MEDITOPES AND MEDITOPE-BINDING ANTIBODIES AND USES THEREOF

PRIORITY CLAIM

[0001] This application claims the benefit of United States Provisional Patent Application Serial Number 61/597,708, filed February 10, 2012; United States Application Serial Number 13/270,207, filed October 10, 2011; and to International Application Number PCT/US2011/055656 filed October 10, 2011, the contents of which are incorporated herein by reference in their entirety.

GOVERNMENT INTEREST

[0002] This invention was made with Government support of NCI comprehensive Cancer Center Grant number CA0335752-28. The government has certain rights in this invention.

BACKGROUND

[0003] Monoclonal antibodies (mAbs) are used in a number of therapeutic, diagnostic, and research applications. Therapeutic and diagnostic areas include cancer, antiviral treatment, autoimmune and inflammatory disease, allergy, cardiovascular disease, osteoporosis, and rheumatology.

[0004] Protein engineering and other efforts have generated mAbs with improved efficacy and targeting (e.g., bispecific mAbs), improved localization, tissue penetration, and blood clearance (e.g., single chain Fab variable fragments (scFvs), diabodies, minibodies, and other fragments), and altered immunostimulatory, safety, toxicity, and/or pharmacokinetic/pharmacodynamics properties, such as those containing modified Fc regions (e.g., through mutation or glycosylation). mAbs have been reengineered to permit site-specific conjugation of small molecules for improved delivery (e.g., ThioMABs) or to irreversibly bind to their antigen (e.g., infinite affinity mAbs). mAbs have also been developed to improve the circulation and presentation of bioactive peptides and other biologics (e.g., CovX-bodies). Conjugation to various agents has allowed targeted immunotherapy and diagnostic methods. Hetero-multimeric scFvs and scFvs or mAbs fused to avidin have been developed for pre-targeted therapy and to improve the detection limits for tumor imaging.

efficacy, synergy, specificity, and safety, and methods and uses of the same. Provided are antibodies, compounds and compositions including peptides and other molecules, and related methods that address such needs.

SUMMARY

[0005] Among the provided embodiments are antibodies, compounds and compositions, including peptides and other molecules for use with the antibodies, as well as methods and uses thereof and for producing the same, as well as compounds, compositions, complexes, mixtures, and systems, e.g., kits, containing the same. In some aspects, the provided embodiments afford improved efficacy, synergy, specificity, and/or safety, compared with available antibodies and associated compounds, compositions, methods and uses.

[0006] Provided are antibodies, including meditope-enabled antibodies (including fragments thereof), which bind to (i.e., are capable of binding to) one or more meditope. In some aspects, the antibodies bind to a meditope that is a cyclic peptide derived from a peptide having an amino acid sequence of SEQ ID NO: 1.

[0007] In some aspects, the antibodies bind to a meditope that is a cyclic peptide derived from a peptide having an amino acid sequence of SEQ ID NO: 2. In some aspects, the antibodies bind to a meditope that is a peptide having an amino acid sequence selected from the group consisting of the sequences set forth in SEQ ID NO: 1, 2, 16-18, 23, 29, 31, 32, 36, 39, 42, 43, 45, 46, 51, 52, 54, and 55, or a cyclic peptide derived therefrom, or the sequences set forth in SEQ ID NOs: 1, 2, and 15-55, or a cyclic peptide derived therefrom, or to any of the meditopes described herein. In some cases, the meditope is a cyclic peptide derived from a peptide of the amino acid sequence set forth in SEQ ID NO: 1 or 2.

[0008] In some aspects, the antibody or fragment binds to the meditope with a particular affinity, such as an affinity that is equal to or substantially equal to one of the exemplary antibodies described herein, such as cetuximab, meditope-enabled trastuzumab, meditope-enabled M5A, or other exemplified antibody. In some examples, the antibodies bind to the meditope or meditopes with a dissociation constant of less than at or about 10 μM, less than at or about 5 μM, or less than at or about 2 μM, less than at or about 1 μM, less than at or about 500, 400, 300, 200, 100 nM, or less, such as at or about 200 picomolar or less, for example, with such a dissociation constant, as measured by a particular technique, such as

surface plasmon resonance (SPR), Isothermal Titration Calorimetry (ITC), fluorescence, fluorescence polarization, NMR, IR, calorimetry titrations; Kinetic exclusion; Circular dichroism, differential scanning calorimetry, or other known method, e.g., by SPR.

[0009] In some aspects, the antibodies include a heavy chain variable (VH) region and/or a light chain variable (VL) region. In some aspects, the VL region has an amino acid sequence comprising a threonine, serine, or aspartate at position 40, a residue other than glycine at position 41, and/or an aspartate or asparagine at position 85, according to Kabat numbering, and/or comprises an isoleucine or leucine at position 10 and isoleucine at position 83, according to Kabat numbering, and/or comprises a valine or isoleucine at position 9 and a residue other than glutamine at position 100, according to Kabat numbering. In some examples, the amino acid sequence of the VL region has a threonine at position 40, an asparagine at position 41, and an aspartate at position 85, according to Kabat numbering.

[0010] In some aspects, the VH region has an amino acid sequence comprising a serine or proline at position 40 and an isoleucine, tyrosine, methionine, phenylalanine, or tryptophan at position 89, according to Kabat numbering. In some examples, the amino acid sequence of the VH region has a serine at position 40 and an isoleucine at position 89, according to Kabat numbering.

[0011] In some examples, the amino acid sequence of the VL region has a valine or isoleucine at position 9, an isoleucine or leucine at position 10, an arginine at position 39, a threonine at position 40, an asparagine at position 41, a glycine at position 42, a serine at position 43, an isoleucine at position 83, an aspartate at position 85, and an alanine at position 100, according to Kabat numbering; and/or the amino acid sequence of the VH region has a serine at position 40 and an isoleucine at position 89, according to Kabat numbering.

[0012] In some aspects of the provided antibodies, the VL region does not contain a proline at position 40, a glycine at position 41, and/or a threonine at position 85, according to Kabat numbering, and/or the VH region does not contain an asparagine or alanine at position 40 and/or a valine at position 89, according to Kabat numbering.

[0013] In some aspects, the VL region does not contain an serine at position 10, a proline at position 40, a glycine at position 41, an phenylalanine at position 83, and/or a threonine at position 85, according to Kabat numbering, and/or the VH region does not

contain an asparagine or alanine at position 40 and/or a valine at position 89, according to Kabat numbering.

[0014] In some aspecst, the antibodies (including fragments) further include one or more constant regions, typically human constant region(s), such as a CL and/or CH1, e.g., human CL and/or CH1.

[0015] In some aspects, the provided antibody or fragment has a VL region with an amino acid sequence comprising a light chain framework region (FR) 1 (FR-L1), an FR-L2, an FR-L3, and/or an FR-L4 of the light chain sequence set forth in SEQ ID NO: 71 or SEQ ID NO: 61 (or an FR-L1, FR-L2, FR-L3, and/or FR-L4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-L1, FR-L2, FR-L3, and/or FR-L4 of the light chain of SEQ ID NO: 71 or 61), and in some aspects at least one complementarity determining region (CDR) that is distinct from the CDRs of the light chain sequence set forth in SEQ ID NO: 71; and/or a VH region with an amino acid sequence having a heavy chain FR1 (FR-H1), an FR-H2, an FR-H3, and/or an FR-H4, of the heavy chain sequence set forth in SEQ ID NO: 72 or SEQ ID NO: 63 (or an FR-H1, FR-H2, FR-H3, and/or FR-H4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-H1, FR-H2, FR-H3, and/or FR-H4 of the heavy chain of SEQ ID NO: 72 or 63), and in some aspects at least one CDR that is distinct from the CDRs of the heavy chain sequence set forth in SEQ ID NO: 72.

[0016] In some aspects, the provided antibody or fragment has a CDR of the light chain sequence set forth in SEQ ID NO: 61, a CDR of the heavy chain sequence set forth in SEQ ID NO: 63.

[0017] In some aspects, the VL comprises the amino acid sequence of SEQ ID NO: 76 and the VH comprises the amino acid sequence of SEQ ID NO: 77.

[0018] In some aspects, the antibody has a VL region with an amino acid sequence comprising a light chain framework region (FR) 1 (FR-L1), an FR-L2, an FR-L3, and/or an FR-L4 of the light chain sequence set forth in SEQ ID NO: 9 (or an FR-L1, FR-L2, FR-L3, and/or FR-L4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-L1, FR-L2, FR-L3, and/or FR-L4 of SEQ ID NO: 9); and/or a VH region with an amino acid sequence having a heavy chain FR1 (FR-H1), an FR-H2, an FR-H3, and/or an FR-H4 of the heavy chain sequence set forth in SEQ ID NO: 6 (or an FR-H1, FR-

H2, FR-H3, and/or FR-H4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-H1, FR-H2, FR-H3, and/or FR-H4 of SEQ ID NO: 9). In some examples, the VL region comprises at least one complementarity determining region (CDR) that is distinct from the CDRs of the VL sequence set forth in SEQ ID NO: 9; and/or the VH region comprises at least one CDR that is distinct from the CDRs of the VH sequence set forth in SEQ ID NO: 6.

[0019] In some examples, the VL region comprises the amino acid sequence of SEQ ID NO: 73. In some examples, the VH region comprises the amino acid sequence of SEQ ID NO: 74.

[0020] In some aspects, the antibody or fragment has a VL region with an amino acid sequence comprising a light chain framework region (FR) 1 (FR-L1), an FR-L2, an FR-L3, and/or an FR-L4 of the light chain sequence set forth in SEQ ID NO: 68 (or an FR-L1, FR-L2, FR-L3, and/or FR-L4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-L1, FR-L2, FR-L3, and/or FR-L4 of the light chain of SEQ ID NO: 68). In some examples, the VL region comprises at least one complementarity determining region (CDR) that is distinct from the CDRs of the light chain sequence set forth in SEQ ID NO: 69; and/or the VH region comprises at least one CDR that is distinct from the CDRs of the heavy chain sequence set forth in SEQ ID NO: 70.

[0021] In some examples, the VL region comprises the amino acid sequence of SEQ ID NO: 75.

[0022] In some aspects, the antibody does not specifically bind to the epitope of an EGFR that is specifically bound by cetuximab, does not contain the CDRs of cetuximab, and/or does not compete for antigen binding with cetuximab. In other aspects, the antibody is cetuximab.

[0023] In some aspects, the antibodies or fragments compete for antigen binding with, specifically bind to the same antigen or epitope as, and/or contain one, more, or all CDRs (or CDRs comprising at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identity to the CDRs), e.g., including a heavy chain CDR 1, 2, and/or 3 and/or a light chain CDR1, 2, and/or 3, of one or more known antibodies, including any commercially available antibody, such as abagovomab, abciximab, adalimumab, adecatumumab, alemtuzumab, altumomab, altumomab pentetate, anatumomab, anatumomab mafenatox,

arcitumomab, atlizumab, basiliximab, bectumomab, ectumomab, belimumab, benralizumab, bevacizumab, brentuximab, canakinumab, capromab, capromab pendetide, catumaxomab, certolizumab, clivatuzumab tetraxetan, daclizumab, denosumab, eculizumab, edrecolomab, efalizumab, etaracizumab, ertumaxomab, fanolesomab, Fbta05, fontolizumab, gemtuzumab, girentuximab, golimumab, ibritumomab, igovomab, infliximab, ipilimumab, labetuzumab, mepolizumab, muromonab, muromonab-CD3, natalizumab, necitumumab, nimotuzumab, ofatumumab, omalizumab, oregovomab, palivizumab, panitumumab, ranibizumab, rituximab, satumomab, sulesomab, ibritumomab, ibritumomab tiuxetan, tocilizumab, tositumomab, trastuzumab, Trbs07, ustekinumab, visilizumab, votumumab, zalutumumab, and/or brodalumab; and/or anrukinzumab, bapineuzumab, dalotuzumab, demcizumab, ganitumab, inotuzumab, mavrilimumab, moxetumomab pasudotox, rilotumumab, sifalimumab, tanezumab, tralokinumab, tremelimumab, urelumab, the antibody produced by the hybridoma 10B5 (see Edelson & Unanue, *Curr Opin Immunol*, 2000 Aug;12(4):425-31), B6H12.2 (abcam) or other anti-CD47 antibody (see Chao et al., *Cell*, 142, 699–713, September 3, 2010); and/or an antibody or fragment thereof having a sequence set forth in any of SEQ ID NOs: 78-124, and/or 125-170.

[0024] In some aspects, the antibody or fragment specifically binds to an antigen selected from the group consisting of: CA-125, glycoprotein (GP) IIb/IIIa receptor, TNF-alpha, CD52, TAG-72, Carcinoembryonic antigen (CEA), interleukin-6 receptor (IL-6R), IL-2, interleukin-2 receptor a-chain (CD25), CD22, B-cell activating factor, interleukin-5 receptor (CD125), VEGF, VEGF-A, CD30, IL-1beta, prostate specific membrane antigen (PSMA), CD3, EpCAM, EGF receptor (EGFR), MUC1, human interleukin-2 receptor, Tac, RANK ligand, a complement protein, e.g., C5, EpCAM, CD11a, e.g., human CD11a, an integrin, e.g., alpha-v beta-3 integrin, vitronectin receptor alpha v beta 3 integrin, HER2, neu, CD3, CD15, CD20 (small and/or large loops), Interferon gamma, CD33, CA-IX, TNF alpha, CTLA-4, carcinoembryonic antigen, IL-5, CD3 epsilon, CAM, Alpha-4-integrin, IgE, e.g., IgE Fc region, an RSV antigen, e.g., F protein of respiratory syncytial virus (RSV), TAG-72, NCA-90(granulocyte cell antigen), IL-6, GD2, GD3, IL-12, IL-23, IL-17, CTAA16.88, IL13, interleukin-1 beta, beta-amyloid, IGF-1 receptor (IGF-1R), delta-like ligand 4 (DLL4), , alpha subunit of granulocyte macrophage colony stimulating factor receptor, hepatocyte growth factor, IFN-alpha, nerve growth factor, IL-13, CD326, Programmed cell death 1 ligand 1 (PD-L1, a.k.a. CD274, B7-H1), CD47, and CD137.

[0025] In some aspects, the antibody or fragment has a light chain having P8 , V9 or I9, I10 or L10, Q38, R39, T40, N41 G42, S43, P44, R45, D82, I83, A84, D85, Y86, Y87, G99, A100, G101, T102, K103, L104, E105, R142, S162, V163, T164, E165, Q166, D167, S168, and/or Y173, according to Kabat numbering, and/or has a heavy chain having Q6, P9, R38, Q39, S40, P41, G42, K43, G44, L45, S84, D86, T87, A88, I89, Y90, Y91, W103, G104, Q105, G106, T107, L108, V111, T110, Y147, E150, P151, V152, T173, F174, P175, A176, V177, Y185, S186, and/or L187, according to Kabat numbering.

[0026] Also provided are complexes containing an antibody or antibodies (e.g., meditope-enabled antibodies) bound to one or more meditope. The antibody or antibody can be any of the meditope-enabled antibodies described herein, such as any of the aforementioned antibodies (including fragments thereof). The one or more meditope can include any one or more of the meditopes described herein, such as those described in this section, including monovalent and multivalent meditopes, and labeled meditopes, as well as meditope fusion proteins.

[0027] Also provided are meditopes, e.g., isolated meditopes. Among the provided meditopes are those described above. Among the provided meditopes are those comprising a peptide that binds to a meditope binding site of a meditope-enabled antibody, wherein the peptide is not a peptide of SEQ ID NO: 1 or 2 or cyclic peptide derived therefrom. In other aspects, the meditope is a peptide of SEQ ID NO: 1 or 2, or a cyclic peptide derived therefrom.

[0028] In some asepcts, the meditope (e.g., the peptide) binds to the meditope binding site with a dissociation constant of of less than at or about 10 μM, less than at or about 5 μM, or less than at or about 2 μM, less than at or about 1 μM, less than at or about 500, 400, 300, 200, 100 nM, or less, such as at or about 200 picomolar or less, for example, with such a dissociation constant, as measured by a particular technique, such as surface plasmon resonance (SPR), Isothermal Titration Calorimetry (ITC), fluorescence, fluorescence polarization, NMR, IR, calorimetry titrations; Kinetic exclusion; Circular dichroism, differential scanning calorimetry, or other known method, e.g., by SPR.

[0029] In some aspects, the meditope binding site includes residues 40, 41, 83, and/or 85 of the light chain of the meditope-enabled antibody, according to Kabat numbering,

and/or residues 39, 89, 105, and/or 108 of the heavy chain of the meditope-enabled antibody, according to Kabat numbering.

[0030] In some aspecst, the meditope binds to a meditope-enabled antibody having: a light chain comprising an amino acid sequence set forth in SEQ ID NO: 71 and a heavy chain comprising an amino acid sequence set forth in SEQ ID NO: 72; to a meditope-enabled antibody having a light chain having an amino acid sequence set forth in SEQ ID NO: 9 and a heavy chain comprising an amino acid sequence set forth in SEQ ID NO: 6; and/or to a meditope-enabled antibody having a light chain having an amino acid sequence set forth in SEQ ID NO: 68 and/or a heavy chain having an amino acid sequence set forth in SEQ ID NO: 70.

[0031] In some aspects, the peptide is between 5 and 16 amino acids in length.

[0032] In some aspects, the peptide has the formula:

X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12 (Formula I)

wherein:

X1 = Cys, Gly, β-alanine, 2,3-diaminopropionic acid, β-azidoalanine, or null;

X2 = Gln or null;

X3 = Phe, Tyr, β-β'-diphenyl-Ala, His, Asp, 2-bromo-L-phenylalanine, 3-bromo-L-phenylalanine, or 4-bromo-L-phenylalanine, Asn, Gln, a modified Phe, a hydratable carbonyl-containing residue; or a boronic acid-containing residue;

X4 = Asp or Asn;

X5 = Leu; β-β'-diphenyl-Ala; Phe; a non-natural analog of phenylalanine, tryptophan, or tyrosine; a hydratable carbonyl-containing residue; or a boronic acid-containing residue;

X6 = Ser or Cys;

X7 = Thr or Ser or Cys;

X8 = Arg, a modified Arg, or a hydratable carbonyl or boronic acid-containing residue;

X9 = Arg, Ala;

X10 = Leu, Gln, Glu, β-β'-diphenyl-Ala; Phe; a non-natural analog of phenylalanine, tryptophan, or tyrosine; a hydratable carbonyl-containing residue; or a boronic acid-containing residue;

X11= Lys; and

X12 = Cys, Gly, 7-aminoheptanoic acid, β-alanine, diaminopropionic acid, propargylglycine, isoaspartic acid, or null,

wherein:

the modified Arg has a structure of the formula shown in Figure 34,

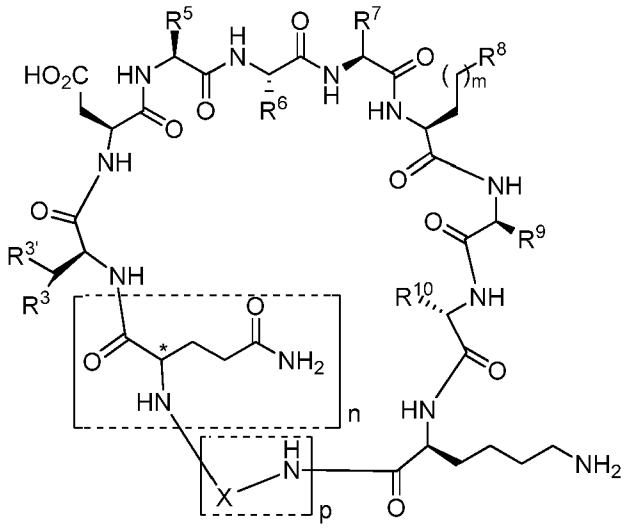
the modified Phe is a Phe with one or more halogen incorporated into the phenyl ring, and

formula I is not SEQ ID NO: 1 or SEQ ID NO: 2 or a cyclic peptide derived therefrom.

[0033] In some aspects, the peptide is a cyclic peptide, such as one in which the cyclization is by disulfide bridge, a thioether bridge, a lactam linkage, cycloaddition. In certain aspects, where the peptide is one of Formula I, the cyclization is via a linkage between X1 and X12, X1 and X11, X3 and X11, X4 and X11, or X2 and X12. In some aspects, where the peptide is one of Formula I, thenon-natural amino acid is β-β'-diphenyl-Ala, a branched alkyl, or an extended aromatic. In some aspects, where the peptide is one of Formula I, each of the one or more halogen is an ortho-, meta-, or para-bromo phenyl substituent.

[0034] Among the provided meditopes are peptides having an amino acid selected from the group consisting of the sequences set forth in SEQ ID NOs: 1, 2, and 15-55, e.g., 1, 2, 16-18, 23, 29, 31, 32, 36, 39, 42, 43, 45, 46, 51, 52, 54, and 55, e.g., 16-18, 23, 29, 31, 32, 36, 39, 42, 43, 45, 46, 51, 52, 54, and 55, or a cyclic peptide derived therefrom.

[0035] In some embodiments, the meditope comprises a compound of Formula (X):



(X)

wherein:

the center marked with "*" is in the "R" or "S" configuration;

R^3 and $R^{3'}$ are each, independently, H or phenyl, optionally substituted with one, two, or three substituents independently selected from C_{1-4}alkyl, -OH, fluoro, chloro, bromo, and iodo;

R^5 is:

(A) C_{1-8}alkyl, optionally substituted with one or more substituents selected from the group consisting of oxo, acetal, ketal, $-B(OH)_2$, boronic ester, phosphonate ester, ortho ester, $-CO_2C_{1-4}$alkyl, -CH=CH-CHO, $-CH=CH-C(O)C_{1-4}$alkyl, $-CH=CH-CO_2C_{1-4}$alkyl, $-CO_2H$, and $-CONH_2$ group; or

(B) a C_{1-4}alkyl group substituted with:

a) one or two phenyl groups, wherein each phenyl is optionally substituted with one, two, or three substituents independently selected from -OH, fluoro, chloro, bromo, and iodo; or

b) a naphthyl, imidazole, or indole group;

R^6 is $-C_{1-4}$alkyl-OH or $-C_{1-4}$alkyl-SH;

R^7 is $-C_{1-4}$alkyl-OH or $-C_{1-4}$alkyl-SH;

m is 0, 1, 2, 3, 4, or 5;

R^8 is:

(a) -OH, $-NR^aR^b$, $-N(R^c)C(O)R^e$, or $-N(R^c)C(=NR^d)R^e$;

wherein:

R^a is H;

R^b is H or C_{1-8}alkyl optionally substituted with one or more substituents selected from the group consisting of oxo, acetal, and ketal,

$-B(OH)_2$, -SH, boronic ester, phosphonate ester, ortho ester, -CH=CH-CHO, $-CH=CH-C(O)C_{1-4}$alkyl, $-CH=CH-CO_2C_{1-4}$alkyl, $-CO_2H$, or $-CO_2C_{1-4}$alkyl group;

R^c is H, C_{1-8}alkyl, C_{3-8}cycloalkyl, branched alkyl, or aryl;

R^d is H or a C_{1-8}alkyl, C_{2-8}alkenyl, C_{2-8}alkynyl, C_{3-8}cycloalkyl, branched alkyl, or aryl group, each optionally substituted with one or more substituents selected from the group consisting of $-N_3$, $-NH_2$, -OH, -SH, halogen, oxo, acetal, ketal, $-B(OH)_2$, boronic ester, phosphonate ester, ortho ester, -CH=CH-CHO, $-CH=CH-C(O)C_{1-4}$alkyl, $-CH=CH-CO_2C_{1-4}$alkyl, $-CO_2H$, and $-CO_2C_{1-4}$alkyl group; and

R^e is H; $-NHR^d$; or a C_{1-12}alkyl, C_{3-8}cycloalkyl, C_{2-12}alkenyl, C_{2-8}alkynyl, or aryl group, each optionally substituted with one or more substituents selected from the group consisting of $-N_3$, $-NH_2$, -OH, -SH, oxo, C_{2-4}acetal, C_{2-4}ketal, $-B(OH)_2$,

boronic ester, phosphonate ester, ortho ester, -CH=CH-CHO, -CH=CH-C(O)C_{1-4}alkyl, -CH=CH-CO_2C_{1-4}alkyl, and -CO_2C_{1-4}alkyl group; or

(b) a C_{1-12} alkyl substituted with an oxo, acetal, ketal, -$B(OH)_2$, boronic ester, -SH, -OH, phosphonate ester, ortho ester, -CH=CH-CHO, -CH=CH-C(O)C_{1-4}alkyl, -CH=CH-CO_2C_{1-4}alkyl, or -CO_2C_{1-4}alkyl group;

R^9 is C_{1-4}alkyl or –C_{1-2}alkylene-R^x;

wherein R^x is -CO_2H, -$CONH_2$, -$CH_2NHC(O)NH_2$, or –$CH_2NHC(=NH)NH_2$;

R^{10} is:

(1) a C_{1-8}alkyl optionally substituted with one or more substituents selected from the group consisting of oxo, acetal, ketal, -$B(OH)_2$, boronic ester, phosphonate ester, ortho ester, -CH=CH-CHO, -CH=CH-C(O)C_{1-4}alkyl, -CH=CH-CO_2C_{1-4}alkyl, -CO_2C_{1-4}alkyl, -CO_2H, and –$CONH_2$ group; or

(2) a C_{1-4}alkyl group substituted with one or two phenyl groups, or one naphthyl, imidazole, or indole group, wherein each phenyl is optionally substituted with one, two, or three substituents independently selected from -OH, fluoro, chloro, bromo, and iodo;

n is 0 or 1;

p is 0 or 1;

X is C_{1-8}alkylene or C_{2-8}alkenylene, each carbon thereof optionally substituted with -CO_2H, -NH_2, or -$NHC(O)R^y$;

wherein one carbon of said alkylene is optionally replaced with -C(O)NH-, a 5-membered heteroaryl ring, or -S-S-; and

R^y is –C_{1-4}alkyl or –$CH(R^z)CO_2H$;

wherein R^z is –H or –C_{1-4}alkyl optionally substituted with –OH, -SH, or –NH_2;

or a pharmaceutically acceptable salt thereof.

[0036] In one embodiment, a meditope contains a cysteine that covalently binds to a cysteine in a meditope binding site. Such a meditope may be conjugated to any substance, molecule or compound, which may be a therapeutic molecule, such as a small molecule diagnostic molecule, such as a marker. In some aspects, the “Cys meditope” directs the conjugate to the Ig and binds via a covalent linkage.

[0037] Also among the provided meditopes are labeled meditopes, such as thosecomprising a meditope (such as any of those described above) and a therapeutic or diagnostic agent. In some aspects, the therapeutic or diagnostic agent is selected from the

group consisting of: a metal chelator bound to a metal ion, a small molecule, a chemotherapeutic agent, a therapeutic antibody or functional fragment, a toxin, a radioisotope, an enzyme, a nuclease, a hormone, an immunomodulator, an oligonucleotide, an organic or inorganic nanoparticle, an RNAi molecule, an siRNA, a chelator, a boron compound, a photoactive agent, a dye, fluorescent or luminescent substance, an enzyme, an enhancing agent, a radioactive substance, and a chelator.

[0038] Also among the provided meditopes are multivalent meditopes, such as those comprising two or more meditopes and one or more linker, e.g., where each of the two or more meditopes is a peptide that binds to a meditope binding site of a meditope-enabled antibody. Such multivalent meditopes may comprise any of the meditopes described herein, e.g., above. In one aspect, the two or more meditopes comprise at least three meditopes, or at least four meditopes. In one aspect, the one or more linker includes a peptide, a small chemical scaffold, a biotin-streptavidin, an organic or inorganic nanoparticle, a polynucleotide sequence, peptide nucleic acid, an organic polymer, or an immunoglobulin Fc domain.

[0039] Also among the provided embodiments are meditope-enabled antibody-meditope complexes, including those containing any of the meditope-enabled antibodies and any of the meditopes described herein, e.g., described above. In some cases, the peptide binds to the meditope binding site with a dissociation constant of less than 10 μM, as measured by surface plasmon resonance (SPR), or another affinity as described above.

[0040] Also provided are methods using the meditopes and/or antibodies, such as any of the above meditopes and/or antibodies. For example, provided are methods for purifying a meditope-enabled antibody or fragment thereof, or a cell expressing the meditope-enabled antibody or fragment thereof. In one aspect, such methods include a step of contacting a composition containing the meditope-enabled antibody, fragment, or cell with a meditope, under conditions whereby the meditope-enabled antibody or fragment binds to the peptide. In some aspect, they further include then isolating the antibody or fragment or cell. Such methods in some aspects therby purify the antibody, fragment, or cell.

[0041] In some aspects, the meditope is coupled to a solid support. In some aspects, the isolation or purification is effected by a change in pH.

[0042] Also provided are compositions, e.g., pharmaceutical compositions, comprising the meditopes (including multivalent and labeled meditopes), meditope-enabled antibodies, and complexes, and/or other compounds described herein, e.g., above. In one example, the composition includes the complex, meditope, and/or meditope-enabled antibody, and a pharmaceutically acceptable carrier.

[0043] Also provided are methods of treatment, such as those carried out by administering to a subject such pharmaceutical compositions, or any of the meditope-enabled antibodies, meditopes, complexes, and/or other compound described herein, e.g., above. In one example, the methods include administering to the subject an antibody or fragment as described above.

[0044] In one example, the method of treatment includes administering to a subject one or more meditope-enabled antibody or fragment, e.g., any of those described above. In one example, the method includes administering to the subject one or more meditope-enabled antibody or fragment as described above, and a meditope, e.g., any of those described above, including multivalent meditopes and meditopes coupled to a therapeutic or diagnostic agent. In one aspect, the meditope-enabled antibody or fragment and one or more meditope are administered sequentially. In another aspect, they are administered simultaneously. Generally, the one or more meditope comprises a peptide that binds to a meditope binding site of the meditope-enabled antibody or fragment. In one aspect, the meditope-enabled antibody or fragment is bound to the one or more meditope, such that administration of the meditope-enabled antibody and the one or more meditope comprises administering a complex of the meditope-enabled antibody and the meditope. In another aspect, the meditope-enabled antibody is administered prior to administration of the one or more meditope.

[0045] In some aspects, the one or more meditope is coupled to a therapeutic agent, such as a therapeutic agent selected from the group consisting of: drugs, chemotherapeutic agents, therapeutic antibodies, toxins, radioisotopes, enzymes, chelators, boron compounds, photoactive agents, dyes, metals, metal alloys, and nanoparticles.

[0046] Also provided are diagnostic methods, such as those carried out by administering to a subject such pharmaceutical compositions, or any of the meditope-enabled antibodies, meditopes, complexes, and/or other compound described herein, e.g., above, and detecting binding of the administered composition, antibody, meditope, complex, and/or

compound to a substance in the subject, e.g., to an antigen. In some aspects, the diagnostic method includes administering to a subject one or more meditope-enabled antibody or fragment thereof, e.g., any of those described above. In some aspects, it further includes detecting binding of the antibody or fragment to an antigen in the subject. In some aspects, the meditope-enabled antibody or fragment and one or more meditope are administered sequentially; in other aspecst, they are administered simultaneously. In one example, the meditope-enabled antibody or fragment is bound to the one or more meditope, such that administration of the meditope-enabled antibody and the one or more meditope comprises administering a complex of the meditope-enabled antibody and the meditope. In another example, the meditope-enabled antibody is administered prior to administration of the one or more meditope. In some aspects, the meditope or meditopes is a multivalent meditope. In some aspects, the meditope is coupled to a diagnostic agent, such as an imaging agent, such as an imaging agent selected from the group consisting of: fluorescent substances, luminescent substances, dyes, indicators, and radioactive substances. In some cases, the imaging agent is DOTA.

[0047] Also provided are methods for generating meditope-enabled antibodies or fragments thereof, e.g., based on template antibodies. In some aspects, such methods include effecting one or more amino acid substitutions in a template antibody or fragment thereof. In one example, the substitutions include a substitution at position 40, 41, or 85 of the VL region, and/or position 40 or position 89 of the VH region, according to Kabat numbering.

[0048] In some aspects, the methods generate a meditope-enabled antibody or fragment thereof which binds to a meditope that is a peptide having an amino acid sequence selected from the group consisting of the sequences set forth in SEQ ID NO: 1, 2, 16-18, 23, 29, 31, 32, 36, 39, 42, 43, 45, 46, 51, 52, 54, and 55, or a cyclic peptide derived therefrom.

[0049] In some aspects, the template antibody or fragment is selected from the group consisting of abagovomab, abciximab, adalimumab, adecatumumab, alemtuzumab, altumomab, altumomab pentetate, anatumomab, anatumomab mafenatox, arcitumomab, atlizumab, basiliximab, bectumomab, ectumomab, belimumab, benralizumab, bevacizumab, brentuximab, canakinumab, capromab, capromab pendetide, catumaxomab, certolizumab, clivatuzumab tetraxetan, daclizumab, denosumab, eculizumab, edrecolomab, efalizumab, etaracizumab, ertumaxomab, fanolesomab, Fbta05, fontolizumab, gemtuzumab, girentuximab, golimumab, ibritumomab, igovomab, infliximab, ipilimumab, labetuzumab,

mepolizumab, muromonab, muromonab-CD3, natalizumab, necitumumab, nimotuzumab, ofatumumab, omalizumab, oregovomab, palivizumab, panitumumab, ranibizumab, rituximab, satumomab, sulesomab, ibritumomab, ibritumomab tiuxetan, tocilizumab, tositumomab, trastuzumab, Trbs07, ustekinumab, visilizumab, votumumab, zalutumumab, and brodalumab, or is the antibody produced by the hybridoma 10B5, or is B6H12.2.

[0050] In some aspects, the meditope-enabled antibody binds to one or more meditopes (e.g., one of SEQ ID NO: 1, 2, or cyclic peptide derived therefrom), e.g., those described above, with a particular affinity, such as with a dissociation constant of less than 10, 5, or 2 (or other number specified above) μM, as measured by SPR, or other method described above.

[0051] In some aspects, each of the one or more amino acid substitutions is at a position selected from the group consisting of: position 8 , 9, 10, 38, 39, 40, 41 42, 43, 44, 45, 82, 83, 84, 85, 86, 87, 99, 100, 101, 102, 103, 104, 105, 142, 162, 163, 164, 165, 166, 167, 168, and 173 of the light chain, and/or 6, 9, 38, 39, 40, 41, 42, 43, 44, 45, 84, 86, 87, 88, 89, 90, 91, 103, 104, 105, 106, 107, 108, 109, 110, 147, 150, 151, 152, 173, 174, 175, 176, 177, 185, 186, and 187 of the heavy chain, according to Kabat numbering. In some aspects, the meditope-enabled antibody contains a light chain having P8 , V9 or I9, I10 or L10, Q38, R39, T40, N41 G42, S43, P44, R45, D82, I83, A84, D85, Y86, Y87, G99, A100, G101, T102, K103, L104, E105, R142, S162, V163, T164, E165, Q166, D167, S168, and Y173, according to Kabat numbering and a heavy chain having Q6, P9, R38, Q39, S40, P41, G42, K43, G44, L45, S84, D86, T87, A88, I89, Y90, Y91, W103, G104, Q105, G106, T107, L108, V111, T110, Y147, E150, P151, V152, T173, F174, P175, A176, V177, Y185, S186, and L187, according to Kabat numbering.

[0052] Also provided are polynucleotides comprising a nucleotide sequence encoding the meditope-enabled antibodies and meditopes, as well as vectors including the same, and libraries comprising the vectors.

[0053] Also provided are screening methods, such as those including the steps of expressing antibodies or fragments thereof from the libraries, and selecting an antibody or fragment thereof from among the expressed antibodies or fragments. In some cases, the selection is based on a binding affinity, pH tolerance, pH dependence, toxicity, PK, or PD of the selected antibody or fragment thereof, and/or is effected by contacting the antibodies or

fragments thereof with a meditope and detecting binding to one or more of the antibodies or fragments thereto.

[0054] Also provided are methods for selecting a meditope analog or meditope variant, including those comprising: (a)combining a meditope and a meditope-enabled antibody or fragment thereof, whereby the meditope non-covalently binds to the antibody or fragment thereof; and (b) adding a candidate meditope variant or analog; and (c) measuring displacement of the meditope by the candidate meditope variant or analog, wherein displacement of the meditope by the candidate variant or analog identifies it as the meditope variant or analog.

[0055] Also provided are methods for modifying meditopes and meditope-enabled antibodies, including a method for modifying a meditope, comprising effecting one or more amino acid substitutions, insertions, or deletions, to a peptide having the sequence set forth in any of SEQ ID NOs: 1, 2, 16-18, 23, 29, 31, 32, 36, 39, 42, 43, 45, 46, 51, 52, 54, and 55, or cyclic peptide derived therefrom, thereby altering the pH-dependence of the binding affinity of the peptide for a meditope-enabled antibody or fragment thereof, such as any of those described above. In some aspects, the method decreases the binding affinity of the peptide for the antibody or fragment at a lysosomal pH level. In other aspects, the method increases the binding affinity in a hypoxic environment.

[0056] Also provided are methods for modifying a meditope-enabled antibody, such as those comprising: effecting one or more modificaitons at position 8 , 9, 10, 38, 39, 40, 41 42, 43, 44, 45, 82, 83, 84, 85, 86, 87, 99, 100, 101, 102, 103, 104, 105, 142, 162, 163, 164, 165, 166, 167, 168, and/or 173 of the light chain, or 6, 9, 38, 39, 40, 41, 42, 43, 44, 45, 84, 86, 87, 88, 89, 90, 91, 103, 104, 105, 106, 107, 108, 109, 110, 147, 150, 151, 152, 173, 174, 175, 176, 177, 185, 186, and/or 187 of the heavy chain, of the meditope-enabled antibody, according to Kabat numbering. In some aspects, the modification methods are provided in tandem, to produce modified meditopes and modified meditope-enabled antibodies that bind to one another, such as by making modifications in the antibody based on modifications in a modified meditope, or vice versa.

[0057] Also provided are meditope analogs, including those that bind to any of the meditope-enabled antibodies described above, and/or with binding properties comparable to any of the meditopes described above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0058] Figure 1 shows meditope peptides binding to framework loops of cetuximab. Figure 1A: The complex of cetuximab Fab (light chain is denoted by V_L and C_L; heavy chain is denoted by V_H and C_H) and cyclic CQFDLSTRRLKC (depicted within the shaded area and labeled with the word "meditope") (SEQ ID NO: 1) indicates that the meditope binds to an interface of the Fab framework, which is distinct from the CDR loops of cetuximab. Figure 1B (top) shows the stick representation of the cQFD meditope and (bottom) the stick representation of the cQYN meditope. The N- and C- terminal cysteines are solvent exposed and display high thermal factors.

[0059] Figure 2 shows certain embodiments of the cetuximab Fab binding interface. cetuximab is a human-murine chimera and, therefore, has a mixture of murine Ig variable domains and human constant Ig domains. Figure 2A shows residues of ch14.18 and the humanized trastuzumab, which correspond to those residues of cetuximab that make contact with the meditope. Figure 2B shows a stereoview of Arg9 of the cQFD meditope that occupies a distinct pocket encoded by the murine sequence of cetuximab (foreground). There is a salt bridge from Asp85 of the cetuximab light chain to the guanidinium group of the meditope Arg9 and the backbone amide of Leu10.

