compound) and solvent. If the solvent is water, the solvate may be conveniently referred to as a hydrate, for example, a mono-hydrate, a di-hydrate, a tri-hydrate, etc.

Isomers

[0358] Certain compounds/ agents of the invention may exist in one or more particular geometric, optical, enantiomeric, diastereomeric, epimeric, atropic, stereoisomeric, tautomeric, conformational, or anomeric forms, including but not limited to, cis- and trans-forms; E- and Z-forms; c-, t-, and r- forms; endo- and exo-forms; R-, S-, and meso-forms; D- and L-forms; d- and l-forms; (+) and (-) forms; keto-, enol-, and enolate-forms;

syn- and anti-forms; synclinal- and anticlinal-forms; α - and β -forms; axial and equatorial forms; boat-, chair-, twist-, envelope-, and halfchair-forms; and combinations thereof, hereinafter collectively referred to as “isomers” (or “isomeric forms”).

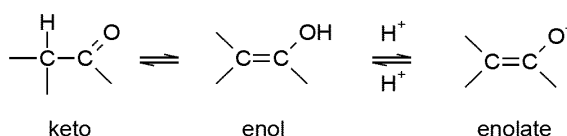
- [0359] The term “chiral” refers to molecules which have the property of non-superimposability of the mirror image partner, while the term “achiral” refers to molecules which are superimposable on their mirror image partner.
- [0360] The term “stereoisomers” refers to compounds which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space.
- [0361] “Diastereomer” refers to a stereoisomer with two or more centers of chirality and whose molecules are not mirror images of one another. Diastereomers have different physical properties, e.g. melting points, boiling points, spectral properties, and reactivities. Mixtures of diastereomers may separate under high resolution analytical procedures such as electrophoresis and chromatography.
- [0362] “Enantiomers” refer to two stereoisomers of a compound which are non-superimposable mirror images of one another.
- [0363] Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., *McGraw-Hill Dictionary of Chemical Terms* (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., “Stereochemistry of Organic Compounds”, John Wiley & Sons, Inc., New York, 1994. The compounds of the invention may contain asymmetric or chiral centers, and therefore exist in different stereoisomeric forms. It is intended that all stereoisomeric forms of the compounds of the invention, including but not limited to, diastereomers, enantiomers and atropisomers, as well as mixtures thereof such as racemic mixtures, form part of the present invention. Many organic compounds exist in optically active forms, i.e., they have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L, or *R* and *S*, are used to denote the absolute configuration of the molecule about its chiral center(s). The prefixes d and l or (+) and (-) are employed to designate the sign of rotation of plane-polarized light by the compound, with (-) or l meaning that the compound is levorotatory. A compound prefixed with (+) or d is dextrorotatory. For a given chemical structure, these stereoisomers are identical except

that they are mirror images of one another. A specific stereoisomer may also be referred to as an enantiomer, and a mixture of such isomers is often called an enantiomeric mixture. A 50:50 mixture of enantiomers is referred to as a racemic mixture or a racemate, which may occur where there has been no stereoselection or stereospecificity in a chemical reaction or process. The terms “racemic mixture” and “racemate” refer to an equimolar mixture of two enantiomeric species, devoid of optical activity.

[0364] “Enantiomerically enriched form” refers to a sample of a chiral substance whose enantiomeric ratio is greater than 50:50 but less than 100:0.

[0365] Note that, except as discussed below for tautomeric forms, specifically excluded from the term “isomers”, as used herein, are structural (or constitutional) isomers (i.e. isomers which differ in the connections between atoms rather than merely by the position of atoms in space). For example, a reference to a methoxy group, -OCH₃, is not to be construed as a reference to its structural isomer, a hydroxymethyl group, -CH₂OH. Similarly, a reference to ortho-chlorophenyl is not to be construed as a reference to its structural isomer, meta-chlorophenyl. However, a reference to a class of structures may well include structurally isomeric forms falling within that class (e.g. C₁₋₇ alkyl includes n-propyl and iso-propyl; butyl includes n-, iso-, sec-, and tert-butyl; methoxyphenyl includes ortho-, meta-, and para-methoxyphenyl).

[0366] The above exclusion does not pertain to tautomeric forms, for example, keto-, enol-, and enolate-forms, as in, for example, the following tautomeric pairs: keto/enol (illustrated below), imine/enamine, amide/imino alcohol, amidine/enediamine, nitroso/oxime, thioketone/enethiol, N-nitroso/hydroxyazo, and nitro/aci-nitro.



[0367] The term “tautomer” or “tautomeric form” refers to structural isomers of different energies which are interconvertible via a low energy barrier. For example, proton tautomers (also known as prototropic tautomers) include interconversions via migration of a proton, such as keto-enol and imine-enamine isomerizations. Valence tautomers include interconversions by reorganization of some of the bonding electrons.

[0368] Note that specifically included in the term “isomer” are compounds with one or more isotopic substitutions. For example, H may be in any isotopic form, including ^1H , ^2H (D), and ^3H (T); C may be in any isotopic form, including ^{12}C , ^{13}C , and ^{14}C ; O may be in any isotopic form, including ^{16}O and ^{18}O ; and the like.

[0369] Examples of isotopes that can be incorporated into compounds of the invention include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorous, fluorine, chlorine and iodine, such as, but not limited to ^2H (deuterium, D), ^3H (tritium), ^{11}C , ^{13}C , ^{14}C , ^{15}N , ^{18}F , ^{31}P , ^{32}P , ^{35}S , ^{36}Cl , and ^{125}I . Various isotopically labeled compounds of the present invention, for example those into which radioactive isotopes such as ^3H , ^{13}C , and ^{14}C are incorporated. Such isotopically labelled compounds may be useful in metabolic studies, reaction kinetic studies, detection or imaging techniques, such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT) including drug or substrate tissue distribution assays, or in radioactive treatment of patients. Deuterium labelled or substituted therapeutic compounds of the invention may have improved DMPK (drug metabolism and pharmacokinetics) properties, relating to distribution, metabolism, and excretion (ADME). Substitution with heavier isotopes such as deuterium may afford certain therapeutic advantages resulting from greater metabolic stability, for example increased in vivo half-life or reduced dosage requirements. An ^{18}F labeled compound may be useful for PET or SPECT studies. Isotopically labeled compounds of this invention and prodrugs thereof can generally be prepared by carrying out the procedures disclosed in the schemes or in the examples and preparations described below by substituting a readily available isotopically labeled reagent for a non-isotopically labeled reagent. Further, substitution with heavier isotopes, particularly deuterium (i.e., ^2H or D) may afford certain therapeutic advantages resulting from greater metabolic stability, for example increased in vivo half-life or reduced dosage requirements or an improvement in therapeutic index. It is understood that deuterium in this context is regarded as a substituent. The concentration of such a heavier isotope, specifically deuterium, may be defined by an isotopic enrichment factor. In the compounds of this invention any atom not specifically designated as a particular isotope is meant to represent any stable isotope of that atom.

- [0370] Unless otherwise specified, a reference to a particular compound includes all such isomeric forms, including (wholly or partially) racemic and other mixtures thereof. Methods for the preparation (e.g. asymmetric synthesis) and separation (e.g. fractional crystallisation and chromatographic means) of such isomeric forms are either known in the art or are readily obtained by adapting the methods taught herein, or known methods, in a known manner.

SEQUENCE HOMOLOGY

- [0371] Any of a variety of sequence alignment methods can be used to determine percent identity, including, without limitation, global methods, local methods and hybrid methods, such as, e.g., segment approach methods. Protocols to determine percent identity are routine procedures within the scope of one skilled in the art. Global methods align sequences from the beginning to the end of the molecule and determine the best alignment by adding up scores of individual residue pairs and by imposing gap penalties. Non-limiting methods include, e.g., CLUSTAL W, see, e.g., Julie D. Thompson et al., CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment Through Sequence Weighting, Position- Specific Gap Penalties and Weight Matrix Choice, 22(22) Nucleic Acids Research 4673-4680 (1994); and iterative refinement, see, e.g., Osamu Gotoh, Significant Improvement in Accuracy of Multiple Protein. Sequence Alignments by Iterative Refinement as Assessed by Reference to Structural Alignments, 264(4) J. Mol. Biol. 823-838 (1996). Local methods align sequences by identifying one or more conserved motifs shared by all of the input sequences. Non-limiting methods include, e.g., Match-box, see, e.g., Eric Depiereux and Ernest Feytmans, Match-Box: A Fundamentally New Algorithm for the Simultaneous Alignment of Several Protein Sequences, 8(5) CABIOS 501 -509 (1992); Gibbs sampling, see, e.g., C. E. Lawrence et al., Detecting Subtle Sequence Signals: A Gibbs Sampling Strategy for Multiple Alignment, 262(5131) Science 208-214 (1993); Align-M, see, e.g., Ivo Van Walle et al., Align-M - A New Algorithm for Multiple Alignment of Highly Divergent Sequences, 20(9) Bioinformatics:1428-1435 (2004).

[0372] Thus, percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-16, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-19, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown below (amino acids are indicated by the standard one-letter codes).

[0373] The "percent sequence identity" between two or more nucleic acid or amino acid sequences is a function of the number of identical positions shared by the sequences. Thus, % identity may be calculated as the number of identical nucleotides / amino acids divided by the total number of nucleotides / amino acids, multiplied by 100. Calculations of % sequence identity may also take into account the number of gaps, and the length of each gap that needs to be introduced to optimize alignment of two or more sequences. Sequence comparisons and the determination of percent identity between two or more sequences can be carried out using specific mathematical algorithms, such as BLAST, which will be familiar to a skilled person.

ALIGNMENT SCORES FOR DETERMINING SEQUENCE IDENTITY

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

[0374] The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{\text{[length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences]}} \times 100$$

[0375] Substantially homologous polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see below) and other substitutions that do not significantly affect the folding or activity of the polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or an affinity tag.

CONSERVATIVE AMINO ACID SUBSTITUTIONS

[0376] Basic: arginine; lysine; histidine

[0377] Acidic: glutamic acid; aspartic acid

[0378] Polar: glutamine; asparagine

[0379] Hydrophobic: leucine; isoleucine; valine

[0380] Aromatic: phenylalanine; tryptophan; tyrosine

[0381] Small: glycine; alanine; serine; threonine; methionine

[0382] In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-N-methyl lysine, 2-aminoisobutyric acid, isovaline and α -methyl serine) may be substituted for amino acid residues of the polypeptides of the present invention. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for polypeptide amino acid residues. The polypeptides of the present invention can also comprise non-naturally occurring amino acid residues.

[0383] Non-naturally occurring amino acids include, without limitation, trans-3-methylproline, 2,4-methano-proline, cis-4-hydroxyproline, trans-4-hydroxy-proline, N-methylglycine, allo-threonine, methyl-threonine, hydroxy-ethylcysteine, hydroxyethylhomo-cysteine, nitro-glutamine, homoglutamine, pipecolic acid, tert-leucine, norvaline, 2-azaphenylalanine, 3-azaphenyl-alanine, 4-azaphenyl-alanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an in vitro system can be employed wherein nonsense mutations are suppressed using chemically

aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., *J. Am. Chem. Soc.* 113:2722, 1991; Ellman et al., *Methods Enzymol.* 202:301, 1991; Chung et al., *Science* 259:806-9, 1993; and Chung et al., *Proc. Natl. Acad. Sci. USA* 90:10145-9, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., *J. Biol. Chem.* 271:19991-8, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the polypeptide in place of its natural counterpart. See, Koide et al., *Biochem.* 33:7470-6, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by in vitro chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci.* 2:395-403, 1993).

[0384] A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for amino acid residues of polypeptides of the present invention.

[0385] Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244: 1081-5, 1989). Sites of biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., *Science* 255:306-12, 1992; Smith et al., *J. Mol. Biol.* 224:899-904, 1992; Wlodaver et al., *FEBS Lett.* 309:59-64, 1992. The

identities of essential amino acids can also be inferred from analysis of homologies with related components (e.g. the translocation or protease components) of the polypeptides of the present invention.

[0386] Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-7, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-6, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-7, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

[0387] Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-7, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-6, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-7, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

[0388] 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 disclosure belongs. Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 20 ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide the skilled person with a general dictionary of many of the terms used in this disclosure.

[0389] This disclosure is not limited by the exemplary methods and materials disclosed herein, and any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of this disclosure. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, any nucleic acid sequences are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

[0390] The headings provided herein are not limitations of the various aspects or embodiments of this disclosure.

[0391] Amino acids are referred to herein using the name of the amino acid, the three letter abbreviation or the single letter abbreviation. The term "protein", as used herein, includes proteins, polypeptides, and peptides. As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". In some instances, the term "amino acid sequence" is synonymous with the term "enzyme". The terms "protein" and "polypeptide" are used interchangeably herein. In the present disclosure and claims, the conventional one-letter and three-letter codes for amino acid residues may be used. The 3-letter code for amino acids as defined in conformity with the IUPACIUB Joint Commission on Biochemical Nomenclature (JCBN). It is also understood that a polypeptide may be coded for by more than one nucleotide sequence due to the degeneracy of the genetic code.

[0392] Other definitions of terms may appear throughout the specification. Before the exemplary embodiments are described in more detail, it is to be understood that this disclosure is not limited to particular embodiments described, and as such 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 disclosure will be defined only by the appended claims.

[0393] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any

other stated or intervening value in that stated range is encompassed within this disclosure. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within this disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in this disclosure.

[0394] It must be noted that as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “an agent” includes a plurality of such agents and reference to “the agent” includes reference to one or more agents and equivalents thereof known to those skilled in the art, and so forth.

[0395] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that such publications constitute prior art to the claims appended hereto.

BRIEF DESCRIPTION OF THE DRAWINGS

[0396] Embodiments of the invention will now be described, by way of example only, with reference to the following Figures and Examples.

[0397] **Figure 1A-1D** shows B7-H4 overexpression in breast cancer. (A) TNBC (re-treatment); (B) HR+ (pre-treatment); (C) HER2+ (pre-treatment, eligible for Herceptin); (D) HER2+ve (Herceptin treated).

[0398] **Figure 2A-2K** shows results for species cross-reactivity ELISA analysis.

[0399] **Figure 3A-3G** shows results of ELISA analysis of clone binding to B7-H4 family members and homologues.

[0400] **Figure 4A-4B** shows results of on cell western analysis for binding of the selected clones (and commercial antibodies) to various cell types. E= E Biosciences 14-5949 anti-Human B7H4 mouse IgG; U= US biological B0000-35B anti Human B7H4 mouse IgG; R= R and D systems AF2514 anti Mouse B7H4 goat IgG1; S= Sigma SAB2500141 anti B7H4 Goat IgG1

- [0401] **Figure 5A-5F** shows results from *in vitro* cytotoxicity assay with the selected clones.
- [0402] **Figure 6** shows results of ELISA analysis performed for the clone ZY0EQD_E02 (as well as a number of variants thereof), demonstrating binding to human, cynomolgus, mouse and rat B7-H4.
- [0403] **Figure 7** shows results of ELISA analysis performed for the clone E02_GL compared with 1D11, demonstrating improved binding of E02_GL to human B7-H4.
- [0404] **Figure 8A-8B** shows results of Flow Cytometry analysis performed for the clone E02_GL compared with 1D11, demonstrating improved binding of E02_GL to human B7-H4 when present on cells. Results for HT29 cells are shown in (A), and results for SKBR-3 cells are shown in (B). The sign $\diamond$ marks the 'E02-GL fraction', and the sign $\square$ marks the '1D11 fraction'. The sign "●" marks a (negative) control 'R347 fraction'.
- [0405] **Figure 9A-9B** shows cytotoxicity of human B7-H4 transfected (and non-transfected control) Ad293 cells following treatment with E02-GL-SG3932 conjugate. Open circles = isotype control ADC (e.g. NIP228-SG3932); filled circles = E02-GL-SG3932.
- [0406] **Figure 10A-10B** shows bystander killing of tumor cells *in vitro* (E02-GL-SG3932).
- [0407] **Figure 11** shows bystander killing of tumor cells *in vivo* (E02-GL-SG3932).
- [0408] **Figure 12** shows that E02-GL-SG3932 has potent *in vivo* activity in patient derived xenograft (PDX) models. Open circles = vehicle only control; Filled circles = E02-GL-SG3932 (7 mg/kg).
- [0409] **Figure 13** shows the anti-tumor activity of E02-GL-SG3932 in PDX models after a single i.v. injection at 7mg/kg.
- [0410] **Figure 14A-14B** shows that E02-GL-topo I inhibitor ADCs have similar potency in MX-1 cells *in vitro* and *in vivo* (A); and in HT29-derived models *in vitro* and *in vivo* (B).
- [0411] **Figure 15A-15B** shows cytotoxicity of cynomolgus B7-H4 transfected (and non-transfected control) HEK293 cells following treatment with E02-GL-SG3249 conjugate.

- [0412] **Figure 16A-16C** shows results of internalisation experiments, demonstrating internalization of bound E02-GL-antigen complex in live cells.
- [0413] **Figure 17A-17B** shows western blot analysis demonstrating that E02-GL-SG3932 treatment leads to double strand DNA breaks *in vitro*; (**Figure 17C**) shows western blot analysis of key double strand break markers following treatment of HCC1569 cells with anti-B7-H4 antibody conjugated to SG3249, more specifically E02-GL-SG3249.
- [0414] **Figure 18** shows caspase 3/7 activity in SKBR3 cells following treatment with E02-GL-SG3249.
- [0415] **Figure 19** shows *in vitro* activity of E02-GL-SG3249 and Warhead SG3199 on tumour cells.
- [0416] **Figure 20A-20D** shows bystander killing of tumor cells *in vitro* (E02-GL-SG3249).
- [0417] **Figure 21A-21C** show E02_GL ADCs have improved cytotoxicity / potency compared with 1D11 ADCs. Comparative cytotoxicity is shown against a HT29 cell line expressing human B7-H4 (A), an SKBR3 cell line (B), and an HCC1569 cell line (C).
- [0418] **Figure 22A-22C** shows *in vivo* activity of E02-GL-SG3249 against tumour grafts of (A) OVCAR4 cells (cisplatin refractory ovarian cancer, high B7-H4), (B) HCC1569 cells (HER2+ breast cancer, heterogeneous expression of B7-H4), and (C) MDA-MB-468 cells (triple negative breast cancer, low B7-H4 expression).
- [0419] **Figure 23** shows bystander killing of tumour cells *in vivo*.
- [0420] **Figure 24A-24O** shows that E02-GL-SG3249 has potent *in vivo* activity in patient derived xenograft (PDX) models. Circles = vehicle only control; Squares = E02-GL-SG3249 (0.3 mg/kg); Triangles = E02-GL-SG3249 (1.0 mg/kg).
- [0421] **Figure 25A-25B** shows results of (A) gamma-H2AX immunohistochemistry (IHC) in a HCC1954 tumour xenograft (with and without E02-GL-SG3249), quantified in (B) as no. of gamma-H2AX positive cells per mm² (within a tissue region of interest) (+/- S.E.M.). Image analysis performed using HALO software (with CRO-OracleBio). Increased numbers of gamma-H2AX positive tumour cells were observed up to 10 days following E02-GL-SG3249 treatment.

- [0422] **Figure 26A-26D** shows retention of B7-H4 Ab in the B7H4 expressing tumour (HT29 cells). Intensity scale shows Epi-fluorescence intensity. Radiant efficiency = $(\text{p/sec/cm}^2/\text{sr})/(\mu\text{W/cm}^2)$; Intensity scale, Min=1.20e8, Max = 1.50e9.
- [0423] **Figure 27A-27D** shows retention of B7-H4 Ab in the B7H4 expressing tumour (CT26 cells). Intensity scale shows Epi-fluorescence intensity. Radiant efficiency = $(\text{p/sec/cm}^2/\text{sr})/(\mu\text{W/cm}^2)$; Intensity scale, Min=1.20e8, Max = 1.50e9.
- [0424] **Figure 28A-28B** shows sequence alignment of the five exemplary antibody clones.
- [0425] **Figure 29A** is a schematic of the B7-H4 targeting TOP1i-ADC. **Figure 29B** shows the key features of the B7-H4 targeting TOP1i-ADC E02-GL-SG3249.
- [0426] **Figure 30** shows example images of representative immunohistochemical staining of B7-H4 expression in normal human and normal cynomolgus monkey breast, pancreas, cervix, endometrium, fallopian tube/oviduct and kidney tissues.
- [0427] **Figure 31** shows example images of representative immunohistochemical staining of B7-H4 expression in human tumor tissues including breast (TNBC and ER+, see individual Figures 1A-1B), cholangiocarcinoma, NSCLC-SCC, endometrial and ovarian tumor.
- [0428] **Figure 32** is a B7-H4 ortholog alignment.
- [0429] **Figure 33** shows binding of antibody intermediate E02-INT and E02-GL-SG3932 to human B7 H4 by DELFIA-ELISA method and anti-human IgG (H+L). E02-INT: antibody intermediate of E02-GL-SG3932; NIP228: isotype-matched control; huB7-H4: recombinant human B7-H4.
- [0430] **Figure 34** shows binding of antibody intermediate E02-INT to HEK 293 cells stably expressing human, murine, or cynomolgus monkey B7-H4. E02-INT: antibody intermediate of E02-GL-SG3932; HEK293 JI TREX: non-transduced HEK293 JI TREX cells; HEK293 JI TREX cynoB7-H4: HEK 293 cells stably expressing cynomolgus monkey B7-H4; HEK293 JI TREX huB7-H4: HEK 293 cells stably expressing human B7-H4; HEK293 JI TREX muB7-H4: HEK 293 cells stably expressing murine B7-H4; MFI: mean fluorescence intensity. The y-axis is the mean fluorescence intensity geometric mean. Data are presented as the average of triplicate determinations $\pm$ SD.

- [0431] **Figure 35** shows binding of antibody intermediate E02-INT and E02-GL-SG3932 to human breast cancer cell lines and to HT29 cells stably expressing human B7-H4. E02-INT: antibody intermediate of E02-GL-SG3932; HT29-huB7-H4 Clone 4 and HT29-huB7-H4 Clone 26: HT29 cells stably expressing human B7-H4; MFI: mean fluorescence intensity. The y-axis is the mean fluorescence intensity geometric mean.
- [0432] **Figures 36A-36C** show the *in vitro* cytotoxic activity of E02-GL-SG3932 in cell lines HT29, HT29-huB7-H4 Clone 26, and MX-1. Data are presented as the average of triplicate determinations $\pm$ SD.
- [0433] **Figure 37** shows *in vitro* antibody-dependent cell-mediated cytotoxicity activity of E02-GL-SG3932. The graph displays mean fold change $\pm$ SEM of six experiments.
- [0434] **Figure 38** shows the time course of E02-INT internalization presented as an image sequence. Human colon cancer cells HT29-huB7-H4 Clone 26 (1st row) and human breast cancer cells MX-1 (2nd row) are labelled with 5 μ g/mL E02-INT conjugated with Alexa Fluor™ 568 (red). The scale bar is 20 μ m.
- [0435] **Figures 39A-39C** show live cell imaging of E02-INT internalization in the human cancer cell lines HT29-huB7-H4 clone 26 and MX-1. (A) Each point represents mean internalization percentage $\pm$ standard deviation of 3 independent wells at 10 minute intervals for 480 minutes. (B) Internalization percentage after 8 hours and (C) The predicted half-life from 3 independent wells is shown. These values were derived using the Dissociation - One phase exponential decay equation. Horizontal bars indicate intragroup arithmetic mean; Statistic significance was evaluated by one-way ANOVA, Tukeys multiple comparison test. ns: not significant, $p > 0.05$; ** $p < 0.05$.
- [0436] **Figures 40A-40B** show that antibody E02-INT colocalizes with lysosomes in HT29-huB7-H4 Clone 26 Cells. (A) HT29-huB7-H4 Clone 26 cells were incubated for 24 hours with 5 μ g/mL E02-INT conjugated with Alexa Fluor™ 568 antibody [(A) red - top and bottom left]. Lysosomes were stained with mouse anti-human LAMP1-Alexa Fluor™ 488 antibodies [(A) green - top middle]. Endosomes were stained with rabbit anti-human EEA1 antibodies and detected with goat anti-rabbit IgG1-DyLight™ 650 [(A) green - bottom middle]. Colocalization of E02-INT with LAMP1 or EEA1 is shown in merged images. (B) Colocalization of E02-INT with EEA1 and LAMP1 analysed by

Pearson's correlation coefficient using Zeiss Zen software. Each spot represents single cell measurement. Statistical significance was evaluated by one-way ANOVA, Tukeys multiple comparison test. ns: not significant, $p > 0.05$; **** $p < 0.0001$.

[0437] **Figure 41** is a picture of a gel showing DNA damage response signaling in MX-1 Cells treated with E02-GL-SG3932 or the TOP1i warhead SG3924. Data presented is a representative of n: 2 experiments.

[0438] **Figure 42** is a picture of a gel showing DNA damage response signaling in HT29-huB7-H4 cells treated with E02-GL-SG3932 or the TOP1i Warhead SG3924. Data presented is a representative of n: 2 experiments.

[0439] **Figures 43A-43F** are images of immunohistochemical (IHC) staining of human IgG, γ H2AX and cleaved Caspase 3 in HT29-huB7-H4 clone 26 xenograft tumors after treatment with E02-GL-SG3932. The images are representative of IHC staining for human IgG, γ H2AX, and cleaved caspase-3 in the HT29-huB7-H4 Clone 26 tumor xenograft model, 168 hours after a single IV administration of 7 mg/kg E02-GL-SG3932 (43D-43F) or isotype-matched control ADC NIP228-SG3932 (43A-43C).

[0440] **Figures 44A-44D** show image analysis data of human IgG, γ H2AX and cleaved Caspase-3 IHC staining across all timepoints and treatments in the HT29 huB7 H4 clone 26 xenograft study. Top panel (44A): Epithelial cell analysis, showing the change in the fraction of human IgG-positive epithelial cells from all epithelial cells over time. Second panel (44B): γ H2AX analysis, showing the fraction of epithelial cells found positive for foci in the γ H2AX assay. Third panel (44C): Cleaved caspase 3 (CC-3), showing the percent of cleaved caspase-3-positive tumor cells in the samples over time. Bottom panel (44D): Cell density of all epithelial cells in the sample over time, indicating cell death resulting from E02-GL-SG3932 treatment compared to the isotype-matched control ADC NIP228-SG3932.

[0441] **Figures 45A-45B** show E02-GL-SG3932 efficacy in the B7-H4 negative HT29 xenograft model (See also Figure 11). Values are mean $\pm$ SEM tumor volumes for n: 8 animals per group. Dotted line denotes the day of dosing.

- [0442] **Figures 46A-46C** show E02-GL-SG3932 efficacy in the HT29-huB7-H4 clone 26 xenograft model. Values are mean $\pm$ SEM tumor volumes for n: 10 or 8 animals per group. Dotted line denotes the day of dosing.
- [0443] **Figures 47A and 47B** show efficacy of E02-GL-SG3932, NIP228-SG3932, and E02-INT in the MX-1 xenograft model. Values are mean $\pm$ SEM tumor volumes for n: 8 animals per group. Dotted line denotes the day of dosing.
- [0444] **Figure 48** shows efficacy of E02-GL-SG3932 and NIP228-SG3932 in the MX-1 xenograft model. Values are mean $\pm$ SEM tumor volumes for n: 3 or 6 animals per group.
- [0445] **Figure 49** shows efficacy of E02-GL-SG3932 and NIP228-SG3932 in the MDA-MB-468 xenograft model. Values are mean $\pm$ SEM tumor volumes for n: 3 or 6 animals per group.
- [0446] **Figure 50** shows quantitative image analysis B7-H4 expression data in patient derived xenograft (PDX) models, sorted by case average of mean of cell membrane OD mean value. Expression is color encoded by IHC cell intensity class (negative: black, 1+: white, 2+: light gray, 3+: dark gray).
- [0447] **Figure 51** shows antitumor activity resulting from a single administration of 1.25 mg/kg E02-GL-SG3932 or NIP228-SG3932 in patient derived xenograft models.
- [0448] **Figures 52A and 52B** show B7-H4 expression in PDX models grouped according to tumor response to E02-GL-SG3932 or NIP228-SG3932 at the 1.25 mg/kg dose level. Models were considered to be responsive (R) to test agents if the percent change in tumor volume from baseline was -30% to -100%, inclusive. Models were considered to be non-responders (NR) if the percent change in tumor volume from baseline was greater than -30%. The y-axis indicates the level of B7-H4 in each model, as determined by H-score.
- [0449] **Figure 53A** shows antitumor activity resulting from a single administration of 3.5 mg/kg E02-GL-SG3932 or NIP228-SG3932 in patient derived xenograft models. **Figures 53B-53E** show results of the study protocol to determine correlation of E02-GL-SG3932 administration, B7-H4 expression level and HR-deficiency at (A) 1.25 mg/kg E02-GL-SG3932, (B) 3.5 mg/kg E02-GL-SG3932, (C) 1.25 mg/kg isotype control ADC, and (D) 3.5 mg/kg isotype control ADC. In **Figures 53B-53E**, the triangle “ Δ ” symbol marks models deficient in homologous recombination (as determined by BRCA mutations or

RAD51 foci assay). The circle “●” symbol marks models without defects in homologous recombination.

[0450]

[0451] **Figure 54A and 54B** show B7-H4 expression in PDX models grouped according to tumor response to E02-GL-SG3932 or NIP228-SG3932 at the 3.5 mg/kg dose level. Models were considered to be responsive (R) to test agents if the percent change in tumor volume from baseline was -30% to -100%, inclusive. Models were considered to be NR if the percent change in tumor volume from baseline was greater than -30%. The y-axis indicates the level of B7 H4 in each model, as determined by H-score.

[0452] **Figures 55A-55G** show the mean tumor volume over time for a first panel of cholangiocarcinoma PDX mouse models treated with a single dose of 1.25 mg/kg or 3.5 mg/kg E02-GL-SG3932, as compared to untreated mice.

[0453] **Figures 56A-56K** show the mean tumor volume over time for a second panel of cholangiocarcinoma PDX mouse models treated with a single dose of 1.25 mg/kg or 3.5 mg/kg E02-GL-SG3932, as compared to untreated mice.

[0454] **Figure 57A-57B** show that the ADC prepared with the cleavable mal-PEG8-val-ala linker-warhead is the most active *in vivo*, as compared to ADCs 2-4. **Figure 57A** is the key to **Figure 57B**.

[0455] **Figure 58** shows that the cleavable mal-PEG8-val-ala linker-warhead ADC exhibits the cleanest safety profile in a rat toxicity study, as compared to ADCs 2-4.

[0456] **Figure 59A-59B** show that the cleavable mal-PEG8-val-ala linker-warhead ADC has good PK properties and widest relative TI, as compared to ADCs 2-4. Relative TI = exposure ratio of AUC at the highest dose tested in rat (NOAEL not HNSTD) to the AUC that provides tumor stasis (MX-1 model). **Figure 59A** is the key to **Figure 59B**.

