

ROR1-SPECIFIC VARIANT ANTIGEN BINDING MOLECULES

FIELD OF INVENTION

- 5 The present invention relates to receptor tyrosine kinase-like orphan receptor 1 (ROR1) specific antigen binding molecules and associated fusion proteins and conjugates. In a further aspect, the present invention relates to conjugated immunoglobulin-like shark variable novel antigen receptors (VNARs).

BACKGROUND

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Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is a 937 amino acid glycosylated type I single pass transmembrane protein. The extracellular region consists of three distinct domains composing an N-terminal immunoglobulin domain (Ig), followed by a cysteine rich fizzled domain (fz) which in turn is linked to the membrane proximal kringle domain (kr). The intracellular region of the protein contains a

15 pseudo kinase domain followed by two Ser/Thr rich domains which are interspersed by a proline-rich region, and this same overall domain architecture is conserved in the closely related family member ROR2, with which it shares high sequence identity.

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ROR1 is expressed during embryonic development, where it is prominently expressed in neural crest cells and in the necrotic and interdigital zones in the later stages of development. However, its expression is quickly silenced after birth, and is largely absent in normal adult tissue. ROR1 expression has been observed at both the mRNA and protein level across a broad range of solid tumours and haematological malignancies including lung, endometrial, pancreatic, ovarian, colon, head and neck and prostate cancers, melanoma and renal cell carcinoma, breast cancer and chronic lymphocytic leukemia

25 (CLL) and acute lymphoblastic leukemia (AML). Additionally, increased ROR1 expression is reported to correlate with poor clinical outcomes for a number of cancer indications including breast cancer, ovarian cancer, colorectal cancer, lung adenocarcinoma and CLL.

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Consistent with ROR1's expression pattern and the link to poor clinical prognosis, a functional role for ROR1 in tumorigenesis and disease progression has been demonstrated for a number of different cancer indications. ROR1 promotes epithelial-mesenchymal transition and metastasis in models of breast cancer and spheroid formation and tumour engraftment in models of ovarian cancer. ROR1 is a transcript target of the NKX2-1/TTF-1 lineage survival factor oncogene in lung adenocarcinoma, where it sustains EGFR signalling and represses pro-apoptotic signalling. ROR1 has also been shown to act

35 as a scaffold to sustain caveolae structures and by-pass signalling mechanism that confer resistance to EGFR tyrosine kinase inhibitors. Signalling through an ROR1-HER3 complex modulates the Hippo-YAP pathway and promotes breast cancer bone metastasis and the protein can promote Met-driven tumorigenesis. ROR1 expression is associated with chemotherapy resistance in breast cancer through activation of Hippo-YAP/TAZ and BMI1 pathways. Whilst in CLL, ROR1 has been reported to hetero-

oligomerise with ROR2 in response to Wnt5a to transduce signalling and enhance proliferation and migration.

Given the functional role of ROR1 in cancer pathology and the general lack of expression on normal adult tissue, this oncofetal protein is an attractive target for cancer therapy. Antibodies to ROR1 have been described in the literature WO2021097313 (4A5 kipps), WO2014031174 (UC961), WO2016187220 (Five Prime) WO2010124188 (2A2), WO2012075158 (R11, R12), WO2011054007 (Oxford Bio), WO2011079902 (Bioinvent) WO2017127664, WO2017127664 (NBE Therapeutics, SCRIPPS), WO2016094847 (Emergent), WO2017127499), and a humanised murine anti-ROR1 antibody, UC961, has entered clinical trials for relapsed or refractory chronic lymphocytic leukemia. Chimeric antigen receptor T-cells targeting ROR1 have also been reported (Hudecek M et al, Clin. Cancer Res., 2013, 19, 3153-64) and preclinical primate studies with UC961 and with CAR-T cells targeting ROR1 showed no overt toxicity, which is consistent with the general lack of expression of the protein on adult tissue (Choi M et al, Clinical Lymphoma, myeloma & leukemia, 2015, S167; Berger C et al, Cancer Immunol. Res., 2015, 3, 206).

Single domain binding molecules can be derived from an array of proteins from distinct species. The immunoglobulin isotope novel antigen receptor (IgNAR) is a homodimeric heavy-chain complex originally found in the serum of the nurse shark (*Ginglymostoma cirratum*) and other sharks and ray species. IgNARs do not contain light chains and are distinct from the typical immunoglobulin structure. Each molecule consists of a single-variable domain (VNAR) and five constant domains (CNAR). The nomenclature in the literature refers to IgNARs as immunoglobulin isotope novel antigen receptors or immunoglobulin isotope new antigen receptors and the terms are synonymous.

There are three main defined types of shark IgNAR known as I, II and III (Kovalena et al, Exp Opin Biol Ther 2014 14(10) 1527-1539). These have been categorized based on the position of non-canonical cysteine residues which are under strong selective pressure and are therefore rarely replaced.

All three types have the classical immunoglobulin canonical cysteines at positions 35 and 107 that stabilize the standard immunoglobulin fold, together with an invariant tryptophan at position 36. There is no defined CDR2 as such, but regions of sequence variation that compare more closely to TCR HV2 and HV4 have been defined in framework 2 and 3 respectively. Type I has germline encoded cysteine residues in framework 2 and framework 4 and an even number of additional cysteines within CDR3. Crystal structure studies of a Type I IgNAR isolated against and in complex with lysozyme enabled the contribution of these cysteine residues to be determined. Both the framework 2 and 4 cysteines form disulphide bridges with those in CDR3 forming a tightly packed structure within which the CDR3 loop is held tightly down towards the HV2 region. To date Type I IgNARs have only been identified in nurse sharks – all other elasmobranchs, including members of the same order have only Type II or variations of this type.

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Type II IgNAR are defined as having a cysteine residue in CDR1 and CDR3 which form intra-molecular disulphide bonds that hold these two regions in close proximity, resulting in a protruding CDR3 that is conducive to binding pockets or grooves. Type I sequences typically have longer CDR3s than type II with an average of 21 and 15 residues respectively. This is believed to be due to a strong selective pressure for two or more cysteine residues in Type I CDR3 to associate with their framework 2 and 4 counterparts. Studies into the accumulation of somatic mutations show that there are a greater number of mutations in CDR1 of type II than type I, whereas HV2 regions of Type I show greater sequence variation than Type II. This evidence correlates well with the determined positioning of these regions within the antigen binding sites. A third IgNAR type known as Type III has been identified in neonates. This member of the IgNAR family lacks diversity within CDR3 due to the germline fusion of the D1 and D2 regions (which form CDR3) with the V-gene. Almost all known clones have a CDR3 length of 15 residues with little or no sequence diversity.

Another structural type of VNAR, termed type (IIb or IV), has only two canonical cysteine residues (in framework 1 and framework 3b regions). So far, this type has been found primarily in dogfish sharks and was also isolated from semisynthetic V-NAR libraries derived from wobbegong sharks.

ROR1-specific antigen binding molecules, including VNARs, are described in WO 2019/122447, hereby incorporated by reference in its entirety. Amongst others, WO 2019/122447 describes the sequences of B1 and P3A1 G1 identified below.

B1

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWLQWYDGAGTVLTVN (SEQ ID NO: 113)

P3A1 G1

TRVDQSPSSLSASVGDRVTITCVLTDTSYGLYSTYWYRK NPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 114).

Conjugates of ROR1-specific antigen binding molecules, including VNARs, are described in PCT/EP2020/067210 filed on 19 June 2020, hereby incorporated by reference in its entirety. PCT/EP2020/067210 describes anthracycline (PNU) derivatives suitable for use in drug conjugates. Specifically, derivatives of PNU159682 are provided, which lack the C14 carbon and attached hydroxyl functionality, and in which an ethylenediamino (EDA) group forms part of a linker region between the C13 carbonyl of PNU159682 and a maleimide group. Alternatively, the same molecules may be described with EDA-PNU as the "warhead" such that the EDA group is not considered part of the linker region. Where the linker comprises val-cit-PAB the maleimide group may be replaced with any reactive group suitable for a conjugation reaction. Such payloads are able to react with a free thiol group on another molecule. Where the free thiol is on a protein a protein-drug conjugate (PDC) may be formed.

The anthracycline derivative PNU-159682 has been described as a metabolite of nemorubicin (Quintieri et al. (2005) *Clin. Cancer Res.* 11, 1608-1617) and has been reported to exhibit extremely high potency for *in vitro* cell killing in the pico- to femtomolar range with one ovarian (A2780) and one breast cancer (MCF7) cell line (WO2012/073217 A1). Derivatives of PNU-159682 have also been described in
5 WO2016/102679.

Conjugation of PNU-159682 derivatives to antibodies is described in WO2009/099741, WO2016/127081 and WO2016/102679, Yu et al, *Clin. Cancer Res* 2015, 21, 3298 and Stefan et al, *Mol. Cancer. Ther.*, 2017, 16,879.

10 Described herein are ROR1-specific variant antigen binding molecules having improved properties and conjugates thereof to derivatives of PNU-159682.

15 SUMMARY OF INVENTION

The present invention generally relates to specific antigen binding molecules.

According to a first aspect, the invention provides a receptor tyrosine kinase-like orphan receptor 1 (ROR1) specific antigen binding molecule comprising an amino acid sequence represented by the
20 formula (I):

FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 (I)

wherein
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CDR3 is a CDR sequence having an amino acid sequence selected from the group consisting of YPWGAGAPYNVQWY (SEQ ID NO: 23), YPWGAGAPYLVQWY (SEQ ID NO: 20), YPWGAGAPWNVQWY (SEQ ID NO: 24), YPSGAGAPRPVQWY (SEQ ID NO: 11), YPWGAGAPCLVQWY (SEQ ID NO: 12), YPWGAGAPRLVQWY (SEQ ID NO: 13),
30 YPWGAGAPRQVQWY (SEQ ID NO: 14), YPWGAGAPRSVQWY (SEQ ID NO: 15), YPWGAGAPSLVQWY (SEQ ID NO: 16), YPWGAGAPSNVQWY (SEQ ID NO: 17), YPWGAGAPSQVQWY (SEQ ID NO: 18), YPWGAGAPSSVQWY (SEQ ID NO: 19), YPWGAGAPWQVQWY (SEQ ID NO: 21), YPWGAGAPWSVQWY (SEQ ID NO: 22), and YPWGAGAPWLVQWY (SEQ ID NO: 10);

35 CDR1 is a CDR sequence having an amino acid sequence selected from the group consisting of GANYGLAA (SEQ ID NO: 1), DANYGLAA (SEQ ID NO: 5), GANYDLA (SEQ ID NO: 2), GANYGLA (SEQ ID NO: 3), and GANYDLAA (SEQ ID NO: 4)

40 FW1 is a framework region;

FW2 is a framework region;

5 HV2 is a hypervariable sequence having an amino acid sequence selected from the group consisting of SSNQERISIS (SEQ ID NO: 6), and SSNKERISIS (SEQ ID NO: 7);

FW3a is a framework region;

10 HV4 is a hypervariable sequence having an amino acid sequence selected from the group consisting of NKRTM (SEQ ID NO: 8) and NKGTM (SEQ ID NO: 9);

FW3b is a framework region;

15 FW4 is a framework region;

wherein if CDR3 is YPWGAGAPWLQWY (SEQ ID NO: 10) then CDR1 is selected from the group consisting of DANYGLAA (SEQ ID NO: 5), GANYGLSA (SEQ ID NO: 3) and GANYDLAA (SEQ ID NO: 4).

20 According to a second aspect, the invention provides a receptor tyrosine kinase-like orphan receptor 1 (ROR1) specific antigen binding molecule comprising an amino acid sequence represented by the formula (I):

FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 (I)

25 wherein

30 CDR1 is a CDR sequence having an amino acid sequence selected from the group consisting of GTRYGLYS (SEQ ID NO: 25), GTRYGLYSS (SEQ ID NO: 26), DTRYALYS (SEQ ID NO: 27), DTRYALYSS (SEQ ID NO: 28), GTKYGLYA (SEQ ID NO: 29) and GTKYGLYAS (SEQ ID NO: 30);

FW1 is a framework region;

35 FW2 is a framework region;

HV2 is a hypervariable sequence having an amino acid sequence selected from the group consisting of SSDEERISIS (SEQ ID NO: 31), STDEERISIG (SEQ ID NO: 32), SPNKDRMIIG (SEQ ID NO: 33), and STDKERIIIG (SEQ ID NO: 34);

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FW3a is a framework region;

HV4 is a hypervariable sequence having an amino acid sequence selected from the group consisting of NKGTK (SEQ ID NO: 35), NKGSK (SEQ ID NO: 36), NNGTK (SEQ ID NO: 37), and NNRSK (SEQ ID NO: 38);

FW3b is a framework region;

CDR3 is a CDR sequence having an amino acid sequence according to REARHPWLRQWY (SEQ ID NO: 39);

FW4 is a framework region.

According to a third aspect, the invention provides a recombinant fusion protein comprising a specific antigen binding molecule according to the first or the second aspects of the invention.

According to a fourth aspect, the invention provides a recombinant fusion protein comprising an antigen binding molecule comprising an amino acid sequence represented by the formula (I):

FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 (I)

wherein

FW1 is a framework region

CDR1 is a CDR sequence

FW2 is a framework region

HV2 is a hypervariable sequence

FW3a is a framework region

HV4 is a hypervariable sequence

FW3b is a framework region

CDR3 is a CDR sequence

FW4 is a framework region

or a functional variant thereof,

5 wherein the antigen binding molecule is fused to a fragment of an immunoglobulin Fc region wherein the fragment of an immunoglobulin Fc region is engineered to dimerize with a second fragment of an immunoglobulin Fc region.

According to a fifth aspect, the invention provides a recombinant fusion protein dimer comprising

- 10 (a) a first recombinant fusion protein, wherein the first recombinant fusion protein is a recombinant fusion protein according to the third or fourth aspects, and
- (b) a second recombinant fusion protein, wherein the second recombinant fusion protein comprises a second antigen binding molecule fused to a second fragment of an immunoglobulin Fc region engineered to dimerize with the first fragment of an immunoglobulin Fc region.

15 According to a sixth aspect, the invention provides a ROR1-specific chimeric antigen receptor (CAR), comprising at least one ROR1-specific antigen binding molecule as defined by the first or second aspects of the invention, fused or conjugated to at least one transmembrane region and at least one intracellular domain.

20 The present invention also provides a cell comprising a chimeric antigen receptor according to the sixth aspect, which cell is preferably an engineered T-cell.

25 In a seventh aspect of the invention, there is provided a nucleic acid sequence comprising a polynucleotide sequence that encodes a specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor according to the first, second, third, fourth, fifth or sixth aspects of the invention.

30 There is also provided a vector comprising a nucleic acid sequence in accordance with the seventh aspect and a host cell comprising such a nucleic acid.

35 A method for preparing a specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor, of the first, second, third, fourth, fifth or sixth aspect is provided, the method comprising cultivating or maintaining a host cell comprising the polynucleotide or vector described above under conditions such that said host cell produces the specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor, optionally further comprising isolating the specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor.

In an eighth aspect of the invention, there is provided a pharmaceutical composition comprising the specific antigen binding molecule, fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth or sixth aspects. The pharmaceutical composition may contain a variety of pharmaceutically acceptable carriers. Pharmaceutical compositions of the invention
5 may be for administration by any suitable method known in the art, including but not limited to intravenous, intramuscular, oral, intraperitoneal, or topical administration. In preferred embodiments, the pharmaceutical composition may be prepared in the form of a liquid, gel, powder, tablet, capsule, or foam.

10 The specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth or sixth aspects may be for use in therapy. More specifically, the specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth or sixth aspects may be for use in the treatment of cancer. Preferably, the cancer is a ROR1-positive cancer type. More
15 preferably, the cancer is selected from the group comprising blood cancers such as lymphomas and leukemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone lymphoma (MZL), non-Hodgkin lymphomas (NHL), acute myeloid leukemia (AML) and solid tumours including neuroblastoma, renal cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, uterine cancer, prostate
20 cancer, thyroid cancer, Head and Neck cancer, bladder cancer, oesophageal cancer, stomach cancer or liver cancer.

Also provided herein is the use of a specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth or
25 sixth aspects in the manufacture of a medicament for the treatment of a disease in a patient in need thereof.

The specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth, sixth aspects or pharmaceutical
30 composition of the eighth aspect may be administered in a single dose. As used herein "single dose" refers to a dosage regiment consisting of one dose. Alternatively, a multi-dose regiment may be used. Without being bound by theory, the advantages of the specific binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth, sixth aspects or pharmaceutical composition of the eighth aspect may be particularly apparent
35 when administered in a single dose.

Furthermore, in accordance with the present invention there is provided a method of treatment of a disease in a patient in need of treatment comprising administration to said patient of a therapeutically effective dosage of a specific antigen binding molecule, recombinant fusion protein, recombinant fusion

protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth or sixth aspects or a pharmaceutical composition of the eighth aspect.

Preferably, the cancer is a ROR1-positive cancer type. More preferably, the cancer is selected from the group comprising blood cancers such as lymphomas and leukemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone lymphoma (MZL), non-Hodgkin lymphomas (NHL), acute myeloid leukemia (AML) and solid tumours including neuroblastoma, renal cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, uterine cancer, prostate cancer, thyroid cancer, Head and Neck cancer, bladder cancer, oesophageal cancer, stomach cancer or liver cancer.

Also provided herein is a method of assaying for the presence of a target analyte in a sample, comprising the addition of a detectably labelled specific antigen binding molecule of the first aspect or second aspect, or a recombinant fusion protein of the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect, to the sample and detecting the binding of the molecule to the target analyte.

In addition, there is provided herein a method of imaging a site of disease in a subject, comprising administration of a detectably labelled specific antigen binding molecule of the first aspect or second aspect, or a detectably labelled recombinant fusion protein of the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect to a subject.

There is also provided herein a method of diagnosis of a disease or medical condition in a subject comprising administration of a specific antigen binding molecule of the first aspect or second aspect, or a recombinant fusion protein of the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect.

Also contemplated herein is an antibody, antibody fragment or antigen-binding molecule that competes for binding to ROR1 with the ROR1-specific antigen binding molecule of the first or second aspect. The term "compete" when used in the context of antigen binding proteins (e.g., neutralizing antigen binding proteins or neutralizing antibodies) means competition between antigen binding proteins as determined by an assay in which the antigen binding protein (e.g., antibody or functional fragment thereof) under test prevents or inhibits specific binding of a the antigen binding molecule defined herein (e.g., specific antigen binding molecule of the first aspect) to a common antigen (e.g., ROR1 in the case of the specific antigen binding molecule of the first or second aspect).

Also described herein is a kit for diagnosing a subject suffering from cancer, or a pre-disposition thereto, or for providing a prognosis of the subject's condition, the kit comprising detection means for detecting the concentration of antigen present in a sample from a test subject, wherein the detection means comprises a ROR1-specific antigen binding molecule of the first or second aspect, a recombinant fusion protein of the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect, a chimeric

antigen receptor of the sixth aspect or a nucleic acid sequence of the seventh aspect, each being optionally derivatized, wherein presence of antigen in the sample suggests that the subject suffers from cancer. Preferably the antigen comprises ROR1 protein, more preferably an extracellular domain thereof. More preferably, the kit is used to identify the presence or absence of ROR1-positive cells in the sample, or determine the concentration thereof in the sample. The kit may also comprise a positive control and/or a negative control against which the assay is compared and/or a label which may be detected.

The present invention also provides a method for diagnosing a subject suffering from cancer, or a pre-disposition thereto, or for providing a prognosis of the subject's condition, the method comprising detecting the concentration of antigen present in a sample obtained from a subject, wherein the detection is achieved using a ROR1-specific antigen binding molecule of the first or second aspect, a recombinant fusion protein of the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect, a chimeric antigen receptor of the sixth aspect or a nucleic acid sequence of the seventh aspect, each being optionally derivatized, and wherein presence of antigen in the sample suggests that the subject suffers from cancer.

Also contemplated herein is a method of killing or inhibiting the growth of a cell expressing ROR1 in vitro or in a patient, which method comprises administering to the cell a pharmaceutically effective amount or dose of (i) ROR1-specific antigen binding molecule of the first or second aspect, a recombinant fusion protein of the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect, a nucleic acid sequence of the seventh aspect, or the CAR or cell according to the sixth aspect, or (ii) of a pharmaceutical composition of the eighth aspect. Preferably, the cell expressing ROR1 is a cancer cell. More preferably, the ROR1 is human ROR1.

According to a ninth aspect, the invention provides a specific antigen binding molecule comprising an amino acid sequence represented by the formula (II):



wherein

FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 is a ROR1-specific antigen binding molecule according to the first or second aspect

X and Y are optional amino acid sequences

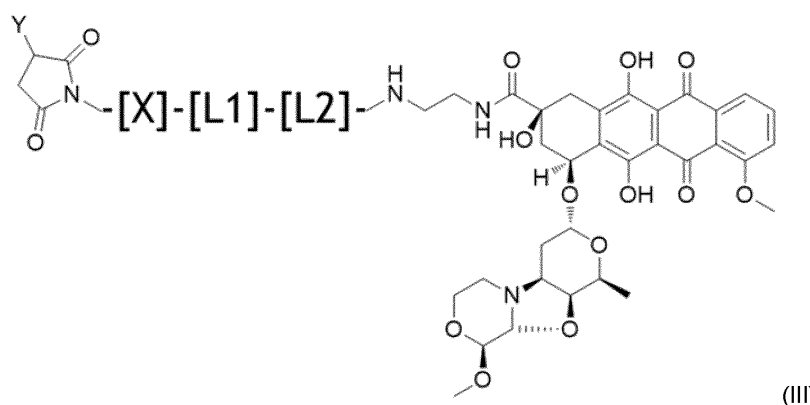
wherein the specific antigen binding molecule is conjugated to a second moiety.

According to a tenth aspect, the invention provides a target-binding molecule-drug conjugate, comprising

- (a) a ROR1 specific antigen binding molecule according to the first, second or ninth aspect, or
 5 a recombinant fusion protein of the third, or fourth aspect or a recombinant fusion protein dimer of the fifth aspect, and

(b) an anthracycline (PNU) derivative,

wherein the target-binding molecule-drug conjugate has the structure of formula (III):



wherein [X] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

- [L1] and [L2] are optional linkers selected from the group consisting of valine (Val), citrulline (Cit), alanine (Ala), asparagine (Asn), a peptide, $-(CH_2)_n-$, $-(CH_2CH_2O)_n-$, p-aminobenzyloxycarbonyl (PAB), Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, Val-Ala, Asn-Ala, any amino acid except glycine, and combinations thereof; and

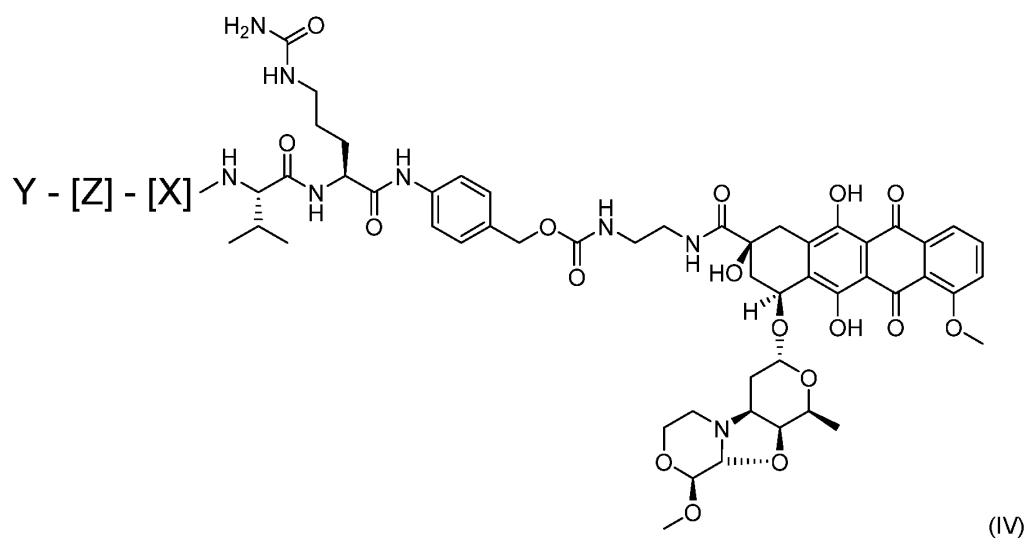
- Y comprises a ROR1 specific antigen binding molecule according to the first, second or ninth aspect, or
 20 a recombinant fusion protein of the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect.

According to an eleventh aspect, the invention provides a target-binding molecule-drug conjugate, comprising

- (a) a ROR1 specific antigen binding molecule according to the first, second or ninth aspect, or
 25 a recombinant fusion protein according to the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect, and

(b) an anthracycline (PNU) derivative,

wherein the target-binding molecule-drug conjugate has the structure of formula (IV):



wherein [X] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

[Z] is a linker derived from a reactive group used to conjugate the anthracycline (PNU) derivative and the target-binding molecule; and

Y comprises a ROR1 specific antigen binding molecule according to the first, second or ninth aspect, or a recombinant fusion protein according to the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect.

DESCRIPTION OF FIGURES

Figure 1: Design of B1 loop library: The sequence of B1 is shown with the "X" indicating amino acids within CDR1 and CDR3 which were randomised.

Figure 2: Cell surface binding of B1 VNAR loop variants (His₆Myc tag) to A549 (ROR1^{hi}) lung cancer cells by flow cytometry.

Figure 3: Cell surface binding of B1 VNAR loop variants (His₆Myc tag) to A427 (ROR1^{low}) lung cancer cells by flow cytometry.

Figure 4: Sequence and loop library design of P3A1 G1. CDR1 diversity results in 448 combinations, HV2 diversity results in 768 combinations and HV4 diversity results in 24 combinations.

Figure 5: Binding of P3A1G1 loop variants to human ROR1 by ELISA. The data is plotted as the OD signal obtained at 450 nm for a fixed concentration (5 ug /mL) of each of the loop variants.

5 Figure 6: Binding of P3A1G1 loop variants and parental P3A1G1 protein to human ROR1 by ELISA.

Figure 7: Linker mouse IgG and linker human IgG sequences used in VNAR IgG Fc fusion proteins. Engineered hlgG1 Fc fusion proteins incorporate an engineered cysteine substitution in the hlgG1 Fc sequence, for example at position S239C or S442C (EU numbering) to enable site specific labelling.

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Figure 8: Cell surface binding of B1 loop variant – hFc fusion proteins to A549 (ROR1^{hi}) lung cancer cells by flow cytometry.

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Figure 9: Analysis of ROR1 bi-paratopic VNAR-hFc fusions by SDS-PAGE (4-12% Bis Tris gel, MOPS buffer, ±50mM DTT). Lane 1 G3CP-P3A1 hFc (S239C+KIH) and lane 2 G3CPG4-P3A1 hFc (S239C+KIH)

20 Figure 10: Cell surface binding of ROR1 bi-paratopic VNAR-hFc fusions to A549 (ROR1^{hi}) and A427 (ROR1^{low}) lung cancer cells by flow cytometry.

Figure 11: Structures of PNU-linker payloads MA-PEG-vc-PAB-EDA-PNU159682 and MA-PEG-va-EDA-PNU159682

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Figure 12: Dose response showing binding of G3CP-hFc and G3CPG4-hFc PNU conjugates and the corresponding parental proteins to human ROR1 by ELISA

Figure 13: Potency of G3CP-hFc PNU and G3CPG4-hFc PNU conjugates in killing the ROR1 positive PA-1 cell-line and a PA-1 cell-line with ROR1 knockout

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Figure 14: *In vivo* efficacy of G3CP-hFc PNU and G3CPG4-hFc PNU conjugates in the ROR1+ HBCx-28 patient-derived TNBC xenograft model. Data plotted until the point when the first animal in the vehicle group reached humane tumour burden.

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Figure 15: An alignment of the sequences for B1, B1 G4, B1V15, G3CP and G3CPG4. Points of variation within the CDRs and HV regions are emphasised in underline. Note B1V15 (SEQ ID NO: 115): is not a loop library variant of B1; they have identical CDR1, HV2, HV4 and CDR3 sequences.

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Figure 16: UV analysis of B1-hFc, B1G4-hFc, G3CP-hFc and G3CPG4-hFc after incubation in PBS pH 7.4 buffer at 37°C for 96h.

5 Figure 17: Size exclusion analysis (SEC) of B1-hFc, B1G4-hFc, G3CP-hFc and G3CPG4-hFc after incubation in PBS pH 7.4 buffer at 37°C for 96h.

Figure 18: Potency of G3CP-hFc PNU and G3CPG4-hFc PNU conjugates in killing ROR1^{low} HEK293 cells and HEK293 cells stably transfected with human ROR1

10 Figure 19: *In vivo* efficacy of G3CP-hFc PNU and G3CPG4-hFc PNU conjugates in the ROR1+ HBCx-10 patient-derived TNBC xenograft model. Data plotted until the point when the first animal in the vehicle group reached humane tumour burden

15 Figure 20: Cell surface binding of ROR1 bi-paratopic VNAR-hFc drug conjugates to A549 (ROR1^{hi}) and A427 (ROR1^{low}) lung cancer cells by flow cytometry.

Figure 21: Potency of bi-paratopic G3CP-P3A1-hFc PNU and G3CPG4-P3A1-hFc PNU conjugates in killing the ROR1 positive PA-1 cell-line and a PA-1 cell-line with ROR1 knockout

20 Figure 22: *In vivo* efficacy of bi-paratopic G3CP-P3A1 hFc PNU and G3CPG4-P3A1-hFc PNU conjugates in the ROR1+ HBCx-28 patient-derived TNBC xenograft model.

DETAILED DESCRIPTION

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The present invention generally relates to specific antigen binding molecules. Specifically, the invention provides immunoglobulin-like shark variable novel antigen receptors (VNARs) specific for receptor tyrosine kinase-like orphan receptor 1 (ROR1) and associated fusion proteins, chimeric antigen receptors, conjugates, and nucleic acids, as well as accompanying methods. The ROR1-specific VNAR domains are described herein as ROR1-specific antigen binding molecules.

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35 The Novel or New antigen receptor (IgNAR) is an approximately 160 kDa homodimeric protein found in the sera of cartilaginous fish (Greenberg A. S., et al., Nature, 1995. 374(6518): p. 168-173, Dooley, H., et al, Mol. Immunol, 2003. 40(1): p. 25-33; Müller, M.R., et al., mAbs, 2012. 4(6): p. 673-685)). Each molecule consists of a single N-terminal variable domain (VNAR) and five constant domains (CNAR). The IgNAR domains are members of the immunoglobulin-superfamily. The VNAR is a tightly folded domain with structural and some sequence similarities to the immunoglobulin and T-cell receptor Variable domains and to cell adhesion molecules and is termed the VNAR by analogy to the N Variable terminal domain of the classical immunoglobulins and T Cell receptors. The VNAR shares limited

sequence homology to immunoglobulins, for example 25-30% similarity between VNAR and human light chain sequences.

- 5 Kovaleva M. et al Expert Opin. Biol. Ther. 2014. 14(10): p. 1527-1539 and Zielonka S. et al mAbs 2015. 7(1): p. 15-25 provided summaries of the structural characterization and generation of the VNARs, which are hereby incorporated by reference.

10 The VNAR does not appear to have evolved from a classical immunoglobulin antibody ancestor. The distinct structural features of VNARs are the truncation of the sequences equivalent to the CDR2 loop present in conventional immunoglobulin variable domains and the lack of the hydrophobic VH/VL interface residues which would normally allow association with a light chain domain, which is not present in the IgNAR structure. Furthermore, unlike classical immunoglobulins some VNAR subtypes include extra cysteine residues in the CDR regions that are observed to form disulphide bridges in addition to the canonical Immunoglobulin superfamily bridge between the Cysteines in the Framework 1 and 3
15 regions N terminally adjacent to CDRs 1 and 3.

To date, there are three defined types of shark IgNAR known as I, II and III. These have been categorized based on the position of non-canonical cysteine residues which are under strong selective pressure and are therefore rarely replaced.

20

All three types have the classical immunoglobulin canonical cysteines at positions 35 and 107 (numbering as in Kabat, E.A. et al. Sequences of proteins of immunological interest. 5th ed. 1991, Bethesda: US Dept. of Health and Human Services, PHS, NIH) that stabilize the standard immunoglobulin fold, together with an invariant tryptophan at position 36. There is no defined CDR2 as
25 such, but regions of sequence variation that compare more closely to TCR HV2 and HV4 have been defined in framework 2 and 3 respectively. Type I has germline encoded cysteine residues in framework 2 and framework 4 and an even number of additional cysteines within CDR3. Crystal structure studies of a Type I IgNAR isolated against and in complex with lysozyme enabled the contribution of these cysteine residues to be determined. Both the framework 2 and 4 cysteines form disulphide bridges with
30 those in CDR3 forming a tightly packed structure within which the CDR3 loop is held tightly down towards the HV2 region. To date Type I IgNARs have only been identified in nurse sharks - all other elasmobranchs, including members of the same order have only Type II or variations of this type.

Type II IgNAR are defined as having a cysteine residue in CDR1 and CDR3 which form intramolecular
35 disulphide bonds that hold these two regions in close proximity, resulting in a protruding CDR3 that is conducive to binding pockets or grooves. Type I sequences typically have longer CDR3s than type II with an average of 21 and 15 residues respectively. This is believed to be due to a strong selective pressure for two or more cysteine residues in Type I CDR3 to associate with their framework 2 and 4 counterparts. Studies into the accumulation of somatic mutations show that there are a greater number
40 of mutations in CDR1 of type II than type I, whereas HV2 regions of Type I show greater sequence

variation than Type II. This evidence correlates well with the determined positioning of these regions within the antigen binding sites.

5 A third IgNAR type known as Type III has been identified in neonates. This member of the IgNAR family lacks diversity within CDR3 due to the germline fusion of the D1 and D2 regions (which form CDR3) with the V-gene. Almost all known clones have a CDR3 length of 15 residues with little or no sequence diversity.

10 Another structural type of VNAR, termed type (IIb or IV), has only two canonical cysteine residues (in framework 1 and framework 3b regions). So far, this type has been found primarily in dogfish sharks and was also isolated from semisynthetic V-NAR libraries derived from wobbegong sharks.

15 The VNAR binding surface, unlike the variable domains in other natural immunoglobulins, derives from four regions of diversity: CDR1, HV2, HV4 and CDR3, joined by intervening framework sequences in the order: FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4. The combination of a lack of a natural light chain partner and lack of CDR2 make VNARs the smallest naturally occurring binding domains in the vertebrate kingdom.

20 The IgNAR shares some incidental features with the heavy chain only immunoglobulin (HCAb) found in camelidae (camels, dromedaries and llamas) Unlike the IgNAR the HCAb is clearly derived from the immunoglobulin family and shares significant sequence homology to standard immunoglobulins. Importantly one key distinction of VNARs is that the molecule has not had at any point in its evolution a partner light chain, unlike classical immunoglobulins or the HCABs. Flajnik M.F. et al PLoS Biol 2011. 9(8): e1001120 and Zielonka S. et al mAbs 2015. 7(1): p. 15-25 have commented on the similarities and
25 differences between, and the possible and distinct evolutionary origins of, the VNAR and the immunoglobulin-derived VHH single binding domain from the camelids.

30 Although antibodies to ROR1 have been reported in the literature, the high sequence identity between the extracellular domain of human, mouse and rat ROR1 and between human ROR1 and ROR2 family members means generating high affinity hROR1-specific binding agents is not trivial. Additionally, the large size of antibodies compromises their ability to penetrate into solid tumours and render regions of target proteins inaccessible due to steric factors, which can be particularly acute for cell-surface proteins where oligomerisation or receptor clustering is observed.

35 As a result there is a need in the art for improved anti-ROR1 binding protein agents with different functional or physical characteristics or properties to antibodies and the development of therapeutics and diagnostic agents for malignancies associated with ROR1 expression. The present invention provides such agents in the form of the ROR1-specific antigen binding molecules described herein.

Without being bound by theory, the presently-described ROR1-specific antigen binding molecules are thought to bind to both human and murine ROR1. A number of variants, including G3CP, 1E5, 1B11, C3CP, 1G9, 1H8, G11CP, D9CP, 1B6, 1 F10, F2CP, B6CP, 1E1 and P3A1G1 NAC6.S, P3A1G1 AE3.S, P3A1G1 NAC6, P3A1G1 AE3 and P3A1G1 NAG8 have been experimentally confirmed to bind to both
 5 hROR1 and mROR1. Furthermore, the ROR1-specific antigen binding molecules of the present invention may bind to deglycosylated forms of ROR1. Furthermore, they may not bind to a number of linear peptides associated with anti-ROR1 antibodies described in the prior art. The presently-described ROR1-specific antigen binding molecules are therefore thought to bind to distinct epitopes in the ROR1 sequence compared to these prior art anti-ROR1 antibodies.

10

Binding of ROR1-specific antigen binding molecules of the invention to cancer cell lines, as well as internalisation, have been demonstrated. This confirms the potential for the use of such molecules in the treatment of cancers, specifically cancers which express ROR1.

15 Various forms of the ROR1-specific antigen binding molecules are described, including fusion proteins of several types. Fusion proteins including an immunoglobulin Fc region are described, as well as both homo and heterodimers. Fusion of proteins to an Fc domain can improve protein solubility and stability, markedly increase plasma half-life and improve overall therapeutic effectiveness.

20 The present inventors have also created VNAR molecules conjugated to a variety of moieties and payloads. The present invention therefore also provides chemically conjugated VNARs. More specifically, ROR1-specific antigen binding molecules in several conjugated formats are provided.

According to a first aspect, the invention provides a receptor tyrosine kinase-like orphan receptor 1
 25 (ROR1) specific antigen binding molecule comprising an amino acid sequence represented by the formula (I):

FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 (I)

30 wherein

CDR3 is a CDR sequence having an amino acid sequence selected from the group consisting of YPWGAGAPYNVQWY (SEQ ID NO: 23), YPWGAGAPYLQWY (SEQ ID NO: 20), YPWGAGAPWNVQWY (SEQ ID NO: 24), YPSGAGAPRPVQWY (SEQ ID NO:11),
 35 YPWGAGAPCLVQWY (SEQ ID NO: 12), YPWGAGAPRLVQWY (SEQ ID NO:13), YPWGAGAPRQVQWY (SEQ ID NO: 14), YPWGAGAPRSVQWY (SEQ ID NO: 15), YPWGAGAPSLVQWY (SEQ ID NO: 16), YPWGAGAPSNVQWY (SEQ ID NO: 17), YPWGAGAPSQVQWY (SEQ ID NO: 18), YPWGAGAPSSVQWY (SEQ ID NO: 19), YPWGAGAPWQVQWY (SEQ ID NO: 21), YPWGAGAPWSVQWY (SEQ ID NO: 22), and
 40 YPWGAGAPWLQWY (SEQ ID NO: 10);

CDR1 is a CDR sequence having an amino acid sequence selected from the group consisting of GANYGLAA (SEQ ID NO: 1), DANYGLAA (SEQ ID NO: 5), GANYDLA (SEQ ID NO: 2), GANYGLSA (SEQ ID NO: 3), and GANYDLAA (SEQ ID NO: 4)

5

FW1 is a framework region;

FW2 is a framework region;

10 HV2 is a hypervariable sequence having an amino acid sequence selected from the group consisting of SSNQERISIS (SEQ ID NO: 6) and SSNKERISIS (SEQ ID NO: 7);

FW3a is a framework region;

15 HV4 is a hypervariable sequence having an amino acid sequence selected from the group consisting of NKRTM (SEQ ID NO: 8) and NKGTM (SEQ ID NO: 9);

FW3b is a framework region;

20 FW4 is a framework region;

wherein if CDR3 is YPWGAGAPWLVQWY (SEQ ID NO: 10) then CDR1 is selected from the group consisting of DANYGLAA (SEQ ID NO: 5), GANYGLSA (SEQ ID NO: 3) and GANYDLAA (SEQ ID NO: 4).

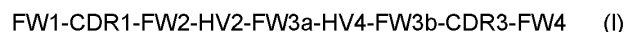
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In one embodiment of the ROR1-specific antigen binding molecule:

CDR3 is a CDR sequence having an amino acid sequence selected from the group consisting of YPWGAGAPYNVQWY (SEQ ID NO: 23), YPWGAGAPYLVQWY (SEQ ID NO: 20), YPWGAGAPWNVQWY (SEQ ID NO: 24), YPSGAGAPRPVQWY (SEQ ID NO: 11), YPWGAGAPCLVQWY (SEQ ID NO: 12), YPWGAGAPRLVQWY (SEQ ID NO: 13), YPWGAGAPRQVQWY (SEQ ID NO: 14), YPWGAGAPRSVQWY (SEQ ID NO: 15), YPWGAGAPSLVQWY (SEQ ID NO: 16), YPWGAGAPSNVQWY (SEQ ID NO: 17), YPWGAGAPSSVQWY (SEQ ID NO: 19), YPWGAGAPWQVQWY (SEQ ID NO: 21), YPWGAGAPWSVQWY (SEQ ID NO: 22), and YPWGAGAPWLVQWY (SEQ ID NO: 10).

35

If CDR3 is not YPWGAGAPWLVQWY (SEQ ID NO: 10) then CDR1 may be a CDR sequence having an amino acid sequence selected from the group consisting of GANYGLAA (SEQ ID NO: 1), DANYGLAA (SEQ ID NO: 5), GANYDLA (SEQ ID NO: 2), GANYGLA (SEQ ID NO: 3), and GANYDLAA (SEQ ID NO: 4). Accordingly, the ROR1 specific antigen binding molecule may be defined as comprising an amino acid sequence represented by the formula (I):



wherein

10

CDR3 is a CDR sequence having an amino acid sequence selected from the group consisting of YPWGAGAPYNVQWY (SEQ ID NO: 23), YPWGAGAPYLVQWY (SEQ ID NO: 20), YPWGAGAPWNVQWY (SEQ ID NO: 24), YPSGAGAPRPVQWY (SEQ ID NO: 11), YPWGAGAPCLVQWY (SEQ ID NO: 12), YPWGAGAPRLVQWY (SEQ ID NO: 13), YPWGAGAPRQVQWY (SEQ ID NO: 14), YPWGAGAPRSVQWY (SEQ ID NO: 15), YPWGAGAPSLVQWY (SEQ ID NO: 16), YPWGAGAPSNVQWY (SEQ ID NO: 17), YPWGAGAPSQVQWY (SEQ ID NO: 18), YPWGAGAPSSVQWY (SEQ ID NO: 19), YPWGAGAPWQVQWY (SEQ ID NO: 21), and YPWGAGAPWSVQWY (SEQ ID NO: 22);

15

20

CDR1 is a CDR sequence having an amino acid sequence selected from the group consisting of GANYGLAA (SEQ ID NO: 1), DANYGLAA (SEQ ID NO: 5), GANYDLA (SEQ ID NO: 2), GANYGLA (SEQ ID NO: 3), and GANYDLAA (SEQ ID NO: 4);

25

FW1 is a framework region;

FW2 is a framework region;

30

HV2 is a hypervariable sequence having an amino acid sequence selected from the group consisting of SSNQERISIS (SEQ ID NO: 6) and SSNKERISIS (SEQ ID NO: 7);

FW3a is a framework region;

35

HV4 is a hypervariable sequence having an amino acid sequence selected from the group consisting of NKRTM (SEQ ID NO: 8) and NKGTM (SEQ ID NO: 9);

FW3b is a framework region; and

FW4 is a framework region.

