

BI-SPECIFIC ANTIGEN BINDING MOLECULES

FIELD OF INVENTION

The present invention relates to bi-specific antigen binding molecules and associated fusion proteins and conjugates. In particular, the present invention relates to bi-specific antigen binding molecules with specificity for both receptor tyrosine kinase-like orphan receptor 1 (ROR1) and epidermal growth factor receptor (EGFR) 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

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 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. (Rebagay G et al, *Frontiers Oncology*, 2012, 2, Borcherdig N et al *Protein Cell*, 2014, 5, 496-502).

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 (Fukada *PNAS*, 2012, Baskar et al *Clin. Cancer Res.*, 2008, 14, 396, Broome HE et al, *Leuk. Res.*, 2011, 35, 1390; Balakrishnan A et al, *Clin. Cancer. Res.* 2017, 23, 3061-3071).

ROR1 expression has been observed at both the mRNA and protein level across a broad range of solid tumours and haematological malignancies including lung, breast, pancreatic, ovarian, colon, head and neck and prostate cancers, melanoma and renal cell carcinoma (Zhang S et al *Am J. Pathol.*, 2012, 181, 1903-1910), breast cancer (Zhang S et al *PLoS One* 2012, 7, e31127; Oxford Biotherapeutics patent application WO2011054007) and Chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia AML (Fukuda T et al, *Proc Natl Acad Sci U S A.* 2008, 105, 3047-3052; Baskar S et al, *Clin Cancer Res.*, 2008, 14, 396-404; Daneshmanesh AH et al, *Int J Cancer.* 2008, 123, 1190-1195; Dave H et al, *PLOS ONE*, 2012, 7, e52655).

Additionally, increased ROR1 expression is reported to correlate with poor clinical outcomes for a number of cancer indications including breast cancer (Chien HP et al, *Virchows Arch.*, 2016, 468, 589-595; Zhang), ovarian cancer (Zhang H et al, *Sci Rep.*, 2014, 4:5811. doi: 10.1038/srep05811),

colorectal cancer (Zhou JK et al, *Oncotarget*, 2017, 8, 32864-32872), lung adenocarcinoma (Zheng YZ et al, *Sci Rep.*, 2016, 6, 36447) and CLL (Cui B et al, *Blood*, 2016, 128, 2931-2940).

Consistent with ROR1's expression pattern and the link to poor clinical prognosis, a functional role for ROR1 in tumourigenesis 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 (Cui B et al *Cancer Res*, 2013, 73, 3649-3660) and spheroid formation and tumour engraftment in models of ovarian cancer (Zhang S et al, *Proc Natl Acad Sci.*, 2014, 11, 17266-17271). 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 and an EGF induced interaction between ROR1 and EGFR has been observed (Yamaguchi T et al, *Cancer Cell*, 2012, 21, 348-361; Ida L et al, *Cancer Science*, 2016, 107, 155-161). Whilst co-expression of EGFR and ROR1 mRNA has been noted from mining breast cancer gene expression database (Peng H et al, *J. Mol. Biol*, 2017, 429, 2954-2973). ROR1 has also been shown to act as a scaffold to sustain caveolae structures and by-pass signalling mechanism that confer resistance to EGFR tyrosine kinase inhibitors (Yamaguchi T et al, *Nat Commun.*, 2016, 7, 10060). Signalling through an ROR1-HER3 complex modulates the Hippo-YAP pathway and promotes breast cancer bone metastasis (Li C et al, *Nature Cell Biol.*, 19, 1206-119) and the protein can promote Met-driven tumourigenesis (Gentile A et al, *Cancer Res.*, 2011, 71, 3132-3140). Whilst in CLL, ROR1 has been reported to hetero-oligomerise with ROR2 in response to Wnt5a to transduce signalling and enhance proliferation and migration (Yu J et al, *J. Clin. Invest.*, 2016, 2, 585-598)

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 kips), 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).

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases. It is a 170kDa transmembrane protein composed of four extracellular domains, a transmembrane region, an intracellular tyrosine kinase domain and a carboxy-terminal tail. The normal function of EGFR relates to regulation of epithelial tissue development, but it is also associated with a number of pathological states. In particular, overexpression of EGFR has been associated with a

number of cancers. Accordingly, it is an important drug target and many therapeutic approaches have been applied. In addition to a number of small molecule-based EGFR inhibitors, such as gefitinib, erlotinib, afatinib, brigatinib, icotinib, and osimertinib a number of antibodies to EGFR have been developed. Anti-EGFR antibodies cetuximab, panitumumab, zalutumumab, nimotuzumab, and matuzumab. These antibodies block the extracellular ligand binding domain, preventing ligand binding and subsequent activation of the tyrosine kinase domain. Single domain antibodies (sdAb) that show competitive binding with cetuximab or matuzumab have also been developed.

Single domain binding molecules can be derived from an array of proteins from distinct species. The immunoglobulin isotype 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 isotype novel antigen receptors or immunoglobulin isotype 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.

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.

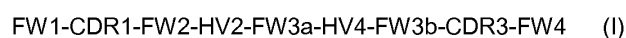
Another structural type of VNAR, termed type (IIb or IV), has only two canonical cysteine residues (in framework 1 and framework 3b regions). So far, this type has been found primarily in dogfish sharks (Liu, J.L., et al. Mol. Immunol. 2007. 44(7): p. 1775–1783; Kovalenko O.V., et al. J Biol Chem. 2013. 288(24): p. 17408-19) and was also isolated from semisynthetic V-NAR libraries derived from wobbegong sharks (Streltsov, V.A. et al. (2004) Proc. Natl. Acad. Sci. U.S.A. 101(34): p. 12444–12449).

SUMMARY OF INVENTION

The present invention generally relates to bi-specific antigen binding molecules. Specifically, the present invention relates to bi-specific molecules having the ability to bind to both ROR1 and EGFR.

In a first aspect, there is provided a bi-specific antigen binding molecule comprising:

(i) a receptor tyrosine kinase-like orphan receptor 1 (ROR1) specific 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

; and

(ii) an epidermal growth factor receptor (EGFR) specific antigen binding molecule.

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 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.

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 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 amino acids in length. In other preferred embodiments, FW2 is 8 amino acids in length.

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 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.

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.

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.

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.

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 molecule binds to deglycosylated ROR1.

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

YMESLHMQGEIENQI (SEQ ID NO: 34)
CQPWNSQYPHTHTFTALRFP (SEQ ID NO: 35)
RSTIYGSRLRIRNLDTTDTGYFQ (SEQ ID NO: 36)
QCVATNGKEVVSSTGVLFVKFGPPPTASPGYSDEYE (SEQ ID NO: 37)

In preferred embodiments of the ROR1-specific antigen binding molecule:

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

CDR1 is a CDR sequence selected from DTSYGLYS (SEQ ID NO: 1), GAKYGLAA (SEQ ID NO: 2), GAKYGLFA (SEQ ID NO: 3), GANYGLAA (SEQ ID NO: 4), or GANYGLAS (SEQ ID NO: 5)

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

HV2 is a hypervariable sequence selected TTDWERMSIG (SEQ ID NO: 6), SSNQERISIS (SEQ ID NO: 7), or SSNKEQISIS (SEQ ID NO: 8)

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

HV4 is a hypervariable sequence selected from NKRAK (SEQ ID NO: 9), NKRTM (SEQ ID NO: 10), NKGAK (SEQ ID NO: 11), or NKGTK (SEQ ID NO: 12)

FW3b is a framework region of from 17 to 24 amino acids

CDR3 is a CDR sequence selected from QSGMAISTGSGHGYNWY (SEQ ID NO: 13), QSGMAIDIGSGHGYNWY (SEQ ID NO: 14), YPWAMWGQWY (SEQ ID NO: 15), VFMPQHWHPAAHWY (SEQ ID NO: 16), REARHPWLRQWY (SEQ ID NO: 17), or YPWGAGAPWLVQWY (SEQ ID NO: 18)

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

or a functional variant with at least 45% sequence identity thereto.

In other preferred embodiments of the ROR1-specific antigen binding molecule, FW1 is selected from: ASVNQTPRTATKETGESLTINCVLT (SEQ ID NO: 19), AKVDQTPRTATKETGESLTINCVLT (SEQ ID NO: 20), TRVDQTPRTATKETGESLTINCVLT (SEQ ID NO: 21), TRVDQTPRTATKETGESLTINCVLT (SEQ ID NO: 22), ASVNQTPRTATKETGESLTINCVLT (SEQ ID NO: 23), or TRVDQSPSSLSASVGDRVTITCVLT (SEQ ID NO: 24), FW2 is selected from: TSWFRKNPG (SEQ ID NO: 25), or TYWYRKNPG (SEQ ID NO: 26), FW3a is selected from: GRYVESV (SEQ ID NO: 27), or GRYSESV (SEQ ID NO: 28), FW3b is selected from: SFSLRIKDLTVADSATYYCKA (SEQ ID NO: 29), SFTLTISLQPEDSATYYCRA (SEQ ID NO: 30), or SFTLTISLQPEDFATYYCKA (SEQ ID NO: 31), and FW4 is selected from DGAGTVLTVN (SEQ ID NO: 32), or DGAGTKVEIK (SEQ ID NO: 33), or functional variants thereof with a sequence identity of at least 45%.

All possible combinations and permutations of the framework regions, complementarity determining regions and hypervariable regions listed above 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, 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 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.

In a preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is ASVNQTPRTATKETGESLTINCVLT (SEQ ID NO: 19); CDR1 is DTSYGLYS (SEQ ID NO: 1); FW2 is TSWFRKNPG (SEQ ID NO: 25); HV2 is TTDWERMSIG (SEQ ID NO: 6); FW3a is GRYVESV (SEQ ID NO: 27); HV4 is NKRAK (SEQ ID NO: 9); FW3b is SFSLRIKDLTVADSATYYCKA (SEQ ID NO: 29); CDR3 is QSGMAISTGSGHGYNWY (SEQ ID NO: 13); and FW4 is DGAGTVLTVN (SEQ ID NO: 32); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is AKVDQTPRTATKETGESLTINCVLT (SEQ ID NO: 20); CDR1 is DTSYGLYS (SEQ ID NO: 1); FW2 is TSWFRKNPG (SEQ ID NO: 25); HV2 is TTDWERMSIG (SEQ ID NO: 6); FW3a is GRYVESV (SEQ ID NO: 27); CDR3 is QSGMAISTGSGHGYNWY (SEQ ID NO: 13); and FW4 is DGAGTVLTVN (SEQ ID NO: 32); or functional variants thereof with a sequence identity of at least 45%.

NO: 27); HV4 is NKRAK (SEQ ID NO: 9); FW3b is SFSRLRIKDLTVADSATYYCKA (SEQ ID NO: 29); CDR3 is QSGMAIDIGSGHGYNWY (SEQ ID NO: 14); and FW4 is DGAGTVLTVN (SEQ ID NO: 32); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is TRVDQTPRTATKETGESLTINC VVT (SEQ ID NO: 21); CDR1 is GAKYGLAA (SEQ ID NO: 2); FW2 is TYWYRKNPG (SEQ ID NO: 26); HV2 is SSNQERISIS (SEQ ID NO: 7); FW3a is GRYVESV (SEQ ID NO: 27); HV4 is NKRTM (SEQ ID NO: 10); FW3b is SFSRLRIKDLTVADSATYYCKA (SEQ ID NO: 29); CDR3 is YPWAMWGQWY (SEQ ID NO: 15); and FW4 is DGAGTVLTVN (SEQ ID NO: 32); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is TRVDQTPRTATKETGESLTINC VVT (SEQ ID NO: 21); CDR1 is GAKYGLFA (SEQ ID NO: 3); FW2 is TYWYRKNPG (SEQ ID NO: 26); HV2 is SSNQERISIS (SEQ ID NO: 7); FW3a is GRYVESV (SEQ ID NO: 27); HV4 is NKRTM (SEQ ID NO: 10); FW3b is SFSRLRIKDLTVADSATYYCKA (SEQ ID NO: 29); CDR3 is VFMPQHWHPAAHWY (SEQ ID NO: 16); and FW4 is DGAGTVLTVN (SEQ ID NO: 32); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is TRVDQTPRTATKETGESLTINC VLT (SEQ ID NO: 22); CDR1 is DTSYGLYS (SEQ ID NO: 1); FW2 is TSWFRKNPG (SEQ ID NO: 25); HV2 is TTDWERMSIG (SEQ ID NO: 6); FW3a is GRYVESV (SEQ ID NO: 27); HV4 is NKGAK (SEQ ID NO: 11); FW3b is SFSRLRIKDLTVADSATYYCKA (SEQ ID NO: 29); CDR3 is REARHPWLRQWY (SEQ ID NO: 17); and FW4 is DGAGTVLTVN (SEQ ID NO: 32); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is ASVNQTPRTATKETGESLTINC VVT (SEQ ID NO: 23); CDR1 is GANYGLAA (SEQ ID NO: 4); FW2 is TYWYRKNPG (SEQ ID NO: 26); HV2 is SSNQERISIS (SEQ ID NO: 7); FW3a is GRYVESV (SEQ ID NO: 27); HV4 is NKRTM (SEQ ID NO: 10); FW3b is SFSRLRIKDLTVADSATYYCKA (SEQ ID NO: 29); CDR3 is YPWGAGAPWLQWY (SEQ ID NO: 18); and FW4 is DGAGTVLTVN (SEQ ID NO: 32); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is TRVDQSPSSLSASVGDRVITIC VLT (SEQ ID NO: 24); CDR1 is GANYGLAS (SEQ ID NO: 5); FW2 is TYWYRKNPG (SEQ ID NO: 26); HV2 is SSNKEQISIS (SEQ ID NO: 8); FW3a is GRYSESV (SEQ ID NO: 28); HV4 is NKGTK (SEQ ID NO: 12); FW3b is SFTLTISLQPEDSATYYCRA (SEQ ID NO: 30); CDR3 is YPWGAGAPWLQWY (SEQ ID NO: 18); and FW4 is DGAGTKVEIK (SEQ ID NO: 33); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is TRVDQSPSSLSASVGDRVTITCVLT (SEQ ID NO: 24); CDR1 is GANYGLAS (SEQ ID NO: 5); FW2 is TYWYRKNP (SEQ ID NO: 26); HV2 is SSNQERISIS (SEQ ID NO: 7); FW3a is GRYSESV (SEQ ID NO: 28); HV4 is NKRTM (SEQ ID NO: 10); FW3b is SFTLTISLQPEDSATYYCRA (SEQ ID NO: 30); CDR3 is YPWGAGAPWLVQWY (SEQ ID NO: 18); and FW4 is DGAGTKVEIK (SEQ ID NO: 33); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is TRVDQSPSSLSASVGDRVTITCVLT (SEQ ID NO: 24); CDR1 is DTSYGLYS (SEQ ID NO: 1); FW2 is TSWFRKNP (SEQ ID NO: 25); HV2 is TTDWERMISG (SEQ ID NO: 6); FW3a is GRYVESV (SEQ ID NO: 27); HV4 is NKGAK (SEQ ID NO: 11); FW3b is SFTLTISLQPEDFATYYCKA (SEQ ID NO: 31); CDR3 is REARHPWLRQWY (SEQ ID NO: 17); and FW4 is DGAGTKVEIK (SEQ ID NO: 33); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is TRVDQSPSSLSASVGDRVTITCVLT (SEQ ID NO: 24); CDR1 is DTSYGLYS (SEQ ID NO: 1); FW2 is TYWYRKNP (SEQ ID NO: 26); HV2 is SSNKEQISIS (SEQ ID NO: 8); FW3a is GRYSESV (SEQ ID NO: 28); HV4 is NKGTK (SEQ ID NO: 12); FW3b is SFTLTISLQPEDSATYYCRA (SEQ ID NO: 30); CDR3 is REARHPWLRQWY (SEQ ID NO: 17); and FW4 is DGAGTKVEIK (SEQ ID NO: 33); or functional variants thereof with a sequence identity of at least 45%.

In another preferred embodiment of the ROR1-specific antigen binding molecule, FW1 is TRVDQSPSSLSASVGDRVTITCVLT (SEQ ID NO: 24); CDR1 is DTSYGLYS (SEQ ID NO: 1); FW2 is TYWYRKNP (SEQ ID NO: 26); HV2 is TTDWERMISG (SEQ ID NO: 6); FW3a is GRYSESV (SEQ ID NO: 28); HV4 is NKGAK (SEQ ID NO: 11); FW3b is SFTLTISLQPEDSATYYCRA (SEQ ID NO: 30); CDR3 is REARHPWLRQWY (SEQ ID NO: 17); and FW4 is DGAGTKVEIK (SEQ ID NO: 33); or functional variants thereof with a sequence identity of at least 45%.

In yet further preferred embodiments, the ROR1-specific antigen binding molecule comprises an amino acid sequence selected from:

ASVNQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMISGGRYVESVNKRAKSFSLRIKDLTVADSATYYCKAQSGMAISTGSGHGYNWYDGAGTVLTVN (SEQ ID NO: 39);
AKVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMISGGRYVESVNKRAKSFSLRIKDLTVADSATYYCKAQSGMAIDIGSGHGYNWYDGAGTVLTVN (SEQ ID NO: 40);
TRVDQTPRTATKETGESLTINCVTGAKYGLAATYWYRKNPSSNQERISISGRYVESVNKRTMSFSLRIKDLTVADSATYYCKAYPWAMWGQWYDGAGTVLTVN (SEQ ID NO: 41);
TRVDQTPRTATKETGESLTINCVTGAKYGLFATYWYRKNPSSNQERISISGRYVESVNKRTMSFSLRIKDLTVADSATYYCKAVFMPQHWHPAAHWYDGAGTVLTVN (SEQ ID NO: 42);
TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMISGGRYVESVNKGAKSFS LRIKDLTVADSATYYCKAREARHPWLRQWYDGAGTVLTVN (SEQ ID NO: 43);

ASVNQTPRTATKETGESLTINC VVTGANYGLAATYWYRK NPGSSNQERISISGRYVESVNKRTMSFSL
 RIKDLTVADSATYYCKAYPWGAGAPWL VQWYDGAGTVLTVN (SEQ ID NO: 44);
 TRVDQSPSSLSASVGDRVITITCVLTGANYGLASTYWYRK NPGSSNKEQISISGRYSESVNKGTKSFTL
 TISSLQPEDSATYYCRAYPWGAGAPWL VQWYDGAGTKVEIK (SEQ ID NO: 45);
 TRVDQSPSSLSASVGDRVITITCVLTGANYGLASTYWYRK NPGSSNQERISISGRYSESVNKR TMSFTL
 TISSLQPEDSATYYCRAYPWGAGAPWL VQWYDGAGTKVEIK (SEQ ID NO: 46);
 TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFT
 LTISSLQPEDFATYYCKAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 47);
 TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTYWYRK NPGSSNKEQISISGRYSESVNKGTKSFTL
 TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 48);
 TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTYWYRK NPGTTDWERMSIGGRYSESVNKGAKSFT
 LTISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 49), or a functional variant
 thereof with a sequence identity of at least 45%.

The EGFR-specific antigen binding molecule may be any molecule which binds to EGFR. In particular, the EGFR-specific antigen binding molecule may be selected from the group comprising an immunoglobulin, an immunoglobulin Fab region, a single chain Fv (scFv), a diabody, a triabody, a tetrabody, a bispecific t-cell engager (BiTE), an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, or a scaffold protein (affibodies, centyrins, darpins etc.). Preferably, the EGFR-specific antigen binding molecule is a single domain antibody (sdAb).

