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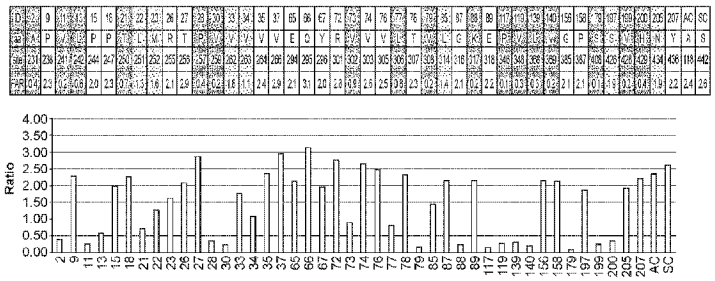

FIG. 7

(57) Abstract: The disclosure provides cysteine substituted immunoglobulins, including polypeptides, antibodies, nucleic acids encoding such polypeptides and antibodies, host cells, vectors and processes for making the same, conjugated derivatives of the antibodies, compositions and methods of making such antibodies and conjugated derivatives, and methods of using the antibodies and conjugated variants for the detection and treatment of cancer and for killing diseased cells. In certain embodiments, the substitution is selected from V266C, G316C, H285C, R301C, V303C, T307C, Y436C and L441C (EU Numbering) or S156 in the hevy chain (under Kabat numbering).

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CYSTEINE-SUBSTITUTED IMMUNOGLOBULINS

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0001] The present patent application claims benefit of priority to US Provisional Patent Application No. 62/193,531, filed July 16, 2015, which is incorporated by reference for all purposes.

REFERENCE TO SUBMISSION OF A SEQUENCE LISTING AS A TEXT FILE

[0002] The Sequence Listing written in file 1014170_ST25.txt created on July 15, 2016, 10,366 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0003] Monoclonal antibodies (mAbs) are an essential tool in research and therapy due to their high specificity and affinity for target antigens. Since the 1990's, therapeutic mAbs have made a substantial impact on medical care for a wide range of diseases, including inflammatory disorders and cancer. A critical feature of mAbs is their ability to bind target antigens in a highly specific manner, marking them for removal by the host immune clearance methods, such as complement-dependent cytotoxicity (CDC) or antibody-dependent cell-mediated cytotoxicity (ADCC). Antibodies can also impart therapeutic benefit by binding an inhibiting the function of target antigens, as in the case of trastuzumab (Herceptin®), bevacizumab (Avastin®) and cetuximab (Erbitux®).

[0004] CLL-1 is a cell surface glycoprotein predominantly expressed in myeloid cells found in hematologic malignancies, such as leukemias (e.g., acute myeloid leukemia (AML)). The currently available therapies for hematologic malignancies carry adverse and often severe side effects. For example, complications arising from MYLOTARG® administration include hepatotoxicity, veno-occlusive disease, severe myelosuppression (in ~98% of patients), tumor lysis syndrome, immune hypersensitivity syndrome and respiratory disorders. Thus, there is a need to identify new therapies for hematologic malignancies that are efficacious with reduced side effects. Since CLL-1 is selectively expressed on myeloid

cells, compositions that recognize and bind to CLL-1 may be useful for such therapies of hematologic malignancies, especially those of myeloid origin.

[0005] Conjugation to cytotoxic drugs or radionuclides can expand the utility of mAbs and improve their potency and effectiveness. This is accomplished because the antibody targets and delivers the cytotoxic payload specifically to the diseased tissue. Antibodies have been conjugated to a number of cytotoxic drugs, through various linker chemistries and these antibody drug conjugates (ADCs) have the ability to selectively and potently kill antigen-expressing tumor cells. ADCs have demonstrated success in the clinic, and there are two such drugs, ado-trastuzumab emtansine (Kadcyla®) and brentuximab vendotin (Adcetris®) are commercially available.

[0006] The successful development of an ADC depends upon the optimization of antibody selection, linker stability, cytotoxic drug potency and mode of linker-drug conjugation to the antibody.

[0007] In a conventional ADC, drug conjugation yields heterogeneous products, containing a mixture of species with different molar ratios of drug to antibody. The conjugation site to the antibody occurs at solvent accessible, reactive amino acid residues such as lysines or cysteines. The heterogeneity occurs as two levels, in that each ADC species differs in both drug load and conjugation site. Panowski et al., mAbs 6:1, 31-45 (2014). Therefore, each species may have distinct properties, resulting in a wide range of in vivo pharmacokinetic (PK) properties as well as batch-to-batch variability. Additionally, the variable drug-to-antibody ratio (DAR) results in a high drug load, high hydrophobicity, fast clearance, lower tolerability and a narrow therapeutic window. Junutula et al., Nat. Biotech. 26(8), 925-932 (2008).

[0008] Site-specific conjugation, in which a known number of linker-drugs are consistently conjugated to defined sites, is one way to overcome these challenges. Heterogeneity is minimized and ADC properties are more predictable, with consistent inter-batch conjugate production.

[0009] The amino acid cysteine provides a reactive thiol group. This group has long been used as the location to label proteins, as well as for the generation of ADCs. While cysteines can be engineered into proteins, this approach is not without challenges. For example, the engineered free cysteine can conjugate with cysteines on other molecules to form protein-dimers. It can also pair intra-molecularly with native cysteine residues to create improper

folding to impair or inhibit protein function. Hence the success of using introduced cysteine residues for site-specific conjugation relies on the ability of select proper sites in which cysteine-introduced substitution does not alter antibody structure or function. Junutula et al., Nat. Biotech. 30(2): 184-191 (2012).

[00010] A further complexity is that solvent accessibility and charge at a substitution site is important for ADC stability. In a study of stability of cysteine, engineered anti-Her2/neu maleimide linker ADCs, high solvent accessibility lost conjugated thiol-reactive linkers in plasma as a result of maleimide exchange with reactive thiols in albumin, free cysteine or glutathione. Shen et al., Nat. Biotech. 30(2): 184-191 (2012). Hence, there is still a great need to identify stable, site-specific ADCs which have consistent drug load, low hydrophobicity, slow clearance, high tolerability and a greater therapeutic index. Furthermore, there is an even greater need to create stable, site-specific ADCs that target CLL-1.

[00011] The statements in this Background are not neither admissions of prior art nor endorsements of the cited references.

BRIEF SUMMARY OF THE INVENTION

[00012] The disclosure provides cysteine substituted immunoglobulins, including polypeptides, antibodies, nucleic acids encoding such polypeptides and antibodies, host cells, vectors and processes for making the same, conjugated derivatives of the antibodies, compositions and methods of making such antibodies and conjugated derivatives, and methods of using the antibodies and conjugated variants for the detection and treatment of cancer and for killing diseased cells.

[00013] In one embodiment, this disclosure provides a cysteine substituted immunoglobulin polypeptide, wherein the substituted residue is one or more residues selected from the group consisting of: V266C, H285C, R301C, V303C, T307C, G316C, Y436C and L441C (EU numbering). In one aspect, the immunoglobulin polypeptides are derived from human IgG heavy chain constant regions. In another aspect, the IgG is isotype IgG1, IgG2, IgG3 or IgG4.

[00014] In another embodiment, the disclosure provides isolated nucleic acid sequences encoding a cysteine substituted immunoglobulin polypeptide, wherein the substituted residue is one or more residues selected from the group consisting of: V266C, H285C, R301C, V303C, T307C, G316C, Y436C and L441C (EU numbering). In one aspect, the nucleic acid

is operably linked with an expression control sequence. In another aspect, the operably linked nucleic acid further comprised in an expression vector. In yet another aspect, the disclosure provides host cells comprising the expression vectors.

[00015] In yet another embodiment, the disclosure provides a process for making a cysteine-substituted immunoglobulin polypeptide comprising culturing a recombinant cell comprising a nucleic acid molecule further comprising a nucleotide sequence encoding a cysteine-substituted immunoglobulin polypeptide, wherein the substituted residue is one or more residues selected from the group consisting of: V266C, H285C, R301C, V303C, T307C, G316C, Y436C and L441C (EU numbering).

[00016] In a further embodiment, the disclosure provides cysteine substituted antibody comprising a cysteine-substituted immunoglobulin polypeptide further comprising a substituted amino acid residue selected from the group consisting of: V266C, H285C, R301C, V303C, T307C, G316C, Y436C and L441C (EU numbering) in a heavy chain constant region. In one aspect the heavy chain constant region is derived from a human IgG isotype selected from the group consisting of IgG1, IgG2, IgG3 and IgG4.

[00017] In another aspect, the antibody further comprises a light chain. In a further aspect, the light chain is selected from the group consisting of kappa and lambda.

[00018] In yet another aspect the antibody binds to CLL-1, GPR114, IL1RAP, TIM-3, CD19, CD20, CD22, ROR1, mesothelin, CD33, CD123/IL3Ra, c-Met, PSMA, prostatic acid phosphatase (PAP), CEA, CA-125, Muc-1, AFP, Glycolipid F77, EGFRvIII, GD-2, NY-ESO-1 TCR, tyrosinase, TRPI/gp75, gp100/pmel-17, Melan-A/MART-1, Her2/neu, WT1, EphA3, telomerase, HPV E6, HPV E7, EBNA1, BAGE, GAGE and MAGE A3 TCRSLITRK6, ENPP3, Nectin-4, CD27, SLC44A4, CAIX, Cripto, CD30, MUC16, GPNMB, BCMA, Trop-2, Tissue Factor (TF), CanAg, EGFR, αv-integrin, CD37, Folate Receptor, CD138, CEACAM5, CD56, CD70, CD74, GCC, 5T4, CD79b, Steap1, Napi2b, Lewis Y Antigen, LIV, c-RET, DLL3,EFNA4, or Endosialin/CD248. In a further aspect, the antibody binds to CLL-1 and comprises a variable light chain and a variable heavy chain, wherein:

(a) the variable light chain further comprises a CDR-L1, CDR-L2 and CDR-L3, further wherein:

a. CDR-L1 is ESVDSYGNSF (SEQ ID NO:1)

b. CDR-L2 is LAS (SEQ ID NO:2)

c. CDR-L3 is QQNNYDPWT (SEQ ID NO:3), and

(b) the variable heavy chain further comprising a CDR-H1, CDR-H2 and CDR-H3, further wherein:

a. CDR-H1 is GYTFTSYV (SEQ ID NO:4)

b. CDR-H2 is INPYNDGT (SEQ ID NO:5), and

c. CDR-H3 is ARPIYFDNDYFDY (SEQ ID NO:6).

[00019] In a further aspect, the antibody binds to CLL-1 and comprises a variable light chain and a variable heavy chain, wherein:

(c) the variable light chain further comprises a CDR-L1, CDR-L2 and CDR-L3, further wherein:

a. CDR-L1 is RATQELSGYLS (SEQ ID NO:13)

b. CDR-L2 is AASTLDS (SEQ ID NO:14)

c. CDR-L3 is LQYAIYPYT (SEQ ID NO:15), and

(d) the variable heavy chain further comprising a CDR-H1, CDR-H2 and CDR-H3, further wherein:

a. CDR-H1 is GYTFTSYFIH (SEQ ID NO:16)

b. CDR-H2 is FINPYNDGSK (SEQ ID NO:17), and

CDR-H3 is DDGYYGYAMDY (SEQ ID NO:18)

[00020] In some embodiments, the anti-CLL-1 antibody comprises a light chain variable region sequence comprises DIQMTQSPSSLSASVGDRVTLTCRATQELSGYLSWLQQKPGKAIKRLIYAASTLDSGV PSRFSGNRAGTDYTLTISSLQPEDFATYYCLQYAIYPYTFGQGTKLEIK (SEQ ID NO:19), a heavy chain variable region sequence comprises EVQLVQSGAEVKKPGASVKMSCKASGYTFTSYFIHWVRQAPGQGLEWIGFINPYND GSKYAQKFQGRATLTSDKSTSTVYMELSSLRSEDTAVYYCTRDDGYYGYAMDYWG QGTLVTVSS (SEQ ID NO:20), or both of the light and heavy chain sequences above.

[00021] In a further aspect, the disclosure provides isolated nucleic acid sequences encoding a cysteine substituted antibody. In one aspect, the nucleic acid is operably linked with an expression control sequence. In another aspect, the operably linked nucleic acid

further comprises an expression vector. In a further aspect, the disclosure provides host cells comprising the expression vectors and methods of making antibodies comprising culturing such host cells. In a further aspect, the disclosure provides isolating the antibody.

[00022] In yet another embodiment, the disclosure provides a cysteine substituted antibody, wherein the substituted cysteine is connected through a linker to a conjugated moiety. In one aspect, the conjugated moiety is selected from the group consisting of: drug, radionucleotide, fluorophore, biotin, RNA, antibiotic, protein and a detectable moiety.

[00023] In another aspect, the conjugated moiety is a drug, biotin (BMCC or HPDP) or fluorophore (Alexa488). In yet another aspect, the drug is selected from the group consisting of: a benzodiazepine derivative (including but not limited to a pyrrolo benzodiazepine, an indolino benzodiazepine or an isoquinolidino benzodiazepine), which can be in monomer or dimer form (e.g., a heterodimer or homodimer, such as pyrrolobenzodiazepine (PBD) dimer, indolinobenzodiazepine dimer, isoquinolidinobenzodiazepine dimer (including but not limited to D202 as described below), a dolastatin, an auristatin, maytansinoid, tubulysin, cryptophycin, alpha-amanitin, trichothene, SN-38, duocarmycin, CC1065, calicheamincin, an enediyne antibioatic, taxane, doxorubicin derivatives, anthracycline and stereoisomers, azanofide, isosteres, analogs or derivatives thereof.

[00024] In a further aspect, the linker is covalently bonded to the drug. In another aspect, the linker is attached to the antibody through a reaction between a thiol and a thiol reactive group, e.g., maleimide, halide and sulfonyl. In yet another aspect, the linker is connected via a disulfide bond to the drug. In a further aspect, the disulfide bond is a pyridyl disulfide moiety. In a further aspect, the linker is cleavable in the microenvironment of the target.

[00025] In a further aspect, the conjugated moiety is a detectable moiety. In a further aspect the detectable moiety is a fluorophore such as A488 or a biotin (e.g., BMCC-biotin or HPDP-biotin).

[00026] In a further embodiment, the disclosure provides compositions comprising the cysteine substituted antibodies and an adjuvant. In one aspect the adjuvant is pharmaceutically acceptable carrier or diluent.

[00027] In a further embodiment, the disclosure provides a method of detecting the presence of a cell of interest, comprising contacting a cell with at least an effective amount of a cysteine-substituted antibody capable of binding the cell, and detecting binding of the antibody to the cell, wherein said binding indicates the cell of interest. In one aspect, the cell

of interest is a cell expressing CLL-1. In another aspect, the cysteine-substituted antibody is conjugated to a detectable moiety.

[00028] In a further embodiment, the disclosure provides a method of diagnosing a disease comprising: (i) contacting a biological sample from an individual with at least an effective amount of a cysteine substituted antibody capable of binding to diseased cells, and (ii) detecting binding of the antibody to a diseased cell, wherein binding indicated the presence of the disease. In one aspect, the substituted cysteine antibody (CYSMAB) is conjugated to a detectable moiety. In another aspect, the disease is cancer, and the antibody bonding to a tumor associated antigen or a cancer stem cell associated antigen. In yet another aspect, the disease is a myeloproliferative disorder. In a further aspect, the myeloproliferative disorder is selected form the group consisting of AML, CML, CMML, multiple myeloma, plasmacytoma and myelofibrosis. In a further aspect, the tumor associated antigen or cancer stem cell antigen is CLL-1, GPR114, IL1RAP, TIM-3, CD19, CD20, CD22, ROR1, mesothelin, CD33, CD123/IL3Ra, c-Met, PSMA, prostatic acid phosphatase (PAP), CEA, CA-125, Muc-1, AFP, Glycolipid F77, EGFRvIII, GD-2, NY-ESO-1 TCR, tyrosinase, TRPI/gp75, gp100/pmel-17, Melan-A/MART-1, Her2/neu, WT1, EphA3, telomerase, HPV E6, HPV E7, EBNA1, BAGE, GAGE and MAGE A3 TCRSLITRK6, ENPP3, Nectin-4, CD27, SLC44A4, CAIX, Cripto, CD30, MUC16, GPNMB, BCMA, Trop-2, Tissue Factor (TF), CanAg, EGFR, αv-integrin, CD37, Folate Receptor, CD138, CEACAM5, CD56, CD70, CD74, GCC, 5T4, CD79b, Steap1, Napi2b, Lewis Y Antigen, LIV, c-RET, DLL3,EFNA4, or Endosialin/CD248.

[00029] In a further embodiment, the disclosure provides a method of inhibiting cell division comprising contacting a cell with at least an effective amount of a cysteine substituted conjugate (CYSMAB) capable of binding to the cell and which is conjugated to a drug that is cytotoxic to the cell. In one aspect, inhibition of cell division results in cell death. In another aspect, the cell is a tumor or cancer stem cell and the antibody binds to a tumor associated antigen or cancer stem cell antigen. In another aspect, the tumor or cancer stem cells are from a myeloproliferative disorder. In yet another aspect, the myeloproliferative disorder is selected from the group consisting of AML, CML, CMML, multiple myeloma, plasmacytoma and myelofibrosis. In a further aspect, the tumor associated antigen or cancer stem cell antigen is CLL-1, GPR114, IL1RAP, TIM-3, CD19, CD20, CD22, ROR1, mesothelin, CD33, CD123/IL3Ra, c-Met, PSMA, prostatic acid phosphatase (PAP), CEA, CA-125, Muc-1, AFP, Glycolipid F77, EGFRvIII, GD-2, NY-ESO-1 TCR, tyrosinase,

TRPI/gp75, gp100/pmel-17, Melan-A/MART-1, Her2/neu, WT1, EphA3, telomerase, HPV E6, HPV E7, EBNA1, BAGE, GAGE and MAGE A3 TCRSLITRK6, ENPP3, Nectin-4, CD27, SLC44A4, CAIX, Cripto, CD30, MUC16, GPNMB, BCMA, Trop-2, Tissue Factor (TF), CanAg, EGFR, αv-integrin, CD37, Folate Receptor, CD138, CEACAM5, CD56, CD70, CD74, GCC, 5T4, CD79b, Steap1, Napi2b, Lewis Y Antigen, LIV, c-RET, DLL3,EFNA4, or Endosialin/CD248.

[00030] In a further embodiment, the disclosure provides a method of treating cancer comprising administering to a patient a therapeutically effective amount of a cysteine substituted antibody conjugate (e.g., an antibody-drug conjugate (ADC) generated using cysteine substituted antibody), wherein the antibody conjugate is capable of binding a tumor associated antigen or cancer stem cell antigen. In one aspect, the cancer is a myeloproliferative disorder. In another aspect, the myeloproliferative disorder is selected form the group consisting of AML, CML, CMML, multiple myeloma, plasmacytoma and myelofibrosis. In yet another further aspect, the tumor associated antigen or cancer stem cell antigen is CLL-1, GPR114, IL1RAP, TIM-3, CD19, CD20, CD22, ROR1, mesothelin, CD33, CD123/IL3Ra, c-Met, PSMA, prostatic acid phosphatase (PAP), CEA, CA-125, Muc-1, AFP, Glycolipid F77, EGFRvIII, GD-2, NY-ESO-1 TCR, tyrosinase, TRPI/gp75, gp100/pmel-17, Melan-A/MART-1, Her2/neu, WT1, EphA3, telomerase, HPV E6, HPV E7, EBNA1, BAGE, GAGE and MAGE A3 TCRSLITRK6, ENPP3, Nectin-4, CD27, SLC44A4, CAIX, Cripto, CD30, MUC16, GPNMB, BCMA, Trop-2, Tissue Factor (TF), CanAg, EGFR, αv-integrin, CD37, Folate Receptor, CD138, CEACAM5, CD56, CD70, CD74, GCC, 5T4, CD79b, Steap1, Napi2b, Lewis Y Antigen, LIV, c-RET, DLL3,EFNA4, or Endosialin/CD248.

[00031] Also provided is an antibody conjugate comprising a cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue at S156 under Kabat numbering (157 under EU numbering) in the antibody heavy chain (the antibody portion having a heavy chain and a light chain) linked via the cysteine to an indolinobenzodiazepine dimer or isoquinolidinobenzodiazepine dimer (including but not limited to D202 as described below). In some embodiments, the indolinobenzodiazepine dimer or isoquinolidinobenzodiazepine dimer (including but not limited to D202 as described below) is attached to the antibody through a linker and the linker is connected via disulfide bond to the drug. In some embodiments, the disulfide bond is a pyridyl disulfide moiety. In some embodiments, the linker is cleavable in the microenvironment of the target.

[00032] Also provided is a composition comprising an antibody conjugate comprising a cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue at S156 under Kabat numbering (157 under EU numbering) in the antibody heavy chain linked via the cysteine to an indolinobenzodiazepine dimer or isoquinolidinobenzodiazepine dimer (including but not limited to D202 as described below) and an adjuvant. In some embodiments, the composition is pharmaceutically acceptable.

[00033] Also provided is a method of inhibiting cell division comprising contacting a cell with at least an effective amount of an antibody conjugate comprising a cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue at S156 under Kabat numbering (157 under EU numbering) in the antibody heavy chain linked via the cysteine to an indolinobenzodiazepine dimer or isoquinolidinobenzodiazepine dimer (including but not limited to D202 as described below). In some embodiments, the inhibition of cell division results in cell death. In some embodiments, the cell is a tumor or cancer stem cell, and the antibody binds to a tumor associated antigen or cancer stem cell antigen. In some embodiments, the tumor or cancer stem cells are from a myeloproliferative disorder. In some embodiments, the myeloproliferative disorder is selected from the group consisting of: AML, CML, CMML, multiple myeloma, plamacytoma myelofibrosis. In some embodiments, the tumor associated antigen or cancer stem cell antigen is CLL-1, GPR114, IL1RAP, TIM-3, CD19, CD20, CD22, ROR1, mesothelin, CD33, CD123/IL3Ra, c-Met, PSMA, prostatic acid phosphatase (PAP), CEA, CA-125, Muc-1, AFP, Glycolipid F77, EGFRvIII, GD-2, NY-ESO-1 TCR, tyrosinase, TRPI/gp75, gp100/pmel-17, Melan-A/MART-1, Her2/neu, WT1, EphA3, telomerase, HPV E6, HPV E7, EBNA1, BAGE, GAGE and MAGE A3 TCRSLITRK6, ENPP3, Nectin-4, CD27, SLC44A4, CAIX, Cripto, CD30, MUC16, GPNMB, BCMA, Trop-2, Tissue Factor (TF), CanAg, EGFR, αv-integrin, CD37, Folate Receptor, CD138, CEACAM5, CD56, CD70, CD74, GCC, 5T4, CD79b, Steap1, Napi2b, Lewis Y Antigen, LIV, c-RET, DLL3,EFNA4, or Endosialin/CD248.

[00034] Also provided is a method of treating cancer comprising administering to a patient a therapeutically effective amount of an an antibody conjugate comprising a cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue at S156 under Kabat numbering (157 under EU numbering) in the antibody heavy chain linked via the cysteine to an indolinobenzodiazepine dimer or isoquinolidinobenzodiazepine dimer (including but not limited to D202 as described below) wherein the antibody conjugate is capable of binding a tumor associated antigen or cancer stem cell antigen. In some embodiments, the cancer is a myeloproliferative disorder. In some embodiments, the

myeloproliferative disorder is selected from the group consisting of: AML, CML, CMML, multiple myeloma, plasmacytoma and myelofibrosis. In some embodiments, the tumor associated antigen or cancer stem cell antigen is CLL-1, GPR114, IL1RAP, TIM-3, CD19, CD20, CD22, ROR1, mesothelin, CD33, CD123/IL3Ra, c-Met, PSMA, prostatic acid phosphatase (PAP), CEA, CA-125, Muc-1, AFP, Glycolipid F77, EGFRvIII, GD-2, NY-ESO-1 TCR, tyrosinase, TRPI/gp75, gp100/pmel-17, Melan-A/MART-1, Her2/neu, WT1, EphA3, telomerase, HPV E6, HPV E7, EBNA1, BAGE, GAGE and MAGE A3 TCRSLITRK6, ENPP3, Nectin-4, CD27, SLC44A4, CAIX, Cripto, CD30, MUC16, GPNMB, BCMA, Trop-2, Tissue Factor (TF), CanAg, EGFR, αv-integrin, CD37, Folate Receptor, CD138, CEACAM5, CD56, CD70, CD74, GCC, 5T4, CD79b, Steap1, Napi2b, Lewis Y Antigen, LIV, c-RET, DLL3,EFNA4, or Endosialin/CD248.

[00035] Other objects of the disclosure may be apparent to one skilled in the art upon reading the following specification and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[00036] FIG.1 shows 3 distinct ELISA assay comparing the specificity of the antibody-fluorophore conjugates (AFCs) of the disclosure. Format 1 is a direct ELISA wherein the CLL-1 extra cellular domain (ECD) fixed, to which the AFC binds, and is then detected by the anti-fluorophore antibody (rabbit anti-A488) and detection reagent (anti-rabbit Fc-HRP). Format 2 is an ELISA wherein the anti-fluorophore antibody (anti-A488 Ab) is bound, and the AFC and biotinylated CLL-1 ECD are sandwiched between the detection reagent (SA-HRP). Format 3 is an alternative ELISA were anti-CLL-1 Ab is immobilized and sandwiches CLL-1 ECD and AFC and the anti- A488 Ab and the detection reagent (anti-rabbit Fc-HRP).

[00037] FIG. 2A-2B displays the assay ELISA specificity assay format 1. FIG. 2A shows similar specificity of the assay for labeled AFC and WT anti-fluorophore antibody, relative to IgG, trastuzumab, naked HuM31, and control labeled IgG. The results show similar specificity between labeled HuM31 and WT. FIG. 2B depicts the effect of interference with human plasma and PBS.

[00038] FIG. 3 is a cartoon showing the format of the stability ELISA assay. In this format, AFC is sandwiched between immobilized CLL-1 ECD and detection reagent. (SA-HRP).

[00039] FIGs. 4A-4E shows an alignment of the HuM31 heavy chain antibody constant chain with other IgG1, IgG2, IgG3 and IgG4 isotypes with residues identified with Kabat, EU

Index and sequential numbering. Light Chain Sequences (FIG. 4A): M31 (SEQ ID NO:7), HuM31 (SEQ ID NO:8), kappa (SEQ ID NO:9) and lambda (SEQ ID NO:10). Heavy Chain Sequences (FIG. 4B): M31 (SEQ ID NO:11) and HuM31 (SEQ ID NO:12).

[00040] FIG. 5 illustrates fluorescence-to-antibody (FAR) ratios for various antibody conjugates.

[00041] FIG. 6 illustrates drug-to-antibody (DAR) ratios for various antibody conjugates.

[00042] FIG. 7 provides results of the conjugation, including amino acid residue and fluorophore-to-antibody ratio ("FAR") for various antibody conjugates.

[00043] FIG. 8 provides results of the conjugation, including amino acid residue and fluorophore-to-antibody ratio ("FAR") for various antibody conjugates.

[00044] FIG. 9 provides results of the conjugation, including amino acid residue and fluorophore-to-antibody ratio ("FAR") for various antibody conjugates.

[00045] FIG. 10 provides results of the conjugation, including amino acid residue and fluorophore-to-antibody ratio ("FAR") for various antibody conjugates.

[00046] FIG. 11 illustrates stability for various antibody conjugates.

[00047] FIG.12A-C provide graphs of FACS Binding data for C6-CYSMAB-ADCs. Circle, C6-S156C-D202; square, C6-A118C-D202; triangle, C6-G316C-D202; open circle, C6-V266C-D202; open square, C6-S239C-D202; open triangle, C0-D202.

DETAILED DESCRIPTION OF THE INVENTION

[00048] This application is not limited to particular methodologies or the specific compositions described, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present application will be limited only by the appended claims and their equivalents.

[00049] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Suggested methods and materials are described, hereafter, although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present application.

I. Definitions

[00050] An "*immunoglobulin*", as used herein, refers to an immunoglobulin polypeptide or an antibody.

[00051] The term "*immunoglobulin polypeptide*" refers to a polypeptide substantially encoded by an immunoglobulin gene.

[00052] The term "*antibody*" refers to a protein having antigen binding activity and an amino acid sequence from or derived from the framework region of an immunoglobulin encoding gene of an animal producing antibodies. The term includes but is not limited to polyclonal or monoclonal antibodies of the isotype classes IgA, IgD, IgE, IgG, and IgM, derived from human or other mammalian cells, including natural or genetically modified forms such as humanized, human, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, and in vitro generated antibodies. The term encompasses conjugates, including but not limited to fusion proteins containing an immunoglobulin moiety (e.g., chimeric or bispecific antibodies or scFv's), and fragments, such as Fab, F(ab')2, Fv, scFv, Fd, single domain (dAb) and other compositions.

[00053] The term "*cysteine substituted immunoglobulin*," as used herein, refers to a cysteine substituted immunoglobulin polypeptide or cysteine substituted antibody.

[00054] The term "*cysteine substituted immunoglobulin polypeptide*" as used herein refers to a polypeptide comprising at least one non-naturally occurring constant region immunoglobulin amino acid residue that has been substituted with cysteine. A non-naturally occurring substitution is one that is not isotypic. In one embodiment, the substituted residues are heavy chain constant regions residues V266C, H285C, R301C, V303C, T307C, G316C, Y436C and L441C. In another embodiment the constant region is of isotype IgG1, IgG2, IgG3 or IgG4.

[00055] The term "*cysteine substituted antibody*" ("CYSMAB") refers to an antibody comprising a cysteine substituted immunoglobulin polypeptide.

[00056] The terms "*immunoglobulin conjugate*" as used herein, refer to an immunoglobulin polypeptide or an antibody or "*antibody conjugate*" that is conjugated to a functional moiety.

[00057] The terms "*immunoglobulin drug conjugate*" as used herein, refers to an immunoglobulin polypeptide or antibody ("*antibody drug conjugate*" ("ADC")), that is

conjugated to a functional moiety, such as a drug moiety or a radiolabel or a detection reagent.

[00058] The term "Cysteine substituted immunoglobulin drug conjugate" or refers to a cysteine substituted immunoglobulin polypeptide or cysteine substituted antibody ("*CYSMAB*") that has been conjugated to a drug moiety, e.g., cysteine substituted antibody drug conjugate ("*CYSMAB ADC*").

[00059] An exemplary antibody immunoglobulin structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively. The variable region contains the antigen-binding region of the antibody (or its functional equivalent) and is most critical in specificity and affinity of binding. See Paul, Fundamental Immunology (2003).

[00060] Antibodies can exist as intact immunoglobulins or as any of a number of well-characterized fragments that include specific antigen-binding activity. For the sake of clarity, a tetrameric antibody with heavy and light chains is referred to herein as an "intact immunoglobulin," and can be naturally occurring, polyclonal, monoclonal, or recombinantly produced. Fragments can be produced by digestion with various peptidases. Pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The F(ab)'2 may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'2 dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region. While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized de novo using recombinant DNA methodologies or those identified using phage display libraries (see, e.g., McCafferty et al., Nature 348:552-554 (1990)).

[00061] As used herein, the term "Fv" refers to a monovalent or bi-valent variable region fragment, and can encompass only the variable regions (e.g., VL and/or VH), as well as

longer fragments, e.g., an Fab, Fab′ or F(ab')2, which also includes CL and/or CH1. Unless otherwise specified, the term "Fc" refers to a heavy chain monomer or dimer comprising CH2 and CH3 regions.

[00062] A single chain Fv (scFv) refers to a polypeptide comprising a VL and VH joined by a linker, e.g., a peptide linker. ScFvs can also be used to form tandem (or di-valent) scFvs or diabodies. Production and properties of tandem scFvs and diabodies are described, e.g., in Asano *et al.* (2011) *J Biol. Chem.* 286:1812; Kenanova *et al.* (2010) *Prot Eng Design Sel* 23:789; Asano et al. (2008) *Prot Eng Design Sel* 21:597.

[00063] The term "*monoclonal antibody*" as used herein refers to a clonal preparation of antibodies with a single binding specificity and affinity for a given epitope on an antigen. A "*polyclonal antibody*" refers to a preparation of antibodies that are raised against a single antigen, but with different binding specificities and affinities.

[00064] As used herein, "*variable region*" or "*V-region*" refers to an antibody variable region domain comprising the segments of Framework 1, CDR1, Framework 2, CDR2, and Framework 3, including CDR3 and Framework 4, which segments are added to the V-segment as a consequence of rearrangement of the heavy chain and light chain V-region genes during B-cell differentiation.

[00065] The term "*framework*" or "*FR*" as used herein refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-HVR1(L1)-FR2-HVR2(L2)-FR3-HVR3(L3)-FR4.

[00066] As used herein, "*complementarity-determining region (CDR)*" refers to the three hypervariable regions in each chain that interrupt the four "framework" regions established by the light and heavy chain variable regions. The CDRs are primarily responsible for binding to an epitope of an antigen. The CDRs of each chain are typically referred to as CDR1, CDR2, and CDR3, numbered sequentially starting from the N-terminus, and are also typically identified by the chain in which the particular CDR is located. Thus, a V_H CDR3 is located in the variable domain of the heavy chain of the antibody in which it is found, whereas a V_L CDR1 is the CDR1 from the variable domain of the light chain of the antibody in which it is found.

[00067] The amino acid sequences of the CDRs and framework regions can be determined using various well known definitions in the art, e.g., Kabat, Chothia, international ImMunoGeneTics database (IMGT), and AbM (see, e.g., Johnson et al., supra; Chothia & Lesk, (1987) *J. Mol. Biol.* 196, 901-917; Chothia et al. (1989) *Nature* 342, 877-883; Chothia et al. (1992) *J. Mol. Biol.* 227, 799-817; Al-Lazikani et al., *J. Mol. Biol.* 1997, 273(4)). A helpful guide for locating CDRs using the Kabat system can be found at the website available at bioinf.org.uk/abs. Definitions of antigen combining sites are also described in the following: Ruiz et al. *Nucleic Acids Res.*, 28, 219-221 (2000); and Lefranc *Nucleic Acids Res.* January 1; 29(1):207-9 (2001); MacCallum et al., *J. Mol. Biol.*, 262: 732-745 (1996); and Martin et al, *Proc. Natl. Acad. Sci. USA*, 86, 9268-9272 (1989); Martin, et al, *Methods Enzymol.*, 203: 121-153, (1991); Pedersen et al, *Immunomethods*, 1, 126, (1992); and Rees et al, In Sternberg M. J. E. (ed.), Protein Structure Prediction. Oxford University Press, Oxford, 141-172 1996). Example CDRs are described as CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2 and CDR-L3 of Figure 7 of US2013/0295118.

[00068] The term "hypervariable region", "HVR" when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six hypervariable regions; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). A number of hypervariable region delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)), whereas Chothia refers to the location of the structural loops (Chothia and Lesk (1987) J. Mol. Biol. 196:901-917). The "contact" hypervariable regions are based on an analysis of the available complex crystal structures. The residues from each of these hypervariable regions are noted below. Unless otherwise denoted, Kabat numbering according to the Kabat Database of aligned sequences of proteins will be employed (Wu and Kabat (1970) J. Exp. Med. 132:211-250; Johnson and Wu (2000) Nuc. Acids Res. 28(1):214-218). Hypervariable region locations are generally as follows: amino acids 24-34 (HVR-L1), amino acids 49-56 (HVR-L2), amino acids 89-97 (HVR-L3), amino acids 26-35A (HVR-H1), amino acids 49-65 (HVR-H2), and amino acids 93-102 (HVR-H3). Hypervariable regions may also comprise "extended hypervariable regions" as follows: amino acids 24-36 (L1), and amino acids 46-56 (L2) in the VL. The variable domain residues are numbered according to Kabat et al., supra for each of these definitions. An

"altered hypervariable region" for the purposes herein is a hypervariable region comprising one or more (e.g. one to about 16) amino acid substitution(s) therein. An "unmodified hypervariable region" for the purposes herein is a hypervariable region having the same amino acid sequence as a non-human antibody from which it was derived, i.e. one which lacks one or more amino acid substitutions therein.

[00069] The term "*chimeric antibody*" as used herein refers to an antibody in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region, CDR, or portion thereof) is linked to a constant region of a different or altered class, effector function and/or species; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity (e.g., CDR and framework regions from different species). Chimeric antibodies can include variable region fragments, e.g., a recombinant antibody comprising two Fab or Fv regions or an scFv. A chimeric can also, as indicated above, include an Fc region from a different source than the attached Fv regions. In some cases, the chimeric antibody includes chimerism within the Fv region. An example of such a chimeric antibody would be a humanized antibody where the FRs and CDRs are from different sources.

[00070] The term "*Humanized antibodies*" as used herein refers to antibodies in which the antigen binding loops, i.e., CDRs, obtained from the V_H and V_L regions of a non-human antibody are grafted to a human framework sequence. Humanization, i.e., substitution of non-human CDR sequences for the corresponding sequences of a human antibody, can be performed following the methods described in, e.g., U.S. Pat. Nos. 5,545,806; 5,569,825; 5,633,425; 5,661,016; Riechmann et al., *Nature* 332:323-327 (1988); Marks et al., *Bio/Technology* 10:779-783 (1992); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14:845-51 (1996). Transgenic mice, or other organisms such as other mammals, may also be used to express humanized or human antibodies, as disclosed in U.S. Pat. No. 6,673,986.

[00071] The terms "*specific for*," "*specifically binds*," and like terms refer to a molecule (e.g., antibody or antibody fragment) that binds to a target with at least 2-fold greater affinity than non-target compounds, e.g., at least any of 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 25-fold, 50-fold, or 100-fold greater affinity. For example, an antibody that specifically binds a primary target will typically bind the primary target with at least a 2-fold

greater affinity than a non-primary antibody target (e.g., an antibody from a different species or of a different isotype, or a non-antibody target).

[00072] The term *"binds"* with respect to an antibody target (e.g., antigen, analyte, immune complex) typically indicates that an antibody binds a majority of the antibody targets in a pure population (assuming appropriate molar ratios). For example, an antibody that binds a given antibody target typically binds to at least ⅔ of the antibody targets in a solution (e.g., at least any of 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100%). One of skill will recognize that some variability will arise depending on the method and/or threshold of determining binding.

[00073] The terms *"label," "detectable moiety,"* and like terms refer to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include fluorescent dyes, luminescent agents, radioisotopes (e.g., ^{32}P, ^{3}H), electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins or other entities which can be made detectable, e.g., by incorporating a radiolabel into a peptide or antibody specifically reactive with a target analyte. Any method known in the art for conjugating an antibody to the label may be employed, e.g., using methods described in Hermanson, *Bioconjugate Techniques* 1996, Academic Press, Inc., San Diego. The term "tag" can be used synonymously with the term "label," but generally refers to an affinity-based moiety, e.g., a "His tag" for purification, or a "streptavidin tag" that interacts with biotin.

[00074] The term *"labeled"* molecule (*e.g.*, nucleic acid, protein, or antibody) as used herein is one that is bound, either covalently, through a linker or a chemical bond, or non-covalently, through ionic, van der Waals, electrostatic, or hydrogen bonds to a label such that the presence of the molecule may be detected by detecting the presence of the label bound to the molecule.

[00075] The term *"C-type lectin-Like molecule 1 (CLL-1),"* also known as CLEC12A, DCAL-2, and MICL, is a type II membrane protein (ITIM domain—TM domain-stalk domain-lectin-like domain). The extracellular domain of CLL-1 is highly glycosylated, and it is expressed exclusively in cells of myeloid lineage. CLL-1 is also expressed on AML, MDS, and CML cells. CLL-1 expression can be used to distinguish between normal hematopoietic stem cells (HSCs), which do not express CLL-1, and leukemic stem cells (LSCs), where it is

expressed. LSCs are CD34+ cells in leukemia patients that lead to production of cancer cells and recurrence of cancer. See Bakker et al. (2004) *Cancer Res.* 64:8443.

[00076] The nucleotide and amino acid sequences of CLL-1 are known for many species. For example, the human sequences can be found as SEQ ID NO:2 in US2013/0295118 and Genbank accession number AF247788.1 and Uniprot accession number Q5QGZ9 (SEQ ID NO:2). For the human CLL-1 protein shown as SEQ ID NO:2, the extracellular domain comprises approximately amino acids 65-265, the transmembrane domain comprises approximately amino acids 44-64, and the cytoplasmic domain comprises approximately amino acids 1-43. The stalk domain of human CLL-1 spans amino acids 65-139, and the C lectin domain spans amino acids 140-249.

[00077] The term "*CLL-1 associated disorder*" as used herein refers to conditions and diseases correlated with non-pathogenic levels (e.g., elevated or reduced cell surface expression of CLL-1) as compared to CLL-1 expression in a standard control (e.g., a normal, non-disease, non-cancer cell). Elevated CLL-1 levels are associated with cancer cells, in particular, leukemias such as AML (acute myelogenous leukemia), MDS (myelodysplastic syndrome), and CML (chronic myelogenous leukemia), and in hematopoietic CSCs (e.g., LSCs).

[00078] The "*cancer stem cell*" hypothesis proposes that only a small portion of a tumor is represented by the "cancer stem cell," which allows the tumor to proliferate and self-renew, and eventually differentiate into the phenotypically diverse and heterogeneous tumor cell population (Bjerkvig *et al., Nat. Rev. Cancer*, 5:899-904, 2005). Cancer stem cells can be isolated from any type of cancers, e.g., leukemias, breast, colon and brain cancers, colon cancers. Cancer stem cells are characterized by their ability to self-renew and proliferate, and recapitulate through differentiation from the parental tumor. Exemplary cancer stem cell antigens include CD133, Bmi-1, Notch, Sonic hedgehog, and Wnt. Additionally, exemplary molecular markers of neural cancer stem cells include CD90, CD44, CXCR4, Nestin, Musashi-1 (Msi1), maternal embryonic leucine zipper kinase (MELK), GLI1, PTCH1, Bmi-1, phosphoserine phosphatase (PSP), Snail, OCT4, BCRP1, MGMT, Bcl-2, FLIP, BCL-XL, XIAP, cIAP1, cIAP2, NAIP, and survivin. One useful cancer stem cell antigen is CLL-1.

[00079] The term "*cytotoxic*" refers to the inhibitory effect that an agent has on a cell, e.g., *necrosis* (a loss of membrane integrity and rapid death as a result of cell lysis); decreased

viability (wherein cells stop proliferating) and *apoptosis* (a genetic program of controlled cell death).

[00080] Cytotoxicity can also be monitored using the 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) or MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS reagent to a colored formazan product. A similar redox-based assay has also been developed using the fluorescent dye, resazurin. In addition to using dyes to indicate the redox potential of cells in order to monitor their viability, researchers have developed assays that use adenosine triphosphate (ATP) content as a marker of viability. Such ATP-based assays include bioluminescent assays in which ATP is the limiting reagent for the luciferase reaction (e.g. CellTiter-Glo Luminescent Cell Viability assay, Promega). Cytotoxicity can also be measured by the sulforhodamine B (SRB) assay, WST assay and chronogenic assay. A label-free approach to follow the cytotoxic response of adherent animal cells in real-time is based on electric impedance measurements when the cells are grown on gold-film electrodes. This technology is referred to as electric cell-substrate impedance sensing (ECIS). Label-free real-time techniques provide the kinetics of the cytotoxic response rather than just a snapshot like many colorimetric endpoint assays.

[00081] The terms "*CLL-1 specific antibody*," "*anti-CLL-1 antibody*," "*CLL-1 antibody*," and "*anti-CLL-1*" are used synonymously herein to refer to an antibody that specifically binds to CLL-1, including variously glycosylated forms of CLL-1. The CLL-1 antibodies described herein specifically bind the CLL-1 polypeptide expressed, e.g., on the surface of certain cancer cells, but not to hematopoietic stem cells (HSCs). As discussed in more detail below, the present anti-CLL-1 antibodies can bind CLL-1 expressing cells, bind a larger percentage of AML cells compared to other AML-targeting antibodies, inhibit AML cell proliferation, and mediate their destruction. Examples of anti-CLL antibodies suitable for use as cysteine substituted antibodies (CYSMABs) of this disclosure are described US2013/0295118, published Nov. 7, 2013. An anti-CLL-1 antibody can have CDRs as disclosed in that publication, in particular, CDRs for antibodies M31 and M26.

[00082] The term "*differentially expressed*" or "*differentially regulated*" refers generally to a protein or nucleic acid biomarker that is overexpressed (upregulated) or under-expressed (downregulated) in one sample compared to at least one other sample. In the context of the present disclosure, the term generally refers to overexpression of CLL-1 on a cancer cell (*e.g.*, an AML cell or AML CSC) compared to a normal, non-cancer cell.

[00083] For example, the terms "*overexpressed*" or "*upregulated*" interchangeably refer to a protein or nucleic acid, generally a biomarker, that is transcribed or translated at a detectably greater than control level. The term includes overexpression due to transcription, post transcriptional processing, translation, post-translational processing, cellular localization (e.g., organelle, cytoplasm, nucleus, cell surface), and RNA and protein stability. Overexpression can be detected using conventional techniques for detecting biomarkers, whether mRNA (i.e., RT-PCR, hybridization) or protein (i.e., flow cytometry, imaging, ELISA, immunohistochemical techniques). Overexpression can be at least any of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more in comparison to a normal cell.

[00084] The term "*control*" sample or value as used herein refers to a sample that serves as a reference, usually a known reference, for comparison to a test sample. For example, a test sample can be taken from a test condition, *e.g.*, in the presence of a test compound, and compared to samples from known conditions, *e.g.*, in the absence of the test compound (negative control), or in the presence of a known compound (positive control). In the context of the present disclosure, an example of a negative control would be a biological sample from a known healthy (non-cancer) individual, and an example of a positive control would be a biological sample from a known AML patient. A control can also represent an average value or a range gathered from a number of tests or results. One of skill in the art will recognize that controls can be designed for assessment of any number of parameters. For example, a control can be devised to compare therapeutic benefit based on pharmacological data (*e.g.*, half-life) or therapeutic measures (e.g., comparison of benefit and/or side effects). Controls can be designed for in vitro applications. One of skill in the art will understand which controls are valuable in a given situation and be able to analyze data based on comparisons to control values. Controls are also valuable for determining the significance of data. For example, if values for a given parameter are widely variant in controls, variation in test samples will not be considered as significant.

[00085] The term "*diagnosis*" refers to a relative probability that a subject has a disorder such as cancer. Similarly, the term "*prognosis*" refers to a relative probability that a certain future outcome may occur in the subject. For example, in the context of the present disclosure, prognosis can refer to the likelihood that an individual will develop cancer, have recurrence, or the likely severity of the disease (e.g., severity of symptoms, rate of functional decline, survival, etc.). The terms are not intended to be absolute, as will be appreciated by any one of skill in the field of medical diagnostics.

[00086] "*Biopsy*" or "*biological sample from a subject*" as used herein refers to a sample obtained from a subject having, or suspected of having a disease, e.g., a CLL-1 associated disorder. The sample can also be a blood sample or blood fraction, *e.g.*, white blood cell fraction, serum, or plasma. In some embodiments, the sample may be a tissue biopsy, such as needle biopsy, fine needle biopsy, surgical biopsy, etc. The sample can comprise a tissue sample harboring a lesion or suspected lesion, although the biological sample may be also be derived from another site, e.g., a site of suspected metastasis, a lymph node, or from the blood. In some cases, the biological sample may also be from a region adjacent to the lesion or suspected lesion.

[00087] A "*biological sample*" can be obtained from a subject, e.g., a biopsy, from an animal, such as an animal model, or from cultured cells, e.g., a cell line or cells removed from a subject and grown in culture for observation. Biological samples include tissues and bodily fluids, e.g., blood, blood fractions, lymph, saliva, urine, feces, etc.

[00088] The EU numbering scheme refers to the number of the US antibody (Edelman *et al., Proc. Natl. Acad. Sci. USA* 63: 78-85 (1969)). The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat *et al.* Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991)). As used herein, EU number refers to the constant chain nomenclature of the antibodies described herein, while Kabat is used to derive the CDRs and HVRs of the variable regions.

[00089] "*Operably linked*" refers to a juxtaposition of two or more components, wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a promoter is operably linked to a coding sequence if it acts in cis to control or modulate the transcription of the linked sequence. Generally, but not necessarily, the DNA sequences that are "*operably linked*" are contiguous and, where necessary to join two protein coding regions or in the case of a secretory leader, contiguous and in reading frame. However, although an operably linked promoter is generally located upstream of the coding sequence, it is not necessarily contiguous with it.