40 In one embodiment of the ROR1-specific antigen binding molecule:

CDR3 is a CDR sequence having an amino acid sequence selected from the group consisting of YPWGAGAPYNVQWY (SEQ ID NO: 23), YPWGAGAPYLVQWY (SEQ ID NO: 20) and YPWGAGAPWNVQWY (SEQ ID NO: 24), and/or

CDR1 is a CDR sequence having an amino acid sequence selected from the group consisting of GANYGLAA (SEQ ID NO: 1) and DANYGLAA (SEQ ID NO: 5).

In one embodiment of the ROR1-specific antigen binding molecule:

CDR3 is a CDR sequence having an amino acid sequence according to YPWGAGAPYNVQWY (SEQ ID NO: 23).

In one embodiment of the ROR1-specific antigen binding molecule:

CDR3 is a CDR sequence having an amino acid sequence according to YPWGAGAPYNVQWY (SEQ ID NO: 23);

CDR1 is a CDR sequence having an amino acid sequence according to GANYGLAA (SEQ ID NO: 1);

HV2 is a hypervariable sequence having an amino acid sequence according to SSNQERISIS (SEQ ID NO: 6); and

HV4 is a hypervariable sequence having an amino acid sequence according to NKRTM (SEQ ID NO: 8).

In one embodiment of the ROR1-specific antigen binding molecule:

CDR3 is a CDR sequence having an amino acid sequence according to YPWGAGAPYNVQWY (SEQ ID NO: 23);

CDR1 is a CDR sequence having an amino acid sequence according to DANYGLAA (SEQ ID NO: 5);

HV2 is a hypervariable sequence having an amino acid sequence according to SSNKERISIS (SEQ ID NO: 7); and

HV4 is a hypervariable sequence having an amino acid sequence according to NKGTM (SEQ ID NO: 9).

In one embodiment of the ROR1-specific antigen binding molecule:

CDR3 is a CDR sequence having an amino acid sequence according to YPWGAGAPWLVQWY (SEQ ID NO: 10);

CDR1 is a CDR sequence having an amino acid sequence according to DANYGLAA (SEQ ID NO: 5);

HV2 is a hypervariable sequence having an amino acid sequence according to SSNKERISIS (SEQ ID NO: 7); and

HV4 is a hypervariable sequence having an amino acid sequence according to NKGTM (SEQ ID NO: 9).

In preferred embodiments the ROR1-specific antigen binding molecule comprises an amino acid sequence selected from the group consisting of:

5 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLTVN (SEQ ID NO: 50) referred to herein as
G3CP;

TRVDQSPSSLSASVGDRVITICVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKG TMSFTL
10 TISSLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 51) referred to herein as
B1G4;

ASVNQTPRTATKETGESLTINC VVTGANYDLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPSGAGAPRPVQWYDGAGTVLTVN (SEQ ID NO: 52) referred to herein as
15 1E2;

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPCLVQWYDGAGTVLTVN (SEQ ID NO: 53) referred to herein as
1E5;

20 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPRLVQWYDGAGTVLTVN (SEQ ID NO: 54) referred to herein as
1B11;

25 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPRQVQWYDGAGTVLTVN (SEQ ID NO: 55) referred to herein as
C3CP;

ASVNQTPRTATKETGESLTINC VVTGANYGLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
30 RIKDLTVADSATYYCKAYPWGAGAPRSVQWYDGAGTVLTVN (SEQ ID NO: 56) referred to herein as
2G5;

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPRSVQWYDGAGTVLTVN (SEQ ID NO: 57) referred to herein as
35 1G12;

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSLVQWYDGAGTVLTVN (SEQ ID NO: 58) referred to herein as
G5CP;

40

- ASVNQTPRTATKETGESLTINC VVTGANYGLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSNVQWYDGAGTVLTVN (SEQ ID NO: 59) referred to herein as
2F4;
- 5 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSQVQWYDGAGTVLTVN (SEQ ID NO: 60) referred to herein as
1G9;
- ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
10 RIKDLTVADSATYYCKAYPWGAGAPSSVQWYDGAGTVLTVN (SEQ ID NO: 61) referred to herein as
1H8;
- ASVNQTPRTATKETGESLTINC VVTGANYGLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWLQWYDGAGTVLTVN (SEQ ID NO: 62) referred to herein as
15 G11CP;
- ASVNQTPRTATKETGESLTINC VVTGANYDLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWLQWYDGAGTVLTVN (SEQ ID NO: 63) referred to herein as
D9CP;
- 20 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWNVQWYDGAGTVLTVN (SEQ ID NO: 64) referred to herein as
1B6;
- 25 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWQVQWYDGAGTVLTVN (SEQ ID NO: 65) referred to herein as
1F10;
- ASVNQTPRTATKETGESLTINC VVTGANYGLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
30 RIKDLTVADSATYYCKAYPWGAGAPWQVQWYDGAGTVLTVN (SEQ ID NO: 66) referred to herein as
E6CP;
- ASVNQTPRTATKETGESLTINC VVTGANYDLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWQVQWYDGAGTVLTVN (SEQ ID NO: 67) referred to herein as
35 F2CP;
- ASVNQTPRTATKETGESLTINC VVTGANYDLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWSVQWYDGAGTVLTVN (SEQ ID NO: 68) referred to herein as
B6CP;
- 40

ASVNQTPRTATKETGESLTINCVVTGANYGLAATYWYRKPNPGSSNQERISISGRYVESVKNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWSVQWYDGAGTVLTVN (SEQ ID NO: 69) referred to herein as
1G1; and

- 5 ASVNQTPRTATKETGESLTINCVVTGANYGLSATYWYRKPNPGSSNQERISISGRYVESVKNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWSVQWYDGAGTVLTVN (SEQ ID NO: 70) referred to herein as
A10CP;

- or a functional variant having CDR1, HV2, HV4 and CDR2 sequences according to any thereof and
10 having FW1, FW2, FW3a, FW3b and FW4 sequences having a combined sequence identity of at least
45% to the combined FW1, FW2, FW3a, FW3b and FW4 sequences of any thereof.

- In a particularly preferred embodiment, the ROR1-specific antigen binding molecule may comprise an
amino acid sequence according to
15 ASVNQTPRTATKETGESLTINCVVTGANYGLAATYWYRKPNPGSSNQERISISGRYVESVKNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPYVQWYDGAGTVLTVN (SEQ ID NO: 50).

- The ROR1-specific antigen binding molecule may comprise an amino acid sequence according to
ASVNQTPRTATKETGESLTINCVVTGANYGLAATYWYRKPNPGSSNQERISISGRYVESVKNKRTMSFSL
20 RIKDLTVADSATYYCKAYPWGAGAPYVQWYDGAGTVLTVN (SEQ ID NO: 50) or a functional variant
thereof having CDR1, HV2, HV4 and CDR2 sequences according to SEQ ID NO: 50 and having FW1,
FW2, FW3a, FW3b and FW4 sequences having a combined sequence identity of at least 45% to the
combined FW1, FW2, FW3a, FW3b and FW4 sequences of SEQ ID NO: 50.

- 25 Particular advantages associated with SEQ ID NO: 50 ("G3CP") and functional variants thereof include
increased expression yields and hydrophilicity and increased ease of analysis, purification and
monomericity in non-optimised aqueous buffer systems for these proteins. Without being bound by
theory, these advantages may be particularly evident in VNAR-hFc fusion proteins comprising the G3CP
sequence or functional variants thereof. The G3CP sequence and functional variants thereof may
30 therefore provide improved manufacturing and/or handling properties. Furthermore, G3CP-hFc shows
excellent *in vivo* efficacy in a patient-derived xenograft model of Triple Negative Breast Cancer (TNBC)
when conjugated to a cytotoxic anthracycline (PNU) derivative. The effect of G3CP-hFc is surprisingly
improved over even B1-hFc which itself shows excellent *in vivo* efficacy.

- 35 In a particularly preferred embodiment, the ROR1-specific antigen binding molecule may comprise an
amino acid sequence according to
TRVDQSPSSLSASVGDRVITCVLTDANYGLAATYWYRKPNPGSSNKERISISGRYSESVNKGTMSFTL
TISSLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 51).

Particular advantages associated with SEQ ID NO: 51 ("B1G4") and functional variants thereof include increased expression yields and monomericity in aqueous buffer systems for fusion proteins comprising the B1G4 sequence or functional variants thereof, such as VNAR-hFc fusion proteins. The B1G4 sequence and functional variants thereof may therefore provide fusion proteins with improved manufacturing and/or handling properties.

The ROR1-specific antigen binding molecule may comprise an amino acid sequence according to TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTFTL TISLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 51) or a functional variant thereof having CDR1, HV2, HV4 and CDR2 sequences according to SEQ ID NO: 51 and having FW1, FW2, FW3a, FW3b and FW4 sequences having a combined sequence identity of at least 45% to the combined FW1, FW2, FW3a, FW3b and FW4 sequences of SEQ ID NO: 51.

In preferred embodiments the ROR1-specific antigen binding molecule comprises an amino acid sequence selected from the group consisting of:

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTFTL TISLQPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIK (SEQ ID NO: 71) referred to herein as G3CP G4;

ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRMTMSFSL RISSLTVEDSATYYCKAYPWGAGAPYNVQWYDGQGTKLEVK (SEQ ID NO: 72) referred to herein as G3CP V15;

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTFTL TISLQPEDSATYYCRAYPWGAGAPSSVQWYDGAGTKVEIK (SEQ ID NO: 73) referred to herein as 1H8 G4;

ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRMTMSFSL RISSLTVEDSATYYCKAYPWGAGAPSSVQWYDGQGTKLEVK (SEQ ID NO: 74) referred to herein as 1H8 V15;

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTFTL TISLQPEDSATYYCRAYPWGAGAPRQVQWYDGAGTKVEIK (SEQ ID NO: 75) referred to herein as C3CP G4; and

ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRMTMSFSL RISSLTVEDSATYYCKAYPWGAGAPRQVQWYDGQGTKLEVK (SEQ ID NO: 76) referred to herein as C3CPV15;

or a functional variant having CDR1, HV2, HV4 and CDR2 sequences according to any thereof and having FW1, FW2, FW3a, FW3b and FW4 sequences having a combined sequence identity of at least 45% to the combined FW1, FW2, FW3a, FW3b and FW4 sequences of any thereof.

5 In a particularly preferred embodiment, the ROR1-specific antigen binding molecule may comprise an amino acid sequence according to TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTSFTL TISSLPEDSATYYCRAYPWGAGAPYVQWYDGAGTKVEIK (SEQ ID NO: 71).

10 The ROR1-specific antigen binding molecule may comprise an amino acid sequence according to TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTSFTL TISSLPEDSATYYCRAYPWGAGAPYVQWYDGAGTKVEIK (SEQ ID NO: 71) or a functional variant thereof having CDR1, HV2, HV4 and CDR2 sequences according to SEQ ID NO: 71 and having FW1, FW2, FW3a, FW3b and FW4 sequences having a combined sequence identity of at least 45% to the
15 combined FW1, FW2, FW3a, FW3b and FW4 sequences of SEQ ID NO: 71.

Particular advantages associated with SEQ ID NO: 71 ("G3CP G4") and functional variants thereof include increased expression yields and hydrophilicity and increased ease of analysis, purification and monomericity in non-optimised aqueous buffer systems for these proteins. Without being bound by
20 theory, these advantages may be particularly evident in VNAR-hFc fusion proteins comprising the G3CP G4 sequence or functional variants thereof. The G3CP G4 sequence and functional variants thereof may therefore provide improved manufacturing and/or handling properties. Furthermore, G3CPG4-hFc shows excellent *in vivo* efficacy in a patient-derived xenograft model of Triple Negative Breast Cancer (TNBC) when conjugated to a cytotoxic anthracycline (PNU) derivative. The effect of G3CPG4-hFc is
25 surprisingly improved over even B1-hFc which itself shows excellent *in vivo* efficacy.

The sequences of G3CP and G3CPG4 have in common two single amino acid changes relative to the sequence of B1. These are both within CDR3 and are the substitution:

1. Of a W residue with a Y residue, and
- 30 2. Of an L residue with a N residue.

Compared to G3CP, G3CPG4 has a further single amino acid change in each of CDR1, HV2 and HV4 relative to B1 (which also appear in B1 G4) and changes to humanise the framework regions (some of which also appear in B1V15, SEQ ID NO: 115, as shown in Figure 15 - B1V15 has the same CDR1,
35 HV2, HV4 and CDR3 sequences as B1 i.e. it is not a loop library variant; the changes to B1V15 relative to B1 are in the framework regions only).

Without being bound by theory, any improvements over B1 shown by both G3CP and G3CPG4 which are not shown by B1 G4 or B1 V15 are thought to result from one or both of the two mutations they

share in CDR3. Accordingly, advantages of G3CP and G3CPG4 are thought to derive from a CDR3 comprising the sequence YPWGAGAPYNVQWY (SEQ ID NO: 23).

Without being bound by theory, the surprising advantages associated with YPWGAGAPYNVQWY (SEQ ID NO: 23) may represent a synergistic effect of both the W to Y and the L to N substitutions. Alternatively, the surprising advantages may derive primarily from the W to Y substitution thus being shared by YPWGAGAPYLVQWY (SEQ ID NO: 20). 1B6, which has the L to N mutation and a CDR3 sequence of YPWGAGAPWNVQWY (SEQ ID NO: 24), has a lower elution volume than B1, therefore the L to N mutation in CDR3 does lead to improved manufacturing and/or handling properties.

According to a second aspect, the invention provides a receptor tyrosine kinase-like orphan receptor 1 (ROR1) specific antigen binding molecule comprising an amino acid sequence represented by the formula (I):

FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 (I)

wherein

CDR1 is a CDR sequence having an amino acid sequence selected from the group consisting of GTRYGLYS (SEQ ID NO: 25), GTRYGLYSS (SEQ ID NO: 26), DTRYALYS (SEQ ID NO: 27), DTRYALYSS (SEQ ID NO: 28), GTKYGLYA (SEQ ID NO: 29) and GTKYGLYAS (SEQ ID NO: 30);

FW1 is a framework region;

FW2 is a framework region;

HV2 is a hypervariable sequence having an amino acid sequence selected from the group consisting of SSDEERISIS (SEQ ID NO: 31), STDEERISIG (SEQ ID NO: 32), SPNKDRMIIG (SEQ ID NO: 33), and STDKERIIG (SEQ ID NO: 34);

FW3a is a framework region;

HV4 is a hypervariable sequence having an amino acid sequence selected from the group consisting of NKGTK (SEQ ID NO: 35), NKGSK (SEQ ID NO: 36), NNGTK (SEQ ID NO: 37), and NNRSK (SEQ ID NO: 38);

FW3b is a framework region;

CDR3 is a CDR sequence having an amino acid sequence according to REARHPWLRQWY (SEQ ID NO: 39);

FW4 is a framework region.

5

In preferred embodiments the ROR1-specific antigen binding molecule comprises an amino acid sequence selected from the group consisting of:

10 TRVDQSPSSLSASVGDRVITITCVLTGTRYGLYSTYWYRKNPGSSDEERISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 77) referred to herein as
P3A1 G1 AE3;

15 TRVDQSPSSLSASVGDRVITITCVLTGTRYGLYSSTYWYRKNPGSSDEERISISGRYSESVNKGTKSFT
LTISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 78) referred to herein as
P3A1 G1 AE3.S;

20 TRVDQSPSSLSASVGDRVITITCVLTDTRYALYSTYWYRKNPGSTDEERISIGGRYSESVNKGSKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 79) referred to herein as
P3A1 G1 NAC6;

TRVDQSPSSLSASVGDRVITITCVLTDTRYALYSSTYWYRKNPGSTDEERISIGGRYSESVNKGSKSFT
LTISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 80) referred to herein as
P3A1 G1 NAC6.S;

25 TRVDQSPSSLSASVGDRVITITCVLTGTYGLYATYWYRKNPGSPNKDRMIIGGRYSESVNNGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 81) referred to herein as
P3A1 G1 NAG8;

30 TRVDQSPSSLSASVGDRVITITCVLTGTYGLYASTYWYRKNPGSPNKDRMIIGGRYSESVNNGTKSF
TLTISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 82) referred to herein as
P3A1 G1 NAG8.S;

35 TRVDQSPSSLSASVGDRVITITCVLTGTYGLYASTYWYRKNPGSTDKERIIIGGRYSESVNNRKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 83) referred to herein as
P3A1 G1 AF7.S;

or a functional variant having CDR1, HV2, HV4 and CDR2 sequences according to any thereof and having FW1, FW2, FW3a, FW3b and FW4 sequences having a combined sequence identity of at least 45% to the combined FW1, FW2, FW3a, FW3b and FW4 sequences of any thereof.

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Particular advantages associated with affinity matured variants of P3A1G1 and functional variants thereof include improved binding to hROR1-Fc compared to parental P3A1G1 as illustrated by the Examples.

The ROR1-specific antigen binding molecule may comprise the CDR and HV sequences of a clone set out in Table 1 below. In preferred embodiments of the first and/or second aspect of the invention, the ROR1-specific antigen binding molecule has the combined sequence of any of the clones set out in Table 1 below.

Table 1 - B1 loop variants

Clone Name	FW1	CDR1	FW2	HV2	FW3a	HV4	FW3b	CDR3	FW4
B1	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWLV QWY (SEQ ID NO: 10)	DGAGTVLT VN (SEQ ID NO: 48)
B1G4	TRVDQSPSSLSASVG DRVITCVLT (SEQ ID NO: 41)	DANYGLA A (SEQ ID NO: 5)	TYWYRKNP G (SEQ ID NO: 43)	SSNKERIS IS (SEQ ID NO: 7)	GRYVESV (SEQ ID NO: 45)	NKGTM (SEQ ID NO: 9)	SFTLTSSSLQPEDSA TYYCRA (SEQ ID NO: 46)	YPWGAGAPWLV QWY (SEQ ID NO: 10)	DGAGTKVE IK (SEQ ID NO: 49)
1E2	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYDLS A (SEQ ID NO: 2)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPSGAGAPRPV QWY (SEQ ID NO: 11)	DGAGTVLT VN (SEQ ID NO: 48)
1E5	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPCLV QWY (SEQ ID NO: 12)	DGAGTVLT VN (SEQ ID NO: 48)
1B11	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPRLV QWY (SEQ ID NO: 13)	DGAGTVLT VN (SEQ ID NO: 48)
C3CP	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPRQV QWY (SEQ ID NO: 14)	DGAGTVLT VN (SEQ ID NO: 48)
2G5	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLS A (SEQ ID NO: 3)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPRSV QWY (SEQ ID NO: 15)	DGAGTVLT VN (SEQ ID NO: 48)

Clone Name	FW1	CDR1	FW2	HV2	FW3a	HV4	FW3b	CDR3	FW4
1G12	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPRSV QWY (SEQ ID NO: 15)	DGAGTVLT VN (SEQ ID NO: 48)
G5CP	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPSLV QWY (SEQ ID NO: 16)	DGAGTVLT VN (SEQ ID NO: 48)
2F4	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLS A (SEQ ID NO: 3)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPSNV QWY (SEQ ID NO: 17)	DGAGTVLT VN (SEQ ID NO: 48)
1G9	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPSQV QWY (SEQ ID NO: 18)	DGAGTVLT VN (SEQ ID NO: 48)
1H8	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPSSV QWY (SEQ ID NO: 19)	DGAGTVLT VN (SEQ ID NO: 48)
G11CP	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLS A (SEQ ID NO: 3)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWL QWY (SEQ ID NO: 10)	DGAGTVLT VN (SEQ ID NO: 48)
D9CP	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYDLA A (SEQ ID NO: 4)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWL QWY (SEQ ID NO: 10)	DGAGTVLT VN (SEQ ID NO: 48)
1B6	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWN VQWY (SEQ ID NO: 24)	DGAGTVLT VN (SEQ ID NO: 48)
1F10	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWQ VQWY (SEQ ID NO: 21)	DGAGTVLT VN (SEQ ID NO: 48)

Clone Name	FW1	CDR1	FW2	HV2	FW3a	HV4	FW3b	CDR3	FW4
E6CP	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLS A (SEQ ID NO: 3)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWQ QWY (SEQ ID NO: 21)	DGAGTVLT VN (SEQ ID NO: 48)
F2CP	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYDLA A (SEQ ID NO: 4)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWQ QWY (SEQ ID NO: 21)	DGAGTVLT VN (SEQ ID NO: 48)
B6CP	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYDLA A (SEQ ID NO: 4)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWSV QWY (SEQ ID NO: 22)	DGAGTVLT VN (SEQ ID NO: 48)
1G1	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWSV QWY (SEQ ID NO: 22)	DGAGTVLT VN (SEQ ID NO: 48)
A10CP	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLS A (SEQ ID NO: 3)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPWSV QWY (SEQ ID NO: 22)	DGAGTVLT VN (SEQ ID NO: 48)
G3CP	ASVNQTPRTATKETG ESLTINCVVT (SEQ ID NO: 40)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYVESV (SEQ ID NO: 44)	NKRTM (SEQ ID NO: 8)	SFSLRIKDLTVADSA TYYCKA (SEQ ID NO: 84)	YPWGAGAPYNV QWY (SEQ ID NO: 23)	DGAGTVLT VN (SEQ ID NO: 48)
G3CP G4	TRVDQSPSSLSASVG DRVITICVLT (SEQ ID NO: 41)	DANYGLA A (SEQ ID NO: 5)	TYWYRKNP G (SEQ ID NO: 43)	SSNKERIS IS (SEQ ID NO: 7)	GRYSES (SEQ ID NO: 45)	NKGT (SEQ ID NO: 9)	SFTLTISLQPEDSA TYYCRA (SEQ ID NO: 46)	YPWGAGAPYNV QWY (SEQ ID NO: 23)	DGAGTKVE IK (SEQ ID NO: 49)
G3CP V15	ASVTQSPRSASKETG ESLTICRVLT (SEQ ID NO: 42)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYSES (SEQ ID NO: 45)	NKRTM (SEQ ID NO: 8)	SFSLRISSLTVEDSA TYYCKA (SEQ ID NO: 47)	YPWGAGAPYNV QWY (SEQ ID NO: 23)	DGAGTKLE VK (SEQ ID NO: 85)
1H8 G4	TRVDQSPSSLSASVG DRVITICVLT (SEQ ID NO: 41)	DANYGLA A (SEQ ID NO: 5)	TYWYRKNP G (SEQ ID NO: 43)	SSNKERIS IS (SEQ ID NO: 7)	GRYSES (SEQ ID NO: 45)	NKGT (SEQ ID NO: 9)	SFTLTISLQPEDSA TYYCRA (SEQ ID NO: 46)	YPWGAGAPSSV QWY (SEQ ID NO: 19)	DGAGTKVE IK (SEQ ID NO: 49)

Clone Name	FW1	CDR1	FW2	HV2	FW3a	HV4	FW3b	CDR3	FW4
1H8 V15	ASVTQSPRSASKETG ESLTITCRVT (SEQ ID NO: 42)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYSESV (SEQ ID NO: 45)	NKRTM (SEQ ID NO: 8)	SFSLRISSLTVEDSA TYYCKA (SEQ ID NO: 47)	YPWGAGAPSSV QWY (SEQ ID NO: 19)	DGQGTKLE VK (SEQ ID NO: 85)
C3CP G4	TRVDQSPSSLSASVG DRVITICVLT (SEQ ID NO: 41)	DANYGLA A (SEQ ID NO: 5)	TYWYRKNP G (SEQ ID NO: 43)	SSNKERIS IS (SEQ ID NO: 7)	GRYSESV (SEQ ID NO: 45)	NKGTM (SEQ ID NO: 9)	SFTLTSSSLQPEDSA TYYCRA (SEQ ID NO: 46)	YPWGAGAPRQV QWY (SEQ ID NO: 14)	DGAGTKVE IK (SEQ ID NO: 49)
C3CP V15	ASVTQSPRSASKETG ESLTITCRVT (SEQ ID NO: 42)	GANYGLA A (SEQ ID NO: 1)	TYWYRKNP G (SEQ ID NO: 43)	SSNQERI SIS (SEQ ID NO: 6)	GRYSESV (SEQ ID NO: 45)	NKRTM (SEQ ID NO: 8)	SFSLRISSLTVEDSA TYYCKA (SEQ ID NO: 47)	YPWGAGAPRQV QWY (SEQ ID NO: 14)	DGQGTKLE VK (SEQ ID NO: 85)

The ROR1-specific antigen binding molecule may comprise the CDR and HV sequences of a clone set out in Table 2 below. In preferred embodiments of the first and/or second aspect of the invention, the ROR1-specific antigen binding molecule has the combined sequence of any of the clones set out in Table 2 below.

Table 2 - P3A1G1 variant sequences

Clone Name	FW1	CDR1	FW2	HV2	FW3a	HV4	FW3b	CDR3	FW4
P3A1 G1 AE3	TRVDQSPSSLSASVG DRVITICVLT (SEQ ID NO: 41)	GTRYGLY S (SEQ ID NO: 25)	TYWYRKNP G (SEQ ID NO: 43)	SSDEERIS IS (SEQ ID NO: 31)	GRYSESV (SEQ ID NO: 45)	NKGTK (SEQ ID NO: 35)	SFTLTSSSLQPEDSA TYYCRA (SEQ ID NO: 46)	REARHPWLRQW Y (SEQ ID NO: 39)	DGAGTKVE IK (SEQ ID NO: 49)
P3A1 G1 AE3.S	TRVDQSPSSLSASVG DRVITICVLT (SEQ ID NO: 41)	GTRYGLY SS (SEQ ID NO: 26)	TYWYRKNP G (SEQ ID NO: 43)	SSDEERIS IS (SEQ ID NO: 31)	GRYSESV (SEQ ID NO: 45)	NKGTK (SEQ ID NO: 35)	SFTLTSSSLQPEDSA TYYCRA (SEQ ID NO: 46)	REARHPWLRQW Y (SEQ ID NO: 39)	DGAGTKVE IK (SEQ ID NO: 49)
P3A1 G1 NAC6	TRVDQSPSSLSASVG DRVITICVLT (SEQ ID NO: 41)	DTRYALY S (SEQ ID NO: 27)	TYWYRKNP G (SEQ ID NO: 43)	STDEERIS IG (SEQ ID NO: 32)	GRYSESV (SEQ ID NO: 45)	NKGSK (SEQ ID NO: 36)	SFTLTSSSLQPEDSA TYYCRA (SEQ ID NO: 46)	REARHPWLRQW Y (SEQ ID NO: 39)	DGAGTKVE IK (SEQ ID NO: 49)

Clone Name	FW1	CDR1	FW2	HV2	FW3a	HV4	FW3b	CDR3	FW4
P3A1 G1 NAC6.S	TRVDQSPSSLSASVG DRVITCVLT (SEQ ID NO: 41)	DTRYALY SS (SEQ ID NO: 28)	TYWYRKNP G (SEQ ID NO: 43)	STDEERIS IG (SEQ ID NO: 32)	GRYSESV (SEQ ID NO: 45)	NKGSK (SEQ ID NO: 36)	SFTLTISLQPEDSA TYYCRA (SEQ ID NO: 46)	REARHPWLRQW Y (SEQ ID NO: 39)	DGAGTKVE IK (SEQ ID NO: 49)
P3A1 G1 NAG8	TRVDQSPSSLSASVG DRVITCVLT (SEQ ID NO: 41)	GTKYGLY A (SEQ ID NO: 29)	TYWYRKNP G (SEQ ID NO: 43)	SPNKDRM IIG (SEQ ID NO: 33)	GRYSESV (SEQ ID NO: 45)	NGTK (SEQ ID NO: 37)	SFTLTISLQPEDSA TYYCRA (SEQ ID NO: 46)	REARHPWLRQW Y (SEQ ID NO: 39)	DGAGTKVE IK (SEQ ID NO: 49)
P3A1 G1 NAG8.S	TRVDQSPSSLSASVG DRVITCVLT (SEQ ID NO: 41)	GTKYGLY AS (SEQ ID NO: 30)	TYWYRKNP G (SEQ ID NO: 43)	SPNKDRM IIG (SEQ ID NO: 33)	GRYSESV (SEQ ID NO: 45)	NGTK (SEQ ID NO: 37)	SFTLTISLQPEDSA TYYCRA (SEQ ID NO: 46)	REARHPWLRQW Y (SEQ ID NO: 39)	DGAGTKVE IK (SEQ ID NO: 49)
P3A1 G1 AF7.S	TRVDQSPSSLSASVG DRVITCVLT (SEQ ID NO: 41)	GTKYGLY AS (SEQ ID NO: 30)	TYWYRKNP G (SEQ ID NO: 43)	STDKERIII G (SEQ ID NO: 34)	GRYSESV (SEQ ID NO: 45)	NNRSK (SEQ ID NO: 38)	SFTLTISLQPEDSA TYYCRA (SEQ ID NO: 46)	REARHPWLRQW Y (SEQ ID NO: 39)	DGAGTKVE IK (SEQ ID NO: 49)

All possible combinations and permutations of the framework regions, complementarity determining regions and hypervariable regions listed herein are explicitly contemplated herein.

Sequence identity referenced in relation to the molecules of the invention may be judged at the level of individual CDRs, HVs or FWs, combined CDRs, HVs or FWs, or it may be judged over the length of the entire molecule. The CDR, HV and FW sequences described may also be longer or shorter, whether
5 that be by addition or deletion of amino acids at the N- or C-terminal ends of the sequence or by insertion or deletion of amino acids with a sequence.

Framework region FW1 is preferably from 20 to 28 amino acids in length, more preferably from 22 to 26 amino acids in length, still more preferably from 23 to 25 amino acids in length. In certain preferred
10 embodiments, FW1 is 26 amino acids in length. In other preferred embodiments, FW1 is 25 amino acids in length. In still other preferred embodiments, FW1 is 24 amino acids in length.

In alternative definitions, CDR region CDR1 is preferably from 7 to 11 amino acids in length, more preferably from 8 to 10 amino acids in length. In certain preferred embodiments, CDR1 is 9 amino acids
15 in length. In other preferred embodiments, CDR1 is 8 amino acids in length.

Framework region FW2 is preferably from 6 to 14 amino acids in length, more preferably from 8 to 12 amino acids in length. In certain preferred embodiments, FW2 is 12 amino acids in length. In other preferred embodiments, FW2 is 10 amino acids in length. In other preferred embodiments, FW2 is 9
20 amino acids in length. In other preferred embodiments, FW2 is 8 amino acids in length.

In alternative definitions, Hypervariable sequence HV2 is preferably from 4 to 11 amino acids in length, more preferably from 5 to 10 amino acids in length. In certain preferred embodiments, HV2 is 10 amino acids in length. In certain preferred embodiments, HV2 is 9 amino acids in length. In other preferred
25 embodiments, HV2 is 6 amino acids in length.

Framework region FW3a is preferably from 6 to 10 amino acids in length, more preferably from 7 to 9 amino acids in length. In certain preferred embodiments, FW3a is 8 amino acids in length. In certain preferred embodiments, FW3a is 7 amino acids in length.
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In alternative definitions, Hypervariable sequence HV4 is preferably from 3 to 7 amino acids in length, more preferably from 4 to 6 amino acids in length. In certain preferred embodiments, HV4 is 5 amino acids in length. In other preferred embodiments, HV4 is 4 amino acids in length.

35 Framework region FW3b is preferably from 17 to 24 amino acids in length, more preferably from 18 to 23 amino acids in length, still more preferably from 19 to 22 amino acids in length. In certain preferred embodiments, FW3b is 21 amino acids in length. In other preferred embodiments, FW3b is 20 amino acids in length.

In alternative definitions, CDR region CDR3 is preferably from 8 to 21 amino acids in length, more preferably from 9 to 20 amino acids in length, still more preferably from 10 to 19 amino acids in length. In certain preferred embodiments, CDR3 is 17 amino acids in length. In other preferred embodiments, CDR3 is 14 amino acids in length. In still other preferred embodiments, CDR3 is 12 amino acids in length. In yet other preferred embodiments, CDR3 is 10 amino acids in length.

Framework region FW4 is preferably from 7 to 14 amino acids in length, more preferably from 8 to 13 amino acids in length, still more preferably from 9 to 12 amino acids in length. In certain preferred embodiments, FW4 is 12 amino acids in length. In other preferred embodiments, FW4 is 11 amino acids in length. In still other preferred embodiments, FW4 is 10 amino acids in length. In yet other preferred embodiments, FW4 is 9 amino acids in length.

In one embodiment of the ROR1-specific antigen binding molecule:

FW1 is a framework region of from 20 to 28 amino acids;

FW2 is a framework region of from 6 to 14 amino acids;

FW3a is a framework region of from 6 to 10 amino acids;

FW3b is a framework region of from 17 to 24 amino acids; and/or

FW4 is a framework region of from 7 to 14 amino acids.

In one embodiment of the ROR1-specific antigen binding molecule:

FW1 has an amino acid sequence selected from the group consisting of: ASVNQTPRTATKETGESLTINCVVT (SEQ ID NO: 40), TRVDQSPSSLSASVGDRVITICVLT (SEQ ID NO: 41) and ASVTQSPRSASKETGESLTITCRVT (SEQ ID NO: 42), or a functional variant of any thereof with a sequence identity of at least 45%;

FW2 has an amino acid sequence according to TYWYRKNPG (SEQ ID NO: 43), or a functional variant of any thereof with a sequence identity of at least 45%;

FW3a has an amino acid sequence selected from the group consisting of: GRYVESV (SEQ ID NO: 44) and GRYSESV (SEQ ID NO: 45), or a functional variant of any thereof with a sequence identity of at least 45%;

FW3b has an amino acid sequence selected from the group consisting of: SFSLRIKDLTVADSATYYCKA (SEQ ID NO: 84), SFTLTISLQPEDSATYYCRA (SEQ ID NO: 46) and SFSLRISSLTVEDSATYYCKA (SEQ ID NO: 47), or a functional variant of any thereof with a sequence identity of at least 45%;
and/or

FW4 has an amino acid sequence selected from the group consisting of: DGAGTVLTVN (SEQ ID NO: 48), DGAGTKVEIK (SEQ ID NO: 49) or DGQGTKLEVK (SEQ ID NO: 85) or a functional variant of any thereof with a sequence identity of at least 45%.

- 5 The ROR1-specific antigen binding molecule of the present invention may be humanized. The ROR1-specific antigen binding molecule of the present invention may be de-immunized. The B1 loop variants on the humanised backbones G4 and V15 described herein are humanised. As P3A1G1 is already humanised all loop variants of P3A1G1 are humanised. Examples of humanised sequences of the invention include, but are not limited to:

10 B1G4
G3CP G4
G3CP V15
1H8 G4
1H8 V15
15 C3CP G4
C3CPV15
P3A1 G1 AE3
P3A1 G1 AE3.S
P3A1 G1 NAC6
20 P3A1 G1 NAC6.S
P3A1 G1 NAG8
P3A1 G1 NAG8.S
P3A1 G1 AF7.S

- 25 It will be appreciated by the skilled person that the humanised ROR1-specific antigen binding molecules described herein may be further humanised, for instance by substituting further FW region amino acids with amino acids of DPK-9.

The ROR1-specific antigen binding molecule of the present invention may also be conjugated to a
30 detectable label, dye, toxin, drug, pro-drug, radionuclide or biologically active molecule.

Preferably, the ROR1-specific antigen binding molecule does not bind to receptor tyrosine kinase-like orphan receptor 2 (ROR2). More preferably, the ROR1-specific antigen binding molecule binds to both human ROR1 and murine ROR1 (mROR1). Yet more preferably, the ROR1-specific antigen binding
35 molecule binds to deglycosylated ROR1.

Certain ROR1-specific antigen binding molecules of the invention may not bind to a linear peptide sequence selected from:

40 YMESLHMQGEIENQI (SEQ ID NO: 91)
CQPWNSQYPHTHTFTALRFP (SEQ ID NO: 92)
RSTIYGSRLRIRNLDTTDTGYFQ (SEQ ID NO: 93)

QCVATNGKEVVSSTGVLFVKFGPPPTASPGYSDEYE (SEQ ID NO: 94)

Preferably, the ROR1-specific antigen binding molecule selectively interacts with ROR1 protein with an affinity constant of approximately 0.01 to 50 nM, preferably 0.1 to 30 nM, even more preferably 0.1 to 10 nM. An affinity constant may be measured by Bio-layer interferometry (BLI). For monomers the interaction is 1:1. For the VNAR-hFc format the inventors have used two approaches. One where the ROR1 is immobilized and thus a bi-valent VNAR-hFc binds with an apparent K_D as the avidity effect comes into play. The other approach is in a 1:1 format whereby the VNAR-hFc is immobilized and ROR1 is flowed across the surface thus giving the K_D for 'true' 1:1 binding. Typically, where used herein affinity constants refer to those measured by Bio-layer interferometry (BLI) using the 1:1 binding format. By this method, for example, G3CP and G3CP G4 are within the 0.1 – 10 nM range. Of the P3A1 G1 loop variants examples have K_D values of 5.0 nM (AE3), 13.8 nM (NAC6) and 12.2 nM (NAG8).

Furthermore, the ROR1-specific antigen binding molecule is preferably capable of mediating killing of ROR1-expressing tumour cells or is capable of inhibiting cancer cell proliferation.

The ROR1-specific antigen binding molecule may also be capable of being endocytosed upon binding to ROR1. In other embodiments, the ROR1-specific antigen binding molecule may not be endocytosed upon binding to ROR1.

In a third aspect of the present invention, there it is provided a recombinant fusion protein comprising a specific antigen binding molecule of the first or second aspect. Preferably, in the recombinant fusion protein of the third aspect, the specific antigen binding molecule is fused to one or more biologically active proteins. The specific antigen binding molecule may be fused to one or more biologically active proteins via one or more linker domains. Preferred linkers include but are not limited to $[G_4S]_x$, where x is 1, 2, 3, 4, 5, or 6. Particular preferred linkers are $[G_4S]_3$ (SEQ ID NO: 86) and $[G_4S]_5$ (SEQ ID NO: 87). Other preferred linkers include the sequences PGVQPSP (SEQ ID NO: 88), PGVQPSPGGGS (SEQ ID NO: 89) and PGVQPAPGGGS (SEQ ID NO: 90). These linkers may be particularly useful when recombinant fusion proteins are expressed in different expression systems that differ in glycosylation patterns, such as CHO and insect, and those that do not glycosylate expressed proteins (e.g. *E. coli*). Any recombinant fusion protein sequence disclosed herein comprising a $[G_4S]_3$ linker may alternatively possess any other linker sequence disclosed herein.

It will also be appreciated that the fusion proteins of the invention can be constructed in any order, i.e., with the ROR1-specific antigen binding molecule at the N-terminus, C-terminus, or at neither terminus (e.g. in the middle of a longer amino acid sequence).

Preferred biologically active proteins include, but are not limited to an immunoglobulin, an immunoglobulin Fc region, a fragment of an immunoglobulin Fc region, an Fc heavy chain, a CH2 region, a CH3 region, an immunoglobulin Fab region, a Fab', a Fv, a Fv-Fc, a single chain Fv (scFv), scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager, an intein, a VNAR domain, a

single domain antibody (sdAb), a VH domain, or a scaffold protein (affibodies, centyrins, darpins etc.). A particularly preferred biologically active protein is an immunoglobulin Fc region. Other preferred fusion proteins include VNAR-VNAR and VNAR-VNAR-VNAR.

- 5 In one embodiment, the at least one biologically active protein is an immunoglobulin Fc region.

Therefore, the recombinant fusion protein may comprise a sequence according to SEQ ID NO: 186, SEQ ID NO: 187, SEQ ID NO: 183, SEQ ID NO: 184, or SEQ ID NO: 185.

10 G3CP-hFc

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLTNNGGGSGGGSGGGSGGGGSEPKSSDKT
HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
15 TKNQVSLTCLVKGFYPDSIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 186)

G3CPG4-hFc

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKGTMSFTL
20 TISSLPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIKGGGSGGGSGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLTCLVKGFYPDSIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
25 SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 187)

1H8-hFc

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSSVQWYDGAGTVLTNNGGGSGGGSGGGSGGGGSEPKSSDKTH
30 TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLTCLVKGFYPDSIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 183)

1H8 G4-hFc

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKGTMSFTL
35 TISSLPEDSATYYCRAYPWGAGAPSSVQWYDGAGTKVEIKGGGSGGGSGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLTCLVKGFYPDSIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
40 SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 184)

1H8 V15-hFc

ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRK NPGSSNQERISISGRYSESVNKRTMSFSL
45 RISSLTVEDSATYYCKAYPWGAGAPSSVQWYDGQGTKLEVKGGGSGGGSGGGSGGGGSEPKSSDKT
HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPDSIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 185)

In a further embodiment, the at least one biologically active protein is an immunoglobulin Fc region further modified to comprise an S to C mutation.

- 5 Therefore, the recombinant fusion protein may comprise a sequence according to SEQ ID NO: 178, SEQ ID NO: 179, SEQ ID NO: 180, SEQ ID NO: 181, or SEQ ID NO: 182.

G3CP-hFc(S239C)

10 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLTVNGGGGSGGGGSGGGGSGGGGSEPKSSDKT
HTCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 178)

15

G3CPG4-hFc(S239C)

20 TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKGTMSFTL
TISSLQPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIKGGGSGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 179)

1H8-hFc (S239C)

25 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSSVQWYDGAGTVLTVNGGGGSGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
30 SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 180)

1H8 G4-hFc (S239C)

35 TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKGTMSFTL
TISSLQPEDSATYYCRAYPWGAGAPSSVQWYDGAGTKVEIKGGGSGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 181)

- 40 1H8 V15-hFc (S239C)

ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRK NPGSSNQERISISGRYSESVNKRTMSFSL
RISSLTVEDSATYYCKAYPWGAGAPSSVQWYDGQGTKEVKGGGSGGGGSGGGGSGGGGSEPKSSDKT
HTCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL

TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 182)

5 In one embodiment, the at least one biologically active protein is a fragment of an immunoglobulin Fc region selected from the group consisting of an Fc heavy chain, a CH2 region and a CH3 region.

In one embodiment, the fragment of an immunoglobulin Fc region is an Fc heavy chain.

10 In one embodiment, the fragment of an immunoglobulin Fc region is engineered to dimerize with a second fragment of an immunoglobulin Fc region.

As used herein, an immunoglobulin Fc region that is “engineered to dimerise” may comprise at least one amino acid substitution. Typically, the at least one amino acid substitution promotes and/or makes more energetically favourable, an interaction and/or association with a second fragment of an immunoglobulin Fc region, which thus promotes dimerization and/or makes dimerization more energetically favourable. Such recombinant fusion proteins may have particular utility in the preparation of bi-specific and/or bi-paratopic binders.

20 Methods for generating Fc based bi-specific and / or bi-paratopic binders, through pairing of two distinct Fc heavy chains that are engineered to dimerize, are known in the art. These methods enable an Fc region to be assembled from two different heavy chains, each fused to a target binding domain or sequence with different binding characteristics. The target binding domains or sequences can be directed to different targets to generate multi-specific binders and/or to different regions or epitopes on the same target to generate bi-paratopic binding proteins. Multiple binding domains or sequences can be fused to the Fc sequences to create multi-specific or multi-paratopic binders or both multi-specific multi-paratopic binders within the same protein. Methods to generate these asymmetric bispecific and/or bi-paratopic binders through heterodimerisation of two different Fc heavy chains, or fragments thereof, include but are not limited to: Knobs-into-holes (Y-T), Knobs-into-holes (CW-CSAV), CH3 charge pair, Fab-arm exchange, SEED technology, BEAT technology, , HA-TF, ZW1 approach, Biclonic approach, 25 EW-RVT and Triomab See for example, Brinkman & Kontermann, (2017) mAbs, 9:2, 182-212; Klein *et al* (2012) mAbs 4:6, 653–663; Wang *et al* (2019) Antibodies, 8, 43; and Dietrich *et al* (2020) BBA - Proteins and Proteomics 1868 140250; each of which is incorporated herein by reference in its entirety.