Cetuximab is an approved monoclonal antibody therapeutic that inhibits epidermal growth factor receptor (EGFR). Cetuximab prevents EGF and other ligands binding EGFR and otherwise activating EGFR (i.e. prevents the extended receptor conformation required for high-affinity ligand binding and dimerization) [Li 2005 Cancer Cell 7 301-311]. Cetuximab binds to a specific epitope within EGFR domain comprising amino acids 384-408.

7C12 and 7D12 are camelid single domain antibodies (nanobodies) that compete for the Cetuximab epitope on EGFR [WO 2007042289 A2]. Both 7C12 and 7D12 demonstrate high affinity EGFR binding (low nM KD) [Roovers 2011 Int J Cancer 129 p2013, Gainkam 2010 Mol Imaging] and block EGF binding to EGFR [Schmitz 2013 Structure 21 p1214]. 7C12 and 7D12 differ by 5 amino acids with 7C12 having a higher off rate for EGFR binding [Roovers 2011 Int J Cancer 129 p2013]

Matuzumab is another approved monoclonal antibody therapeutic that inhibits EGFR. Matuzumab binding sterically blocks the EGFR domain rearrangement required for high affinity ligand binding and receptor dimerization [Schmiedel 2008 Cancer Cell 13(4) 365-373]. Matuzumab binds primarily to the loop preceding the most C-terminal strand of the domain III β -helix (aa 454-464 of EGFR).

9G8 is a sdAb (nanobody) sequence that, although competing for the Matuzumab EGFR epitope [WO 2007042289 A2] has a distinct EGFR epitope, further towards the N terminus of EGFR domain III and

further from the domain II ligand binding site, regions inaccessible to conventional antibodies [Schmitz 2013 Structure 21 p1214].

Examples of sdAbs for use in the bi-specific antigen binding molecule of the first aspect of the invention include but are not limited to molecules that compete for binding with cetuximab or matuzumab. Preferably, the sdAb is selected from the group comprising:

7C12:

AVQLVESGGGSVQAGGSLRLTCAASGRTSRSYGMWFRQAPGKEREFVSGISWRGDSTGYADSVK
GRFTISRDNKNTVDLQMNSLKPEDTAIYYCAAAAGSTWYGTLYEYDYWGQGTQVTVSS (SEQ ID

NO: 83)

7D12:

QVKLEESGGGSVQTGGSLRLTCAASGRTSRSYGMWFRQAPGKEREFVSGISWRGDSTGYADSVK
GRFTISRDNKNTVDLQMNSLKPEDTAIYYCAAAAGSAWYGTLYEYDYWGQGTQVTVSS (SEQ ID

NO: 84)

9G8:

EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREFVVAIWSSGSTYYADSVK
GRFTISRDNKNTMYLQMNSLKPEDTAVYYCAAGYQINSGNYNFKDYDYWGQGTQVTVSS (SEQ

ID NO: 85)

38G7:

EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYVMGWFRQATGKEREFVATIAWDSGSTYYADSVKG
RFTISRDNKNTVHLQMNSLKPEDTAVYYCAASYNVYYNNYYYPISRDEYDYWGQGTQVTVSS (SEQ

ID NO: 86)

It will be appreciated that the ROR1-specific antigen binding molecule and EGFR-specific antigen binding molecule may be combined in any order to form the bi-specific antigen binding molecule of the first aspect, i.e., the ROR1-specific antigen binding molecule may be N-terminal to the EGFR-specific antigen binding molecule or vice versa.

Furthermore, it will be appreciated that higher-order constructs are also contemplated herein, for example constructs composed of multiple ROR1-specific antigen binding molecule and EGFR-specific antigen binding molecules. These may take the form of multiple copies in a single primary amino acid sequence, for example ROR1 binder-EGFR binder-ROR1 binder or EGFR binder-ROR1 binder-EGFR binder.

The bi-specific antigen binding molecule of the first aspect may additionally include a linker region between the ROR1-specific antigen binding molecule and EGFR-specific antigen binding molecule. Preferred linkers include but are not limited to $[G_4S]_x$, where x is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. A particular preferred linker is $[G_4S]_5$. Other linkers may include, but are not limited to PGVQPSPGGGGS (SEQ ID NO: 63) (Wobbe-G4S), PGVQPAPGGGGS (SEQ ID NO: 64) (Wobbe-

G4S GM). It will be appreciated that different combinations of different linkers can be combined within the same construct

The bi-specific antigen binding molecule of the first aspect may also comprise additional domains, which may take the form of N-terminal or C-terminal additions or may be placed between the ROR1-specific antigen binding molecule and EGFR-specific antigen binding molecule in the amino acid sequence of the bi-specific binding molecule. Each domain of the bi-specific antigen binding molecule of the first aspect may be connected via linker regions as described above. Preferred additional domains include, but are not limited to an immunoglobulin, an immunoglobulin Fc region, an immunoglobulin Fab region, a single chain Fv (scFv), a diabody, a triabody, a tetrabody, a bispecific t-cell engager (BiTE), an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, or a scaffold protein (affibodies, centyrins, darpins etc.). A particularly preferred additional domain is an immunoglobulin Fc region, preferably a human Fc region.

Combinations expressly contemplated in the present application include, but are not limited to ::

Monovalent ROR1xEGFR (Fc fusion) bi-specifics

B1-Fc-7C12	P3A1-Fc-9G8	E9-Fc-7C12
7C12-Fc-B1	9G8-Fc-P3A1	7C12-Fc-E9
B1-Fc-9G8	D3-Fc-7C12	E9-Fc-9G8
9G8-Fc-B1	7C12-Fc-D3	9G8-Fc-E9
P3A1-Fc-7C12	D3-Fc-9G8	
7C12-Fc-P3A1	9G8-Fc-D3	

Divalent ROR1xEGFR (Fc fusion) bi-specifics

B1-B1-Fc-7C12	P3A1-7C12-Fc-P3A1	7C12-D3-Fc-D3
B1-7C12-Fc-B1	7C12-P3A1-Fc-P3A1	7C12-7C12-Fc-D3
7C12-B1-Fc-B1	7C12-7C12-Fc-P3A1	7C12-D3-Fc-7C12
7C12-7C12-Fc-B1	7C12-P3A1-Fc-7C12	D3-7C12-Fc-7C12
7C12-B1-Fc-7C12	P3A1-7C12-Fc-7C12	7C12-Fc-D3-D3
B1-7C12-Fc-7C12	7C12-Fc-P3A1-P3A1	D3-Fc-7C12-7C12
7C12-Fc-B1-B1	P3A1-Fc-7C12-7C12	7C12-Fc-D3-7C12
B1-Fc-7C12-7C12	7C12-Fc-P3A1-7C12	7C12-Fc-7C12-D3
7C12-Fc-B1-7C12	7C12-Fc-7C12-P3A1	D3-Fc-7C12-D3
7C12-Fc-7C12-B1	P3A1-Fc-7C12-P3A1	D3-Fc-D3-7C12
B1-Fc-7C12-B1	P3A1-Fc-P3A1-7C12	E9-E9-Fc-7C12
B1-Fc-B1-7C12	D3-D3-Fc-7C12	E9-7C12-Fc-E9
P3A1-P3A1-Fc-7C12	D3-7C12-Fc-D3	7C12-E9-Fc-E9

7C12-7C12-Fc-E9	7C12-Fc-E9-E9	7C12-Fc-7C12-E9
7C12-E9-Fc-7C12	E9-Fc-7C12-7C12	E9-Fc-7C12-E9
E9-7C12-Fc-7C12	7C12-Fc-E9-7C12	E9-Fc-E9-7C12

Monovalent ROR1xEGFR (non-Fc) bi-specifics

B1-7C12	9G8-P3A1	E9-7C12
7C12-B1	P3A1-9G8	7C12-E9
9G8-B1	D3-7C12	9G8-E9
B1-9G8	7C12-D3	E9-9G8
P3A1-7C12	9G8-D3	
7C12-P3A1	D3-9G8	

Divalent ROR1xEGFR (non-Fc) bi-specifics

B1-B1-7C12	7C12-P3A1-P3A1	D3-9G8-D3
B1-7C12-B1	P3A1-P3A1-9G8	9G8-D3-D3
7C12-B1-B1	P3A1-9G8-P3A1	E9-E9-7C12
B1-B1-9G8	9G8-P3A1-P3A1	E9-E9-D3
B1-9G8-B1	D3-D3-7C12	7C12-E9-E9
9G8-B1-B1	D3-7C12-D3	E9-E9-9G8
P3A1-P3A1-7C12	7C12-D3-D3	E9-9G8-E9
P3A1-7C12-P3A1	D3-D3-9G8	9G8-E9-E9

Monovalent ROR1, half life extended ROR1xEGFR (non-Fc) bi-specifics

B1-BA11-7C12	BA11-P3A1-7C12	7C12-D3-BA11
B1-7C12-BA11	BA11-7C12-P3A1	7C12-BA11-D3
BA11-B1-7C12	7C12-P3A1-BA11	D3-BA11-9G8
BA11-7C12-B1	7C12-BA11-P3A1	D3-9G8-BA11
7C12-B1-BA11	P3A1-BA11-9G8	BA11-D3-9G8
7C12-BA11-B1	P3A1-9G8-BA11	BA11-9G8-D3
B1-BA11-9G8	BA11-P3A1-9G8	9G8-D3-BA11
B1-9G8-BA11	BA11-9G8-P3A1	9G8-BA11-D3
BA11-B1-9G8	9G8-P3A1-BA11	E9-BA11-7C12
BA11-9G8-B1	9G8-BA11-P3A1	E9-7C12-BA11
9G8-B1-BA11	D3-BA11-7C12	BA11-E9-7C12
9G8-BA11-B1	D3-7C12-BA11	BA11-7C12-E9
P3A1-BA11-7C12	BA11-D3-7C12	7C12-E9-BA11
P3A1-7C12-BA11	BA11-7C12-D3	7C12-BA11-E9

E9-BA11-9G8

BA11-E9-9G8

9G8-E9-BA11

E9-9G8-BA11

BA11-9G8-E9

9G8-BA11-E9

Where the linkers between domains are preferentially, but not limited to $(G_4S)_x$, where X is 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, PGVQPSPGGGGS (SEQ ID NO: 63) (Wobbe-G4S), PGVQPAPGGGGS (SEQ ID NO: 64) (Wobbe-G4S GM) and wherein different combinations of different linkers can be combined within the same construct.

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: 68)) 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: 69)

QACGAHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 67)

QACKAHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 70)

AAAHHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 71)

ACAHHHHHHHGAEFEQKLISEEDL (SEQ ID NO: 72)

QASGAHHHHHH (SEQ ID NO: 73)

QACGAHHHHHH (SEQ ID NO: 74)

QACKAHHHHHH (SEQ ID NO: 75)

AAAHHHHHHH (SEQ ID NO: 76)

ACAHHHHHHH (SEQ ID NO: 77)

QASGA (SEQ ID NO: 78)

QACGA (SEQ ID NO: 79)

QACKA (SEQ ID NO: 80)

ACA (SEQ ID NO: 81)

SAPSA (SEQ ID NO: 82)

Domains may also be combined via N-terminal, C-terminal or both N- and C-terminal fusion to an Fc domain, including but not limited to:

hIgG1

EPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 87)

hlgG1 (S252C)

EPKSSDKTHTCPPCPAPELLGGPCVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 88)

hlgG1 (S473C)

EPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 89)

Wherein:

- 7C12 is
AVQLVESGGGSVQAGGSLRLTCAASGRTSRSYGMGWFQRQAPGKEREFVSGISWRGDSTGYADSVK
GRFTISRDNKNTVDLQMNLSLKPEDTAIYYCAAAGSTWYGTLYEYDYWGQGTQVTVSSAAHHHH
HHGAEFEQKLISEEDL (SEQ ID NO: 90)
- 9G8 is
MEVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFQRQAPGKEREFVAINWSSGSTYYADSV
KGRFTISRDNKNTMYLQMNLSLKPEDTAVYYCAAGYQINSGNYNFKDYEYDYWGQGTQVTVSSAA
HHHHHHGAEFEQKLISEEDL (SEQ ID NO: 91)
- B1 is
ASVNQTPRTATKETGESLTINCVTGANYGLAATYWYRKNPGSSNQERISISGRYVESVNKRTMSFSL
RIKDLTVADSATYYCKAYPWGAGAPWLQWYDGAGTVLTVN (SEQ ID NO: 44)
- 2V is
TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKAQSLAISTRSYWYDGAGTVLTVN (SEQ ID NO: 65)
- P3A1 is
TRVDQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKGAKSFS
LRIKDLTVADSATYYCKAREARHPWLRQWYDGAGTVLTVN (SEQ ID NO: 43)
- D3 is
ASVNQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKRAKSFS
LRIKDLTVADSATYYCKAQSGMAISTGSGHGYNWDGAGTVLTVN (SEQ ID NO: 39)
- D3D3 is
ASVNQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKRAKSFS
LRIKDLTVADSATYYCKAQSGMAISTGSGHGYNWDGAGTVLTVNNGGGSGGGSGGGSGGGG
SGGGGSASVNQTPRTATKETGESLTINCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVN
KRAKSFS LRIKDLTVADSATYYCKAQSGMAISTGSGHGYNWDGAGTVLTVN (SEQ ID NO: 92)
- BA11 is
TRVDQSPSSLSASVGDRTITCVLTDTSYPLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAMSTNIWTGDGAGTKVEIK (SEQ ID NO: 66)

It will be clear to those of skill in the relevant art that bi-specific antigen binding molecules comprising additional domains as described herein may, in some situations, include additional specificity beyond ROR1 and EGFR. Such configurations are also within the scope of the present invention.

The ROR1-specific antigen binding molecule may be humanized. The ROR1-specific antigen binding molecule may be de-immunized. Examples of humanised sequences of the invention include, but are not limited to:

B1 G1

TRVDQSPSSLSASVGDRVITITCVLTGANYGLASTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 45);

B1 G2

TRVDQSPSSLSASVGDRVITITCVLTGANYGLASTYWYRKNPGSSNQERISISGRYSESVNKRTMSFTL
TISSLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 46);

P3A1 V1

TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSSIGGRYVESVNKGAKSFT
LTISSLQPEDFATYYCKAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 47);

P3A1 G1

TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTYWYRKNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 48);

P3A1 G2

TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTYWYRKNPGTTDWERMSSIGGRYSESVNKGAKSFT
LTISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 49);

D3 humanised ADV1

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSSIGGRYSESVNKGAKSFT
LTISSLQPEDSATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 50);

D3 humanised ADV2

TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSSIGGRYSESVNKGAKSFT
LTISSLQPEDSATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 51);

D3 humanised ADV3

ASVNQSPSSASASVGDRLTITCVLTDTSYGLYSTSWFRKNPGTTDWERMSSIGGRYSESVNKGAKSFT
LTISSLQPEDSATYYCKAQSGMAISTGSGHGYNWYDGAGTKLEVK (SEQ ID NO: 52);

B1 humanised V5

ASVDQSPSSLSASVGDRVITITCVVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRTMSFTL
TISSLQPEDSATYYCKAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 53);

B1 humanised V7

ASVDQSPSSASASVGDRLTITCVVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRTMSFTL
TISSLQPEDSATYYCKAYPWGAGAPWLQWYDGAGTKLEVK (SEQ ID NO: 54);

D3 humanised EL V1

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKRAKSFS
LRIKDLTVADSATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 55);

D3 humanised EL V2

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKRAKSFT
LTISSLQPEDFATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 56);

D3 humanised EL V3

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRFSGSGSKRAKSFT
LTISSLQPEDFATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 57);

D3 humanised EL V4

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWYQQKPGTTDWERMSIGGRYVESVNKRAKSFT
LTISSLQPEDFATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 58); and

D3 humanised EL V5

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWYQQKPGTTDWERMSIGGRFSGSGSKRAKSFT
LTISSLQPEDFATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 59).

The EGFR-specific antigen binding molecule may be humanised or de-immunised, independently of the humanisation or de-immunization of the ROR1-specific antigen binding molecule.

The bi-specific antigen binding molecule of the present invention may also be conjugated to a detectable label, dye, toxin, drug, pro-drug, radionuclide or biologically active molecule. The conjugation may be via either the ROR1-specific antigen binding molecule and EGFR-specific antigen binding molecule. Additionally, conjugation may be via sequences or domains fused to the ROR1-specific antigen binding molecule and EGFR-specific antigen binding molecule. In particular, conjugation is via Fc domains or short thiol containing sequences fused to the ROR1-specific antigen binding molecule and EGFR-specific antigen binding molecule.

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.

Furthermore, the bi-specific antigen binding molecule is preferably capable of mediating killing of ROR1-expressing tumour cells or is capable of inhibiting cancer cell proliferation. In addition, the bi-specific antigen binding molecule is preferably capable of mediating killing of EGFR-expressing tumour cells or is capable of inhibiting cancer cell proliferation. Furthermore, the bi-specific antigen binding molecule is preferably capable of mediating killing of tumour cells expressing both ROR1 and EGFR or is capable of inhibiting proliferation of such cells.

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

The bi-specific antigen binding molecule may also be capable of down-regulating cell-surface levels or total protein levels of ROR1 or EGFR upon binding to ROR1 and / or EGFR. Furthermore, the bi-specific antigen binding molecule may also be capable of down regulating ROR1 or EGFR signalling. The bi-specific antigen binding molecule may also be capable of down regulating ROR1 and EGFR signalling

The components of the bi-specific antigen binding molecule may be connected 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, 6, 7, 8, 9, or 10. Particular preferred linkers are $[G_4S]_3$ (SEQ ID NO: 60) and $[G_4S]_5$ (SEQ ID NO: 61). Other preferred linkers include the sequences PGVQPSP (SEQ ID NO: 62), PGVQPSPGGGGS (SEQ ID NO: 63) and PGVQPAPGGGGS (SEQ ID NO: 64). These linkers may be particularly useful when 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*).

It will also be appreciated that the bi-specific antigen binding molecule of the invention can be constructed in any order, i.e., with the ROR1-specific antigen binding molecule at the N-terminus or C-terminus

In a second aspect of the present invention, there it is provided a recombinant fusion protein comprising a bi-specific antigen binding molecule of the first aspect. Preferably, in the recombinant fusion protein of the second aspect, the bi-specific antigen binding molecule is fused to one or more biologically active proteins. The bi-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, 6, 7, 8, 9, or 10. Particular preferred linkers are $[G_4S]_3$ (SEQ ID NO: 60) and $[G_4S]_5$ (SEQ ID NO: 61). Other preferred linkers include the sequences PGVQPSP (SEQ ID NO: 62), PGVQPSPGGGGS (SEQ ID NO: 63) and PGVQPAPGGGGS (SEQ ID NO: 64). 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*).

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). Furthermore, fusion proteins in which the ROR1 and EGFR components of the bi-specific antigen binding molecule are separated by the biologically

active protein are described. A non-limiting example would be the presence of an Fc domain between the two components of the bi-specific antigen binding molecule. Depending on the exact configuration desired, one or more linker domains as described herein may be included.

Preferred biologically active proteins include, but are not limited to an immunoglobulin, an immunoglobulin Fc region, an immunoglobulin Fab region, a single chain Fv (scFv), a diabody, a triabody, a tetrabody, a bispecific t-cell engager (BiTE), 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.

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 accordance with the second 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.

Also in accordance with the second aspect, recombinant fusions are provided which include a bi-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 (Kovalenko et al, J.Biol. Chem., 2013 JBC).

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 such molecules with a variety of N- or C-terminal tags, which tags may also include additional cysteines for conjugation.