[0457] **Figures 60A-60B** show that E02-GL-SG3932 has robust activity in breast and ovarian PDX mouse models.

[0458] **Figure 61** shows that E02-GL-SG3932 has robust activity in HR-deficient tumors and in HR-proficient tumors with elevated B7-H4.

[0459] **Figures 62A-62B** show the tumor response in HR-deficient (A) and HR-proficient (B) PDX models grouped according to tumor response to E02-GL-SG3932 at

the 3.5 mg/kg dose level. (R) indicates that models were considered to be responsive to test agents. (NR) indicates that models were considered to be non-responders. The y-axis indicates the level of B7-H4 in each model, as determined by H-score.

[0460] **Figures 62C-62D** show the tumor response in HR-deficient (A) and HR-proficient (B) PDX models grouped according to tumor response to E02-GL-SG3932 at the 1.25 mg/kg dose level. (R) indicates that models were considered to be responsive to test agents. (NR) indicates that models were considered to be non-responders. The y-axis indicates the level of B7-H4 in each model, as determined by H-score.

[0461] **Figure 63** shows the results of 6-day cytotoxicity assays of different warheads in DLD1 wt or BRCA2^{-/-} cells. MMAE = negative control, microtubule-inhibitor warhead.

EXAMPLES

MATERIALS AND METHODS

Protein Reagents

[0462] Protein reagents (e.g. constructs) produced are shown in Table 1. The table indicates what species the protein is from (human, mouse or cynomolgus), the vector used to clone the construct and whether the leader sequence used was either native or the Human CD33 leader sequence.

Table 1. Protein constructs made for B7H4 project

Cloned insert	Vector	Comments
Human B7H4 ECD	pDest12.2 oriP_Fc_6His	Native Leader
Mouse B7H4 ECD	pDest12.2 oriP_Fc_6His	Native Leader
Human B7H4 ECD	pDest12.2 oriP_Flag_10His	Native Leader
Mouse B7H4 ECD	pDest12.2 oriP_Flag_10His	Native Leader
Cyno B7H4 ECD	pDest12.2 oriP_Flag_10His	Native Leader
Human B7H4 ECD	pDest12.2 oriP_FcTM_6His	Native Leader Triple mutant Fc
Mouse B7H4 ECD	pDest12.2 oriP_FcTM_6His	Native Leader

Cyno B7H4 ECD	pDest12.2 oriP_FcTM_6His	Triple mutant Fc Native Leader Triple mutant Fc
Human B7H4 ECD	pDest12.2 oriP N CD33 10His FLAG	Human CD33 leader
Human B7H4 ECD	pDest12.2 oriP N CD33 C mIgG2a Fc 10His	Human CD33 leader
Human B7H4 ECD (SV)	pDest12.2 oriP N CD33 10His FLAG	Human CD33 leader Splice variant

[0463] A “triple mutant Fc” (TM) comprises the mutations triple mutation L234F/L235E/P331S within the Fc region, as previously described in Acta Crystallogr D Biol Crystallogr. 2008 Jun 1; 64(Pt 6): 700–704 (incorporated herein by reference). For the avoidance of any doubt, the inventors have the antibodies hereinafter described retain their advantageous binding properties/ profiles also in the absence of such TM (e.g. where there is a WT Fc).

[0464] All the proteins in the Table were purified using standard conditions. In brief, expression was carried out in HEK EBNAs, concentrated using a TFF concentration setup, purified using a His trap column or a Protein G column (depending on whether a 10His Flag tag was present or an Fc tag was present), followed by final polish step of a SEC S200 column.

ELISA

[0465] The binding of the anti-B7-H4 antibodies (e.g. intermediate ZY0EQD-E02-GL and anti-B7-H4 mAb D11) to B7-H4 (e.g. human and mouse B7-H4) was measured by ELISA. Recombinant B7-H4-Fc (e.g. Human B7-H4-Fc and mouse B7-H4-Fc) proteins were diluted into DPBS to give 5 ug/ml solution. 50 µL of the diluted stocks was then added Nunc Maxisorp 96 well plates. 50 µL/well DPBS was added to control wells. The antigens were allowed to adsorb to the plates overnight at 4°C, washed once with DPBS and incubated with blocking buffer (3% w/v Marvel in DPBS) at room temperature for 1 hour. The plates were then washed once with PBS and incubated for 1 hour with 6.4 pM-100 nM anti-B7-H4 antibody (e.g. intermediate ZY0EQD-E02-GL or anti-B7-H4 mAb D11) diluted in DPBS containing 1% BSA, 0.3% Triton X-100. The plates were then

washed three times with PBS containing 0.1% Tween and incubated with peroxidase-conjugated goat anti-human light chain antibody (Sigma Aldrich, Poole, UK) for 1 hour at room temperature. The plates were then washed five times with PBS containing 0.1% Tween. Following a 5-minute incubation with TMB peroxidase substrate, the reaction was stopped by the addition of 0.5 M H₂SO₄. Absorbance at 450 nm (A₄₅₀) was measured using an Envision multilabel plate reader (Perkin Elmer, Seer Green, UK).

Flow cytometry (for detecting antibody binding to B7-H4 positive cells)

[0466] Cells were detached from tissue culture flasks by accutase (Gibco, Paisley, UK), pelleted by centrifugation, and resuspended in ice cold DPBS (Gibco, Paisley, UK). Viable cells were counted by trypan blue exclusion using a haemocytometer. Cell densities were adjusted to 5×10^6 cells/mL in DPBS. 100 μ L of the cell suspension (5×10^5 cells) was added to 96-well V-bottomed plates and placed on ice. Then, the cells were incubated with Live/Dead fixable violet stain (ThermoFisher Scientific, Loughborough, UK) for 20 minutes on ice. Following a wash with flow cytometry buffer (eBiosciences, Hatfield, UK), the cells were incubated on ice for 30 minutes in 100 μ L flow cytometry buffer alone (unstained control cells) or supplemented with AF647-labelled anti-B7H4 antibodies (e.g. E02-GL or 1D11) or isotype R347 at a concentration ranging from 10 μ g/ml to 78 ng/ml. The cells were then washed three times with 200 μ L ice cold flow cytometry buffer, fixed with 200 μ L of 4 % paraformaldehyde (Sigma Aldrich, Poole, UK) for 20 minutes, and suspended in DPBS on ice for flow cytometry analysis on a FACSCanto II instrument (BD Biosciences, San Jose, CA, USA). FlowJo cytometry analysis software (Treestar, Ashland, OR, USA) was used to quantify antibody binding to cells as follows. Live, single cells were gated based on forwards scatter, side scatter, and Live/Dead violet fluorescence intensity and the AF647 geometric mean fluorescence intensity (MFI) was determined.

Cytotoxicity Assay

[0467] Cell lines were detached from tissue culture flasks by accutase (Gibco, Paisley, UK), pelleted by centrifugation, and resuspended in growth media (McCoy's 5A, 10 % FBS, 400 μ g/mL G418 for HT29-hB7H4 clone 44, McCoy's 5A, 10 % FBS for SKBR3

and RPMI-1640, 10 % FBS for HCC1569). Viable cells were counted by trypan blue exclusion using a haemocytometer. Cell densities were adjusted to 2.7×10^4 cells/mL in growth media. 75 μ L/well of the cell suspension (2×10^3 cells) was added to 96-well white walled clear bottom tissue culture treated plates and cultured overnight in a humidified tissue culture incubator at 37 °C in 5 % CO₂.

[0468] ADCs (e.g. 1D11-MMAE, E02-GL-MMAE and E02-GL-SG3249) were diluted into DPBS to give 400 μ g/mL or 16 μ g/mL stocks respectively. Four-fold serial dilutions of the stocks were prepared in DPBS. 25 μ L of the diluted stocks was then added to duplicate wells of the cultured cells, with a ten-point, four-fold serial dilution of antibody (e.g. 1D11-MMAE or E02-GL-MMAE or E02-GL-SG3249). 25 μ L/well DPBS was added to mock-treated control cells.

[0469] Cells were cultured in the presence of ADCs or DPBS (mock-treated control cells) for six days. Cell viability was assessed using CellTiter-Glo® assay: 100 μ L of CellTiter-Glo® (Promega, Southampton, UK) was added to each well. Plates were agitated on a benchtop shaker for 2 minutes and were then incubated at room temperature for an additional 10 minutes. Luminescence was measured using an Envision multilabel plate reader (Perkin Elmer, Seer Green, UK). The potency for test article antibodies (e.g. 1D11-MMAE or E02-GL-MMAE or E02-GL-SG3249) was determined by generating half-maximal inhibitory concentration (IC₅₀) values using a nonlinear regression model [log agonist vs. response – variable slope (three parameters)] in GraphPad Prism, version 7 (GraphPad Software, La Jolla, CA) and presented as percent cell viability relative to Mock-treated control cells –

$$\left(\frac{[(\text{Treated cells} - \text{Background}) / (\text{Mock-treated control cells} - \text{Background})]}{1} \right) \times 100.$$

On-cell western

[0470] The on-cell western method was developed and used to run 5 exemplary antibody clones with the following cells: SKBR3, A549, OVCAR4 (all minus transfections with B7-H4 vector), CHO and HEK cells (plus and minus transfection with full length B7-H4 vector).

[0471] In addition to the exemplary antibody clones, the following antibodies (for comparative purposes) were also used:

- E Biosciences 14-5949 anti-Human B7H4 mouse IgG
- US biological B0000-35B anti Human B7H4 mouse IgG
- *R and D systems* AF2514 anti Mouse B7H4 goat IgG1
- *Sigma* SAB2500141 anti B7H4 Goat IgG1
- *Isotype 1* CAT004 SP06-003
- *Isotype 2* R and D Normal goat IgG control (AB-108C)

Affinity analysis KinExA 3200

[0472] EO2_GL Fab: EO2_GL Fab SEC fraction (33.4-34.5 min, 07031802.D),

[0473] B7H4: hB7H4-ECD-Flag-His10 (4.34 mg mL⁻¹, J Watson, 31/10/17).

KinExA buffers:

[0474] D-PBS with added 0.02% sodium azide (VWR/Merck 103692K, lot: K35580906), 1 Litre, 0.20 µm sterile filtered.

[0475] D-PBS with added 0.02% sodium azide (VWR/Merck 103692K, lot: K35580906) and 1 mg mL⁻¹ bovine serum albumin (Sigma A-2058, lot: 108H0573). 1.0 Litre, 0.20 µm sterile filtered.

Secondary detection reagent:

[0476] DyLight649 labelled Mouse anti-human H+L chain secondary detection reagent (Jackson ImmunoResearch, 209-495-088, lot 91003) was used for detection of whole IgGs or Fab. Vial (~1 mg) reconstituted with 800 µL Milli-Q water.

Minimal Amine Biotinylation of r human B7H4 ECD:

- Protein: r human B7H4 ECD-FlagHis10 (4.34 mg mL⁻¹, batch 1, 31 Oct 17)
- Source: JWPur006
- Volume/buffer: 0.100 mL /PBS
- Mass of protein (Da): 29,053.57 Da
- Mass of protein to be biotinylated = 0.434 mg

- Amount protein to be biotinylated (pmoles) 0.000434 g/29,054 Da =
1.494 E-8 moles (14,938 pmoles)

Biotinylation:

- 10 μL saturated NaHCO_3 in D-PBS added.
- Reagent: EZ link Sulfo-NHS-LC-Biotin (Perbio/Pierce, product no. 21335) in
(1.0 mg mL^{-1} in DMF).
- First pulse protein:biotin ratio: 1:0.5 $14,938 \times 0.5 = 14,938$
 - $7,468/1,797 \text{ pmol } \mu\text{L}^{-1} = 4.16 \text{ } \mu\text{L}$
- Start: 16:07 p.m.; sampled: 16:35 p.m.
- All applied to a Dulbecco's PBS equilibrated PD-10 column.

Imaging studies *in vivo*

[0477] Antibody clones (e.g. E02_GL) were labelled with 800CW (LI-COR Biosciences). 800 CW labelled R347 was used in control experiments.

[0478] B7-H4 expressing CT26/4TI/HT29 cancer cells were grafted (e.g. inoculated subcutaneously) to the left flank 3-5 day old nude mice (Charles River Laboratories, Wilmington, MA), and non-B7-H4 expressing CT26/4TI/HT29 cancer cells were grafted to the right flank to provide an internal control. Mice were kept for a week to develop tumours, 800CW-labelled E02_GL was injected. 800CW-labelled R347 was injected into control mice. *In vivo* imaging of tumours was performed at days 1, 3, 7 and 9 following injection of labelled antibodies, by imaging radiance from the label.

EXAMPLE 1

B7-H4 is over-expressed in multiple cell types

[0479] Immunohistochemistry was carried out on sections from a number of sections of tumours taken from human subjects, representing a number of tumour types (as outlined in Table 2). Expression of B7-H4 was found to be particularly pronounced in breast cancer (e.g. hormone-receptor-positive (HR+)) breast cancer, as well as non-small-cell

lung carcinoma (NSCLC) (see Figures 1A-1D). Interestingly, the majority of tumours showed heterogeneous expression.

[0480] Expression was maintained in a proportion of patients following treatment (where HER2+ breast cancer patients were treated with Herceptin; and ovarian cancer patients were treated with platinum-based chemotherapy).

Table 2.

Tumor Type		% Pos ¹	% High Pos ²	% Low Pos ³	N Evaluated
Breast	HR+	85	23	51	39
	TNBC	70	32	23	230
	HER2+	87	42	28	98
	- Herceptin eligible	83	43	20	30
	- Herceptin treated	88	41	31	68
Ovarian (Serous)		72	24	33	71
Endometrial		84	35	48	39
NSCLC	Squamous	63	15	48	160
	Adenocarcinoma	19	2	15	143
Pancreatic		31	1	18	90
Gastric		10	0	10	21
Cholangiocarcinoma		13	38	38	13

¹ Positive: Tumors with staining at any intensity and frequency

² High Positive: Tumors with membrane staining with intensity of >2+ in >50% of tumor cells

³ Low Positive: Tumors with membrane staining with intensity of ≤2+ in <50% of tumor cells

Data based on Tissue MicroArray analysis

EXAMPLE 2

Generation of anti-B7-H4 antibodies

[0481] A repetitive immunisation multiple site (RIMS) strategy was taken, in which VelocImmune II mice (Regeneron, Tarrytown, NY) were immunised as follows:

- Minus 4 days: pre-bleed
- 0 days: prime immunisation
- 7 days: second boost
- 13 days: first bleed
- 15 days: third boost
- 20 days: second bleed
- 22 days: fourth boost
- 24 days: fifth boost
- 28 days: terminal bleed and spl. and LN fusions

[0482] For immunisation, sixteen V2 mice were split into 4 groups, each group containing 4 animals. Animals were immunised with human and mouse recombinant B7-H4, as well as SkBr3 cells (e.g. which express B7-H4). Details of immunogen can be found in Table 3 below (TT = tetanus toxin; DTA = diphtheria toxin; KLH = Keyhole limpet haemocyanin).

Table 3.

Group N =4	Prime	2	3	4	5
G1	KLH-mB7H4	KLH-hB7H4	KLH-mB7H4	KLH-hB7H4	KLH-hB7H4
G2	KLH-mB7H4	SkBr3	KLH-mB7H4	SkBr3	SkBr3
G3	mB7H4-TT	KLH-hB7H4	mB7H4-TT	KLH-hB7H4	mB7H4-TT
G4	mB7H4-DTA	KLH-hB7H4	mB7H4-DTA	KLH-hB7H4	mB7H4-DTA

Hybridoma generation

[0483] Lymphoid cells were harvested from 10 mice, cells from mice 3 and 9 were enriched for B cells, no selection was performed on cells from the other 8 mice. Lymphoid cells and Sp2/0 Ag14 myeloma cells were mixed at 5:1 ratio, washed in serum-free medium, and fusions were performed using PEG either manually or by the tecan robot. Following fusion, the cells were resuspended in 200ml of complete HM20 medium and 100µl added to columns 1-11 of 20 plates. After 3 days, a further 100µl of medium was added to each well. Fusion details for each mouse are shown in Table 4.

Table 4.

Mouse	Group	Spleen cells x 10 ⁶	LN cells x 10 ⁶	Total x 10 ⁶	% LNC	Fusion Type	Selection
1	1	57.72	13.78	71.50	19.27	Tecan	None
3	1	73.42	10.35	83.77	12.36	Manual	B cell
4	1	27.78	11.47	39.25	29.21	Tecan	None
5	2	67.92	23.45	91.37	25.66	Tecan	None
7	2	78.20	25.70	103.90	24.74	Tecan	None
9	3	62.47	11.98	74.45	16.09	Manual	B cell
10	3	53.43	5.25	58.68	8.95	Manual	None
11	3	50.80	9.25	60.05	15.40	Tecan	None
13	4	41.33	11.98	53.30	22.47	Manual	None
14	4	38.20	34.30	72.50	47.31	Tecan	None

Hybridoma screening and cloning

[0484] Supernatants harvested 13 days following fusion were screened in a bead based IgG/IgM screen by the hybridoma group and in human, cynomolgus and mouse B7H4 HTRF biochemical binding assays, and in a SkBr3 FMAT assay by HTS.

[0485] Following screening positive hits were picked of which 58 were cloned out into semi-solid media. IgG positive clones were picked from each well line using the ClonePix-FL, which were subsequently screened in the primary assays. A maximum of 4 clones were then picked from each well line, which were grown up and small scale IgG purifications performed (Phytip- protein A).

[0486] Following biological screens of the Phytip material, 5 exemplary antibodies were chosen for further characterisation (details of which can be found in Table 5).

Table 5.

Clone ID	Mouse ID	Immunogen
ZY0EPQ-E02	5	Group 2 (SkBr3 cells/mB7H4-KLH)
ZY0EQD-E02	7	Group 2 (SkBr3 cells/mB7H4-KLH)
ZY0EP0-C07	4	Group 1 (mB7H4-KLH/h-B7H4-KLH)
ZY0EOB-F05	1	Group 1 (mB7H4-KLH/h-B7H4-KLH)
ZY0EO5-E07	1	Group 1 (mB7H4-KLH/h-B7H4-KLH)

- [0487] ZY0EPQ-E02 (and germlined versions thereof, e.g. connotated by the abbreviation “GL”) comprises a CHDR1-3 of SEQ ID NO: 1-3 (respectively), and a CLDR1-3 of SEQ ID NO: 4-6 (respectively). ZY0EPQ-E02 comprises a VH chain of SEQ ID NO: 31, and a VL of SEQ ID NO: 32.
- [0488] ZY0EQD-E02 comprises a CHDR1-3 of SEQ ID NO: 7-9 (respectively), and a CLDR1-3 of SEQ ID NO: 10-12 (respectively). ZY0EQD-E02 comprises a VH chain of SEQ ID NO: 33, and a VL of SEQ ID NO: 34. A germlined version of ZY0EQD-E02 is referred to later e.g. by inclusion of the abbreviation “GL”, and referred to e.g. as EQD-E02_GL (having a VH chain of SEQ ID NO: 45, and a VL of SEQ ID NO: 34).
- [0489] ZY0EOB-F05 comprises a CHDR1-3 of SEQ ID NO: 13-15 (respectively), and a CLDR1-3 of SEQ ID NO: 16-18 (respectively). ZY0EOB-F05 comprises a VH chain of SEQ ID NO: 35, and a VL of SEQ ID NO: 36.
- [0490] ZY0EO5-E07 comprises a CHDR1-3 of SEQ ID NO: 19-21 (respectively), and a CLDR1-3 of SEQ ID NO: 22-24 (respectively). ZY0EO5-E07 comprises a VH chain of SEQ ID NO: 37, and a VL of SEQ ID NO: 38.
- [0491] ZY0EP0-C07 comprises a CHDR1-3 of SEQ ID NO: 25-27 (respectively), and a CLDR1-3 of SEQ ID NO: 28-30 (respectively). ZY0EP0-C07 comprises a VH chain of SEQ ID NO: 39, and a VL of SEQ ID NO: 40.
- [0492] These 5 exemplary antibodies were reformatted onto human IgG1, human IgG1-TM (triple mutation) and murine IgG1 backbones.
- [0493] Sequence analysis of the 5 reformatted exemplary antibodies shows identity between ZY0EPQ-E02 and ZY0EQD-E02 as well as clones ZY0EOB-F05 and ZY0EO5-E07 (see Figure 28).

EXAMPLE 3

Antigen binding assays with selected clones

[0494] Concentration-effect binding of the 5 exemplary antibodies as murine IgG1, human IgG1 and human IgG1-TM were performed using human, cynomolgus and murine B7-H4-Fc via HTRF assay. For the murine IgG1 antibodies, anti-murine IgG conjugated with Dylight-649 was used for detection. A similar assay format was used with the human IgG1 and IgG1-TM antibodies; however anti-human kappa conjugated with Dylight-649 was used for detection.

[0495] Antibodies ZY0EPQ-E02 and ZY0EQD-E02 have a higher EC₅₀ and a lower max binding to murine B7-H4-Fc than to human or cynomolgus B7-H4-Fc, indicating a lower affinity to the murine B7-H4. All the other antibodies have a similar EC₅₀ and max binding to the human, cynomolgus and murine B7-H4-Fc (see Tables 6-8).

Table 6. HTRF Data using human B7-H4-Fc

Antibody	Human B7-H4-Fc					
	Murine IgG1		Human IgG1		Human IgG1-TM	
	EC50 (nM)	Max	EC50 (nM)	Max	EC50 (nM)	Max
ZY0EPQ-E02	0.38	2741	0.38	1127	0.41	1092
ZY0EQD-E02	0.22	3166	0.35	1237	0.33	1272
ZY0EOB-F05	0.30	3025	0.15	1816	0.15	1873
ZY0E05-E07	0.68	2150	0.19	1708	NT	NT
ZY0EP0-C07	0.95	1736	0.30	1221	0.31	1207

Table 7. HTRF Data using cynomolgus B7-H4-Fc

Antibody	Cyno B7-H4-Fc					
	Murine IgG1		Human IgG1		Human IgG1-TM	
	EC50 (nM)	Max	EC50 (nM)	Max	EC50 (nM)	Max
ZY0EPQ-E02	0.38	2236	0.33	987	0.35	934
ZY0EQD-E02	0.22	2494	0.28	1127	0.28	1100
ZY0EOB-F05	0.32	2398	0.14	1483	0.15	1483
ZY0E05-E07	0.71	1698	0.18	1359	NT	NT
ZY0EP0-C07	1.28	1247	0.33	946	0.39	890

Table 8. HTRF Data using murine B7-H4-Fc

Antibody	Murine B7-H4-Fc					
	Murine IgG1		Human IgG1		Human IgG1-TM	
	EC50 (nM)	Max	EC50 (nM)	Max	EC50 (nM)	Max
ZY0EPQ-E02	2.03	790	1.49	279	1.76	256
ZY0EQD-E02	3.64	447	1.40	167	1.44	224
ZY0EOB-F05	0.38	2190	0.19	1319	0.19	1342
ZY0E05-E07	0.73	1697	0.21	1300	NT	NT
ZY0EP0-C07	0.49	1910	0.18	1321	0.20	1252

Binding affinity measurements

[0496] Antibody affinity to the human, murine and splice variant B7-H4 ECD's was measured with the ForteBio Octet system. Human IgG1-TM antibodies were captured by Protein G and the monomeric species B7-H4-FLAG ECD were measured binding to the antibody. Affinity to human B7-H4 was in the 10-25nM range, whereas the murine affinity was between 10-600nM. All antibodies except ZY0EP0-C07 could bind the splice variant, with affinities between 200-1600nM. Kinetic binding parameters are summarised in Table 9 below.

Table 9. Octet Affinity Summary

Antibody	Protein	hIgG1-TM (n=4-7)			hIgG1 (n=1)		
		KD (nM)	kon(1/Ms)	kdis(1/s)	KD (nM)	kon(1/Ms)	kdis(1/s)
ZY0EPQ-E02	HumanB7-H4	25.7	7.09E+04	1.82E-03	24	8.59E+04	2.08E-03
	MurineB7-H4	396	7.67E+04	3.04E-02	498	1.00E+05	4.98E-02
	B7-H4-SV	1660	1.38E+03	2.29E-03	1030	2.46E+03	2.52E-03
ZY0EQD-E02	HumanB7-H4	14.4	1.93E+05	2.78E-03	14.9	2.16E+05	3.21E-03
	MurineB7-H4	621	2.03E+05	1.26E-01	625	3.41E+05	2.13E-01
	B7-H4-SV	801	3.36E+03	2.69E-03	834	4.19E+03	3.49E-03
ZY0EOB-F05	HumanB7-H4	13.4	2.65E+04	3.55E-04	11.7	3.10E+04	3.62E-04
	MurineB7-H4	25	1.87E+04	4.76E-04	19.8	2.49E+04	4.93E-04
	B7-H4-SV	174	3.35E+03	5.84E-04	127	4.71E+03	5.98E-04
ZY0E05-E07	HumanB7-H4	19.8	1.71E+04	3.38E-04	14.6	2.17E+04	3.18E-04
	MurineB7-H4	30	1.21E+04	3.61E-04	23.2	1.79E+04	4.15E-04
	B7-H4-SV	264	1.89E+03	4.99E-04	182	2.91E+03	5.28E-04
	HumanB7-H4	9.4	4.16E+04	3.97E-04	7	4.88E+04	3.63E-04

ZY0EP0-C07	MurineB7-H4	11	4.30E+04	5.24E-04	9	6.02E+04	5.51E-04
	B7-H4-SV	No Binding			No Binding		

Epitope Binning

- [0497]** Epitope binning was performed in an HTRF assay format using the exemplary antibodies (IgG1-TM) conjugated with DyLight-649 binding to the monomeric B7-H4-FLAG ECD with detection with europium conjugated anti-FLAG antibody.
- [0498]** Bin 1 was defined with the ZY0EPQ-E02, ZY0EQD-E02 and ZY0EO5-E07 antibodies fully inhibiting each other, and only partial inhibiting ZY0EP0-C07. Bin 2 was defined with the ZY0EOB-F05 antibody fully competing with all antibodies and all antibodies fully competing with it. Bin 3 was defined with ZY0EP0-C07 partially competing with antibodies ZY0EPQ-E02, ZY0EQD-E02 and ZY0EO5-E07 (Bin 1).

Table 10. HTRF Epitope Summary Table

Competing Antibody	Dy649 conjugated antibody				
	ZY0EPQ-E02	ZY0EQD-E02	ZY0EOB-F05	ZY0EO5-E07	ZY0EP0-C07
ZY0EPQ-E02	Full	Full	Full	Not Tested	Partial
ZY0EQD-E02	Full	Full	Full		Partial
ZY0EOB-F05	Full	Full	Full		Full
ZY0EO5-E07	Full	Full	Full		Partial
ZY0EP0-C07	Partial	Partial	Full		Full

Species cross-reactivity ELISA

- [0499]** Each of the above-mentioned 5 VelocImmune derived anti-B7-H4 exemplary antibodies were tested for binding to in house derived murine B7-H4 (ECD). All exemplary antibodies were tested as human IgG1-TMs, alongside 4/5 murine IgG1s (ZY0EP0-C07 was not available as a murine IgG1 at this time). Binding to monomeric and dimeric murine and human B7-H4 variants were compared (FlagHis10 or FcHis6 tagged, respectively).

[0500] All IgGs, irrespective of isotype, retained cross-reactivity to both murine and human B7-H4 antigens. The dimeric nature of the FcHis6-tagged B7-H4 antigens produced binding curves that were uniformly shifted to the left of those curves provided by the corresponding monomeric FlagHis10 B7-H4 antigens. This effect was more marked for murine B7-H4.

[0501] No significant binding to irrelevant antigen controls was observed with any of the VelocImmune exemplary antibodies. Isotype control IgGs did not bind non-specifically to the B7-H4 antigens (NIP228 – human IgG1-TM and MOPC-21 - murine IgG1) – see Figure 2.

Commercial anti-B7-H4 Polyclonal and Monoclonal Specificity ELISA

[0502] A panel of commercial monoclonal and polyclonal antisera were tested for binding to human and murine B7-H4 antigens by ELISA. Each antiserum was also tested for binding to a truncated human B7-H4 splice variant (essentially missing its extracellular IgV domain). Equivalent data was obtained irrespective of whether FlagHis10 or FcHis6 B7-H4 antigens were used. The different cross reactivity profiles are summarised within Tables 11 and 12.

Table 11. Monoclonals

<i>Company</i>	<i>Cat #</i>	<i>Lot #</i>	<i>B7-H4</i>		
			<i>Human</i>	<i>Murine</i>	<i>Splice Variant</i>
AdD serotec	<u>MCA2632</u>	707	Y	N	N
eBioscience	<u>14-5949</u>	E021763	Y	Y	Y
Epitomics	<u>2516-1</u>	YD-08-23-02	N	N	n/a
US Biologicals	<u>B0000-35B</u>	L10061519	Y	N	N
eBioscience	<u>145972-82</u>	E010563	Y	Y	Y
eBioscience	<u>145970-85</u>	E010556	N	N	n/a
R & D	<u>MAB2154</u>	WVP0107101	Y	Y	Y

Table 12. Polyclonals

<i>Company</i>	<i>Cat #</i>	<i>Lot #</i>	<i>B7-H4</i>		
			<i>Human</i>	<i>Murine</i>	<i>Splice Variant</i>
Santa Cruz	<u>sc68872 (H108)</u>	L1008	Y	Y	n/a
Santa Cruz	<u>sc68254 (G-18)</u>	C1910	N	N	n/a

R & D	AF2154	WVP016011	Y	Y	Y
Abbiotec	250473	10051104	N	N	n/a
Sigma Aldrich	SAB2500141	7942P1	Y	Y	N

B7-H4 Homolog Specificity ELISA

[0503] Each of the five selected clones (VelocImmune IgGs) were tested for non-specific binding to B7-H4 family members and homologs (murine IgG1 ZY0EP0-C07 was available to test). The choice of which antigens to test was guided by undertaking a BLASTP search using the ECD of human B7-H4 as test sequence. Advantageous hits from this list were then aligned using CLUSTALW analysis and those antigens displaying the highest percentage identity at the primary amino acid level were sourced (see Table 13).

Table 13.