[00090] The term "*promoter*" as used herein refers to a polynucleotide sequence that controls transcription of a gene or sequence to which it is operably linked. A promoter includes signals for RNA polymerase binding and transcription initiation. The promoters

used will be functional in the cell type of the host cell in which expression of the selected sequence is contemplated.

[00091] The term "*vector*," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors").

[00092] The term "*host cell*" (or "*recombinant host cell*"), as used herein, is intended to refer to a cell that has been genetically altered, or is capable of being genetically altered by introduction of an exogenous polynucleotide, such as with a recombinant plasmid or vector. It should be understood that such terms refer not only to the particular subject cell but also to its progeny.

[00093] The terms "*therapy*," "*treatment*," and "*amelioration*" refer to any reduction in the severity of symptoms. In the case of treating cancer (*e.g.*, AML), treatment can refer to, e.g., reducing tumor size, number of cancer cells, growth rate, metastatic activity, reducing cell death of non-cancer cells, reduced nausea and other chemotherapy or radiotherapy side effects, etc. The terms "treat" and "prevent" are not intended to be absolute terms. Treatment and prevention can refer to any delay in onset, amelioration of symptoms, improvement in patient survival, increase in survival time or rate, etc. Treatment and prevention can be complete (undetectable levels of neoplastic cells) or partial, such that fewer neoplastic cells are found in a patient than would have occurred without the present invention. The effect of treatment can be compared to an individual or pool of individuals not receiving the treatment, or to the same patient prior to treatment or at a different time during treatment. In some aspects, the severity of disease is reduced by at least 10%, as compared, *e.g.*, to the individual before administration or to a control individual not undergoing treatment. In some aspects

the severity of disease is reduced by at least 25%, 50%, 75%, 80%, or 90%, or in some cases, no longer detectable using standard diagnostic techniques.

[00094] An "*effective amount*" of an agent, *e.g.*, a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired cellular response, therapeutic or prophylactic result. For example, in a method for inhibiting cell proliferation, an effective amount of a cysteine substituted immunoglobulin drug conjugate (e.g., CYSMAB ADC) is a concentration which noticeably attenuates, inhibits, or prevents cell division in a cell relative to a control cell.

[00095] The phrase "*therapeutically effective amount*" means an amount of a compound of the present invention that (i) treats or prevents the particular disease, condition or disorder, (ii) attenuates, ameliorates or eliminates one or more symptoms of the particular disease, condition, or disorder, or (iii) prevents or delays the onset of one or more symptoms of the particular disease, condition or disorder described herein. In one embodiment, the therapeutic effective amount is an amount sufficient to decrease or alleviate the symptoms of a disorder responsive to the modulation of CLL-1. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP) and/or determining the response rate (RR). In one embodiment, the therapeutic effective amount is an amount sufficient to decrease or alleviate the symptoms of a disorder responsive to the modulation of CLL-1. In the case of immunological disorders, the therapeutic effective amount is an amount sufficient to decrease or alleviate an allergic disorder, the symptoms of an autoimmune and/or inflammatory disease, or the symptoms of an acute inflammatory reaction. In some embodiments, a therapeutically effective amount is an amount of a chemical entity described herein sufficient to significantly decrease the activity or number of myeloproliferative cancer stem cells.

[00096] As used herein, the term "*pharmaceutically acceptable*" is used synonymously with physiologically acceptable and pharmacologically acceptable. A pharmaceutical composition will generally comprise agents for buffering and preservation in storage, and can

include buffers and carriers for appropriate delivery, depending on the route of administration.

[00097] The phrase "*pharmaceutically acceptable salt*," as used herein, refers to pharmaceutically acceptable organic or inorganic salts of an ADC. Exemplary salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, and pamoate (i.e., 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. A pharmaceutically acceptable salt may involve the inclusion of another molecule such as an acetate ion, a succinate ion or other counterion. The counterion may be any organic or inorganic moiety that stabilizes the charge on the compound. Furthermore, a pharmaceutically acceptable salt may have more than one charged atom in its structure. Instances where multiple charged atoms are part of the pharmaceutically acceptable salt can have multiple counter ions. Hence, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counterion.

[00098] "*Pharmaceutically acceptable solvate*" refers to an association of one or more solvent molecules and an ADC. Examples of solvents that form pharmaceutically acceptable solvates include, but are not limited to, water, isopropanol, ethanol, methanol, DMSO, ethyl acetate, acetic acid, and ethanolamine.

[00099] "*Carriers*" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS®. The terms "*dose*" and "*dosage*" are used interchangeably herein. A dose refers to the amount of active ingredient given to an individual at each administration. For the

present invention, the dose can refer to the concentration of the antibody or associated components, e.g., the amount of therapeutic agent or dosage of radiolabel. The dose will vary depending on a number of factors, including frequency of administration; size and tolerance of the individual; severity of the condition; risk of side effects; the route of administration; and the imaging modality of the detectable moiety (if present). One of skill in the art will recognize that the dose can be modified depending on the above factors or based on therapeutic progress. The term "dosage form" refers to the particular format of the pharmaceutical, and depends on the route of administration. For example, a dosage form can be in a liquid, e.g., a saline solution for injection.

[000100] "*Subject*," "*patient*," "*individual*" and like terms are used interchangeably and refer to, except where indicated, mammals such as humans and non-human primates, as well as rabbits, rats, mice, goats, pigs, and other mammalian species. The term does not necessarily indicate that the subject has been diagnosed with a particular disease. The term "patient" refers to a subject under medical supervision. A patient can be an individual that is seeking treatment, monitoring, adjustment or modification of an existing therapeutic regimen, etc. A "cancer patient" or "AML patient" can refer to an individual that has been diagnosed with cancer, is currently following a therapeutic regimen, or is at risk of recurrence, e.g., after surgery to remove a tumor. In some embodiments, the cancer patient has been diagnosed with cancer and is a candidate for therapy. Cancer patients can include individuals that have not received treatment, are currently receiving treatment, have had surgery, and those that have discontinued treatment.

[000101] In the context of treating cancer, a subject in need of treatment can refer to an individual that has cancer or a pre-cancerous condition, has had cancer and is at risk of recurrence, is suspected of having cancer, is undergoing standard treatment for cancer, such as radiotherapy or chemotherapy, etc.

[000102] "*Cancer*", "*tumor*," and like terms include precancerous, neoplastic, and cancerous cells, and can refer to a solid tumor, or a non-solid cancer (see, e.g., Edge et al. *AJCC Cancer Staging Manual* (7th ed. 2009); Cibas and Ducatman *Cytology: Diagnostic principles and clinical correlates* (3rd ed. 2009)). Cancer includes both benign and malignant neoplasms (abnormal growth).

[000103] The term "*cancer*" can refer to leukemias, carcinomas, sarcomas, adenocarcinomas, lymphomas, solid and lymphoid cancers, etc. Examples of different types

of cancer include, but are not limited to, acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), B-cell lymphoma, non-Hodgkin's lymphoma, Burkitt's lymphoma, Small Cell lymphoma, Large Cell lymphoma, monocytic leukemia, myelogenous leukemia, acute lymphocytic leukemia, multiple myelomas, lung cancer (e.g., non-small cell lung cancer or NSCLC), ovarian cancer, prostate cancer, colorectal cancer, liver cancer (i.e., hepatocarcinoma), renal cancer (i.e., renal cell carcinoma), bladder cancer, breast cancer, thyroid cancer, pleural cancer, pancreatic cancer, uterine cancer, cervical cancer, testicular cancer, anal cancer, pancreatic cancer, bile duct cancer, gastrointestinal carcinoid tumors, esophageal cancer, gall bladder cancer, appendix cancer, small intestine cancer, stomach (gastric) cancer, cancer of the central nervous system, skin cancer, choriocarcinoma; head and neck cancer, osteogenic sarcoma, fibrosarcoma, neuroblastoma, glioma, and melanoma.

[000104] A "*cancer target*" or "*cancer marker*" is a molecule that is differentially expressed or processed in cancer, e.g., on a cancer cell or in the cancer milieu. Exemplary cancer targets are cell surface proteins such as CLL-1 (also, e.g., cell adhesion molecules and receptors), intracellular receptors, hormones, and molecules such as proteases that are secreted by cells into the cancer milieu. Markers for specific cancers are known in the art, e.g., CD45 for AML, CD34+CD38− for AML CSCs, MUC1 expression on colon and colorectal cancers, bombesin receptors in lung cancer, and prostate specific membrane antigen (PSMA) on prostate cancer.

[000105] In some embodiments, the cancer target can be associated with a certain type of cancer cell, e.g., AML, leukemia, myeloma, lymphoma, non-small cell lung cancer cells, prostate cancer, colorectal cancer, breast cancer or ovarian cancer. A cell type specific target is typically expressed at levels at least 2 fold greater in that cell type than in a reference population of cells. In some embodiments, the cell type specific marker is present at levels at least any of 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, or 1000 fold higher than its average expression in a reference population. Thus, the target can be detected or measured to distinguish the cell type or types of interest from other cells. For example, AML cancer targets include CLL-1, Ly86, LILRA1, and CD180.

[000106] A cancer stem cell (CSC) is a cell found in a tumor or blood cancer that can give rise to the cells that make up the bulk of the cancer. The CSC can also be self-renewing, similar to a normal (non-cancer) stem cell. CSCs can thus mediate metastasis by migrating to a non-tumor tissue in an individual and starting a "new" tumor. CSCs make up a very small percentage of any given cancer, depending on the stage that the cancer is detected. For

example, the average frequency of CSCs in a sample of AML cells is believed to be about 1:10,000. Hematopoietic CSCs can be identified as CD34+, similar to normal hematopoietic stem cells (HSCs).

[000107] *"Conservatively modified variants"* applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode most proteins. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to another of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are *"silent variations,"* which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes silent variations of the nucleic acid. One of skill will recognize that in certain contexts each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, silent variations of a nucleic acid which encodes a polypeptide are implicit in a described sequence with respect to the expression product, but not with respect to actual probe sequences.

[000108] The term *"recombinant"* when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

[000109] The term *"heterologous,"* with reference to a polynucleotide or polypeptide, indicates that the polynucleotide or polypeptide comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, a heterologous polynucleotide or polypeptide is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional unit, *e.g.*, a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (*e.g.*, a fusion protein).

[000110] A "*thiol reactive reagent*" is a reagent having a moiety that reacts with a thiol to form a covalent bond. Thiol reactive reagents can have a group selected from halide, maleimide and sulfonyl. Non-limiting examples include biotin-PEO-maleimide ((+)-biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctainediamine, Oda *et al* (2001) *Nature Biotechnology* 19:379-382, Pierce Biotechnology, Inc.) Biotin-BMCC, PEO-Iodoacetyl Biotin, Iodoacetyl-LC-Biotin, and Biotin-HPDP (Pierce Biotechnology, Inc.), and Nα-(3-maleimidylpropionyl)biocytin (MPB, Molecular Probes, Eugene, OR). Other commercial sources for biotinylation, bifunctional and multifunctional linker reagents include Molecular Probes, Eugene, Oreg., and Sigma, St. Louis, Mo.

II. Polynucleotides Encoding Cysteine Substituted Immunoglobulins (e.g., CYSMAB)

[000111] Also provided are polynucleotides (e.g., DNA) encoding the cysteine substituted immunoglobulins described herein, or constant domains thereof having the cysteine substitution. Polynucleotides encoding cysteine substituted immunoglobulins can be prepared by site-directed mutagenesis on polynucleotides encoding immunoglobulin polypeptides. Kits for performing site directed mutagenesis are commercially available from a variety of sources. These include, for example, Phusion available from Life Technologies, QuikChange, available from Agilent Technologies, and Q5, available from New England Biolabs. In general, site directed mutagenesis involves primer extension of a target immunoglobulin-encoding polynucleotide using a primer that includes a mutant inserting a cys codon at the desired site.

[000112] Also provided are expression cassettes comprising a promoter operably linked to a polynucleotide encoding the cysteine substituted immunoglobulins described herein, or a constant domains thereof having the cysteine substitution. In some embodiments, the promoter is heterologous, i.e., not found in nature operably-linked to the coding sequence. In some embodiments, vectors (including but not limited to expression vectors or shuttle vectors) comprising a polynucleotide encoding the cysteine substituted immunoglobulins described herein, or a constant domains thereof having the cysteine substitution. Also provided are cells comprising, and optionally expressing, a polynucleotide encoding the cysteine substituted immunoglobulins described herein, or a constant domains thereof having the cysteine substitution. Exemplary cells include prokaryotic cells, including but not limited

to *E. coli*, and eukaryotic cells, including but not limited to mammalian (e.g., human, hamster, rat, mouse, etc.), fungal (e.g., yeast), or plant cells.

III. Method of Making Antibodies

[000113] For preparation of the presently described immunoglobulins, e.g., recombinant, monoclonal, or polyclonal antibodies, many techniques known in the art can be used (see, e.g., Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor et al., *Immunology Today* 4: 72 (1983); Cole et al., pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985); Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies, A Laboratory Manual* (1988); and Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986)). The genes encoding the heavy and light chains of an antibody of interest can be cloned from a cell, e.g., the genes encoding a monoclonal antibody can be cloned from a hybridoma and used to produce a recombinant monoclonal antibody. Gene libraries encoding heavy and light chains of monoclonal antibodies can also be made from hybridoma or plasma cells. Random combinations of the heavy and light chain gene products generate a large pool of antibodies with different antigenic specificity (see, e.g., Kuby, *Immunology*(3^{rd} ed. 1997)). Techniques for the production of single chain antibodies or recombinant antibodies (U.S. Pat. No. 4,946,778, U.S. Pat. No. 4,816,567) can be adapted to produce antibodies to polypeptides of this disclosure. Also, transgenic mice, or other organisms such as other mammals, can be used to express humanized or human antibodies (see, e.g., U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, Marks et al., *Bio/Technology*10:779-783 (1992); Lonberg et al., *Nature* 368:856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14:845-51 (1996); Neuberger, *Nature Biotechnology* 14:826 (1996); and Lonberg & Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995)). Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (see, e.g., McCafferty et al., *Nature* 348:552-554 (1990); Marks et al., *Biotechnology* 10:779-783 (1992)). Antibodies can also be made bispecific, i.e., able to recognize two different antigens (see, e.g., WO 93/08829, Traunecker et al., *EMBO J.* 10:3655-3659 (1991); and Suresh et al., *Methods in Enzymology* 121:210 (1986)). Antibodies can also be heteroconjugates, e.g., two covalently joined antibodies, or immunotoxins (see, e.g., U.S. Pat. No. 4,676,980, WO 91/00360; WO 92/200373; and EP 03089).

[000114] Antibodies can be produced using any number of expression systems, including prokaryotic and eukaryotic expression systems. In some embodiments, the expression system is a mammalian cell expression, such as a hybridoma, or a CHO cell expression system. Many such systems are widely available from commercial suppliers. In embodiments in which an antibody comprises both a V_H and V_L region, the V_H and V_L regions may be expressed using a single vector, e.g., in a di-cistronic expression unit, or under the control of different promoters. In other embodiments, the V_H and V_L region may be expressed using separate vectors. A V_H or V_L region as described herein may optionally comprise a methionine at the N-terminus.

[000115] An antibody of the disclosure can also be produced in various formats, including as a Fab, a Fab', a $F(ab')_2$, a scFv, or a dAb. The antibody fragments can be obtained by a variety of methods, including, digestion of an intact antibody with an enzyme, such as pepsin (to generate $(Fab')_2$ fragments) or papain (to generate Fab fragments); or de novo synthesis. Antibody fragments can also be synthesized using recombinant DNA methodology. In some embodiments, the CLL-1 antibody comprises $F(ab')_2$ fragments that specifically bind CLL-1. An antibody of the disclosure can also include a human constant region. See, e.g., Fundamental Immunology (Paul ed., 4d ed. 1999); Bird, et al., *Science* 242:423 (1988); and Huston, et al., *Proc. Natl. Acad. Sci. USA* 85:5879 (1988).

[000116] Methods for humanizing non-human antibodies (i.e., using CDRs from non-human antibodies) are also known in the art. Generally, a humanized antibody has one or more amino acid residues from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers (see, e.g., Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-327 (1988); Verhoeyen et al., *Science* 239:1534-1536 (1988) and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[000117] In some cases, the antibody or antibody fragment can be conjugated to another molecule, e.g., polyethylene glycol (PEGylation) or serum albumin, to provide an extended half-life in vivo. Examples of PEGylation of antibody fragments are provided in Knight et al. *Platelets* 15:409, 2004 (for abciximab); Pedley et al., *Br. J. Cancer* 70:1126, 1994 (for an anti-CEA antibody); Chapman et al., *Nature Biotech.* 17:780, 1999; and Humphreys, et al., *Protein Eng. Des.* 20: 227 2007). The antibody or antibody fragment can also be labeled, or conjugated to a therapeutic agent as described below.

IV. Preparation of Cysteine Substituted Immunoglobulin (e.g., CYSMAB) Drug Conjugates

[000118] Antibody-Drug Conjugates prepared from the cysteine substituted immunoglobulins (CYSMABs) of the disclosure may be prepared by several routes, employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a cysteine group of a cysteine engineered antibody with a linker reagent, to form antibody-linker intermediate Ab-L, via a covalent bond, followed by reaction with an activated drug moiety D; and (2) reaction of a nucleophilic group of a drug moiety with a linker reagent, to form drug-linker intermediate D-L, via a covalent bond, followed by reaction with a cysteine group of a cysteine engineered antibody (CYSMAB). Conjugation methods (1) and (2) may be employed with a variety of cysteine engineered antibodies (CYSMABs), drug moieties, and linkers to prepare the antibody-drug conjugates (ADCs).

[000119] Antibody cysteine thiol groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker reagents and drug-linker intermediates including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides, such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups; and (iv) disulfides, including pyridyl disulfides, via sulfide exchange. Nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents.

[000120] Cysteine engineered antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (Cleland's reagent, dithiothreitol) or TCEP (tris(2-carboxyethyl)phosphine hydrochloride; Getz et al (1999) Anal. Biochem. Vol

273:73-80; Soltec Ventures, Beverly, Mass.), followed by reoxidation, e.g., with DHAA to reform inter-chain and intra-chain disulfide bonds (Example 2).

A. Linkers

[000121] "Linker", "Linker Unit", or "link" means a chemical moiety comprising a covalent bond or a chain of atoms that covalently attaches an antibody to a drug moiety. In various embodiments, a linker is specified as L. A "Linker" (L) is a bifunctional or multifunctional moiety which can be used to link one or more Drug moieties (D) and an antibody unit (Ab) to form antibody-drug conjugates (ADCs). Antibody-drug conjugates (ADCs) can be conveniently prepared using a Linker having reactive functionality for binding to the Drug and to the Antibody. A cysteine thiol of a cysteine engineered antibody (CYSMAB) can form a bond with an electrophilic functional group of a linker reagent, a drug moiety or drug-linker intermediate.

[000122] In one aspect, a Linker has a reactive site, which has an electrophilic group that is reactive to a nucleophilic cysteine present on an antibody. The cysteine thiol of the antibody is reactive with an electrophilic group on a Linker and forms a covalent bond to a Linker. Useful electrophilic groups include, but are not limited to, maleimide and haloacetamide groups.

[000123] Linkers include a divalent radical such as an alkyldiyl, an arylene, a heteroarylene, moieties such as: —$(CR_2)_nO(CR_2)_n$—, repeating units of alkyloxy (e.g. polyethylenoxy, PEG, polymethyleneoxy) and alkylamino (e.g. polyethyleneamino, Jeffamine™); and diacid ester and amides including succinate, succinamide, diglycolate, malonate, and caproamide.

[000124] Cysteine engineered antibodies (CYSMABs) react with linker reagents or drug-linker intermediates, with electrophilic functional groups such as maleimide or α-halo carbonyl, according to the conjugation method at page 766 of Klussman, et al (2004), Bioconjugate Chemistry 15(4):765-773.

[000125] The linker may be composed of one or more linker components. Exemplary linker components include 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), valine-citrulline ("val-cit" or "vc"), alanine-phenylalanine ("ala-phe" or "af"), p-aminobenzyloxycarbonyl ("PAB"), N-succinimidyl 4-(2-pyridylthio) pentanoate ("SPP"), N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1 carboxylate ("SMCC"), N-Succinimidyl (4-iodo-acetyl) aminobenzoate ("SIAB"), ethyleneoxy —CH2CH2O— as one or more

repeating units ("EO" or "PEO"). Additional linker components are known in the art and some are described herein.

[000126] In another embodiment, a Linker has a reactive functional group, which has a nucleophilic group that is reactive to an electrophilic group present on an antibody. Useful electrophilic groups on an antibody include, but are not limited to, aldehyde and ketone carbonyl groups. The heteroatom of a nucleophilic group of a Linker can react with an electrophilic group on an antibody and form a covalent bond to an antibody unit. Useful nucleophilic groups on a Linker include, but are not limited to, hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide. The electrophilic group on an antibody provides a convenient site for attachment to a Linker.

[000127] Typically, peptide-type Linkers can be prepared by forming a peptide bond between two or more amino acids and/or peptide fragments. Such peptide bonds can be prepared, for example, according to the liquid phase synthesis method (E. Schröder and K. Lübke (1965) "The Peptides", volume 1, pp 76-136, Academic Press), which is well known in the field of peptide chemistry. Linker intermediates may be assembled with any combination or sequence of reactions including spacer, stretcher, and amino acid units. The spacer, stretcher, and amino acid units may employ reactive functional groups which are electrophilic, nucleophilic, or free radical in nature. Reactive functional groups include, but are not limited to carboxyls, hydroxyls, para-nitrophenylcarbonate, isothiocyanate, and leaving groups, such as O-mesyl, O-tosyl, —Cl, —Br, —I; or maleimide.

[000128] In another embodiment, the Linker may be substituted with groups, which modulated solubility or reactivity. For example, a charged substituent such as sulfonate (—SO3 −) or ammonium, may increase water solubility of the reagent and facilitate the coupling reaction of the linker reagent with the antibody or the drug moiety, or facilitate the coupling reaction of Ab-L (antibody-linker intermediate) with D, or D-L (drug-linker intermediate) with Ab, depending on the synthetic route employed to prepare the ADC.

[000129] An exemplary phe-lys(Mtr, mono-4-methoxytrityl)dipeptide linker reagent comprising a maleimide moiety and a PAB self-immolative moiety can be prepared according to Dubowchik, et al. (1997) Tetrahedron Letters, 38:5257-60.

B. Linker Reagents

[000130] Conjugates of the antibody and auristatin may be made using a variety of bifunctional linker reagents such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP),

succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene).

[000131] Linker reagents useful for the antibody drug conjugates (ADCs) of the disclosure include, but are not limited to: BMPEO, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate), and including bis-maleimide reagents: DTME, BMB, BMDB, BMH, BMOE, 1,8-bis-maleimidodiethyleneglycol (BM(PEO)2), and 1,11-bis-maleimidotriethyleneglycol (BM(PEO)3), which are commercially available from Pierce Biotechnology, Inc., ThermoScientific, Rockford, Ill., and other reagent suppliers. Bis-maleimide reagents allow the attachment of a free thiol group of a cysteine residue of an antibody to a thiol-containing drug moiety, label, or linker intermediate, in a sequential or concurrent fashion. Other functional groups besides maleimide, which are reactive with a thiol group of an antibody, nemorubicin metabolite and analog drug moiety, or linker intermediate include iodoacetamide, bromoacetamide, vinyl pyridine, disulfide, pyridyl disulfide, isocyanate, and isothiocyanate.

V. Methods of Use

[000132] Cysteine substituted immunoglobulins of this disclosure are useful, among other things, in the preparation cysteine substituted immunoglobulin conjugates, including molecules conjugated to detectable moieties or drugs, such as cytotoxic agents.

A. Treatment of disease

[000133] Cysteine substituted immunoglobulin drug conjugates (e.g., CYSMAB ADCs) are useful in the treatment of any disease treatable by targeting a cell to which such a conjugate binds. This includes any form of cancer.

[000134] Cysteine substituted immunoglobulin drug conjugates may be used to treat various diseases or disorders, e.g. characterized by the overexpression of a tumor antigen. Exemplary conditions or hyperproliferative disorders include benign or malignant tumors; leukemia and lymphoid malignancies. Others include neuronal, glial, astrocytal, hypothalamic, glandular,

macrophagal, epithelial, stromal, blastocoelic, inflammatory, angiogenic and immunologic, including autoimmune, disorders.

[000135] Cysteine substituted immunoglobulin drug conjugates can be further tested in tumor-bearing higher primates and human clinical trials. Human clinical trials can be designed similar to the clinical trials testing the efficacy of the anti-HER2 monoclonal antibody HERCEPTIN® in patients with HER2 overexpressing metastatic breast cancers that had received extensive prior anti-cancer therapy as reported by Baselga et al. (1996) J. Clin. Oncol. 14:737-744. The clinical trial may be designed to evaluate the efficacy of an ADC in combinations with known therapeutic regimens, such as radiation and/or chemotherapy involving known chemotherapeutic and/or cytotoxic agents.

[000136] Generally, the disease or disorder to be treated is a hyperproliferative disease such as cancer. Examples of cancer to be treated herein include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g. epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, as well as head and neck cancer.

[000137] The cancer may comprise HER2-expressing cells, such that the ADC of the present invention are able to bind to the cancer cells. To determine ErbB2 expression in the cancer, various diagnostic/prognostic assays are available. In one embodiment, ErbB2 overexpression may be analyzed by IHC, e.g. using the HERCEPTEST (Dako). Paraffin embedded tissue sections from a tumor biopsy may be subjected to the IHC assay and accorded a ErbB2 protein staining intensity criteria as follows: Score 0, no staining is observed or membrane staining is observed in less than 10% of tumor cells; Score 1+, a faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells, the cells are only stained in part of their membrane; Score 2+, a weak to moderate complete membrane staining is observed in more than 10% of the tumor cells; Score 3+, a moderate to strong complete membrane staining is observed in more than 10% of the tumor cells. Those

tumors with 0 or 1+ scores for ErbB2 overexpression assessment may be characterized as not overexpressing ErbB2, whereas those tumors with 2+ or 3+ scores may be characterized as overexpressing ErbB2.

[000138] Alternatively, or additionally, FISH assays such as the INFORM™ (Ventana Co., Ariz.) or PATHVISION™ (Vysis, Ill.) may be carried out on formalin-fixed, paraffin-embedded tumor tissue to determine the extent (if any) of ErbB2 overexpression in the tumor.

[000139] Autoimmune diseases for which the ADC compounds may be used in treatment include rheumatologic disorders (such as, for example, rheumatoid arthritis, Sjögren's syndrome, scleroderma, lupus such as SLE and lupus nephritis, polymyositis/dermatomyositis, cryoglobulinemia, anti-phospholipid antibody syndrome, and psoriatic arthritis), osteoarthritis, autoimmune gastrointestinal and liver disorders (such as, for example, inflammatory bowel diseases (e.g., ulcerative colitis and Crohn's disease), autoimmune gastritis and pernicious anemia, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and celiac disease), vasculitis (such as, for example, ANCA-associated vasculitis, including Churg-Strauss vasculitis, Wegener's granulomatosis, and polyarteriitis), autoimmune neurological disorders (such as, for example, multiple sclerosis, opsoclonus myoclonus syndrome, myasthenia gravis, neuromyelitis optica, Parkinson's disease, Alzheimer's disease, and autoimmune polyneuropathies), renal disorders (such as, for example, glomerulonephritis, Goodpasture's syndrome, and Berger's disease), autoimmune dermatologic disorders (such as, for example, psoriasis, urticaria, hives, pemphigus vulgaris, bullous pemphigoid, and cutaneous lupus erythematosus), hematologic disorders (such as, for example, thrombocytopenic purpura, thrombotic thrombocytopenic purpura, post-transfusion purpura, and autoimmune hemolytic anemia), atherosclerosis, uveitis, autoimmune hearing diseases (such as, for example, inner ear disease and hearing loss), Behcet's disease, Raynaud's syndrome, organ transplant, and autoimmune endocrine disorders (such as, for example, diabetic-related autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM), Addison's disease, and autoimmune thyroid disease (e.g., Graves' disease and thyroiditis)). More preferred such diseases include, for example, rheumatoid arthritis, ulcerative colitis, ANCA-associated vasculitis, lupus, multiple sclerosis, Sjögren's syndrome, IDDM, pernicious anemia, thyroiditis, and glomerulonephritis.

[000140] The presently described cysteine substituted immunoglobulin conjugates can also be used to detect and treat CLL-1 associated disorders, i.e., diseases correlated with elevated or reduced cell surface expression of CLL-1 as compared to CLL-1 expression in a standard

control (e.g., a normal, non-disease, non-cancer cell). CLL-1 expression is normally limited to myeloid lineage cells, e.g., dendritic cells, granulocytes, and monocytes in the peripheral blood and spleen. Elevated CLL-1 levels are associated with cancer, in particular, in hematopoietic CSCs (e.g., LSCs), and in myeloproliferative disorders, including leukemias such as AML (acute myelogenous or myeloproliferative leukemia), MDS (myelodysplastic syndrome), myelofibrosis, CMML (chronic myelomonocytic leukemia), multiple myeloma, plasmacytoma, and CML (chronic myelogenous or myeloproliferative leukemia). See Bakker et al. (2004) Cancer Res. 64:8443; Van Rhenen et al. (2007) Blood 110:2659-66; Zhao et al. (2010) Haematologica (2010) 95:71; Van Rhenen et al. (2007) Leukemia 21:1700; and Herrmann et al. (2012)Haematologica 97:219.

[000141] AML cells can be characterized and distinguished from other cells by detecting cell surface marker expression. Aside from being CLL-1+, AML cells can be CD33+ (though some are CD33−), CD45+, and CDw52+. AML blasts (including LSCs) are typically CD34+CD38−. HSCs and LSCs can be characterized by expression of CD34, but the former do not express CLL-1. MDS cells can be characterized by expression of CD5, CD7, CD13, and CD34. CML cells can be characterized by expression of 7-ADD, CD33, CD34, and CD38.

[000142] Myelodysplastic Syndromes (MDS) include a group of closely-related blood formation disorders, in which the bone marrow shows qualitative and quantitative changes suggestive of a preleukemic process, but having a chronic course that does not necessarily terminate as acute leukemia. A variety of terms, including preleukemia, refractory anemia, refractory dysmyelopoietic anemia, smoldering or subacute leukemia, dysmyelopoietic syndrome (DMPS), and myelodysplasia, have all been used to describe MDS. These conditions are all characterized by a cellular marrow with impaired maturation (dysmyelopoiesis) and a reduction in the number of blood cells. DMPS is characterized by presence of megablastoids, megarkaryocyte dysplasia, and an increase in number of abnormal blast cells, reflective of enhanced granulocyte maturation process. Patients with DMPS show chromosomal abnormalities similar to those found in acute myeloid leukemia and progress to acute myeloid leukemia in a certain fraction of afflicted patients.

[000143] Chronic myeloproliferative disorders are a collection of conditions characterized by increased number of mature and immature granulocytes, erythrocytes, and platelets. Chronic myeloproliferative disorders can transition to other forms within this group, with a tendency to terminate in acute myeloid leukemia. Specific diseases within this group include

polycythemia vera, chronic myeloid leukemia, agnogenic myeloid leukemia, essential thrombocythemia, and chronic neutrophilic leukemia.

[000144] Myelofibrosis is characterized by scarring of the bone marrow that results in reduced number of red and white blood cells, and platelets. Myelofibrotic scarring can result from leukemia, but can have other causes, such as thrombocytosis or adverse drug effects.

B. CDC, ADCC, and ADC Assays

[000145] The effectiveness of the cysteine substituted immunoglobulin drug conjugates (e.g., CYSMAB ADCs) of the disclosure can be evaluated in by complement dependent cytotoxicity (CDC), Antibody dependent cell-mediated cytotoxicity (ADCC) assays of cells that express a target antigen, such as CLL-1. Exemplary cells that express CLL-1 include cell lines that express heterologous, recombinant CLL-1 (e.g., human CLL-1); human AML cell lines such as HL60, THP1, TF1-alpha, U937, and OCI AML-5 (the first four of which are available from ATCC); primary cells from one or more AML patients (e.g., PBMC or engrafted tumor cells); human CML cell lines such as K562 and KU812 (available from ATCC); and primary cells from one or more CML or MDS patients.

[000146] An antibody is described as having CDC activity and mediating CDC if it results in complement dependent killing of cells that express the antibody target. CDC assays are known in the art, and are described, e.g., in Gazzano-Santoro et al. (1997) J. Immunol. Methods 202:163; Idusogie et al. (2000) J. Immunol. 164:4178; and in Example 6 below. CDC kits and services are commercially available, e.g. from GeneScript® and Cell Technology Inc.

[000147] In brief, the assay is typically carried out in vitro, and includes antibody binding to a cell expressing the antibody target on its surface. Complement components, including C1q,22 which binds to the Ch region of the antibody, are added. The complement components then interact to kill the targeted cell. CDC is measured after a period of incubation of generally between 4 and 24 hours, for example, by determining the release of intracellular enzyme or granules known to be present in the targeted cell, by comparing the starting and ending target cell population, etc.

[000148] An antibody is described as having ADCC activity and mediating ADCC if it results in killing of antibody-bound cells (e.g., CLL-1 expressing cells) by effector cells. Effector cells are typically natural killer cells, but can also be macrophages, neutrophils, or eosinophils.

[000149] Genetically engineered effector cell lines have also been developed for use in ADCC assays (see, e.g., Schnueriger et al. (2011) Mol. Immunol. 48:1512). ADCC assays are known in the art, and are described, e.g., in Perussia and Loza (2000)Methods in Mol. Biol. 121:179; Bretaudeau and Bonnaudet (2011) BMC Proceedings 5(Suppl 8):P63; ADCC kits and services are commercially available, e.g. from GeneScript® and Promega®, and in the Example below.

[000150] In brief, the assay is typically carried out in vitro, and includes antibody binding to a cell expressing the antibody target on its surface. Effector cells are added that recognize antibody-bound cells, typically through an Fc receptor such as CD 16. The effector cells kill the antibody-bound cell, e.g., by releasing cytotoxins that cause apoptosis. Cell death is detected by release of a detectable element within the target cells (e.g., Cr51) or by detection of an element involved in the cell mediated toxicity (e.g., activation of NFAT signaling in effector cells).

[000151] An antibody is described as having antibody-drug conjugate (ADC) activity (or mediating ADC) if the antibody, when conjugated with a cytotoxic agent (drug), results in killing (inhibiting survival) a cell that expresses the target of the antibody, in this case, CLL-1. Appropriate cytotoxic agents are known in the art, e.g., saporin, doxorubicin, daunomycin, vinca-alkaloids, taxoids, tubulin agents (e.g., Maytansin, auristatin), and DNA agents (e.g., calicheamicin, duocarmycin, pyrrolobenzodiazepine dimers), etc. ADC assays are known in the art, e.g., as described in Gerber *et al.* (2009) 3:247, and in the Examples below.

C. Diagnostic Applications

[000152] The cysteine substituted immunoglobulin conjugates can thus be used for in vitro and in vivo diagnostic assays to detect cancer cells. This includes antibodies specific to CLL-1 described herein specifically bind CLL-1-expressing cells ("CLL-1 antibody" – for this section only) for detection of CLL-1-expressing cells (e.g., AML cells and AML CSCs). For example, a sample (e.g., blood sample or tissue biopsy) can be obtained from a patient and contacted with a CLL-1 antibody, and the presence of a CLL-1-expressing cell in the patient sample can be determined by detecting antibody binding. Antibody binding can be detected directly (e.g., where the antibody itself is labeled) or by using a second detection agent, such as a secondary antibody. The detectable label can be associated with an antibody of the disclosure, either directly, or indirectly, e.g., via a chelator or linker.

[000153] In some embodiments, the CLL-1 antibody is contacted with a biological sample from an individual having or suspected of having a CLL-1 associated disorder, and antibody binding to a cell in the sample is determined, wherein higher or lower than normal antibody binding indicates that the individual has a CLL-1 associated disorder. In some embodiments, the biological sample is a blood sample or blood fraction (e.g., serum, plasma, platelets, red blood cells, white blood cells, PBMCs). In some embodiments, the biological sample is a tissue sample (biopsy), e.g., from a suspected tumor site, or from a tissue that is known to be affected, e.g., to determine the boundaries of a known tumor.

[000154] Biopsies are typically performed to obtain samples from tissues, i.e., non-fluid cell types. The biopsy technique applied will depend on the tissue type to be evaluated (e.g., breast, skin, colon, prostate, kidney, lung, bladder, lymph node, liver, bone marrow, airway or lung). In the case of a cancer the technique will also depend on the size and type of the tumor (e.g., solid, suspended, or blood), among other factors. Representative biopsy techniques include, but are not limited to, excisional biopsy, incisional biopsy, needle biopsy, surgical biopsy, and bone marrow biopsy. An "excisional biopsy" refers to the removal of an entire tumor mass with a small margin of normal tissue surrounding it. An "incisional biopsy" refers to the removal of a wedge of tissue that includes a cross-sectional diameter of the tumor. A diagnosis or prognosis made by endoscopy or fluoroscopy can require a "core-needle biopsy" of the tumor mass, or a "fine-needle aspiration biopsy" which generally obtains a suspension of cells from within the tumor mass. Biopsy techniques are discussed, for example, in Harrison's Principles of Internal Medicine, Kasper, et al., eds., 16th ed., 2005, Chapter 70, and throughout Part V.

[000155] Any method of detecting antibody binding to a cell in a sample can be used for the present diagnostic assays. Methods of detecting antibody binding are well known in the art, e.g., flow cytometry, fluorescent microscopy, ELISAs, etc. In some embodiments, the method comprises preparing the biological sample for detection prior to the determining step. For example, a subpopulation of cells (e.g., white blood cells, CD34+ cells, CD45+ cells, etc.) can be separated from the rest of the sample from the individual (e.g., other blood components) or cells in a tissue can be suspended for easier detection.

[000156] In some embodiments, the percentage of CLL-1-expressing cells in the sample is determined and compared to a control, e.g., a sample from an individual or group of individuals that are known to have a CLL-1 associated disorder (positive control) or from an individual or group of individuals that are known not to have a CLL-1 associated disorder

(normal, healthy, non-disease, or negative control). In some embodiments, the control is a standard range of CLL-1 expression established for a given tissue. A higher or lower than normal percentage of CLL-1 expressing cells, or higher or lower expression level, indicates that the individual has a CLL-1 associated disorder.

[000157] In some embodiments, a labeled CLL-1 antibody can be provided (administered) to an individual to determine the applicability of an intended therapy. For example, a labeled antibody may be used to detect CLL-1 density within a diseased area, where the density is typically high relative to non-diseased tissue. A labeled antibody can also indicate that the diseased area is accessible for therapy. Patients can thus be selected for therapy based on imaging results. Anatomical characterization, such as determining the precise boundaries of a cancer, can be accomplished using standard imaging techniques (e.g., CT scanning, MRI, PET scanning, etc.).

[000158] In some embodiments, labeled CLL-1 antibodies as described herein can be further associated with a therapeutic compound, e.g., to form a "theranostic" composition. For example, an CLL-1 antibody can be linked (directly or indirectly) to both a detectable label and a therapeutic agent, e.g., a cytotoxic agent to kill CLL-1-expressing cancer cells. In some embodiments, a labeled CLL-1 antibody is used for diagnosis and/or localization of a CLL-1 expressing cancer cell, and the CLL-1 expressing cancer cell is then targeted with a separate therapeutic CLL-1 specific antibody. In some embodiments, the diagnostic CLL-1 specific antibody is one that is not internalized into CLL-1-expressing cells at a high rate or percentage. In some embodiments, the therapeutic CLL-1 antibody is internalized into CLL-1-expressing cells at a high rate or percentage.

1. Labels

[000159] A diagnostic agent comprising an antibody capable of binding a target of interest can include any diagnostic agent known in the art, as provided, for example, in the following references: Armstrong et al., Diagnostic Imaging, 5th Ed., Blackwell Publishing (2004); Torchilin, V. P., Ed., Targeted Delivery of Imaging Agents, CRC Press (1995); Vallabhajosula, S., Molecular Imaging: Radiopharmaceuticals for PET and SPECT, Springer (2009). A diagnostic agent can be detected by a variety of ways, including as an agent providing and/or enhancing a detectable signal. Detectable signals include, but are not limited to, gamma-emitting, radioactive, echogenic, optical, fluorescent, absorptive, magnetic, or tomography signals. Techniques for imaging the diagnostic agent can include, but are not

limited to, single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), optical imaging, positron emission tomography (PET), computed tomography (CT), x-ray imaging, gamma ray imaging, and the like. The terms "detectable agent," "detectable moiety," "label," "imaging agent," and like terms are used synonymously herein.

[000160] In some embodiments, the label can include optical agents such as fluorescent agents, phosphorescent agents, chemiluminescent agents, and the like. Numerous agents (e.g., dyes, probes, labels, or indicators) are known in the art and can be used in the present disclosure. (See, e.g., Invitrogen, The Handbook—A Guide to Fluorescent Probes and Labeling Technologies, Tenth Edition (2005)). Fluorescent agents can include a variety of organic and/or inorganic small molecules or a variety of fluorescent proteins and derivatives thereof. For example, fluorescent agents can include but are not limited to cyanines, phthalocyanines, porphyrins, indocyanines, rhodamines, phenoxazines, phenylxanthenes, phenothiazines, phenoselenazines, fluoresceins, benzoporphyrins, squaraines, dipyrrolo pyrimidones, tetracenes, quinolines, pyrazines, corrins, croconiums, acridones, phenanthridines, rhodamines, acridines, anthraquinones, chalcogenopyrylium analogues, chlorins, naphthalocyanines, methine dyes, indolenium dyes, azo compounds, azulenes, azaazulenes, triphenyl methane dyes, indoles, benzoindoles, indocarbocyanines, benzoindocarbocyanines, and BODIPY™ derivatives. Fluorescent dyes are discussed, for example, in U.S. Pat. No. 4,452,720, U.S. Pat. No. 5,227,487, and U.S. Pat. No. 5,543,295.

[000161] The label can also be a radioisotope, e.g., radionuclides that emit gamma rays, positrons, beta and alpha particles, and X-rays. Suitable radionuclides include but are not limited to 225Ac, 72As, 211At, 11B, 128Ba, 212Bi, 75Br, 77Br, 14C, 109Cd, 2Cu, 64Cu, 67Cu, 18F, 67Ga, 68Ga, 3H, 166Ho, 123I, 124I, 125I, 130I, 131I, 111In, 177Lu, 13N, 15O, 32P, 33P, 212Pb, 103Pd,186Re, 188Re, 47Sc, 153Sm, 89Sr, 99mTc, 88Y and 90Y. In some embodiments, radioactive agents can include 111In-DTPA,99mTc(CO)3-DTPA, 99mTc(CO)3-ENPy2, 62/64/67Cu-TETA, 99mTc(CO)3-IDA, and 99mTc(CO)3-triamines (cyclic or linear). In some embodiments, the agents can include DOTA and its various analogs with 111In, 177Lu, 153Sm, 62/64/67Cu, or67/68Ga. In some embodiments, a nanoparticle can be labeled by incorporation of lipids attached to chelates, such as DTPA-lipid, as provided in the following references: Phillips et al., Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 1(1): 69-83 (2008); Torchilin, V. P. & Weissig, V., Eds. Liposomes 2nd Ed.: Oxford Univ. Press (2003); Elbayoumi, T. A. & Torchilin, V. P.,

Eur. J. Nucl. Med. Mol. Imaging. 33:1196-1205 (2006); Mougin-Degraef, M. et al., Int'l J. Pharmaceutics 344:110-117 (2007).

[000162] In some embodiments, the diagnostic agent can be associated with a secondary binding ligand or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of suitable enzymes include urease, alkaline phosphatase, (horseradish) hydrogen peroxidase and glucose oxidase. Secondary binding ligands include, e.g., biotin and avidin or streptavidin compounds as known in the art.

[000163] In some embodiments, the labeled antibody can be further associated to a composition that improves stability in vivo, e.g. PEG or a nanoparticle such as a liposome, as described in more detail below.

2. Methods of Labeling

[000164] Techniques for conjugating detectable and therapeutic agents to antibodies are well known (see, e.g., Amon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery" in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review" in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev., 62:119-58 (1982)).

[000165] Typically, the antibody is attached to detectable moiety in an area that does not interfere with binding to the epitope. Thus in some cases, the detectable moiety is attached to the constant region, or outside the CDRs in the variable region. One of skill in the art will recognize that the detectable moiety can be located elsewhere on the antibody, and the position of the detectable moiety can be adjusted accordingly. In some embodiments, the ability of the antibody to associate with the epitope is compared before and after attachment to the detectable moiety to ensure that the attachment does not unduly disrupt binding.

[000166] In some embodiments, the antibody can be associated with an additional targeting moiety. For example, an antibody fragment, peptide, or aptamer that binds a different site on the target molecule or target cell can be conjugated to the antibody to optimize target binding, e.g., to a cancer cell.

D. Therapeutic Applications

[000167] CLL-1-expressing cells such as AML cells can be targeted using the cysteine substituted CLL-1 ADC antibodies described herein ("CLL-1 Antibodies" – for this section only). CLL-1 expression is elevated on AML cells and CSCs (e.g., AML CSCs). CLL-1 is not significantly expressed on normal CD34+ hematopoietic stem cells (HSCs), thus CSCs can be distinguished from HSCs using the present CLL-1 antibodies. High affinity CLL-1 antibodies that recognize a CLL-1 epitope common to AML cells, and thus able to universally bind to AML cells, is particularly valuable, as AML has a very high rate of recurrence. As noted above, a therapeutic composition comprising CLL-1 antibody can further include a detectable label to form a theranostic composition, e.g., for detection and localization of CLL-1 expressing cells, and monitoring of therapeutic effect. Sequences of antibodies that bind CLL-1 are described in USSN 62/259,100, filed November 24, 2015 (Jiang et al., "Humanized Anti-") and in US 2013/0295118 published November 7, 2013 (Jiang et al., "Antibodies Specific For CLL-1), incorporated by reference in their entirety.

[000168] Antibodies that bind targets other than CLL-1 can also be used in the cysteine-substituted antibody and antibody conjugates described herein. In some embodiments, the antibody targets can be selected from GPR114, CLL-1, IL1RAP, TIM-3, CD19, CD20, CD22, ROR1, mesothelin, CD33, CD123/IL3Ra, c-Met, PSMA, prostatic acid phosphatase (PAP), CEA, CA-125, Muc-1, AFP, Glycolipid F77, EGFRvIII, GD-2, NY-ESO-1 TCR, tyrosinase, TRPI/gp75, gp100/pmel-17, Melan-A/MART-1, Her2/neu, WT1, EphA3, telomerase, HPV E6, HPV E7, EBNA1, BAGE, GAGE and MAGE A3 TCRSLITRK6, ENPP3, Nectin-4, CD27, SLC44A4, CAIX, Cripto, CD30, MUC16, GPNMB, BCMA, Trop-2, Tissue Factor (TF), CanAg, EGFR, αv-integrin, CD37, Folate Receptor, CD138, CEACAM5, CD56, CD70, CD74, GCC, 5T4, CD79b, Steap1, Napi2b, Lewis Y Antigen, LIV, c-RET, DLL3,EFNA4, or Endosialin/CD248.

[000169] As demonstrated herein, the present CLL-1 antibodies can inhibit cancer cell growth (proliferation and/or engraftment) and thus can be considered chemotherapeutic agents alone. The following disclosure provides examples of chemotherapeutic and cytotoxic agents that can be linked to CLL-1 antibody for additional effect on CLL-1-expressing cells.