35 In one embodiment, the fragment of an immunoglobulin Fc region is engineered to dimerize with the second fragment of an immunoglobulin Fc region by a method selected from the group consisting of knobs-into-holes (Y-T), knobs-into-holes (CW-CSAV), CH3 charge pairing, Fab-arm exchange, SEED technology, BEAT technology, HA-TF, ZW1 approach, Biclonic approach, EW-RVT and Triomab. Features of each of these methods are described in detail in connection with the fourth aspect of the invention and are likewise contemplated as relating to the third aspect of the invention.

40

In one embodiment, one or more residues of the fragment of the immunoglobulin Fc region comprises one or more amino acid substitution suitable for heterodimerization with a second fragment of an immunoglobulin Fc region comprising one or more corresponding amino acid mutation.

- 5 In one embodiment, the one or more amino acid substitution is selected from the group consisting of T366Y, Y407T, S354C, T366W, Y349C, T366S, L368A and Y407V.

In one embodiment, the one or more amino acid substitution is selected from the group consisting of T366Y and Y407T.

10

Any part of the fusion protein of the invention may be engineered to enable conjugation. In a preferred example, where an immunoglobulin Fc region is used, it may be engineered to include a cysteine residue as a conjugation site. Preferred introduced cysteine residues include, but are not limited to S252C and S473C (Kabat numbering), which correspond to S239C and S442C in EU numbering, respectively. In some embodiments, any of the fusion proteins disclosed herein may comprise the S239C point mutation. In some embodiments, any of the fusion proteins disclosed herein may comprise the S442C point mutation. In some embodiments, any of the fusion proteins disclosed herein may comprise both S239C and S442C point mutations. It is explicitly contemplated herein that sequence of any of the fusion proteins disclosed herein may be modified to include an S239C and/or S442C point mutation.

20

In accordance with the third aspect, recombinant fusions comprising multiple VNAR domains are provided. Accordingly, the recombinant fusions of the invention may be dimers, trimers or higher order multimers of VNARs. In such recombinant fusions, the specificity of each VNAR may be the same or different. Recombinant fusions of the invention include, but are not limited to, bi-specific or tri-specific molecules in which each VNAR domain binds to a different antigen, or to different epitopes on a single antigen (bi-paratopic binders). The term "bi-paratopic" as used herein is intended to encompass molecules that bind to multiple epitopes on a given antigen. Molecules that bind three or more epitopes on a given antigen are also contemplated herein and where the term "bi-paratopic" is used, it should be understood that the potential for tri-paratopic or multi-paratopic molecules is also encompassed.

25

30

Also in accordance with the third aspect, recombinant fusions are provided which include a ROR1-specific antigen binding molecule of the first aspect and a humanised VNAR domain. Humanised VNAR domains may be referred to as soloMERs and include but are not limited to the VNAR BA11, which is a humanised VNAR that binds with high affinity to human serum albumin.

35

Examples of bi-paratopic and multivalent fusion proteins include, but are not limited to:

- B1-G3CP
- G3CP-BA11
- BA11-G3CP
- 1H8-BA11
- 5 • BA11-1H8
- G3CP V15-BA11
- G3CP G4-BA11
- B1-G3CP Cys
- G3CP-BA11 Cys
- 10 • BA11-G3CP Cys
- 1H8-BA11 Cys
- BA11-1H8 Cys
- G3CP V15-BA11 Cys
- G3CP G4-BA11 Cys
- 15 • P3A1G1AE3-(L₂)-G3CPG4
- G3CPG4 (L₂)-P3A1G1AE3
- P3A1G1AE3-(L₂)-G3CPG4 Cys
- G3CPG4-(L₂)-P3A1G1AE3 Cys
- P3A1-(L₂)-BA11-(L₂)-G3CP
- 20 • P3A1-(L₂)-G3CP-(L₂)-BA11
- BA11-(L₂)-G3CP-(L₂)-P3A1
- BA11-(L₂)-P3A1-(L₂)-G3CP
- P3A1-(L₂)-BA11-(L₂)-1H8
- BA11-(L₂)-P3A1-(L₂)-1H8
- 25 • P3A1-(L₂)-BA11-(L₂)-G3CP Cys
- P3A1-(L₂)-G3CP-(L₂)-BA11 Cys
- BA11-(L₂)-G3CP-(L₂)-P3A1 Cys
- BA11-(L₂)-P3A1-(L₂)-G3CP Cys
- P3A1-(L₂)-BA11-(L₂)-1H8 Cys
- 30 • BA11-(L₂)-1P3A1-(L₂)-1H8 Cys

Wherein:

G3CP is

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESV NKRTMSFSL
 35 RIKDLTVADSATYYCKAYPWGAGAPY NVQWYDGAGTVLTVN (SEQ ID NO: 50)

1H8 is

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESV NKRTMSFSL
 RIKDLTVADSATYYCKAYPWGAGAPSSVQWYDGAGTVLTVN (SEQ ID NO: 61)

G3CP G4 is

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTSFTL
TISSLQPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIK (SEQ ID NO: 71)

5

G3CP V15 is

ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRTMSFSL
RISSLTVEDSATYYCKAYPWGAGAPYNVQWYDGQGTKLEVK (SEQ ID NO: 72)

10 BA11 is
TRVDQSPSSLSASVGDRVITITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAMSTNIWTGDGAGTKVEIK (SEQ ID NO: 95)

P3A1 G1 AE3 is

15 TRVDQSPSSLSASVGDRVITITCVLTGTRYGLYSTYWYRKNPGSSDEERISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK
(SEQ ID NO: 77)

and

20 where no linker is defined (-) corresponds to the linker Wobbe-G₅S, which in turn is PGVQPSPGGGGGS
(SEQ ID NO: 96)

-(L₂)- corresponds to the linker Wobbe-G₄S-GM, which in turn is PGVQPAPGGGGGS (SEQ ID NO: 90)

Cys – corresponds to a Cys containing C-terminal tag – for example
QACKAHHHHHGAEFEQKLISEEDL (SEQ ID NO: 97)

25

Recombinant bi-paratopic fusion protein dimers can also be made by fusing any recombinant fusion protein disclosed herein, in particular the loop library variants disclosed herein, onto one arm of an Fc fusion and by fusing binders to a different ROR1 epitope onto the other.

30 In certain embodiments, the specific binding molecules or recombinant fusions of the invention may be expressed with N- or C-terminal tags to assist with purification. Examples include but are not limited to His₆ and/or Myc. In addition, the N- or C-terminal tag may be further engineered to include additional cysteine residues to serve as conjugation points. It will therefore be appreciated that reference to specific binding molecules or recombinant fusions in all aspects of the invention is also intended to encompass
35 such molecules with a variety of N- or C-terminal tags, which tags may also include additional cysteines for conjugation.

Additional recombinant fusions are listed below. It will be appreciated that not every combination of linker and VNAR or fusion partner is listed below. However, all such combinations are expressly
40 encompassed by the present invention.

Monovalent-BA11 fusions

BA11-G3CP
 G3CP-BA11
 BA11-G3CPG4
 G3CPG4-BA1
 P3A1G1 AE3-BA11
 BA11-P3A1G1 AE3

Divalent-BA11 fusions

P3A1G1 AE3-P3A1G1 AE3-BA11
 BA11-P3A1G1 AE3-P3A1G1 AE3
 P3A1G1 AE3-BA11-P3A1G1 AE3
 G3CP-G3CP-BA11
 G3CP-BA11-G3CP
 BA11-G3CP-G3CP
 G3CPG4-G3CPG4-BA11
 G3CPG4-BA11-G3CPG4
 BA11-G3CPG4-G3CPG4
 B1G4-B1G4-BA11
 B1G4-BA11-B1G4
 BA11-B1G4-B1G4

Biparatopic Dimers

G3CP-P3A1G1 AE3
 P3A1G1 AE3-G3CP
 G3CPG4-P3A1G1 AE3
 P3A1G1 AE3-G3CPG4

Dimeric biparatopic BA11 fusions

G3CP-P3A1G1 AE3-BA11
 P3A1G1 AE3-G3CP-BA11
 G3CP-BA11-P3A1G1 AE3
 P3A1G1 AE3-BA11-G3CP
 G3CPG4-P3A1G1 AE3-BA11
 P3A1G1 AE3-G3CPG4-BA11
 G3CPG4-BA11-P3A1G1 AE3
 P3A1G1 AE3-BA11-G3CPG4
 B1G4-P3A1G1 AE3-BA11
 P3A1G1 AE3-B1G4-BA11
 B1G4-BA11-P3A1G1 AE3
 P3A1G1 AE3-BA11-B1G4

- Where the linkers between the VNAR domains are preferentially, but not limited to (G₄S)₅ (SEQ ID NO: 87), (G₄S)₃ (SEQ ID NO: 86), (G₄S)₇ (SEQ ID NO: 116), PGVQPSPGGGGS (SEQ ID NO: 89) (Wobbe-G₄S), PGVQPAPGGGGS (SEQ ID NO: 90) (Wobbe-G₄S GM), PGVQPCPGGGGGS (SEQ ID NO: 177) (WobbeCys-G₄S) and wherein different combinations of different linkers can be combined within the same construct. The WobbeCys-G₄S sequence also contains a single cysteine residue to facilitate site-selective bioconjugation of payloads to the proteins, in this linker, using thiol mediated chemical coupling strategies. The use of this linker sequence for bioconjugation is advantageous as reoxidation and capping of the reduced cysteine is minimal, leading to high yielding conversion of the protein to the corresponding conjugate in bioconjugation reactions.

Whereby, additional C-terminal (or N-terminal) tag sequences may or may not be present.

C-terminal tags include, but are not limited to, tags that contain poly-Histidine sequences to facilitate purification (such as His₆), contain c-Myc sequences (such as EQKLISEEDL (SEQ ID NO: 112)) to enable detection and / or contain Cysteine residues to enable labelling and bioconjugation using thiol reactive payloads and probes and combinations thereof. Preferential C-terminal tags include but are not limited to:

QASGAHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 98)

QACGAHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 99)

10 QACKAHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 97)

AAAHHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 100)

ACAHHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 101)

QASGAHHHHHH (SEQ ID NO: 102)

15 QACGAHHHHHH (SEQ ID NO: 103)

QACKAHHHHHH (SEQ ID NO: 104)

AAAHHHHHHH (SEQ ID NO: 105)

ACAHHHHHHH (SEQ ID NO: 106)

20 QASGA (SEQ ID NO: 107)

QACGA (SEQ ID NO: 108)

QACKA (SEQ ID NO: 109)

ACA (SEQ ID NO: 110)

SAPSA (SEQ ID NO: 111)

25

Wherein:

G3CP is

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL

30 RIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLTVN (SEQ ID NO: 50)

G3CP G4 is

TRVDQSPSSLSASVGDRVITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKG TMSFTL

TISSLQPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIK (SEQ ID NO: 71)

35

BA11

is

TRVDQSPSSLSASVGDRVITCVLTDTSYPLYSTYWYRK NPGSSNKEQISISGRYSESVNKG TKSFTL

TISSLQPEDSATYYCRAMSTNIWTGDGAGTKVEIK (SEQ ID NO: 95)

40 P3A1 G1 AE3 is

TRVDQSPSSLSASVGDRVITITCVLTGTRYGLYSTYWYRKNPGSSDEERISISGRYSESVNKGTKSFTL
 TISSLPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK
 (SEQ ID NO: 77)

5 B1G4 is

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTL
 TISSLPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 51)

As stated above, all combinations of VNAR and linker are expressly encompassed herein.

10 Humanised derivatives of the VNARs are also encompassed herein.

Also in accordance with the third aspect, recombinant fusions are provided which include a ROR1-specific antigen binding molecule of the first aspect and a recombinant toxin. Examples of recombinant toxins include but are not limited to *Pseudomonas* exotoxin PE38 and diphtheria toxin.

15

Also in accordance with the third aspect, recombinant fusions are provided which include a ROR1-specific antigen binding molecule of the first aspect and a recombinant CD3 binding protein. Examples of recombinant ROR1 and CD3 binding agents include but are not limited to:

20 B1G4-[WGM]-CD3

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTL
 TISSLPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIKPGVQAPGGGGSDIKLQSGAELAR
 PGASVKMSCKTSGYTFTRYTMHWWKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTA
 YMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSGGGGSGGGSGGGGSDIQLTQSPAI
 25 MSASPGEKVTMTCRASSSVSYMNWYQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISS
 MEAEDAATYYCQWSSNPLTFGAGTKLELKSHHHHHH (SEQ ID NO: 117)

G3CP-[WGM]-CD3

ASVNQTPRTATKETGESLTINCVTGANYGLAATYWYRKNPGSSNQERISISGRYVESVNKRTMSFSL
 30 RIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLTVPVQAPGGGGSDIKLQSGAELAR
 PGASVKMSCKTSGYTFTRYTMHWWKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTA
 YMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSGGGGSGGGSGGGGSDIQLTQSPAI
 MSASPGEKVTMTCRASSSVSYMNWYQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISS
 MEAEDAATYYCQWSSNPLTFGAGTKLELKSHHHHHH (SEQ ID NO: 118)

35 G3CPG4-[WGM]-CD3

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTL
 TISSLPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIKPGVQAPGGGGSDIKLQSGAELAR
 PGASVKMSCKTSGYTFTRYTMHWWKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTA
 YMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSGGGGSGGGSGGGGSDIQLTQSPAI
 40 MSASPGEKVTMTCRASSSVSYMNWYQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISS
 MEAEDAATYYCQWSSNPLTFGAGTKLELKSHHHHHH (SEQ ID NO: 119)

P3A1G1AE3-[WGM]-CD3

TRVDQSPSSLSASVGDRVITITCVLTGTRYGLYSTYWYRKNPGSSDEERISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIKPGVQAPAGGGGSDIKLQQSGAELARPG
5 ASVKMSCKTSGYTFTRYTMHWWKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYM
QLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMSA
SPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEA
EDAATYYCQQWSSNPLTFGAGTKLELKSHHHHHH (SEQ ID NO: 120)

10 B1G4-[WGM]-BA11-[G4S]-CD3

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTFTL
TISSLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIKPGVQAPAGGGGSTRVDQSPSSLSAS
VGDRVITITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTLTISSLQPEDSATYY
CRAMSTNIWTGDGAGTKVEIKGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWWKQR
15 PGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLD
YWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMASAPGEKVTMTCRASSSVSYMNWYQQ
KSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLE
LKSHHHHHH (SEQ ID NO: 121)

20 G3CP-[WGM]-BA11-[G4S]-CD3

ASVNQTPRTATKETGESLTINCVTGANYGLAATYWYRKNPGSSNKERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLTVNPGVQAPAGGGGSTRVDQSPSSLSA
SVGDRVITITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTLTISSLQPEDSATY
YCRAMSTNIWTGDGAGTKVEIKGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWWKQR
25 RQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCL
DYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMASAPGEKVTMTCRASSSVSYMNWYQQ
QKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKL
ELKSHHHHHH (SEQ ID NO: 122)

30 G3CPG4-[WGM]-BA11-[G4S]-CD3

TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTFTL
TISSLQPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIKPGVQAPAGGGGSTRVDQSPSSLSAS
VGDRVITITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTLTISSLQPEDSATYY
CRAMSTNIWTGDGAGTKVEIKGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWWKQR
35 PGQGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLD
YWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMASAPGEKVTMTCRASSSVSYMNWYQQ
KSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLE
LKSHHHHHH (SEQ ID NO: 123)

40 P3A1G1AE3-[WGM]-BA11-[G4S]-CD3

TRVDQSPSSLSASVGDRVITITCVLTGTRYGLYSTYWYRKNPGSSDEERISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIKPGVQAPAGGGGSTRVDQSPSSLSASVG
DRVITITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTLTISSLQPEDSATYYCR
45 AMSTNIWTGDGAGTKVEIKGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWWKQRPQ
QGLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYW

GQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMASASPGEKVTMTCRASSSVSYMNWYQQKSG
TSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELKS
HHHHHH (SEQ ID NO: 124)

5 P3A1-[WGM]-G3CP-[G4S]-CD3

TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKAREARHPWLRQWYDGAGTVLTVNPGVQPAPGGGGGSASVNQTPRTATKET
GESLTINCVTGANYGLAATYWYRKNPGSSNQRERISISGRYVESVNKRMTSFSRLIKDLTVADSATYY
CKAYPWGAGAPYNVQWYDGAGTVLTVNGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYT
10 MHWVKRPGQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARY
YDDHYCLDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMASASPGEKVTMTCRASSSVS
YMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLT
FGAGTKLELKSHHHHHH (SEQ ID NO: 125)

15 P3A1-[WGM]-G3CPG4-[G4S]-CD3

TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKAREARHPWLRQWYDGAGTVLTVNPGVQPAPGGGGSTRVDQSPSSLSASV
GDRVTITCVLTDANYGLAATYWYRKNPGSSNKRERISISGRYSESVNKGTMSTLTISLQPEDSATYYC
RAYPWGAGAPYNVQWYDGAGTKVEIKGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTM
20 HWWKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYD
DHYCLDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMASASPGEKVTMTCRASSSVSYM
NWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFG
AGTKLELKSHHHHHH (SEQ ID NO: 126)

25 P3A1G1AE3-[WGM]-G3CP-[G4S]-CD3

TRVDQSPSSLSASVGDRVTITCVLTGTRYGLYSTYWYRKNPGSSDEERISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIKPGVQPAPGGGGGSASVNQTPRTATKETG
ESLTINCVTGANYGLAATYWYRKNPGSSNQRERISISGRYVESVNKRMTSFSRLIKDLTVADSATYYC
30 KAYPWGAGAPYNVQWYDGAGTVLTVNGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTM
HWWKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYD
DHYCLDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMASASPGEKVTMTCRASSSVSYM
NWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFG
AGTKLELKSHHHHHH (SEQ ID NO: 127)

35

P3A1G1AE3-[WGM]-G3CPG4-[G4S]-CD3

TRVDQSPSSLSASVGDRVTITCVLTGTRYGLYSTYWYRKNPGSSDEERISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIKPGVQPAPGGGGSTRVDQSPSSLSASVG
40 DRVTITCVLTDANYGLAATYWYRKNPGSSNKRERISISGRYSESVNKGTMSTLTISLQPEDSATYYCR
AYPWGAGAPYNVQWYDGAGTKVEIKGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMH
WVKRPGQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYD
HYCLDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMASASPGEKVTMTCRASSSVSYMN
WYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGA
45 GTKLELKSHHHHHH (SEQ ID NO: 128)

P3A1G1AE3-[WGM]-D3-[G4S]-CD3

TRVDQSPSSLSASVGDRVITITCVLTGTRYGLYSTYWYRKPNPGSSDEERISISGRYSESVNKGTKSFTL
 TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIKPGVQPAPGGGGSASVNQTPRTATKETG
 5 ES LTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKRAKSFSLRIKDLTVADSATYYC
 KAQSGMAISTGSGHGYNWYDGAGTVLTVNNGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRY
 TMHWWKQRPQGQLEWIGYINPSRGYTNYNQKFKDKATLTDDKSSSTAYMQLSSLTSEDSAVYYCAR
 YYDDHYCLDYWGQGTTLTVSSGGGGSGGGGGSGGGGSDIQLTQSPAIMSASPGEKVTMTCRASSSV
 SYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFGSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPL
 TFGAGTKLELKSHHHHHH (SEQ ID NO:129)

10

Any CD3 binding sequence, and variants thereof, known in the art can be substituted in above. For examples:

UCL OKT3 sequence (WO2019008379)

15 QVQLVQSGAEVKKPGSSVKVCKASGYTFTRYTMHWWRQAPGQGLEWMGYINPSRGYTNYNQKFK
 DRVTITADKSTSTAYMELSSLRSED TAVYYCARYYDDHYCLDYWGQGTMTVSSVEGGSGGSGGSG
 GSGGVDDIQMTQSPSSLSASVGDRVITITCSASSSVSYMNWYQQKPGKAPKRLIYDTSKLASGVPSRF
 SGSGSGTEFTLTISLQPEDFATYYCQQWSSNPFTFGQGTKVEIK (SEQ ID NO: 130)

20 Harpoon ID20 (WO2016187594)

DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWWKQRPQGQLEWIGYINPSRGYTNYNQKFKD
 KATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSVEGGSGGSGGSGG
 SGGVDDIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRF
 SGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELK (SEQ ID NO: 131)

25

According to a fourth aspect, the invention provides a recombinant fusion protein comprising an antigen binding molecule comprising an amino acid sequence represented by the formula (I):

FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 (I)

30

wherein

FW1 is a framework region

35

CDR1 is a CDR sequence

FW2 is a framework region

HV2 is a hypervariable sequence

40

FW3a is a framework region

HV4 is a hypervariable sequence

FW3b is a framework region

CDR3 is a CDR sequence

5

FW4 is a framework region

or a functional variant thereof,

10 wherein the antigen binding molecule is fused to a fragment of an immunoglobulin Fc region wherein the fragment of an immunoglobulin Fc region is engineered to dimerize with a second fragment of an immunoglobulin Fc region.

15 In one embodiment, the fragment of an immunoglobulin Fc region selected from the group consisting of an Fc heavy chain, a CH2 region and a CH3 region.

In one embodiment, the fragment of an immunoglobulin Fc region is an Fc heavy chain.

20 Fc regions may be engineered to reduce FcγR binding. Therefore, the Fc regions disclosed herein may be engineered to reduce FcγR binding.

25 As used herein, an immunoglobulin Fc region that is “engineered to dimerise” may comprise at least one amino acid substitution. Typically, the at least one amino acid substitution promotes and/or makes more energetically favourable, an interaction and/or association with a second fragment of an immunoglobulin Fc region, which thus promotes dimerization and/or makes dimerization more energetically favourable. Such recombinant fusion proteins may have particular utility in the preparation of bi-specific and/or bi-paratopic binders.

30 Methods for generating Fc based bi-specific and / or bi-paratopic binders, through pairing of two distinct Fc heavy chains that are engineered to dimerize, are known in the art. These methods enable an Fc region to be assembled from two different heavy chains, each fused to a target binding domain or sequence with different binding characteristics. The target binding domains or sequences can be directed to different targets to generate multi-specific binders and/or to different regions or epitopes on the same target to generate bi-paratopic binding proteins. Multiple binding domains or sequences can be fused to the Fc sequences to create multi-specific or multi-paratopic binders or both multi-specific multi-paratopic binders within the same protein. Methods to generate these asymmetric bispecific and/or bi-paratopic binders through heterodimerisation of two different Fc heavy chains, or fragments thereof, include but are not limited to: Knobs-into-holes (Y-T), Knobs-into-holes (CW-CSAV), CH3 charge pair, Fab-arm exchange, SEED technology, BEAT technology, , HA-TF, ZW1 approach, Biclonic approach, 35 EW-RVT and Triomab See for example, Brinkman & Kontermann, (2017) mAbs, 9:2, 182-212; Klein *et* 40

al (2012) *mAbs* 4:6, 653–663; Wang *et al* (2019) *Antibodies*, 8, 43; and Dietrich *et al* (2020) *BBA - Proteins and Proteomics* 1868 140250; each of which is incorporated herein by reference in its entirety.

In one embodiment, the fragment of an immunoglobulin Fc region is engineered to dimerize with the second fragment of an immunoglobulin Fc region by a method selected from the group consisting of knobs-into-holes (Y-T), knobs-into-holes (CW-CSAV), CH3 charge pairing, Fab-arm exchange, SEED technology, BEAT technology, HA-TF, ZW1 approach, Biclonic approach, EW-RVT and Triomab.

Knobs-into-holes (Y-T) may comprise a T366Y substitution in a first CH3 domain and a Y407T substitution in a second CH3 domain.

Knobs-into-holes (CW-CSAV) may comprise one or more (preferably all) of the following substitutions in a first CH3 domain: S354C, T366W. Knobs-into-holes (CW-CSAV) may comprise one or more (preferably all) of the following substitutions in a second CH3 domain: Y349C, T366S, L368A, Y407V.

Knobs-into-holes (CW-CSAV) may comprise a disulphide bond in CH3.

CH3 charge pairing, may comprise one or more (preferably all) of the following substitutions in a first CH3 domain: K392D, K409D. CH3 charge pairing may comprise one or more (preferably all) of the following substitutions in a second CH3 domain: E356K, D399K.

Fab-arm exchange, may comprise a K409R substitution in a first CH3 domain and a F405L substitution in a second CH3 domain. Fab arm exchange and DuoBody capture the same Fc changes. DuoBody technology, may therefore comprise a K409R substitution in a first CH3 domain and a F405L substitution in a second CH3 domain.

SEED technology may incorporate known substitutions and/or result in an IgG/A chimera. Complementarity in the CH3 interface allowing for a heterodimeric assembly of Fc chains was developed by designing strand-exchange engineered domain (SEED) heterodimers. These SEED CH3 domains are composed of alternating segments derived from human IgA and IgG CH3 sequences (AG SEED CH3 and GA SEED CH3) and were used to generate so-called SEEDbodies, Davis *et al* (2010) *PEDS* 23, 4, 195-202 hereby incorporated by reference in its entirety. Because molecular models suggested that interaction with FcRn is impaired in the AG SEED CH3, residues at the CH2-CH3 junction were returned to IgG sequences. Pharmacokinetic studies confirmed that the half-life of SEEDbodies was comparable to other Fc fusion proteins and IgG1.

BEAT technology engineers the constant α and β domains of the human T cell receptor into the IgG1 CH3 dimer interface to drive heterodimerisation (Skegro *et al* (2017) *JBC* 292(23) 9745-9759). An additional D410Q mutation can further increase heterodimer formation in this system (Stutz & Blein 2020 *JBC* 295(28) 9392-9408).

- HA-TF, may comprise one or more (preferably all) of the following substitutions in a first CH3 domain: S364H, F405A. HA-TF may comprise one or more (preferably all) of the following substitutions in a second CH3 domain: Y349T, T394F.
- 5 ZW1 approach, may comprise one or more (preferably all) of the following substitutions in a first CH3 domain: T350V, L351Y, F405A, Y407V. ZW1 approach, may comprise one or more (preferably all) of the following substitutions in a second CH3 domain: T350V, T366L, K392L, T394W.
- Biclonic approach, may comprise one or more (preferably all) of the following substitutions in a first CH3 domain: 366K (+351K). Biclonic approach, may comprise one or more (preferably all) of the following substitutions in a second CH3 domain: 351D or E or D at 349, 368, 349, or 349 + 355.
- 10 EW-RVT, may comprise one or more (preferably all) of the following substitutions in a first CH3 domain: K360E, K409W. EW-RVT, may comprise one or more (preferably all) of the following substitutions in a second CH3 domain: Q347R, D399V, F405T. EW-RVT may comprise a disulphide bond in CH3. A disulphide bridge may be supported by the further incorporation of Y349C to a first CH3 domain and S354C to a second CH3 domain.
- 15 Triomabs may be formed by fusing a mouse hybridoma with a rat hybridoma, resulting in production of a bispecific, assymmetric hybrid IgG molecule. Preferential pairing of light chains with its corresponding heavy chain may then occur.
- 20 In one embodiment, one or more residues of the fragment of the immunoglobulin Fc region comprises one or more amino acid substitution suitable for knobs-in-holes (KIH) dimerization with a second fragment of an immunoglobulin Fc region comprising one or more corresponding amino acid mutation.
- 25 In one embodiment, the one or more amino acid substitution is selected from the group consisting of T366Y, Y407T, S354C, T366W, Y349C, T366S, L368A and Y407V.
- 30 In one embodiment, the one or more amino acid substitution is selected from the group consisting of T366Y and Y407T.
- In one embodiment, the antigen binding molecule is a ROR1 specific antigen binding molecule.
- 35 The recombinant fusion protein may comprise a sequence according to SEQ ID NO: 146 or SEQ ID NO: 147.
- G3CP hFc(S239C+Y407T) SEQ ID NO: 146
- 40 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESV NKR TMSFSL RIKDLTVADSATYYCKAYPWGAGAPYNVQWYD GAGTVLTVNGGGGSGGGGSGGGGSGGGGSEPKSSDKT

HTCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
 PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKTVDKSRWQQGNVFSC
 5 SVMHEALHNHYTQKSLSLSPGK

G3CPG4 hFc(S239C+Y407T) SEQ ID NO: 147

10 TRVDQSPSSLSASVGDRTITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTL
 TISSLPEDSATYYCAYPWGAGAPYNVQWYDGAGTKVEIKGGGGSGGGGSGGGGSEPKSSDKTH
 TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
 REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 15 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKTVDKSRWQQGNVFSC
 VMHEALHNHYTQKSLSLSPGK

The recombinant fusion protein may comprise a sequence according to SEQ ID NO: 194, SEQ ID NO:
 195 or SEQ ID NO 196:

1H8 hFc (S239C+Y407T) SEQ ID NO: 194
 20 ASVNQTPRTATKETGESLTINCVTGANYGLAATYWYRKNPGSSNQERISISGRYVESVNKRTMSFSL
 RIKDLTVADSATYYCKAYPWGAGAPSSVQWYDGAGTVLTVNGGGGSGGGGSGGGGSEPKSSDKTH
 TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
 REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 25 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKTVDKSRWQQGNVFSC
 VMHEALHNHYTQKSLSLSPGK

1H8 G4 hFc (S239C+Y407T) SEQ ID NO: 195

30 TRVDQSPSSLSASVGDRTITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTL
 TISSLPEDSATYYCAYPWGAGAPSSVQWYDGAGTKVEIKGGGGSGGGGSGGGGSEPKSSDKTH
 TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
 REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 35 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKTVDKSRWQQGNVFSC
 VMHEALHNHYTQKSLSLSPGK

1H8 v15 hFc (S239C+Y407T) SEQ ID NO: 196

40 ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRTMSFSL
 RISSLTVEDSATYYCKAYPWGAGAPSSVQWYDGQGTKLEVKGGGGSGGGGSGGGGSEPKSSDKT
 HTCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK
 PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKTVDKSRWQQGNVFSC
 45 SVMHEALHNHYTQKSLSLSPGK

The recombinant fusion protein may comprise a sequence according to SEQ ID NO: 148.

P3A1 hFc(S239C+T366Y) SEQ ID NO: 148

50 TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMISIGGRYVESVNKGAKSFS
 LRIKDLTVADSATYYCKAREARHPWLRQWYDGAGTVLTVNGGGGSGGGGSGGGGSEPKSSDKTH
 CPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
 EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK

NQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
VMHEALHNHYTQKSLSPGK

The recombinant fusion protein may comprise a sequence according to SEQ ID NO: 191 or SEQ ID NO:
5 192:

G3CP hFc(S239C+T366Y) SEQ ID NO: 191

10 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYKAYPWGAGAPYNVQWYDGAGTVLT VNGGGGSGGGGSGGGGSGGGGSEPKSSDKT
HTCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
CSVMHEALHNHYTQKSLSPGK

15 G3CPG4 hFc (S239C+T366Y) SEQ ID NO: 192

20 TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKGTMSFTL
TISSLQPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIKGGGSGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
SVMHEALHNHYTQKSLSPGK

25 The recombinant fusion protein may comprise a sequence according to SEQ ID NO: 197, SEQ ID NO:
198, or SEQ ID NO: 199

1H8 hFc (S239C+T366Y) SEQ ID NO: 197

30 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSSVQWYDGAGTVLT VNGGGGSGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
35 SVMHEALHNHYTQKSLSPGK

1H8 G4 hFc (S239C+T366Y) SEQ ID NO: 198

40 TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKGTMSFTL
TISSLQPEDSATYYCRAYPWGAGAPSSVQWYDGAGTKVEIKGGGSGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
45 SVMHEALHNHYTQKSLSPGK

1H8 v15 hFc (S239C+T366Y) SEQ ID NO: 199

50 ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRK NPGSSNQERISISGRYSESVNKRTMSFSL
RISSLTVEDSATYYCKAYPWGAGAPSSVQWYDGQGTKLEVKG GGGGSGGGGSGGGGSGGGGSEPKSSDKT
HTCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
55 CSVMHEALHNHYTQKSLSPGK

The recombinant fusion protein may comprise a sequence according to SEQ ID NO: 193:

P3A1 hFc (S239C+Y407T) SEQ ID NO: 193:

5

TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKAREARHPWLRQWYDGAGTVLTVNGGGGSGGGGSGGGGSEPKSSDKTHT
CPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK
10 NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGSSFFLTSKLTVDKSRWQQGNVFSCSV
MHEALHNHYTQKSLSLSPGK

The recombinant fusion protein may be a bi-paratopic dimer comprising any one or any two of SEQ ID
15 NOs 146, 147, 194, 195, 196, 148, 191, 191, 192, 193, 197, 198 and 199. The bi-paratopic dimer may
comprise one of SEQ ID NOs 146, 147, 194, 195, 196 and 193 comprising the Y407T point mutation.
The bi-paratopic dimer may comprise one of SEQ ID NOs 148, 191, 192, 197, 198 and 199 comprising
the T366Y point mutation. The bi-paratopic dimer may comprise SEQ ID NO: 146 and SEQ ID NO: 148
or SEQ ID NO: 147 and SEQ ID NO: 148. Any of the recombinant fusion proteins disclosed herein may
20 be associated with any of the linkers and payloads disclosed herein, Any of the bi-paratopic dimers
disclosed herein may be associated with any of the linkers and payloads disclosed herein, Conjugation
may be by any one or more S239C residue in the bi-paratopic dimer. Preferably, the bi-paratopic dimer
may be associated with the linker and payload vc-PAB-EDA-PNU. Preferably, the bi-paratopic dimer
comprises G3CP hFc(S239C+Y407T) (SEQ ID NO: 146) and P3A1 hFc(S239C+T366Y) (SEQ ID NO:
25 148), conjugated to vc-PAB-EDA-PNU, or G3CPG4 hFc(S239C+Y407T) (SEQ ID NO: 147) and P3A1
hFc(S239C+T366Y) (SEQ ID NO: 148), conjugated to vc-PAB-EDA-PNU which have been shown to be
highly efficacious *in vivo*.

SEQ ID Nos: 146, 147, 194, 195, 196, 148, 191, 191, 192, 193, 197, 198 and 199 include an S239C
30 mutation, for use in conjugation reactions. Where the recombinant fusion protein is not conjugated (for
example to an anthracycline (PNU) derivative) the S239C mutation is not needed and position 239 may
be an S rather than a C. Accordingly, in alternative embodiments the recombinant fusion protein or bi-
paratopic dimer may comprise a sequence according to any one of SEQ ID Nos: 146, 147, 194, 195,
196, 148, 191, 191, 192, 193, 197, 198 and 199 except that each sequence does not include an S239C
35 mutation.

Therefore, the recombinant fusion protein may comprise a sequence according to SEQ ID NO: 165 or
SEQ ID NO: 166.

40 G3CP-hFc (Y407T) SEQ ID NO: 165:

ASVNQTPRTATKETGESLTINCVTGANYGLAATYWYRKPNPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLTVNGGGGSGGGGSGGGGSEPKSSDKT

HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTK
 PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKLTVDKSRWQQGNVFSC
 SVMHEALHNHYTQKSLSLSPGK

5

G3CP G4-hFc (Y407T) SEQ ID NO: 166:

TRVDQSPSSLSASVGDRTITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTSFSL
 TISSLPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIKGGGGSGGGGSGGGGSEPKSSDKTH
 10 TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTKP
 REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKLTVDKSRWQQGNVFSCS
 VMHEALHNHYTQKSLSLSPGK

15 The recombinant fusion protein may comprise a sequence according to SEQ ID NO: 200, SEQ ID NO:
 201 or SEQ ID NO 202.

1H8 hFc (Y407T) SEQ ID NO: 200

20 ASVNQTPRTATKETGESLTINCVTGANYGLAATYWYRKNPGSSNQERISISGRYVESVNKRTMSFSL
 RIKDLTVADSATYYCKAYPWGAGAPSSVQWYDGAGTVLTVNGGGGSGGGGSGGGGSEPKSSDKTH
 TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTKP
 REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 25 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKLTVDKSRWQQGNVFSCS
 VMHEALHNHYTQKSLSLSPGK

1H8 G4 hFc (Y407T) SEQ ID NO: 201

30 TRVDQSPSSLSASVGDRTITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTSFSL
 TISSLPEDSATYYCRAYPWGAGAPSSVQWYDGAGTKVEIKGGGGSGGGGSGGGGSEPKSSDKTH
 TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTKP
 REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKLTVDKSRWQQGNVFSCS
 35 VMHEALHNHYTQKSLSLSPGK

1H8 v15 hFc (Y407T) SEQ ID NO: 202

40 ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRTMSFSL
 RISSLTVEDSATYYCKAYPWGAGAPSSVQWYDGQGTKLEVKGGGGSGGGGSGGGGSEPKSSDKT
 HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTK
 PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKLTVDKSRWQQGNVFSC
 SVMHEALHNHYTQKSLSLSPGK

45 The recombinant fusion protein may comprise a sequence according to SEQ ID NO: 167:

P3A1 hFc (T366Y) SEQ ID NO: 167:

50 TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
 LRIKDLTVADSATYYCKAREARHPWLRQWYDGAGTVLTVNGGGGSGGGGSGGGGSEPKSSDKTH
 CTPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
 EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK

The recombinant fusion protein may comprise a sequence according to SEQ ID NO: 188 or SEQ ID NO: 189:

10 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLTVNGGGSGGGGSGGGGSEPKSSDKT
HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYCKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLVSDGGSFFLYSKLTVDKSRWQQGNVFS
CSVMHEALHNHYTQKSLSIAPGK

20 TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKPNPGSSNKERISISGRYSESVNKGTMSTFL
TISSLQPEDSATYYCRAYPWGAGAPYNVQWYDAGATKVEIKGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLSDSGSFFLYSKLTVDKSRWQQGNVFC
SVMHEALHNHYTQKSLSLSPGK

30 ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKPNPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSSVQWYDGAGTVLT VNGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPSVFLFPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQVNSLYTRVVSFLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLYCLVKGFPSYDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFC
35 SVMHEALHNHYTKQSLSLSPGK

40 TRVDQSPSSLSASVGDRVITICVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTFL
TISSLQPEDSATYYCRAYPWGAGAPSSVQWYDGAGTKVEIKGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLSDSGSFFLYSKLTVDKSRWQQGNVFC
SVMVHEALHNHYTQKSLSLSPGK

ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKPNPGSSNQERISISGRYSESVNKRMTMSFSL
 50 RISSLTVEDSATYYCKAYPWGAGAPSSVQWYDGGQTKLEVKGSSGGGGSGGGGGSEPKSSDKT
 HTPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTK
 PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
 TKNQVSLYCLVKGYFSDIAHDEVESNGQPENNYKTTTPVLDSGGSFFLYSKLTVDKSRWQQGNVFS
 CSVMHEALHNHYTQKSLSLSPGK

57

P3A1 hFc (Y407T) SEQ ID NO: 190

5 TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKAREARHPWLRQWYDGAGTVLTVNGGGGSGGGGSGGGGSEPKSSDKTHT
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
EEQYNSTYRVVSVLTVLHQDWLNGKEYCKKVSNAKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLTSLKTVDKSRWQQGNVFCSSV
MHEALHNHYTQKSLSLSPGK

10

According to a fifth aspect, the invention provides a recombinant fusion protein dimer comprising

- (a) a first recombinant fusion protein, wherein the first recombinant fusion protein is a recombinant fusion protein according to the third or fourth aspects, and
- 15 (b) a second recombinant fusion protein, wherein the second recombinant fusion protein comprises a second antigen binding molecule fused to a second fragment of an immunoglobulin Fc region engineered to dimerize with the first fragment of an immunoglobulin Fc region.

20 In one embodiment, the second fragment of an immunoglobulin Fc region selected from the group consisting of an Fc heavy chain, a CH2 region and a CH3 region.

In one embodiment, the second fragment of an immunoglobulin Fc region is an Fc heavy chain.

25 In one embodiment, the second fragment of an immunoglobulin Fc region is engineered to dimerize with the second fragment of an immunoglobulin Fc region by a method selected from the group consisting of knobs-into-holes (Y-T), knobs-into-holes (CW-CSAV), CH3 charge pairing, Fab-arm exchange, SEED technology, BEAT technology, HA-TF, ZW1 approach, Biclonic approach, EW-RVT and Triomab.

30 In one embodiment, one or more residues of the fragment of the immunoglobulin Fc region comprises one or more amino acid substitution suitable for knobs-in-holes (KIH) dimerization with a second fragment of an immunoglobulin Fc region comprising one or more corresponding amino acid mutation.

In one embodiment, the one or more amino acid substitution is selected from the group consisting of T366Y, Y407T, S354C, T366W, Y349C, T366S, L368A and Y407V.

35

In one embodiment, the one or more amino acid substitution is selected from the group consisting of T366Y and Y407T.

40 Any sequence of a recombinant fusion protein disclosed herein may comprise any one or more amino acid substitution selected from the group consisting of T366Y, Y407T, S354C, T366W, Y349C, T366S, L368A and Y407V. SEQ ID NO: 145 (human Fc region) may therefore be modified by the incorporation of any one or more amino acid substitution selected from the group consisting of T366Y, Y407T, S354C,

T366W, Y349C, T366S, L368A and Y407V and incorporated into a recombinant fusion protein as described herein in place of the human Fc region sequence.

In one embodiment, the second antigen binding molecule is a ROR1 specific antigen binding molecule.

5

In one embodiment, the second specific antigen binding molecule is an immunoglobulin, an immunoglobulin Fab region, a Fab', a Fv, a Fv-Fc, a single chain Fv (scFv), scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager (BiTE), an intein, a VNAR domain, a single domain antibody (sdAb) or a VH domain.

10

In one embodiment:

- (a) the first recombinant fusion protein comprises a sequence according to SEQ ID NO: 146 or SEQ ID NO: 147, and
- (b) the second recombinant fusion protein comprises a sequence according to SEQ ID NO: 148.

15

According to a sixth aspect, the invention provides a ROR1-specific chimeric antigen receptor (CAR), comprising at least one ROR1-specific antigen binding molecule as defined by the first or second aspects of the invention, fused or conjugated to at least one transmembrane region and at least one intracellular domain.

20

The present invention also provides a cell comprising a chimeric antigen receptor according to the sixth aspect, which cell is preferably an engineered T-cell.

25

In a seventh aspect of the invention, there is provided a nucleic acid sequence comprising a polynucleotide sequence that encodes a specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor according to the first, second, third, fourth, fifth, or sixth aspects of the invention.

30

There is also provided a vector comprising a nucleic acid sequence in accordance with the seventh aspect and a host cell comprising such a nucleic acid.

35

A method for preparing a specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor, of the first, second, third, fourth, fifth, or sixth aspect is provided, the method comprising cultivating or maintaining a host cell comprising the polynucleotide or vector described above under conditions such that said host cell produces the specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor, optionally further comprising isolating the specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor.