[0060] Figure 3 shows surface plasmon resonance (SPR) traces of cQFD and cQYN with an immobilized Fab. The cetuximab Fab used for crystallographic studies was coupled to a CM5 chip at low densities. Traces at increasing concentrations of the cQFD (Figure 3A) and cQYN (Figure 3B) meditopes are shown. The series of traces are fit to a simple 1:1 Langmuir binding model. The residuals of the fit are shown below each.

[0061] Figure 4 illustrates that the cQFD and cQYN meditopes do not bind to the CDRs of cetuximab, as was previously hypothesized. Figure 4A shows an overlay of two crystal structures, one of a cetuximab Fab and its antigen, EGFR domain III, the other showing the cQFD meditope and cetuximab Fab, in which the cQFD meditope binds to the central cavity of the cetuximab Fab. The antigen, EGFR domain III, binds at the complementarity determining regions at a significant distance from the meditope binding site. Figure 4B shows SDS-PAGE results on the left-hand side and the corresponding size exclusion chromatography results on the right-hand side. Size exclusion experiments of the individual components, Fab, EGFR domain III, and SMT-cQFD meditope, as well as an

admixture of all three, indicate the formation of a hetero-trimeric complex that coeluted. The non-reducing SDS-PAGE gel shows the fraction that eluted first, indicating the presence of all three components within the new peak (the left-most peak for "complex," shaded light gray) observed from the admixture. Figure 4C shows the results of a FACS analysis, indicating that the cQFD meditope bound to EGFR positive MD-MBA-468 cells only in the presence of cetuximab (arrows). The meditope alone or the meditope in the presence of M425, a murine EGFR antibody, did not bind. Figure 4D shows results of surface plasmon resonance experiments using a sensor chip coupled with a cetuximab scFv or Fab. The experiments indicate that saturation of the scFv could not be achieved at concentrations as high as 100 μM of the cQFD meditope. The same experiments using the cetuximab Fab coupled sensor chip indicate full saturation. The dissociation constant from this experiment is 660 nM. Control SPR experiments (bottom panel) show that the cetuximab scFv readily binds to the soluble EGFR domain III fragment, indicating that the CDR loops were functional.

[0062] Figure 5 is a graph of fluorescence intensity compared to cell count and shows fluorescently-labeled cetuximab binding to MDA-MB-468 cells in the presence of cQFD. Cetuximab binds to MDA-MB-468 cells without meditope, and in the presence of 6 μM and 60 μM meditope. As expected, the Figure shows that there was no binding of the isotype IgG control to EGFR on MDA-MB468 cells.

[0063] Figure 6 shows how meditope and EGFR bind to distinct sites. The top images show superposition of the cQFD-Fab complex and the EGFR-Fab complex (1YY8) in stereo. The bottom images show that residues 39-46 of the heavy chain are flexible and accommodate the meditope.

[0064] Figure 7 shows Fab framework binders. Superposition of Fabs bound to meditope, Protein A, and Protein L indicate that each binds to a unique site on the cetuximab Fab.

[0065] Figure 8 illustrates a mechanism of action in one embodiment for enhancing tumor therapy. In the exemplified embodiment, bivalent antibodies bound to antigen (e.g., ErbB receptor family) overexpressed on tumor cells (left panel) and blocks receptor signaling, alters endocytosis and receptor recycling and/or elicits an immune response. The addition of an antibody such as matuzumab which recognizes a different domain of EGFR in

combination with cetuximab can, in some cases be more effective due to daisy chaining of the surface receptor (right panel 1). A multivalent meditope (in this particular example, trivalent) tethers/cross-links the therapeutic mAb and can enhance its therapeutic potential (right). In addition, the multivalent meditope (shown here as a trivalent version) can also carry an imaging agent, which can allow for both enhanced therapeutics as well as imaging.

[0066] Figure 9 shows an scFv-meditope linker. scFvs are created by fusing the light chain variable domain to the heavy chain variable domain (or *vice versa*) through a 12-20 amino acid linker (typically $\{GGGS\}_{3-5}$). In this example, a portion of the flexible linker sequence can be replaced with the meditope sequence.

[0067] Figure 10 shows results from an SDS-PAGE showing that the cQFD meditope coupled to beads could bind to cetuximab. Biotinylated cQFD peptide was added to avidin-coupled beads, thoroughly washed, and equilibrated in PBS. Cetuximab was then added to the beads (lane 1), washed (lanes 2-4), and then eluted (lane 5). The top band is the IgG heavy chain and bottom band is the IgG light chain.

[0068] Figure 11 is a depiction of an exemplary embodiment of a steric mask. In this exemplary embodiment, the meditope is fused to the N-terminus of the Fab light or heavy chain through a flexible linker containing a tumor associated protease site, to sterically occlude the antigen binding site.

[0069] Figure 12 shows SPR-determined binding kinetics for monovalent (left panel) and tetravalent (right panel) meditopes to cetuximab. The panel above the surface plasmon resonance traces depicts a cartoon of the monovalent meditopes being passed over the bivalent IgG. As shown in the right panel, avidin was saturated with biotinylated meditope and passed over the same immobilized cetuximab IgG. The off-rate of the multivalent meditope was reduced by at least 36 fold. (Note the time scales between the two experiments are different.)

[0070] Figure 13 illustrates the synthesis of dimeric and trimeric meditopes according to some embodiments, for meditopes containing lactam linkages.

[0071] Figure 14 illustrates the characterization of a fluorescein isothiocyanate (FITC)-labeled meditope dimer ("**14**" in Figure 13), with an HPLC trace of final bivalent meditope and its mass spectrum.

[0072] Figure 15 shows the nucleic acid sequence (SEQ ID NO:3) and the corresponding amino acid sequence (SEQ ID NO:4) for a meditope-Fc fusion protein according to some embodiments.

[0073] Figure 16 shows an exemplary bivalent meditope. The meditope is directly fused to the N-terminus of a peptide linker that is directly fused to the Fc Region of an IgG. In this example, as the Fc is naturally homodimerized during expression, the end product from the meditope-Fc construct is bivalent.

[0074] Figure 17 shows the results of a series of exemplary FACS analyses monitoring binding of monovalent and bivalent meditopes to cetuximab pretreated EGFR-expressing cells. MDA-MB-468 cells that over-express EGFR were pre-treated with 10 nM of cetuximab for 30 minutes, rinsed, and then treated with either the meditope-Fc construct or the monomeric meditope at four concentrations. The bottom trace is a negative control (no antibody). The next four traces show that a monomeric meditope binds to cells pre-treated with cetuximab in a concentration dependent manner. The top four traces also show that the bivalent, meditope Fc binds to cells pre-treated with cetuximab in a concentration dependent manner, but with higher affinity (i.e., more shifted to the right). This is predicted and consistent with a multivalent interaction.

[0075] Figure 18 is a depiction of an alternative meditope-Fc linker, a coiled coil, which may be used in accordance with certain embodiments.

[0076] Figure 19: Fragment screening by NMR. Representative NMR spectra of a fragment pool before (top trace) and after (bottom trace) cetuximab addition. Using this method, lead compounds (e.g., those shown herein) have been identified and the binding site is being determined using diffraction studies.

[0077] Figure 20 illustrates exemplary steps to alter and/or enhance the binding affinity (or alter another property) of a mAb for a meditope or compound binding at the meditope site using a directed random library. Specifically, a gene library where codons for mAb residues that line the meditope binding site are replaced with NNK (where N is any nucleotide and K is a thymidine or guanosine) can be selected using FACS sorting (where the meditope and the antigen are labeled with distinct fluorophores). In another embodiment, the codons are substituted with NNN, where N is any nucleotide. The GPI linker can ensure the selected mAb remains associated with the cell transfected with vectors encoding the gene

sequences. Sequencing the genes encoding the light and heavy chain from the selected cells will identify high affinity mAbs. The method can be repeated to select for higher affinity meditope or meditope analogs.

[0078] Figure 21 shows surface representation of sequence differences of cetuximab and trastuzumab. The dark grey regions in the top panel indicate amino acid differences between cetuximab and the fully humanized trastuzumab framework. Certain residues inside the box have been mutated onto the trastuzumab framework. The CDR loops of trastuzumab have been identified (dark regions; bottom panel) and grafted onto the cetuximab framework.

[0079] Figure 22 Illustrates that a meditope-enabled trastuzumab binds to a meditope-Fc fusion protein. The meditope binding site was created on trastuzumab, a humanized mAb that binds to the tumor antigen, HER2. FACS analysis in the top panel shows that meditope-enabled trastuzumab binds to SKBR3 cells that overexpress HER2 (top two traces). In the bottom graph, the meditope-Fc binds to meditope-enabled trastuzumab (top two traces – peak shifted right), but not to wild-type trastuzumab (second from bottom) or to the negative control (bottom trace).

[0080] Figure 23A shows the nucleic acid (SEQ ID NO:5) sequence of a meditope-enabled trastuzumab heavy chain sequence. Signal peptide sequence is shaded in grey. Figure 23B shows the amino acid (SEQ ID NO:6) sequence of a meditope-enabled trastuzumab heavy chain sequence as compared to the wild-type (SEQ ID NO:7). Differences are highlighted in grey. After the amino acids shown in Figure 23B, there are no differences remaining in the human sequence of the heavy chain shown. Figure 23C shows the nucleic acid (SEQ ID NO:8) sequence of a meditope-enabled trastuzumab light chain sequence. Signal peptide sequence is shaded in grey. Figure 23D shows the amino acid (SEQ ID NO:9) sequence of a meditope-enabled trastuzumab light chain sequence as compared to the wild-type (SEQ ID NO:10). Differences are highlighted in grey.

[0081] Figure 24 shows that an antibody containing HER2-binding CDR loops grafted on cetuximab-like framework binds to HER2 and a meditope-Fc. In Figure 24A, FACS analysis shows that HER2-CDR grafted meditope-enabled mAb binds to SKBR3 cells, which overexpresses HER2 (top three traces at different concentrations). Figure 24B shows that while the meditope-Fc bound to the HER2-CDR grafted, meditope-enabled mAb (top

three traces – peak shifted right in concentration dependent manner), it did not bind to wild-type trastuzumab (second from bottom) or to the negative control (bottom trace).

[0082] Figure 25A shows nucleic acid (SEQ ID NO: 11) and amino acid (SEQ ID NO: 12) sequences for the heavy chain of a trastuzumab with secretion sequence and engineered restriction sites (underlined). Figure 25B shows nucleic acid (SEQ ID NO: 13) and amino acid (SEQ ID NO: 14) sequences for a light chain of trastuzumab with secretion sequence and engineered restriction sites (underlined). Shaded areas indicate exemplary residues that in some aspects could be altered to enhance or alter the specificity of the meditope, or could participate in binding interactions with a modified epitope.

[0083] Figure 26 illustrates examples of sequence and structural alignment of the IgG and IgE Fab domains, with shaded areas indicating certain residues corresponding to resiues near a meditope binding site.

[0084] Figure 27 is a graph showing surface plasmon resonance (SPR) studies. The binding affinity of different meditopes to cetuximab [cQFD (meditope 1, SEQ ID NO: 1), cQYN (meditope 2, SEQ ID NO: 2), and cQYD (meditope 16, SEQ ID NO: 16)] was determined from pH=4.0 to pH=8.0.

[0085] Figure 28 illustrates the results of an MTT assay comparing the efficacy of a bivalent meditope-Fc to that of a monomeric meditope to inhibit MDA-MB-468 cell growth. Figure 28A shows results of a study in which a meditope-Fc, but not a monomeric meditope, inhibited cell growth when combined with cetuximab. Figure 28B shows that the meditope-Fc enhances the cell-killing capacity of cetuximab, similar to the combination of M425 and cetuximab.

[0086] Figure 29 illustrates binding of a meditope to a cetuximab meditope binding site ("Cetuximab Binding" as shown in Figure), but not to a fully humanized framework ("Trastuzumab No binding" as shown in Figure) or another murine-chimera framework ("Rituximab No binding" as shown in Figure). The cQFD meditope (meditope 1, SEQ ID NO: 1) was conjugated to a CM5 chip for surface plasmon resonance studies and cetuximab (meditope binding Fab), trastuzumab (fully humanized framework), and rituximab (murine-human chimeric framework) were tested at concentrations of 0.01, 0.05, 0.1, 0.5 and 1 μM. Only cetuximab bound to the meditope-conjugated chip.

[0087] Figure 30 shows biophysical data obtained for the meditopes. Figure 30A shows a SPR sensogram of an unmodified meditope cQFD (SEQ ID NO:1) and a QYN meditope (SEQ ID NO:2) using a cetuximab Fab chip. Figure 30B shows a representative binding isotherm of the meditope (SEQ ID NO: 1) and cetuximab Fab (top) and integration (bottom). Figure 30C shows superposition of both meditopes. Oval 1 indicates the Phe/Tyr position. The arrow indicates a shift of the backbone that leads to a favorable hydrogen bond network and may account for a favorable change in the enthalpy. The hydrophobic groups, F/Y3 L5, and L10 are nearly identically positioned, but the hydroxyl group of Y5 in the cQYN meditope prevents R8 from interacting with the Q105 backbone, as observed in the cQFD meditope ("steric clash"). This rearrangement also results in a concomitant change in the backbone residues of the β-turn ('backbone rotation"). Figure 30D shows individual thermodynamic parameters determined by ITC of different meditope variants. The Phe3Tyr variant (meditope 2, SEQ ID NO: 2) shows a significant change in ΔH though lower affinity. Based on the structure, Gln2 in the meditope of SEQ ID NO: 1 was replaced with the D-stereomer. ITC analysis of this meditope, (SEQ ID NO:15), revealed a significant increase in entropy and loss in enthalpy.

[0088] Figure 31 shows the chemical structure of a meditope according to some embodiments. The circles indicate positions that can be modified, e.g., to improve meditope affinity for the Fab. The boxes indicate cyclization strategies. 'Click' chemistry and olefin metathesis, top and bottom far right boxes, respectively, are additional routes to cyclize the meditope.

[0089] Figure 32 illustrates the synthesis of lactam (2, SEQ ID NO: 32) and fluorescein-labeled lactam (3).

[0090] Figure 33 shows the sites of optimization according to some embodiments. Ovals indicate cavities in the Fab region of the mAb that may be exploited to optimize the affinity of a meditope.

[0091] Figure 34 shows a modified Arg8 residue that may be used to optimize meditope binding parameters, and may be used in accordance with the embodiments described herein (R = alkyl, substituted alkyl, aromatic or NHR'", where R'" = alkyl, substituted alkyl, or aromatic).

[0092] Figure 35 shows the hydrophilic interface in the vicinity of .Phe3 of a meditope. Halogens incorporated in the phenyl ring may form strong noncovalent bonds to the hydroxyl group of cetuximab Tyr87 or Tyr91, as well as to backbone carbonyls.

[0093] Figure 36 illustrates covalent interactions between a meditope and the backbone of cetuximab. A hydratable side chain may be incorporated at Arg8 (left) or Leu10 (right) to form a covalent bond to the hydroxyl groups of Ser or Tyr of the Fab.

[0094] Figure 37 illustrates a fluorescence polarization assay of dose-dependent inhibition of the interaction between cetuximab and a fluorescein-labeled peptide by unlabeled peptide. The assay identified small molecule compounds that could compete with the meditope for mAb binding and thus can be developed to function in a similar manner as a meditope. As a control, it was demonstrated that a non-labeled meditope could displace the fluorescently-labeled meditope. Equilibration of the displacement at three times points indicates that the assay is robust and amendable to high throughput screening.

[0095] Figure 38 shows five lead compounds identified from the fluorescence polarization screen.

[0096] Figure 39 shows stick structures for eighteen modified meditopes, corresponding to SEQ ID NO:22 (A), SEQ ID NO:16 (B), SEQ ID NO:17 (C), SEQ ID NO:18 (D), SEQ ID NO:15 (E), SEQ ID NO:23 (F), SEQ ID NO:24 (G), SEQ ID NO:25 (H), SEQ ID NO:32 (I), SEQ ID NO:33 (J), SEQ ID NO:34 (K), SEQ ID NO:35 (L), SEQ ID NO:36 (M), SEQ ID NO:37 (N), SEQ ID NO:38 (O), SEQ ID NO:39 (P), SEQ ID NO:40 (Q) and SEQ ID NO:41 (R), SEQ ID NO:42 (S), SEQ ID NO:43 (T), SEQ ID NO:44 (U), SEQ ID NO:46 (V) SEQ ID NO:49 (W), SEQ ID NO:51 (X), SEQ ID NO:52 (Y), SEQ ID NO:53 (Z), SEQ ID NO:54 (AA), and SEQ ID NO:55 (BB). The sequence of these structures is shown in Tables 3 and 4.

[0097] Figure 40 is a table showing the X-ray diffraction data for the structures shown in Figure 39 and additional meditopes in Tables 3 and 4.

[0098] Figure 41 shows representative surface plasmon resonance studies of meditope variants. Based on the structural information of the Phe3His mutation in the cQFD meditope, a variant meditope was synthesized with β,β'-diphenylalanine at position 3 (top trace). A significant improvement in the binding affinity for cetuximab of ~4 fold, was observed by

surface plasmon resonance. Aminohexanoic acid was used to replace the disulfide bridge of the original meditope (bottom trace). While the binding affinity was decreased, these data indicate that modifications can be made to meditopes to address potential issues with pharmacokinetics, pharmacodynamics and toxicity in animal and human studies. It is noted that this combination can be combined with unnatural amino acids at other positions within a meditope.

[0099] Figure 42 illustrates structural information that can be useful, e.g., to improve meditope affinity. The top panel shows a crystal structure of a meditope bound cetuximab, showing Phe3 bound within the Fab cavity of cetuximab. Substitution of this position with histidine and subsequent structural analysis indicates the side chain (imidazole) takes on a new conformation (middle panel). Based on this observation, β,β'-diphenylalanine which could place one phenyl side chain at the original position and one phenyl side chain at the imidazole ring was substituted at position 3. The crystal structure indicates that this substitution mimics both side chain conformations. Surface plasmon resonance studies shows that this substitute binds with higher affinity (Figure 41, top panel).

[00100] Figure 43 shows results of a study demonstrating that a meditope-enabled trastuzumab provided herein has a similar binding affinity for antigen as wild-type trastuzumab, as described in Example 4.

[00101] Figure 44 shows results of a study described in Example 4, demonstrating that HER2-expressing cells pre-bound with meditope-enabled trastuzumab (TM), but not those pre-bound with wild-type trastuzumab (WT T), bound to a cQFD (SEQ ID NO: 1) meditope-Protein L fusion (MPL). Control cells, without pre-bound antibody (MPL (WT) only) also did not bind to MPL.

[00102] Figure 45 shows results of a study described in Example 4, demonstrating that HER2-expressing cells (SKBR3) pre-bound with meditope-enabled trastuzumab (TM), but not those pre-bound with wild-type trastuzumab (WT T), bound to a cQFD (SEQ ID NO: 1) meditope-Protein L fusion in which all but one lysine of Protein L were mutated to arginine and asparagine (MPL-5K). Control cells, not pre-bound with any antibody (MPL-5K only) also did not bind to MPL-5K.

[00103] Figure 46 shows results of a study described in Example 4, demonstrating that meditope binds to meditope-enabled trastuzumab to a similar degree whether the antibody is

pre-bound to cells expressing antigen prior to incubation with the meditope (left panel), or meditope-
Protein L- and antibody are pre-mixed and then incubated with cells expressing antigen (right panel). TM = meditope-enabled trastuzumab. The percentage of MPL-positive cells relative to no-treatment control is shown in the legend.

[00104] Figure 47A shows light chain nucleic acid (SEQ ID NO: 61) and light chain amino acid (SEQ ID NO: 61) sequences of a CDR-grafted meditope-enabled trastuzumab (with trastuzumab-like CDRs grafted onto a cetuximab-like framework), with the signal sequence and other residues shaded. Figure 47B shows heavy chain nucleic acid (SEQ ID NO: 62) and heavy chain amino acid (SEQ ID NO: 63) of this antibody.

[00105] Figure 48 shows the structure of meditope 54 (see Table 4) bound to a cetuximab Fab fragment (backside view), with structures of the 5-position β,β'-diphenylalanine meditope (SEQ ID NO: 18, meditope 18) and cQFD (meditope 1, SEQ ID NO: 1) superimposed.

[00106] Figure 49 shows the results of a study in which an Alexa488-labeled meditope-Fc fusion protein (600 nM, 180 nM, or 60 nM) was incubated with MDA-MB-468 cells, pre-labeled with cetuximab or M425 (a mouse anti-EGFR antibody) for 30 minutes, and antibody binding and meditope binding analyzed by FACS analysis.

[00107] Figure 50 (top panel) shows stick representations of the structures of meditope 18 (SEQ ID NO: 18, shown in Table 3, with a β,β'-diphenylalanine at position 5) bound to cetuximab (dark grey sticks), the same meditope (18) bound to meditope-enabled trastuzumab (white sticks), and wild-type trastuzumab (outline), superimposed. The bottom panel shows a ribbon cartoon comparing wild-type and meditope-enabled trastuzumab.

[00108] Figure 51, in the upper-left panel, shows a superposition of the structures of trastuzumab and trastuzumab memab (in this figure, the trastuzumab memab is labeled as "Meditope-enabled Memab") with certain residues involved in meditope-binding in the meditope-enabled antibody illustrated by sticks. The top right panel shows a superposition of the structures of meditope-enabled trastuzumab (memab) and cetuximab, with the same residues labeled. The bottom panel shows a "cartoon/ribbon diagram" of all three of these structures superimposed.

[00109] Figure 52 shows the structure of meditope (position 3 β,β' diphenyl), Protein L (left), Protein A (right) and Fab (grey cartoon). Lysines in Protein L and the meditope that were mutated in MPLs described herein are shown in black.

[00110] Figure 53 shows surface plasmon resonance (SPR) data for a meditope-Protein L fusion (MPL): Top panel shows the traces of MPL being added to the meditope-enabled trastuzumab at concentrations up to 10 nM. The fit data indicates a binding affinity of 165pM. The bottom panel shows the traces of Protein L (only) added at the same concentrations to the meditope-enabled trastuzumab, showing that there is not binding at this concentration.

[00111] Figure 54 shows that a GPI-linked meditope-enabled trastuzumab binds to a meditope-Protein L (MPL) fusion protein. 1 x 10^6 cells/sample were used. Cells were removed from plates by gentle pipetting and were washed once with 0.1% BSA (w/v) in PBS. AF647-labeled-MPL was diluted to 10nM in wash buffer and incubated with cells for 30 min at RT. Cells were washed twice and then analyzed by FACS.

[00112] Figure 55A shows a sequence comparison between the CH1 domain of a human IgG2, IgG4, IgG3, and IgG1 (SEQ ID NOs: 64-67). Figure 55B shows the differences between these sequences mapped onto the co-crystal structure of cetuximab and meditope 1 (SEQ ID NO:1, cQFD).

[00113] Figure 56 shows the amino acid sequence of the light chain of a meditope-enabled ("medi") anti-CEA antibody (meditope-enabled M5A) (SEQ ID NO: 68) compared to the wild-type M5A light chain sequence (SEQ ID NO: 69). Shaded residues represent eight point mutations that were introduced in the light chain of the M5A antibody, allowing it to bind to meditopes (for reference the heavy chain M5A sequence is set forth in SEQ ID NO: 70).

[00114] Figure 57 shows results of FACS analysis, demonstrating binding of the meditope-enabled M5A antibody (M5A 8M) to the M5A antigen (CEA) on LS174T cells.

[00115] Figure 58 shows surface plasmon resonance (SPR) data for a 5-diphenyl meditope bound to an M5A 8M meditope enabled antibody.

[00116] Figure 59 illustrates illustrates the characterization of meditope 54, with an HPLC trace and its mass spectrum.

[00117] Figure 60 illustrates the mass spectrum of DOTA-NHS-MPL conjugates formed from a starting material ratio of 120:1 (DOTA-NHS:MPL).

[00118] Figure 61A shows MDA-MB-468 cells that were pre-incubated with Alexa 555-cetuximab and then incubated with Alexa 488- meditope-Fc. DAPI was used as counter stain. Images were taken with an Olympus AX70 Automatic Upright Microscope. White arrows indicate positive meditope-Fc staining. Figure 61B shows quantification of meditope-Fc- positive cells. Bar graph showed shows percentage of meditope-Fc- positive cells of total cells from cetuximab pre-treated or untreated samples counted from two fields.

DETAILED DESCRIPTION

A. Definitions

[00119] An "antibody," as used herein, refers to an immunoglobulin molecule that specifically binds to, or is immunologically reactive with an antigen or epitope, and includes both polyclonal and monoclonal antibodies, as well as functional antibody fragments , including but not limited to fragment antigen binding (Fab) fragments, $F(ab')_2$ fragments, Fab' fragments, Fv fragments, recombinant IgG (rIgG) fragments, single chain variable fragments (scFv) and single domain antibodies (e.g., sdAb, sdFv, nanobody) fragments. The term "antibody" includes genetically engineered or otherwise modified forms of immunoglobulins, such as intrabodies, peptibodies, chimeric antibodies, fully human antibodies, humanized antibodies, meditope-enabled antibodies and heteroconjugate antibodies (e.g., bispecific antibodies, diabodies, triabodies, tetrabodies, tandem di-scFv, tandem tri-scFv). Unless otherwise stated, the term "antibody" should be understood to encompass functional antibody fragments thereof.

[00120] The terms "complementarity determining region," and "CDR," are known in the art to refer to non-contiguous sequences of amino acids within antibody variable regions, which confer antigen specificity and binding affinity. In general, there are three CDRs in each heavy chain variable region (CDR-H1, CDR-H2, CDR-H3) and three CDRs in each light chain variable region (CDR-L1, CDR-L2, CDR-L3). "Framework regions" and "FR" are known in the art to refer to the non-CDR portions of the variable regions of the heavy and

light chains. In general, there are four FRs in each heavy chain variable region (FR-H1, FR-H2, FR-H3, and FR-H4), and four FRs in each light chain variable region (FR-L1, FR-L2, FR-L3, and FR-L4).

[00121] The precise amino acid sequence boundaries of a given CDR or FR can be readily determined using any of a number of well-known schemes, including those described by Kabat et al. (1991), "Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD ("Kabat" numbering scheme), Al-Lazikani et al., (1997) JMB 273,927-948 ("Chothia" numbering scheme), MacCallum et al., J. Mol. Biol. 262:732-745 (1996), "Antibody-antigen interactions: Contact analysis and binding site topography," J. Mol. Biol. 262, 732-745." (Contact" numbering scheme), Lefranc MP et al., "IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains," Dev Comp Immunol, 2003 Jan;27(1):55-77 ("IMGT" numbering scheme), and Honegger A and Plückthun A, "Yet another numbering scheme for immunoglobulin variable domains: an automatic modeling and analysis tool," J Mol Biol, 2001 Jun 8;309(3):657-70, (AHo numbering scheme).

[00122] The boundaries of a given CDR or FR may vary depending on the scheme used for identification. For example, the Kabat scheme is based structural alignments, while the Chothia scheme is based on structural information. Numbering for both the Kabat and Chothia schemes is based upon the most common antibody region sequence lengths, with insertions accommodated by insertion letters, for example, "30a," and deletions appearing in some antibodies. The two schemes place certain insertions and deletions ("indels") at different positions, resulting in differential numbering. The Contact scheme is based on analysis of complex crystal structures and is similar in many respects to the Chothia numbering scheme.

[00123] Table 1, below, lists the positions of CDR-L1, CDR-L2, CDR-L3 and CDR-H1, CDR-H2, CDR-H3 as identified by the Kabat, Chothia, and Contact schemes, respectively. For CDR-H1, residue numbering is given listed using both the Kabat and Chothia numbering schemes. It is noted that because the Kabat numbering scheme places insertions at H35A and H35B, the end of the Chothia CDR-H1 loop when numbered using the Kabat numbering convention varies between H32 and H34, depending on the length of the loop.

Table 1.

CDR	Kabat	Chothia	Contact
CDR-L1	L24--L34	L24--L34	L30--L36
CDR-L2	L50--L56	L50--L56	L46--L55
CDR-L3	L89--L97	L89--L97	L89--L96
CDR-H1 (Kabat Numbering[1])	H31--H35B	H26--H32..34	H30--H35B
CDR-H1 (Chothia Numbering[2])	H31--H35	H26--H32	H30--H35
CDR-H2	H50--H65	H52--H56	H47--H58
CDR-H3	H95--H102	H95--H102	H93--H101

1 - Kabat et al. (1991), "Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD
2 - Al-Lazikani et al., (1997) JMB 273,927-948

[00124] Thus, unless otherwise specified, a "CDR" or "complementary determining region," or individual specified CDRs (e.g., "CDR-H1, CDR-H2), of a given antibody or region thereof, such as a variable region thereof, should be understood to encompass a (or the specific) complementary determining region as defined by any of the known schemes. Likewise, unless otherwise specified, a "FR" or "framework region," or individual specified FRs (e.g., "FR-H1, FR-H2), of a given antibody or region thereof, such as a variable region thereof, should be understood to encompass a (or the specific) framework region as defined by any of the known schemes. In some instances, the scheme for identification of a particular CDR, FR, or FRs or CDRs is specified, such as the CDR as defined by the Kabat, Chothia, or Contact method. In other cases, the particular amino acid sequence of a CDR or FR is given.

[00125] The term "meditope-enabled" antibody and "meMAb" refer to an antibody or functional fragment thereof that is able to bind to a meditope, via a meditope binding site. Examples of meditope-enabled antibodies include, but are not limited to, cetuximab and others described herein. A "meditope binding site" is a region of the meditope-enabled antibody containing the amino acid residues that interact with a bound meditope, which residues include framework region (FR) residues of the heavy and light chains. With reference to a Fab fragment or a Fab portion of an antibody, the meditope binding site is located within the central cavity of the Fab fragment or portion.

[00126] The "central cavity," with respect to the three-dimensional structure of a Fab, refers to the internal cavity of the Fab, lined by portions of the heavy and light chain variable

and constant regions. The central cavity thus is lined by residues of the VH, VL, CH1, and CL regions and does not include the antigen binding site.

[00127] In some embodiments, the meditope binding site includes residues 40, 41, 83, and 85 of the light chain of a meditope-enabled antibody, according to Kabat numbering, and/or residues 39, 89, 105, and 108 of the heavy chain of the meditope-enabled antibody, according to Kabat numbering.

[00128] In some embodiments, the meditope binding site is located within a cavity formed by residues 8, 9, 10, 38, 39, 40, 41 42, 43, 44, 45, 82, 83, 84, 85, 86, 87, 99, 100, 101, 102, 103, 104, 105, 142, 162, 163, 164, 165, 166, 167, 168, and 173 of the light chain and 6, 9, 38, 39, 40, 41, 42, 43, 44, 45, 84, 86, 87, 88, 89, 90, 91, 103, 104, 105, 106, 107, 108, 111, 110, 147, 150, 151, 152, 173, 174, 175, 176, 177, 185, 186, and 187 of the heavy chain of the antibody, according to Kabat numbering.

[00129] With respect to a Fab portion of a meditope-enabled antibody, the meditope binding site includes residues within the central cavity. The meditope-binding site typically further includes constant region residues.

[00130] The terms "meditope", as used herein, refers to a peptide or peptides that binds to a meditope-binding site of a meditope-enabled antibody, which antibody has a threonine at position 40, an asparagine at position 41, and an aspartage at position 85 of its light chain, according to Kabat numbering, or contains a meditope binding site containing residues that correspond to those within the meditope-binding site of cetuximab, meditope-enabled trastuzumab, or meditope-enabled M5A, disclosed herein. Exemplary meditopes include, but are not limited to, the cQFD and cQYN peptides and variants thereof ("meditope variants" or "variant meditopes"), as well as multivalent and labeled meditopes. Other molecules may also bind to meditope binding sites of meditope-enabled antibodies, with functional characteristics similar to those of a meditope. Such molecules, meditope analogs, may include, but are not limited to, small molecules, aptamers, nucleic acid molecules, peptibodies and any other substance able to bind to the same meditope binding site as a meditope.

[00131] A "therapeutic agent," as used herein, is an atom, molecule, or compound that is useful in treatment of a disease or condition.

[00132] A "therapeutically effective amount," "therapeutically effective concentration" or "therapeutically effective dose" is the amount of a compound that produces a desired therapeutic effect in a subject, such as preventing or treating a target condition, alleviating symptoms associated with the condition, producing a desired physiological effect, or allowing imaging or diagnosis of a condition that leads to treatment of the disease or condition. The precise therapeutically effective amount is the amount of the composition that will yield the most effective results in terms of efficacy of treatment in a given subject. This amount will vary depending upon a variety of factors, including, but not limited to, the characteristics of the therapeutic compound (including activity, pharmacokinetics, pharmacodynamics, and bioavailability), the physiological condition of the subject (including age, sex, disease type and stage, general physical condition, responsiveness to a given dosage, and type of medication), the nature of the pharmaceutically acceptable carrier or carriers in the formulation, and the route of administration. One skilled in the clinical and pharmacological arts will be able to determine a therapeutically effective amount through routine experimentation, namely by monitoring a subject's response to administration of a compound and adjusting the dosage accordingly. For additional guidance, see Remington: The Science and Practice of Pharmacy 21st Edition, Univ. of Sciences in Philadelphia (USIP), Lippincott Williams & Wilkins, Philadelphia, PA, 2005.

[00133] A "pharmaceutically acceptable carrier" refers to a pharmaceutically acceptable material, composition, or vehicle that is involved in carrying or transporting a compound of interest from one tissue, organ, or portion of the body to another tissue, organ, or portion of the body. For example, the carrier may be a liquid or solid filler, diluent, excipient, solvent, or encapsulating material, or some combination thereof. Each component of the carrier must be "pharmaceutically acceptable" in that it must be compatible with the other ingredients of the formulation. It also must be suitable for contact with any tissue, organ, or portion of the body that it may encounter, meaning that it must not carry a risk of toxicity, irritation, allergic response, immunogenicity, or any other complication that outweighs its therapeutic benefits.

[00134] A "route of administration" may refer to any administration pathway known in the art, including but not limited to aerosol, enteral, nasal, ophthalmic, oral, parenteral, rectal, transdermal, or vaginal. "Transdermal" administration may be accomplished using a topical cream or ointment or by means of a transdermal patch. "Parenteral" refers to a route of

administration that is generally associated with injection, including infraorbital, infusion, intraarterial, intracapsular, intracardiac, intradermal, intramuscular, intraperitoneal, intrapulmonary, intraspinal, intrasternal, intrathecal, intrauterine, intravenous, subarachnoid, subcapsular, subcutaneous, transmucosal, or transtracheal.

[00135] "In combination" or "in combination with," when used herein in the context of multiple agents, therapeutics, or treatments, means in the course of treating the same disease or condition in a subject administering two or more agents, drugs, treatment regimens, treatment modalities or a combination thereof (e.g., an antibody in combination with a meditope or a multivalent tethering agent), in any order. This includes simultaneous administration (or "coadministration"), administration of a first agent prior to or after administration of a second agent, as well as in a temporally spaced order of up to several days apart. Such combination treatment may also include more than a single administration of any one or more of the agents, drugs, treatment regimens or treatment modalities. Further, the administration of the two or more agents, drugs, treatment regimens, treatment modalities or a combination thereof may be by the same or different routes of administration.

[00136] A "therapeutic antibody" may refer to any antibody or functional fragment thereof that is used to treat cancer, autoimmune diseases, transplant rejection, cardiovascular disease or other diseases or conditions such as those described herein. Examples of therapeutic antibodies that may be used according to the embodiments described herein include, but are not limited to murine antibodies, murinized or humanized chimera antibodies or human antibodies including, but not limited to, Erbitux (cetuximab), ReoPro (abciximab), Simulect (basiliximab), Remicade (infliximab); Orthoclone OKT3 (muromonab-CD3); Rituxan (rituximab), Bexxar (tositumomab) Humira (adalimumab), Campath (alemtuzumab), Simulect (basiliximab), Avastin (bevacizumab), Cimzia (certolizumab pegol), Zenapax (daclizumab), Soliris (eculizumab), Raptiva (efalizumab), Mylotarg (gemtuzumab), Zevalin (ibritumomab tiuxetan), Tysabri (natalizumab), Xolair (omalizumab), Synagis (palivizumab), Vectibix (panitumumab), Lucentis (ranibizumab), and Herceptin (trastuzumab).

[00137] "Treating" or "treatment" of a condition may refer to preventing the condition, slowing the onset or rate of development of the condition, reducing the risk of developing the condition, preventing or delaying the development of symptoms associated with the condition, reducing or ending symptoms associated with the condition, generating a complete or partial regression of the condition, or some combination thereof.

[00138] As used herein, the terms "including," "containing," and "comprising" are used in their open, non-limiting sense.

[00139] As used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

[00140] To provide a more concise description, some of the quantitative expressions given herein are not qualified with the term "about". It is understood that, whether the term "about" is used explicitly or not, every quantity given herein is meant to refer to the actual given value, and it is also meant to refer to the approximation to such given value that would reasonably be inferred based on the ordinary skill in the art, including equivalents and approximations due to the experimental and/or measurement conditions for such given value.

[00141] As used herein, "alkyl" refers to a saturated, straight- or branched-chain hydrocarbon group having from 1 to 12 carbon atoms. Representative alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, 2-methyl-1-propyl, 2-methyl-2-propyl, 2-methyl-1-butyl, 3-methyl-1-butyl, 2-methyl-3-butyl, 2,2-dimethyl-1-propyl, 2-methyl-1-pentyl, 3-methyl-1-pentyl, 4-methyl-1-pentyl, 2-methyl-2-pentyl, 3-methyl-2-pentyl, 4-methyl-2-pentyl, 2,2-dimethyl-1-butyl, 3,3-dimethyl-1-butyl, 2-ethyl-1-butyl, butyl, isobutyl, t-butyl, n-pentyl, isopentyl, neopentyl, n-hexyl, and the like, and longer alkyl groups, such as heptyl, octyl, and the like. The term "C_{x-y}alkyl," where x and y are integers, refers to an alkyl with x-y carbon atoms.

[00142] As used herein, an "alkenyl" refers to a straight- or branched-chain hydrocarbon group having one or more double bonds therein and having from 2 to 12 carbon atoms. Illustrative alkenyl groups include, but are not limited to, ethylenyl, vinyl, allyl, butenyl, pentenyl, hexenyl, butadienyl, pentadienyl, hexadienyl, 2-ethylhexenyl, 2-propyl-2-butenyl, 4-(2-methyl-3-butene)-pentenyl, and the like. The term "C_{x-y}alkenyl," where x and y are integers, refers to an alkenyl with x-y carbon atoms.

[00143] The term "alkylenyl" or "alkylene" refers to a divalent alkyl group. The term "alkenylene" or "alkenylene" refers to a divalent alkenyl group.

[00144] As used herein, "alkynyl" refers to a straight- or branched-chain hydrocarbon group having one or more triple bonds therein and having from 2 to 10 carbon atoms. Exemplary alkynyl groups include, but are not limited to, ethynyl, propynyl, butynyl,

pentynyl, hexynyl, methylpropynyl, 4-methyl-1-butynyl, 4-propyl-2-pentynyl, 4-butyl-2-hexynyl, and the like.

[00145] "Aryl" means a mono-, bi-, or tricyclic aromatic group, wherein all rings of the group are aromatic. For bi- or tricyclic systems, the individual aromatic rings are fused to one another. Exemplary aryl groups include, but are not limited to, phenyl, naphthalene, and anthracene.