[000170] A chemotherapeutic (anti-cancer) agent can be any agent capable of reducing cancer growth, interfering with cancer cell replication, directly or indirectly killing cancer cells, reducing metastasis, reducing tumor blood supply, etc. Chemotherapeutic agents thus

include cytotoxic agents. Cytotoxic agents include but are not limited to saporin, taxanes, vinca alkaloids, anthracycline, and platinum-based agents. Classes of chemotherapeutic agents include but are not limited to alkylating agents, antimetabolites, e.g., methotrexate, plant alkaloids, e.g., vincristine, and antibiotics, e.g., doxorubicin as well as miscellaneous drugs that do not fall in to a particular class such as hydroxyurea. Platinum-based drugs, exemplified by cisplatin and oxaliplatin, represent a major class of chemotherapeutics. These drugs bind to DNA and interfere with replication. Taxanes, exemplified by taxol, represent another major class of chemotherapeutics. These compounds act by interfering with cytoskeletal and spindle formation to inhibit cell division, and thereby prevent growth of rapidly dividing cancer cells. Other chemotherapeutic drugs include hormonal therapy. Drug moieties can include cytotoxic agents, such as a monomeric or dimeric benzodiazepine derivative (*see, e.g.*, U.S Patent Application No 15/048,865, which is incorporated for reference), dolastatins, auristatins, maytansinoid, dolastatin, tubulysin, cryptophycin, pyrrolobenzodiazepine (PBD) dimer, indolinobenzodiazepine dimer, isoquinolidinobenzodiazepine dimer (including but not limited to D202 as described below), alpha-amanitin, trichothene, SN-38, duocarmycin, CC1065, calicheamincin, an enediyne antibioatic, taxane, doxorubicin derivatives, anthracycline and stereoisomers, azanofide, isosteres, analogs or derivatives thereof.

[000171] More than one therapeutic agent can be combined, either in the same composition, or in separate compositions. The therapeutic agent(s) can also be combined with additional therapeutic agents as appropriate for the particular individual. Common therapeutic agents provided to cancer patients include medications to address pain, nausea, anemia, infection, inflammation, and other symptoms commonly experienced by cancer patients.

[000172] Antibodies can be attached to a therapeutic agent, detectable agent, or nanocarrier using a variety of known cross-linking agents. Methods for covalent or non-covalent attachment of polypeptides are well known in the art. Such methods may include, but are not limited to, use of chemical cross-linkers, photoactivated cross-linkers and/or bifunctional cross-linking reagents. Exemplary methods for cross-linking molecules are disclosed in U.S. Pat. No. 5,603,872 and U.S. Pat. No. 5,401,511. Non-limiting examples of cross-linking reagents include glutaraldehyde, bifunctional oxirane, ethylene glycol diglycidyl ether, carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide or dicyclohexylcarbodiimide, bisimidates, dinitrobenzene, N-hydroxysuccinimide ester of

suberic acid, disuccinimidyl tartarate, dimethyl-3,3′-dithio-bispropionimidate, azidoglyoxal, N-succinimidyl-3-(2-pyridyldithio)propionate and 4-(bromoadminoethyl)-2-nitrophenylazide.

[000173] In some embodiments, the CLL-1 antibody is associated with a nanocarrier. For antibodies conjugated to nanocarriers (e.g., liposomes), a certain number of antibodies will be present on the surface, i.e., at a given surface density. In some embodiments, the nanocarrier will have at least 5 antibodies per nanocarrier, e.g., at least 10, 30, 40, 50, 75, 100 or higher antibodies per nanocarrier. One of skill in the art will understand that surface density represents an average range, as the number of antibodies per nanocarrier will not be absolutely uniform for all members of the population.

[000174] Nanocarriers include vesicles such as liposomes and micelles, as well as polymeric nanoparticles, etc. Nanocarriers are useful for delivery of therapeutic and diagnostic agents, but can be particularly useful for shielding cytotoxic agents used to treat cancer. The nanocarrier can comprise lipids (e.g., phospholipids), hydrophilic polymers, hydrophobic polymers, amphipathic compounds, cross-linked polymers, and a polymeric matrix (see, e.g., WO2009/110939). Depending on the application, the nanocarrier can be designed to have a particular size, half-life, shelf life, and leakage rate.

[000175] Preparation of nanocarriers, such as an antibody targeted liposome, polymeric nanoparticle, or extended shelf-life liposome, is described, e.g., in U.S. Pat. Nos. 6,465,188, 7,122,202, 7462603 and 7550441.

[000176] In some embodiments, the antibody is linked to a stabilizing moiety such as PEG, or a liposome or other nanocarrier. U.S. Pat. Nos. 4,732,863 and 7,892,554 and Chattopadhyay et al. (2010) Mol Pharm 7:2194 describe methods for attaching the selected antibody to PEG, PEG derivatives, and nanoparticles (e.g., liposomes). Liposomes containing phosphatidyl-ethanolamine (PE) can be prepared by established procedures as described herein. The inclusion of PE provides an active functional site on the liposomal surface for attachment.

[000177] The antibody conjugate can also be formulated to provide more than one active compound, e.g., additional chemotherapeutic or cytotoxic agents, cytokines, or growth inhibitory agents. The active ingredients may also prepared as sustained-release preparations (e.g., semi-permeable matrices of solid hydrophobic polymers (e.g., polyesters, hydrogels (for example, poly (2-hydroxyethyl-methacrylate), or poly (vinylalcohol)), polylactides. The antibodies and immunoconjugates can be entrapped in a nanoparticle prepared, for example,

by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions.

[000178] The CLL-1 antibodies described herein can kill CLL-1-expressing cells alone, or in combination with a cytotoxic agent. In some embodiments, the method of treatment comprises administering to an individual an effective amount of a therapeutic CLL-1 antibody or CLL-1 antibody conjugate, e.g., a CLL-1 antibody attached to a therapeutic agent. In some embodiments, the individual has been diagnosed with cancer, e.g., AML. In some embodiments, the individual is receiving or has received cancer therapy, e.g., surgery, radiotherapy, or chemotherapy. In some embodiments, the individual has been diagnosed, but the cancer is in remission.

[000179] In some embodiments, the method further comprises monitoring the individual for progression of the cancer. In some embodiments, the dose of the CLL-1 antibody or CLL-1 antibody conjugate for each administration is determined based on the therapeutic progress of the individual, e.g., where a higher dose of chemotherapeutic is administered if the individual is not responding sufficiently to therapy.

[000180] In some embodiments, the disclosure can include an antibody or antibody-targeted composition and a physiologically (i.e., pharmaceutically) acceptable carrier. The term "carrier" refers to a typically inert substance used as a diluent or vehicle for a diagnostic or therapeutic agent. The term also encompasses a typically inert substance that imparts cohesive qualities to the composition. Physiologically acceptable carriers can be liquid, e.g., physiological saline, phosphate buffer, normal buffered saline (135-150 mM NaCl), water, buffered water, 0.4% saline, 0.3% glycine, glycoproteins to provide enhanced stability (e.g., albumin, lipoprotein, globulin, etc.), and the like. Since physiologically acceptable carriers are determined in part by the particular composition being administered as well as by the particular method used to administer the composition, there are a wide variety of suitable formulations of pharmaceutical compositions of the present disclosure (See, e.g., Remington's Pharmaceutical Sciences, 17th ed., 1989).

[000181] The compositions of the present disclosure may be sterilized by conventional, well-known sterilization techniques or may be produced under sterile conditions. Aqueous

solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, and the like, e.g., sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, and triethanolamine oleate. Sugars can also be included for stabilizing the compositions, such as a stabilizer for lyophilized antibody compositions.

[000182] Dosage forms can be prepared for mucosal (e.g., nasal, sublingual, vaginal, buccal, or rectal), parenteral (e.g., subcutaneous, intravenous, intramuscular, or intraarterial injection, either bolus or infusion), oral, or transdermal administration to a patient. Examples of dosage forms include, but are not limited to: dispersions; suppositories; ointments; cataplasms (poultices); pastes; powders; dressings; creams; plasters; solutions; patches; aerosols (e.g., nasal sprays or inhalers); gels; liquid dosage forms suitable for oral or mucosal administration to a patient, including suspensions (e.g., aqueous or non-aqueous liquid suspensions, oil-in-water emulsions, or a water-in-oil liquid emulsions), solutions, and elixirs; liquid dosage forms suitable for parenteral administration to a patient; and sterile solids (e.g., crystalline or amorphous solids) that can be reconstituted to provide liquid dosage forms suitable for parenteral administration to a patient.

[000183] Injectable (e.g., intravenous) compositions can comprise a solution of the antibody or antibody-targeted composition suspended in an acceptable carrier, such as an aqueous carrier. Any of a variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.9% isotonic saline, 0.3% glycine, 5% dextrose, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc. Often, normal buffered saline (135-150 mM NaCl) will be used. The compositions can contain pharmaceutically acceptable auxiliary substances to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, e.g., sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc. In some embodiments, the antibody-targeted composition can be formulated in a kit for intravenous administration.

[000184] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intratumoral, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile

injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

[000185] The pharmaceutical preparation can be packaged or prepared in unit dosage form. In such form, the preparation is subdivided into unit doses containing appropriate quantities of the active component, e.g., according to the dose of the therapeutic agent or concentration of antibody. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, in unit-dose or multi-dose sealed containers, such as ampoules and vials. The composition can, if desired, also contain other compatible therapeutic agents.

[000186] The antibody (or antibody-targeted composition) can be administered by injection or infusion through any suitable route including but not limited to intravenous, subcutaneous, intramuscular or intraperitoneal routes. An example of administration of a pharmaceutical composition includes storing the antibody at 10 mg/mL in sterile isotonic aqueous saline solution for injection at 4° C., and diluting it in either 100 mL or 200 mL 0.9% sodium chloride for injection prior to administration to the patient. The antibody is administered by intravenous infusion over the course of 1 hour at a dose of between 0.2 and 10 mg/kg. In other embodiments, the antibody is administered by intravenous infusion over a period of between 15 minutes and 2 hours. In still other embodiments, the administration procedure is via sub-cutaneous bolus injection.

[000187] The dose of antibody is chosen in order to provide effective therapy for the patient and is in the range of less than 0.1 mg/kg body weight to about 25 mg/kg body weight or in the range 1 mg-2 g per patient. In some cases, the dose is in the range 1-100 mg/kg, or approximately 50 mg-8000 mg/patient. The dose may be repeated at an appropriate frequency which may be in the range once per day to once every three months, depending on the pharmacokinetics of the antibody (e.g., half-life of the antibody in the circulation) and the pharmacodynamic response (e.g., the duration of the therapeutic effect of the antibody). In some embodiments, the in vivo half-life of between about 7 and about 25 days and antibody dosing is repeated between once per week and once every 3 months.

[000188] Administration can be periodic. Depending on the route of administration, the dose can be administered, e.g., once every 1, 3, 5, 7, 10, 14, 21, or 28 days or longer (e.g.,

once every 2, 3, 4, or 6 months). In some cases, administration is more frequent, e.g., 2 or 3 times per day. The patient can be monitored to adjust the dosage and frequency of administration depending on therapeutic progress and any adverse side effects, as will be recognized by one of skill in the art.

[000189] Thus in some embodiments, additional administration is dependent on patient progress, e.g., the patient is monitored between administrations. For example, after the first administration or round of administrations, the patient can be monitored for rate of tumor growth, recurrence (e.g., in the case of a post-surgical patient), or general disease-related symptoms such as weakness, pain, nausea, etc.

[000190] For the treatment of cancer, an antibody or antibody-targeted composition (e.g., including a therapeutic and/or diagnostic agent) can be administered at the initial dosage of about 0.001 mg/kg to about 1000 mg/kg daily and adjusted over time. A daily dose range of about 0.01 mg/kg to about 500 mg/kg, or about 0.1 mg/kg to about 200 mg/kg, or about 1 mg/kg to about 100 mg/kg, about 5 to about 10 mg/kg, or about 10 mg/kg to about 50 mg/kg, can be used. The in vivo xenograft results described herein indicate that a dose between 5-20 mg antibody/kg body weight is effective for dramatic reduction of tumor growth.

[000191] The dosage is varied depending upon the requirements of the patient, the severity of the condition being treated, and the targeted composition being employed. For example, dosages can be empirically determined considering the type and stage of cancer diagnosed in a particular patient. The dose administered to a patient, in the context of the present disclosure, should be sufficient to affect a beneficial therapeutic response in the patient over time. The size of the dose will also be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular targeted composition in a particular patient, as will be recognized by the skilled practitioner.

[000192] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entireties for all purposes.

[000193] All publications and patent applications mentioned in this specification are herein expressly incorporated by reference in their entirety to the same extent as if each individual

publication or patent application were specifically and individually indicated to be incorporated by reference.

[000194] From the foregoing it will be appreciated that, although specific embodiments of the Invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

[000195] The following examples are offered by way of illustration and not by way of limitation.

VI. EXAMPLES

[000196] A cysteine residue was engineered at selected position (EU numbering) of CLL-1 antibody (HuM31) heavy chain to produce corresponding CYSMAB variant using QuickChange II Site-Directed Mutagenesis Kit (Agilent). Authenticity of the cysteine substitution was verified by DNA sequencing. CYSMAB light and heavy chain construct were transiently transfected into HEK-293 cells. Expressed CYSMAB variant was purified using MabSelectsuRe beads and further characterized with various CLL-1 functional assays.

Example 1 -- Conjugation

[000197] To demonstrate conjugation at selected residues of the heavy chain constant region, antibody-fluorophore conjugates were created. The following procedure was used: Purified HuM31 or CYSMAB variants (1.5mg each) were dialyzed against PBS overnight at 4 °C. Antibodies were incubated with 200µL of MabSelectsuRe beads at room temperature for 1 hour. After washing beads three times with 2mL PBS each time, antibodies were reduced in 2mM DTT at RT overnight in 150mM NaCl-50mM Tris, pH 8.0 buffer. Beads were washed three times then antibodies were re-oxidized in 1mM Dehydroascorbic acid (DHAA) at room temperature for three hours. Antibodies were washed three times and conjugated with 10 molar excess Alexa488-C5-maleimide at room temperature for two hours. Beads were washed three times and Alexa488 conjugated antibodies were eluted with 500µL of 0.1M Glycine, pH 2.7. The antibody concentration and Alexa488 conjugation efficiency (number of Alexa488 per antibody) was determined by using NanoDrop 2000.

[000198] To demonstrate that the conjugation was not variable with the date or amount of fluorophore, the conjugation procedure was repeated on different dates, and with different

concentrations. The results (Figure 5 and Figure 6, respectively) show that the neither the conjugation ratio (e.g., DAR, FAR) vary appreciably in the procedure.

[000199] The results of the conjugation, including amino acid residue and fluorophore-to-antibody ratio ("FAR") are reported in Figures 7-9.

[000200] Figure 7 shows of 45 total conjugations, 21 (47%) displayed high conjugation (>2), 7 (16%) medium (1-2) and 17 (38%) low (<1).

[000201] Figure 8 shows of 20 total conjugates, 10 (50%) display high conjugation (>2), 1 (5%) medium (1-2) and 9 (45%) low (<1).

Example 2 -- Specificity ELISA

[000202] Figure 1: An ELISA assay specifically to detect Alexa 488 (A488) conjugated to HuM31 (HuM31-A488 AFC) was developed. Three different formats of ELISA were designed to detect the A488 conjugated to HuM31 in human plasma. Unconjugated HuM31, HuM31-A488 and IgG-A488 were tested in these three ELISA methods. The results showed ELISA format #1 has best signal to noise ratio. And format #2 and #3 showed higher background binding. Format #1 ELISA was moved forward to detect HuM31-A488.

[000203] Figure 2a: Specificity of the ELISA assay. Format #1 ELISA method was used to specifically detect HuM31-A488 and site-specific CYSMAB-A488 conjugates instead of control samples (relative to IgG (Isotype control), IgG-A488 AFC, trastuzumab and unconjugated HuM31) used. The result demonstrated that this ELISA method only detected HuM31-A488 and site-specific CYSMAB-A488 conjugates. In contrast controls: isotype human IgG, trastuzumab, HuM31 or IgG-A488 conjugates showed no binding.

[000204] Figure 2b: Testing human plasma interference in the ELISA assay. Under the optimal ELISA conditions, the presence of 1% human plasma only marginally enhanced the binding of HuM31-A488 conjugate to anti-A488 antibody. Therefore the ELISA method can be used to analyze antibody conjugate samples that had prior exposure to human plasma.

Example 3 -- Stability of HuM31-A488 Conjugate (AFC)

[000205] The stability in human plasma of the AFCs of the disclosure was tested by incubation in human plasma. AFC (50 µg/mL) was spiked into pooled human plasma *or* 0.5% BSA in PBS. Each sample was then incubated at 37°C with 5% CO_2, and then transferred to -80°C at 0, 24, 48, 72 and 96 hour time points. The samples were diluted 1:5000 in sample Diluent (PBS buffer containing 0.5% BSA, 0.05% Tween 20, 5mM EDTA, 0.35M NaCl,

0.25% CHAPS and 0.2% BGG). The samples at various time points were then assayed by the ELISA.

[000206] **ELISA procedure**: CLL-1 extra cellular domain protein in PBS (1 µg/mL) was coated on 96-well plate and incubated overnight at 4 °C. Plate then washed three times with 0.1% Tween 20 PBS followed by 1 hour of blocking with 1% BSA in 0.1% Tween 20 PBS at room temperature. After six times washes with 0.1% Tween 20 in PBS, serial diluted Alexa488 conjugated human M31 and its controls were added to the plate and incubated for 1 hour at room temperature. Then plate was washed with 0.1% Tween 20 in PBS. Rabbit anti-Alexa 488 secondary antibody (1 µg/mL) was added to the plate and incubated for 1 hour at RT. After six times washes with 0.1% Tween 20 PBS plate was detected by HRP conjugated goat anti-rabbit Fc polyclonal antibody at 1:50,000 dilution. The percent (%) values were then evaluated by comparing the OD value of each time point to time 0.

[000207] The stability of tested samples after 5 days are shown in Figures 9 and 10:

[000208] Figures 9 and 10 show that samples 58, 64, 73, 81, 86, and 206 have stability >85% after 5 days of incubation.

Example 4 -- Stability of HuM31-Biotin conjugates

[000209] HuM31-Biotin conjugates were generated by conjugating CYSMABs with HPDP-biotin and BMCC-biotin.

[000210] Purified human M31 or CYSMAB variants (1.5mg each) were dialyzed against PBS overnight at 4 °C. Antibodies were incubated with 200 µL of MabSelectsuRe beads at room temperature for 1 hour. After washing beads three times with 2mL PBS each time, antibodies were reduced in 2mM DTT at RT overnight in 150mM NaCl-50mM Tris, pH 8.0 buffer. Beads were washed three times then antibodies were re-oxidized in 1mM DHAA at room temperature for three hours. Antibodies were washed three times and conjugated with 10 molar excess HPDP-biotin or BMCC biotin at room temperature for two hours. Beads were washed three times and biotin conjugated antibodies were eluted with 500µL of 0.1M Glycine, pH 2.7. The antibody concentration and biotin conjugation efficiency was determined by using NanoDrop 2000 and ELISA based assay, respectively.

[000211] An ELISA assay was developed to determine stability of ADCs in human plasma.: CLL-1 extra cellular domain (1µg/mL) in PBS was coated on 96-well plate and incubated overnight at 4 °C. ELISA plate was washed three times with washing buffer (0.1% Tween 20

in PBS) followed by 1 hour of blocking with 1% BSA in 0.1% Tween 20 in PBS at room temperature. After washing the plate six times with washing buffer, serially diluted HuM31-Biotin and its corresponding control samples were added to the plate and incubated for 1 hour at room temperature. The plate was washed six time with washing buffer followed by detection using streptavidin-HRP conjugate (used at 1:100,000 dilution).

[000212] The stability of the HPDP and BMCC linked antibody conjugates after day 5 are reported in Figure 11.

The Stability of Biotin-BMCC Conjugate Samples in Human Plasma:

[000213] Figure 11 shows that samples V266, V303, T307, G316, Y436, L441, H285, R301, Q295 have stability > 80% after 5 days of incubation.

Example 5 -- Affinity Testing

[000214] The binding affinity for the cysteine-substituted CLL-1 CYSMABs can be tested for comparative binding affinity to their naked, unconjugated counterparts. Briefly, biotinylated CLL-1 (25 µg/mL) is loaded onto streptavidin sensor tips for 2 hours at 22 °C. Ab-Ag dissociation curves were generated at three different concentrations for each antibody with either Fortebio or BIAcore analysis (10, 30, and 90 µg/mL) using a global 1:1 curve fitting.

Example 6 -- Binding to AML Cell Lines and AML Patient Samples

[000215] The cysteine-substituted CYSMABs can be tested for comparative binding to recombinant 293 cells expressing human CLL-1, and two AML cell lines, HL60 and OCI AML-5. The percentage of live cells with antibody binding can be detected by any suitable means, e.g., FACS. The binding consistency, i.e., interpatient variability, can also be evaluated.

Example 7 -- Antibody-Drug Conjugate (ADC) Assays

[000216] Antibody-Drug Conjugate (ADC) assays can be carried out on a suitable AML cell lines (e.g., HL60 and OCI AML-5), as well as recombinant 293 cells expressing CLL-1. Briefly, cells are incubated with various concentrations of ADCs for 72-120 hours at 37 °C. Cell viability is determined by CellTiter-Glo (Promega) luminescent cell viability assay to determine IC50 values.

Example 8 -- In Vivo Inhibition of AML Tumor Growth

[000217] The CLL-1 CYSMAB ADCs can be evaluated for *in vivo* efficacy. Suitable studies include both (1) subcutaneous (SC) tumor engraftment and growth model utilizing the CLL-1 positive HL60 AML human cell line in mice, and (2) an orthotopic (bone marrow, blood, spleen and lymph node) tumor engraftment and outgrowth model utilizing the CLL-1 positive HL60 or OCI AML-5 human AML cell line.

[000218] An established SC HL60 study can be carried out as follows. Animals (nu/nu mice) were inoculated with of 5×10^6 or 10^7HL60 cells. Tumor-bearing mice were randomized to a mean tumor volume of 100-150 mm^3 in each group (8 animals/group). CLL-1 CYSMAB ADC, or an IgG control ADC, is administered i.p. at a dose of 5-200 µg/animal. Mean tumor volumes were plotted over a time (post-dose).

[000219] The OCI AML-5 cell orthotopic studies can be carried out as follows. Immunodeficient NSG mice are split into 5 groups of 8 animals/group. CLL-1 CYSMAB ADC, or an IgG control ADC, are administered i.p. at a dose of 5-200 µg/animal at post (day-6) intravenous inoculation of 5×1^{06} or 1^{07} OCI AML-5 cells. Animals then receive additional antibody doses once per week for the next 2 weeks. The study terminates 4 weeks after administration of the OCI AML-5 cells.

Example 9 -- Specificity for AML Stem Cells in ADC Assays

[000220] The specificity of the CLL-1 CYSMAB ADCs prepared according to the disclosure can be tested for specific killing in an ADC assay. Primary patient AML cells or normal CD34 positive hematopoietic stem cells are isolated from the bone marrow of human subjects, and are seeded into a soft agar colony formation assay (100,000 cells/plate). The cells are then incubated in the presence CLL-1 CYSMAB ADCs 14 days. The ADC can cause selective, specific inhibition of AML stem cell clonogenic growth, while normal HSCs should not be affected. The effect of the conjugation can be compared to the naked parent antibody. The negative controls are untreated or treated with an unrelated IgG-ADC.

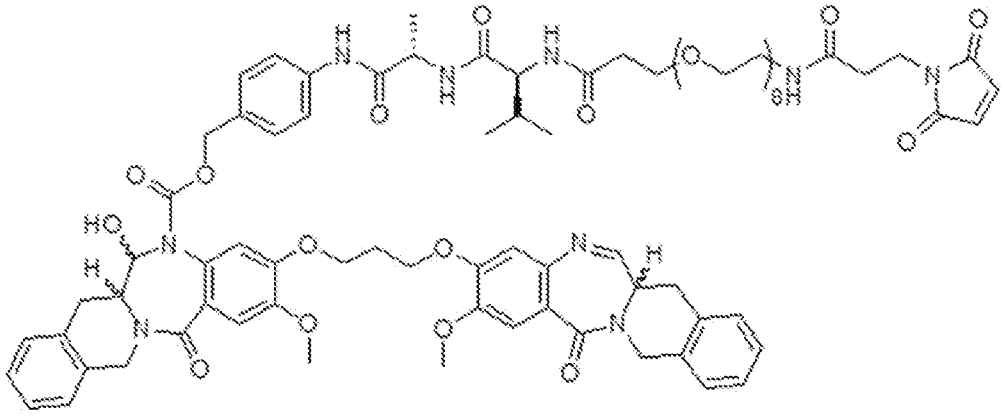
Example 10 – Stability of various drug conjugates

[000221] A variety of antibody-drug conjugates were made as follows:

Name	cysMAB	Concentration	Amount	DAR
cc-Cys-02-D202	S156C	3.20 mg/ml	3.1 mg	>1.8

Cys-01-D202	A118C	2.44 mg/ml	2.8 mg	>1.8
Cys-87-D202	G316C	2.63 mg/ml	2.9 mg	< 1.0
Cys-37-D202	V266C	4.28 mg/ml	2.7 mg	>1.8
Cys-10-D202	S239C	3.72 mg/ml	2.6 mg	>1.8

All of the conjugates were made with an anti-CLL-1 antibody having the following variable regions: light chain variable region sequence comprising: DIQMTQSPSSLSASVGDRVTLTCRATQELSGYLSWLQQKPGKAIKRLIYAASTLDSGV PSRFSGNRAGTDYTLTISSLQPEDFATYYCLQYAIYPYTFGQGTKLEIK (SEQ ID NO:19) and a heavy chain variable region sequence comprising: EVQLVQSGAEVKKPGASVKMSCKASGYTFTSYFIHWVRQAPGQGLEWIGFINPYND GSKYAQKFQGRATLTSDKSTSTVYMELSSLRSEDTAVYYCTRDDGYYGYAMDYWG QGTLVTVSS (SEQ ID NO:20). The antibodies were conjugated to the indicated cysteine substitution via a cysteine reactive linker to an isoquinolidinobenzodiazepine dimer as follows:



(designated "D202").

[000222] A humanized, cys-substituted anti-CLL1 antibodies in PBS were exchanged into borate buffer (50 mM, pH 8.5, 1mM diethylene triamine pentaacetic acid (DTPA)) via 2 cycles of molecular weight cut-off filtration (MWCO) using a Millipore, 15 mL, 30 kDa device. To the new solution of the antibodies (5.0 mg/mL, borate buffer (50 mM, pH 8.5, 1mM DTPA)) was added a solution of Dithiothreitol (DTT) (33 μL, 50.0 equiv., 50 mM) and the resultant solution was shaken gently overnight.

[000223] Complete reduction of the interchain disulfide bridges and removal of the substituted cysteine cysteine/glutathione adducts were confirmed by rp-LCMS as described earlier (Junutula et al., 2008, *Nature Biotech*, 26, 925-932). DTT was then removed from the

solution via 3 cycles of molecular weight cut-off filtration (MWCO) using a Millipore, 15mL, 30 kDa device, using PBS as the exchange buffer. To a 5 mg/ml solution of the fully reduced antibody was added a solution of dehydro ascorbic acid (dhAA) (33 μL, 50.0 equiv., 50 mM). The resultant solution was shaken gently for 3 hrs. The re-oxidation was monitored via rp-LCMS. Once the re-oxidation was deemed complete, the reaction mixture was diluted up to 50% v/v with propylene glycol and D202 was added as a solution in DMSO (10.0 equiv., 10 mM in DMSO).

[000224] The reaction was allowed to stir at ambient temperature for 1 hr. The mixture was then treated with activated charcoal for 1 hr at ambient temperature. The activated charcoal was then removed via filtration. The conjugate was then exchanged into PBS via multiple cycles of molecular weight cut-off filtration (MWCO) using Millipore, 15mL, 30 kDa devices. The solution was then subjected to a sterile filtration to yield the desired conjugate.

[000225] Starting at 30 μg/mL, C6-CYSMAB-D202 ADCs and C0-D202 were subjected to 8-point, 6-fold serial dilutions using cell binding buffer (PBS, with 2% fetal bovine serum). HL60, OCI-AML5 and OCI-AML5-CLL1 knockout cells were washed by staining media and incubated with 5% normal mouse serum on ice for 30 minutes to block Fcγ receptors. The cells were then dispensed into 96-well plate in a density of $0.1e^6$ cells per well and medium was removed by centrifugation. The cell plates were incubated with 100 μL ADC sample dilutions for 30 minutes on ice followed by three times washing and further stained with Alexa-488 conjugated Goat anti human IgG as secondary antibody for 30 minutes on ice. The cells were then washed three times and resuspended in 100 μL cell binding buffer using propidium iodide as cell viability dye. The ADC binding to the cell samples were analyzed by flow cytometry and data analysis by Flowjo. The MFI (Geom. Mean) of FITC signal were plotted using Graphpad Prizm 6. *See*, FIG. 12A-C.

[000226] Stability of the conjugates was determined as follows. On day 0, dilute C6-CYSMAB-D202 ADC samples in human plasma to 200mg/mL. Perform 9-point, 6-fold serial dilution using human plasma as a diluent . Seal the sample dilution plate and incubate at 37 °C in the CO_2 incubator for 5 days as a sample D5. Prepare Sample D3, D1 and D0 by repeating this plasma dilution and 37 °C incubation procedure on day 2, 4 and 5. On day 5, set up cell killing assay by adding 5mL of sample D0, D1, D3 and D5 into 95mL of OCI-AML2 and HL60 cells, incubate at 37 °C for 5 days and quantify the cell viability by Cell-Titer-Glo.

[000227] C6-CYSMAB-D202 ADC sample dilutions were carried out in 96-well plates using human plasma as diluent and incubated at 37 °C in the CO_2 incubator for plasma stability study. 9-point, 6-fold serial dilutions, starting at 200 µg/mL ADC, were performed on day 0, 2, 4, and day 5 and the sealed sample dilution plates plus no ADC, plasma only control, were incubated at 37 °C, 5% CO_2 incubator for 5, 3, 1 and 0 days, respectively, as sample D5, D3, D1 and D0. In day 5, OCI-AML2 and HL60 cells were seeded in 96-well plates at a density of 2,000 cells in 95 µL Alpha-MEM and IMDM cell culture media, supplemented with 20% fetal bovine serum (FBS) and treated with 5 µL samples from D0, D1, D3, D5 sample dilution plates in triplicates. The assay plates were then incubated at 37 °C, 5% CO_2 incubator for 5 days. Live cells (percent of viable cells) were assayed using CellTiter-Glo kit (Promega) and the luminescence measured by a plate reader (Molecular Device Spetramax M5). The results were expressed in percentage of viable cells relative to no ADC plasma only control cells. Individual dose response curves and inhibitory drug concentrations (IC_{50}) were derived by nonlinear regression using Graphpad Prizm 6.

[000228] Stability Test of C6-CYSMAB-D202 ADCs on AML2, HL60 Cell Killing is exemplified in the following tables:

	AML2			
IC50 µg/mL	D0	D1	D3	D5
S156C	0.0016	0.0012	0.0011	0.0058
A118C	0.0017	0.0010	0.0028	0.0043
G316C	0.0037	0.0019	0.0084	0.0128
V266C	0.0041	0.0028	0.0043	0.0070
S239C	0.0021	0.0009	0.0008	0.0021

	HL60			
IC50 µg/mL	D0	D1	D3	D5
S156C	0.0072	0.0070	0.0092	0.0399
A118C	0.0100	0.0116	0.0245	0.0608
G316C	0.0256	0.0315	0.1153	0.1772
V266C	0.0154	0.0115	0.0200	0.0394
S239C	0.0087	0.0047	0.0043	0.0120

WHAT IS CLAIMED IS:

1. A cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue selected from the group consisting of: V266C, G316C, H285C, R301C, V303C, T307C, Y436C and L441C under EU numbering.

2. The polypeptide of Claim 1, wherein the immunoglobulin polypeptides are derived from human IgG heavy chain constant regions.

3. The polypeptide of Claim 2, wherein the IgG is an isotype selected from the group consisting of IgG1, IgG2, IgG3 or IgG4.

4. The polypeptide of Claim 3, wherein the isotype is IgG1.

5. A nucleic acid molecule comprising a nucleotide sequence encoding a cysteine-substitute

6. d immunoglobulin polypeptide comprising a substituted amino acid residue selected from the group consisting of: V266C, G316C, H285C, R301C, V303C, T307C, Y436C and L441C under EU numbering.

7. The nucleic acid molecule of Claim 5 further comprising an expression control sequence operably linked with the nucleotide sequence.

8. The nucleic acid molecule of Claim 6, comprised in an expression vector.

9. A recombinant cell comprising a nucleic acid molecule comprising a nucleotide sequence encoding a cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue selected from the group consisting of: V266C, G316C, H285C, R301C, V303C, T307C, Y436C and L441C under EU numbering.

10. A process for making a cysteine-substituted polypeptide comprising culturing recombinant cell comprising a nucleic acid molecule comprising a nucleotide sequence encoding a cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue selected from the group consisting of: V266C, G316C, H285C, R301C, V303C, T307C, Y436C and L441C under EU numbering.

11. A cysteine-substituted antibody comprising a cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue selected from the group consisting of: V266C, G316C, H285C, R301C, V303C, T307C, Y436C and L441C in a heavy chain constant region under EU numbering.

12. The antibody of Claim 10, wherein the heavy chain constant region is derived from a human IgG isotype selected from the group consisting of: IgG1, IgG2, IgG3 and IgG4.

13. The antibody of Claim 10 comprising an immunoglobulin light chain selected from the group consisting of kappa and lambda.

14. The antibody of Claim 12, wherein the light chain is kappa.

15. The antibody of Claim 10 that binds to CLL-1, GPR114, IL1RAP, TIM-3, CD19, CD20, CD22, ROR1, mesothelin, CD33, CD123/IL3Ra, c-Met, PSMA, prostatic acid phosphatase (PAP), CEA, CA-125, Muc-1, AFP, Glycolipid F77, EGFRvIII, GD-2, NY-ESO-1 TCR, tyrosinase, TRPI/gp75, gp100/pmel-17, Melan-A/MART-1, Her2/neu, WT1, EphA3, telomerase, HPV E6, HPV E7, EBNA1, BAGE, GAGE and MAGE A3 TCRSLITRK6, ENPP3, Nectin-4, CD27, SLC44A4, CAIX, Cripto, CD30, MUC16, GPNMB, BCMA, Trop-2, Tissue Factor (TF), CanAg, EGFR, αv-integrin, CD37, Folate Receptor, CD138, CEACAM5, CD56, CD70, CD74, GCC, 5T4, CD79b, Steap1, Napi2b, Lewis Y Antigen, LIV, c-RET, DLL3,EFNA4, or Endosialin/CD248.

16. The antibody of Claim 14, comprising a variable light chain and a variable heavy chain, wherein:

(a) the variable light chain comprises a CDR-L1, CDR-L2 and CDR-L3, further wherein:

CDR-L1 is ESVDSYGNSF (SEQ ID NO:1)

CDR-L2 is LAS (SEQ ID NO:2)

CDR-L3 is QQNNYDPWT (SEQ ID NO:3), and

(b) the variable heavy chain comprises a CDR-H1, CDR-H2 and CDR-H3, further wherein:

CDR-H1 is GYTFTSYV (SEQ ID NO:4)

CDR-H2 is INPYNDGT (SEQ ID NO:5), and

CDR-H3 is ARPIYFDNDYFDY (SEQ ID NO:6); or wherein:

(c) the variable light chain further comprises a CDR-L1, CDR-L2 and CDR-L3, further wherein:

CDR-L1 is RATQELSGYLS (SEQ ID NO:13)

CDR-L2 is AASTLDS (SEQ ID NO:14)

CDR-L3 is LQYAIYPYT (SEQ ID NO:15), and

(d) the variable heavy chain further comprising a CDR-H1, CDR-H2 and CDR-H3, further wherein:

CDR-H1 is GYTFTSYFIH (SEQ ID NO:16)

CDR-H2 is FINPYNDGSK (SEQ ID NO:17), and

CDR-H3 is DDGYYGYAMDY (SEQ ID NO:18)

17. A nucleic acid molecule comprising a nucleotide sequence encoding the antibody of Claims 10-17.

18. The nucleic acid molecule of Claim 17 operably linked with an expression control sequence.

19. The nucleic acid molecule of Claim 18 further comprising an expression vector.

20. A recombinant cell comprising a nucleic acid molecule of claim 19.

21. A process for making an antibody comprising culturing the recombinant cell of Claim 1.

22. The process of claim **1** further comprising isolating the antibody.

23. An antibody conjugate comprising the antibody of any of Claims **1-4 or 10-15**, conjugated from the substituted amino acid residue (cysteine) in the antibody through a linker to a moiety selected from the group consisting of: drug, radionucleotide, fluorophore, biotin, RNA, antibiotic, protein and a detectable moiety.

24. The antibody conjugate of Claim 1, which is conjugated to a drug.

25. The antibody conjugate of Claim 1, wherein the drug is selected from the group consisting of: a monomeric or dimeric benzodiazepine derivative, maytansinoid, auristatin, dolastatin, tubulysin, cryptophycin, pyrrolobenzodiazepine (PBD) dimer, indolinobenzodiazepine dimer, isoquinolidinobenzodiazepine dimer, alpha-amanitin, trichothene, SN-38, duocarmycin, CC1065, calicheamincin, an enediyne antibioatic, taxane, doxorubicin derivatives, anthracycline, azanofide and stereoisomers, isosteres, analogs or derivatives thereof.

26. The antibody conjugate of Claim 1, wherein the drug is an isoquinolidinobenzodiazepine dimer.

27. The antibody conjugate of any of Claims 22-25, wherein the linker is covalently bonded to the drug.

28. The antibody conjugate of any of Claims 22-25, wherein the linker is attached to the antibody through a reaction between a thiol and a thiol reactive group selected from maleimide, halide and sulfonyl.

29. The antibody conjugate of any of Claims 23-25, wherein the linker is connected via disulfide bond to the drug.

30. The antibody conjugate of Claim 28, wherein the disulfide bond is a pyridyl disulfide moiety.

31. The antibody conjugate of any of Claims 22-25, wherein the linker is cleavable in the microenvironment of the target.

32. The antibody conjugate of Claim 22, which is conjugated to a detectable moiety.

33. The antibody conjugate of Claim 31, wherein the detectable moiety is selected form the group consisting of A488, BMCC-biotin and HPDP-biotin.

34. A composition comprising the antibody or conjugated antibody of any of Claims 10-32 and an adjuvant.

35. The composition of Claim 1, which is pharmaceutically acceptable.

36. A method of detecting a cell of interest comprising:

(a) contacting a cell with an effective amount of an antibody of any of claims 1-4 or 10-15 capable of binding the cell, and

(b) detecting binding of the antibody to the cell,

wherein said binding indicates the cell of interest.

37. The method of Claim 1, wherein the cell of interest expresses CLL-1.

38. The method of Claim 1, wherein the antibody is conjugated to a detectable moiety.

39. A method of diagnosing a disease comprising:

(a) contacting a biological sample from an individual with an effective amount of an antibody of any of claims 1-4 or 10-15 capable of binding to diseased cells; and

(b) detecting binding of the antibody to a disease cell,

wherein binding indicates the presence of the disease.

40. The method of Claim 1, wherein the antibody is conjugated to a detectable moiety.

41. The method of Claim 1, wherein the disease is cancer and the antibody binds to a tumor associated antigen or a cancer stem cell associated antigen.

42. The method of Claim 1, wherein the tumor associated antigen or cancer stem cell associated antigen is CLL-1.

43. The method of Claim 1, wherein the disease is a myeloproliferative disorder.

44. The method of Claim 42, wherein the myeloproliferative disorder is selected from the group consisting of: AML, CML, CMML, multiple myeloma, plasmacytoma and myelofibrosis.

45. A method of inhibiting cell division comprising contacting a cell with at least an effective amount of an antibody conjugate of any of claims 22-32 capable of binding the cell and which is conjugated to a drug that is cytotoxic to the cell.

46. The method of Claim 1, wherein the inhibition of cell division results in cell death.

47. The method of Claim 1 wherein the cell is a tumor or cancer stem cell, and the antibody binds to a tumor associated antigen or cancer stem cell antigen.

48. The method of Claim 1, wherein the tumor or cancer stem cells are from a myeloproliferative disorder.

49. The method of Claim 1, wherein the myeloproliferative disorder is selected from the group consisting of: AML, CML, CMML, multiple myeloma, plamacytoma myelofibrosis.

50. The method of Claim 1, wherein the tumor associated antigen or cancer stem cell antigen is CLL-1.

51. A method of treating cancer comprising administering to a patient a therapeutically effective amount of an antibody conjugate of any of claims 22-32 wherein the antibody conjugate is capable of binding a tumor associated antigen or cancer stem cell antigen.

52. The method of Claim 1, wherein the cancer is a myeloproliferative disorder.

53. The method of Claim 1, wherein the myeloproliferative disorder is selected from the group consisting of: AML, CML, CMML, multiple myeloma, plasmacytoma and myelofibrosis.

54. The method of Claim 1, wherein the tumor associated antigen or cancer stem cell antigen is CLL-1.

55. An antibody conjugate comprising a cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue at S156 (under Kabat numbering) in the heavy chain linked via the cysteine to a monomeric or dimeric benzodiazepine derivative, maytansinoid, auristatin, dolastatin, tubulysin, cryptophycin, pyrrolobenzodiazepine (PBD) dimer, indolinobenzodiazepine dimer, isoquinolidinobenzodiazepine dimer, alpha-amanitin, trichothene, SN-38, duocarmycin, CC1065, calicheamincin, an enediyne antibioatic, taxane,

doxorubicin derivatives, anthracycline, azanofide and stereoisomers, isosteres, analogs or derivatives thereof.

56. The antibody conjugate of claim 54, wherein the indolinobenzodiazepine dimer or isoquinolidinobenzodiazepine dimer is attached to the antibody through a linker and the linker is connected via disulfide bond to the drug.

57. The antibody conjugate of Claim 54, wherein the disulfide bond is a pyridyl disulfide moiety.

58. The antibody conjugate of Claim 54, wherein the linker is cleavable in the microenvironment of the target.

59. A composition comprising the antibody or conjugated antibody of any of Claims 54-57 and an adjuvant.

60. The composition of Claim 58, which is pharmaceutically acceptable.

61. A method of inhibiting cell division comprising contacting a cell with at least an effective amount of an antibody conjugate of any of claims **54-57**.

62. The method of Claim 60, wherein the inhibition of cell division results in cell death.

63. The method of Claim 60 wherein the cell is a tumor or cancer stem cell, and the antibody binds to a tumor associated antigen or cancer stem cell antigen.

64. The method of Claim **62**, wherein the tumor or cancer stem cells are from a myeloproliferative disorder.

65. The method of Claim 63, wherein the myeloproliferative disorder is selected from the group consisting of: AML, CML, CMML, multiple myeloma, plamacytoma myelofibrosis.

66. The method of Claim **62**, wherein the tumor associated antigen or cancer stem cell antigen is CLL-1.

67. A method of treating cancer comprising administering to a patient a therapeutically effective amount of an antibody conjugate of any of claims **54-57** wherein the

antibody conjugate is capable of binding a tumor associated antigen or cancer stem cell antigen.

68. The method of Claim **66**, wherein the cancer is a myeloproliferative disorder.

69. The method of Claim 67, wherein the myeloproliferative disorder is selected from the group consisting of: AML, CML, CMML, multiple myeloma, plasmacytoma and myelofibrosis.