40

In an eighth aspect of the invention, there is provided a pharmaceutical composition comprising the specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth, or sixth aspects. The pharmaceutical

composition may contain a variety of pharmaceutically acceptable carriers. Pharmaceutical compositions of the invention may be for administration by any suitable method known in the art, including but not limited to intravenous, intramuscular, oral, intraperitoneal, or topical administration. In preferred embodiments, the pharmaceutical composition may be prepared in the form of a liquid, gel, powder, tablet, capsule, or foam.

The specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of the first, second, third, fourth, fifth, or sixth aspects may be for use in therapy. More specifically, the specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth, or sixth aspects may be for use in the treatment of cancer. Preferably, the cancer is a ROR1-positive cancer type. More preferably, the cancer is selected from the group comprising blood cancers such as lymphomas and leukemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone lymphoma (MZL), non-Hodgkin lymphomas (NHL), acute myeloid leukemia (AML) and solid tumours including neuroblastoma, renal cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, uterine cancer, prostate cancer, thyroid cancer, Head and Neck cancer, bladder cancer, oesophageal cancer, stomach cancer or liver cancer.

Also provided herein is the use of a specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth, or sixth aspects in the manufacture of a medicament for the treatment of a disease in a patient in need thereof.

Furthermore, in accordance with the present invention there is provided a method of treatment of a disease in a patient in need of treatment comprising administration to said patient of a therapeutically effective dosage of a specific antigen binding molecule, recombinant fusion protein, recombinant fusion protein dimer or chimeric antigen receptor of the first, second, third, fourth, fifth, or sixth aspects or a pharmaceutical composition of the sixth aspect.

Preferably, the cancer is a ROR1-positive cancer type. More preferably, the cancer is selected from the group comprising blood cancers such as lymphomas and leukemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone lymphoma (MZL), non-Hodgkin lymphomas (NHL), acute myeloid leukemia (AML) and solid tumours including neuroblastoma, renal cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, uterine cancer, prostate cancer, thyroid cancer, Head and Neck cancer, bladder cancer, oesophageal cancer, stomach cancer or liver cancer.

Also provided herein is a method of assaying for the presence of a target analyte in a sample, comprising the addition of a detectably labelled specific antigen binding molecule of the first or second aspect, or a recombinant fusion protein or recombinant fusion protein of the third or fourth aspect, or a recombinant

fusion protein dimer of the fifth aspect, to the sample and detecting the binding of the molecule to the target analyte.

5 In addition, there is provided herein a method of imaging a site of disease in a subject, comprising administration of a detectably labelled specific antigen binding molecule of the first or second aspect or a detectably labelled recombinant fusion protein or recombinant fusion protein of the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect to a subject.

10 There is also provided herein a method of diagnosis of a disease or medical condition in a subject comprising administration of a specific antigen binding molecule of the first aspect or second aspect, or a recombinant fusion protein or recombinant fusion protein of the third or fourth aspect, or a recombinant fusion protein dimer of the fifth aspect.

15 Also contemplated herein is an antibody, antibody fragment or antigen-binding molecule that competes for binding to ROR1 with the ROR1-specific antigen binding molecule of the first or second aspect. The term "compete" when used in the context of antigen binding proteins (e.g., neutralizing antigen binding proteins or neutralizing antibodies) means competition between antigen binding proteins as determined by an assay in which the antigen binding protein (e.g., antibody or functional fragment thereof) under test prevents or inhibits specific binding of a the antigen binding molecule defined herein (e.g., specific
20 antigen binding molecule of the first aspect) to a common antigen (e.g., ROR1 in the case of the specific antigen binding molecule of the first or second aspect).

Also described herein is a kit for diagnosing a subject suffering from cancer, or a pre-disposition thereto, or for providing a prognosis of the subject's condition, the kit comprising detection means for detecting
25 the concentration of antigen present in a sample from a test subject, wherein the detection means comprises a ROR1-specific antigen binding molecule of the first or second aspect, a recombinant fusion protein or recombinant fusion protein dimer of the third, fourth or fifth aspect, a chimeric antigen receptor of the sixth aspect or a nucleic acid sequence of the seventh aspect, each being optionally derivatized, wherein presence of antigen in the sample suggests that the subject suffers from cancer. Preferably the
30 antigen comprises ROR1 protein, more preferably an extracellular domain thereof. More preferably, the kit is used to identify the presence or absence of ROR1-positive cells in the sample, or determine the concentration thereof in the sample. The kit may also comprise a positive control and/or a negative control against which the assay is compared and/or a label which may be detected.

35 The present invention also provides a method for diagnosing a subject suffering from cancer, or a pre-disposition thereto, or for providing a prognosis of the subject's condition, the method comprising detecting the concentration of antigen present in a sample obtained from a subject, wherein the detection is achieved using a ROR1-specific antigen binding molecule of the first or second aspect, a recombinant fusion protein or recombinant fusion protein dimer of the third, fourth or fifth aspect, a
40 chimeric antigen receptor of the sixth aspect or a nucleic acid sequence of the seventh aspect, each

being optionally derivatized, and wherein presence of antigen in the sample suggests that the subject suffers from cancer.

Also contemplated herein is a method of killing or inhibiting the growth of a cell expressing ROR1 in vitro or in a patient, which method comprises administering to the cell a pharmaceutically effective amount or dose of (i) ROR1-specific antigen binding molecule of the first or second aspect, a recombinant fusion protein or recombinant fusion protein dimer of the third, fourth or fifth aspect, a nucleic acid sequence of the sixth aspect, or the CAR or cell according the seventh aspect, or (ii) of a pharmaceutical composition of the eighth aspect. Preferably, the cell expressing ROR1 is a cancer cell. More preferably, the ROR1 is human ROR1.

According to a ninth aspect, the invention provides a specific antigen binding molecule comprising an amino acid sequence represented by the formula (II):

X-FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4-Y (II)

wherein

FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 is a ROR1-specific antigen binding molecule according to the first or second aspect

X and Y are optional amino acid sequences

wherein the specific antigen binding molecule is conjugated to a second moiety.

In certain preferred embodiments, the specific antigen binding molecule according to this aspect of the invention may additionally be conjugated to a third, fourth or fifth moiety. Conjugation of further moieties is also contemplated. In some cases, a third, fourth or fifth moiety may be conjugated to the second moiety. Accordingly, it will be understood that any of the moieties according to this aspect of the invention may have additional moieties conjugated thereto. Description of preferred features of the second moiety as set out below apply to the third, fourth, fifth or higher order moiety *mutatis mutandis*.

Preferably X or Y are individually either absent or selected from the group comprising an immunoglobulin, an immunoglobulin Fc region, a fragment of an immunoglobulin Fc region, an Fc heavy chain, a CH2 region, a CH3 region, an immunoglobulin Fab region, a Fab', a Fv, a Fv-Fc, a single chain Fv (scFv), scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager, an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, a scaffold protein (affibodies, centyrins, darpins etc.), or a toxin including but not limited to *Pseudomonas* exotoxin PE38, diphtheria toxin.

Preferably, the conjugation is via a cysteine residue in the amino acid sequence of the specific antigen binding molecule. The cysteine residue may be anywhere in the sequence, including in optional sequences X or Y (if present).

- 5 The conjugation may be via a thiol, aminoxy or hydrazinyl moiety incorporated at the N-terminus or C-terminus of the amino acid sequence of the specific antigen binding molecule.

Preferably, the second moiety is selected from the group comprising detectable label, dye, toxin, drug, pro-drug, radionuclide or biologically active molecule.

10

More preferably, the second moiety is at least one toxin selected from the group comprising:

- maytansinoids,
- auristatins,
- 15 • anthracyclines, preferably PNU-derived anthracyclines
- calicheamicins,
- amanitin derivatives, preferably α -amanitin derivatives
- tubulysins
- duocarmycins
- 20 • radioisotopes for example alpha-emitting radionuclide, such as ^{227}Th or ^{225}Ac
- liposomes comprising a toxic payload,
- protein toxins
- taxanes,
- pyrrolbenzodiazepines
- 25 • indolinobenzodiazepine pseudodimers and/or
- spliceosome inhibitors
- CDK11 inhibitors
- Pyridinobenzodiazepines
- Irinotecan and its derivatives

30

In other preferred embodiments in accordance with this aspect, the second moiety may be from the group comprising an immunoglobulin, an immunoglobulin Fc region, a fragment of an immunoglobulin Fc region, an Fc heavy chain, a CH2 region, a CH3 region, an immunoglobulin Fab region, a Fab', a Fv, a Fv-Fc, a single chain Fv (scFv), scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager, an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, a scaffold protein (affibodies, centyrins, darpins etc.), or a toxin including but not limited to *Pseudomonas* exotoxin PE38, diphtheria toxin.

35

In particularly preferred embodiments, the second moiety is a VNAR domain, which may be the same or different to the specific antigen binding molecule according to this aspect. Accordingly, dimers, trimers

40

or higher order multimers of VNAR domains linked by chemical conjugation are explicitly contemplated herein. In such embodiments, each individual VNAR domain may have the same antigen specificity as the other VNAR domains, or they may be different.

5 In accordance with this aspect, the specific antigen binding molecule may comprise, for example, bi-paratopic specific antigen binding molecules as described in relation to the first to fifth aspects fused to further biologically active molecules (including but not limited to molecules for half-life extension, for example BA11) and then further conjugated to a second moiety, including but not limited to cytotoxic payloads

10

In accordance with this aspect, the specific antigen binding molecule may be a receptor tyrosine kinase-like orphan receptor 1 (ROR1) specific antigen binding molecule. This may be a ROR1-specific antigen binding molecule of the first or second aspect of the invention. Accordingly, any of the preferred features described in relation to the first, second and third aspects apply *mutatis mutandis* to the sixth aspect.

15

The specific antigen binding molecule of the ninth aspect may be for use in therapy. More specifically, the specific antigen binding molecule of the ninth aspect may be for use in the treatment of cancer. Preferably, the cancer is a ROR1-positive cancer type. More preferably, the cancer is selected from the group comprising blood cancers such as lymphomas and leukemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone lymphoma (MZL), non-Hodgkin lymphomas (NHL), acute myeloid leukemia (AML) and solid tumours including neuroblastoma, renal cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, uterine cancer, prostate cancer, thyroid cancer, Head and Neck cancer, bladder cancer, oesophageal cancer, stomach cancer or liver cancer.

20

Also provided herein is the use of a specific antigen binding molecule of the ninth aspect in the manufacture of a medicament for the treatment of a disease in a patient in need thereof.

25

Pharmaceutical compositions comprising the specific antigen binding molecule of the ninth aspect are also provided. The pharmaceutical composition may contain a variety of pharmaceutically acceptable carriers

30

Furthermore, in accordance with the present invention there is provided a method of treatment of a disease in a patient in need of treatment comprising administration to said patient of a therapeutically effective dosage of a specific antigen binding molecule of the ninth aspect or a pharmaceutical composition comprising a specific antigen binding molecule of the ninth aspect.

35

Preferably, the cancer is a ROR1-positive cancer type. More preferably, the cancer is selected from the group comprising blood cancers such as lymphomas and leukemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone

40

lymphoma (MZL), non-Hodgkin lymphomas (NHL), acute myeloid leukemia (AML) and solid tumours including neuroblastoma, renal cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, uterine cancer, prostate cancer, thyroid cancer, Head and Neck cancer, bladder cancer, oesophageal cancer, stomach cancer or liver cancer.

5

Also provided herein is a method of assaying for the presence of a target analyte in a sample, comprising the addition of a detectably labelled specific antigen binding molecule of the ninth aspect to the sample and detecting the binding of the molecule to the target analyte.

10 In addition, there is provided herein a method of imaging a site of disease in a subject, comprising administration of a detectably labelled specific antigen binding molecule of the ninth aspect to a subject.

There is also provided herein a method of diagnosis of a disease or medical condition in a subject comprising administration of a specific antigen binding molecule of the ninth aspect.

15

Furthermore, any of the features described in respect of any of the above-mentioned aspects of the invention may be combined *mutatis mutandis* with the other aspects of the invention.

20 In addition to the sequences mentioned the following sequences are expressly disclosed. Certain of these sequences relate to examples of molecules of the invention described herein:

Table 3 – additional sequences

SEQ ID NO:	Sequence name	Sequence
115	B1V15	ASVTQSPRSASKETGESLTITCRVTGAINYGAAATYWYRKKNPGSSNQERISGRYSESVNKRMTMSFSLSLTVEDSATYY CKAYPWGAGAPWLQWYDGGQTKLEVK
151	human ROR1-His (CHO)	QETELSVSAELVPTSSWNISSELNKKDSYLTLDPEMNNITSLGQTAEHLCKVSGNPPPTI RWFKNDAAPVWQEPRLSFRSTIYGSRLRIRNLDTTDTGYFQCQVATNGKEVVSSTGVLVFK FGPPTASPGYSDEYEEDGFCQPYRGACARFIGNRTVYMESLHMQGEIENQITAAFTMI GTSSHLSDKCSQFAIPSLCHYAFPYCDETSSVPKPRDLCRDECEILENVLCQTEYIFARS NPMILMRLKLPNCEDLPQPESPEAANCIRIGIPMADPINKNHKCYNSTGVDYRGTVSVTK SGRQCQPWNSQYPHTHTFTALRFPELNGGHSYCRNPGNQKEAPWCFTLDENFKSDLCDIP ACDSKDSKEKNKMEHHHHH
152	human ROR1 (lg domain)	SELNKDSYLTLDPEMNNITSLGQTAEHLCKVSGNPPPTIRWFKNDAPVWQEPRLSFRS TIYGSRLRIRNLDTTDTGYFQCQVATNGKEVVSSTGVLVFKAAHHHHH
153	mouse ROR1-His (CHO)	QETELSVSAELVPTSSWNTSSEIDKGSYLTLDPEMNNITSLGQTAEHLCKVSGNPPPSI RWFKNDAAPVWQEPRLSFRATNYGSRLRIRNLDTTDTGYFQCQVATNGKVVSTTGVLVFK FGPPTASPGSDEYEEDGFCQPYRGACARFIGNRTVYMESLHMQGEIENQITAAFTMI GTSSHLSDKCSQFAIPSLCHYAFPYCDETSSVPKPRDLCRDECEILENVLCQTEYIFARS NPMILMRLKLPNCEDLPQPESPEAANCIRIGIPMADPINKNHKCYNSTGVDYRGTVSVTK SGRQCQPWNSQYPHTHTFTALRFPELNGGHSYCRNPGNQKEAPWCFTLDENFKSDLCDIP ACDSKDSKEKNKMEHHHHH
154	human ROR1-Fc (CHO)	QETELSVSAELVPTSSWNISSELNKKDSYLTLDPEMNNITSLGQTAEHLCKVSGNPPPTI RWFKNDAAPVWQEPRLSFRSTIYGSRLRIRNLDTTDTGYFQCQVATNGKEVVSSTGVLVFK FGPPTASPGYSDEYEEDGFCQPYRGACARFIGNRTVYMESLHMQGEIENQITAAFTMI GTSSHLSDKCSQFAIPSLCHYAFPYCDETSSVPKPRDLCRDECEILENVLCQTEYIFARS NPMILMRLKLPNCEDLPQPESPEAANCIRIGIPMADPINKNHKCYNSTGVDYRGTVSVTK

SEQ ID NO:	Sequence name	Sequence
		SGRQCQPWNSQYPHTHTFTALRFPELNGHSGYCRNPNGNQKEAPWCFTLDENFKSDLCDIP ACDSKDSKEKNKMEGGGGGGGGGGGGGSDKTHTCPPCPAPELLGGPSVFLFPPKPKDRTL MISRTPEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTPREEQYNSTYRVVSVLTVLHQ DWLNKEYCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTTPPVLDSDGSFFLYSKLTVDKSRWQQGQNVFSCSVMH EA LHNHYTQKSLSLSPGK
155	human ROR2-Fc (CHO)	EVEVLDPNPLGPDGQDGIPTLKGFLNLEPNNITIVGGQTALHCKVAGNPPPNV RWLKNDAPVWQEPRIIRKTEYGSRLRIQDLDTDTGYQCQVATNGMKTITATGVLFVR LGPTHSPNHFQDDYHEDGFCQPYRGIAACARFIGNRTIYVDSLQMQGEIENRITAAFTMI GTSTHLSQCSQFAIPSFCHFVPLCDARSRTPKPRELCRDECEVLESDLCRQEYTIARS NPLILMRLQLPKCEALPMPESPDAANCMRIGIPAERLGRYHQCYNHSGMDYRGTAATTKS GHQCQPWALQHPHSHHLSSTDFPELGGGHAYCRNPNGGMEGPWCFTQNKNNRMELCDVPS CSPRDSKMGGGGGGGGGGGGGGSDKTHTCPPCPAPELLGGPSVFLFPPKPKDRTLMISRT PEVTCVWVDVSHEDPEVKFNWYVDGVEVHNAKTPREEQYNSTYRVVSVLTVLHQDWLNG KEYCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSD IAVEWESNGQPENNYKTTTPPVLDSDGSFFLYSKLTVDKSRWQQGQNVFSCSVMH EA LHNHYTQKSLSLSPGK
156	mouse ROR1-Fc (CHO)	QETELSVSAELVPTSSWNTSSEIDKGSYLTLDPEMNNITSLGQTAEHLCKVSGNPPPSI RWFKNDAPVWQEPRIIRKTEYGSRLRIQDLDTDTGYQCQVATNGMKTITATGVLVFK FGPPPTASPGSSDEYEEGFCQPYRGIAACARFIGNRTIYVMSLHMQGEIENQITAAFTMI GTSSHLSDKCSQFAIPSLCHYAFPYCDETSSVPKPRDLCDRDECEVLENLVLCQTEYIFARS NPMLILMRLKLPNCEDLPQPESPEAANCIRIGIPMADPINKNHKCYNSTGVDRGTVSVTK SGRQCQPWNSQYPHTHTFTALRFPELNGHSGYCRNPNGNQKEAPWCFTLDENFKSDLCDIP ACDSKDSKEKNKMEGGGGGGGGGGGGGSDKTHTCPPCPAPELLGGPSVFLFPPKPKDRTL

SEQ ID NO:	Sequence name	Sequence
		MISRTPEVTCWVDVSHEDPEVKFNWYVDGVEVHNAKTPREEQYNSTYRWSVLTVLHQ DWLNGKEYKCKVSNKALPAIEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMHEA LHNHYTQKSLSLSPGK
157	rat ROR1-Fc (CHO)	QETELSVSAELVPTSSWNTSSEIDKDSYLTLDPEMNNITSLGQTAEHLCKVSGNPPPNII RWFKNDAVWQEPRRISFRATNYGSRRLRIRNLDTTDTGYFQC VATSGKKVSVSTTGVLVFK FGPPPTASPGSSDEYEEDGFCQPYRGIACARFIGNRTVYMESLHMQGEIENQITAAFTMI GTSSHLSDKCSQFAIPSLCHYAFPYCDETSSVPKPRDLCRDECEVLENVLCHEIYIFARS NPMILMRLKLPNCEDLPQPESPEAANCIRIGIPMADPINKNHKCYNSTGVDYRGTVSVTK SGRQCQPWNSQYPHTHSFTALRFPELNGGHSYCRNPGNQKEAPWCFTLDENFKSDLCDIP ACDSKDSKEKNKMEGGGGGGGGGGGGSDKTHTCPAPPELLGGPSVFLFPPKPKDTL MISRTPEVTCWVDVSHEDPEVKFNWYVDGVEVHNAKTPREEQYNSTYRWSVLTVLHQ DWLNGKEYKCKVSNKALPAIEKTIKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSVMHEA LHNHYTQKSLSLSPGK

A highly interesting class of DNA intercalating toxins for use as payloads for drug conjugates are anthracyclines, because of their proven clinical validation as chemotherapeutic drugs in cancer therapy.

5 Stability of chemically-conjugated protein drug conjugates is an important consideration, since unintended release of a highly potent anthracycline toxin, like PNU-159682, in the circulation of a patient prior to targeting of the tumour cells would lead to off target effects and undesirable side effects. Some example molecules released from PNU conjugates include release of PNU159682 derivative from different Val-Cit-PAB containing drug linkers.

10

Potent toxins that can be linked to targeting proteins with high stability are therefore required in order to avoid, or at least reduce, unwanted side effects. Alternatively, linker payloads are designed such that extracellular cleavage releases derivatives of the payload with attenuated potency. However, sufficient potency needs to be retained in order to avoid any reduction in side effect being negated due to the need to administer higher doses to achieve efficacy.

15

Ease of conjugation is an important factor in producing easily manufacturable products. Payloads of the present disclosure may use a maleimide group, which can react to any available thiol group on a conjugation partner using straightforward and standard conditions. Furthermore, the use of maleimide/thiol chemistry for conjugation allows for site-specific conjugation to introduced thiol groups, for example on the side-chain of an engineered cysteine residue in a protein sequence. In some cases described herein, a cysteine may be introduced via the introduction of his-myc tag containing an engineered cysteine (example sequences include, but are not limited to, QACKAHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 97) or QACGAHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 99)) at the C- or N-terminal of a protein.

20

25

Antibody / protein drug conjugates generated using non-selective labelling methods, such as through reaction with amino functionalities within proteins, deliver products containing multiple different species with differing drug to antibody ratios. This impacts the properties of the conjugate including potency and PK properties which impacts *in vivo* efficacy and toxicities. Therefore, thiol reactive payloads are of great importance, as these can be reacted in high yield, in a simple process, with naturally occurring cysteine residues in proteins or with a cysteine residue engineered into a specific site at any point within the sequence of proteins using molecular biology / recombinant protein expression or chemical synthesis or through chemical modification of expressed, synthetic or natural proteins. In some cases described herein, the cysteine is engineered into the Fc region of an Fc fusion protein.

30

35

The present disclosure provides anthracycline (PNU) derivatives suitable for use in drug conjugates. Specifically, derivatives of PNU159682 are provided, which lack the C14 carbon and attached hydroxyl functionality, and are functionalised with an ethylenediamino (EDA) group at the C13 carbonyl of PNU159682. This EDA-PNU159682 can in turn be functionalised, through the amino group of the EDA

40

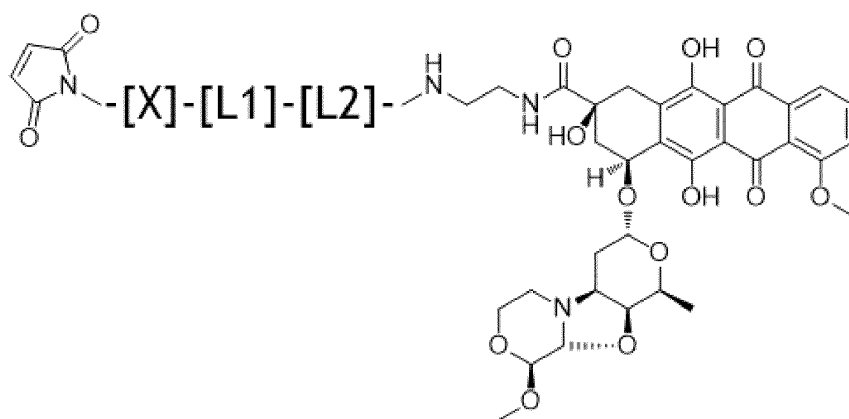
moiety, with a maleimide containing linker. A maleimide group is present in the anthracycline (PNU) derivatives of formula (V) and may also be present in the anthracycline (PNU) derivatives of formula (VI). Such payloads are able to react with a free thiol group on another molecule. Where the free thiol is on a protein, a protein-drug conjugate (PDC) may be formed.

5

Surprisingly, derivatives of PNU159682 functionalised with an ethylenediamino (EDA) group and linked to a thiol group via a maleimide group show higher stability compared to non-EDA payloads or liberated payload derivatives with slightly less potency. More stable payloads may be advantageous because of reduced off-target effects, which in turn may lead to reduced side effects and increased patient

10

PCT/EP2020/067210 describes anthracycline (PNU) derivatives of formula (V):



(V)

15

wherein [X] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

20

[L1] and [L2] are optional linkers selected from the group consisting of valine (Val), citrulline (Cit), alanine (Ala), asparagine (Asn), a peptide, $-(CH_2)_n-$, $-(CH_2CH_2O)_n-$, p-aminobenzyloxycarbonyl (PAB), Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, Val-Ala, Asn-Ala, any amino acid except glycine, and combinations thereof.

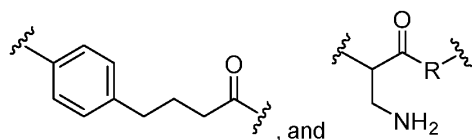
25

The anthracycline (PNU) derivative of formula (V) may comprise [L1], [L2] or [L1] and [L2].

Preferably, where [L1] and/or [L2] are peptides, said peptides do not contain glycine.

It will be clear to those of skill in the art that when optional spacers and/or optional linkers are absent a bond remains in their place.

Preferably, [X] is selected from the group comprising polyethylene glycol,



- 5 of the molecule and wherein [R] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof.

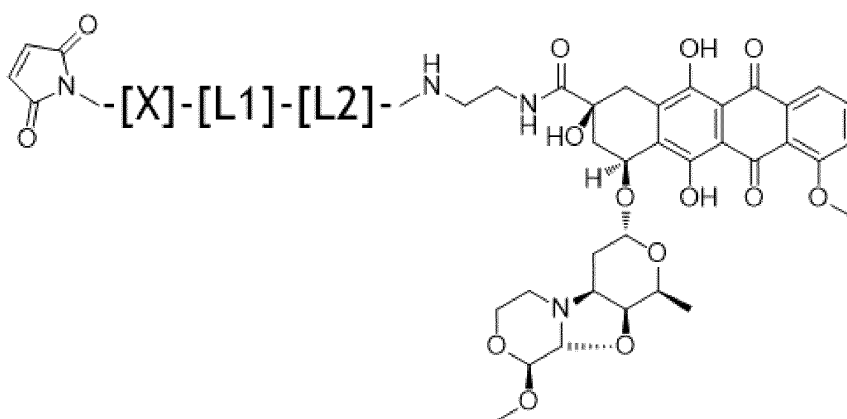
- 10 Most preferably, [X] is polyethylene glycol. The polyethylene glycol may be PEG4.

Preferably, [L2] is p-aminobenzyloxycarbonyl (PAB) or Alanine.

- 15 Preferably, the anthracycline (PNU) derivative comprises [L1] and/or [L2] and [X] is optional. Accordingly, [L1] and/or [L2] may be linkers selected from the group consisting of valine (Val), citrulline (Cit), alanine (Ala), asparagine (Asn), a peptide, $-(CH_2)_n-$, $-(CH_2CH_2O)_n-$, p-aminobenzyloxycarbonyl (PAB), Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, Val-Ala, Asn-Ala, any amino acid except glycine, and combinations thereof. The anthracycline (PNU) derivative of formula (V) may comprise [L1], [L2] or [L1] and [L2]. The anthracycline (PNU) derivative of formula (V) may comprise [L1] and/or [L2].

20

PCT/EP2020/067210 describes anthracycline (PNU) derivatives of formula (V):



(V)

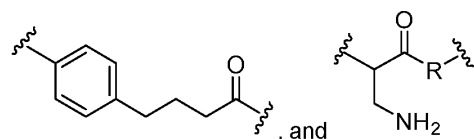
wherein [X] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

- 5 [L1] and/or [L2] are linkers selected from the group consisting of valine (Val), citrulline (Cit), alanine (Ala), asparagine (Asn), a peptide, $-(CH_2)_n-$, $-(CH_2CH_2O)_n-$, p-aminobenzyloxycarbonyl (PAB), Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, Val-Ala, Asn-Ala, any amino acid except glycine, and combinations thereof;

wherein the anthracycline (PNU) derivative of formula (V) comprises [L1], [L2] or [L1] and [L2].

10

Preferably, [X] is selected from the group comprising polyethylene glycol,



, and , wherein represents the point of attachment to the rest

of the molecule and wherein [R] is an optional spacer selected from the group comprising substituted or

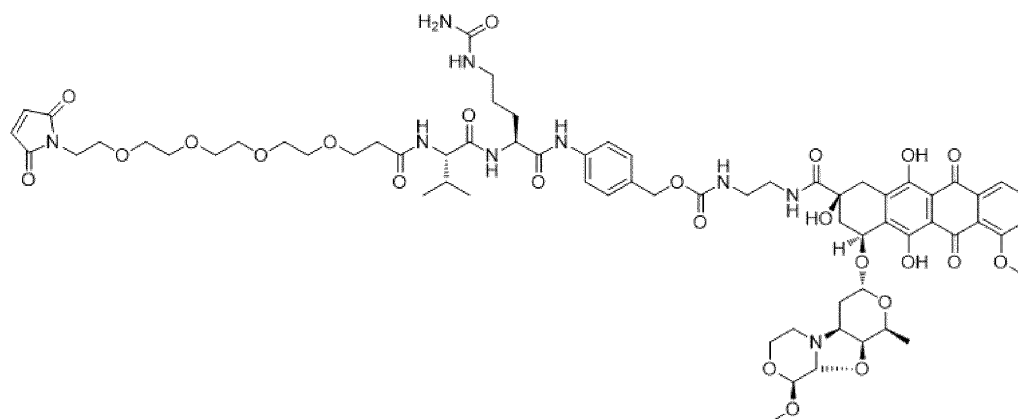
15 unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof.

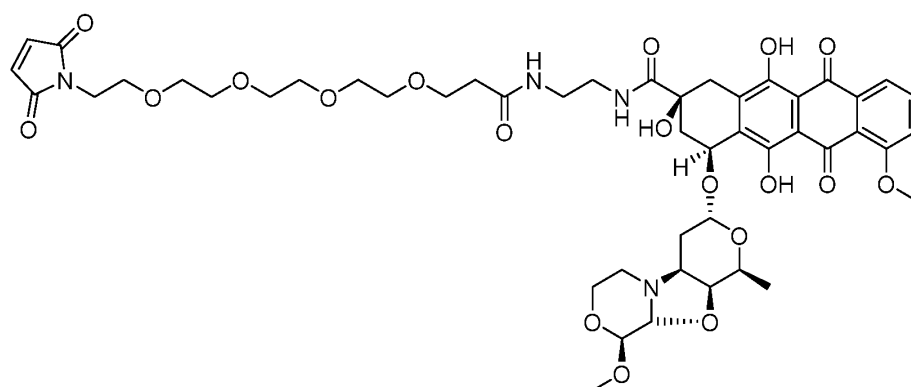
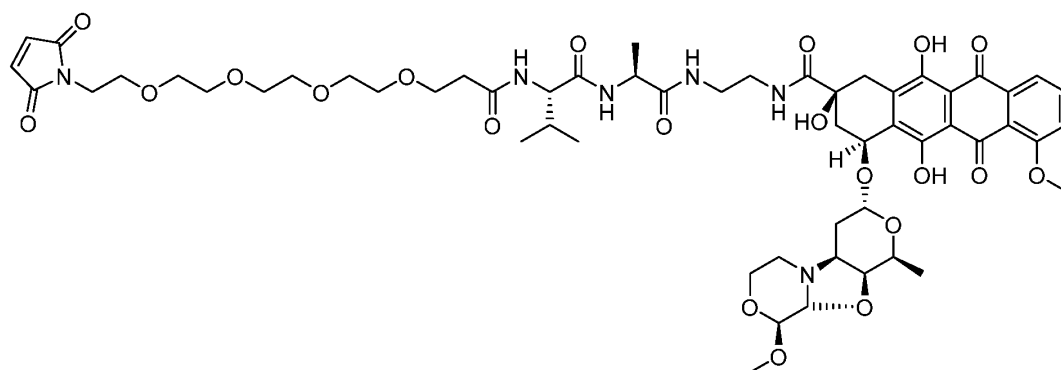
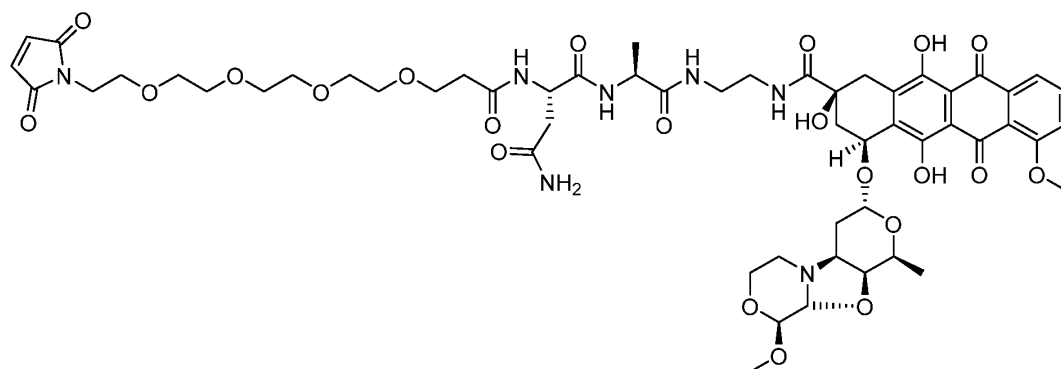
Most preferably, [X] is polyethylene glycol. The polyethylene glycol may be PEG4.

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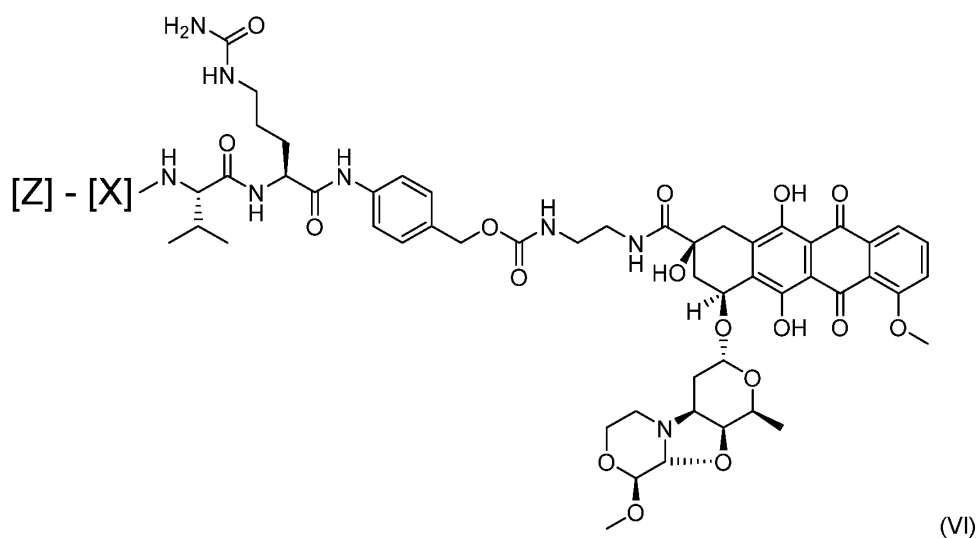
Preferably, [L2] is p-aminobenzyloxycarbonyl (PAB) or Alanine.

Preferably, the PNU derivative has a structure selected from:





5 PCT/EP2020/067210 also describes anthracycline (PNU) derivatives of formula (VI):



wherein [X] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

wherein [Z] is a reactive group. The reactive group may be any reactive group suitable for use in a conjugation reaction, particularly a conjugation reaction to a target binding molecule.

[Z] may therefore be a moiety comprising a functional group for use in bioconjugation reactions.

Functional groups for use in bioconjugation reactions include but are not limited to,

- maleimides or alkyl halides for reaction with thiol groups or selenol groups on proteins through thioether and selenoether reactions;
- sulphydryl groups for reaction with maleimide, alkyl halide or thiol functionalised molecules including the thiol groups of protein cysteine residues;
- activated disulphides such as pyridyl dithiols (Npys thiols) or TNB thiols (5-thiol-2-nitrobenzoic acid) for reaction with thiol groups to form disulphide linkages through thiol disulphide exchange;
- amino groups for attachment to carboxyl groups on proteins and biomolecules through amide bond forming reactions;
- alkyne groups, particularly ring constrained alkynes such as dibenzocyclooctyne (DBCO) or bicyclo[6.1.0]nonyne (BCN) for the reaction with azido functionalised biomolecules through strain promoted alkyne-azide cycloaddition copper free chemistry. Azido functionalities can be introduced into proteins through, for example, the incorporation of the unnatural amino acid para-azidomethyl-L-phenylalanine or into protein glycans using enzyme mediated glycoengineering to attach azido-containing sugar analogues;
- azido groups for reaction with alkyne functionalised target-binding molecule through strain promoted alkyne-azide cycloaddition copper free chemistry;
- aminoxy groups for reactions with aldehyde and ketone groups on biomolecules through oxime forming ligations. Ketones can be introduced into proteins through the use of amber stop codon

technologies such as the incorporation of the non-natural amino acid, para-acetyl phenylalanine. Aldehydes can be found on biomolecules through the presence of reducing sugars and can be introduced into proteins through periodate oxidation of N-terminal serine residues or periodate oxidation of cis-glycol groups of carbohydrates. Aldehyde groups can also

5 be incorporated into proteins through the conversion of protein cysteines, within specific sequences, to formyl glycine by formylglycine generating enzyme. In addition formylglycine containing proteins have been conjugation to payloads via the Hydrazino-Pictet-Spengler (HIPS) ligation;

- aldehyde or ketone groups for the reaction with aminoxy or hydrazide or hydrazinyl functionalized biomolecules through oxime or hydrazine bond forming ligation reactions. Protein aminoxy and hydrazide functionalized proteins can be generated through cleavage of intein-fusion proteins.

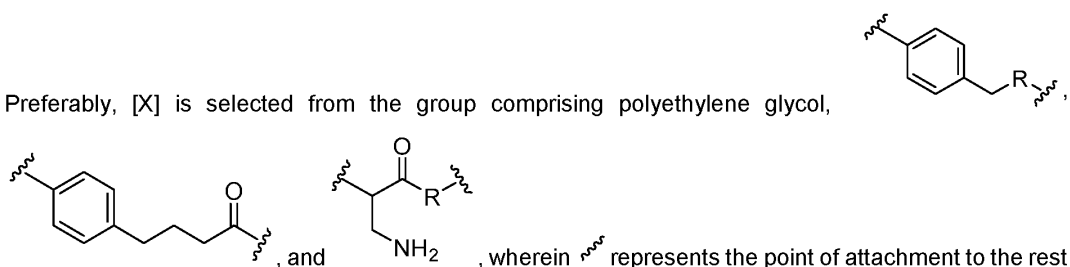
[Z] may therefore be selected from the group consisting of a maleimide, an alkyl halide, a sulphhydryl group, an activated disulphide (such as pyridyl dithiols (Npys thiols) or TNB thiols (5-thiol-2-nitrobenzoic acid)), an amino group, an alkyne group (such as ring constrained alkynes such as dibenzocyclooctyne (DBCO) or bicyclo[6.1.0]nonyne (BCN)), an azido group, an aminoxy group, an aldehyde group and a ketone group.

[Z] may also be a moiety for enzyme mediated bioconjugation reactions. Moieties for use in enzyme mediated conjugation reactions include but are not limited to polyGly [(Gly)_n] for use in sortase-enzyme mediated antibody conjugation or an appropriate primary amine for bacterial transglutaminase mediated conjugation to glutamine γ-carboxyamide groups contained with sequences such as Lys-Lys-Gln-Gly and Lys-Pro-Glu-Thr-Gly.

[Z] may therefore be selected from the group consisting of polyGly and a primary amine.

The PNU derivative according to formula (VI) may therefore correspond to a PNU derivative of formula (V) wherein L1 is Val-Cit-PAB, L2 is absent and wherein the maleimide group may be replaced with another Reactive Group as defined above.

Preferably, [X] is selected from the group comprising polyethylene glycol,



30 of the molecule and wherein [R] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof.

Most preferably, [X] is polyethylene glycol. The polyethylene glycol may be PEG4.

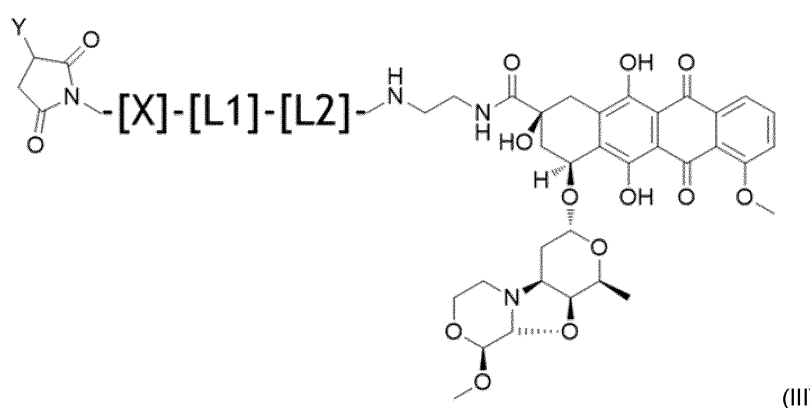
The PNU derivative according to formula (V) or formula (VI) may be conjugated to a ROR1 specific antigen binding molecule according to the present invention or to a recombinant fusion protein or recombinant fusion protein dimer of the invention.

- 5 According to a tenth aspect, the invention provides a target-binding molecule-drug conjugate, comprising

(a) a ROR1 specific antigen binding molecule according to the first, second or ninth aspect, or a recombinant fusion protein or recombinant fusion protein of the third or fourth aspect or a recombinant fusion protein dimer of the fifth aspect, and

- 10 (b) an anthracycline (PNU) derivative,

wherein the target-binding molecule-drug conjugate has the structure of formula (III):



- 15 wherein [X] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

- [L1] and [L2] are optional linkers selected from the group consisting of valine (Val), citrulline (Cit), alanine (Ala), asparagine (Asn), a peptide, $-(CH_2)_n-$, $-(CH_2CH_2O)_n-$, p-aminobenzyloxycarbonyl (PAB), Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, Val-Ala, Asn-Ala, any amino acid except glycine, and combinations thereof; and
- 20

Y comprises a ROR1 specific antigen binding molecule according to the first, second or ninth aspect, or a recombinant fusion protein or recombinant fusion protein of the third or fourth aspect or a recombinant fusion protein dimer of the fifth aspect.