[00146] The term "boronic ester" refers to a substituent $-B(OR)_2$, wherein each R group is independently a C_{1-4}alkyl, or the two R groups taken together form a C_{2-6}alkylene.

[00147] The term "acetal" refers to a $-CH(OR)_2$ group, wherein wherein each R group is independently a C_{1-4}alkyl, or the two R groups taken together form a C_{2-6}alkylene. Exemplary acetal groups include dimethylacetal or diethylacetal, or a cyclic acetal. The term "ketal" refers to a $-C(OR)_2-$ group, wherein each R group is independently a C_{1-4}alkyl, or the two R groups taken together form a C_{2-6}alkylene. Exemplary ketals include dimethylketal or diethylketal, or a cyclic ketal.

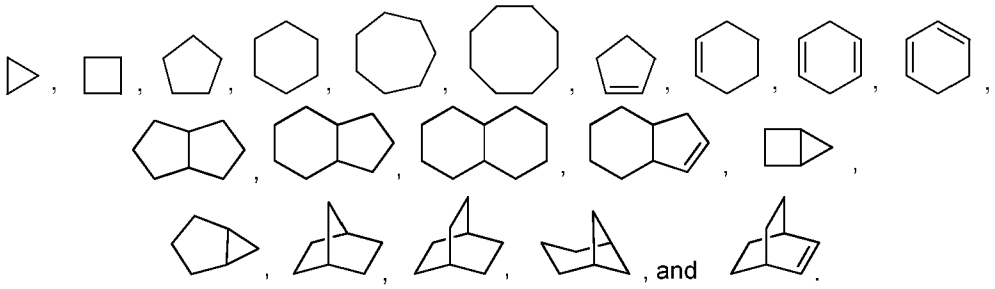
[00148] The term "halo" represents chloro, fluoro, bromo, or iodo. In some embodiments, halo is chloro, fluoro, or bromo. The term "halogen" as used herein refers to fluorine, chlorine, bromine, or iodine.

[00149] The term "ortho ester" refers to a $-C(OR)_3$ group, wherein each R group is independently a C_{1-4}alkyl, or two of the R groups taken together form a C_{2-6}alkylene

[00150] The term "oxo" means an =O group and may be attached to a carbon atom or a sulfur atom.

[00151] The term "phosphonate ester" refers to a $-P(O)(OR)_2$ group, wherein each R group is independently a C_{1-4}alkyl, or the two R groups taken together form a C_{2-6}alkylene

[00152] As used herein, the term "cycloalkyl" refers to a saturated or partially saturated, monocyclic, fused polycyclic, bridged polycyclic, or spiro polycyclic carbocycle having from 3 to 15 ring carbon atoms. A non limiting category of cycloalkyl groups are saturated or partially saturated, monocyclic carbocycles having from 3 to 6 carbon atoms. Illustrative examples of cycloalkyl groups include, but are not limited to, the following moieties:



[00153] As used herein, the term "5-membered heteroaryl" refers to a monocyclic, aromatic heterocycle having five ring atoms that are selected from carbon, oxygen, nitrogen, and sulfur. Examples of 5-membered heteroaryl groups include, but are not limited to, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, furanyl, thienyl, isoxazolyl, thiazolyl, oxazolyl, isothiazolyl, pyrrolyl, and thiadiazolyl. Particular examples of 5-membered heteraryls include those that may be formed by 1,3-cycloaddition reactions such as a Huisgen reaction between an azide and a propargyl group.

[00154] As used herein, the term "substituted" means that the specified group or moiety bears one or more suitable substituents. As used herein, the term "unsubstituted" means that the specified group bears no substituents. As used herein, the term "optionally substituted" means that the specified group is unsubstituted or substituted by the specified number of substituents. Where the term "substituted" is used to describe a structural system, the substitution is meant to occur at any valency-allowed position on the system.

[00155] As used herein, the expression "one or more substituents" denotes one to maximum possible number of substitution(s) that can occur at any valency-allowed position on the system. In a certain embodiment, "one or more substituents" means 1, 2, 3, 4, or 5 substituents. In another embodiment, one or more substituent means 1, 2, or 3 substituents.

[00156] Any atom that is represented herein with an unsatisfied valence is assumed to have the sufficient number of hydrogen atoms to satisfy the atom's valence.

[00157] When any variable, such as alkyl, R^3, or R^5, appears in more than one place in any formula or description provided herein, the definition of that variable on each occurrence is independent of its definition at every other occurrence.

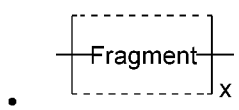
[00158] Numerical ranges, as used herein, are intended to include sequential whole numbers. For example, a range expressed as "from 0 to 4" or "0-4" includes 0, 1, 2, 3 and 4.

[00159] When a multifunctional moiety is shown, the point of attachment to the core is indicated by a line or hyphen. For example, -OH refers to a moiety in which an oxygen atom is the point of attachment of the hydroxyl group to the remainder of the molecule.

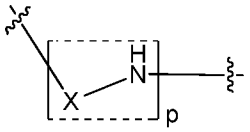
[00160] Any formula given herein is intended to represent compounds having structures depicted by the structural formula as well as certain variations or forms. For example, compounds of any formula given herein may have asymmetric or chiral centers and therefore exist in different stereoisomeric forms. All stereoisomers, including optical isomers, enantiomers, and diastereomers, of the compounds of the general formula, and mixtures thereof, are considered to fall within the scope of the formula. Furthermore, certain structures may exist as geometric isomers (i.e., *cis* and *trans* isomers), as tautomers, or as atropisomers. All such isomeric forms, and mixtures thereof, are contemplated herein as part of the present invention. Thus, any formula given herein is intended to represent a racemate, one or more enantiomeric forms, one or more diastereomeric forms, one or more tautomeric or atropisomeric forms, and mixtures thereof.

[00161] Diastereomeric mixtures may be separated into their individual diastereomers on the basis of their physical chemical differences by methods well known to those skilled in the art, such as, for example, by chromatography and/or fractional crystallization. Enantiomers may be separated by converting the enantiomeric mixture into a diastereomeric mixture by reaction with an appropriate optically active compound (e.g., chiral auxiliary such as a chiral alcohol or Mosher's acid chloride, or formation of a mixture of diastereomeric salts), separating the diastereomers and converting (e.g., hydrolyzing or de-salting) the individual diastereomers to the corresponding pure enantiomers. Enantiomers may also be separated by use of chiral HPLC column. The chiral centers of compounds of the present invention may be designated as "R" or "S" as defined by the IUPAC 1974 Recommendations, or by "D" or "L" designations consistent with the peptide literature.

[00162] As used herein, a box around a portion of a structure, drawn with a subscript, indicates that the structural fragment that appears within the box is repeated according to the subscript. For example, the substructure:



[00163] where x is 0, 1, or 2, indicates the fragment is absent from the structure, or is –Fragment-, or is
–Fragment-Fragment-. For example, within formula (X), the following substructure:



[00164] where p is 0 or 1, means that the –X-NH- group within the box is absent from the structure (p is 0), or is present once (p is 1).

[00165] The compounds of the invention can form pharmaceutically acceptable salts, which are also within the scope of this invention. A "pharmaceutically acceptable salt" refers to a salt of a free acid or base of a compound described herein that is non-toxic, is physiologically tolerable, is compatible with the pharmaceutical composition in which it is formulated, and is otherwise suitable for formulation and/or administration to a subject. Reference to a compound herein is understood to include reference to a pharmaceutically acceptable salt of said compound unless otherwise indicated.

[00166] Compound salts include acidic salts formed with inorganic and/or organic acids, as well as basic salts formed with inorganic and/or organic bases. In addition, where a given compound contains both a basic moiety, such as, but not limited to, a pyridine or imidazole, and an acidic moiety, such as, but not limited to, a carboxylic acid, one of skill in the art will recognize that the compound may exist as a zwitterion ("inner salt"); such salts are included within the term "salt" as used herein. Salts of the compounds of the invention may be prepared, for example, by reacting a compound with an amount of a suitable acid or base, such as an equivalent amount, in a medium such as one in which the salt precipitates or in an aqueous medium followed by lyophilization.

[00167] 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 ("mesylate"), 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 parent 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 counterions. Hence, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counter ion.

[00168] Exemplary acid addition salts include acetates, ascorbates, benzoates, benzenesulfonates, bisulfates, borates, butyrates, citrates, camphorates, camphorsulfonates, fumarates, hydrochlorides, hydrobromides, hydroiodides, lactates, maleates, methanesulfonates, naphthalenesulfonates, nitrates, oxalates, phosphates, propionates, salicylates, succinates, sulfates, tartarates, thiocyanates, toluenesulfonates (also known as tosylates,) and the like.

[00169] Exemplary basic salts include ammonium salts, alkali metal salts such as sodium, lithium, and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salts with organic bases (for example, organic amines) such as dicyclohexylamines, t-butyl amines, and salts with amino acids such as arginine, lysine and the like. Basic nitrogen-containing groups may be quarternized with agents such as lower alkyl halides (e.g. methyl, ethyl, and butyl chlorides, bromides and iodides), dialkyl sulfates (e.g. dimethyl, diethyl, and dibutyl sulfates), long chain halides (e.g. decyl, lauryl, and stearyl chlorides, bromides and iodides), aralkyl halides (e.g. benzyl and phenethyl bromides), and others.

[00170] Additionally, acids and bases which are generally considered suitable for the formation of pharmaceutically useful salts from pharmaceutical compounds are discussed, for example, by P. Stahl et al, Camille G. (eds.) Handbook of Pharmaceutical Salts. Properties, Selection and Use. (2002) Zurich: Wiley-VCH; S. Berge et al, Journal of Pharmaceutical Sciences (1977) 66(1) 1-19; P. Gould, International J. of Pharmaceutics (1986) 33 201-217; Anderson et al, The Practice of Medicinal Chemistry (1996), Academic Press, New York; and in The Orange Book (Food & Drug Administration, MD, available from FDA). These disclosures are incorporated herein by reference thereto.

[00171] Additionally, any compound described herein is intended to refer also to any unsolvated form, or a hydrate, solvate, or polymorph of such a compound, and mixtures thereof, even if such forms are not listed explicitly. "Solvate" means a physical association of a compound of the invention with one or more solvent molecules. This physical association involves varying degrees of ionic and covalent bonding, including hydrogen bonding. In certain instances the solvate will be capable of isolation, for example when one or more solvent molecules are incorporated in the crystal lattice of a crystalline solid. "Solvate" encompasses both solution-phase and isolatable solvates. Suitable solvates include those formed with pharmaceutically acceptable solvents such as water, ethanol, and the like. In some embodiments, the solvent is water and the solvates are hydrates.

[00172] Any formula given herein is also intended to represent unlabeled forms as well as isotopically labeled forms of the compounds. Isotopically labeled compounds have structures depicted by the formulas given herein except that one or more atoms are replaced by an atom having a selected atomic mass or mass number. Examples of isotopes that can be incorporated into compounds of the invention include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorous, fluorine, chlorine, and iodine, such as ^{2}H, ^{3}H, ^{11}C, ^{13}C, ^{14}C, ^{15}N, ^{18}O, ^{17}O, ^{31}P, ^{32}P, ^{35}S, ^{18}F, ^{36}Cl, and ^{125}I, respectively. Such isotopically labelled compounds are useful in metabolic studies (for example with ^{14}C), reaction kinetic studies (with, for example ^{2}H or ^{3}H), detection or imaging techniques [such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT)] including drug or substrate tissue distribution assays, or in radioactive treatment of patients. In particular, an ^{18}F or ^{11}C labeled compound may be particularly suitable for PET or SPECT studies. Further, substitution with heavier isotopes such as deuterium (i.e., ^{2}H) may afford certain therapeutic advantages resulting from greater metabolic stability, for example increased *in vivo* half-life or reduced dosage requirements. Isotopically labeled compounds of this invention and prodrugs thereof can generally be prepared by carrying out the procedures disclosed in the schemes or in the examples and preparations described below by substituting a readily available isotopically labeled reagent for a non-isotopically labeled reagent.

B. Antibodies and antibody-binding substances

[00173] Provided herein are antibodies, including monoclonal antibodies (mAbs), and fragments, e.g., functional fragments, thereof, and compounds and compositions, including peptides, such as meditopes, and meditope analogs, that bind to the antibodies. The

substances, e.g., meditopes, generally bind to regions/binding sites of the antibodies and fragments other than (e.g., separate from) the complementarity determining regions (CDRs), typically to residues within the framework regions (FRs) and/or constant regions of the antibodies and fragments. Also provided are complexes, compounds, and compositions containing the antibodies and substances, e.g., meditopes, as well as methods and uses of the same, including therapeutic, diagnostic, research, and other uses of the antibodies and substances, and methods for producing the same.

[00174] In certain aspects, the provided embodiments are based on the discovery described herein that certain peptides, C-QFDLSTRRLK-C (cQFD; SEQ ID NO: 1) and C-QYNLSSRALK-C (cQYN; SEQ ID NO: 2), non-covalently bind to a murine-human chimeric antibody, cetuximab, by interacting with residues outside the complementarity determining regions (CDRs), including residues in the framework and constant region. Also as described herein, the ability to bind to these peptides was based on certain aspects specific to cetuximab and in the studies herein binding was not observed, for example, to fully human antibodies, murine antibodies, or other chimeric antibodies). As demonstrated herein, these meditopes are capable of binding the chimeric antibody simultaneously with its antigen. See Figure 4. As demonstrated herein, the binding site for these meditopes on cetuximab also are distinct from the binding sites of other framework-binding antigens such as the superantigens Staphylococcal Protein A (SpA) and Peptostreptococcus magnus Protein L (PpL) (Figure 7). In certain aspects, the provided embodiments build upon this discovery, for example, by modifying other mAbs to allow their binding to these and other meditopes (i.e., "meditope-enabling") and generating variant meditopes and/or altered meditope-enabled antibodies, for example, those having altered properties, including improved binding affinity, toxicity, PK/PD, and/or pH dependence.

C. Meditope-enabled antibodies and complexes

[00175] Provided are meditope-enabled antibodies that are capable of binding to one or more meditopes via meditope-binding sites. In some cases, the meditope-enabled antibody binds to a cyclic peptide of SEQ ID NO: 1 or 2 (meditope 1 or 2) and/or to one or more variants thereof, such as meditopes 1, 2, 16-18, 23, 29, 31, 32, 36, 39, 42, 43, 45, 46, 51, 52, 54, or 55 (meditopes based on peptides having the sequences set forth in SEQ ID NOs: 1, 2, 16-18, 23, 29, 31, 32, 36, 39, 42, 43, 45, 46, 51, 52, 54, or 55), or in some cases, any of meditopes 1, 2, or 15-55. Among the provided meditope-enabled antibodies are those that

bind to a meditope or meditopes with an affinity similar to that of cetuximab. For example, in certain aspects, the antibodies bind to the meditope(s) with a dissociation constant of less than at or about 10 μM, less than at or about 5 μM, or less than at or about 2 μM, less than at or about 1 μM, less than at or about 500, 400, 300, 200, 100 nM, or less, such as at or about 200 picomolar or less. In some cases, the dissociation constant, such as any of those listed herein, is that measured using a particular technique, such as surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), fluorescence, fluorescence polarization, NMR, IR, calorimetry titrations; kinetic exclusion; circular dichroism, differential scanning calorimetry, or other known method. For example, in some cases, the analog or meditope exhibits a binding constant of less than at or about 10 μM, less than at or about 5 μM, or less than at or about 2 μM, less than at or about 1 μM, less than at or about 500, 400, 300, 200, 100 nm, or less, as measured by SPR or as measured by ITC or as measured by any of these methods.

[00176] In some examples, the meditope-binding site is a structural feature of the monoclonal antibody, cetuximab, a human-murine chimeric antibody used for the treatment of EGFR-expressing metastatic colorectal cancer and head and neck cancers. Thus, in some cases, the meditope-binding site contains residues corresponding to those within the meditope binding site of cetuximab. In a study reported herein, x-ray crystallographic analysis has revealed that the peptides of SEQ ID NO: 1 binds to a meditope-binding site within the central cavity of the cetuximab Fab fragment, defined by various residues of the heavy and light chains (see Figures 1 and 4A), with a binding constant of ~700 nM (see Figures 30A-30B).

[00177] Several interactions between cetuximab's meditope binding site and the cQFD (SEQ ID NO: 1, meditope 1) and cQYN (SEQ ID NO: 2, meditope 2) meditopes are based on particular structural features of cetuximab, particularly within the framework and constant regions of the central cavity of the Fab fragment. The regions of cetuximab that constitute the meditope binding site are unique and appear to be the result of the chimeric nature of this antibody. Specifically, the Fab variable regions (Fvs) are murine and the Fab constant regions (CH1 and CL) are human. The engineering of this Fab produced a combination of residues not found in a sequence alignment of murine and human chimeric antibodies. For example, data herein show that the meditopes did not bind to fully human IgG framework (e.g., trastuzumab), to other chimeric antibodies such as rituximab (Figure 29), or to mouse

antibodies, confirming that the cetuximab meditope-binding site is highly specific. In addition to these results, superposition of the molecular structure of trastuzumab (1N8Z; Cho et al., *Nature*, "Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab," 2003 Feb 13;421(6924):756-60) and rituxumab (2OSL;Du et al., *J Biol Chem*, 2007 May 18;282(20):15073-80. Epub 2007 Mar 29) Fabs on to the meditope bound cetuximab Fab structure further highlighted the uniqueness of the framework. Superposition of multiple human and murine Fabs onto the cetuximab-cyclic peptide (meditope 1) structure indicated that the key interactions between this peptide and murine-human chimeric Fab are absent in both human-only and murine-only IgG structures compared in this study. Point mutations of key residues within the cQFD cyclic peptide (meditope 1) reduced its binding affinity for the cetuximab Fab, further confirming the high specificity and structural model. Thus, the interaction appears to be specific to the central cavity of this specific murine-human chimera Fab and the selected meditope.

[00178] In certain embodiments, the unique interactions between the meditopes and the cetuximab binding site are exploited to generate additional meditope-enabled antibodies. Meditope-enabled antibodies are useful, for example, to improve antibody and cell purification methods, improving the therapeutic efficacy of mAbs, enhancing targeted delivery of imaging or therapeutic agents, including in pre-targeted delivery and imaging, and improving mAb-based imaging methods, and in some aspects are broadly applicable to any monoclonal antibody.

[00179] In some embodiments, the meditope-enabled antibodies are generated by modifying an antibody other than cetuximab (sometimes referred to as the template antibody), such as an antibody having one or more CDRs distinct from those of cetuximab, to confer the ability to bind to one or more of the provided meditopes, such as a meditope of SEQ ID NO: 1 or 2, or variant thereof. The template antibody can be a human or humanized antibody or a mouse antibody. In one aspect, the modifications include substituting residues within the central cavity of the Fab fragment, typically within the framework regions (FRs) of the heavy and light chain variable regions and/or the constant regions to render the template antibody meditope-enabled. For example, where the template antibody is a human or humanized antibody, the modifications generally include substitutions at residues within the heavy and light chain variable region FRs. In some embodiments, such residues are replaced with the corresponding residue present in cetuximab, or comparable amino acid. Thus, in

certain embodiments, residues within the FRs of a human or humanized antibody are replaced with corresponding murine residues; in certain embodiments, they are replaced by other residues, such as those having similar functional groups or moieties for interacting with the meditopes. Typically, the residues replaced by corresponding murine (or other) residues are found within the central Fab cavity, and thus are not exposed to the immune system. As such, in some embodiments, introducing these amino acid substitutions in a human or humanized antibody do not increase or do not substantially increase the antigenicity of the modified template antibody, in the context of delivery to a human subject. In addition, antigenicity prediction algorithms may be further used to indicate that the human sequence with the point mutations should not be antigenic.

[00180] In some embodiments, the one or more residues that are replaced, are selected from light chain framework residues 10, 39-43, 83, 85, 100 and 104, according to Kabat numbering (see Kabat E.A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition. NIH Publication No. 91-3242, incorporated herein by reference in its entirety), and/or heavy chain framework residue numbers 40, 89 and 105, according to Kabat numbering (see Figure 2). In general, unless otherwise specified, amino acid positions in the heavy or light chain of an antibody refer to Kabat numbering. Also encompassed within the present disclosure are residues in other antibodies corresponding to the residues of cetuximab described herein, such as those within the cetuximab meditope-binding site. In some embodiments, residues in the template antibody corresponding to light chain residues 9, 10, 39, 40, 41, 42, 43, 83, 85, 100, and/or 104, and/or heavy chain residues 40, 89, and/or 105, are replaced, for example, with amino acids present at those positions within cetuximab.

[00181] In one embodiment, the one or more residues replaced are light chain framework residues including, but not limited to, 40, 41, 83 and 85, according to Kabat numbering. In one embodiment, light chain residue 40 is replaced with threonine; light chain residue 41 is replaced with asparagine, light chain residue 83 is replaced with isoleucine or valine, and/or light chain residue 85 is replaced with aspartate. In a particular example, light chain framework Pro40 is replaced with Thr (P40T) or Ser (P40S), light chain framework Gly41 is replaced with Asn (G41N), light chain framework residue Phe83 is replaced with Ile (F83I) or Val (F83V) and light chain framework residue Thr85 is replaced with Asp (T85D) or Asn (T85N).

[00182] Thus, among the provided meditope-enabled antibodies are antibodies having one or more modifications, typically amino acid substitutions, at residues that correspond to positions within the meditope binding site of cetuximab or other meditope-enabled antibody, such as those described herein, including meditope-enabled trastuzumab and meditope-enabled M5A. Among the antibodies are those having a VL region with a threonine, serine, or aspartate at position 40, a residue other than glycine at position 41, and an aspartate or asparagine at position 85, according to Kabat numbering, for example, an antibody with a VL region having a threonine at position 40, an asparagine at position 41, and an aspartate at position 85. In some embodiments, the antibody has a VH region with a serine at position 40 and an isoleucine at position 89 and/or a VH region with a serine or proline at position 40 and an isoleucine, tyrosine, methionine, phenylalanine, or tryptophan at position 89, according to Kabat numbering. In some embodiments, the VL region has an isoleucine or leucine at position 10, and/or an isoleucine at position 83. In some embodiments, the VL region has a valine or isoleucine at position 9 and/or a residue other than glutamine at position 100.

[00183] In some examples, the VL region has a valine or isoleucine at position 9, an isoleucine or leucine at position 10, an arginine at position 39, a threonine at position 40, an asparagine at position 41, a glycine at position 42, a serine at position 43, an isoleucine at position 83, an aspartate at position 85, and an alanine at position 100; and the VH region has a serine at position 40 and an isoleucine at position 89, according to Kabat numbering.

[00184] In some examples, the VL region does not contain a proline at position 40, a glycine at position 41, or a threonine at position 85, according to Kabat numbering, and/or the VH region does not contain an asparagine or alanine at position 40 or a valine at position 89, according to Kabat numbering. In some examples, the VL region does not contain an serine at position 10, a proline at position 40, a glycine at position 41, an phenylalanine at position 83, or a threonine at position 85, according to Kabat numbering, and/or the VH region does not contain an asparagine or alanine at position 40 or a valine at position 89, according to Kabat numbering.

[00185] In some aspects, the antibody has a light chain having P8 , V9 or I9, I10 or L10, Q38, R39, T40, N41 G42, S43, P44, R45, D82, I83, A84, D85, Y86, Y87, G99, A100, G101, T102, K103, L104, E105, R142, S162, V163, T164, E165, Q166, D167, S168, and Y173, according to Kabat numbering, and/or a heavy chain having Q6, P9, R38, Q39, S40, P41, G42, K43, G44, L45, S84, D86, T87, A88, I89, Y90, Y91, W103, G104, Q105, G106,

T107, L108, V109, T110, V111, Y147, E150, P151, V152, T173, F174, P175, A176, V177, Y185, S186, and L187, according to Kabat numbering.

[00186] In other embodiments, the meditope-enabled antibodies are generated via CDR grafting, typically by modifying one or more complementarity determining region (CDR) (e.g., one or more of CDRs 1-3) of the heavy and/or light chain of a meditope-enabled antibody, such as any of the meditope-enabled antibodies described herein, to replace them with other CDRs, such as CDRs of existing or new antibodies. CDR grafting is standard practice for producing humanized monoclonal antibodies, e.g., by grafting CDRs of an antibody generated in a non-human species, such as mouse, onto a human antibody framework. See U.S. Patent Nos. 5,558,864 and 8,133,982; Kettleborough et al., "Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation," *Protein Eng.*, 4:773-783 (1991). Thus, in certain embodiments, the antigen specificity of a meditope-enabled antibody is altered by grafting the CDRs of preexisting or newly-generated antibodies of interest. Also among the provided meditope-enabled antibodies are such CDR-grafted meditope-enabled antibodies.

[00187] In some embodiments, the meditope-enabled antibodies are generated, using one of the antibodies disclosed herein (e.g., cetuximab, meditope-enabled trastuzumab, or meditope-enabled M5A (anti-CEA) antibody) as a template sequence, and carrying out one or more known antibody engineering methods to alter it, for example, to alter its antigen-binding characteristics, producing a meditope-enabled antibody with distinct characteristics. Known antibody engineering methods typically employed to alter antigen binding and other properties include various *in vitro* randomization, affinity maturation, and selection methods, including error-prone PCR, spiked PCR, site-directed mutagenesis, phage display and other selection methods. Also provided are constructs, libraries, and expression systems, including GPI-linked expression systems, for carrying out such methods.

[00188] Thus, in certain embodiments, the provided meditope-enabled antibody has a light chain and/or heavy chain variable region with the framework region or regions (FRs) of a meditope-enabled antibody, such as cetuximab, a meditope-enabled trastuzumab, or a meditope-enabled M5A (or FR(s) with at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identity to the FR(s) of such an antibody). In some aspects, such an antibody has one or more CDRs that are distinct from the CDRs of that meditope-enabled antibody.

[00189] For example, in some embodiments, the VL region has an amino acid sequence comprising a light chain framework region (FR) 1 (FR-L1), an FR-L2, an FR-L3, and/or an FR-L4 of the light chain sequence set forth in SEQ ID NO: 71 (or an FR-L1, FR-L2, FR-L3, and/or FR-L4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-L1, FR-L2, FR-L3, and/or FR-L4 of SEQ ID NO: 71), and in some aspects at least one CDR that is distinct from the CDRs of the light chain sequence set forth in SEQ ID NO: 71; and/or a VH region with an amino acid sequence having a heavy chain FR1 (FR-H1), an FR-H2, an FR-H3, and/or an FR-H4, of the heavy chain sequence set forth in SEQ ID NO: 72 (or an FR-H1, FR-H2, FR-H3, and/or FR-H4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-H1, FR-H2, FR-H3, and/or FR-H4 of SEQ ID NO: 72), and in some aspects at least one CDR that is distinct from the CDRs of the heavy chain sequence set forth in SEQ ID NO: 72.

[00190] In some embodiments, the VL region has an amino acid sequence comprising a light chain framework region (FR) 1 (FR-L1), an FR-L2, an FR-L3, and/or an FR-L4 of the light chain sequence set forth in SEQ ID NO: 9 (or an FR-L1, FR-L2, FR-L3, and/or FR-L4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-L1, FR-L2, FR-L3, and/or FR-L4 of SEQ ID NO: 9), and in some aspects at least one CDR that is distinct from the CDRs of the light chain sequence set forth in SEQ ID NO: 9; and/or a VH region with an amino acid sequence having a heavy chain FR1 (FR-H1), an FR-H2, an FR-H3, and/or an FR-H4, of the heavy chain sequence set forth in SEQ ID NO: 6 (or an FR-H1, FR-H2, FR-H3, and/or FR-H4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-H1, FR-H2, FR-H3, and/or FR-H4 of SEQ ID NO: 6), and in some aspects at least one CDR that is distinct from the CDRs of the heavy chain sequence set forth in SEQ ID NO: 6.

[00191] In some embodiments, the VL region has an amino acid sequence comprising a light chain framework region (FR) 1 (FR-L1), an FR-L2, an FR-L3, and/or an FR-L4 of the light chain sequence set forth in SEQ ID NO: 68 (or an FR-L1, FR-L2, FR-L3, and/or FR-L4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-L1, FR-L2, FR-L3, and/or FR-L4 of SEQ ID NO: 68), and in some aspects at least one CDR that is distinct from the CDRs of the light chain sequence set forth in SEQ ID NO: 68; and/or a VH region with an amino acid sequence having a heavy chain FR1 (FR-H1), an FR-H2, an FR-H3, and/or an FR-H4, of the heavy chain sequence set forth in SEQ ID NO: 70 (or

an FR-H1, FR-H2, FR-H3, and/or FR-H4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-H1, FR-H2, FR-H3, and/or FR-H4 of SEQ ID NO: 70), and in some aspects at least one CDR that is distinct from the CDRs of the heavy chain sequence set forth in SEQ ID NO: 70.

[00192] In some embodiments, the VL region has an amino acid sequence comprising a light chain framework region (FR) 1 (FR-L1), an FR-L2, an FR-L3, and/or an FR-L4 of the light chain sequence set forth in SEQ ID NO: 61 (or an FR-L1, FR-L2, FR-L3, and/or FR-L4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-L1, FR-L2, FR-L3, and/or FR-L4 of SEQ ID NO: 61), and in some aspects at least one CDR that is distinct from the CDRs of the light chain sequence set forth in SEQ ID NO: 61; and/or a VH region with an amino acid sequence having a heavy chain FR1 (FR-H1), an FR-H2, an FR-H3, and/or an FR-H4, of the heavy chain sequence set forth in SEQ ID NO: 63 (or an FR-H1, FR-H2, FR-H3, and/or FR-H4 that is at least at or about 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99 % identical to the FR-H1, FR-H2, FR-H3, and/or FR-H4 of SEQ ID NO: 63), and in some aspects at least one CDR that is distinct from the CDRs of the heavy chain sequence set forth in SEQ ID NO: 63.

[00193] In some embodiments, the meditope-enabled antibody has one or more CDRs distinct from the CDRs set forth in SEQ ID NO: 6, 7, 9, 10, 12, 14, 61, 63, 68, 69, 70, 71, and/or 72.

[00194] In some embodiments, the meditope is an antibody other than cetuximab, does not specifically bind to an EGFR, binds to an antigen other than EGFR, and/or does not specifically bind to the epitope on EGFR that is specifically bound by cetuximab.

[00195] In some examples, the meditope-enabled antibody is generated based on a template antibody that is selected from among abagovomab, abciximab, adalimumab, adecatumumab, alemtuzumab, altumomab, altumomab pentetate, anatumomab, anatumomab mafenatox, arcitumomab, atlizumab, basiliximab, bectumomab, ectumomab, belimumab, benralizumab, bevacizumab, brentuximab, canakinumab, capromab, capromab pendetide, catumaxomab, certolizumab, clivatuzumab tetraxetan, daclizumab, denosumab, eculizumab, edrecolomab, efalizumab, etaracizumab, ertumaxomab, fanolesomab, Fbta05, fontolizumab, gemtuzumab, girentuximab, golimumab, ibritumomab, igovomab, infliximab, ipilimumab, labetuzumab, mepolizumab, muromonab, muromonab-CD3, natalizumab, necitumumab

nimotuzumab, ofatumumab, omalizumab, oregovomab, palivizumab, panitumumab, ranibizumab, rituximab, satumomab, sulesomab, ibritumomab, ibritumomab tiuxetan, tocilizumab, tositumomab, trastuzumab, Trbs07, ustekinumab, visilizumab, votumumab, zalutumumab, brodalumab, anrukinzumab, bapineuzumab, dalotuzumab, demcizumab, ganitumab, inotuzumab, mavrilimumab, moxetumomab pasudotox, rilotumumab, sifalimumab, tanezumab, tralokinumab, tremelimumab, the antibody produced by the hybridoma 10B5, B6H12.2, and urelumab, fragments thereof, antibodies having the CDRs and/or antigen-binding regions thereof, and/or antibodies that compete for binding with such antibodies; and/or antibodies having a sequence set forth in any of SEQ ID NOs: 78-124, and/or 125-170, fragments thereof antibodies having the CDRs and/or antigen-binding regions thereof, and/or antibodies that compete for binding with such antibodies.

[00196] Table 2 lists CAS® Registry Numbers (see www.cas.org/expertise/cascontent/registry/regsys.html) for certain antibodies.

Table 2.

Antibody	CAS Registry number
abagovomab	792921-10-9
abciximab	143653-53-6
adalimumab	331731-18-1
adecatumumab	503605-66-1
alemtuzumab	216503-57-0
indium (111In) altumomab pentetate	156586-92-4
arcitumomab	154361-48-5
arcitumumab	154361-48-5
atlizumab	375823-41-9
basiliximab	152923-56-3
bectumomab	158318-63-9
belimumab	356547-88-1
benralizumab	1044511-01-4
bevacizumab	216974-75-3
brentuximab	914088-09-8
canakinumab	914613-48-2
capromab pendetide	145464-28-4
capromab	151763-64-3
catumaxomab	509077-98-9
certolizumab	428863-50-7
certolizumab	428863-50-7
cetuximab	205923-56-4
clivatuzumab tetraxetan	943976-23-6
daclizumab	152923-56-3

denosumab	615258-40-7
eculizumab	219685-50-4
edrecolomab	156586-89-9
efalizumab	214745-43-4
etaracizumab	892553-42-3
etrumaxomab	509077-99-0
fanolesomab	225239-31-6
FBTA05	Lymphomun/FBTA05
fontolizumab	326859-36-3
gemtuzumab	220578-59-6
girentuximab	916138-87-9
golimumab	476181-74-5
ibritumomab	174722-31-7
igovomab	171656-50-1
Infliximab	170277-31-3
ipilimumab	477202-00-9
labetuzumab	219649-07-7
mepolizumab	196078-29-2
muromonab-CD3	140608-64-6
natalizumab	189261-10-7
nimotuzumab	828933-61-3
ofatumumab	679818-59-8
omalizumab	242138-07-4
oregovomab	213327-37-8
palivizumab	188039-54-5
panitumumab	339177-26-3
ranibizumab	347396-82-1
rituximab	174722-31-7
satumomab	138955-26-7
sulesomab	167747-19-5
tiuxetan (ibritumomab)	174722-31-7
tocilizumab	375823-41-9
tositumomab	192391-48-3
trastuzumab	180288-69-1
ustekinumab	815610-63-0
votumumab	148189-70-2
zalutumumab	667901-13-5
brodalumab	1174395-19-7
anrukinzumab	910649-32-0
bapineuzumab	648895-38-9
dalotuzumab	1005389-60-5
demcizumab (OMP-21M18)	1292853-12-3
ganitumab	905703-97-1
inotuzumab	635715-01-4
mavrilimumab	1085337-57-0
moxetumomab	1020748-57-5
moxetumomab pasudotox	1020748-57-5
rilotumumab	872514-65-3

sifalimumab	1143503-67-6
tanezumab	880266-57-9
tralokinumab	1044515-88-9
tremelimumab	745013-59-6
urelumab	934823-49-1
necitumumab	906805-06-9

[00197] In other examples, the template antibody is selected from among: abagovomab, abciximab, adalimumab, adecatumumab, alemtuzumab, altumomab, altumomab pentetate, anatumomab, anatumomab mafenatox, arcitumomab, atlizumab, basiliximab, bectumomab, ectumomab, belimumab, benralizumab, bevacizumab, brentuximab, canakinumab, capromab, capromab pendetide, catumaxomab, certolizumab, clivatuzumab tetraxetan, daclizumab, denosumab, eculizumab, edrecolomab, efalizumab, etaracizumab, ertumaxomab, fanolesomab, Fbta05, fontolizumab, gemtuzumab, girentuximab, golimumab, ibritumomab, igovomab, infliximab, ipilimumab, labetuzumab, mepolizumab, muromonab, muromonab-CD3, natalizumab, necitumumab, nimotuzumab, ofatumumab, omalizumab, oregovomab, palivizumab, panitumumab, ranibizumab, rituximab, satumomab, sulesomab, ibritumomab, ibritumomab tiuxetan, tocilizumab, tositumomab, trastuzumab, Trbs07, ustekinumab, visilizumab, votumumab, zalutumumab, the antibody produced by the hybridoma 10B5, and brodalumab, or tiuzetan. In some such examples, the one or more CDRs are CDRs present in these template antibodies, and/or the antibodies specifically bind to the same antigen or epitope as such antibodies, and/or compete for binding with such antibodies to their antigens.

[00198] Thus, in some cases, the meditope-enabled antibodies (including fragments thereof) specifically binds to an antigen selected from the group consisting of: CA-125, glycoprotein (GP) IIb/IIIa receptor, TNF-alpha, CD52, TAG-72, Carcinoembryonic antigen (CEA), interleukin-6 receptor (IL-6R), IL-2, interleukin-2 receptor a-chain (CD25), CD22, B-cell activating factor, interleukin-5 receptor (CD125), VEGF, VEGF-A, CD30, IL-1beta, prostate specific membrane antigen (PSMA), CD3, EpCAM, EGF receptor (EGFR), MUC1, human interleukin-2 receptor, Tac, RANK ligand, a complement protein, e.g., C5, EpCAM, CD11a, e.g., human CD11a, an integrin, e.g., alpha-v beta-3 integrin, vitronectin receptor alpha v beta 3 integrin , HER2, neu, CD3, CD15, CD20 (small and/or large loops), Interferon gamma, CD33, CA-IX, TNF alpha, , CTLA-4, carcinoembryonic antigen, IL-5, CD3 epsilon, CAM, Alpha-4-integrin, IgE, e.g., IgE Fc region, an RSV antigen, e.g., fusion protein of

respiratory syncytial virus (RSV), TAG-72, NCA-90(granulocyte cell antigen), IL-6, GD2, GD3, IL-12, IL-23, IL-17, CTAA16.88, IL13, interleukin-1 beta, beta-amyloid, IGF-1 receptor (IGF-1R), delta-like ligand 4 (DLL4), alpha subunit of granulocyte macrophage colony stimulating factor receptor, hepatocyte growth factor, IFN-alpha, nerve growth factor, IL-13, PD-L1, CD326, CD47, and CD137. In some examples, the meditope-enabled antigen binds to another antigen identified as a target in a disease or condition of interest, such as cancer or other disease.

[00199] The meditope-enabled antibodies generally further include a constant region, typically a heavy and light chain constant region, which generally are human or partially human constant regions. In some aspects, the heavy chain constant region includes a CH1, or a portion thereof. In some aspects, the light chain constant region includes a CL, or a portion thereof. In some embodiments, the portion of the constant region is sufficient to render the antibody capable of binding to the meditope, e.g., with the requisite binding affinity. In some aspects, the constant regions are the constant regions of cetuximab or trastuzumab. Thus, in some aspects, the heavy chain constant regions are one or more human IgG1 constant regions; in some aspects, the light chain constant regions are kappa constant chain regions. In other examples, the constant regions can include those of other isotypes, including human (or other organism, e.g., murine or chicken) IgG1, IgG2, IgG3, IgG4, IgEs, IgA1, IgA2, IgD, or IgM, and can include kappa or lambda constant regions. Thus, among the provided meditope-enabled antibodies are those generated by mutation of residues in other IgGs, such as human, murine, or chicken, or other immunoglobulins. In other words, the meditope-enabling methods provided herein may be used for any antibody, including IgA, IgE, IgD, and IgM and from any organism that produces antibodies including but not limited to chicken, murine, rat, bovine, rabbit, primates, and goat.