Also in accordance with the second aspect, recombinant fusions are provided which include a bi-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.

In a third aspect of the present invention, there is provided a chimeric antigen receptor (CAR), comprising at least one bi-specific antigen binding molecule as defined by the first aspect of the invention, fused or conjugated to at least one transmembrane region and at least one intracellular domain.

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

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

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

A method for preparing a bi-specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor, of the first, second or third 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 bi-specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor, optionally further comprising isolating the bi-specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor.

In a fifth aspect of the invention, there is provided a pharmaceutical composition comprising the bi-specific antigen binding molecule, fusion protein or chimeric antigen receptor of the first, second or third 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 bi-specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of the first, second or third aspects may be for use in therapy. More specifically, the bi-specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of the first, second or third 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, stomach cancer or liver cancer.

Also provided herein is the use of a bi-specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of the first, second or third 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 bi-specific antigen binding molecule, recombinant fusion protein or chimeric antigen receptor of the first, second or third aspects or a pharmaceutical composition of the fifth 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, 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 bi-specific antigen binding molecule of the first aspect, or a recombinant fusion protein of the second 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 bi-specific antigen binding molecule of the first aspect or a detectably labelled recombinant fusion protein of the second 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 bi-specific antigen binding molecule of the first aspect or a recombinant fusion protein of the second 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 aspect.

Also contemplated herein is an antibody, antibody fragment or antigen-binding molecule that competes for binding to ROR1 and EGFR with the bi-specific antigen binding molecule of the first 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., the bi-specific antigen binding molecule of the first aspect) to a common antigen (e.g., ROR1 or EGFR in the case of the bi-specific antigen binding molecule of the first 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 bi-specific antigen binding molecule of the first aspect, a recombinant fusion protein of the second aspect, a chimeric antigen receptor of the third aspect or a nucleic acid sequence of the fourth 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 bi-specific antigen binding molecule of the first aspect, a recombinant fusion protein of the second aspect, a chimeric antigen receptor of the third aspect or a nucleic acid sequence of the fourth aspect, each being optionally derivatized, and wherein presence of antigen in the sample suggests that the subject suffers from cancer.

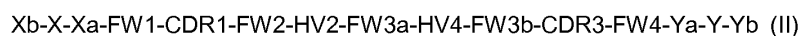
Also contemplated herein is a method of killing or inhibiting the growth of a cell expressing ROR1 in vitro or in a patient, which method comprises administering to the cell a pharmaceutically effective amount or dose of (i) bi-specific antigen binding molecule of the first aspect, a recombinant fusion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

Also contemplated herein is a method of killing or inhibiting the growth of a cell expressing EGFR in vitro or in a patient, which method comprises administering to the cell a pharmaceutically effective amount or dose of (i) bi-specific antigen binding molecule of the first aspect, a recombinant fusion protein of the second aspect, a nucleic acid of the third aspect, or the CAR or cell according to the cell

aspect, or (ii) of a pharmaceutical composition according to the fifth aspect. Preferably, the cell expressing EGFR is a cancer cell.

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

In a sixth aspect of the present invention, there is provided a bi-specific antigen binding molecule comprising an amino acid sequence represented by the formula (II):



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

wherein Xa, Xb, Ya and Yb are either absent or an EGFR-specific binding molecule, wherein at least one of Xa, Xb, Ya and Yb is an EGFR-specific binding molecule,

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 bi-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, an immunoglobulin Fab region, a single chain Fv (scFv), a diabody, a triabody, a tetrabody, a bispecific t-cell engager (BiTE), an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, or 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).

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.

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

- maytansinoids,
- auristatins,
- anthracyclins, preferably PNU-derived anthracyclins
- calicheamicins,
- amanitin derivatives, preferably α -amanitin derivatives
- tubulysins
- duocarmycins
- radioisotopes for example alpha-emitting radionuclide, such as ^{227}Th or ^{225}Ac
- liposomes comprising a toxic payload,
- protein toxins
- taxanes,
- pyrrolbenzodiazepines
- indolinobenzodiazepine pseudodimers and/or

- spliceosome inhibitors
- CDK11 inhibitors
- Pyridinobenzodiazepines

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

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 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.

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 as described above in relation to the first aspect of the invention. Accordingly, any of the preferred features described above in relation to the first, second and third aspects apply *mutatis mutandis* to the sixth aspect.

Preferably, the EGFR-specific antigen binding molecule is selected from the group comprising an immunoglobulin, an immunoglobulin Fab region, a single chain Fv (scFv), a diabody, a triabody, a tetrabody, a bispecific t-cell engager (BiTE), an intein, a VNAR domain, a single domain antibody (sdAb), a VH domain, or a scaffold protein (affibodies, centyrins, darpins etc.).

The bi-specific antigen binding molecule of the sixth aspect may be for use in therapy. More specifically, the bi-specific antigen binding molecule of the sixth 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, stomach cancer or liver cancer.

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

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

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 bi-specific antigen binding molecule of the sixth aspect or a pharmaceutical composition comprising a specific antigen binding molecule of the sixth aspect.

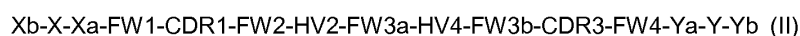
Preferably, the cancer is a ROR1-positive cancer type. More preferably, the cancer is selected from the group comprising blood cancers such as lymphomas and leukemias, chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), B-cell acute lymphoblastic leukaemia (B-ALL), marginal zone lymphoma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

In a seventh aspect of the present invention, there is provided there is provided a bi-specific antigen binding molecule comprising an amino acid sequence represented by the formula (II):



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

wherein Xa, Xb, Ya and Yb are either absent or an EGFR-specific binding molecule,
wherein at least one of Xa, Xb, Ya and Yb is an EGFR-specific binding molecule,

X and Y are optional amino acid sequences.

All features described in relation to the sixth aspect of the invention apply *mutatis mutandis* to the molecule of the seventh aspect.

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.

DESCRIPTION OF FIGURES

Figure 1: anti-ROR1 phage monoclonals displaying VNAR domains: binding to human or mouse recombinant ROR1-Fc in ELISA. B1, P3A1 and E7- specific ROR1 binders, H2 – non-specific phage.

Figure 2: ROR1 binding sequences obtained from screening the synthetic VNAR library using human ROR1 (B1 and E7) and mouse ROR1 (P3A1 and CPF7). Sequences shown without and with the C-terminal His₆Myc tag (His₆ Myc sequence in italics).

Figure 3: Generation of the immunised VNAR library using human ROR1: analysis of three spiny dogfish pre- and post-immunisation plasma binding to murine or human ROR1.

Figure 4: anti-ROR1 phage monoclonals from immunised VNAR library: binding to human or mouse recombinant ROR1-Fc in ELISA. E9 and D3 - specific ROR1 binders, H1 – non-specific VNAR binder displayed on phage.

Figure 5: ROR1 binding sequences E9 and D3 obtained from screening the immunised VNAR library using mouse ROR1. Sequences shown without and with the C-terminal His₆Myc tag (His6 Myc sequence in italics).

Figure 6: Far UV CD spectra of VNAR no tag, VNAR 6xHis and VNAR- His₆-Myc in 50mM NaCl 20mM NaP buffer pH 6.0 at room temperature.

Figure 7: VNAR reformatting A: monomeric VNAR, B: homodimers, C: conjugated homodimers via C-terminal intermolecular disulphide bond, D: heterodimers, E: VNAR IgG Fc fusions, F: IgG Fc – VNAR fusions, G: VNAR- (IgG Fc) – VNAR fusions.

Figure 8: Binding of B1 C-terminally linked homodimer to hROR1. B1, B1 C-terminal thiol (B1 SH) and B1 C-terminal disulphide dimer (B1 S-S B1) binding to human ROR1 by ELISA.

Figure 9: Cell surface binding of VNAR (His₆Myc tag) molecules to A549 (ROR1^{hi}) lung cancer cells by flow cytometry. B1 and E7 monomers and P3A1-P3A1 dimer bind strongly to A549 cells at all concentrations tested. CPF7 and P3A1 monomers bind at 50µg/ml to A549 cells. VNAR binding was detected using PE-anti Myc tag Ab (CST) and analysed using a BD Biosciences FACSCalibur flow cytometer.

Figure 10: Linker mouse IgG and linker human IgG sequences used in VNAR IgG Fc fusion proteins. Engineered hIgG1 Fc fusion proteins incorporate an engineered cysteine substitution in the hIgG1 Fc sequence, for example at position S252C (Kabat numbering) to enable site specific labelling.

Figure 11: Intein cleavage reagents and the corresponding VNAR C-terminal derivatives.

Figure 12: VNAR binding to human, mouse and rat ROR1 and human ROR2 by ELISA. All VNARs were found to be species cross-reactive to ROR1. None of the VNAR clones cross-reacted with human ROR2.

Figure 13: VNAR cell surface binding to A549 (ROR1^{hi}) vs A427 (ROR1^{low}) lung cancer cell lines by flow cytometry. VNAR binding was detected using a PE-anti-Myc Ab (CST) and a ThermoFisher Attune NxT flow cytometer.

Figure 14: Cell surface binding of VNARs to MDA-MB-231 breast cancer cells for 2 hrs at 4°C or 37°C. Loss of cell surface signal at 37°C is suggestive of ROR1 internalisation. VNAR binding was detected using PE-anti Myc tag Ab (CST) and analysed using a BD Biosciences FACS Calibur (B1) or a ThermoFisher Attune NxT flow cytometer.

Figure 15: Bar chart depicting VNAR-hFc molecule cell surface binding to A549 (ROR1^{hi}) vs A427 (ROR1^{low}) lung cancer cell lines. VNAR hFc binding was detected using a PE-anti-human antibody (Jackson ImmunoResearch Labs/Strattech) and a ThermoFisher Attune NxT flow cytometer.

Figure 16: Internalisation of VNAR-Fc fusions. Cell surface binding of VNAR-Fc to MDA-MB-231 breast cancer cells for 2 hrs at 4°C or 37°C. Loss of cell surface signal at 37°C is suggestive of ROR1 internalisation. VNAR-Fc binding was detected using a PE-anti-human antibody (JacksonImmunoResearch) and a ThermoFisher Attune NxT flow cytometer.

Figure 17: VNARs bind to human ROR1 independent of glycosylation. A, SDS PAGE analysis of hROR1 (lane 2) and deglycosylated hROR1 (lane 3). Mwt markers (lane 1). B, ROR1 binding VNARs B1, P3A1-P3A1 and D3-D3 bind equally well to deglycosylated hROR1 by ELISA. C, B1 mFc binds equally well to glycosylated and deglycosylated hROR1 by ELISA. Binding to unfolded hROR1 (reduced with 28mM DTT, 0.5% Sarkosyl) was significantly reduced, consistent with B1 VNAR binding to conformational epitope(s).

Figure 18: B1 forms a complex with ROR1 Ig domain by SEC. A, Overlayed SEC analysis (Superdex 200 Increase 10/300, GE Healthcare) of human ROR1 Ig domain with and without B1 his (orange and blue traces, respectively). B, SDS PAGE analysis of peak fractions.

Figure 19: SPR sensograms depicting binding of VNARs to hROR1 +/- previously captured B1 His₆Myc VNAR. 2V monomer or dimer did not bind under any of these conditions.

Figure 20: B1 and P3A1 do not bind to selected linear ROR1 peptides by ELISA. Binding to human ROR1 is included as a positive control.

Figure 21: B1, P3A1, D3 and D3-D3 do not bind to selected linear ROR1 peptides by ELISA. Binding to human ROR1 is included as a positive control.

Figure 22: Competition ELISA experiments.

Figure 23: Competition ELISA experiments.

Figure 24: Binding of B1, P3A1, D3 monomer and D3-D3 dimer to different ROR1 domains.

Figure 25: Schematic of BA11 aminoxy conjugation to benzaldehyde fluorescein.

Figure 26: Schematic of BA11 thiol conjugation to maleimide fluorescein.

Figure 27: Schematic of BA11 C-terminal cysteine derivative conjugation to maleimide fluorescein

Figure 28: Examples of labels and payloads used for conjugation.

Figure 29: Analysis of B1 MMAE conjugates. A, SDS PAGE analysis of B1 his myc derivatives and conjugates - lanes 1, B1 aminoxy; 2, B1 oxime MMAE; 3, B1 oxime vc MMAE; 4, B1 SH vc MMAE. B-F, electrospray mass spectra of B1 his myc derivatives and conjugates – B, B1 SH (expected mass; 14908.9 Da, observed mass 14908.4 Da); C, B1 SH vc MMAE (expected mass 16225.5 Da, observed mass 16225.5 Da); D, B1 aminoxy (expected mass 14937.4 Da, observed mass 14936.5 Da); E, B1 oxime MMAE (expected mass 16015.4 Da, observed mass 16016.7 Da); F, B1 oxime vc MMAE (expected mass 16334.4 Da, observed mass 16334.2 Da).

Figure 30: Cell surface binding of B1-, P3A1- and 2V- hFc molecules vs the MMAE-conjugated versions in A549 (ROR1^{hi}) vs A427 (ROR1^{low}) lung cancer cell lines. VNAR hFc binding was detected using a PE-anti-human antibody (Jackson ImmunoResearch Labs/Strattech) and a ThermoFisher Attune NxT flow cytometer.

Figure 31: Analysis of VNAR hFc conjugates. A&B, SDS PAGE analysis of VNAR hFc (S252C) proteins and conjugates (4-12% and 12% Bis Tris gel, respectively). Lanes 1, untreated protein, 2, refolded protein and 3, MMAE conjugate (+/- reduction with DTT). C & D, Example of mass spec analysis of deglycosylated, reduced VNAR hFc (S252C) fusion proteins before and after MMAE conjugation, respectively. Expected masses: unconjugated 38,997.8 Da and MMAE conjugate (DAR 2) 40,310.0 Da. E&F SDS PAGE analysis of VNAR hFc (S473C) protein conjugates. Lanes 3, MMAE conjugates and 4, AF488 conjugates (+/- reduction with DTT). G&H Mass spec analysis of deglycosylated, reduced B1- and P3A1 hFc (S473C) MMAE conjugates, respectively. Expected masses: B1 conjugate 40,170.5 Da and P3A1 conjugate 40,308.5 Da (DARs of 2) [* corresponds to MS artefact due to in source fragmentation]. I&J Mass spec analysis of deglycosylated, reduced B1- and P3A1 hFc (S473C) AF488 conjugates, respectively. Expected masses: B1 conjugate 39,552.4 Da and P3A1 conjugate 39,690.4 Da (DARs of 2).

Figure 32: Schematic of VNAR hFc PBD dimer, amanitin and PNU conjugates.

Figure 33: Cell viability following treatment with B1 mFc MMAE or 2V mFc-MMAE molecules (72hr) in a panel of different human cancer cell lines. Cell Titre Glo reagent (Promega) was used to quantify ATP which correlates with the number of metabolically active cells in culture. IC50 values were determined using GraphPad Prism software.

Figure 34: Cell viability following treatment with VNAR hFc PBD conjugates (96hr) in 2 different human cancer cell lines (DU145 and Jeko-1). Cell Titre Glo reagent (Promega) was used to quantify ATP which correlates with the number of metabolically active cells in culture. IC50 values were determined

using GraphPad Prism software. VNAR hFc conjugates were generated by reacting VNAR hlgG1 Fc(S252C) fusions with MA PEG4 va PBD (see Figure 32).

Figure 35: Cell viability following treatment with VNAR hFc PBD, SG3199 PBD and PNU (PEG4 vc PAB DMAE PNU159682) conjugates (96hr) in 2 different human cancer cell lines (PA-1 and Kasumi-2). Cell Titre Glo reagent (Promega) was used to quantify ATP which correlates with the number of metabolically active cells in culture. IC50 values were determined using GraphPad Prism software. Whereby VNAR hFc conjugates were generated by reacting VNAR hlgG1 Fc(S252C) fusions with MA PEG4 va PBD, MA PEG8 va PAB SG3199, MA PEG4 vc PAB DMAE PNU 159682 (see Figure 32).

Figure 36A and 36B: Cell surface binding of bispecific molecule B1hFc7C12 to a variety of cell lines compared to parental molecules. B1hFc7C12 shows an uplifting in binding. Performed using an Attune NxT flow cytometer (ThermoFisher) and a PE-conjugated anti-human secondary antibody to detect (Biolegend). A: Proteins applied at 66 nM B: Proteins applied at 357 nM.

Figure 36C: Cell surface binding of bispecific molecule P3A1hFc7C12 to a variety of cell lines compared to parental molecules. P3A1hFc7C12 shows an uplifting in binding. Performed using an Attune NxT flow cytometer (ThermoFisher) and a PE-conjugated anti-human secondary antibody to detect (Biolegend).

Figure 37A: Cell surface binding of ROR1-, EGFR-, and EGFR-ROR1 bispecific molecules to A549 cells (high ROR1, high EGFR). Binding of the His6Myc tagged proteins was assessed by flow cytometry using a PE-anti-Myc tag antibody to detect (CST). Analyses was performed using an Attune NxT flow cytometer (Thermo).

Figure 37B: Cell surface binding of ROR1-, EGFR-, and EGFR-ROR1 bispecific molecules to PA-1 cells (high ROR1, medium/low EGFR). Binding of the His6Myc tagged proteins was assessed by flow cytometry using a PE-anti-Myc tag antibody to detect (CST). Analyses was performed using an Attune NxT flow cytometer (Thermo).

Figure 37C: Cell surface binding of ROR1-, EGFR-, and EGFR-ROR1 bispecific molecules to A427 cell (low ROR1, low EGFR). Binding of the His6Myc tagged proteins was assessed by flow cytometry using a PE-anti-Myc tag antibody to detect (CST). Analyses was performed using an Attune NxT flow cytometer (Thermo). A427 are ROR1 low, so as expected the ROR1xEGFR bi-specific shows little increase in binding wrt parental EGFR binder only.

Figure 38: Comparison of cell surface binding of 7C12hFc fusion and hFc-7C12 fusion to a variety of cell lines. Fusion of 7C12 to the C-terminus of the hFc shows a decrease in binding to EGFR on cell-lines as compared to when 7C12 is fused to the N-terminus of the hFc protein. Performed using an

Attune NxT flow cytometer (ThermoFisher) and a PE-conjugated anti-human secondary antibody to detect (Biolegend)

Figure 39: B1hFc7C12 microscopy

Increased cell surface binding at 4°C of bispecific molecule B1hFc7C12 and internalisation after incubation at 37°C for 2 hours was observed in A549 cells compared to parental molecules. Images were obtained using a GE Healthcare InCell 2000. Hoechst dye was used to stain nuclei (blue), AF488-anti-human Ab (Thermo) was used to detect VNAR hFc molecules (green) and AF647-anti-rabbit Ab (CST) was used to detect Lamp-1 or EEA1 (red).

Figure 40: P3A1hFc7C12 microscopy

Increased cell surface binding at 4°C of bispecific molecule P3A1hFc7C12 and internalisation after incubation at 37°C for 2 hours was observed in A549 cells compared to parental molecules. Images were obtained using a GE Healthcare InCell 2000. Hoechst dye was used to stain nuclei (blue), AF488-anti-human Ab (Thermo) was used to detect VNAR hFc molecules (green) and AF647-anti-rabbit Ab (CST) was used to detect Lamp-1 or EEA1 (red).

Figure 41: B1hFc7C12 co-localisation

Bispecific molecule B1hFc7C12 appears to co-localise with EEA1 (early endosome antigen 1) and to some extent with Lamp-1 (lysosomal marker-1) following incubation at 37°C for 2hrs in A549 cells.