25

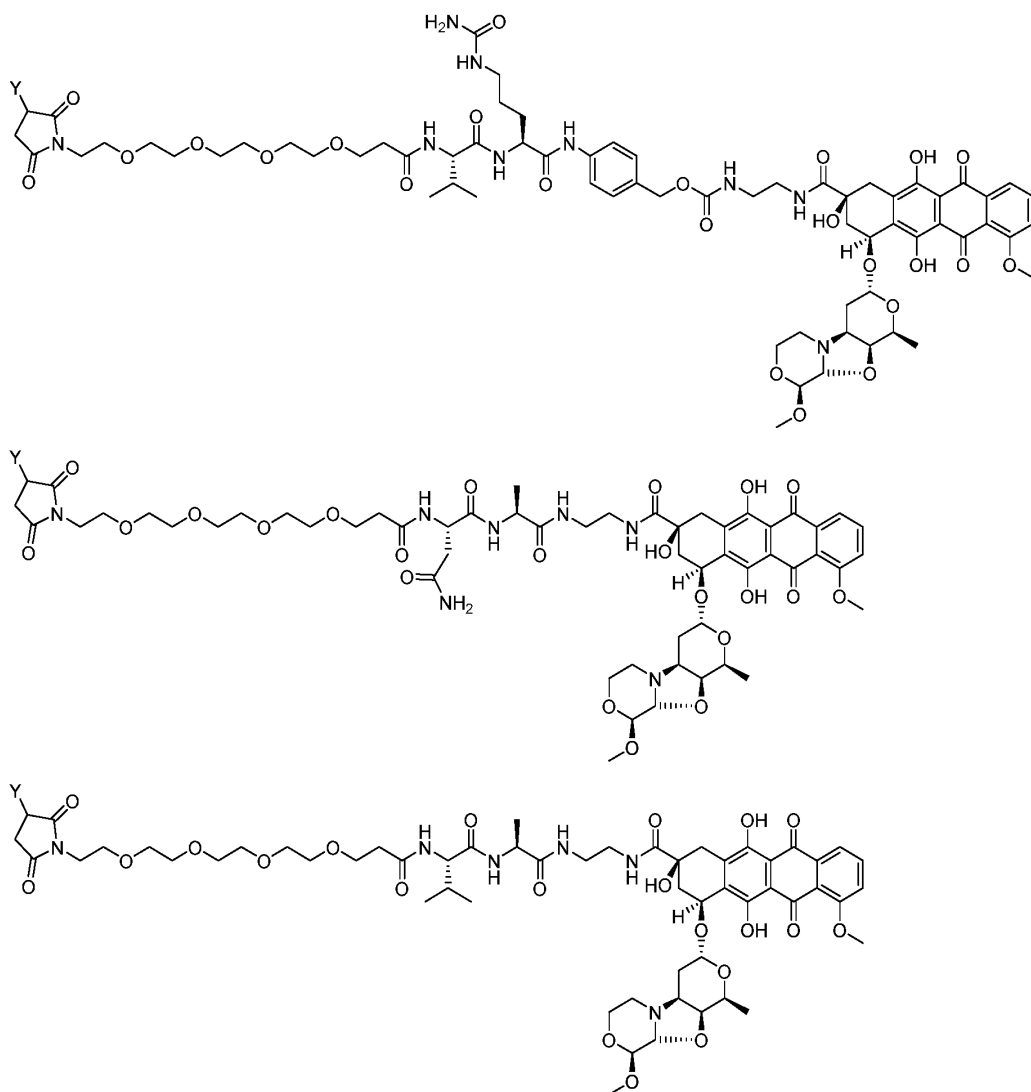
The target-binding molecule-drug conjugate of formula (III) may comprise [L1], [L2] or [L1] and [L2].

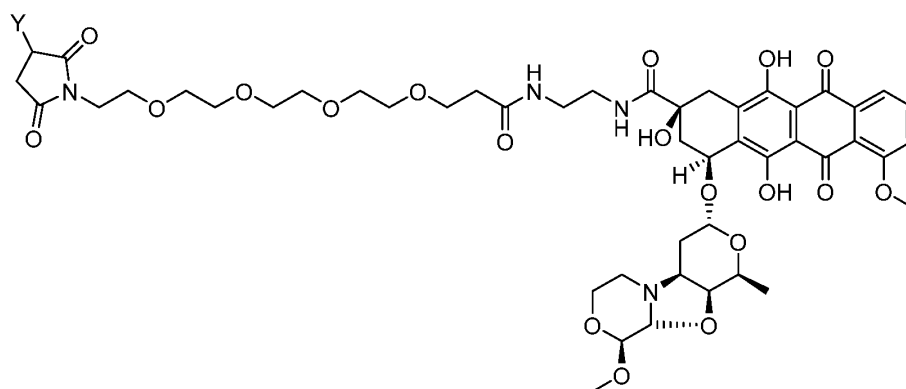
Preferably, target-binding molecule-drug conjugate where [L1] and/or [L2] are peptides, said peptides do not contain glycine.

30

It will be clear to those of skill in the art that when optional spacers and/or optional linkers are absent a bond remains in their place.

- 5 Preferably, the target-binding molecule-drug conjugate has a structure selected from:

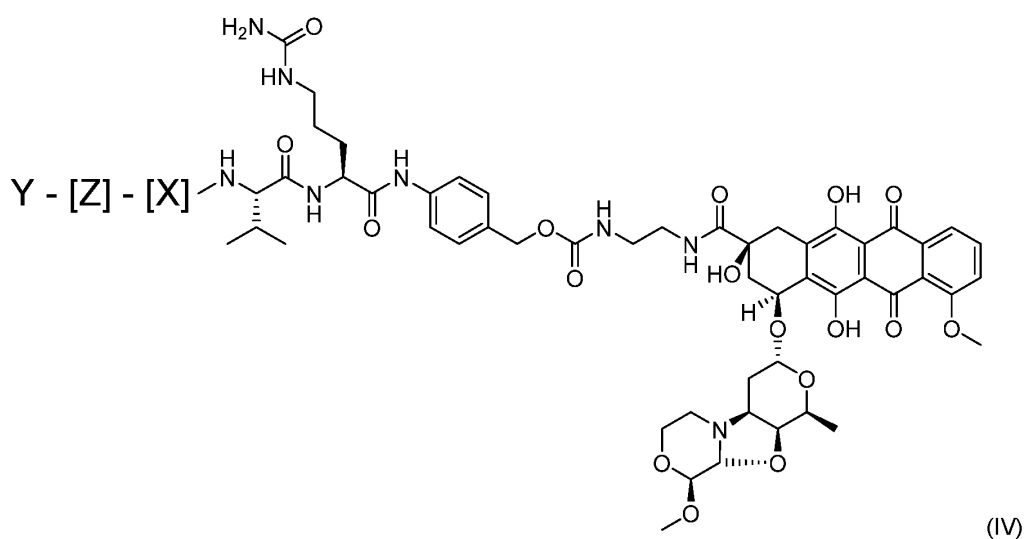




According to an eleventh aspect, the invention provides a target-binding molecule-drug conjugate, comprising

- 5 (a) a ROR1 specific antigen binding molecule according to the first, second or ninth aspect, or a recombinant fusion protein or recombinant fusion protein dimer according to the third, fourth or fifth aspect, and
- (b) an anthracycline (PNU) derivative,

wherein the target-binding molecule-drug conjugate has the structure of formula (IV):



(IV)

wherein [X] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

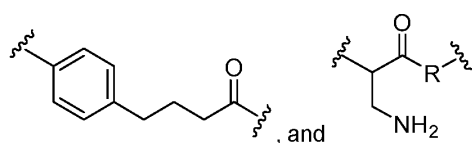
- 15 [Z] is a linker derived from a reactive group used to conjugate the anthracycline (PNU) derivative and the target-binding molecule; and

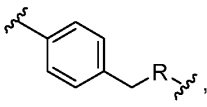
Y comprises a ROR1 specific antigen binding molecule according to the first, second or ninth aspect, or a recombinant fusion protein or recombinant fusion protein dimer according to the third, fourth or fifth aspect.

[Z] is typically a moiety derived from a reactive group used to conjugate the anthracycline (PNU) derivative and the target-binding molecule. [Z] may be a moiety derived from a reactive group selected from the group consisting of a maleimide, an alkyl halide, a sulphhydryl group, an activated disulphide, an amino group, an alkyne group, an azido group, an aminoxy group, an aldehyde group and a ketone group.

[Z] may therefore be selected from the group consisting of a disulphide bond, an amide bond, an oxime bond, a hydrazone bond, a thioether bond, a 1, 2, 3 triazole and polyGly.

10 Preferably, [X] is selected from the group comprising polyethylene glycol,



and , wherein  represents the point of attachment to the rest of the molecule and wherein [R] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof.

Most preferably, [X] is polyethylene glycol. The polyethylene glycol may be PEG4.

20 Preferably, the target-binding molecule is a protein or a nucleic acid. Examples of target-binding proteins (which may also be referred to as specific antigen binding proteins) include but are not limited to an immunoglobulin or antibody, an immunoglobulin Fc region, a fragment of an immunoglobulin Fc region, an Fc heavy chain, a CH2 region, a CH3 region, an immunoglobulin Fab region, a Fab', a Fv, a Fv-Fc, a single chain Fv (scFv), a scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager, an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, a scaffold protein (affibodies, centyrins, darpins etc.). Examples of target-binding nucleic acids include but are not limited to aptamers.

30 Preferably, the target-binding molecule-drug conjugate is a protein and the anthracycline (PNU) derivative is conjugated to a thiol-containing amino acid residue in the amino acid sequence of a protein or to a thiol group introduced by chemical modification of the protein, for example incorporated at the N-terminus or C-terminus of the amino acid sequence of the specific antigen binding protein. Thiol groups may also be introduced into other target-binding molecules, such as nucleic acids.

35 In one embodiment of the tenth or eleventh aspect, the target-binding molecule-drug conjugate, Y comprises a ROR1 specific antigen binding molecule according to the first or second aspects of the invention, conjugated to the PNU derivative via a human immunoglobulin Fc region or fragment thereof.

In one embodiment the fragment of the human immunoglobulin Fc region may be selected from the group consisting of an Fc heavy chain, a CH2 region and a CH3 region.

- 5 Also provided herein is the target-binding molecule-drug conjugate according to the above aspects, for use in therapy.

Also provided herein is the target-binding molecule-drug conjugate according to the above aspects, for use in the treatment of cancer.

10

Also provided herein is the use of a target-binding molecule-drug conjugate according to the above aspects in the manufacture of a medicament for the treatment of a disease in a patient in need thereof.

- 15 Also provided herein is a method of treatment of a disease in a patient in need of treatment comprising administration to said patient of a therapeutically effective dosage of a target-binding molecule-drug conjugate according to the above aspects. The disease may be cancer.

- Preferably, the cancer is a ROR1-positive cancer type. More preferably, the cancer is selected from the group comprising blood cancers such as lymphomas and leukemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone lymphoma (MZL), non-Hodgkin lymphomas (NHL), acute myeloid leukemia (AML) and solid tumours including neuroblastoma, renal cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, uterine cancer, prostate cancer, thyroid cancer, Head and Neck cancer, bladder cancer, oesophageal cancer, stomach cancer or liver cancer. The cancer may be mesothelioma or triple negative breast cancer (TNBC). The mesothelioma may be pleural mesothelioma.
- 20
25

- Also provided herein is a pharmaceutical composition comprising a target-binding molecule-drug conjugate according to any of the above aspects, and at least one other pharmaceutically acceptable ingredient.

30

Definitions

An antigen specific binding molecule of the invention comprises amino acid sequence derived from a synthetic library of VNAR molecules, or from libraries derived from the immunization of a cartilaginous fish. The terms VNAR, IgNAR and NAR may be used interchangeably also.

35

Amino acids are represented herein as either a single letter code or as the three letter code or both.

- The term "affinity purification" means the purification of a molecule based on a specific attraction or binding of the molecule to a chemical or binding partner to form a combination or complex which allows the molecule to be separated from impurities while remaining bound or attracted to the partner moiety.
- 40

The term "Complementarity Determining Regions" or CDRs (i.e., CDR1 and CDR3) refers to the amino acid residues of a VNAR domain the presence of which are typically involved in antigen binding. Each VNAR typically has two CDR regions identified as CDR1 and CDR3. Additionally, each VNAR domain comprises amino acids from a "hypervariable loop" (HV), which may also be involved in antigen binding. In some instances, a complementarity determining region can include amino acids from both a CDR region and a hypervariable loop. In other instances, antigen binding may only involve residues from a single CDR or HV. According to the generally accepted nomenclature for VNAR molecules, a CDR2 region is not present.

10

"Framework regions" (FW) are those VNAR residues other than the CDR residues. Each VNAR typically has five framework regions identified as FW1, FW2, FW3a, FW3b and FW4.

15

The boundaries between FW, CDR and HV regions in VNARs are not intended to be fixed and accordingly some variation in the lengths and compositions of these regions is to be expected. This will be understood by those skilled in the art, particularly with reference to work that have been carried out in analyzing these regions. (Anderson et al., PLoS ONE (2016) 11 (8); Lui et al., Mol Immun (2014) 59, 194-199; Zielonka et al., Mar Biotechnol (2015). 17, (4) 386-392; Fennell et al., J Mol Biol (2010) 400, 155-170; Kovalenko et al., J Biol Chem (2013) 288. 17408-17419; Dooley et al., (2006) PNAS 103 (6). 1846-1851). The molecules of the present invention, although defined by reference to FW, CDR and HV regions herein, are not limited to these strict definitions. Variation in line with the understanding in the art as the structure of the VNAR domain is therefore expressly contemplated herein.

20

A "codon set" refers to a set of different nucleotide triplet sequences used to encode desired variant amino acids. A set of oligonucleotides can be synthesized, for example, by solid phase synthesis, including sequences that represent all possible combinations of nucleotide triplets provided by the codon set and that will encode the desired group of amino acids. A standard form of codon designation is that of the IUB code, which is known in the art and described herein.

25

A codon set is typically represented by 3 capital letters in italics, e.g. *NNK*, *NNS*, *XYZ*, *DVK* etc. A "non-random codon set" therefore refers to a codon set that encodes select amino acids that fulfill partially, preferably completely, the criteria for amino acid selection as described herein. Synthesis of oligonucleotides with selected nucleotide "degeneracy" at certain positions is well known in that art, for example the TRIM approach (Knappek *et al.*; J. Mol. Biol. (1999), **296**, 57-86); Garrard & Henner, Gene (1993), **128**, 103). Such sets of oligonucleotides having certain codon sets can be synthesized using commercial nucleic acid synthesizers (available from, for example, Applied Biosystems, Foster City, CA), or can be obtained commercially (for example, from Life Technologies, Rockville, MD). A set of oligonucleotides synthesized having a particular codon set will typically include a plurality of oligonucleotides with different sequences, the differences established by the codon set within the overall sequence. Oligonucleotides used according to the present invention have sequences that allow for

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hybridization to a VNAR nucleic acid template and also may where convenient include restriction enzyme sites.

“Cell”, “cell line”, and “cell culture” are used interchangeably (unless the context indicates otherwise) and such designations include all progeny of a cell or cell line. Thus, for example, terms like “transformants” and “transformed cells” include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

“Control sequences” when referring to expression means DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, etc. Eukaryotic cells use control sequences such as promoters, polyadenylation signals, and enhancers.

The term “coat protein” means a protein, at least a portion of which is present on the surface of the virus particle. From a functional perspective, a coat protein is any protein which associates with a virus particle during the viral assembly process in a host cell, and remains associated with the assembled virus until it infects another host cell.

The “detection limit” for a chemical entity in a particular assay is the minimum concentration of that entity which can be detected above the background level for that assay. For example, in the phage ELISA, the “detection limit” for a particular phage displaying a particular antigen binding fragment is the phage concentration at which the particular phage produces an ELISA signal above that produced by a control phage not displaying the antigen binding fragment.

A “fusion protein” and a “fusion polypeptide” refer to a polypeptide having two portions covalently linked together, where each of the portions is a polypeptide having a different property. The property may be a biological property, such as activity *in vitro* or *in vivo*. The property may also be a simple chemical or physical property, such as binding to a target antigen, catalysis of a reaction, etc. The two portions may be linked directly by a single peptide bond or through a peptide linker containing one or more amino acid residues. Generally, the two portions and the linker will be in reading frame with each other. Preferably, the two portions of the polypeptide are obtained from heterologous or different polypeptides.

The term “fusion protein” in this text means, in general terms, one or more proteins joined together by chemical means, including hydrogen bonds or salt bridges, or by peptide bonds through protein synthesis or both. Typically fusion proteins will be prepared by DNA recombination techniques and may be referred to herein as recombinant fusion proteins.

“Heterologous DNA” is any DNA that is introduced into a host cell. The DNA may be derived from a variety of sources including genomic DNA, cDNA, synthetic DNA and fusions or combinations of these. The DNA may include DNA from the same cell or cell type as the host or recipient cell or DNA from a different cell type, for example, from an allogenic or xenogenic source. The DNA may, optionally, include
5 marker or selection genes, for example, antibiotic resistance genes, temperature resistance genes, etc.

A “highly diverse position” refers to a position of an amino acid located in the variable regions of the light and heavy chains that have a number of different amino acid represented at the position when the amino acid sequences of known and/or naturally occurring antibodies or antigen binding fragments are
10 compared. The highly diverse positions are typically in the CDR or HV regions.

“Identity” describes the relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. Identity also means the degree of sequence relatedness (homology) between polypeptide or polynucleotide sequences, as the case
15 may be, as determined by the match between strings of such sequences. While there exist a number of methods to measure identity between two polypeptide or two polynucleotide sequences, methods commonly employed to determine identity are codified in computer programs. Preferred computer programs to determine identity between two sequences include, but are not limited to, GCG program package (Devereux, *et al.*, Nucleic acids Research, **12**, 387 (1984), BLASTP, BLASTN, and FASTA
20 (Atschul *et al.*, J. Molec. Biol. (1990) **215**, 403).

Preferably, the amino acid sequence of the protein has at least 45% identity, using the default parameters of the BLAST computer program (Atschul *et al.*, J. Mol. Biol. (1990) **215**, 403-410) provided by HGMP (Human Genome Mapping Project), at the amino acid level, to the amino acid sequences
25 disclosed herein.

More preferably, the protein sequence may have at least 45%, 46%, 47%, 48%, 49%, 50%, 55%, 60%, 65%, 66%, 67%, 68%, 69%, 70%, 75%, 80%, 85%, 90% and still more preferably 95% (still more preferably at least 96%, 97%, 98% or 99%) identity, at the nucleic acid or amino acid level, to the amino
30 acid sequences as shown herein.

The protein may also comprise a sequence which has at least 45%, 46%, 47%, 48%, 49%, 50%, 50%, 55%, 60%, 65%, 66%, 67%, 68%, 69%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity with a sequence disclosed herein, using the default parameters of the BLAST computer program
35 provided by HGMP, thereto

A “library” refers to a plurality of VNARs or VNAR fragment sequences (for example, polypeptides of the invention), or the nucleic acids that encode these sequences, the sequences being different in the combination of variant amino acids that are introduced into these sequences according to the methods
40 of the invention.

“Ligation” is the process of forming phosphodiester bonds between two nucleic acid fragments. For ligation of the two fragments, the ends of the fragments must be compatible with each other. In some cases, the ends will be directly compatible after endonuclease digestion. However, it may be necessary
5 first to convert the staggered ends commonly produced after endonuclease digestion to blunt ends to make them compatible for ligation. For blunting the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenol- chloroform extraction and ethanol precipitation or by silica purification. The DNA fragments that
10 are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 µg of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase or calf intestinal phosphatase to prevent self-ligation during the ligation step.

15 A “mutation” is a deletion, insertion, or substitution of a nucleotide(s) relative to a reference nucleotide sequence, such as a wild type sequence.

“Natural” or “naturally occurring” VNARs, refers to VNARs identified from a non-synthetic source, for
20 example, from a tissue source obtained ex vivo, or from the serum of an animal of the *Elasmobranchii* subclass. These VNARs can include VNARs generated in any type of immune response, either natural or otherwise induced. Natural VNARs include the amino acid sequences, and the nucleotide sequences that constitute or encode these antibodies. As used herein, natural VNARs are different than “synthetic VNARs”, synthetic VNARs referring to VNAR sequences that have been changed from a source or
25 template sequence, for example, by the replacement, deletion, or addition, of an amino acid, or more than one amino acid, at a certain position with a different amino acid, the different amino acid providing an antibody sequence different from the source antibody sequence.

The term “nucleic acid construct” generally refers to any length of nucleic acid which may be DNA, cDNA
30 or RNA such as mRNA obtained by cloning or produced by chemical synthesis. The DNA may be single or double stranded. Single stranded DNA may be the coding sense strand, or it may be the non-coding or anti-sense strand. For therapeutic use, the nucleic acid construct is preferably in a form capable of being expressed in the subject to be treated.

35 “Operably linked” when referring to nucleic acids means that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding
40 sequence if it is positioned so as to facilitate translation. Generally, “operably linked” means that the

DNA sequences being linked are contiguous and, in the case of a secretory leader, contingent and in reading frame. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adapters or linkers are used in accord with conventional practice.

5

The term "protein" means, in general terms, a plurality of amino acid residues joined together by peptide bonds. It is used interchangeably and means the same as peptide, oligopeptide, oligomer or polypeptide, and includes glycoproteins and derivatives thereof. The term "protein" is also intended to include fragments, analogues, variants and derivatives of a protein wherein the fragment, analogue, variant or derivative retains essentially the same biological activity or function as a reference protein. Examples of protein analogues and derivatives include peptide nucleic acids, and DARPin (Designed Ankyrin Repeat Proteins).

10

A fragment, analogue, variant or derivative of the protein may be at least 25 preferably 30 or 40, or up to 50 or 100, or 60 to 120 amino acids long, depending on the length of the original protein sequence from which it is derived. A length of 90 to 120, 100 to 110 amino acids may be convenient in some instances.

15

The fragment, derivative, variant or analogue of the protein may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably, a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or auxiliary sequence which is employed for purification of the polypeptide. Such fragments, derivatives, variants and analogues are deemed to be within the scope of those skilled in the art from the teachings herein.

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"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid-phase techniques). Further methods include the polymerase chain reaction (PCR) used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available. Alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue. The oligonucleotides can be purified on polyacrylamide gels or molecular sizing columns or by precipitation. DNA is "purified" when the DNA is separated from non-nucleic acid impurities (which may be polar, non-polar, ionic, etc.).

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A "source" or "template" VNAR, as used herein, refers to a VNAR or VNAR antigen binding fragment whose antigen binding sequence serves as the template sequence upon which diversification according

to the criteria described herein is performed. An antigen binding sequence generally includes within a VNAR preferably at least one CDR, preferably including framework regions.

5 A "transcription regulatory element" will contain one or more of the following components: an enhancer element, a promoter, an operator sequence, a repressor gene, and a transcription termination sequence.

"Transformation" means a process whereby a cell takes up DNA and becomes a "transformant". The DNA uptake may be permanent or transient. A "transformant" is a cell which has taken up and maintained DNA as evidenced by the expression of a phenotype associated with the DNA (e.g.,
10 antibiotic resistance conferred by a protein encoded by the DNA).

A "variant" or "mutant" of a starting or reference polypeptide (for example, a source VNAR or a CDR thereof), such as a fusion protein (polypeptide) or a heterologous polypeptide (heterologous to a phage), is a polypeptide that (1) has an amino acid sequence different from that of the starting or reference
15 polypeptide and (2) was derived from the starting or reference polypeptide through either natural or artificial mutagenesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. For example, a fusion polypeptide of the invention generated using an oligonucleotide comprising a nonrandom codon set that encodes a sequence with a variant amino acid (with respect to the amino acid found at the
20 corresponding position in a source VNAR or antigen binding fragment) would be a variant polypeptide with respect to a source VNAR or antigen binding fragment. Thus, a variant CDR refers to a CDR comprising a variant sequence with respect to a starting or reference polypeptide sequence (such as that of a source VNAR or antigen binding fragment). A variant amino acid, in this context, refers to an amino acid different from the amino acid at the corresponding position in a starting or reference
25 polypeptide sequence (such as that of a source VNAR or antigen binding fragment). Any combination of deletion, insertion, and substitution may be made to arrive at the final variant or mutant construct, provided that the final construct possesses the desired functional characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites.

30 A "wild-type" or "reference" sequence or the sequence of a "wild-type" or "reference" protein/polypeptide, such as a coat protein, or a CDR of a source VNAR, may be the reference sequence from which variant polypeptides are derived through the introduction of mutations. In general, the "wild-type" sequence for a given protein is the sequence that is most common in nature. Similarly, a "wild-type" gene sequence
35 is the sequence for that gene which is most commonly found in nature. Mutations may be introduced into a "wild-type" gene (and thus the protein it encodes) either through natural processes or through man induced means. The products of such processes are "variant" or "mutant" forms of the original "wild-type" protein or gene.

A "humanised" antigen specific antigen binding molecule may be modified at one or more amino acid sequence position to reduce the potential for immunogenicity *in vivo*, while retaining functional binding activity for the specific epitopes on the specific antigen.

- 5 Humanization of antibody variable domains is a technique well-known in the art to modify an antibody which has been raised, in a species other than humans, against a therapeutically useful target so that the humanized form may avoid unwanted immunological reaction when administered to a human subject. The methods involved in humanization are summarized in Almagro J.C and William Strohl W. *Antibody Engineering: Humanization, Affinity Maturation, and Selection Techniques in Therapeutic*
 10 *Monoclonal Antibodies: From Bench to Clinic*. Edited by An J. 2009 John Wiley & Sons, Inc and in Strohl W.R. and Strohl L.M., *Therapeutic Antibody Engineering*, Woodhead Publishing 2012.

- Although IgNARs have distinct origins compared to immunoglobulins and have very little sequence homology compared to immunoglobulin variable domains there are some structural similarities between
 15 immunoglobulin and IgNAR variable domains, so that similar processes can be applied to the VNAR domain. For example, WO2013/167883, incorporated by reference, provides a description of the humanization of VNARs, see also Kovalenko O.V., *et al. J Biol Chem.* 2013. 288(24): p. 17408-19.

- A humanised antigen specific binding molecule may differ from a wild-type antigen specific binding molecule by substituting one or more framework amino acid residues with a corresponding framework amino acid residue of DPK-9. DPK-9 is a human germline VL scaffold, a member of the variable kappa subgroup 1 (Vk1). DPK-9 has a sequence according to:

- DIQMTQSPSSLSASVGDRVITICRASQSISSYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSG
 25 TDFTLTISLQPEDFATYYCQQSYSTPNTFGQGKVEIK
 (SEQ ID NO:132)

- The term "chimeric antigen receptors (CARs)," as used herein, may refer to artificial T-cell receptors, chimeric T-cell receptors, or chimeric immunoreceptors, for example, and encompass engineered
 30 receptors that graft an artificial specificity onto a particular immune effector cell. CARs may be employed to impart the specificity of an antigen-specific binding protein, such as a monoclonal antibody or VNAR, onto a T cell, thereby allowing a large number of specific T cells to be generated, for example, for use in adoptive cell therapy. CARs may direct the specificity of the cell to a tumour associated antigen, for example. CARs may comprise an intracellular activation domain, a transmembrane domain, and an
 35 extracellular domain comprising a tumour associated antigen binding region. In particular aspects, CARs comprise fusions of single-chain variable fragments (scFv) derived from monoclonal antibodies fused to CD3-zeta transmembrane and endodomains. In other particular aspects, CARs comprise fusions of the VNAR domains described herein with CD3-zeta transmembrane and endodomains. The specificity of other CAR designs may be derived from ligands of receptors (e.g., peptides) or from pattern-recognition
 40 receptors, such as Dectins. In particular embodiments, one can target malignant B cells by redirecting

the specificity of T cells by using a CAR specific for the B-lineage molecule, CD 19. In certain cases, the spacing of the antigen-recognition domain can be modified to reduce activation-induced cell death. In certain cases, CARs comprise domains for additional co-stimulatory signalling, such as CD3-zeta, FcR, CD27, CD28, CD 137, DAP 10, and/or OX40. In some cases, molecules can be co- expressed with the
5 CAR, including co-stimulatory molecules, reporter genes for imaging (e.g., for positron emission tomography), gene products that conditionally ablate the T cells upon addition of a pro-drug, homing receptors, chemokines, chemokine receptors, cytokines, and cytokine receptors.

The term "conjugation" as used herein may refer to any method of chemically linking two or more
10 chemical moieties. Typically, conjugation will be via covalent bond. In the context of the present invention, at least one of the chemical moieties will be a polypeptide and in some cases the conjugation will involve two or more polypeptides, one or more of which may be generated by recombinant DNA technology. A number of systems for conjugating polypeptides are known in the art. For example, conjugation can be achieved through a lysine residue present in the polypeptide molecule using N-
15 hydroxy-succinimide or through a cysteine residue present in the polypeptide molecule using maleimidobenzoyl sulfosuccinimide ester. In some embodiments, conjugation occurs through a short-acting, degradable linkage including, but not limited to, physiologically cleavable linkages including ester, carbonate ester, carbamate, sulfate, phosphate, acyloxyalkyl ether, acetal, and ketal, hydrazone, oxime and disulphide linkages. In some embodiments linkers that are cleavable by intracellular or
20 extracellular enzymes, such as cathepsin family members, cleavable under reducing conditions or acidic pH are incorporated to enable releases of conjugated moieties from the polypeptide or protein to which it is conjugated.

A particularly preferred method of conjugation is the use of intein-based technology (US2006247417)
25 Briefly, the protein of interest is expressed as an N terminal fusion of an engineered intein domain (Muir 2006 Nature 442, 517–518). Subsequent N to S acyl shift at the protein-intein union results in a thioester linked intermediate that can be chemically cleaved with bis-aminoxy agents or amino-thiols to give the desired protein C-terminal aminoxy or thiol derivative, respectively. These C-terminal aminoxy and thiol derivatives can be reacted with aldehyde / ketone and maleimide functionalised moieties, respectively,
30 in a chemoselective fashion to give the site-specific C-terminally modified protein.

In another preferred method of conjugation the VNARs are directly expressed with an additional cysteine at or near the C-terminal region of the VNAR or incorporated within a short C-terminal tag sequence enabling conjugation with thiol reactive payloads such as maleimide functionalised moieties.

35 Conjugation as referred to herein is also intended to encompass the use of a linker moiety, which may impart a number of useful properties. Linker moieties include, but are not limited to, peptide sequences such as poly-glycine, gly-ser, val-cit or val-ala. In certain cases, the linker moiety may be selected such that it is cleavable under certain conditions, for example via the use of enzymes, nucleophilic/basic
40 reagents, reducing agents, photo-irradiation, electrophilic/acidic reagents, organometallic and metal

reagents, or oxidizing reagents, or the linker may be specifically selected to resist cleavage under such conditions.

Polypeptides may be conjugated to a variety of functional moieties in order to achieve a number of goals.

- 5 Examples of functional moieties include, but are not limited to, polymers such as polyethylene glycol in order to reduce immunogenicity and antigenicity or to improve solubility. Further non-limiting examples include the conjugation of a polypeptide to a therapeutic agent or a cytotoxic agent.

- 10 The term "detectable label" is used herein to specify that an entity can be visualized or otherwise detected by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, chemical or other means. The detectable label may be selected such that it generates a signal which can be measured and whose intensity is proportional to the amount of bound entity. A wide variety of systems for labelling and/or detecting proteins and peptides are known in the art. A label may be directly detectable (i.e., it does not require any further reaction or manipulation to be detectable, e.g., a
- 15 fluorophore is directly detectable) or it may be indirectly detectable (i.e., it is made detectable through reaction or binding with another entity that is detectable, e.g., a hapten is detectable by immunostaining after reaction with an appropriate antibody comprising a reporter such as a fluorophore). Suitable detectable agents include, but are not limited to, radionuclides, fluorophores, chemiluminescent agents, microparticles, enzymes, colorimetric labels, magnetic labels, haptens, molecular beacons, and aptamer
- 20 beacons.

- Methods of killing or inhibiting the growth of a cells expressing ROR1 *in vitro* or in a patient are contemplated herein, in general, the term "killing" as used herein in the context of cells means causing a cell death. This may be achieved by a number of mechanisms, such as necrosis or other cells injury,
- 25 or the induction of apoptosis. The phrases "inhibiting the growth" or "inhibiting proliferation" when used herein are intended to encompass the prevention of cell development, more specifically the prevention of cell division.

- As used herein, an alkyl group is a straight chain or branched, substituted or unsubstituted group
- 30 (preferably unsubstituted) containing from 1 to 40 carbon atoms. An alkyl group may optionally be substituted at any position. The term "alkenyl," as used herein, denotes a group derived from the removal of a single hydrogen atom from a straight- or branched-chain aliphatic moiety having at least one carbon-carbon double bond. The term "alkynyl," as used herein, refers to a group derived from the removal of a single hydrogen atom from a straight- or branched-chain aliphatic moiety having at least one carbon-
- 35 carbon triple bond.

- The term 'alkyl', 'aryl', 'heteroaryl' etc also include multivalent species, for example alkylene, arylene, 'heteroarylene' etc. Examples of alkylene groups include ethylene (-CH₂-CH₂-), and propylene (-CH₂-CH₂-CH₂-). An exemplary arylene group is phenylene (-C₆H₄-), and an exemplary heteroarylene group
- 40 is pyridinylene (-C₅H₃N-).

Aromatic rings are cyclic aromatic groups that may have 0, 1, 2 or more, preferably 0, 1 or 2 ring heteroatoms. Aromatic rings may be optionally substituted and/or may be fused to one or more aromatic or non-aromatic rings (preferably aromatic), which may contain 0, 1, 2, or more ring heteroatoms, to form a polycyclic ring system.

Aromatic rings include both aryl and heteroaryl groups. Aryl and heteroaryl groups may be mononuclear, i.e. having only one aromatic ring (like for example phenyl or phenylene), or polynuclear, i.e. having two or more aromatic rings which may be fused (like for example naphthyl or naphthylene), individually covalently linked (like for example biphenyl), and/or a combination of both fused and individually linked aromatic rings. Preferably the aryl or heteroaryl group is an aromatic group which is substantially conjugated over substantially the whole group. Aryl groups may contain from 5 to 40 ring carbon atoms, from 5 to 25 carbon atoms, from 5 to 20 carbon atoms, or from 5 to 12 carbon atoms. Heteroaryl groups may be from 5 to 40 membered, from 5 to 25 membered, from 5 to 20 membered or from 5 to 12 membered rings, containing 1 or more ring heteroatoms selected from N, O, S and P. An aryl or heteroaryl may be fused to one or more aromatic or non-aromatic rings (preferably an aromatic ring) to form a polycyclic ring system.

Aryl and heteroaryl preferably denote a mono-, bi- or tricyclic aromatic or heteroaromatic group with up to 25 ring atoms that may also comprise condensed rings and is optionally substituted.

Preferred aryl groups include, without limitation, benzene, biphenylene, triphenylene, [1,1':3',1'']terphenyl-2'-ylene, naphthalene, anthracene, binaphthylene, phenanthrene, pyrene, dihydropyrene, chrysene, perylene, tetracene, pentacene, benzopyrene, fluorene, indene, indenofluorene, spirobifluorene, etc.

Preferred heteroaryl groups include, without limitation, 5-membered rings like pyrrole, pyrazole, silole, imidazole, 1,2,3-triazole, 1,2,4-triazole, tetrazole, furan, thiophene, selenophene, oxazole, isoxazole, 1,2-thiazole, 1,3-thiazole, 1,2,3-oxadiazole, 1,2,4-oxadiazole, 1,2,5-oxadiazole, 1,3,4-oxadiazole, 1,2,3-thiadiazole, 1,2,4-thiadiazole, 1,2,5-thiadiazole, 1,3,4-thiadiazole, 6-membered rings like pyridine, pyridazine, pyrimidine, pyrazine, 1,3,5-triazine, 1,2,4-triazine, 1,2,3-triazine, 1,2,4,5-tetrazine, 1,2,3,4-tetrazine, 1,2,3,5-tetrazine, and fused systems like carbazole, indole, isoindole, indolizine, indazole, benzimidazole, benzotriazole, purine, naphthimidazole, phenanthrimidazole, pyridimidazole, pyrazinimidazole, quinoxalinimidazole, benzoxazole, naphthoxazole, anthroxazole, phenanthroxazole, isoxazole, benzothiazole, benzofuran, isobenzofuran, dibenzofuran, quinoline, isoquinoline, pteridine, benzo-5,6-quinoline, benzo-6,7-quinoline, benzo-7,8-quinoline, benzoisoquinoline, acridine, phenothiazine, phenoxazine, benzopyridazine, benzopyrimidine, quinoxaline, phenazine, naphthyridine, azacarbazole, benzocarboline, phenanthridine, phenanthroline, thieno[2,3b]thiophene, thieno[3,2b]thiophene, dithienothiophene, dithienopyridine, isobenzothiophene, dibenzothiophene, benzothiadiazothiophene, 2,5-dihydropyrrolo[3,4-c]pyrrol-1,4-dione (diketopyrrolopyrrole, DPP), 2-oxo-1H-indol-3-ylidene, [3,3'-bipyrrolo[2,3-b]pyridinylidene]-2,2'-(1H,1'H)-dione (pyridine isoindigo) and (3E)-

3-(2-oxo-1H-indol-3-ylidene)-1H-indol-2-one (isoindigo), or combinations thereof. The heteroaryl groups may be substituted with alkyl, alkoxy, thioalkyl, fluoro, fluoroalkyl or further aryl or heteroaryl substituents. Preferably a heteroaryl group is thiophene.

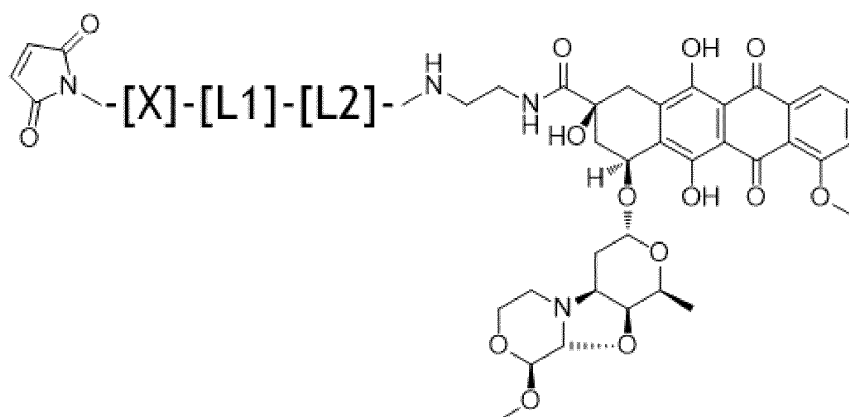
- 5 Particularly preferred heteroatoms are selected from O, S, N, P and Si. Typically, hydrogen will complete the valency of a heteroatom included in the molecules of the invention, e.g. for N there may be -NH- or -NH₂ where one or two other groups are involved.

- 10 As used herein, the term "optionally substituted" means that one or more of the hydrogen atoms in the optionally substituted moiety is replaced by a suitable substituent. Unless otherwise indicated, an "optionally substituted" group may have a suitable substituent at each substitutable position of the group, and when more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at every position. Combinations of substituents envisioned by this invention are preferably those that result in the formation of stable compounds. The term "stable", as used herein, refers to compounds that are chemically feasible and can exist for long enough at room temperature (i.e. 16-25°C) to allow for their detection, isolation and/or use in chemical synthesis.

- 20 Any of the above groups (for example, those referred to herein as "optionally substituted", including alkyl, aryl and heteroaryl groups) may optionally comprise one or more substituents, preferably selected from silyl, sulfo, sulfonyl, formyl, amino, imino, nitrilo, mercapto, cyano, nitro, halogen, -NCO, -NCS, -OCN, -SCN, -C(=O)NR⁰R⁰⁰, -C(=O)X⁰, -C(=O)R⁰, -NR⁰R⁰⁰, C₁₋₁₂alkyl, C₁₋₁₂alkenyl, C₁₋₁₂alkynyl, C₆₋₁₂aryl, C₃₋₁₂cycloalkyl, heterocycloalkyl having 4 to 12 ring atoms, heteroaryl having 5 to 12 ring atoms, C₁₋₁₂ alkoxy, hydroxy, C₁₋₁₂ alkylcarbonyl, C₁₋₁₂ alkoxy-carbonyl, C₁₋₁₂ alkylcarbonyloxy or C₁₋₁₂ alkoxycarbonyloxy wherein one or more H atoms are optionally replaced by F or Cl and/or combinations thereof; wherein X⁰ is halogen and R⁰ and R⁰⁰ are, independently, H or optionally substituted C₁₋₁₂alkyl. The optional substituents may comprise all chemically possible combinations in the same group and/or a plurality of the aforementioned groups (for example amino and sulfonyl if directly attached to each other represent a sulfamoyl radical). In one embodiment, the substituent is not acyl. As used herein acyl refers to an acyl group which is a moiety derived by the removal of one or more hydroxyl groups from an oxoacid, such as a carboxylic acid. It contains a double-bonded oxygen atom and an alkyl group.

- 30 In some embodiments the groups may be unsubstituted. For example, the anthracycline (PNU) derivative may be of formula (V):

35



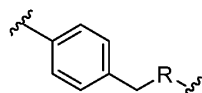
(V)

wherein [X] is an optional spacer selected from the group comprising unsubstituted alkyl groups, unsubstituted heteroalkyl groups, unsubstituted aryl groups, unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

[L1] and [L2] are optional linkers selected from the group consisting of valine (Val), citrulline (Cit), alanine (Ala), asparagine (Asn), a peptide, $-(CH_2)_n-$, $-(CH_2CH_2O)_n-$, p-aminobenzyloxycarbonyl (PAB), Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, Val-Ala, Asn-Ala, any amino acid except glycine, and combinations thereof.

10

In embodiments wherein the groups are unsubstituted, [X] is preferably selected from the group



comprising polyethylene glycol and $-\text{CH}_2\text{CH}_2\text{O}-$, wherein $\sim$ represents the point of attachment to the rest of the molecule and wherein [R] is an optional spacer selected from the group comprising unsubstituted alkyl groups, unsubstituted heteroalkyl groups, unsubstituted aryl groups, unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof.

15

In general, the term PAB is intended to mean p-aminobenzyloxycarbonyl. Occasionally in the literature, the term PAB may be used to indicated p-aminobenzyl. In the present specification, PAB is intended to indicate p-aminobenzyloxycarbonyl.

20

The term "target-binding molecule" refers to any molecule that binds to a given target. In this context, "target" and "antigen" may be used interchangeably. Examples of target-binding molecules include natural or recombinant proteins including immunoglobulins or antibodies, immunoglobulin Fc regions, immunoglobulin Fab regions, Fab, Fab', Fv, Fv-Fc, single chain Fv (scFv), scFv-Fc, (scFv)₂, diabodies, triabodies, tetrabodies, bispecific t-cell engagers, inteins, intein fusions, VNAR domains, single domain antibodies (sdAb), VH domains, scaffold proteins (affibodies, centyrins, darpins etc.) and nucleic acids

25

including aptamers or small molecules or natural products that have been developed to bind to the target or naturally bind to the target.

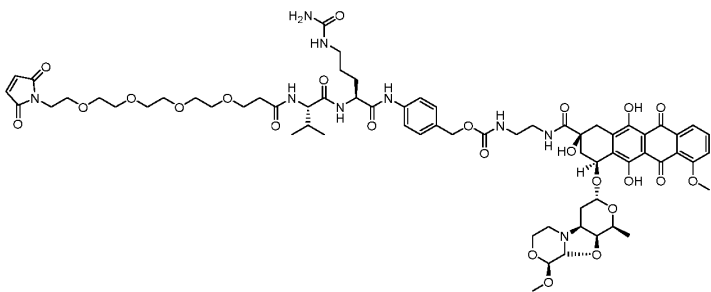
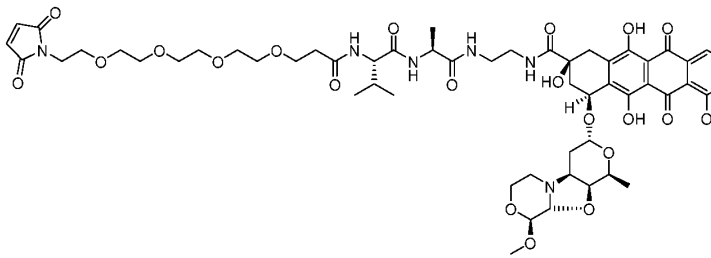
Chemical modification of proteins and biomolecules to introduce thiols is well established. Methods include reaction of amine groups with 2-iminothiolane (Traut's reagent), modification of amine groups with NHS-ester containing heterobifunctional agents such as N-succinimidyl S-acetylthiolate (SATA) or N-succinimidyl-4-(2-pyridyldithio)butanoate (SPDB), followed by treatment with hydroxylamine and reducing agents respectively and cleavage of engineered intein-fusion proteins with cysteamine to generate C-terminal thiol proteins and peptides.