Percent Identity											
	1	2	3	4	5	6	7	8	9	10	
D i v e r g e n c e	1		13.2	21.5	21.4	25.9	21.6	20.7	25.4	25.2	1
	2	377.0		11.7	12.1	13.1	10.3	11.0	13.7	9.7	2
	3	229.0	426.0		19.2	27.8	21.5	20.1	18.2	18.1	3
	4	229.0	414.0	255.0		21.2	20.8	17.7	21.4	18.2	4
	5	190.5	377.0	174.9	231.0		25.6	19.5	23.4	24.1	5
	6	228.0	498.0	229.0	236.0	192.5		42.3	45.8	34.8	6
	7	238.0	461.0	244.0	279.0	253.0	103.0		41.0	33.6	7
	8	194.4	362.0	270.0	229.0	209.0	91.9	107.6		32.5	8
	9	196.4	538.0	271.0	270.0	203.0	133.7	139.3	145.3		9
	10	191.3	661.0	360.0	368.0	245.0	330.0	311.0	282.0	426.0	10
	1	2	3	4	5	6	7	8	9	10	

human B7-H4 ECD.pro
human MOG1.pro
human B7-H1.pro
human B7-H2.pro
human B7-H3.pro
human BTN1A1.pro
human BTN2A1.pro
human BTN3A2.pro
human BTNL3.pro
human HHLA2.pro

[0504] The five exemplary antibodies do not bind measurably to homologues huB7-H1, huB7-H2, huB7-H3, huBTN1A1, huHHLA2 or huBTN3A2, irrespective of antibody isotype. Note that binding was not seen when testing huMOG1, huBTN2A1 or huBTNL3 antigens either (see Figure 3).

On-cell western binding analysis

[0505] Cells were detached from T175 flasks using Accutase and counted. Transfection method used was essentially that stated in the Invitrogen Lipofectamine LTX protocol

(high throughput protocol using the reverse transfection method). 100µl of cells (4.5×10^4 per 100µl) was added to 96 well plates containing the DNA- lipofectamine mix. Plates incubated at 37°C for 18 hours and fixed using 10% buffered formalin overnight. Plates were blocked with Odyssey blocking buffer, incubated with appropriate primary antibodies overnight followed by washing and detection with the appropriate Odyssey secondary antibody.

[0506] Plates were air dried and scanned using the Odyssey Imager using the recommended scan levels provided by the manufacturer.

[0507] QD-E02 and PQ-E02 high levels of non-specific binding, such that the antibodies surprisingly bind a wide spectrum of cell types in a manner which goes beyond that seen for the control antibodies (lanes E, U, R, S) - see Figure 4.. OB-F05 and O5-E07 give good results with transfected HEKs and also bind to transfected CHOs. Exemplary antibodies QD-E02, OB-F05 and O5-E07 and PQ-E02 bind to the OVCAR4 cells.

SUMMARY

[0508] Table 14 shows a summary of properties of the five exemplary antibodies.

Table 14.

Antibody	Protein	Octet KD (nM)	Epitope Bin	ZAP		
ZY0EPQ-E02	HumanB7-H4	26	1	Cell	IC50 (M)	% Viability
	MurineB7-H4	396		SKBR3	4.E-09	65
	B7-H4-SV	1660		OVCAR4	2.E-08	46
ZY0EQD-E02	HumanB7-H4	14	1	Cell	IC50 (M)	% Viability
	MurineB7-H4	621		SKBR3	1.E-08	48
	B7-H4-SV	801		OVCAR4	2.E-08	40
ZY0EOB-F05	HumanB7-H4	13	2	Cell	IC50 (M)	% Viability
	MurineB7-H4	25		SKBR3	4.E-18	84
	B7-H4-SV	174		OVCAR4	3.E-17	67
ZY0E05-E07	HumanB7-H4	20	1	Cell	IC50 (M)	% Viability
	MurineB7-H4	30		SKBR3	2.E-16	79
	B7-H4-SV	264		OVCAR4	7.E-17	58
ZY0EP0-C07	HumanB7-H4	9	3	Cell	IC50 (M)	% Viability
	MurineB7-H4	11		SKBR3	4.E-09	72
	B7-H4-SV	No Binding		OVCAR4	1.E-08	57

EXAMPLE 4

Screening cell lines for B7-H4 surface expression by flow cytometry

[0509] The following human cell lines have been tested positive for B7-H4 expression using exemplary antibodies and/or commercial anti-B7-H4 antibodies (relative B7-H4 expression levels indicated by '+'):

- SK-BR-3 (+++)
- T47D (+)
- MDA-MB-468 (++)
- OVCAR4 (+++)
- NIH:OVCAR3 (+)
- Calu-3 (+)

[0510] The following human cell lines have been tested negative: NCI-H322, Raji (+/- IFN γ activation), Ramos (+/- IFN γ activation) and Du145.

EXAMPLE 5

In vitro Cytotoxicity assay

[0511] Internalisation of the five human IgG1-TM exemplary antibodies by B7-H4 expressing cell lines have been confirmed experimentally (SK-BR-3, MDA-MB-468, OVCAR4 and JumpIn CHO Fl-B7H4). ADC cytotoxicity was demonstrated for the five IgG1-TM exemplary antibodies using saporin-conjugated anti-human IgG secondary antibody (see Figure 5). Cell viability at 20 μ g/ml 'antibody-alone' control was around 100%.

EXAMPLE 6

Clone ZY0EQD_E02 Specificity ELISA

[0512] Due to the superior performance of clone ZY0EQD_E02, this clone was chosen for more detailed analysis. The ZY0EQD_E02 comprises a CHDR1 of SEQ ID NO: 7; a CHDR2 of SEQ ID NO: 8; a CHDR3 of SEQ ID NO: 9; a CLDR1 of SEQ ID NO: 10; a CLDR1 of SEQ ID NO: 11; a CLDR1 of SEQ ID NO: 12. Said clone comprises a VH

chain of SEQ ID NO: 33, and a VL chain of SEQ ID NO: 34. Said clone comprises a heavy chain of SEQ ID NO: 48, and light chain of SEQ ID NO: 44. A germlined version (E02-GL) comprises a VH chain of SEQ ID NO: 45, and a VL chain of SEQ ID NO: 34; e.g. a heavy chain of SEQ ID NO: 51, and light chain of SEQ ID NO: 44.

[0513] ELISA analysis was performed to determine binding to B7-H4 from human (including a splice variant), cynomolgus macaque (cyno), mouse and rat. Percentage sequence identity of human B7-H4 in said species is: Domain FL – cynomolgus macaque (98.6%); rabbit (91.6%); mouse (87.9%); rat (86.9%); Domain ECD – cynomolgus macaque (99.6%); rabbit (94.3%); mouse (90%); rat (89.6%).

[0514] Maia format mAbs (e.g. comprising a C insertion, as shown, for example, in SEQ ID NO.: 41) of the clone were tested for binding. Wildtype, as well as a number of variants (germlined (GL), GLQ, GLY) were assayed, together with R347-maia human IgG1 as a control (see Fig 6).

[0515] R347-maia isotype control shows no binding to any antigen tested. E02-maia binding profile is similar to previous experiments. E02-maia and E02-GL-maia have similar binding profiles. Advantageously, this demonstrates that both germlined (GL) and non-germlined (e.g. WT) versions of the antibody retain the advantageous binding properties/ profiles of the antibody.

EXAMPLE 7

Comparison of E02-GL binding to the mAb “1D11”

[0516] Clone ZY0EQD_E02, germlined (referred to here as E02-GL) binding affinity was directly compared to that of the known anti-B7-H4 antibody “1D11” (Genentech; described in WO2016040724, which is incorporated herein by reference) by ELISA (see Materials & Methods, above).

[0517] “E02-GL” antibody has the CDR sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined). For example, E02-GL in these examples comprises a VH chain sequence of SEQ ID NO: 45, e.g. a germlined version of SEQ ID NO: 43.

- [0518] Direct comparison demonstrates that clone E02-GL demonstrates significantly better binding (affinity) than 1D11 (see Figure 7). Consistent with Example 7, R347-maia isotype control shows no binding to any antigen tested.
- [0519] Furthermore, E02-GL was shown (via flow cytometry experiments, see Materials and Methods above) to have superior binding to the B7-H4 expressed on cancer cells, when compared with 1D11. Thus, not only does E02-GL show superior binding, but superior targeting of cancer cells. Binding to both hB7-H4 expressing HT29 cells and SK-BR-3 cells (which express B7-H4) was tested. Antibody binding at concentrations of 31 ng/ml, 156 ng/ml and 78 ng/ml were tested. Results for HT29 cells is shown in Figure 8A, and results for SK-BR-3 cells is shown in Figure 8B. The sign $\diamond$ marks the 'E02-GL fraction', and the sign $\square$ marks the '1D11 fraction'. The sign "●" marks a (negative) control 'R347 fraction'.
- [0520] As can be seen in Figures 8A-8B, the number of cells stained cells in the 'E02-GL fraction' was significantly higher than the number of stained cells in the '1D11 fraction'.

EXAMPLE 8

In vitro cytotoxicity of Ad293 cells, with and without B7-H4 transfection (E02-GL-SG3932)

- [0521] The clone E02-GL was conjugated to the topoisomerase I payload SG3932 at an average Drug Antibody Ratio (DAR) of 8 (providing E02-GL-SG3932) and tested for the ability to target and kill both human B7-H4 transfected and non-transfected Ad293 cells (e.g. the latter representing a negative control). The antibody NIP228 conjugated to SG3932 was used as a control. The transfected cells were readily targeted and killed by the E02-GL-SG3932 conjugate, which had an IC₅₀ of 53.3 ng/ml (see Figure 9A). No significant killing following addition of the E02-GL-SG3932 conjugate was observed in the non-transfected cells (see Figure 9B). In all experiments described herein involving E02-GL conjugated to a topoisomerase inhibitor, the E02-GL clones used typically had a heavy chain sequence of SEQ ID NO.: 51.

METHOD:

[0522] Cell lines were detached from tissue culture flasks by TrypLE Express (Gibco, Paisley, UK), pelleted by centrifugation, and resuspended in growth media (RPMI-1640, 10 % FBS). Viable cells were counted by trypan blue exclusion using a Vi-CELL XR Cell Viability Analyzer (Beckman Coulter Life Sciences, Indianapolis, Indiana). Cell densities were adjusted to 3.33×10^4 cells/mL in growth media. 75 μ L/well of the cell suspension (2.5×10^3 cells) was added to 96-well white walled clear bottom tissue culture treated plates and cultured overnight in a humidified tissue culture incubator at 37 °C in 5 % CO₂.

[0523] ADCs (e.g. NIP228-SG3932 and E02-GL-SG3932) were diluted into growth media (RPMI-1640, 10 % FBS) at a concentration of 240 μ g/mL. Five-fold serial dilutions were prepared in growth media (RPMI-1640, 10 % FBS), and 25 μ L was then added to triplicate wells of the cultured cells, with a nine-point, five-fold serial dilution of antibody (e.g. NIP228-SG3932 or E02-GL-SG3932). 25 μ L/well of growth media (RPMI-1640, 10 % FBS) was added to mock-treated control cells. Cells were cultured in a humidified tissue culture incubator at 37 °C in 5 % CO₂ for six days at which time cell viability was assessed using CellTiter-Glo® assay (Promega, Southampton, UK) according to the manufacturer's protocol. Luminescence was measured using an Envision multilabel plate reader (Perkin Elmer, Seer Green, UK). The potency for test article antibodies (e.g. NIP228-SG3932 or E02-GL-SG3932) was determined by generating half-maximal inhibitory concentration (IC₅₀) values using a nonlinear regression model [log agonist vs. response – variable slope (four parameters)] in GraphPad Prism, version 8 (GraphPad Software, La Jolla, CA) and presented as percent cell viability relative to Mock-treated control cells - $\left(\frac{(\text{Treated cells} - \text{Background})}{(\text{Mock-treated control cells} - \text{Background})} \times 100 \right)$.

EXAMPLE 9

E02-GL- SG3932 causes bystander killing of tumor cells *in vitro*

[0524] HT29-huB7-H4 clone 26 target positive and GFP-labelled HT29 target negative cells, plated either individually or mixed at a ratio of 1:1 and cultured for 1 day, were treated with 200 ng/mL E02-GL-SG3932 or NIP228-SG3932 isotype control ADC for 6

days. At the end of treatment, the number of live GFP-negative HT29-huB7-H4 clone 26 cells or GFP-positive HT29 cells was determined using flow cytometry. Results are shown in Figure 10: A) Dot plots show representative images from flow cytometry analyses at the end of treatment for media treated samples of HT29-GFP and HT29+huB7-H4 clone 26 cells cultured alone or together. Numbers shown in the upper-left and lower-left quadrants reflect the percentage of HT29-GFP and HT29+huB7-H4 clone 26 cells, respectively. B) When plated individually, a decrease in cell count was observed following treatment with E02-GL-SG3932 in HT29+huB7H4 clone 26 cells which was not observed in the target negative HT29-GFP cells. When cells were plated in co-culture, cell counts for both the target negative HT29-GFP and target positive HT29+huB7H4 clone 26 cells were reduced, demonstrating a bystander killing effect.

METHOD:

[0525] A lentiviral expression system was used to prepare the HT29-GFP cell line, which stably expresses green fluorescent protein (GFP). Cells were plated at a total cell density of 15,000 cells per well in 24-well plates, cultured individually or mixed at a ratio of 1:1 and cultured for 1 day. Media was then removed and replaced with fresh media alone or media containing 200 ng/mL of NIP228-SG3932 or E02-GL-SG3932 ADCs, and cells were incubated for an additional 6 days. At the end of treatment, the number of live GFP-negative HT29+huB7-H4 clone 26 cells or GFP-positive HT29 cells was determined using flow cytometry and FlowJo software.

EXAMPLE 10

E02-GL- SG3932 causes bystander killing of tumor cells *in vivo*

[0526] Tumor cells were implanted subcutaneously into female SCID mice between 8 to 10 weeks of age. When tumors reached the appropriate tumor volume range (typically 150-250 mm³), animals were randomized into treatment and control groups and dosing was initiated. Tumor-bearing mice were administered a single dose of test articles via intravenous injection. Animals were observed daily and tumor dimensions and body weight were measured and recorded two to three times weekly. Results are shown in Figure 11. Tumor volumes were measured by caliper and the volumes of tumors were

calculated using the following formula: tumor volume = length (mm) x width (mm)²/2, where the length and width are the longest and shortest diameters of the tumor, respectively.

EXAMPLE 11

E02-GL-SG3932 has potent *in vivo* activity
in patient derived xenograft (PDX) models

[0527] Tumor tissue fragments were implanted subcutaneously into female athymic nude mice between 6 to 8 weeks of age. When tumors reached the appropriate tumor volume range (typically 150-300 mm³), animals were randomized into treatment and control groups and dosing was initiated. Tumor-bearing mice were administered a single dose of test articles via intravenous injection. Animals were observed daily and tumor dimensions and body weight were measured and recorded twice weekly. Results are shown in Figure 12, and demonstrate that E02-GL-SG3932 has potent *in vivo* activity in patient derived xenograft (PDX) models. Tumor volumes were measured by digital caliper and the volumes of tumors were calculated using the following formula: tumor volume = [length (mm) x width (mm)² x 0.52, where the length and width are the longest and shortest diameters of the tumor, respectively.

[0528] Figure 13 shows the results of further assessment of the *in vivo* anti-tumor response to E02-GL-SG3932 by determining the tumor volume changes following treatment, again demonstrating anti-tumor activity of E02-GL-SG3932 in PDX models after a single i.v. injection. Tumor volume at the beginning of treatment is referred as the initial tumor volume (ITV); tumor volume at the time showing maximal response to the ADC treatment is referred to as the end tumor volume (ETV). If ETV is less than ITV, the anti-tumor response is calculated as follows: [(ETV-ITV)/ITV] x 100. Otherwise, the anti-tumor response is expressed as percent tumor volume change in the treatment arm relative to the vehicle control arm: 100-[1-((ETV-ITV)_{treatment}/(ETV-ITV)_{vehicle}) x 100].

Example 12

E02-GL-topo I inhibitor ADCs have similar potency
in MX-1 cells and HT29-derived models *in vitro* and *in vivo*

MX-1 Model

[0529] The MX-1 xenograft model was performed at Crown Biosciences, (Taicang, China). The study was conducted in accordance with both the CRO and AstraZeneca IACUC guidelines. The MX-1 tumor cells were maintained *in vitro* in RPMI-1640 medium supplemented with 10 % fetal bovine serum at 37 °C in an atmosphere of 5% CO₂ in air. Exponentially growing cells were harvested and 5×10⁶ cells (in 0.1 ml volume of PBS:matrigel=1:1) were implanted in the right flank of female BALB/c nude mice. Immediately prior to start of treatment, mice were randomized into treatment groups (mean tumor volume = 153mm³) using the “Matched distribution” randomization method (Study DirectorTM software). All antibody-drug conjugates were diluted in buffer (25mM Histidine, 7% Sucrose, 0.02% PS80, pH 6.0), immediately prior to administration. The ADC’s were administered as a single i.v. dose. Tumor and body weight measurements were recorded twice a week and tumor volume calculated using the equation length (mm) x width (mm)²/2. Animals were monitored daily for morbidity and mortality. Results are shown in Figure 14A.

HT29+huB7-H4 (clone 4)

[0530] The HT29+huB7-H4 clone 4 cell line was maintained *in vitro* in McCoy’s Modified 5A medium supplemented with 10 % fetal bovine serum at 37 °C in an atmosphere of 5% CO₂ in air. Exponentially growing HT29+B7-H4 Clone 4 cells were harvested and 5×10⁶ cells (in 0.2 ml volume of PBS:Cultrex=1:1) implanted into the right flank of female CB-17 SCID mice. Mice were randomized into treatment groups immediately prior to treatment (mean tumor volume = 250 mm³) using the “Deterministic” randomization method (Study DirectorTM software). All antibody-drug conjugates were diluted in buffer (25mM Histidine, 7% Sucrose, 0.02% PS80, pH 6.0), immediately prior to administration. The ADC’s were administered i.v. as a single dose. Tumor and body weight measurements were recorded twice a week and tumor volume

calculated using the equation length (mm) x width (mm)²/2. Animals were monitored daily for morbidity and mortality. Results are shown in Figure 14B.

EXAMPLE 13

In vitro cytotoxicity of cyno B7-H4 transfected cells (E02-GL-SG3249)

- [0531] The clone E02-GL was conjugated to the cytotoxin SG3249 (providing E02-GL-SG3249), and tested for the ability to target and kill both cynomolgus B7-H4 transfected and non-transfected B7-H4 cells. For convenience, the antibody tested in this example comprised the Maia heavy chain backbone (e.g. having a cysteine insertion providing an additional site to which SG3249 can conjugate, see SEQ ID NO: 48) was used. No loss of efficacy occurs where other heavy chain backbones (e.g. lacking such cysteine insertion, for example see SEQ ID NO: 52) is used. The antibody R347 conjugated to SG3249 was used as a control.
- [0532] “E02-GL” antibody has the CDR sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined). For example, E02-GL in these examples comprises a VH chain sequence of SEQ ID NO: 45, e.g. a germlined version of SEQ ID NO: 43.
- [0533] The transfected cells were readily targeted and killed by the E02-GL-SG3249 conjugate, which had an IC₅₀ of 0.6721ng/ml. No significant killing following addition of the E02-GL-SG3249 conjugate was observed in the non-transfected cells (see Figure 15).

EXAMPLE 14

E02-GL mode of action

Monitoring internalisation kinetics of E02-GL in live cells

- [0534] E02-GL conjugated to fluorescence marker AF647 was used to treat live cells. At 0 mins following treatment, clusters of fluorescence were noted on the cell membranes (indicative of binding to B7-H4 present on the membrane). Time course analysis showed that the number of fluorescent spots within the cells increased steadily over time,

indicating internalisation of the antibody together with the receptor antigen. Co-visualisation with Lamp1-AF488 (a marker of lysosomes) showed significant overlap, indicating that the bound antibody is internalised by endocytosis (see Figure 16A-16C).

[0535] The observed internalisation of the antibody upon binding with the target antigen is highly advantageous, as such internalisation is generally considered a prerequisite to achieving the desired ADC effect.

[0536] “E02-GL” antibody has the CDR sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined). For example, E02-GL in these examples comprises a VH chain sequence of SEQ ID NO: 45, e.g. a germlined version of SEQ ID NO: 43.

E02-GL-SG3932 ADC: Mechanism of action

[0537] Following treatment of HT29+huB7-H4 clone26 cells with NIP228, E02-GL, NIP228-SG3932, E02-GL-SG3932, or the warhead SG3924 as a control (10 ug/ml for the mAbs or ADCs and 10nM for the warhead), lysate was prepared and subjected to western blot analysis with antibodies to pATR, ATR, pChk1, Chk1, pATM, ATM, pChk2, Chk2, pH2AX, H2AX and actin (loading control) – see Figure 17A. Results demonstrate that the Topoisomerase I poison warhead (SG3924) of E02-GL-SG3932 activates ATM and ATR signalling, indicating E02-GL-SG3932 treatment leads to double strand DNA breaks *in vitro*.

[0538] Following treatment of MX-1 cells with NIP228, E02-GL, NIP228-SG3932, E02-GL-SG3932, or the warhead SG3924 as a control (10 ug/ml for the mAbs or ADCs and 10nM for the warhead), lysate was prepared and subjected to western blot analysis with antibodies to pATR, ATR, pATM, ATM, pH2AX, H2AX and actin (loading control) – see Figure 17B. Results demonstrate that the Topoisomerase I poison warhead (SG3924) of E02-GL-SG3932 activates ATM and ATR signalling, indicating E02-GL-SG3932 treatment leads to double strand DNA breaks *in vitro*.

METHOD:

[0539] HT29+huB7-H4 clone 26 and MX-1 cells were plated in 6-well plates at a density of 500,000 and 1,500,000 cells per well respectively in medium containing 10% heat-

inactivated FBS. The next day, the plating medium was removed and cells were subjected to incubation with HT29+huB7-H4 clone26 cells with NIP228, E02-GL, NIP228-SG3932, and E02-GL-SG3932 in complete medium at a concentration of 10 µg/mL. The warhead SG3924 was used at 10 nM as a control. After 72 hours, cells were washed once with phosphate-buffered saline (PBS) and then lysed by adding Laemmli Reducing buffer (loading buffer Boston BioProducts). After a brief incubation, cell lysates were collected, equal amounts were loaded onto Bis NuPAGE Novex Bis-Tris gels (Invitrogen) and proteins transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen). Membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 (Sigma) in Tris-buffered saline pH 7.4 (TBST) and incubated overnight at 4°C with antibodies from Cell Signaling to pATM-Ser1981 (#4526), ATM (#2873), pATR-Thr1989 (#58014), ATR (#13934), pChk1-Ser345 (#2348), Chk1 (#2360), pChk2-Thr68 (#2197), Chk2 (#3440), pH2AX-Ser139 (#2577) and H2AX (#2595). An antibody to actin (A1978, Sigma) was used to ensure equal amount of protein was loaded across all wells. Membranes were washed in 0.1% Tween 20 in TBS and then incubated for 1 hour with horseradish peroxidase (HRP)-conjugated streptavidin secondary antibodies (GE Healthcare). After washing, protein bands were detected by using SuperSignal West Femto Chemiluminescent substrate and SuperSignal West Pico Chemiluminescent substrate (Pierce/Thermo Scientific). The ImageQuant LAS4000 instrument (GE Healthcare) was used to capture and analyze images.

E02-GL-SG3249 ADC: Mechanism of action

[0540] Following treatment of HCC1569 cells with E02-GL-SG3249, and SG3199 as a control (at 100ng/ml and 100pM, respectively), lysate was prepared and subjected to western blot analysis with antibodies to pATR, ATR, pChk1, Chk1, pRPA32, RPA32, pATM, ATM (all involved in ATR signalling), pChk2, Chk2, pKAP1, KAP1 (all involved in ATM signalling), pDNA-PK, DNA-PK, pH2AX, H2AX, pBRCA1, BRCA1 (all involved in DNA double strand break), pFANCD2 and GAPDH (load control). Negative controls were PBS treatment alone.

[0541] Results demonstrate that the PBD dimer warhead (SG3199) of E02-GL-SG3249 activates ATM and ATR signalling, indicating E02-GL-SG3249 treatment leads to double strand DNA breaks *in vitro* (see Figure 17C).

Caspase 3/7 activity

[0542] SKBR-3 cells were treated with E02-GL-SG3249, SG3199 and Olaparib (control) and monitored for caspase 3/7 activity (e.g. apoptosis) by IncuCyte. A dose dependent increase in the level of caspase 3/7 activity was observed (see Figure 18).

EXAMPLE 15

In vitro activity of E02-GL-SG3249 and Warhead SG3199 on tumour cells

[0543] A number of cancer cell lines were treated with clone E02-GL-SG3249 (e.g. conjugated to a cytotoxin), as outlined in Table 15. Results are shown in Figure 19.

Table 15.

Cell line	COMMENTS	E02-GL MESF
HT29	B7-H4 negative	2,101
HT29 hB7H4 clone 4		71,928
HT29 hB7H4 clone 44		147,125
HT29 hB7H4 clone 26		429,398
HEK293 JI parental	B7-H4 negative	3,266
HEK 293 JI hB7H4 pool	Has some negative cells in pool	2,481,266
HCC1954		47,173
SKBr3		78,343
Zr75-1		80,699
HCC1569		112,608
MDA-MB-468		92,157
OVCAR4		261,997

EXAMPLE 16

E02-GL-SG3249 causes bystander killing of tumor cells *in vitro*

[0544] HT29 B7-H4 expressing cells were treated with E02-GL-SG3249 for 4 days, after which the conditioned medium was removed and added to HT29 WT (i.e. not expressing B7-H4). A rapid decrease in cell viability was observed following addition of the conditioned medium, which was not observed in the non-treated control (see Figure 20).

[0545] “E02-GL” antibody has the CDR / VH sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined).

EXAMPLE 17

E02-GL-SG3249 suppresses growth of tumour xenografts

[0546] Tumour xenografts were prepared on mice using the following cancer cell lines:

- OVCAR4 (Cisplatin refractory ovarian cancer; high B7-H4 expression)
- HCC1569 (HER2 positive breast cancer; heterogeneous B7-H4 expression)
- MDA-MB-468 (Triple negative breast cancer; low B7-H4 expression)

[0547] “E02-GL” antibody has the CDR / VH sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined).

[0548] A significant decrease in tumour volume was observed compared to the control (vehicle only), which was surprisingly also the case for low-level B7-H4 expressing TNBC tumours (see Figure 22), indicating high potency of E02-GL-SG3249 at suppressing tumour growth.

EXAMPLE 18

E02-GL-SG3249 causes bystander killing of tumour cells *in vivo*

[0549] B7-H4 heterogeneous tumours were generated by co-implantation of B7-H4 expressing HT29 cell lines with non-expressing HT29 cells (1:1 ratio). The heterogeneous nature of these xenografts did not prevent their growth suppression by E02-GL-SG3249, which was pronounced (see Figure 23). This is highly advantageous, as the inventors have found B7-H4 to be heterogeneously expressed within tumours.

EXAMPLE 19

E02-GL ADCs have superior *in vitro* cytotoxicity of B7-H4 expressing cells compared with “1D11”

[0550] The clone E02-GL (ZY0EQD_E02-germlined (GL)) was conjugated to the cytotoxin SG3249 (providing an E02-GL-SG3249 ADC), or to AZ1508 (providing E02-GL-AZ1508) and compared with a Genentech “1D11” ADC (1D11 conjugated to (A114C-) MMAE) for the ability to target and kill B7-H4 expressing cells.

[0551] “E02-GL” antibody has the CDR / VH sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined).

[0552] Cytotoxicity assays (see Materials and Methods, above) demonstrated that E02-GL ADCs (E02-GL-SG3249) had superior cytotoxicity potency when compared with 1D11 ADCs (1D11 conjugated to (A114C-) MMAE) – see Figure 21.

[0553] ADC titrations were as follows:

1D11-A114C-MMAE	100µg/ml	25 µg/ml	6.25 µg/ml	1.56 µg/ml	390 ng/ml	98 ng/ml	24.5 ng/ml	6.12 ng/ml	1.53 ng/ml	382 pg/ml
E02-maia-GL-SG3249	4 µg/ml	1 µg/ml	250 ng/ml	65 ng/ml	16 ng/ml	4 ng/ml	1 ng/ml	250 pg/ml	62.5 pg/ml	16 pg/ml

EXAMPLE 20

E02-GL-SG3249 has potent *in vivo* activity in patient derived xenograft (PDX) models

[0554] Patient derived xenograft models were generated using cancer cell lines with varying levels of B7-H4 expression, as outlined in Table 16. Suppressed tumour growth was observed in all models (see Figure 24). “E02-GL” antibody has the CDR / VH sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined).

Table 16.

Model	Cancer type	% Tumour Growth Inhibition (TGI) *
HBCx-10	Breast (Triple negative)	90
CTG-012	Breast (Triple negative)	90
HBCx-11	Breast (Triple negative)	80
T330	Breast (Triple negative)	70
HBCx-9	Breast (Triple negative)	70
T168	Breast (HR+)	60
T272	Breast (HR+)	30
CTG-1106	Breast (Triple negative)	30
CTG-052	Breast (Triple negative)	30
CTG-1086	Ovarian	20
CTG-1153	Breast (Triple negative)	<10
CTG-0897	Ovarian	<10
CTG-017	Breast (Triple negative)	<10
T226	Breast (Her2+)	<10
CTG-0992	Ovarian	0

* % Tumour Growth Inhibition (TGI) 0.3 mg/kg E02-GL-SG3249 vs vehicle; Least square mean (LSM) & TGI = 100*(LSM vehicle - LSM 'E02-GL-SG3249')/LSM Vehicle

+++ = high positive expression; ++ & + = low positive expression

EXAMPLE 21

E02-GL-SG3249 causes double strand breaks in tumor xenografts

[0555] Tumour xenograft models were generated (using HCC1954 cells), and treated with E02-GL-SG3249. "E02-GL" antibody has the CDR / VH sequences (e.g. corresponds to) of ZY0EQD-E02 ("GL" means the antibody has been germlined).

[0556] Immunohistochemistry was then performed to determine gamma H2AX (marker of double strand breaks) following treatment. Increased numbers of gamma H2AX positive tumour cells were observed up to 10 days following E02-GL-SG3249 treatment (see Figure 25).

EXAMPLE 22

Analysis of the affinity of E02_GL using KinExA 3200

[0557] Experiments to obtain estimates of affinity (K_D) of clone E02_GL anti-B7H4 Fab with human B7H4 was performed using KinExA technology. “E02-GL” antibody has the CDR / VH sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined).

[0558] A range of human B7H4 concentrations were equilibrated overnight in the presence of 5, 10 or 45 nM fixed concentrations of the Fab at 25°C. These were analysed on the KinExA 3200 and the data sets fitted globally (N-curve analysis). Results are summarised in Table 17.

Table 17.

Antibody	B7H4	Best estimate of K_D	N-curve analysis, comments
E02_GL	human	1.2 nM	$K_D = 1.21$ nM with 95% Confidence Intervals = 0.556 - 2.22 nM. 62% of h B7H4 epitopically active, 95% Confidence Intervals = 51-77%

[0559] The 62% h B7H4 activity figure matches well with R_{max} calculations in accompanying Biacore based affinity assessments of E02_GL.

EXAMPLE 23

Comparison of E02_GL binding to B7-H4 with mAb “1D11”

[0560] Clone E02_GL was subjected to ELISA analysis for binding to human and mouse B7-H4. Binding was compared directly with the mAb 1D11 (Genentech), and R347 isotype control. “E02-GL” antibody has the CDR / VH sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined).