Figure 42: A549 internalisation assay data for (A) 9G8 containing bispecifics and (B) 7C12 containing bispecifics by flow cytometry analysis at 4 °C and 37 °C. Performed using an Attune NxT flow cytometer (ThermoFisher) and a PE-anti-Myc Ab (CST) to detect.

Figure 43: (A) Down-regulation of ROR1 by B1hFc, hFc7C12 and B1hFc7C12; (B) Down-regulation of EGFR by B1hFc, hFc7C12 and B1hFc7C12. Cell surface expression of ROR1 or EGFR receptors was assessed using PE-ROR1 2A2 mAb (Biolegend) and PE-AY13 EGFR mAb (Biolegend), respectively. An Attune NxT flow cytometer (ThermoFisher) was used to perform the analyses. Data are presented as % of 0 h control levels.

Figure 44: (A) Down-regulation of ROR1 by P3A1hFc, hFc7C12 and P3A1hFc7C12; (B) Down-regulation of EGFR by P3A1hFc, hFc7C12 and P3A1hFc7C12. Cell surface expression of ROR1 or EGFR receptors was assessed using PE-ROR1 2A2 mAb (Biolegend) and PE-AY13 EGFR mAb (Biolegend), respectively. An Attune NxT flow cytometer (ThermoFisher) was used to perform the analyses. Data are presented as % of 0 h control levels.

Figure 45: (A) Down-regulation of ROR1 by D3D3hFc, hFc7C12 and D3D3hFc7C12; (B) Down-regulation of EGFR by D3D3hFc, hFc7C12 and D3D3hFc7C12. Cell surface expression of ROR1 or EGFR receptors was assessed using PE-ROR1 2A2 mAb (Biolegend) and PE-AY13 EGFR mAb

(Biolegend), respectively. An Attune NxT flow cytometer (ThermoFisher) was used to perform the analyses. Data are presented as % of 0 h control levels.

Figure 46: Examples of simultaneous ROR1 and EGFR binding of ROR1xEGFR bi-specific molecules using BLI. Constructs are shown to bind immobilised hROR1, which then in turn bind EGFR as it is passed over the sensor surface. Mono-specific ROR1 binding VNAR B1 does not contain an EGFR binding moiety, and so no additional increase in signal is observed when EGFR is flowed over the surface of the sensor.

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:

SEQ ID NO:	Sequence Name	SEQ ID NO:	Sequence Name
111	7C12 hFc (S252C)	142	B1 QASGA his myc
112	hFc (S252C) 7C12	143	D3 QASGA his myc
113	B1 hFc (S252C) 7C12	144	D3-D3 QASGA his myc
114	P3A1 hFc (S252C) 7C12	145	P3A1 QASGA his myc
115	D3D3 hFc (S252C) 7C12	146	7C12 hFc (S252C)
116	B1-7C12 AAA his myc	147	hFc (S252C) 7C12
117	B1-7C12 ACA his myc	148	B1 hFc (S252C) 7C12
118	7C12-B1 QASGA his myc	149	P3A1 hFc (S252C) 7C12
119	7C12-B1 QACGA his myc	150	D3D3 hFc (S252C) 7C12
120	B1-9G8 AAA his myc	151	B1-7C12
121	9G8-B1 QASGA his myc	152	7C12-B1
122	D3-7C12 AAA his myc	153	B1-9G8
123	D3-7C12 ACA his myc	154	9G8-B1
124	7C12-D3 QASGA his myc	155	D3-7C12
125	7C12-D3 QACGA his myc	156	7C12-D3
126	D3-9G8 AAA his myc	157	D3-9G8
127	9G8-D3 QASGA his myc	158	9G8-D3
128	D3D3-7C12 AAA his myc	159	D3D3-7C12
129	D3D3-7C12 ACA his myc	160	7C12-D3D3
130	7C12-D3D3 QASGA his myc	161	P3A1-7C12
131	7C12-D3D3 QACGA his myc	162	7C12-P3A1
132	P3A1-7C12 AAA his myc	163	2V-7C12
133	P3A1-7C12 ACA his myc	164	7C12-2V
134	7C12-P3A1 QASGA his myc	165	2V-9G8
135	7C12-P3A1 QACGA his myc	166	9G8-2V
136	2V-7C12 ACA his myc	167	7C12
137	7C12-2V QACGA his myc	168	9G8
138	2V-9G8 ACA his myc	169	B1

139	9G8-2V QACGA his myc	170	D3
140	7C12 AAA his myc	171	D3-D3
141	9G8 AAA his myc	172	P3A1

DETAILED DESCRIPTION

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.

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 (Dooley, H. and Flajnik, M. F., *Eur. J. Immunol.*, 2005. 35(3): p. 936-945).

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.

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 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.

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 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.

Type II IgNAR are defined as having a cysteine residue in CDR1 and CDR3 which form intramolecular disulphide bonds that hold these two regions in close proximity, resulting in a protruding CDR3 (Figure 2) 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 (Liu, J.L., et al. Mol. Immunol. 2007. 44(7): p. 1775–1783; Kovalenko O.V., et al. J Biol Chem. 2013. 288(24): p. 17408-19) and was also isolated from semisynthetic V-NAR libraries derived from wobbegong sharks (Streltsov, V.A. et al. (2004) Proc. Natl. Acad. Sci. U.S.A. 101(34): p. 12444–12449).

It has been shown however specific VNARs isolated from synthetic libraries formed from the VNAR sequences can bind with high affinity to other proteins (Shao C.Y. et al. Mol Immunol. 2007. 44(4): p. 656-65; WO2014/173959) and that the IgNAR is part of the adaptive immune system as cartilaginous fish can be immunized with antigen and responsive IgNARs obtained that bind to the antigen (Dooley, H., et al, Mol. Immunol, 2003. 40(1): p. 25-33; WO2003/014161). It has been shown that the IgNAR has a mechanism for combinatorial joining of V like sequences with D and J sequences similar to that of immunoglobulins and the T cell receptor (summarized by Zielonka S. et al mAbs 2015. 7(1): p. 15-25).

The VNAR binding surface, unlike the variable domains in other natural immunoglobulins, derives from four regions of diversity: CDR1, HV2, HV4 and CDR3 (see also Stanfield, R. L., et al, Science, 2004. 305(5691): p. 1770-1773; Streltsov, V.A., et al, Protein Sci., 2005. 14(11): p. 2901-2909; Stanfield, R. L., et al., J Mol. Biol., 2007. 367(2): p. 358-372), 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.

The IgNAR shares some incidental features with the heavy chain only immunoglobulin (HCAb) found in camelidae (camels, dromedaries and llamas, Hamers-Casterman, C. et al. Nature, 1993. 363, 446-448; Wesolowski, J., et al., Med Microbiol Immunol, 2009. 198(3): p. 157-74) 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 differences between, and the possible and distinct evolutionary origins of, the VNAR and the immunoglobulin-derived VHH single binding domain from the camelids.

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.

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.

The presently-described ROR1-specific antigen binding molecules have been shown to bind to both human and murine ROR1. Furthermore, the ROR1-specific antigen binding molecules of the present invention bind to deglycosylated forms of ROR1 and do 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 novel epitopes in the ROR1 sequence.

Binding of the 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.

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases. It is a 170kDa transmembrane protein composed of four extracellular domains, a transmembrane region, an intracellular tyrosine kinase domain and a carboxy-terminal tail. The normal function of EGFR relates to regulation of epithelial tissue development, but it is also associated with a number of pathological states. In particular, overexpression of EGFR has been associated with a number of cancers. Accordingly, it is an important drug target and many therapeutic approaches have been applied. In addition to a number of small molecule-based EGFR inhibitors, such as gefitinib, erlotinib, afatinib, brigatinib, icotinib, and osimertinib a number of antibodies to EGFR have been developed. Anti-EGFR antibodies cetuximab, panitumumab, zalutumumab, nimotuzumab, and matuzumab. These antibodies block the extracellular ligand binding domain, preventing ligand binding and subsequent activation of the tyrosine kinase domain. Single domain antibodies (sdAb) that show competitive binding with cetuximab or matuzumab have also been developed.

The present inventors have created a bi-specific antigen binding molecule based on an ROR1-specific VNAR and an EGFR binding molecule. Various forms of the ROR1-specific antigen binding molecules are described, including fusion proteins of several types, which may be used in the bi-specific antigen binding molecule. 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.

Surprisingly, the bi-specific antigen binding molecules described show marked improvement in binding and internalisation compared to the equivalent constituent molecules.

The present inventors have also, for the first time, 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 molecules in several conjugated formats are provided. Such molecules may be included in the bi-specific antigen binding molecules described. Furthermore, there are provided herein bi-specific ROR1/EGFR-specific antigen molecules chemically conjugated to various payloads in various formats. Specifically, the present inventors have provided ROR1/EGFR bi-

specific recombinant fusion proteins which include an Fc region, in which conjugates are provided via the Fc region. In specific examples, the S239C or S442C mutations (EU numbering, equivalent to S252C and S473C in Kabat numbering) in the Fc domain are used as conjugation sites.

Bi-specific ROR1/EGFR-specific antigen molecules have also been generated appended with short cysteine containing tag sequences to facilitate conjugation with thiol reactive payloads and labels.

Furthermore, the inventors have found that, surprisingly, the increase in binding to A549 cells and PA1 cells is observed dependent on the orientation of the EGFR binding domain (9G8 or 7C12) with respect to the ROR1 binding agent. When the EGFR binding agent is fused C-terminal to the ROR1 binding agent the cell-surface binding is compromised as compared to the same construct but with the EGFR binding agent fused N-terminal to the ROR1 binding agent. Changing the orientation of the domains within the construct therefore provides a method for altering the apparent affinity of the bi-specific agent to the cell-surface.

A similar surprising observation was within the context of Fc fusion proteins (Figure 38). When the EGFR binding agent 7C12 was fused to the C-terminus of the Fc fragment (hFc 7C12) the binding to the EGFR+ve cell-lines A549, PA-1 and A427 was consistently lower as compared to the corresponding N-terminal fusion (7C12 hFc). Thereby, enabling the cell-surface binding characteristics of ROR1-EGFR bispecific binding agents to be modulated through appropriate design of the corresponding Fc fusion proteins.

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.

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.

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.

“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.

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.

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.

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 (Knappke *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 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 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 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 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 (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 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 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 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 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 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 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.

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 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 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 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.

"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 promotor 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 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.

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 DARPinS (Designed Ankyrin Repeat Proteins).

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.

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.

“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.).

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.

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., 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 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 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 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.

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 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.

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 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 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 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 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 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-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 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). 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 (Figure 11). These C-terminal aminoxy and thiol derivatives can be reacted with aldehyde / ketone and maleimide functionalised moieties, respectively, in a chemoselective fashion to give the site-specific C-terminally modified protein (Figures 25-27).

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.

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 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. 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.

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 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 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 “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, 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.

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

EXAMPLES

EXAMPLE 1 – Generation of specific anti-ROR1 VNAR sequences

Specific VNAR sequences from synthetic library

Two selection campaigns were adopted for screening a VNAR synthetic domain library (WO2014173959) for specific ROR1 binders. The first campaign made use of human ROR1 antigen and the second used mouse ROR1 antigen. Both recombinant ROR1 proteins were biotinylated as per manufacturer's instructions (Thermo Scientific Sulfo-NHS-LC-Biotin protocol, Cat N 21327) to aid the antigen presentation and selection process. VNAR domains were isolated after 3 rounds of selection using these biotinylated ROR1 antigens immobilised on streptavidin-coated beads. Post selection and following the screening of individual clones, 70% of monoclonal phage displaying VNAR domains (selected against human ROR1 protein) were found to be specific to human and mouse ROR1, but not a closely related ROR2 protein (the lead clones from this selection were called B1 - 40 % and E7 – 30% (Figure1). Similarly, 45% of monoclonal phage selected with mouse ROR1 were specific to human and mouse ROR1, but not ROR2 (lead clone from this selection was called P3A1, Figure 1). Another specific clone obtained from mouse ROR1 screening was CPF7 which was present as a single sequence out of 200 screened clones.

The sequences obtained from screening with human ROR1 are B1 and E7, and from screening with mouse ROR1 is P3A1 and CPF7. (Figure 2)

Specific VNAR sequences from immunised libraries

Libraries construction.

Three spiny dogfish were immunised with extracellular domain of recombinant human ROR1 protein and a target-specific IgNAR immune response was monitored through the analysis of post-immunised sera as described in Müller M.R. et al. Generation and Isolation of Target-Specific Single-Domain Antibodies from Shark Immune Repertoires, Humana Press 2012. Sera samples pre- and post-immunisation were taken from animals and tested for antigen binding in ELISA. An IgNAR titre increase, specific for human ROR1, was observed after 16 weeks in all animals (Figure 3). The specificity of post-immune sera to mouse ROR1 was also observed indicating the presence in immunised animals of species cross-reactive ROR1 specific IgNAR binders (Figure 3).

The VNAR repertoire (binding sites of IgNAR) was amplified from dogfish blood using specific PCR primers and cloned into a phage display vector, which contained an in-frame coat protein pIII of the bacteriophage M13 gene as described in Müller M.R. et al. Generation and Isolation of Target-Specific Single-Domain Antibodies from Shark Immune Repertoires, Humana Press 2012. The library sizes were calculated and are shown in Table 1:

Table 1:

Fish #	Library	Size (unique transformants)
154	ELSI 5	6 x 10 ⁷
156	ELSI 6	1.7 x 10 ⁷
161	ELSI 7	2 x 10 ⁷

Screening of the immunised libraries for antigen specific VNAR sequences.

Recombinant mouse ROR1 protein was used for screening the immunised libraries (ELSI 5–7). Following a protocol similar to that used to screen the synthetic library, VNAR domains were isolated after 3 rounds of selection using biotinylated ROR1 antigen immobilised on streptavidin-coated beads. Following the selection process, 45% of monoclonal phage displaying a VNAR domain (from the combined output from the 3 libraries) was specific to human and mouse ROR1. One third of the ROR1 specific VNAR were found to have the sequence D3 (Figures 4 and 5) and the remaining two thirds - to the sequence E9 (Figures 4 and 5).

The sequences obtained from screening with mouse ROR1 are E9 and D3. (Figure 5)

All lead anti-ROR1 VNAR proteins were expressed in TG1 *E.coli* or HEK293 mammalian cells and IMAC purified from the periplasmic fraction or the cell supernatant, respectively.

Methods

IgNAR titre in sera ELISA

ELISA were carried out using the following protocol:

1. Coat an ELISA plate with 100 µl/well of 1 mg/ml of human ROR1-Fc or mouse ROR1-Fc in or PBS. Incubate at 4 °C overnight.
2. Wash plates 3x with PBST.
3. Block plates by adding 200 µl/well 2% (w/v) M-PBS and incubate at 37 °C for 1 h.
4. Wash plates 3x with PBST.
5. Serially dilute dogfish sera in PBS from no less than 1:10 up to 1:1000 and add 100 µl/well. Incubate at room temperature for 1h.
6. Wash plates 3x with PBST.
7. Add 100 µl/well primary antibody (mouse monoclonal anti- IgNAR antibody, GA8) diluted as hybridoma tissue culture supernatant in PBST.
8. Wash plates 3x with PBST.

9. Add 100 µl/well of a suitable secondary anti-mouse IgG HRP conjugate diluted in PBS. Incubate for 1 h.
10. Wash plates 2x with PBST followed by 2x with PBS.
11. Add 100 µl/well of TMB substrate to the plate and incubate until the appearance of signal/onset of saturation. Stop the colour development by adding 100 µl/well of 0.18 M H₂SO₄.
12. Read at 450 nm with a microtiter plate reader.

Library screening

1. To rescue library phage for selections, cultures from library glycerol stocks were grown at 37 °C and 250 rpm, in 2xTY, 2% glucose, 100 µg/ml ampicillin to an OD₆₀₀ of 0.5.
2. Cells were super-infected with 109 M13K07 helper phage (NEB) and then incubated overnight in 2xTY, 100 µg/ml ampicillin, 50 µg/ml kanamycin at 25 °C and 250 rpm.
3. The phage was PEG-precipitated (20% PEG/2.5 M NaCl) twice from the bacterial culture and the resulting phage pellets were resuspended in 1 ml PBS.
4. 200 µl of Dynabeads M-280 Streptavidin (Invitrogen #11205D), pre-blocked with 2% (w/v) MPBS, were coated with 400 nM biotinylated mouse ROR1 rotating at 20 rpm, at room temperature for 1 h.
5. Library phage was de-selected by incubation with Dynabeads for 1 h rotating at room temperature and then added to the antigen-coated beads.
6. Beads were washed 5-10 times with PBST and 5-10 times with PBS, eluted by rotating for 8 min in 400 µl 100 mM TEA and neutralised by the addition of 200 µl 1 M Tris-HCl pH 7.5.
7. *E. coli* TG1 cells (10 ml) were infected with 300 µl of eluted phage for 30 min at 37 °C and grown overnight at 37 °C on TYE agar plates containing 2 % (w/v) glucose and 100 µg/ml ampicillin.
8. Three further rounds of selection were conducted and outputs were screened for antigen-specific binding by monoclonal phage and periplasmic extract ELISAs against human or mouse ROR1. Phage binders were detected using HRP-conjugated anti-M13 antibody (GE Healthcare, 27942101) and periplasmic protein was detected using HRP-conjugated anti-c-Myc antibody (Roche, 118 141 50 001).

VNAR expression in E.coli

1. Dilute the overnight culture 1:50 in TB media with phosphate salts, 1% glucose, 100 µg/ml Ampicillin and incubate at 37 °C with vigorous shaking (250 rpm) all day.
2. Pellet the cells by centrifugation at 3,000 x g for 20 min at 20 °C.
3. Re-suspend the cells in the same volume of TB media with phosphate salts, 100 µg/ml Ampicillin (no glucose).
4. Add IPTG to a final concentration of 1 mM IPTG and incubate at 16 °C overnight (16 h) with shaking at 250rpm.
5. Collect the cells by centrifugation at 6,000 x g for 30 min (the pellet could be frozen at this point at -20 °C).

6. Re-suspend the pellet in 10% culture volume ice-cold TES and shake gently on ice for 15 min.
7. 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.
8. Pellet the suspension by centrifugation at 15,000 x g for 30 min at 4 °C and carefully decant the supernatant containing released periplasmic proteins into a clean falcon.
9. Add 10x PBS pH 7.4 [final concentration of 1xPBS] to peri-prep extract prior to IMAC incubation.

VNAR expression in HEK293

10 µg DNA in water (sterile filtrated) for 10 ml culture.

Use 10 ml of cells (~106/ml) in a 50ml bioreactor tube (exponentially growing cells in fresh media)

Add OptiMEM media to DNA to a total volume of 500 µl.

Add 25 µl of PEI (1mg/ml stock made up in water) to a separate 500 µl OptiMEM media.

Incubated DNA and PEI at room temperature for up to 15 min.

Mix 500 µl of PEI in media to each 500 µl of DNA in media.

Incubated at room temperature for 20-30 min facilitating complex formation.

Add 1 ml of mixture to the cells and incubate at 37 °C, 5%CO₂ shaking 140rpm.

Next day feed cells by addition of 250 µl of 20% (w/v) tryptone to 10 ml of cells to obtain the final concentration of tryptone 0.5%

Leave cells to express for 3-5 days.

Spin the cells and assess supernatant for secreted protein to determine productivity.

Add 10xPBS pH 7.4 [final concentration of 1xPBS] to peri-prep extract prior to IMAC incubation.