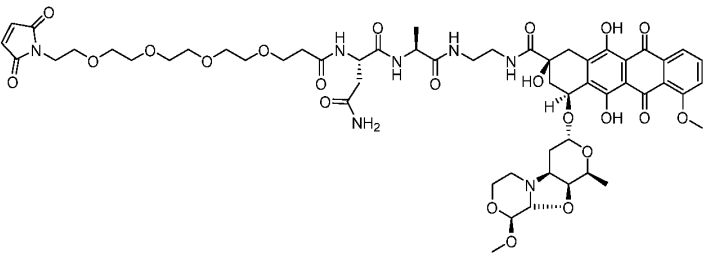
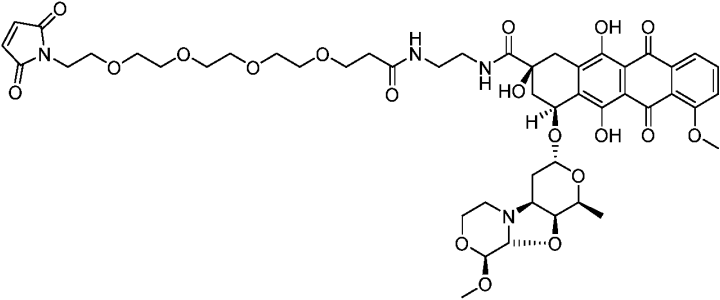
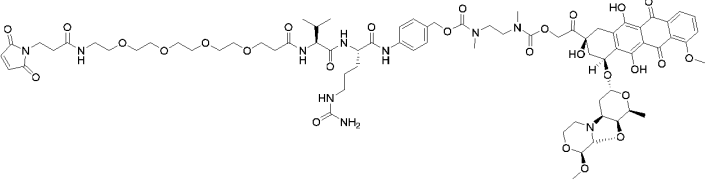
The phrase "selected from the group comprising" may be substituted with the phrase "selected from the group consisting of" and *vice versa*, wherever they occur herein.

The PNU derivatives described herein may be prepared accordingly to standard synthesis methods.

Mass spectrometry may be used to verify that the correct molecules have been produced (Table 4).

Table 4: Characterisation of PNU derivatives by mass spectrometry

Name	Structure	Exp. mass, Da	Obs. mass, Da
MA-PEG4-vc-PAB-EDA-PNU159682		1402.5	1402.1
MA-PEG4-va-EDA-PNU159682		1167.2	1167.6

MA-PEG4- na-EDA- PNU159682		1182.2	1182.4
MA-PEG4- EDA- PNU159682		997.01	996.8
MA-PEG4- vc-PAB- DMAE- PNU159682		1559.6	1558.7

The present invention will be further understood by reference to the following examples.

5 EXAMPLES

EXAMPLE 1 – Generation of the anti-ROR1 VNAR B1 loop library sequences

B1 protein library design.

- 10 To gain a better understanding of the interaction between B1 and ROR1, we solved the crystal structure of B1 in complex with the ROR1 Ig domain (data not shown). This crystal structure informed which positions to change in the protein library that was expressed and screened. We had previously noted that mutation of B1 Tryptophan residues at positions 88 and 94 to Alanine (standard alanine scanning

approach) caused loss of function or expression of the protein. From the crystal structure it was observed that these residues in the CDR3 loop appear to be important for ROR1 binding. A B1 loop library was therefore designed to modify the biophysical properties of the protein through changing selected positions within the CDR1 and CDR3 regions. The set of mutations made at each particular
 5 loop position was informed from the structural analysis of the B1:ROR1 complex, with a view to changing the biophysical properties whilst maintaining structural integrity and high affinity binding.

Library construction

Sequence and loop library design of B1 are shown in Figure 1. Library was synthesised by controlled
 10 mutagenesis of CDR1 and CDR3. Residues 30, 32, 88, 94 and 95 located within CDR loops were randomised.

Libraries construction.

15 B1 loop library DNA was amplified by PCR using specific primers to introduce SfiI restriction sites for cloning into pEDV1 phagemid vector. Library DNA ligated into pEDV1 was transformed into electrocompetent TG1 *E.coli* (Lucigen). The library size was calculated to be 8×10^4 .
 84 single clones were picked and sequenced as a quality control of the library. One sequence has been found to be WT B1 clone. In total 70 unique clones based on CDR1 and CDR3 diversity were identified.
 20 These sequences contain a C-terminal HisMyc tag to enable purification by IMAC chromatography and assessment of ROR1 binding by ELISA and flow cytometry

Screening of the library for ROR1 specific VNAR sequences

As B1 binds to both human and mouse ROR1, recombinant human and mouse ROR1-Fc protein was
 25 used for screening CDR loop library. In total 928 clones were expressed in 96 well format; periplasmic fractions were extracted and binding to ROR1 analysed in ELISA. 23 unique sequences additional to B1 WT sequence which binds to ROR1 were found.

Expression of ROR1 VNAR binders

30 23 clones were expressed in TG1 *E.coli* bacteria and IMAC purified using Ni-NTA Sepharose. Proteins were dialysed to PBS pH 7.4, absorbance Abs₂₈₀ was measured and concentrations calculated. Yields obtained were in a range of 1.5 and 9 mg/L. Purity of proteins was analysed by SDS-PAGE.

Binding to human and mouse ROR1 by ELISA for the different loop variants is summarised in Table 5.
 35

Methods

Library synthesis

CDR loops library was synthesized by GeneArt Gene Synthesis according with provided design.
 Library subcloning into pEDV1

PCR amplification of 11.4 ng (10 µl) of synthesised library was performed in the total reaction volume 1 ml using Phusion High-Fidelity PCR Master Mix and the following primers:

280: 5'-CTACCGTGGCCCAGGCGGCC-3' (SEQ ID NO: 133)

287: 5'-GGTGATGGTGGGCCCCTGAGGCCT-3' (SEQ ID NO: 134)

- 5 Amplicons were purified with Promega PCR purification Kit, digested with SfiI and ligated into pEDV1 vector opened with SfiI restriction enzyme as well. Ligation performed at ratio 1:3 (0.54 µg vector ligated with 1.62 µg library DNA).

Screening of CDR loop library: Periplasmic expression of single clones in 96 well format and binding

10 ELISA

1. Inoculate Greiner 96 deep-well plate containing 1ml 2xTY/0.1% glucose/100µg/µl Amp. Grow for 5h at 37 °C, 180 rpm in incubation chamber until faintly turbid.
2. Induced with 110 µl/well 1 mM IPTG in 2xTY/Amp (final concentration of IPTG =100µM); same shaking speed at 28°C overnight.
- 15 3. Spin cultures for 15 min at 4°C and 3500 rpm. Decant supernatant and tap dry on paper towels.
4. Add 250 µl/well ice-cold TES buffer (50 mM Tris/HCl, pH8.0/1 mM EDTA, pH8.0/20% Sucrose) to the pellets. Vortex.
5. Add 250 µl 1:5 diluted in water TES buffer (ice-cold). Keep on ice (or in the ridge) for 30 min. Spin as above. Keep supernatants on ice until ready to use.

20

ELISA

1. Coat 96 well plates with 1 µg/ml of huROR1-Fc, mouse ROR1-Fc, human ROR2-Fc or HSA and incubated overnight at 4°C.
2. Wash plates 3xPBST.
- 25 3. Block coated plates with 200µl/well 4% MPBS. Incubate for 1h at room temperature.
4. Wash 3xPBST.
5. Incubate plates with 100 µl/well of peri-prep for 1 h at room temperature.
6. Add 100 µl of anti-His-HRP (1:1000 in PBST) and incubate 1h at room temperature.
7. Wash 2xPBST and 2x PBS.
- 30 8. Add 100 µl/well of TMB substrate. Stop reaction with 50 µl/well 1M H₂SO₄.

Large scale expression and purification of ROR1 VNAR binders

1. Inoculate clones from glycerol stock into 20 ml of 2xTY/0.1% glucose/100 µg/µl Amp. Grow overnight at 37°C shaking at 250 rpm in incubation chamber.
- 35 2. Dilute the overnight culture 1:50 in TB + phosphate salt + 1% glucose +100 µg/ml Amp media (10 ml o/n culture into 500ml media; 450 ml TB + 50 ml phosphate salt) and incubate at 37°C with vigorous shaking (250 rpm) all day or as long as possible.
3. Pellet the cells by centrifugation at 4,000 x g for 15 min at 20°C.
4. Re-suspend the cells in the same volume of TB + phosphate salt + 1% glucose +100 µ g/ml
- 40 Amp media and incubate at 30°C overnight with shaking.

5. Pellet the cells by centrifugation at 4,000 x g for 15 min at 20°C and re-suspend the cells in the same volume of TB + phosphate salt +100 µg/ml Amp media (NO GLUCOSE). Add IPTG to a final conc. of 1 mM IPTG. Incubate at 30°C for 4-5 h with shaking.
6. Collect the cells by centrifugation at 4500 x g for 20 min [the pellet could be frozen at this point at -20°C]
7. Re-suspend the pellet in 10% culture volume ice-cold TES buffer (50 ml for 500 ml culture) and shake gently on ice for 15 min.
8. Add an equal volume ice-cold 5 mM MgSO₄ (for 2.5 mM final concentration of MgSO₄) and continue shaking gently on ice for a further 15 min.
9. Pellet the suspension by centrifugation at 8000 x g for 30 min at 4°C. Supernatant contains released periplasmic proteins.
10. Add 10xPBS (pH 7.4) [final conc. of 1xPBS] to peri-prep extract prior to IMAC purification.

Immobilised Metal Affinity Chromatography (IMAC) purification

1. Add 2-3 ml Nickel-resin (His Pur Ni-NTA Resin, Thermo Fisher#88222) to 100 ml osmotic shock solution (periplasmic extract) and incubated on roller for 1 hour at room temperature.
2. Allow periplasmic extract to pass through the column (Poly prep chromatography columns 10 ml, Bio-Rad# 7321010)
3. Wash the resin with 50 - 100ml PBS.
4. Eluted protein with 5 x 1 ml 500 mM imidazole (pH 8).
5. Dialyze eluates in 3x5 liters PBS with agitation in dialysis cassette (Slide A Lyzer Dialysis cassette 7.000 MWCO, Thermo Fisher#66707)
6. Analyse proteins by SDS-PAGE.

- Concentrations of purified proteins were determined from absorbance at 280 nm using the theoretical extinction coefficient predicted from the amino acid sequence. All proteins were characterised by reducing and non-reducing SDS PAGE analysis and mass spectrometry. The formation of the desired disulphide bond was confirmed by mass spectrometry methods.

Size exclusion chromatography

- Loop engineered variants were assessed by size exclusion chromatography. The monomericity and biophysical properties of B1 loop variants were assessed by size-exclusion chromatography (SEC) using an analytical SEC column (Superdex 75 10/300 GL). Chromatography was carried out in PBS pH 7.4. The elution volume on SEC can be a measure of the relative hydrophobicity of the different proteins. With increased elution volume, as a result of interactions with the column matrix, an indication of increasing hydrophobicity.
- The SEC elution volumes run under identical conditions are shown in Table 5.

Binding to human and mouse ROR1 by BLI

Binding kinetics were determined using the Biolayer Interferometry (BLI) Octet K2 system (ForteBio). Human or mouse ROR1-hFc fusion proteins (extracellular domains) were immobilised in sodium acetate pH5 buffer to COOH₂ chips or AR2G sensors using amine coupling. VNARs were tested at various concentrations and the K_a ($M^{-1}s^{-1}$), K_d (s^{-1}) and K_D (nM) values were determined using Octet Data Analysis High Throughput software (ForteBio) for Biolayer Interferometry.

Table 5. summarise the BLI data for the affinity of these molecules for human and mouse ROR1.

Table 5: Characterisation of B1 loop library variants

Name	SEC RT (ml)	hROR1				mROR1				ELISA	
		K_D (nM)	K_M ($M^{-1} s^{-1}$)	K_J (s^{-1})	K_D (nM)	K_M ($M^{-1} s^{-1}$)	K_J (s^{-1})	K_D (nM)	K_M ($M^{-1} s^{-1}$)	hROR1	mROR1
B1	27.79	2.1	120000	0.000252							
1E2	17.74	8.11	162600	0.001319						**	-
1E5	29.1	1.02	40200	0.0000411						***	***
1B11	18.76	12.3	84500	0.00104	4.74E-09	2.35E+05	1.11E-03			***	*
C3CP	18.65	4.56	165000	0.000753	3.21E-08	1.70E+05	5.44E-03			***	*
2G5	18.16	9.49	452000	0.00429						**	-
1G12	17.72	39.9	225000	0.00897						**	-
G5CP	18.58	8.56	122000	0.00104	1.00E-07	1.00E+04	1.00E-03			**	-
2F4	18	2.58	374000	0.000966						*	-
1G9	18.52	13.4	112000	0.00151	1.78E-08	2.23E+05	3.96E-03			***	**
1H8	18.47	12.6	41300	0.000519	4.58E-09	1.82E+05	8.35E-04			***	***/*
G11CP	25.3	0.592	87900	0.0000521						**	*
D9CP	21.99	13.4	57200	0.000766						***	*
1B6	25.24	0.262	506000	0.000133						***	***
1F10	25.65	0.602	147300	0.0000886						***	***
E6CP	23.45	2.05	66500	0.000136						**	-
F2CP	20.6	9.43	48500	0.000458						***	**
B6CP	20.76	2.1	79900	0.000168	5.13E-09	1.10E+05	5.63E-04			***	**
1G1	26.67	0.662	127400	0.0000844						***	***
A10CP	18.07	11.6	157000	0.00182						*	-
G3CP	20.58	0.796	369000	0.000294	2.80E-09	2.23E+05	6.23E-04			***	***

Binding of loop variant VNARs to cell-surface ROR1 by flow cytometry

Loop variant VNARs were re expressed using intein technology. For expression as intein fusions, DNA encoding VNARs was optimised for *E. coli* expression (GeneArt, Thermo) and cloned in frame into an intein expression vector. This results in a gene encoding the VNAR protein of interest fused to an engineered intein domain which in turn is fused to a chitin binding domain (CBD) to enable purification on a chitin column.

Transformed *E.coli* cells were grown in 1L shaker flasks until $OD_{600nm} = \sim 0.6$, cold shocked 4 °C for 2 hours then protein expression induced with 0.5mM IPTG at 18 °C overnight. Cells were lysed by sonication in lysis buffer (50mM sodium phosphate pH7.4, 0.5M NaCl, 15% glycerol, 0.5mM EDTA, 0.1% Sarkosyl, 1mM AEBSF) and centrifuged to remove cell debris. VNAR intein fusion protein was purified from clarified cell lysate by immobilising on chitin beads (NEB, S6651). Beads were washed extensively with lysis buffer followed by cleavage buffer (50mM sodium phosphate pH6.9, 200mM NaCl) and VNARs released from the beads by overnight chemical cleavage in 400mM dioxamine, or O,O'-1,3-propanediylbis(hydroxylamine), or 100mM cysteine or cysteamine to generate the corresponding C-terminal aminoxy, C-terminal cysteine or C-terminal thiol derivative of the VNARs.

Cleaved VNAR supernatant was then further purified by SEC (Superdex75 26/60 GE healthcare) and / or IMAC (HisTrap HP, GE Healthcare). Concentrations were determined from absorbance at 280 nm using the theoretical extinction coefficient predicted from the amino acid sequence. All proteins were characterised by reducing and non-reducing SDS PAGE analysis and mass spectrometry. The formation of the desired disulphide bond in the VNAR domain was confirmed by mass spectrometry methods. These C-terminal HisMyc tagged proteins were then assessed for ROR1 cell-surface binding by flow cytometry

Cell surface binding of test agents to hROR1 was characterized in different cell lines (A549 and A427) and the resulting K_{Dapp} values determined. Adherent cancer cells were detached from tissue culture flasks by incubating with 0.1% EDTA/PBS solution at 37 °C for ~10 minutes or until cells detached easily. Cells were re-suspended in ice-cold PBS/2%FCS in 15ml tubes and centrifuged at 1500rpm for 5 mins at 4 °C. Supernatant was removed and the cell pellet re-suspended in PBS/2%FCS. A cell count was performed using a Z1 Coulter Particle Counter (Beckman Coulter) or Chemometec Nucleocounter NC-202 and 5×10^5 cells were aliquoted per test sample into a 96 well plate. Cells were incubated with 100µl of test agents at a range of concentrations, plus controls for 1hr on ice. The sample plate was centrifuged at 2000 rpm for 5mins. The supernatant was removed and a wash performed by re-suspending the cell pellets in 0.25mL of ice-cold PBS/2%FCS using a multichannel pipette. Samples were again centrifuged at 2000rpm for 5min at 4°C. Supernatant was removed and two further washes performed as described. After the final wash and centrifugation step, excess liquid was removed by blotting the plate on tissue paper. Binding of VNARs was determined by adding 100µl of anti-x6His tag Ab (Abcam) per cell pellet sample as appropriate and incubated on ice for 30mins. Wash steps were performed as described previously. PE-anti-mouse antibody (JIR) was used to detect

binding of the VNAR (His6 tagged) agents and corresponding drug-conjugates by incubating with the appropriate samples for 30min on ice in the dark. Wash steps were performed as described previously. All cell pellets were finally re-suspended in 0.3ml of ice-cold PBS/2%FCS and left on ice in the dark prior to analysis on a Merck-Millipore Guava EasyCyte HT or Thermo Fisher Attune NxT flow cytometer.

As shown in Figure 2 and Figure 3, the loop library variants bind to the ROR1^{hi} human cancer cell-line A549 but not to the ROR1^{low} human cancer cell-line A427. 2V is a control VNAR sequence, derived from a naïve VNAR library, so is representative of this protein class but has no known target.

50 **EXAMPLE 2 - Humanisation and further engineering of B1 loop library variants**

Humanised sequence derivatives of three lead ROR1 binding B1 loop library VNARs were generated using two different strategies.

55 Humanised sequences were designed based on the human germ line Vk1 sequence, DPK-9. For example, in P3A1 V1 the framework regions 1, 3 and 4 of the VNAR were mutated to align with the framework regions of DPK-9.

The second strategy involved grafting the binding loops of the ROR1 binding VNARs onto a previously humanised VNAR framework (Kovalenko et al JBC 2013 288(24) 17408-17419; WO2013/167883). But with further positions engineered based on the structure of the VNAR B1 in complex with the ROR1 Ig domain.

Additional sites of engineering include amino acid changes in the CDR1, HV2 and HV4 regions of the protein.

Similarly, a humanised variant of B1 was developed using this approach, which accordingly contains amino acid changes in its CDR1, HV2 and HV4 regions as well as the framework regions. So B1G4 is, by de facto, a loop library derivative of B1 or a loop library variant of humanised variants of B1 whereby the CDR1, HV2, HV4 and CDR3 sequences are the same as in the parental protein.

Examples of humanised / grafted loop library VNAR sequences are below:

G3CP G4

75 TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTFTL
TISSLQPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIK (SEQ ID NO: 71)

G3CP V15

ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRMTMSFSL
80 RISSLTVEDSATYYCKAYPWGAGAPYNVQWYDGQGTKLEVK (SEQ ID NO: 72)

1H8 G4

TRVDQSPSSLSASVGDRVITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTL
 TISLQPEDSATYYCRAYPWGAGAPSSVQWYDGAGTKVEIK (SEQ ID NO: 73)

85

1H8 V15

ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRMTMSFSL
 RISSLTVEDSATYYCKAYPWGAGAPSSVQWYDGQGTKLEVK (SEQ ID NO: 74)

90 C3CP G4

TRVDQSPSSLSASVGDRVITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTL
 TISLQPEDSATYYCRAYPWGAGAPRQVQWYDGAGTKVEIK (SEQ ID NO: 75)

C3CPV15

95 ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRMTMSFSL
 RISSLTVEDSATYYCKAYPWGAGAPRQVQWYDGQGTKLEVK (SEQ ID NO: 76)

B1G4

TRVDQSPSSLSASVGDRVITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTL
 100 TISLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 51).

DNA encoding the humanised constructs was codon optimised for expression in *E. coli* and synthesised by GeneArt (Thermo). All humanised sequences were generated with the following C terminal His₆ tag: QASGAHHHHHH (SEQ ID NO: 102)

105

G4 sequences were made without an additional C-terminal tag.

DNA encoding these proteins was sub cloned into the intein expression vectors, expressed in *E. coli* and purified as described previously in "Typical method for expression of VNAR intein fusion proteins" section.

110

Humanised ROR1 binding VNAR variants demonstrated high affinity binding to human ROR1 by BLI, good thermal stability and little evidence of aggregation by SEC. BLI was performed as described previously using human ROR1 ECD – Fc immobilised to the chip surface. SEC was performed as previously described. Thermal stability assays used Applied Biosystems StepOne Real Time PCR system with the Protein Thermal Shift™ dye kit (Thermo). The assay mix was set up so that the protein was at a final concentration of 20 μM in 20 μL. 5 μL of Thermal Shift™ buffer was added alongside 2.5 μL 8x Thermal Shift™ Dye. Assays were run using the StepOne software and data analysed using Protein Thermal Shift™ software. All data are from first derivative analysis. BLI data for hROR1 binding and thermal stability by protein thermal shift is shown in Table 6.

115

120

Table 6: Thermal stability and hROR1 binding data for humanised VNAR loop variants

Construct	T _m (°C)	hROR1 binding K _D (nM)
G3CP G4	57.4	8.5
G3CP V15	57.2	<0.5
1H8 G4	58.7	33.7
1H8 V15	57.4	<0.5
C3CP G4	58.2	6.8
C3CP V15	57.2	1.4
B1G4	56.0	9.2

125

Either grafting the HV and CDR loops of G3CP, 1H8 and C3CP onto a humanised VNAR framework coupled with additional mutations in the CDR1, HV2 and HV4 regions or substituting VNAR framework sequences with regions from the human DPK-9 sequence, yielded substantially engineered proteins that are stable, monomeric and maintain high affinity binding to hROR1.

130

EXAMPLE 3 – Generation of the anti-ROR1 VNAR P3A1 G1 loop library sequences

Library design

135 P3A1 G1 is a humanised version of the ROR1 binding VNAR P3A1. The P3A1 G1 loop library was designed to improve ROR1 binding affinity of this humanised variant via randomisation of CDR1, HV2 and HV4 regions without any changes within frameworks. Choice of mutations was made based on the data analysis of VNAR sequences from *Squalus acanthus*. Sequence of P3A1 G1 and library design are shown in Figure 4.

140

Library was synthesised by controlled mutagenesis of CDR1, HV2 and HV4. Residues 26-33, 44-52 and 61-65 located within CDR1, HV2 and HV4 loops respectively were changed to selected amino acids as specified in Fig. 4 resulting in total library diversity of 8.2×10^6 combinations.

145

Libraries construction.

P3A1 G1 library DNA was amplified by PCR using specific primers to introduce SfiI restriction sites for cloning into pEDV1 phagemid vector. This introduces an additional Ser residue into CD1. Library DNA ligated into pEDV1 was transformed into electrocompetent *TG1 E.coli* (Lucigen). The library size was calculated to be 2×10^8 . 192 single clones were picked and sequenced as a quality control of the library.

150

Screening of P3A1 G1 library for antigen specific VNAR sequences.

155 Recombinant human ROR1 protein was used for selections and screening of the P3A1 G1 library. Two strategies were utilised to isolate ROR1 specific binders: selections on biotinylated antigen immobilised on pre-decorated streptavidin-coated beads and selections with antigen directly immobilised to the immunotube. Selection on pre-decorated with biotinylated antigen beads involved 3 rounds of panning with low stringency in first and second rounds (3xPBST and 3xPBS washes for both rounds, 100 nM and 10 nM of biotinylated huROR1 for round 1 and 2 respectively), but high stringency for third round
160 (10xPBST and 10xPBS washes, 0.5 nM of biotinylated huROR1). Selection on immunotubes consists of 2 rounds of panning with constant antigen concentration of 2 ng/ml. Following the selection process, outputs were screened for antigen-specific binding by monoclonal phage and periplasmic extract ELISAs against human or mouse ROR1. 95% of monoclonal phage displaying the VNARs were specific to human and mouse ROR1 from selections with antigen directly immobilised to the immunotube and
165 4% for selections on biotinylated antigen immobilised on pre-decorated streptavidin-coated beads. In total 9 unique sequences from each selection campaign were expressed and analysed for binding and selectivity (Table 7 and 8).

Table 7: P3A1 G1 loop variants isolated from selections with antigen directly immobilised to the immunotube.

Clone ID	Sequence - including C-terminal tag: QASGAHHHHHGAFFEQLISEEDL (SEQ ID NO: 98)
P3A1 G1	TRVDQTPSSLSASVGDRVTITCVLTDSYGLYSTYWYRKNPGSSNKEQISGRYSESVNKGTSFTLTISSLQPEDSATYYCRAREARHPWLRQ YDGAGTKVEIKQASGAHHHHHGAFFEQLISEEDL (SEQ ID NO:135)
NAG8.S	TRVDQSPSSLSASVGDRVTITCVLTGTYGLYASTYWYRKNPGSPNKMIDRMIIIGGRYSESVNNGTKSFTLTISSLQPEDSATYYCRAREARHPWLRQ WYDGAGTKVEIKQASGAHHHHHGAFFEQLISEEDL (SEQ ID NO: 136)
AC5.S	TRVDQSPSSLSASVGDRVTITCVLTDSYGLYSSTYWYRKNPGSSNKMERSISGRYSESVNKGSKSFTLTISSLQPEDSATYYCRAREARHPWLRQ WYDGAGTKVEIKQASGAHHHHHGAFFEQLISEEDL (SEQ ID NO: 137)
AG5.S	TRVDQSPSSLSASVGDRVTITCVLTDTNYALYSSTYWYRKNPGSTNKMESMISIGGRYSESVNKRKSFTLTISSLQPEDSATYYCRAREARHPWLRQ WYDGAGTKVEIKQASGAHHHHHGAFFEQLISEEDL (SEQ ID NO: 138)
AF1.S	TRVDQSPSSLSASVGDRVTITCVLTDKYGLYSSTYWYRKNPGSPDKERIINGGRYSESVNNRKTSFTLTISSLQPEDSATYYCRAREARHPWLRQ WYDGAGTKVEIKQASGAHHHHHGAFFEQLISEEDL (SEQ ID NO: 139)
AB11.S	TRVDQSPSSLSASVGDRVTITCVLTDARYGLFASTYWYRKNPGSPDKDSISIGGRYSESVNKRKSFTLTISSLQPEDSATYYCRAREARHPWLRQ WYDGAGTKVEIKQASGAHHHHHGAFFEQLISEEDL (SEQ ID NO: 140)
NAE1.S	TRVDQSPSSLSASVGDRVTITCVLTGTYGLFSSTYWYRKNPGSSDKERISIGGRYSESVNKGPKSFTLTISSLQPEDSATYYCRAREARHPWLRQ WYDGAGTKVEIKQASGAHHHHHGAFFEQLISEEDL (SEQ ID NO: 141)
AA6.S	TRVDQSPSSLSASVGDRVTITCVLTGTRYGLYASTYWYRKNPGSPDEESMINGGRYSESVNKRKTSFTLTISSLQPEDSATYYCRAREARHPWLRQ WYDGAGTKVEIKQASGAHHHHHGAFFEQLISEEDL (SEQ ID NO: 142)
AB7.S	TRVDQSPSSLSASVGDRVTITCVLTDKYALFSSTYWYRKNPGSSNEERISIGGRYSESVNNRKTSFTLTISSLQPEDSATYYCRAREARHPWLRQ WYDGAGTKVEIKQASGAHHHHHGAFFEQLISEEDL (SEQ ID NO: 143)

AF7.S	TRVDQSPSSLSASVGDRTITCVLTGTGYLYASTYWKPNPGSTDKERIIGGRYSESVNNRSKSFLLTISSLQPEDSATYYCRAREARHPWLRQW YDGAGTKVEIKQASGAHHHHHGAEEFQKLISEEDL (SEQ ID NO: 144)
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Table 8: P3A1 G1 loop variants isolated from selections on biotinylated antigen immobilised on pre-decorated streptavidin-coated beads.

Clone ID	Sequence - including C-terminal tag: QASGAHHHHHGAEEFQKLISEEDL (SEQ ID NO: 98)
P3A1 G1	TRVDQTPSSLSASVGDRTITCVLTDTSYGLYSTYWKPNPGSSNKEQISGRYSESVNKGTSFLLTISSLQPEDSATYYCRAREARHPWLRQW DGAGTKVEIKQASGAHHHHHGAEEFQKLISEEDL (SEQ ID NO: 135)
NAC 1.S	TRVDQSPSSLSASVGDRTITCVLTDTKYGLYSTYWKPNPGSTDEERIIGGRYSESVNKGSKSFLLTISSLQPEDSATYYCRAREARHPWLRQW DGAGTKVEIKQASGAHHHHHGAEEFQKLISEEDL (SEQ ID NO: 168)
NAC 6.S	TRVDQSPSSLSASVGDRTITCVLTDTRYALYSTYWKPNPGSTDEERISIGGRYSESVNKGSKSFLLTISSLQPEDSATYYCRAREARHPWLRQW YDGAGTKVEIKQASGAHHHHHGAEEFQKLISEEDL (SEQ ID NO: 169)
NAE9 .S	TRVDQSPSSLSASVGDRTITCVLTGTGYLYASTYWKPNPGSTDEERMIISGRYSESVNKRKTSFLLTISSLQPEDSATYYCRAREARHPWLRQW YDGAGTKVEIKQASGAHHHHHGAEEFQKLISEEDL (SEQ ID NO: 170)
NAF3 .S	TRVDQSPSSLSASVGDRTITCVLTDTKYGLYSTYWKPNPGSPDEERIISGRYSESVNKGSKSFLLTISSLQPEDSATYYCRAREARHPWLRQW DGAGTKVEIKQASGAHHHHHGAEEFQKLISEEDL (SEQ ID NO: 171)
NAF5 .S	TRVDQSPSSLSASVGDRTITCVLTDTKYALFSTYWKPNPGSTNEDRISIGGRYSESVNKGTSFLLTISSLQPEDSATYYCRAREARHPWLRQW YDGAGTKVEIKQASGAHHHHHGAEEFQKLISEEDL (SEQ ID NO: 172)

AA5. S	TRVDQSPSSLSASVGDVRTITCVLTGTGYGLYSSTYWYRKNPGSSDEERIIIGGRYSESVNKGSKSFTLTISLQPEDSATYYCRAREARHPWLRQW YDGAGTKVEIKQASGAHHHHHGAEEQKLISEEDL (SEQ ID NO: 173)
AD10 .S	TRVDQSPSSLSASVGDVRTITCVLTGTGYGLYSSTYWYRKNPGSSDKESIIGGRYSESVNNGTKSFTLTISLQPEDSATYYCRAREARHPWLRQWY DGAGTKVEIKQASGAHHHHHGAEEQKLISEEDL (SEQ ID NO: 174)
AE3. S	TRVDQSPSSLSASVGDVRTITCVLTGTTRYGLYSSTYWYRKNPGSSDEERISIGRYSESVNKGTKSFTLTISLQPEDSATYYCRAREARHPWLRQW YDGAGTKVEIKQASGAHHHHHGAEEQKLISEEDL (SEQ ID NO: 175)
AF4. S	TRVDQSPSSLSASVGDVRTITCVLTDTRYALYASTYWYRKNPGSTDDEDRIIIGGRYSESVNKGSKSFTLTISLQPEDSATYYCRAREARHPWLRQWY DGAGTKVEIKQASGAHHHHHGAEEQKLISEEDL (SEQ ID NO: 176)

Expression of ROR1 P3A1 G1 loop variants

Clones were expressed in *TG1 E.coli* bacteria and the resulting C-terminally HisMyc-tagged proteins were purified by IMAC using Ni-NTA Sepharose. Proteins were dialysed to PBS pH 7.4, absorbance Abs₂₈₀ was measured and concentrations calculated. Yields obtained were in a range of 0.5 and 6.5 mg/L. Purity of proteins was analysed by SDS-PAGE.

All proteins were characterised by reducing and non-reducing SDS PAGE analysis and mass spectrometry. The formation of the desired disulphide bond was confirmed by mass spectrometry methods.

10 *Binding of P3A1 G1 loop variants to hROR1 by ELISA*

The binding of P3A1 G1 loop variants to human ROR1 was initially assessed by ELISA. In brief, ELISA method as follows. Wells coated with 100ng of ROR1-hFc antigen and incubated, covered, at room temperature for 2hr. Plates washed 3x 400ul per well with PBST (PBS + 0.05% Tween 20 (v/v)), then blocked with 4% skimmed milk powder (w/v) in PBST for 1 hour at 37°C. Plates washed as before plus additional wash in PBS alone. HisMyc-tagged binding proteins were diluted in 4% milk PBST and incubated overnight at 4 °C. Plates washed 3x with PBST, 3x PBS and binding detected using appropriate secondary detection antibody in 4% milk PBST, room temperature 1 hour. Secondary antibodies used include:

20 Anti-c-Myc, HRP (Invitrogen #R951-25)

Mouse anti-polyHis, HRP (Sigma #A7058)

Plates washed 3x with PBST. 100µL TMB substrate (Thermo #34029) added and reaction allowed to proceed at r.t. for 10mins. 100 µL of 2M H₂SO₄ added to quench the reaction. Plate centrifuged briefly before absorbance at 450nm read on a CLARIOstar plate reader (BMG Labtech).

25

Figure 5 shows the relative binding of different variants to human ROR1 with sequences NAG8.S, AF7.S, NAC6.S and AE3.S showing the strongest signal for binding.

The same ELISA method was also used to compare binding of variants NAG8.S, AF7.S, NAC6.S and AE3.S to human ROR1 with that of the parental P3A1 G1 sequence.

The dose response data shown in Figure 6 shows that these loop library sequences bind stronger to human ROR1 than the parental P3A1 G1 protein.

35 *Characterisation of mouse ROR1 and ROR2 binding of P3A1 G1 loop variants by ELISA*

A selection of P3A1 G1 loop variants were further characterised for binding to mouse ROR1 and human ROR2 by ELISA. The same ELISA procedure was employed as described above but with either

mROR1-hFc or hROR2-hFc coated on the plates. None of the variants tested bound to human ROR2. Of the variants that were tested, NAC6.S and AE3.S bound to mouse ROR1

Expression of P3A1 G1 loop library variants as intein fusion proteins

5

P3A1 G1 loop variant VNARs NAG8.S, NAC6.S and AE3.S were re-expressed using intein technology but with a Ser deletion from the CDR1 loop. Expression as intein fusions was performed as described above with either a His tag QACKAHHHHHHG (SEQ ID NO: 163) or HisMyc tag QACKAHHHHHHGAEFEQKLISEEDLG (SEQ ID NO: 164) incorporated at the C-terminus of the VNAR

10

domain.

Following expression and capture on chitin beads the intein VNARs were released from the beads by overnight chemical cleavage in 400mM dioxyamine, or O,O'-1,3-propanediylbis hydroxylamine, or 100mM cysteine or cysteamine to generate the corresponding C-terminal aminoxy, C-terminal cysteine

15

or C-terminal thiol derivative of the VNARs.

Cleaved VNAR supernatant was then further purified by SEC (Superdex75 26/60 GE healthcare) and / or IMAC (HisTrap HP, GE Healthcare) to give the proteins NAG8, NAC6 and AE3. Concentrations were determined from absorbance at 280 nm using the theoretical extinction coefficient predicted from the amino acid sequence. All proteins were characterised by reducing and non-reducing SDS PAGE analysis and mass spectrometry. The formation of the desired disulphide bond in the VNAR domain was confirmed by mass spectrometry methods.

20

These C-terminal HisMyc or His tagged proteins were then further assessed for ROR1 binding by BLI, thermal stability and biophysical properties by SEC,

25

Binding to human and mouse ROR1 by BLI

Binding kinetics were determined using the Biolayer Interferometry (BLI) Octet K2 system (ForteBio). Human or mouse ROR1-hFc fusion proteins (extracellular domains) were immobilised in sodium acetate pH5 buffer to COOH₂ chips or AR2G sensors using amine coupling. VNARs were tested at various concentrations and the K_a (M⁻¹s⁻¹), K_d (s⁻¹) and K_D (nM) values were determined using Octet Data Analysis High Throughput software (ForteBio) for Biolayer Interferometry. Binding parameters are shown in Table 9.

30

Thermal stability assays

Thermal stability assays used Applied Biosystems StepOne Real Time PCR system with the Protein Thermal Shift™ dye kit (Thermo). The assay mix was set up so that the protein was at a final concentration of 20 μM in 20 μL in PBS pH 7.4. 2.5 μL 8x Thermal Shift™ Dye was added. Assays were run using the StepOne software and data analysed using Protein Thermal Shift™ software. All data are from first derivative analysis with the T_m values detailed in Table 9.

40

Size exclusion chromatography

The monomericity and biophysical properties of P3A1 loop variants were assessed by size-exclusion chromatography (SEC) using an analytical SEC column (Superdex 75 increase 10/300 GL).

5 Chromatography was carried out in PBS pH 7.4.

The % monomericity and SEC elution volumes run under identical conditions are shown in Table 9.

Table 9: Summary of ROR1 binding and physical properties of P3A1 G1 variants NAG8, NAC6 and AE3

Parameter	P3A1 G1	P3A1 G1 AE3 His	P3A1 G1 NAC6 His	P3A1 G1 NAG8 HisMyc
hROR1 affinity K_D (nM)	Weak binder	5	13.8	12.2
hROR1 affinity k_{on} ($M^{-1}s^{-1}$)	-	1.32E+06	1.38E+06	1.48E+06
hROR1 affinity k_{off} (s^{-1})	-	6.60E-03	1.90E-02	1.80E-02
% monomer by SEC	100	100	100	100
SEC elution volume (mL)	15.47	17.3	17.3	14.8
T_m ($^{\circ}C$)	58.01	63.2	58.9	53.8
mouseROR1 K_D (nM)	-	4.4	26.1	13.3

10

EXAMPLE 4 – VNAR Reformatting as multimers

ROR1 binding loop variant VNARs were successfully reformatted into hetero dimers and trimers by genetic fusion using different GlySer based linkers to generate bi-specific binders, ROR1 bi-paratopic binders and ROR1 bi-paratopic bi-specific binders.

15

Bispecific binders

Several bi-specific VNAR-based binders were developed by combining ROR1 loop-variant VNAR binders with the humanised VNAR BA11, which binds with high affinity to serum albumins, using a PGVQPSPGGGGGS (SEQ ID NO: 96) linker

20

Proteins were expressed with a C-terminal tag QACKAHHHHHGAEFQKLISEEDL (SEQ ID NO: 97) or QACKAHHHHH (SEQ ID NO: 104) to aid purification and characterisation. This tag also contains a single cysteine residue to facilitate site-selective bioconjugation of payloads to the proteins using thiol mediated chemical coupling strategies

25

Binding kinetics were determined using Biolayer interferometry (K2 Octet instrument / Pall ForteBio) as previously described. For BLI experiments ROR1-hFc, (extracellular domain) and HSA were immobilised in sodium acetate pH5 buffer to AR2G sensors using amine coupling. VNAR-based molecules were tested at various concentrations and the K_a ($M^{-1}s^{-1}$), K_d (s^{-1}) and K_D (nM) values were determined using the Octet data analysis HT software (Pall ForteBio).

30

Binding kinetics for hROR1 binding were also performed with saturating levels of HSA (200 nM) in the baseline, association and dissociation conditions.

- 5 Binding to the ROR1hi A549 cancer-cell lines was determined by flow cytometry. A dose response was performed and the K_{Dapp} for cell-surface ROR1 binder determined using the change in mean fluorescence intensity (background corrected) as a function of VNAR concentration.
- The characterisation of these bi-specific VNARs is shown in Table 10.

Table 10: Characterisation of bi-specific proteins containing ROR1 VNAR loop library variants

	BA11-B1	BA11-G3CP	G3CP-BA11	BA11-1H8	1H8-BA11	G3CP V15-BA11	G3CP G4-BA11
Expected Mass (Da)	27121.0	27170.0	27170.0	27066.9	27066.9	27264.1	27160.9
Observed Mass (Da)	27116.5	27165.9	27165.7	27062.6	27062.4	27260.1	27156.8
hROR1 affinity K_D (nM)	0.21	2.61	1.74	6.83	4.74	1.47	10.2
hROR1 affinity k_{on} ($M^{-1}s^{-1}$)	9.70E+04	1.04E+05	1.13E+05	7.02E+04	8.26E+04	9.00E+04	1.52E+05
hROR1 affinity k_{off} (s^{-1})	2.03E-05	2.73E-04	1.96E-04	4.79E-04	3.92E-04	1.33E-04	1.55E-03
HSA affinity K_D (nM)	3.15	2.11	5.36	3.17	3.07	1.49	3.28
HSA affinity k_{on} ($M^{-1}s^{-1}$)	8.48E+04	2.91E+05	1.17E+05	2.09E+05	2.57E+05	2.59E+05	2.15E+05
HSA affinity k_{off} (s^{-1})	2.67E-04	6.12E-04	6.25E-04	6.63E-04	7.88E-04	3.85E-04	7.06E-04
hROR1 affinity in presence of HSA K_D (nM)	3.05	26.7	6.21	50.8	29.4	11.0	23
hROR1 affinity in presence of HSA k_{on} ($M^{-1}s^{-1}$)	1.00E+05	4.87E+04	1.12E+05	4.59E+04	4.17E+04	6.20E+04	4.22E+03
hROR1 affinity in presence of HSA k_{off} (s^{-1})	3.06E-04	1.30E-03	6.95E-04	2.33E-03	1.22E-03	6.80E-04	1.15E-02
Binding to ROR1 ⁺ A549 cells [K_D app]	5.42 nM	2.22nM	1.74 nM	2.61nM	6.61 nM	ND	4.30 nM

10

Bi-specific VNAR binders were further modified through conjugation to the single cysteine residue in the C-terminal tag.

Bi-paratopic binders

- 15 Several ROR1 bi-paratopic binders were developed combining different ROR1 loop-variant VNAR binders with or without additional insertion of the serum albumin binding humanised VNAR BA11. The VNAR domains were joined together using a PGVQPAPGGGGS (SEQ ID NO: 90) linker and proteins were expressed with a C-terminal tag QACKAHHHHHHGAEFQKLISEEDL (SEQ ID NO: 97) or QACKAHHHHHHH (SEQ ID NO: 104) to aid purification and characterisation. This tag also contains a single cysteine residue to facilitate site-selective bioconjugation of payloads to the proteins using thiol
- 20 mediated chemical coupling strategies

Binding kinetics were determined using Biolayer interferometry (K2 Octet instrument / Pall ForteBio) as previously described. For BLI experiments ROR1-hFc, (extracellular domain) was immobilised in sodium acetate pH5 buffer to AR2G sensors using amine coupling. VNAR-based molecules were tested at

various concentrations and the K_a ($M^{-1}s^{-1}$), K_d (s^{-1}) and K_D (nM) values were determined using the Octet data analysis HT software (Pall ForteBio).