[0561] E02_GL shows higher binding affinity for human B7-H4 compared to mouse B7-H4 (see e.g. Figure 2E). Advantageously, E02-GL shows significantly stronger binding (affinity) to human B7-H4 than the Genentech “1D11” mAb (which shows much weaker binding), as demonstrated by both ELISA and FACs analysis – see Figures 7 and 8,

respectively. Indeed, Genentech “1D11” mAb binds human B7-H4 at similar levels (low) to mouse B7-H4 (showing that “1D11” has less specificity for the human protein, when compared with E02_GL).

EXAMPLE 24

Imaging studies showing tumour localisation of E02_GL

- [0562] *In vivo* imaging studies were performed as outlined under Materials and Methods (above), namely with 800CW labelled antibody (E02_GL). “E02-GL” antibody has the CDR / VH sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined).
- [0563] Preferential localization of B7-H4 Ab (E02_GL) to the B7-H4 expressing tumour compared to the WT tumour was observed for the HT29 on days 3, 7, 14; the CT26 on day 3 and 7, and $p=0.06$ on day 10; and the 4T1 on day 7 - see Figures 26 and 27.
- [0564] Total radiant efficiency produced the clearest results. Similar trends were observed when normalizing to tumour volume.

EXAMPLE 25

Toxicology studies with E02-GL-SG3249

- [0565] E02-GL-SG3249 was administered to male cynomolgus monkeys (N = 2/dose level). The “E02-GL” antibody has the CDR / VH sequences (e.g. corresponds to) of ZY0EQD-E02 (“GL” means the antibody has been germlined).
- [0566] No abnormal toxicities were observed, and toxicities were consistent with other similar PBD-ADCs (by monitoring standard target organs: kidney, bone marrow, skin; no evidence of target-related effects).

PHARMACOLOGY STUDIES

- [0567] *In vitro* and *in vivo* pharmacology studies were undertaken to further characterize the effects and mechanism of action of the E02-GL antibody (the E02-GL antibody and antigen-binding Fab intermediate thereof, are referred to herein as “E02-INT”). The E02-

GL-SG3932 ADC -GL-SG3932 is an ADC directed against B7-H4 and is comprised of an anti-B7-H4 human IgG1 κ monoclonal antibody (i.e., E02-GL) conjugated via a cleavable maleimide-PEG8-valine-alanine linker (cleavable mal-PEG8-val-ala linker) to a TOP1i warhead. The TOP1i warhead is referred to herein as SG3924. The TOP1i drug is covalently bound to native cysteines in the antibody through a thiosuccinimide linkage, with approximately 8 drugs bound per antibody (i.e., DAR of 8). A schematic of the E02-GL-SG3932 ADC is shown in Figure 29A. The key features that differentiate E02-GL-SG3932 ADC from competitor ADCs are shown in Figure 29B.

[0568] The E02-GL-SG3932 characteristics are set forth below and further described elsewhere herein:

- mAb E02-INT: Specific binding to human and cynomolgus monkey B7-H4 (3.7 nM, 3.94 nM affinity respectively)
- Warhead SG3932
- Linker-warhead: cleavable mal-PEG8-val-ala linker-SG3932

In Vitro Studies

EXAMPLE 26

B7-H4 Expression Profiling by Immunohistochemistry

[0569] The expression profile of B7-H4 was assessed using a validated IHC protocol to demonstrate B7-H4 expression in FFPE normal cynomolgus monkey and human tissues and human tumor tissues.

[0570] B7-H4 was shown to be expressed in a limited number of normal human tissues, and when present, was generally expressed in < 10% of the total cells in the sample (except for fallopian tube and lung bronchus), restricted towards ductal or tubular epithelium, and primarily located on the apical luminal membrane (Table 18). A similar B7-H4 expression pattern was detected in normal cynomolgus monkey tissues (Tables 2 and 19), where B7-H4 was shown to be expressed in a limited number of tissues, with an expression pattern that is restricted towards ductal or tubular epithelium and primarily located on the apical luminal membrane and within cytoplasm. Representative images of

immunohistochemical staining of B7-H4 in selected normal human and cynomolgus monkey tissues are shown in Figure 30.

Table 18. Results of B7-H4 Expression in Human Normal Tissue Using Immunohistochemistry

Tissue	Donor Number demonstrating B7-H4 IHC Staining	Relative proportion of Tissue demonstrating positive B7-H4 staining	Intensity ^a	Cellular Localisation	Details
Breast	6/6	< 10%	+ /+++	Luminal m	Ductal epithelial cells
Epididymis	3/3	< 5%	++	Luminal m	Luminal membrane in ducts
Fallopian Tube	3/3	< 30%	++ /+++	Luminal m	Ductal epithelial staining
Kidney	11/11	< 5%	++ /+++	Luminal m > c	Luminal membrane and some cytoplasmic staining in occasional tubules
Liver	2/3	1%	+ /++	Luminal m	Luminal membrane of occasional small bile ducts
Lung - Bronchus	7/7	< 20%	++ /+++	Luminal m > c	Basal cells in bronchial epithelium and occasional ductal epithelium from bronchial glands

Tissue	Donor Number demonstrating B7-H4 IHC Staining	Relative proportion of Tissue demonstrating positive B7-H4 staining	Intensity ^a	Cellular Localisation	Details
Oesophagus	1/3	1%	+	Luminal m	Basolateral membrane of occasional epithelial cells in sub-mucosal glands
Ovary	3/6	1%	+/++	c > m	Individual mature oocyte showing granulosa cells
Pancreas	8/8	< 5%	+/+++	Luminal m	Luminal membrane and cytoplasm of centroacinar cells and luminal staining of intercalated ducts
Pituitary	3/3	< 5%	++/+++	m > c	Occasional localised cells within Pars Intermedia cells showing staining of colloid in follicles and Rathke's Cleft epithelial cells
Prostate	3/3	< 10%	++/+++	m > c	Basal epithelial cells in some ducts
Seminal Gland	3/3	< 10%	++/+++	Luminal m	Several ductular epithelial cells

Tissue	Donor Number demonstrating B7-H4 IHC Staining	Relative proportion of Tissue demonstrating positive B7-H4 staining	Intensity ^a	Cellular Localisation	Details
Skin	15/16	< 5%	++/+++	Luminal m	Membrane of occasional sweat gland duct epithelium, membrane of hair follicle epithelium
Ureter	3/3	< 5%	+ / ++	m > c	Cytoplasmic staining in some basal urothelial cells
Urinary Bladder	8/8	< 10%	++/+++	Luminal m > c	Apical aspect of urothelial cells
All other tissues demonstrated no positive B7-H4 IHC staining			Adrenal, Aorta, Artery, Bone Marrow, Brain, Caecum, Colon, Duodenum, Eye, Heart, Ileum, Jejunum, Lung, Lymph Node, Nerve, Rectum, Salivary Gland, Skeletal muscle, Spinal cord, Spleen, Stomach, Testis, Thymus, Thyroid, Tongue, Vagina		

c: cytoplasmic; m: membrane.

^a All stained slides were reviewed and scored by a Pathologist assessing both the proportion of cells expressing B7-H4, the intensity of staining, and cellular localisation of staining. Intensity is reported as weak (+), moderate (++), or strong (+++).

[0571] B7-H4 expression was assessed in a range of human tumor tissues using IHC and scored by a pathologist. A summary of the human tumor tissue expression is detailed in Table 19, with representative images shown in Figure 31.

Table 19. Results of B7-H4 Expression in Human Tumor Tissue Using Immunohistochemistry

Tumor Indication	Proportion of Donors (%) with HIGH B7-H4+ tumors ^a	Proportion of Donors (%) with LOW B7-H4+ tumors ^b	Proportion of Donors (%) with Positive B7-H4+ tumors ^c	Total Proportion of Donors (%) with B7-H4+ tumors ^d	Total Number of Donors
Endometrial Adenocarcinoma	42.4	45.5	6.1	93.9	66
Breast (TNBC)	28.8	35.1	12.6	76.6	111
Breast (ER+)	32.6	32.3	10.8	75.7	288
Breast (HER2+)	46.7	23.3	13.3	83.3	30
Ovarian (Serous Papillary)	49.3	28.0	6.7	84.0	75
Cholangio carcinoma	33.3	30.3	21.2	84.8	66
NSCLC-SCC	15.0	27.5	23.5	66.0	153

^a HIGH B7-H4: human tumor tissue samples containing 50% to 100% of B7-H4 positive cells.

^b LOW B7-H4: human tumor tissue samples containing 5% to 49% of B7-H4 positive cells.

^c Positive B7-H4: human tumor tissue samples containing 1% to 4% of B7-H4 positive cells.

^d Indicates the proportion of human tumor tissue samples containing 1% to 100% of B7-H4 positive cells.

[0572] This example demonstrates that B7-H4 was expressed in many types of human cancers, including breast cancer, cholangiocarcinoma, endometrial carcinoma, non-small cell lung cancer squamous cell carcinoma, and ovarian serous carcinoma.

EXAMPLE 27

Sequence Homology Comparison of Human B7-H4 with Orthologs in Cynomolgus Monkey, Rhesus Monkey, Mouse, and Rat

[0573] Amino acid sequences for human B7-H4 were identified and aligned to orthologs in cynomolgus monkey, rhesus monkey, mouse and rat. Percentage identity was calculated based on the results of these alignments. B7-H4 is well conserved among non human primates; human B7-H4 (hB7-H4) shares 98% and 99% sequence identity for both full-length and extracellular domain regions, respectively, in both cynomolgus monkey (cyB7 H4) and rhesus monkey (rhB7-H4) (Figure 32). Rodent species are less conserved; mouse and rat B7-H4 share 87% and 86% sequence identity with hB7-H4 for full length B7-H4, respectively, and 90% and 89% sequence identity in the extracellular domain with hB7-H4, respectively.

[0574] This example demonstrates that the high amino acid sequence identity in B7-H4 across human and non-human primates suggests that E02-GL-SG3932 is likely to bind to cynomolgus monkey and rhesus monkey B7-H4. In contrast, the amino acid identity comparison between human, mouse and rat B7-H4 is lower, indicating that binding of E02-GL-SG3932 to murine or rat B7-H4 is less likely.

EXAMPLE 28

E02-GL-SG3932 Stability After Fifteen Days Incubation in Mouse, Rat, Cynomolgus Monkey, and Human Serum

[0575] ADCs bearing drug linked to cysteines via a thiosuccinimide are known to exhibit some drug loss in physiological milieu due to the retro-Michael reaction. This process regenerates the cysteine used for conjugation and the maleimide-bearing drug, thus reducing the DAR of the ADC over time. This deconjugation process is a known property of ADCs containing drug linked to antibodies through thiosuccinimides.

[0576] The stability of E02-GL-SG3932 in cynomolgus monkey, mouse, and rat serum was evaluated using immunoprecipitation followed by reduced reverse phase mass spectrometry (rLCMS). Measurements indicate that less than 20% drug loss occurs from E02-GL-SG3932 after incubation in mouse, rat and cynomolgus monkey serum, with

84.5%, 83.5% and 82.0% of SG3924 remaining attached to E02-GL-SG3932 at day 15 in mouse, rat, and cynomolgus monkey serum samples, respectively. The stability of E02-GL-SG3932 in human serum was evaluated by immunocapture using human B7-H4 coated resin, followed by rLCMS (ONC8205-012). Measurements indicate that drug loss after incubation in human serum was modest, with 81% of SG3924 remaining attached to E02-GL-SG3932 at day 15.

[0577] This example indicates the mechanism of drug release is deconjugation through the retro-Michael reaction and not linker cleavage, consistent with other ADCs prepared by maleimide conjugation to cysteine amino acids involved with interchain disulfides.

EXAMPLE 29

Binding Affinity of Antibody Intermediate E02-INT Fab for Recombinant B7-H4 Antigens

[0578] The binding affinity of anti-B7-H4 antibody E02-INT Fab for recombinant human, cynomolgus monkey, and mouse B7-H4 variants were determined by surface plasmon resonance (SPR). The dissociation constant (K_D) values, shown in Table 20, demonstrate that E02-INT Fab binds to immobilized human and cynomolgus B7-H4 with similar affinities. In contrast, the affinity of E02-INT Fab for mouse B7-H4 antigen is approximately 100-fold lower than for human B7-H4 antigen.

Table 20. Binding Affinity of Anti-B7-H4 Antibody Intermediate E02-INT Fab to Human, Cynomolgus Monkey and Mouse B7-H4 Determined by SPR

Species	K_D
Human	3.70 nM
Cynomolgus Monkey	3.94 nM
Mouse	405 ($\pm$ 15) nM

Fab: antigen binding fragment

EXAMPLE 30

Binding Affinity of E02-GL-SG3932 for Human FcRn and Fc γ Receptors

[0579] The binding affinity of E02-GL-SG3932 to human FcRn and Fc γ receptors was assessed by SPR. The steady state binding affinity (K_D) of human FcRn to E02-GL-SG3932 is 4360 nM. The equilibrium K_D of E02-GL-SG3932 to huFc γ RI was 4.35 nM. The equilibrium K_D of E02-GL-SG3932 to huFc γ RIIa, huFc γ RIIb, huFc γ RIIIA-158V, and huFc γ RIIIA-158F ranged from 3307 to 21640 nM.

EXAMPLE 31

Comparative Binding Affinity of Antibody Intermediate E02-INT and E02-GL-SG3932 for Recombinant Human B7-H4 Antigen

[0580] To assess whether conjugation of the topoisomerase 1 linker-warhead affected binding properties of the E02-INT antibody, the binding affinity of E02-INT and E02-GL-SG3932 were measured using a DELFIA-ELISA method and an SPR method. As shown in Figure 33, results from the DELFIA-ELISA assay indicate that E02-INT and E02-GL-SG3932 bind similarly to immobilized recombinant human B7-H4, with EC50 values of 1.98 nM and 1.71 nM, respectively. The kinetic rate constants (k_{on} and k_{off}) and equilibrium dissociation constants (K_D) of E02-INT and E02-GL-SG3932 for human B7-H4 antigen was also determined by SPR, using an antibody capture method. As shown in Table 21, E02-INT and E02-GL-SG3932 bind similarly to human B7-H4 with K_D values of 31.1 nM and 29.3 nM, respectively.

Table 21. Binding Affinity of Antibody Intermediate E02-INT and E02-GL-SG3932 to Human B7-H4 Determined by SPR

Species	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (nM)	R_{max} (RU)
E02-INT	9.38×10^4	2.92×10^{-3}	31.1	56.8
E02-GL-SG3932	9.55×10^4	2.80×10^{-3}	29.3	49.7

RU: resonance unit

[0581] The results for Examples 30 and 31 show that binding properties of the E02-INT antibody are maintained after conjugation to the topoisomerase 1 linker-warhead.

EXAMPLE 32

Cellular Binding of Antibody Intermediate E02-INT to Engineered HEK 293 Cells Expressing Human, Cynomolgus Monkey, or Mouse B7-H4

[0582] Flow cytometry was used to measure binding of antibody E02-INT to non-transduced HEK 293 Jump In TREX cells and to HEK 293 Jump In TREX cells stably expressing human, murine, or cynomolgus monkey B7-H4. Antibody E02-INT bound to HEK 293 Jump In TREX cells stably expressing human, murine and cynomolgus monkey B7-H4 but did not bind to the B7-H4-negative non-transduced HEK 293 Jump In TREX cells (Figure 34). Binding to HEK 293 Jump In TREX cells expressing murine B7-H4 was reduced compared to cells expressing human or cynomolgus monkey B7-H4.

EXAMPLE 33

Cellular Binding of Antibody Intermediate E02-INT and E02-GL-SG3932 to Human Breast Cancer Cell Lines and to HT29 Cells Stably Expressing Human B7-H4

[0583] Flow cytometry was used to measure binding of antibody E02-INT and E02-GL-SG3932 to the human breast cancer cell lines MX-1 and MDA-MB-468, and to engineered HT29 colon cancer cells stably expressing human B7-H4. As shown in Figure 35, E02-INT and E02-GL-SG3932 bound similarly to HT29 cells stably expressing human B7-H4 (HT29-huB7-H4 Clone 4 and HT29-huB7-H4 Clone 26) but did not bind to the B7-H4-negative non-transduced HT29. E02-INT and E02-GL-SG3932 also bound to MX-1 and MDA-MB-468 cells, demonstrating that the antibody intermediate and ADC can recognize endogenously-expressed B7-H4 in human cancer cell lines. The binding of E02-INT and E02-GL-SG3932 was comparable,

[0584] Examples 32 and 33 demonstrate that the cellular binding properties of the parental antibody are maintained after conjugation to the topoisomerase 1 linker-warhead.

EXAMPLE 34

In-vitro Cytotoxicity of E02-GL-SG3932

[0585] The effect of E02-GL-SG3932 treatment on cell viability was determined using a target-negative human colon cancer cell line HT29, the engineered human colon cancer cell line HT29-huB7-H4 Clone 26, and a human breast cancer cell line, MX-1, using a CellTiter-Glo assay. As shown in Figure 36, E02-GL-SG3932 was cytotoxic to the B7-H4 expressing HT29-huB7-H4 Clone 26 and MX-1 cells, with an IC₅₀ value of 0.036 µg/mL and 0.029 µg/mL, respectively. In contrast, no difference in activity was observed between E02-GL-SG3932 and the isotype-matched control ADC (NIP228-SG3932) in the B7-H4 negative HT29 cell line, suggesting that E02-GL-SG3932 can specifically kill cancer cells expressing human B7-H4.

EXAMPLE 35

In-vitro Cytotoxicity of E02-GL-SG3932

[0586] When IgG antibodies bind cell surface antigens via their Fab domains, the Fc portion of the antibodies can engage with FcγRIIIa on natural killer cells. The interaction of the Fc domain with FcγRIIIa induces cross-linking of the FcγRs, which triggers the release of cytotoxic granules containing perforin and granzymes, leading to the death of the target cell, a process called antibody-dependent cellular cytotoxicity (ADCC). The potential of antibody E02-INT and E02-GL-SG3932 to initiate ADCC activity was evaluated utilizing isolated primary human NK cells and the human breast cancer SKBR3 cell line as the target cell. In this assay, both E02-INT and E02-GL-SG3932 yielded a significant increase in ADCC activity beyond untreated co-cultured cells (Figures 36A-36C). E02-GL-SG3932 activity is slightly reduced when compared to E02-INT, however this difference is not statistically significant, suggesting that E02-INT and E02-GL-SG3932 can elicit ADCC activity *in vitro*.

[0587] Next, human breast cancer SK-BR-3 cells were co-cultured with NK cells isolated from six healthy donors in the presence of 1 µg/mL E02-GL-SG3932, antibody intermediate E02-INT, isotype-matched control antibody NIP228, and the isotype-matched control ADC NIP228-SG3932 (Figure 37). ADCC activity was evaluated by

Incycyte® Annexin V Dye binding of extracellular surface exposure of phosphatidyl serine on apoptotic cells, yielding a bright and stable red fluorescent signal. The fold-change was based on maximum cell death of non-drug treated NK/SK-BR-3 co-cultured cells. Maximum cell death was calculated by dividing the average red objects in experimental wells by the average maximum cell death in Staurosporine treated SK-BR-3 cells.

EXAMPLE 36

Internalization and Lysosomal Trafficking of Antibody E02-INT

[0588] The internalization and intracellular trafficking properties of antibody E02-INT was evaluated using a quantitative live cell imaging assay in MX-1 human breast cancer cells and HT29 huB7-H4 Clone 26 human colon cancer cells overexpressing B7-H4. Time-lapse sequence images showed that in both human breast cancer (MX-1) and colon cancer (HT29-huB7-H4 Clone 26) cell lines, intense E02-INT membrane signal persists from 0 minutes to 120 minutes, with increased internalization of E02-INT by 240 minutes (Figure 38, Figure 39A and Figure 39B). Measurement of internalization kinetics across two cell lines showed internalization half-life of 127 ($\pm$ 35 SD) minutes and 102 ($\pm$ 18 SD) minutes for the HT29-huB7-H4 Clone 26 and MX-1 cells, respectively (Figure 39C).

[0589] Confocal microscopy was used to determine intracellular trafficking of E02-INT by measuring co-localization with the early endosomal marker, Early Endosome Antigen 1 (EEA1) and the lysosomal marker, lysosomal-associated membrane protein 1 (LAMP1). As shown in Figure 40, E02-INT was enriched in the LAMP1 decorated subcellular compartment with limited colocalization with EEA1, indicating that after internalization, E02-INT is trafficked to the lysosomal compartment of cells.

EXAMPLE 37

In Vitro Activation of DNA Damage Response Signaling by E02-GL-SG3932 and SG3924

[0590] Western blotting was used to evaluate DDR pathway activation in the MX-1 human breast cancer cell line and the engineered colon cancer cell line HT29-huB7-H4

Clone 26 following treatment with either E02-GL-SG3932 or its TOP1i warhead, SG3924. As shown in Figure 41, treatment of MX-1 cells with either 10 µg/mL E02-GL-SG3932 or 10 nM SG3924 resulted in activation of the ATM signalling pathway, evident by an increase in phosphorylation of ATM (Ser 1981) as early as 24-hours and persisting over the 72-hour treatment period. Similarly, E02-GL-SG3932 and SG3924 induced activation of ATR (Thr 1989 phosphorylation), observed at the 48-hour and 72-hour timepoint. An increase in γH2AX was observed following treatment after 24 hours and persisting through the 72-hour treatment period, indicating DNA damage.

[0591] The effect of E02-GL-SG3932 or its TOP1i warhead on DDR signalling was also examined in the colorectal cancer cell line HT29-huB7-H4 Clone 26, engineered to express B7-H4. As shown in Figure 42, treatment with either 10 µg/mL E02-GL-SG3932 or 10 nM SG3924 resulted in an activation of the DDR signalling pathway, evidenced by an increase in phosphorylation of ATR (Thr 1989), its downstream target Chk1 (Ser 345) and Chk2 (Thr 68). This increase in phosphorylation persisted over the 72-hour treatment period. Similarly, activation of ATM (Ser 1981) and its downstream target KAP1 (Ser 824), was observed after treatment with E02-GL-SG3932 and TOP1i warhead at the 48-hour and 72-hour timepoint. An increase in γH2AX was observed following treatment after 48 hours and persisting through the 72-hour treatment period, indicating DNA damage.

[0592] Taken together, these results confirmed, in two different cell lines, that E02-GL-SG3932 activates a DDR pathway consistent with the mechanism of action of its TOP1i warhead.

In Vivo Studies

[0593] The *in vitro* studies demonstrated the ability of E02-GL-SG3932 to bind to human and cynomolgus monkey B7-H4 with similar affinity, that E02-GL-SG3932 specifically binds, and is cytotoxic to, B7-H4 expressing tumor cells and could elicit modest ADCC activity in an isolated primary NK cell co-culture assay, that the antibody intermediate of E02-GL-SG3932 (E02-INT) is internalized into tumor cells, and is trafficked to the lysosomal compartment, and that treatment with E02-GL-SG3932 or its TOP1i warhead

activates the DDR signaling pathway in B7-H4 expressing cell lines. To further elucidate the mechanism of action of E02-GL-SG3932 and determine if these *in vitro* findings translate into antitumor activity, *in vivo* mouse models were employed.

EXAMPLE 38

Pharmacodynamic Study of E02-GL-SG3932 in the HT29-huB7-H4 Clone 26 Xenograft Model

- [0594] Pharmacodynamic effects following E02-GL-SG3932 treatment were evaluated in a human tumor xenograft mouse model using immunodeficient CB-17 SCID mice. Animals were inoculated subcutaneously (SC) with the human colon cancer cell line HT29-huB7-H4 Clone 26, engineered to express human B7-H4 and after tumors grew in volume to approximately 250 to 300 mm³, animals were randomized and each mouse received an IV injection of either E02-GL-SG3932 or control articles. Tumors were collected at designated timepoints, fixed in 10% neutral buffered formalin and subsequently processed and embedded into paraffin blocks. IHC and image analysis techniques were used to examine human IgG, γ H2AX foci, cleaved caspase-3, and epithelial cell density in tumor samples over time. Representative IHC images of human IgG, γ H2AX, and cleaved caspase-3 in tumors collected 168 hours after a single IV administration of 7 mg/kg E02-GL-SG3932 or isotype-matched control ADC NIP228 SG3932 are shown in Figures 43A-43F.
- [0595] As shown in Figures 44A-44D, dose-dependent accumulation of E02-GL-SG3932 was observed in tumor cells over time, as visualized by a human IgG IHC assay. Accumulation of E02-GL-SG3932 correlated with increased positive staining for γ H2AX foci, signifying induction of DNA damage. Elevated cleaved caspase-3 and an overall decrease in epithelial cell density were observed over time in E02-GL-SG3932-treated tumors compared to the control.
- [0596] Taken together, these data suggest that E02-GL-SG3932 binds to B7-H4 on tumor cells, causing DNA damage and apoptotic cell death.

EXAMPLE 39

Antitumor Efficacy of E02-GL-SG3932 in Subcutaneous Human Breast and Colon Cancer Xenograft Models *In vivo* efficacy, PDX

[0597] The antitumor activity of antibody intermediate E02-INT and E02-GL-SG3932 was investigated in human tumor xenograft mouse models using immunodeficient CB-17 SCID mice.

HT29 or HT29-huB7-H4 Clone 26 xenograft models

[0598] E02-GL-SG3932 was first evaluated in a pair of colon cancer cell line xenograft models in two separate studies; HT29, which is B7-H4 negative, and HT29-huB7-H4 Clone 26, which is derived from the HT29 cell line and engineered to express human B7-H4. In both studies, animals were injected SC with either the HT29 or HT29-huB7-H4 Clone 26 cells and after tumors grew in volume to a mean of 178 mm³ (HT29) or 194 mm³ (HT29-huB7-H4 Clone 26), animals were randomized and each mouse received an IV injection of either E02-GL-SG3932 or control articles. As shown in Figures 45A and 45B, compared to the vehicle treated control group, neither E02-GL-SG3932 nor the isotype-matched control ADC NIP228-SG3932 significantly inhibited the growth of HT29 xenograft tumors when administered as a single IV dose at 10 mg/kg, demonstrating 12% ($p = 0.7006$) and 14% ($p = 0.6593$) TGI, respectively. In contrast, compared to the vehicle treated control group or the isotype-matched control ADC NIP228-SG3932, E02-GL-SG3932 significantly inhibited the growth of HT29-huB7-H4 Clone 26 xenograft tumors when dosed as a single IV dose at 5 mg/kg, 2.5 mg/kg and 1.25 mg/kg (Figures 46A-46C), with TGI relative to vehicle of 42% ($p < 0.001$), 37% ($p = 0.0005$), and 31% ($p = 0.0039$), respectively.

MX-1 breast cancer xenograft model

[0599] The effect of E02-GL-SG3932, the isotype-matched control ADC NIP228-SG3932, and E02-INT, the antibody intermediate of E02-GL-SG3932 was evaluated in the MX-1 breast cancer xenograft model. Animals were injected SC with MX-1 cells and after tumors grew in volume to a mean of 270 mm³, animals were randomized and each mouse received an IV injection of test or control articles. Compared to the vehicle treated

control group, E02-GL-SG3932 significantly inhibited the growth of MX-1 xenograft tumors when dosed as a single IV dose at 5 mg/kg, 2.5 mg/kg and 1.25 mg/kg (Figures 47A and 47B), resulting in TGI of 100% ($p < 0.001$), 96% ($p < 0.001$) and 98% ($p < 0.001$), respectively.

[0600] The antibody intermediate, E02-INT, dosed at 10 mg/kg, did not significantly inhibit tumor growth (TGI = -1%, $p > 0.9999$). The lack of activity of E02-INT at this high dose level in SCID mice suggests that ADCC may not be a significant contributor to the activity of E02-GL-SG3932 *in vivo* and that the antitumor effects of E02-GL-SG3932 are driven through its TOP1i warhead.

MX-1 and MDA-MB-468 breast cancer xenograft models

[0601] To further elucidate the dose-dependent effect of E02-GL-SG3932 treatment *in vivo*, dose levels ranging from 0.125 mg/kg to 2 mg/kg were evaluated in MX-1 and MDA-MB-468 breast cancer xenograft models. In both studies, animals were injected SC with either the MX-1 or MDA-MB-468 cells and after tumors grew in volume to a mean of approximately 138 mm³ (MX-1) or 120 mm³ (MDA-MB-468), animals were randomized and each mouse received an IV injection of either E02-GL-SG3932 or control articles. As shown in Figure 48, a single intravenous dose of E02-GL-SG3932 resulted in dose-dependent inhibition of MX-1 xenografts. When compared to the untreated group at day 40, E02-GL-SG3932 treatment resulted in 100% TGI at the 2 mg/kg, 1.5 mg/kg and 1 mg/kg dose levels. E02-GL-SG3932 doses of 0.75 mg/kg, 0.5 mg/kg, 0.25 mg/kg and 0.125 mg/kg resulted in dose-dependent %TGI of 99.7%, 80.1%, 52.1% and -5.5%, respectively. Similarly, a single intravenous dose of E02-GL-SG3932 resulted in dose-dependent inhibition of MDA-MB-468 xenografts (Figure 49). When compared to the untreated group at day 55, E02-GL-SG3932 treatment resulted in 97.7%, 75.2% and 66.6% TGI at the 2 mg/kg, 1.5 mg/kg and 1 mg/kg dose levels, respectively. E02-GL-SG3932 doses of 0.75 mg/kg, 0.5 mg/kg, 0.25 mg/kg and 0.125 mg/kg resulted in dose-dependent %TGI of 52.8%, 58.4%, 25.5% and 31.8%, respectively.