This protocol can be scaled up or down as required for protein production.

Protein expression (scale up)

ROR1 binding VNAR proteins expressed well in many different forms in several different expression systems. The addition of standard C terminal tags, including His and His₆Myc, to aid protein purification, handling and protein analysis, did not affect the binding of ROR1 VNARs to target ROR1 (Table 2).

Table 2: SPR data for binding of VNARs with different C-terminal tags to human ROR1 and ROR2

VNAR	C-terminal tag	hROR1			hROR2
		K _a (M ⁻¹ s ⁻¹)	K _d (s ⁻¹)	K _D (nM)	
B1	6xHis	2.33E+06	1.91E-04	0.11	No binding
	6xHis myc	7.47E+05	6.09E-04	0.83	No binding
P3A1	6xHis	2.92E+06	2.06E-02	7.8	No binding
	6xHis myc	9.8E+05	2.5E-02	25.6	No binding
P3A1 dimer	No tag	1.67E+06	5.98E-04	0.36	No binding
	6xHis myc	2.08E+06	6.37E-04	0.35	No binding

In addition, VNAR C-terminal tags do not affect VNAR structure as measured by circular dichroism (Figure 6 – CD spectra of VNARs) (Glasgow University, UK).

VNARs were also expressed genetically fused to mouse and human IgG Fc sequences, and as N-terminal fusions to engineered inteins, enabling site specific conjugation to labels and drugs. Expression systems used include *E. coli* (periplasmic and cytoplasmic expression), HEK 293 and CHO (Evitria Fc fusion proteins).

EXAMPLE 2 – VNAR Reformatting

Homodimers

ROR1 binding VNARs were successfully reformatted into homodimers by genetic fusion using standard GlySer based linkers (Figure 7B). Homodimers were shown to have increased affinity for recombinant hROR1 by SPR and ELISA, and increased binding to cell surface ROR1 on ROR1 positive cancer cell lines by flow cytometry (Figure 9). Flow cytometry experiments are described in more detail in Example 4.

In addition, ROR1 binding VNAR homodimers were successfully generated through chemical conjugation. VNARs were expressed as intein fusion proteins and cleaved with cysteamine to generate C-terminal thiol derivatives, which then self-associated into homodimers via C terminal intermolecular disulphide formation (Figure 7C). These disulphide-linked homodimers showed increased binding affinity to recombinant hROR1 by ELISA (Figure 8). Production of intein fusion proteins is discussed in more detail in Example 8.

Heterodimers

ROR1 binding VNAR heterodimers were generated by genetic fusion with standard GlySer linkers (Figure 7D) and demonstrated high affinity specific binding to recombinant ROR1 and ROR1 positive cells. Heterodimeric VNAR proteins can also be generated by chemical conjugation.

Results for binding characterisation experiments are tabulated in Table 3 and 4 (see Example 3).

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. ROR1 binding VNARs were genetically fused to the N terminus of mouse IgG2a Fc (mFc) and both the N and C termini of human IgG1 (hFc) via standard GlySer linkers (Figure 7 E, F, G). Examples of Fc sequences

Mouse IgG2a Fc (mFc)

EPRGPTIKPCPPCKCPAPNLLGGPSVFIFFPKIKDVLMSLSPIVTCVVVDVSEDDPDVQISWVFNNEV
HTAQTQTHREDYNSTLRVVSALPIQHQDWMMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVL
PPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNW
VERNSYSCSVVHEGLHNHHTTKSFSRTPGK (SEQ ID NO: 93)

Human IgG1 Fc (hFc)

EPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVY
TLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 94)

VNAR Fc fusion proteins were expressed as secreted protein in CHO K1 cells and purified from the media using MabSelect™ SuRe™ (Evitria, Switzerland). Purified proteins were analysed by SEC (AdvanceBio, Agilent), SDS PAGE and mass spectrometry to confirm sequence and protein integrity. The resulting VNAR Fc fusion proteins bind recombinant human ROR1 by SPR (Table 6) and ROR1 positive cells with high affinity (Figure 15) and were shown to internalise into ROR1 positive cells. ROR1 binding VNARs were also genetically fused to engineered hIgG1 Fc fusion proteins that incorporated an engineered cysteine substitution in the hIgG1 Fc sequence, for example at position S252C or S473C (Kabat numbering) to enable site specific labelling. (Figure 10)

Typical method for expression of VNAR intein fusion proteins

For expression as intein fusions, DNA encoding VNARs was optimised for *E. coli* expression (GeneArt, Thermo) and cloned into the NdeI/SapI sites of the pTXB1 vector (NEB) and derivatives thereof. 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. pTXB1 vector derivatives encode alternative inteins as the fusion proteins.

Transformed *E. coli* cells were grown in 1L shaker flasks until OD600 = ~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 immobilizing 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 dioxyamine, or O,O'-1,3-propanediylbishydroxylamine, or 100mM cysteine or cysteamine to generate the

corresponding C-terminal aminoxy, C-terminal cysteine or C-terminal thiol derivative of the VNARs (Figure 11).

Cleaved VNAR supernatant was then further purified by SEC (Superdex75 26/60 GE healthcare) 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 characterized by reducing and non-reducing SDS PAGE analysis and mass spectrometry. The formation of the desired disulphide bond was confirmed by mass spectrometry methods.

EXAMPLE 3 – Anti-ROR1 VNAR characterisation – binding to ROR1 and ROR2 by SPR and ELISA

Species cross-reactivity of ROR1 VNAR binders

Soluble VNAR protein clones (B1, P3A1 and D3) were analysed for species cross-reactivity with human, mouse and rat ROR1 along with a positive control antibody 2A2 and an anti ROR2 specific antibody control. 2A2 is an anti-human ROR1 specific mouse monoclonal antibody (BioLegend Cat # 357802) and the anti ROR2 antibody is a commercial monoclonal mouse antibody from R&D (Cat # MAB2064).

VNAR B1 was observed to be a very strong binder to both mouse and human ROR1. All VNARs are species cross-reactive to ROR1 derived from a human, mouse and rat origin (Table 3 and Table 4). None of the VNAR clones cross-reacted with human ROR2 (Table 3).

Determination of binding kinetics to human ROR1, human ROR2, mouse ROR1 or rat ROR1

Binding kinetics were determined using a Pioneer Surface Plasmon Resonance (SPR) instrument (SensiQ/Pall ForteBio). ROR1-hFc or ROR2-hFc fusion proteins (extracellular domain) were immobilised in sodium acetate pH5 buffer to COOH₂ chips using amine coupling. VNARs and VNAR-Fc 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 Qdat software (SensiQ/Pall ForteBio). ROR1 2A2 mAb (Biolegend) and ROR2 mAb (R&D Systems) were included as controls for positive/negative binding to ROR1 and ROR2. 2V is a control VNAR sequence, derived from a naïve VNAR library, so is representative of this protein class but has no known target.

Table 3: SPR data for binding of VNAR molecules to human ROR1 (hROR1) and human ROR2 (hROR2). C-terminal His₆ or His₆Myc tagged VNARs were expressed.

VNAR	Expression System	hROR1			hROR2
		Ka (M ⁻¹ s ⁻¹)	Kd (s ⁻¹)	KD (nM)	
B1	<i>E. coli</i>	6.29E+05	7.93E-04	1.6	No binding
B1	HEK293	5.36E+05	2.26E-03	0.63	No binding
P3A1	<i>E. coli</i>	2.47E+06	4.42E-02	19.1	No binding
CPF7	<i>E. coli</i>	2.33E+06	2.96E-02	13.6	No binding
E7	<i>E. coli</i>	1.11E+06	1.18E-02	11.1	No binding
D3	<i>E. coli</i>	2.09E+05	3.24E-02	159.1	No binding
D3	HEK293	1.39E+06	7.52E-02	54.5	No binding
E9	HEK293	4.23E+05	4.45E-02	136.6	No binding
P3A1-[G4S] ₅ -P3A1	HEK293	4.9E+06	1.12E-03	0.30	No binding
D3-[G4S] ₅ -D3	<i>E. coli</i>	2.95E+06	3.38E-03	2.33	No binding
P3A1-[G4S] ₅ -B1	<i>E. coli</i>	3.13E+06	2.08E-03	1.0	No binding
P3A1-[G4S] ₃ -B1	<i>E. coli</i>	1.09E+06	2.84E-03	2.7	No binding
P3A1-[G4S] ₇ -B1	<i>E. coli</i>	1.49E+06	6.44E-03	4.3	No binding
2V	<i>E. coli</i>	No binding	No binding	No binding	No binding
2V-[G4S] ₅ -2V	<i>E. coli</i>	No binding	No binding	No binding	No binding

Table 4 – SPR data for binding to mouse ROR1 (mROR1) and rat ROR1 (rROR1)

VNAR	Expression System	mROR1			rROR1		
		Ka (M ⁻¹ s ⁻¹)	Kd (s ⁻¹)	KD (nM)	Ka (s ⁻¹)	Kd (M ⁻¹ s ⁻¹)	KD (nM)
B1	<i>E. coli</i>	4.32E+05	2.09E-03	5.2	1.2E+05	1.11E-02	94.5
B1	HEK 293	7.2E+05	1.51E-03	2.18	1.16E+05	6.51E-03	56.5
P3A1	<i>E. coli</i>	2.95E+06	4.08E-02	14.3	2.86E+06	4.5E-02	17.7
CPF7	<i>E. coli</i>	2.26E+06	3.2E-02	19.1	7.72E+05	3.66E-02	68.6
E7	<i>E. coli</i>	1.41E+06	2.0E-03	1.4	ND	ND	ND
P3A1-[G ₄ S] ₅ -P3A1	HEK 293	4.17E+06	1.45E-03	0.396	3.18E+06	1.73E-03	0.57
2V	<i>E. coli</i>	No binding	No binding	No binding	No binding	No binding	No binding
2V-[G ₄ S] ₅ -2V	<i>E. coli</i>	No binding	No binding	No binding	No binding	No binding	No binding

VNAR proteins have been developed, which bind with high affinity to human ROR1 ECD in monomeric and multimeric formats (both homo and hetero dimeric forms), show no binding to the closely related family member human ROR2 and cross react with high affinity to mouse and rat orthologues of ROR1. Reformating the P3A1 and D3 proteins as dimers significantly increased the binding affinity to human ROR1 with a significant reduction in the dissociation rate constants being observed.

The binding of a chemically conjugated B1 homodimer to hROR1 was also assessed by ELISA. To generate this molecule a B1 derivative was generated with a unique C-terminal thiol functionality through chemical cleavage of the corresponding B1-intein fusion protein precursor with cysteamine. Intermolecular disulphide bond formation was used to covalently link the C-termini of the two proteins to generate a homodimer of unnatural but defined topology (B1-S-S-B1, Figure 7C). Binding of the B1-S-S-B1 to hROR1 was compared to the B1 monomer by ELISA.

In brief, ELISA method as follows. Wells coated with 100ng 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. 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:

- Anti-c-Myc, HRP (Invitrogen #R951-25)
- Rabbit anti-Human IgG H&L, HRP (Abcam #ab6759)
- Rabbit anti-Mouse IgG H&L, HRP (Abcam #ab97046)
- 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).

Whilst B1 monomer and the C-terminal thiol derivative binds strongly to human ROR1, an increase in human ROR1 binding was observed for the chemically linked B1-S-S-B1 dimer (Figure 8).

EXAMPLE 4 – Anti-EGFR-ROR1 VNAR characterisation – cell binding and internalisation by flow cytometry

Cell Surface Binding

Adherent human 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 5ml 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 1-2ml of PBS/2%FCS. A cell count was performed using a Z1 Coulter Particle Counter (Beckman Coulter) and 5 x 10⁵ cells were aliquoted per test sample. Cells were incubated with 100µl of either VNAR (His₆Myc tagged), VNAR-Fc molecules or ROR1 mAb, EGFR mAb and IgG controls for 1 hour on ice. Excess VNAR, VNAR-Fc or mAb was removed by adding 5ml of ice-cold PBS/2% FCS, followed by centrifugation at 1500rpm for 5 mins at 4°C. The supernatant was removed and a second wash performed by re-suspending the cell pellet in 1ml of ice-cold PBS/2%FCS and adding a further 4ml of ice-cold PBS/2%FCS. Samples were again centrifuged at 1500rpm for 5min at 4°C. Supernatant was removed and excess liquid removed by blotting the tubes on tissue paper. Appropriate secondary antibodies were used to detect bound VNAR (His₆Myc), VNAR-hFc, VNAR-mFc or ROR mAb (PE-anti-Myc tag antibody (CST), PE-anti-human antibody (JIR labs/Strattech), and PE-anti-mouse antibody (JIR/Strattech) respectively). Cells were incubated with chosen secondary antibody for 30min on ice. Cells were washed to remove excess antibody as described earlier. Cell pellets were re-suspended in 0.5ml of ice-cold PBS/2%FCS and left on ice in the dark prior to analysis on either a FACS Calibur (BD Biosciences) or an Attune NxT (ThermoFisher) flow cytometer.

Cell-surface staining following incubation at 37 °C vs 4 °C.

Briefly, 5×10^5 cells were incubated with VNAR, VNAR-Fc, ROR1 2A2 mAb, EGFR AY13 mAb or IgG1 control for 1 hr on ice. Cells were washed twice by addition of 5ml of ice-cold PBS/2%FCS followed by centrifugation at 1500rpm for 5mins at 4 °C. Following the final centrifugation step, excess supernatant was removed and the tubes blotted on tissue paper. Each cell pellet was re-suspended in 200µl of PBS/2%FCS and either placed on ice or at 37 °C for 2 hours. Bound VNAR (His₆Myc tagged), VNAR-hFc, VNAR, ROR1 2A2 mAb or EGFR AY13 mAb was detected using either PE-conjugated anti-Myc tag antibody (CST), PE-conjugated anti-human antibody (JIR/Stratech) or PE-conjugated anti-mouse antibody (JIR/Stratech). Loss of signal at 37 °C with respect to samples incubated on ice is indicative of ROR1 internalisation.

A decrease in cell-surface binding after incubation at 37 °C versus 4 °C was observed for anti-ROR1 VNAR constructs (Figure 14) and anti-EGFR-ROR1 VNAR molecules (Figure 42A and 42B), which is consistent with binding and internalisation of the proteins by ROR1 and EGFR.

Binding of VNARs to a panel of cancer cell-lines

Figure 9 shows representative flow cytometry histograms for binding of anti ROR1 VNARs binding to the ROR1^{hi} A549 lung adenocarcinoma cells.

Figure 13 shows the binding of different VNARs to the ROR1^{hi} A549 lung adenocarcinoma cells and the ROR1^{low} lung cancer cell-line A427 by flow cytometry..

Table 5 shows a summary of flow cytometry data for binding of VNAR proteins to a variety of ROR1^{hi} and ROR1^{low} cancer cell-lines.

Table 5: Relative ranking of VNAR cell surface binding in human cancer cell lines, ascertained by flow Cytometry. Number of '+' corresponds to binding strength. '-' indicates no binding. '/' not determined in this cell line.

	Based on Median (YL1-PE) or Geo Mean (FL2-PE)					
Molecule	A549 (ROR1 ^{hi})	A427 (ROR1 ^{low})	MDA-MB-231 (ROR1 ^{hi})	T47D (ROR1 ^{low})	HT-29 (ROR1 ^{hi})	Colo205 (ROR1 ^{low})
B1	+++++	+	+++	++	+++	+
E7	++++	+	+++	++	+++	+
P3A1	+	-	+	+/-	+	-
CPF7	++	-	+	+/-	+	-
P3A1-[G ₄ S] ₅ - P3A1 dimer	+++	-	++	/	/	/

CPF7-[G ₄ S] ₅ - CPF7 dimer	+++	++	/	/	/	/
P3A1- [G ₄ S] ₅ - B1	++++	-	+++	/	/	/
D3	+	-	/	/	/	/
D3-[G ₄ S] ₅ -D3 dimer	++++	-	++	/	/	/
2V	-	-	-	-	-	-
2V-[G ₄ S] ₅ -2V dimer	-	-	-	-	-	-

Robust binding of the VNARs to ROR1 expressing cancer cell-lines is observed as compared to the ROR1^{low} cancer cell-lines where little to no staining was observed for the majority of the ROR1 binding VNARs tested.

The cell-surface staining for P3A1-P3A1 is not as strong as for B1 or D3-D3 proteins, which may reflect differences in the epitopes of these binders and that in the cellular context some regions of the extracellular domain of ROR1 are potentially more accessible for binding than others.

EXAMPLE 5 - Characterisation of anti-ROR1 VNAR-Fc fusion proteins - binding to ROR1 and ROR2 by SPR and cell surface binding and internalisation

ROR1 binding VNARs were expressed fused to the N terminus of mouse IgG2a Fc (mFc) and the N terminus and C-terminus of human IgG1 (hFc) via standard GlySer linkers. Fusion of the human IgG1 Fc were also generated whereby Ser235 in the Fc region (Kabat numbering) was replaced with a Cys (Figure 10).

Binding to ROR1 and ROR2 by SPR

Using the procedures outlined above the binding of VNAR-Fc fusions to human, mouse and rat ROR1 and human ROR2 were determined by SPR.

Table 6 – SPR data for binding of VNAR-Fc fusions to human ROR1 and human ROR2

Molecule	hROR1			hROR2
	Ka ($M^{-1}s^{-1}$)	Kd (s^{-1})	KD (nM)	
B1 mFc	4.19E+05	3.356E-04	0.8	No binding
2V mFc	No binding	No binding	No binding	No binding
B1 hFc	3.08E+06	9.53E-05	0.032	No binding
P3A1 hFc	1.07E+07	5.64E-04	0.084	No binding
D3 hFc	1.21E+06	2.88E-03	2.6	No binding
E9 hFc	7.07E+05	3.64E-03	5.3	No binding
D3-D3 hFc	4.96E+06	9.88E-04	0.25	No binding
hFc - P3A1	2.38E+06	7.76E-04	0.35	No binding
hFc - D3	1.10E+06	2.35E-03	2.37	No binding
hFc – D3-D3	2.35E+06	1.01E-03	0.49	No binding
2V hFc	No binding	No binding	No binding	No binding
2V-2V hFc	No binding	No binding	No binding	No binding

As shown in Table 6 anti ROR1 VNAR-Fc proteins bind with high affinity to human ROR1, with no binding to human ROR2 observed. Strong binding to mouse and rat ROR1 ECD was also observed. As VNAR-Fc fusions, a significant decrease in the K_D apparent values for ROR1 binding is observed with respect to the corresponding VNAR monomers. This is consistent with these VNAR-Fc fusions binding in a bivalent fashion to the ROR1-chip surface in the SPR experiments. Both N- and C-terminal VNAR Fc fusions bind with high affinity to human ROR1 but do not bind to human ROR2.

Binding of VNARs to cancer cell-lines

Binding of the VNAR-Fc fusions to the surface of a panel of cancer cell lines was measured by flow cytometry using the methods outlined previously. Figure 15 shows the binding of different VNAR-Fc fusions to the ROR1^{hi} A549 lung adenocarcinoma cells and the ROR1^{low} lung cancer cell-line A427 by flow cytometry at a fixed concentration of protein.

Table 7 summarises the binding data for VNAR-Fc proteins with a variety of ROR1^{hi} cancer cell-lines.

Table 7: Relative ranking of VNAR hFc molecule cell surface binding in ROR1^{hi} human cancer cell lines. The number of '+' indicates the strength of binding. '-' indicates no binding. '/' indicates that it has not been determined. hFc molecules were detected using a PE-anti-human antibody (Jackson Immune Research/Strattech) and a ThermoFisher Attune NxT flow cytometer.