The characterisation of these bi-specific VNARs is shown in Table 11.

5 **Table 11: Characterisation of bi-paratopic proteins containing ROR1 VNAR loop library variants**

Construct	Linker	Expected Mass (reduced); Da	Observed Mass; Da	hROR1 binding (BLI)		
				K_D (nM)	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})
P3A1G1AE3-G3CPG4	PGVQPAPGGGGGS (SEQ ID NO: 90)	26288.0	26284.4	0.58	4.46E+05	2.58E-04
G3CPG4-P3A1G1AE3	PGVQPAPGGGGGS (SEQ ID NO: 90)	26288.0	26284.5	0.53	4.11E+05	2.17E-04
P3A1-BA11-G3CP	PGVQPAPGGGGGS (SEQ ID NO: 90)	40146.51	ND	0.22	5.82E+04	1.28E-05
P3A1-G3CP-BA11	PGVQPAPGGGGGS (SEQ ID NO: 90)	40146.51	ND	0.20	5.74E+04	1.14E-05
BA11-G3CP-P3A1	PGVQPAPGGGGGS (SEQ ID NO: 90)	40146.51	40142.16	0.30	2.07E+05	6.15E-05
BA11-P3A1-G3CP	PGVQPAPGGGGGS (SEQ ID NO: 90)	40146.51	ND	0.14	2.94E+05	4.09E-05

Bi-paratopic binders show increased affinity for binding ROR1 as compared to the individual ROR1 binding monomers.

The constructs containing BA11 are examples of bi-paratopic bi-specific protein binders.

10

Furthermore, several bi-specific and bi-paratopic VNAR-based binders were developed by combining ROR1 loop-variant VNAR binders with the humanised VNAR BA11 or by combining different ROR1 loop-variant VNAR binders using a PGVQPCPGGGGS (SEQ ID NO: 177) linker. This linker sequence also contains a single cysteine residue to facilitate site-selective bioconjugation of payloads to the proteins, in this linker, using thiol mediated chemical coupling strategies.

15

Proteins were expressed with a C-terminal tag QASGAHHHHHH (SEQ ID NO: 102) or QACKAHHHHHH (SEQ ID NO: 104) to aid purification and characterisation.

20 **Table 12: Characterisation of bi-specific and bi-paratopic proteins containing ROR1 VNAR loop library variants with cysteine containing linker sequences**

Construct	Linker	Expected Mass (reduced); Da	Observed Mass; Da	hROR1 binding (BLI)		
				K _D (nM)	K _a (M ⁻¹ s ⁻¹)	K _d (s ⁻¹)
P3A1G1AE3-G3CPG4	PGVQPCPGGGGGS (SEQ ID NO: 177)	26232.9	26228.9	0.24	1.10E+06	2.60E-04
G3CPG4-P3A1G1AE3	PGVQPCPGGGGGS (SEQ ID NO: 177)	26232.9	26228.8	0.28	6.41E+05	1.76E-04

Bi-specific VNAR binders were further modified through conjugation to the single cysteine residue in the linker sequence.

- 5 Prior to conjugation, 20 equivalents of TCEP were added to the bispecific proteins to remove cysteine/glutathione capping of the linker thiol. After incubation at room temperature for one hour the TCEP was removed by purification on a HiTrap SP cation exchange chromatography column (Cytiva). To load onto the column the protein was diluted three-fold in 50 mM Na Phosphate buffer pH 6.0. The protein was then eluted by an increasing gradient of elution buffer consisting of 50 mM Na phosphate pH 6.0, 1 M NaCl. To conjugate, 4 equivalents of a maleimide containing payload was added and left to incubate at room temperature for 1 hour. Free payload was then removed by cation exchange using the same protocol as above.
- 10

15 **Table 13: Characterisation of bi-specific and bi-paratopic protein drug conjugates containing ROR1 VNAR loop library variants with cysteine containing linker sequences**

Protein	Payload	Deglycosylated, reduced mass, Da		DAR by MS	Conjugate Yield (overall)	hROR1 binding (BLI)
		Expected	Observed		%	K _D (nM)
BA11-G3CP	MA-vc-PAB-MMAE	26825.8	26823.3	1.0	35	0.54
G3CP G4-P3A1G1AE3	MA-vc-PAB-MMAE	27549.5	27544.9	1.0	28	0.76

Without being bound by any particular theory, it is expected that conjugate yields can be improved by increasing scale of production and by employing optimised purification processes.

20

EXAMPLE 5 – VNAR Reformatting as Fc fusion proteins

VNAR Fc Fusion Proteins

Fusion of proteins to an Fc domain can improve protein solubility and stability, markedly increase plasma half-life and improve overall therapeutic effectiveness. A human IgG1 Fc sequence is shown below and further examples are shown in Figure 7.

5

Human IgG1 Fc (hFc)

EPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
10 TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 145)

VNAR loop variants were genetically fused via standard $[G_4S]_3$ linkers to engineered hIgG1 Fc domains that contained a cysteine substitution in the hIgG1 Fc sequence, S239C (EU numbering). The VNAR
15 Fc fusion proteins were transiently expressed as secreted protein in CHO K1 cells and purified from the media using MabSelect™ SuRe™ (Evitria, Switzerland). Purified proteins were exchanged into PBS pH 7.4 or PBS + 100 mM Arg pH 7.4 and analysed by SEC (AdvanceBio, Agilent, running buffer DPBS pH 7.4), SDS PAGE and mass spectrometry to confirm sequence and protein integrity.

20 Binding kinetics were determined using a Pioneer Surface Plasmon Resonance (SPR) instrument (SensiQ/Pall ForteBio), or the Biolayer Interferometry (BLI) Octet K2 system (ForteBio). ROR1-hFc fusion proteins (extracellular domains) were immobilised in sodium acetate pH5 buffer to COOH₂ chips or AR2G sensors using amine coupling. VNAR-Fc molecules were tested at various concentrations and the K_a ($M^{-1}s^{-1}$), K_d (s^{-1}) and K_{Dapp} (nM) values were determined using Octet Data Analysis High
25 Throughput software (ForteBio) for Biolayer Interferometry. Alternatively, the kinetic parameters for binding were determined by immobilising the VNAR-hFc fusion onto AHC sensors. Human ROR1 (ECD) was tested at various concentrations and the K_a ($M^{-1}s^{-1}$), K_d (s^{-1}) and K_D (nM) values for 1:1 binding were determined using Octet Data Analysis High Throughput software (ForteBio) for Biolayer Interferometry. ROR1 2A2 mAb (Biolegend) was included as a control for positive/negative binding to
30 ROR1. 2V is a control VNAR sequence, derived from a naïve VNAR library, so is representative of this protein class but has no known target. SEC analysis was performed as described previously. The data, summarised in Table 14, demonstrates advantageous properties of the loop library hFc variants versus the parental B1-hFc protein.

Binding of the VNAR-Fc fusions to ROR1 on the surface of cancer cell lines was measured by flow
35 cytometry using the methods described previously, with binding of VNAR-hFc fusion molecules determined by adding 100µL of PE-anti-human antibody (JIR) and incubating on ice for 30mins. K_{Dapp} values were calculated from the increase in fluorescence intensity as a function of VNAR-hFc concentration. Figure 8 shows the binding of different VNAR-Fc fusions to the ROR1^{hi} A549 lung adenocarcinoma cells.

Table 14 - Summarises the expression yield (based on final purified, buffer exchanged protein), SEC analysis of the hFc fusions, the affinity of these molecules for ROR1 by BLI and the K₀app for binding ROR1^{hi} A549 cells.

Table 14: Characteristics of ROR1 loop variants VNAR-Fc fusions

	B1-hFc	G3CP-hFc	G3CP G4-hFc	1H8-hFc	1H8 G4 - hFc	1H8 V15-hFc
Overall expression yield (transient CHO expression)	50 mg/L	> 100 mg/L	>100 mg/L	80 mg/L	>100 mg/L	>100 mg/L
% monomer by SEC	81	>98	95.2	90.5	96.1	92.5
hROR1 affinity K ₀ app (nM)	<0.05	<0.05	<0.05	<0.05	0.3	0.19
hROR1 affinity K ₀ (1:1) (nM)	3.78	5.74	17.9	26.9	2506	16.9
ratROR1 affinity K ₀ app (nM)	0.04	0.7	1.9	4.4	NB	2.5
Cell surface hROR1 binding (flow cytometry titration A549 cells) K ₀ app (nM)	1.7	0.72	0.75	ND	ND	ND

The relative stabilities of VNAR-hFc fusion proteins in PBS buffer were assessed. G3CP-hFc, G3CPG4-hFc and B1G4-hFc and parental B1-hFc were incubated at 2 mg / mL in sterile PBS buffer pH 7.4 containing 0.05% sodium azide at 37°C for 96h. The UV absorbance was measured at 280nm and 320nm at t=0 and t=96h. The monomericity of the proteins at t=0 and t=96 h was assessed by size-exclusion chromatography (S200 Increase 10/300GL with PBS pH 7.4 running buffer). As shown in Figure 16 the UV absorbance at both 280nm and 320nm was increased after 96h incubation for B1-hFc but not for the loop library variants (i.e. G3CP-hFc and G3CPG4-hFc). The absorbance at 320nm in particular is attributed to the scattering of light by aggregate particles. The protein concentrations for the loop library variants (i.e. G3CP-hFc and G3CPG4-hFc), calculated from the absorbance at 280 nm, remain constant throughout the experiment. The SEC analysis at t=0 versus t=96h (Figure 17) shows that loop library variants (i.e. G3CP-hFc and G3CPG4-hFc) have good stability and are more stable than the parental protein B1-hFc.

Bi-paratopic VNAR Fc Fusion Proteins

VNAR loop variants were genetically fused via standard [G₄S]₃ linkers to hIgG1 Fcs engineered for heterodimerisation (Ridgway 1996 Protein Engineering 9(7):617-21). The Knob variant has a tryptophan substitution at position 336 (T366Y) and the Hole variant has a Threonine substitution at position 407 (Y407T) (EU numbering). This approach was used to generate bi-paratopic ROR1 binders where one arm comprises a VNAR loop variant and the other arm comprises a second ROR1 binding VNAR. In addition, a cysteine substitution was incorporated in the hIgG1 Fc sequence [S239C (EU numbering)] of both Knob and Hole variants to facilitate bioconjugation with different payloads.

The VNAR Fc fusion proteins were transiently co-expressed as secreted protein in CHO K1 cells and purified from the media using MabSelect™ SuRe™ (Evitria, Switzerland). Purified proteins were exchanged into PBS pH 7.4 and analysed by SEC (AdvanceBio, Agilent, running buffer DPBS), SDS PAGE and mass spectrometry to confirm sequence and protein integrity.

G3CP hFc(S239C+Y407T)

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLT VNGGGGSGGGGSGGGGSGGGGSEPKSSDKT
HTCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTK
PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT PTVLSDSGSFLLTSKLTVDKSRWQQGNVFSC
SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 146)

G3CPG4 hFc(S239C+Y407T)

TRVDQSPSSLSASVGDRVITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKGTMSTFL
TISSLQPEDSATYYCRAYPWGAGAPYNVQWYDGAGTKVEIKGGGSGGGGSGGGGSGGGGSEPKSSDKTH
TCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKP
REEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT PTVLSDSGSFLLTSKLTVDKSRWQQGNVFSC
VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 147)

P3A1 hFc(S239C+T366Y)

TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
 LRIKDLTVADSATYYCKAREARHPWLRQWYDGAGTVLTVNGGGGSGGGGSGGGGSEPKSSDKTHT
 CPPCPAPPELLGGPCVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
 EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK
 5 NQVSLYCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
 VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 148)

All proteins were characterised by reducing and non-reducing SDS PAGE analysis (Figure 9) and mass spectrometry (Table 15).

10

Binding to ROR1 was determined using Biolayer interferometry (K2 Octet instrument / Pall ForteBio) as previously described. For BLI experiments ROR1-hFc, (extracellular domain) was immobilised on the sensors. Data is shown in Table 15.

15 **Table 15: MS characterisation of bi-paratopic VNAR-Fc fusions and binding to human ROR1 by BLI**

Protein	Expected MW Δ C term Lys, Da			Observed MW, Da			ROR1 binding (BLI)
	monomer 1	monomer 2	dimer	monomer 1	monomer 2	dimer	K_D app (nM)
G3CP-P3A1 hFc (S239C+KIH)	38,775.5	39,059.9	77,827.3	38,770.0	39,054.5	77,820.2	<0.05
G3CPG4-P3A1 hFc (S239C+KIH)	38,766.4	39,059.9	77,818.2	38,760.9	39,054.5	77,812.1	<0.05

20

Binding of the bi-paratopic VNAR-Fc fusions to ROR1 on the surface of cancer cell lines was measured by flow cytometry using the methods described previously. Binding of VNAR-hFc fusion molecules determined by adding 100 μ L of PE-anti-human antibody (JIR) and incubating on ice for 30mins. K_{Dapp} values were calculated from the increase in fluorescence intensity as a function of VNAR-hFc concentration. Figure 10 shows the binding of the bi-paratopic VNAR-Fc fusions to the ROR1^{hi} A549 lung adenocarcinoma cells and the ROR1^{low} A427 cells. G3CP-P3A1 hFc (S239C+KIH) and G3CPG4-P3A1 hFc (S239C+KIH) bind strongly to A549 cells with K_D app of 0.06 nM and 0.20 nM respectively but show little binding to A427 cells.

25

30

EXAMPLE 6 – Loop variant VNAR drug conjugates

VNAR-hFc drug conjugates

Another approach for generating ADCs is to engineer cysteine substitutions or additions at positions on the light and heavy chains of antibodies and these cysteines provide reactive thiol groups for site specific labelling (Junutula 2008 Nature Biotechnology 26, 925 – 932, Jeffrey 2013, Sutherland 2016).

- 5 The anti ROR1 loop library VNAR -hFc fusions were generated with an additional cysteine engineered into the Fc region as described previously, which enabled site specific labelling with maleimide derivatives of fluorescent labels (AF488) and cytotoxic drugs (MA PEG4 vc PAB EDA PNU 159682 and MA PEG4 va PAB EDA PNU 159682) (Figure 11).

10 *Generation of VNAR-hFc – drug conjugates*

Using a partial reduction, refolding and labelling method adapted from the literature [Junutula et al, 2008 Nat Biotech, Jeffrey et al, 2013 Bioconj Chem], these proteins were site specific labelled with the maleimide PNU derivatives. Briefly, 1mg/ml VNAR hFc solutions were prepared in PBS +100mM L-Arginine pH7.4 with 1mM EDTA. 20 molar equivalents TCEP added and incubated at 4°C for a minimum of 48 hours. 30 molar equivalents DHAA added, pH adjusted to 6.5 and incubated at room temperature for 1 hour. Refolded VNAR Fc S239C was extensively dialysed or buffer exchanged into PBS +50mM L-Arginine and quantified by UV before reacting with 4 or 5 molar equivalents maleimide PNU solution, room temperature overnight. Conjugates were purified by SEC and analysed by analytical HIC, analytical SEC, and LC-MS. Table 16 summaries the conjugates prepared.

Table 16: Summary of characteristics of VNAR-PNU conjugates

Protein	Payload	Deglycosylated, reduced mass, Da		DAR by MS	Conjugate Yield (overall)	HMW aggregate
		Expected	Observed		%	%
G3CP hFc	MA-PEG4-vc-PAB-EDA-PNU159682	40,239.6	40,239.8	2.1	52	<3
	MA-PEG4-va-EDA-PNU159682	40,003.6	40,005.3	2.0	61	<3
G3CPG4 hFc	MA-PEG4-vc-PAB-EDA-PNU159682	40,229.5	40,230.0	2.2	49	<1.5
	MA-PEG4-va-EDA-PNU159682	39,994.5	39,995.4	2.0	61	<1
B1G4 hFc	MA-PEG4-vc-PAB-EDA-PNU159682	40,253.0	40,251.8	2.0	37	<1
	MA-PEG4-va-EDA-PNU159682	40,017.0	40,016.2	2.0	43	<1

Protein	Payload	Deglycosylated, reduced mass, Da		DAR by MS	Conjugate Yield (overall)	HMW aggregate
		Expected	Observed		%	%
B1 hFc	MA-PEG4-vc-PAB-EDA-PNU159682	40,262.2	40,263.3	2.1	46	<1
	MA-PEG4-va-EDA-PNU159682	40,026.9	40,027.6	2.0	50	<1

SDS-PAGE and mass spectrometry analysis of the final conjugates determined that the labelling had proceeded in a quantitative fashion to give highly pure homogenous protein drug conjugates with drug
5 to antibody ratio (DAR) of 2.

Binding of VNAR-hFc – drug conjugates to hROR1 by ELISA

The binding of G3CP hFc and G3CPG4 hFc and their respective drug conjugates to human ROR1 was
10 assessed by ELISA. In brief, ELISA method as follows. Wells were coated with 100ng of ROR1-his antigen and incubated, covered, at room temperature for 2hr. Plates washed 3x 400ul per well with PBST (PBS + 0.05% Tween 20 (v/v)), then blocked with 4% skimmed milk powder (w/v) in PBST for 1 hour at 37°C. Plates washed as before plus additional wash in PBS alone. B1 loop variants (VNAR-hFc fusion) binding proteins were diluted in 4% milk PBST and incubated overnight at 4 °C. Plates
15 washed 3x with PBST, 3x PBS and binding detected using appropriate secondary detection antibody in 4% milk PBST, room temperature 1 hour. The secondary antibody used for detection was a Rabbit anti-human IgG H&L (HRP), Abcam Cat No. ab6759. Plates were washed 3x with PBST and then 100µL TMB substrate (Thermo #34029) added and the reaction allowed to proceed at r.t. for 10mins. 100 µL of 2M H₂SO₄ was then added to quench the reaction. The plate was centrifuged briefly before
20 absorbance at 450nm read on a CLARIOstar plate reader (BMG Labtech).

Figure 12 shows that G3CP hFc and G3CPG4 hFc PNU conjugates bind strongly to human ROR1 and there is no loss in binding activity after conjugation of the different PNU linker payloads to the parental proteins.
25

In vitro cell viability assays for cancer cells treated with anti ROR1 VNAR drug conjugates

Cells were seeded into white, clear bottom 96 well plates (Costar) and incubated at 37°C, 5% CO₂ for 24 hours. On the following day, dilution series were set up for each test agent at x10 working stocks. The dose response X10 stock was: 10000, 5000, 1000, 500, 100, 50, 10, 5, 1, 0.5nM etc. 10µL of the
30 X10 stock solutions were added to the cell plates (90µl per well) using a multichannel pipette. This resulted in a 1:10 dilution into the well and dose responses ranging from 1000nM (column 1) to 0.05nM (column 10) or continued to 0.5fM, if required, for the most sensitive cells lines. 10µl of vehicle control

(PBS) was added to the control wells (columns 11 and 12). Plates were incubated at 37°C, 5% CO₂ for 72-96 hours. Promega Cell Titre Glo reagent was used as per the manufacturer's instructions to assess cell viability. Briefly, assay plates were removed from incubator and allowed to equilibrate to room temperature before adding 100µl of room temperature Cell Titre Glo reagent to each 100µl assay well.

5 Plates were placed on a plate shaker for 2 minutes at 600rpm. Plates were allowed to sit for a further 10 minutes at room temperature prior to measuring luminescence read-out using a Clariostar plate-reader (BMG). Data was analysed by calculating the average for untreated (vehicle only) control wells and determining the % of control for each treated well. % of control data was then plotted against Log [Treatment] concentration and the IC₅₀ value derived using non-linear regression fitting in GraphPad

10 Prism software.

The following cell lines were used

PA-1 – human ovarian cancer cell line: EMEM, 10% hiFCS

PA-1 ROR1 ko - human ovarian cancer cell line with ROR1 knock-out: EMEM, 10% hiFCS

HEK293 – human embryonic kidney cell line: EMEM, 10% FCS

15 HEK293 stably transfected with human ROR1 (HEK293.hROR1) – human embryonic kidney cell line stably expressing hROR1: EMEM, 10% FCS

Figure 13 shows dose response curves, with corresponding IC₅₀ values (Table 17), for cell-killing of the ROR1 positive PA-1 ovarian cancer cells and PA-1 ROR1 ko cells by G3CP-hFc-PNU conjugates

20 (PEG4-vc PAB EDA PNU159682 and PEG4-va-EDA-PNU159682) and G3CPG4-hFc-PNU conjugate (PEG4-vc PAB EDA PNU159682). PA-1 ROR1 ko is PA-1 cancer cell-line where ROR1 expression has been knocked out.

Table 17: Calculated IC₅₀ values (nM) for the cell-killing of PA-1 and PA1 ROR1 ko cancer cells by

25 G3CP-hFc conjugates.

	IC ₅₀ (nM) 96hr		
	PA-1 cells	PA-1 ROR1 ko	PA-1/PA-1 ROR1 ko window
G3CP-hFc(S239C)-vcPAB-EDA-PNU	0.0028	6.3	x2250
G3CP-hFc(S239C)-va-EDA-PNU	0.11	11.3	x103
G3CPG4-hFc(S239C)-vcPAB-EDA-PNU	0.77	7.8	X10

The ROR1 targeting VNAR-hFc conjugates show potent killing of PA-1 cell-lines, which is abrogated upon knockdown of the ROR1 receptor. There is > 100 fold window in the IC₅₀ values for both of the G3CP-hFc PNU conjugates.

- 5 Figure 18 shows dose response curves, with corresponding IC₅₀ values (Table 18), for cell-killing of the ROR1^{low} HEK293 cells and HEK293 cells stably transfected with human ROR1 (HEK293.hROR1) by G3CP-hFc-PNU, G3CPG4-hFc-PNU and 2V-hFc-PNU conjugates (PEG4-vc PAB EDA PNU159682). 2V is a control VNAR sequence, derived from a naïve VNAR library, so is representative of this protein class but has no known target.

10

Table 18: Calculated IC₅₀ values (nM) for the cell-killing of HEK293 WT and HEK293.hROR1 cells by G3CP-hFc, G3CPG4-hFc and 2V-hFc conjugates.

	IC ₅₀ (nM) 96hr		
	HEK293 cells	HEK293.hROR1	HEK293/HEK293.hROR1 window
G3CP-hFc(S239C)-vcPAB-EDA-PNU	18.1	0.008	x2263
G3CPG4-hFc(S239C)-vcPAB-EDA-PNU	23.5	0.002	x11750
2V-hFc(S239C)-vcPAB-EDA-PNU	10.6	9.2	X1.2

- 15 The ROR1 targeting VNAR-hFc conjugates show potent killing of the HEK293.hROR1 cell-line, which is stably transfected with the ROR1 receptor, but not the ROR1^{low} wild-type HEK293 cells. There is > 2000-fold window in the IC₅₀ values for both the G3CP-hFc PNU and G3CPG4-hFc-PNU conjugates for killing HEK293 vs HEK293.hROR1 cells but no window for the 2V-hFc-PNU non-binding control conjugate.

20

Example 7 - *In vivo* efficacy of protein-drug conjugates in patient-derived xenograft model of Triple Negative Breast Cancer (TNBC)

- 25 An efficacy study in the ROR1+ HBCx-28 patient-derived TNBC xenograft model was performed by XenTech (Paris).

Outbred athymic (*nu/nu*) female mice (*HSD: Athymic Nude-Foxn1^{nu}*) were implanted subcutaneously with tumours of the same *in vivo* passage. Mice were monitored until the tumour implants reached the study volume recruitment criteria of 60–200 mm³, preferably 75–196 mm³ in a sufficient number of animals. Mice were randomised to treatment groups such that there was no statistical difference

between tumour volumes in each group. Randomisation was designated as Day 0 of the experiment. Mice were treated with vehicle or with the protein-drug conjugates B1-hFc-vc-PAB-EDA-PNU, B1G4-hFc-vc-PAB-EDA-PNU, G3CP-hFc-vc-PAB-EDA-PNU or G3CPG4-hFc-vc-PAB-EDA-PNU by single dose 0.3 mg / kg i.v. injection on day 2. All mice pre-primed with mouse IgG 20h before first PDC dose.

5 Tumour volume was evaluated by measuring perpendicular tumour diameters, with a calliper, three times a week during the experimental period. Absolute tumour volume (ATV) was calculated using the formula $TV\ (mm^3) = [length\ (mm) \times width\ (mm)^2] \times 0.5$, where the length and the width are the longest and the shortest perpendicular diameters of the tumour measured perpendicularly, respectively. All animals were weighed at the same time as tumour size measurement. Mice were observed and

10 documented daily for changes in physical appearance, behaviour, adverse clinical signs and general welfare in line with local welfare and best veterinary practice guidelines.

Figure 14 shows the effect of the protein-drug conjugates on tumour growth versus vehicle control. All protein drug conjugates were well tolerated and show highly statistically significant *in vivo* efficacy in this ROR1+ TNBC PDX model. B1G4-hFc-vc-PAB-EDA-PNU retains comparable levels of *in vivo*

15 efficacy to B1-hFc-vc-PAB-EDA-PNU (data not shown). Loop library variants G3CP-hFc-vc-PAB-EDA-PNU and G3CPG4-hFc-vc-PAB-EDA-PNU show improved efficacy over the parental B1 fusion with complete and durable regressions observed for both loop library variants for the 0.3 mg / kg single dose regimen.

An efficacy study was also performed in the ROR1+ HBCx-10 patient-derived TNBC xenograft model

20 by XenTech (Paris).

Outbred athymic (*nu/nu*) female mice (*HSD: Athymic Nude-Foxn1^{nu}*) were implanted subcutaneously with tumours of the same *in vivo* passage. Mice were monitored until the tumour implants reached the study volume recruitment criteria of 75–196 mm³ in a sufficient number of animals. Mice were randomised to treatment groups such that there was no statistical difference between tumour volumes

25 in each group. Randomisation was designated as Day 0 of the experiment. Mice were treated with vehicle or with the protein-drug conjugates B1-hFc-vc-PAB-EDA-PNU, B1G4-hFc-vc-PAB-EDA-PNU, G3CP-hFc-vc-PAB-EDA-PNU or G3CPG4-hFc-vc-PAB-EDA-PNU or G3CP-hFc-va-EDA-PNU at a dose of 0.3 mg / kg i.v. injection, three times, four days apart (3 x Q4D on day 2, 6 and 10). All mice were pre-primed with mouse IgG 20h before first PDC dose. Tumour volume was evaluated by

30 measuring perpendicular tumour diameters, with a calliper, three times a week during the experimental period. Absolute tumour volume (ATV) was calculated using the formula $TV\ (mm^3) = [length\ (mm) \times width\ (mm)^2] \times 0.5$, where the length and the width are the longest and the shortest perpendicular diameters of the tumour measured perpendicularly, respectively. All animals were weighed at the same time as tumour size measurement. Mice were observed and documented daily for changes in physical

35 appearance, behaviour, adverse clinical signs and general welfare in line with local welfare and best veterinary practice guidelines.

Figure 19 shows the effect of the protein-drug conjugates on tumour growth versus vehicle control. All protein drug conjugates were well tolerated and show highly statistically significant *in vivo* efficacy in this ROR1+ TNBC PDX model with complete and durable regressions observed for this dosing regimen.

EXAMPLE 8 – Bi-paratopic loop variant VNAR drug conjugates*Bi-paratopic VNAR-hFc drug conjugates*

5

Bi-paratopic anti-ROR1 loop library VNAR-hFc fusions, as described in Example 5, were generated with an additional cysteine engineered into the Fc region as described previously, which enabled site specific labelling with maleimide derivatives of labels and cytotoxic drugs.

10 *Generation of Bi-paratopic VNAR-hFc – drug conjugates*

Bi-paratopic ROR1 binding proteins G3CP-P3A1 hFc (S239C+KIH) and G3CPG4-P3A1 hFc (S239C+KIH) were conjugated with MC-vc-PAB-MMAE or MA-PEG4-vc-PAB-EDA-PNU159682 using a partial reduction, refolding and labelling method as described in Example 6. Conjugates were purified by SEC and analysed by analytical HIC, analytical SEC, and LC-MS. Table 19 summaries the conjugates prepared.

Table 19: Summary of characteristics of Bi-paratopic VNAR-PNU conjugates

Protein	Payload	Deglycosylated, reduced mass, Da		DAR by MS	Conjugate Yield (overall)	HMW aggregate
		Expected	Observed		%	%
G3CP-P3A1 hFc (S239C+KIH)	MA-PEG4-vc-PAB-EDA-PNU159682	40,178.0 & 40,462.4	40,180.7 & 40,465.1	2.0	50	<1
G3CP-P3A1 hFc (S239C+KIH)	MC-vc-PAB-MMAE	40,091.3 & 39,059.9	40,087.3 & 40,371.7	2.0	63	<1
G3CPG4-P3A1 hFc (S239C+KIH)	MA-PEG4-vc-PAB-EDA-PNU159682	40,168.9 & 40,462.4	40,170.9 & 40,464.8	2.15	44	<1
G3CPG4-P3A1 hFc (S239C+KIH)	MC-vc-PAB-MMAE	40,082.2 & 40,375.7	40,077.7 & 40,371.3	2.0	65	<1

Protein	Payload	Deglycosylated, reduced mass, Da		DAR by MS	Conjugate Yield (overall)	HMW aggregate
		Expected	Observed		%	%
P3A1 hFc (S239C)	MA-PEG4-vc-PAB-EDA- PNU159682	40,398.8	40,402.6	2.16	60	<1

Binding of the bi-paratopic VNAR-Fc-PNU conjugates to ROR1 on the surface of cancer cell lines was measured by flow cytometry using the methods described previously. Binding of VNAR-hFc-PNU molecules was determined by adding 100 μ L of PE-anti-human antibody (JIR) and incubating on ice for 30mins. K_{Dapp} values were calculated from the increase in fluorescence intensity as a function of VNAR-hFc concentration. Figure 20 a and b shows the binding of the bi-paratopic VNAR-Fc-PNU conjugates (PEG4-vc PAB EDA PNU159682) to the ROR1^{hi} A549 lung adenocarcinoma cells and the ROR1^{low} A427 cells along with the corresponding mono-paratopic PNU conjugates. G3CP-P3A1 hFc (S239C+KIH)-PNU and G3CPG4-P3A1 hFc (S239C+KIH)-PNU bind strongly to A549 cells with K_D app of 0.92 nM and 1.83 nM respectively but show little binding to A427 cells. G3CP-P3A1 hFc (S239C+KIH)-PNU demonstrates a greater level of saturation binding to A549 cells as compared to the corresponding G3CP-hFc-PNU and P3A1-hFc-PNU conjugates (Figure 20a). Similarly, G3CPG4-P3A1 hFc (S239C+KIH)-PNU demonstrates a greater level of saturation binding to A549 cells as compared to the corresponding G3CPG4-hFc-PNU and P3A1-hFc-PNU conjugates (Figure 20b)

15

In vitro cell viability assays for cancer cells treated with anti ROR1 Bi-paratopic VNAR drug conjugates

Cell Titre Glo assays were performed as describe din Example 6. Cells were incubated with VNAR-hFc conjugates at 37°C, 5% CO₂ for 96 hours and the % of cell viability determined as a function of dose response. The % of control data was plotted against Log [Treatment] concentration and the IC₅₀ value derived using non-linear regression fitting in GraphPad Prism software.

20

The following cell lines were used

PA-1 – human ovarian cancer cell line: EMEM, 10% hiFCS

PA-1 ROR1 ko - human ovarian cancer cell line with ROR1 knock-out: EMEM, 10% hiFCS

Kasumi-2 – human B cell precursor leukaemia cell line: RPMI 1640, 10% hiFCS

25 MHH-ES1 – human Ewings sarcoma cell line: RPMI 1640, 10% hiFCS

Figure 21 shows dose response curves for cell-killing of the ROR1 positive PA-1 ovarian cancer cells and PA-1 ROR1 ko cells by G3CP-P3A1 hFc (S239C+KIH)-PNU and G3CPG4-P3A1 hFc (S239C+KIH)-PNU conjugates (PEG4-vc PAB EDA PNU159682). PA-1 ROR1 ko is PA-1 cancer cell-line where ROR1 expression has been knocked out.

30

Table 20 shows IC₅₀ values, for cell-killing of Kasumi-2, MHH-ES1, PA-1 and PA-1 ROR1 ko cells by G3CP-P3A1 hFc (S239C+KIH)-PNU and G3CPG4-P3A1 hFc (S239C+KIH)-PNU conjugates (PEG4-vc PAB EDA PNU159682). The cell-surface ROR1 receptor number was determined for each cell-line by flow cytometry using BD Biosciences Quantibrite beads.

5

Table 20: Calculated IC₅₀ values (nM) for the cell-killing of PA-1 and PA1 ROR1 ko cancer cells by G3CP-P3A1 hFc (S239C+KIH)-PNU and G3CPG4-P3A1 hFc (S239C+KIH)-PNU conjugates.

	IC50 (nM)			
	Kasumi-2	MHH-ES1	PA1	PA1.ROR1ko
ROR1 receptor / cell	5,086	2,342	12,249	0
G3CP-P3A1 hFc (S239C + KIH) vcPAB-EDA-PNU	0.016	0.3	0.003	6.3
G3CPG4-P3A1 hFc (S239C + KIH) vcPAB-EDA-PNU	0.141	1	0.009	7.8

10

The ROR1 targeting bi-paratopic VNAR-hFc conjugates show potent killing of the ROR1+ cancer cell-lines, but not the ROR1 negative PA1.ROR1ko cell-line.

15 **EXAMPLE 9 - *In vivo* efficacy of bi-paratopic protein-drug conjugates in patient-derived xenograft model of Triple Negative Breast Cancer (TNBC)**

An efficacy study in the ROR1+ HBCx-28 patient-derived TNBC xenograft model was performed by XenTech (Paris).

20 Outbred athymic (*nu/nu*) female mice (*HSD: Athymic Nude-Foxn1^{nu}*) were implanted subcutaneously with tumours of the same *in vivo* passage. Mice were monitored until the tumour implants reached the study volume recruitment criteria of 100–200 mm³ in a sufficient number of animals. Mice were randomised to treatment groups such that there was no statistical difference between tumour volumes in each group. Randomisation was designated as Day 0 of the experiment. Mice were treated with

25 vehicle or with the Bi-paratopic protein-drug conjugates G3CP-P3A1 hFc (S239C+KIH)-vc-PAB-EDA-PNU and G3CPG4-P3A1 hFc (S239C+KIH) vc-PAB-EDA-PNU either by single dose 0.3 mg / kg i.v. injection on day 2 or by 3 x 0.1 mg / kg i.v. injections four days apart (3 x Q4D on day 2, 6 and 10). All mice were pre-primed with mouse IgG 20h before first PDC dose. Tumour volume was evaluated by

measuring perpendicular tumour diameters, with a caliper, three times a week during the experimental period. Absolute tumour volume (ATV) was calculated using the formula $TV\ (mm^3) = [length\ (mm) \times width\ (mm)^2] \times 0.5$, where the length and the width are the longest and the shortest perpendicular diameters of the tumour measured perpendicularly, respectively. All animals were weighed at the same time as tumour size measurement. Mice were observed and documented daily for changes in physical appearance, behaviour, adverse clinical signs and general welfare in line with local welfare and best veterinary practice guidelines.

Figure 22 shows the effect of the protein-drug conjugates on tumour growth versus vehicle control. All protein drug conjugates were well tolerated and both bi-paratopic loop library variants G3CP-P3A1-hFc-vc-PAB-EDA-PNU and G3CPG4-P3A1-hFc-vc-PAB-EDA-PNU show excellent *in vivo* efficacy in this ROR1+ TNBC PDX model, with tumour regressions observed for both agents.

EXAMPLE 10 - ROR1 VNAR Bi-specifics

Bispecific target combinations for ROR1 binding VNARs include, for example, HSA for half-life extension; bispecific engagement of ROR1 and serum albumin RTKs e.g. EGFR, Her3; bispecific targeting both EGFR and ROR1 or HER3 and ROR1 on the surface of cells.

The VNAR BA11, already discussed and exemplified herein, is an example of a HSA-binding VNAR. Bi-specific molecules comprising a HSA-binding VNAR (such as BA11) and another specific binding molecule are discussed.

ROR1 x CD3 bispecific sequences combining N-terminal ROR1 VNARs with a C-terminal anti-CD3 scFv (clone OKT3) via 2 different length G4S linkers were expressed in CHO cells (Evitria) and purified by IMAC (HisTrap Excel, GE Healthcare) followed by SEC (Superdex 200 26/60, GE Healthcare). Similarly, biparatopic ROR1 x CD3 bispecific sequences combining N-terminal biparatopic ROR1 VNARs with the C-terminal anti-CD3 scFv were also expressed in CHO (Evitria).

CD3 BiTE-like approach; examples of CD3 binding sequences for use as an ROR1 VNAR bispecific Anti CD3 scFv clone OKT3 (WO 2014028776 ZynGenia) and orientation and humanised derivatives thereof

VH-[G₄S]₃-VL

DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWWKQRPQGGLWIGYINPSRGYTNYNQKFCD
KATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYDDHYCLDYWGQGTTLTVSSGGGGSGGGSGG
GGSDIQLTQSPAIMASPGKVTMTCRASSSVSYMNWYQKSGTSPKRWIYDTSKVASGVPYRFSG
SGSGTSYSLTISSMEAEDAATYCYQQWSSNPLTFGAGTKLELKS (SEQ ID NO: 149)

Humanised anti CD3 scFv UCHT1 (Arnett et al PNAS 2004 101(46) 16268-16273) and derivatives thereof

VL-[G₄S]₃-VH

MDIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDGTVKLLIYYTSRLHSGVPSKFSGSGS

5 GTDYSLTISNLEQEDIATYFCQQGNTLPWTFAGGKLEIKGGGSGGGGSGGGGSEVQLQQSGPEL
VKPGASMKISCKASGYSFTGYTMNWVKQSHGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSS
TAYMELLSLTSEDSAVYYCARSGYYGDSDWYFDVWGQGTTLTVFS (SEQ ID NO: 150)

EXAMPLE 11 – ROR1 CAR-T approaches

10 Chimeric antigen receptors (CARs) based on the ROR1-specific antigen binding molecules described in the present application may be generated. Furthermore, engineered T cells expressing such a CAR may also be generated, which may then be used in, for example, adoptive cell therapy.

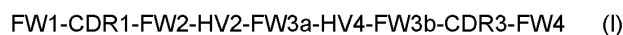
15 In brief, a nucleic acid construct encoding a ROR1-specific CAR may be produced. The ROR1-specific CAR may include an intracellular activation domain, a transmembrane domain, and an extracellular domain comprising the ROR1-specific antigen binding molecule described herein. The nucleic acid construct may then be incorporated into a viral vector, such as a retroviral vector (e.g., a lentiviral vector).

20 T cells may be isolated from a patient in need of treatment, which may then be modified to express the nucleic acid construct encoding the CAR, for example by retroviral transfection or gene-editing using approaches such as CRISPR-CAS-9.

The engineered T cells may then be re-infused into the patient in order to treat the condition, such as
25 treatment of cancer.

CLAIMS

1. A receptor tyrosine kinase-like orphan receptor 1 (ROR1) specific antigen binding molecule comprising an amino acid sequence represented by the formula (I):



wherein

CDR3 is a CDR sequence having an amino acid sequence selected from the group consisting of YPWGAGAPYNNVQWY (SEQ ID NO: 23), YPWGAGAPYLVQWY (SEQ ID NO: 20), YPWGAGAPWNVQWY (SEQ ID NO: 24), YPSGAGAPRPVQWY (SEQ ID NO: 11), YPWGAGAPCLVQWY (SEQ ID NO: 12), YPWGAGAPRLVQWY (SEQ ID NO: 13), YPWGAGAPRQVQWY (SEQ ID NO: 14), YPWGAGAPRSVQWY (SEQ ID NO: 15), YPWGAGAPSLVQWY (SEQ ID NO: 16), YPWGAGAPSNVQWY (SEQ ID NO: 17), YPWGAGAPSQVQWY (SEQ ID NO: 18), YPWGAGAPSSVQWY (SEQ ID NO: 19), YPWGAGAPWQVQWY (SEQ ID NO: 21), YPWGAGAPWSVQWY (SEQ ID NO: 22), and YPWGAGAPWLQWY (SEQ ID NO: 10);

CDR1 is a CDR sequence having an amino acid sequence selected from the group consisting of GANYGLAA (SEQ ID NO: 1), DANYGLAA (SEQ ID NO: 5), GANYDLA (SEQ ID NO: 2), GANYGLA (SEQ ID NO: 3), and GANYDLAA (SEQ ID NO: 4)

FW1 is a framework region;

FW2 is a framework region;

HV2 is a hypervariable sequence having an amino acid sequence selected from the group consisting of SSNQERISIS (SEQ ID NO: 6), and SSNKERISIS (SEQ ID NO: 7);

FW3a is a framework region;

HV4 is a hypervariable sequence having an amino acid sequence selected from the group consisting of NKRTM (SEQ ID NO: 8) and NKGTM (SEQ ID NO: 9);

FW3b is a framework region;

FW4 is a framework region;

wherein if CDR3 is YPWGAGAPWLVQWY (SEQ ID NO: 10) then CDR1 is selected from the group consisting of DANYGLAA (SEQ ID NO: 5), GANYGLSA (SEQ ID NO: 3) and GANYDLAA (SEQ ID NO: 4).

2. The ROR1-specific antigen binding molecule of claim 1 wherein:

CDR3 is a CDR sequence having an amino acid sequence selected from the group consisting of YPWGAGAPYNVQWY (SEQ ID NO: 23), YPWGAGAPYLVQWY (SEQ ID NO: 20) and YPWGAGAPWNVQWY (SEQ ID NO: 24), and/or

CDR1 is a CDR sequence having an amino acid sequence selected from the group consisting of GANYGLAA (SEQ ID NO: 1) and DANYGLAA (SEQ ID NO: 5).

3. The ROR1-specific antigen binding molecule of claim 1 or claim 2 wherein:

CDR3 is a CDR sequence having an amino acid sequence according to YPWGAGAPYNVQWY (SEQ ID NO: 23).

4. The ROR1-specific antigen binding molecule of any preceding claim wherein:

CDR3 is a CDR sequence having an amino acid sequence according to YPWGAGAPYNVQWY (SEQ ID NO: 23);

CDR1 is a CDR sequence having an amino acid sequence according to GANYGLAA (SEQ ID NO: 1);

HV2 is a hypervariable sequence having an amino acid sequence according to SSNQERISIS (SEQ ID NO: 6); and

HV4 is a hypervariable sequence having an amino acid sequence according to NKRTM (SEQ ID NO: 8).

5. The ROR1-specific antigen binding molecule of any one of claims 1 to 3 wherein:

CDR3 is a CDR sequence having an amino acid sequence according to YPWGAGAPYNVQWY (SEQ ID NO: 23);

CDR1 is a CDR sequence having an amino acid sequence according to DANYGLAA (SEQ ID NO: 5);

HV2 is a hypervariable sequence having an amino acid sequence according to SSNKERISIS (SEQ ID NO: 7); and

HV4 is a hypervariable sequence having an amino acid sequence according to NKGTM (SEQ ID NO: 9).