EXAMPLE 40
Antitumor Efficacy of E02-GL-SG3932 in Patient-derived Xenograft
Models of Triple-Negative Breast Cancer

- [0602] The antitumor activity of E02-GL-SG3932 was investigated in a panel of 26 human TNBC PDX models using immunocompromised athymic nude mice. These PDX models have been established from human tumor samples without prior *in vitro* culture and have been studied for histology, cytogenetics, genetic and other biological markers, and for their response to standard-of-care therapies. Tumor fragments were subcutaneously transplanted into mice, and once tumors grew in volume to approximately 94 to 189 mm³, animals were randomized and each mouse received a single IV injection of either E02-GL-SG3932 or control articles, at a dose of 1.25 mg/kg or 3.5 mg/kg. To assess the relationship between B7-H4 expression and efficacy, fresh untreated tumors with a volume of around 500 to 1183 mm³ from 3 additional mice from each model were collected, fixed in 10% neutral buffered formalin and subsequently processed and embedded into paraffin blocks. IHC and image analysis techniques were then used to characterize the expression of B7-H4 on tumor cell membranes in each model.
- [0603] IHC analysis demonstrated that these PDX models represented heterogenous tumor expression of B7-H4, with different levels of IHC staining intensity and proportion of tumor staining, including one model with nearly undetectable levels of B7-H4 (HBCx-15) (Figure 50).
- [0604] Tumor growth inhibition was observed after a single dose of 1.25 mg/kg E02-GL-SG3932, with 46.2% of models (12 of 26) exhibiting a reduction in tumor volume from baseline of 30% or greater (Figure 51). Of these, 75% (9 of 12) expressed elevated levels of B7-H4, with an H-score of 100 or greater (Figures 52A and 52B). A significant association between H-score classification and responder status was identified for E02-GL-SG3932 at the 1.25 mg/kg dose level (Fisher's exact tests, $p = 0.047$), suggesting that elevated levels of B7-H4 are associated with response to treatment with 1.25 mg/kg E02-GL-SG3932.
- [0605] Compared with the 1.25 mg/kg dose level, greater antitumor activity was observed after a single dose of 3.5 mg/kg E02-GL-SG3932, with 69.2% (18 of 26) of the

models tested exhibiting a reduction in tumor growth from baseline of 30% or greater (Figure 53). Of these, 66.7% (12 of 18) expressed elevated levels of B7-H4, with an H-score of 100 or greater (Figures 54A and 54B). At this dose level, the association between H-score classification and responder status was not statistically significant (Fisher's exact tests, $p = 0.073$).

[0606] Increased activity was also observed for the NIP228-SG3932 isotype ADC at this dose level, with 5 models (T330, BCX-017-LOP, T168, HBCx-15, and HBCx-6) exhibiting a reduction in tumor growth from baseline of 30% or greater (Figure 53). These 5 models have a shared characteristic; defective homologous DNA repair, defined by the presence of either a BRCA1 mutation or a negative score in a RAD51 foci formation assay, proposed as a functional measure of homologous DNA repair capacity.

[0607] This example demonstrates that administration of E02-GL-SG3932 in tumors that have elevated levels of B7-H4 expression and are defective in homologous DNA repair, defined by (1) the presence of a BRCA1 mutation, (2) a negative score in a RAD51 foci formation assay, can reduce tumor volume. Figures 53B-53E show that at low doses, E02-GL-SG3932 response correlates with B7-H4 expression level and HR-deficiency. In a separate study, PDX models treated at 7 mg/kg showed a high level of isotype-control ADC activity (Figures 60A-60B and Figure 61).

[0608] This example also demonstrates that E02-GL-SG3932 shows robust activity in HR-deficient tumors and in HR-proficient tumors with elevated B7-H4, and that HR-deficient tumors are more sensitive to damage by TOP1i and will have a lower B7-H4 expression threshold for activity.

EXAMPLE 41

Antitumor Efficacy of E02-GL-SG3932 in Patient-derived Xenograft Models of Cholangiocarcinoma

[0609] The antitumor activity of E02-GL-SG3932 was investigated in a panel of 37 human cholangiocarcinoma PDX models. In the pre-study phase, female mice of various backgrounds (athymic nude mice, Balb/c Nude or NOD/SCID) were implanted with human cholangiocarcinoma PDX fragments and allowed to grow to approximately 1000-

1500mm³ in size. These tumors were then harvested and reimplanted into study mice. When tumors reached an average tumor volume of 150-300mm³, animals were then matched by tumor volume into treatment or control groups and dosing initiated on Day 0. Each mouse received a single IV injection of E02-GL-SG3932 or a control (untreated), at a dose of 1.25 mg/kg or 3.5 mg/kg. Figures 55A-55G provide results for a first investigated panel. The results shown in Figures 56A-56K provide results for a second investigated panel.

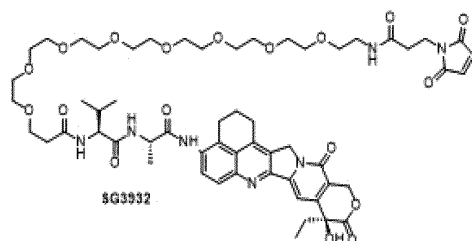
EXAMPLE 42

E02-GL-SG3932 TOP1i Linker-Warhead is Related to Wider Therapeutic Index (TI)

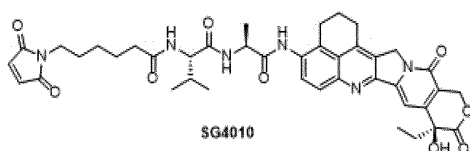
[0610] Head to head comparative studies of the AZ'0133 linker-warhead selected for E02-GL-SG3932 were conducted to determine efficacy, pharmacokinetics, and toxicity of the SG3932 warhead with four alternative linkers. The cleavable mal-PEG8-val-ala linker-SG3932 linker-warhead ADC offers the widest relative TI and compares favorably to benchmark. The four comparative linker-warheads are provided below. Data collected from the comparative studies is provided set forth in Table 22.

Comparative Linker-Warheads

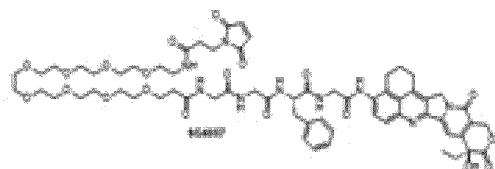
E02-INT- cleavable mal-PEG8-val-ala linker-SG3932



E02-INT-SG4010



E02-INT-SG4057



E02-INT-SG4052

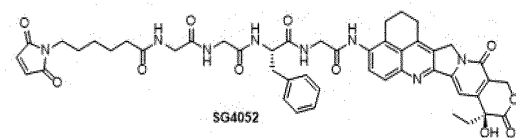


Table 22: Head-to-head comparative studies of efficacy, PK and toxicity of the E02-INT antibody with four alternative linkers

	E02-INT-SG3932	E02-INT-SG4010	E02-INT-SG4057	E02-INT-SG4052
Linker + Warhead	mp-PEG8-Val-Ala-SG3932	mc-Val-Ala-SG3932	mp-PEG8-GGFG-SG3932	mc-GGFG-SG3932
Hydrophobicity (HIC), Retention time (min)	3.41	3.37	3.76	3.53
In vitro serum stability (avg % deconjug. at day 15 vs 0)	24	68.4	28.4	69
In vitro cytotoxicity IC50 MX1 / HT29 clone 26 (ng/mL)	8.81 / 53.3	5.54 / 40.6	14.4 / 44.0	6.21 / 67.6
In vivo efficacy-MX-1, dose for tumor stasis / 30% regression (mg/kg)	0.57 / 1.27	0.83 / 1.18	0.92 / 1.02	1.02 / 1.85
In vivo efficacy-MDA-MB-468, dose for tumor stasis / 30% regression (mg/kg)	0.89 / 1.35	0.71 / 1.47	1.12 / 1.85	1.16 / 1.98
SCID mouse PK – CL (ml/day/kg) @ 1.25 mg/kg	11.3	20.4	14.6	19.0
Rat PK – CL (ml/day/kg) @ 60 mg/kg (HNSTD)	7.57	13.3	10.3	24.3
Rat toxicology study (# of findings with severity over background/control after 20 or 60 mg/kg dose)	1/7	3/7	3/7	4/7
Relative TI for tumor stasis in MX-1	152	101	82	44
Relative TI for 30% tumor	68	71	45	39

regression at day 7 in MX-1				
--------------------------------	--	--	--	--

[0611] The mp-PEG8-Val-Ala- SG3932 linker-warhead for E02-GL-SG3932 performed better than each comparator. The ADC prepared with the cleavable mal-PEG8-val-ala linker-SG3932 linker-warhead is the most active *in vivo*, when conjugated to the E02-INT antibody (Figure 57A and 57B). The cleavable mal-PEG8-val-ala linker-SG3932 linker-warhead ADC exhibits the cleanest safety profile in rat toxicity study, as shown in Figure 58. The cleavable mal-PEG8-val-ala linker-SG3932 linker-warhead ADC has good PK properties and widest relative TI, as shown in figure 59. Relative TI = exposure ratio of AUC at the highest dose tested in rat (NOAEL not HNSTD) to the AUC that provides tumor stasis (MX-1 model).

[0612] As shown in Figure 29B, the cleavable mal-PEG8-val-ala linker-SG3932 linker-warhead can be differentiated from competitor TOP1i conjugated ADC and contains key features thought to contribute to TI and provide advantages. In particular, the mp-PEG in the cleavable mal-PEG8-val-ala linker-SG3932 stabilizes the conjugate and contributes to ADC stability, and the lactone switch in cleavable mal-PEG8-val-ala linker-SG3932 can open while attached to the ADC, which increases warhead potency following internalization by receptor-mediated endocytosis (RME). (Figure 29B)

[0613] Figures 60A-60B show that E02-GL-SG3932 has robust activity in Breast and Ovarian PDX. Panel A: The antitumor activity of E02-GL-SG3932 was investigated in a panel of breast and ovarian PDX models. Each mouse (N=1 per group) received a single IV injection of E02-GL-SG3932 or a control (untreated), at a dose of 7 mg/kg or 3.5 mg/kg. Figure 60B shows antitumor activity in a panel of TNBC PDX models. Each mouse received a single IV injection of E02-GL-SG3932 or a vehicle control at a dose of 3.5 mg/kg or 1.25 mg/kg.

[0614] E02-GL-SG3932 also shows robust activity in HR-deficient tumors and in HR-proficient tumors with elevated B7-H4 (Figures 61 and 62A-D).

[0615] Additionally, HR-deficiency increases sensitivity to the SG3932 warhead. As shown in Figure 63, a shift in potency with the SG3932 warhead as compared to Dimethylsulfoxide (DMSO) treatment and a negative control microtubule-inhibitor

warhead (MMAE). As shown in Figure 63, cell viability was over 100% when wild-type and BRCA2^{-/-} cells were treated with DMSO. Cell viability was reduced in both wild-type and BRCA2^{-/-} cells when treated with the MMAE warhead. A large decrease in viability was seen when wild-type cells were treated with the SG3932 warhead as compared to the MMAE warhead. However, the largest decrease in viability (~349 fold shift in potency) was seen when BRCA2^{-/-} cells were treated with the SG3932 warhead. This demonstrates that HR-deficiency increases sensitivity to the SG3932 warhead.

SYNTHESIS OF TOPOISOMERASE I INHIBITORS

General Information

[0616] Flash chromatography was performed using a Biotage® Isolera™ and fractions checked for purity using thin-layer chromatography (TLC). TLC was performed using Merck Kieselgel 60 F254 silica gel, with fluorescent indicator on aluminium plates. Visualisation of TLC was achieved with UV light.

[0617] Extraction and chromatography solvents were bought and used without further purification from VWR U.K.

[0618] All fine chemicals were purchased from Sigma-Aldrich unless otherwise stated. Pegylated reagents were obtained from Quanta biodesign US via Stratech UK.

LC/MS conditions

Method A

[0619] Positive mode electrospray mass spectrometry was performed using a Waters Aquity H-class SQD2. Mobile phases used were solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). Initial composition 5% B held over 25 seconds, then increased from 5% B to 100% B over a 1 minute 35 seconds' period. The composition was held for 50 seconds at 100% B, then returned to 5% B in 5 seconds and held there for 5 seconds. The total duration of the gradient run was 3.0 minutes. Flow rate was 0.8 mL/minute. Detection was at 254 nm. Columns: Waters Acquity UPLC® BEH Shield RP18 1.7µm 2.1 x 50 mm at 50 °C fitted with Waters Acquity UPLC® BEH Shield RP18 VanGuard Pre-column, 130A, 1.7µm, 2.1 mm x 5 mm.

Method B

[0620] The HPLC (Waters Alliance 2695) was run using a mobile phase of water (A) (formic acid 0.1%) and acetonitrile (B) (formic acid 0.1%). Initial composition 5% B held over 25 seconds, then increased from 5% B to 100% B over a 1 minute 35 seconds' period. The composition was held for 50 seconds at 100% B, then returned to 5% B in 5 seconds and held there for 5 seconds. The total duration of the gradient run was 3.0 minutes. Flow rate was 0.8 mL/minute. Wavelength detection range: 190 to 800 nm. Columns: Waters Acquity UPLC® BEH Shield RP18 1.7µm 2.1 x 50 mm at 50 °C fitted with Waters Acquity UPLC® BEH Shield RP18 VanGuard Pre-column, 130A, 1.7µm, 2.1 mm x 5 mm.

Method C

[0621] The HPLC (Waters Alliance 2695) was run using a mobile phase of water (A) (formic acid 0.1%) and acetonitrile (B) (formic acid 0.1%).

[0622] Initial composition 5% B held over 1 min, then increase from 5% B to 100% B over a 9 min period. The composition was held for 2 min at 100% B, then returned to 5% B in 0.10 minutes and hold there for 3 min. Total gradient run time equals 15 min. Flow rate 0.6 mL/min. Wavelength detection range: 190 to 800 nm. Oven temperature: 50°C. Column: ACE Excel 2 C18-AR, 2 µ, 3.0 x 100mm.

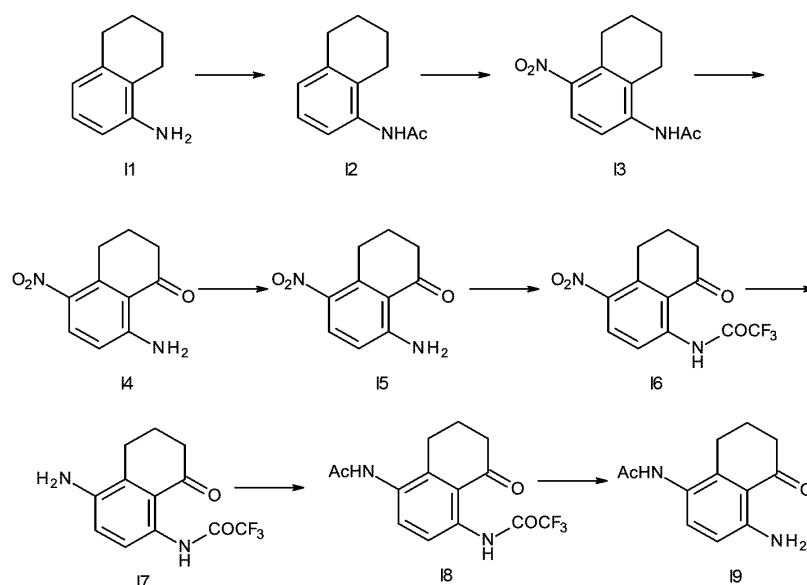
HPLC conditions

[0623] Reverse-phase ultra-fast high-performance liquid chromatography (UFLC) was carried out on a Shimadzu Prominence™ machine using a Phenomenex™ Gemini NX 5µ C18 column (at 50 °C) dimensions: 150 x 21.2 mm. Eluents used were solvent A (H₂O with 0.1% formic acid) and solvent B (CH₃CN with 0.1% formic acid). All UFLC experiments were performed with gradient conditions: Initial composition 13% B increased to 30% B over a 3 minutes period, then increased to 45% B over 8 minutes and again to 100% over 6 minutes before returning to 13% over 2 min and hold for 1 min. The total duration of the gradient run was 20.0 minutes. Flow rate was 20.0 mL/minute and detection was at 254 and 223 nm.

NMR Method

[0624] Proton NMR chemical shift values were measured on the delta scale at 400 MHz using a Bruker AV400. The following abbreviations have been used: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; m, multiplet; br, broad. Coupling constants are reported in Hz.

Synthesis of key intermediates

a) N-(5,6,7,8-tetrahydronaphthalen-1-yl)acetamide (**I2**)

[0625] 5,6,7,8-tetrahydronaphthalen-1-amine **I1** (8.54 g, 58.0 mmol) was dissolved in dichloromethane (80 mL). Triethylamine (18 mL, 129 mmol) was added and the mixture cooled to 0°C. Dropwise, acetic anhydride (11.5 mL, 122 mmol) was added, upon completion of the addition, the reaction mixture was warmed to rt and stirred for 45 min, whereupon LCMS indicated the reaction was complete. The mixture was diluted with CH₂Cl₂, washed with H₂O, sat. NaHCO₃, 10% citric acid, the organic phase dried over MgSO₄ and concentrated *in vacuo*. The off-white solid was triturated with 1:3 Et₂O/isohexane to afford **I2** (10.8 g, 57.1 mmol, 98% Yield) as a white solid which was

used without further purification. LC/MS (method A): retention time 1.44 mins (ES+) m/z 190 $[M + H]^+$

b) *N*-(4-nitro-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide (**I3**)

[0626] *N*-(5,6,7,8-tetrahydronaphthalen-1-yl)acetamide **I2** (1.00 g, 5.2840 mmol) was added portion-wise to sulfuric acid (15 mL, 281 mmol) at -5°C. Sodium nitrate (450 mg, 5.2945 mmol) was added portion-wise to the reaction mixture and stirred for 30 min at -5°C whereupon LCMS indicated no further reaction progress. The reaction mixture was poured onto ice with external cooling, the aqueous mixture extracted with CH₂Cl₂, the organic phase dried over MgSO₄ and purified by Isolera (10-80% EtOAc in *isohexane*) to afford a mixture of *N*-(4-nitro-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide **I3** and *N*-(2-nitro-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide (956 mg, 4.0811 mmol, 77% Yield) as a white/yellow solid. LC/MS (method A): retention time 1.53 mins (ES+) m/z 235 $[M + H]^+$.

c) *N*-(4-nitro-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide (**I4**)

[0627] *N*-(4-nitro-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide **I3** (1.01 g, 4.31 mmol) was dissolved in acetone (30 mL). Magnesium sulfate in water (3.9 mL, 5.9 mmol, 1.5 mol/L) was added and the mixture was cooled to 0°C. Potassium permanganate (2.07 g, 13.0 mmol) was added portionwise to the reaction mixture and the mixture warmed to rt and stirred for 50 min, whereupon TLC indicated the reaction was complete. The reaction mixture was filtered through Celite, the solids washed with CHCl₃ and the resulting organic mixture washed with H₂O, brine, dried over MgSO₄ and purified by isolera (20-50% EtOAc in *isohexane*) to afford a mixture of *N*-(4-nitro-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide **I4** and *N*-(2-nitro-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide (709 mg, 2.86 mmol, 66%) as a white/yellow solid. LC/MS (method A): retention time 1.44 mins (ES+) m/z 190 $[M + H]^+$

d) 8-amino-5-nitro-3,4-dihydronaphthalen-1(2H)-one (**I5**)

[0628] A mixture of *N*-(4-nitro-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide **I4** and *N*-(2-nitro-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide (709 mg, 2.8561 mmol) and 6N hydrochloric acid (7 mL) were stirred at 80°C for 2.5 h, whereupon

LCMS indicated the reaction was complete. The reaction mixture was cooled in an ice bath and 6N NaOH solution was added until the pH was basic. The aqueous mixture was extracted with CH₂Cl₂, the organic phase dried over MgSO₄ and concentrated in vacuo. Isolera (0-50% EtOAc in *isohexane*) afforded 8-amino-5-nitro-3,4-dihydronaphthalen-1(2H)-one **I5** (320 mg, 1.552 mmol, 54% Yield) as a yellow/orange solid. LC/MS (method A): retention time 1.54 mins (ES+) m/z 207 [$M + H$]⁺

e) 2,2,2-trifluoro-*N*-(4-nitro-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide (**I6**)
[0629] 8-amino-5-nitro-3,4-dihydronaphthalen-1(2H)-one **I5** (430 mg, 2.0854 mmol) was dissolved in dichloromethane (20 mL). Pyridine (340 μ L, 4.20 mmol) was added and the mixture cooled to 0°C. Trifluoroacetic anhydride (590 μ L, 4.197 mmol) was added and stirred for 30 min, whereupon LCMS indicated the reaction was complete. The mixture was diluted with CH₂Cl₂, washed with H₂O, the organic phase dried over MgSO₄ and concentrated *in vacuo* to afford 2,2,2-trifluoro-*N*-(4-nitro-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide **I6** (630 mg, 2.0846 mmol, >99% Yield) as a yellow solid, which was used without further purification. LC/MS (method A): retention time 1.86 min (ES+) m/z 301X [$M - H$]⁻

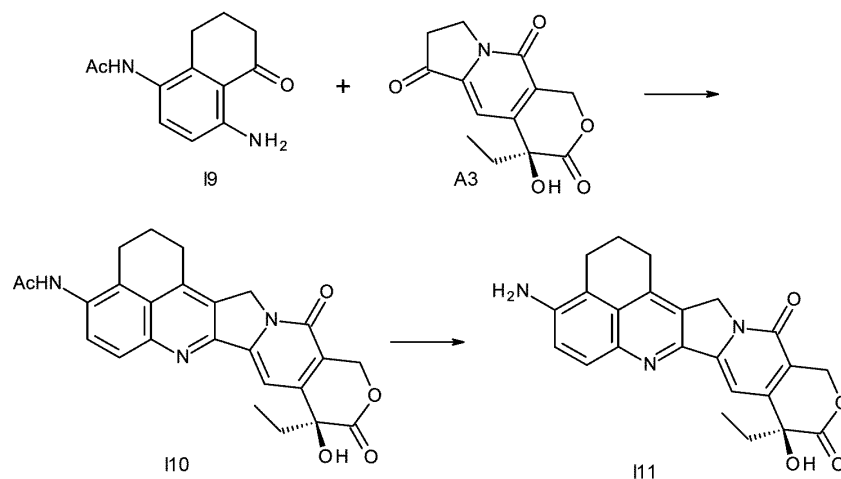
f) *N*-(4-amino-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)-2,2,2-trifluoroacetamide (**I7**)
[0630] Zinc (2.73 g, 41.7 mmol) was suspended in methanol (80 mL), formic acid (4 mL) and water (4 mL) and the mixture cooled to 0°C. 2,2,2-trifluoro-*N*-(4-nitro-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide **I6** (568 mg, 2.0865 mmol) was added portion-wise and the mixture stirred at 0°C for 30 min, whereupon LCMS indicated the reaction was complete. The reaction mixture was filtered, the filtrate diluted with EtOAc and washed with sat NaHCO₃. The organic phase was dried over MgSO₄ and concentrated in vacuo to afford *N*-(4-amino-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)-2,2,2-trifluoroacetamide **I7** (568 mg, 2.0865 mmol, >99% Yield) as a yellow solid, which was used without further purification. LC/MS (method A): retention time 1.65 min (ES+) m/z 273 [$M + H$]⁺

g) *N*-(4-acetamido-8-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)-2,2,2-trifluoroacetamide (**I8**)
[0631] *N*-(8-amino-4-oxo-tetralin-5-yl)-2,2,2-trifluoro-acetamide **I7** (568 mg, 2.0865 mmol) was dissolved in dichloromethane (20 mL). Triethylamine (580 μ L, 4.16 mmol)

then acetyl chloride (297 μ L, 4.173 mmol) were added and the mixture stirred for 30 min, whereupon LCMS indicated the reaction was complete. The reaction mixture was diluted with CH_2Cl_2 , washed with H_2O , the organic phase dried over MgSO_4 and concentrated *in vacuo* to afford *N*-(8-acetamido-4-oxo-tetralin-5-yl)-2,2,2-trifluoro-acetamide **18** (655 mg, 2.084 mmol, >99% yield) as a yellow solid, which was used without further purification. LC/MS (method A): retention time 1.55 min (ES+) m/z 315 [$M + \text{H}$]⁺

h) *N*-(4-amino-5-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)acetamide (**19**)

[0632] *N*-(8-acetamido-4-oxo-tetralin-5-yl)-2,2,2-trifluoro-acetamide **18** (2.77 g, 8.81 mmol) was dissolved in methanol (240 mL) and water (17 mL). Potassium carbonate (4.88 g, 35.3 mmol) was added and the mixture stirred for 1.5 h at 50°C, whereupon LCMS indicated the reaction was complete. The reaction mixture was cooled, concentrated *in vacuo*, dissolved in 10% MeOH in CH_2Cl_2 and washed with H_2O . The organic phase was dried over MgSO_4 and purified by isolera chromatography (2-15% MeOH in CH_2Cl_2) to afford *N*-(8-amino-1-oxo-tetralin-5-yl)acetamide **19** (1.20 g, 5.50 mmol, 62.3% Yield) as a yellow solid. LC/MS (method A): retention time 0.98 min (ES+) m/z 219 [$M + \text{H}$]⁺



i) (S)-N-(9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)acetamide (**110**)

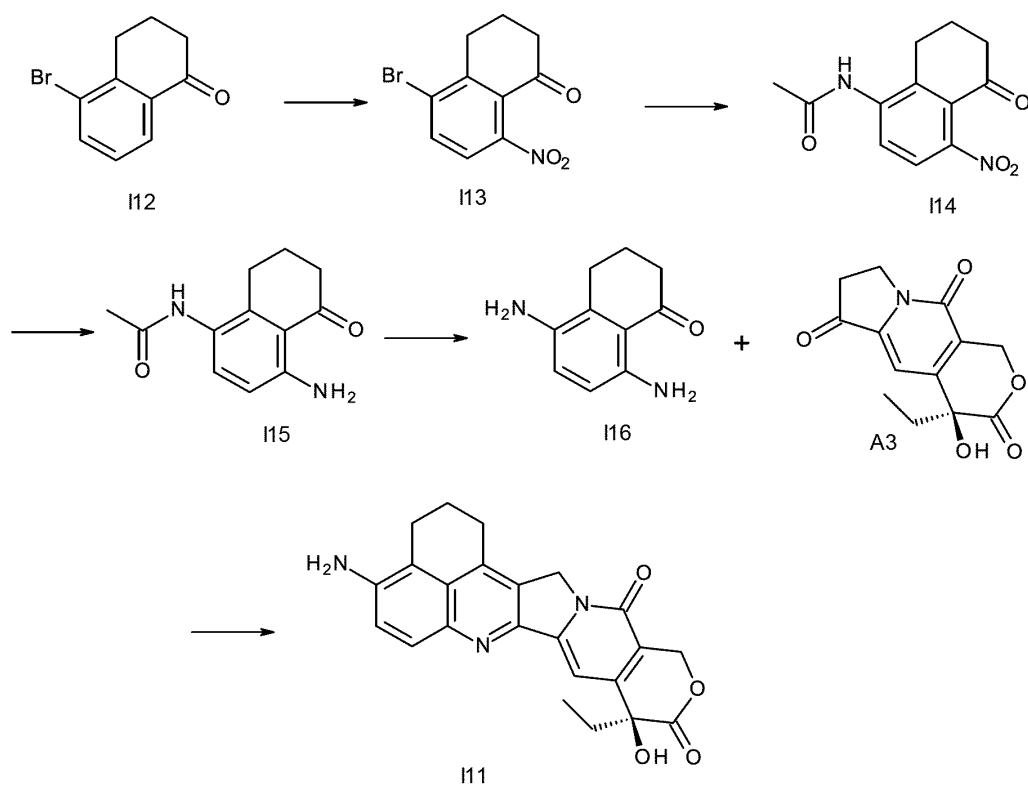
[0633] *N*-(8-amino-1-oxo-tetralin-5-yl)acetamide **I9** (641 mg, 2.94 mmol, 1.0 eq.), (*S*)-4-ethyl-4-hydroxy-7,8-dihydro-1*H*-pyrano[3,4-*f*]indolizine-3,6,10(4*H*)-trione A3 (840 mg, 3.19 mmol, 1.1 eq.) and PPTS (740 mg, 2.95 mmol, 1.0 eq.) were dissolved in toluene (60 mL) and stirred at reflux for 3 h, whereupon LCMS indicated **I9** had been consumed. The reaction mixture was cooled and concentrated *in vacuo*. The resulting solids were triturated with acetonitrile, then acetone to afford **I10** as a brown solid with minor TsOH contamination (1.26 g, 96%). LC/MS (method A): retention time 1.32 mins (ES+) *m/z* 447 [*M* + *H*]⁺

j) (*S*)-4-amino-9-ethyl-9-hydroxy-1,2,3,9,12,15-hexahydro-10*H*,13*H*-benzo[de]pyrano[3',4':6,7]indolizino[1,2-*b*]quinoline-10,13-dione (**I11**)

[0634] (*S*)-*N*-(9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1*H*,12*H*-benzo[de]pyrano[3',4':6,7]indolizino[1,2-*b*]quinolin-4-yl)acetamide (**I10**) (1.26 g, 2.83 mmol, 1.0 eq.) was dissolved in hydrochloric acid (6 mol/L) in H₂O (12 mL) and the mixture stirred for 5 h at 80°C, whereupon LCMS indicated **I10** had been consumed. The reaction mixture was diluted with H₂O and concentrated *in vacuo* to afford (*S*)-4-amino-9-ethyl-9-hydroxy-1,2,3,9,12,15-hexahydro-10*H*,13*H*-benzo[de]pyrano[3',4':6,7]indolizino[1,2-*b*]quinoline-10,13-dione **I11** (1.51 g, 2.85 mmol, 90 mass%, 101% Yield) as a red crystalline solid. LC/MS (method A): retention time 1.36 mins (ES+) *m/z* 405 [*M* + *H*]⁺.

179

Alternate synthesis of I11



IPC, purity and assay method for this synthesis

Instrument	Thermo U-3000		
Column	ACE Excel 3 C18- PFP (3.0 mm×150 mm)		
Oven	40°C		
Mobile phase	A: 10mM Ammonium Formate in water pH=3.5		
	B: CAN		
Gradient program	Time (min)	A%	B%
	0.0	90	10
	20.0	10	90
	23.0	10	90
	24.0	90	10
	30.0	90	10
	Re-equilibration time: 6 min		
Flow rate	1.0 ml/min		
Detector	UV 220 nm		
Diluent	CAN		

a) 5-bromo-8-nitro-tetralin-1-one (**I13**)

[0635] A solution of potassium nitrate (1.15 eq., 13.83 g) dissolved in sulphuric acid (Conc., 5.0 rel. vol., 160 mL), was added (addition time 4-12 h, maintaining the temperature below 10°C) to a solution of 5-bromotetralin-1-one (**I12**) (1.0 equiv., 26.77 g) in sulfuric acid (Conc., 5.0 rel. vol., 160 mL) under nitrogen. When the reaction was complete the reaction mixture was transferred to flask containing water (36 rel. vol., 1.15 L) adjusting the transfer rate to keep the temperature below 10°C. The resulting solid was filtered, washed with water (4.0 rel. vol., 128 mL) three times and then dried at ~40°C for 24h. The dry cake was dissolved in a mixture of acetone (2.5 rel. vol., 80 mL) and water (0.38 rel. vol., 12.2 mL) heated to ~75°C and then cooled to ~20°C. The resulting solid was removed by filtration. The solvent was swapped to ethanol by distillation and the solution volume reduced to a 2.0 rel. vol. (64 mL). The solution was cooled to ~25°C and the resulting solid collected by filtration. The solid was washed with ethanol (1.0 Rel. Vol., 32mL) then dried under vacuum at 40°C to give 5-bromo-8-nitro-tetralin-1-one **I13** (15.36g, 40%) as a brown solid; RT 14.0 min

Method 1 IPC, purity and assay method for bromo-8-nitro-tetralin-1-one.