70. The method of Claim 66, wherein the tumor associated antigen or cancer stem cell antigen is CLL-1.

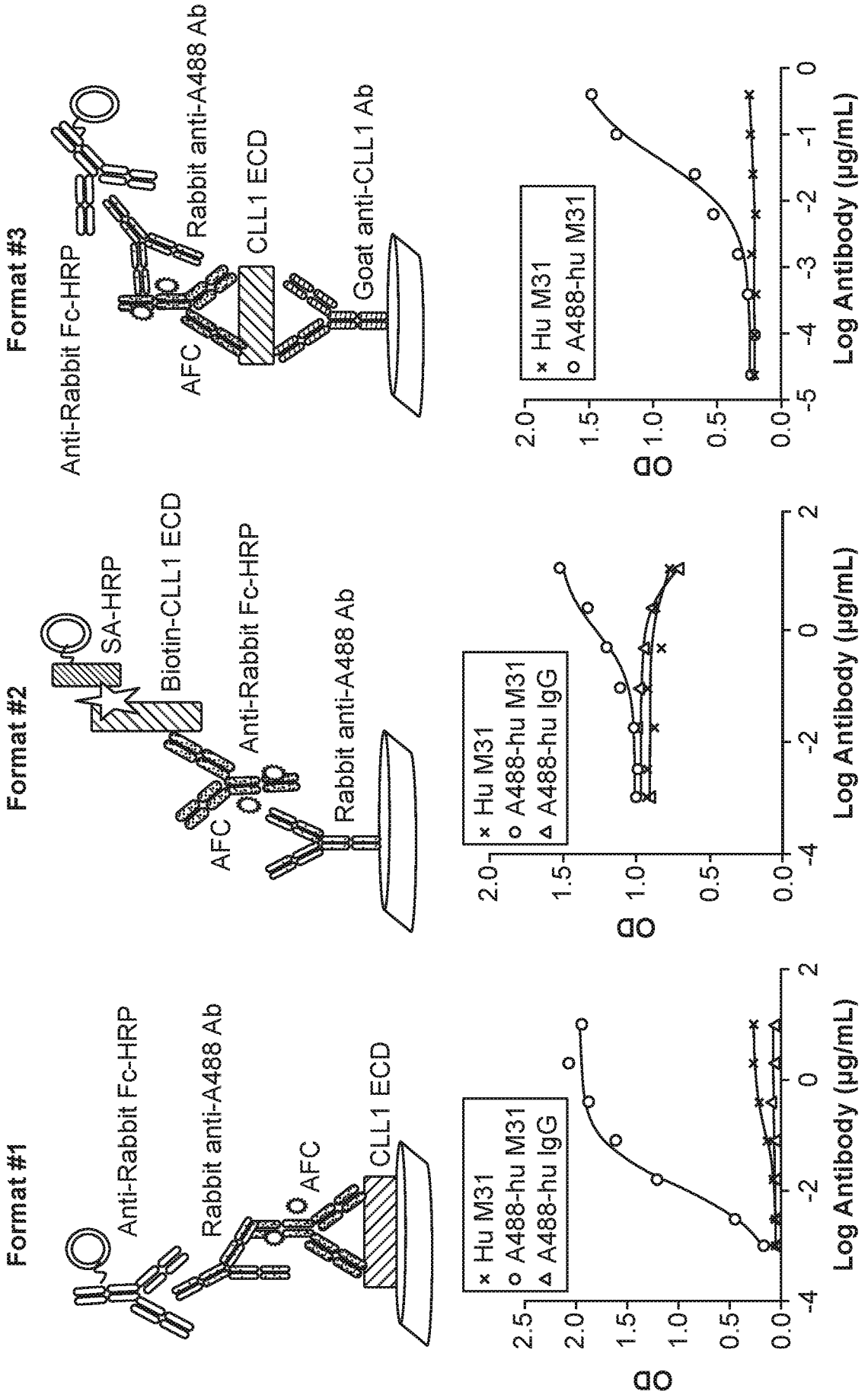

Format #1
Anti-Rabbit Fc-HRP
Rabbit anti-A488 Ab
AFC
CLL1 ECD
Format #2
SA-HRP
Biotin-CLL1 ECD
AFC
Anti-Rabbit Fc-HRP
Rabbit anti-A488 Ab
Format #3
Anti-Rabbit Fc-HRP
Rabbit anti-A488 Ab
AFC
CLL1 ECD
Goat anti-CLL1 Ab
× Hu M31
o A488-hu M31
Δ A488-hu IgG
OD
Log Antibody (µg/mL)

FIG. 1

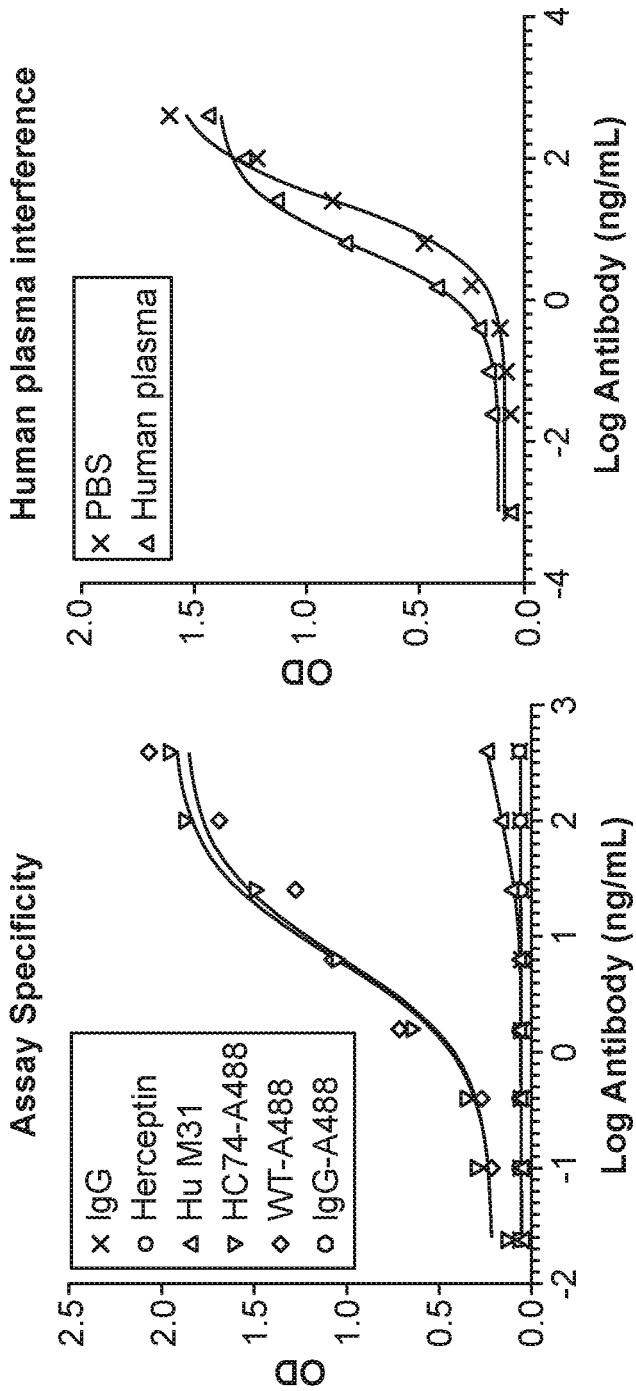

Assay Specificity
Human plasma interference
OD
Log Antibody (ng/mL)
IgG
Herceptin
Hu M31
HC74-A488
WT-A488
IgG-A488
PBS
Human plasma

FIG. 2

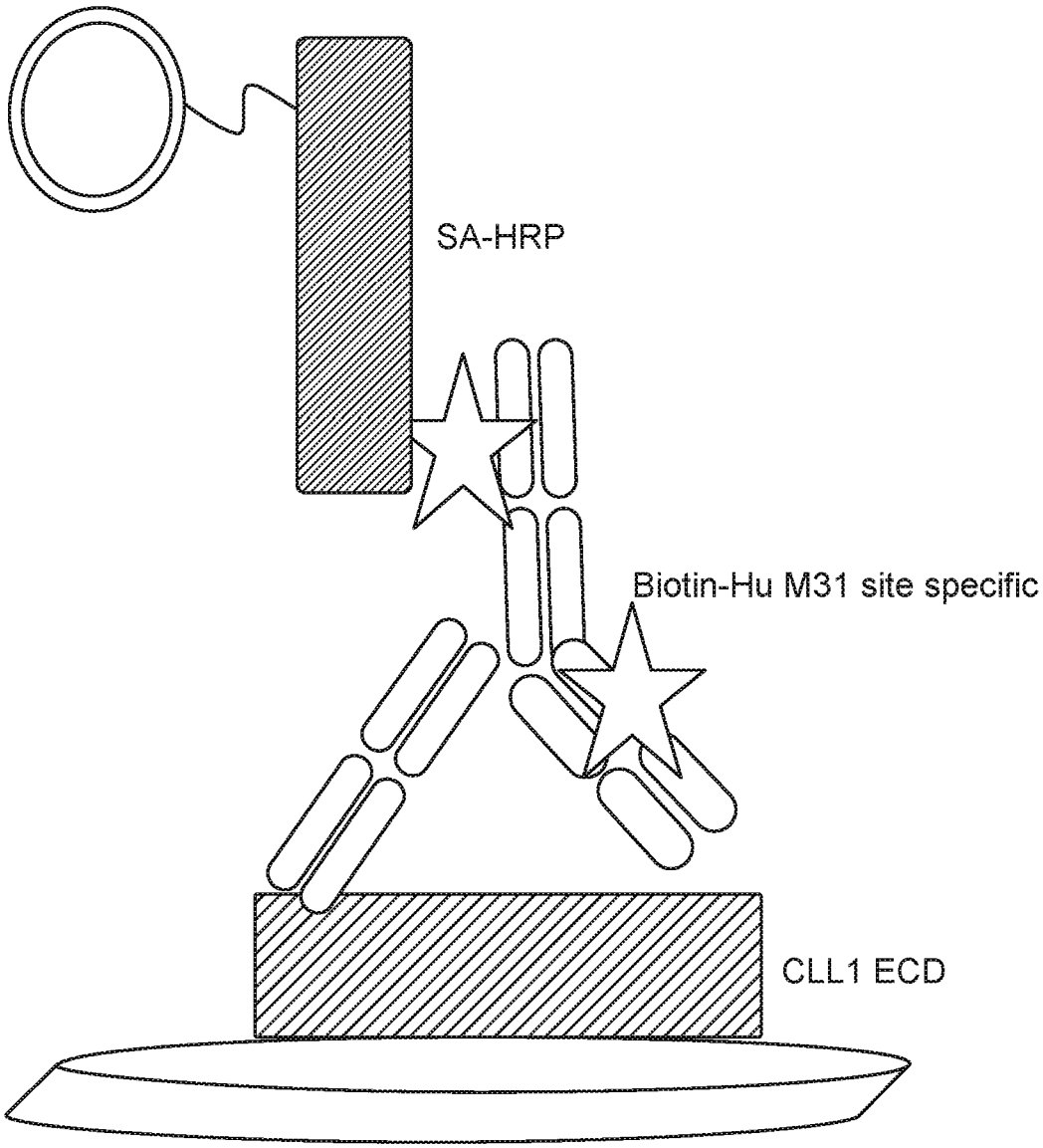


FIG. 3

M31 Ab v. HuM31 Light Chain Sequences

Light Chain variable:

CDR-LC1

CDR-LC2

```
                1       10        20        30        40        50        60
M31         TIVLTQSPAS LAVSLGQRAT ISCRASESVD SYGNSFMHWY QQKPGQPPKL LIYLASNLES
HuM31       DIVMTQSPDS LAVSLGERAT INCRASESVD SYGNSFMHWY QQKPGQPPKL LIYLASNLES

                      70        80        90       100       110
M31         GVPARFSGSG SRTDFTLTID PVEADDAATY YCQQNNYDPW TFGGGTKLEI K
HuM31       GVPDRFSGSG SGTDFTLTIS SLQAEDVAVY YCQQNNYDPW TFGGGTKVEI K
                                                CDR-LC3
```

Light chain constant (kappa)

```
       120       130       140       150       160       170       180
RTVAAPSVF IFPPSDEQLK SGTASVVCLL NNFYPREAKV QWKVDNALQS GNSQESVTEQ DSKDSTYSLS

                190       200       210       218
STLTLSKADY EKHKVYACEV THQGLSSPVT KSFNRGEC
```

Lambda light chain

```
PKANPTVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEK
TVAPTECS
```

FIG. 4A

M31 Ab v. HuM31 Heavy Chain Sequences

```
           Heavy Chain Variable:                                  CDR-HC1
                                   CDR-HC2
      1       10         20         30         40         50         60         70
M31        EVQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY INPYNDGTKY NEKFKGKATL
HuM31      QVQLVQSGAE VKKPGASVKV SCKASGYTFT SYVMHWVRQA PGQRLEWIGY INPYNDGTKY NEKFKGKATI

                      80         90        100        110        120
            M31       TSDTSSSTAY MELNSLTSED SAVYFCARPI YFDNDYFDYW GQGTTLKVSS
            HuM31     TSDTSASTAY MELSSLRSED TAVYYCARPI YFDNDYFDYW GQGTLVTVSS
                                                          CDR-HC3
```

FIG. 4B

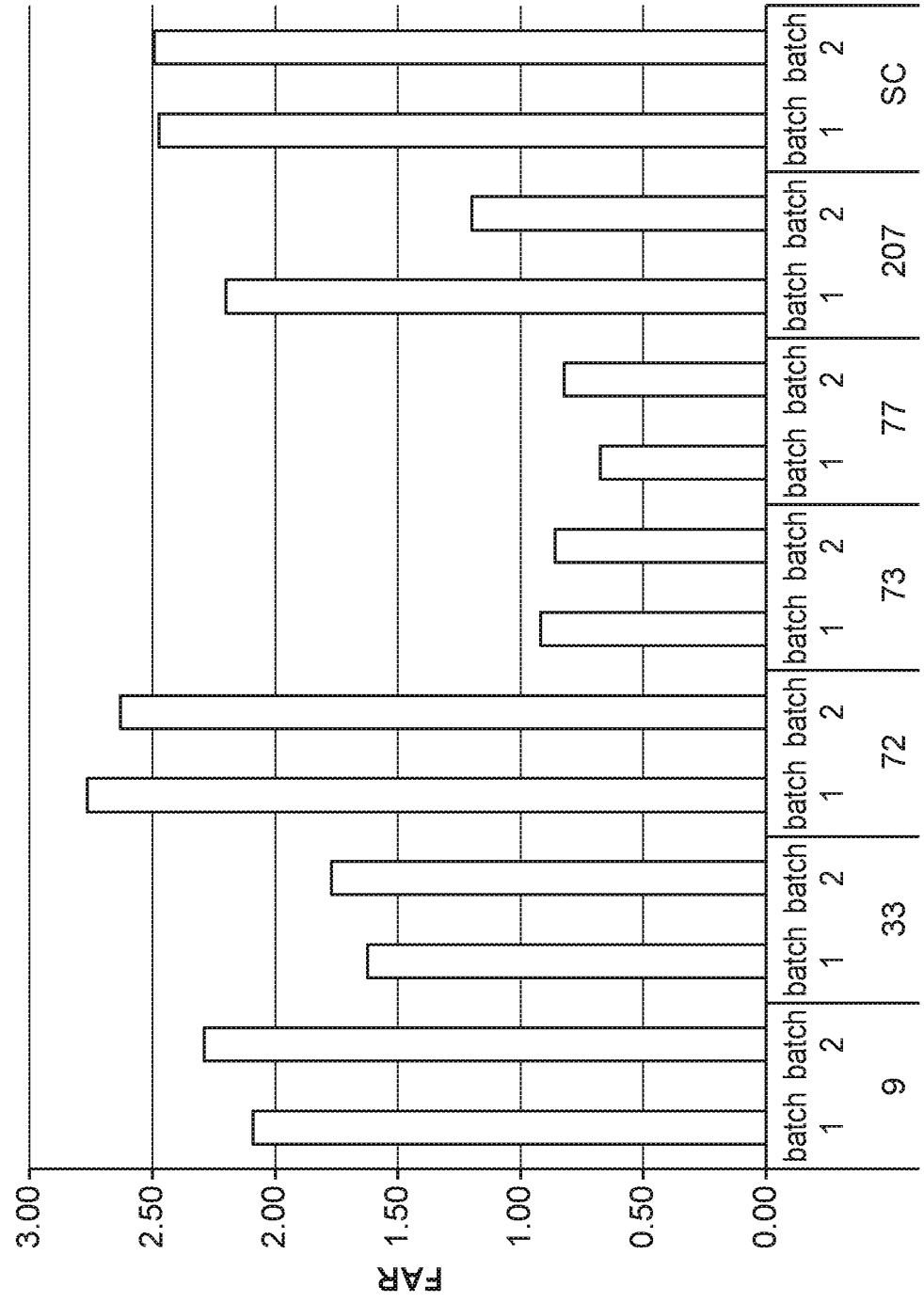

FAR
3.00
2.50
2.00
1.50
1.00
0.50
0.00
batch 1
batch 2
9
batch 1
batch 2
33
batch 1
batch 2
72
batch 1
batch 2
73
batch 1
batch 2
77
batch 1
batch 2
207
batch 1
batch 2
SC

FIG. 5

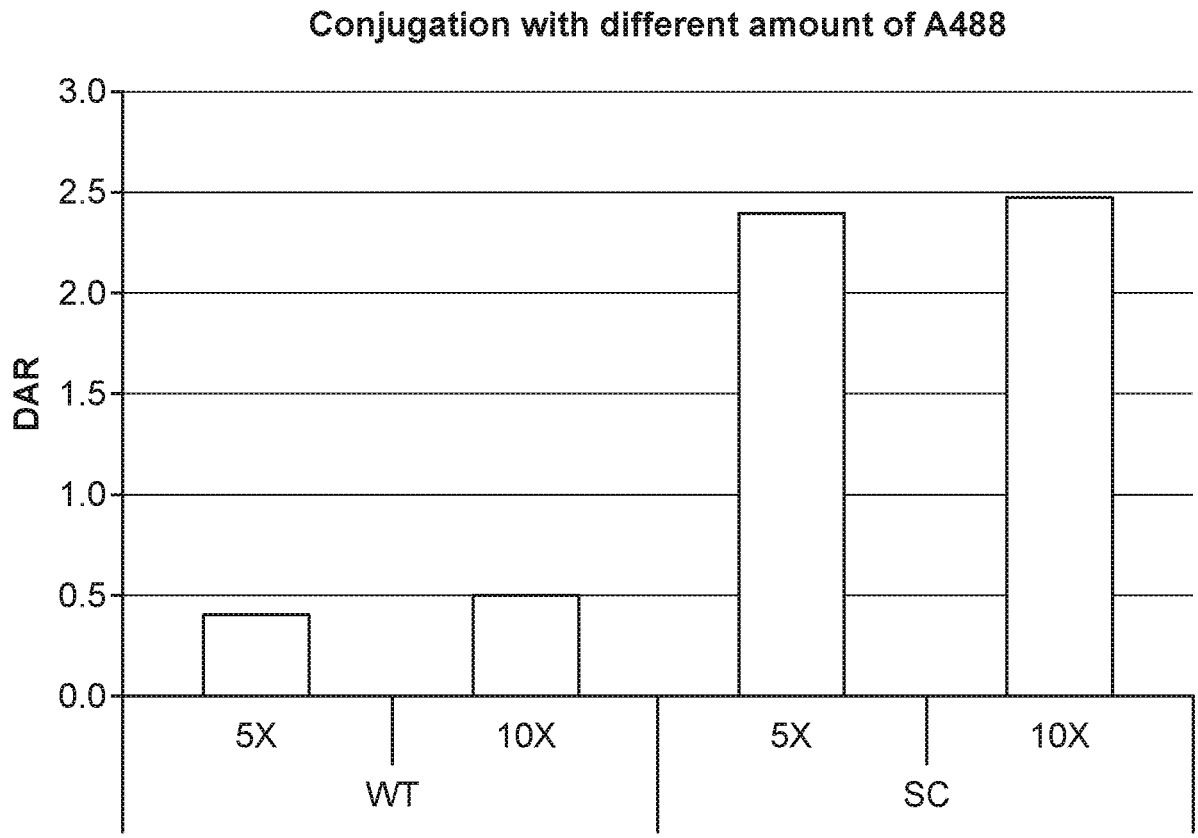

Conjugation with different amount of A488
DAR
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5X
10X
5X
10X
WT
SC

FIG. 6

ID	2	9	11	13	15	18	21	22	23	26	27	28	30	33	34	35	37	65	66	67	72	73	74	76	77	78	79	85	87	89	117	119	139	140	156	158	179	197	199	200	205	207	AC	SC
aa	A	P	V	L	P	P	T	L	M	R	T	P	V	V	V	V	V	E	Q	Y	R	V	V	V	L	T	V	L	G	K	E	P	V	L	G	P	S	S	M	H	N	Y	A	S
site	231	238	241	242	244	247	250	251	252	255	256	257	259	262	263	264	266	294	295	296	301	302	303	305	306	307	308	314	316	317	318	346	368	369	385	387	408	426	428	429	434	436	118	442
FAR	04	23	02	06	2.0	2.3	07	13	16	2.1	2.9	04	02	18	11	2.4	2.9	2.1	3.1	2.0	2.8	09	2.6	2.5	0.8	2.3	02	14	2.1	02	2.2	01	03	03	02	2.1	2.1	01	19	02	04	1.9	2.2	2.4	2.6

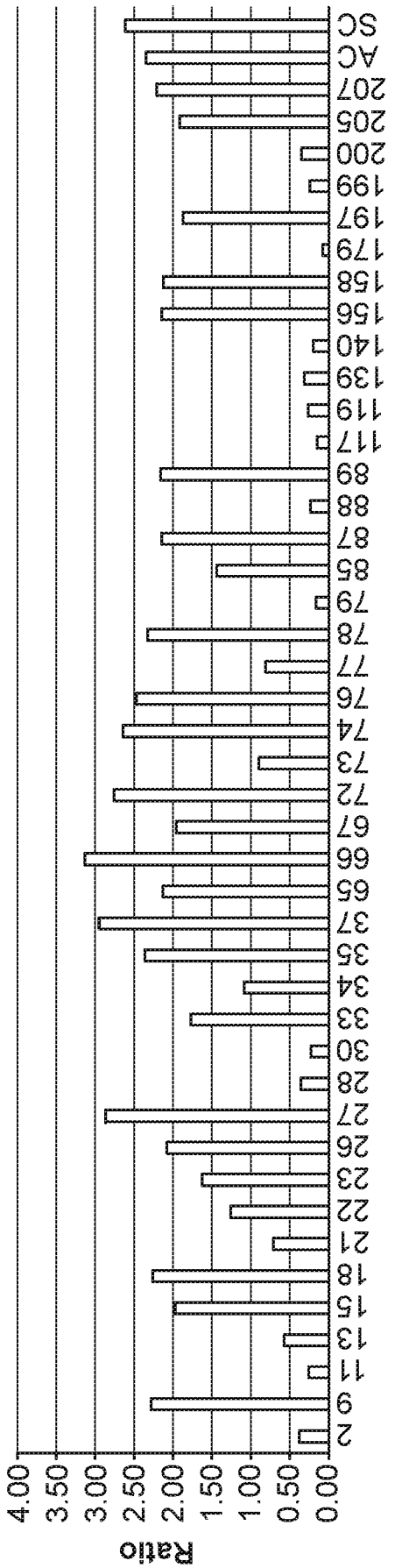


FIG. 7

ID	2	14	16	21	50	56	58	64	73	80	81	82	86	198	206	207	212	AC	SC
aa	A	F	P	T	V	H	A	E	V	L	H	Q	N	V	H	Y	L	A	S
site	231	243	245	250	279	285	287	293	302	309	310	311	315	427	435	436	441	118	442
FAR	0.4	0.3	0.3	0.5	0.5	2.2	2.4	2.1	0.9	2.1	0.6	2.0	2.0	0.3	0.9	1.2	2.1	2.3	2.5

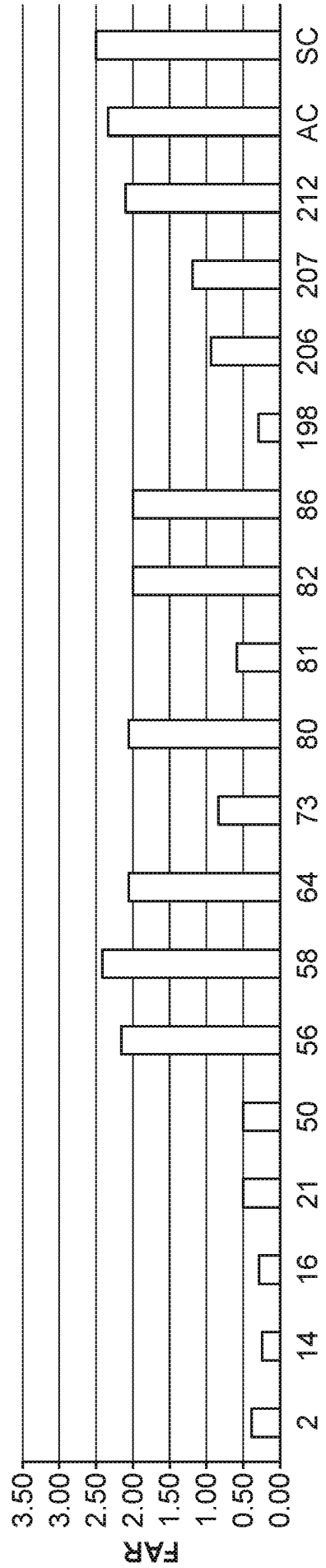
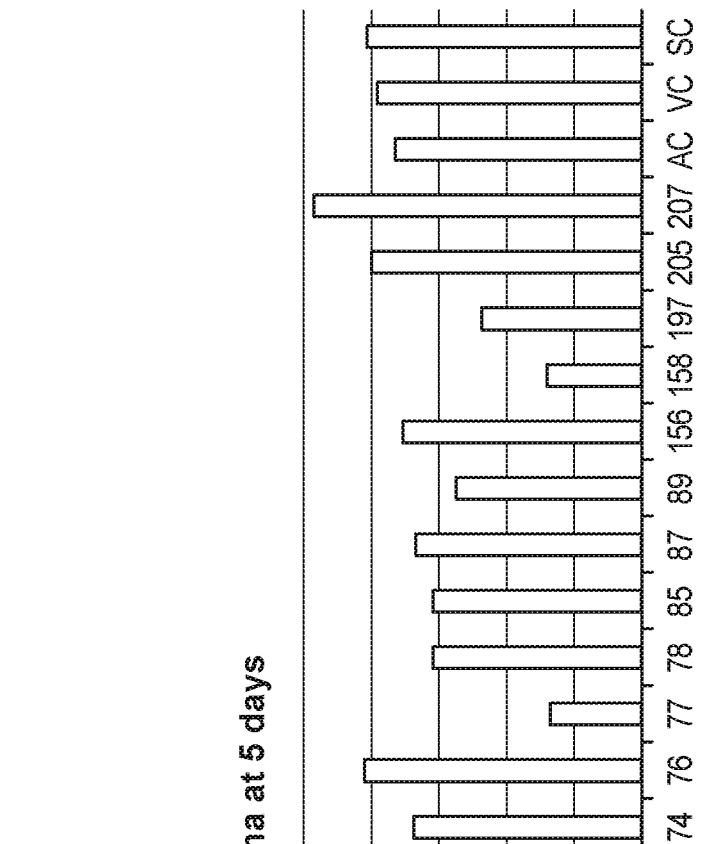
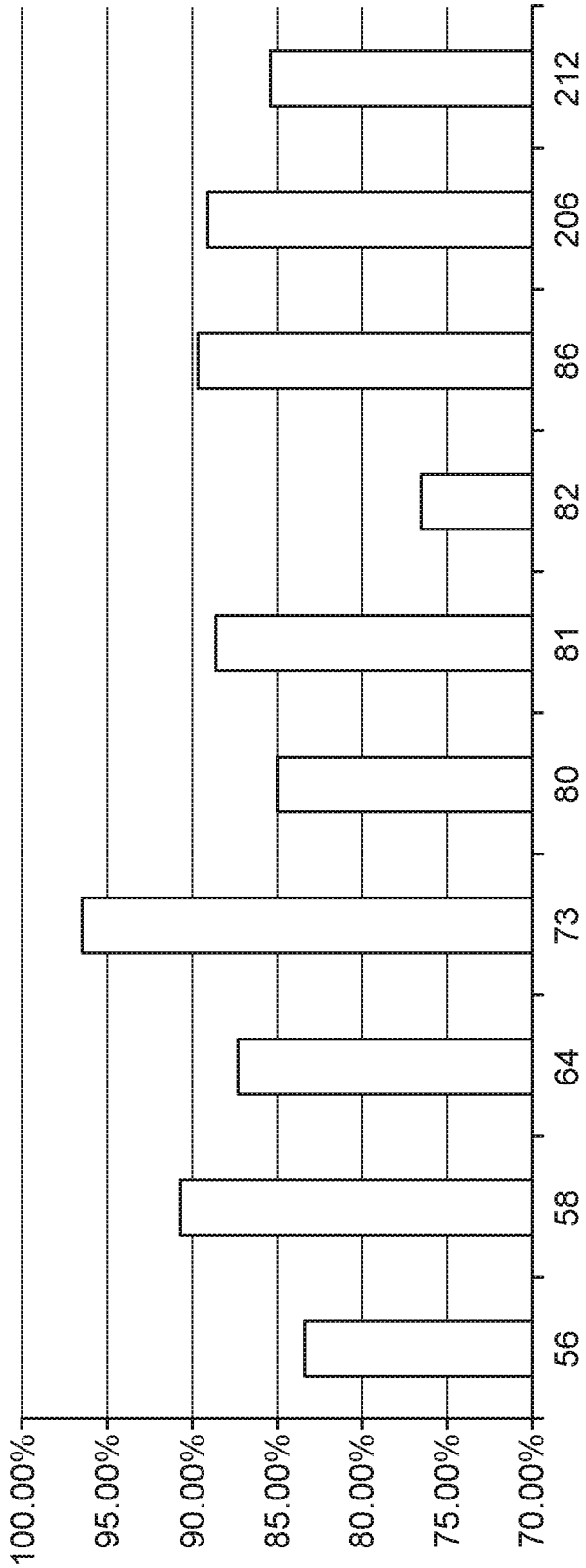


FIG. 8



ID:	9	15	18	22	23	26	27	33	34	35	37	65	66	67	72	73	74	76	77	78	85	87	89	156	158	197	205	207	AC	VC	SC
aa:	P	P	P	L	M	R	T	V	V	V	V	E	Q	Y	R	V	V	V	L	T	L	G	E	G	P	S	N	Y	A	V	S
site:	238	244	247	251	252	255	256	262	263	264	266	294	295	296	301	302	303	305	306	307	314	316	318	385	387	426	434	436	118	205	442
FAR:	22	2.1	2.3	1.3	1.7	2.1	2.9	1.8	1.1	2.4	3.0	2.1	3.1	1.9	2.8	0.9	2.6	2.4	0.9	2.3	1.4	2.1	2.2	2.2	2.1	1.9	1.9	2.2	2.3	2.0	2.5
Stability:	G	G	M	L	L	G	M	G	L	M	G	L	G	L	G	G	G	G	L	G	G	G	M	G	L	M	G	G	G	G	G

FIG. 9



ID	56	58	64	73	80	81	82	86	206	212
aa	H	A	E	V	L	H	Q	N	H	L
site	285	287	293	302	309	310	311	315	435	441
FAR	2.2	2.4	2.1	0.9	2.1	0.6	2.0	2.0	0.9	2.1
Stability	M	G	M	G	M	G	L	M	M	M

FIG. 10

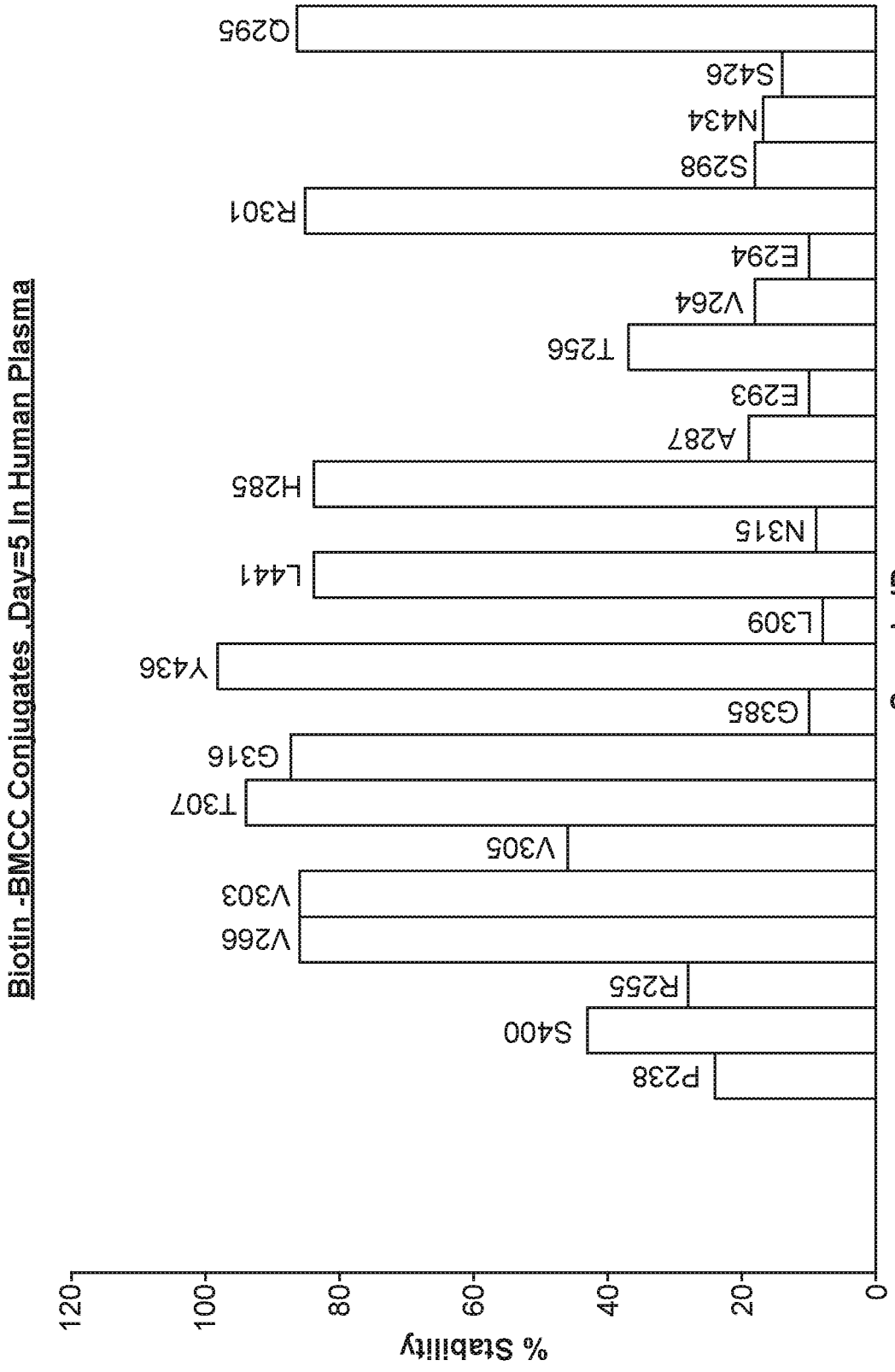

Biotin -BMCC Conjugates ,Day=5 In Human Plasma
% Stability
0
20
40
60
80
100
120
Sample ID
P238
S400
R255
V266
V303
V305
T307
G316
G385
Y436
L309
L441
N315
H285
A287
E293
T256
V264
E294
R301
S298
N434
S426
Q295

FIG. 11

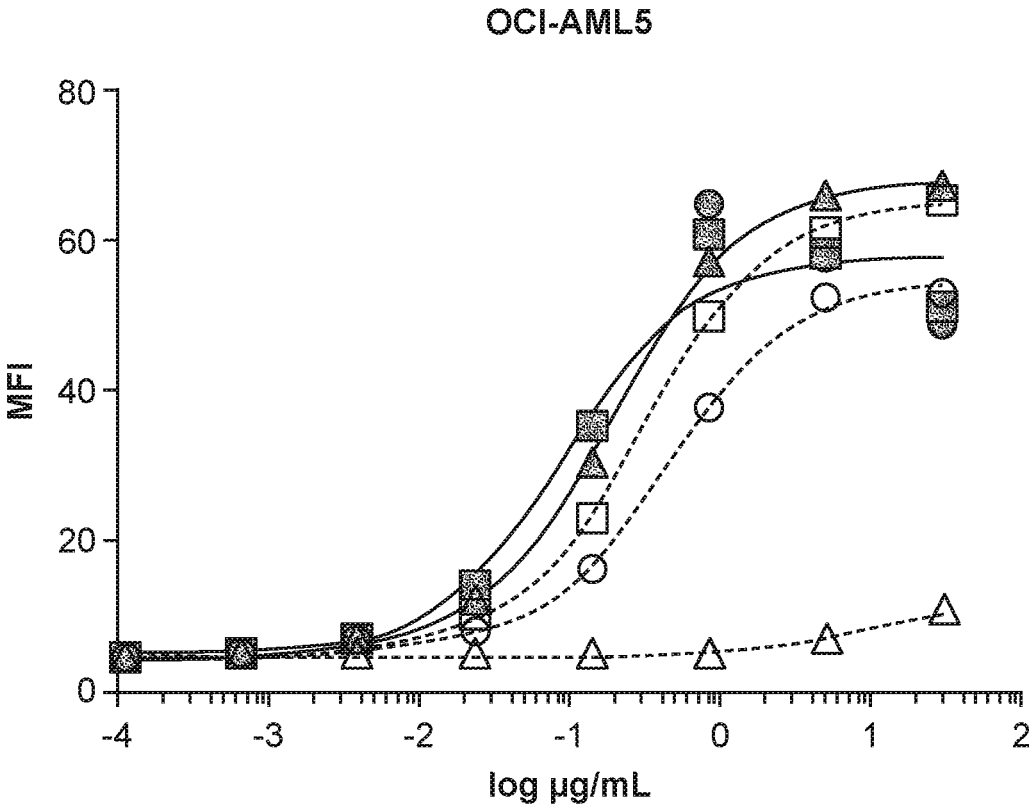


FIG. 12A

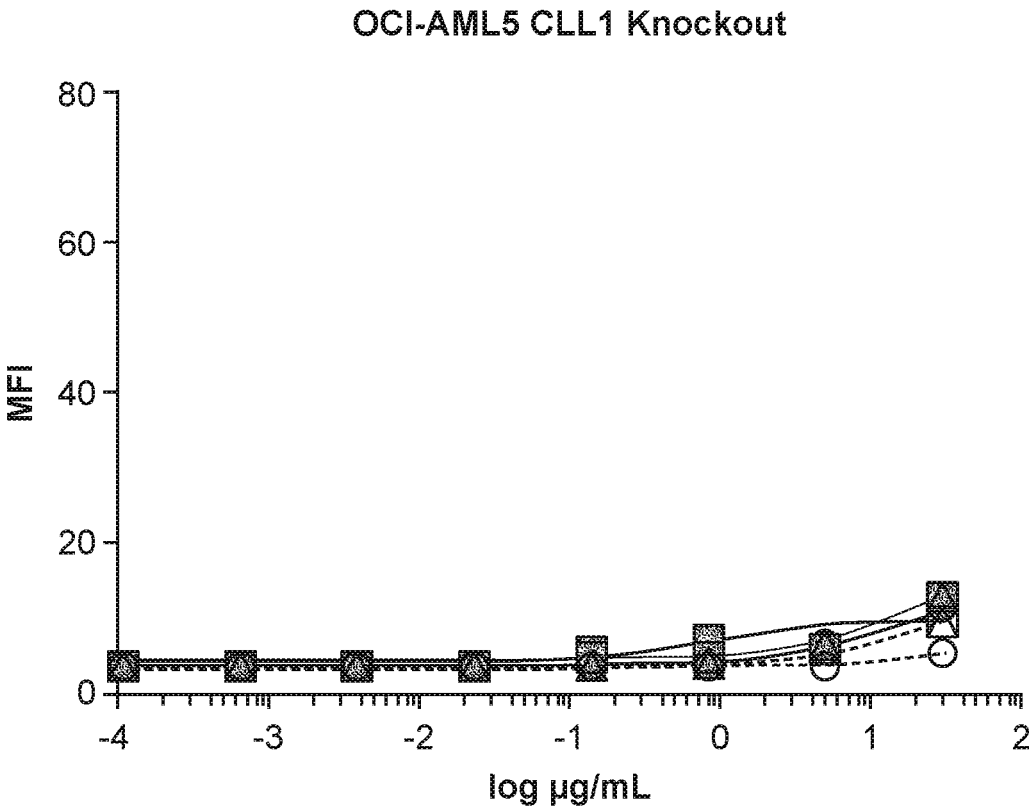


FIG. 12B

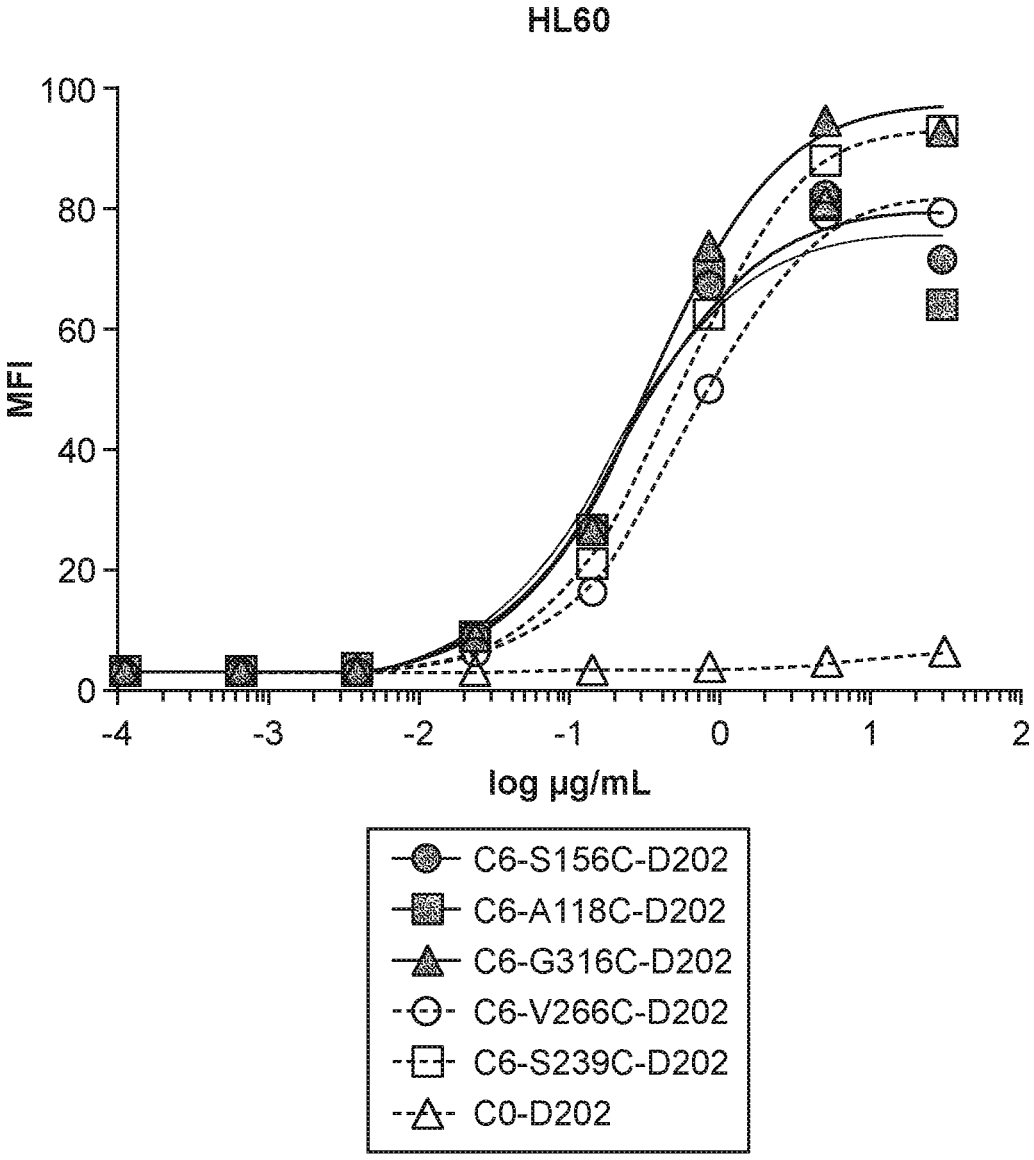

HL60
MFI
100
80
60
40
20
0
-4
-3
-2
-1
0
1
2
log µg/mL
C6-S156C-D202
C6-A118C-D202
C6-G316C-D202
C6-V266C-D202
C6-S239C-D202
C0-D202

FIG. 12C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/042645

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48 A61K51/00 A61P35/00 C07K16/00 C07K16/28 C07K16/30
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/100565 A2 (RES CORP TECHNOLOGIES INC [US]; BRAMHILL DAVID [US]; GEHLSEN KURT R [U) 18 August 2011 (2011-08-18) table 8; sequence 20	1-54
X	WO 2008/070593 A2 (SEATTLE GENETICS INC [US]; MCDONAGH CHARLOTTE [US]; CARTER PAUL [US];) 12 June 2008 (2008-06-12) claims 1-47; tables 1-9	1-54
X	WO 2010/141902 A2 (NOVARTIS AG [CH]; MASSACHUSETTS INST TECHNOLOGY [US]; CHENNAMSETTY NAR) 9 December 2010 (2010-12-09) claims 1-80; examples 1-5	1-54
A	WO 2013/169625 A1 (CELLERANT THERAPEUTICS INC [US]) 14 November 2013 (2013-11-14) table 3	1-54

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 2016	16/12/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cilensek, Zoran

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/042645

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011100565 A2	18-08-2011	AU 2011215684 A1	30-08-2012
		CA 2789328 A1	18-08-2011
		EP 2533811 A2	19-12-2012
		US 2013189247 A1	25-07-2013
		WO 2011100565 A2	18-08-2011
WO 2008070593 A2	12-06-2008	CA 2699837 A1	12-06-2008
		DK 2099823 T3	15-12-2014
		EP 2099823 A2	16-09-2009
		EP 2609932 A2	03-07-2013
		ES 2523915 T3	02-12-2014
		PT 2099823 E	22-12-2014
		SI 2099823 T1	30-01-2015
		US 2010158909 A1	24-06-2010
		US 2014086942 A1	27-03-2014
		US 2014105922 A1	17-04-2014
		WO 2008070593 A2	12-06-2008
WO 2010141902 A2	09-12-2010	AU 2010256410 A1	08-12-2011
		BR PI1013016 A2	29-03-2016
		CA 2763935 A1	09-12-2010
		CN 102458479 A	16-05-2012
		CN 106117348 A	16-11-2016
		EP 2437785 A2	11-04-2012
		EP 2896404 A2	22-07-2015
		ES 2537566 T3	09-06-2015
		JP 5739880 B2	24-06-2015
		JP 2012528601 A	15-11-2012
		KR 20120031267 A	02-04-2012
		MX 338775 B	02-05-2016
		PT 2437785 E	20-04-2015
		RU 2011153327 A	20-07-2013
		US 2012148580 A1	14-06-2012
		US 2015056220 A1	26-02-2015
		WO 2010141902 A2	09-12-2010
WO 2013169625 A1	14-11-2013	AU 2013259850 A1	20-11-2014
		CA 2872513 A1	14-11-2013
		CN 104736562 A	24-06-2015
		EP 2847222 A1	18-03-2015
		JP 2015519336 A	09-07-2015
		KR 20150023355 A	05-03-2015
		US 2013295118 A1	07-11-2013
		US 2015376290 A1	31-12-2015
		WO 2013169625 A1	14-11-2013

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/042645

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/042645

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-54(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-54(partially)

A cysteine-substituted immunoglobulin polypeptide comprising the substituted amino acid residue V266C under EU numbering.

2-8. claims: 1-54(partially)

A cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue selected from the group consisting of G316C, H285C, R301C, V303C, T307C, Y436C and L441C, respectively, under EU numbering.

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9. claims: 55-70

An antibody conjugate comprising a cysteine-substituted immunoglobulin polypeptide comprising a substituted amino acid residue at S156 (under Kabat numbering) in the heavy chain linked via the cysteine to a monomeric or dimeric benzodiazepine derivative, maytansinoid, auristatin, dolastatin, tubulysin, cryptophycin, pyrrolobenzodiazepine (PBD) dimer. indolinobenzodiaze<p>ine dimer, isoquinolidinobenzodiazepine dimer, alpha-amanitin, trichothene, SN-38, duocarmycin, CC1065, calicheamincin, an enediyne antibioatic, taxane, doxorubicin derivatives, anthracycline, azanofide and stereoisomers, isosteres, analogs or derivatives thereof.