6. The ROR1-specific antigen binding molecule of any one of claims 1 to 3 wherein:

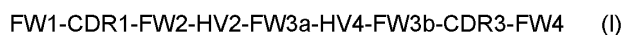
CDR3 is a CDR sequence having an amino acid sequence according to YPWGAGAPWLVQWY (SEQ ID NO: 10);

CDR1 is a CDR sequence having an amino acid sequence according to DANYGLAA (SEQ ID NO: 5);

HV2 is a hypervariable sequence having an amino acid sequence according to SSNKERISIS (SEQ ID NO: 7); and

HV4 is a hypervariable sequence having an amino acid sequence according to NKGTM (SEQ ID NO: 9).

7. A receptor tyrosine kinase-like orphan receptor 1 (ROR1) specific antigen binding molecule comprising an amino acid sequence represented by the formula (I):



wherein

CDR1 is a CDR sequence having an amino acid sequence selected from the group consisting of GTRYGLYS (SEQ ID NO: 25), GTRYGLYSS (SEQ ID NO: 26), DTRYALYS (SEQ ID NO: 27), DTRYALYSS (SEQ ID NO: 28), GTKYGLYA (SEQ ID NO: 29) and GTKYGLYAS (SEQ ID NO: 30);

FW1 is a framework region;

FW2 is a framework region;

HV2 is a hypervariable sequence having an amino acid sequence selected from the group consisting of SSDEERISIS (SEQ ID NO: 31), STDEERISIG (SEQ ID NO: 32), SPNKDRMIIG (SEQ ID NO: 33), and STDKERIIG (SEQ ID NO: 34);

FW3a is a framework region;

HV4 is a hypervariable sequence having an amino acid sequence selected from the group consisting of NKGTK (SEQ ID NO: 35), NKGSK (SEQ ID NO: 36), NNGTK (SEQ ID NO: 37), and NNRSK (SEQ ID NO: 38);

FW3b is a framework region;

CDR3 is a CDR sequence having an amino acid sequence according to REARHPWLRQWY (SEQ ID NO: 39);

FW4 is a framework region.

8. The ROR1-specific antigen binding molecule of any preceding claim, wherein
- FW1 is a framework region of from 20 to 28 amino acids;
- FW2 is a framework region of from 6 to 14 amino acids;
- FW3a is a framework region of from 6 to 10 amino acids;
- FW3b is a framework region of from 17 to 24 amino acids; and/or
- FW4 is a framework region of from 7 to 14 amino acids.
9. The ROR1-specific antigen binding molecule of claim 8, wherein
- FW1 has an amino acid sequence selected from the group consisting of: ASVNQTPRTATKETGESLTINCVVT (SEQ ID NO: 40), TRVDQSPSSLSASVGDRVITITCVLT (SEQ ID NO: 41) and ASVTQSPRSASKETGESLTITCRVT (SEQ ID NO: 42), or a functional variant of any thereof with a sequence identity of at least 45%;
- FW2 has an amino acid sequence according to TYWYRKNPG (SEQ ID NO: 43), or a functional variant of any thereof with a sequence identity of at least 45%;
- FW3a has an amino acid sequence selected from the group consisting of: GRYVESV (SEQ ID NO: 44) and GRYSESV (SEQ ID NO: 45), or a functional variant of any thereof with a sequence identity of at least 45%;
- FW3b has an amino acid sequence selected from the group consisting of: SFSLRIKDLTVADSATYYCKA (SEQ ID NO: 84), SFTLTISLQPEDSATYYCRA (SEQ ID NO: 46) and SFSLRISSLTVEDSATYYCKA (SEQ ID NO: 47), or a functional variant of any thereof with a sequence identity of at least 45%;
- and/or
- FW4 has an amino acid sequence selected from the group consisting of: DGAGTVLTVN (SEQ ID NO: 48), DGAGTKVEIK (SEQ ID NO: 49) or DGQGTKLEVK (SEQ ID NO: 85) or a functional variant of any thereof with a sequence identity of at least 45%.
10. The ROR1-specific antigen binding molecule of claim 1, wherein the ROR1-specific antigen binding molecule comprises an amino acid sequence selected from the group consisting of:
- ASVNQTPRTATKETGESLTINCVVTGANYGLAATYWYRKNPGSSNQERISISGRYVESVNKRTMSFSLRIKDLTVADSATYYCKAYPWGAGAPYNVQWYDGAGTVLTVN (SEQ ID NO: 50);
- TRVDQSPSSLSASVGDRVITITCVLTDANYGLAATYWYRKNPGSSNKERISISGRYSESVNKGTMSTFTLTISLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 51);
- ASVNQTPRTATKETGESLTINCVVTGANYDLSATYWYRKNPGSSNQERISISGRYVESVNKRTMSFSLRIKDLTVADSATYYCKAYPSGAGAPRPVQWYDGAGTVLTVN (SEQ ID NO: 52);
- ASVNQTPRTATKETGESLTINCVVTGANYGLAATYWYRKNPGSSNQERISISGRYVESVNKRTMSFSL

RIKDLTVADSATYYCKAYPWGAGAPCLVQWYDGAGTVLTVN (SEQ ID NO: 53);
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPRLVQWYDGAGTVLTVN (SEQ ID NO: 54);
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPRQVQWYDGAGTVLTVN (SEQ ID NO: 55);,
ASVNQTPRTATKETGESLTINC VVTGANYGLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPRSVQWYDGAGTVLTVN (SEQ ID NO: 56);
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPRSVQWYDGAGTVLTVN (SEQ ID NO: 57);
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSLVQWYDGAGTVLTVN (SEQ ID NO: 58);
ASVNQTPRTATKETGESLTINC VVTGANYGLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSNVQWYDGAGTVLTVN (SEQ ID NO: 59);
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSQVQWYDGAGTVLTVN (SEQ ID NO: 60);
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPSSVQWYDGAGTVLTVN (SEQ ID NO: 61);
ASVNQTPRTATKETGESLTINC VVTGANYGLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWL VQWYDGAGTVLTVN (SEQ ID NO: 62);
ASVNQTPRTATKETGESLTINC VVTGANYDLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWL VQWYDGAGTVLTVN (SEQ ID NO: 63);
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWNVQWYDGAGTVLTVN (SEQ ID NO: 64);
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWQVQWYDGAGTVLTVN (SEQ ID NO: 65);
ASVNQTPRTATKETGESLTINC VVTGANYGLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWQVQWYDGAGTVLTVN (SEQ ID NO: 66);
ASVNQTPRTATKETGESLTINC VVTGANYDLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWQVQWYDGAGTVLTVN (SEQ ID NO: 67);
ASVNQTPRTATKETGESLTINC VVTGANYDLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWSVQWYDGAGTVLTVN (SEQ ID NO: 68);
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWSVQWYDGAGTVLTVN (SEQ ID NO: 69); and
ASVNQTPRTATKETGESLTINC VVTGANYGLSATYWYRKNPGSSNQERISISGRYVESV NKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWSVQWYDGAGTVLTVN (SEQ ID NO: 70);
or a functional variant having CDR1, HV2, HV4 and CDR2 sequences according to any thereof and
having FW1, FW2, FW3a, FW3b and FW4 sequences having a combined sequence identity of at least
45% to the combined FW1, FW2, FW3a, FW3b and FW4 sequences of any thereof.

11. The ROR1-specific antigen binding molecule of claim 1, wherein the ROR1-specific antigen binding molecule comprises an amino acid sequence according to
ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPY NVQWYDGAGTVLTVN (SEQ ID NO: 50).
12. The ROR1-specific antigen binding molecule of claim 1, wherein the ROR1-specific antigen binding molecule comprises an amino acid sequence according to
TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKG TMSFTL
TISSLQPEDSATYYCRAYPWGAGAPWL VQWYDGAGTKVEIK (SEQ ID NO: 51).
13. The ROR1-specific antigen binding molecule of claim 1, wherein the ROR1-specific antigen binding molecule comprises an amino acid sequence selected from the group consisting of:
TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKG TMSFTL
TISSLQPEDSATYYCRAYPWGAGAPY NVQWYDGAGTKVEIK (SEQ ID NO: 71);
ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRK NPGSSNQERISISGRYSESVNKR TMSFSL
RISSLTVEDSATYYCKAYPWGAGAPY NVQWYDGQGTKLEV K (SEQ ID NO: 72);
TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKG TMSFTL
TISSLQPEDSATYYCRAYPWGAGAPSSVQWYDGAGTKVEIK (SEQ ID NO: 73);
ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRK NPGSSNQERISISGRYSESVNKR TMSFSL
RISSLTVEDSATYYCKAYPWGAGAPSSVQWYDGQGTKLEV K (SEQ ID NO: 74);
TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKG TMSFTL
TISSLQPEDSATYYCRAYPWGAGAPRQVQWYDGAGTKVEIK (SEQ ID NO: 75); and
ASVTQSPRSASKETGESLTITCRVTGANYGLAATYWYRK NPGSSNQERISISGRYSESVNKR TMSFSL
RISSLTVEDSATYYCKAYPWGAGAPRQVQWYDGQGTKLEV K (SEQ ID NO: 76);
or a functional variant having CDR1, HV2, HV4 and CDR2 sequences according to any thereof and having FW1, FW2, FW3a, FW3b and FW4 sequences having a combined sequence identity of at least 45% to the combined FW1, FW2, FW3a, FW3b and FW4 sequences of any thereof.
14. The ROR1-specific antigen binding molecule of claim 1, wherein the ROR1-specific antigen binding molecule comprises an amino acid sequence according to
TRVDQSPSSLSASVGDRVTITCVLTDANYGLAATYWYRK NPGSSNKERISISGRYSESVNKG TMSFTL
TISSLQPEDSATYYCRAYPWGAGAPY NVQWYDGAGTKVEIK (SEQ ID NO: 71).
15. The ROR1-specific antigen binding molecule of claim 7, wherein the ROR1-specific antigen binding molecule comprises an amino acid sequence selected from the group consisting of:
TRVDQSPSSLSASVGDRVTITCVLTGTRYGLYSTYWYRK NPGSSDEERISISGRYSESVNKG TKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 77);
TRVDQSPSSLSASVGDRVTITCVLTGTRYGLYSTYWYRK NPGSSDEERISISGRYSESVNKG TKSFT
LTISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 78);

TRVDQSPSSLSASVGDRVITITCVLTDTRYALYSTYWYRKNPGSTDEERISIGGRYSESVNKGSKSFTL
 TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 79);
 TRVDQSPSSLSASVGDRVITITCVLTDTRYALYSSTYWYRKNPGSTDEERISIGGRYSESVNKGSKSFTL
 LTISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 80);
 TRVDQSPSSLSASVGDRVITITCVLTGTTYGLYATYWYRKNPGSPNKDRMIIGGRYSESVNNGTKSFTL
 TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 81);
 TRVDQSPSSLSASVGDRVITITCVLTGTTYGLYASTYWYRKNPGSPNKDRMIIGGRYSESVNNGTKSF
 TLTISLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 82);
 TRVDQSPSSLSASVGDRVITITCVLTGTTYGLYASTYWYRKNPGSTDKERIIIGGRYSESVNNRSKSFTL
 TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 83);
 or a functional variant having CDR1, HV2, HV4 and CDR2 sequences according to any thereof and
 having FW1, FW2, FW3a, FW3b and FW4 sequences having a combined sequence identity of at least
 45% to the combined FW1, FW2, FW3a, FW3b and FW4 sequences of any thereof.

16. The ROR1-specific antigen binding molecule of any preceding claim, wherein the ROR1-specific antigen binding molecule does not bind to receptor tyrosine kinase-like orphan receptor 2 (ROR2).
17. The ROR1-specific antigen binding molecule of any preceding claim, wherein the ROR1-specific antigen binding molecule binds to both human ROR1 and murine ROR1 (mROR1).
18. The ROR1-specific antigen binding molecule of any preceding claim, wherein the ROR1-specific antigen binding molecule binds to deglycosylated ROR1.
19. The ROR1-specific antigen binding molecule of any preceding claim, wherein the ROR1-specific antigen binding molecule is humanized.
20. The ROR1-specific antigen binding molecule of any one of claims 1 to 18, wherein the ROR1-specific antigen binding molecule is de-immunized.
21. The ROR1-specific antigen binding molecule of any one of claims 1 to 20, wherein the ROR1-specific antigen binding molecule is conjugated to a detectable label, dye, toxin, drug, pro-drug, radionuclide or biologically active molecule.
22. The ROR1-specific antigen binding molecule of any one of claims 1 to 21, wherein the specific antigen binding molecule selectively interacts with ROR1 protein with an affinity constant of approximately 0.01 to 50 nM, preferably 0.1 to 30 nM, even more preferably 0.1 to 10 nM.
23. The ROR1-specific antigen binding molecule of any one of claims 1 to 22, wherein the specific antigen binding molecule is capable of mediating killing of ROR1-expressing tumour cells.

24. The ROR1-specific antigen binding molecule of any one of claims 1 to 22, wherein the specific antigen binding molecule is capable of inhibiting cancer cell proliferation.
25. The ROR1-specific antigen binding molecule of any one of claims 1 to 22, wherein the specific antigen binding molecule is capable of being endocytosed upon binding to ROR1.
26. A recombinant fusion protein comprising a specific antigen binding molecule as claimed in any one of claims 1 to 25.
27. A recombinant fusion protein as claimed in claim 26, in which the specific antigen binding molecule is fused to one or more biologically active proteins.
28. A recombinant fusion protein as claimed in claim 27, wherein the specific antigen binding molecule is fused to one or more biologically active proteins via one or more linker domains.
29. The recombinant fusion protein as claimed in either claim 27 or 28, wherein at least one biologically active protein is an immunoglobulin, an immunoglobulin Fc region, a fragment of an immunoglobulin Fc region, an Fc heavy chain, a CH2 region, a CH3 region, an immunoglobulin Fab region, a Fab', a Fv, a Fv-Fc, a single chain Fv (scFv), scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager, an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, or a scaffold protein.
30. The recombinant fusion protein as claimed in claim 29, wherein the at least one biologically active protein is an immunoglobulin Fc region.
31. The recombinant fusion protein as claimed in claim 29, wherein the at least one biologically active protein is a fragment of an immunoglobulin Fc region selected from the group consisting of an Fc heavy chain, a CH2 region and a CH3 region.
32. The recombinant fusion protein as claimed in claim 31, wherein the fragment of an immunoglobulin Fc region is an Fc heavy chain, optionally wherein the Fc heavy chain is engineered to comprise one or more cysteine residues suitable for bioconjugation.
33. The recombinant fusion protein as claimed in any one of claims 31 to claim 32 wherein the fragment of an immunoglobulin Fc region is engineered to dimerize with a second fragment of an immunoglobulin Fc region.
34. The recombinant fusion protein as claimed in any one of claims 31 to 33 wherein the fragment of an immunoglobulin Fc region is engineered to dimerize with the second fragment of an

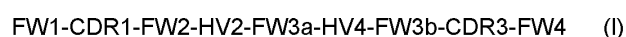
immunoglobulin Fc region by a method selected from the group consisting of knobs-into-holes (Y-T), knobs-into-holes (CW-CSAV), CH3 charge pairing, Fab-arm exchange, SEED technology, BEATtechnology, HA-TF, ZW1 approach, Biclonic approach, EW-RVT and Triomab.

35. The recombinant fusion protein as claimed in any one of claims 31 to claim 34 wherein one or more residues of the fragment of the immunoglobulin Fc region comprises one or more amino acid substitution suitable for heterodimerization with a second fragment of an immunoglobulin Fc region comprising one or more corresponding amino acid mutation.

36. The recombinant fusion protein as claimed in claim 35 wherein the one or more amino acid substitution is selected from the group consisting of T366Y, Y407T, S354C, T366W, Y349C, T366S, L368A and Y407V.

37. The recombinant fusion protein as claimed in claim 35 or claim 36 wherein the one or more amino acid substitution is selected from the group consisting of T366Y and Y407T.

38. A recombinant fusion protein comprising an antigen binding molecule comprising an amino acid sequence represented by the formula (I):



wherein

FW1 is a framework region

CDR1 is a CDR sequence

FW2 is a framework region

HV2 is a hypervariable sequence

FW3a is a framework region

HV4 is a hypervariable sequence

FW3b is a framework region

CDR3 is a CDR sequence

FW4 is a framework region

or a functional variant thereof,

wherein the antigen binding molecule is fused to a fragment of an immunoglobulin Fc region wherein the fragment of an immunoglobulin Fc region is engineered to dimerize with a second fragment of an immunoglobulin Fc region.

39. The recombinant fusion protein as claimed in claim 38, wherein the fragment of an immunoglobulin Fc region selected from the group consisting of an Fc heavy chain, a CH2 region and a CH3 region.

40. The recombinant fusion protein as claimed in claim 39, wherein the fragment of an immunoglobulin Fc region is an Fc heavy chain.

41. The recombinant fusion protein as claimed in any one of claims 38 to 40 wherein the fragment of an immunoglobulin Fc region is engineered to dimerize with the second fragment of an immunoglobulin Fc region by a method selected from the group consisting of knobs-into-holes (Y-T), knobs-into-holes (CW-CSAV), CH3 charge pairing, Fab-arm exchange, SEED technology, BEATtechnology, HA-TF, ZW1 approach, Biclonic approach, EW-RVT and Triomab.

42. The recombinant fusion protein as claimed in any one of claims 38 to claim 41 wherein one or more residues of the fragment of the immunoglobulin Fc region comprises one or more amino acid substitution suitable for knobs-in-holes (KIH) dimerization with a second fragment of an immunoglobulin Fc region comprising one or more corresponding amino acid mutation.

43. The recombinant fusion protein as claimed in claim 42 wherein the one or more amino acid substitution is selected from the group consisting of T366Y, Y407T, S354C, T366W, Y349C, T366S, L368A and Y407V.

44. The recombinant fusion protein as claimed in claim 42 or claim 43 wherein the one or more amino acid substitution is selected from the group consisting of T366Y and Y407T.

45. The recombinant fusion protein as claimed in any one of claims 29 to 44 wherein the antigen binding molecule is a ROR1 specific antigen binding molecule.

46. The recombinant fusion protein as claimed in any one of claims 29 to 45 comprising a sequence according to SEQ ID NO: 146 or SEQ ID NO: 147.

47. The recombinant fusion protein as claimed in claim 38 comprising a sequence according to SEQ ID NO: 148.

48. A recombinant fusion protein dimer comprising
- (a) a first recombinant fusion protein, wherein the first recombinant fusion protein is a recombinant fusion protein as claimed in any one of claims 30 to 47, and
 - (b) a second recombinant fusion protein, wherein the second recombinant fusion protein comprises a second antigen binding molecule fused to a second fragment of an immunoglobulin Fc region engineered to dimerize with the first fragment of an immunoglobulin Fc region.
49. The recombinant fusion protein dimer as claimed in claim 48, wherein the second fragment of an immunoglobulin Fc region selected from the group consisting of an Fc heavy chain, a CH2 region and a CH3 region.
50. The recombinant fusion protein dimer as claimed in claim 49, wherein the second fragment of an immunoglobulin Fc region is an Fc heavy chain.
51. The recombinant fusion protein dimer as claimed in any one of claims 48 to 50 wherein the second fragment of an immunoglobulin Fc region is engineered to dimerize with the second fragment of an immunoglobulin Fc region by a method selected from the group consisting of knobs-into-holes (Y-T), knobs-into-holes (CW-CSAV), CH3 charge pairing, Fab-arm exchange, SEED technology, BEAT technology, HA-TF, ZW1 approach, Biclonic approach, EW-RVT and Triomab.
52. The recombinant fusion protein dimer as claimed in any one of claims 48 to claim 51 wherein one or more residues of the fragment of the immunoglobulin Fc region comprises one or more amino acid substitution suitable for knobs-in-holes (KIH) dimerization with a second fragment of an immunoglobulin Fc region comprising one or more corresponding amino acid mutation.
53. The recombinant fusion protein dimer as claimed in claim 52 wherein the one or more amino acid substitution is selected from the group consisting of T366Y, Y407T, S354C, T366W, Y349C, T366S, L368A and Y407V.
54. The recombinant fusion protein dimer as claimed in claim 52 or claim 53 wherein the one or more amino acid substitution is selected from the group consisting of T366Y and Y407T.
55. The recombinant fusion protein dimer as claimed in any one of claims 48 to 54 wherein the second antigen binding molecule is a ROR1 specific antigen binding molecule.
56. The recombinant fusion protein dimer according to any one of claims 48 to 55 wherein the second specific antigen binding molecule is an immunoglobulin, an immunoglobulin Fab region, a Fab', a

Fv, a Fv-Fc, a single chain Fv (scFv), scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager, an intein, a VNAR domain, a single domain antibody (sdAb) or a VH domain.

57. The recombinant fusion protein dimer according to any one of claims 48 or 56 wherein
- (a) the first recombinant fusion protein comprises a sequence according to SEQ ID NO: 146 or SEQ ID NO: 147, and
 - (b) the second recombinant fusion protein comprises a sequence according to SEQ ID NO: 148.
- 58.. A ROR1-specific chimeric antigen receptor (CAR), comprising at least one ROR1-specific antigen binding molecule as defined in any one of claims 1 to 20, fused or conjugated to at least one transmembrane region and at least one intracellular domain.
59. A cell comprising a chimeric antigen receptor according to claim 58, which cell is preferably an engineered T-cell.
60. A nucleic acid sequence comprising a polynucleotide sequence that encodes a specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor according to any one of claims 1 to 58.
61. A vector comprising a nucleic acid sequence as claimed in claim 60, optionally further comprising one or more regulatory sequences.
62. A host cell comprising a vector as claimed in claim 61.
63. A method for preparing a specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor, comprising cultivating or maintaining a host cell comprising the polynucleotide of claim 60 under conditions such that said host cell produces the binding molecule, optionally further comprising isolating the binding molecule.
- 64.. A pharmaceutical composition comprising the specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of any one of claims 1 to 58.
- 65.. The specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of any one of claims 1 to 58, for use in therapy.
- 66.. The specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of any one of claims 1 to 58, for use in the treatment of cancer.
67. The specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of claim 66, wherein the cancer is a ROR1-positive cancer type.

68. The specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of claim 66, wherein the cancer is selected from the group comprising blood cancers such as lymphomas and leukemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone lymphoma (MZL), non-Hodgkin lymphomas (NHL), acute myeloid leukemia (AML) and solid tumours including neuroblastoma, renal cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, uterine cancer, prostate cancer, thyroid cancer, Head and Neck cancer, bladder cancer, oesophageal cancer, stomach cancer or liver cancer.

69. The use of a specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of any one of claims 1 to 58 in the manufacture of a medicament for the treatment of a disease in a patient in need thereof.

70. A method of treatment of a disease in a patient in need of treatment comprising administration to said patient of a therapeutically effective dosage of a specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of any one of claims 1 to 58 or a pharmaceutical composition of claim 64.

71. The method of claim 70, wherein the disease is cancer.

72. The method of claim 71 wherein the cancer is a ROR1-positive cancer type.

73. The method of claim 71, wherein the cancer is selected from the group consisting of blood cancers such as lymphomas and leukaemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone lymphoma (MZL), non-Hodgkin lymphomas (NHL), acute myeloid leukemia (AML) and solid tumours including neuroblastoma, renal cancer, lung cancer, colon cancer, ovarian cancer, pancreatic cancer, breast cancer, skin cancer, uterine cancer, prostate cancer, thyroid cancer, Head and Neck cancer, bladder cancer, oesophageal cancer, stomach cancer or liver cancer.

74. A method of assaying for the presence of a target analyte in a sample, comprising the addition of a detectably labelled specific antigen binding molecule of any one of claims 1 to 25 or a recombinant fusion protein of claim 26 to 47, or a recombinant fusion protein dimer of any one of claims 48 to 57 to the sample and detecting the binding of the molecule to the target analyte.

75. A method of imaging a site of disease in a subject, comprising administration of a detectably labelled specific antigen binding molecule as claimed in any one of claims 1 to 25 or a detectably labelled recombinant fusion protein of any one of claim 26 to 47, or a detectably labelled recombinant fusion protein dimer of any one of claims 48 to 57 to a subject.

76. A method of diagnosis of a disease or medical condition in a subject comprising administration of a specific antigen binding molecule as claimed in any one of claims 1 to 25 or a recombinant fusion protein of claim 26 to 47, or a recombinant fusion protein dimer of any one of claims 48 to 57 .

77. An antibody, antibody fragment or antigen-binding molecule that competes for binding to ROR1 with the ROR1-specific antigen binding molecule of any one of claims 1 to 25.

78. A kit for diagnosing a subject suffering from cancer, or a pre-disposition thereto, or for providing a prognosis of the subject's condition, the kit comprising detection means for detecting the concentration of antigen present in a sample from a test subject, wherein the detection means comprises a ROR1-specific antigen binding molecule as defined in any one of claims 1 to 25, a recombinant fusion protein as defined in any one of claims 26 to 47, a recombinant fusion protein dimer as defined in any one of claims 48 to 57, a CAR as defined in claim 58, or a nucleic acid as defined in claim 60, each being optionally derivatized, wherein presence of antigen in the sample suggests that the subject suffers from cancer.

79. The kit according to claim 78, wherein the antigen comprises ROR1 protein, more preferably an extracellular domain thereof.

80. The kit according to claim 78, wherein the kit is used to identify the presence or absence of ROR1-positive cells in the sample, or determine the concentration thereof in the sample.

81. The kit according to claim 78, wherein the kit comprises a positive control and/or a negative control against which the assay is compared.

82. The kit according to claim 78, wherein the kit further comprises a label which may be detected.

83. A method for diagnosing a subject suffering from cancer, or a pre-disposition thereto, or for providing a prognosis of the subject's condition, the method comprising detecting the concentration of antigen present in a sample obtained from a subject, wherein the detection is achieved using a ROR1-specific antigen binding molecule as defined in any one of claims 1 to 25, a recombinant fusion protein as defined in any one of claims 26 to 47, a recombinant fusion protein dimer as defined in any one of claims 48 to 57, a CAR as defined in claim 58, or a nucleic acid sequence as defined in claim 60, each being optionally derivatized, and wherein presence of antigen in the sample suggests that the subject suffers from cancer.

84. A method of killing or inhibiting the growth of a cell expressing ROR1 in vitro or in a patient, which method comprises administering to the cell a pharmaceutically effective amount or dose of (i) ROR1-specific antigen binding molecule as defined in any one of claims 1 to 25, a recombinant fusion protein as defined in any one of claims 26 to 47, a recombinant fusion protein dimer as defined in any

one of claims 48 to 57, a nucleic acid as defined in claim 60, or the CAR or cell according to claim 58 or 59, or (ii) of a pharmaceutical composition according to claim 64.

85. The method of claim 84, wherein the cell expressing ROR1 is a cancer cell.
86. The method according to either claim 84 or 85, wherein the ROR1 is human ROR1.
87. A specific antigen binding molecule comprising an amino acid sequence represented by the formula (II):



wherein

FW1-CDR1-FW2-HV2-FW3a-HV4-FW3b-CDR3-FW4 is a ROR1-specific antigen binding molecule according to any one of claims 1 to 25

X and Y are optional amino acid sequences

wherein the specific antigen binding molecule is conjugated to a second moiety.

88. The specific antigen binding molecule of claim 87, wherein X or Y are individually either absent or selected from the group comprising an immunoglobulin, an immunoglobulin Fc region, an immunoglobulin Fab region, a Fab', a Fv, a Fv-Fc, a single chain Fv (scFv), scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager, an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, or a scaffold protein.
89. The specific antigen binding molecule of either claim 87 or claim 88, wherein the conjugation is via a cysteine residue in the amino acid sequence of the specific antigen binding molecule.
90. The specific antigen binding molecule of any one of claims 87 to 89, wherein the second moiety is selected from the group comprising an immunoglobulin or antibody, an immunoglobulin Fc region, an immunoglobulin Fab region, a Fab', a Fv, a Fv-Fc, a single chain Fv (scFv), scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager, an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, or a scaffold protein.
91. The specific antigen binding molecule of any one of claims 87 to 89, wherein the second moiety is selected from the group comprising detectable label, dye, toxin, drug, pro-drug, radionuclide or biologically active molecule.

92. The specific antigen binding molecule according to any one of claims 87 to 89 or 91, wherein the second moiety is at least one toxin selected from the group comprising:

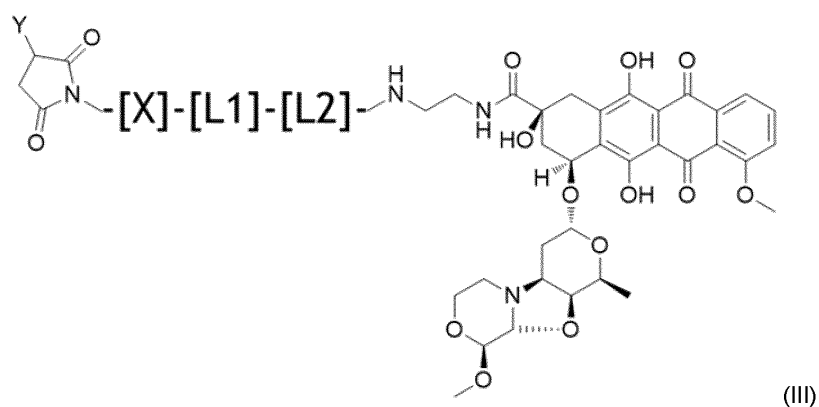
- maytansinoids,
- auristatins,
- anthracyclines, preferably PNU-derived anthracyclines
- amanitin derivatives, preferably α -amanitin derivatives
- calicheamicins,
- tubulysins
- duocarmycins
- radioisotopes - such as an alpha-emitting radionuclide, such as ^{227}Th or ^{225}Ac label
- liposomes comprising a toxic payload,
- protein toxins
- taxanes
- pyrrolbenzodiazepines and/or
- indolinobenzodiazepine pseudodimers and/or
- spliceosome inhibitors
- CDK11 inhibitors
- Pyridinobenzodiazepines
- Irinotecan and its derivatives

93. A target-binding molecule-drug conjugate, comprising

(a) a ROR1 specific antigen binding molecule according to any one of claims 1 to 25 or 87 to 90, or a recombinant fusion protein of any one of claims 26 to 47, or a recombinant fusion protein dimer of any one of claims 48 to 57, and

(b) an anthracycline (PNU) derivative,

wherein the target-binding molecule-drug conjugate has the structure of formula (III):



wherein [X] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups,

substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

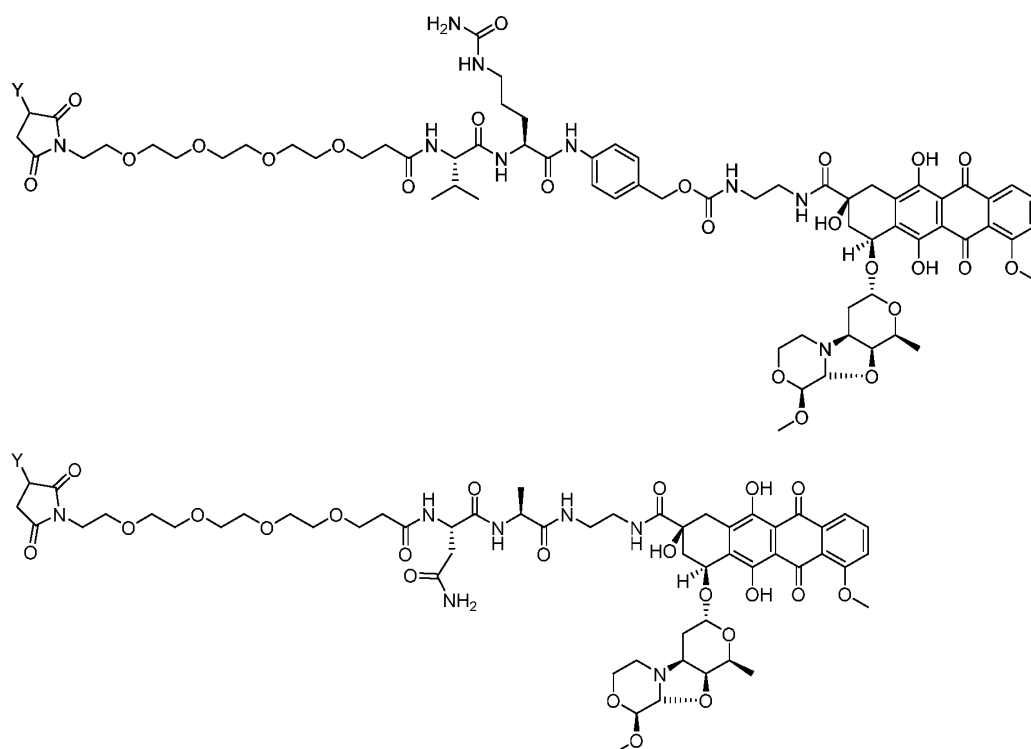
[L1] and [L2] are optional linkers selected from the group consisting of valine (Val), citrulline (Cit), alanine (Ala), asparagine (Asn), a peptide, $-(CH_2)_n-$, $-(CH_2CH_2O)_n-$, p-aminobenzoyloxycarbonyl (PAB), Val-Cit-PAB, Val-Ala-PAB, Ala-Ala-Asn-PAB, Val-Ala, Asn-Ala, any amino acid except glycine, and combinations thereof; and

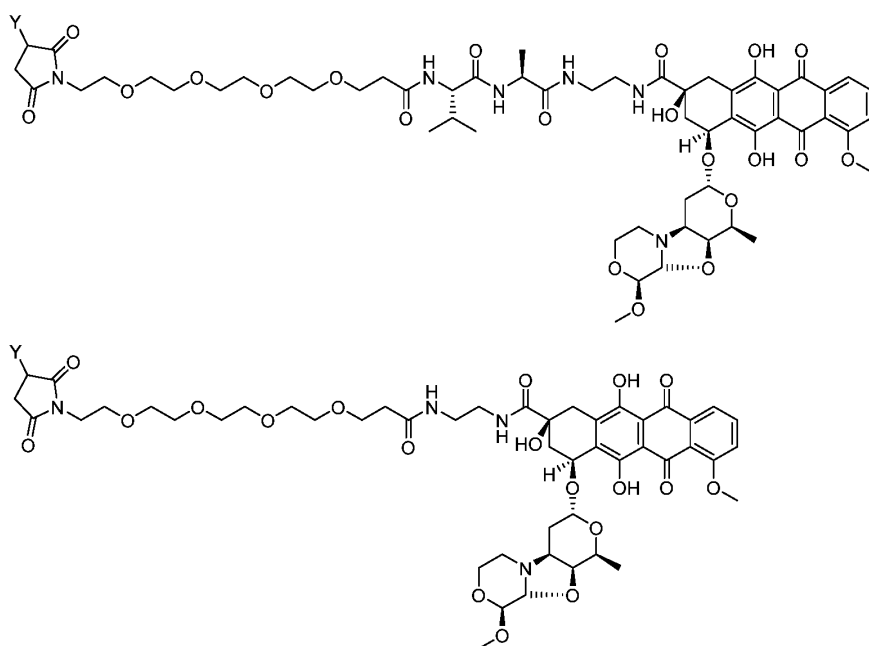
Y comprises a ROR1 specific antigen binding molecule according to any one of claims 1 to 25 or 87 to 90, or a recombinant fusion protein of any one of claims 26 to 47, or a recombinant fusion protein dimer of any one of claims 48 to 57.

94. The target-binding molecule-drug conjugate of claim 93, wherein the target-binding molecule-drug conjugate of formula (III) comprises [L1], [L2] or [L1] and [L2].

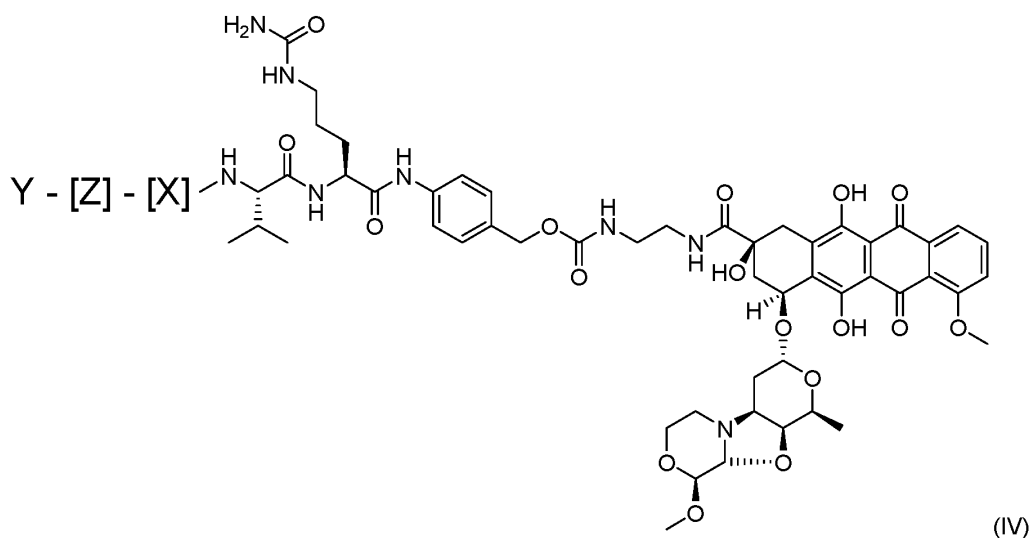
95. The target-binding molecule-drug conjugate of any one of claims 93 to 94, wherein [L2] is p-aminobenzoyloxycarbonyl (PAB) or alanine.

96. The target-binding molecule-drug conjugate of claim 93, wherein the target-binding molecule-drug conjugate has a structure selected from:





97. A target-binding molecule-drug conjugate, comprising
- (a) a ROR1 specific antigen binding molecule according to any one of claims 1 to 25 or 87 to 90, or a recombinant fusion protein of any one of claims 26 to 47, or a recombinant fusion protein dimer of any one of claims 48 to 57, and
 - (b) an anthracycline (PNU) derivative,
- wherein the target-binding molecule-drug conjugate has the structure of formula (IV):



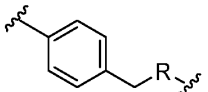
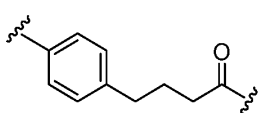
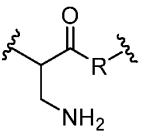
wherein [X] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof;

[Z] is a linker derived from a reactive group used to conjugate the anthracycline (PNU) derivative and the target-binding molecule; and

Y comprises a ROR1 specific antigen binding molecule according to any one of claims 1 to 25 or 87 to 90, or a recombinant fusion protein of any one of claims 26 to 47, or a recombinant fusion protein dimer of any one of claims 48 to 57.

98. The target-binding molecule-drug conjugate of claim 97, wherein [Z] is selected from the group consisting of a disulphide bond, an amide bond, an oxime bond, a hydrazone bond, a thioether bond, a 1, 2, 3 triazole and polyGly.

99. The target-binding molecule-drug conjugate of any one of claims 93 to 95 or 97 to 98, wherein

[X] is selected from the group comprising polyethylene glycol, , , and , wherein  represents the point of attachment to the rest of the molecule and wherein [R] is an optional spacer selected from the group comprising substituted or unsubstituted alkyl groups, substituted or unsubstituted heteroalkyl groups, substituted or unsubstituted aryl groups, substituted or unsubstituted heteroaryl groups, one or more heteroatoms, polyethylene glycol, or a combination thereof.

100. The target-binding molecule-drug conjugate of any one of claims 93 to 95 or 97 to 98, wherein [X] is polyethylene glycol.

101. The target-binding molecule-drug conjugate of any one of claims 93 to 100, wherein the target-binding molecule is a protein and the anthracycline (PNU) derivative is conjugated to a thiol-containing amino acid residue in the amino acid sequence of the protein or wherein the PNU derivative is conjugated via a thiol moiety incorporated by chemical modification at the N-terminus or C-terminus of the amino acid sequence of the protein.

102. The target-binding molecule-drug conjugate according to any one of claims 93 to 101, wherein Y comprises a ROR1 specific antigen binding molecule according to any one of claims 1 to 25 conjugated to the PNU derivative via a human immunoglobulin Fc region or fragment thereof.

103. The recombinant fusion protein of any one of claims 26 to 47 or any claim dependent thereon, or recombinant fusion protein dimer of any one of claims 48 to 57 or any claim dependent thereon, wherein the recombinant fusion protein comprises SEQ ID NO: 186 and/or SEQ ID NO: 187.

104. The recombinant fusion protein as claimed in any one of claims 29 to 45 or any claim dependent thereon, comprising a sequence according to any one or more of SEQ ID NO: 194, SEQ ID NO: 195, SEQ ID NO: 196, SEQ ID NO: 191, SEQ ID NO: 192, SEQ ID NO: 197, SEQ ID NO: 198, SEQ ID NO: 199, SEQ ID NO: 165, SEQ ID NO: 166, SEQ ID NO: 200, SEQ ID NO: 201, SEQ ID NO: 202, SEQ ID NO: 167, SEQ ID NO: 188, SEQ ID NO: 189, SEQ ID NO: 203, SEQ ID NO: 204, or SEQ ID NO: 205.

105. The specific antigen binding molecule of claim 89 or any claim dependent thereon, wherein the specific antigen binding molecule comprises SEQ ID NO: 178 and/or SEQ ID NO: 179.

106. The specific antigen binding molecule of claim 87 or any claim dependent thereon, wherein X or Y are individually either absent or selected from the group comprising an immunoglobulin, an immunoglobulin Fc region, a fragment of an immunoglobulin Fc region, an Fc heavy chain, a CH2 region, a CH3 region, an immunoglobulin Fab region, a Fab', a Fv, a Fv-Fc, a single chain Fv (scFv), scFv-Fc, (scFv)₂, a diabody, a triabody, a tetrabody, a bispecific t-cell engager, an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, or a scaffold protein.

107. The specific antigen binding molecule of claim 87 or any claim dependent thereon or claim 106, wherein X or Y are individually either absent or an immunoglobulin Fc region.

108. The specific antigen binding molecule of claim 106, wherein X or Y are individually either absent or a fragment of an immunoglobulin Fc region selected from the group consisting of an Fc heavy chain, a CH2 region and a CH3 region.

109. The specific antigen binding molecule of claim 108, wherein X or Y are individually either absent or a fragment of an immunoglobulin Fc region is an Fc heavy chain, optionally wherein the Fc heavy chain is engineered to comprise one or more cysteine residues suitable for bioconjugation.

110. The specific antigen binding molecule of claim 108 or 109, wherein X or Y are individually either absent or a fragment of an immunoglobulin Fc region engineered to dimerize with a second fragment of an immunoglobulin Fc region.

111. The specific antigen binding molecule of claim 108 to 110, wherein X or Y are individually either absent or a fragment of an immunoglobulin Fc region engineered to dimerize with the second fragment of an immunoglobulin Fc region by a method selected from the group consisting of knobs-into-holes (Y-T), knobs-into-holes (CW-CSAV), CH3 charge pairing, Fab-arm exchange, SEED technology, BEATtechnology, HA-TF, ZW1 approach, Biclonic approach, EW-RVT and Triomab.

112. The specific antigen binding molecule of claim 108 to 111, wherein X or Y are individually either absent or a fragment of the immunoglobulin Fc region that comprises one or more amino acid

substitution suitable for heterodimerization with a second fragment of an immunoglobulin Fc region comprising one or more corresponding amino acid mutation.

113. The specific antigen binding molecule of claim 112 wherein the one or more amino acid substitution is selected from the group consisting of T366Y, Y407T, S354C, T366W, Y349C, T366S, L368A and Y407V.

114. The specific antigen binding molecule of claim 112 or claim 113 wherein the one or more amino acid substitution is selected from the group consisting of T366Y and Y407T.