	Based on Median (YL1-PE)			
Molecule	A549	MDA-MB-231	PC-9	NCI-H1975
B1 hFc	++++	+++++	+++++	+++++
P3A1 hFc	++	+++	++	++
D3 hFc	+	++	/	/
E9 hFc	+	/	/	/
D3-D3 hFc	++	++++	/	/
2V hFc	-	-	-	-
2V-2V hFc	+	/	/	/

Robust binding of the VNARs to ROR1 expressing cancer cell-lines is observed as compared to the ROR1^{low} cancer cell-lines, where little to no staining was detected for the majority of the ROR1 binding VNARs tested.

Differences in the mean cell-surface staining may indicate that different regions of ROR1 may be more accessible than others when the protein is expressed on the cell surface. For targeting less accessible regions of ROR1 on cancer cells, it would be advantageous to use small protein binders such as VNARs as opposed to large antibodies that will be sterically occluded.

Cell-surface staining following incubation at 37°C vs 4°C.

The binding of VNAR-Fc fusions to MDA-MB-231 cells after incubation at 37°C or 4°C was determined by flow cytometry using the methods described previously. For the B1-hFc, P3A1-hFc, D3-hFc and D3D3-hFc proteins tested, there was a loss of cell-surface staining after incubation at 37°C versus 4°C (Figure 16), consistent with binding and internalisation of these VNAR-hFc fusion proteins.

Internalisation by immunofluorescence following incubation at 37°C vs 4°C.

The cellular localisation of human IgG1 Fc and mouse IgG2a Fc fusion proteins can be detected by immunofluorescence using fluorescently labelled secondary antibodies targeting these domains. Immunofluorescence methods were used to detect internalisation of VNAR-Fc by ROR1 on cancer cells.

Black, clear bottom 96-well plates (Greiner) were coated with 100 µg/ml Collagen I (Sigma) to aid cell attachment. Cells were seeded in complete growth media (Gibco) into the coated 96 well plates and incubated at 5% CO₂, 37°C for 24hr. The media was removed and replaced with serum-free media (Gibco) on the following day and left overnight. On the following morning, media was removed and cells were treated with various concentrations of VNAR-Fc molecules. Plates were incubated on ice for 30 minutes. Treatments were removed and replaced with 100µl of PBS/2%FCS per well. One plate was kept on ice and the other was placed at 37°C, 5% CO₂ for 2 hours. Following this 2 hour incubation, the PBS/2%FCS solution was removed and cells were fixed with 4% Paraformaldehyde in ice cold PBS for 20min on ice. The PFA solution was removed and replaced with 0.05% Saponin (Sigma) made up in PBS/2% FCS for 15min at room temperature. This step permeabilises the cell membranes. Secondary antibody staining was performed using; AF488-anti-human Ab (1:250; ThermoFisher) to detect VNAR-hFc fusion proteins. All secondary antibody working stocks were made up in 0.05% Saponin/PBS/2% FCS. Plates were incubated at 4°C overnight in the dark. On the following day, secondary AF488-conjugated antibodies were removed and the cells were washed X3 using 0.05% Saponin/PBS/2% FCS. Lamp-1 antibody (1:200; CST) or EEA1 antibody (1:50; CST) were added to detect lysosome and early endosome compartments respectively. Plates were incubated in the dark at room temperature for 2 hours. The Lamp-1 and EEA1 antibodies were then removed and the cells were washed X3 with 0.05% Saponin/PBS/2% FCS. AF647-anti rabbit antibody (1:1000; CST) was then added to detect Lamp1 and EEA1 antibody binding. A further incubation in the dark at room temperature for 2 hours was performed before removing the AF647-secondary antibody and washing the cells X3 with 0.05% Saponin/PBS/2% FCS. Cell nuclei were stained using 10µM Hoechst reagent (Sigma) in 0.05% Saponin/PBS/2% FCS for 20 min at room temperature in the dark. Finally, this solution was removed and replaced with PBS. Plates were stored at 4°C in the dark prior to imaging using a GE Healthcare InCell 2000 instrument.

Internalisation of B1 hFc and B1 mFc was observed in MDA-MB-231 breast cancer cells following incubation at 37°C for 2 hours. The VNAR-Fc-ROR1 complex appears to overlay with Lamp-1 and EEA1 staining following internalisation which is suggestive of ROR1 cellular trafficking via early endosomal and lysosomal compartments. ROR1-VNAR-Fc staining remained predominantly at the cell surface when the samples were incubated on ice for 2 hours. No cell surface binding or internalisation was observed following incubation with 2V Fc protein (non-binding negative control VNAR). B1-hFc and B1-mFc were not internalised by the ROR1^{low} lung cancer cell-line A427.

EXAMPLE 6 - Humanisation and further engineering

A number of humanised sequence derivatives of two lead ROR1 binding VNARs were generated using two different strategies.

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). For the first construct (G1) only the CDR1 and CDR3 loops were grafted. The second construct (G2) had both the CDRs and HV loops grafted.

Examples of humanised / grafted VNAR sequences:

B1 G1

TRVDQSPSSLSASVGDRVITITCVLTGANYGLASTYWYRKPNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 45)

B1 G2

TRVDQSPSSLSASVGDRVITITCVLTGANYGLASTYWYRKPNPGSSNQERISISGRYSESVNKRTMSFTL
TISSLQPEDSATYYCRAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 46)

P3A1 V1

TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSSIGGRYVESVNKGAKSFT
LTISSLQPEDFATYYCKAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 47)

P3A1 G1

TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTYWYRKPNPGSSNKEQISISGRYSESVNKGTKSFTL
TISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 48)

P3A1 G2

TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTYWYRKPNPGTTDWERMSSIGGRYSESVNKGAKSFT
LTISSLQPEDSATYYCRAREARHPWLRQWYDGAGTKVEIK (SEQ ID NO: 49)

D3 humanised ADV1

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYSESVNKGAKSFT
LTISSLQPEDSATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 50)

D3 humanised ADV2

TRVDQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYSESVNKGAKSFT
LTISSLQPEDSATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 51)

D3 humanised ADV3

ASVNQSPSSASASVGDRLTITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYSESVNKGAKSFT
LTISSLQPEDSATYYCKAQSGMAISTGSGHGYNWYDGAGTKLEVK (SEQ ID NO: 52)

B1 humanised V5

ASVDQSPSSLSASVGDRVITITCVVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRTMSFTL
TISSLQPEDSATYYCKAYPWGAGAPWLQWYDGAGTKVEIK (SEQ ID NO: 53)

B1 humanised V7

ASVDQSPSSASASVGDRLTITCVVTGANYGLAATYWYRKNPGSSNQERISISGRYSESVNKRTMSFTL
TISSLQPEDSATYYCKAYPWGAGAPWLQWYDGAGTKLEVK (SEQ ID NO: 54)

DNA encoding the humanised constructs was codon optimised for expression in *E. coli* and synthesised by GeneArt (Thermo). P3A1 sequences were designed as dimers with a [G₄S]₅ linker connecting the VNAR domains. All humanised sequences were generated with the following C terminal His₆myc tag:

QASGAHHHHHHGAEFEQKLISEEDLG (SEQ ID NO: 95)

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.

Further humanised versions of D3 were created as follows:

D3 humanised EL V1

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKRAKSFS
LRIKDLTVADSATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 55)

D3 humanised EL V2

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRYVESVNKRAKSFT
LTISSLQPEDFATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 56)

D3 humanised EL V3

ASVNQSPSSLSASVGDRVITITCVLTDTSYGLYSTSWFRKNPGTTDWERMSIGGRFSGSGSKRAKSFT
LTISSLQPEDFATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 57)

D3 humanised EL V4

ASVNQSPSSLSASVGDRVITCVLTDTSYGLYSTSWYQQKPGTTDWERMSSIGGRYVESVNKRAKSFT
LTISSLQPEDFATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 58)

D3 humanised EL V5

ASVNQSPSSLSASVGDRVITCVLTDTSYGLYSTSWYQQKPGTTDWERMSSIGGRFSGSGSKRAKSFT
LTISSLQPEDFATYYCKAQSGMAISTGSGHGYNWYDGAGTKVEIK (SEQ ID NO: 59)

Humanised ROR1 binding VNAR variants demonstrated high affinity binding to human ROR1 by SPR and improved thermal stability. SPR was performed as described previously using human ROR1 ECD – Fc immobilised to the chip surface. 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.

Table 8 – Thermal stability and hROR1 binding data for humanised VNAR variants

Construct	T _m (°C)	hROR1 binding (SPR)		
		K _a (M ⁻¹ s ⁻¹)	K _d (s ⁻¹)	K _D (nM)
B1	54.2	7.45E+05	6.09E-04	0.83
B1G1	58.0	2.20E+05	1.62E-02	82.8
B1G2	59.9	1.85E+05	7.90E-03	45.9
B1V5	46.05	5.20E+04	3.85E-05	0.74
B1V7	43.91	7.74E+04	5.54E-05	0.77
P3A1 dimer	60.7	3.78E+05	1.17E-03	0.30
P3A1 V1 dimer	48.5	4.78E+05	8.46E-04	0.18
P3A1 G1 dimer	57.1	4.30E+05	1.47E-03	0.43
P3A1 G2 dimer	54.0	1.88E+05	1.19E-03	0.77
B1G1-hFc	ND	2.4E+05	2.66E-03	11.8
B1G2-hFc	ND	6.26E+05	1.41E-03	2.55
D3 ADV1 dimer	54.66	6.36E+05	5.67E-03	8.92
D3 ADV2 dimer	56.18	6.09E+05	1.60E-02	26.2
D3 WT	64.4	1.21E+06	9.43E-05	15.5
D3 AD V2	56.98	4.58E+04	2.36E-03	51.6
D3 EL V1	53.25	1.50E+06	1.58E-04	16.1
D3 EL V2	56.50	1.85E+06	1.63E-04	18.9
D3 EL V4	54.1	1.38E+06	5.45E-04	58.5

Grafting the HV and / or CDR loops of B1 onto a humanised VNAR framework and substituting P3A1 sequences with regions from the human DPK-9 sequence, yielded substantially engineered proteins that are stable and maintain hROR1 binding with nanomolar and picomolar affinity respectively.

EXAMPLE 7 - Epitope mapping

Binding of proteins to deglycosylated human ROR1

ELISA was used to compare VNAR binding to glycosylated and deglycosylated human ROR1 protein. To generate deglycosylated human ROR1, 0.2 mg/ml protein was incubated overnight at room temperature with 1U PNGaseF (Roche) per 2 μ g ROR1 protein. Control, glycosylated human ROR1 was prepared in parallel without adding PNGaseF. SDS PAGE analysis showed shift on PNGaseF treatment, consistent with ROR1 deglycosylation (Figure 17A).

These ROR1 proteins were used to coat ELISA plates and ELISAs were performed as previously described in the "Anti ROR1 VNAR characterisation" section. VNARs (B1, P3A1-P3A1, D3-D3, B1 mFc) bound equally well to both glycosylated and deglycoylated ROR1 proteins by ELISA (Figures 17B & 17C) indicating ROR1 binding is independent of ROR1 glycosylation.

Binding of B1 to unfolded hROR1 (reduced with 28mM DTT, 0.5% Sarkosyl) was significantly reduced, consistent with B1 VNAR binding to conformational epitope(s) (Figure 17C)

Binding of B1 to ROR1 Ig domain by SEC

B1 VNAR forms a complex with ROR1 Ig domain by SEC (Figure 18). 1:1 VNAR:ROR1 domain or ROR1 domain pairs was incubated on ice for 30 mins then run on a the Superdex 200 increase 10/300 column (GE Healthcare) in PBS and fractions analysed by SDS-PAGE. Under these conditions, B1 formed a complex with the ROR1 Ig domain.

Epitope binning experiments

Competition of binding studies were completed using SPR. Human ROR1 (hROR1) was immobilised to flow channels 1 and 3 (FC1 and FC3) of a COOH₂ chip by amine coupling. FC2 was used as the reference channel. A chosen VNAR e.g. B1, P3A1 dimer; or ROR1 2A2 mAb (BioLegend) was then captured to hROR1 on FC1. Test analytes were then assessed for binding to i) hROR1 with either VNAR or ROR1 2A2 mAb previously captured, or ii) to hROR1 in the absence of bound VNAR or mAb. The hROR1 chip surface was regenerated following each test analyte using Glycine pH2. Prior to testing the next analyte, VNAR or ROR1 2A2 mAb was again captured to hROR1 in FC1 and so on. Binding kinetics were determined using QDat software. For non-competing molecules, binding kinetics and sensogram profiles were similar/unaffected to hROR1 +/- captured binder. For competing molecules, the sensogram profile and binding kinetics were significantly altered.

Figure 19 shows representative sensograms and binding kinetics for binding of the VNARs to human ROR1 without and with prior incubation with B1. The results demonstrated that B1 and P3A1 VNARs do not compete with each other, nor with the ROR1 mAb 2A2 for binding to hROR1. When B1 VNAR was captured to hROR1 on the chip surface, further binding of B1 was significantly hindered, however the binding profiles of P3A1 monomer, P3A1 dimer or ROR1 2A2 mAb to hROR1 were the same in the absence and presence of pre-captured B1 (Figure 19). The kinetic parameters derived for binding of these molecules to hROR1 in the presence or absence of captured B1 VNAR confirm that they do not compete with B1 (with the exception of B1, which competes with itself as expected).

Binding of VNARs to hROR1 with and without pre-capture of P3A1 derivatives or 2A2 mAb was similarly assessed. The results are summarised in (Table 9), which showed that B1, P3A1 and 2A2 do not compete with each other, but compete with themselves as anticipated, and therefore bind different regions of hROR1.

Table 9 - Binding kinetic data derived by SPR analysis of VNARs or ROR1 2A2 mAb to hROR1 +/- previously captured B1 VNAR. Data demonstrates that B1 binding does not compete with P3A1 or 2A2. VNARs were expressed with C-terminal His₆Myc tags.

Molecule	hROR1 binding			B1 pre- captured to hROR1		
	K _a (M ⁻¹ s ⁻¹)	K _d (s ⁻¹)	K _D (nM)	K _a (M ⁻¹ s ⁻¹)	K _d (s ⁻¹)	K _D (nM)
B1	1.04E+06	4.40E-04	0.424nM	No/poor binding		
P3A1-P3A1	1.63E+06	6.28E-04	0.385nM	1.52E+06	5.36E-04	0.352nM
P3A1	2.58E+06	4.11E-02	15.9nM	1.94E+06	3.20E-02	16.45nM
ROR1 2A2 mAb (Biolegend)	9.79E+05	2.11E-04	0.21nM	8.35E+05	8.47E-05	0.101nM

Epitope Mapping of anti-ROR1 VNARs using anti-ROR1 peptides

ELISA analysis was used to determine whether the lead anti-ROR1 VNAR domains, B1, P3A1 and D3 bound to the same or overlapping epitopes on ROR1 (defined here as four ECD peptides). Initial analysis of direct binding with peptides (in PBS and DMSO) immobilised onto ELISA plates indicated that none of the VNARs bound any of the peptides but did bind to the immobilised ECD hROR1-Fc protein control included as part of the same ELISA (Figure 20 and Figure 21). To interrogate this further, a competition assay was designed where VNARs were incubated with increasing concentrations of the four test peptides (or human ROR1 ECD-Fc) in solution and an assessment of residual binding to ROR1-Fc immobilised on an ELISA plate was then observed. Competition was evident between the VNARs and human ROR1 ECD-Fc, which was used as a positive control. However, no decrease signal was evident in the presence of the peptides, clearly indicating that no binding of VNAR to these specific ECD peptides had occurred (Figure 22 and Figure 23).

Further, B1, P3A1 and D3 VNARs do not bind any overlapping linear 15mer peptides spanning the entire ECD of hROR1. Nor do they bind to hROR1 previously sonicated in SDS containing buffer under reducing conditions, conditions that typically denature protein (Pepscan data not shown). Together this indicates B1, P3A1 and D3 VNARs bind to distinct conformational epitope(s) on human ROR1 ECD protein.

Direct binding of VNARs to ECD peptides

The following peptides were synthesised and dissolved in PBS pH 7.4:

Peptide 1 — YMESLHMQGEIENQI (SEQ ID NO: 34)
 Peptide 2 — RSTIYGSRLRINLDTTDTGYFQ (SEQ ID NO: 38)
 Peptide 3 — CQPWNSQYPHTHTFTALRFP (SEQ ID NO: 35)
 Peptide 4 — QCVATNGKEVVSSTGVLFVKFGPPPTASPGYSDEYE (SEQ ID NO: 37)
 Peptide 5 — RSTIYGSRLRIRNLDTTDTGYFQ (SEQ ID NO: 36)

Clones B1 and P3A1 isolated from ELSS1 were assessed as monomers and D3 from an immunized library as both a monomer and a homodimer.

Both B1 and P3A1 demonstrated binding to ROR1 with no binding evident to any of the four peptides. HSA was included as a non-specific control (Figure 20).

However as peptide 2 was insoluble in PBS, the direct binding ELISAs were repeated with the peptides dissolved in 25% DMSO. D3 and D3-D3 as a protein dimer fusion were included in these datasets and again no binding to the peptides was observed (Figure 21).

Methods

Direct Peptide Binding ELISA

1. Coated 96 well plates with 10 or 50 nM huROR1-Fc in PBS or 10 μ M of peptides in PBS or 25% DMSO. Incubated o/n at 4°C
2. Washed 2xPBS
3. Blocked with 200 μ l/well of 4% MPBS for 1h at RT.
4. Washed 2xPBS
5. Added B1 or P3A1 at 1 μ g/ml (67nM); D3 and D3-D3 at 10 μ g/ml (670 nM) and 1:3 serial dilutions across the plate. Incubated for 1 h at RT.
6. Washed 3xPBST
7. Incubated plates with 100 μ l of anti-his-HRP SIGMA (1:1000 in PBST) for 1h at RT
8. Washed 2xPBST and 2xPBS
9. Added 100 μ l/well of TMB substrate. Stopped reaction with 1 M H₂SO₄

Competition assays of VNARs and ROR1 peptides

Competition assays were conducted as described in the methods with all four peptides reconstituted in PBS. In these assays no binding was observed by VNARs B1 or P3A1 to any of the four peptides immobilised in typical binding ELISA format (Figure 22). Therefore, there was no evidence that these peptides represented epitopes on ROR1 that are recognised by B1 or P3A1.

Following the conditions used in Figure 21 (due to peptide 2 being insoluble in PBS), all the competition assays were repeated with peptides dissolved in 25% DMSO. For the assay D3 and D3-D3 dimer were also included in these datasets. These results confirmed that the VNAR domains B1, P3A1 and D3 recognise a different epitope (or epitopes) from those represented by the 4 peptides tested.