[00200] For example, the sequences of the first constant region (CH1) of a human IgG1, IgG2, IgG3, and IgG4, are compared in Figure 55A. Figure 55B shows the sequence differences in the IgG2-4 CH1, compared to the IgG1 CH1, mapped onto the co-crystal structure of cetuximab and the cQFD meditope (meditope 1, SEQ ID NO: 1). As shown in the Figure, the residues that are different among these isotypes are not within the meditope-binding region of cetuximab, confirming that the meditope-enabling technology is applicable to isotypes other than the IgG1 of cetuximab. As another example, the sequence and

structural alignment of an IgG1 and an IgE Fab domain indicates residues on the IgE near the meditope binding site (Figure 26).

[00201] The provided methods for meditope-site graftingof the Fab cavity within monoclonal antibodies can be used to create a unique handle for meditope binding and used with the technology previously disclosed and for newly-generated antibodies. In certain embodiments, the meditope binding site can be created on pre-existing and all future monoclonal antibodies.

[00202] As described below in section F, also provided are methods for modifying the meditope-enabled antibodies, for example, to alter various properties of the meditope-enabled antibodies, including aspects of the interaction with the meditopes, including affinity, avidity, pH-dependence, as well as other aspects, including pharmacokinetics (PK) and pharmacodynamics (PD) of the antibodies. Thus, also among the provided meditope-enabled antibodies are those modified antibodies generated according to those methods, e.g., by generating a pharmacophore binding model, including antibodies having any one or more of the modifications described in section F, below.

[00203] Also among the antibodies used as template antibodies to generate the meditope-enabled antibodies are modified antibodies and portions thereof, such as a CovX-Body™. Thus, among the provided meditope-enabled antibodies are CovX-bodies, modified to allow their binding to one or more of the provided meditopes, e.g., with an affinity as described herein.

[00204] Also provided are complexes containing one or more meditope bound to a meditope-enabled antibody, such as any of the antibodies described herein.

[00205] Also provided are nucleic acids, such as cDNA and RNA molecules, encoding the meditope-enabled antibodies, and vectors and libraries containing the same, as well as methods for their use, including selection and expression methods and methods for generating transgenic animals using such constructs.

D. Meditopes

[00206] Also provided are meditopes, including variant, modified, and multivalent meditopes, that bind to meditope-enabled antibodies, and complexes and compositions

containing the same, and methods and uses thereof. In certain embodiments, the meditopes include meditopes 1 and 2 (cQFD (SEQ ID NO: 1) and cQYN (SEQ ID NO: 2)), which were originally identified as candidate peptides for binding to the CDR region of cetuximab, as described by Riemer, et al. (2004); *J Immunol* 173, 394-401; Riemer, et al. (2005); *J Natl Cancer Inst* 97, 1663-1670. As demonstrated herein, the cQFD and cQYN meditopes bind to sites within cetuximab distinct from the cetuximab CDRs, and thus were not likely candidates for specific cetuximab-like antibody immunogens for use as a vaccine.

[00207] Nucleic acids encoding the meditopes, including meditope variants and multivalent meditopes, as well as vectors, cells, libraries, and other systems containing the same, also are provided.

I. Variant meditopes

[00208] Among the provided meditopes are meditope variants (also called variant meditopes), having one or more modifications, e.g., structural modifications, as compared to meditope 1 or 2 (meditope of SEQ ID NO: 1 or 2), and methods for producing the same. In some embodiments, cQFD and cQYN meditopes are used as starting points in the design of meditope variants. In some aspects, the meditope variants are designed to have altered properties, such as increased or altered affinity, altered pH dependence, or different affinities under different physiological conditions for one or more of the provided meditope-enabled antibodies, including cetuximab and other antibodies described herein, e.g., as compared to the unmodified meditopes, cQFD and cQYN. Meditope variants are designed and produced using various chemical and biophysical methods.

[00209] Meditope variants include, but are not limited to, variants incorporating modifications to meditopes, e.g., cQFD and cQYN and others described herein. Suitable modifications include, but are not limited to, any peptide modification known in the art, such as, but not limited to, modifications to the manner and/or position of peptide cyclization, modifications to one or more amino acid components of the cyclic peptide, or adding or deleting one or more amino acid from the cyclic peptide. In a particular example, cQFD may be altered with one or more of the following modifications: a modification of Arg8, a modification of Phe3, a modification of Leu5, a modification of Leu10, change to the mode of peptide cyclization, and/or an incorporation of hydratable carbonyl functionality at one or more positions, and one or more amino acid deletions or additions. In the case of cQYN,

suitable modifications may include one or more of the following: a modification of Arg8, a modification of Leu5, a modification of Leu10, change to the mode of peptide cyclization, and/or an incorporation of hydratable carbonyl functionality at one or more positions, and one or more deletions or additions. Certain amino acid positions within the meditope may be deleted or replaced with a different natural amino acid or an unnatural amino acid, or the meditope may be chemically conjugated with a fragment. It is shown herein that a meditope in which Arg9 of SEQ ID NO: 1 has been mutated to citrulline binds to cetuximab. In addition, the amino and carboxy termini can be extended with further amino acids beyond (i.e., in addition to) the cyclic portion of the meditope variant in order to make additional contact to the Fab. For example, Protein L has been added to the C-terminus of the cQFD meditope and preliminary data shows that this binds with much higher affinity. Such modifications are discussed further in Examples 6 and 7.

[00210] In some embodiments, the meditopes include those listed in Tables 3 and 4, as well as such meditopes with additional amino acids, such as those up to 16 amino acids in length. For example, in some aspects, the meditope is one of meditopes 1, 2, or 15-55, further including a serine before the first residues, i.e., at position zero. The meditopes listed in Table 3 employ a disulfide linkage to connect the C and N termini (except that meditope 31 contained an additional tail, meaning that the disulfide linkage is not between the two terminal residues); for the peptides in Table 4, a lactam bridge, a linkage other than disulfide (such as [3+2] cycloaddition), or no linkage is used (e.g., an acyclic or linear variant). Additional meditopes that may be used according to the embodiments described herein include any meditope as defined herein peptide that binds to an antibody framework binding interface (i.e., between the Fab light and heavy chains) of cetuximab or any other therapeutic antibody. For example, in addition to the cyclic peptides cQFD and cQYN, some embodiments include one or more variants of cQFD and cQYN.

Table 3.

SEQ ID NO	Meditope Number	Sequence	Modification (red)	Linkage method
1	1	C-QFDLSTRRLK-C	original	Disulfide 1-Cys : 12-Cys
2	2	C-QYNLSSRALK-C	original	Disulfide 1-Cys : 12-Cys
15	15	C-qFDLSTRRLK-C	q = D-glutamine	Disulfide 1-Cys : 12-Cys
16	16	C-QYDLSTRRLK-C	Y = tyrosine	Disulfide 1-Cys : 12-Cys
17	17	C-QXDLSTRRLK-C	X = β-β'-di-phenyl-Ala	Disulfide 1-Cys : 12-Cys
18	18	C-QFDXSTRRLK-C	X = β-β'-di-phenyl-Ala	Disulfide 1-Cys : 12-Cys
19	19	C-QFDFSTRXLK-C	F = phenylalanine, X = citrulline	Disulfide 1-Cys : 12-Cys
20	20	C-QFDFSTRRLK-C	F = phenylalanine	Disulfide 1-Cys : 12-Cys
21	21	C-QFDESTRRLK-C	E = glutamic acid	Disulfide 1-Cys : 12-Cys
22	22	C-QFDYSTRRLK-C	Y = tyrosine	Disulfide 1-Cys : 12-Cys

23	23	C-QFDLSTRR$QK-C	Q = glutamine	Disulfide 1-Cys : 12-Cys
24	24	C-QFDLSTR$QLK-C	Q = glutamine	Disulfide 1-Cys : 12-Cys
25	25	C-Q$NLST$RLK-C	Y = tyrosine; N = asparagine; A = alanine	Disulfide 1-Cys : 12-Cys
26	26	C-Q$DLSTRRLK-C	A = alanine	Disulfide 1-Cys : 12-Cys
27	27	C-QFD$STRRLK-C	A = alanine	Disulfide 1-Cys : 12-Cys
28	28	C-QFDLST$RLK-C	A = alanine	Disulfide 1-Cys : 12-Cys
29	29	C-QFDLSTRR$K-C	A = alanine	Disulfide 1-Cys : 12-Cys
30	30	C-QFDLSTRR$K-C	E = glutamic acid	Disulfide 1-Cys : 12-Cys
31	31	AcC-QFDLSTRRL$-CGGGSK	AcC-N-acetylcysteine R = arginine	Disulfide 1-AcCys : 12-Cys

Table 4

SEQ ID NO		Sequence	Modification (red)	Linkage method
32	32	G-QFDLSTRRLK-G	G = glycine	Lactam 1-Gly : 12-Gly
33	33	G-Q$DLSTRRLK-G	H = histidine	Lactam 1-Gly : 12-Gly
34	34	G-Q$DLSTRRLK-G	N = asparagine	Lactam 1-Gly : 12-Gly
35	35	G-Q$DLSTRRLK-G	Q = glutamine	Lactam 1-Gly : 12-Gly
36	36	G-Q$DLSTRRLK-G	X = 2-bromo-L-phenylalanine	Lactam 1-Gly : 12-Gly
37	37	G-Q$DLSTRRLK-G	X = 3-bromo-L-phenylalanine	Lactam 1-Gly : 12-Gly
38	38	G-Q$DLSTRRLK-G	X = 4-bromo-L-phenylalanine	Lactam 1-Gly : 12-Gly
39	39	G-QFDLSTR$LK-G	X = citrulline	Lactam 1-Gly : 12-Gly
40	40	G-QFDLST$$LK-G	X = citrulline	Lactam 1-Gly : 12-Gly
41	41	G-QFDLST$RLK-G	X = citrulline	Lactam 1-Gly : 12-Gly
42	42	Q-FDLSTRRLK-$	X = 7-aminoheptanoic acid	Lactam 1-Gln: 11-X
43	43	$-QFDLSTRRLK-$	X = β -alanine	Lactam 1-X: 12-X
44	44	$-QFDLSTRRLK-$'	X = diaminopropionic acid; X' = iso-aspartic acid	Lactam 1-X: 12-X'
45	45	$-QFDLSTRRLK-$'	X = β -alanine; X' = iso-aspartic acid	Lactam 1-X: 12-X'
46	46	$-QFDLSTRRLK-$'	X = diaminopropionic acid; X' = β -alanine	Lactam 1-X: 12-X'
47	47	F-DLSTRRL-K		Lactam 1-Phe : 9-Lys
48	48	C-QFDLSTRRLK-C		Disulfide 1-Cys : 12-Cys; Lactam 4-Asp to 11-Lys
49	49	Q-$DLSTRRLK-$	Y = tyrosine, X = 7-aminoheptanoic acid	Lactam 1-Gln: 11-X
50	50	$-QFDLSTRRLK-$'	X = β-azidoalanine, X' = propargylglycine	[3+2] cycloaddition Azide-1-X: alkyne-12-X'
51	51	Q-$DLSTRRLK-$'	X = β-β'-di-phenyl-Ala, X' = 7-aminoheptanoic acid	Lactam 1-Gln: 11-X'
52	52	qFDLSTRRLK-$	q = D-glutamine, X = 7-aminoheptanoic acid	Lactam 1-Gln: 11-X
53	53	Q-DSTRRLK-$'	X = β-β'-di-phenyl-Ala, X' = 7-aminoheptanoic acid	Lactam 1-Gln: 11-X'
54	54	Q-FDLST$RLK-$'	X = n-butyl-arginine, X' = 7-aminoheptanoic acid	Lactam 1-Gln: 11-X'
55	55	$QFDLSTRRLK$		No linkage

[00211] The meditope variants typically have an amino acid sequence length of between 5 and 16 amino acids, e.g., 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 amino acids in length, such as between 8 and 13 amino acids in length, e.g., between 9 and 12 amino acids in length. The meditope can additionally be conjugated to or associated with (e.g., as part of a fusion protein or peptide) with another molecule, such as another peptide, including a linker or agent. Thus, in this case, the compound containing the meditope may contain additional

amino acid residues beyond the lengths described in this paragraph, where the meditope portion contains between 5 and 16 amino acids and the complex or compound contains additional amino acids. Examples are described herein, e.g., SEQ ID NO: 31 above.

[00212] In some embodiments, the variant meditopes are cyclic peptides. In other embodiments, they are linear or acyclic peptides.

[00213] The meditopes can include peptides, or cyclic peptides derived from such peptides, for example, where the peptides have the formula:

X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12 (Formula I),

[00214] for example, where:

[00215] X1 = Cys, Gly, β-alanine, diaminopropionic acid, β-azidoalanine, or null;

[00216] X2 = Gln or null;

[00217] X3 = Phe, Tyr, β-β'-diphenyl-Ala, His, Asp, 2-bromo-L-phenylalanine, 3-bromo-L-phenylalanine, or 4-bromo-L-phenylalanine, Asn, Gln, a modified Phe, a hydratable carbonyl-containing residue; or a boronic acid-containing residue;

[00218] X4 = Asp or Asn;

[00219] X5 = Leu; β-β'-diphenyl-Ala; Phe; a non-natural analog of phenylalanine, tryptophan, or tyrosine; a hydratable carbonyl-containing residue; or a boronic acid-containing residue;

[00220] X6 = Ser;

[00221] X7 = Thr or Ser;

[00222] X8 = Arg, a modified Arg, or a hydratable carbonyl or boronic acid-containing residue;

[00223] X9 = Arg, Ala;

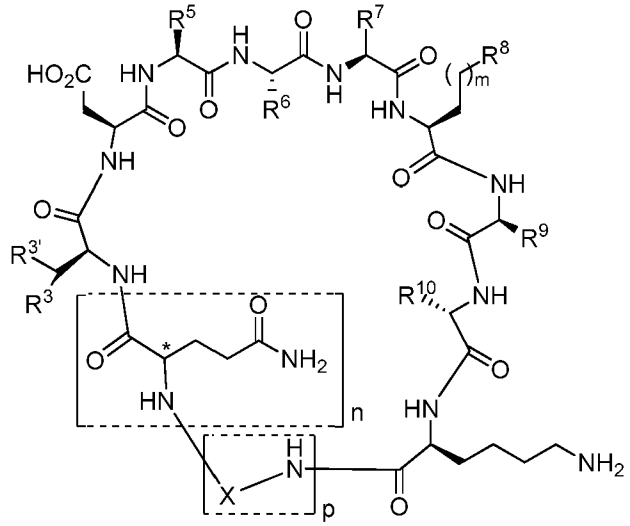
[00224] X10 = Leu, Gln, Glu, β-β'-diphenyl-Ala; Phe; a non-natural analog of phenylalanine, tryptophan, or tyrosine; a hydratable carbonyl-containing residue; or a boronic acid-containing residue;

[00225] X11 = Lys; and

[00226] X12 = Cys, Gly, 7-aminoheptanoic acid, β-alanine, diaminopropionic acid, propargylglycine, isoaspartic acid, or null.

[00227] In some aspects, the modified Arg has a structure of the formula shown in Figure 34. In some aspects, the modified Phe is a Phe with one or more halogen incorporated into the phenyl ring. In some aspects, formula I is not SEQ ID NO: 1 or SEQ ID NO: 2.

[00228] In some embodiments, the meditopes are peptides having the structure of Formula (X):



(X)

wherein:

the center marked with "*" is in the "R" or "S" configuration;

R^3 and $R^{3'}$ are each, independently, H or phenyl, optionally substituted with one, two, or three substituents independently selected from C_{1-4}alkyl, -OH, fluoro, chloro, bromo, and iodo;

R^5 is:

(A) C_{1-8}alkyl, optionally substituted with one or more substituents selected from the group consisting of oxo, acetal, ketal, $-B(OH)_2$, boronic ester, phosphonate ester, ortho ester, $-CO_2C_{1-4}$alkyl, -CH=CH-CHO, $-CH=CH-C(O)C_{1-4}$alkyl, $-CH=CH-CO_2C_{1-4}$alkyl, $-CO_2H$, and $-CONH_2$ group; or

(B) a C_{1-4}alkyl group substituted with:

a) one or two phenyl groups, wherein each phenyl is optionally substituted with one, two, or three substituents independently selected from -OH, fluoro, chloro, bromo, and iodo; or

b) a naphthyl, imidazole, or indole group;

R^6 is $-C_{1-4}$alkyl-OH or $-C_{1-4}$alkyl-SH;

R^7 is $-C_{1-4}$alkyl-OH or $-C_{1-4}$alkyl-SH;

m is 0, 1, 2, 3, 4, or 5;

R^8 is:

(a) -OH, $-NR^aR^b$, $-N(R^c)C(O)R^e$, or $-N(R^c)C(=NR^d)R^e$;

wherein:

R^a is H;

R^b is H or C_{1-8}alkyl optionally substituted with one or more substituents selected from the group consisting of oxo, acetal, and ketal,

$-B(OH)_2$, -SH, boronic ester, phosphonate ester, ortho ester, -CH=CH-CHO, -CH=CH-C(O)C_{1-4}alkyl, -CH=CH-CO_2C_{1-4}alkyl, $-CO_2H$, or -CO_2C_{1-4}alkyl group;

R^c is H, C_{1-8}alkyl, C_{3-8}cycloalkyl, branched alkyl, or aryl;

R^d is H or a C_{1-8}alkyl, C_{2-8}alkenyl, C_{2-8}alkynyl, C_{3-8}cycloalkyl, branched alkyl, or aryl group, each optionally substituted with one or more substituents selected from the group consisting of $-N_3$, $-NH_2$, -OH, -SH, halogen, oxo, acetal, ketal, $-B(OH)_2$, boronic ester, phosphonate ester, ortho ester, -CH=CH-CHO, -CH=CH-C(O)C_{1-4}alkyl, -CH=CH-CO_2C_{1-4}alkyl, $-CO_2H$, and -CO_2C_{1-4}alkyl group; and

R^e is H; $-NHR^d$; or a C_{1-12}alkyl, C_{3-8}cycloalkyl, C_{2-12}alkenyl, C_{2-8}alkynyl, or aryl group, each optionally substituted with one or more substituents selected from the group consisting of $-N_3$, $-NH_2$, -OH, -SH, oxo, C_{2-4}acetal, C_{2-4}ketal, $-B(OH)_2$, boronic ester, phosphonate ester, ortho ester, -CH=CH-CHO, -CH=CH-C(O)C_{1-4}alkyl, -CH=CH-CO_2C_{1-4}alkyl, and -CO_2C_{1-4}alkyl group; or

(b) a C_{1-12} alkyl substituted with an oxo, acetal, ketal, $-B(OH)_2$, boronic ester, -SH, -OH, phosphonate ester, ortho ester, -CH=CH-CHO, -CH=CH-C(O)C_{1-4}alkyl, -CH=CH-CO_2C_{1-4}alkyl, or -CO_2C_{1-4}alkyl group;

R^9 is C_{1-4}alkyl or $-C_{1-2}$alkylene-R^x;

wherein R^x is -CO_2H, -$CONH_2$, -$CH_2NHC(O)NH_2$, or $-CH_2NHC(=NH)NH_2$;

R^{10} is:

(1) a C_{1-8}alkyl optionally substituted with one or more substituents selected from the group consisting of oxo, acetal, ketal, $-B(OH)_2$, boronic ester, phosphonate ester,

ortho ester, -CH=CH-CHO, -CH=CH-C(O)C_{1-4}alkyl, -CH=CH-CO_2C_{1-4}alkyl, -CO_2C_{1-4}alkyl, -CO_2H, and –$CONH_2$ group; or

(2) a C_{1-4}alkyl group substituted with one or two phenyl groups, or one naphthyl, imidazole, or indole group, wherein each phenyl is optionally substituted with one, two, or three substituents independently selected from -OH, fluoro, chloro, bromo, and iodo;

n is 0 or 1;

p is 0 or 1;

X is C_{1-8}alkylene or C_{2-8}alkenylene, each carbon thereof optionally substituted with -CO_2H, -NH_2, or -NHC(O)R^y;

wherein one carbon of said alkylene is optionally replaced with -C(O)NH-, a 5-membered heteroaryl ring, or -S-S-; and

R^y is –C_{1-4}alkyl or –$CH(R^z)CO_2H$;

wherein R^z is –H or –C_{1-4}alkyl optionally substituted with –OH, -SH, or –NH_2;

or a pharmaceutically acceptable salt thereof.

[00229] In some cases, such meditopes are not SEQ ID NO: 1 or 2, or are not cyclic peptides derived from such sequences, and/or are not meditope 1 or 2.

[00230] In some embodiments of the meditope of Formula (X), m is 0, 1, or 2. In other embodiments, R^3 is H or phenyl and $R^{3'}$ is phenyl, 2-bromophenyl, 3-bromophenyl, or 4-bromophenyl. In further embodiments, R^5 is methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, or tert-butyl, each optionally substituted with an oxo, -$B(OH)_2$, -CO_2H, or –$CONH_2$ group, or with one or two phenyl groups each optionally substituted with a bromo or chloro substituent. In further embodiments, R^8 is -OH, -NH_2, -$N(R^c)C(O)R^e$, or -$N(R^c)C(=NR^d)R^e$. In still further embodiments, R^c is H or methyl, R^d is H or C_{1-4}alkyl, and R^e is C_{1-4}alkyl, or –$NH(C_{1-4}$alkyl). In other embodiments, R^9 is methyl or ethyl, optionally substituted with -CO_2H, -$CONH_2$, -$CH_2NHC(O)NH_2$, or –$CH_2NHC(=NH)NH_2$. In still other embodiments, R^{10} is methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, or tert-butyl, each optionally substituted with an oxo, -$B(OH)_2$, -CO_2H, or –$CONH_2$ group. In still other embodiments, –X-NH- is –Cys-Cys-, -Gly-Gly-, -$C(O)(CH_2)_6$-NH-, -β-Ala-β-Ala-, -$C(O)CH(NH_2)CH_2CH{=}CHCH_2CH(CO_2H)$-NH-,

-C(O)CH(NH_2)CH_2NHC(O)CH_2CH(CO_2H)-NH-, -β-Ala-C(O)CH_2CH(CO_2H)-NH-, or –C(O)CH(NH_2)CH_2-triazinyl-CH_2-CH(CO_2H)-NH-.

Modifications

[00231] Based on the structural and thermodynamic data, multiple positions within the meditopes 1 and 2 described herein have been identified as target sites for modification, e.g., with different natural or non-natural amino acids, to enhance the overall binding affinity and/or to alter another property as described herein. Such modifications include, but are not limited to, modification of cQFD or cQYN to generate a head-to-tail cyclic lactam peptide, modification of Arg8, modification of position 3 (e.g., Phe3 of cQFD or variant thereof), modification of Leu5, modification of Leu10, and/or incorporation of hydratable carbonyl functionality (see Figure 31). As demonstrated herein, mutation of each of Phe3, Leu5, and Arg8 to alanine in the original meditope of cQFD reduced the affinity of the resulting compounds for the meditope-enabled antibody binding interface by 10–140-fold. In some aspects, the variant meditopes include those having modifications at one or more of position 1, 3, 5, 8, 10, and 12 of the meditope of SEQ ID NO: 1 or 2, or other meditope listed in Table 3 or 4.

Position 8

[00232] In some embodiments, the meditope variants contain a modification in the position corresponding to position 8 (Arg8) of meditope 1 or 2. In the unmodified meditope (cQFD; SEQ ID NO: 1), Arg8 is extended, making a hydrogen bond with the heavy chain carbonyl of Q105 of the meditope-enabled antibody heavy chain. The immediate area about this residue is hydrophobic yet solvent exposed (Figure 33A). In some aspects, the meditopes contain a modified residue at this position (e.g., modified Arg8). In some examples, the modified residue maintains the iminium functionality of the Arg8 residue useful for meditope-enabled antibody H-bonding, and introduces a substituted or unsubstituted hydrophobic arm to fill the cavity. Such modifications result in significant gains in binding, due to entropic increases, as supported by ligand docking calculations. Such modifications may be incorporated by using an N-alkyl guanidinium group, or an alkyl-amidine functionality. In either case, the substituted group of the terminal N-atom can be alkyl or aryl, wherein each position of the alkyl or aryl group may be optionally substituted with additional functionalities within the group including the terminal position. In one example, a

modified Arginine (modified Arg8), having a structure as shown in Figure 34, is substituted for Arg8 of the meditope, e.g., of SEQ ID NO: 1 or 2 with the butyl group on the NH_2 (shown in Figure 34 as NHR). In some aspects, the variant meditope contains an n-butyl-arginine or butylamidine modification at position 8.

Position 3

[00233] In some embodiments, the meditope variants contain a modification in the position corresponding to position 3, such asPhe3 of meditope 1. As shown herein with structural data, the hydroxyl group of the meditope variant Phe3Tyr cQYN (SEQ ID NO: 2) has an alteration in the extended conformation of the Arg8 side chain as compared to cQFD (SEQ ID NO: 1) (see Figures 30C and 35). Data herein suggest the formation of a favorable hydrogen bond network, with water bound to the Fab. Enthalpy-driven optimization has proven successful in many small-molecule approaches in drug design and there are opportunities in the provided meditopes for engineering increases in entropy. In some embodiments, approaches resulting in enthalpic and/or entropic gains in meditope designs are used to generate the variant meditopes, e.g., to optimize binding.

[00234] For example, when bound to a meditope-enabled antibody, the hydrophobic phenyl ring of Phe3 is surrounded by a fairly polar array of side chain residues of the meditope-enabled antibody Fab (Figure 35). In some embodiments, one or more halogens is introduced on the phenyl ring of this residue, to allow for a halogen bonding interaction with the polar side chain residues. A halogen bond is a relatively strong non-covalent bond, similar to a hydrogen bond but involving the interaction of a halogen such as bromine or chlorine (or other halogen), with an oxygen atom. In some aspects, the residue at this position is modified to incorporate a halogen substituent. In some aspects, Phe3 is replaced with 2-bromo-, 3-bromo-, or 4-bromophenylalanine, in order to place a bromine atom in a position suitable for halogen bonding with a meditope-enabled antibody, e.g., at positions Tyr87 (light chain), Gln38, and/or Tyr91 (heavy chain) of a meditope-enabled antibody, respectively. Such phenylalanine derivatives are commercially available and in some aspects are incorporated into the cyclic peptide meditope variant by solid phase peptide synthesis (SPPS). Exemplary variant meditopes include those containing 2-bromo-L-phenylalanine, 3-bromo-L-phenylalanine, or 4-bromo-L-phenylalanine in the position corresponding to Phe3 of meditope 1.

[00235] In another example, the meditope incorporates an additional phenyl group at this position, for example, by replacing Phe3 with β,β'-diphenylalanine.

Positions 5 and 10 (e.g., Leu5, Leu10 of meditopes 1 or 2)

[00236] In some embodiments, the meditope variants contain a modification in the position corresponding to position 5 or 10 (Leu5 or Leu10) of meditopes 1 or 2. As shown herein, the side chains of Leu5 and Leu10 of meditope 1 make hydrophobic contacts to the meditope-enabled Fab, cetuximab (see Figure 36, right panel; Leu10). In certain embodiments, one or more properties of the meditopes, e.g., affinity, are altered by incorporating a different natural amino acid, or a non-natural amino acid at one or both of these positions, e.g., thereby changing the amount of surface area that can be extended. In one embodiment, natural amino acids (Phe/Tyr/Trp) and non-natural analogs (e.g., β,β'-diphenyl-L-alanine, or amino acids incorporating side chains that include branched alkyl, extended aromatics such as napthyl, or other hydrophobic groups) are systematically introduced via SPPS at one or both positions.

"Hydratable" functionality

[00237] In certain embodiments, one or more Fab hydroxyl-bearing side chains surrounding the meditope-binding site of a meditope-enabled antibody is/are exploited through selective trapping, by formation of a covalent bond with a meditope that incorporates a hydratable functionality. Thus, in some embodiments, the meditope contains one or more residues with hydratable substituents, e.g., in order to create a highly selective but irreversible interaction with a meditope-enabled antibody or other substance. For example, Arg8 of the meditope 1 extends in proximity to Ser43 of the light chain (3.5 Å) and Tyr91 (3.8 Å) of the meditope-enabled antibody heavy chain, according to Kabat numbering (Figure 36, left panel). Incorporation of a hydratable functionality at the end of Arg8 or Leu10 of the meditope would allow for selective formation of a serine or tyrosine hemi-acetal. Such a covalent adduct would essentially afford irreversible binding. In addition, residues containing boronic acid may also be integrated into the meditope as a hydratable group. Boronic acid plays an important role in the structural activity of bortezamib (Velcade®), which is used to treat multiple myeloma. Further representative examples of hydratable residues are also shown in Figure 34, where R = $-CH_2CHO$ or $-CH_2B(OH)_2$. In some

examples, such variants are prepared by incorporating residues containing such groups using SPPS.

[00238] Such hydratable strategies can be applied to engineering new amino acid residues within the antibody Fab-meditope binding site and introducing the requisite complementary "hydratable" functionalities within different positions of the meditope. A similar strategy can be applied by introducing cysteine residues into the Fab region and use this functionality for nucleophilic attack on a "hydratable" functionality such as an electrophilic carbonyl group or derivative thereof contained within the meditope or for making selective disulfide bonds (-S-S-) between the Fab region and the meditope containing the requisite thiol or thiol equivalent functionalities. Other embodiments of this idea would include introducing Michael acceptors contained in the meditope such as α,β-unsaturated carbonyl functionalities. These functionalities are well-known to selectively react with thiols to form stable covalent carbon-sulfur bonds.

Alternative cyclization strategies and replacement of disulfide bridge

[00239] In certain embodiments, the variant meditopes include a disulfide bridge, as in cQFD and cQYN. Disulfide bridges may be formed by the reaction of the side chains of two cysteine residues. In certain embodiments, the disulfide bridge in a meditope, e.g., meditope 1 or 2, is replaced with an alternative linkage or is removed. Thus, among the variant meditopes are those having alternative linkages or lacking the disulfide bridge of the original meditopes.

[00240] In some aspects, the linkage is made between one or more unnatural amino acids within the amino acid chain. Examples of linkages that may be made with unnatural amino acids include linkages comprising (i) stable hydrazone or oxime-based linkages made by reaction of a residue comprising an aldehyde or ketone with a residue comprising an amine group, where the amine nitrogen is substituted with -NH2 or alkyloxy group (e.g., reaction of a p-acetylphenylalanine, m-acetylphenylalanine, or p-(3-oxobutanoyl)-L-phenylalanine residue with a p-(2-amino-3-hydroxyethyl) -phenylalanine residue), (ii) thiol reactive by incorporating phenylselenidylalanine, (iii) a UV crosslinker containing benzophenone by incorporating p-benzoyl-L-phenylalanine, (iv) amine reactive by incorporating p-isopropylthiocarbonyl-phenylalanine or p-ethylthiocarbonyl-phenylalanine, (v) heterocyclic linkages, such as a triazine, thiazole, thiazolidine, or oxazole linkage, made,

for example, by reaction of a residue containing an azide group with a residue containing an alkyne group via Huisgen cycloaddition (e.g., reaction of a p-propargyloxyphenylalanine residue with a p-azidophenylalanine residue); (v) an amide bond made by reaction of an acid group in one residue with an amine group in another residue; (vi) an ester bond made by reaction of an acid group in one residue with an alcohol in another residue, such as a serine; (vii) a double bond, made by reaction of two residues each containing a terminal olefin, e.g., by olefin metathesis (e.g., reaction of two allylglycine residues or two N-allyl substituted amino acids), or (viii) by reaction of any other pair of suitable residues known in the art. For a review, see, for example, Davies, J.S., "The Cyclization of Peptides and Depsipeptides," *J. Peptide Sci.* 2003, 9, 471-501. In one embodiment, the meditope may direct a reactive group to an unnatural amino acid incorporated into the Fab, such as p-acetylphenylalanine.

[00241] Various methods for cyclization of a meditope may be used, e.g., to address *in vivo* stability and to enable chemoselective control for subsequent conjugation chemistry. In some embodiments, the cyclization strategy is a lactam cyclization strategy, including head-to-tail (head-tail) lactam cyclization (between the terminal residues of the acyclic peptide) and/or lactam linkage between other residues. Lactam formation may also be effected by incorporating residues such as glycine, β-Ala, 7-aminoheptanoic acid, and the like, into the acyclic meditope cyclization precursors to produce different lactam ring sizes and modes of connectivity. Additional cyclization strategies such as "click" chemistry and olefin metathesis also can be used (see Figure 31, right boxes). Such methods of peptide and peptidomimetic cyclization are well known in the art.

[00242] In some embodiments, the meditopes containing lactam linkages are more stable, *in vivo*, e.g., have a linkage that is more stable *in vivo* compared to meditopes with other linkages.

[00243] In some embodiments, the terminal residues of an acyclic peptide are reacted to form a cyclic meditope, e.g., cyclic meditope variant. In other embodiments, other positions are amenable to cyclization, including between residues 3 and 11 and 4 and 11. Thus, in some aspects, the meditopes contain a linkage formed between residues other than the N-terminal and C-terminal residues, such as between residues 3 and 11 and/or 4 and 11, e.g., of a 12-amino acid peptide.

[00244] In some embodiments, the meditopes, e.g., variant meditopes, contain a reactive amine functionality (e.g., Lys11), which can be used for subsequent conjugation of the meditope variant, e.g., to a scaffold or linker or to an agent, such as a diagnostic, e.g., imaging, agent or therapeutic agent as described herein. For example, Figure 13 shows a procedure for conjugation of a meditope variant with fluorescein for FACS analysis; this strategy can be applied to other imaging and other agents, including DOTA for *in vivo* PET imaging.

[00245] In some embodiments, thiol functionalities can be introduced in any suitable position on the meditope and can be selectively modified using a number of external reagents containing imagining agents, other proteins and peptides, metal chelators, siRNAs, nanoparticles, and cytotoxic drugs.

Characterization of meditopes

[00246] In some embodiments, the meditopes, such as variant meditopes, are characterized, for example, by ITC, SPR and/or diffraction and/or other methods, such as those described herein in the Examples. In one example, the synthesis of meditopes and characterization thereof is carried out in an iterative fashion, e.g., such that the meditope variant with the most desirable property, e.g., highest affinity for one or more meditope-enabled antibodies or other desired property, such as pH dependence, is subsequently modified to improve the desired property.

[00247] In one example, for characterization of binding of meditopes to meditope-enabled antibodies, the meditope is purified to >95% homogeneity and structurally characterized by mass spectrometry. Peptides are dialyzed in water, their concentrations measured by UV-Vis and calibrated with elemental analysis, and diluted (>100x) into the appropriate buffer. Binding to a meditope-enabled antibody is rigorously characterized by ITC, SPR, X-ray diffraction, or a combination thereof. ITC measurements may be performed on a TA Instruments nanoITC, with only 1–2 mg of peptide per measurement. In one example, for SPR measurements, low density and high density chips are conjugated with a meditope-enabled antibody, e.g., a Fab or a full IgG. In some cases, the chips are first characterized using an antigen, such as a soluble fragment of the entire extracellular domain of EGFR (residues 1–621) in the case of cetuximab. In a study reported herein, the cQFD meditope bound with similar enthalpy and entropy to the cetuximab Fab fragment as

compared to the fully intact cetuximab IgG, e.g., as measured by SPR and ITC. Accordingly, binding measurements may be carried out using the full IgG or Fab fragment of cetuximab or other meditope-enabled antibody. In one example, for diffraction, the co-crystallization conditions of the cetuximab Fab fragment and the meditope of SEQ ID NO: 1 are well-established and diffraction quality crystals are typically obtained in 1 to 3 days, typically 1 day. Full data sets are collected in 8 to 12 hours with an in-house source (Rigaku 007-HF and an R-Axis IV++) and in 10 min at the Stanford Synchrotron Radiation Lightsource, which allows for rapid characterization of the interactions of the meditope variants with meditope-enabled antibodies.

[00248] In some aspects, ITC, SPR and X-ray diffraction data, e.g., collectively, provide atomic detail to guide subsequent chemical modifications and ultimately improve the affinity of the meditopes and/or make other alterations to the meditopes. A calculation based on $\Delta G = -RT \ln Ka$ shows that the difference between micromolar and nanomolar affinity of a meditope for cetuximab results from a change in free energy at 300 K of ~4 kCal/mol, which is on the order of a strong hydrogen bond. Thus, the loss of an ordered water molecule from a protein binding pocket or the reorientation of an amino acid residue-chain may be sufficient to alter binding by orders of magnitude.

[00249] In some examples, other approaches are used to alter properties of the meditopes, e.g., to improve the affinity of the meditope-Fab interaction. In one example, structural data, such as those obtained in the studies described above, may be used to replace residues in the Fab, by mutagenesis, for example, to add additional hydrogen bonds, substitute amino acids for unnatural amino acids or alter the hydrophobic interface, for example, in ways that might better complement meditope binding. In some examples, fluorescence polarization assays are used to identify meditope variants that can displace a given meditope, such as SEQ ID NO: 1. In other examples, the same technique is used to identify small molecules that can displace the meditope and then use these small molecules as templates to further improve the binding affinity of the meditopes.

[00250] In some examples, the meditope variants are designed based on pH dependence, e.g., to have differing affinities at different pH conditions. Thus, in certain embodiments, the meMAb-meditope interaction is tailored with respect to pH. Examples of such meditopes are described herein. In some aspects, the binding affinities of the meditope variants are measured as a function of buffer pH. For example, variant meditopes include

meditopes with a binding affinity for one or more meditope-enabled antibody or fragment that is decreased at a lysosomal pH level or is increased in a hypoxic environment. For example, provided are variant meditopes that exhibit higher binding affinity for a meditope-enabled antibody at a neutral pH (e.g., pH 7-8, e.g., pH 7.3-7.5) and exhibit a relatively lower binding affinity for the antibody at a more acidic pH, such as a pH between at or about 4 and 6.5, e.g., at an endosomal pH (e.g., between at or about 5 and at or about 6.5) or at a lysosomal pH (e.g., between at or about 4.5 and at or about 5). See Figure 27, showing several examples. In another example, the meditopes have increased binding affinity for the antibody in a hypoxic environment, such as a tumor environment, for example, as compared to the affinity observed under other physiological conditions, such as in the blood. In some embodiments, modification of meditope variants or meditope "analogs" for the specific release at low pHs (e.g., in lysosomes for drug delivery) is provided; and modification of meditopes so that they bind with higher affinity in a hypoxic environment (e.g., tumor stroma pH is often lower than normal tissues). Also provided are methods for generating such meditope variants.

[00251] According to some embodiments, the meditope binding site may be exploited or optimized to enhance binding of, purification of, and/or imaging or other method using the meditope-enabled antibodies andfunctional fragments thereof. In a separate embodiment, a meditope may contain one or more cysteine residue that binds to one or more engineered cysteine in the Fab at the meditope binding site (e.g., ThioMAbs). The meditope is thereby conjugated to any diagnostic and/or therapeutic substance, molecule or compound. For example, the substance may be a small molecule diagnostic molecule, such as a marker. The "Cys meditope" directs the conjugate to the antibody and binds via a covalent linkage. Alternatively, the meditope may be conjugated to the Fab to one or more unnatural amino acids that are incorporated into the meditope binding site.

II. Multivalent meditopes

[00252] Also among the provided meditopes are multivalent meditopes. In certain aspects, conjugation of a meditope, to a linker or scaffold (e.g., to form a multivalent tethering entity), significantly improves overall affinity for and targeting to a meditope-enabled mAb bound to an antigen, e.g., a tumor-associated antigen.