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(19)中华人民共和国国家知识产权局

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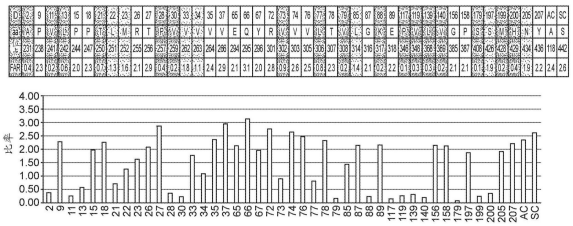
权利要求书4页 说明书34页
序列表7页 附图15页

(54)发明名称

经半胱氨酸取代的免疫球蛋白

(57)摘要

本公开提供了经半胱氨酸取代的免疫球蛋白，包括多肽、抗体、编码此类多肽和抗体的核酸，用于制备其的宿主细胞、载体和方法，抗体的经缀合的衍生物，制备此类抗体和经缀合的衍生物的组合物和方法，及使用抗体和经缀合的变体用于检测和治疗癌症以及用于杀伤患病细胞的方法。在某些实施方案中，该取代选自重链中的V266C、G316C、H285C、R301C、V303C、T307C、Y436C和L441C(EU编号)或S156(根据Kabat编号)。



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1.经半胱氨酸取代的免疫球蛋白多肽,其包含选自以下的经取代的氨基酸残基:根据EU编号的V266C、G316C、H285C、R301C、V303C、T307C、Y436C和L441C。

2.如权利要求1所述的多肽,其中所述免疫球蛋白多肽源自人IgG重链恒定区。

3.如权利要求2所述的多肽,其中所述IgG是选自以下的同种型:IgG1、IgG2、IgG3或IgG4。

4.如权利要求3所述的多肽,其中所述同种型是IgG1。

5.核酸分子,其包含编码半胱氨酸取代物的核苷酸序列。

6.免疫球蛋白多肽,其包含选自以下的经取代的氨基酸残基:根据EU编号的V266C、G316C、H285C、R301C、V303C、T307C、Y436C和L441C。

7.如权利要求5所述的核酸分子,其还包含与所述核苷酸序列可操作地连接的表达控制序列。

8.如权利要求6所述的核酸分子,其包含在表达载体中。

9.重组细胞,其包含含有编码经半胱氨酸取代的免疫球蛋白多肽的核苷酸序列的核酸分子,所述经半胱氨酸取代的免疫球蛋白多肽包含选自以下的经取代的氨基酸残基:根据EU编号的V266C、G316C、H285C、R301C、V303C、T307C、Y436C和L441C。

10.用于制备经半胱氨酸取代的多肽的方法,其包括培养含有核酸分子的重组细胞,所述核酸分子包含编码经半胱氨酸取代的免疫球蛋白多肽的核苷酸序列,所述经半胱氨酸取代的免疫球蛋白多肽包含选自以下的经取代的氨基酸残基:根据EU编号的V266C、G316C、H285C、R301C、V303C、T307C、Y436C和L441C。

11.经半胱氨酸取代的抗体,其包含含有选自以下的经取代的氨基酸残基的经半胱氨酸取代的免疫球蛋白多肽:根据EU编号的重链恒定区中的V266C、G316C、H285C、R301C、V303C、T307C、Y436C和L441C。

12.如权利要求10所述的抗体,其中重链恒定区源自人IgG同种型,所述人IgG同种型选自:IgG1、IgG2、IgG3和IgG4。

13.如权利要求10所述的抗体,其包含选自κ和λ的免疫球蛋白轻链。

14.如权利要求12所述的抗体,其中轻链是κ。

15.如权利要求10所述的抗体,其能结合CLL-1、GPR114、IL1RAP、TIM-3、CD19、CD20、CD22、ROR1、间皮素、CD33、CD123/IL3Ra、c-Met、PSMA、前列腺酸性磷酸酶(PAP)、CEA、CA-125、Muc-1、AFP、糖脂F77、EGFRvIII、GD-2、NY-ESO-1TCR、酪氨酸酶、TRPI/gp75、gp100/pmel-17、Melan-A/MART-1、Her2/neu、WT1、EphA3、端粒酶、HPV E6、HPV E7、EBNA1、BAGE、GAGE和MAGE A3TCRSLITRK6、ENPP3、连接蛋白-4、CD27、SLC44A4、CAIX、Cripto、CD30、MUC16、GPNMB、BCMA、Trop-2、组织因子(TF)、CanAg、EGFR、αv-整联蛋白、CD37、叶酸受体、CD138、CEACAM5、CD56、CD70、CD74、GCC、5T4、CD79b、Steap1、Napi2b、Lewis Y抗原、LIV、c-RET、DLL3、EFNA4或内皮唾酸蛋白/CD248。

16.如权利要求14所述的抗体,其包含可变轻链和可变重链,其中:

(a)所述可变轻链包含CDR-L1、CDR-L2和CDR-L3,此外其中:

CDR-L1是ESVDSYGNSF(SEQ ID NO:1)

CDR-L2是LAS(SEQ ID NO:2)

CDR-L3是QQNNYDPWT(SEQ ID NO:3),以及

(b)所述可变重链包含CDR-H1、CDR-H2和CDR-H3,此外其中:

CDR-H1是GYTFTSYV(SEQ ID NO:4)

CDR-H2是INPYNDGT(SEQ ID NO:5),和

CDR-H3是ARPIYFDNDYFDY(SEQ ID NO:6);或其中:

(c)所述可变轻链还包含CDR-L1、CDR-L2和CDR-L3,此外其中:

CDR-L1是RATQELSGYLS(SEQ ID NO:13)

CDR-L2是AASTLDS(SEQ ID NO:14)

CDR-L3是LQYAIYPYT(SEQ ID NO:15),以及

(d)所述可变重链还包含CDR-H1、CDR-H2和CDR-H3,此外其中:

CDR-H1是GYTFTSYFIH(SEQ ID NO:16)

CDR-H2是FINPYNDGSK(SEQ ID NO:17),和

CDR-H3是DDGYYGYAMDY(SEQ ID NO:18)。

17.核酸分子,其包含编码权利要求10-17所述的抗体的核苷酸序列。

18.如权利要求17所述的核酸分子,其与表达控制序列可操作地连接。

19.如权利要求18所述的核酸分子,其还包含表达载体。

20.重组细胞,其包含权利要求19所述的核酸分子。

21.用于制备抗体的方法,其包括培养权利要求1所述的重组细胞。

22.如权利要求1所述的方法,其还包括分离所述抗体。

23.抗体缀合物,其包含权利要求1-4或10-15中任一项所述的抗体,所述抗体由所述抗体中经取代的氨基酸残基(半胱氨酸)通过接头缀合至选自以下的部分:药物、放射性核苷酸、荧光团、生物素、RNA、抗生素、蛋白和可检测部分。

24.如权利要求1所述的抗体缀合物,其缀合至药物。

25.如权利要求1所述的抗体缀合物,其中所述药物选自:单体的或二聚体的苯二氮䓬衍生物、类美登素、阿里他汀、多拉司他汀、小管素、念珠藻素、吡咯并苯二氮䓬(PBD)二聚体、吲哚啉并苯二氮䓬二聚体、异喹啉烷并苯二氮䓬二聚体、α-鹅膏蕈碱、单端胞菌毒素、SN-38、倍癌霉素、CC1065、加利车霉素、烯二炔抗生素、紫杉烷、阿霉素衍生物、蒽环霉素、azanofide及它们的立体异构体、等排体、类似物或衍生物。

26.如权利要求1所述的抗体缀合物,其中所述药物是异喹啉烷并苯二氮䓬二聚体。

27.如权利要求22-25中任一项所述的抗体缀合物,其中所述接头共价键合至所述药物。

28.如权利要求22-25中任一项所述的抗体缀合物,其中所述接头通过硫醇与选自马来酰亚胺、卤化物和磺酰基的硫醇反应性基团反应附接至所述抗体。

29.如权利要求23-25中任一项所述的抗体缀合物,其中所述接头经由二硫键连接至所述药物。

30.如权利要求28所述的抗体缀合物,其中所述二硫键是吡啶基二硫化物部分。

31.如权利要求22-25中任一项所述的抗体缀合物,其中所述接头在靶标的微环境中是可切割的。

32.如权利要求22所述的抗体缀合物,其缀合至可检测部分。

33.如权利要求31所述的抗体缀合物,其中所述可检测部分选自:A488、BMCC-生物素和HPDP-生物素。

34.组合物,其包含权利要求10-32中任一项所述的抗体或经缀合的抗体以及佐剂。

35.如权利要求1所述的组合物,其是药学上可接受的。

36.检测目标细胞的方法,其包括:

(a)使细胞与有效量的能够结合所述细胞的权利要求1-4或10-15中任一项所述的抗体接触,和

(b)检测所述抗体与所述细胞的结合,

其中所述结合指示所述目标细胞。

37.如权利要求1所述的方法,其中所述目标细胞表达CLL-1。

38.如权利要求1所述的方法,其中所述抗体缀合至可检测部分。

39.诊断疾病的方法,其包括:

(a)使来自个体的生物样品与有效量的能够结合患病细胞的权利要求1-4或10-15中任一项所述的抗体接触;和

(b)检测所述抗体与疾病细胞的结合,

其中结合指示存在所述疾病。

40.如权利要求1所述的方法,其中所述抗体缀合至可检测部分。

41.如权利要求1所述的方法,其中所述疾病是癌症并且所述抗体能结合肿瘤相关的抗原或癌症干细胞相关的抗原。

42.如权利要求1所述的方法,其中所述肿瘤相关的抗原或癌症干细胞相关的抗原是CLL-1。

43.如权利要求1所述的方法,其中所述疾病是骨髓增生性病症。

44.如权利要求42所述的方法,其中所述骨髓增生性病症选自:AML、CML、CMML、多发性骨髓瘤、浆细胞瘤和骨髓纤维化。

45.抑制细胞分裂的方法,其包括使细胞与至少有效量的能够结合所述细胞且缀合至对所述细胞有细胞毒性的药物的权利要求22-32中任一项所述的抗体缀合物接触。

46.如权利要求1所述的方法,其中细胞分裂的抑制导致细胞死亡。

47.如权利要求1所述的方法,其中所述细胞是肿瘤或癌症干细胞,并且所述抗体能结合肿瘤相关的抗原或癌症干细胞抗原。

48.如权利要求1所述的方法,其中所述肿瘤或癌症干细胞来自骨髓增生性病症。

49.如权利要求1所述的方法,其中所述骨髓增生性病症选自:AML、CML、CMML、多发性骨髓瘤、浆细胞瘤和骨髓纤维化。

50.如权利要求1所述的方法,其中所述肿瘤相关抗原或癌症干细胞抗原是CLL-1。

51.治疗癌症的方法,其包括向患者施用治疗有效量的权利要求22-32中任一项所述的抗体缀合物,其中所述抗体缀合物能够结合肿瘤相关的抗原或癌症干细胞抗原。

52.如权利要求1所述的方法,其中所述癌症是骨髓增生性病症。

53.如权利要求1所述的方法,其中所述骨髓增生性病症选自:AML、CML、CMML、多发性骨髓瘤、浆细胞瘤和骨髓纤维化。

54.如权利要求1所述的方法,其中所述肿瘤相关的抗原或癌症干细胞抗原是CLL-1。

55.抗体缀合物，其包含经半胱氨酸取代的免疫球蛋白多肽，所述经半胱氨酸取代的免疫球蛋白多肽包含在重链中S156(根据Kabat编号)处的经取代的氨基酸残基并经由所述半胱氨酸连接至：单体的或二聚体的苯二氮䓬衍生物、类美登素、阿里他汀、多拉司他汀、小管素、念珠藻素、吡咯并苯二氮䓬(PBD)二聚体、吲哚啉并苯二氮䓬二聚体、异喹啉烷并苯二氮䓬二聚体、α-鹅膏蕈碱、单端胞菌毒素、SN-38、倍癌霉素、CC1065、加利车霉素、烯二炔抗生素、紫杉烷、阿霉素衍生物、蒽环霉素、azanofide及它们的立体异构体、等排体、类似物或衍生物。

56.如权利要求54所述的抗体缀合物，其中所述吲哚啉并苯二氮䓬二聚体或异喹啉烷并苯二氮䓬二聚体通过接头附接至所述抗体并且所述接头经由二硫键连接至所述药物。

57.如权利要求54所述的抗体缀合物，其中所述二硫键是吡啶基二硫化物部分。

58.如权利要求54所述的抗体缀合物，其中所述接头在所述靶标的微环境中是可切割的。

59.组合物，其包含权利要求54-57中任一项所述的抗体或经缀合的抗体以及佐剂。

60.如权利要求58所述的组合物，其是药学上可接受的。

61.抑制细胞分裂的方法，其包括使细胞与至少有效量的权利要求54-57中任一项所述的抗体缀合物接触。

62.如权利要求60所述的方法，其中细胞分裂的抑制导致细胞死亡。

63.如权利要求60所述的方法，其中所述细胞是肿瘤或癌症干细胞，并且所述抗体能结合肿瘤相关的抗原或癌症干细胞抗原。

64.如权利要求62所述的方法，其中所述肿瘤或癌症干细胞来自骨髓增生性病症。

65.如权利要求63所述的方法，其中所述骨髓增生性病症选自：AML、CML、CMML、多发性骨髓瘤、浆细胞瘤和骨髓纤维化。

66.如权利要求62所述的方法，其中所述肿瘤相关的抗原或癌症干细胞抗原是CLL-1。

67.治疗癌症的方法，其包括向患者施用治疗有效量的权利要求54-57中任一项所述的抗体缀合物，其中所述抗体缀合物能够结合肿瘤相关的抗原或癌症干细胞抗原。

68.如权利要求66所述的方法，其中所述癌症是骨髓增生性病症。

69.如权利要求67所述的方法，其中所述骨髓增生性病症选自：AML、CML、CMML、多发性骨髓瘤、浆细胞瘤和骨髓纤维化。

70.如权利要求66所述的方法，其中所述肿瘤相关的抗原或癌症干细胞抗原是CLL-1。

经半胱氨酸取代的免疫球蛋白

[0001] 相关申请的交叉引用

[0002] 本专利申请要求2015年7月16日提交的美国临时专利申请第62/193,531号的优先权权益,其出于所有目的通过引用并入本文。

[0003] 以文本文件提交的序列表的引用

[0004] 序列表写于2016年7月15日创建的文件1014170_ST25.txt,其为10,366字节,机器形式IBM-PC,MS-Windows操作系统,出于所有目的通过引用以其整体并入本文。

背景技术

[0005] 单克隆抗体(mAb)由于它们对靶抗原的高特异性和亲和力而在研究和疗法中是必不可少的工具。自二十世纪九十年代以来,治疗性mAb已经对包括炎性病症和癌症的各种疾病的医疗保健产生重大影响。mAb的一个关键特征是其以高度特异性的方式结合靶抗原,将其标记以通过宿主免疫清除法(诸如补体依赖性细胞毒性(CDC)或抗体依赖性细胞介导的细胞毒性(ADCC))去除的能力。还可通过结合靶抗原并抑制其功能来赋予抗体治疗性益处,如在曲妥珠单抗(Herceptin(赫赛汀)®)、贝伐珠单抗(Avastin®)和西妥昔单抗(Erbitux®)的情况下。

[0006] CLL-1是一种主要在血液恶性肿瘤,诸如白血病(如急性髓性白血病(AML))中存在的髓性细胞中表达的细胞表面糖蛋白。目前可用的血液恶性肿瘤疗法有不良且往往是严重的副作用。例如,由MYLOTARG®施用引起的并发症包括肝毒性、静脉闭塞性疾病、严重骨髓抑制(在~98%患者中)、肿瘤溶解综合征、免疫超敏性综合征及呼吸病症。因此,需要鉴定有效且副作用降低的新型血液恶性肿瘤疗法。由于CLL-1在髓性细胞上选择性表达,识别并结合CLL-1的组合物可用于此类血液恶性肿瘤,尤其是骨髓来源的血液恶性肿瘤的疗法。

[0007] 与细胞毒性药物或放射性核素缀合可以扩大mAb的效用并改善其效力和有效性。这是因为抗体特异性地靶向并递送细胞毒性有效载荷至患病组织而实现的。抗体已通过多种接头化合物缀合至多种细胞毒性药物,且这些抗体药物缀合物(ADC)具有选择性且有效地杀灭抗原-表达性肿瘤细胞的能力。ADC经证实已在临床上取得成功,且有两种此类药物,ado-曲妥珠单抗美坦新(ado-trastuzumab emtansine)(Kadcyla®)和布仑妥昔单抗维汀(brentuximab vendotin)(Adcetris®)可商购获得。

[0008] ADC的成功开发取决于对抗体选择性、接头稳定性、细胞毒性药物效力以及接头-药物缀合至抗体的模式的优化。

[0009] 在常规ADC中,药物缀合产生异质性产物,其含有具有不同药物与抗体摩尔比的种类的混合物。抗体的缀合位点存在于溶剂可及的反应性氨基酸残基诸如赖氨酸或半胱氨酸处。异质性以两个水平存在,因为每个ADC种类在药物载量和缀合位点上都不同。Panowski等人,mAbs6:1,31-45(2014)。因此,每个种类可能具有不同的性质,导致广泛的体内药代动力学(PK)性质以及批次间变异。此外,可变的药物与抗体比(DAR)导致高药物载量、高疏水性、快速清除、较低的耐受性和狭窄的治疗窗口。Junutula等人,Nat.Biotech.26(8),925-

932(2008)。

[0010] 位点-特异性缀合(其中已知数量的接头-药物始终缀合至确定的位点)是克服这些挑战的一种方式。异质性被最小化,并且ADC性质更具可预测性,产生批次间一致的缀合物。

[0011] 氨基酸半胱氨酸提供反应性硫醇基团。该基团长久以来一直被用作定位以标记蛋白以及用于产生ADC。虽然半胱氨酸可以工程化成蛋白,但这种方法并非没有挑战。例如,经工程化的游离半胱氨酸可与其它分子上的半胱氨酸共轭以形成蛋白-二聚体。它也可以与天然半胱氨酸残基分子内配对以产生不适当的折叠而损害或抑制蛋白功能。因此,成功使用引入的半胱氨酸残基作为位点-特异性缀合是依赖于选择合适位点的能力,其中引入半胱氨酸的取代不改变抗体结构或功能。Junutula等人,Nat.Biotech.30(2):184-191(2012)。

[0012] 另一个复杂之处是取代位点处的溶剂可及性和电荷对于ADC稳定性是重要的。在经半胱氨酸工程化的抗-Her2/neu马来酰亚胺接头ADC的稳定性研究中,高溶剂可及性由于马来酰亚胺与白蛋白中的反应性硫醇、游离半胱氨酸或谷胱甘肽的交换而丧失血浆中的缀合的硫醇-反应性接头。Shen等人,Nat.Biotech.30(2):184-191(2012)。因此,仍然有很大需求来鉴定具有一致的药物载量、低疏水性、缓慢清除、高耐受性和较好的治疗指数的稳定的位点特异性ADC。此外,甚至还更需要创造靶向CLL-1的稳定的位点特异性ADC。

[0013] 本背景中的陈述既不是对现有技术的承认,也不是对所引用参考文献的认可。

[0014] 发明概述

[0015] 本公开提供了经半胱氨酸取代的免疫球蛋白,其包括多肽、抗体、编码此类多肽和抗体的核酸,用于制备其的宿主细胞、载体和方法,抗体的经缀合的衍生物,制备此类抗体和经缀合的衍生物的组合物和方法,以及使用抗体和经缀合的变体检测和治疗癌症以及杀灭患病细胞的方法。

[0016] 在一个实施方案中,本公开提供了经半胱氨酸取代的免疫球蛋白多肽,其中经取代的残基选自如下的一种或多种残基:V266C、H285C、R301C、V303C、T307C、G316C、Y436C和L441C(EU编号)。在一方面,免疫球蛋白多肽源自人IgG重链恒定区。在另一方面,IgG是同种型IgG1、IgG2、IgG3或IgG4。

[0017] 在另一个实施方案中,本公开提供了编码经半胱氨酸取代的免疫球蛋白多肽的分离的核酸序列,其中经取代的残基选自如下的一种或多种残基:V266C、H285C、R301C、V303C、T307C、G316C、Y436C和L441C(EU编号)。在一方面,核酸与表达控制序列可操作地连接。在另一方面,可操作地连接的核酸进一步包含在表达载体中。在另一方面,本公开提供了包含表达载体的宿主细胞。

[0018] 在另一个实施方案中,本公开提供了用于制备经半胱氨酸取代的免疫球蛋白多肽的方法,其包括培养包含核酸分子(还包括编码经半胱氨酸取代的免疫球蛋白多肽的核苷酸序列)的重组细胞,其中经取代的残基选自如下的一种或多种残基:V266C、H285C、R301C、V303C、T307C、G316C、Y436C和L441C(EU编号)。

[0019] 在另一个实施方案中,本公开提供了经半胱氨酸取代的抗体,所述抗体包含经半胱氨酸取代的免疫球蛋白多肽,所述多肽进一步包含选自如下的经取代的氨基酸残基:重链恒定区中的V266C、H285C、R301C、V303C、T307C、G316C、Y436C和L441C(EU编号)。在一方

面，重链恒定区源自人IgG同种型，所述人IgG同种型选自IgG1、IgG2、IgG3和IgG4。

[0020] 在另一方面，抗体还包含轻链。在另一方面，轻链选自κ和λ。

[0021] 在另一方面，抗体能结合CLL-1、GPR114、IL1RAP、TIM-3、CD19、CD20、CD22、ROR1、间皮素、CD33、CD123/IL3Ra、c-Met、PSMA、前列腺酸性磷酸酶(PAP)、CEA、CA-125、Muc-1、AFP、糖脂F77、EGFRvIII、GD-2、NY-ESO-1 TCR、酪氨酸酶、TRPI/gp75、gp100/pmel-17、Melan-A/MART-1、Her2/neu、WT1、EphA3、端粒酶、HPV E6、HPV E7、EBNA1、BAGE、GAGE和MAGE A3TCRSLITRK6、ENPP3、连接蛋白-4、CD27、SLC44A4、CAIX、Cripto、CD30、MUC16、GPNMB、BCMA、Trop-2、组织因子(TF)、CanAg、EGFR、αv-整联蛋白、CD37、叶酸受体、CD138、CEACAM5、CD56、CD70、CD74、GCC、5T4、CD79b、Steap1、Napi2b、Lewis Y抗原、LIV、c-RET、DLL3、EFNA4或内皮唾酸蛋白/CD248。在另一方面，抗体能结合CLL-1且包含可变轻链和可变重链，其中：

[0022] (a)可变轻链进一步包含CDR-L1、CDR-L2和CDR-L3，其中：

[0023] a.CDR-L1是ESVDSYGNSF(SEQ ID NO:1)

[0024] b.CDR-L2是LAS(SEQ ID NO:2)

[0025] c.CDR-L3是QQNNYDPWT(SEQ ID NO:3)，以及

[0026] (b)可变重链还包含CDR-H1、CDR-H2和CDR-H3，其中：

[0027] a.CDR-H1是GYTFTSYV(SEQ ID NO:4)

[0028] b.CDR-H2是INPYNDGT(SEQ ID NO:5)，和

[0029] c.CDR-H3是ARPIYFDNDYFDY(SEQ ID NO:6)。

[0030] 在另一方面，抗体能结合CLL-1且包含可变轻链和可变重链，其中：

[0031] (c)可变轻链进一步包含CDR-L1、CDR-L2和CDR-L3，其中：

[0032] a.CDR-L1是RATQELSGYLS(SEQ ID NO:13)

[0033] b.CDR-L2是AASTLDS(SEQ ID NO:14)

[0034] c.CDR-L3是LQYAIYPYT(SEQ ID NO:15)，以及

[0035] (d)可变重链进一步包含CDR-H1、CDR-H2和CDR-H3，其中：

[0036] a.CDR-H1是GYTFTSYFIH(SEQ ID NO:16)

[0037] b.CDR-H2是FINPYNDGSK(SEQ ID NO:17)，和

[0038] c.CDR-H3是DDGYYGYAMDY(SEQ ID NO:18)

[0039] 在一些实施方案中，抗CLL-1抗体包含轻链可变区序列，其包含DIQMTQSPSSLSASVGDRVTLTCRATQELSGYLSWLQQKPGKAIKRLIYAASTLDSGVPSRFSGNRAGTDYTLTISSLQPEDFATYYCLQYAIYPYTFGQGTKLEIK(SEQ ID NO:19)，重链可变区序列，其包含EVQLVQSGAEVKKPGASVKMSCKASGYTFTSYFIHWVRQAPGQGLEWIGFINPYNDGSKYAQKFQGRATLTSDKSTSTVYMELSSLRSEDTAVYYCTRDDGYYGYAMDYWGQGTLVTVSS(SEQ ID NO:20)或上述轻链和重链序列两者。

[0040] 在另一方面，本公开提供了编码经半胱氨酸取代的抗体的经分离的核酸序列。在一方面，核酸与表达控制序列可操作地连接。在另一方面，可操作地连接的核酸还包含表达载体。在另一方面，本公开提供了包含表达载体的宿主细胞，及包括培养此类细胞的制备抗体的方法。在另一方面，本公开提供了分离抗体。

[0041] 在另一个实施方案中，本公开提供了经半胱氨酸取代的抗体，其中将取代的半胱氨酸通过接头连接至缀合的部分。在一方面，缀合的部分选自：药物、放射性核苷酸、荧光

团、生物素、RNA、抗生素、蛋白和可检测的部分。

[0042] 在另一方面，缀合的部分是药物、生物素(BMCC或HPDP)或荧光团(Alexa488)。在另一方面，药物选自：苯二氮䓬衍生物(包括但不限于吡咯并苯二氮䓬、吲哚啉并苯二氮䓬或异喹啉烷并苯二氮䓬)，其可呈单体或二聚体形式(如异二聚体或同二聚体，诸如吡咯并苯二氮䓬(PBD)二聚体、吲哚啉并苯二氮䓬二聚体、异喹啉烷并苯二氮䓬二聚体(包括但不限于如下所述的D202)、多拉司他汀(dolastatin)、阿里他汀(auristatin)、类美登素(maytansinoid)、小管素(tubulysin)、念珠藻素(cryptophycin)、α-鹅膏蕈碱(alpha-amanitin)、单端胞菌毒素(trichothene)、SN-38、倍癌霉素(duocarmycin)、CC1065、加利车霉素(calicheamincin)、烯二炔抗生素、紫杉烷、阿霉素衍生物、蒽环霉素及立体异构体、azanofide以及它们的等排体、类似物或衍生物。

[0043] 在另一方面，将接头共价键合至药物。在另一方面，将接头通过硫醇和硫醇反应性基团(如马来酰亚胺、卤化物和磺酰基)之间的反应附接至药物。在另一方面，将接头经由二硫键连接至药物。在另一方面，二硫键是吡啶基二硫化物部分。在另一方面，接头在靶标的微环境中是可切割的。

[0044] 在另一方面，缀合的部分是可检测的部分。在另一方面，可检测的部分是荧光团诸如A488或生物素(如BMCC-生物素或HPDP-生物素)。

[0045] 在另一个实施方案中，本公开提供了包含经半胱氨酸取代的抗体和佐剂的组合物。在一方面，佐剂是药学上可接受的载剂或稀释剂。

[0046] 在另一个实施方案中，本公开提供了检测目标细胞的存在的方法，其包括使细胞接触能够结合细胞的至少有效量的经半胱氨酸取代的抗体，以及检测抗体与细胞的结合，其中所述结合指示目标细胞。在一方面，目标细胞是表达CLL-1的细胞。在另一方面，将经半胱氨酸取代的抗体缀合至可检测的部分。

[0047] 在另一个实施方案中，本公开提供了诊断疾病的方法，其包括：(i)使来自个体的生物样品与能够结合患病细胞的至少有效量的经半胱氨酸取代的抗体接触，以及(ii)检测抗体与患病细胞的结合，其中结合指示疾病的存在。在一方面，将经取代的半胱氨酸抗体(CYSMAB)缀合至可检测的部分。在另一方面，疾病是癌症，且抗体能结合肿瘤相关抗原或癌症干细胞相关抗原。在又一方面，疾病是骨髓增生性病症。在另一方面，骨髓增生性病症选自：AML、CML、CMML、多发性骨髓瘤、浆细胞瘤和骨髓纤维化。在另一方面，肿瘤相关抗原或癌症干细胞抗原是CLL-1、GPR114、IL1RAP、TIM-3、CD19、CD20、CD22、ROR1、间皮素、CD33、CD123/IL3Ra、c-Met、PSMA、前列腺酸性磷酸酶(PAP)、CEA、CA-125、Muc-1、AFP、糖脂F77、EGFRvIII、GD-2、NY-ESO-1TCR、酪氨酸酶、TRPI/gp75、gp100/pmel-17、Melan-A/MART-1、Her2/neu、WT1、EphA3、端粒酶、HPV E6、HPV E7、EBNA1、BAGE、GAGE和MAGE A3TCRSLITRK6、ENPP3、连接蛋白-4、CD27、SLC44A4、CAIX、Cripto、CD30、MUC16、GPNMB、BCMA、Trop-2、组织因子(TF)、CanAg、EGFR、αv-整联蛋白、CD37、叶酸受体、CD138、CEACAM5、CD56、CD70、CD74、GCC、5T4、CD79b、Steap1、Napi2b、Lewis Y抗原、LIV、c-RET、DLL3、EFNA4或内皮唾酸蛋白/CD248。

[0048] 在另一个实施方案中，本公开提供了抑制细胞分裂的方法，其包括使细胞与能够结合细胞且缀合至对细胞有细胞毒性的药物的至少有效量的经半胱氨酸取代的缀合物(CYSMAB)接触。在一方面，细胞分裂的抑制导致细胞死亡。在另一方面，细胞是肿瘤或癌症

干细胞，且抗体能结合肿瘤相关抗原或癌症干细胞抗原。在另一方面，肿瘤或癌症干细胞来自骨髓增生性病症。在又一方面，骨髓增生性病症选自：AML、CML、CMML、多发性骨髓瘤、浆细胞瘤和骨髓纤维化。在另一方面，肿瘤相关抗原或癌症干细胞抗原是CLL-1、GPR114、IL1RAP、TIM-3、CD19、CD20、CD22、ROR1、间皮素、CD33、CD123/IL3Ra、c-Met、PSMA、前列腺酸性磷酸酶(PAP)、CEA、CA-125、Muc-1、AFP、糖脂F77、EGFRvIII、GD-2、NY-ESO-1 TCR、酪氨酸酶、TRPI/gp75、gp100/pmel-17、Melan-A/MART-1、Her2/neu、WT1、EphA3、端粒酶、HPV E6、HPV E7、EBNA1、BAGE、GAGE和MAGE A3TCRSLITRK6、ENPP3、连接蛋白-4、CD27、SLC44A4、CAIX、Cripto、CD30、MUC16、GPNMB、BCMA、Trop-2、组织因子(TF)、CanAg、EGFR、αv-整联蛋白、CD37、叶酸受体、CD138、CEACAM5、CD56、CD70、CD74、GCC、5T4、CD79b、Steap1、Napi2b、Lewis Y抗原、LIV、c-RET、DLL3、EFNA4或内皮唾酸蛋白/CD248。

[0049] 在另一个实施方案中，本公开提供了治疗癌症的方法，其包括向患者施用治疗有效量的经半胱氨酸取代的抗体缀合物(如使用经半胱氨酸取代的抗体产生的抗体-药物缀合物(ADC))，其中抗体缀合物能够结合肿瘤相关抗原或癌症干细胞抗原。在一方面，癌症是骨髓增生性病症。在另一方面，骨髓增生性病症选自：AML、CML、CMML、多发性骨髓瘤、浆细胞瘤和骨髓纤维化。在另一方面，肿瘤相关抗原或癌症干细胞抗原是CLL-1、GPR114、IL1RAP、TIM-3、CD19、CD20、CD22、ROR1、间皮素、CD33、CD123/IL3Ra、c-Met、PSMA、前列腺酸性磷酸酶(PAP)、CEA、CA-125、Muc-1、AFP、糖脂F77、EGFRvIII、GD-2、NY-ESO-1 TCR、酪氨酸酶、TRPI/gp75、gp100/pmel-17、Melan-A/MART-1、Her2/neu、WT1、EphA3、端粒酶、HPV E6、HPV E7、EBNA1、BAGE、GAGE和MAGE A3TCRSLITRK6、ENPP3、连接蛋白-4、CD27、SLC44A4、CAIX、Cripto、CD30、MUC16、GPNMB、BCMA、Trop-2、组织因子(TF)、CanAg、EGFR、αv-整联蛋白、CD37、叶酸受体、CD138、CEACAM5、CD56、CD70、CD74、GCC、5T4、CD79b、Steap1、Napi2b、Lewis Y抗原、LIV、c-RET、DLL3、EFNA4或内皮唾酸蛋白/CD248。

[0050] 还提供了包含经半胱氨酸取代的免疫球蛋白多肽的抗体缀合物，所述经半胱氨酸取代的免疫球蛋白多肽包含在抗体重链(具有重链和轻链的抗体部分)中根据Kabat编号的S156(157，根据EU编号)处的经取代的氨基酸残基并经由半胱氨酸连接至吲哚啉并苯二氮䓬二聚体或异喹啉烷并苯二氮䓬二聚体(包括但不限于如下所述的D202)。在一些实施方案中，使吲哚啉并苯二氮䓬二聚体或异喹啉烷并苯二氮䓬二聚体(包括但不限于如下所述的D202)通过接头附接至抗体，并且使接头经由二硫键连接至药物。在一些实施方案中，二硫键是吡啶基二硫化物部分。在一些实施方案中，接头在靶标的微环境中是可切割的。

[0051] 还提供了包含含有经半胱氨酸取代的免疫球蛋白多肽的抗体缀合物和佐剂的组合物，所述经半胱氨酸取代的免疫球蛋白多肽包含在抗体重链中根据Kabat编号的S156(根据EU编号的157)处的经取代的氨基酸残基并经由半胱氨酸连接至吲哚啉并苯二氮䓬二聚体或异喹啉烷并苯二氮䓬二聚体(包括但不限于如下所述的D202)。在一些实施方案中，组合物是药学上可接受的。

[0052] 还提供了抑制细胞分裂的方法，其包括使细胞与至少有效量的包含经半胱氨酸取代的免疫球蛋白多肽的抗体缀合物接触，所述经半胱氨酸取代的免疫球蛋白多肽包含抗体重链中根据Kabat编号的S156(根据EU编号的157)处的经取代的氨基酸残基并经由半胱氨酸连接至吲哚啉并苯二氮䓬二聚体或异喹啉烷并苯二氮䓬二聚体(包括但不限于如下所

述的D202)。在一些实施方案中,细胞分裂的抑制导致细胞死亡。在一些实施方案中,细胞是肿瘤或癌症干细胞,且抗体能结合肿瘤相关的抗原或癌症干细胞抗原。在一些实施方案中,肿瘤或癌症干细胞来自骨髓增生性病症。在一些实施方案中,骨髓增生性病症选自:AML、CML、CMML、多发性骨髓瘤、浆细胞瘤骨髓纤维化。在一些实施方案中,肿瘤相关抗原或癌症干细胞抗原是CLL-1、GPR114、IL1RAP、TIM-3、CD19、CD20、CD22、ROR1、间皮素、CD33、CD123/IL3Ra、c-Met、PSMA、前列腺酸性磷酸酶(PAP)、CEA、CA-125、Muc-1、AFP、糖脂F77、EGFRvIII、GD-2、NY-ESO-1 TCR、酪氨酸酶、TRPI/gp75、gp100/pmel-17、Melan-A/MART-1、Her2/neu、WT1、EphA3、端粒酶、HPV E6、HPV E7、EBNA1、BAGE、GAGE和MAGE A3TCRSLITRK6、ENPP3、连接蛋白-4、CD27、SLC44A4、CAIX、Cripto、CD30、MUC16、GPNMB、BCMA、Trop-2、组织因子(TF)、CanAg、EGFR、αv-整联蛋白、CD37、叶酸受体、CD138、CEACAM5、CD56、CD70、CD74、GCC、5T4、CD79b、Steap1、Napi2b、Lewis Y抗原、LIV、c-RET、DLL3、EFNA4或内皮唾酸蛋白/CD248。

[0053] 还提供了治疗癌症的方法,其包括向患者施用治疗有效量的包含经半胱氨酸取代的免疫球蛋白多肽的抗体缀合物,所述经半胱氨酸取代的免疫球蛋白多肽包含在抗体重链中根据Kabat编号的S156(根据EU编号的157)处的经取代的氨基酸残基并经由半胱氨酸连接至吲哚啉并苯二氮䓬二聚体或异喹啉烷并苯二氮䓬二聚体(包括但不限于如下所述的D202),其中所述抗体缀合物能够结合肿瘤相关的抗原或癌症干细胞抗原。在一些实施方案中,癌症是骨髓增生性病症。在一些实施方案中,骨髓增生性病症选自:AML、CML、CMML、多发性骨髓瘤、浆细胞瘤和骨髓纤维化。在一些实施方案中,肿瘤相关抗原或癌症干细胞抗原是CLL-1、GPR114、IL1RAP、TIM-3、CD19、CD20、CD22、ROR1、间皮素、CD33、CD123/IL3Ra、c-Met、PSMA、前列腺酸性磷酸酶(PAP)、CEA、CA-125、Muc-1、AFP、糖脂F77、EGFRvIII、GD-2、NY-ESO-1 TCR、酪氨酸酶、TRPI/gp75、gp100/pmel-17、Melan-A/MART-1、Her2/neu、WT1、EphA3、端粒酶、HPV E6、HPV E7、EBNA1、BAGE、GAGE和MAGE A3TCRSLITRK6、ENPP3、连接蛋白-4、CD27、SLC44A4、CAIX、Cripto、CD30、MUC16、GPNMB、BCMA、Trop-2、组织因子(TF)、CanAg、EGFR、αv-整联蛋白、CD37、叶酸受体、CD138、CEACAM5、CD56、CD70、CD74、GCC、5T4、CD79b、Steap1、Napi2b、Lewis Y抗原、LIV、c-RET、DLL3、EFNA4或内皮唾酸蛋白/CD248。

[0054] 在阅读以下说明书和权利要求后,本公开的其它目的对本领域技术人员是显而易见的。

[0055] 附图简述

[0056] 图1显示了比较本公开的抗体-荧光团缀合物(AFC)的特异性的3种不同的ELISA测定。形式1是直接的ELISA,其中将CLL-1胞外结构域(ECD)固定(AFC结合其上),然后通过抗-荧光团抗体(兔抗-A488)和检测试剂(抗-兔Fc-HRP)检测。形式2是ELISA,其中抗-荧光团抗体(抗-A488Ab)被结合,且AFC和生物素化的CLL-1ECD夹在检测试剂(SA-HRP)之间。形式3是可替代的ELISA,其中抗-CLL-1Ab被固定化且夹有CLL-1ECD和AFC以及抗-A488Ab和检测试剂(抗-兔Fc-HRP)。

[0057] 图2A-2B展示了测定ELISA特异性测定形式1。图2A显示了相对于IgG、曲妥珠单抗、裸HuM31及对照标记的IgG,对标记的AFC和WT抗-荧光团抗体的类似的特异性测定。结果显示标记的HuM31与WT之间类似的特异性。图2B描绘了人血浆和PBS的干扰作用。

[0058] 图3是显示稳定性ELISA测定形式的动画图。在此形式下,AFC夹在经固定的CLL-1ECD和检测试剂之间。(SA-HRP)。

[0059] 图4A-4E显示了HuM31重链抗体恒定链与其它IgG1、IgG2、IgG3和IgG4同种型的比对，其中残基用Kabat、EU索引和顺序编号鉴定。轻链序列(图4A)：M31(SEQ ID NO:7)、HuM31(SEQ ID NO:8)、κ(SEQ ID NO:9)和λ(SEQ ID NO:10)。重链序列(图4B)：M31(SEQ ID NO:11)和HuM31(SEQ ID NO:12)。

[0060] 图5说明了各种抗体缀合物的荧光-与-抗体(FAR)比。

[0061] 图6说明了各种抗体缀合物的药物-与-抗体(DAR)比。

[0062] 图7提供了缀合的结果，包括氨基酸残基和各种抗体缀合物的荧光团-与-抗体比(“FAR”)。

[0063] 图8提供了缀合的结果，包括氨基酸残基和各种抗体缀合物的荧光团-与-抗体比(“FAR”)。

[0064] 图9提供了缀合的结果，包括氨基酸残基和各种抗体缀合物的荧光团-与-抗体比(“FAR”)。

[0065] 图10提供了缀合的结果，包括氨基酸残基和各种抗体缀合物的荧光团-与-抗体比(“FAR”)。

[0066] 图11说明了各种抗体缀合物的稳定性。

[0067] 图12A-C提供了C6-CYSMAB-ADC的FACS结合数据的图。圆形，C6-S156C-D202；方形，C6-A118C-D202；三角形，C6-G316C-D202；空心圆形，C6-V266C-D202；空心方形，C6-S239C-D202；空心三角形，C0-D202。

[0068] 发明详述

[0069] 本申请不限于所描述的具体方法或特定组合物，因为它们可以变化。还应该理解，本文使用的术语仅仅是出于描述具体实施方案的目的，而不意在是限制性的，因为本申请的范围将仅由所附的权利要求及其等同物来限制。

[0070] 除非另外定义，否则本文使用的所有技术和科学术语具有与本公开所属领域的普通技术人员通常理解的相同的含义。下文描述了建议的方法和材料，然而在本申请的实践或测试中可以使用与本文所述的那些方法和材料相似或等效的任何方法和材料。

[0071] I.定义

[0072] 如本文所用的“免疫球蛋白”是指免疫球蛋白多肽或抗体。

[0073] 术语“免疫球蛋白多肽”是指基本上由免疫球蛋白基因编码的多肽。

[0074] 术语“抗体”是指具有抗原结合活性的蛋白和来自或来源于产生抗体的动物的免疫球蛋白编码基因的框架区的氨基酸序列。术语包括但不限于来源于人或其它哺乳动物细胞的同种型类别IgA、IgD、IgE、IgG和IgM的多克隆或单克隆抗体，其包括天然或遗传修饰形式，诸如人源化抗体、人抗体、单链抗体、嵌合抗体、合成抗体、重组抗体、杂合抗体、突变抗体、移植抗体和体外生成的抗体。术语涵盖缀合物，包括但不限于含有免疫球蛋白部分的融合蛋白(如嵌合或双特异性抗体或scFv')及片段，诸如Fab、F(ab′)2、Fv、scFv、Fd、单结构域(dAb)和其它组合物。

[0075] 如本文所用的术语“经半胱氨酸取代的免疫球蛋白”是指经半胱氨酸取代的免疫球蛋白多肽或经半胱氨酸取代的抗体。

[0076] 如本文所用的术语“经半胱氨酸取代的免疫球蛋白多肽”是指经半胱氨酸取代的包含至少一种非天然存在的恒定区免疫球蛋白氨基酸残基的多肽。非天然存在的取代是并

非同种型的取代。在一个实施方案中，经取代的残基是重链恒定区残基V266C、H285C、R301C、V303C、T307C、G316C、Y436C和L441C。在另一个实施方案中，恒定区是同种型IgG1、IgG2、IgG3或IgG4的恒定区。

[0077] 术语“经半胱氨酸取代的抗体”(“CYSMAB”)是指包含经半胱氨酸取代的免疫球蛋白多肽的抗体。

[0078] 如本文所用的术语“免疫球蛋白缀合物”是指缀合至功能部分的免疫球蛋白多肽或抗体或“抗体缀合物”。

[0079] 如本文所用的术语“免疫球蛋白药物缀合物”是指缀合至功能部分(诸如药物部分或放射标记物或检测试剂)的免疫球蛋白多肽或抗体(“抗体药物缀合物”(“ADC”))。

[0080] 术语“经半胱氨酸取代的免疫球蛋白药物缀合物”或者是指已缀合至药物部分的经半胱氨酸取代的免疫球蛋白多肽或经半胱氨酸取代的抗体(“CYSMAB”)，如经半胱氨酸取代的抗体药物缀合物(“CYSMAB ADC”)。

[0081] 示例性抗体免疫球蛋白结构单元包括四聚体。每个四聚体包含两对相同的多肽链，每对具有一条“轻”链(约25kD)和一条“重”链(约50-70kD)。每条链的N-末端限定了主要负责抗原识别的约100至110个或更多个氨基酸的可变区。术语可变轻链(VL)和可变重链(VH)分别是指这些轻链和重链。可变区含有抗体(或其功能等效物)的抗原结合区，且在结合的特异性和亲和力方面是关键的。参见Paul,Fundamental Immunology(2003)。

[0082] 抗体可以作为完整的免疫球蛋白存在，或作为包含特定抗原-结合活性的许多被充分表征的片段中的任一种存在。为了清楚起见，具有重链和轻链的四聚体抗体在本文中被称为“完整免疫球蛋白”，并且可以是天然存在的、多克隆的、单克隆的或重组产生的。可以通过各种肽酶消化来产生片段。胃蛋白酶消化在铰链区中的二硫键下的抗体以产生F(ab)′2，即Fab的二聚体，其本身是通过二硫键与VH-CH1连结的轻链。在温和条件下，F(ab)′2可以被还原以断裂铰链区中的二硫键，由此将F(ab)′2二聚体转化为Fab’单体。Fab’单体实质上是具有部分铰链区的Fab。尽管根据完整抗体的消化来定义各种抗体片段，但是技术人员将会理解，此类片段可以通过化学方法或通过使用重组DNA方法从头合成。因此，术语抗体，如本文所用，还包括通过修饰整个抗体产生的抗体片段，或者使用重组DNA方法从头合成的那些抗体片段，或者使用噬菌体展示文库鉴定的那些抗体片段(参见如McCafferty等人,Nature 348:552-554(1990))。

[0083] 如本文所用，术语“Fv”是指单价或二价可变区片段，并且可以仅涵盖可变区(如VL和/或VH)，以及更长的片段，如Fab、Fab′或F(ab′)2，其还包括CL和/或CH1。除非另有说明，否则术语“Fc”是指包含CH2和CH3区域的重链单体或二聚体。

[0084] 单链Fv(scFv)是指包含通过接头(如肽接头)连结的VL和VH的多肽。ScFv还可用于形成串联(或二价)scFv或双链抗体。串联scFv和双链抗体的产生和性质描述于如Asano等人(2011)J Biol.Chem.286:1812;Kenanova等人(2010)Prot Eng Design Sel 23:789;Asano等人(2008)Prot Eng Design Sel 21:597中。

[0085] 如本文所用的术语“单克隆抗体”是指对抗原上给定表位具有单一的结合特异性和亲和力的抗体的克隆制备物。“多克隆抗体”是指针对单一抗原产生的、但具有不同的结合特异性和亲和力的抗体制备物。

[0086] 如本文所用，“可变区”或“V-区”是指抗体可变区结构域，其包含框架1(CDR1)、框

架2(CDR2)和框架3(包括CDR3和框架4)的区段,所述区段作为在B细胞分化期间重链和轻链V-区基因的重排的结果被添加到V-区段。

[0087] 如本文所用的术语“框架”或“FR”是指除高变区(HVR)残基外的可变结构域残基。可变结构域的FR通常由四个FR结构域组成:FR1、FR2、FR3和FR4。因此,HVR和FR序列通常出现在VH(或VL)中的以下序列中:FR1-HVR1(L1)-FR2-HVR2(L2)-FR3-HVR3(L3)-FR4。

[0088] 如本文所用,“互补决定区(CDR)”是指每条链中的中断了由轻链和重链可变区所建立的四个“框架”区的三个高变区。CDR主要负责与抗原的表位结合。每条链的CDR通常被称为CDR1、CDR2和CDR3,其从N-末端开始顺序编号,并且也通常由特定CDR所处的链来标识。因此,V_H CDR3位于其存在的抗体的重链的可变结构域中,而V_L CDR1是来自其存在的抗体的轻链的可变结构域的CDR1。

[0089] CDR和框架区的氨基酸序列可使用本领域熟知的定义测定,如Kabat,Chothia,国际ImMunoGeneTics数据库(IMGT)和AbM(参见,如Johnson等人,同上;Chothia&Lesk,(1987)J.Mol.Biol.196,901-917;Chothia等人(1989)Nature 342,877-883;Chothia等人(1992)J.Mol.Biol.227,799-817;Al-Lazikani等人,J.Mol.Biol.1997,273(4))。有关使用Kabat系统定位CDR的有用指南,可见于bioinf.org.uk/abs可获得的网站。抗原组合位点的定义也描述如下:Ruiz等人Nucleic Acids Res.,28,219-221(2000);和Lefranc Nucleic Acids Res.1月1日;29(1):207-9(2001);MacCallum等人,J.Mol.Biol.,262:732-745(1996);和Martin等人,Proc.Natl.Acad.Sci.USA,86,9268-9272(1989);Martin,等人,Methods Enzymol.,203:121-153,(1991);Pedersen等人,Immunomethods,1,126,(1992);和Rees等人,In Sternberg M.J.E.(编),Protein Structure Prediction.Oxford University Press,Oxford,141-172 1996)。示例性CDR描述为US 2013/0295118的图7的CDR-H1、CDR-H2、CDR-H3、CDR-L1、CDR-L2和CDR-L3。

[0090] 术语“高变区”、“HVR”当在本文中使用时是指抗体可变结构域的在序列中高变的和/或形成在结构上限定的环的区域。一般而言,抗体包含六个高变区;VH中的三个(H1、H2、H3)和VL中的三个(L1、L2、L3)。许多高变区的描述正在使用并涵盖在本文中。Kabat互补决定区(CDR)是基于序列可变性的并且是最常用的(Kabat等人,Sequences of Proteins of Immunological Interest,第5版Public Health Service,National Institutes of Health,Bethesda,Md.(1991)),而Chothia是指结构环的位置(Chothia和Lesk(1987)J.Mol.Biol.196:901-917)。“完整”高变区基于对可用的复杂晶体结构的分析。下面示出了来自这些高变区中的各个的残基。除非另有说明,否则将采用根据蛋白的比对序列的Kabat数据库的Kabat编号(Wu和Kabat(1970)J.Exp.Med.132:211-250;Johnson和Wu(2000)Nuc.Acids Res.28(1):214-218)。高变区位置一般如下:氨基酸24-34(HVR-L1)、氨基酸49-56(HVR-L2)、氨基酸89-97(HVR-L3)、氨基酸26-35A(HVR-H1)、氨基酸49-65(HVR-L2)和氨基酸93-102(HVR-H3)。高变区还可包括如下的“扩展高变区”:VL中的氨基酸24-36(L1)和氨基酸46-56(L2)。可变结构域残基根据Kabat等人,同上,进行编号用于这些定义中的各个。用于本文目的的“改变的高变区”是在其中包含一个或多个(如1个至约16个)氨基酸取代的高变区。用于本文目的的“未经修饰的高变区”是具有与其来源的非-人类抗体相同的氨基酸序列的高变区,即其中缺少一个或多个氨基酸取代的高变区。

[0091] 如本文所用的术语“嵌合抗体”是指这样的抗体,其中(a)改变、替代或交换恒定区

或其一部分，使得抗原结合位点(可变区、CDR或其部分)连接至不同的或改变的类别、效应子功能和/或物种的恒定区；或(b)用具有不同或改变的抗原特异性的可变区(如不同物种的CDR和框架区)改变、替代或交换可变区或其一部分。嵌合抗体可包括可变区片段，如包含两个Fab或Fv区或scFv的重组抗体。如上所指示，嵌合也可以包括来自与所附接的Fv区不同来源的Fc区。在一些情况下，嵌合抗体包括Fv区域内的嵌合体。此类嵌合抗体的实例是人源化抗体，其中FR和CDR来自不同来源。

[0092] 如本文所用的术语“人源化抗体”是指其中使从非人抗体的V_H和V_L区获得的抗原结合环即CDR移植到人框架序列的抗体。人源化，即将非人CDR序列取代为相应的人抗体序列，可按照以下文献中描述的方法进行，如美国专利第5,545,806号；第5,569,825号；第5,633,425号；第5,661,016号；Riechmann等人，Nature 332:323-327(1988)；Marks等人，Bio/Technology 10:779-783(1992)；Morrison,Nature 368:812-13(1994)；Fishwild等人，Nature Biotechnology 14:845-51(1996)。转基因小鼠或其它生物体，诸如其它哺乳动物，也可用于表达人源化或人抗体，如美国专利第6,673,986号所公开。

[0093] 术语“对……具有特异性”、“特异性结合”及类似术语是指以比非靶标化合物高至少2倍的亲和力(如高至少4倍、5倍、6倍、7倍、8倍、9倍、10倍、20倍、25倍、50倍或100倍亲和力中的任一种)结合靶标的分子(如抗体或抗体片段)。例如，特异性结合首要靶标的抗体将通常以比非首要抗体靶标(如来自不同物种的或不同同种型的抗体，或非抗体靶标)高至少2倍的亲和力结合首要靶标。

[0094] 术语“结合”就抗体靶标(如抗原、分析物、免疫复合物)而言，通常指抗体结合纯群体中大多数抗体靶标(假定适当的摩尔比)。例如，结合给定抗体靶标的抗体通常结合溶液中至少2/3的抗体靶标(如75、80、85、90、91、92、93、94、95、96、97、98、99或100％中的至少任一种)。技术人员将认识到根据确定结合的方法和/或阈值，会出现一些可变性。