Instrument	Thermo U-3000		
Column	ACE Excel 3 C18- PFP (3.0 mm×150 mm)		
Oven	40°C		
Mobile phase	A: 10mM Ammonium Formate in water pH=3.5		
	B: CAN		
Gradient program	Time (min)	A%	B%
	0.0	90	10
	20.0	10	90
	23.0	10	90
	24.0	90	10
	30.0	90	10
	Re-equilibration time: 6 min		
Flow rate	1.0 ml/min		
Detector	UV 220 nm		
Diluent	CAN		

b) N-(8-nitro-1-oxo-tetralin-5-yl)acetamide (**I14**)

[0636] A solution of bromo-8-nitro-tetralin-1-one (**I13**) (1.0 eq., 18.0 g, 90.6% ww), acetamide (1.2 eq., 4.72 g), tris(dibenzylideneacetone)dipalladium(0) (0.01 eq., 0.61 g)

and potassium phosphate (1.4 eq., 19.8 g) in dioxane (15 rel. vol., 270 mL) under nitrogen was heated to ~70°C. When the reaction was complete the solution was cooled to ~20°C and diluted with dioxane (5 rel. vol., 90.0 mL) and filtered. The solvent was swapped to ethanol and the volume reduced to a total reaction volume of 3 rel. vol. (54.0 mL). the solution was cooled to ~20°C and the resulting solid collected by filtration and washed with MTBE (methyl tert-butyl ether)(1.0 rel. vol., 18.0 mL). The solid was dried under vacuum at 40°C to give N-(8-nitro-1-oxo-tetralin-5-yl)acetamide **I14** (10.0 g, 60.6%) as a dark yellow solid; RT 8.86 min.

c) N-(8-amino-1-oxo-tetralin-5-yl)acetamide (I15)

[0637] Palladium hydroxide on carbon (20% w/w, 0.15 eq., 5.25g) was added to a solution of N-(8-nitro-1-oxo-tetralin-5-yl)acetamide (**I14**)(1.0 eq., 32.6g) in methanol (40 rel. vol., 1250mL). The reaction mixture was placed under a hydrogen atmosphere at ~40 psi, at ~40°C for 8h. The hydrogen was removed and replaced with nitrogen and the catalyst was removed by filtration over cellulose, washing the cellulose with methanol (4.0 rel. vol., 130mL). The solution volume was reduced to 4.0 rel. vol. by distillation and then diluted with MTBE (4 rel. vol, 130mL). The resulting solid was collect by filtration, washed with MTBE (2 rel. vol., 65mL) and dried under vacuum at 40°C to give N-(8-amino-1-oxo-tetralin-5-yl)acetamide **I15** (21.1g, 77.8%) as a grey green solid; RT 5.44 min.

d) 5,8-diaminotetralin-1-one (I16)

[0638] A solution of N-(8-amino-1-oxo-tetralin-5-yl)acetamide (**I15**)(1.0 eq., 10.0 g) in hydrochloric acid (5M, 6.0 rel. vol., 60 mL), was held at ~90°C for 3h. The temperature was reduced to 25°C and sodium hydroxide (2M, 4.0 rel. vol., 40mL) was added until pH 10.0 was achieved, maintaining the temperature 25°C. The resulting solid was collected by filtration and washed with water (2.0 rel. vol., 20 mL). The wet cake was dissolved in tetrahydrofuran (60 rel. vol., 600 mL) and filtered. The solution was concentrated to 5.0 rel. vol. and heptane (20 rel. vol., 200 mL) added. The solution was concentrated to 10.0 rel. vol. and further heptane (20 rel. vol., 200 mL) added, and then the volume reduce dto 10.0 rel. vol. again. The resulting solid was collected by filtration and washed with heptane (5.0 rel. vol., 50 mL). The solid was dried under vacuum at 40°C for 17h to give

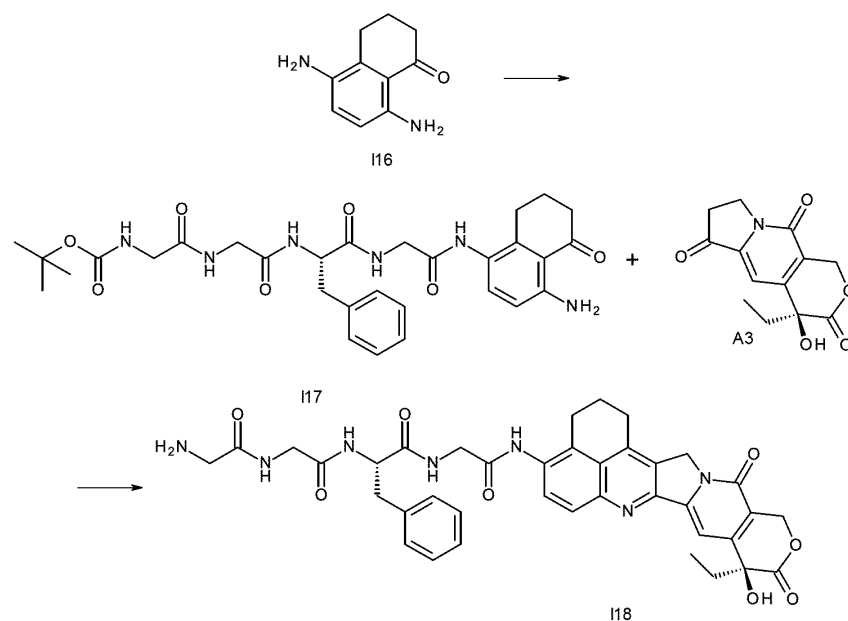
5,8-diaminotetralin-1-one (**I16**)(6.90g, 82.7%) as a yellow solid; ¹H NMR (400 MHz DMSO-d₆) δ ppm 1.82 (m, 2H), 2.38 (t, *J*=2.0 Hz, 2H), 2.47 (t, *J*=2.0 Hz, 2H), 6.34 (d, *J*=2.0 Hz, 1H), 6.68 (d, *J*=2.0 Hz, 1H); RT 3.90

e) (S)-4-amino-9-ethyl-9-hydroxy-1,2,3,9,12,15-hexahydro-10H,13H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13-dione (**I11**)

[0639] A solution of 5,8-diaminotetralin-1-one (**I16**)(1.0 eq., 5.0g), (4S)-4-ethyl-4-hydroxy-7,8-dihydro-1H-pyrano[3,4-f]indolizine-3,6,10-trione (**A3**)(1.06 eq., 7.9g), and pyridinium para-toluenesulfonate (1.0 eq., 7.2g) in toluene (50.0 rel. vol., 250 mL) was held at 120°C for 15 h. The volume of the solution was reduced to 2.0 rel. vol. and then diluted with acetonitrile (20 rel. vol., 100 mL) and water (20 rel. vol., 100 mL). The resulting slurry was filtered and the solid washed with aqueous acetonitrile (1:1, 20 rel. vol., 100 mL). The solid was slurried with aqueous methanol (water:MeOH 3:1, 40 rel. vol., 200 mL), filtered and washed with aqueous methanol (1:1, 20 rel. vol., 100 mL). The solid was slurried with water (60 rel. vol., 300 mL) at 50°C, filtered and washed with water (10 rel. vol., 50 mL). The solid was slurried with aqueous acetonitrile (water: acetonitrile, 1:3, 40 rel. vol., 200 mL) at 30°C, filtered and washed with aqueous acetonitrile (water: acetonitrile, 1:3, 5 rel. vol., 50 mL) and then dried under vacuum at 40°C to give (S)-4-amino-9-ethyl-9-hydroxy-1,2,3,9,12,15-hexahydro-10H,13H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13-dione (**I11**) as white solid (5.0g, 43.7%); RT 5.13.

183

Synthesis of I18



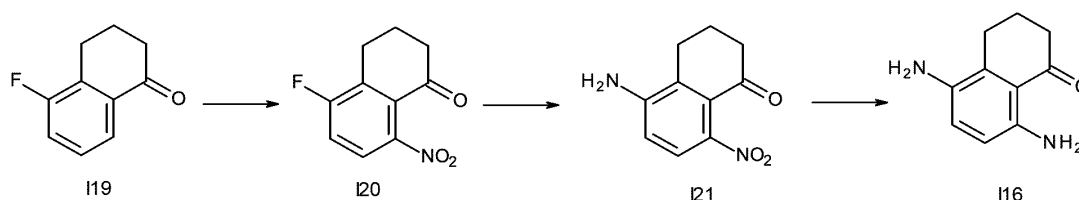
a) tert-butyl (S)-(2-((2-((1-((2-((4-amino-5-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)amino)-2-oxoethyl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)amino)-2-oxoethyl)carbamate (I17)

[0640] Boc-GGFG-OH (227 mg, 0.52 mmol) and EEDQ (157 mg, 0.634 mmol) were solubilised in CH_2Cl_2 (25 mL) and the mixture stirred for 15 min, until the peptide has gone into solution. Compound **I16** (100 mg, 0.56747 mmol) was subsequently added and the mixture left to stir until complete. After 1h, the reaction looked 90% complete by LVMC. The mixture has gone thicker as the product is crashing out. The mixture was left for another hour before vaccing down to dryness. The crude was taken up in Et_2O (50 mL). The solid was filtered and subsequently taken up in CH_2Cl_2 (50 mL) to purify further. The solid was filtered and dried to give product **I17** (273 mg, 0.459 mmol, 80.9% Yield) as a grey solid. Analytical data: LCMS 3min: $\text{ES}^+ = 1.46$ min, m/z 595.7 $[\text{M} + \text{H}]^+$.

b) (S)-2-(2-(2-aminoacetamido)acetamido)-N-(2-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-2-oxoethyl)-3-phenylpropanamide (I18)

[0641] Aniline **I17** (450 mg, 1.045 mMol), lactone **A5** (280 mg, 1.064 mMol) and pyridinium *p*-toluenesulfonate (273 mg, 1.086 mMol) were solubilised in toluene (20 mL) and the mixture was heated to 150°C (high reflux). MeOH (4 mL) was added to help solubilise the mixture. After 7h the crude reaction was vacced down to dryness. The crude product was purified by silica gel chromatography (CHCl₃/MeOH, 100% to 65:35) to give product **I18** (259 mg, 0.359 mMol, 78.1 yield). Analytical data: LCMS 3min: ES⁺ = 1.17 min, *m/z* 722.8 [M + H]⁺.

Alternative Synthesis of I16



a) 5-Fluoro-8-nitro-tetralin-1-one (I20)

[0642] 5-fluorotetralin-1-one **I19** (4.7 g, 29 mmol) was solubilised in 1/2 the amount of sulfuric acid (120 mL) in a 3 neck round bottom flask. The mixture was stirred until all the solid has dissolved and then cooled to 0-5 °C. In a dropping funnel, dissolve potassium nitrate (3 g, 29.6730 mmol) into the remaining half of sulfuric acid (120 mL) at 0-5 °C. Slowly add to the SM mixture making sure to maintain the solution cool (45 min). Stir at 0-5 °C until complete. The reaction mixture was subsequently quenched with water (250 mL) and left to stir at 0-5 °C. The solid was filtered and washed with water (50 mL). The solid was dried in a vaccum oven for 2h at 50 °C. The crude solid was slurried in Et₂O overnight before being cooled to 0 °C and filtered. The wet cake was washed with more cold Et₂O (50 mL) and left to dry in a vaccum oven at 50 °C to give pure product **I20** (5.5 g, 26 mmol, 92% yield) as a light pink fine powder. LCMS (Method B): ES⁺ = 1.55 min, *m/z* 210.1 [M + H]⁺.

b) 5-Amino-8-nitro-tetralin-1-one (I21)

[0643] Compound **I20** (2.7 g, 13 mmol) was solubilised in CH₃CN (2.5 mL) and NH₄OH (21 mass%) in H₂O (8 mL, 40 mmol) was added to a sealed pressure resistant tube and

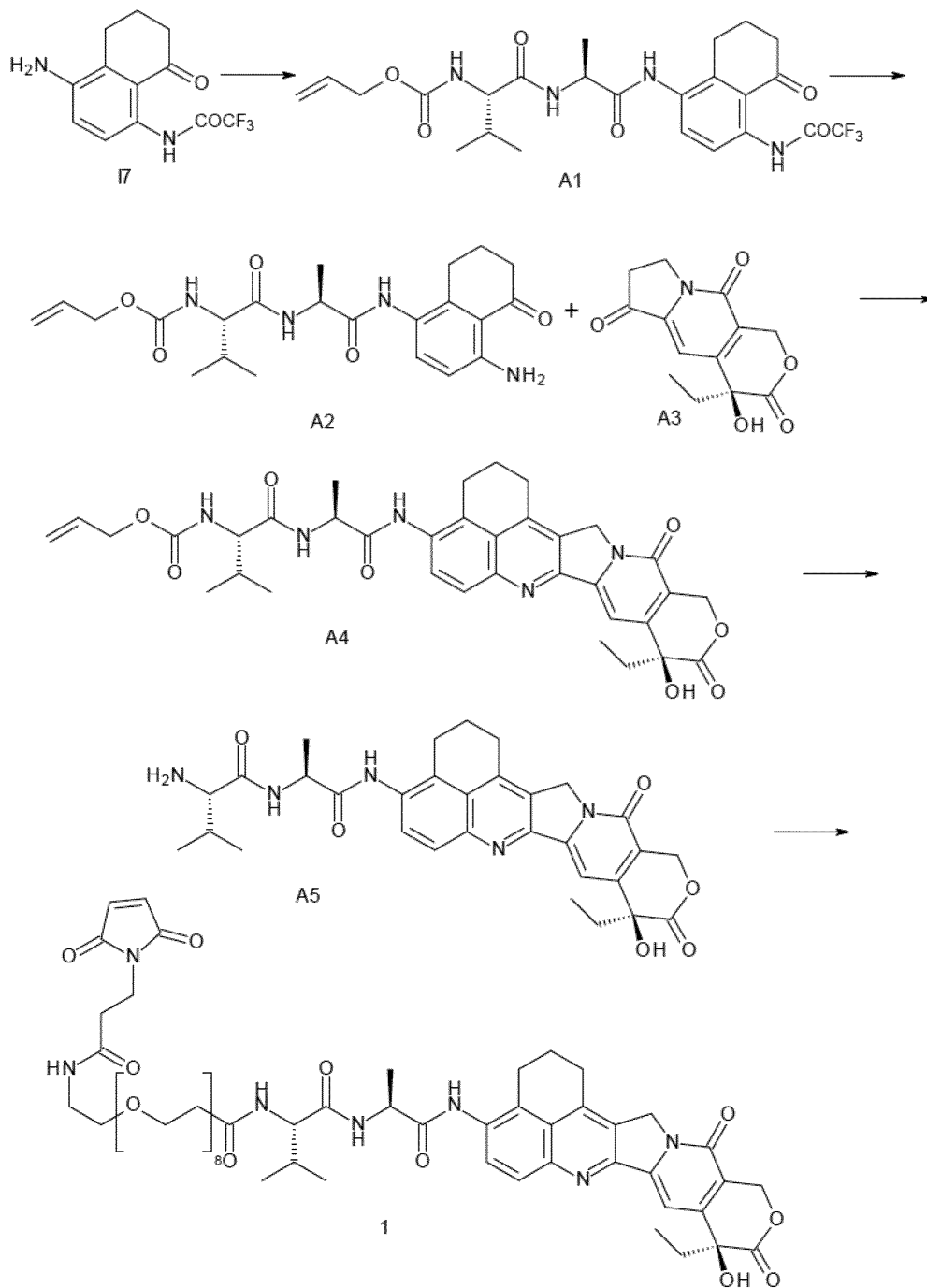
heated to 185 °C. Once complete, the mixture was transferred to a round bottom flask and vacced down. The crude was purified by silica gel column chromatography (CHCl₃/MeOH; 100 to 99:1) to give pure product **I21** (1.1 g, 5.3 mmol, 41% yield) as a black solid. LCMS (Method B): ES⁺ = 1.34 min, *m/z* 207.1 [M + H]⁺.

c) 5,8-diaminotetralin-1-one (I16)

[0644] Compound **I21** (1.35 g, 6.55 mmol) was dissolved in a mixture of methanol (20 mL), H₂O (1 mL) and formic acid (1 mL) at 0 °C. Zinc (8.5 g, 130 mmol) was slowly added, making sure to keep the temperature below 40 °C. A little more formic acid/H₂O (0.5 mL) was added to push the reaction to completion. The reaction mixture was filtered, and the filtrate diluted with EtOAc and CH₂Cl₂ before being vacced down. The crude was dry loading onto silica gel column chromatography (CHCl₃/EtOAc; 100 to 7:3 then CHCl₃/MeOH; 99:1 to 98:2) to give pure product **I16** (1.015 g, 5.760 mmol, 88.0% Yield). LCMS (Method B): ES⁺ = 0.2 min, *m/z* not observed.

186

Synthesis Example 1 (e.g. synthesis of SG3932)



a) Allyl ((S)-3-methyl-1-oxo-1-(((S)-1-oxo-1-((5-oxo-4-(2,2,2-trifluoroacetamido)-5,6,7,8-tetrahydronaphthalen-1-yl)amino)propan-2-yl)amino)butan-2-yl)carbamate (**A1**)

[0645] DCC (6.54 g, 31.7 mMol) and HOPO (3.36 g, 30.2 mMol) were added to a solution of alloc-Val-Ala-OH (9.09 g, 31.7 mmol) and **I7** (7.85 g, 28.8 mMol) in CH₂Cl₂ (300 mL) at 25°C. The resulting mixture was left to stir overnight. The white solid that formed during the reaction was filtered out and washed with cold CH₂Cl₂. The filtrate was washed with water (150 mL) and brine (150 mL). The organic layer was dried over MgSO₄, filtered and evaporated. The crude product was purified by silica gel chromatography (Hex/EtOAc, 60:40). Product **A1** isolated was contaminated with co-eluting DCU (21.1 g, 140% yield). LC/MS (Method B): ES⁺ = 1.81 min, *m/z* 527.6 [M + H]⁺.

b) Allyl ((S)-1-(((S)-1-((4-amino-5-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**A2**)

[0646] Protected aniline **A1** (18 g, 34.19 mMol) was solubilised in a mixture of MeOH and H₂O 10:1 (165 mL) and K₂CO₃ was added (10 g, 72.36 mMol). The mixture was stirred at 50°C until complete. The mixture was vacced down to almost dryness and the residue was taken up with CH₂Cl₂ and washed with H₂O and brine, before being dried over MgSO₄, filtered and evaporated. The crude product was purified by silica gel chromatography (CHCl₃/MeOH, 100% to 7:3). The isolated product **A2** was contaminated with a co-eluting impurity (10.71 g, 73% yield). LC/MS (Method B): ES⁺ = 1.46 min, *m/z* 431.7 [M + H]⁺.

c) Allyl ((S)-1-(((S)-1-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-1-oxopropan-2-yl)amino)-3-methylbutan-2-yl)carbamate (**A4**)

[0647] Aniline **A2** (450 mg, 1.045 mMol), lactone **A3** (280 mg, 1.064 mMol) and pyridinium *p*-toluenesulfonate (273 mg, 1.086 mMol) were solubilised in toluene (20 mL) and the mixture was heated to 130°C (high reflux). Every now and then a few drops of MeOH is added to help solubilise the mixture. After 7h the crude reaction was vacced down to dryness. The crude product was purified by silica gel chromatography (CHCl₃/MeOH, 100% to 95:5) to give product **A4** (360 mg, 52.3% yield). LC/MS (Method B): ES⁺ = 1.51 min, *m/z* 658.8 [M + H]⁺.

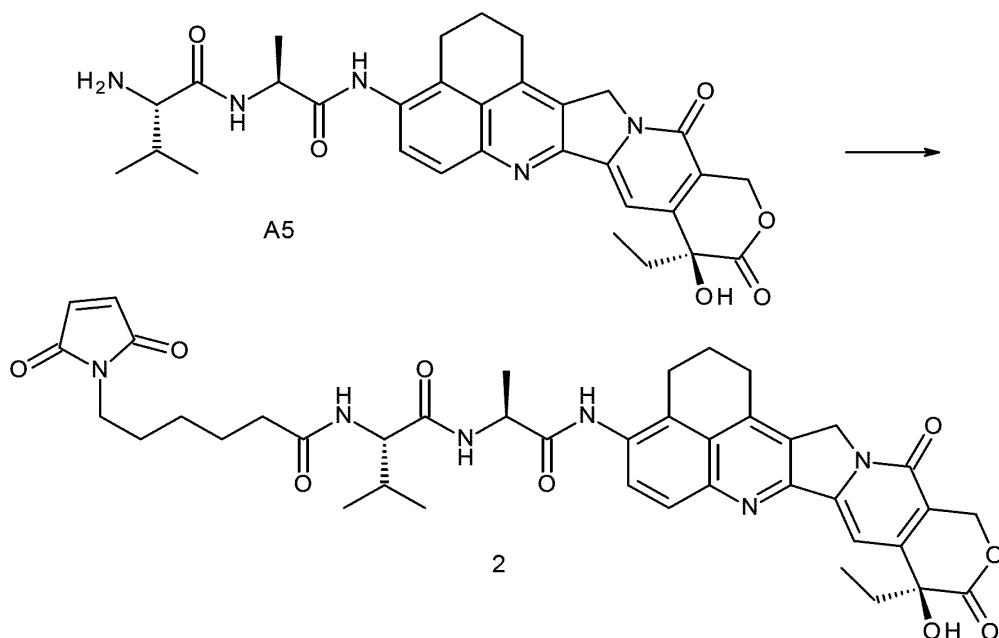
d) Allyl (S)-2-amino-N-((S)-1-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-1-oxopropan-2-yl)-3-methylbutanamide (**A5**)

[0648] Excess piperidine was added (642 μ L) to a solution of **A4** (543 mg, 0.82 mMol) and PdP(Ph₃)₄ (89 mg, 0.08 mMol) in CH₂Cl₂ (15 mL). The mixture was allowed to stir at room temperature for 20 min, at which point the reaction had gone to completion (as monitored by LC/MS). The reaction mixture was diluted with CH₂Cl₂ (25 mL) and the organic phase was washed with H₂O (25 mL) and brine (25 mL). The organic phase was dried over MgSO₄, filtered and excess solvent removed by rotary evaporation under reduced pressure to afford crude product **A5** which was used as such in the next step. LC/MS (Method B): ES⁺ = 1.15 min, *m/z* 574.6 [M + H]⁺.

e) 1-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanamido)-N-((S)-1-(((S)-1-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-3,6,9,12,15,18,21,24-octaohaheptacosan-27-amide (**1**)

[0649] Pyridine (83 μ L, 1.03 mMol) and Mal-dPEG₈-OTFP (767 mg, 1.03 mMol) were added to a solution of crude **A5** (assumed 1.03 mMol) in dry CH₂Cl₂ (50 mL) under an argon atmosphere. The reaction was stirred overnight and as the reaction was not complete 0.5 eq. of Mal-dPEG₈-OTFP was added to try to push the reaction. The reaction was diluted with CH₂Cl₂ (25 mL) and the organic phase was washed with H₂O (2 x 50 mL) and brine before being dried over MgSO₄, filtered and excess solvent removed by rotary evaporation under reduced pressure by rotary evaporation under reduced pressure. The crude was purified by reverse phase HPLC (gradient of H₂O/CH₃CN +0.05% FA) and freeze-dried to give **1** (1.189 g, 31% yield over 2 steps). LC/MS (Method B): ES⁺ =1.43 min, *m/z* 1149.3 [M + H]⁺. LC/MS (Method C): ES⁺ =5.37 min, *m/z* 1149.4 [M + H]⁺.

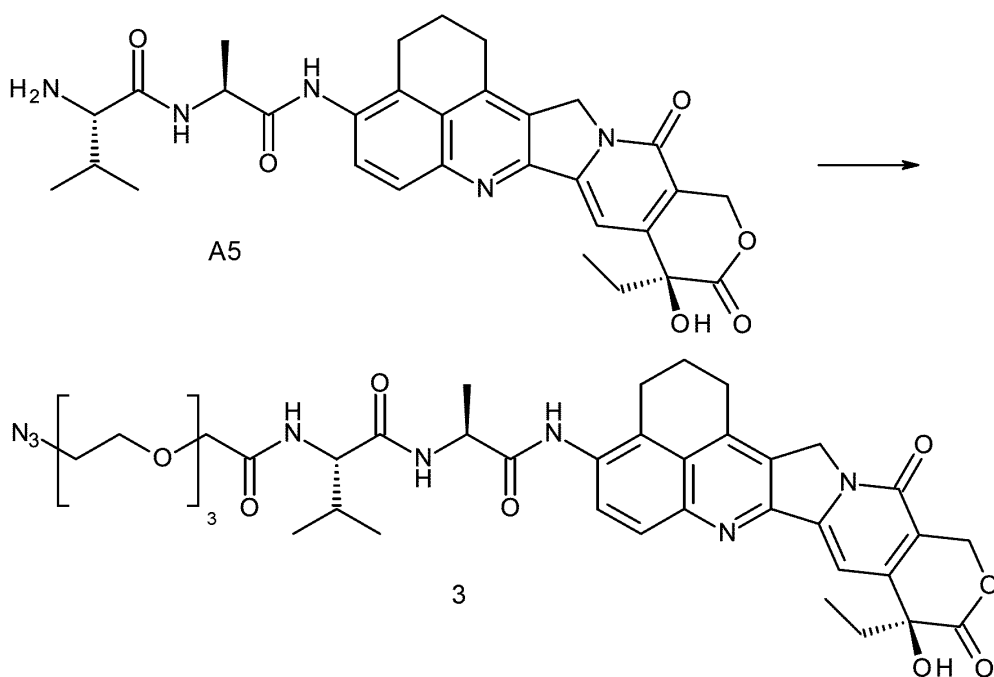
Synthesis Example 2 (e.g. synthesis of SG4010)



6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-((S)-1-(((S)-1-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)hexanamide (2)

[0650] Mal-caproic acid (56 mg, 0.26 mMol) and EDCl.HCl (51 mg, 0.26 mMol) were added to a solution of crude **A5** (assumed 0.26 mMol) in dry CH₂Cl₂ (20 mL) under an argon atmosphere. The reaction was stirred overnight and as the reaction was incomplete, another 0.5 eq of Mal-caproic acid and EDCl.HCl were added. The reaction was diluted with CH₂Cl₂ (25 mL) and the organic phase was washed with H₂O (2 x 50 mL) and brine before being dried over MgSO₄, filtered and excess solvent removed by rotary evaporation under reduced pressure by rotary evaporation under reduced pressure. The crude was purified by silica gel column chromatography (CHCl₃/MeOH 95:5) to give **2** (31.6 mg, 20% yield over 2 steps). LC/MS (Method B): ES⁺=1.56 min, *m/z* 767.8 [M + H]⁺. LC/MS (Method C) 15min: ES⁺=6.05 min, *m/z* 767.8 [M + H]⁺.

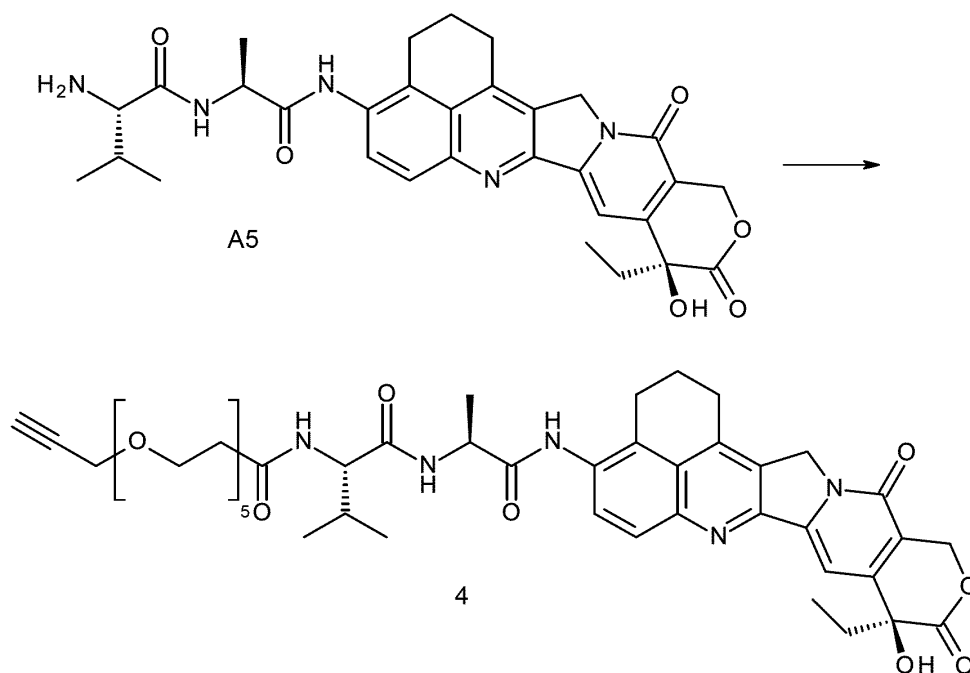
Synthesis Example 3



(S)-2-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetamido)-N-(((S)-1-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-1-oxopropan-2-yl)-3-methylbutanamide (**3**)

[0651] Azido-dPEG₃-acid (77.5 mg, 0.31 mMol) and EDCI.HCl (60 mg, 0.31 mMol) were added to a solution of crude **A5** (assumed 0.31 mMol) in dry CH₂Cl₂ (20 mL) under an argon atmosphere. The reaction was stirred overnight and as the reaction was incomplete, another 0.5 eq. of azido-dPEG₃-OH and EDCI.HCl were added. The reaction was diluted with CH₂Cl₂ (25 mL) and the organic phase was washed with H₂O (2 x 50 mL) and brine before being dried over MgSO₄, filtered and excess solvent removed by rotary evaporation under reduced pressure by rotary evaporation under reduced pressure. The crude was purified by preparative HPLC and the fractions were freeze-dried to give pure **3** (92.2 mg, 24.7% yield over 2 steps). LC/MS (Method B): ES⁺ = 1.69 min, *m/z* 789.9 [M + H]⁺. LC/MS (Method C): ES⁺ = 6.68 min, *m/z* 790.0 [M + H]⁺.