Methods

Competition ELISA

1. Coated 96well plates with 50 nM of huROR1-Fc for P3A1; 10 nM of huROR1-Fc for B1, D3 and D3-D3 dimer in PBS. Incubated o/n at 4°C
2. Washed 2xPBS
3. Blocked with 200 µl/well 4% MPBS for 1h at RT
4. Washed 2xPBS
5. Pre-incubated for 30 min at RT
 - B1= 15 nM
 - Plus peptides (in PBS or 25% DMSO) at start concentration of 1 µM (then 1:3 serial dilutions across the plate)
 - or huROR1-Fc at start concentration of 100 nM (then 1:3 serial dilutions across the plate)
 - P3A1= 670 nM
 - Plus peptides (in PBS or 25% DMSO) at starting concentration of 50 µM (then 1:3 serial dilutions across the plate)
 - or of huROR1-Fc at a starting concentration 1 µM (then 1:3 serial dilutions across the plate)
 - D3 = 67 nM
 - Plus peptides or huROR1-Fc (in PBS or 25% DMSO) at starting concentration of 500 nM (then 1:3 serial dilutions across the plate)
 - D3-D3 = 0.67 nM
 - Plus peptides or huROR1-Fc (in PBS or 25% DMSO) at starting concentration of 500 nM (then 1:3 serial dilutions across the plate)
6. Add 100 µl/well of pre-incubated samples. Incubated 1 h at RT
7. Washed 3 x PBST
8. Incubated plates with 100 µl/well of anti-His-HRP (1:1000 in PBST). Incubated 1 h at RT
9. Washed 2 x PBST and 2 x PBS
10. Added 100 µl/well of TMB substrate. Stopped reaction with 50 µl/well 1 M H₂SO₄

Epitope Mapping of anti-ROR1 VNARs using recombinant ROR1 domains

The ROR1 ECD is made up of three distinct protein domains: Ig-like, Frizzles and Kringle. To determine if the epitope recognised by each of these VNARs was within a specific sub-domain of the whole ROR1 protein the following ELISA analysis was performed.

Direct binding of VNARs to ROR1 domains

Anti-ROR1 VNARs B1, P3A1 and D3 were assessed for binding to the three extracellular domains of human ROR1 (Ig-like, Frizzles and Kringle) by direct binding ELISA. B1 and P3A1 were assessed as monomers and D3 as both a monomer and a homodimer (D3-D3). 2A2 anti-ROR1 antibody was also incorporated into the assay as a positive control.

B1 and 2A2 recognised the Ig-like domain, however this binding to Ig-like domain was much weaker compared to their binding of the whole extracellular huROR1. P3A1 recognised the Frizzled domain but again weaker binding than to the intact ROR1 protein (Figure 24 and Table 10). D3 and D3-D3 homodimer bound full length ROR1 ECD but no binding to individual ROR1 ECD sub domains was observed (Figure 24 and Table 10).

All results are summarised in a Table 10.

Table 10:

	B1	P3A1	D3	2A2
rhROR1-Fc	+++	+++	+++	+++
Ig-like domain	+	-	-	+
Frizzle domain	-	+	-	-
Kringle domain	-	-	-	-

Methods

Direct Binding ELISA to ROR1 domains

1. Coated 96 well plates with 1 µg/ml of huROR1-Fc or huROR1 domains in PBS. Incubated o/n at 4 °C.
2. Washed 2xPBS
3. Blocked with 200 µl/well of 4% MPBS for 1h at RT.
4. Washed 2 x PBS
5. Added D3, D3-D3 dimer or 2A2 mAb at start concentration 10 µg/ml for VNAR and 1:150 dilution for mAb. Made 3-fold serial dilutions across the plate. Incubated for 1 h at RT.
6. Washed 3 x PBST
7. Incubated plates with 100 µl of anti-c-myc-HRP (1:1000 in PBST) for 1h at RT.
8. Washed 2 x PBST and 2 x PBS
9. Added 100 µl/well of TMB substrate. Stopped reaction with 1 M H₂SO₄.

EXAMPLE 8 – VNAR conjugation chemistries

Labelling of BA11 as proof of concept for site-specific VNAR conjugation

Currently there are no methods for the site-specific conjugation of labels and drugs to VNARs, therefore there is a need to establish such conjugation methods. The VNAR BA11 is a humanised variant of E06 that binds with high affinity to human serum albumin (Kovalenko et al, J.Biol. Chem., 2013 JBC) and has applications as a half-life extension technology. BA11 was used as a model VNAR to determine whether site-specifically conjugated VNARs can be generated in good yield without compromising the binding activity of the VNAR domain. The C-terminus of VNARs is distal to

the CDR1 & 3 and HV2 & 4 regions, which are the regions of the VNAR generally used to bind its target.

Therefore, intein based technology (US2006247417) was used to assess the site-specific conjugation of payloads to the C-terminus of VNARs via different chemistries. Briefly, the protein of interest is expressed as an N terminal fusion of an engineered intein domain (Muir TW 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 (Figure 11). These C-terminal aminoxy and thiol derivatives can be reacted with aldehyde / ketone and maleimide functionalised moieties, respectively, in a chemoselective fashion to give the site-specific C-terminally modified protein (Figures 25-27). Using this approach BA11 fluorescein conjugates were generated via oxime and thioether forming chemistry in good yields and these conjugates maintained binding to human serum albumin protein.

Initially, the BA11 intein-CBD fusion protein, immobilised on chitin beads, was generated as described previously with typical yields $\geq 10\text{mg/L}$ from cytosolic expression in *E. coli*. This precursor fusion protein was then cleaved under aqueous buffered conditions with different small molecule agents to generate BA11 with unique chemically reactive functionalities at its C-terminus.

Generation of BA11-aminoxy (Figure 11)

Immobilised BA11 intein-CBD fusion protein was cleaved overnight in 400mM dioxyamine ($\text{NH}_2\text{-O-}(\text{CH}_2)_2\text{-O-NH}_2$) in cleavage buffer pH6.9 resulting in $\sim 75\%$ cleavage.

Cleavage supernatant containing BA11 aminoxy was drained and purified on a Superdex75 26/60 (GE Healthcare) in 20mM sodium phosphate pH6.9, 200mM NaCl. This yielded soluble, derivatised, folded protein with yields of $>2\text{mg/L}$ *E. coli*. All protein was characterised by reducing and non-reducing SDS PAGE analysis and mass spectrometry. The formation of the desired disulphide bond was confirmed by mass spec methods.

Generation of BA11-oxime-fluorescein (Figure 25)

Purified BA11 aminoxy was mixed with 3 molar equivalents benzaldehyde-peg-fluorescein in pH5.5 buffer with 10% acetonitrile and 10mM aniline catalyst, room temperature overnight. SDS PAGE and mass spectrometry showed $\geq 98\%$ reaction and conjugate was purified by SEC as above, and confirmed by reducing and non-reducing SDS PAGE analysis and mass spectrometry.

Generation of BA11 C-terminal thiol derivatives (Figure 11)

BA11 intein-CBD fusion protein immobilised on chitin beads was cleaved overnight in 100mM cysteamine (Sigma) in cleavage buffer with 2mM TCEP to generate the corresponding C-terminal thiol derivative of the VNAR. The cleavage supernatant containing BA11 thiol was drained, treated with 2mM TCEP to reduce any cysteamine adducts on the introduced C-term thiol group, and protein purified on a Superdex75 26/60 (GE Healthcare) in 20mM sodium phosphate pH6.9, 200mM NaCl. Yields ~1.6mg / L *E. coli* for BA11 SH were obtained. All proteins were characterised by reducing and non-reducing SDS PAGE analysis and mass spectrometry. The formation of the desired disulphide bond and free C-terminal thiol were confirmed by mass spec methods.

Generation of BA11-C term thiol-maleimide-peg-fluorescein (Figure 26)

BA11 generated with a C-terminal thiol (BA11 SH) was mixed with 4 molar equivalents maleimide-peg-fluorescein in pH6.9 buffer with 0.3% DMF final, room temperature 0.5-1 hour. SDS PAGE and mass spectrometry showed ≥98% reaction. Conjugate was purified by SEC as above, and confirmed by reducing and non-reducing SDS PAGE analysis and mass spectrometry.

Generation of BA11 C-terminal Cysteine derivatives (Figure 11)

BA11 Intein-CBD fusion protein immobilised on chitin beads was cleaved overnight in 100mM cysteine in cleavage buffer with 2mM TCEP to generate the corresponding C-terminal cysteine derivative of the VNAR. The cleavage supernatant containing BA11 Cys was drained, treated with 2mM TCEP to reduce any cysteine adducts on the introduced C-term thiol group, and protein purified on a Superdex75 26/60 (GE Healthcare) in 20mM sodium phosphate pH6.9, 200mM NaCl. Yields ~ >3mg / L *E. coli* for BA11-cys were obtained. All proteins were characterised by reducing and non-reducing SDS PAGE analysis and mass spectrometry. The formation of the desired disulphide bond and free C-terminal cysteine thiol were confirmed by mass spec methods.

Generation of BA11-C terminal cysteine-maleimide-peg-fluorescein (Figure 27)

BA11 generated with a C-terminal cysteine (BA11 cys,) was mixed with 4 molar equivalents maleimide-peg-fluorescein in pH6.9 buffer with 0.3% DMF final, room temperature 0.5-1 hour. SDS PAGE and mass spectrometry showed 60-80% reaction for BA11 cys, lower reaction was due to significant BA11 cys dimer formation. Conjugate was purified by SEC as above, and confirmed by reducing and non-reducing SDS PAGE analysis and mass spectrometry.

The binding of BA11 and the corresponding C-terminal derivatives and conjugates to serum albumins was determined by SPR

Determination of the binding kinetics of the half-life extension VNAR (BA11) or Fluorescein-conjugated-BA11 to human, mouse, rat and cynomolgous serum albumin

Binding kinetics were determined using SPR. The serum albumins or negative control protein were immobilised to COOH₂ chips by amine coupling using optimised buffer conditions as follows:- Human serum albumin (HSA) and mouse serum albumin (MSA) were immobilised in sodium acetate pH5 buffer. Rat serum albumin (RSA) and cynomolgous serum albumin (CSA) in sodium acetate pH 4.5 buffer and the negative control hen egg lysozyme (HEL) protein was immobilised in sodium acetate pH 5.5 buffer.

Analytes (BA11, BA11-Fluorescein or 2V negative control binder) 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 QDat software (SensiQ/Pall ForteBio). For each analyte test experiment, binding to the chosen serum albumin protein was assayed alongside the negative control protein (HEL).

Table 11. Summary of SPR data (K_D nM) for BA11 C terminal derivatives and subsequent fluorescein conjugates with different conjugation chemistries binding to serum albumin proteins. FI, fluorescein; cys, cysteine; mal, maleimide; SH, thiol; 2V, non-binding VNAR negative control.

Serum Albumin	Human		Rat		Mouse		Cyano	
pH	7.4	5.5	7.4	5.5	7.4	5.5	7.4	5.5
BA11	0.636	0.910	2.969	4.389	1.902	4.804	3.360	1.109
BA11-aminooxy	1.118	ND	10.85	ND	8.767	21.20	7.970	ND
BA11-oxime-Fl	0.677	1.296	5.725	5.928	4.238	7.442	1.748	5.540
BA11-cys	0.756	1.956	3.370	3.215	ND	3.775	2.103	ND
BA11-cys-mal-Fl	1.097	3.160	4.775	7.240	5.064	13.645	3.681	8.205
BA11-SH	0.774	2.229	7.671	12.10	4.764	11.08	3.414	6.738
BA11-S-mal-Fl	1.417	1.912	5.297	7.925	5.004	10.60	2.300	7.010
2V	Did not bind							

All BA11 derivatives and conjugates showed high affinity binding to the different serum albumin proteins at both pH7.4 and pH5.5. Therefore the methodologies described provide robust high yielding approaches for the site-specific modification and conjugation of VNARs that maintain the binding activity of the protein.

ROR1 binding VNARs - AF488 and MMAE conjugates

Expression of ROR1 binding VNARs as C-terminal intein fusion proteins enabled generation of ROR1 binding VNARs with unique C-terminal aminooxy and C-terminal thiol groups. This in turn enabling site specific, C-terminal conjugation to fluorescent labels and cytotoxic payloads via oxime forming

conjugation chemistry and maleimide chemistry, respectively. Examples of labels and payloads used are shown in Figure 28.

ROR1 binding VNAR intein CBD fusion protein immobilised on chitin beads was generated as described above

Generation of VNAR-ox-vcMMAE and VNAR-ox-MMAE

The immobilised VNAR intein fusion protein was cleaved with 400mM O,O'-1,3-propanediylbishydroxylamine ($\text{NH}_2\text{-O-(CH}_2\text{)}_3\text{-O-NH}_2$) in cleavage buffer pH 6.9, room temperature overnight. The resulting VNAR containing a C-terminal aminoxy group (VNAR aminoxy) was purified by IMAC or SEC and reacted with 3 molar equivalents of benzaldehyde PEG2 vc PAB MMAE or benzaldehyde PEG4 MMAE in 10% acetonitrile with 10mM aniline catalyst final, room temperature overnight. Conjugates were purified by IMAC or SEC, sterile filtered and formation of the desired material and final purity confirmed by reducing and non-reducing SDS PAGE analysis and mass spectrometry (Figure 29)

Generation of VNAR-S-mal-vcMMAE

The immobilised VNAR intein fusion protein was cleaved with 100mM cysteamine in cleavage buffer pH 6.9 with 2mM TCEP, room temperature overnight. The resulting VNAR containing a C-terminal thiol group (VNAR SH) was purified by IMAC or SEC and reacted with 4 molar equivalents of MC vc PAB MMAE or malAF488. Conjugates were purified by IMAC or SEC, and sterile filtered and formation of the desired material and final purity confirmed by reducing and non-reducing SDS PAGE analysis and mass spectrometry (Figure 29)

Characterisation of anti ROR1 VNAR-MMAE conjugates - binding to ROR1 and ROR2 by SPR and cell surface binding by flow cytometry

Binding of VNAR conjugates to ROR1 and ROR2 by SPR

The ability of the VNAR-MMAE conjugates and VNAR-fluorescein conjugates to bind to human ROR1 ECD was determined by SPR using the procedures described above.

As shown in Table 12 VNAR conjugates that were prepared through oxime ligation of benzaldehyde payloads to C-terminal aminoxy VNARs; through thioether ligation of maleimide functionalised payloads to C-terminal thiol VNARs and through thioether ligation of maleimide functionalised payloads to C-terminal Cysteine VNARs all maintain high affinity for human ROR1 but do not bind to human ROR2. Conjugates were prepared using enzyme cleavable linkers (Val-Cit) or non-cleavable linkers and showed similar binding to human ROR1.

Table 12: SPR data for binding of VNARs and corresponding Fluorescein and MMAE conjugates to human ROR1 and ROR2. VNARs were expressed with C-terminal His₆Myc tags.

	hROR1			hROR2
VNAR	K _a (M ⁻¹ s ⁻¹)	K _d (s ⁻¹)	K _D (nM)	
B1	4.75E+05	7.56E-04	1.65	No binding
B1 -S- mal-Fluorescein	4.7E+05	3.67E-04	0.81	No binding
2V	No binding	No binding	No binding	No binding
2V -S-mal-vcMMAE	No binding	No binding	No binding	No binding
2V-Ox-vcMMAE	No binding	No binding	No binding	No binding
2V-Ox-MMAE	No binding	No binding	No binding	No binding
P3A1-P3A1	1.86E+06	2.96E-03	1.61	No binding
P3A1-P3A1-S-mal-vcMMAE	4.96E+06	2.6E-03	0.59	No binding
P3A1-P3A1-Ox-vcMMAE	2.07E+06	2.77E-03	1.43	No binding
P3A1-P3A1-Ox-MMAE	4.20E+06	3.20E-03	0.78	No binding
2V-2V	No binding	No binding	No binding	No binding
2V-2V-S-mal-vcMMAE	No binding	No binding	No binding	No binding
2V-2V-Ox-vcMMAE	No specific binding	No specific binding	No specific binding	No binding
2V-2V-Ox-MMAE	No binding	No binding	No binding	No binding

Binding of VNAR conjugates to cancer cell-lines

Binding of B1 and P3A1 MMAE conjugates to cancer cell-lines was determined by flow cytometry using methods described above. B1 and P3A1 conjugates maintain binding to the ROR1^{hi} A549 lung

adenocarcinoma cells and do not bind the ROR1^{low} lung cancer cell-line A427 by flow cytometry at a fixed concentration of protein.

VNAR mFc fusion protein conjugates

B1 mlgG2a Fc and nonbinding 2V mlgG2a Fc fusion proteins were labelled with mal AF488 and mc vc PAB MMAE via protocols adapted from the partial reduction and labelling of antibody interchain disulfides (Methods in Molecular Biology vol 1045 chapter 9; Sun et al, Bioconj Chem 2005). Briefly VNAR mlgG2a Fc proteins at 1mg/ml in PBS +100mM L-Arg with 1mM EDTA added were partially reduced with 2.75 molar equivalents fresh TCEP; 37°C 2hours. 1.1 molar equivalents maleimide label to free protein thiol was added, incubated on ice 45 mins and L-cysteine added to stop the reaction. Reactions were dialysed to remove unreacted label / drug, sterile filtered and analysed by SDS PAGE. Typical DAR of 4.4 for B1-mFc-AF488, and 3.9 for 2V-mFc-AF488.

VNAR hFc fusion protein 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).

Anti ROR1 VNARs were genetically fused to engineered hlgG1 Fc domains that contained a cysteine substitution in the hlgG1 Fc sequence, S252C or S473C (Kabat numbering). This enabled site specific labelling with maleimide derivatives of fluorescent labels (AF488) and cytotoxic drugs (MC vc PAB MMAE, MC vc PAB NHC₆ α -amanitin, MA PEG4 va PBD, MA PEG8 va PAB SG3199, MA PEG4 vc PAB DMAE PNU 159682) (Figure 32).

Generation of VNAR-hFc – drug conjugates

A partial reduction, refolding and labelling method to label the VNAR Fc S252C was adapted from the literature (Junutula et al, 2008 Nat Biotech, Jeffrey et al, 2013 Bioconj Chem). 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 S252C was extensively dialysed or buffer exchanged into PBS +50mM L-Arginine and quantified by UV before reacting with 4 molar equivalents maleimide label / drug solution, room temperature 1 hour to overnight depending on label / drug. Conjugates were dialysed / buffer exchanged directly or purified further by SEC or IEX before dialysis / buffer exchange.

This approach was used to generate MMAE conjugates of B1, P3A1 and 2V Fc fusion proteins whereby the corresponding hlgG1 Fc (S252C) derivative was labelled with a maleimide functionalised MMAE payload incorporating an enzyme cleavable (Cathepsin B) linker.

A similar approach was used to generate MMAE conjugates of B1-hFc-7C12 and P3A1-hFc-7C12 fusion proteins.

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 VNAR-hFc – MMAE conjugates with drug to antibody ratio (DAR) of 2 (Figure 31). Similar procedures were used to generate PBD dimer, α -amanitin and PNU conjugates of cysteine engineered VNAR-hFc fusion proteins (Levena Biopharma, San Diego). Whereby VNAR (B1, P3A1, 2V) hIgG1 Fc(S252C) fusions were reacted with MC vc PAB NHC₆ α -amanitin, MA PEG4 va PBD, MA PEG8 va PAB SG3199, MA PEG4 vc PAB DMAE PNU 159682 (Figure 32).

Binding of VNAR-hFc – MMAE conjugates to hROR1 and cancer cell-lines

The ability of the VNAR-hFc conjugates to bind to human ROR1 ECD was determined by SPR using the procedures described above.

Table 13: SPR data for binding of VNAR human Fc (hFc) and MMAE conjugated versions to human ROR1 and human ROR2

Molecule set	hROR1			hROR2
	Ka (M ⁻¹ s ⁻¹)	Kd (s ⁻¹)	KD (nM)	
B1 hFc	3.08E+06	9.53E-05	0.032	No binding
B1 hFc-MMAE	1.22E+06	1.29E-04	0.105	No binding
P3A1 hFc	1.07E+07	5.64E-04	0.084	No binding
P3A1 hFc-MMAE	2.68E+06	1.00E-03	0.38	No binding
2V hFc	No binding	No binding	No binding	No binding
2V hFc -MMAE	No binding	No binding	No binding	No binding
2V-2V hFc	No binding	No binding	No binding	No binding

B1 and P3A1 VNAR -hIgG Fc (S252C) – vcMMAE conjugates demonstrated high affinity binding to ROR1 but do not bind to human ROR2. 2V is a non-binding VNAR and the corresponding 2V-hFc drug conjugates were generated as non-binding controls.