[0095] 术语“标记物”、“可检测的部分”及类似术语是指通过光谱学、光化学、生物化学、免疫化学、化学或其它物理手段检测的组合物。例如，有用的标记物包括荧光染料、发光剂、放射性同位素(如^{32}P、^{3}H)、电子致密试剂、酶(如通常用于ELISA)、生物素、地高辛或半抗原和蛋白或其它实体(如通过将放射性标记物掺入与靶标分析物特异性反应的肽或抗体中而变得可检测)。可以采用本领域已知的将抗体缀合至标记物的任何方法，如使用Hermanson,Bioconjugate Techniques 1996,Academic Press,Inc.,San Diego中所述的方法。术语“标签”可以与术语“标记物”同义使用，但通常是指基于亲和力的部分，如用于纯化的“His标签”或与生物素相互作用的“链霉亲和素标签”。

[0096] 如本文所用的术语“标记的”分子(如核酸、蛋白质或抗体)是通过接头或化学键共价结合或通过离子键、范德华键、静电键或氢键非共价键结合到标记物，使得分子的存在可以通过检测与分子结合的标记物的存在来检测的分子。

[0097] 术语“C型凝集素样分子1(CLL-1)”，也被称为CLEC12A、DCAL-2和MICL，是II型膜蛋白(ITIM结构域—TM结构域-茎结构域-凝集素样结构域)。CLL-1的胞外结构域是高度糖基化的，并且仅在髓系细胞中表达。CLL-1也于AML、MDS和CML细胞表达。CLL-1表达可用来区分不表达CLL-1的正常造血干细胞(HSC)和表达CLL-1的白血病干细胞(LSC)。LSC是白血病患者中的CD34+细胞，其导致癌细胞产生和癌症复发。参见Bakker等人(2004)Cancer Res.64:8443。

[0098] CLL-1的核苷酸和氨基酸序列对于许多物种是已知的。例如，人序列可作为US2013/0295118中的SEQ ID NO:2和Genbank登录号AF247788.1和Uniprot登录号Q5QGZ9(SEQ ID NO:2)存在。对于如SEQ ID NO:2所示的人CLL-1蛋白，胞外结构域包含大约氨基酸65-265，跨膜结构域包含大约氨基酸44-64，且细胞质结构域包含大约氨基酸1-43。人CLL-1的茎结构域跨越氨基酸65-139，且C凝集素结构域跨越氨基酸140-249。

[0099] 如本文所用的术语“CLL-1相关的病症”是指与同标准对照(如正常、非疾病、非癌细胞)中的CLL-1表达相比的非致病性水平(如CLL-1的升高或降低的细胞表面表达)相关的病况和疾病。升高的CLL-1水平与癌细胞相关，特别是白血病诸如AML(急性骨髓性白血病)、MDS(骨髓增生异常综合征)和CML(慢性骨髓性白血病)和造血CSC(如LSC)。

[0100] “癌症干细胞”假说认为，由“癌症干细胞”代表的一小部分肿瘤使得肿瘤增殖和自我更新，并最终分化成表型多样性和异质性的肿瘤细胞群(Bjerkvig等人，Nat.Rev.Cancer,5:899-904,2005)。癌症干细胞可以从任何类型的癌症中分离出来，如白血病、乳腺癌、结肠癌和脑癌、结肠癌。癌症干细胞的特征在于其自我更新和增殖的能力，并通过从亲本肿瘤分化而重现。示例性癌症干细胞抗原包括CD133、Bmi-1、Notch、音猬因子(Sonic hedgehog)和Wnt。此外，神经癌症干细胞的示例性分子标记物包括CD90、CD44、CXCR4、巢蛋白、Musashi-1(Msi1)、母系胚胎亮氨酸拉链蛋白激酶(MELK)、GLI1、PTCH1、Bmi-1、磷酸丝氨酸磷酶(PSP)、Snail、OCT4、BCRP1、MGMT、Bcl-2、FLIP、BCL-XL、XIAP、cIAP1、cIAP2、NAIP和存活素。一种有用的癌症干细胞抗原是CLL-1。

[0101] 术语“细胞毒性”是指药剂对细胞的抑制作用，如坏死(由于细胞裂解导致的细胞膜完整性丧失和快速死亡)；存活力降低(其中细胞停止增殖)和细胞凋亡(受控的细胞死亡的遗传程序)。

[0102] 细胞毒性还可以使用3-(4,5-二甲基-2-噻唑基)-2,5-二苯基-2H-四唑鎓溴化物(MTT)或MTS测定来监测。这种测定使用比色反应来测量细胞的还原电位。存活细胞将MTS试剂还原成有色的甲臜产物。也使用荧光染料刃天青来开发类似的基于氧化还原的测定。除了使用染料来指示细胞的氧化还原电位以监测它们的存活力之外，研究人员还开发了使用三磷酸腺苷(ATP)含量作为存活力标记物的测定。这种基于ATP的测定包括生物发光测定，其中ATP是萤光素酶反应的限制试剂(如CellTiter-Glo发光细胞存活力测定，Promega)。细胞毒性也可以通过磺酰罗丹明B(SRB)测定、WST测定和发色测定(chronogenic assay)来测量。实时追踪粘附动物细胞的细胞毒性响应的无标记物方法是基于细胞在金膜电极上生长时的电阻抗测量。这种技术被称为电子细胞基质阻抗判断(electric cell-substrate impedance sensing,ECIS)。无标记物实时技术提供了细胞毒性响应的动力学，而不仅仅是许多比色终点测定的快照。

[0103] 术语“CLL-1特异性抗体”、“抗CLL-1抗体”、“CLL-1抗体”和“抗CLL-1”在本文中同义地使用，以指代特异性结合CLL-1(包括各种糖基化形式的CLL-1)的抗体。本文所述的CLL-1抗体特异性结合例如在某些癌细胞表面上表达的CLL-1多肽，而不是结合造血干细胞(HSC)。如下文更详细讨论的，本发明抗-CLL-1抗体可以结合CLL-1表达细胞，与其它AML-靶向抗体相比，结合更大百分比的AML细胞，抑制AML细胞增殖并介导它们的破坏。适合用作本公开的经半胱氨酸取代的抗体(CYSMAB)的抗CLL抗体的实例描述于2013年11月7日公布的US2013/0295118。抗CLL-1抗体可具有如此出版物中所公开的CDR，特别是抗体M31和M26的

CDR。

[0104] 术语“差异表达的”或“差异调控的”通常是指在一个样品中与至少一个其它样品相比，过表达(上调)或低表达(下调)的蛋白或核酸生物标志物。在本公开的上下文中，术语通常是指与正常的非癌细胞相比，癌细胞(如AML细胞或AML CSC)上CLL-1的过表达。

[0105] 例如，术语“过表达的”或“上调控的”可互换地指代以可检测的高于对照的水平转录或翻译的蛋白或核酸，通常是生物标记物。术语包括由于转录、转录后加工、翻译、翻译后加工、细胞定位(如细胞器、细胞质、核、细胞表面)以及RNA和蛋白质稳定性而导致的过表达。过表达可以使用用于检测生物标记物的常规技术来检测，无论是mRNA(即RT-PCR，杂交)还是蛋白(即流式细胞术、成像、ELISA、免疫组织化学技术)。与正常细胞相比，过表达可以是至少10％、20％、30％、40％、50％、60％、70％、80％、90％或更大的任一种。

[0106] 如本文所用的术语“对照”样品或值是指，用作参考、通常是已知的参考以用于与测试样品进行比较的样品。例如，测试样品可以取自测试条件，如在测试化合物的存在下，并与来自已知条件的样品比较，如在不存在测试化合物(阴性对照)下，或在已知的化合物(阳性对照)的存在下。在本公开的上下文中，阴性对照的实例是来自已知健康(非癌症)个体的生物样品，并且阳性对照的实例是来自已知AML患者的生物样品。对照也可以代表从多个测试或结果中收集的平均值或范围。本领域的技术人员将认识到，可以将对照设计用于评估任何数量的参数。例如，可以设计对照来比较基于药理学数据(如半衰期)或治疗措施(如比较益处和/或副作用)的治疗性益处。可以设计对照用于体外应用。本领域技术人员将理解哪些对照在给定情况下是有价值的，并且能够基于与对照值的比较来分析数据。对照对于确定数据的重要性也是有价值的。例如，如果给定参数的值在对照中变化很大，则测试样本的变化不会被认为是显著的。

[0107] 术语“诊断”是指受试者患有诸如癌症的疾病的相对概率。类似地，术语“预后”是指受试者中可能发生某种未来结果的相对概率。例如，在本公开的上下文中，预后可以指个体将发展癌症、具有复发或疾病的可能严重程度(如症状的严重程度、功能衰退速率、存活率等)的可能性。术语并非旨在是绝对的，正如医学诊断领域的技术人员所理解的那样。

[0108] 如本文所用的“活检组织”或“来自受试者的生物样品”是指从患有或怀疑患有疾病、如CLL-1相关的病症的受试者获得的样品。样品也可以是血液样品或血液级分，如白细胞级分、血清或血浆。在一些实施方案中，样品可以是组织的活检组织，诸如针活检组织、细针活检组织、手术活检组织等。样品可以包括携带有病变或疑似病变的组织样品，然而生物样品也可来源于另一个部位，如疑似转移的部位、淋巴结或血液。在某些情况下，生物样品也可能来自邻近病变或疑似病变的区域。

[0109] “生物样品”可以从受试者(如活检组织)、动物(诸如动物模型)或培养的细胞(如从受试者中取出并培养生长以观察的细胞系或细胞)获得。生物样品包括组织和体液，如血液、血液级分、淋巴、唾液、尿液、粪便等。

[0110] EU编号体系是指美国抗体的编号(Edelman等人，Proc.Natl.Acad.Sci.USA 63:78-85(1969))。Kabat互补决定区(CDR)是基于序列可变性，并且是最常用的(Kabat等人Sequences of Proteins of Immunological Interest，第5版，Public Health Service，National Institutes of Health，Bethesda，Md.(1991))。如本文所用，EU编号是指本文所述的抗体的恒定链命名法，而Kabat则用于获得可变区的CDR和HVR。

[0111] “可操作地连接的”是指两个或多个组分并置，其中如此描述的组分处于允许它们以其预期的方式起作用的关系。例如，如果启动子以顺式方式作用以控制或调节连接序列的转录，则启动子可操作地连接至编码序列。通常但不一定，“可操作地连接的”DNA序列是连续的，并且必要时连结两个蛋白编码区或在分泌型前导序列的情况下是连续的且在阅读框中。然而，尽管可操作地连接的启动子一般位于编码序列的上游，但它不一定与其相邻。

[0112] 如本文所用的术语“启动子”是指控制其可操作地连接的基因或序列的转录的多核苷酸序列。启动子包括RNA聚合酶结合和转录起始的信号。所使用的启动子将在宿主细胞的预期所选序列在其中表达的细胞类型中起作用。

[0113] 如本文所用的术语“载体”意指能够转运与其连接的另一核酸的核酸分子。一种类型的载体是″质粒″，其是指可以连接附加DNA区段于其中的环状双链DNA环。另一种类型的载体是噬菌体载体。另一种类型的载体是病毒载体，其中额外的DNA区段可以连接到病毒基因组中。某些载体能够在所引入它们的宿主细胞中自主复制(如具有细菌复制起点的细菌载体和附加型哺乳动物载体)。其它载体(如非附加型哺乳动物载体)可以在引入到宿主细胞中之后整合到宿主细胞的基因组中，并从而与宿主基因组一起复制。而且，某些载体能够指导它们可操作地连接的基因的表达。此类载体在本文中被称为″重组表达载体″(或简称为″表达载体″)。

[0114] 如本文所用的术语“宿主细胞”(或“重组宿主细胞”)意指已被遗传改变，或能够通过引入外源多核苷酸诸如用重组质粒或载体来遗传改变的细胞。应该理解，此类术语不仅是指特定的受试者细胞，而且还是指其后代。

[0115] 术语“疗法”、“治疗”和“改善”是指症状的严重程度减轻。在治疗癌症(如AML)的情况下，治疗可以是指如减小肿瘤大小、癌细胞数量、生长速率、转移活性，减少非癌细胞的细胞死亡，减少恶心和其它化疗或放疗副作用等。术语“治疗”和“预防”并非意在为绝对的术语。治疗和预防可以是指任何发作延迟、症状改善、患者存活提高、存活时间或存活率增加等。治疗和预防可以是完全的(不可检测的肿瘤细胞水平)或部分的，使得(相比于没有本发明时所发生的情况而言)较少的肿瘤细胞存在于患者体内。治疗的效果可以与未接受治疗的个体或个体库，或治疗前或治疗期间的不同时间点的相同患者相比较。在一些方面，疾病的严重程度相较于如施用前的个体或不经历治疗的对照个体减少至少10％。在一些方面，疾病的严重程度减少了至少25％、50％、75％、80％或90％，或在一些情况下，使用标准诊断技术不再是可检测的。

[0116] “有效量”的药剂，如药物制剂，是指在获得所需的细胞响应、治疗性或预防性结果所必需的剂量下和时间段内有效的量。例如，在抑制细胞增殖的方法中，有效量的经半胱氨酸取代的免疫球蛋白药物缀合物(如CYSMAB ADC)是相对于对照细胞显著减弱、抑制或防止细胞中细胞分裂的浓度。

[0117] 短语“治疗有效量”意指本发明的化合物发挥以下作用的量：(i)治疗或预防特定疾病、病况或病症，(ii)减弱、改善或消除特定疾病、病况或病症中的一种或多种症状，或(iii)预防或延迟本文所述的特定疾病、病况或病症中的一种或多种症状的发作。在一个实施方案中，治疗性有效量是足以减小或减轻对CLL-1的调节有响应的病症的症状的量。在癌症的情况下，治疗有效量的药物可以减少癌细胞的数量；减小肿瘤的大小；抑制(即在一定程度上减缓，且优选停止)癌细胞向外周器官的浸润；抑制(即在一定程度上减缓，且优选停

止)肿瘤转移;在一定程度上抑制肿瘤生长;和/或在一定程度上减轻与癌症相关的一种或多种症状。在药物可防止和/或杀灭存在的癌细胞的程度上,其可能是细胞生长抑制和/或细胞毒性的。对于癌症疗法,效力可以通过评估疾病进展时间(TTP)和/或确定响应率(RR)来测量。在一个实施方案中,治疗有效量是足以减少或减轻对CLL-1的调节有响应的病症的症状的量。在免疫病症的情况下,治疗有效量是足以减少或减轻过敏性病症、自身免疫性和/或炎性疾病的症状或急性炎性反应的症状的量。在一些实施方案中,治疗有效量是本文所述的化学实体足以显著降低骨髓增生性癌症干细胞的活性或数量的量。

[0118] 如本文所用,术语"药学上可接受的"与生理上可接受的和药理学上可接受的同义使用。药物组合物通常将包含用于缓冲和储存保护的试剂,并且可根据施用途径包含用于适合递送的缓冲剂和载剂。

[0119] 如本文所用的短语"药学上可接受的盐"是指ADC的药学上可接受的有机或无机盐。示例性盐包括但不限于硫酸盐、柠檬酸盐、乙酸盐、草酸盐、氯化物、溴化物、碘化物、硝酸盐、硫酸氢盐、磷酸盐、酸式磷酸盐、异烟酸盐、乳酸盐、水杨酸盐、酸式柠檬酸盐、酒石酸盐、油酸盐、单宁酸盐、泛酸盐、酒石酸氢盐、抗坏血酸盐、琥珀酸盐、马来酸盐、龙胆酸盐、富马酸盐、葡糖酸盐、葡糖醛酸盐、糖酸盐、甲酸盐、苯甲酸盐、谷氨酸盐、甲磺酸盐、乙磺酸盐、苯磺酸盐、对甲苯磺酸盐和双羟萘酸盐(即1,1'-亚甲基-双-(2-羟基-3-萘甲酸盐))。药学上可接受的盐可能包括纳入另一种分子,诸如乙酸根离子、琥珀酸根离子或其它抗衡离子。抗衡离子可以是使化合物上的电荷稳定的任何有机或无机部分。此外,药学上可接受的盐在其结构中可能具有多于一个带电原子。多个带电原子是药学上可接受的盐的一部分的情况可以有多个抗衡离子。因此,药学上可接受的盐可以具有一个或多个带电原子和/或一个或多个抗衡离子。

[0120] "药学上可接受的溶剂化物"是指一种或多种溶剂分子与ADC的缔合。形成药学上可接受的溶剂化物的溶剂的实例包括但不限于水、异丙醇、乙醇、甲醇、DMSO、乙酸乙酯、乙酸和乙醇胺。

[0121] 如本文所用的"载剂"包括在以采用的剂量和浓度暴露于细胞或哺乳动物时对细胞或哺乳动物无毒的药学上可接受的载剂、赋形剂或稳定剂。通常生理上可接受的载剂是pH缓冲水溶液。生理上可接受的载剂的实例包括缓冲剂,诸如磷酸盐、柠檬酸盐和其它有机酸;抗氧化剂,包括抗坏血酸;低分子量(小于约10个残基)多肽;蛋白质,诸如血清白蛋白、明胶或免疫球蛋白;亲水性聚合物,诸如聚乙烯吡咯烷酮;氨基酸,诸如甘氨酸、谷氨酰胺、天冬酰胺、精氨酸或赖氨酸;单糖、二糖和其它碳水化合物,包括葡萄糖、甘露糖或糊精;螯合剂,诸如EDTA;糖醇,诸如甘露糖醇或山梨糖醇;成盐抗衡离子,诸如钠;和/或非离子表面活性剂,诸如TWEEN®、聚乙二醇(PEG)和PLURONICS®。术语"剂量(dose)"和"剂量(dosage)"在本文中可互换使用。剂量是指在每次施用时给予个体的活性成分的量。对于本发明,剂量可以是指抗体或相关组分的浓度,如治疗剂的量或放射性标记物的剂量。剂量将根据多种因素而变化,包括施用频率;个体的大小和耐受性;病况的严重程度;副作用的风险;施用途径;以及可检测部分的成像方式(如果存在的话)。本领域技术人员将认识到可以根据上述因素或基于治疗进展来调整剂量。术语"剂型"是指药物的特定形式,并且取决于施用途径。例如,剂型可以是液体,如注射用盐水溶液。

[0122] "受试者"、"患者"、"个体"及类似术语可互换使用,并且除非另有说明,否则是指

哺乳动物诸如人和非人灵长类，以及兔、大鼠、小鼠、山羊、猪和其它哺乳动物物种。术语并不一定指示受试者已经被诊断出患有特定疾病。术语“患者”是指处于医疗监督下的受试者。患者可以是寻求治疗、监测、调整或修改现有治疗方案等的个体。“癌症患者”或“AML患者”可以是指被诊断为患有癌症、目前正在接受治疗性方案或者如在去除肿瘤手术后处于复发风险的个体。在一些实施方案中，癌症患者已经被诊断为患有癌症并且是疗法的候选人。癌症患者可以包括未接受治疗、目前正在接受治疗、已经进行手术的个体及已经停止治疗的那些个体。

[0123] 在治疗癌症的上下文中，需要治疗的受试者可以是指患有癌症或癌前病况、已经患有癌症且处于复发风险、疑似患有癌症、正在经历标准癌症治疗诸如放疗或化疗等的个体。

[0124] “癌症”、“肿瘤”及类似术语包括癌前、赘生性和癌性细胞，并且可以是指实体瘤或非实体癌(参见，如Edge等人AJCC Cancer Staging Manual(第7版，2009)；Cibas和Ducatman Cytology:Diagnostic principles and clinical correlates(第3版，2009))。癌症包括良性和恶性赘生物(异常生长)。

[0125] 术语“癌症”可以是指白血病、癌、肉瘤、腺癌、淋巴瘤、实体瘤和淋巴癌等。不同类型的癌症的实例包括但不限于急性骨髓性白血病(AML)、慢性骨髓性白血病(CML)、B-细胞淋巴瘤、非霍奇金氏淋巴瘤(non-Hodgkin’s lymphoma)、伯基特氏淋巴瘤(Burkitt’s lymphoma)、小细胞淋巴瘤、大细胞淋巴瘤、单核细胞性白血病、骨髓性白血病、急性淋巴细胞性白血病、多发性骨髓瘤、肺癌(如非小细胞肺癌或NSCLC)、卵巢癌、前列腺癌、结肠直肠癌、肝癌(liver cancer)(即肝癌(hepatocarcinoma))、肾癌(即肾细胞癌)、膀胱癌、乳腺癌、甲状腺癌、胸腔癌、胰腺癌、子宫癌、宫颈癌、睾丸癌、肛门癌、胰腺癌、胆管癌、胃肠道类癌肿瘤、食管癌、胆囊癌、阑尾癌、小肠癌、胃(胃部)癌、中枢神经系统癌、皮肤癌、绒毛膜癌；头颈癌、骨原性肉瘤、纤维肉瘤、神经母细胞瘤、神经胶质瘤和黑素瘤。

[0126] “癌症靶标”或“癌症标记物”是在癌症中、如在癌细胞上或在癌症环境中差异表达或加工的分子。示例性癌症靶标是细胞表面蛋白，诸如CLL-1(如也是细胞粘附分子和受体)、细胞内受体、激素和分子，诸如由细胞分泌到癌症环境中的蛋白酶。用于特定癌症的标记物在本领域中是已知的，如用于AML的CD45，用于AML CSC的CD34+CD38-，结肠和结肠直肠癌上的MUC1表达，肺癌中的铃蟾肽受体，及前列腺癌上的前列腺特异性膜抗原(PSMA)。

[0127] 在一些实施方案中，癌症靶标可以与某种类型的癌细胞有关，如AML、白血病、骨髓瘤、淋巴瘤、非小细胞肺癌细胞、前列腺癌、结肠直肠癌、乳腺癌或卵巢癌。细胞类型特异性靶标通常在此细胞类型中以比在参考细胞群体中高至少2倍的水平表达。在一些实施方案中，细胞类型特异性标记物的存在水平比其在参考群体中的平均表达高至少3、4、5、6、7、8、9、10、20、50、100或1000倍中的任一个。因此，可以检测或测量靶标以区分细胞类型或目标类型与其它细胞。例如，AML癌症靶标包括CLL-1、Ly86、LILRA1和CD180。

[0128] 癌症干细胞(CSC)是在肿瘤或血液癌症中存在的细胞，其可以产生构成大部分癌症的细胞。CSC还可以自我更新，类似于正常(非癌症)干细胞。因此，CSC可以通过迁移到个体中的非肿瘤组织并开始“新”肿瘤来介导转移。根据检测到癌症的阶段，CSC占任何给定癌症的非常小的百分比。例如，AML细胞样本中CSC的平均频率据信约为1:10,000。造血CSC可被鉴定为CD34+，与正常的造血干细胞(HSC)类似。

[0129] “经保守修饰的变体”适用于氨基酸和核酸序列。就具体的核酸序列而言，经保守修饰的变体是指那些编码相同氨基酸序列的核酸。由于遗传密码的简并性，大量功能相同的核酸编码大部分蛋白。例如，密码子GCA、GCC、GCG和GCU都编码氨基酸丙氨酸。因此，在密码子指定丙氨酸的每个位置处，密码子可以被改变为所述相应密码子中的另一个，而不改变编码的多肽。这种核酸变异是“沉默变异”，这是一种经保守修饰的变异。本文中编码多肽的每条核酸序列也描述了核酸的沉默变异。技术人员将认识到，在某些上下文中，核酸中的每个密码子(除了通常为甲硫氨酸的唯一密码子的AUG和通常为色氨酸的唯一密码子的TGG外)可被修饰以产生功能相同的分子。因此，编码多肽的核酸的沉默变异隐含在就表达产物而不是就实际探针序列而言的所述序列中。

[0130] 术语“重组”当涉及如细胞或核酸、蛋白或载体使用时，表示细胞、核酸、蛋白质或载体已经通过引入异源的核酸或蛋白或者改变的天然核酸或蛋白来修饰，或者细胞源自如此修饰的细胞。因此，例如，重组细胞表达在天然(非重组)形式的细胞内未发现的基因，或表达另外异常表达、低表达或完全不表达的天然基因。

[0131] 术语“异源的”当涉及多核苷酸或多肽时，表示多核苷酸或多肽包含在自然界中不存在彼此处于同一关系的两条或多条子序列。例如，异源的多核苷酸或多肽通常是重组产生的，具有来自不相关基因的两条或多条序列排列成新的功能单元，如来自一个来源的启动子和来自另一个来源的编码区。类似地，异源蛋白质表示该蛋白包含在自然界中未发现彼此处于同一关系的两条或多条子序列(如融合蛋白)。

[0132] “硫醇反应性试剂”是具有与硫醇反应形成共价键的部分的试剂。硫醇反应性试剂可具有选自卤化物、马来酰亚胺和磺酰基的基团。非限制性实例包括生物素-PEO-马来酰亚胺((+)-生物素基-3-马来酰亚胺基丙酰胺基-3,6-二氧杂辛二胺，Oda等人(2001)Nature Biotechnology 19:379-382,Pierce Biotechnology,Inc.)、生物素-BMCC、PEO-碘代乙酰基生物素、碘代乙酰基-LC-生物素和生物素-HPDP(Pierce Biotechnology,Inc.)以及Nα-(3-马来酰亚胺基丙酰基)生物胞素(MPB,Molecular Probes,Eugene,OR)。生物素化、双功能和多功能接头试剂的其它商业来源包括Molecular Probes,Eugene,Oreg.和Sigma,St.Louis,Mo。

[0133] II.编码经半胱氨酸取代的免疫球蛋白(如CYSMAB)的多核苷酸

[0134] 本文还提供了编码本文所述的经半胱氨酸取代的免疫球蛋白或其具有经半胱氨酸取代的恒定结构域的多核苷酸(如DNA)。编码经半胱氨酸取代的免疫球蛋白的多核苷酸可通过编码免疫球蛋白多肽的多核苷酸上的定点突变来制备。用于进行定点突变的试剂盒可从多个来源商购获得。这些包括例如，可从Life Technologies获得的Phusion，可从Agilent Technologies获得的QuikChange，及可从New England Biolabs获得的Q5。通常，定点突变涉及使用在所需位点处包含突变插入cys密码子的引物进行的靶标免疫球蛋白-编码多核苷酸的引物延伸。

[0135] 本文还提供了表达盒，其包含可操作连接至编码本文所述的经半胱氨酸取代的免疫球蛋白或其具有经半胱氨酸取代的恒定结构域的多核苷酸的启动子。在一些实施方案中，启动子是异源的，即并非天然存在的可操作地连接至编码序列。在一些实施方案中，包含编码本文所述的经半胱氨酸取代的免疫球蛋白或其具有经半胱氨酸取代的恒定结构域的多核苷酸的载体(包括但不限于表达载体或穿梭载体)。本文还提供了细胞，其包含且任

选地表达编码本文所述的经半胱氨酸取代的免疫球蛋白或其具有经半胱氨酸取代的恒定结构域的多核苷酸。示例性细胞包括原核细胞(包括但不限于大肠埃希氏杆菌(E.coli))和真核细胞(包括但不限于哺乳动物(如人、仓鼠、大鼠、小鼠等)细胞、真菌(如真菌)或植物细胞)。

[0136] III.制备抗体的方法

[0137] 为了制备本文所述的免疫球蛋白,如重组、单克隆或多克隆抗体,可使用本领域已知的许多技术(参见,如Kohler&Milstein,Nature 256:495-497(1975);Kozbor等人,Immunology Today 4:72(1983);Cole等,第77-96页,in Monoclonal Antibodies and Cancer Therapy,Alan R.Liss,Inc.(1985);Coligan,Current Protocols in Immunology(1991);Harlow&Lane,Antibodies,A Laboratory Manual(1988);以及Goding,Monoclonal Antibodies:Principles and Practice(第2版,1986))。编码目标抗体的重链和轻链的基因可以从细胞中克隆,如编码单克隆抗体的基因可以从杂交瘤中克隆并用于产生重组单克隆抗体。编码单克隆抗体的重链和轻链的基因文库也可以由杂交瘤或浆细胞制成。重链和轻链基因产物的随机组合产生具有不同抗原特异性的大型抗体库(参见,如Kuby,Immunology(第3版,1997))。用于单链抗体或重组抗体产生的技术(美国专利第4,946,778号,美国专利第4,816,567号)可经调整以产生针对本公开的多肽的抗体。此外,转基因小鼠或其它生物体,诸如其它哺乳动物,可用于表达人源化或人抗体(参见,如美国专利第5,545,807号;第5,545,806号;第5,569,825号;第5,625,126号;第5,633,425号;第5,661,016号,Marks等人,Bio/Technology 10:779-783(1992);Lonberg等人,Nature 368:856-859(1994);Morrison,Nature 368:812-13(1994);Fishwild等人,Nature Biotechnology 14:845-51(1996);Neuberger,Nature Biotechnology 14:826(1996);以及Lonberg&Huszar,Intern.Rev.Immunol.13:65-93(1995))。可替代地,噬菌体展示技术可用于鉴定特异性结合所选抗原的抗体和异聚Fab片段(参见,如McCafferty等人,Nature 348:552-554(1990);Marks等人,Biotechnology 10:779-783(1992))。抗体也可以制成双特异性的,即能识别两种不同的抗原(参见,如WO 93/08829,Traunecker等人,EMBO J.10:3655-3659(1991);以及Suresh等人,Methods in Enzymology 121:210(1986))。抗体也可以是异源缀合物,如两个共价连接的抗体或免疫毒素(参见,如美国专利第4,676,980号,WO 91/00360;WO 92/200373;和EP 03089)。

[0138] 可以使用任何数量的表达系统来产生抗体,这包括原核和真核表达系统。在一些实施方案中,表达系统是哺乳动物细胞表达,诸如杂交瘤或CHO细胞表达系统。许多此类系统可普遍获得自商业供应商。在抗体包含V_H和V_L区两者的实施方案中,可以使用单一载体,例如在双顺反子表达单元中,或者在不同的启动子的控制下表达V_H和V_L。在其它实施方案中,V_H和V_L区可以使用分开的载体来表达。如本文所述的V_H和V_L区可以任选地在N-末端处包含甲硫氨酸。

[0139] 本公开的抗体还可以以各种形式产生,包括Fab、Fab′、$F(ab')_2$、scFv或dAb。抗体片段可以通过多种方法获得,包括用酶(如胃蛋白酶)消化完整抗体(以产生$(Fab')_2$片段)或木瓜蛋白酶消化完整抗体(以产生Fab片段);或者从头合成。抗体片段也可以使用重组DNA方法来合成。在一些实施方案中,CLL-1抗体包含特异性结合CLL-1的$F(ab')_2$片段。本公开的抗体还可以包含人恒定区。参见,如Fundamental Immunology(Paul编,第4版,1999);

Bird,等人,Science 242:423(1988);以及Huston,等人,Proc.Natl.Acad.Sci.USA 85:5879(1988)。

[0140] 用于人源化非人抗体(即,使用来自非人抗体的CDR)的方法也是本领域已知的。通常,人源化抗体具有来自非人来源的一个或多个氨基酸残基。这些非人的氨基酸残基通常被称为导入残基,其通常取自导入可变结构域。人源化基本上可以按照Winter和同事的方法通过将啮齿类动物CDR或CDR序列取代为相应的人抗体序列进行(参见,如Jones等人,Nature 321:522-525(1986);Riechmann等人,Nature 332:323-327(1988);Verhoeyen等人,Science 239:1534-1536(1988)以及Presta,Curr.Op.Struct.Biol.2:593-596(1992))。此类人源化抗体是嵌合抗体(美国专利第4,816,567号),其中基本上小于一个完整的人可变结构域已被来自非人物种的相应序列取代。在实践中,人源化抗体通常是人抗体,其中一些CDR残基和可能的一些FR残基被来自啮齿类动物抗体中类似位点的残基取代。

[0141] 在一些情况下,抗体或抗体片段可以缀合至另一分子,如聚乙二醇(PEG化)或血清白蛋白,以提供延长的体内半衰期。抗体片段的PEG化的实例提供于Knight等人Platelets 15:409,2004(对于阿昔单抗);Pedley等人,Br.J.Cancer 70:1126,1994(对于抗-CEA抗体);Chapman等人,Nature Biotech.17:780,1999;以及Humphreys,等人,Protein Eng.Des.20:227 2007中。抗体或抗体片段还可如下所述标记至或缀合至治疗剂。

[0142] IV.经半胱氨酸取代的免疫球蛋白(如CYSMAB)药物缀合物的制备

[0143] 由本公开的经半胱氨酸取代的免疫球蛋白(CYSMAB)制备的抗体-药物缀合物可以采用本领域技术人员已知的有机化学反应、条件和试剂通过几种途径制备,包括:(1)经半胱氨酸工程化的抗体的半胱氨酸基团与接头试剂反应,以经由共价键形成抗体-接头中间体Ab-L,然后与激活的药物部分D反应;和(2)药物部分的亲核基团与接头试剂反应,以经由共价键形成药物-接头中间体D-L,然后与经半胱氨酸工程化的抗体(CYSMAB)的半胱氨酸基团反应。缀合方法(1)和(2)可与多种经半胱氨酸工程化的抗体(CYSMAB)、药物部分和接头一起使用来制备抗体-药物缀合物(ADC)。

[0144] 抗体半胱氨酸硫醇基团是亲核性的并且能够与接头试剂和药物-接头中间体上的亲电子基团反应以形成共价键,包括:(i)活性酯,诸如NHS酯、HOBt酯、卤代甲酸酯和酸性卤化物;(ii)烷基和苄基卤化物,诸如卤代乙酰胺;(iii)醛、酮、羧基和马来酰亚胺基团;和(iv)二硫化物,包括吡啶基二硫化物,经由硫化物交换。药物部分上的亲核基团包括但不限于:能够与接头部分和接头试剂上的亲电子基团反应形成共价键的胺、硫醇、羟基、酰肼、肟、肼、缩氨基硫脲、肼羧酸酯和芳基酰肼基团。

[0145] 通过用还原剂诸如DTT(克莱兰氏试剂(Cleland's reagent),二硫苏糖醇)或TCEP(三(2-羧乙基)膦盐酸盐;Getz等(1999)Anal.Biochem.第273卷:73-80;Soltec Ventures,Beverly,Mass.)处理可使经半胱氨酸工程化的抗体具有反应性以与接头试剂缀合,然后如用DHAA再氧化以重新形成链间和链内二硫键(实施例2)。

[0146] A.接头

[0147] “接头”、“接头单元”或“连接物”意指包含将抗体共价附接至药物部分的共价键或原子链的化学部分。在各种实施方案中,接头被指定为L。“接头”(L)是双功能部分或多功能部分,其可用于连接一个或多个药物部分(D)和抗体单元(Ab)以形成抗体-药物缀合物(ADC)。抗体-药物缀合物(ADC)可以使用具有用于与药物和抗体结合的反应性官能团的接

头来方便地制备。经半胱氨酸工程化的抗体(CYSMAB)的半胱氨酸硫醇可以与接头试剂、药物部分或药物-接头中间体的亲电子官能团形成键。

[0148] 在一方面，接头具有反应性位点，其具有与存在于抗体上的亲核半胱氨酸反应的亲电子基团。抗体的半胱氨酸硫醇与接头上的亲电子基团反应并与接头形成共价键。有用的亲电子基团包括但不限于马来酰亚胺和卤代乙酰胺基团。

[0149] 接头包括二价基团，诸如烷基二基、亚芳基、亚杂芳基、诸如—$(CR_2)_nO(CR_2)_n$—的部分、烷基氧基的重复单元(如聚乙烯氧基、PEG、聚亚甲基氧基)和烷基氨基的重复单元(如聚乙烯氨基、Jeffamine™)；以及二酸酯和酰胺，包括琥珀酸酯、琥珀酰胺、二甘醇酸酯、丙二酸酯和己酰胺。

[0150] 根据Klussman,等人(2004),Bioconjugate Chemistry 15(4):765-773的第766页的缀合方法，使经半胱氨酸工程化的抗体(CYSMAB)与具有亲电子官能团诸如马来酰亚胺或α-卤代羰基的接头试剂或药物-接头中间体反应。

[0151] 接头可由一种或多种接头组分组成。示例性接头组分包括6-马来酰亚胺基己酰基(“MC”)、马来酰亚胺基丙酰基(“MP”)、缬氨酸-瓜氨酸(“val-cit”或“vc”)、丙氨酸-苯丙氨酸(“ala-phe”或“af”)、对氨基苯甲基氧羰基(“PAB”)、N-琥珀酰亚胺基4-(2-吡啶基硫代)戊酸酯(“SPP”)、N-琥珀酰亚胺基4-(N-马来酰亚胺基甲基)环己烷-1羧酸酯(“SMCC”)、N-琥珀酰亚胺基(4-碘代-乙酰基)氨基苯甲酸酯(“SIAB”)、乙烯氧基—CH2CH2O—作为一个或多个重复单元(“EO”或“PEO”)。另外的接头组分是本领域已知的且一些描述于本文中。

[0152] 在另一个实施方案中，接头具有反应性官能团，其具有对存在于抗体上的亲电子基团具有反应性的亲核基团。抗体上有用的亲电子基团包括但不限于醛和酮羰基基团。接头的亲核基团的杂原子可以与抗体上的亲电子基团反应并与抗体单元形成共价键。有用的亲核基团包括但不限于酰肼、肟、氨基、肼、缩氨基硫脲、羧酸肼和芳基酰肼。抗体上的亲电子基团提供了用于附接至接头的方便的位点。

[0153] 通常，肽型接头可通过在两个或更多个氨基酸和/或肽片段之间形成肽键来制备。可以例如根据肽化学领域熟知的液相合成方法(E.Schröder和K.Lübke(1965)“The Peptides”,第1卷，第76-136页,Academic Press)制备此类肽键。可将接头中间体与任何包括间隔子、拉伸子和氨基酸单位的反应的组合或序列装配。间隔子、拉伸子和氨基酸单元可采用本质上是亲电、亲核或自由基的反应性官能团。反应性官能团包括但不限于羧基、羟基、对硝基苯基碳酸酯、异硫氰酸酯和离去基团，诸如O-甲磺酰基、O-甲苯磺酰基、—Cl、—Br、—I;或马来酰亚胺。

[0154] 在另一个实施方案中，接头可能被调节溶解度或反应性的基团所取代。例如，带电取代基诸如磺酸根(—SO3-)或铵可以增加试剂的水溶性，并促进接头试剂与抗体或药物部分的偶联反应，或促进Ab-L(抗体-接头中间体)与D的偶联反应或者D-L(药物-接头中间体)与Ab的偶联反应，这取决于用于制备ADC的合成路线。

[0155] 可根据Dubowchik,等人(1997)Tetrahedron Letters,38:5257-60制备包含马来酰亚胺部分和PAB自分解性部分的示例性phe-lys(Mtr,单-4-甲氧基三苯甲基)二肽接头试剂。

[0156] B.接头试剂

[0157] 抗体和阿里他汀的缀合物可使用多种双功能接头试剂来制备，诸如N-琥珀酰亚胺

基-3-(2-吡啶基二硫代)丙酸酯(SPDP)、琥珀酰亚胺基-4-(N-马来酰亚胺基甲基)环己烷-1-羧酸酯(SMCC)、亚胺硫醇烷(IT)、亚胺酯的双功能衍生物(诸如二亚胺代己二酸二甲酯HCl)、活性酯(诸如辛二酸二琥珀酰亚胺酯)、醛(诸如戊二醛)、双-叠氮化合物(诸如双(对叠氮苯甲酰基)己二胺)、双-重氮衍生物(诸如双-(对重氮苯甲酰基)-乙二胺)、二异氰酸酯(诸如甲苯2,6-二异氰酸酯)和双活性氟化合物(诸如1,5-二氟-2,4-二硝基苯)。

[0158] 可用于本公开的抗体药物缀合物(ADC)的接头试剂包括但不限于:BMPEO、BMPS、EMCS、GMBS、HBVS、LC-SMCC、MBS、MPBH、SBAP、SIA、SIAB、SMCC、SMPB、SMPH、磺基-EMCS、磺基-GMBS、磺基-KMUS、磺基-MBS、磺基-SIAB、磺基-SMCC和磺基-SMPB以及SVSB(琥珀酰亚胺基-(4-乙烯砜)苯甲酸酯),并且包括双-马来酰亚胺试剂:DTME、BMB、BMDB、BMH、BMOE、1,8-双-马来酰亚胺基二乙二醇(BM(PEO)2)和1,11-双-马来酰亚胺基三乙二醇(BM(PEO)3),其可从Pierce Biotechnology,Inc.,ThermoScientific,Rockford,Ill.和其它试剂供应商处商购获得。双马来酰亚胺试剂允许将抗体的半胱氨酸残基的游离硫醇基团以依序或同时形式附接至含巯基的药物部分、标记物或接头中间体。除马来酰亚胺之外的与抗体的硫醇基团、奈莫柔比星代谢物和类似物药物部分或接头中间体反应的其它官能团包括碘乙酰胺、溴乙酰胺、乙烯基吡啶、二硫化物、吡啶基二硫化物、异氰酸酯和异硫氰酸酯。

[0159] V.使用方法

[0160] 本公开的经半胱氨酸取代的免疫球蛋白尤其用于制备经半胱氨酸取代的免疫球蛋白缀合物,包括缀合至可检测部分或药物(诸如细胞毒性剂)的分子。

[0161] A.疾病治疗

[0162] 经半胱氨酸取代的免疫球蛋白药物缀合物(如CYSMAB ADC)可用于治疗可通过靶向此类缀合物结合的细胞来治疗的任何疾病。这包括任何形式的癌症。

[0163] 经半胱氨酸取代的免疫球蛋白药物缀合物可用于治疗例如特征为肿瘤抗原的过表达的各种疾病或病症。示例性病况或过度增生性病症包括良性或恶性肿瘤;白血病和淋巴样恶性肿瘤。其它包括神经元病症、神经胶质病症、星形胶质细胞病症、下丘脑病症、腺体病症、巨噬细胞病症、上皮细胞病症、间质细胞病症、囊胚腔病症、炎性病症、血管生成病症和免疫性病症,包括自身免疫性病症。

[0164] 经半胱氨酸取代的免疫球蛋白药物缀合物可在携带肿瘤的高等灵长类动物和人类临床试验中进一步检测。人临床试验可经设计为类似于测试已经接受广泛的前期抗癌疗法的过表达HER2的转移性乳腺癌患者中抗-HER2单克隆抗体HERCEPTIN®的效力的临床试验,如Baselga等人(1996)J.Clin.Oncol.14:737-744所报道。可以设计临床试验以评价ADC与已知的治疗方案(诸如涉及已知化疗剂和/或细胞毒性剂的放疗和/或化疗)的组合的效力。

[0165] 通常,待治疗的疾病或病症是过度增殖疾病,诸如癌症。本文中待治疗的癌症的实例包括但不限于癌、淋巴瘤、母细胞瘤、肉瘤和白血病或淋巴样恶性肿瘤。此类癌症的更具体的实例包括鳞状细胞癌(如上皮鳞状细胞癌),包括小细胞肺癌、非小细胞肺癌、肺癌腺癌和肺鳞癌的肺癌,腹膜癌,肝细胞癌,包括胃肠癌的胃部(gastric)癌或胃(stomach)癌,胰腺癌,胶质母细胞瘤,宫颈癌,卵巢癌,肝癌,膀胱癌,肝细胞瘤,乳腺癌,结肠癌,直肠癌,结肠直肠癌,子宫内膜癌或子宫癌,唾液腺癌,肾(kidney)癌或肾部(renal)癌,前列腺癌,外阴癌,甲状腺癌,肝癌,肛门癌,阴茎癌以及头颈癌。

[0166] 癌症可以包含HER2-表达细胞，使得本发明的ADC能够结合癌细胞。为了确定癌症中的ErbB2表达，可以使用各种诊断/预后测定。在一个实施方案中，可通过IHC例如使用HERCEPTEST(Dako)分析ErbB2过表达。可使来自肿瘤活检组织的石蜡包埋的组织切片经受IHC测定，并赋予如下的ErbB2蛋白染色强度标准：评分0，未观察到染色或在小于10％的肿瘤细胞中观察到膜染色；评分1+，在超过10％的肿瘤细胞中检测到微弱/几乎不可察觉的膜染色，细胞仅在其部分膜中染色；评分2+，在超过10％的肿瘤细胞中观察到弱至中度完全膜染色；评分3+，在超过10％的肿瘤细胞中观察到中度至强度的完全膜染色。那些ErbB2过表达评估评分为0或1+的肿瘤可被表征为不过表达ErbB2，而具有2+或3+评分的那些肿瘤可被表征为过表达ErbB2。

[0167] 可替代地或此外，FISH测定诸如INFORM™(Ventana Co.,Ariz.)或PATHVISION™(Vysis,Ill.)可以在福尔马林固定的石蜡包埋的肿瘤组织上进行以确定ErbB2过表达在肿瘤中的程度(如果有的话)。

[0168] ADC化合物可用于治疗的自身免疫性疾病包括类风湿性病症(诸如，例如，类风湿性关节炎、舍格伦氏综合征(Sjögren'ssyndrome)、硬皮病、狼疮诸如SLE和狼疮肾炎、多肌炎/皮肌炎、冷球蛋白血症、抗磷脂抗体综合征和银屑病性关节炎)，骨关节炎，自身免疫性胃肠和肝脏病症(例如，炎性肠病(如溃疡性结肠炎和克罗恩氏病(Crohn's disease))、自身免疫性胃炎和恶性贫血、自身免疫性肝炎、原发性胆汁性肝硬化、原发性硬化性胆管炎和乳糜泻)，血管炎(例如，ANCA-相关的血管炎，包括丘-施二氏血管炎(Churg-Strauss vasculitis)、韦格纳氏肉芽肿(Wegener's granulomatosis)和多动脉炎)，自身免疫性神经系统病症(诸如，例如，多发性硬化、斜视眼阵挛-肌阵挛综合征、重症肌无力、视神经脊髓炎、帕金森氏病(Parkinson's disease)、阿尔茨海默氏病(Alzheimer's disease)和自身免疫性多神经病)，肾病症(例如，肾小球性肾炎、古德帕斯彻氏综合征(Goodpasture's syndrome)和伯杰氏病(Berger's disease)，自身免疫性皮肤病症(例如，银屑病、荨麻疹、麻疹、寻常性天疱疮、大疱性类天疱疮和皮肤红斑狼疮)，血液学病症(例如，血小板减少性紫癜、血栓性血小板减少性紫癜、输注后紫癜和自身免疫性溶血性贫血)，动脉粥样硬化，葡萄膜炎，自身免疫性听力疾病(例如，内耳病和听力丧失)，白塞氏病(Behcet's disease)，雷诺氏综合征(Raynaud's syndrome)，器官移植和自身免疫性内分泌病症(例如，糖尿病-相关的自身免疫性疾病，诸如胰岛素依赖性糖尿病(IDDM)、艾迪生氏病(Addison's disease)和自身免疫性甲状腺疾病(如格雷夫斯病(Graves'disease)和甲状腺炎))。更优选的此类疾病包括例如类风湿性关节炎、溃疡性结肠炎、ANCA-相关的血管炎、狼疮、多发性硬化、舍格伦氏综合征、IDDM、恶性贫血、甲状腺炎和肾小球肾炎。

[0169] 本文所述的经半胱氨酸取代的免疫球蛋白缀合物可用于检测和治疗CLL-1相关的病症，即与标准对照(如正常、非疾病、非癌细胞)中的CLL-1表达相比，CLL-1的细胞表面表达升高或降低相关的疾病。CLL-1表达通常限于髓系细胞，如外周血和脾中的树突细胞、粒细胞和单核细胞。升高的CLL-1水平与癌症相关，尤其是在造血CSC(如LSC)和骨髓增生性病症中，包括白血病诸如AML(急性骨髓性或骨髓增生性白血病)、MDS(骨髓增生异常综合征)、骨髓纤维化、CMML(慢性骨髓单核细胞性白血病)、多发性骨髓瘤、浆细胞瘤和CML(慢性骨髓性或骨髓增生性白血病)。参见Bakker等人(2004)Cancer Res.64:8443;Van Rhenen等(2007)Blood 110:2659-66;Zhao等人(2010)Haematologica(2010)95:71;Van Rhenen等人

(2007)Leukemia 21:1700;以及Herrmann等人(2012)Haematologica 97:219。

[0170] AML细胞可通过检测细胞表面标记物表达来表征和区别于其它细胞。除了CLL-1+之外,AML细胞可以是CD33+(虽然有些是CD33-)、CD45+和CDw52+。AML胚细胞(包括LSC)通常是CD34+CD38-。HSC和LSC可通过表达CD34来表征,但前者不表达CLL-1。MDS细胞可以通过表达CD5、CD7、CD13和CD34来表征。CML细胞可通过表达7-ADD、CD33、CD34和CD38来表征。