[00253] Full-length monoclonal antibodies (mAbs) and F(Ab)'$_2$ fragments include two Fab domains (in the case of full-length mAbs, coupled to a dimeric Fc). Such bivalent antibodies (e.g., IgGs) preferentially bind to cells expressing antigen at high densities. Combinations of monoclonal antibodies (mAbs) that recognize unique epitopes on the same antigen can produce synergistic effects, including enhancement of various antibody effector functions (e.g., ADCC, complement-dependent lysis, signaling inhibition), enhancement of cell death, and in the case of cancer-targeting antibodies, enhancement of tumor growth inhibition. See, for example, Dechant M et al., "Complement-dependent tumor cell lysis triggered by combinations of epidermal growth factor receptor antibodies," *Cancer Res*, 2008 Jul 1;68(13):4998-5003; Scheuer W et al., "Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models," *Cancer Res*, 2009 Dec 15;69(24):9330-6; Cardarelli PM **et al.,** "Binding to CD20 by anti-B1 antibody or F(ab')(2) is sufficient for induction of apoptosis in B-cell lines," *Cancer Immunol Immunother*, 2002 Mar;51(1):15-24. Epub 2001 Dec 18. While the precise mechanism of this enhanced cell death remains debated, studies indicate that both mAbs should be multivalent (e.g., full antibodies or F(ab)'$_2$) to achieve enhanced effects, suggesting that the second bivalent mAb (which binds to a unique epitope on the same antigen) can cluster cell surface antigens and act more efficiently, e.g., more efficiently kill tumor cells. See Figures 8 and 16.

[00254] In some embodiments herein, such clustering is recapitulated using multivalent meditopes. In some aspects, the multivalent meditope used instead of a second antibody, in some aspects to achieve synergy in combination with a meditope-enabled antibody. In some aspects, the multivalent meditopes provide advantages compared to use of a second antibody recognizing a separate epitope. For example, production of the multivalent meditopes can be more efficient, and cost-effective when compared to a second antibody. For example, although a number of preclinical/clinical trials are investigating the co-administration of two monoclonal antibodies, the costs of producing and marketing such a therapeutic is likely to be prohibitive. In some aspects, the multivalent meditopes also are comparatively more easily targeted to disease sites, such as tumors. Given the nature of the meditope binding site (within the meditope binding site, separate from the antibody CDRs), and the broadly applicable methods provided herein for meditope-enabling any antibody of choice, the multivalent meditopes also have the advantage of being readily applicable to a large range of therapeutic antibodies, without the need to identify a second antibody or

epitope with therapeutically acceptable characteristics. Thus, in some aspects, use of a multivalent meditope avoids the need to identify a second mAb with acceptable characteristics, and the associated and significant cost of its development thereof.

[00255] Specificity and affinity are often achieved through multivalency. For a bivalent ligand with a linker, this can be expressed as $\Delta G_{Total} = \Delta G1 + \Delta G2 - \Delta G_{linker}$. In other words, $K_{Total} = K_1 * K_2 / K_{linker}$. In the case where the linker makes no contribution to the free energy ($K_{linker} \sim 1$), the apparent affinity of the bivalent ligand for the bivalent target is the product of the monomeric binding constants. Thus, significant gains in affinity can be achieved through multivalency in general (e.g., for a meditope with $K_D = 1$ μM, the affinity of a 'theoretical' bivalent meditope is 1 pM). While such large gains are rarely seen in general (primarily due to the geometry of bivalent/trivalent/multivalent receptors), synergy is observed. The geometry of a cell surface-expressed antigen can place strict constraints on the linker of a multivalent meditope, but can also ensure specificity, which can be an important goal in some contexts, such as for targeted delivery, e.g., by minimizing the risk of off-target effects.

[00256] In one example, the multivalent meditope contains an Fc region of an antibody or portion thereof, such as substantially all of the Fc region. The use of the Fc region to 'dimerize' ligands is established and described, for example, by Jazayeri JA & Carroll GJ., "Fc-based cytokines: prospects for engineering superior therapeutics," *BioDrugs*, 22(1):11-26 (2008) In some examples, to generate a bivalent meditope, the meditope is fused to an Fc region (e.g., to the N-terminus of the Fc region of an IgG) through a linker, typically a peptide linker, e.g., a flexible peptide linker. In one example, the length of the linker is chosen to roughly match the distance between the Fabs of an IgG, such as of 17 amino acids in length. In some aspects, the linker contains glycines, serines, and/or a combination thereof. Exemplary "meditope-Fc" fusions are shown in Figure 15 (SEQ ID NO: 3 and 4, respectively). See also Figure 16.

[00257] In some embodiments, the composition of the linker and/or the distance between the Fc and the meditope is systematically altered, e.g., to optimize affinity and specificity. In one embodiment, each natural or unnatural residue can be substituted at any position within the linker for optimization. In some aspects, the linker is between at or about 2 and 100 residues in length, e.g., at or about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85,

90, 95, 100, 125, 150, 175, or 200 residues (amino acids) in length, or more. In one example, such linkers are generated using current and future DNA synthesizers, and inserted between the meditope and the Fc regions. The linker may also be 'rigidified' to limit the radius of gyration and to enhance the affinity and specificity of the Fc-meditope. For example, a coiled coil domain may be placed between the meditope and the Fc (Figure 18). In other examples, inert protein domains (e.g., immunoglobulin folds) are substituted for the linker. Multiple immunoglobulin folds can be placed between the meditope and the Fc domain. In certain embodiments, the composition of the linker is of human origin to mitigate potential antigenicity.

[00258] In some examples, the provided meditopes are tethered to a scaffold to create a multivalent meditope, e.g., for enhanced selectivity and binding affinity. In some embodiments, multivalent meditope scaffolds are used to scaffold or "daisy-chain" meditope-enabled mAbs bound to tumor associated antigen to enhance ligand antagonism, alter receptor endocytosis, and/or improve an immune response through ADCC/CDC (see Figure 8). Thus, in some embodiments, the meditopes are used to tether two or more antibodies or functional fragments thereof. Such meditopes may be part of a multivalent tethering agent, for example, to enhance cancer or tumor therapy and imaging. A multivalent tethering agent may include two or more meditopes coupled through linker(s), such as through a long linker and biotin to streptavidin, to create a multivalent meditope tethering entity.

[00259] In one embodiment, the multivalent meditope tethering entity is a tetravalent meditope tethering agent. In one aspect, the tetrameric tethering entity is shown by surface plasmon resonance to have enhanced binding to an antibody as compared to the monovalent peptide, which is consistent with a multivalent interaction. In one example, use of such multivalent meditopes (e.g., tetravalent meditope) produces enhanced binding affinity of the meditope-enabled antibody to the antigen (e.g., in the case of cetuximab, binding affinity for EGFR positive cells), compared to use of the antibody alone or monovalent meditope alone. Such binding affinities can be determined using well-known methods, e.g., by FACS analysis. See Figure 12.

[00260] In some embodiments, to address the receptor constraints on the linker, unmodified or modified, e.g., variant, e.g., optimized, meditopes, such as those obtained as described in Example 6, are coupled to a multivalent scaffold, such as by optimizing the linker. In some aspects, the multivalent meditope is able to "latch-on" to adjacent antibodies,

e.g., IgGs, to form a "daisy-chain"-like array (see Figure 8), which can be used, for example, in antibodies targeting tumor antigens, given the high antigen density of tumor cells. While an intramolecular association of a bivalent meditope and antibody is possible, the C2 symmetry of the antibody, e.g., IgG, can place severe geometrical constraints on the linker for such an interaction. Thus, in some aspects, the meditope is based on a trivalent or higher valency scaffold, ensuring that more than one antibody would be "daisy chained". By including a third meditope arm, the lifetime of the initial encounter of a trivalent meditope to antigen-bound antibody can increase. This, in turn, can increase the probability that an additional arm will bind to a neighboring antigen-bound antibody, thus stabilizing the overall complex. In other embodiments, a multifunctional meditope may be constructed to simultaneously bind a memab and other targets, such as other B and T-cell surface proteins, such as T cell receptor and costimulatory molecules. In some embodiments, multiple meditope-enabled antibodies (for example, including one or more modified meditope-enabled antibodies) having specificities for different meditopes are used together with such different meditopes in such multivalent embodiments.

[00261] Various linkers and scaffolds are known in the art and may be used in connection with these embodiments. An exemplary scaffold synthesis scheme is shown in Figure 13 and discussed in the Examples herein. In some aspects, the use of templates **4** and **5** shown in Figure 13 allows for the formation of both bi- and trivalent meditopes, respectively. In some embodiments, different length polyethylene glycol (PEG) and/or other linkers are used, for example, to improve or alter binding or other properties. In some aspects, the PEG length is between at or about 10 A and at or about 1000 A. The synthetic approach is also amenable to DOTA incorporation for radionuclide imaging. For example, a 30 Å PEG bifunctional arm has been incorporated in the synthesis of a FITC-labeled divalent meditope, namely compound **13** (Figure 13). The distance between the CDR regions within an IgG is ~130 Å. The length of the PEG linker may be systematically varied to ensure this approach is optimal. End-to-end distances of commercially available PEGs extend to 90 Å (Pierce), which would exceed the IgG distance.

[00262] In other embodiments, other scaffolds and/or linkers are used, e.g., to generate high affinity multivalent meditopes and/or to create synergy. For example, DNA may be used as a scaffold for the meditopes to create a more rigid scaffold. For example, different scaffolds of biological and chemical origin may also be used to achieve multivalency. This

includes, but is not limited to, constructing a bivalent or trivalent scaffold, using streptavidin or (see European Application, Publication No.: EP 2065402 A1), strepavidin as a tetravalent scaffold, unique scaffolds (http://www.sciencedirect.com/science/article/pii/S0022283611000283), Origami DNA (see Gu et al., *Nature Nanotechnology* **4**, 245 - 248 (2009)) and the like. A chemical scaffold may also be created using molecules including, but not limited to, DNA (single strand, duplex, Holliday junctions, aptamers and the like), RNA (single strand, hairpin, stem loop, aptamers and the like), PNA (peptide nucleic acids), DNA/PNA duplexes and triplexes for rigidity, organic and or inorganic nanoparticles (directly coupled or coupled through organic polymers such as PEG), organic polymers that can form duplexes with themselves and/or with DNA or PNA.

Characterization of multivalent meditopes

[00263] Binding properties of the multivalent meditopes to meditope-enabled antibodies can be characterized by any of a number of known methods, including SPR and ITC, for example, to ensure that conjugation to the multivalent scaffold does not affect the meditope-Ig interaction. In some cases, such measurements can be limited in their ability to determine mutlivalency and synergistic effects, given that these approaches generally do not involve antigen present on a cell surface (such as on the surface of a tumor cell). Thus, in some aspects, FACS analysis and/or cell viability assays are used to quantify the effect of the multivalent meditope directly on cells expressing antigen recognized by the meditope-enabled antibody (e.g., cells that overexpress EGFR in the context of cetuximab). Exemplary protocols are described in Example 7. In general, a cell line expressing (e.g., over-expressing) the antigen recognized by the meditope-enabled antibody is incubated with the meditope-enabled antibody under conditions whereby the antibody binds to the antigen expressed on the cells. In some cases, varying concentrations of the antibody are used. Either simultaneously or subsequently, the cells are incubated with the multivalent meditope, in some cases in varying concentrations. Appropriate incubation times and washes are carried out. A second antibody and monovalent meditopes may be used as positive and negative controls, respectively. The antibodies and meditopes may be labeled with agents detectable by flow cytometry or microscopy, which are well known. In some examples, a shift (in the case of FACS) or increased signal in the presence of multivalent meditope, compared to monovalent meditope, indicates a synergistic or additive effect. In another

example, to further confirm the additive effects of the multivalent meditope, the non-labeled, monovalent meditope is used in a competition assay, to determine whether it can compete with the labeled multivalent meditope for the antigen-bound meditope-enabled antibody.

[00264] In other examples, cell viability assays are used to determine the ability of a multivalent meditope to enhance cell-killing effects of a meditope-enabled antibody. For example, a cell expressing the antigen of interest may be incubated with varying concentrations of the meditope-enabled antibody and/or multivalent meditope. Monovalent meditopes and second antibodies again are useful as controls. In some examples, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), is used to quantify the number or percentage of viable cells. Other approaches for measuring cell viability, proliferation, and/or death are known in the art and may be used.

[00265] In another example, for multivalent meditopes that demonstrate activity in such assays, Western blot analysis or other biochemical or signaling approach is performed to investigate inhibition of signaling associated with a particular antigen, such as a cell surface receptor (e.g., in the case of cetuximab, to follow the phosphorylation status of EGFR, AKT, MAP which are part of the EGFR signaling pathway). Data from such studies may be compared with data from cells treated only with meditope-enabled antibody (i.e., without meditope), with monovalent meditopes, and/or with tyrosine kinase or other known inhibitors (AG1478, etc.). In some examples, an increase in cell death as a function of multivalent meditope concentration is observed, demonstrating synergistic cell killing effects of the multivalent meditopes.

III. Fusion proteins

[00266] Also provided are fusion proteins containing one or more meditopes. As demonstrated herein by diffraction on the meditope-enabled Fab fragment bound to a cQFD meditope (meditope 1), the N- and C-termini of that antibody-bound meditope were shown to be juxtaposed to the location of bound Protein L, a bacterial protein that binds to human antibodies, including IgMs, IgDs, IgGs, IgEs, and IgAs. In some embodiments, provided are meditope-Protein L fusion proteins (MPLs). In some aspects, the Protein L-meditope fusions exhibit binding to meditope-enabled antibodies with greater affinity as compared to meditopes alone and/or Protein alone, e.g., via energy additivity. The MPLs also can be advantageous compared to Protein L alone and other multivalent conjugates of Protein L,

Protein A, and/or similar antibody-binding proteins, in that they provide specificity for meditope-enabled antibodies and will not target endogenous immunoglobulin molecules when administered therapeutically.

[00267] Originally isolated from bacterium *Peptostreptococcus magnus*, Protein L is a protein that binds to the kappa light chain of human and other antibodies. Exemplary meditope-Protein L (MPL) fusion proteins include those in which a provided meditope is coupled to a Protein L, which can be wild-type Protein L or a variant thereof, such as one of several variants known in the art, or a variant modified to improve one or more property, such as to reduce antigenicity. Methods for modifying similar proteins, such as to reduce antigenicity or immunogenicity, or to improve "immune tolerance," are known in the art and described, for example, by Ahlgren et al., *J Nucl Med*, 2010; 51:1131-1138; Feldwisch J et al., *J Mol Biol*, 2010 Apr 30;398(2):232-47. Epub 2010 Mar 10. For example, amino acid substitutions can be made, e.g., in an iterative process, randomly or based on structural modeling, following by analysis of the desired property, e.g., antigenicity. In another example, mutations are introduced to improve specificity. In other embodiments, the meditopes are fused with other antibody-binding substances, such as other antibody-binding proteins, for example, Protein A or Protein G.

[00268] The MPLs generally include a linker, linking the meditope portion to the Protein L portion. The linker typically includes at least 2, more typically at least 3, e.g., 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues (e.g., four glycines). In some aspects, such residues link the C-terminus of the meditope and the N-terminus of Protein L. In one example, the meditope-Protein L fusion protein has the sequence set forth in SEQ ID NO: 58 (S C Q F D L S T R R L K C G G G G S E V T I K V N L I F A D G K I Q T A E F K G T F E E A T A E A Y R Y A A L L A K V N G E Y T A D L E D G G N H M N I K F A G).

[00269] In some embodiments, the MPLs are used in connection with meditope-enabled antibodies, in the methods and uses described herein, including those involving the conjugation of the meditopes to agents, such as therapeutic or diagnostic agents, such as cytotoxins, radionuclides, DOTA, proteins or other biological molecules, e.g., to target disease and/or image disease sites. In some aspects, to facilitate such conjugation, one or more lysines in the Protein L fusion are mutated, for example, to arginine or asparagine, to generate a Protein L fusion protein that can be specifically conjugated to other molecules, for example, by leaving the N-terminal amine and the epsilon amine available for conjugation,

the latter being solvent exposed and more reactive. An exemplary fusion protein has the sequence set forth in SEQ ID NO: 59 (S C Q F D L S T R R L R C G G G G S E V T I R V N L I F A D G N I Q T A E F R G T F E E A T A E A Y R Y A A L L A R V N G E Y T A D L E D G G N H M N I K F A G), in which all lysines but one have been removed.

[00270] The coding sequence of an exemplary meditope-Protein L fusion protein is set forth in SEQ ID NO: 56. The sequence of exemplary meditope-Protein L fusion proteins are set forth in SEQ ID NO: 57 (His6-Smt3-meditope-ProteinL) and SEQ ID NO: 58 (containing two cysteins, set forth in bold text, which in some aspects are used to cyclize the MPL, e.g., by peroxide or overnight with air: SCQFDLSTRRLKCGGGGSEVTIKVNLIFADGKIQTAEFKGTFEEATAEAYRYAALLA KVNGEYTADLEDGGNHMNIKFAG (SEQ ID NO: 58).

[00271] In some embodiments, the meditope-Protein L (MPL) construct exhibits improved binding affinity for a meditope-enabled antibody as compared to meditope alone. For example, the improvement can be an at least or an at or about 10,000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10-fold improvement in affinity, e.g., in dissociation constant, for the meditope-enabled antibody as compared with the corresponding meditope alone and/or with the Protein L alone. In one example, the binding constants for interactions between Protein L and the meditope alone with the meditope-enabled antibody are approximately 0.5 μM and 1 μM, respectively, whereas the binding constant for the MPL to the antibody is approximately 165 pM.

IV. Meditope-agent conjugates

[00272] In certain embodiments, the meditopes, including one or more meditope, meditope variants, multivalent meditopes, meditope-Protein L fusions (MPLs), multivalent tethering agents or multivalent meditope variant tethering agents are conjugated to one or more therapeutic or diagnostic agent, e.g., imaging agents, therapeutically effective agents or compounds in therapeutically effective amounts or both. Provided are such complexes containing the meditope and one or more agent.

[00273] In some aspects, the binding of the meditopes or variants thereof to one or more meditope-enabled antibody conjugated to a therapeutic or diagnostic (e.g., imaging) agent or compound is used to treat, prevent, diagnose or monitor a disease or condition. In

one aspect, such conjugation of a meditope, such as a multivalent meditope, to an agent, when used in connection with meditope-enabled monoclonal antibodies, provides a highly versatile platform technology that will significantly improve mAb based therapeutics and imaging methods to treat and detect disease (see Figure 8).

[00274] The diagnostic and therapeutic agents include any such agent, which are well-known in the relevant art. Among the imaging agents are fluorescent and luminescent substances, including, but not limited to, a variety of organic or inorganic small molecules commonly referred to as "dyes," "labels," or "indicators." Examples include fluorescein, rhodamine, acridine dyes, Alexa dyes, and cyanine dyes. Enzymes that may be used as imaging agents in accordance with the embodiments of the disclosure include, but are not limited to, horseradish peroxidase, alkaline phosphatase, acid phoshatase, glucose oxidase, β-galactosidase, β-glucoronidase or β-lactamase. Such enzymes may be used in combination with a chromogen, a fluorogenic compound or a luminogenic compound to generate a detectable signal.

[00275] Radioactive substances that may be used as imaging agents in accordance with the embodiments of the disclosure include, but are not limited to, ^{18}F, ^{32}P, ^{33}P, ^{45}Ti, ^{47}Sc, ^{52}Fe, ^{59}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{77}As, ^{86}Y, ^{90}Y. ^{89}Sr, ^{89}Zr, ^{94}Tc, ^{94}Tc, ^{99m}Tc, ^{99}Mo, ^{105}Pd, ^{105}Rh, ^{111}Ag, ^{111}In, ^{123}I, ^{124}I, ^{125}I, ^{131}I, ^{142}Pr, ^{143}Pr, ^{149}Pm, ^{153}Sm, $^{154\text{-}1581}Gd$, ^{161}Tb, ^{166}Dy, ^{166}Ho, ^{169}Er, ^{175}Lu, ^{177}Lu, ^{186}Re, ^{188}Re, ^{189}Re, ^{194}Ir, ^{198}Au, ^{199}Au, ^{211}At, ^{211}Pb, ^{212}Bi, ^{212}Pb, ^{213}Bi, ^{223}Ra and ^{225}Ac. Paramagnetic ions that may be used as additional imaging agents in accordance with the embodiments of the disclosure include, but are not limited to, ions of transition and lanthanide metals (e.g. metals having atomic numbers of 21-29, 42, 43, 44, or 57-71). These metals include ions of Cr, V, Mn, Fe, Co, Ni, Cu, La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb and Lu.

[00276] When the imaging agent is a radioactive metal or paramagnetic ion, the agent may be reacted with another long-tailed reagent having a long tail with one or more chelating groups attached to the long tail for binding to these ions. The long tail may be a polymer such as a polylysine, polysaccharide, or other derivatized or derivatizable chain having pendant groups to which the metals or ions may be added for binding. Examples of chelating groups that may be used according to the disclosure include, but are not limited to, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), DOTA, NOTA, NETA, TETA, porphyrins, polyamines, crown ethers, bis-

thiosemicarbazones, polyoximes, and like groups. The chelate is normally linked to the PSMA antibody or functional antibody fragment by a group, which enables the formation of a bond to the molecule with minimal loss of immunoreactivity and minimal aggregation and/or internal cross-linking. The same chelates, when complexed with non-radioactive metals, such as manganese, iron and gadolinium are useful for MRI, when used along with the antibodies and carriers described herein. Macrocyclic chelates such as NOTA, DOTA, and TETA are of use with a variety of metals and radiometals including, but not limited to, radionuclides of gallium, yttrium and copper, respectively. Other ring-type chelates such as macrocyclic polyethers, which are of interest for stably binding nuclides, such as ^{223}Ra for RAIT may be used. In certain embodiments, chelating moieties may be used to attach a PET imaging agent, such as an Al-^{18}F complex, to a targeting molecule for use in PET analysis.

[00277] Exemplary therapeutic agents include, but are not limited to, drugs, chemotherapeutic agents, therapeutic antibodies and antibody fragments, toxins, radioisotopes, enzymes (e.g., enzymes to cleave prodrugs to a cytotoxic agent at the site of the tumor), nucleases, hormones, immunomodulators, antisense oligonucleotides, RNAi molecules (e.g., siRNA or shRNA), chelators, boron compounds, photoactive agents and dyes. The therapeutic agent may also include a metal, metal alloy, intermetallic or core-shell nanoparticle bound to a chelator that acts as a radiosensitizer to render the targeted cells more sensitive to radiation therapy as compared to healthy cells. Further, the therapeutic agent may include paramagnetic nanoparticles for MRI contrast agents (e.g., magnetite or Fe_3O_4) and may be used with other types of therapies (e.g., photodynamic and hyperthermal therapies and imaging (e.g., fluorescent imaging (Au and CdSe)).

[00278] Chemotherapeutic agents are often cytotoxic or cytostatic in nature and may include alkylating agents, antimetabolites, anti-tumor antibiotics, topoisomerase inhibitors, mitotic inhibitors hormone therapy, targeted therapeutics and immunotherapeutics. In some embodiments the chemotherapeutic agents that may be used as therapeutic agents in accordance with the embodiments of the disclosure include, but are not limited to, 13-cis-retinoic acid, 2-chlorodeoxyadenosine, 5-azacitidine, 5-fluorouracil, 6-mercaptopurine, 6-thioguanine, actinomycin-D, adriamycin, aldesleukin, alemtuzumab, alitretinoin, all-transretinoic acid, alpha interferon, altretamine, amethopterin, amifostine, anagrelide, anastrozole, arabinosylcytosine, arsenic trioxide, amsacrine, aminocamptothecin, aminoglutethimide, asparaginase, azacytidine, bacillus calmette-guerin (BCG),

bendamustine, bevacizumab, etanercept, bexarotene, bicalutamide, bortezomib, bleomycin, busulfan, calcium leucovorin, citrovorum factor, capecitabine, canertinib, carboplatin, carmustine, cetuximab, chlorambucil, cisplatin, cladribine, cortisone, cyclophosphamide, cytarabine, darbepoetin alfa, dasatinib, daunomycin, decitabine, denileukin diftitox, dexamethasone, dexasone, dexrazoxane, dactinomycin, daunorubicin, decarbazine, docetaxel, doxorubicin, doxifluridine, eniluracil, epirubicin, epoetin alfa, erlotinib, everolimus, exemestane, estramustine, etoposide, filgrastim, fluoxymesterone, fulvestrant, flavopiridol, floxuridine, fludarabine, fluorouracil, flutamide, gefitinib, gemcitabine, gemtuzumab ozogamicin, goserelin, granulocyte - colony stimulating factor, granulocyte macrophage-colony stimulating factor, hexamethylmelamine, hydrocortisone hydroxyurea, ibritumomab, interferon alpha, interleukin – 2, interleukin-11, isotretinoin, ixabepilone, idarubicin, imatinib mesylate, ifosfamide, irinotecan, lapatinib, lenalidomide, letrozole, leucovorin, leuprolide, liposomal Ara-C, lomustine, mechlorethamine, megestrol, melphalan, mercaptopurine, mesna, methotrexate, methylprednisolone, mitomycin C, mitotane, mitoxantrone, nelarabine, nilutamide, octreotide, oprelvekin, oxaliplatin, paclitaxel, pamidronate, pemetrexed, panitumumab, PEG Interferon, pegaspargase, pegfilgrastim, PEG-L-asparaginase, pentostatin, plicamycin, prednisolone, prednisone, procarbazine, raloxifene, rituximab, romiplostim, ralitrexed, sapacitabine, sargramostim, satraplatin, sorafenib, sunitinib, semustine, streptozocin, tamoxifen, tegafur, tegafur-uracil, temsirolimus, temozolamide, teniposide, thalidomide, thioguanine, thiotepa, topotecan, toremifene, tositumomab, trastuzumab, tretinoin, trimitrexate, alrubicin, vincristine, vinblastine, vindestine, vinorelbine, vorinostat, or zoledronic acid.

[00279] Therapeutic antibodies and functional fragments thereof, that may be used as therapeutic agents in accordance with the embodiments of the disclosure include, but are not limited to, alemtuzumab, bevacizumab, cetuximab, edrecolomab, gemtuzumab, ibritumomab tiuxetan, panitumumab, rituximab, tositumomab, and trastuzumab and other antibodies associated with specific diseases listed herein.

[00280] Toxins that may be used as therapeutic agents in accordance with the embodiments of the disclosure include, but are not limited to, ricin, abrin, ribonuclease (RNase), DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtheria toxin, Pseudomonas exotoxin, and Pseudomonas endotoxin.

[00281] Radioisotopes that may be used as therapeutic agents in accordance with the embodiments of the disclosure include, but are not limited to, ^{32}P, ^{89}Sr, ^{90}Y. ^{99m}Tc, ^{99}Mo, ^{131}I, ^{153}Sm, ^{177}Lu, ^{186}Re, ^{213}Bi, ^{223}Ra and ^{225}Ac.

E. Methods of use and compositions

[00282] Also provided are methods and uses of the meditopes, meditope-enabled antibodies, and complexes containing the same, including therapeutic and diagnostic uses, as well as other uses, including antibody purification. Also provided are pharmaceutical compositions containing the meditopes (including variant and multivalent meditopes and meditope fusion proteins) for use in such diagnostic and therapeutic methods.

I. Therapeutic and diagnostic uses and pharmaceutical compositions

[00283] In one embodiment, the specificity and binding of the meditopes, meditope variants, multivalent meditopes, MPLs, multivalent meditope tethering agents and multivalent meditope variant tethering agents, etc., are used to deliver therapeutic agents, diagnostic agents (e.g., imaging agents), or a combination thereof for treatment, diagnosis (e.g., imaging) a disease or condition, typically when administered in combination with (either simultaneously or separately) one or more meditope-enabled monoclonal antibodies.

[00284] In one example, meditopes, e.g., multivalent meditopes, are used for pre-targeted therapy or pre-targeted imaging, as described further below, by administering a meditope-enabled monoclonal antibody before administering the meditopes, meditope variants, multivalent meditope tethering agents or multivalent meditope variant tethering agents. Further, the use of multivalent meditopes can enhance selectivity and improve tumor detection as has been demonstrated for engineered scFvs or chemically conjugated mAbs, but avoids potential immunogenicity inherent in these non-human constructs.

[00285] Thus, the platform technology described herein has a broad impact on the mAb delivery field and provides useful methods for the treatment and diagnosis of various diseases and conditions, including cancers, such as those against which use of an antibody is indicated. For example, meditope-enabled antibodies directed against EGFR-positive cancers, including colorectal and squamous cell carcinoma head and neck cancers, would benefit from use of where cetuximab and a meditope. Additionally, modifying other therapeutic antibodies to generate meditope-enabled versions of such antibodies allows for

the platform technology to be utilized in methods for the treatment and diagnosis of several other cancers, diseases and other conditions.

[00286] Use of the meditopes in such methods is advantageous, for example, by way of specificity, e.g., as compared to other means for binding or conjugation to antibodies. For example, PpL (Protein L) and SpA (Protein A) are not murine-specific or chimeric antibody-specific. PpL binds to ~66% of murine and ~50% of human IgG Kappa light chains and SpA binds to 12% of murine and 50% of human variable heavy chains (Graille et al., 2002). In contrast, as demonstrated herein, the interaction of meditopes with meditope-enabled antibodies is highly specific, which can avoid adverse effects, including off-target effects, e.g., avoiding binding of the therapeutic compound to immunoglobulins endogenous to the subject.

[00287] In some embodiments, a meditope administered in combination with a meditope enabled antibody, an antibody-meditope complex, a multivalent tethering agent administered in combination with a meditope enabled antibody, or a combination thereof may be conjugated to one or more imaging agent. In one aspect, an imaging agent may include, but is not limited to a fluorescent, luminescent, magnetic protein, or radionuclide protein, peptide or derivatives thereof (e.g., genetically engineered variants). Fluorescent proteins that may be expressed by the mRNA component include green fluorescent protein (GFP), enhanced GFP (EGFP), red, blue, yellow, cyan, and sapphire fluorescent proteins, and reef coral fluorescent protein. Luminescent proteins that may be expressed by the mRNA component include luciferase, aequorin and derivatives thereof. Numerous fluorescent and luminescent dyes and proteins are known in the art (see, e.g., U.S. Patent Application Publication 2004/0067503; Valeur, B., "Molecular Fluorescence: Principles and Applications," John Wiley and Sons, 2002; Handbook of Fluorescent Probes and Research Products, Molecular Probes, 9.sup.th edition, 2002; and The Handbook--A Guide to Fluorescent Probes and Labeling Technologies, Invitrogen, 10th edition, available at the Invitrogen web site; both of which are hereby incorporated by reference as if fully set forth herein.)

[00288] In other aspects, the meditope administered in combination with a meditope enabled antibody, the antibody-meditope complex, the multivalent tethering agent administered in combination with a meditope enabled antibody, or a combination thereof may be further conjugated to or otherwise associated with a non-protein imaging agent or a

delivery vehicle such as a nanoparticle, radioactive substances (e.g., radioisotopes, radionuclides, radiolabels or radiotracers), dyes, contrast agents, fluorescent compounds or molecules, bioluminescent compounds or molecules, enzymes and enhancing agents (e.g., paramagnetic ions). In addition, it should be noted that some nanoparticles, for example quantum dots and metal nanoparticles (described below) may also be suitable for use as an imaging agent or a therapeutic agent (e.g., using hyperthermal and photodynamic therapies as well as imaging agents through fluorescence and or MRI contrast).

[00289] The meditope-mAb technology allows for a system that may be used to generate an antibody-meditope complex that may be further conjugated to one or more meditope-enabled antibody, a therapeutic agent, an imaging agent or a combination thereof. Thus, a set of meditopes or high affinity meditope variants, each conjugated to a unique cytotoxic or imaging agent, would allow for the co-administration of a desired meditope conjugate and meditope-enabled mAb for treatment. The meditope conjugates are an improvement over the current antibody-drug conjugates, which have drawbacks such as a reduced specificity due to chemical modification or release of payload. A method for enhancing the binding affinity of a therapeutic antibody or functional fragment thereof is provided herein. Such a method may include administering to a subject a therapeutically effective amount of a pharmaceutical composition via any suitable route of administration. The pharmaceutical composition may include a meditope or meditope variant in combination with a meditope enabled antibody, a multivalent meditope or meditope variant tethering entity in combination with a meditope enabled antibody, a meditope-enabled therapeutic antibody or functional fragment thereof, a pharmaceutically acceptable carrier, and any combination thereof. The enhanced binding affinity of the multivalent meditope may be attributed to the multivalent cross-linking of IgGs bound to the cell surface. Crosslinking IgGs (through parental murine M425 antibody or using anti-IgG IgM) significantly affects signaling, receptor endocytosis and recycling, and cell death. Thus, multivalent peptides may act synergistically with a therapeutic monoclonal antibody to enhance its therapeutic efficacy.

[00290] In some embodiments, the meditope, alone or as part of the tethering entity, may contain a cysteine or other suitable akylating agent that binds to a Fab cysteine at the binding site, thus creating a cysteine-cysteine interaction. Alternatively, the meditope may bind to the Fab at an unnatural amino acid (e.g., p-acetylphenylalanine). The Cys meditope is conjugated to any substance and directs the conjugate to the IgG.

[00291] An antibody-meditope complex may also be used in a method for directing treatment to a particular type of cell or population of cells in a disease or condition that can be targeted by a therapeutic antibody. Such a method of treatment may include administering a therapeutically effective amount of a pharmaceutical composition to a subject having the disease or condition via any suitable route of administration. The pharmaceutical composition may include a meditope or meditope variant in combination with a meditope enabled antibody, a multivalent meditope or meditope variant tethering entity in combination with a meditope enabled antibody, a meditope-enabled therapeutic antibody or functional fragment thereof.

[00292] In other embodiments, a method for imaging tumors or other tissues is provided. In such methods, an unmodified therapeutic antibody may be administered to target a tumor or other tissue that overexpress the corresponding antigen. Subsequently, a multivalent meditope tethering entity that is labeled with an imaging agent is administered via any suitable route of administration and will bind to the therapeutic antibodies that are bound to the target tumor or tissue. See Figure 8. Examples of imaging agents include but are not limited to radiolabels (e.g., ^{3}H, ^{14}C, ^{35}S, ^{90}Y, ^{99m}Tc, ^{125}I, ^{131}I, ^{177}Lu, ^{166}Ho, and ^{153}Sm), metal or magnetic labels (e.g., gold, iron, gadolinium), biotin, chelating agents (e.g., 1,4,7,10-tetraazacyclododecane-N,N', N",N"'-tetraacetic acid ("DOTA")) or any agent described above. In one embodiment, the imaging agent used with the method described herein is DOTA.

[00293] There are several advantages over known methods to the imaging or treatment methods described above. First, the bivalent character of a therapeutic monoclonal antibody enhances the selectivity to the tumor. As discussed further below, this enhanced selectivity is lost with the use of scFvs and is not entirely compensated for by enhanced affinity. Second, the mass of the labeled multivalent meditope tethering entity will be below the renal threshold for filtering (less than 60 kDa, may be as low as ~10 kDa), allowing it to be easily filtered out of the body. In contrast, direct labeling of a therapeutic antibody with an imaging agent or other therapeutic agent is typically avoided because it will circulate for extended periods (days to weeks) in the body. Thus, imaging of tumors or other diseased organs is often accomplished using less selective scFvs.

[00294] In further embodiments, the meditopes may be used to join two or more therapeutic molecules to form a pharmaceutical compound that may be administered, as part

of a pharmaceutical composition, in a therapeutically effective amount to a subject for treatment of cancer, autoimmune disease or other conditions. The two or more therapeutic molecules may include, but are not limited to, functional antibody fragments (e.g., $F(ab')_2$ or Fab fragments), peptides or other small molecules that can target tumor or disease-specific receptors such as those described above. The therapeutic molecules may be two or more of the same therapeutic molecule or alternatively, may be two or more different molecules that target the same tumor or diseased tissue.

[00295] In some embodiments, the pharmaceutical compound or composition may include a proprietary antibody, or portion thereof, such as a CovX-Body™. In one example, the meditopes are used as linkers to join two or more therapeutic molecules to a specially designed CovX antibody. In one example, a small molecule, peptide or scFv associated with such a meditope is recognized by the meditope-binding site (e.g., framework binding interface) of the CovX antibody. When these components are combined, the resulting bivalent CovX-Body™ possesses the biologic actions of the small molecule, peptide or scFv while also retaining an extended half-life of the antibody.

[00296] In some embodiments, the provided meditopes, meditope-enabled antibodies, and complexes thereof, are used in treatment, diagnosis or imaging of a disease or condition, including any cancer, disease or other condition that may be treated or targeted using a therapeutic antibody. Cancers avoid immune surveillance by actively suppressing the immune system. One method envisioned for counteracting this immunosuppression is through vaccination using epitopes of antigens that are either uniquely expressed or over-expressed by the tumor cells. For example, monoclonal antibodies (mAbs) that block signaling pathways, sequester growth factor and/or induce an immune response have been successfully implemented in the clinic to treat cancer and other diseases.

[00297] Thus, the diseases and conditions include any cancer, as well as other diseases and conditions, such as those targeted using therapeutic mAbs, including, but not limited to, leukemia and lymphomas (which can be treated or imaged using, e.g., meditope-enabled versions of alemtuzumab, bectumomab, gemtuzumab, FBTA05, ibritumomab tiuzetan, ofatumumab, rituximab, tositumomab), breast cancer (which can be treated or imaged using, e.g., meditope-enabled versions of trastuzumab, adecatumumab, ertumaxomab) prostate cancer (which can be treated or imaged using, e.g., meditope-enabled versions of adecatumumab, capromab pendetide, etaracizumab), colorectal cancer (which can be treated

or imaged using, e.g., meditope-enabled versions of labetuzumab, panitumumab, altumumab pentetate, votumumab), gastrointestinal cancers (which can be treated or imaged using, e.g., meditope-enabled versions of arcitumumab, catumaxomab), ovarian cancer (which can be treated or imaged using, e.g., meditope-enabled versions of abagovomab, catumaxomab, etaracizumab, igovomab, oregovomab), lung cancer (which can be treated or imaged using, e.g., meditope-enabled versions of anatumumab mafenatox), pancreatic cancer (which can be treated or imaged using, e.g., meditope-enabled versions of clivatuzumab tetraxetan), renal cancer (which can be treated or imaged using, e.g., meditope-enabled versions of girentuximab), melanoma cancer (which can be treated or imaged using, e.g., meditope-enabled versions of etaracizumab, ipilimumab, TRBS07), glioma (which can be treated or imaged using, e.g., meditope-enabled versions of nimotuzumab), bone metastases (which can be treated or imaged using, e.g., meditope-enabled versions of denosumab), head and neck cancer (which can be treated or imaged using, e.g., meditope-enabled versions of zalutumumab), cardiovascular disease (which can be treated or imaged using, e.g., meditope-enabled versions of abciximab), autoimmune disorders (which can be treated or imaged using, e.g., meditope-enabled versions of adalimumab, infliximab), rheumatoid arthritis (which can be treated or imaged using, e.g., meditope-enabled versions of atlizumab, golimumab, infliximab), transplant rejection (which can be treated or imaged using, e.g., meditope-enabled versions of basiliximab, daclizumab, muromonab-CD3), Crohn's disease (which can be treated or imaged using, e.g., meditope-enabled versions of certolizumab, fontolizumab, natalizumab, infliximab, visilizumab), hemoglobinuria (which can be treated or imaged using, meditope-enabled versions of eculizumab), psoriasis (which can be treated or imaged using, e.g., meditope-enabled versions of efalizumab, infliximab, ustekinumab), multiple sclerosis (which can be treated or imaged using, e.g., meditope-enabled versions of natalizumab, ustekinumab), asthma (which can be treated or imaged using, e.g., meditope-enabled versions of benralizumab, mepolizumab, omalizumab), respiratory syncytial virus (RSV) (which can be treated or imaged using, e.g., meditope-enabled versions of palivizumab), macular degeneration (which can be treated or imaged using, e.g., meditope-enabled versions of ranibizumab), appendicitis (which can be treated or imaged using, e.g., meditope-enabled versions of fanolesomab) and any other condition that may be targeted or treated with an antibody. The above-listed antibodies and related diseases or disorders are examples only and do not limit the platform.