Synthesis Example 4

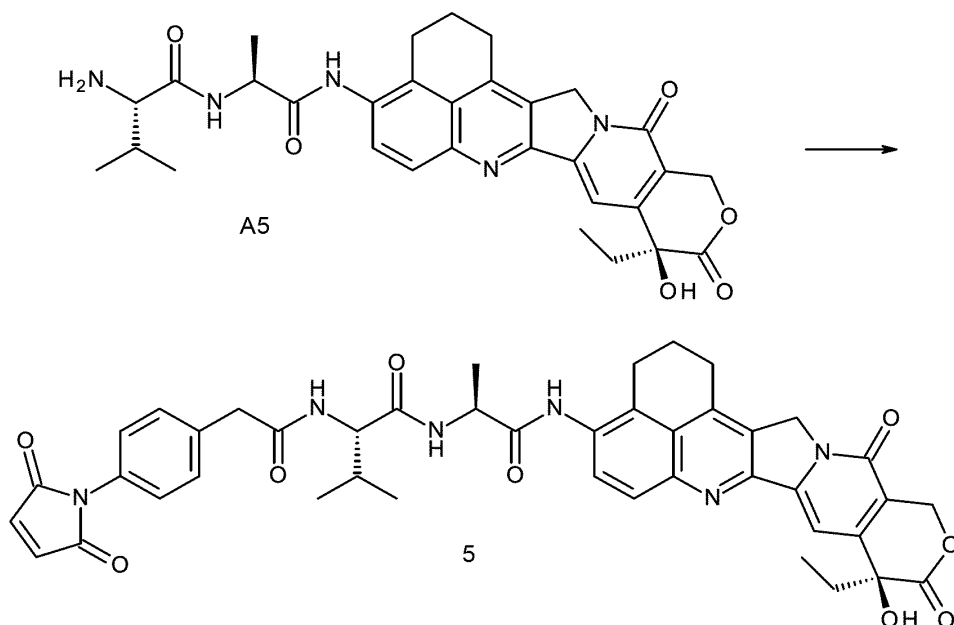


N-((S)-1-(((S)-1-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-4,7,10,13,16-pentaoxanonadec-18-ynamide (**4**)

[0652] Propargyl-dPEG₅-acid (56 mg, 0.19 mMol) and EDCI.HCl (37 mg, 0.19 mMol) were added to a solution of crude **A5** (assumed 0.19 mMol) in dry CH₂Cl₂ (10 mL) under an argon atmosphere. The reaction was stirred overnight and as the reaction was incomplete, another 0.5 eq. of Propargyl-dPEG₅-OH and EDCI.HCl were added. The reaction was diluted with CH₂Cl₂ (25 mL) and the organic phase was washed with H₂O (2 x 50 mL) and brine before being dried over MgSO₄, filtered and excess solvent removed by rotary evaporation under reduced pressure by rotary evaporation under reduced pressure. The crude was purified by preparative HPLC and the fractions were freeze-dried to give pure **4** (22 mg, 16.7% yield over 2 steps). LC/MS (Method B): ES⁺=1.54 min, *m/z* 860.9 [M + H]⁺. LCMS (Method C): ES⁺=5.57 min, *m/z* 860.9 [M + H]⁺.

192

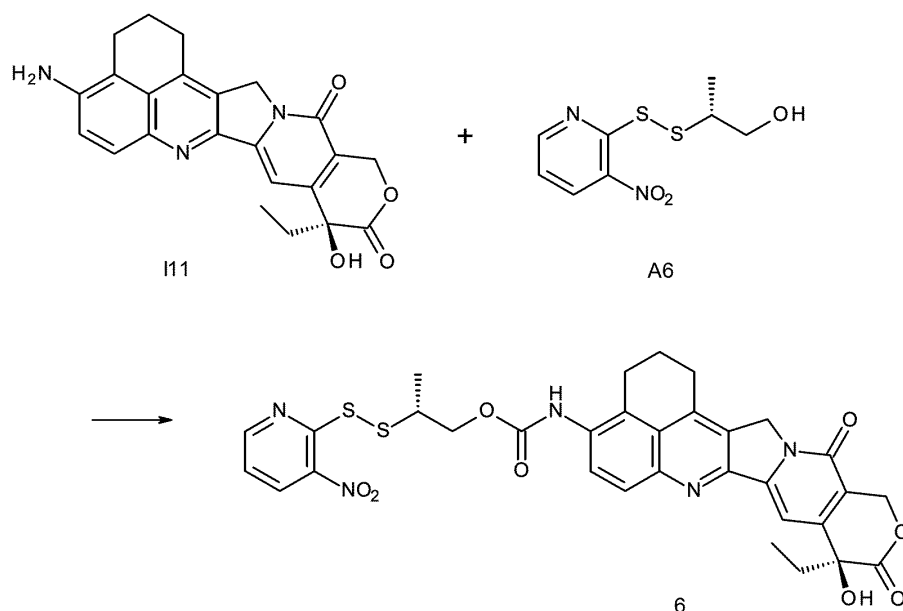
Synthesis Example 5



(S)-2-(2-(4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)acetamido)-N-(((S)-1-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-1-oxopropan-2-yl)-3-methylbutanamide (**5**)

[0653] PM-acetic-OSu (64 mg, 0.19 mMol) was added to a solution of crude **A5** (assumed 0.19 mMol) in dry CH₂Cl₂ (10 mL) under an argon atmosphere. The reaction was not proceeding so DIPEA (51 μL, 0.28 mMol) was added. The reaction was stirred until complete. The mixture was diluted with CH₂Cl₂ (25 mL) and the organic phase was washed with H₂O (2 x 50 mL) and brine before being dried over MgSO₄, filtered and excess solvent removed by rotary evaporation under reduced pressure by rotary evaporation under reduced pressure. The crude was purified by preparative HPLC and the fractions were freeze-dried to give pure **5** (2.5 mg, 1.6% yield over 2 steps). LC/MS (Method B): ES⁺ = 1.54 min, *m/z* 787.7 [M + H]⁺. LC/MS (Method C): ES⁺ = 5.61 min, *m/z* 787.8 [M + H]⁺.

Synthesis Example 6



(R)-2-((3-nitropyridin-2-yl)disulfanyl)propyl ((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)carbamate (**6**)

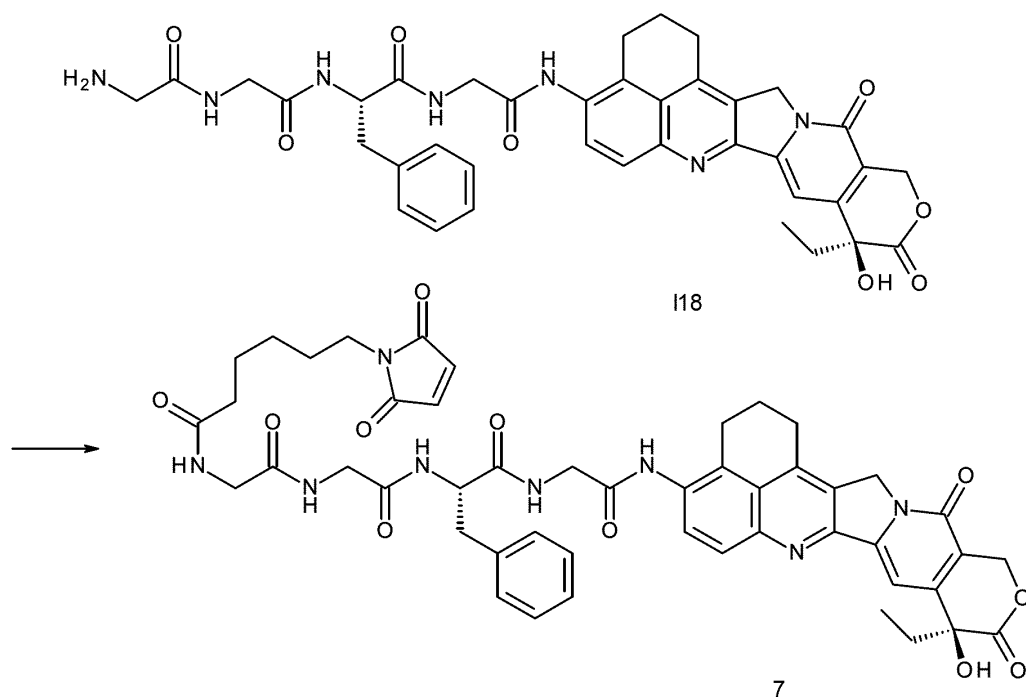
[0654] (i) (2R)-2-[(3-nitro-2-pyridyl)disulfanyl]propan-1-ol **A6** (25 mg, 0.1015 mmol, 1.0 eq.) was dissolved in dichloromethane (1 mL). Pyridine (8.5 μ L, 0.11 mmol, 1.0 eq.), then triphosgene (11 mg, 0.0370685 mmol, 0.33 eq.) were added and the mixture stirred under Ar for 45 min, whereupon LCMS (Et_2NH quench) indicated the formation of the corresponding carbamate.

[0655] (ii) (S)-4-amino-9-ethyl-9-hydroxy-1,2,3,9,12,15-hexahydro-10H,13H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13-dione (**111**) (43 mg, 0.09026 mmol, 1.0 eq.) was dissolved in dichloromethane (2 mL), *N,N*-diisopropylethylamine (42 μ L, 0.241 mmol, 2.7 eq.) and pyridine (25 μ L, 0.309 mmol, 3.4 eq.). The reaction mixture from step (i) was added and the mixture stirred for 30 min, whereupon LCMS indicated the reaction was complete. The reaction mixture was concentrated *in vacuo* and purified by isolera chromatography (0-4% MeOH in CH_2Cl_2) to afford **6** (22 mg, 0.03256

194

mmol, 36% Yield, QC = 96.8%) as a yellow solid. LC/MS (Method B): RT = 1.86 min, 676.6 [M+H]⁺.

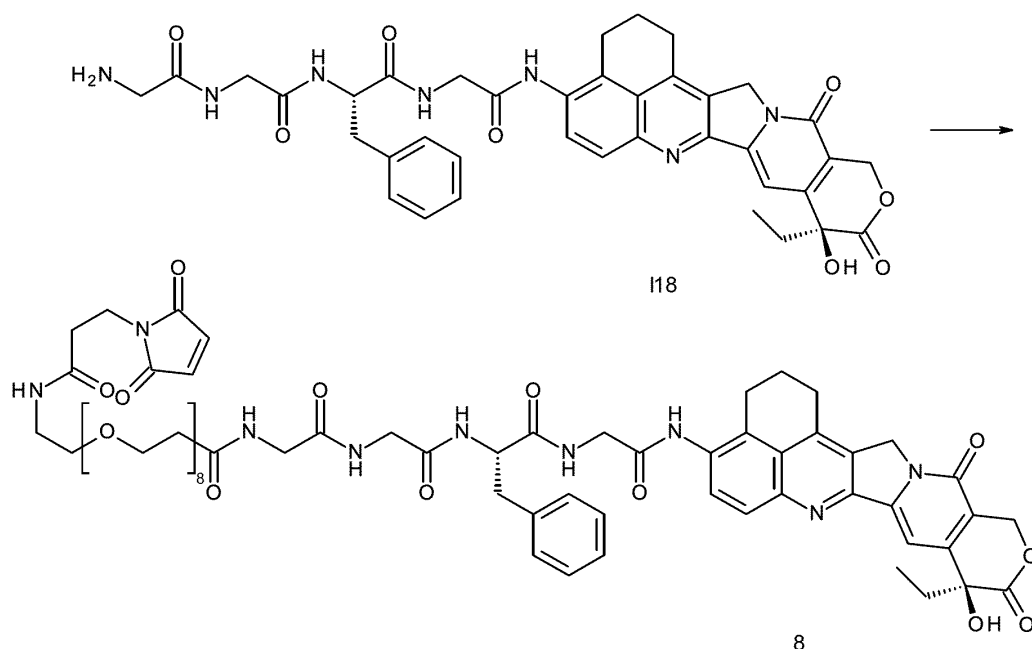
Synthesis Example 7 (e.g. synthesis of SG4052)



6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-(2-(((S)-1-((2-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-2-oxoethyl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)amino)-2-oxoethyl)hexanamide (7)

[0656] Compound **118** (259 mg, 0.3588 mmol) was solubilised in CH₂Cl₂ (25 mL). The starting material was not soluble at all so DMA (1 mL) was added. As no improvement was observed, DIPEA (68 µL, 0.390 mmol) was added and all the solid went in solution. Maleimide caproic acid (69 mg, 0.358 mmol) was added and the mixture left to stir at r.t. overnight and which point LCMS analysis revealed the reaction to be complete. The reaction mixture was quenched with MeOH (2 mL) and vacced down to dryness. The crude product was purified by preparative HPLC and subsequently freeze-dried to give compound **7** as an ochre solid (38.2 mg, 11% yield). Analytical data: LCMS 3min: ES⁺ = 1.47 min, *m/z* 916.2 [M + H]⁺ LCMS 15min: ES⁺ = 5.46 min, *m/z* 916.1 [M + H]⁺.

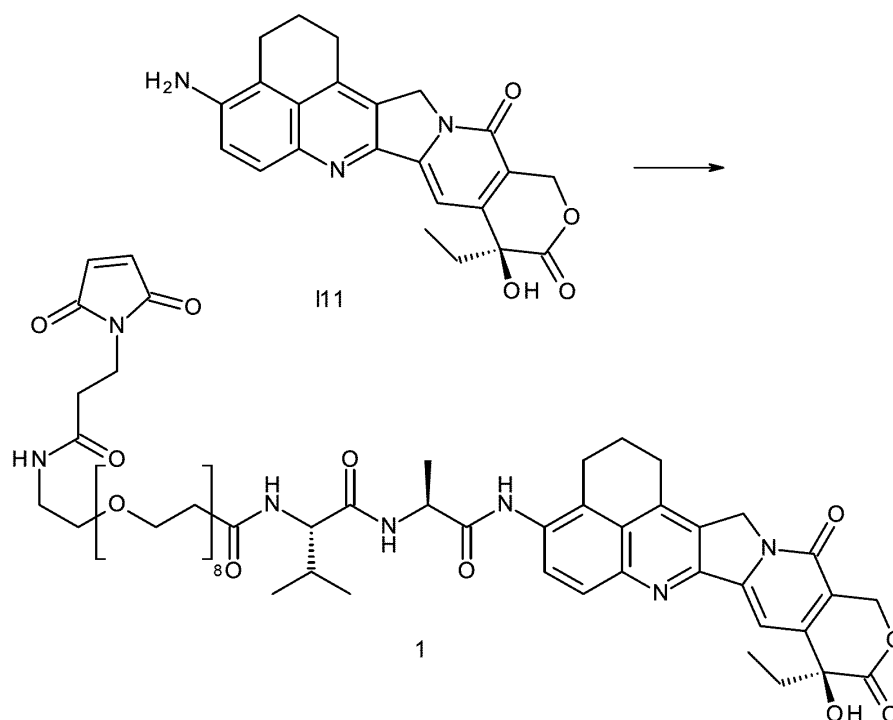
Synthesis Example 8 (e.g. synthesis of SG4057)



1-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanamido)-N-(2-(((2-(((S)-1-((2-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-2-oxoethyl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)amino)-2-oxoethyl)-3,6,9,12,15,18,21,24-octaoxaheptacosan-27-amide (8)

[0657] Compound **118** (70 mg, 0.096 mmol) was solubilised in CH₂Cl₂ (5 mL). The starting material was not soluble at all so DMA (0.5 mL) was added. As no improvement was observed, DIPEA (19 µL, 0.106 mmol) was added and all the solid went in solution. Mal-dPEG₈-OH (63 mg, 0.106 mmol) and EDCl.HCl (19mg, 0.099 mMol) were added and the mixture left to stir at r.t. overnight and which point LCMS analysis revealed the reaction to be complete. The reaction mixture was quenched with MeOH (2 mL) and vatted down to dryness. The crude product was purified by preparative HPLC and subsequently freeze-dried to give **8** as an ochre solid (30 mg, 24% yield). LCMS 3min: ES⁺ = 1.44 min, *m/z* 1297.6 [M + H]⁺.

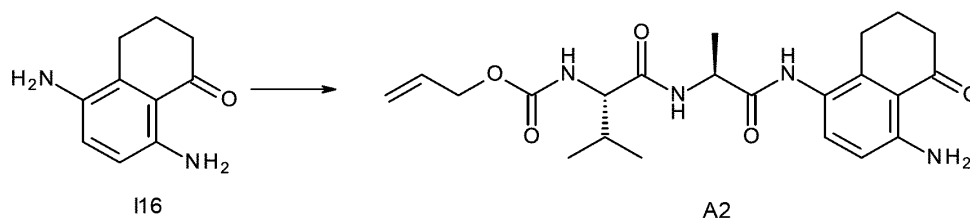
Synthesis Example 9 – Alternative synthesis of 1
(e.g. alternative synthesis of SG3932)



[0658] (S)-4-amino-9-ethyl-9-hydroxy-1,2,3,9,12,15-hexahydro-10H,13H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinoline-10,13-dione **111** (371 mg, 0.779 mmol, 1.0 eq.) was dissolved in dichloromethane (30 mL). *N,N*-diisopropylethylamine (69 μ L, 0.396 mmol, 0.51 eq.), and (2*S*)-2-[[[(2*S*)-2-[3-[2-[2-[2-[2-[2-[2-[2-[2-[3-(2,5-dioxopyrrol-1-yl)propanoylamino]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoylamino]-3-methyl-butanoyl]amino]propanoic acid (664 mg, 0.871 mmol, 1.1 eq.) in *N,N*-dimethylacetamide (10 mL) were added, followed by EDCI.HCl (226 mg, 1.18 mmol, 1.5 eq.) and the mixture stirred for 2 h, whereupon LCMS indicated good conversion, but that the reaction had stalled. The reaction mixture was warmed to 30°C and stirred for 30 min, LCMS indicated no change so CH₂Cl₂ was removed in vacuo and Et₂O added to the resulting DMA solution. The precipitated oil was collected, Et₂O

removed *in vacuo* and the precipitation process repeated. The combined precipitates were purified by HPLC (10-60% B in A over 13 min) to afford **1** (200 mg, 0.174 mmol, 98% purity, 22% Yield) as a yellow residue after freeze-drying. LC/MS (method A): retention time 1.44 mins (ES+) m/z 1149 [$M + H$]⁺. ¹H NMR (600 MHz, Chloroform-*d*) δ 8.81 (s, 1H), 7.83 (s, 2H), 7.48 (s, 1H), 7.18 (dd, $J = 18.7, 7.5$ Hz, 2H), 6.69 (s, 2H), 6.43 (s, 1H), 5.68 (d, $J = 16.1$ Hz, 1H), 5.27 (d, $J = 16.1$ Hz, 1H), 5.03 (d, $J = 18.4$ Hz, 1H), 4.90 (d, $J = 18.4$ Hz, 1H), 4.75 (p, $J = 7.2$ Hz, 1H), 4.32 (dd, $J = 7.4, 5.8$ Hz, 1H), 4.05 (s, 1H), 3.83 (t, $J = 7.2$ Hz, 3H), 3.78 – 3.68 (m, 3H), 3.68 – 3.57 (m, 31H), 3.53 (t, $J = 5.1$ Hz, 3H), 3.40 (q, $J = 5.3$ Hz, 2H), 3.06 – 2.91 (m, 3H), 2.84 (dt, $J = 16.3, 6.2$ Hz, 1H), 2.63 (ddd, $J = 14.8, 8.5, 4.2$ Hz, 1H), 2.57 – 2.44 (m, 4H), 2.30 (dq, $J = 13.4, 6.7$ Hz, 1H), 2.10 (p, $J = 6.4$ Hz, 3H), 1.91 (ddt, $J = 16.8, 14.3, 7.2$ Hz, 3H), 1.54 (d, $J = 7.1$ Hz, 3H), 1.02 (dd, $J = 15.5, 6.9$ Hz, 10H).

Synthesis Example 10 – Alternate synthesis of A2

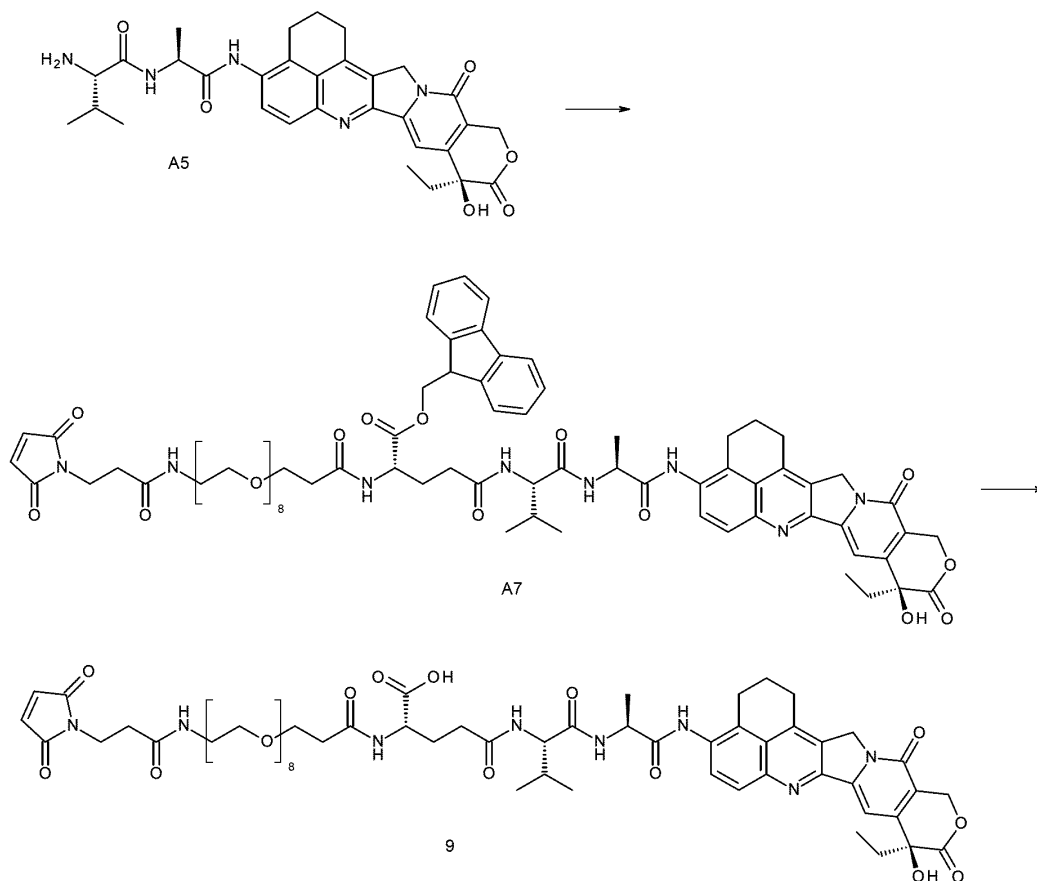


Allyl ((S)-1-(((S)-1-((4-amino-5-oxo-5,6,7,8-tetrahydronaphthalen-1-yl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**A2**)

[0659] EDCl.HCl (7.71 g, 31.2 mMol) was added to a solution of alloc-Val-Ala-OH (8.49 g, 31.2 mmol) in CH₂Cl₂ (200 mL) and stirred for 15 min or until solubilised. **116** (5 g, 28.3 mMol) was subsequently added and the resulting mixture was left to stir until the reaction was completed. The volatiles were removed under reduced pressure. The crude product was taken up in Et₂O (50 mL) and the mixture sonicated for 3 min. The solid was filtrated and taken up again in CH₂Cl₂ (50 mL), sonicated for 3 min and filtered again to give pure product **A2** as a grey solid (12.21 g, 79% yield). LC/MS (Method B): ES⁺ = 1.47 min, *m/z* 431.5 [M + H]⁺.

200

Synthesis Example 11



a) (9H-fluoren-9-yl)methyl N2-(1-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-oxo-7,10,13,16,19,22,25,28-octaoxa-4-azahentriacontan-31-oyl)-N5-((S)-1-(((S)-1-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-L-glutamate (A7)

[0660] EDCI.HCl (0.10 mmol, 1.2eq) was added to a solution of **A5** (0.087 mmol, 1.0eq) and Mal-PEG₈-Glu-OH (0.10 mmol, 1.2eq) in DCM (5 mL) and the resulting mixture stirred at room temperature overnight. The reaction mixture was evaporated to dryness and purified by column (8-12% MeOH / DCM) to leave the product as a white solid. Yield = 80 mg (63%). LC/MS (Method B) rt 1.66 min *m/z* (1456.2) M+H.

b) N2-(1-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-3-oxo-7,10,13,16,19,22,25,28-octaoxa-4-azahentriacontan-31-oyl)-N5-((S)-1-(((S)-1-(((S)-9-ethyl-9-hydroxy-10,13-dioxo-2,3,9,10,13,15-hexahydro-1H,12H-benzo[de]pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-L-glutamine (9)

[0661] 1-Methylpyrrolidine (200 μ L) was added to a solution of **A7** (0.06 mmol) in DMF (0.8 mL) and stirred at room temperature for 10 mins. The solvent was removed under vacuum and the residue purified by prep HPLC (30% MeCN / water + 0.05% formic acid over 8.5 mins). Fractions containing product were freeze dried to give the product as an off-white solid. Yield = 23 mg (30%). LC/MS (Method B) rt 1.43 min m/z (1278.4) M+H.

[0662] All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

SEQUENCES

Identifier	Sequence
ZY0EPQ-E02; CHDR1	SEQ ID NO: 1: GYYWN
ZY0EPQ-E02; CHDR2	SEQ ID NO: 2: EINHSGSTNYNPSLKS
ZY0EPQ-E02; CHDR3	SEQ ID NO: 3: NLYNWNLDS
ZY0EPQ-E02; CLDR1	SEQ ID NO: 4: RASQGIRNDLG
ZY0EPQ-E02; CLDR2	SEQ ID NO: 5: VASSLQS
ZY0EPQ-E02; CLDR3	SEQ ID NO: 6: LQHNSYPRT
ZY0EQD-E02; CHDR1	SEQ ID NO: 7: GYYWN
ZY0EQD-E02; CHDR2	SEQ ID NO: 8: EINHSGSTSYNPSLKS
ZY0EQD-E02; CHDR3	SEQ ID NO: 9: VLYNWNVDS
ZY0EQD-E02; CLDR1	SEQ ID NO: 10: RASQDIRNDVG
ZY0EQD-E02; CLDR2	SEQ ID NO: 11: AASRLQS
ZY0EQD-E02; CLDR3	SEQ ID NO: 12: LQHNSYPRT
ZY0EOB-F05; CHDR1	SEQ ID NO: 13: SGGYYWS
ZY0EOB-F05; CHDR2	SEQ ID NO: 14: NIYYSGSTYYNPSLKS
ZY0EOB-F05; CHDR3	SEQ ID NO: 15: EKALATVTPSGYENYYTVDV
ZY0EOB-F05; CLDR1	SEQ ID NO: 16: WASQGISSYLA
ZY0EOB-F05; CLDR2	SEQ ID NO: 17: AASTLQS
ZY0EOB-F05; CLDR3	SEQ ID NO: 18: QHLNSYPLT
ZY0EO5-E07; CHDR1	SEQ ID NO: 19: SGGYYWS
ZY0EO5-E07; CHDR2	SEQ ID NO: 20: NIYYSGSTYYNPSLKS
ZY0EO5-E07; CHDR3	SEQ ID NO: 21: EKALASVIPSGYENYYVVDV
ZY0EO5-E07; CLDR1	SEQ ID NO: 22: WASQGIAGYLA
ZY0EO5-E07; CLDR2	SEQ ID NO: 23: AASTLQS
ZY0EO5-E07; CLDR3	SEQ ID NO: 24: QHLNSYPLT
ZY0EP0-C07; CHDR1	SEQ ID NO: 25: DYYMS
ZY0EP0-C07; CHDR2	SEQ ID NO: 26: YISSSGSTIYYTDSVKG
ZY0EP0-C07; CHDR3	SEQ ID NO: 27: DGVGFDY
ZY0EP0-C07; CLDR1	SEQ ID NO: 28: RASQSVSSSYLA
ZY0EP0-C07; CLDR2	SEQ ID NO: 29: AASSRAT
ZY0EP0-C07; CLDR3	SEQ ID NO: 30: QQYGSSPLYT

SEQ ID NO: 31 (ZY0EPQ-E02, variable heavy chain)

QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWNWIRQPPGKGLEWIGEINHSGST
 NYNPSLKS RVTILVDT SKNQFSLKLSSVTAADTAVYYCARNLYNWNLDSWGQGTLTV
 SS

SEQ ID NO: 32 (ZY0EPQ-E02, variable light chain)

DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGRAPKRLIYVASSLQSGV
PSRFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPRTFGQGGTKVEIK

SEQ ID NO: 33 (ZY0EQD-E02, variable heavy chain, e.g. pre-germlining)

QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWNWIRQPPGKGLEWIGEINHSGST
SYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARVLYNWNVDSWGQGTLLTVV
SS

SEQ ID NO: 34 (ZY0EQD-E02, variable light chain)

DIQMTQSPSSLSASVGDRVTITCRASQDIRNDVGWYQQKPGKAPKRLIYAASRLQSGV
PSRFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPRTFGQGGTKVEIK

SEQ ID NO: 35 (ZY0EOB-F05, variable heavy chain)

QVQLQESGPGLVKPSQTLSTCTVSDGSISGGYYWSWIRQHPGKGLEWIGNIYYSG
STYYNPSLKSRTISVDTSKNQFSLKLNSVTAADTAVYYCATEKALATVTPSGYENYYT
VDVWGQGTTTVTVSS

SEQ ID NO: 36 (ZY0EOB-F05, variable light chain)

DIQLTQSPSFLSASVGDRVTITCWAQQGISSYLAWYQQKPGKAPKLLIYAASRLQSGVP
SRFSGSGSGTEFTLTISSLQPEDFATYYCQHLNSYPLTFGGGGTKVEIK

SEQ ID NO: 37 (ZY0EO5-E07, variable heavy chain)

QVQLQESGPGLVKPSQTLSTCTVSGGISGGYYWSWIRQHPGKGLEWIGNIYYSG
STYYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCAREKALASVIPSGYENYYT
VDVWGQGTTTVTVSS

SEQ ID NO: 38 (ZY0EO5-E07, variable light chain)

DIQLTQSPSFLSASVGGRVTITCWAQQGIAGYLAWYQQKPGKAPKLLIYAASRLQSGVP
SRFSGSGSGTEFTLTISSLQPEDFATYYCQHLNSYPLTFGGGGTKVEIK

SEQ ID NO: 39: (ZY0EP0-C07, variable heavy chain)

QVQLVESGGVLVKPGGSLRLSCAASGFTLSDYYMSWIRQAPGMGLEWVSYYSSSGST
IYYTDSVKGRFTISRDSAKNSLYLQMNSLRAEDTAVYYCARDGVGFDYWGQGTLLTVS
S

SEQ ID NO: 40 (ZY0EP0-C07, variable light chain)

EIVLTQSPGTLSTLFPGERATLSCRASQSVSSSYLAWYQQKPGQSPRLIYAASSRATGI
PDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPLYTFGGGGTKLEIK

SEQ ID NO: 41 (Maia heavy chain constant region, cysteine insertion underlined):

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ
SSGLYSLSVVTPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE
LLGGPSCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV

YTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFL
YSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 42 (light chain constant region)

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE
QDSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 43 (ZY0EQD-E02, variable heavy chain, e.g. pre-germlining, e.g. variant of SEQ ID NO: 33/ SEQ ID NO: 45)