[0171] 骨髓增生异常综合征(MDS)包括一组密切相关的血液形成病症,其中骨髓显示出定性和定量的改变,指示白血病前期过程但具有不一定终止为急性白血病的慢性过程。多个术语,包括白血病、难治性贫血、难治性骨髓细胞生成障碍性贫血、郁积性或亚急性白血病、骨髓细胞生成障碍综合征(DMPS)和脊髓发育不良都被用来描述MDS。这些病况的特征都是细胞骨髓成熟受损(骨髓细胞生成障碍)和血细胞数量减少。DMPS的特征是存在巨型母细胞样细胞(megablastoids)、巨核细胞发育异常和异常的母细胞数量增加,反映出增强的粒细胞成熟过程。DMPS患者显示出与急性髓性白血病中发现的染色体异常相似的染色体异常,并且在一定比例的患病患者中进展为急性髓性白血病。

[0172] 慢性骨髓增生性病症是以成熟和未成熟粒细胞、红细胞和血小板数量增加为特征的一组病况。慢性骨髓增生性病症可以在这一组内转变为其它形式,倾向于终止于急性髓性白血病。该组中的特定疾病包括真性红细胞增多症、慢性髓性白血病、原因不明的髓性白血病、原发性血小板增多症和慢性嗜中性粒细胞性白血病。

[0173] 骨髓纤维化的特征在于骨髓瘢痕形成,导致红细胞和白细胞以及血小板的数量减少。骨髓纤维化瘢痕形成可由白血病引起,但也可有其它原因,诸如血小板增多症或不良药物作用。

[0174] B.CDC、ADCC和ADC测定

[0175] 可通过表达靶标抗原的细胞(诸如CLL-1)的补体依赖性细胞毒性(CDC)、抗体依赖性细胞-介导的细胞毒性(ADCC)测定评价本公开的经半胱氨酸取代的免疫球蛋白药物缀合物(如CYSMAB ADC)的效果。表达CLL-1的示例性细胞包括表达异源的重组CLL-1(如人CLL-1)的细胞系;人AML细胞系,诸如HL60、THP1、TF1-α、U937和OCI AML-5(其中前四种可从ATCC获得);来自一个或多个AML患者的原代细胞(如PBMC或移植的肿瘤细胞);人CML细胞系,诸如K562和KU812(可从ATCC获得);以及来自一个或多个CML或MDS患者的原代细胞。

[0176] 将抗体描述为具有CDC活性,并且如果其导致表达抗体靶标的细胞的补体依赖性杀灭,则介导CDC。CDC测定在本领域是已知的,且描述于如Gazzano-Santoro等人(1997)J.Immunol.Methods 202:163;Idusogie等人(2000)J.Immunol.164:4178;和以下实施例6中。CDC试剂盒和服务可例如从GeneScript®和Cell Technology Inc处商购获得。

[0177] 简言之,测定通常在体外进行,并且包括抗体结合在细胞表面上表达抗体靶标的细胞。添加补体组分,包括结合抗体的Ch区的C1q,22。然后,补体组分相互作用以杀灭所靶向的细胞。通常在4至24小时之间的孵育时间段后,例如通过测定已知存在于所靶向的细胞中的胞内酶或颗粒的释放,通过比较起始和终止靶标细胞群等来测量CDC。

[0178] 如果抗体导致效应细胞杀灭抗体-结合的细胞(如CLL-1表达细胞),则将抗体描述为具有ADCC活性并介导ADCC。效应细胞通常是自然杀伤细胞,但也可以是巨噬细胞、嗜中性粒细胞或嗜酸性粒细胞。

[0179] 经遗传工程化的效应细胞系也被开发用于ADCC测定(参见,如Schnueriger等人

(2011)Mol.Immunol.48:1512)。ADCC测定在本领域是已知的,并且描述于如Perussia和Loza(2000)Methods in Mol.Biol.121:179;Bretaudeau和Bonnaudet(2011)BMC Proceedings 5(增刊8):P63;ADCC试剂盒和服务可例如从GeneScript®和Promega®商购获得,及以下实施例中。

[0180] 简言之,测定通常在体外进行,并且包括抗体结合在细胞表面上表达抗体靶标的细胞。添加效应细胞,其通常通过Fc受体诸如CD 16识别抗体-结合的细胞。效应细胞杀灭抗体结合的细胞,如通过释放导致细胞凋亡的细胞毒素。通过释放靶细胞内的可检测元素(如Cr51)或通过检测参与细胞介导的毒性的元素(如效应细胞中NFAT信号传导的激活)来检测细胞死亡。

[0181] 当抗体与细胞毒性剂(药物)缀合,导致杀灭(抑制存活)表达抗体靶标的细胞(此情况下是CLL-1)时,抗体被描述为具有抗体-药物缀合物(ADC)活性(或介导ADC)。适当的细胞毒性剂是本领域已知的,如皂草素(saporin)、阿霉素(doxorubicin)、道诺霉素(daunomycin)、长春花生物碱、紫杉烷类、微管蛋白剂(如美登新(Maytansin)、阿里他汀)和DNA剂(如卡奇霉素(calicheamicin)、倍癌霉素(duocarmycin)、吡咯并苯二氮䓬二聚体)等。ADC测定在本领域中是已知的,如Gerber等人(2009)3:247和以下实施例中所述。

[0182] C.诊断性应用

[0183] 因此,经半胱氨酸取代的免疫球蛋白缀合物可用于体外和体内诊断性测定以检测癌细胞。这包括本文所述的对CLL-1具有特异性的抗体特异性结合CLL-1-表达细胞("CLL-1抗体"-仅用于本节)以用于检测CLL-1表达细胞(如AML细胞和AML CSC)。例如,可以从患者获得样品(如血液样品或组织活检组织)并使其与CLL-1抗体接触,并且患者样品中CLL-1-表达细胞的存在可以通过检测抗体结合来确定。可以直接(如当标记抗体本身时)或通过使用第二种检测剂诸如二级抗体来检测抗体结合。可检测的标记物可以直接或间接地如经由螯合剂或接头与本公开的抗体缔合。

[0184] 在一些实施方案中,将CLL-1抗体与来自患有或疑似患有CLL-1相关的病症的个体的生物样品接触,并且确定样品中结合细胞的抗体,其中高于或低于正常的抗体结合表明个体患有CLL-1相关的病症。在一些实施方案中,生物样品是血液样品或血液级分(如血清、血浆、血小板、红细胞、白细胞、PBMC)。在一些实施方案中,生物样品是组织样品(活检组织),如来自疑似的肿瘤位点或来自已知受感染的组织,以例如确定已知肿瘤的边界。

[0185] 通常进行活检以从组织中获得样品,即非流体细胞类型。应用的活检技术将取决于待评价的组织类型(如乳房、皮肤、结肠、前列腺、肾、肺、膀胱、淋巴结、肝、骨髓、气道或肺)。在癌症的情况下,该技术还将取决于肿瘤的大小和类型(如实体、混悬的或血液)等因素。代表性活检技术包括但不限于切除活检、切开活检、针活检、手术活检和骨髓活检。"切除活检"是指去除具有围绕其的正常组织的小边缘的整个肿瘤块。"切开活检"是指去除包含横截面直径的肿瘤的楔形组织。通过内窥镜检查或荧光镜检查进行的诊断或预后可能需要肿瘤块的"核心-针活检"或通常从肿瘤块内获得细胞混悬液的"细针抽吸活检"。在例如Harrison's Principles of Internal Medicine,Kasper,等人编,第16版,2005,第70章以及第五部分全文中讨论了活检技术。

[0186] 检测样品中结合细胞的抗体的任何方法都可以用于本诊断性测定。检测抗体结合的方法在本领域是公知的,如流式细胞术、荧光显微术、ELISA等。在一些实施方案中,该方

法包括在确定步骤之前制备用于检测的生物样品。例如，细胞(如白细胞、CD34+细胞、CD45+细胞等)亚群可以从来自个体的样品的其余部分(如其它血液组分)中分离出来，或者可混悬组织中的细胞以用于更容易的检测。

[0187] 在一些实施方案中，确定样品中CLL-1-表达细胞的百分比并将其与对照比较，如来自已知患有CLL-1相关的病症的个体或个体组的样品(阳性对照)或来自已知不患有CLL-1相关的病症的个体或个体组(正常、健康、非疾病或阴性对照)。在一些实施方案中，对照是针对给定组织建立的CLL-1表达的标准范围。高于或低于CLL-1表达细胞的正常百分比，或更高或更低的表达水平，指示该个体患有CLL-1相关的病症。

[0188] 在一些实施方案中，经标记的CLL-1抗体可以被提供(施用)给个体以确定预期疗法的适用性。例如，经标记的抗体可用于检测患病区域内的CLL-1密度，其中所述密度相对于未患病组织通常较高。经标记的抗体也可以指示病变区域是可用于治疗的。因此，可根据成像结果选择用于治疗的患者。可以使用标准成像技术(如CT扫描、MRI、PET扫描等)来完成解剖学表征，诸如确定癌症的精确边界。

[0189] 在一些实施方案中，如本文所述的经标记的CLL-1抗体可以进一步与治疗性化合物缔合，如以形成“治疗诊断”组合物。例如，CLL-1抗体可(直接地或间接地)连接至可检测的标记物和治疗剂，如细胞毒性剂以杀灭CLL-1-表达癌细胞。在一些实施方案中，将经标记的CLL-1抗体用于表达CLL-1的癌细胞的诊断和/或定位，然后用单独的治疗性CLL-1特异性抗体靶向表达CLL-1的癌细胞。在一些实施方案中，诊断性CLL-1特异性抗体是不以高比率或高百分比内化到CLL-1-表达细胞中的抗体。在一些实施方案中，治疗性CLL-1抗体以高比率或高百分比内化到CLL-1-表达细胞中。

[0190] 1.标记物

[0191] 包含能够结合靶标目标的抗体的诊断剂可包括本领域已知的任何诊断剂，例如，如在以下参考文献中提供:Armstrong等人，Diagnostic Imaging，第5版，Blackwell Publishing(2004)；Torchilin，V.P.，编，Targeted Delivery of Imaging Agents，CRC Press(1995)；Vallabhajosula，S.，Molecular Imaging:Radiopharmaceuticals for PET and SPECT，Springer(2009)。可通过多种方式检测诊断剂，包括作为提供和/或增强可检测信号的试剂。可检测的信号包括但不限于γ-发射信号、放射性信号、回声信号、光学信号、荧光信号、吸收信号、磁性信号或断层摄影信号。用于对诊断剂成像的技术可包括但不限于单光子发射计算机断层摄影(SPECT)、磁共振成像(MRI)、光学成像、正电子发射断层摄影(PET)、计算机断层摄影(CT)、X射线成像、伽马射线成像等。术语“可检测试剂”、“可检测部分”、“标记物”、“成像剂”和类似术语在本文中同义使用。

[0192] 在一些实施方案中，标记物可以包括光学剂，诸如荧光剂、磷光剂、化学发光剂等。许多试剂(如染料、探针、标记物或指示剂)是本领域已知的并且可用于本公开。(参见如Invitrogen，The Handbook—A Guide to Fluorescent Probes and Labeling Technologies，第十版(2005))。荧光剂可以包括多种有机和/或无机小分子或多种荧光蛋白及其衍生物。例如，荧光剂可以包括但不限于菁、酞菁、卟啉、吲哚菁、罗丹明、吩噁嗪、苯基呫吨、吩噻嗪、吩硒嗪、荧光素、苯并卟啉、方酸菁、二吡咯并嘧啶酮、并四苯、喹啉、吡嗪、呫吖、克酮酸、吖啶酮、菲啶、罗丹明、吖啶、蒽醌、硫属元素的吡喃盐(chalcogenopyrylium)类似物、二氢卟酚、萘酞菁、次甲基染料、吲哚鎓染料、偶氮化合物、甘菊蓝、氮杂甘菊蓝、三

苯基甲烷染料、吲哚、苯并吲哚、吲哚羰花青、苯并吲哚羰花青和BODIPY™衍生物。荧光染料讨论于例如，美国专利第4,452,720号、美国专利第5,227,487号和美国专利第5,543,295号中。

[0193] 标记物还可为放射性同位素，如发射γ射线、正电子、β和α颗粒及X-射线的放射性核素。适合的放射性核素包括但不限于225Ac、72As、211At、11B、128Ba、212Bi、75Br、77Br、14C、109Cd、2Cu、64Cu、67Cu、18F、67Ga、68Ga、3H、166Ho、123I、124I、125I、130I、131I、111In、177Lu、13N、150、32P、33P、212Pb、103Pd、186Re、188Re、47Sc、153Sm、89Sr、99mTc、88Y和90Y。在一些实施方案中，放射活性剂可包括111In-DTPA、99mTc(CO)3-DTPA、99mTc(CO)3-ENPy2、62/64/67Cu-TETA、99mTc(CO)3-IDA和99mTc(CO)3-三胺(环形或线性)。在一些实施方案中，该剂可包括具有111In、177Lu、153Sm、62/64/67Cu或67/68Ga的DOTA及其各种类似物。在一些实施方案中，纳米颗粒可以通过掺入附接至螯合物的脂质(诸如DTPA-脂质)而被标记，如在以下参考文献中提供：Phillips等人，Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology,1(1):69-83(2008)；Torchilin,V.P.&Weissig,V.,编Liposomes第2版:Oxford Univ.Press(2003)；Elbayoumi,T.A.&Torchilin,V.P.,Eur.J.Nucl.Med.Mol.Imaging.33:1196-1205(2006)；Mougin-Degraef,M.等人，Int'l J.Pharmaceutics 344:110-117(2007)。

[0194] 在一些实施方案中，诊断剂可以与次级结合配体或与在与发色底物接触后将会产生有色产物的酶(酶标签)缔合。适合的酶的实例包括脲酶、碱性磷酸酶、(辣根)过氧化氢酶和葡萄糖氧化酶。次级结合配体包括例如如本领域已知的生物素和抗生物素蛋白或链霉亲和素化合物。

[0195] 在一些实施方案中，经标记的抗体可以进一步与改善体内稳定性的组合物，例如PEG或纳米颗粒诸如脂质体缔合，如下面更详细所描述。

[0196] 2.标记方法

[0197] 用于缀合可检测剂和治疗剂至抗体的技术是熟知的(参见，如Amon等人，“Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy”于Monoclonal Antibodies And Cancer Therapy,Reisfeld等人(编)，第243-56页(Alan R.Liss,Inc.1985)；Hellstrom等人，“Antibodies For Drug Delivery”于Controlled Drug Delivery(第2版)，Robinson等人(编)，第623-53页(Marcel Dekker,Inc.1987)；Thorpe，“Antibody Carriers Of Cytotoxic Agents In Cancer Therapy:A Review”于Monoclonal Antibodies'84:Biological And Clinical Applications,Pinchera等人(编)，第475-506页(1985)；以及Thorpe等人，“The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates”,Immunol.Rev.,62:119-58(1982))。

[0198] 通常，抗体附接至不干扰表位结合的区域中的可检测部分。因此，在一些情况下，将可检测部分附接至恒定区，或在可变区中的CDR之外。本领域技术人员将认识到，可检测部分可位于抗体上的其它位置，并且可检测部分的位置可以相应地调整。在一些实施方案中，比较附接至可检测部分之前和之后的抗体与表位缔合的能力，以确保附接不会不适当地干扰结合。

[0199] 在一些实施方案中，抗体可以与另外的靶向部分缔合。例如，结合靶标分子或靶标细胞上不同位点的抗体片段、肽或适配体可缀合至抗体以优化靶标结合，例如结合癌细胞。

[0200] D.治疗性应用

[0201] 可使用本文所述的经半胱氨酸取代的CLL-1ADC抗体(“CLL-1抗体”-仅用于本节)靶向CLL-1-表达细胞,如AML细胞。CLL-1表达在AML细胞和CSC(如AML CSC)上升高。CLL-1在正常CD34+造血干细胞(HSC)上不显著表达,因此可使用本发明的CLL-1抗体将CSC与HSC区分。识别AML细胞共有的CLL-1表位并因此能够普遍结合AML细胞的高亲和力CLL-1抗体是特别有价值的,因为AML具有非常高的复发率。如上所述,包含CLL-1抗体的治疗性组合物还可包含可检测的标记物以形成治疗诊断组合物,例如用于CLL-1表达细胞的检测和定位,以及监测治疗效果。在2015年11月24日提交的USSN 62/259,100(Jiang等人,“Humanized Anti-”)和2013年11月7日公开的US2013/0295118(Jiang等人,“Antibodies Specific For CLL-1)中描述了抗体的结合CLL-1的序列,其通过引用将其整体并入本文。

[0202] 结合除CLL-1之外的靶标的抗体还可用于本文所述的经半胱氨酸取代的抗体和抗体缀合物中。在一些实施方案中,抗体靶标可选自GPR114、CLL-1、IL1RAP、TIM-3、CD19、CD20、CD22、ROR1、间皮素、CD33、CD123/IL3Ra、c-Met、PSMA、前列腺酸性磷酸酶(PAP)、CEA、CA-125、Muc-1、AFP、糖脂F77、EGFRvIII、GD-2、NY-ESO-1TCR、酪氨酸酶、TRPI/gp75、gp100/pmel-17、Melan-A/MART-1、Her2/neu、WT1、EphA3、端粒酶、HPV E6、HPV E7、EBNA1、BAGE、GAGE和MAGE A3TCRSLITRK6、ENPP3、连接蛋白-4、CD27、SLC44A4、CAIX、Cripto、CD30、MUC16、GPNMB、BCMA、Trop-2、组织因子(TF)、CanAg、EGFR、αv-整联蛋白、CD37、叶酸受体、CD138、CEACAM5、CD56、CD70、CD74、GCC、5T4、CD79b、Steap1、Napi2b、Lewis Y抗原、LIV、c-RET、DLL3、EFNA4或内皮唾酸蛋白/CD248。

[0203] 如本文所证明,本发明的CLL-1抗体可以抑制癌细胞生长(增殖和/或移植),并因此可以被认为是单独的化疗剂。以下公开提供了可与CLL-1抗体连接以用于对CLL-1-表达细胞的额外作用的化疗剂和细胞毒性剂的实例。

[0204] 化疗(抗-癌)剂可以是能够减少癌症生长、干扰癌细胞复制、直接或间接杀伤癌细胞、减少转移、减少肿瘤血液供应等的任何试剂。因此,化疗剂包括细胞毒素剂。细胞毒素剂包括但不限于皂草素、紫杉烷、长春花生物碱、蒽环霉素和铂基剂。化疗剂的种类包括但不限于烷化剂,抗代谢物如甲氨蝶呤,植物生物碱如长春新碱以及抗生素如阿霉素,以及不属于特定类别的其它药物如羟基脲。以顺铂和奥沙利铂为代表的铂基药物代表了一类主要的化疗剂。这些药物结合DNA并干扰复制。以紫杉酚为代表的紫杉烷代表另一种主要的化疗剂。这些化合物通过干扰细胞骨架和纺锤体形成来抑制细胞分裂,从而防止迅速分裂的癌细胞的生长来起作用。其它化疗治疗药物包括荷尔蒙疗法。药物部分可包括细胞毒性剂,诸如单体的或二聚体的苯二氮䓬衍生物(参见,如美国专利申请第15/048,865号,其通过引用并入本文)、多拉司他汀、阿里他汀、类美登素、多拉司他汀、小管素、念珠藻素、吡咯并苯二氮䓬(PBD)二聚体、吲哚啉并苯二氮䓬二聚体、异喹啉烷并苯二氮䓬二聚体(包括但不限于如下所述的D202)、α-鹅膏蕈碱、单端胞菌毒素、SN-38、倍癌霉素、CC1065、加利车霉素、烯二炔抗生素、紫杉烷、阿霉素衍生物、蒽环霉素和立体异构体、azanofide以及它们的等排体、类似物或衍生物。

[0205] 多于一种的治疗剂可在相同的组合物中或在分开的组合物中进行组合。治疗剂还可以与另外的治疗剂组合,以适合于特定个体。为癌症患者提供的常见治疗剂包括用于解决癌症患者常经历的疼痛、恶心、贫血、感染、炎症和其它症状的医药。

[0206] 抗体可以使用各种已知的交联剂附接至治疗剂、可检测剂或纳米载体上。用于共价或非共价附接多肽的方法在本领域是公知的。此类方法可包括但不限于使用化学交联-接头、光活化交联-接头和/或双功能交联剂。用于交联分子的示例性方法公开于美国专利第5,603,872号和美国专利第5,401,511号中。交联试剂的非限制性实例包括戊二醛、双功能环氧乙烷、乙二醇二缩水甘油醚、碳二亚胺如1-乙基-3-(3-二甲基氨基丙基)碳二亚胺或二环己基碳二亚胺、双酰亚胺酯、二硝基苯、辛二酸的N-羟基琥珀酰亚胺酯、酒石酸二琥珀酰亚胺酯、二甲基-3,3'-二硫代-双丙酰亚胺酸酯、叠氮基乙二醛、N-琥珀酰亚胺基-3-(2-吡啶基二硫代)丙酸酯和4-(溴代乙基氨基乙基)-2-硝基苯基叠氮化物。

[0207] 在一些实施方案中,CLL-1抗体与纳米载剂相缔合。对于缀合至纳米载剂(如脂质体)的抗体,表面上将会存在一定数量的抗体,即以给定的表面密度。在一些实施方案中,纳米载剂将具有每纳米载剂至少5个抗体,如每纳米载剂至少10、30、40、50、75、100或更高个的抗体。本领域技术人员将理解,表面密度代表平均范围,因为每纳米载剂的抗体数目对于所有群体成员不是绝对一致的。

[0208] 纳米载剂包括囊泡如脂质体和胶束,以及聚合物纳米颗粒等。纳米载剂可用于递送治疗剂和诊断剂,但可特别用于屏蔽用于治疗癌症的细胞毒性剂。纳米载剂可以包含脂质(如磷脂)、亲水性聚合物、疏水性聚合物、两亲性化合物、交联聚合物和聚合物基质(参见,如WO2009/110939)。根据应用,纳米载剂可被设计成具有特定尺寸、半衰期、保质期和泄漏速率。

[0209] 如在美国专利第6,465,188号、第7,122,202号、第7462603号和第7550441号中描述了纳米载剂如抗体靶向脂质体、聚合物纳米颗粒或延长保质期的脂质体的制备。

[0210] 在一些实施方案中,使抗体连接到稳定部分如PEG,或脂质体或其它纳米载剂。美国专利第4,732,863号和第7,892,554号及Chattopadhyay等人(2010)Mol Pharm 7:2194描述了将选择的抗体附接至PEG、PEG衍生物和纳米颗粒(如脂质体)的方法。含有磷脂酰乙醇胺(PE)的脂质体可通过如本文所述的已建立的程序来制备。包含PE使提供了在脂质体表面用于附接的活性功能位点。

[0211] 还可以配制抗体缀合物以提供多于一种的活性化合物,如另外的化疗剂或细胞毒性剂、细胞因子或生长抑制剂。活性成分还可以制备为持续释放的制剂(如半固体疏水性聚合物的半透性基质(如聚酯,水凝胶(例如,聚(2-羟乙基-甲基丙烯酸酯)或聚(乙烯醇)),聚交酯。抗体和免疫缀合物可以包埋在例如通过凝聚技术或通过界面聚合制备的纳米颗粒(例如分别为羟甲基纤维素或明胶微胶囊和聚-(甲基丙烯酸甲酯)微胶囊)中、包埋在胶体药物递送系统(例如,脂质体、白蛋白微球、微乳剂、纳米颗粒和纳米胶囊)中或包埋在粗乳剂中。

[0212] 本文所述的CLL-1抗体可单独或与细胞毒剂组合杀伤CLL-1-表达细胞。在一些实施方案中,治疗方法包括向个体施用有效量的治疗性CLL-1抗体或CLL-1抗体缀合物,如附接至治疗剂的CLL-1抗体。在一些实施方案中,该个体被诊断为患有癌症,如AML。在一些实施方案中,该个体正在接受或已经接受癌症疗法,如手术、放疗或化疗。在一些实施方案中,该个体已被诊断,但是癌症已得到缓解。

[0213] 在一些实施方案中,该方法进一步包括监测个体癌症的进展。在一些实施方案中,CLL-1抗体或CLL-1抗体缀合物用于每次施用的剂量基于个体的治疗进度来确定,例如,如

果个体对疗法的响应不够，则施用更高剂量的化疗剂。

[0214] 在一些实施方案中，本公开可以包括抗体或抗体靶向的组合物和生理上(即药学上)可接受的载剂。术语“载剂”通常是指用作稀释剂或媒介物以用于诊断剂或治疗剂的惰性物质。术语还涵盖赋予组合物粘附性的典型惰性物质。生理上可接受的载剂可以是液体，如生理盐水、磷酸盐缓冲液、生理缓冲盐水(135-150mM的NaCl)、水、缓冲水、0.4％盐水、0.3％甘氨酸、糖蛋白以提供增强的稳定性(如白蛋白、脂蛋白、球蛋白等)等。由于生理上可接受的载剂部分是由所施用的特定组合物以及用于施用组合物的特定方法来确定，因此，存在本公开的药物组合物的各种适合的制剂(参见，如Remington's Pharmaceutical Sciences,第17版,1989)。

[0215] 本公开的组合物可以通过常规的熟知的灭菌技术进行灭菌，或者可以在无菌条件下产生。可将水溶液包装使用或在无菌条件下过滤并冻干，在施用前将冻干制剂与无菌水溶液合并。该组合物可以根据需要包含药学上可接受的辅助物质以接近生理条件，如pH调节剂和缓冲剂、张力调节剂、润湿剂等，如乙酸钠、乳酸钠、氯化钠、氯化钾、氯化钙、脱水山梨糖醇单月桂酸酯和三乙醇胺油酸酯。也可以包含糖用于稳定组合物，诸如用于冻干抗体组合物的稳定剂。

[0216] 剂型可经制备用于经粘膜(如经鼻、经舌下、经阴道、经颊或经直肠)、肠胃外(如皮下、静脉内、肌内或动脉内的注射，推注或输注)、经口或经皮施用给患者。剂型的实例包括但不限于：分散剂；栓剂；软膏剂；糊剂(泥敷剂)；糊剂；粉剂；敷料剂；乳膏剂；硬膏剂；溶液；贴剂；气雾剂(如鼻腔喷雾剂或吸入剂)；凝胶剂；适用于经口或经粘膜施用给患者的液体剂型，包括混悬剂(如水性或非水性液体混悬剂、水包油乳剂或油包水液体乳剂)、溶液和酏剂；适用于肠胃外施用给患者的液体剂型；及无菌固体(如结晶或无定形固体)，其可复溶以提供适用于肠胃外施用给患者的液体剂型。

[0217] 可注射(如静脉内)组合物可包括抗体或抗体靶向组合物混悬于可接受的载剂(诸如水性载剂)中的溶液。可以使用各种水性载剂中的任一种，如水、缓冲水、0.4％盐水、0.9％等渗盐水、0.3％甘氨酸、5％右旋糖等，并且可以包括用于增强稳定性的糖蛋白，诸如白蛋白、脂蛋白、球蛋白等。通常，将使用生理缓冲盐水(135-150mM的NaCl)。该组合物可含有药学上可接受的辅助物质以接近生理条件，诸如pH调节剂和缓冲剂、张力调节剂、润湿剂如乙酸钠、乳酸钠、氯化钠、氯化钾、氯化钙、脱水山梨糖醇单月桂酸酯、三乙醇胺油酸酯等。在一些实施方案中，抗体靶向组合物可在用于静脉施用的试剂盒中配制。

[0218] 适用于例如通过关节内(关节中)、静脉内、肌内、肿瘤内、皮内、腹膜内和皮下途径来肠胃外施用的制剂包括水性和非水性等渗无菌注射溶液，其可包含抗氧化剂、缓冲剂、抑菌剂和使制剂与预期接受者血液等渗的溶质，以及可包含混悬剂、增溶剂、增稠剂、稳定剂和防腐剂的水性和非水性无菌混悬液。

[0219] 药物制剂可以以单位剂型包装或制备。在此类形式下，如根据治疗剂的剂量或抗体的浓度，制剂被细分成含有适量活性组分的单位剂量。单位剂型可以是包装制剂，所述包装含有在单位-剂量或多剂量密封容器(诸如安瓿和小瓶)中的分散量的制剂。如果需要，该组合物还可以含有其它相容的治疗剂。

[0220] 抗体(或抗体靶向组合物)可通过注射或输注通过任何适合的途径施用，包括但不限于静脉内、皮下、肌内或腹膜内途径。施用药物组合物的实例包括在4℃下将抗体以10mg/

mL储存于注射用无菌等渗盐水溶液中，并且在施用给患者前将其在100mL或200mL注射用0.9％氯化钠中稀释。在1小时的过程内通过以0.2至10mg/kg的剂量静脉内输注施用抗体。在其它实施方案中，抗体通过静脉内输注经15分钟至2小时的时间段施用。在其它的实施方案中，施用程序是经由皮下团注注射进行的。

[0221] 选择抗体的剂量用以为患者提供有效的疗法，并且所述剂量在每位患者小于0.1mg/kg体重至约25mg/kg体重的范围内或在1mg-2g范围内。在一些情况下，剂量在1-100mg/kg的范围内，或者约50mg-8000mg/患者。可根据抗体的药代动力学(如循环中抗体的半衰期)和药效动力学响应(如抗体的治疗性作用的持续时间)，以适当的频率重复剂量，所述频率在每天一次至每三个月一次的范围内。在一些实施方案中，约7天和约25天之间的体内半衰期以及抗体给药在每周一次和每三个月一次之间重复。

[0222] 施用可以是周期性的。根据施用途径，可以施用剂量，例如每1、3、5、7、10、14、21或28天或者更久施用一次(如每2、3、4或6个月施用一次)。在一些情况下，施用更频繁，如每天2次或3次。如本领域技术人员将认识到，可根据治疗进展和任何不良副作用监测患者以调整施用的剂量和频率。

[0223] 因此，在一些实施方案中，额外的施用依赖于患者的进展，如患者在施用之间受到监测。例如，第一次施用或若干轮施用后，可监测患者的肿瘤生长速率、复发(如在手术后患者的情况下)或一般疾病相关症状诸如无力、疼痛、恶心等。

[0224] 为了治疗癌症，可以每天以约0.001mg/kg至约1000mg/kg的初始剂量施用抗体或抗体靶向组合物(如包含治疗剂和/或诊断剂)，并随着时间的推移调整。可使用约0.01mg/kg至约500mg/kg，或约0.1mg/kg至约200mg/kg，或约1mg/kg至约100mg/kg，约5至约10mg/kg或约10mg/kg至约50mg/kg的每日剂量范围。本文所述的体内异种移植结果表明，5-20mg抗体/kg体重之间的剂量对于肿瘤生长显著减少是有效的。

[0225] 剂量根据患者的要求、所治疗病况的严重程度以及所使用的靶向组合物而变化。例如，考虑到在特定患者中诊断的癌症的类型和阶段，剂量可以凭经验确定。在本公开的上下文中施用给患者的剂量应该足以影响患者中随着时间推移的有益的治疗性响应。如技术人员将认识到，剂量的大小还将由与特定患者中的特定靶向组合物的施用所伴随的任何不良副作用的存在、性质和程度来确定。

[0226] 应理解，本文描述的实施例和实施方案仅用于说明目的，并且对于本领域技术人员表明了对其的各种修改或改变，并且将所述修改或改变包括在本申请的精神和范围及所附权利要求的范围内。本文引用的所有出版物、专利和专利申请在此为了所有目的通过引用整体并入本文。

[0227] 本说明书中提及的所有出版物和专利申请通过引用整体明确地并入本文，其程度如同每个单独的出版物或专利申请被具体和单独地指示通过引用并入本文。

[0228] 从前述应当理解的是，虽然本文为了说明的目的已经描述了本发明的特定实施方案，但是可以在不偏离本发明的精神和范围的情况下进行各种修改。因此，除了所附权利要求之外，本发明不受限制。

[0229] 以下实施例是以说明而非限制的方式提供的。

VI.实施例

[0230] 使用QuickChange II定点突变试剂盒(Agilent),在CLL-1抗体(HuM31)重链的选定位置(EU编号)处将半胱氨酸残基工程化以产生相应的CYSMAB变体。通过DNA测序验证了半胱氨酸取代的真实性。将CYSMAB轻链和重链构建体瞬时转染到HEK-293细胞中。使用MabSelectsuRe珠纯化表达的CYSMAB变体,并将其用各种CLL-1功能测定进一步表征。

[0231] 实施例1--缀合

[0232] 为了证明在重链恒定区的所选残基处的缀合,建立了抗体-荧光团缀合物。使用以下程序:将经纯化的HuM31或CYSMAB变体(各1.5mg)在4℃下对PBS透析过夜。将抗体与200μL的MabSelectsuRe珠在室温下孵育1小时。每次用2mL PBS洗涤珠三次后,将在150mM NaCl-50mM Tris,pH 8.0缓冲液中于2mM DTT中在室温下还原过夜。将珠洗涤三次,然后将抗体在1mM脱氢抗坏血酸(DHAA)中在室温下再氧化3小时。将抗体洗涤三次,并与10摩尔过量的Alexa488-C5-马来酰亚胺在室温下缀合2小时。将珠洗涤三次,并用500μL的0.1M甘氨酸,pH 2.7洗脱Alexa488缀合的抗体。通过使用NanoDrop 2000测定抗体浓度和Alexa488缀合效率(每个抗体Alexa488的数量)。

[0233] 为了证明缀合不随荧光团的日期或量而变化,在不同的日期和不同的浓度下重复缀合程序。结果(分别地,图5和图6)显示,任何缀合比(如DAR、FAR)都在程序中都没有明显变化。

[0234] 包括氨基酸残基和荧光团-与-抗体比(“FAR”)的缀合结果报道于图7-图9中。

[0235] 图7显示了45种总缀合,21种(47%)展现出高缀合(>2)、7种(16%)中缀合(1-2)和17种(38%)低缀合(<1)。

[0236] 图8显示了20种总缀合物,10种(50%)展现出高缀合(>2)、1种(5%)中缀合(1-2)和9种(45%)低缀合(<1)。

[0237] 实施例2--特异性ELISA

[0238] 图1:开发了特异性检测缀合至HuM31的Alexa 488(A488)(HuM31-A488AFC)的ELISA测定。设计三种形式的ELISA以检测缀合至人血浆中的HuM31的A488。在这三种ELISA方法中测试未经缀合的HuM31、HuM31-A488和IgG-A488。结果显示了ELISA形式#1具有最佳的信噪比。并且,形式#2和形式#3显示出更高的本底结合。进一步用形式#1ELISA以检测HuM31-A488。

[0239] 图2a:ELISA测定的特异性。形式#1将ELISA方法用于特异性检测HuM31-A488和位点-特异性CYSMAB-A488缀合物,而不是对照样品(相对于IgG(同种型对照)、IgG-A488AFC、曲妥珠单抗和未经缀合的HuM31)。结果证明这种ELISA方法仅检测HuM31-A488和位点-特异性CYSMAB-A488缀合物。相对之下,对照:同种型人IgG、曲妥珠单抗、HuM31或IgG-A488缀合物显示无结合。

[0240] 图2b:在ELISA测定中测试人血浆干扰。在最佳的ELISA条件下,1%人血浆的存在仅略微增强了HuM31-A488缀合物与抗-A488抗体的结合。因此,ELISA方法可用于分析先前暴露于人血浆的抗体缀合物样品。

[0241] 实施例3--HuM31-A488缀合物(AFC)的稳定性

[0242] 通过在人血浆中孵育来测试本公开的AFC的在人血浆中的稳定性。将AFC(50μg/mL)加入(spike)到PBS中的汇集的人血浆或0.5%BSA中。然后将各样品在37°7,5%CO_2下孵育,然后在0、24、48、72和96小时的时间点转移至-80°8。将样品在样品稀释液(含有0.5%

BSA、0.05％Tween 20、5mM EDTA、0.35M NaCl、0.25％CHAPS和0.2％BGG的PBS缓冲液)中以1:5000稀释。然后通过ELISA测定不同时间点的样品。

[0243] ELISA程序:将PBS(1μg/mL)中的CLL-1胞外结构域蛋白在96孔板上包被并在4°在下孵育过夜。然后将板用0.1％Tween 20的PBS洗涤三次,然后在室温下用在0.1％Tween 20的PBS中的1％BSA封闭1小时。用0.1％Tween 20的PBS洗涤6次后,将连续稀释的Alexa488缀合的人M31及其对照加入平板并在室温下孵育1小时。然后将板用0.1％Tween 20的PBS洗涤。将兔抗-Alexa 488二级抗体(1μg/mL)加入板并在室温孵育1小时。用0.1％Tween 20的PBS洗涤6次后,将板用以1:50,000稀释的HRP缀合的山羊抗-兔Fc多克隆抗体检测。然后通过比较每个时间点与时间0的OD值来评价百分比(％)值。

[0244] 图9和10中显示了5天后测试样品的稳定性:

[0245] 图9和10显示了样品58、64、73、81、86和206在孵育5天后具有>85％的稳定性。

[0246] 实施例4--HuM31-生物素缀合物的稳定性

[0247] 通过将CYSMAB与HPDP-生物素和BMCC-生物素缀合产生HuM31-生物素缀合物。

[0248] 将经纯化的人M31或CYSMAB变体(各1.5mg)在4°g下对PBS透析过夜。将抗体与200μL的MabSelectsuRe珠在室温下孵育1小时。每次用2mL的PBS洗涤珠三次后,将抗体在150mM NaCl-50mM Tris,pH 8.0缓冲液中于2mM DTT中在室温下过夜还原。将珠洗涤三次,然后将抗体在1mM DHAA中在室温下再氧化3小时。将抗体洗涤三次,并与10摩尔过量的HPDP-生物素或BMCC生物素在室温下缀合2小时。将珠洗涤三次,并将生物素缀合的抗体用500μL的0.1M甘氨酸,pH2.7进行洗脱。使用NanoDrop 2000和基于ELISA的测定分别确定抗体浓度和生物素缀合效率。

[0249] 开发ELISA测定以确定人血浆中的ADC的稳定性:将PBS中的CLL-1胞外结构域(1μg/mL)在96孔板上包被并在4°在下孵育过夜。将ELISA平板用洗涤缓冲液(0.1％Tween 20于PBS中)洗涤三次,随后在室温下用在含有0.1％Tween 20的PBS溶液中的1％BSA封闭1小时。用洗涤缓冲液洗涤平板6次后,将连续稀释的HuM31-生物素及其相应的对照样品添加至板并在室温下孵育1小时。将板用洗涤缓冲液洗涤6次,随后使用链霉亲和素-HRP缀合物(以1:100,000稀释度使用)检测。

[0250] 图11报道了第5天后HPDP和BMCC连接的抗体缀合物的稳定性。

[0251] 人血浆中生物素-BMCC缀合物样品的稳定性:

[0252] 图11显示了样品V266、V303、T307、G316、Y436、L441、H285、R301、Q295在5天的孵育后具有>80％的稳定性。

[0253] 实施例5--亲和力测试

[0254] 针对与它们的裸露的未缀合的对应物的比较性结合亲和力,可测试经半胱氨酸取代的CLL-1CYSMAB的结合亲和力。简言之,将经生物素化的CLL-1(25μg/mL)在22°2下加载到链霉亲和素传感器尖端上2小时。使用整体1:1曲线拟合,通过Fortebio或BIAcore分析(10、30和90μg/mL),针对每种抗体在三种不同浓度下产生出Ab-Ag解离曲线。

[0255] 实施例6-结合AML细胞系和AML患者样品

[0256] 可测试经半胱氨酸取代的CYSMAB与表达人CLL-1的重组293细胞和两种AML细胞系(HL60和OCI AML-5)的比较性结合。具有抗体结合的活细胞的百分比可以通过任何合适的手段如FACS来检测。也可以评估结合一致性,即患者之间的变异性。

[0257] 实施例7--抗体-药物缀合物(ADC)测定

[0258] 抗体-药物缀合物(ADC)测定可在适合的AML细胞系(如HL60和OCI AML-5)以及表达CLL-1的重组293细胞上进行。简言之,将细胞与不同浓度的ADC在37°7下孵育72-120小时。通过CellTiter-Glo(Promega)发光细胞存活力测定来确定细胞存活力以确定IC50值。

[0259] 实施例8--AML肿瘤生长的体内抑制

[0260] 可评价CLL-1CYSMAB ADC的体内效力。适合的研究包括(1)在小鼠中利用CLL-1阳性HL60AML人细胞系的皮下(SC)肿瘤植入和生长模型,和(2)利用CLL-1阳性HL60或OCI AML-5人AML细胞系的原位(骨髓、血液、脾脏和淋巴结)肿瘤植入和赘疣模型。

[0261] 可如下进行所建立的SC HL60研究。用5×10^{6}或10^{7}个HL60细胞接种动物(nu/nu小鼠)。将荷瘤小鼠随机分组,每组平均肿瘤体积为100-150mm^{3}(8只动物/组)。将CLL-1CYSMAB ADC或IgG对照ADC以5-200μg/动物的剂量i.p.施用。将经一段时间(给药后)的平均肿瘤体积进行作图。

[0262] 可如下进行OCI AML-5细胞原位研究。将免疫缺陷的NSG小鼠分成5组,每组8只动物。将CLL-1CYSMAB ADC或IgG对照ADC在静脉内接种5×1^{06}或1^{07}个OCI AML-5细胞后(第6天)以5-200μg/动物的剂量i.p.施用。然后使动物在后续2周内每周接受一次另外的抗体剂量。该研究在施用OCI AML-5细胞后4周终止。

[0263] 实施例9--ADC测定中对AML干细胞的特异性

[0264] 可以在ADC测定中测试根据本公开制备的CLL-1CYSMAB ADC的特异性杀灭的特异性。将原代患者AML细胞或正常CD34阳性造血干细胞从人受试者的骨髓中分离出来,并接种到软琼脂集落形成测定(100,000个细胞/板)中。然后将细胞在CLL-1CYSMAB ADC的存在下孵育14天。ADC可引起AML干细胞克隆形成生长的选择性、特异性抑制,而正常的HSC不应该受到影响。可将缀合的效果与裸露的亲本抗体相比较。将阴性对照未经处理或用不相关的IgG-ADC处理。

[0265] 实施例10-各种药物缀合物的稳定性

[0266] 多种抗体-药物缀合物如下制备:

[0267]

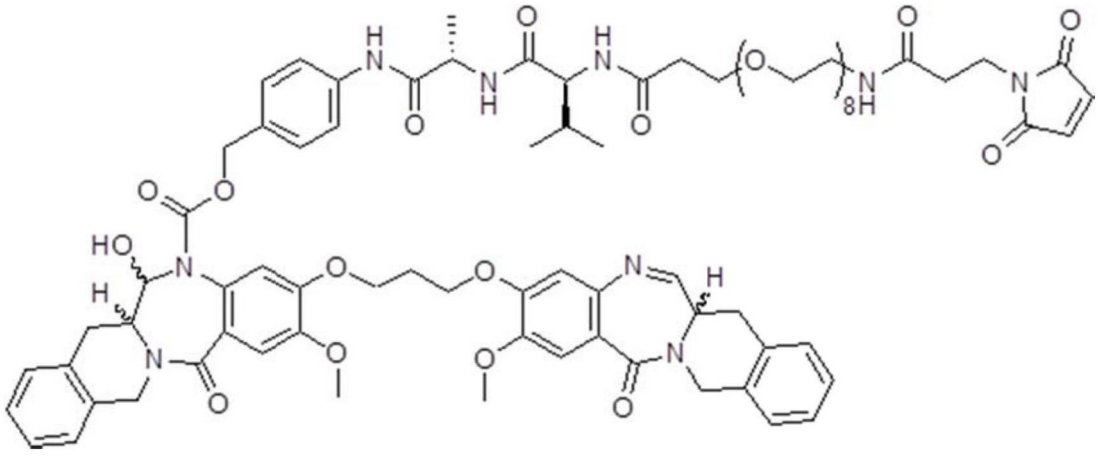
名称	CYSMAB	浓度	量	DAR
C6-Cys-02-D202	S156C	3.20 mg/ml	3.1 mg	>1.8
Cys-01-D202	A118C	2.44 mg/ml	2.8 mg	>1.8
Cys-87-D202	G316C	2.63 mg/ml	2.9 mg	< 1.0
Cys-37-D202	V266C	4.28 mg/ml	2.7 mg	>1.8

[0268]

Cys-10-D202	S239C	3.72 mg/ml	2.6 mg	>1.8

[0269] 用具有以下可变区的抗-CLL-1抗体制备所有缀合物::轻链可变区序列,其包含:DIQMTQSPSSLSASVGDRVTLTCRATQELSGYLSWLQQKPGKAIKRLIYAASTLDSGVPSRFSGNRAGTDYTLTISS

LQPEDFATYYCLQYAIYPYTFGQGTKLEIK(SEQ ID NO:19)以及重链可变区序列,其包含:EVQLVQSGAEVKKPGASVKMSCKASGYTFTSYFIHWVRQAPGQGLEWIGFINPYNDGSKYAQKFQGRATLTSDKSTSTVYMELSSLRSEDTAVYYCTRDDGYYGYAMDYWGQGTLVTVSS(SEQ ID NO:20)。如下将抗体经由至异喹啉烷并苯二氮䓬二聚体的半胱氨酸反应性接头缀合至指定的半胱氨酸取代:

[0270] 

[0271] (被命名为"D202")。

[0272] 使用Millipore,15mL,30kDa装置,经由2轮的分子量截留过滤(MWCO)将PBS中的人源化的经cys取代的抗-CLL1抗体交换到硼酸盐缓冲液(50mM,pH 8.5,1mM二乙烯三胺五乙酸(DTPA))中。向新的抗体溶液(5.0mg/mL,硼酸盐缓冲液(50mM,pH 8.5,1mM DTPA))中加入二硫苏糖醇(DTT)溶液(33μL,50.0当量,50mM),并将所得的溶液轻轻振摇过夜。

[0273] 如前所述,通过rp-LCMS证实链间二硫桥的完全还原以及去除经取代的半胱氨酸半胱氨酸/谷胱甘肽加合物(Junutula等人,2008,Nature Biotech,26,925-932)。然后使用Millipore,15mL,30kDa装置,使用PBS作为交换缓冲液,通过3轮的分子量截留过滤(MWCO)从溶液中除去DTT。向完全还原的抗体的5mg/ml溶液中加入脱氢抗坏血酸(dhAA)溶液(33μL,50.0当量,50mM)。将所得的溶液轻轻振摇3小时。经由rp-LCMS监测再氧化。一旦认为再氧化完全,用丙二醇将反应混合物稀释至50%v/v,并将D202作为DMSO中的溶液(10.0当量,10mM于DMSO中)进行添加。

[0274] 使反应在环境温度下搅拌1小时。然后将混合物用活性炭在环境温度下处理1小时。然后经由过滤去除活性炭。然后使用Millipore,15mL,30kDa装置经由多轮的分子量截留过滤(MWCO)将缀合物交换到PBS中。然后将溶液进行无菌过滤,得到所需的缀合物。

[0275] 以30μg/mL开始,使用细胞结合缓冲液(PBS,含有2%胎牛血清)将C6-CYSMAB-D202 ADC和CO-D202进行8个点的、6倍的连续稀释。将HL60、OCI-AML5和OCI-AML5-CLL1敲除细胞用染色培养基洗涤,并用5%正常小鼠血清在冰上孵育30分钟以阻断Fcγ受体。然后将细胞以每孔0.1e^6个细胞的密度分配到96孔板中,并通过离心去除培养基。将细胞板与100μL ADC样品稀释液一起在冰上孵育30分钟,然后洗涤3次并进一步用Alexa-488缀合的山羊抗人IgG作为二级抗体在冰上染色30分钟。然后将细胞洗涤三次并使用碘化丙啶作为细胞存活力染料重悬于100μL细胞结合缓冲液中。通过流式细胞术和Flowjo数据分析来分析与细胞样品结合的ADC。使用Graphpad Prizm 6对FITC信号的MFI(几何平均值)进行作图。参见图12A-图12C。

[0276] 如下测定缀合物的稳定性。在第0天,在人血浆中稀释C6-CYSMAB-D202 ADC样品至

200mg/mL。使用人血浆作为稀释剂进行9-点,6倍连续稀释。密封样品稀释板并在37°7下在CO_2孵育箱中孵育5天,作为样品D5。通过在第2、4和5天重复该血浆稀释和37°7孵育程序来制备样品D3、D1和D0。在第5天,通过将5mL样品D0、D1、D3和D5添加到95mL的OCI-AML2和HL60细胞中建立细胞杀灭测定,在37°7下孵育5天,并通过Cell-Titer-Glo定量细胞存活力。