Binding of B1 and P3A1 hFc - vcMMAE conjugates to ROR1^{hi} A549 lung adenocarcinoma cell-line and the ROR1^{low} A427 lung cancer cell-line was determined by flow cytometry using methods described above.

Figure 30 shows that B1 and P3A1 hFc-vcMMAE conjugates bind strongly to the ROR1^{hi} cancer cells but not the ROR1^{low} cancer cells. Whilst the 2V-hFc-vcMMAE conjugate does not bind to either cell-line.

In vitro cell viability assays for cancer cells treated with anti EGFR-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. 10µL of the 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 a top concentration 1000nM (column 1) to 0.05nM (column 10). 10µl of vehicle control (PBS) was added to the control wells (columns 11 and 12). Plates were incubated at 37°C, 5% CO₂ for 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 the incubator and allowed to equilibrate to room temperature before adding 100µl of room temperature Cell Titre Glo reagent to each 100µl assay well. 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 Prism software.

Figure 33 shows dose response curves, with corresponding IC₅₀ values, for cell-killing of the ROR1 positive cancer cell-lines A549 (lung adenocarcinoma), MDA-MB-231 (breast cancer), DU145 (prostate cancer), Kasumi-2 (ALL cells) and Jeko1 (MCL cells) by B1-mFc-vcMMAE and 2V-mFc-vcMMAE conjugates. B1-hFc-vcMMAE conjugates show potent cell-killing of the ROR1 positive cancer cells and show superior potency to the corresponding 2V-mFc-vcMMAE conjugate across each of the cell-lines.

Table 14: IC₅₀ values for cell-killing by B1-mFc-MMAE and 2V-mFc-MMAE per cell line.

Cell line	IC ₅₀ (nM)	
	B1 mFc MMAE	2V mFc MMAE
A549	24.2	228
MDA-231	36.6	212
DU145	15	75
Kasumi-2	26	240
JeKo-1	8.1	66

Figure 34 shows dose response curves, with corresponding IC₅₀ values, for cell-killing of A) the ROR1 positive DU145 prostate cancer cells by B1-hFc-PBD, D3-hFc-PBD and 2V-hFc-PBD conjugates and B) ROR1 positive Jeko1 MCL cells by B1-hFc-PBD, P3A1-hFc-PBD, D3-hFc-PBD and 2V-hFc-PBD conjugates. B1-hFc-vcMMAE conjugates show potent cell-killing of the ROR1 positive cancer cells and are significantly more potent than the corresponding 2V-mFc-vcMMAE conjugates.

Table 15: IC₅₀ values (nM) determined for VNAR hFc-PBD molecules in DU145 and Jeko-1 cancer cell lines at 96hr.

Cell Line	IC ₅₀ (nM)			
	B1 hFc-PBD	P3A1 hFc-PBD	D3 hFc-PBD	2V hFc-PBD
DU145	4.6	/	29.2	226.2
JeKo-1	0.36	1.9	12.6	25.4

The ROR1 targeting VNAR-PBD conjugates show potent killing of both cancer cell-lines and show increased potency with respect to the 2V-hFc-PBD conjugate, with the IC₅₀ values for the B1-hFc conjugate at least 49 fold lower than 2V-hFc conjugate.

Figure 35 shows dose response curves, with corresponding IC₅₀ values, for cell-killing of the ROR1 positive PA-1 ovarian cancer cells (A,C,E) and Kasumi-2 B-cell precursor leukaemia cells (B, D, F) by B1-hFc-PNU, 2V-hFc-PNU conjugates (PEG4-vc PAB DMAE PNU 159682), P3A1-hFc-PBD, D3-hFc-PBD and 2V-hFc-PBD conjugates and B1-hFc SG3199 PBD and 2V-hFc SG3199 PBD conjugates.

Table 16: Calculated IC₅₀ values (nM) for the cell-killing of PA-1 and Kasumi-2 cancer cells by VNAR-hFc conjugates. PA-1 ROR1 ko is a PA-1 cancer cell-line where ROR1 expression has been knocked out.

Cell Line	P3A1 hFc-va-PBD-SGD1882 IC ₅₀ (nM)	D3 hFc-va-PBD-SGD1882 IC ₅₀ (nM)	2V hFc-va-PBD-SGD1882 IC ₅₀ (nM)	B1 hFc-va-PAB-SG3199 IC ₅₀ (nM)	2V hFc-va-PAB-SG3199 IC ₅₀ (nM)	B1 hFc-vc-PAB-DMAE-PNU159682 IC ₅₀ (nM)	P3A1 hFc-vc-PAB-DMAE-PNU159682 IC ₅₀ (nM)	2V-hFc-vc-PAB-DMAE-PNU159682 IC ₅₀ (nM)
PA-1	0.065	0.34	2.5	0.03	5.9	0.028	0.0027	3.13
PA-1 ROR1 ko	ND	ND	ND	0.79	10.5	1.5	3.4	4.5
Kasumi-2	0.52	0.25	6.6	0.06	4.4	0.8	5.1	11

The ROR1 targeting VNAR- conjugates show potent killing of both PA-1 and Kasumi-2 cancer cell-lines and show increased potency with respect to the corresponding 2V-hFc conjugates, with the IC50 values for a number of ROR1 targeting conjugates > 100 fold lower than the corresponding 2V-hFc conjugate controls.

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.

CD3 BITE 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

DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKD
KATLTDDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSGGGGSGGGGSGG
GGSDIQLTQSPAIMASAPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPIRFSG
SGSGTSYSLTSSMEADAATYYCQWSSNPLTFGAGTKLELKS (SEQ ID NO: 96)

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

VL-[G₄S]₃-VH

MDIQMTQTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDGTVKLLIYYTSRLHSGVPSKFSGSGS
GTDYSLTISNLEQEDIATYFCQQGNTLPWTFAGGTKEIKGGGGSGGGGSGGGGSEVQLQQSGPEL
VKPGASMKISCKASGYSTGYTMNWVKQSHGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSS
TAYMELLSTSEDSAVYYCARSGYYGDSWYFDVWGQGTTLTVFS (SEQ ID NO: 97)

EXAMPLE 11 – ROR1 CAR-T approaches

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.

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).

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 treatment of cancer.

EXAMPLE 12 – Characterisation of ROR1xEGFR bi-specific molecules

Construction of ROR1xEGFR bispecific antigen binding molecule

ROR1xEGFR bispecific antigen binding molecules were constructed using the EGFR nanobody binders 7C12 or 9G8. 7C12 was chosen because it blocks EGF binding, shows high EGFR affinity (low nM), and has a higher off rate than the related 7D12 (5aa seq difference). 9G8, which binds to a slightly different EGFR epitope and elicits EGFR inhibition via a slightly different mechanism was also chosen.

The EGFR nanobodies were fused to the ROR1-specific VNARs B1, D3 & D3D3, P3A1 using a [G₄S]₅ linker sequence. A His6Myc tag was included for purification and detection.

Fusions were also generated containing a short sequence of QACGA (SEQ ID NO: 79) between the VNAR and the His6Myc tag or alternatively the sequence -ACA- (SEQ ID NO: 81) between the nanobody and the His6Myc tag to facilitate conjugation with thiol reactive payloads and labels.

In addition, fusion proteins comprising a ROR1-specific antigen binding molecule, an EGFR-specific nanobody and a human Fc region were constructed.

ROR1 and EGFR receptor number determination

ROR1 and EGFR cell receptor numbers were determined for a number of human cancer cell lines using a PE-conjugated ROR1 (2A2) mAb and a PE-conjugated EGFR (AY13) mAb (both Biolegend). Briefly, 5×10^5 cells were incubated with PE-conjugated ROR1 2A2 mAb at 5µg/ml or with 5µg/ml of PE-conjugated EGFR (AY13) mAb, for 1 hour on ice in the dark. Cells were washed twice by re-suspending into 5ml of ice-cold PBS/2% FCS and centrifuging at 1500rpm for 5min at 4°C. Quantibrite beads (BD Biosciences) were used as per the manufacturer's instructions. Analysis was performed on an Attune NxT flow cytometer (ThermoFisher). Receptor numbers (average of n=2) are displayed in Table 17.

*Cell panel characterisation**Cell Surface Binding*

Adherent human 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 5ml 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 1-2ml of PBS/2%FCS. A cell count was performed using a Z1 Coulter Particle Counter (Beckman Coulter) and 5×10^5 cells were aliquoted per test sample. Suspension cells were treated similarly but did not require the initial detachment step. Cells were incubated with 100µl of either VNAR (His₆Myc tagged), VNAR-Fc molecules or ROR1 mAb, EGFR mAb and IgG controls for 1 hour on ice. Excess VNAR, VNAR-Fc or mAb was removed by adding 5ml of ice-cold PBS/2% FCS, followed by centrifugation at 1500rpm for 5 mins at 4°C. The supernatant was removed and a second wash performed by re-suspending the cell pellet in 1ml of ice-cold PBS/2%FCS and adding a further 4ml of ice-cold PBS/2%FCS. Samples were again centrifuged at 1500rpm for 5min at 4°C. Supernatant was removed and excess liquid removed by blotting the tubes on tissue paper. Appropriate secondary antibodies were used to detect bound VNAR (His₆Myc), VNAR-hFc, or mAb (PE-anti-Myc tag antibody (CST), PE-anti-human antibody (JIR labs/Stratech), and PE-anti-mouse antibody (JIR/Stratech). Cells were incubated with chosen secondary antibody for 30min on ice. Cells were washed to remove excess antibody as described earlier. Cell pellets were re-suspended in 0.5ml of ice-cold PBS/2%FCS and left on ice in the dark prior to analysis on an Attune NxT (ThermoFisher) flow cytometer.

A panel of 5 cell lines expressing different levels of ROR1- EGFR was selected for screening bispecific molecules. These cell lines are set out in Table 17

Table 17

	Receptor Number		EGFR Status
	EGFR	ROR1	
High ROR1 – High EGFR			
A549	118793	9316	wt amplification (K-Ras G12S mutation)
Equivalent levels of ROR1/EGFR			
PA-1	6334	12249	* wt
High ROR1 – Low EGFR			
Kasumi-2	0	8087	
Low ROR1 – Medium/Low EGFR			
A427	27662	222	wt (K-Ras G12D mutation)
Low ROR1-Low EGFR			
Mv4-11	0	0	
Cell Lines with EGFR Mutations			
MDA-MB231	84540	6997	EGFR L468W (K-Ras G13D mutation)
PC9	122293	9827	EGFR del.E746_A750
H1975	32584	7987	EGFR L858R+T790M

Cell surface binding of the B1hFc7C12 bispecific molecule was investigated in A549, PA-1, A427, Kasumi-2 and Mv4-11 cells using flow cytometry at 4°C as described earlier. A signal uplifting was observed in A549 and PA-1 cell lines between B1hFc7C12 bispecific molecule and its parental single domain molecules B1hFc and hFc7C12 (Figure 36A & 36B). A signal uplifting was also observed in the ROR1 low, EGFR positive cell line A427. No binding to the receptor negative Mv4-11 cells was observed.

Binding of the P3A1hFc7C12 bispecific molecule was investigated in A549, PA-1, A427 and Kasumi-2 cells again using flow cytometry at 4°C. A signal uplifting is observed in A549 cell line between P3A1hFc7C12 bispecific molecule and its parental single domain molecules P3A1hFc and hFc7C12 (Figure 36C).

ROR1 and EGFR bi-specific binding agents, without an Fc portion, were also assessed for cell-surface binding in the two different orientations ie ROR1-EGFR and EGFR-ROR1 (Figure 37A, 37B and 37C) Figure 37A shows cell surface binding to A549 cells (high ROR1, high EGFR). An uplift in binding for the ROR1-EGFR bi-specific binding agents with respect to the individual ROR1 and EGFR binding domains is clearly observed for certain constructs.

Figure 37B shows cell surface binding to PA-1 cells (high ROR1, medium/low EGFR. Again, an uplift in binding for the ROR1-EGFR bi-specific binding agents with respect to the individual ROR1 and EGFR binding domains is clearly observed.

However, as shown in Figure 37C no uplift in binding of the ROR1-EGFR bi-specific binding agents is observed for A427 cells (low ROR1, low EGFR), which is consistent with the bi-specific agents targeting both ROR1 and EGFR on the surface of ROR1+EGFR+ cells.

Surprisingly, the increase in binding to A549 cells and PA1 cells is dependent on the orientation of the EGFR binding domain (9G8 or 7C12) with respect to the ROR1 binding agent. When the EGFR binding agent is fused C-terminal to the ROR1 binding agent the cell-surface binding is compromised as compared to the same construct but with the EGFR binding agent fused N-terminal to the ROR1 binding agent. Changing the orientation of the domains within the construct therefore provides a method for altering the apparent affinity of the bi-specific agent to the cell-surface.

A similar observation was observed within the context of Fc fusion proteins (Figure 38). When the EGFR binding agent 7C12 was fused to the C-terminus of the Fc fragment (hFc 7C12) the binding to the EGFR+ve cell-lines A549, PA-1 and A427 was consistently lower as compared to the corresponding N-terminal fusion (7C12 hFc). Thereby, enabling the cell-surface binding characteristics of ROR1-EGFR bispecific binding agents to be modulated through appropriate design of the corresponding Fc fusion proteins.

Internalisation Experiments

Internalisation of ROR1xEGFR bi-specific antigen binding molecules was investigated using Immunofluorescence (IF) microscopy in A549 cells.

Black, clear bottom 96-well plates (Greiner) were coated with 100 µg/ml Collagen I (Sigma) to aid cell attachment. Cells were seeded in complete growth media (Gibco) into the coated 96 well plates and incubated at 5% CO₂, 37°C for 24hr. The media was removed and replaced with serum-free media (Gibco) on the following day and left overnight. On the following morning, media was removed and cells were treated with VNAR-hFc molecules. Plates were incubated on ice for 30 minutes. Treatments were removed and replaced with 100µl of PBS/2%FCS per well. One plate was kept on ice and the other was placed at 37°C, 5% CO₂ for 2 hours. Following this 2 hour incubation, the PBS/2%FCS solution was removed and cells were fixed with 4% Paraformaldehyde in ice cold PBS for 20min on ice. The PFA solution was removed and replaced with 0.05% Saponin (Sigma) made up in PBS/2% FCS for 15min at room temperature. This step permeabilises the cell membranes. Secondary antibody staining was performed using; AF488-anti-human Ab (1:250; ThermoFisher) to detect VNAR-hFc fusion proteins. All secondary antibody working stocks were made up in 0.05% Saponin/PBS/2% FCS. Plates were incubated at 4°C overnight in the dark. On the following day, secondary AF488-conjugated antibodies were removed and the cells were washed X3 using 0.05% Saponin/PBS/2% FCS. Lamp-1 antibody (1:200; CST) or EEA1 antibody (1:50; CST) were added to detect lysosome and early endosome compartments respectively. Plates were incubated in the dark at room temperature for 2 hours. The Lamp-1 and EEA1 antibodies were then removed and the cells were

washed X3 with 0.05% Saponin/PBS/2% FCS. AF647-anti rabbit antibody (1:1000; CST) was then added to detect Lamp1 and EEA1 antibody binding. A further incubation in the dark at room temperature for 2 hours was performed before removing the AF647-secondary antibody and washing the cells X3 with 0.05% Saponin/PBS/2% FCS. Cell nuclei were stained using 10µM Hoechst reagent (Sigma) in 0.05% Saponin/PBS/2% FCS for 20 min at room temperature in the dark. Finally, this solution was removed and replaced with PBS. Plates were stored at 4°C in the dark prior to imaging using a GE Healthcare InCell 2000 instrument.

As shown in Figure 39, B1hFc7C12 is clearly and strongly internalised by A549 cells after 2h at 37°C. By contrast, the parental molecules B1hFc and hFc7C12 show lower levels of internalisation and for hFc7C12 only in a few cells.

Similarly, P3A1hFc7C12 is internalised by A549 cells after 2h at 37°C (Figure 40). Parental molecule hFc7C12 show low levels of internalisation and only in a few cells.

Additionally, B1hFc7C12 was shown to co-localise with Early Endosome Antigen 1 (EEA1) and Lysosomal-associated membrane protein 1 (LAMP-1) in A549 cells (Figure 41).

Internalisation of non-Fc ROR1xEGFR bi-specific antigen binding molecules was investigated using flow cytometry as previously described (Figure 42A and 42B). The fluorescence signal for the ROR1xEGFR bi-specific antigen binding molecules was significantly lower after incubation at 37°C versus 4°C indicative of internalisation of the bi-specific proteins upon target binding.

Surface Receptor Downregulation

Expression of ROR1 and EGFR in A549 cells was monitored over a 24 hour period following treatment with B1hFc7C12. As shown in Figure 43A, B1hFc7C12 temporarily downregulates ROR1. B1hFc7C12 also downregulates EGFR with a more prolonged effect: EGFR levels do not return to untreated levels even after 24 hours (Figure 43B).

Downregulation of ROR1 and EGFR by P3A1hFc7C12 and D3D3hFc7C12 is observed, whilst downregulation of EGFR by these bi-specific constructs is less obvious (Figures 44 to 45).

Binding to ROR1 and EGFR proteins

The ability of ROR1xEGFR bi-specific antigen binding molecules to simultaneously engage with both ROR1 and EGFR targets was confirmed by BLI (Figure 46). hROR1 was immobilised on a sensor and the bi-specific constructs or the constituent mono-specific binders were flowed over the sensor to confirm ROR1 binding. EGFR was then immediately passed over the sensor and binding of the ROR1 immobilised bi-specific binding agent to EGFR then assessed by a second increase in signal.

Representative examples for a selection of different bi-specific constructs are shown in Figure 46. Mono-specific ROR1 binding VNAR B1 does not contain an EGFR binding moiety, and so no additional increase in signal is observed when EGFR is flowed over the surface of the sensor.

Cell Killing by ROR1xEGFR Fc Fusion Molecules

The potency of ROR1xEGFR Fc fusion molecules was investigated in a number of cell lines. The results are summarised in table 18. Both the B1hFc-7C12-MMAE and P3A1hFc-7C12-MMAE molecules were shown to be more potent than hFc7C12-MMAE alone

Table 18: B1hFc-7C12-, P3A1hFc-7C12 bispecific-MMAE conjugates and hFc7C12-MMAE were tested in a 96h cell viability assay using Cell Titer Glo read-out. IC50 (nM) are reported. B1hFc-7C12-MMAE and P3A1hFc-7C12 were more potent than hFc7C12-MMAE alone

Cell line	ROR1 receptor #	EGFR receptor #	IC50 (nM) 96hr			IC50 (nM) 96hr	
			B1hFc-7C12- MMAE	P3A1hFc-7C12- MMAE	hFc7C12- MMAE	B1hFc- MMAE	2VhFc- MMAE
A549	9316	118,793	11	197	257	83	>1000
PA-1	12,249	6334	14	32	148	46	641
MDA-MB-468	4107	1,825,000	5.2	25	45	ND	ND
NCI-H1975	7987	35,800	18.3	83	255	ND	ND
PC-9	9827	122,293	3.4	15	42	98	692