QVQLQQWGAGLLKPSETLSLTCTVYGGSFSGYYWNWIRQPPGRGLEWIGEINHSGST
SYNPSLKSRITISIDTSKNQFSLKLSSVTAADTAVYYCARVLYNWNVDSWGQGTTLVTVS
S

SEQ ID NO: 44 (ZY0EQD-E02, light chain):

DIQMTQSPSSLSASVGDRVTITCRASQDIRNDVGWYQQKPGKAPKRLIYAASRLQSGV
PSRFSGSGSGTEFTLTISSLQPEDFATYYCLQHNSYPRTFGQGTKVEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSTY
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 45 (EQD-E02_GL, variable heavy chain, GL = germlined)

QVQLQQWGAGLLKPSETLSLACTVYGGSFSGYYWNWIRQPPGKGLEWIGEINHSGST
SYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARVLYNWNVDSWGQGTTLVTV
SS

SEQ ID NO: 46 (EQD-E02-GLY, variable heavy chain, GLY = germlined with a Y substitution)

QVQLQQWGAGLLKPSETLSLACTVYGGSFSGYYWNWIRQPPGKGLEWIGEIYHSGST
SYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARVLYNWNVDSWGQGTTLVTV
SS

SEQ ID NO: 47 (EQD-E02-GLQ, variable heavy chain, GLQ = germlined with a Q substitution)

QVQLQQWGAGLLKPSETLSLACTVYGGSFSGYYWNWIRQPPGKGLEWIGEIQHSGST
SYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARVLYNWNVDSWGQGTTLVTV
SS

SEQ ID NO: 48 (E02-GL-Maia-heavy chain, cysteine insertion underlined)

QVQLQQWGAGLLKPSETLSLACTVYGGSFSGYYWNWIRQPPGKGLEWIGEINHSGST
SYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARVLYNWNVDSWGQGTTLVTV
SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVTVPPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPA
PELLGGPSCVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP

QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGS
FFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 49 (E02-GLY-Maia-heavy chain, GLY = germlined with a Y substitution)

QVQLQQWGAGLLKPSETLSLACTVYGGSFSGYYWNWIRQPPGKGLEWIGEIYHSGST
SYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARVLYNWNVDSWGQGTLLTV
SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPA
PELLGGPSCVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP
QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGS
FFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 50 (E02-GLQ-Maia-heavy chain, GLQ = germlined with a Q substitution)

QVQLQQWGAGLLKPSETLSLACTVYGGSFSGYYWNWIRQPPGKGLEWIGEIQHSGST
SYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARVLYNWNVDSWGQGTLLTV
SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPA
PELLGGPSCVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP
QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGS
FFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 51 (E02-GL-WT-heavy chain)

QVQLQQWGAGLLKPSETLSLACTVYGGSFSGYYWNWIRQPPGKGLEWIGEINHSGST
SYNPSLKSRTISVDTSKNQFSLKLSSVTAADTAVYYCARVLYNWNVDSWGQGTLLTV
SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPA
PELLGGPSCVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKT
KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ
VYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFF
LYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 52 (heavy chain constant region)

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ
SSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE
LLGGPSCVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV
YTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFL
YSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 53 (Human B7H4 nucleic acid sequence, including 5' and 3' UTR)

GCCACCatggcttccctggggcagatcctcttctggagcataattagcatcatcattattctggctggagcaattgcactc
atcattggctttggtatttcagggagacactccatcacagtcactactgtcgctcagctgggaacattggggaggatggaa
tcctgagctgcacttttgaacctgacatcaaactttctgatatcgtgatacaatggctgaaggaagggttttaggcttggcca
tgagttcaaagaaggcaaatgagctgtcggagcaggatgaaatgttcagaggccggacagcagtggttctgatcaa
gtgatagttggcaatgcctctttgcggtgaaaaacgtgcaactcacagatgctggcacctacaaatgttatatcatcacttct
aaaggcaaggggaatgctaaccttgagtataaaactggagccttcagcatgccggaagtgaatgtggactataatgcc
gctcagagaccttgcggtgtgaggctccccgatggttccccagcccacagtggtctgggcatcccaagttgaccaggga
gccaaacttctcgaagtctcaataccagctttgagctgaactctgagaatgtgacctgaaggttgtctgtctctacaat
gttacgatcaacaacacatactcctgtatgattgaaaatgacattgccaaagcaacaggggatataaagtacagaatc
ggagatcaaaaggcggagtcacctacagctgctaaactcaaaggcttctgtgtgtctcttcttcttccatcagctgggc
acttctgcctctcagcccttacctgatgctaaaaTAATAA

SEQ ID NO: 54 (Human B7H4 nucleic acid sequence, coding sequence)

atggcttccctggggcagatcctcttctggagcataattagcatcatcattattctggctggagcaattgcactcatcattggctt
tggatttcagggagacactccatcacagtcactactgtcgctcagctgggaacattggggaggatggaatcctgagctg
cacttttgaacctgacatcaaactttctgatatcgtgatacaatggctgaaggaagggttttaggcttggccatgagtcaaa
gaaggcaaatgagctgtcggagcaggatgaaatgttcagaggccggacagcagtggttctgatcaagtatagttg
gcaatgcctctttgcggtgaaaaacgtgcaactcacagatgctggcacctacaaatgttatatcatcacttctaaaggcaa
ggggaatgctaaccttgagtataaaactggagccttcagcatgccggaagtgaatgtggactataatgccagctcagaga
ccttgcggtgtgaggctccccgatggttccccagcccacagtggtctgggcatcccaagttgaccaggggagccaacttct
cggaagtctcaataccagctttgagctgaactctgagaatgtgacctgaaggttgtctgtctctacaatgttacgatc
aacaacacatactcctgtatgattgaaaatgacattgccaaagcaacaggggatataaagtacagaatcggagatca
aaaggcggagtcacctacagctgctaaactcaaaggcttctgtgtgtctcttcttcttccatcagctgggcacttctgcct
ctcagcccttacctgatgctaaaa

SEQ ID NO: 55 (Human B7H4 polypeptide sequence; UniProt Accession No.: Q7Z7D3)

MASLGQILFWSIIIIILAGAIALIIIGFGISGRHSITVTTVASAGNIGEDGILSCTFEPDIKLS
D
IVIQWLKEGVLGLVHEFKEGKDELSEQDEMFRGRTAVFADQVIVGNASRLKKNVQLTD
AGTYKCYIITSKGKGNANLEYKTGAFSMPEVNVDYNASSETLRCEAPRWFPPQPTVVW
ASQVDQGANFSEVSNTSFELNSENVTMKVVSVLNVNTINNTYSCMIENDIAKATGDIKV
TESEIKRRSHLQLLNSKASLCVSSFFAISWALLPLSPYLMK

EMBODIMENTS

- E1. An antibody or antigen binding fragment thereof which binds to B7-H4, comprising:
- i. a heavy chain CDR1 (HCDR1), a heavy chain CDR2 (HCDR2), a heavy chain CDR3 (HCDR3), a light chain CDR1 (LCDR1), a light chain CDR2 (LCDR2), and a light chain CDR3 (LCDR3) comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof;
 - ii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof;
 - iii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof;
 - iv. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof; or
 - v. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof.
- E2. The antibody or antigen binding fragment thereof according to E1, wherein the antibody or antigen binding fragment thereof comprises:
- i. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 45 and SEQ ID NO: 34, respectively, or a functional variant thereof;

- ii. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 33 and SEQ ID NO: 34, respectively, or a functional variant thereof;
 - iii. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 43 and SEQ ID NO: 34, respectively, or a functional variant thereof;
 - iv. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 46 and SEQ ID NO: 34, respectively, or a functional variant thereof;
 - v. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 47 and SEQ ID NO: 34, respectively, or a functional variant thereof;
 - vi. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 31, and SEQ ID NO: 32, respectively, or a functional variant thereof;
 - vii. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 35 and SEQ ID NO: 36, respectively, or a functional variant thereof;
 - viii. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 37 and SEQ ID NO: 38, respectively, or a functional variant thereof; or
 - ix. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 39 and SEQ ID NO: 40, respectively, or a functional variant thereof.
- E3. The antibody or antigen binding fragment thereof according to E1 or E2, wherein the antibody or antigen binding fragment thereof comprises:
- i. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof.
- E4. The antibody or antigen binding fragment thereof according to E1-E3, wherein the antibody or antigen binding fragment thereof comprises:

- i. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 45 and SEQ ID NO: 34, respectively, or a functional variant thereof.
- E5. The antibody or antigen binding fragment thereof according to E1-E4, wherein the antibody or antigen binding fragment thereof binds an OVCAR4 cell line.
- E6. The antibody or antigen binding fragment thereof according to E1-E5, wherein the antibody or antigen binding fragment thereof comprises a heavy chain constant region comprising the amino acid sequence of SEQ ID NO: 41.
- E7. The antibody or antigen binding fragment thereof according to E1-E5, wherein the antibody or antigen binding fragment thereof comprises a heavy chain constant region comprising the amino acid sequence of SEQ ID NO: 52.
- E8. The antibody or antigen binding fragment thereof according to E1-E7, wherein the antibody or antigen binding fragment thereof comprises a light chain constant region comprising the amino acid sequence of SEQ ID NO: 42.
- E9. The antibody or antigen binding fragment thereof according to any one of E1-E5, wherein the antibody or antigen binding fragment thereof comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 51; and a light chain comprising the amino acid sequence of SEQ ID NO: 44.
- E10. The antibody or antigen binding fragment thereof according to any one of E1-E5, wherein the antibody or antigen binding fragment thereof comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 48; and a light chain comprising the amino acid sequence of SEQ ID NO: 44.

- E11. The antibody or antigen binding fragment thereof according to any one of E1-E10, wherein the antibody or antigen binding fragment thereof is conjugated to a heterologous agent.
- E12. The antibody or antigen binding fragment thereof according to any one of E1-E11, wherein the antibody or antigen binding fragment thereof is conjugated to one or more heterologous agent selected from the group consisting of a topoisomerase I inhibitor, a tubulysin derivative, a pyrrolobenzodiazepine, an antimicrobial agent, a therapeutic agent, a prodrug, a peptide, a protein, an enzyme, a lipid, a biological response modifier, a pharmaceutical agent, a lymphokine, a heterologous antibody, a fragment of a heterologous antibody, a detectable label, a polyethylene glycol (PEG), a radioisotope, or a combination thereof.
- E13. The antibody or antigen binding fragment thereof according to any one of E1-E12, wherein the antibody or antigen binding fragment thereof is conjugated to one or more heterologous agent selected from a topoisomerase I inhibitor, tubulysin derivative, a pyrrolobenzodiazepine, or a combination thereof.
- E14. The antibody or antigen binding fragment thereof according to any one of E1-E13, wherein the antibody or antigen binding fragment thereof is conjugated to a heterologous agent selected from the group consisting of tubulysin AZ1508, pyrrolobenzodiazepine SG3315, pyrrolobenzodiazepine SG3249, or a combination thereof.
- E15. The antibody or antigen binding fragment thereof according to any one of E1-E14, wherein the antibody or antigen binding fragment thereof is conjugated to a pyrrolobenzodiazepine SG3249 cytotoxin: (SG3249).
- E16. The antibody or antigen binding fragment thereof according to any one E1-E14, wherein the antibody or antigen binding fragment thereof is conjugated to:
(SG3932); (SG4010); (SG4057); and/or (SG4052).

- E17. The antibody or antigen binding fragment thereof according to any one of E1-E14, or E16, wherein the antibody or antigen binding fragment thereof is conjugated to: (SG3932).
- E18. The antibody or antigen binding fragment thereof according to any one of E1-E17, wherein said antibody or antigen binding fragment thereof is a monoclonal antibody.
- E19. The antibody or antigen binding fragment thereof according to any one of E1-E18, wherein said antibody or antigen binding fragment thereof is a humanised monoclonal antibody.
- E20. A pharmaceutical composition comprising an antibody or antigen binding fragment thereof according to any one of E1-E19.
- E21. A polynucleotide encoding the antibody or antigen binding fragment thereof according to any one of E1-E19.
- E22. A host cell comprising the polynucleotide of E21.
- E23. A method for producing an antibody or antigen binding fragment thereof that binds to B7-H4, comprising expressing a polynucleotide according to E22 in a host cell.
- E24. An antibody or antigen binding fragment thereof obtainable by the method of E23.
- E25. A method of treating a cancer comprising a cancer cell which expresses B7-H4, the method comprising administering to a subject the antibody or antigen binding fragment of any one of E1-E19 or E24, the pharmaceutical composition of E20, or a combination thereof.

- E26. An antibody or antigen binding fragment thereof according to any one of E1-E19 or 24, or the pharmaceutical composition of E20, for use in treating a cancer, wherein said cancer comprises a cancer cell which expresses B7-H4.
- E27. The method according to E25, or antibody or antigen binding fragment thereof or pharmaceutical composition for use according to E26, where said cancer is selected from breast cancer, ovarian cancer, endometrial cancer, cholangiocarcinoma, NSCLC (squamous and/or adenocarcinoma), pancreatic cancer, and gastric cancer.
- E28. The method, or antibody or antigen binding fragment thereof or pharmaceutical composition for use according to any one of E25-E27, wherein said cancer is selected from breast cancer, ovarian cancer, endometrial cancer, and cholangiocarcinoma.
- E29. The method, or antibody or antigen binding fragment thereof or pharmaceutical composition for use according to any one of E25-E28, wherein said cancer is a breast cancer selected from hormone receptor-positive (HR+) breast cancer, human epidermal growth factor receptor 2 positive (HER2+) breast cancer, and triple negative breast cancer (TNBC).
- E30. A method for detecting the presence or absence of a B7-H4 polypeptide in a sample, comprising:
- i. contacting a sample with an antibody or antigen binding fragment thereof according to any one of E1-E19 or E24, or a pharmaceutical composition according to E20, to provide an antibody-antigen complex;
 - ii. detecting the presence or absence of said antibody-antigen complex;
 - iii. wherein the presence of the antibody-antigen complex confirms the presence of a B7-H4 polypeptide;
 - iv. wherein the absence of the antibody-antigen complex confirms the absence of B7-H4 polypeptide.

- E31. The method according to E30, wherein the presence of said antibody-antigen complex is indicative of the presence of a cancer cell, and wherein the absence of said antibody-antigen complex is indicative of the absence of a cancer cell.
- E32. The method according to E30 or E31, wherein the sample is an isolated sample obtainable from a subject.
- E33. The method according to any one of E30-E32, wherein the B7-H4 polypeptide is an integral component of a cancer cell.
- E34. An antibody-drug conjugate (ADC) comprising:
(i) antibody or antigen binding fragment thereof which binds to human B7-H4 comprising: a HCDR1 comprising the amino acid sequence of SEQ ID NO: 7; a HCDR2 comprising the amino acid sequence of SEQ ID NO: 8; a HCDR3 comprising the amino acid sequence of SEQ ID NO: 9; and a LCDR1 comprising the amino acid sequence of SEQ ID NO: 10; a LCDR2 comprising the amino acid sequence of SEQ ID NO: 11; and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 12;
(ii) a linker; and
(iii) a cytotoxic agent wherein the cytotoxic agent is SG3932, wherein the ADC has a drug to antibody ratio (DAR) of 8.
- E35. The ADC of E34, wherein the antibody or antigen binding fragment thereof comprises a variable heavy (VH) chain comprising the amino acid sequence of SEQ ID NO: 45 and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 34.
- E36. The ADC of E34 or E35 comprising a heavy chain (HC) comprising the amino acid sequence of SEQ ID NO: 51, and light chain (LC) comprising the amino acid sequence of SEQ ID NO: 44.

- E37. A pharmaceutical composition comprising the ADC of any one of E34-E37.
- E38. A method of treating a cancer comprising a cancer cell which expresses B7-H4, the method comprising administering to a subject the ADC of any one of E34-E37, or the pharmaceutical composition of E37, or a combination thereof.

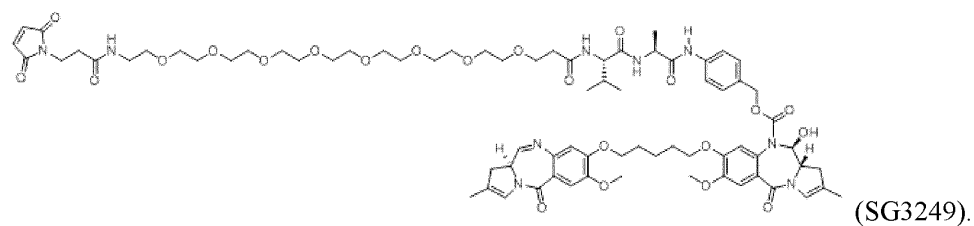
CLAIMS

1. An antibody or antigen binding fragment thereof which binds to B7-H4, comprising:
 - i. a heavy chain CDR1 (HCDR1), a heavy chain CDR2 (HCDR2), a heavy chain CDR3 (HCDR3), a light chain CDR1 (LCDR1), a light chain CDR2 (LCDR2), and a light chain CDR3 (LCDR3) comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof;
 - ii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6, respectively, or a functional variant thereof;
 - iii. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, and SEQ ID NO: 18, respectively, or a functional variant thereof;
 - iv. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24, respectively, or a functional variant thereof; or
 - v. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, and SEQ ID NO: 30, respectively, or a functional variant thereof.
2. The antibody or antigen binding fragment thereof according to claim 1, wherein the antibody or antigen binding fragment thereof comprises:
 - i. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 45 and SEQ ID NO: 34, respectively, or a functional variant thereof;

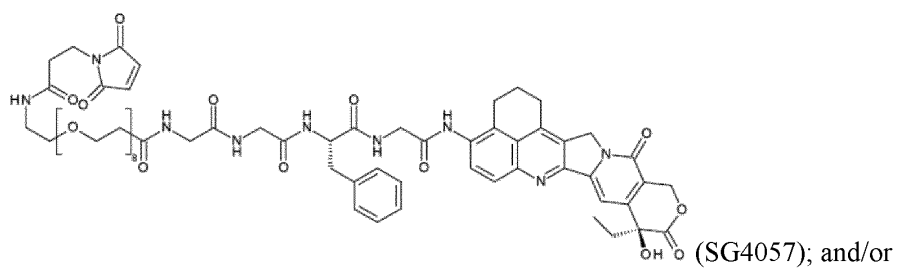
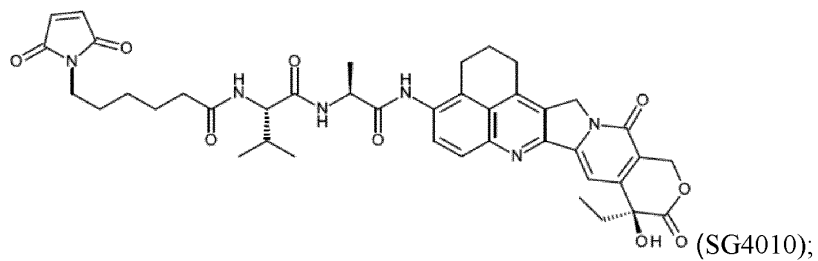
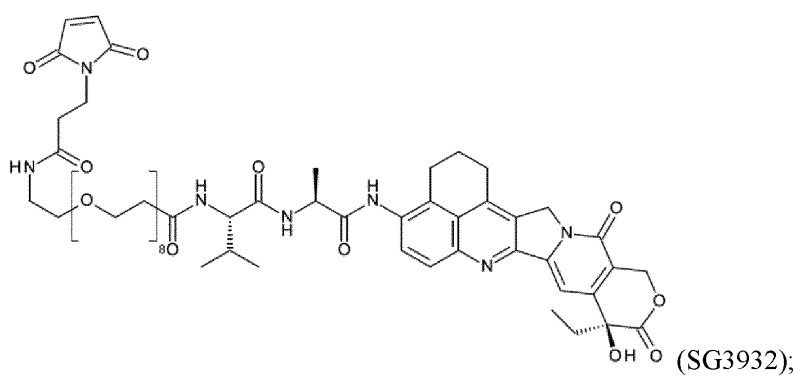
- ii. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 33 and SEQ ID NO: 34, respectively, or a functional variant thereof;
 - iii. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 43 and SEQ ID NO: 34, respectively, or a functional variant thereof;
 - iv. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 46 and SEQ ID NO: 34, respectively, or a functional variant thereof;
 - v. a variable heavy (VH) chain and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 47 and SEQ ID NO: 34, respectively, or a functional variant thereof;
 - vi. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 31, and SEQ ID NO: 32, respectively, or a functional variant thereof;
 - vii. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 35 and SEQ ID NO: 36, respectively, or a functional variant thereof;
 - viii. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 37 and SEQ ID NO: 38, respectively, or a functional variant thereof; or
 - ix. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 39 and SEQ ID NO: 40, respectively, or a functional variant thereof.
3. The antibody or antigen binding fragment thereof according to claim 1 or claim 2, wherein the antibody or antigen binding fragment thereof comprises:
- i. a HCDR1, a HCDR2, a HCDR3, a LCDR1, a LCDR2, and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO: 12, respectively, or a functional variant thereof.
4. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein the antibody or antigen binding fragment thereof comprises:

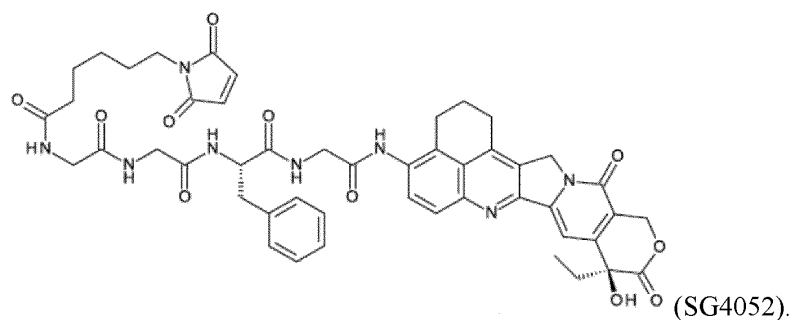
- i. a VH chain and a VL chain comprising the amino acid sequence of SEQ ID NO: 45 and SEQ ID NO: 34, respectively, or a functional variant thereof.
5. The antibody or antigen binding fragment thereof according any one of the preceding claims, wherein the antibody or antigen binding fragment thereof binds an OVCAR4 cell line.
6. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein the antibody or antigen binding fragment thereof comprises a heavy chain constant region comprising the amino acid sequence of SEQ ID NO: 41.
7. The antibody or antigen binding fragment thereof according to any one of claims 1-5, wherein the antibody or antigen binding fragment thereof comprises a heavy chain constant region comprising the amino acid sequence of SEQ ID NO: 52.
8. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein the antibody or antigen binding fragment thereof comprises a light constant region comprising the amino acid sequence of SEQ ID NO: 42.
9. The antibody or antigen binding fragment thereof according to any one of claims 1-5, wherein the antibody or antigen binding fragment thereof comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 51; and a light chain comprising the amino acid sequence of SEQ ID NO: 44.
10. The antibody or antigen binding fragment thereof according to any one of claims 1-5, wherein the antibody or antigen binding fragment thereof comprises a heavy chain comprising the amino acid sequence of SEQ ID NO: 48; and a light chain comprising the amino acid sequence of SEQ ID NO: 44.

11. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein the antibody or antigen binding fragment thereof is conjugated to a heterologous agent.
12. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein the antibody or antigen binding fragment thereof is conjugated to one or more heterologous agent selected from the group consisting of a topoisomerase I inhibitor, a tubulysin derivative, a pyrrolobenzodiazepine, an antimicrobial agent, a therapeutic agent, a prodrug, a peptide, a protein, an enzyme, a lipid, a biological response modifier, a pharmaceutical agent, a lymphokine, a heterologous antibody, a fragment of a heterologous antibody, a detectable label, a polyethylene glycol (PEG), a radioisotope, or a combination thereof.
13. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein the antibody or antigen binding fragment thereof is conjugated to one or more heterologous agent selected from a topoisomerase I inhibitor, tubulysin derivative, a pyrrolobenzodiazepine, or a combination thereof.
14. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein the antibody or antigen binding fragment thereof is conjugated to a heterologous agent selected from the group consisting of tubulysin AZ1508, pyrrolobenzodiazepine SG3315, pyrrolobenzodiazepine SG3249, or a combination thereof.
15. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein the antibody or antigen binding fragment thereof is conjugated to a pyrrolobenzodiazepine SG3249 cytotoxin:

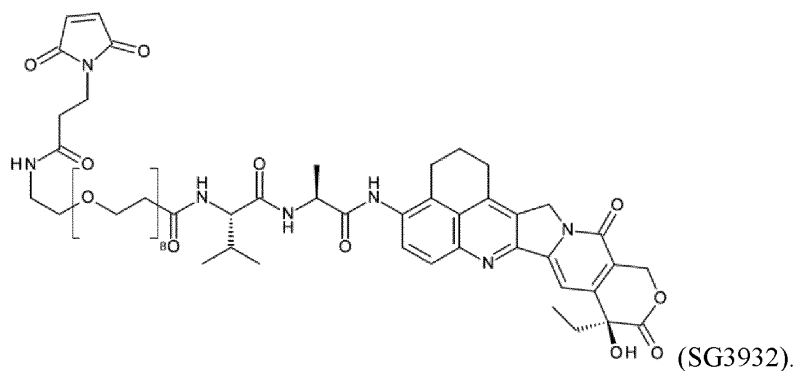


16. The antibody or antigen binding fragment thereof according to any one claims 1-14, wherein the antibody or antigen binding fragment thereof is conjugated to:





17. The antibody or antigen binding fragment thereof according to any one of claims 1-14 or 16, wherein the antibody or antigen binding fragment thereof is conjugated to:



18. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein said antibody or antigen binding fragment thereof is a monoclonal antibody.
19. The antibody or antigen binding fragment thereof according to any one of the preceding claims, wherein said antibody or antigen binding fragment thereof is a humanised monoclonal antibody.
20. A pharmaceutical composition comprising an antibody or antigen binding fragment thereof according to any one of the preceding claims.

21. A polynucleotide encoding the antibody or antigen binding fragment thereof according to any one of claims 1-19.
22. A host cell comprising the polynucleotide of claim 21.
23. A method for producing an antibody or antigen binding fragment thereof that binds to B7-H4, comprising expressing a polynucleotide according to claim 22 in a host cell.
24. An antibody or antigen binding fragment thereof obtainable by the method of claim 23.
25. A method of treating a cancer comprising a cancer cell which expresses B7-H4, the method comprising administering to a subject the antibody or antigen binding fragment of any one of claims 1-19 or 24, the pharmaceutical composition of claim 20, or a combination thereof.
26. An antibody or antigen binding fragment thereof according to any one of claims 1-19 or 24, or the pharmaceutical composition of claim 20, for use in treating a cancer, wherein said cancer comprises a cancer cell which expresses B7-H4.
27. The method according to claim 25, or antibody or antigen binding fragment thereof or pharmaceutical composition for use according to claim 26, where said cancer is selected from breast cancer, ovarian cancer, endometrial cancer, cholangiocarcinoma, NSCLC (squamous and/or adenocarcinoma), pancreatic cancer, and gastric cancer.
28. The method, or antibody or antigen binding fragment thereof or pharmaceutical composition for use according to any one of claims 25-27, wherein said cancer is selected from breast cancer, ovarian cancer, endometrial cancer, and cholangiocarcinoma.

29. The method, or antibody or antigen binding fragment thereof or pharmaceutical composition for use according to any one of claims 25-28, wherein said cancer is a breast cancer selected from hormone receptor-positive (HR+) breast cancer, human epidermal growth factor receptor 2 positive (HER2+) breast cancer, and triple negative breast cancer (TNBC).
30. A method for detecting the presence or absence of a B7-H4 polypeptide in a sample, comprising:
- i. contacting a sample with an antibody or antigen binding fragment thereof according to any one of claims 1-19 or 24, or a pharmaceutical composition according to claim 20, to provide an antibody-antigen complex;
 - ii. detecting the presence or absence of said antibody-antigen complex;
 - iii. wherein the presence of the antibody-antigen complex confirms the presence of a B7-H4 polypeptide;
 - iv. wherein the absence of the antibody-antigen complex confirms the absence of B7-H4 polypeptide.
31. The method according to claim 30, wherein the presence of said antibody-antigen complex is indicative of the presence of a cancer cell, and wherein the absence of said antibody-antigen complex is indicative of the absence of a cancer cell.
32. The method according to claim 30 or claim 31, wherein the sample is an isolated sample obtainable from a subject.
33. The method according to any one of claims 30-32, wherein the B7-H4 polypeptide is an integral component of a cancer cell.
34. An antibody-drug conjugate (ADC) comprising:

(i) antibody or antigen binding fragment thereof which binds to a B7-H4 polypeptide comprising: a HCDR1 comprising the amino acid sequence of SEQ ID NO: 7; a HCDR2 comprising the amino acid sequence of SEQ ID NO: 8; a HCDR3 comprising the amino acid sequence of SEQ ID NO: 9; and a LCDR1 comprising the amino acid sequence of SEQ ID NO: 10; a LCDR2 comprising the amino acid sequence of SEQ ID NO: 11; and a LCDR3 comprising the amino acid sequence of SEQ ID NO: 12;

(ii) a cleavable mp-PEG8-val-ala linker; and

(iii) a cytotoxic agent wherein the cytotoxic agent is SG3932,
and wherein the ADC has a drug to antibody ratio (DAR) of about 8.

35. The ADC of claim 34, wherein the antibody or antigen binding fragment thereof comprises a variable heavy (VH) chain comprising the amino acid sequence of SEQ ID NO: 45 and a variable light (VL) chain comprising the amino acid sequence of SEQ ID NO: 34.
36. The ADC of claim 34 or 35 comprising a heavy chain (HC) comprising the amino acid sequence of SEQ ID NO: 51, and light chain (LC) comprising the amino acid sequence of SEQ ID NO: 44.
37. A pharmaceutical composition comprising the ADC of any one of claims 34-37.
38. A method of treating a cancer comprising a cancer cell which expresses B7-H4, the method comprising administering to a subject the ADC of any one of claims 34-37, or the pharmaceutical composition of claim 37, or a combination thereof.
39. The method of claim 25 or claim 38, wherein the cancer cell has a homologous DNA repair defect.

40. The method of claim 39, wherein the homologous DNA repair defect is defined by the presence of a BRCA1 mutation.
41. The method of claim 39 or claim 40, wherein the homologous DNA repair defect is defined by a negative score in a RAD51 foci formation assay.
42. A method for reducing the volume of a tumor which expresses B7-H4, the method comprising administering to a subject the the antibody or antigen binding fragment of any one of claims 1-19 or 24, the pharmaceutical composition of claim 20, the ADC of of any one of claims 34-37, the pharmaceutical composition of claim 37, or a combination thereof.
43. The method of claim 42, wherein the tumor has a homologous DNA repair defect.
44. The method of claim 43, wherein the homologous DNA repair defect is defined by the presence of a BRCA1 mutation.
45. The method of claim 43 or 44, wherein the homologous DNA repair defect is defined by a negative score in a RAD51 foci formation assay.