[00298] In certain embodiments, one or more meditopes, meditope variants, multivalent meditope tethering agents or multivalent meditope variant tethering agents may be conjugated to one or more imaging agents, therapeutically effective agents or compounds in therapeutically effective amounts or both, such that the binding of the meditopes or variants thereof to one or more meditope-enabled antibody with the therapeutically effective compound may treat, prevent, diagnose or monitor a disease or condition. Such conjugation of a high affinity and/or multivalent meditope coupled to meditope-enabled mAbs provides a highly versatile platform technology that will significantly improve mAb based therapeutics and imaging methods to treat and detect disease (see Figure 8).

III. Other uses and compositions

[00299] Also provided are methods and compositions for stabilization of scFv (single chain Fab variable) fragments. In general, scFv fragments (e.g., the scFv format of a given Fab) bind antigen with a substantially lower affinity than full-length mAbs and other fragments, such as the Fab itself. Such reduced affinity is attributed, at least in part, to absence of the Fab constant domain that directly affects the orientation, conformational fluctuations of the Fv domains, and possibly, to poor linker design. On the other hand, scFv and other smaller fragments can have other advantages, including better tissue, e.g., tumor penetration.

[00300] In some embodiments, provided is a method for improving the stability and affinity of scFv fragments. In one aspect, such methods are carried out by incorporating a meditope into an scFv fragment by facilitating its interaction with residues in the VH and VL regions corresponding to residues within the meditope binding site of the corresponding Fab or whole antibody, for example, by introducing a linker between the scFv and the meditope, to stabilize the scFv. Also provided are complexes containing the scFv bound to one or more meditopes, and compositions containing the same, and uses thereof in the provided methods. In some aspects, such methods will help to enforce the proper orientation of the variable domains and thus enhance the affinity (Figure 9).

[00301] Also provided are isolation and purification methods using the meditopes, including methods for purifying meditope-enabled antibodies and fragments thereof, and/or cells expressing such antibodies, and compositions and kits for use in the same. In one embodiment, such methods are carried out by coupling the meditope (e.g., any of the

provided meditopes) to a solid support, such as a bead, e.g., magnetic bead, plate, column, or other solid support. See Figure 10. In another example, a meditope having the sequence CQFDLSTRRLRCGGGSK was successfully linked through amine coupling to a solid support, and is useful, for example, in biacore/surface plasmon resonance studies. See Figure 29. Such biacore studies in certain embodiments are performed by flowing antibody over an amine coupled long meditope chip.

[00302] Methods for coupling proteins to solid supports are well known to those skilled in the art and can be used in connection with this embodiment. Meditope-enabled antibodies or cells expressing the same then can be exposed to the solid support, whereby they are isolated and purified.

[00303] There are several advantages to such purification methods compared to other available methods. For example, the provided meditopes are easily synthesized and can be readily added to common solid supports (including magnetic beads). Second, the affinity of the meditopes is easily modulated by point mutations, which enables the fine-tuning of the purification procedure and avoids harsh conditions such as low pH that is commonly used to elute antibodies from Protein A or Protein L. In some embodiments, these Protein A or Protein L resins usually contain the full length (e.g., wildtype) sequence where there are up to 5 domains. One or more of these domains are the same as Protein L and Protein A being used herein. In some examples, the meditopes are made bivalent or multivalent (such as those described in Example 7 below) for use in extracting meditope-enabled antibodies, such as intact meditope-enabled antibodies.

[00304] In some aspects, the provided purification and isolation methods have additional advantages over other purification methods using Protein A or Protein L, including reduction in the high cost associated with the production of Protein A or Protein L, improvement compared to the limited life cycle of these proteins, and avoidance of the risk of introducing extraneous biological material such as bacterial pathogens, associated with use of Protein L or A.

F. Modification and screening of meditopes and meditope-enabled antibodies

I. Modification of meditope-enabled antibodies

[00305] Also provided are methods for modifying meditope-enabled antibodies to alter one or more properties of the antibodies, such as binding affinity or specificity with respect to the meditope, and/or other properties. Also provided are modified antibodies produced by the methods and libraries for producing the same. In one embodiment, residues that line the meditope-binding site of a meditope-enabled antibody and/or that are otherwise important for binding of such an antibody to a meditope, are systematically or randomly altered (e.g., using degenerate libraries and selection), for example, to enhance and/or change the specificity of the meditope or meditope analogs (see, for example, Sheedy et al. 2007 and Akamatsu et al. 2007, hereby incorporated herein by reference, for methods of making alterations). In some aspects, the residues are substituted with natural or non-natural amino acids or both, in order to improve the affinity of the meditope interaction and/or alter another property of the meditope-antibody interaction. The incorporation of a non-natural amino acid in an antibody to generate a bi-specific antibody was described by Hutchins et al. 2011).

[00306] Residues of meditope-enabled antibodies that make contact with the meditope and/or are otherwise important, e.g., line the cavity, for example, residues within 8 Å of any atom of a bound meditope, that can be systematically or randomly changed to improve the meditope affinity through hydrogen bonding, ionic, electrostatic or steric interaction can include, but are not limited to, one or more light chain residues (e.g., P8 , V9 or I9, I10 or L10, S14, E17, Q38, R39, T40, N41 G42, S43, P44, R45, D82, I83, A84, D85, Y86, Y87, G99, A100, G101, T102, K103, L104, E105, K107, R142, S162, V163, T164, E165, Q166, D167, S168, or Y173 of the light chain, based on Kabat numbering and with reference to cetuximab, meditope-enabled trastuzumab, or meditope-enabled M5A, or analogous residues in other meditope-enabled antibodies), and/or one or more heavy chain residues (e.g., Q6, P9, R38, Q39, S40, P41, G42, K43, G44, L45, S84, D86, T87, A88, I89, Y90, Y91, W103, G104, Q105, G106, T107, L108, V109, T110, V111, Y147, E150, P151, V152, T173, F174, P175, A176, V177, Y185, S186, or L187 of the heavy chain, based on Kabat numbering and with reference to cetuximab, meditope-enabled trastuzumab, or meditope-enabled M5A, or analogous residues in other meditope-enabled antibodies), or a combination thereof.

[00307] For example, in some aspects, one or more of P8 , V9 or I9, I10 or L10, Q38, R39, T40, N41 G42, S43, P44, R45, D82, I83, A84, D85, Y86, Y87, G99, A100, G101, T102, K103, L104, E105, R142, S162, V163, T164, E165, Q166, D167, S168, and Y173 of the light chain, and/or one or more of Q6, P9, R38, Q39, S40, P41, G42, K43, G44, L45, S84, D86, T87, A88, I89, Y90, Y91, W103, G104, Q105, G106, T107, L108, V109, T110, V111, Y147, E150, P151, V152, T173, F174, P175, A176, V177, Y185, S186, and L187 (with reference to cetuximab, or analogous residues in other meditope-enabled antibodies) of the heavy chain are mutated.

[00308] Other residues within or in proximity to the meditope binding site may be mutated to allow a meditope group to hydrate it and bind with high affinity. For example, the Tyr87 of the light chain residue, the Tyr91 of the heavy chain residue, or both, may be used to form a hydrate with an aldehyde or boron containing compound originating from a meditope analog. In some aspects, as shown in the Examples herein, meditopes do not affect antigen binding and can be used to deliver drugs, daisy chain/crosslink antibodies, to increase the efficiency and/or efficacy of therapeutic antibodies, and in targeted diagnostic, e.g., imaging, methods, among other uses as described herein. Thus, in some embodiments, the meditope binding site of a provided meditope-enabled antibody is altered, for example, such that it can bind to a meditope with higher affinity and/or can bind with one or more variant meditopes, meditope analogs, and/or small molecules such as DOTA or a DOTA derivative (for radioactive delivery and/or pre-targeted imaging).

[00309] In some embodiments, alteration of the residues of the meditope binding site is by systematic alteration. In some aspects, mutating all sites would involve $>20^6$, potentially $>20^{16}$, combinations of mutations. Thus, in some aspects, desired mutations are efficiently identified using a library, in which mutations in the antibody are generated at the DNA level. In one aspect, the library contains individual members that collectively express each individual natural amino acid at one or more positions targeted for mutation. In one example, a DNA library is created using degenerate oligonucleotides at sites of interest (e.g., any one or more of the sites described herein, e.g., Ile89, Thr87, Leu108 and Thr110 of the heavy chain and Lys103 and Glu165 light chain), producing a library the members of which collectively encode all 20 naturally occurring amino acids at these sites.

[00310] In some aspects, an anchor, e.g., a GPI domain, is coupled to the antibody members of the library, such as to the C-terminus of the antibody (e.g., IgG) heavy chain, to

facilitate selection of the expressed antibodies. The library can be transfected using standard methods and members of the library expressed.

[00311] Mutant antibodies of the library having one or more desired characteristics then can be selected. In some aspects, members of the library are selected for their ability to bind to an antigen, e.g., to select for mutations that do not affect antigen binding. In one example, cells expressing antibodies of the library that bind to a fluorescently labeled antigen (e.g., HER2, EGFR, IGFR, CLTA-4, etc) are selected, e.g., by FACS (Figure 20). In some aspects (either after or instead of antigen-binding selection), cells expressing antibodies of the library are selected for another characteristic, such as binding to a specific meditope, meditope analog, or other molecule of interest (e.g., DOTA). In another example, two or more characteristics are simultaneously selected for.

[00312] Selected members of the library, e.g., cells expressing the antibodies, are evaluated and characterized, for example, to determine the desired mutation or combination of mutations for achieving a particular property. In one aspect, sorted cells are characterized by PCR to identify the resulting mutations that facilitate or enhance meditope/analog/small molecule binding.

In some aspects, the methods are carried out in an iterative fashion, for example, by repeating the mutation, selection, and characterization process, e.g., multiple times, to "evolve/optimize" the binding or other desired characteristic(s).

II. Meditope Analogs and Fragments

[00313] Also provided are meditope analogs, and methods for identifying, producing, and screening such analogs. Thus, in some embodiments, other molecules also bind to meditope binding sites of meditope-enabled antibodies, with functional characteristics similar to those of a meditope. Such molecules are called meditope analogs and include, but are not limited to, small molecules, aptamers, nucleic acid molecules, peptibodies and any other substance able to bind to the same binding interface as a meditope. In one example, the analog is a DOTA derivative.

[00314] In one embodiment, provided are screening methods for identifying such analogs, including small molecule analogs that mimic the meditopes. Such methods include

fluorescence polarization assays, diffraction based and NMR based fragment screening, and tethering dynamic combinatorial methods.

[00315] In other embodiments, a fragment-based drug discovery approach is used to identify fragments, small molecules, such as chemical groups, that bind to the meditopes, meditope binding sites, and/or meditope-enabled antibodies near the meditope binding sites. See, for example, Erlanson, *Top Curr Chem* (2012) 317:1-32. In some examples, the identified fragments are coupled to the meditopes, for example, to improve their affinity or other properties of the interaction with the meditope binding site. In other examples, they are expanded and/or linked together to generate compounds for coupling to meditopes or meditope-enabled antibodies, or for use as meditope analogs. Methods for fragment and/or meditope analog discovery include, but are not limited to, the following. In some examples, the fragments are between about or at 100 and about or at 1000 Da.

Fluorescence polarization assays

[00316] In some aspects, the methods include fluorescence polarization assays and/or other competition-based assays. In one example, to identify alternative molecules that can bind at the meditope site and be used for similar functions, a fluorescent marker (e.g., Alexafluor, rhodamine, fluorescein) is conjugated to a meditope, e.g., using a suitable method (e.g., amines, sulfhydryl, carboxylate, sugars or other known methods), and allowed to interact with a meditope-enabled antibody. In general, interaction between the labeled meditope and meditope-enabled antibody causes a change in the fluorescence polarization/intensity of the fluorescent tag. Once established, test compounds (potential analogs), such as small molecule compounds (MW < 1000 Da), are added and equilibrated, e.g., with fluorescent tagged meditope-antibody complex, and the fluorescence polarization is monitored. Test compounds that block the meditope-antibody interaction will alter the fluorescent polarization properties. Accordingly, another embodiment is a method of identifying compounds that can be optimized and used for target delivery. The analogs or potential analogs may be screened and further characterized, e.g., by crystallography or other methods described herein for characterization of the meditopes.

Diffraction methods

[00317] Diffraction based methods to identify lead compounds are well established (Shuker et al. 1996; Erlanson et al. 2001; Hughes et al. 2011). Since cetuximab and other

meditope-enabled Fabs diffract beyond 2.5 Å, this approach is used in certain embodiments to identify lead compounds or small molecule fragments that can be coupled to a meditope or used as analogs. For example, such compounds include fragments that are then linked together to form synthetic meditope analogs. In one example, a compound library is developed to soak into crystals of cetuximab or other meditope-enabled antibody. Diffraction data from these soaks are collected and data sets analyzed. Such methods can identify additional sites on meditope-enabled antibodies that are amendable for fragment growth and optimization.

[00318] The fragments, can be grown (chemically derivatized) to enhance their binding and specificity. The fragments can also be chemically tethered to the meditope. Optimization of this chemical coupling can significantly enhance the overall binding affinity of the meditope for the meditope binding site. Additionally, analogs found by diffraction methods can be optimized and used in lieu of the meditope for drug delivery, multivalent scaffolding and other functions. Further, mutations in the light and heavy chains may be made to change the specificity of the ligand (meditope) and that these diffraction methods (including fluorescence polarization, NMR screening, and phage display methods) can be used to optimized alternative ligands.

NMR screening

[00319] NMR can also be used to identify analogs and fragments (e.g., peptide fragments, non-peptide based small molecules) that can be optimized and used as described herein. In one example, to identify such leads, one dimensional (1D) spectra of pools containing fragments, e.g., 15 to 20 fragments, are collected. In one embodiment, the fragments used are non-peptide based small molecules. Next, a meditope-enabled antibody is added to each pool and a second 1D spectra collected. Compounds that bind (transiently) to a meditope-enabled antibody undergo rapid magnetization transfer, resulting in a loss of intensity. Thus, in some aspects, comparing the spectra before and after meditope-enabled antibody binding and identifying peaks that are altered, indicates an interaction. These peaks can be pre-assigned to a specific compound and thus immediately known or the pools can be subdivided and the spectra recollected. After several rounds the exact identity of the compound is known. In these experiments, the precise position of the interaction is not known. The binding site can be determined by NMR or the fluorescence polarization assay. Alternatively, the Fab fragment can be labeled with NMR active and inactive nuclei (e.g.,

^{13}C, ^{15}N and ^{2}H), multiple NMR experiments performed to assign the spectrum, and then used with the fragment library to identify the binding position.

Virtual ligand screening

[00320] Virtual ligand screening is another method that can be used to identify lead meditope analogs, fragments, and other compounds. Using crystal structures, standard programs (e.g., Schroerdinger Glide) can define a "box' about a site of a macromolecule (the meditope binding site) and dock known ligands to this site. Potential lead compounds are scored by a select energy function and the top 50 to 200 compounds can be purchased. In our initial studies, approximately 100 lead compounds have been identified, and using crystallography, these lead compounds should be shown to demonstrate that they bind to the meditope site.

[00321] In one embodiment, a method of screening for meditopes or meditope analogs is provided herein. Such a method may include, but is not limited to, steps of contacting a library of putative meditopes or small molecules with a meditope-enabled antibody; determining whether the putative meditopes of small molecules bind the meditope-enabled antibody at a framework binding interface; identifying one or more candidate meditopes or meditope analogs; determining binding affinity of the one or more candidates; and identifying one or more of the candidates as a meditope or meditope analogs when the binding dissociation constant is less than 0.70 μM. In other contexts, a low affinity meditope is desired, such that a minimum dissociation constant is specified, for example, 0.70 μM. In some examples, the candidate is identified as a meditope or analog thereof if it exhibits binding constant of less than at or about 10 μM, less than at or about 5 μM, or less than at or about 2 μM, less than at or about 1 μM, less than at or about 500, 400, 300, 200, 100 nm, or less. In some cases, the dissociation constant, such as any of those listed herein, is that measured using a particular technique, such as SPR, Isothermal Titration Calorimetry (ITC), fluorescence, fluorescence polarization, NMR, IR, calorimetry titrations; kinetic exclusion; circular dichroism, differential scanning calorimetry, or other known method. For example, in some cases, the analog or meditope exhibits a binding constant of less than at or about 10 μM, less than at or about 5 μM, or less than at or about 2 μM, less than at or about 1 μM, less than at or about 500, 400, 300, 200, 100 nm, or less, as measured by SPR or as measured by ITC or as measured by any of these methods.

[00322] Additionally, methods of screening for novel framework binding interfaces are also provided, and are described further in the examples below.

[00323] In another embodiment, provided is a method for identifying and optimizing fragments useful in connection with the meditopes, such as in building meditope analogs and/or in coupling to the meditopes to improve their function. Also provided are such analogs and fragments and compounds.

[00324] Having described the invention with reference to the embodiments and illustrative examples, those in the art may appreciate modifications to the invention as described and illustrated that do not depart from the spirit and scope of the invention as disclosed in the specification. The Examples are set forth to aid in understanding the invention but are not intended to, and should not be construed to limit its scope in any way. The examples do not include detailed descriptions of conventional methods. Such methods are well known to those of ordinary skill in the art and are described in numerous publications. Further, all references cited above and in the examples below are hereby incorporated by reference in their entirety, as if fully set forth herein.

EXAMPLE 1: DETERMINATION OF MEDITOPE-ANTIBODY FAB CRYSTAL STRUCTURES

[00325] Meditopes were discovered that bind with high specificity and affinity within a central cavity of the Fab region and Fab fragment of cetuximab. These meditopes were bound to cetuximab Fabs and the structures determined crystalographically.

Materials and Methods

[00326] *Reagents.* An antigen binding fragment (Fab) of cetuximab was obtained by digestion of the cetuximab IgG with immobilized papain (Pierce), followed by reverse purification with Protein A and size exclusion chromatography (SEC) on a Superdex 75 column (GE Healthcare). The single chain variable fragment of cetuximab (scFvC225) was synthesized with a twenty amino acid linker between the light chain and heavy chain. ScFvC225 and soluble epidermal growth factor receptor domain III (sEGFRdIII) were expressed in Sf9 cells and purified as previously described (Donaldson, J.M., Kari, C., Fragoso, R.C., Rodeck, U. & Williams, J.C. Design and development of masked therapeutic antibodies to limit off-target effects: Application to anti-EGFR antibodies. *Cancer Biol Ther* **8** (2009)).

[00327] Meditopes CQFDLSTRRLKC (cQFD; SEQ ID NO:1) and CQYNLSSRALKC (cQYN; SEQ ID NO:2), isolated from a phage display biopanning experiment as described by Riemer, A.B. et al., Vaccination with cetuximab mimotopes and biological properties of induced anti-epidermal growth factor receptor antibodies, *J Natl Cancer Inst* **97**, 1663-70 (2005), were synthesized, oxidized and purified at the City of Hope Synthetic and Polymer Chemistry Core Facility.

[00328] *Crystallization and Diffraction Data.* Cetuximab Fabs (5 mg/mL) were mixed with cQFD and cQYN meditopes at a 1:10 molar ratio and screened for crystal formation using the Qiagen JCSG Core Suites (IIV) at 20 °C. Co-crystals that diffracted beyond 2.2 Å were grown in 100 mM sodium phosphate/citrate, pH 4.5, 2.5 M sodium/potassium phosphate and 1.6 % w/v mesoerythritol. The crystals were wicked through 14% w/v mesoerythritol and flash frozen in liquid nitrogen. Crystallization trials and initial screening studies were carried out in the X-ray facility at City of Hope. Diffraction data were collected at the Stanford Synchrotron Radiation Lab, beam lines 9.1 and 11.1. The initial phases were determined by molecular replacement using the program Phaser (McCoy, A.J. et al. Phaser crystallographic software. *J Appl Crystallogr* **40**, 658-674 (2007)) with the unliganded structure of cetuximab (pdb:1YY8 – chains A and B) (Li, S. et al. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* **7**, 301-11 (2005)). Two Fabs were placed in the asymmetric unit with a Matthews Coefficient of 3.26 and solvent content of 62.4%. The Z scores (standard deviation of the solution over the mean) were 27 and 25 for the rotational search and 38 and 71 for the translational search. A third Fab fragment could not be placed (three Fabs in the asymmetric unit cell produces a reasonable Matthews coefficient of 2.18 at 43% solvent). The cQFD and cQYN meditopes were built into the density manually through multiple iterations using Coot (Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **60**, 2126-32 (2004)) and Phenix (Adams, P.D. et al. PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr D Biol Crystallogr* **58**, 1948-54 (2002)).

Crystallization and structure determination

[00329] As noted above, cetuximab Fabs were generated and purified and mixed with cQFD meditope (cyclic peptide of SEQ ID NO: 1) at a 1:10 ratio; commercial factorials were used to screen for crystal formation. Crystals formed after 1 day at 20 °C. Initial diffraction

analysis of these crystals indicated that the unit cell dimensions and space group were similar to those for the cetuximab Fab already deposited in the Protein Data Bank (1YY8.pdb) (Li, S. et al. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* **7**, 301-11 (2005)). The observation that the CDR loops in the deposited structure make extensive crystal contacts could have suggested that the cQFD meditope was not present in the crystal. Nonetheless, the structure was solved by molecular replacement and the experimental maps were examined to identify unmodeled electron density consistent with the meditope. The initial Fo-Fc map clearly indicated an area in the middle of the Fab fragment as a potential binding site (Fig 1). After an initial round of refinement using the Fab model only, a continuous stretch of unmodeled density consistent with the meditope was observed. The meditope was built into the density and the R and R_{Free} dropped accordingly. Water molecules were added during refinement using Phenix (Adams, P.D. et al. PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr D Biol Crystallogr* **58**, 1948-54 (2002)). The diffraction data and refinement statistics are given in Table 5 below.

Table 5: Diffraction Data and Refinement Statistics for Meditope-Antibody Co-Crystals for Meditopes cQFD, cQYN

	cQYD	cQYN
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions		
a, *b*, *c* (Å)	64.26, 82.59, 211.63	64.16, 82.52, 211.88
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	19.85-2.24 (2.30-2.24) *	29.61-2.20 (2.26-2.20)*
$R_{merge-F}$	0.078 (0.50)	0.067 (0.26)
$I / \sigma I$	17.0 (3.1)	25.5 (6.0)
Completeness (%)	97.4 (83.3)	99.9 (100)
Redundancy	4.2 (3.2)	7.9 (8.2)
Refinement		
Resolution (Å)	19.85-2.24	29.61-2.20
No. reflections	53791	58047
R_{work} / R_{free}	18.2/22.6	17.4/22.2
No. atoms		
Protein	6602	6528
Ligand/ion	200/15	188/15
Water	581	618

B-factors		
Protein	31.4	32.0
Ligand/ion	50.3/67.4	64.0/44.2
Water	37.5	38.7
R.m.s. deviations		
Bond lengths (Å)	0.004	0.007
Bond angles (°)	0.913	1.100

* values in parenthesis are for the highest resolution shell

[00330] Based on these observations and as a point of comparison, crystals of the cetuximab Fab bound to the meditope cQYN (SEQ ID NO: 2) were produced. As for the meditope of SEQ ID NO: 1, clear unmodeled electron density was observed in the center of the Fab. Using the first structure, the differences in sequences were modeled accordingly and multiple rounds of refinement were carried out. Representative electron density maps of both meditopes are shown in Figure 1 B. The diffraction data and refinement statistics for the cQYN-Fab complex also are presented in Table 5, above.

EXAMPLE 2: CHARACTERIZATION OF THE MEDITOPE BINDING SITE OF CETUXIMAB FAB

[00331] The meditope binding site of cetuximab was characterized as discussed below. It was demonstrated that this meditope binding site is unique and has not been found to exist in the immunoglobulins examined to date.

Materials and Methods

[00332] In addition to those described in Example 1 above, the following materials and methods were used.

[00333] *Meditopes and point mutations.* As described in Example 1, CQFDLSTRRLKC (cQFD; SEQ ID NO:1) and CQYNLSSRALKC (cQYN; SEQ ID NO:2), were synthesized, oxidized and purified at the City of Hope Synthetic and Polymer Chemistry Core Facility. Alanine point mutations in the cQFD meditope were generated at residues 3 (Phe3 to Ala), 5 (Leu5 to Ala), 8 (Arg8 to Ala) and 10 (Leu10 to Ala) and the mutants produced bacterially by encoding the peptides at the C-terminus of SMT3 (Mossessova, E. & Lima, C.D. Ulp1-SUMO crystal structure and genetic analysis reveal conserved interactions and a regulatory element essential for cell growth in yeast. *Mol Cell* **5**, 865-76 (2000)). The peptides were oxidized by dialysis into buffer without DTT and purified by SEC to obtain

monomers. Before surface plasmon resonance (SPR) analysis, ubiquitin-like protease (Ulp1) was added to the samples to release the peptides. Each of the biosynthesized mutant peptides contained an additional serine residue at the N-terminus due to the ULP1 cleavage site. Ulp1 and SMT3 were run as controls and did not interact with the cetuximab Fab.

[00334] *Characterization of the meditope-Fab interface.* Affinity analysis by SPR was performed as previously described (Donaldson et al., 2009; Li et al., 2005, *supra*). Briefly, scFvC225 or FabC225 (cetuximab Fab) was immobilized on a CM5 chip using amine chemistry. Peptide or sEGFRdIII affinities were assessed by equilibrium methods at 20 °C and fit to the equation $RU = \{Rmax * [L]\}/\{[L] * K_d\} + R_{offset}$. SEC was performed using a Superdex 200 10/300 column (GE Healthcare). The proteins were mixed, incubated at room temperature for 20 min and applied to the column at 4 °C.

[00335] The EGFR-expressing MDA-MB-468 cell line was used to test cetuximab binding in the presence of peptide meditope. Labeled cetuximab (AF488, Invitrogen) was added for 20 min with or without 60 μM cQFD peptide at 4 °C. Labeled MOPS-21 was used as an isotype control. Cell fluorescence was determined using a FACS Calibur instrument (BD Biosciences).

Analysis of meditope/Fab interface

[00336] The interface of the binding site between the meditope (SEQ ID NO: 1) and the cetuximab Fab was determined to be formed by all four domains of the IgG Fab (e.g., the variable heavy and light chain domains, the light chain constant region, and the heavy chain CH1). Using the PISA server, the buried surface area at the cQFD or cQYN meditope-Fab interface was 904 (± 28) Å2 and 787 (± 42) Å2, respectively, and approximately equally distributed between the light and heavy chains. Figures 2, 35, and 36 show the residues and the loops from the Fab that were determined to contact the meditope.

[00337] Both meditopes (SEQ ID NOs: 1 and 2) were determined to make multiple hydrogen bonds and hydrophobic contacts with the cetuximab Fab. The amino acid sequences of cetuximab (murine chimeric IgG) were aligned with a chimeric monoclonal IgG (ch14.18) used as an isotype control in the phage display experiments that originally identified the meditopes of SEQ ID NOs: 1 and 2, and trastuzumab (a humanized monoclonal antibody that binds to ErbB2) (see Figure 2A). The structure of trastuzumab Fab also was superimposed onto the structure of cetuximab Fab bound to the meditope of SEQ ID NO: 1.

Figure 2A shows amino acid differences between the residues within the central cavity meditope-binding interface of the cetuximab Fab (murine chimera IgG) and ch14.18 and trastuzumab.

[00338] Superposition of the humanized trastuzumab Fab (1NZ8.bdb) on the cetuximab Fab indicated that Arg9 of the cQFD meditope binds to a unique cavity created by the murine variable light chain. Specifically, Asp85, Ile83 and Thr40 of the murine variable light chain of cetuximab, based on Kabat numbering, were shown to be important with respect to binding to the Arg9 residue of the cQFD meditope (Figure 2B). Asp85 in the murine framework region of the variable light chain was shown to make a salt bridge to the guanidinium group of Arg9 of the cQFD meditope ($d_{NE\cdots OD1}$ = 2.7 and 2.9 Å & $d_{NH2\cdots OD2}$ = 3.0 and 3.1 Å). The carboxyl group of Asp85 also was shown to make a hydrogen bond to the backbone amide nitrogen of Leu10 of the cQFD meditope ($d_{OD2\cdots HN}$ = 2.7 and 2.8 Å). The hydroxyl group of Thr40 from the light chain also was shown to make a hydrogen bond to guanidinium group of the meditope Arg9 ($d_{OG1\cdots NH1}$ = 3.2 Å for both). Superimposition of the structures also showed that the phenyl ring in Phe83 and the pyrrolidine ring of Pro40 found in the human variable light chain sequence (corresponding to Ile and Thr, respectively, in cetuximab) are positioned such that they would sterically occlude the side chain of Arg9 in the cQFD meditope.

[00339] Although the Arg9 side chain in the cQFD meditope mapped to the differences between the murine and human Fab sequences, the cQYN meditope encodes an alanine at the same position as Arg9 in the cQFD meditope, and thus could potentially have bound to the human Fab. Although the hydrogen bond to the guanidinium group of Arg9 of the cQFD meditope to Asp85 of the light chain was not present for the cQYN meditope, the carboxyl group of Asp85 of the light chain was shown to retain its hydrogen bond to the backbone amide nitrogen of Leu10 of the cQYN meditope. Differences between the cQFD and cQYN meditopes and their interaction with the cetuximab Fab were determined. Superposition of the structures of the two meditopes (cQFD and cQYN) bound to cetuximab Fab (specifically of the Cα atoms of the heavy and light Fab chains) showed that the hydrophobic side chains of residues Phe/Tyr3, Leu5 and Leu10 of each meditope were positioned nearly identically, indicating that interaction of these residues with the antibody were important for binding (Figure 2B). The phenyl ring was shown to pack against the conserved Gln39 of the heavy chain variable region (Kabat numbering); the Leu5 side chain was shown to pack against Ile

89 and Leu108 of the heavy chain variable region (Kabat numbering), as well as the phenyl ring from Phe3/Tyr3 of the meditopes. Leu10 of the meditopes was shown to bind to an exposed pocket located on the variable light chain.

[00340] The backbone traces of the cQFD and cQYN peptides, however, did deviate. Specifically, the Arg8 side chain structure of the cQFD meditope is extended and makes a strong backbone hydrogen bond to the backbone carbonyl of Gln105 of the Fab heavy chain ($d_{NH1\cdots O=C}$ = 2.6 and 2.8; $d_{NH2\cdots O=C}$ = 2.8 and 2.9 Å). The hydroxyl group of Tyr3 in the cQYN peptide, however, sterically interferes with the Arg8 side chain (Figure 2B) and blocks the interaction between Arg8 of the cQYN meditope and Gln105 of the heavy chain. Consistent with this observation, both Arg8 side chains in the cQYN complex are poorly defined in the electron density map and takes on at least two different rotamers. (There are two Fab-meditope complexes in the asymmetric unit.) Concomitant with this change, a shift in the backbone hydrogen bond pattern was observed. The amide carbonyl of Thr7 in the cQFD meditope makes a hydrogen bond to the amide Asn41 in the cetuximab Fab light chain ($d_{N\text{-}H\cdots O=C}$ = 2.7 and 2.8 Å). This hydrogen bond shifts to the carbonyl of the Arg8 backbone in the amide of backbone of Asn41 in the cQYN peptide ($d_{C=O\cdots H\text{-}N}$ = 3.0 and 3.1 Å).

[00341] The superposition indicated that the change from Pro40 and Gly41 in the human light chain sequence to Thr and Asn in the cetuximab light chain relieves a steric constraint and affords two additional points for forming hydrogen bonds to the meditopes. In the cQFD meditope-cetuximab Fab structure, the amide nitrogen of Fab Asn41 was shown to interact with the carbonyl of Thr7 ($d_{N\text{-}H\cdots O=C}$ = 2.7 and 2.8 Å) of the meditope and the side chain amide of Fab Asn41 was shown to interact with the carbonyl of Ser6 ($d_{ND2\cdots O=C}$ = 3.2 and 3.2 Å) of the meditope. In the cQYN meditope-cetuximab Fab structure, the shift of the backbone results in a shift of the hydrogen bond pattern. The amide nitrogen of Fab Asn41 interacts with carbonyl of Arg8 of cQYN ($d_{N\text{-}H\cdots O=C}$ = 3.0 and 3.1 Å) but the side chain interaction with Fab Asn41 is lost.

[00342] In addition to these results, superposition of the molecular structure of trastuzumab (1N8Z; Cho et al., *Nature*, "Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab," 2003 Feb 13;421(6924):756-60) and rituxumab (2OSL;Du et al., *J Biol Chem*, 2007 May 18;282(20):15073-80. Epub 2007 Mar 29) Fabs on to the meditope bound cetuximab Fab structure further highlighted the uniqueness of the framework.

[00343] Collectively, these results suggest that the differences in the framework region of cetuximab and the control IgG (as opposed to the differences in the CDRs) accounted for the selection of the meditope to the central cavity of the Fab (i.e., cetuximab and the phage display control antibody differed in regions other than CDRs).

Meditopes do not induce large conformational changes in the Fab

[00344] Based on the location of the meditope-cetuximab Fab interface, it was determined whether the meditopes perturbed the relative orientation of the IgG domains to the unligated and/or ligated structure. First, the light and heavy chains within the asymmetric unit cell of both meditope complexes were compared and then each chain was compared to the unligated and EGFR-ligated structures. The variable domains of the light chains bound to either meditope were essentially identical to the unliganded structure of cetuximab (r.m.s.d. average: cQFD, 0.231 ± 0.014 Å, cQYN, 0.18 ± 0.01 Å). However, the variable domains of the heavy chain showed a greater divergence (r.m.s.d. average: cQFD, 0.85 ± 0.01 Å, cQYN, 0.88 ± 0.01 Å). This divergence mainly stemmed from the position of framework region 2 (residues 39-46), since deleting the residues in this region and recalculating the r.m.s.d. resulted in a much lower value: cQFD, 0.18 ± 0.01 Å; cQYN, 0.31 ± 0.01 Å) (Figure 6). In addition, this region was also shown to be displaced in the Fab C225-EGFR (cetuximab Fab-antigen) co-crystal structure, and its relative B-factor value suggested that it is flexible (Figure 6). Finally, the presence of the meditope did not result in significant changes to the CDR structure relative to the EGFR bound or unbound structure. Although the backbone of Tyr98 in the heavy chain CDR loop 3 of the EGFR-liganded structure was shown to be flipped as compared to the Fab structure bound to either meditope, this flip is also observed in the unliganded cetuximab Fab structure (Li et al., 2005, supra).

Contribution of meditope residues to antibody binding

[00345] Based on the structure of the cQFD meditope-Fab complex, as well as the sequence similarity of cQYN to cQFD, several point mutations in the cQFD meditope were generated (Phe3→Ala, Leu5→Ala, Arg8→Ala and Leu10→Ala) to characterize the role of these residues to the overall binding affinity of the meditope. To assess binding affinity, the cetuximab Fab fragment was coupled to a CM5 chip using standard amine chemistry and surface plasmon resonance (SPR) was used to measure the binding properties of the various meditopes. The affinities of the synthetic cQFD and cQYN meditopes to the cetuximab Fab

were measured to be 950 ± 30 nM and 3.5 ± 0.1 μM, respectively (n=3). The binding kinetics were also measured (Figure 3). The association constants, modeled as a bimolecular interaction, were 4.2 (± 0.1) x 10^4 $M^{-1}s^{-1}$ and 1.8 (± 0.1) x 10^4 $M^{-1}s^{-1}$ for cQFD and cQYN, respectively. The dissociation constants were 2.5 (± 0.1) x 10^{-2} s^{-1} and 8.6 (± 0.1) x 10^{-2} s^{-1} for cQFD and cQYN, respectively. The K_D values based on these measurements, 430 (± 30) nM for cQFD and 3.5 (± 0.1) μM for cQYN, are in close agreement with the equilibrium measurements.

[00346] Next, the affinity of each mutated cQFD meditope was measured. The point mutation and wild type cQFD meditopes were generated as C-terminal fusions to SMT3 and cleaved with Ulp1 before the analysis. The biologically-produced, wild type cQFD meditope bound with an affinity of 770 nM, similar to the synthetically-produced cQFD, whereas the mutations Phe3→Ala, Leu5→Ala and Arg8→Ala significantly reduced the affinity for the cetuximab Fab (Table 6, below). In particular, the Arg8→Ala mutation resulted in an approximately 183-fold loss in binding affinity.

[00347] In Table 6, WT = cQFD (SEQ ID NO: 1), F3A = SEQ ID NO: 26 with an additional serine before the cysteine, L5A = SEQ ID NO: 27 with an additional serine before the cysteine, R8A = SEQ ID NO: 28 with an additional serine before the cysteine, and L10A = SEQ ID NO: 29 with an additional serine before the cysteine.

Table 6: Dissociation constants for "WT" (cQFD) and mutant meditopes

Dissociation constants of cQFD mimotope mutants

Ligand	K_D (μM)	ΔΔG (kcal/mol)
WT	0.77	-
F3A	34	2.3
L5A	57	2.6
R8A	141	3.1
L10A	2.2	0.63

[00348] Finally, the Fab of trastuzumab, a humanized therapeutic monoclonal antibody, was coupled to a CM5 chip to characterize the affinity of the cQFD and cQYN meditopes to a human framework. Equilibrium measurements revealed that the dissociation constants for both meditopes exceed 150 μM.

EXAMPLE 3: BINDING OF MEDITOPES TO REGIONS OTHER THAN CDRs

Materials and Methods

[00349] It was demonstrated that binding of the meditopes cQFD and cQYN, and others, to a meditope-enabled antibody (cetuximab), does not affect the ability of the antibody to bind to antigen, meaning that the meditope-enabled antibodies can bind to antigen and a meditope simultaneously.

[00350] In addition to those described in Examples 1 and 2 above, the following materials and methods were used.

[00351] *Reagents.* As described above, the single chain variable fragment of cetuximab (scFvC225) was synthesized with a twenty amino acid linker between the light chain and heavy chain. ScFvC225 and soluble epidermal growth factor receptor domain III (sEGFRdIII) were expressed in Sf9 cells and purified as previously described (Donaldson et al., 2009).