[0277] 使用人血浆作为稀释剂在96孔板中进行C6-CYSMAB-D202 ADC样品稀释,并在37°7下在CO_2孵育箱中孵育以进行血浆稳定性研究。在第0天、第2天、第4天和第5天以200μg/mL ADC开始进行9点、6倍连续稀释,并且密封的样品稀释平板加上无ADC仅血浆对照,在37°7,5%CO_2孵育箱内孵育5天、3天、1天和0天,,分别作为样品D5、D3、D1和D0。在第5天,将OCI-AML2和HL60细胞以2,000个细胞的密度接种在96孔板内的补充有20%胎牛血清(FBS)的95μL的Alpha-MEM和IMDM细胞培养基中,并用5μL来自D0、D1、D3、D5样品稀释板的样品以一式三份进行处理。然后将测定板在37°7,5%CO_2孵育箱中孵育5天。使用CellTiter-Glo试剂盒(Promega)测定活细胞(活细胞的百分比),并通过读板仪(Molecular Device Spetramax M5)测量发光。结果以相对于无ADC仅血浆对照细胞的活细胞的百分比表示。通过使用Graphpad Prizm 6的非线性回归推导出各个剂量响应曲线和抑制药物浓度(IC_{50})。

[0278] 将C6-CYSMAB-D202 ADC对AML2,HL60细胞杀灭的稳定性测试示列于下表中:

[0279]

	AML2			
IC50 μg/mL	D0	D1	D3	D5
S156C	0.0016	0.0012	0.0011	0.0058
A118C	0.0017	0.0010	0.0028	0.0043
G316C	0.0037	0.0019	0.0084	0.0128
V266C	0.0041	0.0028	0.0043	0.0070
S239C	0.0021	0.0009	0.0008	0.0021

[0280]

	HL60			
IC50 μg/mL	D0	D1	D3	D5
S156C	0.0072	0.0070	0.0092	0.0399
A118C	0.0100	0.0116	0.0245	0.0608
G316C	0.0256	0.0315	0.1153	0.1772
V266C	0.0154	0.0115	0.0200	0.0394
S239C	0.0087	0.0047	0.0043	0.0120

序列表

<110> 塞勒兰特治疗公司 (Cellerant Therapeutics, Inc.)
杰格塔·R·祖奴图拉 (Junutula, Jagath R.)
蒋英萍 (Jiang, Ying Ping)
黄姜清 (Huang, Jianqing)
马德哈维·米什拉 (Mishra, Madhavi)
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35 40 45
Lys Leu Leu Ile Tyr Leu Ala Ser Asn Leu Glu Ser Gly Val Pro Ala
50 55 60
Arg Phe Ser Gly Ser Gly Ser Arg Thr Asp Phe Thr Leu Thr Ile Asp
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35 40 45
Lys Leu Leu Ile Tyr Leu Ala Ser Asn Leu Glu Ser Gly Val Pro Asp
50 55 60
Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
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Ala Ala Ser Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser His
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50 55 60
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35 40 45
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50 55 60
Lys Gly Lys Ala Thr Ile Thr Ser Asp Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80
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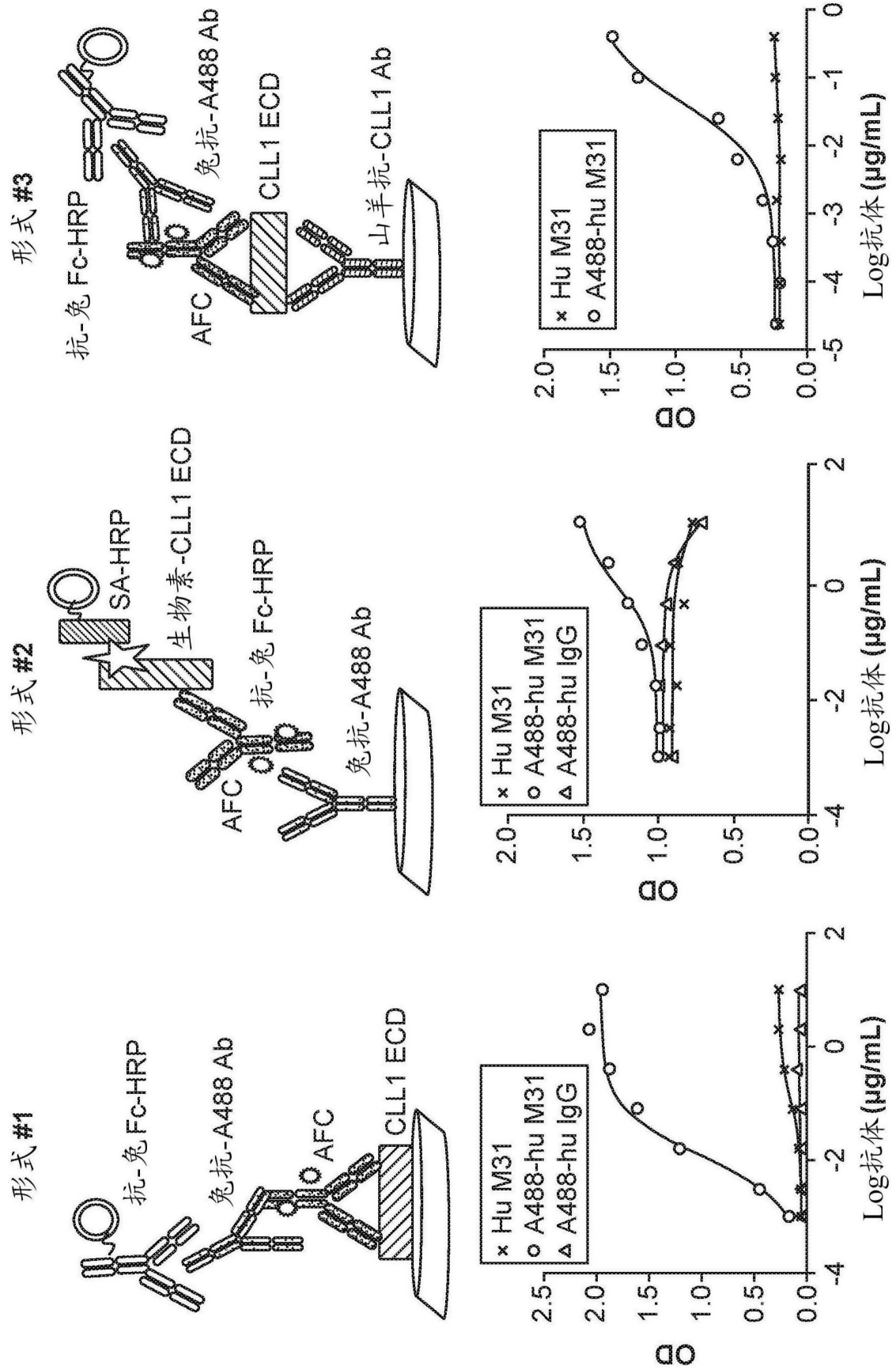
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AFC
CLL1 ECD
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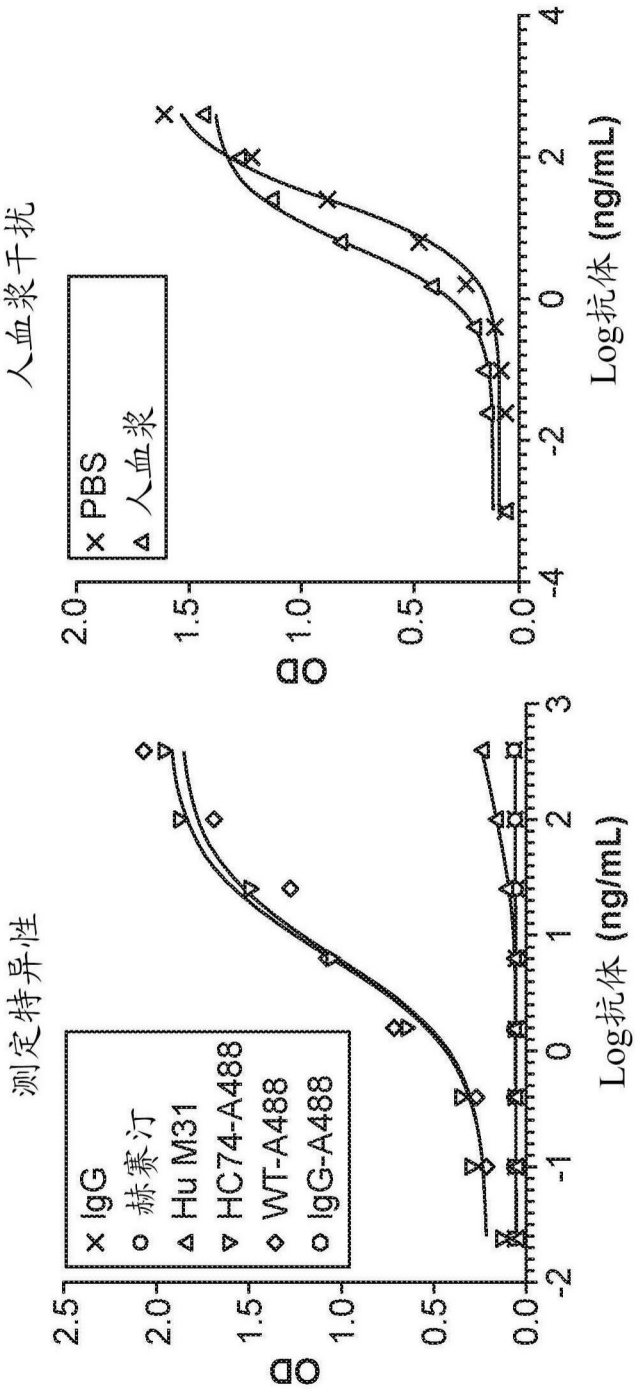
× Hu M31
o A488-hu M31
Δ A488-hu IgG
OD
Log抗体 (μg/mL)

图1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测定特异性
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赫赛汀
Hu M31
HC74-A488
WT-A488
IgG-A488
OD
Log抗体 (ng/mL)

图2

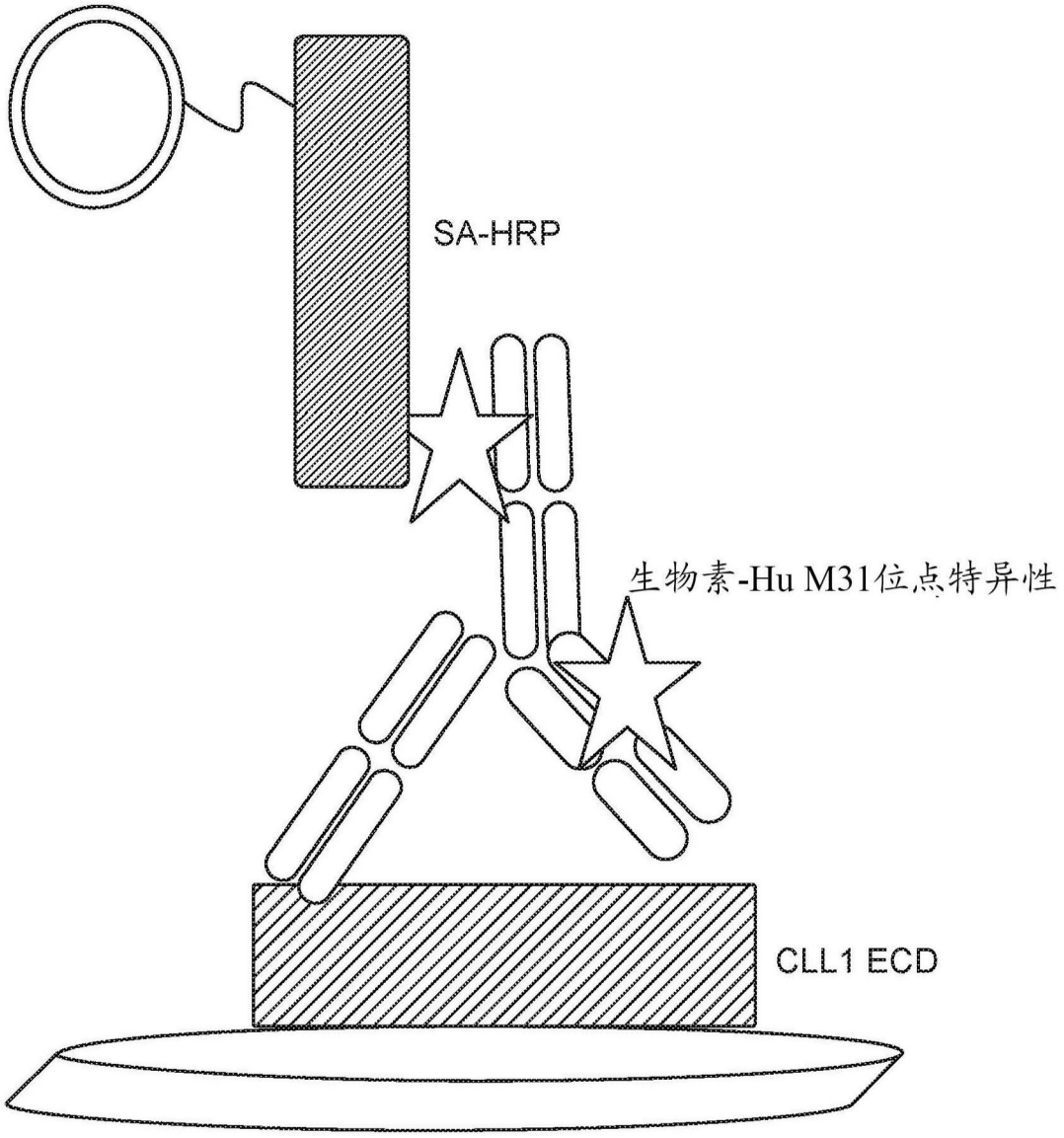


图3

M31 Ab v. HuM31 轻链序列

轻链可变区:

CDR-LC1 / CDR-LC2

```
             1        10        20        30        40        50        60
M31          TIVLTQSPAS LAVSLGQRAT ISCRASESVD SYGNSFMHWY QQKPGQPPKL LIYLASNLES
HuM31        DIVMTQSPDS LAVSLGERAT INCRASESVD SYGNSFMHWY QQKPGQPPKL LIYLASNLES

                     70        80        90       100       110
M31          GVPARFSGSG SRTDFTLTID PVEADDAATY YCQQNNYDPW TFGGGTKLEI K
HuM31        GVPDRFSGSG SGTDFTLTIS SLQAEDVAVY YCQQNNYDPW TFGGGTKVEI K
```

CDR-LC3

轻链恒定区(κ)

```
       120        130        140        150        160        170        180
RTVAAPSVF IFPPSDEQLK SGTASVVCLL NNFYPREAKV QWKVDNALQS GNSQESVTEQ DSKDSTYSLS

       190        200        210     218
STLTLSKADY EKHKVYACEV THQGLSSPVT KSFNRGEC
```

λ轻链

PKANPTVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEK
TVAPTECS

图4A

M31 Ab v. HuM31 重链序列

重链可变区:

CDR-HC1

CDR-HC2

1 10 20 30 40 50 60 70

M31 EVQLQQSGPE LVKPGASVKM SCKAS**GYTFT** **SYV**MHWVKQK PGQGLEWIGY **INPYNDGT**KY NEKFKGKATL

HuM31 QVQLVQSGAE VKKPGASVKV SCKASGYTFT SYVMHWVRQA PGQRLEWIGY INPYNDGTKY NEKFKGKATI

80 90 100 110 120

M31 TSDTSSSTAY MELNSLTSED SAVYFC**ARPI** **YFDNDYFDY**W GQGTTLKVSS

HuM31 TSDTSASTAY MELSSLRSED TAVYYCARPI YFDNDYFDYW GQGTLVTVSS

CDR-HC3

图4B

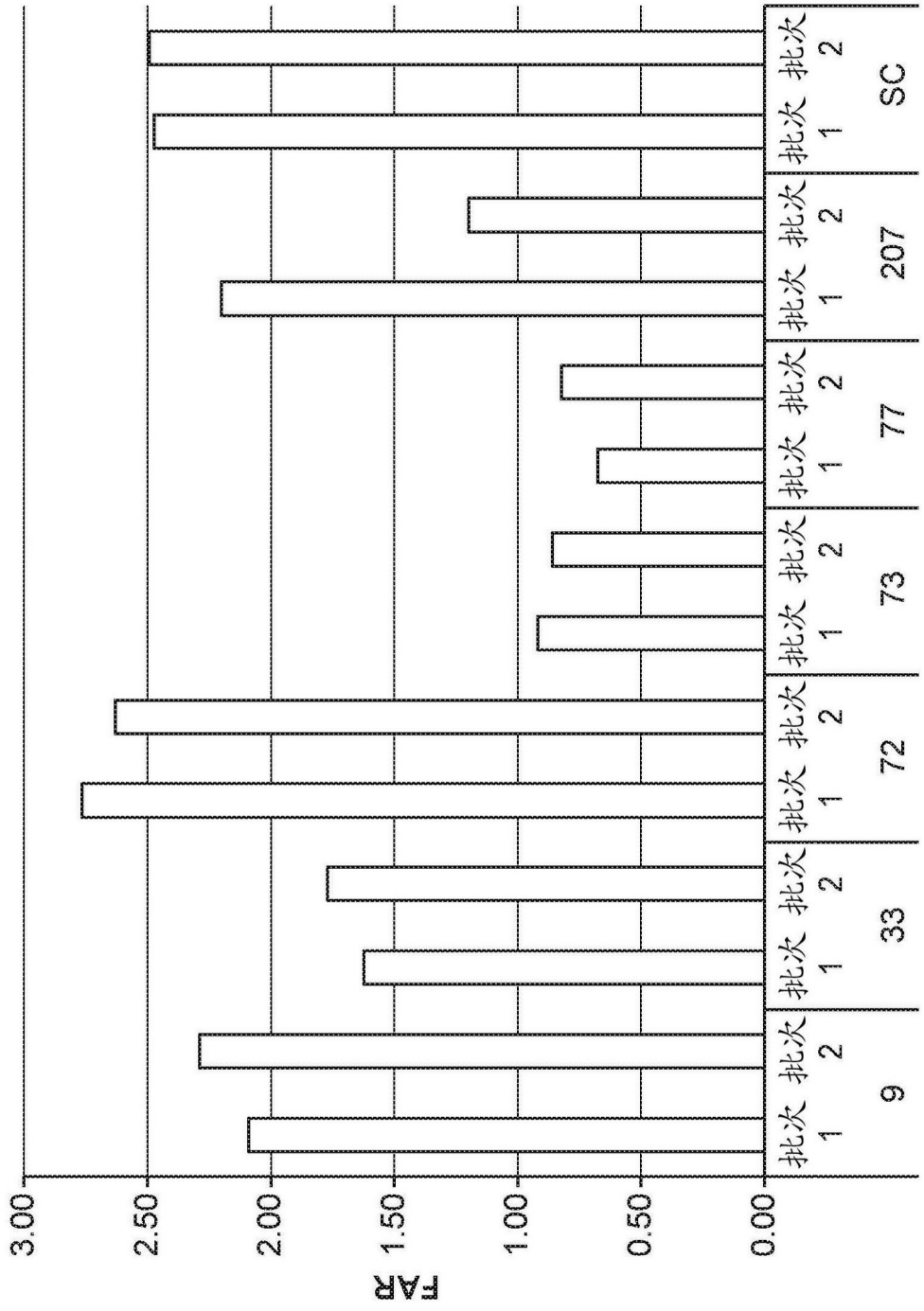
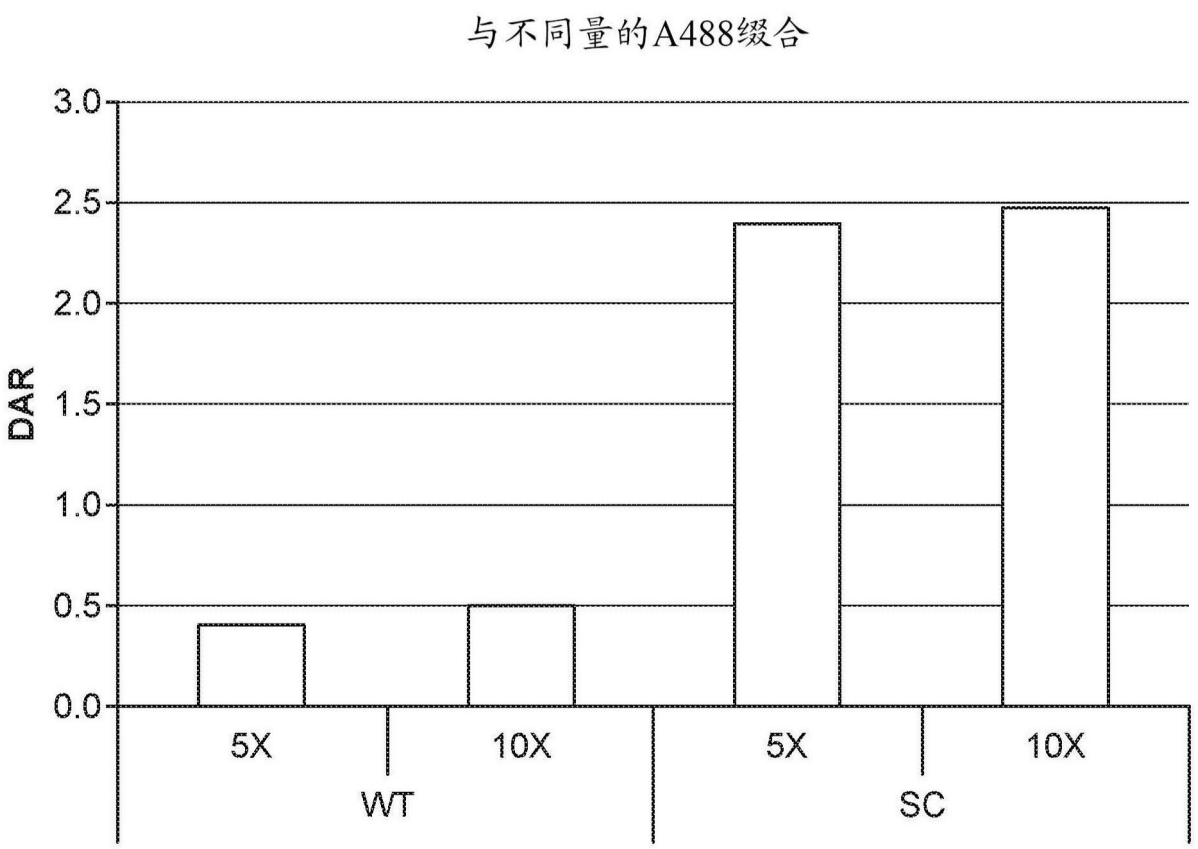


图5



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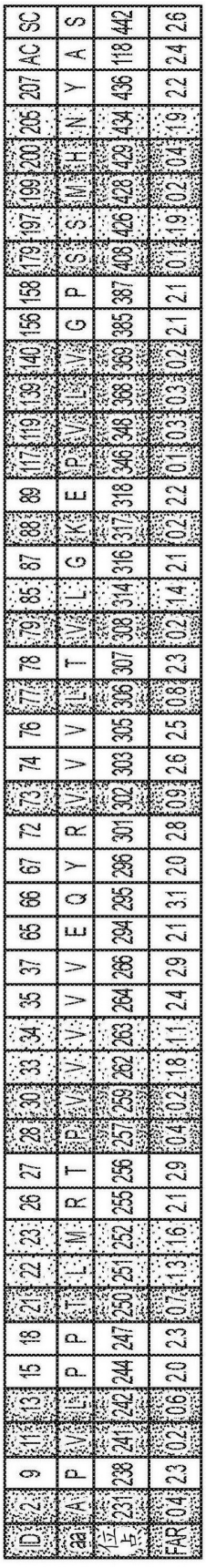

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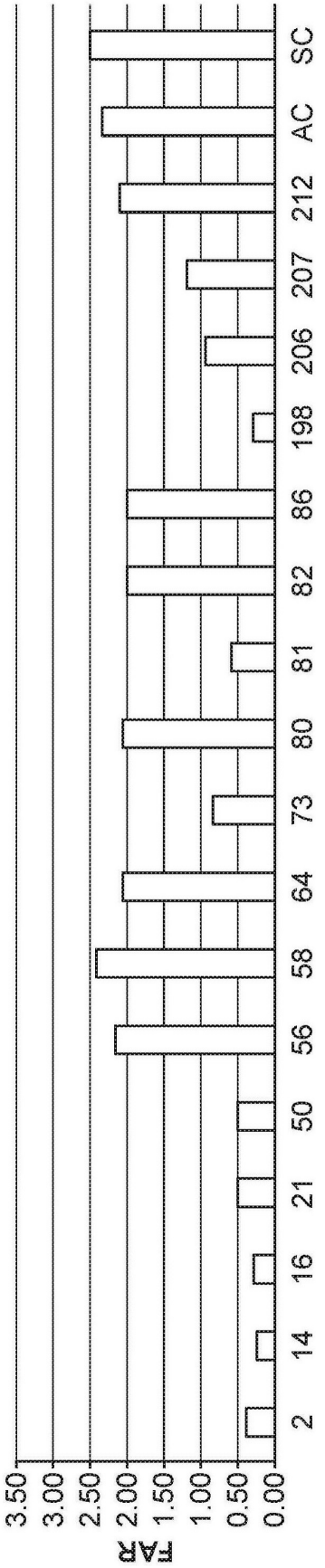
aa	A	P	V	L	P	P	T	L	M	R	T	P	V	V	V	V	V	E	Q	Y	R	V	V	V	V	L	T	V	L	G	K	E	P	V	L	G	P	S	S	M	N	Y	A	S
位点	231	238	241	242	244	247	250	251	252	255	256	257	259	262	263	264	266	294	295	296	301	302	303	305	306	307	308	314	316	317	318	346	348	368	369	385	387	408	426	428	429	434	436	118	442
FAR	0.4	2.3	0.2	0.6	2.0	2.3	0.7	1.3	1.6	2.1	2.9	0.4	0.2	1.8	1.1	2.4	2.9	2.1	3.1	2.0	2.8	0.9	2.6	2.5	0.8	2.3	0.2	1.4	2.1	0.2	2.2	0.1	0.3	0.3	0.2	2.1	2.1	0.1	1.9	0.2	0.3	1.9	2.2	2.4	2.6

图7

ID	2	14	16	21	50	56	58	64	73	80	81	82	86	198	206	207	212	AC	SC
aa	A	F	P	T	V	H	A	E	V	L	H	Q	N	V	H	Y	L	A	S
位点	231	243	245	250	279	285	287	293	302	309	310	311	315	427	435	436	441	118	442
FAR	0.4	0.3	0.3	0.5	0.5	2.2	2.4	2.1	0.9	2.1	0.6	2.0	2.0	0.3	0.9	1.2	2.1	2.3	2.5



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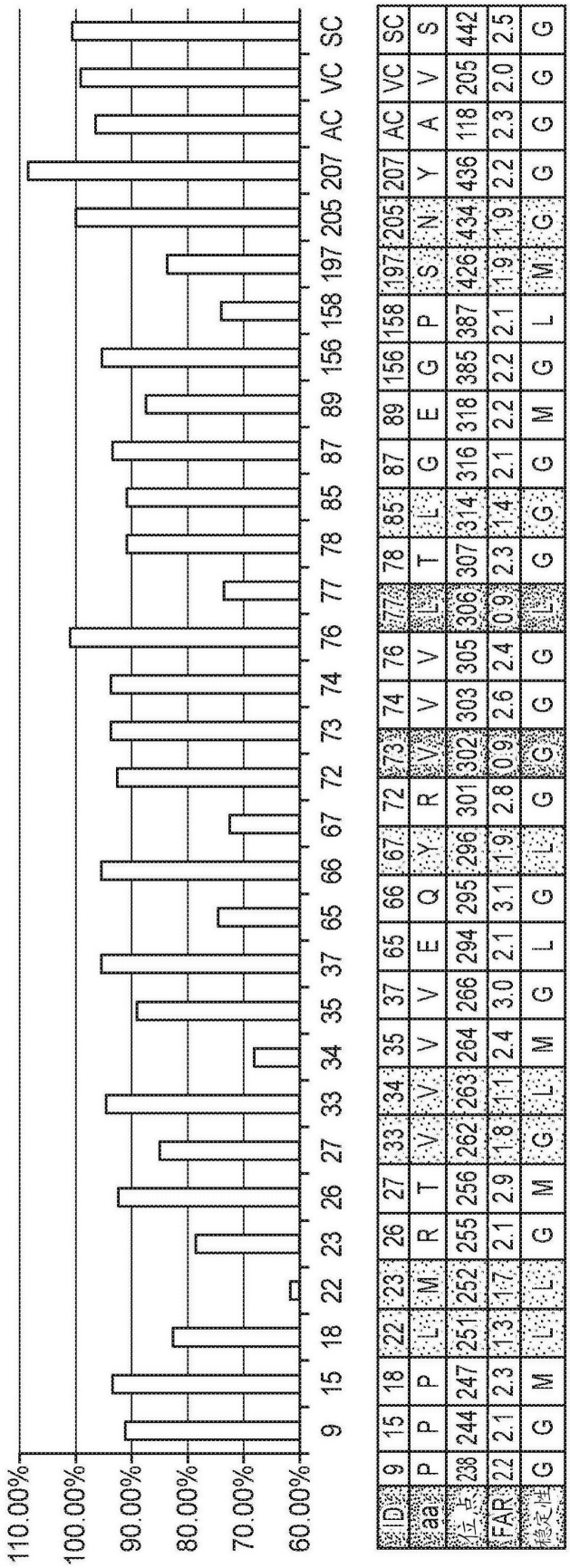
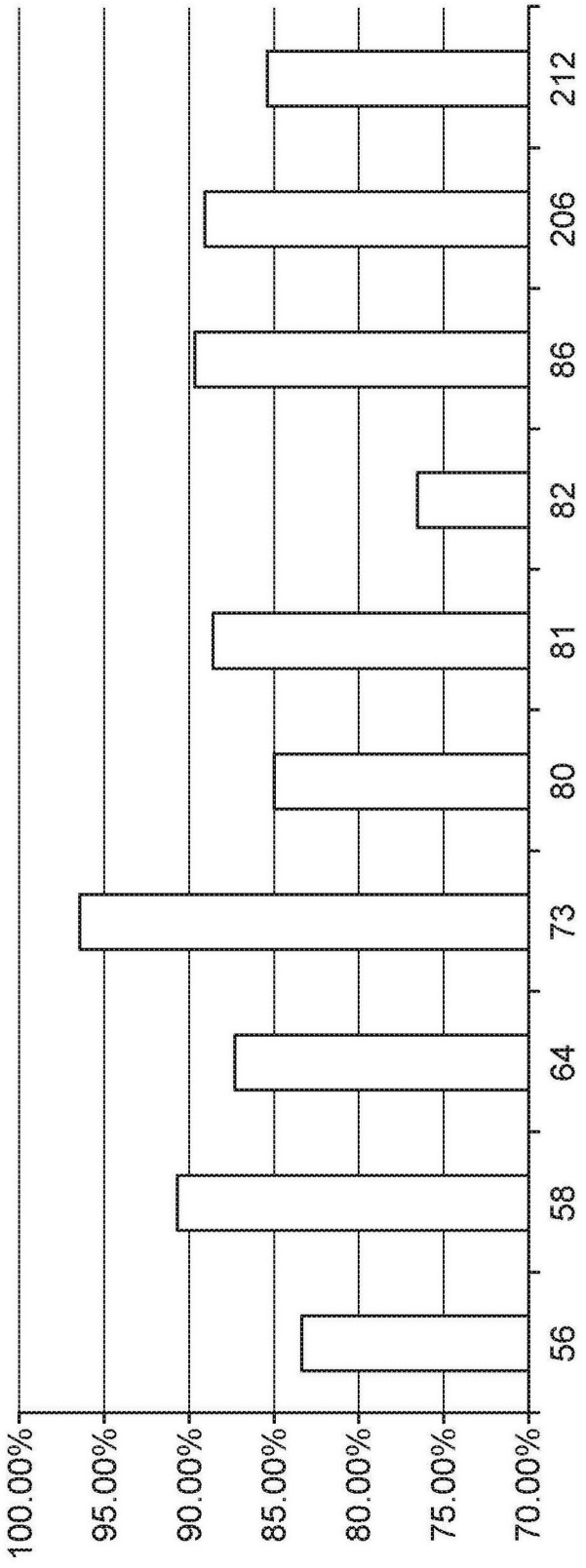

5天时血浆中的稳定性
110.00%
100.00%
90.00%
80.00%
70.00%
60.00%
9 15 18 22 23 26 27 33 34 35 37 65 66 67 72 73 74 76 77 78 85 87 89 156 158 197 205 207 AC VC SC
ID 9 15 18 22 23 26 27 33 34 35 37 65 66 67 72 73 74 76 77 78 85 87 89 156 158 197 205 207 AC VC SC
aa P P P L M R T V V V E Q Y R V V L T L G E G P S N Y A V S
位点 238 244 247 251 252 255 256 262 263 264 266 294 295 296 301 302 303 305 306 307 314 316 318 385 387 426 434 436 118 205 442
FAR 2.2 2.1 2.3 1.3 1.7 2.1 2.9 1.8 1.1 2.4 3.0 2.1 3.1 1.9 2.8 0.9 2.6 2.4 0.9 2.3 1.4 2.1 2.2 2.2 2.1 1.9 1.9 2.2 2.3 2.0 2.5
稳定性 G G M L L G M G L M G L G L G G G G L T G G M G L M G G G G

图9



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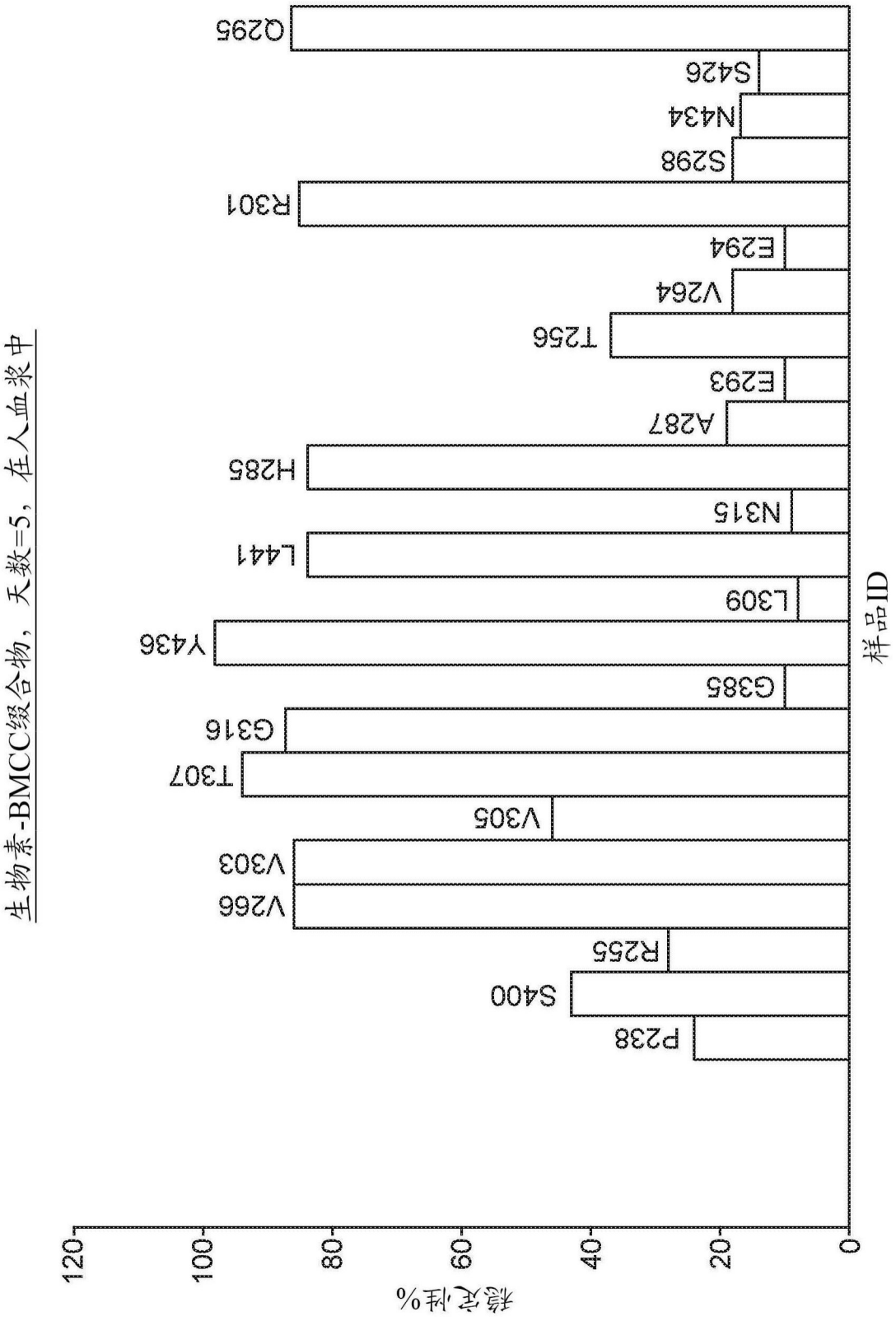
位点	285	287	293	302	309	310	311	315	435	441
FAR	2.2	2.4	2.1	0.9	2.1	0.6	2.0	2.0	0.9	2.1
稳定性	M	G	M	G	M	G	L	M	M	M

图10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稳定性%
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40
60
80
100
120
样品ID
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R255
V266
V303
V305
T307
G316
G385
Y436
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V264
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S426
Q295

图11

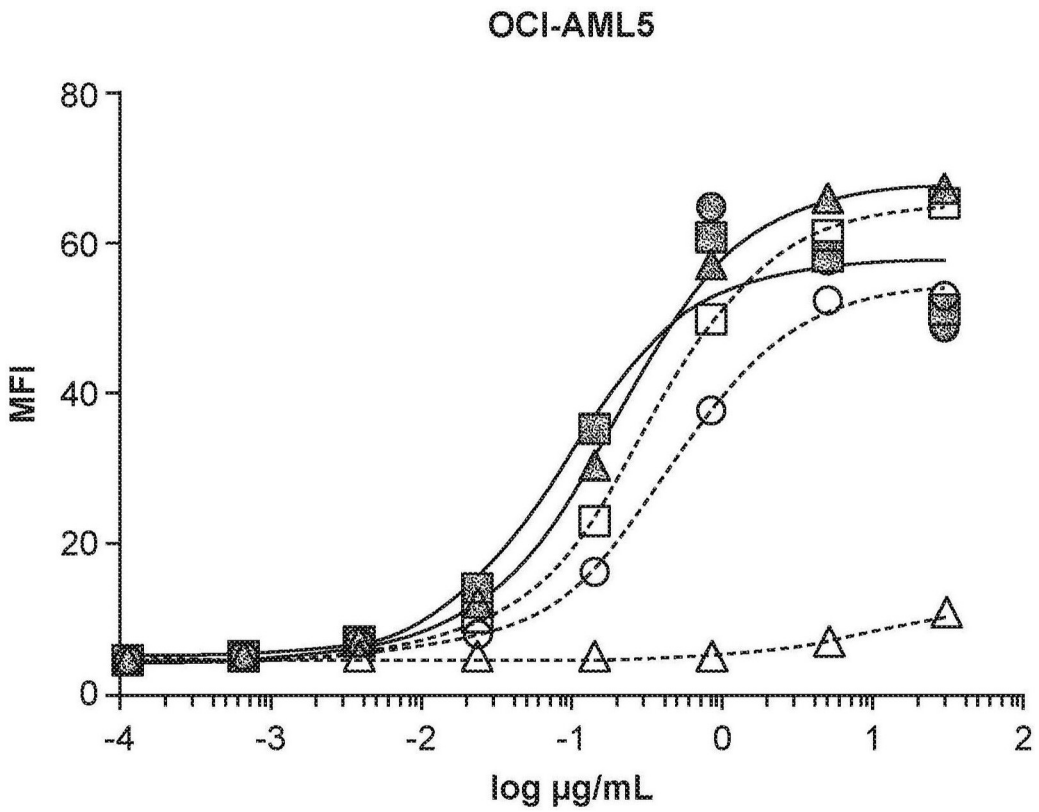

图12A

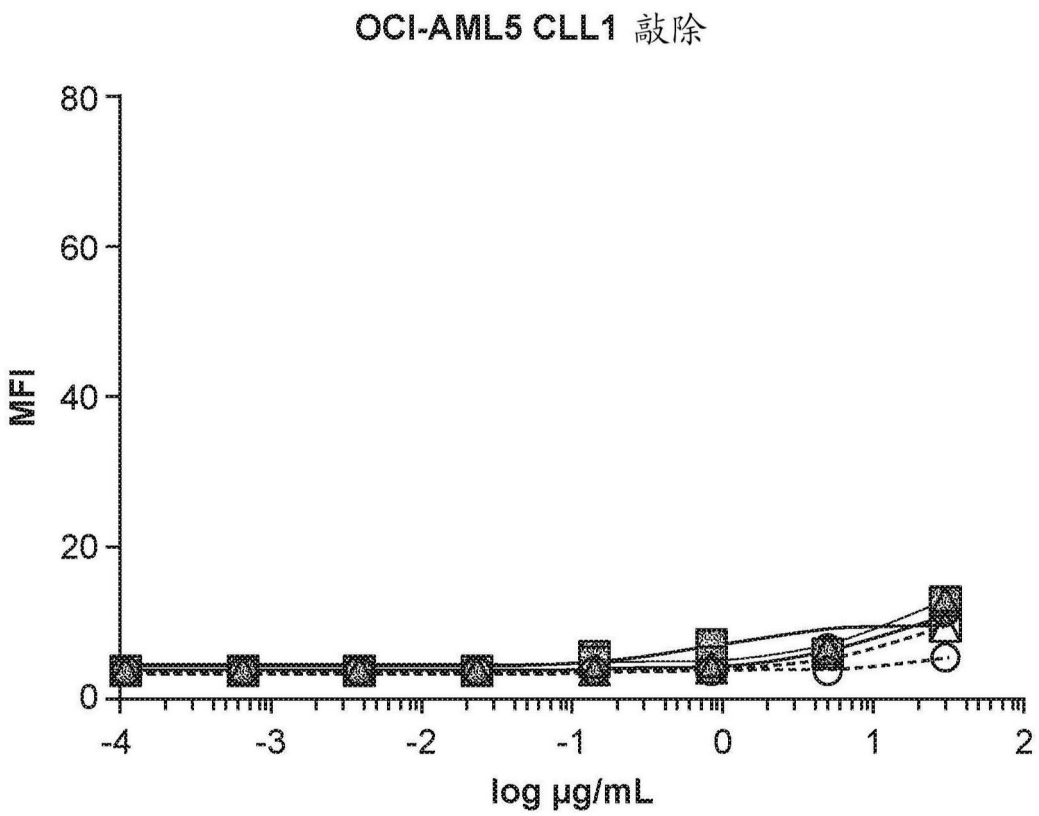

图12B

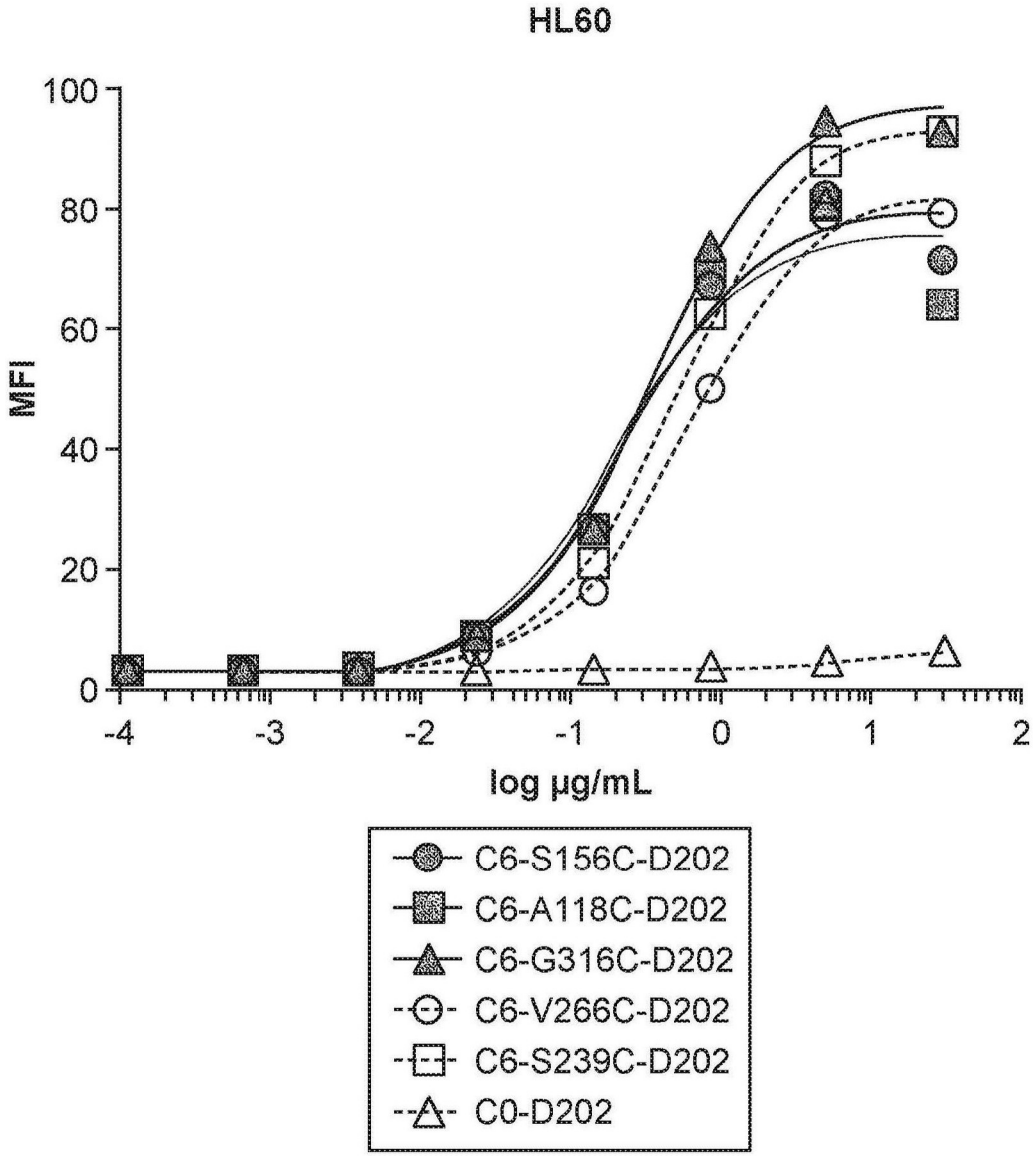
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图12C

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<110> Cellerant Therapeutics, Inc.
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Jiang, Ying Ping
Huang, Jianqing
Mishra, Madhavi

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<151> 2015-07-16

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Lys Leu Leu Ile Tyr Leu Ala Ser Asn Leu Glu Ser Gly Val Pro Ala
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Arg Phe Ser Gly Ser Gly Ser Arg Thr Asp Phe Thr Leu Thr Ile Asp
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Lys Leu Leu Ile Tyr Leu Ala Ser Asn Leu Glu Ser Gly Val Pro Asp
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Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser
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Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
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Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
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Ala Gly Val Glu Thr Thr Lys Pro Ser Lys Gln Ser Asn Asn Lys Tyr
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Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Lys Tyr Asn Glu Lys Phe
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Lys Gly Lys Ala Thr Leu Thr Ser Asp Thr Ser Ser Ser Thr Ala Tyr
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Met Glu Leu Asn Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
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Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Lys Tyr Asn Glu Lys Phe
50 55 60

Lys Gly Lys Ala Thr Ile Thr Ser Asp Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
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Tyr Ala Ala Ser Thr Leu Asp Ser Gly Val Pro Ser Arg Phe Ser Gly
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Asn Arg Ala Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
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Gly Phe Ile Asn Pro Tyr Asn Asp Gly Ser Lys Tyr Ala Gln Lys Phe
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Gln Gly Arg Ala Thr Leu Thr Ser Asp Lys Ser Thr Ser Thr Val Tyr
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Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
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Thr Arg Asp Asp Gly Tyr Tyr Gly Tyr Ala Met Asp Tyr Trp Gly Gln
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Gly Thr Leu Val Thr Val Ser Ser
115 120