[00352] *Meditopes and point mutations.* As described above, CQFDLSTRRLKC (cQFD; SEQ ID NO:1) and CQYNLSSRALKC (cQYN; SEQ ID NO:2), were cyclized through the oxidation of the cysteines to create a disulfide bond and purified at the City of Hope Synthetic and Polymer Chemistry Core Facility. Alanine point mutations in the cQFD meditope were generated at residues 3 (Phe3 to Ala), 5 (Leu5 to Ala), 8 (Arg8 to Ala) and 10 (Leu10 to Ala) and were produced bacterially by encoding the peptides at the C-terminus of SMT3 (Mossessova et al., 2000). Before surface plasmon resonance (SPR) analysis, ubiquitin-like protease (Ulp1) was added to the samples to release the peptides. An N-terminal serine residue remained as a remnant of the protease cleavage site.

Simultaneous binding of EGFR and meditope to Fab

[00353] The cetuximab Fab was incubated with sEGFRdIII and cQFD and applied to an analytical SEC column. A peak at 13.9 mL was observed, indicating the formation of a

heterotrimeric complex between these three components. Non-reducing SDS-PAGE of the peak showed the presence of all three components (Figure 4B). In comparison, the individual components eluted at 15.2 mL (Fab C225), 15.6 mL (sEGFRdIII) and 16.3 mL (SMT-CQFDLSTRRLKC; SEQ ID NO:1). The results showed that the meditopes and EGFR could bind to cetuximab simultaneously, indicating that meditope binding did not occlude antigen binding by cetuximab.

[00354] In addition, Förster resonance energy transfer (FRET) measurements were used to confirm that the meditope did not affect antigen binding by cetuximab. The fluorescent signal emitted from Alexfluor 488-labeled cetuximab Fab was quenched upon binding of Alexafluor 555-labeled sEGFRdIII. Titration of labeled cetuximab with labeled sEGFRdIII afforded a binding constant of 20 nM, essentially the same value as was observed using SPR (data not shown). To test for allostery, labeled cetuximab Fab was incubated with sEGFRdIII at concentrations ranging from 5 to 1000 nM in the presence of the cQFD meditope (66 μM). The experiment was performed in triplicate. Similar to the SEC studies, no substantial change in the binding constant of the Fab-sEGFRdIII interaction was observed.

[00355] Taken together, these biochemical studies indicate that the meditope-Fab interaction does not substantially affect antigen binding. These observations are similar to results with Protein L and Protein A, which also bind to the Fab framework region without affecting antigen binding to Fab.

cQFD meditope does not bind to CDR loops of cetuximab

[00356] To further confirm that the meditopes did not bind to CDRs, binding between the cQFD meditope and the scFv fragment of cetuximab was assessed. In the scFv, the CDR loops remain intact, but the Fab variable domains are directly connected through a short peptide linker, eliminating the Fab constant domains. In other words, much of the meditope binding pocket is eliminated in the scFv, while the CDRs are minimally affected. SPR demonstrated that EGFR domain III and the cQFD meditope bound to cetuximab Fab tethered to a CM5 chip (See Figure 4D). In addition, EGFR domain III bound with a minimal affinity loss to the scFv tethered to a second CM5 chip. However, relative to Fab binding, the cQFD meditope did not saturate the scFv at concentrations as high as 100 μM of meditope. This indicates minimal, if any, affinity of the meditope for the CDRs, consistent with the crystallographic studies.

cQFD meditope binding does not affect cetuximab binding to EGFR-expressing cells

[00357] As described above, the cetuximab Fab could bind to the cQFD meditope and EGFR domain III simultaneously. It was further shown that this meditope did not affect cetuximab (full IgG) binding to EGFR-expressing cells. FACS analysis was used to follow the binding of the IgG, as a function of meditope concentration, to MDA MB-468 cells, which overexpress EGFR. Cells were incubated with cetuximab in the presence of increasing cQFD meditope concentrations. Even with meditope concentrations greater than 60 μM, no significant changes in cetuximab binding to the cells were observed. This observation is consistent with the SEC studies described above, and indicates that the meditope does not act as an allosteric regulator of antigen binding.

[00358] Simultaneous binding to the Fab of EGFR domain III and the meditope is shown at concentrations significantly above the K_D of the meditope. Like the cQFD and cQYN meditopes, superantigens SpA and PpL, bind to the Fab framework region and do not affect antigen binding (see Graille, M. et al. Crystal structure of a Staphylococcus aureus Protein A domain complexed with the Fab fragment of a human IgM antibody: structural basis for recognition of B-cell receptors and superantigen activity. *Proc Natl Acad Sci U S A* **97**, 5399-404 (2000)); Graille, M. et al. Complex between Peptostreptococcus magnus Protein L and a human antibody reveals structural convergence in the interaction modes of Fab binding proteins. *Structure* **9**, 679-87 (2001); Young, W.W., Jr., Tamura, Y., Wolock, D.M. & Fox, J.W. Staphylococcal Protein A binding to the Fab fragments of mouse monoclonal antibodies. *J Immunol* **133**, 3163-6 (1984); Graille, M. et al. Evidence for plasticity and structural mimicry at the immunoglobulin light chain-Protein L interface. *J Biol Chem* **277**, 47500-6 (2002)).

Meditope binding in relation to other Fab-binding proteins

[00359] The meditope binding site on the Fab is distinct from that of other proteins that bind to the framework region of a Fab. For example, domain D of Protein A, isolated from *Staphylococcus aureus*, binds to the framework of heavy chain (V_H) of human IgG; the B_1 domain of Protein L, isolated from *Peptostreptococcus magnus*, binds to the framework region of kappa light chain (V_L) of human IgG; and domain II of Protein G, isolated from a *Streptococcal* strain, binds to the framework of heavy chain (V_C) of human IgG. Unlike these interactions, in which the interface is predominantly confined to a single Fab domain that is

solvent-exposed, the meditope-Fab interface was shown to be formed by all four domains (e.g., the variable and constant domains of the heavy and light chains).

[00360] The buried surface areas at the cQFD and cQYN meditope-cetuximab Fab interface were shown to be 904 (± 28) $Å^2$ and 787 (± 42) $Å^2$, respectively, and generally distributed equally between the light and heavy chains. These values are roughly similar to those for the interfaces of the Protein A, L and G domains bound to their Fab domains: 580 $Å^2$ (1DEE.pdb), 714 $Å^2$ (1HEZ.pdb), and 518 $Å^2$ (1QKZ.pdb), respectively. There are two unique interfaces between the variable region of the Fab and Protein L, which buries 646 $Å^2$, and a minor interaction between the constant region of the Fab and Protein G, which buries 184 $Å^2$.

Steric Mask

[00361] The meditope can be tethered to the N-terminus of either the light chain or heavy chain N-terminus of a murine chimera or meditope-enabled human mAb through a flexible linker (Figure 11). The N-termini of mAb IgGs are juxtaposed to the antigen binding site and the extension from the N-termini through the flexible linker will sterically interfere with antigen binding. By encoding a tumor specific protease site (e.g., MMP9, MMP14, prostate-specific antigen (PSA) serine protease or other suitable site) in the linker, the steric constraint of intramolecular "masked" IgG construct will be severed at the tumor site and permit antibody binding. This design principle would avoid binding of the intramolecularly 'masked' IgG to healthy tissues and avoid adverse side effects due to off-target binding. Off-rate determination for avidin-peptide mask on cetuximab showed that a multivalent meditope bound with higher affinity than a monovalent meditope, but did not mask.

EXAMPLE 4: GENERATION OF MEDITOPE-ENABLED ANTIBODIES

[00362] Based on structural information, additional meditope-enabled antibodies were generated.

A. Generation of meditope-enabled trastuzumab by mutation

[00363] A meditope-enabled HER2-binding antibody was generated by mutating trastuzumab. The sequence differences between the heavy and light chain variable region framework regions of a human IgG (trastuzumab – 1N8Z.pdb) compared to those of

cetuximab were mapped onto the crystal structure of cetuximab Fab bound to cQFD meditope. Residues in the human framework corresponding to residues lining the meditope binding site in cetuximab were mutated to contain the corresponding residues present in cetuximab; additionally, S9 and S10 of the light chain, according to Kabat numbering, were mutated to isoleucine and leucine, respectively. Figures 23A and 23C show nucleic acid sequences of a portion of the heavy chain (SEQ ID NO: 5) and the light chain (SEQ ID NO: 8), respectively, of the mutant (meditope-enabled) trastuzumab. Figures 23B and 23D show the amino acid sequences of a portion of the heavy chain (SEQ ID NO:6) and light chain (SEQ ID NO:9), respectively, of the meditope-enabled trastuzumab. Figures 23B and 23D also show a comparison of these amino acid sequences and those of a portion of the heavy and light chain of wild type trastuzumab (SEQ ID NO:7 and SEQ ID NO:10, respectively).

[00364] The nucleic acids encoding the heavy and light chains of this mutant, meditope-enabled trastuzumab were synthesized using standard methods and subcloned into a standard vector for mammalian expression. The meditope-enabled trastuzumab IgG was purified using standard methods and characterized for the ability to bind to HER2 and the cQFD meditope.

Meditope-enabled trastuzumab binds to antigen and meditope and has indistinguishable PK/PD properties as wild-type trastuzumab

[00365] In one study, to characterize antigen binding, wild-type trastuzumab and meditope-enabled trastuzumab were labeled with Alexa 647 using standard protocols. A cQFD (SEQ ID NO: 1) meditope-Fc fusion protein (produced as described in Example 7 and shown in Figures 15 and 16) was labeled with Alexa488 using the same protocols. To show that the meditope-Fc binds to the meditope-enabled trastuzumab and not to the wild-type trastuzumab, SKBR3 cells (0.5×10^6), which over-express HER2, were incubated with labeled wild-type and meditope-enabled trastuzumab for 30 minutes. Unbound antibody was washed and the cells were incubated with the meditope-Fc construct for 30 minutes. Antibody binding and meditope binding were analyzed by FACS analysis. The FACS data demonstrate that meditope-enabled trastuzumab binds to HER2 expressed on the SKBR3 cells (e.g., the meditope site can be grafted onto an antibody without loss of antigen specificity) (Figure 22A), and that the meditope-Fc binds to the meditope-enabled trastuzumab, but not to wild-type trastuzumab (Figure 22B).

[00366] In another study, wild-type and meditope-enabled trastuzumab were labeled with Alexafluor 488 using standard protocols. SKBR3 cells (0.5×10^6) were trypsinized, washed once with 0.1 % BSA, and incubated with 1, 10, or 100 nM wild-type or meditope-enabled trastuzumab, for 30 minutes. Unbound antibody was washed twice and analyzed by flow cytometry. The results are shown in Figure 43, demonstrating that meditope-enabled trastuzumab had a similar affinity for antigen-expressing cells as did wild-type trastuzumab.

[00367] In another study, a cQFD (SEQ ID NO: 1) meditope-Protein L fusion protein (MPL) was labeled with Alexafluor 647 using the same standard protocols. SKBR3 cells were trypsinized, washed once with 0.1% BSA, and then pre-bound to wild-type trastuzumab (WT T) or meditope-enabled trastuzumab (TM) (by incubation for 30 minutes). The cells then were washed, followed by another 30min incubation with 0.1μM or 1μM MPL (right). Cells were washed twice and analyzed by flow cytometry (FACS). Cells not pre-bound antibody (MPL (WT) only) were used as a negative control. The results are shown in Figure 44. Histograms demonstrate that cells bound to meditope-enabled trastuzumab, but not unbound cells or cells bound to wild-type trastuzumab, bound to the meditope-Protein L fusion protein. Dot plots show percentage of cells in each quadrant relative to the MPL only control.

[00368] As shown in Figure 45, the same results were obtained using a meditope-Protein L in which all but one lysine in the Protein L were mutated to Arg/Asn residues (MPL-5K).

[00369] These data demonstrate that meditope-enabled trastuzumab is able to bind to antigen to the same degree as the wild-type antibody, and that that the meditope-enabled trastuzumab (but not wild-type trastuzumab) binds to the meditope. Thus, these data confirm that binding of the meditopes to the meditope binding site of this antibody did not affect antigen binding in this study, demonstrating the tripartite interaction.

[00370] Figure 50 (top panel) shows stick representations of the structures of meditope 18 (SEQ ID NO: 18, shown in Table 3, with a β,β'-diphenylalanine at position 5) bound to cetuximab (dark grey sticks), the same meditope (18) bound to meditope-enabled trastuzumab (white sticks), and wild-type trastuzumab (outline), superimposed. The bottom panel shows a ribbon cartoon comparing wild-type and meditope-enabled trastuzumab. The results demonstrate that the position of residues important for binding with the meditope are

in nearly identical places in cetuximab and the meditope-enabled trastuzumab. The right panel of Figure 50 shows a ribbon cartoon of wild-type and meditope-enabled trastuzumab, demonstrating little difference in structure.

[00371] Likewise, Figure 51, in the upper-left panel, shows a superposition of the structures of trastuzumab and trastuzumab memAb (labeled "Meditope-enabled Memab") with certain residues involved in meditope-binding in the meditope-enabled antibody illustrated by sticks. The top right panel shows a superposition of the structures of meditope-enabled trastuzumab (memAb) and cetuximab, with the same residues labeled. As shown, Ile83 takes on two rotamers in the respective structures, which was determined not to be problematic, given that in the meditope bound meditope-enabled trastuzumab crystal structure, it assumes the same rotamer as observed in cetuximab. The bottom panel is a 'cartoon/ribbon diagram' figure of all three structures superimposed, demonstrating no significant differences overall. Some differences in CDR loops were observed, as expected (given binding of these antibodies to different antigens).

[00372] Additionally, animal studies indicated that the biodistribution of the meditope-enabled trastuzumab and wild-type trastuzumab were indistinguishable.

[00373] These data demonstrate that antibodies can be effectively meditope-enabled, while retaining other functions, by mutating residues to correspond with those in the meditope-binding interface of cetuximab.

Meditope-enabled trastuzumab binds to meditope and antigen, whether contacted sequentially or pre-mixed with meditope

[00374] Another study demonstrated that meditope bound to meditope-enabled trastuzumab with similar efficiency whether the cells were pre-bound with antibody (sequential) or a pre-formed meditope-antibody complex (pre-mixed) was applied to cells. The results are shown in Figure 46. In this study, SKBR3 cells were trypsinized, washed once with 0.1% BSA, then incubated with 10 nM meditope-enabled trastuzumab (TM) for 30 min, washed, and then incubated for another 30min with 4, 20, or 100 nM MPL (left panel). Alternatively, 10 nM of TM was premixed with 4, 20, or 100 nM of MPL for 30 min on ice, followed by application of the mixture to the cells for 1 hr (right panel). Cells were washed twice and analyzed by FACS. Percentage of MPL-positive cells relative to no-treatment control is shown in the legend.

B. Generation of meditope-enabled HER2-binding antibody by CDR-grafting

[00375] A meditope-enabled HER2-binding antibody was generated by grafting the CDRs of trastuzumab onto cetuximab.

[00376] Nucleic acid and amino acid sequences of a heavy chain of trastuzumab are shown in Figure 25A (SEQ ID NOs: 11 and 12, respectively), with signal sequence and other sequences. Nucleic acid and amino acid sequence of a light chain of trastuzumab are shown in Figure 25B (SEQ ID NOs: 13 and 14, respectively), with signal sequence and other sequences.

[00377] While the "boundaries" of the CDR loops of mAbs have been clearly defined by sequence homology structural methods in general, the crystal structure of trastuzumab was superimposed onto cetuximab and the position of each residue examined to address potential differences outside of the CDR loops that may have a secondary effect on the conformation of the CDR loops; based on this information, additional modifications beyond modifying the CDRs. The amino acid sequences of the light and heavy chains of the antibody designed to contain trastuzumab-like CDRs on a cetuximab-like framework were translated into DNA sequences and the genes encoding each were synthesized. The amino acid and nucleic acid sequences of the resulting heavy and light chains of antibodies containing trastuzumab-like CDRs grafted onto cetuximab-like framework are shown in Figure 47. Specifically, Figure 47A shows light chain nucleic acid (SEQ ID NO: 60) and light chain amino acid (SEQ ID NO: 61) sequences of a CDR-grafted meditope-enabled trastuzumab (with trastuzumab-like CDRs grafted onto a cetuximab-like framework), with the signal sequence and other residues shaded. Figure 47B shows heavy chain nucleic acid (SEQ ID NO: 62) and heavy chain amino acid (SEQ ID NO: 63) of this antibody.

[00378] The genes were then subcloned in frame into the remaining IgG DNA sequence, confirmed by DNA sequencing and placed in individual expression vectors. The resulting expression vectors were transfected into NS0 cells for co-expression of the heavy and light chains. As the expressed full-length CDR-grafted IgG was secreted, the supernatant was clarified by centifugation, concentrated, and passed over a Protein A column. IgG was eluted using a low pH solution and immediately neutralized. SDS-PAGE (poly acrylamide gel electrophoresis) was carried out on the full-length CDR-grafted IgG, under reducing conditions. The results indicated two protein bands with apparent masses consistent with the

light and heavy chain. The position of the bands migrated at similar positions compared to wild-type cetuximab.

[00379] To characterize antigen binding, wild-type trastuzumab and the trastuzumab CDR-grafted, meditope-enabled mAb (memAb) were labeled with Alexafluor 647 using standard protocols. As described above, cQFD (SEQ ID NO: 1) meditope-Fc (produced as described in Example 7 and shown in Figures 15 and 16); Meditope-Fc was labeled with Alexafluor 488 using the same protocols. To show that the meditope-Fc binds to the trastuzumab CDR-grafted, meditope-enabled mAb and not to the wild-type trastuzumab, SKBR3 cells (0.5×10^6) which over-express HER2, were incubated with labeled wild-type or CDR-grafted meditope-enabled trastuzumab as produced in this example, for 30 minutes. Unbound antibody was washed and the cells were incubated with the meditope-Fc construct for 30 minutes. Antibody binding and meditope binding were analyzed by FACS analysis. As an important component of the "CDR-grafting." The results are shown in Figure 24. The FACS data showed that the trastuzumab CDR-grafted, meditope-enabled mAb bound to HER2 expressed on the SKBR3 cells, demonstrating that CDR loops of one antibody (trastuzumab) grafted onto a meditope-enabled antibody (cetuximab) framework retain the ability to bind to antigen (HER2) (Figure 24A). The FACS data also showed that the meditope-Fc bound to the trastuzumab CDR-grafted, meditope-enabled mAb, but not to wild-type trastuzumab (Figure 24B), demonstrating that a CDR-grafted meditope-enabled antibody binds to meditope.

[00380] It is noted that optimizing the production of the CDR-grafted meditope-enabled trastuzumab would produce more material for more rigorous/quantitative characterization. The low amount of material and thus less rigorous/quantitative characterization (e.g., uncertainty in the final concentration of the meditope-enabled trastuzumab and its labeling with Alexafluor 647) in this study likely accounted for the apparent reduced affinity. There are art-known methods to optimize antigen binding. The data show that the CDR-grafted meditope-enabled trastuzumab bind the trastuzumab antigen (i.e., bind to HER2 overexpressing SKBR3 cells) and bind to the meditope of SEQ ID NO: 1 (i.e., antigen-expressing cells pretreated with the CDR-grafted meditope-enabled trastuzumab bind to the meditope).

C. Generation of meditope-enabled CEA-binding antibody (meditope-enabled M5A) by mutation

[00381] Additionally, an anti-CEA antibody (M5A) was meditope-enabled by mutation of residues to correspond to those of cetuximab. Specifically, as shown with shading in Figure 56, eight point mutations were introduced in the light chain of the M5A antibody, allowing it to bind to meditopes. The wild-type heavy chain sequence and the meditope-enabled light chain sequence were cloned into a Lonza glutamine selection expression vector and transfected in NS0 cells. Two stable lines were obtained, expressing the meditope-enabled mAb at ~5 mg/L.

[00382] Binding of the meditope-enabled M5A to the M5A antigen (CEA) was demonstrated using LS174T cells by FACS. 500 nM of Alexa Fluor 488 labeled wild-type or meditope-enabled M5A was incubated with trypsinized cells and 9 μM of Alexa Fluor 647 labeled meditope-Protein L (see Example 10) for 30 min, room temperature. Cells were washed twice with 0.1% BSA and FACS analysis was performed. The results are shown in Figure 57. Given that M5A is a humanized antibody with the same backbone as trastuzumab, results showed that the meditope-Protein L bound readily to the wild-type M5A. However, an enhanced binding was observed with the meditope-enabled M5A (M5A 8M), which is due to avidity gained from simultaneous binding of both the meditope and Protein L.

[00383] Additionally, SPR measurements were carried out as described herein using surface plasmon resonance, confirming binding of various meditope variants to this meditope-enabled antibody, including CQA(diphenyl)DLSTRRLKC, CQFDA(diphenyl)STRRLKC, meditope 31, and the cQYN meditope (Figure 58).

EXAMPLE 5: MODIFICATION OF THE MEDITOPE BINDING SITE

[00384] The residues lining the meditope-binding site of one or more of the provided meditope-enabled antibodies, such as cetuximab, are systematically or randomly altered, for example, using degenerate libraries and selection, to enhance and/or change the specificity of the meditope or meditope analogs (see Sheedy et al. 2007 and Akamatsu et al. 2007, for methods of making alterations). Residues at these positions are substituted with natural or non-natural amino acids or both, for example, to improve the affinity of the meditope interaction and/or alter another property of the meditope-antibody interaction.

[00385] Residues of meditope-enabled antibodies that make contact with the meditope, line the cavity, and/or are otherwise important (such as residues described for modification herein), are mutated. In one example, structural data, such as those obtained in the studies described above, are used to replace residues in the Fab, by mutagenesis, for example, to add additional hydrogen bonds, substitute amino acids for unnatural amino acids or alter the hydrophobic interface, for example, in ways that might better complement meditope binding. (See Figures 21).

[00386] In one example, individual residues are systematically altered, followed by production and characterization of the mutant antibodies. In another example, a library of IgGs is generated at the DNA level using degenerate oligos at sites of interest to produce members that collectively encode all 20 naturally-occurring amino acids at the site or sites of interest, such that the library produces individual members of the library having each amino acid substituted at one or more given site.

[00387] A GPI domain was added, e.g., to the C-terminus of the Ig heavy chain, of the antibodies in the library. In one example, the library is transfected using standard methods; antibodies (e.g., IgGs) from the library are expressed.

[00388] To demonstrate binding to a GPI-linked meditope-enabled antibody according to these methods, a GPI-linked meditope-enabled trastuzumab was produced. Figure 54 shows that this GPI-linked meditope-enabled trastuzumab bound to a meditope-Protein L (MPL), produced as described in Example 10, below. In this study, 1 x 10^6 cells/sample were used. Cells were removed from plates by gentle pipetting and were washed once with 0.1% BSA (w/v) in PBS. AF647 MPL was diluted to 10 nM in wash buffer and incubated with cells for 30 min at room temperature. Cells were washed twice and then analyzed by FACS.

[00389] Antibodies produced from the library are screened for one or more desired traits. In one example, to select mutations that do not affect antigen binding, antibodies from the library (e.g., cells expressing the antibodies) that bind to a fluorescently labeled antigen to which the antibodies specifically bind (e.g., HER2, EGFR, IGFR, CLTA-4, etc.) are selected, e.g., by FACS (Figure 20). In one example, antibodies selected for their antigen-binding capabilities are subjected to another round of selection, for example, to select for mutant antibodies that bind to a specific meditope, meditope analog, or other molecule of interest.

[00390] Following selection, the selected antibodies are characterized to determine the desired combination of mutations. In one example, following cell sorting, PCR is used to identify the resulting mutations that facilitate or enhance meditope/analog/small molecule binding. In one example, this process is repeated multiple times to 'evolve/optimize' the desired characteristic, e.g., binding, pH dependency, PK, and/or PD.

EXAMPLE 6: VARIANT MEDITOPES

[00391] Several variant meditopes were generated. For example, certain meditope variants in Tables 3 and 4 (above) were synthesized, with demonstrated binding affinities to cetuximab. For the peptides in Table 3, a disulfide linkage was used to connect the C and N termini (except that meditope 31 contained an additional tail, meaning that the disulfide linkage is not between the two terminal residues). Meditopes 26, 27, 28, and 29 were biosynthesized and thus in this example, contained an additional serine before the first cysteine, i.e., position zero. Further, in some embodiments, meditope 31 may optionally include a GGSK linker. For the peptides in Table 4, a lactam bridge, a linkage other than disulfide (such as [3+2] cycloaddition), or no linkage was used as the connector. For example, meditope 55 is a linear peptide that binds within the meditope-binding site. Individual meditopes in these tables are discussed below.

[00392] As described above, the cyclic peptide cQFD (SEQ ID NO: 1) and cQYN (SEQ ID NO: 2) were identified and co-crystallized with cetuximab Fab, and shown to bind in a cavity created by the light and heavy chains of the Fab. Biophysical and biochemical methods were used to characterize this interaction. Specifically, mutation of Phe3, Leu5, and Arg8 to alanine reduced the affinity of the meditope for the binding interface by 44–183-fold. See Tables 6 and 7.

Meditope Modification and Chemistry Design

[00393] Based on the structural and thermodynamic data, multiple positions within the provided meditopes were identified as targets for modification, e.g., with substitutions and/or non-natural amino acids, for example, to enhance the overall binding affinity and/or to alter another property. Modifications included generation of head-to-tail cyclic lactam peptides, modification of Arg8, modification of Phe3, modification of Leu5, modification of Leu10, and incorporation of hydratable carbonyl functionality (see Figure 31).

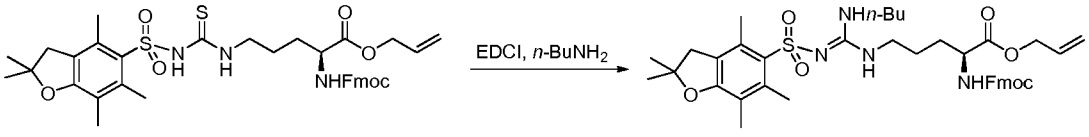
Modification of Arg8.

[00394] Modifications were made to Arg8. Based on structural data, it was determined that Arg8 of the unmodified meditope (cQFD; SEQ ID NO: 1) is extended, making a hydrogen bond with the heavy chain carbonyl of Q105 of the meditope-enabled antibody heavy chain. The immediate area about this residue is hydrophobic, yet solvent-exposed (Figure 33A).

[00395] Structural data indicated that incorporation of a modified Arg8 residue that maintains the guanidinium functionality for meditope-enabled antibody H-bonding, while simultaneously introducing a hydrophobic arm to partially fill the cavity, could produce significant gains in binding, due to entropic increases, as supported by ligand docking calculations.

[00396] A variant meditope was generated containing an n-butyl-arginine at position 8, meditope 54 (SEQ ID NO: 54, shown in Table 4), as follows:

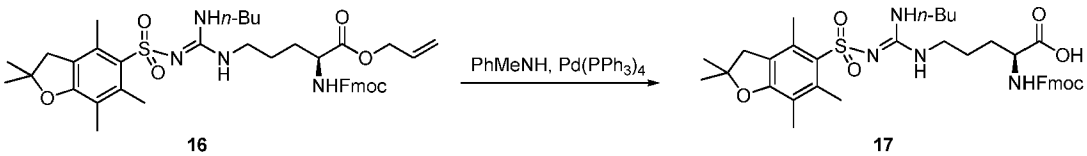
Synthesis of Fmoc-N-butyl Arg derivative 3 (meditope 54)



15 16

[00397] To a stirred solution of **15**, above, (23 mg, 0.03 mmol) in CH_2Cl_2 (0.6 mL) were added EDCI (12 mg, 0.06 mmol, 2 equiv) and n-butylamine (4.4 mg, 0.06 mmol, 2 equiv). After 5 min at room temperature (rt), the solvent was removed in vacuum. The residue was purified by silica gel column chromatography (40-50% EtOAc/Hex) to afford the product **16**, above, (23 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78-7.70 (m, 2H), 7.60-7.54 (m, 2H), 7.42-7.37 (m, 2H), 7.35-7.27 (m, 2H), 5.95-5.83 (m, 1H), 5.52-5.42 (m, 1H), 5.36-5.22 (m, 2H), 4.61(d, J = 5.4 Hz, 2H), 4.44-4.30 (m, 3H), 4.16 (t, J = 6.4 Hz, 1H), 3.30-3.02 (m, 4H), 2.92 (s, 2H), 2.54 (s, 3H), 2.48 (s, 3H), 2.07 (s, 3H), 1.98-1.80 (m, 1H), 1.76-1.50 (m, 3H), 1.45 (s, 6H), 1.36-1.26 (m, 4H), 0.85 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.9, 158.7, 156.4, 155.0, 144.0, 143.8, 141.5, 138.5, 133.8, 132.4, 131.5, 128.0, 127.3, 125.2, 124.6, 120.3, 119.6, 117.6, 86.5, 67.4, 66.5, 53.3, 47.3, 43.5, 41.5, 41.0, 30.9,

30.7, 28.8, 25.4, 20.2, 19.5, 18.2, 13.9, 12.7; HRMS $C_{41}H_{52}N_4O_7S$ $[M+Na]^+$ calc'd 767.3449, found. 767.3455.

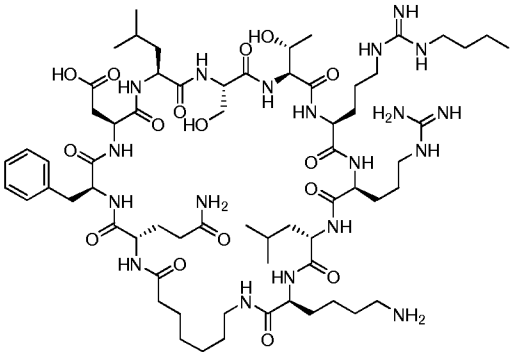


16 17

[00398] To a stirred solution of **16**, above, (23 mg, 0.03 mmol) in THF (0.8 mL) were added N-methylaniline (10 mg, 0.09 mmol, 3 equiv) and $Pd(PPh_3)_4$ (2 mg, 0.0015 mmol, 0.05 equiv). After 45 min at rt, the solvent was removed in vacuum. The residue was purified by silica gel column chromatography ($MeOH:CH_2Cl_2$:HOAc = 25:1:0.1) to afford the product **17**, above, (20 mg, 90%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.78-7.70 (m, 2H), 7.60-7.54 (m, 2H), 7.40-7.34 (m, 2H), 7.30-7.24 (m, 2H), 5.95-5.83 (m, 1H), 4.48-4.10 (m, 5H), 3.30-3.02 (m, 4H), 2.90 (s, 2H), 2.58 (s, 3H), 2.50 (s, 3H), 2.07 (s, 3H), 2.00-1.86 (m, 1H), 1.86-1.74 (m, 1H), 1.72-1.60 (m, 2H), 1.45 (s, 6H), 1.30-1.22 (m, 4H), 0.82 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.9, 158.9, 156.6, 155.2, 144.0, 143.8, 141.5, 138.5, 133.3, 132.4, 129.3, 128.5, 128.0, 127.3, 125.3, 124.8, 120.2, 117.7, 86.6, 67.4, 53.4, 47.4, 43.4, 41.6, 41.1, 31.4, 29.9, 28.8, 25.2, 20.1, 19.5, 18.2, 13.9, 12.7;HRMS $C_{38}H_{48}N_4O_7S$ $[M+Na]^+$ calc'd 727.3136, found. 727.3144.

[00399] See Martin, N. I., and Liskamp, R. M. *J. Org. Chem.* 2008, 73, 7849–7851.

Synthesis of Arg-butyl meditope 54.



[00400] Additionally, meditope 54 ((SEQ ID NO: 54), in the above structure) was prepared as according to solid phase Fmoc synthesis protocol using Fmoc-N-butyl Arg derivative **17**. Figure 59 shows an HPLC trace and mass spectrum of meditope 54.

[00401] The structure of meditope 54 bound to a cetuximab Fab fragment (backside view) is shown in Figure 48, with structures of the 5-position β,β'-diphenylalanine meditope (SEQ ID NO: 18, meditope 18) and cQFD (meditope 1, SEQ ID NO: 1) superimposed. As shown in Table 7, below, meditope 54 was determined by SPR to bind to cetuximab with an average K_D of 745.2 nM.

[00402] In some examples, this meditope-enabled antibody binding pocket identified in these studies is explored as described herein for new contacts (meditopes or analogs) that may increase affinity of a meditope-meditope-enabled antibody interaction.

Modification of Phe3.

[00403] Various modifications were made to Phe3 of cQFD. Structural data demonstrated that the hydroxyl group of the meditope variant Phe3Tyr cQYN (SEQ ID NO: 2) has an alteration in the extended conformation of the Arg8 side chain as compared to cQFD (meditope 1) (see Figures 30C and 35). SPR demonstrated that the overall affinity of this variant for the cetuximab Fab was reduced. ITC measurements indicated a significant decrease in entropy for this Phe3Tyr cQYN variant upon binding that was off-set by a favorable increase in enthalpy compared to unmodified meditope (SEQ ID NO: 1) (from -2.1 kCal/mol to -7.9 kCal/mol [n=3]) (Figure 30D). Structural data suggested the formation of a favorable hydrogen bond network, with water bound to the Fab.

[00404] It was determined that when bound to a meditope-enabled antibody, the hydrophobic phenyl ring of Phe3 was surrounded by a fairly polar array of side chain residues of a meditope-enabled antibody (cetuximab) Fab (Figure 35). It was desired to introduce one or more halogen atom onto the phenyl ring, which could participate in halogen bonding (relatively strong non-covalent bonding, similar to a hydrogen bond but involving the interaction of a halogen such as bromine or chlorine with an oxygen atom), such as by incorporation of an ortho- meta- and/or para-bromo phenyl substituent to favorably place a bromine atom for halogen bonding with Tyr87 (light chain), Gln39, and/or Tyr91 (heavy chain) of a meditope-enabled antibody, respectively. Meditopes 36 (SEQ ID NO: 36), 37 (SEQ ID NO: 37), and 38 (SEQ ID NO: 38) were generated, containing 2-bromo-L-

phenylalanine, 3-bromo-L-phenylalanine, and 4-bromo-L-phenylalanine, respectively, in place of the Phe at position 3. These meditopes were co-crystalized with cetuximab Fab. These commercially available derivatives were incorporated by SPPS. Structures are shown in Figure 39. Diffraction data are shown in Figure 40. Affinities of some of these meditopes for the cetuximab Fab (average K_D values) were determined by SPR and are listed in Table 7, below.

[00405] Additionally, based on the structural information for the meditope containing a Phe3His mutation (meditope 33, SEQ ID NO: 33), a meditope was synthesized with β,β'-diphenylalanine at position 3 (meditope 17, SEQ ID NO: 17). As shown in Figure 41, a significant improvement in the binding affinity for cetuximab was observed by SPR, representing a roughly 4-fold increase compared to cQFD (increase in affinity by a factor of approximately 4 to 5 (~200 nM)).

Modification of Leu5 and Leu10.

[00406] Modifications were made to Leu5 and Leu10. It was determined that the Leu5 and Leu10 side chains make hydrophobic contacts to the meditope-enabled Fab (Figure 36, right panel; Leu10). In one example, natural amino acids (Phe/Tyr/Trp) and/or non-natural analogs (e.g., β,β'-diphenylalanine, branched alkyl, extended aromatics such as napthyl, etc.) are systematically introduced via SPPS at one or both of these positions. The observation, above, that introduction of β,β'-diphenylalanine at position three increased the overall affinity for the cetuximab Fab (see Figures 41-42), demonstrated success by introducing such non-natural amino acids in the meditopes. Accordingly, a β,β'-diphenylalanine was introduced at position 5 and the average affinity of the resulting meditope (meditope 18, SEQ ID NO: 18) determined by SPR to be 687 nM (see Table 7, below). These data confirm that use of structural biology to identify regions for alteration and mutation of residues are useful to alter characteristics of the meditopes, such as to improve their binding kinetics. In another embodiment, the same modification may be made to Leu10.

Alternative cyclization strategies and replacement of disulfide bridge

[00407] Alternative cyclization strategies were used to replace the disulfide bridge in cQFD and other meditopes. As shown in Table 4, various lactam cyclization strategies were used, including those involving natural and non-natural amino acids, generated based on different starting materials, including glycine, β-Ala, 7-aminoheptanoic acid,

diaminopropionic acid, and isoaspartic acid, to produce different lactam ring sizes (see Figure 31, left and middle boxes).

[00408] In one example, a 7-aminoheptanoic acid was used to replace the disulfide bridge of the original cQFD meditope, and the affinity of the resulting meditope for the cetuximab Fab fragment determined by SPR (meditope 42, SEQ ID NO: 42). The SPR data are shown in Figure 41, bottom panel. Although the binding affinity was decreased compared to cQFD, these data indicate that modifications can be made to the meditope to address potential issues with pharmacokinetics, pharmacodynamics and toxicity in animal and human studies. It is noted that alternative linkers can be combined with unnatural amino acids at other positions within the meditope.

[00409] Other variant meditopes with lactam linkages were generated using glycine, 7-aminoheptanoic acid, iso-aspartic acid, β-alanine, and diaminopropionic acid (see meditopes 42, 43, 44, 45, 46, 49, 51, 52, 53, and 54, listed in Table 4 and others). Affinities for some of these meditope variants for cetuximab Fab are listed in Table 7.

[00410] An azide alkyne Huisgen cycloaddition was used to produce a triazole as a linkage in meditope 50, by incorporating propargylglycine at position 12 and beta-azidoalanine at position 1 and carrying out a cyclization between these termini.

[00411] Additional cyclization strategies, such as 'click' chemistry and olefin metathesis, also were used (Figure 31, right boxes). For example, head-to-tail lactam peptides were designed and synthesized by solid phase peptide synthesis (SPPS) starting from Fmoc-Asp (Wang resin LL)-Oall (Figure 31, lower left box & Figure 32). One such variant, meditope 32 (SEQ ID NO: 32), with FITC conjugation, was shown to bind to cetuximab in a similar manner but with slightly reduced affinity compared to the unmodified meditope of SEQ ID NO: 1 (meditope 1). Other head-to-tail lactam variant meditopes produced include meditopes 33-47, 49, and 50-54 (see Tables 4 and 7). Meditope 48 in Table 4 was engineered with a disulfide linkage and a 4:11 lactam linkage.

[00412] Meditope 32 was conjugated with fluorescein for FACS analysis. In another example, this strategy is applied to conjugate the meditope with DOTA for *in vivo* PET imaging.

[00413] Structural data demonstrated that additional positions are amendable to cyclization, such as by cyclization between residues 3 and 11 or 4 and 11.

[00414] Meditope 55, a linear peptide, was produced and demonstrated to bind to the meditope-enabled antibody, cetuximab, albeit with reduced affinity.

Hydratable carbonyl functionality.

[00415] In another example, a meditope with hydratable carbonyl capabilities is developed to create a highly selective but irreversible interaction. Several Fab hydroxyl-bearing side chains in a meditope-enabled antibody that surround the meditope cavity are exploited through selective trapping, by formation of their corresponding hemi-acetal or -ketal, using a hydratable-enabled meditope. For example, Arg8 of the meditope extends in proximity to Ser43 of the light chain (3.5 Å) and Tyr91 of the heavy chain (3.8 and 4.0 Å) of the meditope-enabled antibody light chain, according to Kabat numbering (Figure 36, left panel). In one example, incorporation of a hydratable carbonyl functionality at the end of Arg8 or Leu10 of the meditope allows selective formation of a serine or tyrosine hemi-acetal, which essentially affords irreversible binding. In another example, a residue containing boronic acid is integrated into the meditope as an alternative to a hydratable carbonyl group. Boronic acid plays an important role in the structural activity of bortezamib (Velcade®), which is used to treat multiple myeloma. Representative examples of such hydratable residues are also shown in Figure 36 or 34, where R = $-CH_2CHO$ or $-CH_2B(OH)_2$. In some examples, such analogs are modified using SPPS (Duggan, P. J. and D. A. Offermann (2007). "The Preparation of Solid-Supported Peptide Boronic Acids Derived from 4-Borono-L-phenylalanine and their Affinity for Alizarin," *Australian Journal of Chemistry*, 60(11): 829-834.

Other methods

[00416] In some examples, fluorescence polarization assays are used to identify meditope variants that can displace a given meditope, such as SEQ ID NO: 1. In other examples, the same technique is used to identify small molecules that can displace the meditope and then use these small molecules as templates to further improve the binding affinity of the meditopes.

Characterization of meditopes

[00417] For characterization, variant meditope peptide lyophilized powders were suspended in 500 µL of 10 mM Tris pH 8.0 buffer and dialyzed 3 times into 1 L of H_2O each time. The final volume after dialysis was carefully measured and absorbance measurements were taken to estimate the concentration (typically 1-10 mM). These stock solutions were used to make dilutions into HBS-EP buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.05% v/v surfactant P20) for SPR measurements. SPR measurements were carried out on the GE Biacore T100 instrument using a CM5 chip with cetuximab IgG or cetuximab Fab ligand immobilized using amine coupling chemistry. Ligands were immobilized at low levels suitable for kinetic data. Typical kinetics SPR experiments were carried out at a flow rate of 30 µL/min using HBS-EP as both running and regeneration buffer. Kinetic parameters were calculated using the Biacore T100 evaluation software version 2.0.1.

[00418] Isothermal titration calorimetry experiments were performed in 100 mM Hepes, pH 7.4 at 25°C using Nano ITC calorimeter (TA Instruments). In a typical experiment, 250 µl of protein (Fab or IgG) at 0.03-0.06 mM were loaded into the calorimeter cell (163 µl or 185µl) and the titrant (meditope, at 0.3-0.8 mM) was loaded into a 50 µl syringe. The cell solution was stirred at 250 rpm and upon equilibration the titrant was added in 2-2.5 µl increments. Heat of the reaction was measured and the data was processed using NanoAnalyze software (TA Instruments). Background heat was subtracted by averaging the last four measurements or by subtracting heat of reaction obtained from titration of the meditope at the same concentration into buffer containing no protein.

[00419] A number of the meditope variants were co-crystallized with cetuximab Fab fragment. Meditopes were purified to >95% homogeneity and structurally characterized by mass spectrometry. Peptides were dialyzed in water. Their concentrations were measured, for example, in some cases, by UV-Vis and calibrated with elemental analysis, and diluted (>100x) into the appropriate buffer. The structures of some of these meditope variants, corresponding to SEQ ID NOs: 15-18, 22-25 and 31-40, are shown in Figure 39.

[00420] Interactions of various meditopes with the meditope-enabled antibody cetuximab were characterized by X-Ray diffraction. Since the co-crystallization conditions of the cetuximab Fab fragment and the meditope of SEQ ID NO: 1 are well-established,

diffraction quality crystals were typically obtained in 1 to 3 days, typically 1 day. Full data sets were collected in 8 to 12 hours with an in-house source (Rigaku 007-HF and an R-Axis IV++) and in less than 10 min at the Stanford Synchrotron Radiation Lightsource, which allows for rapid characterization of the interactions of the meditope variants with cetuximab.

[00421] Diffraction data were collected for various different meditope-Fab (cetuximab) complexes, including complexes containing the cQFD (SEQ ID NO: 1) and cQYN (SEQ ID NO: 2) meditopes and modified meditopes. X-ray diffraction data for several meditopes are shown in Figure 40. Most of these co-crystal structures were shown to diffract beyond 2.4 Å and be well refined, with R and R_{Free} less than 20 and 24%, respectively. All of these meditope variants have very good stereochemical values (Molprobity scores above 89th percentile).

[00422] For SPR measurements, low density and high density chips were conjugated with the cetuximab Fab or full IgG. Each chip was first characterized using a soluble fragment of the entire extracellular domain of EGFR (residues 1–621). Similar kinetics and binding affinities were observed as previously reported. Using the low density chips, on and off rates were measured for the unmodified meditope and determined to be $k_{on} = 9.2x10^{-4}$ $M^{-1}s^{-1}$ and $k_{off} = 9.9x10^{-3}$ s^{-1}, respectively. With meditopes of SEQ ID NO: 1 and 2, consistent with ITC, similar values for the Fab-conjugated chip and the IgG-conjugated chip were observed, demonstrating that either can be used for binding assessments.

[00423] Table 7, below, lists information on the average dissociation constants (K_Ds), which were determined by SPR, for several of the meditopes listed in Tables 3 and 4.

Table 7: Dissociation Constants Determined by SPR

SEQ ID NO / Meditope No.	Average K_D (nM)
1	898.8
2	3500
15	18300
16	2828
17	624.9
18	687
21	11180
22	54820
23	9460
24	14180
25	39340
26	34000
27	57000

28	140000
29	2200
31	102.5
32	5041
33	No binding observed
34	No binding observed
35	No binding observed
36	1791
37	29130
39	8186
40	No binding observed
41	No binding observed
42	1520
43	1619
44	16490
45	4634
46	5467
48	517.1**
49	23800
50	21002
51	433.3
52	1264
53	No binding observed
54	745.2
55	8684

[00424] ** In this study, meditope 48 hydrolyzed to the original meditope.

[00425] Binding of several meditopes to the meditope-enabled antibody cetuximab was rigorously characterized by ITC, SPR, X-ray diffraction and combinations thereof. ITC measurements were performed on a TA Instruments nanoITC, with as little as 1–2 mg of peptide per measurement. ITC, SPR and X-ray diffraction data, e.g., collectively, provide atomic detail to guide subsequent chemical modifications and ultimately improve the affinity of the meditopes and/or make other alterations to the meditopes. A calculation based on $\Delta G = -RT \ln Ka$ shows that the difference between micromolar and nanomolar affinity of a meditope for cetuximab results from a change in free energy at 300 K of ~4 kCal/mol, which is on the order of a strong hydrogen bond. Thus, the loss of an ordered water molecule from a protein binding pocket or the reorientation of an amino acid residue-chain may be sufficient to alter binding by orders of magnitude.

[00426] The data in this example demonstrate that a large number of meditope permutations may be systematically and efficiently introduced, indicating that a large number of meditope variant permutations may be generated, for example, to produce altered meditope-meditope enabled antibody binding affinity, pharmacokinetics (PK), pharmacodynamics (PD), toxicity, and/or ability to bind or strength of binding under differing conditions, such as pH, e.g., pH dependence, for example, to produce high affinity meditopes.

[00427] In one example, after characterization by ITC, SPR and diffraction methods, the meditope with the highest affinity (or other desired property, e.g., pH dependence) is subsequently modified, e.g., to further improve the desired property (e.g., overall affinity or pH dependence).

EXAMPLE 7: GENERATION OF MULTIVALENT MEDITOPES

[00428] Bivalent and other multivalent meditopes were generated, e.g., for use in enhancing selectivity and/or binding affinity by "cross-linking" meditope-enabled antibodies on the surface of cells expressing antigen.

Bivalent meditope-Fc

[00429] The use of the Fc region to 'dimerize' ligands is established and described, for example, by Jazayeri JA & Carroll GJ., "Fc-based cytokines: prospects for engineering superior therapeutics," *BioDrugs*, 22(1):11-26 (2008) To generate a bivalent meditope, the meditope cQFD (SEQ ID NO: 1) was fused to the N-terminus of the Fc region of an IgG through a flexible peptide linker of 17 amino acids in length, comprised of glycine and serines. The length of the linker was chosen to roughly match the distance between the Fabs of an IgG. The nucleic acid and amino acid sequences of the resulting "meditope-Fc" are shown in Figure 15 (SEQ ID NO: 3 and 4, respectively). The structure of the meditope-Fc is shown in Figure 16.

[00430] To demonstrate enhanced binding to antigen afforded by the multivalency of the meditope-Fc, 0.5 x 10^6 MDA-MB-468 cells were labeled with 10 nM cetuximab for 30 min at room temperature, washed, then incubated with 0.1, 0.3, 1 and 3 μM of bivalent meditope-Fc or monomeric meditope for 30 min at room temperature, washed, and then analyzed by FACS. As shown in Figure 17, FACS analysis demonstrated that the meditope-

Fc, corrected for the stoichiometry, bound to cells pre-treated with cetuximab with higher affinity compared to the meditope monomer. The interaction was demonstrated to be specific to the meditope-enabled mAb (cetuximab). These data demonstrate synergy using meditope-Fc, combined with a meditope enabled mAb, and thus that a bivalent meditope can be substituted for a second antibody to produce synergistic effects.

[00431] In another study, the meditope-Fc fusion protein was labeled with Alexa488 as described above. MDA-MB-468 cells were labeled with cetuximab or M425 (a murine anti-EGFR antibody) for 30 minutes. Unbound antibody was washed and the cells were incubated with the meditope-Fc (600 nM, 180 nM, or 60 nM) for 30 minutes. Antibody binding and meditope binding were analyzed by FACS analysis. The FACS data, shown in Figure 49 demonstrate that the meditope-Fc bound to the cells incubated with cetuximab, but not to cells incubated with M425 or those not incubated with any antibody, with increasing signal at higher concentrations of meditope-Fc.

[00432] Similar to the FACS experiments described above, MDA-MB-468 cells were treated with Alexa 555-labeled cetuximab, washed, incubated with Alexa 488-labeled meditope-Fc, and washed. The cells were then imaged at 20Xby microscopy (Figure 61A). Strong surface-associated staining of Cetuximab was observed. The meditope-FC also localized to the cell surface and appeared to co-localize with cetuximab (Figure 61A). Concomitant with this staining, we also observe the co-localization of the labeled meditope-Fc was observed (Figure 61A, white arrows). This co-localization, however, was absent in cells which that were not pre-treated with cetuximab (Figure 61B).

[00433] Taken together, these experiments show that the meditope binds to EGFR-expressing cells that are pre-treated with cetuximab, that using a multivalent scaffold affords creates a higher apparent affinity for cetuximab pre-treated cells, and that the apparent binding affinity, in this study, was more sensitive.

[00434] The ability of a bivalent meditope-Fc to induce cell death in conjunction with the meditope-enabled antibody, cetuximab, was confirmed using an MTT assay. 4000 MDA-MB-468 cells were placed in each well of a 96 well plate in 80 µl of medium. 10 µl of 1 µM cetuximab was added along with 10 µl of 0.1, 1 or 10 µM of monovalent meditope (cQFD – (control) or bivalent meditope-Fc (two cQFDs) to a final concentration of 0.1 µM cetuximab and 0.01, 0.1 and 1µM meditope or meditope-Fc. Each component was also added alone with

PBS as control. After a 48-hour incubation, 10 µl of MTT reagent was added and allowed to incubate for another 4 hours. The culture supernatant was then removed, 100 µl of MTT crystal dissolving reagent was added, and the plate was read at 630 nm. Neither addition of monovalent meditope (alone or with cetuximab), or meditope-Fc alone altered cell growth significantly. Addition of meditope-Fc together with cetuximab, however, inhibited cell growth, as shown in Figure 28A.

[00435] The ability of the multivalent meditope-Fc to enhance cell killing by cetuximab to a degree comparable to a second anti-EGFR antibody was demonstrated by MTT assay. The assay compared the enhancement of cetuximab-mediated inhibition of antigen-expressing tumor cell growth by meditope-Fc and by M425 (a mouse anti-EGFR antibody). 4000 MDA-MB-468 cells were placed in each well of a 96 well plate in 80 µl of medium. 10 µl of 1µM cetuximab was added, along with 10 µl of either 2, 4 or 8 µM of meditope-Fc or M425, to a final concentration of 0.1 µM cetuximab and either 0.2, 0.4 or 0.8 µM meditope-Fc or M425. cetuximab added with PBS alone was used as control. After a 48-hour incubation period, 10µl MTT reagent was added and the mixture allowed to incubate for an additional 4 hours. The culture supernatant was removed, 100 µl of MTT crystal dissolving reagent added, and the plate read at 630 nm. As shown in Figure 28B, meditope-Fc and M425 enhanced the cell-killing capacity of cetuximab to a similar degree.

[00436] In some examples, the composition of and the distance between the Fc and meditope are systematically explored to optimize affinity and specificity. In one example, each natural or unnatural residue is substituted at any position within the linker, for optimization. In another example, the linker is 'rigidified' to limit the radius of gyration and to enhance the affinity and specificity of the Fc-meditope. In one example, a coiled coil domain is placed between the meditope and the Fc (Figure 18). In another example, inert protein domains (e.g., immunoglobulin folds) are substituted for the linker. In one example, multiple immunoglobulin folds are placed between the meditope and the Fc domain. In certain examples, the composition of the linker is of human origin, e.g., to mitigate potential antigenicity.

Multivalent scaffolds

[00437] To address the receptor constraints on the linker, meditopes were coupled to multivalent scaffolds.

[00438] Multivalent meditopes were designed to "latch-on" to adjacent IgGs to form a "daisy-chain"-like array (see Figure 8). Synthesis of a FITC-labeled bivalent meditope was developed using "Click" chemistry, using compound **2** (SEQ ID NO: 32). Templates **4** and **5** of Figure 13, were used to form bi- and trivalent meditopes, respectively. A 30 Å PEG bifunctional arm was incorporated in the synthesis of a FITC-labeled bivalent meditope containing meditopes of SEQ ID NOs: 32 (meditopes 32), namely compound **13**, shown in Figure 13. As also shown in Figure 13, a trivalent meditope (compound **14**) also was successfully synthesized. Figure 14 illustrates the characterization of this fluorescein isothiocyanate (FITC)-labeled meditope compound **13**.

[00439] In other examples, differing lengths of polyethylene glycol (PEG) (and other) linkers are used, for example, to optimize binding. In other examples, this synthetic approach is used to incorporate DOTA for radionuclide imaging. The distance between the CDR regions within an IgG is ~130 Å. End-to-end distances of commercially available PEGs extend to 90 Å (Pierce), which would exceed the IgG distance. In one example, the length of the PEG linker is systematically varied, bearing in mind this constraint.

[00440] In some examples, trivalent or higher valency scaffolds are used, with the goal of having more than one antibody "daisy chained". In some example, different scaffolds and linkers are used to generate high affinity multivalent meditopes. In one example, DNA is used as a more rigid scaffold.

[00441] Different scaffolds of biological and chemical origin also are used to achieve multivalency. This includes, but is not limited to, constructing a bivalent or trivalent scaffold, using streptavidin or collagen (http://ip.com/patapp/EP2065402A1), strepavidin as a tetravalent scaffold, unique scaffolds (http://www.sciencedirect.com/science/article/pii/S0022283611000283), Origami DNA (http://www.nature.com/nnano/journal/v4/n4/abs/nnano.2009.5.html) and the like. A chemical scaffold may also be created using molecules including, but not limited to, DNA (single strand, duplex, Holliday junctions, aptamers and the like), RNA (single strand, hairpin, stem loop, aptamers and the like), PNA (peptide nucleic acids), DNA/PNA duplexes

and triplexes for rigidity, inorganic or organic nanoparticles (directly coupled or coupled through organic polymers such as PEG), organic polymers that can form duplexes with themselves and/or with DNA or PNA.

Multivalent Meditope Characterization

[00442] In one example, the multivalent meditopes are characterized by SPR and ITC, to verify that conjugation to the multivalent scaffold does not affect the meditope-IgG interaction.

[00443] In other examples, FACS analysis, cell viability assays, and other assays are used. For example, cell viability assays are used as described above for meditope-Fc to quantify the effect of the multivalent meditope directly on cells that overexpress the antigen recognized by the meditope-enabled antibody of choice, such as EGFR when the meditope-enabled antibody is cetuximab. For example, MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is used to quantify the number or percentage of viable cells. In some examples, a shift at far lower concentrations than observed for the corresponding monovalent meditope and/or a relative increase in percentage of cells that shift, is observed.

[00444] In some examples, for multivalent meditopes that demonstrate activity in such assays, Western blot analysis is performed to follow other parameters, such as phosphorylation status of EGFR, AKT, and MAPK in the case of antibodies targeting the EGFR signaling pathway, such as cetuximab. In one example, the data are compared with data from antibody (e.g., cetuximab)-only treated cells and cells treated with inhibitors (e.g., tyrosine kinase inhibitors (AG1478)). An increase in cell death as a function of multivalent meditope concentration is observed.

[00445] In one example, to further confirm the additive effects of the multivalent meditope, the non-labeled, monovalent meditope is used to compete with the labeled multivalent meditope for the antigen-bound cetuximab.

EXAMPLE 8: MEDITOPE BINDING AFFINITY AS A FUNCTION OF pH

[00446] The composition of the meditope was altered to affect the binding affinity as a function of pH. As shown in Figure 27, the binding affinity of three different meditope variants was measured as a function of buffer pH. The results demonstrated that cQYD (SEQ ID NO: 16, meditope 16) meditope variant had a marked decrease in affinity for the meditope-enabled antibody cetuximab at lower pH. Substitution of the aspartate to

asparagine in the cQYN variant produced a flat pH dependence. The affinity of the cQFD meditope (SEQ ID NO: 1) was determined to be slightly greater at higher pHs. Collectively, these data demonstrate that the affinity of the meditope-memAb interaction can be tailored to pH, which is used, for example, to generate meditope variants for the specific release at low pH, such as in lysozymes for drug delivery, and/or to bind with higher affinity in a hypoxic environment, e.g., tumor stroma.

EXAMPLE 9: MEDITOPE ANALOGS, FRAGMENTS, AND OTHER COMPOUNDS

[00447] Screening methods were carried out to identify meditope analogs and compounds, such as small molecules, fragments, aptamers, nucleic acid molecules, peptibodies and/or other substances that bind meditope-enabled antibodies near meditope-binding sites and in some aspects can be linked to meditopes, for example, to improve their affinities for the meditope-binding sites.

[00448] *Fluorescence polarization assays*: To identify compounds, including alternative molecules that could bind at the meditope binding site of meditope-enabled antibodies and thus be used for similar functions, a displacement assay was established. A cyclic meditope was synthesized and chemically attached to a fluorescein, generating a fluorescein-labeled meditope of the following sequence: AcCQFDLSTRRLRCGGGSK (SEQ ID NO: 31, cysteines form a disulfide linkage)-Fluorescein. This fluorescently-labeled peptide was then titrated with cetuximab and the fluorescence polarization measured. The interaction between the labeled meditope and mAb cause a change in the fluorescence polarization/intensity of the fluorescent tag. The dissociation constant, 1 μM, closely matched values obtained from surface plasmon resonance and isothermal titration calorimetry. A non-labeled meditope, AcCQFDLSTRRLRCGGGSK (SEQ ID NO: 31), was used to displace the fluorescein-label peptide pre-bound to cetuximab. The fluorescence polarization was monitored. Compounds that blocked the meditope-antibody interaction altered the fluorescent polarization properties. As shown in Figure 37, a sigmoid curve indicative of a competition reaction was observed. Accordingly, this method is useful for identifying meditope analogs.

[00449] Based on these data, an initial screen to identify small molecules capable of displacing the meditope was carried out. In this study, 42 lead compounds at concentrations

of 50 μM were identified from a library of 30,000 small molecule compounds. Figure 38 shows five such lead compounds.

[00450] In another example, these compounds are characterized further, for example, by crystallography.

[00451] *Diffraction methods.* Cetuximab Fab was shown to diffract beyond 2.5 Å, as shown above. Established diffraction-based methods (well-established for identifying lead compounds (Shuker et al. 1996; Erlanson et al. 2001; Hughes et al. 2011)) were used to identify candidate compounds, including compounds that can be coupled to a meditope, for example, to improve affinity for the meditope binding site. A library of small molecules was developed to soak into crystals of cetuximab. Diffraction data from these soaks was collected and several data sets analyzed. In these initial studies, two additional sites were identified on cetuximab that are amendable for fragment growth and optimization.

[00452] In another example, such fragments (small molecules that can serve as building blocks for larger entities), such as chemical groups, e.g., imidazole or other chemical group, are grown (chemically derivatized) to enhance their binding and specificity and/or are chemically tethered to the meditope. Optimization of this chemical coupling can significantly enhance the overall binding affinity.

[00453] *NMR screening:* NMR was used to identify fragments for optimization of meditopes, e.g., by linkage to the meditope, and/or use in lieu of the meditope (i.e., as meditope analogs). To identify these leads, one dimensional (1D) spectra of pools containing 15 to 20 fragments were collected. Cetuximab was added to each pool and second 1D spectra were collected. Compounds that bound (transiently) with cetuximab underwent rapid magnetization transfer, resulting in a loss of intensity. The spectra were compared before and after addition of cetuximab and altered peaks identified, indicating interactions.

[00454] In one example, these peaks are pre-assigned to a specific compound, and thus immediately known. Alternatively, the pools are subdivided and the spectra recollected. After several rounds, the exact identity of the compound is known. In these experiments, the precise position of the interaction is not known. The binding site is determined by NMR or the fluorescence polarization assay. Alternatively, the Fab fragment is labeled with NMR active and inactive nuclei (e.g., ^{13}C, ^{15}N and ^{2}H), followed by multiple NMR experiments performed to assign the spectrum, and use of the fragment library to identify the binding

position. Using this procedure, a set of initial lead compounds has been identified (Figure 19, bottom).

[00455] *Virtual ligand screening:* Virtual ligand screening was used to identify lead compounds to function as meditopes. Using crystal structure, standard programs (e.g., Schroerdinger Glide) were used to define a "box' about a site of the macromolecule (the meditope binding site); known ligands were docked to this site. Potential lead compounds were scored by a select energy function. In this study, approximately 100 lead compounds were identified.

[00456] In another example, additional analogs found by diffraction methods are optimized and used in lieu of the meditope for drug delivery, multivalent scaffolding and other functions.

[00457] In another example, mutations in the light and heavy chains are made to change the specificity of the ligand (meditope) and all the above-described methods (including fluorescence polarization, NMR screening, phage display, and diffraction methods are used to optimized alternative ligands.

EXAMPLE 10: MEDITOPE-PROTEIN L FUSION

[00458] Characterization by diffraction of the cQFD meditope-enabled Fab fragment bound to a meditope demonstrated that the N- and C-termini of the meditope were juxtaposed to the location of bound Protein L, a bacterial protein that binds to human IgGs. See Figure 52, showing the crystal structure of meditope 18 (5- β,β'-diphenyl), Protein L (left), Protein A (right) and Fab (grey cartoon) and meditope-enabled trastuzumab Fab.

[00459] To generate a meditope that binds to meditope-enabled antibodies with greater affinity via energy additivity, a meditope-Protein L fusion polypeptide was produced. Based on structural data information, four glycines were introduced to link the C-terminus of the cQFD meditope and the N-terminus of Protein L. The coding sequence of a meditope-Protein L fusion protein is set forth in SEQ ID NO: 56.

[00460] The amino acid sequence of an encoded protein, including a His6-Smt3 tag (plain text), meditope (underlined), and Protein L (bold) is set forth below and in SEQ ID NO: 57:

HHHHHHSSGLVPRGSHMASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKI
KKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQI
GGSCQFDLSTRRLKCGGGGSEVTIKVNLIFADGKIQTAEFKGTFEEATAEAYRYA
ALLAKVNGEYTADLEDGGNHMNIKFAG (SEQ ID NO: 57)

[00461] The amino acid sequence of the meditope-Protein L fusion protein, after cleavage of the tag, is set forth below (meditope underlined; two cysteins that are cyclized (e.g., by peroxide or overnight with air) set forth in bold text) SEQ ID NO: 58:

SCQFDLSTRRLKCGGGGSEVTIKVNLIFADGKIQTAEFKGTFEEATAEAYRYAALLA
KVNGEYTADLEDGGNHMNIKFAG (SEQ ID NO: 58).

[00462] Binding constants for the interaction of this fusion protein (and separately, the interaction of each individual component) with meditope-enabled antibodytrastuzumab were measured by surface plasmon resonance (SPR). The binding constant for the interaction between Protein L and the meditope-enabled trastuzumab was approximately 0.5 μM. For the interaction between the meditope and the meditope-enabled trastuzumab Fab fragment, it was approximately 1 μM. The binding constant for the interaction between the meditope-Protein L fusion protein and the IgG, however, was 165 pM. Figure 53 shows surface plasmon resonance (SPR) data for a meditope-Protein L fusion (MPL): The top panel shows the traces of MPL being added to the meditope-enabled trastuzumab at concentrations up to 10 nM. The fit data indicates a binding affinity of 165 pM. The bottom panel shows the traces of Protein L (only) added at the same concentrations to the meditope-enabled trastuzumab, showing that there was no binding at this concentration in this study. This result demonstrated far stronger binding between the fusion protein and meditope-enabled antibody compared to the individual interactions, indicating improved affinity via additivity.

[00463] Point mutations were created in Protein L and the meditope to ensure specificity. As expected, in both cases, the binding constant was reduced.

[00464] To facilitate conjugation of various molecules, such as therapeutic and diagnostic agents, e.g., cytotoxins, radionucleotides, for example, DOTA, proteins, solid supports, and other molecules, e.g., for drug delivery, imaging, or purification, all lysines in the Protein L-meditope fusion protein but one were mutated to arginine or another residue. The crystal structure shown in Figure 52 was used to identify all lysines on Protein L and the meditope that, if conjugated, would sterically occlude the meditope-Protein L / meditope-

enabled antibody interaction. Based on this information, the meditope-Protein L fusion (MPL) having the sequence set forth in SEQ ID NO: 59 (SCQFDLSTRRLRCGGGGSEVTI**R**VNLIFADG**N**IQTAEF**R**GTFEEATAEAYRYAALLA **R**VNGEYTADLEDGGNHMNI<u>K</u>FAG) was produced, in which certain lysines in Protein L/meditope (shown in black in Figure 52 – all but one lysine) were mutated to Arg or Asn. In the sequence shown above, the residues mutated from lysine to arginine/asparagine are shown in bold text. The lysine that was not mutated (underlined above) is pointed into the solvent. Thus, in the resulting meditope-Protein L fusion protein, the N-terminal amine and epsilon amine were left for conjugation, the latter being solvent exposed and likely more reactive.

[00465] In another example, this unique lysine residue in this mutant is used to PEGylate the MPL, e.g., to address potential antigenicity.

<u>MPL-DOTA-NHS conjugates</u>

[00466] MPL having the sequence set forth in SEQ ID NO: 59 was conjugated to DOTA-NHS in a reaction carried out with various ratios (0.5:1, 2:1, 10:1, 30:1, 60:1, 120:1, 240:1) of starting material (DOTA-NHS:MPL) in phosphate buffer (80mM) for 10 minutes at a pH=10, which favors conjugation to lysine as compared to N-terminal conjugation. The mono-DOTA-conjugate product increases with increasing DOTA-NHS. A significant amount of MPL-mono-DOTA-MPL was formed at a 60:1 ratio, and a significant amount of both MPL-mono-DOTA and MPL-di-DOTA formed at a 120:1 ratio (Figure 60).

[00467] The foregoing examples and methods of the invention are illustrative only and are not intended to be limiting of the invention in any way. Those of ordinary skill in the art will recognize that various modifications of the foregoing are within the intended scope of the invention.

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All references below and cited in the specification above are hereby incorporated by reference in their entirety, as if fully set forth herein.

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CLAIMS

1. An isolated meditope-enabled antibody or fragment thereof, comprising: a heavy chain variable (VH) region, a heavy chain constant region (CH) or portion thereof, a light chain variable (VL) region comprising a threonine, serine, or aspartate at position 40, a residue other than glycine at position 41, a residue other than phenylalanine at position 83, and an aspartate or asparagine at position 85, according to Kabat numbering, and a light chain constant region (CL) or portion thereof, wherein

the meditope-enabled antibody is a meditope-enabled variant of a human or humanized template antibody, which variant contains a plurality of framework region (FR) modifications, according to Kabat numbering, compared to the template antibody, said FR modifications being only at positions selected from the group consisting of positions 8, 9, 10, 38, 39, 40, 41, 42, 43, 44, 45, 82, 83, 84, 85, 86, 87, 99, 100, 101, 102, 103, 104, and 105 of the light chain of said template antibody, according to Kabat numbering, and positions 6, 9, 38, 39, 40, 41, 42, 43, 44, 45, 84, 86, 87, 88, 89, 90, 91, 103, 104, 105, 106, 107, 108, 109, and 110 of the heavy chain of said template antibody, according to Kabat numbering; and

the meditope-enabled antibody or fragment binds to a meditope comprising a peptide of SEQ ID NO: 1, via a meditope binding site.

2. The meditope-enabled antibody or fragment of claim 1, wherein the VH region comprises a serine or proline at position 40 and an isoleucine at position 89, according to Kabat numbering.

3. The meditope-enabled antibody or fragment of claim 1, wherein the VL region further comprises an isoleucine or leucine at position 10, according to Kabat numbering, and the residue at position 83 is an isoleucine.

4. The meditope-enabled antibody or fragment of claim 1, wherein the VL region further comprises a valine or isoleucine at position 9 and a residue other than glutamine at position 100, according to Kabat numbering.

5. The meditope-enabled antibody or fragment of claim 1, wherein the VL region has a threonine at position 40, an asparagine at position 41, and an aspartate at position 85, according to Kabat numbering.

6. The meditope-enabled antibody or fragment of claim 1, wherein the VH region has a serine at position 40 and an isoleucine at position 89, according to Kabat numbering.

7. The meditope-enabled antibody or fragment of claim 1, wherein the peptide is a cyclic peptide.

8. The meditope-enabled antibody or fragment of claim 1, wherein the meditope binds to the antibody or fragment with a dissociation constant of less than 10 μM, as measured by surface plasmon resonance (SPR).

9. The meditope-enabled antibody or fragment of claim 1, wherein the meditope binds to residues 40, 41, 83, and 85 of the VL region of the meditope-enabled antibody or fragment, according to Kabat numbering, and residues 39, 89, 105, and 108 of the VH region of the meditope-enabled antibody or fragment, according to Kabat numbering.

10. The meditope-enabled antibody or fragment of claim 1, comprising a light chain comprising the amino acid sequence set forth in SEQ ID NO:9 and a heavy chain comprising the amino acid sequence set forth in SEQ ID NO:6.

11. The meditope-enabled antibody or fragment of claim 1, wherein the FR modifications are at positions selected from the group consisting of positions 9, 10, 39, 40, 41, 42, 43, 83, and 85 of the light chain of said template antibody, according to Kabat numbering, and positions 40, 89, and 105 of the heavy chain of said template antibody, according to Kabat numbering.

12. An isolated meditope-enabled antibody or fragment thereof, comprising: a heavy chain variable (VH) region, a heavy chain constant region (CH) or portion thereof, a light chain

variable (VL) region comprising a threonine or serine at position 40, an asparagine at position 41, a residue other than phenylalanine at position 83, and an aspartate or asparagine at position 85, and a light chain constant region (CL) or portion thereof, wherein:

the meditope-enabled antibody is a meditope-enabled variant of a human or humanized template antibody, which variant contains a plurality of framework region (FR) modifications, according to Kabat numbering, compared to the template antibody, said FR modifications being only at positions selected from the group consisting of positions 8, 9, 10, 38, 39, 40, 41, 42, 43, 44, 45, 82, 83, 84, 85, 86, 87, 99, 100, 101, 102, 103, 104, and 105 of the light chain of said template antibody, according to Kabat numbering, and positions 6, 9, 38, 39, 40, 41, 42, 43, 44, 45, 84, 86, 87, 88, 89, 90, 91, 103, 104, 105, 106, 107, 108, 109, and 110 of the heavy chain of said template antibody, according to Kabat numbering; and

the antibody or fragment binds to a meditope comprising a peptide of SEQ ID NO: 1 via a meditope binding site and does not specifically bind to the epitope of EGFR that is specifically bound by cetuximab.

13. The isolated meditope-enabled antibody or fragment of claim 1, wherein the VL region comprises the amino acid sequence of SEQ ID NO: 73.

14. The isolated meditope-enabled antibody or fragment of claim 1, wherein the VH region comprises the amino acid sequence of SEQ ID NO: 74.

15. The isolated meditope-enabled antibody or fragment of claim 1, wherein the VL region comprises the amino acid sequence of SEQ ID NO: 75.

16. The isolated meditope-enabled antibody or fragment of claim 1, wherein:

The VL region has a light chain framework (FR-L)1, an FR-L2, an FR-L3, and an FR-L4 of the light chain sequence set forth as SEQ ID NO: 9; and

the VH region has a heavy chain framework (FR-H)1, an FR-H2, an FR-H3, and an FR-H4 of the heavy chain sequence set forth as SEQ ID NO: 6.

17. An isolated meditope-enabled antibody or fragment thereof, comprising: a heavy chain variable (VH) region, a heavy chain constant region (CH) or portion thereof, a light chain variable (VL) region comprising a valine or isoleucine at position 9, a threonine, serine, or aspartate at position 40, a residue other than glycine at position 41, a residue other than phenylalanine at position 83, and an aspartate or asparagine at position 85, according to Kabat numbering, and a light chain constant region (CL) or portion thereof, wherein:

the meditope-enabled antibody is a meditope-enabled variant of a template antibody, wherein the template antibody is a human or humanized antibody and the meditope-enabled antibody contains a plurality of framework region (FR) modifications, according to Kabat numbering, compared to the template antibody, said FR modifications being only at positions selected from the group consisting of positions 8, 9, 10, 38, 39, 40, 41, 42, 43, 44, 45, 82, 83, 84, 85, 86, 87, 99, 100, 101, 102, 103, 104, and 105 of the light chain of said template antibody, according to Kabat numbering, and positions 6, 9, 38, 39, 40, 41, 42, 43, 44, 45, 84, 86, 87, 88, 89, 90, 91, 103, 104, 105, 106, 107, 108, 109, and 110 of the heavy chain of said template antibody, according to Kabat numbering; and

the meditope-enabled antibody or fragment binds to a meditope comprising a peptide of SEQ ID NO: 1, via a meditope binding site and does not specifically bind to the epitope of EGFR that is specifically bound by cetuximab.

18. The meditope-enabled antibody or fragment thereof of claim 1, wherein:

the meditope-enabled antibody or fragment competes for antigen binding with, binds to the same epitope as an antibody or antigen-binding fragment thereof selected from the group consisting of abagovomab, abciximab, adalimumab, adecatumumab, alemtuzumab, altumomab, altumomab pentetate, anatumomab, anatumomab mafenatox, arcitumomab, atlizumab, basiliximab, bectumomab, ectumomab, belimumab, benralizumab, bevacizumab, brentuximab, canakinumab, capromab, capromab pendetide, catumaxomab, certolizumab, clivatuzumab tetraxetan, daclizumab, denosumab, eculizumab, edrecolomab, efalizumab, etaracizumab, ertumaxomab, fanolesomab, ~~Fbta05,~~ fontolizumab, gemtuzumab, girentuximab, golimumab, ibritumomab, igovomab, infliximab, ipilimumab, labetuzumab, mepolizumab, muromonab, muromonab-CD3, natalizumab, necitumumab nimotuzumab, ofatumumab, omalizumab, oregovomab, palivizumab, panitumumab, ranibizumab, rituximab, satumomab, sulesomab, ibritumomab, ibritumomab tiuxetan, tocilizumab, tositumomab, trastuzumab, Trbs07, ustekinumab, visilizumab, votumumab, zalutumumab, brodalumab, anrukinzumab, bapineuzumab, dalotuzumab, demcizumab, ganitumab, inotuzumab,

mavrilimumab, moxetumomab pasudotox, rilotumumab, sifalimumab, tanezumab, tralokinumab, tremelimumab, the antibody produced by the hybridoma 10B5, and urelumab; or

the meditope-enabled antibody or fragment specifically binds to an antigen selected from the group consisting of: CA-125, glycoprotein (GP) IIb/IIIa receptor, TNF-alpha, CD52, TAG-72, Carcinoembryonic antigen (CEA), interleukin-6 receptor (IL-6R), IL-2, interleukin-2 receptor a-chain (CD25), CD22, B-cell activating factor, interleukin-5 receptor (CD125), VEGF, VEGF-A, CD30, IL-1beta, prostate specific membrane antigen (PSMA), CD3, EpCAM, EGF receptor (EGFR), MUC1, human interleukin-2 receptor, Tac, RANK ligand, C5 or other complement proteins, CD11a, alpha-v beta-3 integrin, HER2, neu, CD15, CD20, Interferon gamma, CD33, CA-IX, CTLA-4, IL-5, CD3 epsilon, CAM, Alpha-4-integrin, IgE, IgE Fc region, an RSV antigen, F (or fusion) protein of respiratory syncytial virus (RSV), NCA-90 (granulocyte cell antigen), IL-6, GD2, GD3, IL-12, IL-23, IL-17, CTAA16.88, beta-amyloid, IGF-1 receptor (IGF-1R), delta-like ligand 4 (DLL4), alpha subunit of granulocyte macrophage colony stimulating factor receptor, hepatocyte growth factor, IFN-alpha, nerve growth factor, IL-13, CD326, PD-L1, CD47, and CD137.

19. An isolated meditope-enabled antibody or fragment thereof, comprising:-a heavy chain variable (VH) region, a heavy chain constant region (CH) or portion thereof, a light chain variable (VL) region comprising an isoleucine or leucine at position 10, a threonine, serine, or aspartate at position 40, a residue other than glycine at position 41, an isoleucine at position 83, and an aspartate or asparagine at position 85, according to Kabat numbering, and a light chain constant region (CL) or portion thereof, wherein:

the meditope-enabled antibody is a meditope-enabled variant of a template antibody, wherein the template is a human or humanized antibody and the meditope-enabled antibody contains a plurality of framework region (FR) modifications, according to Kabat numbering, compared to the template antibody, said FR modifications being only at positions selected from the group consisting of positions 8, 9, 10, 38, 39, 40, 41, 42, 43, 44, 45, 82, 83, 84, 85, 86, 87, 99, 100, 101, 102, 103, 104, and 105 of the light chain of said template antibody, according to Kabat numbering, and positions 6, 9, 38, 39, 40, 41, 42, 43, 44, 45, 84, 86, 87, 88, 89, 90, 91, 103, 104, 105, 106, 107, 108, 109, and 110 of the heavy chain of said template antibody, according to Kabat numbering; and

the meditope-enabled antibody or fragment binds to a peptide meditope comprising SEQ ID NO: 1 via a meditope binding site and does not specifically bind to the epitope of EGFR that is specifically bound by cetuximab.

20. The isolated meditope-enabled antibody of claim 1, wherein the VL region comprises an isoleucine or leucine at position 10, a threonine at position 40, an asparagine at position 41, an isoleucine at position 83, and an aspartate at position 85, according to Kabat numbering; and the VH region comprises an isoleucine at position 89, according to Kabat numbering.

21. The isolated meditope-enabled antibody of claim 1, wherein the VL region comprises an isoleucine or valine at position 9, an isoleucine or leucine at position 10, an arginine at position 39, a threonine at position 40, an asparagine at position 41, a glycine at position 42, a serine at position 43, an isoleucine at position 83, an aspartate at position 85, and an alanine at position 100, according to Kabat numbering; and the VH region comprises a serine at position 40 and an isoleucine at position 89, according to Kabat numbering.

22. The meditope-enabled antibody or fragment thereof of claim 1, wherein the VH region comprises an isoleucine, tyrosine, methionine, phenylalanine, or tryptophan at position 89, according to Kabat numbering.