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(54) Title: SMALL MOLECULE SURROGATE FOR A NEUTRALIZATION EPITOPE ON THE ZIKA VIRUS ENVELOPE PROTEIN

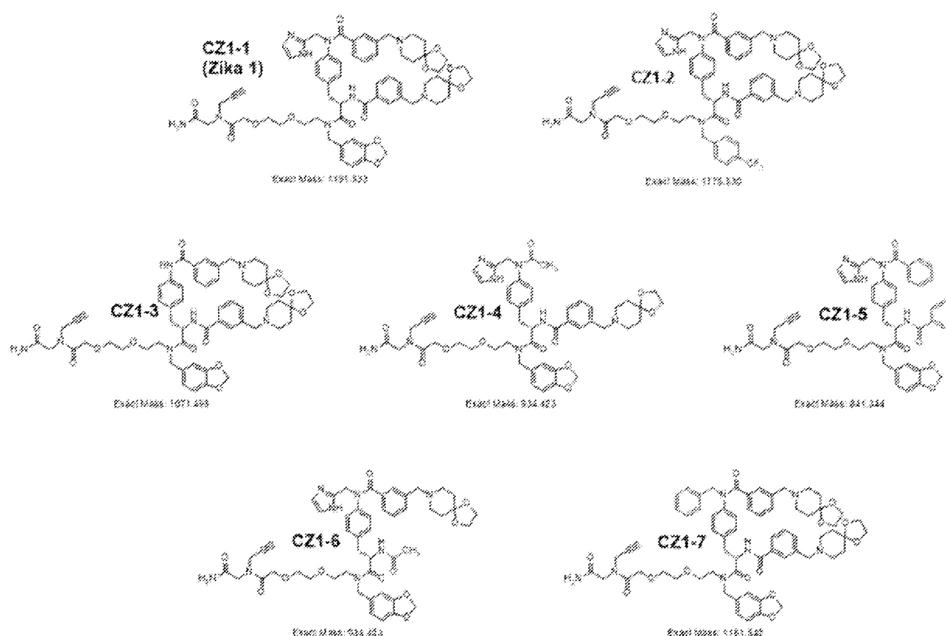

FIG. 1

(57) Abstract: Provided herein are compositions including a binding reagent for a Zika virus (ZIKV) epitope, pharmaceutical compositions including such binding reagents, and methods of detecting ZIKV binding antibodies and ZIKV neutralizing antibodies in patients.

WO 2025/137264 A1

[Continued on next page]

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SMALL MOLECULE SURROGATE FOR A NEUTRALIZATION EPITOPE ON THE ZIKA VIRUS ENVELOPE PROTEIN

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Patent Application No. 63/612,746, filed December 20, 2023, the disclosure of which is hereby incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under Grant No. AI127677 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

<u>Field of the Invention</u>

[0003] Provided herein are small molecules capable of mimicking viral epitopes, and methods of using the same for detecting antibodies to a virus and for eliciting an immune response to a virus.

<u>Description of Related Art</u>

[0004] Zika virus (ZIKV) is a global health threat that causes adverse pregnancy and fetal outcomes, as well as Guillain-Barré syndrome in healthy adults. Reduced, but nonetheless sustained, the transmission of ZIKV has been documented in recent years, particularly in regions that are highly endemic for other mosquito-borne flaviviruses such as dengue viruses (DENV1-4). Antigenic similarities between ZIKV and DENV lead to the development of both virus-specific and cross-reactive antibodies following infection with either of these viruses. Immune interactions can modulate transmission and risk for subsequent enhanced disease by ZIKV and DENV, which poses challenges to the development of virus-specific diagnostic tools and effective vaccines.

[0005] Recently, there has been considerable interest in the identification of unique antigenic determinants for highly specific detection and differentiation of flavivirus infections as well as for defining immune correlates of protection against ZIKV. However, antigenic similarities between ZIKV and other flaviviruses pose challenges to the development of virus-specific diagnostic tools and effective vaccines. Accordingly, there is a need in the art

for effective surrogates that can mimic viral epitopes and aid in accurate detection, diagnosis, and ultimately treatment of flaviviruses.

SUMMARY OF THE INVENTION

[0006] Provided herein are compositions including a binding reagent for a Zika virus (ZIKV) epitope, pharmaceutical compositions including such binding reagents, and methods of detecting ZIKV binding antibodies and ZIKV neutralizing antibodies in patients.

[0007] Provided herein is a non-peptide binding reagent for a Zika virus (ZIKV) antibody paratope.

[0008] Also provided herein is a method of detecting an antibody to ZIKV in serum obtained from a patient, including incubating a binding reagent as described herein with a sample obtained from the patient and subjecting the binding reagent and serum to a binding assay.

[0009] Also provided herein is a method of eliciting an immune response to ZIKV in a patient, including administering a binding reagent as described herein to the patient in one or more doses such that an immune response to ZIKV is raised in the patient.

[0010] Also provided herein is a method of identifying an antibody fragment specific to ZIKV, including screening a display library, such as a phage display library, with a binding reagent as described herein and identifying one or more antibody fragment clones from the display library that specifically bind to ZIKV.

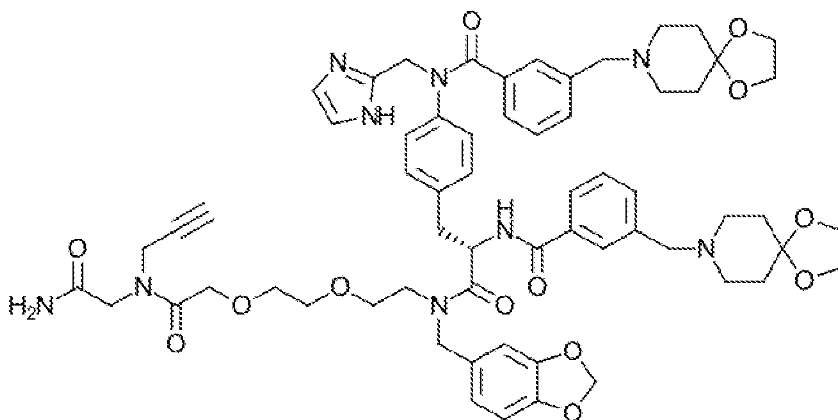
[0011] Also provided herein is a method of isolating an antibody to ZIKV in serum obtained from a patient, including linking a binding reagent as described herein to a surface, incubating the binding reagent with a solution containing antibodies, and washing the surface-linked binding agent to dissociate bound antibodies from the binding reagent.

[0012] Further non-limiting embodiments are set forth in the following numbered clauses:

[0013] 1. A non-peptide binding reagent for a Zika virus (ZIKV) antibody paratope.

[0014] 2. The binding reagent of clause 1, wherein the binding reagent binds a paratope of an antibody to a protein in the ZIKV envelope DIII C-C’ loop region.

[0015] 3. The binding reagent of clause 1 or clause 2, having the following structure:



,

or a pharmaceutically-acceptable salt thereof, including stereoisomers and enantiomeric mixtures thereof.

[0016] 4. The binding reagent of any of clauses 1-3, linked to a hapten carrier to produce an immunogen for use in eliciting antibodies directed to the binding reagent.

[0017] 5. The binding reagent of any of clauses 1-4, wherein the hapten carrier comprises: gelatin, serum globulin, albumin, keyhole limpet hemocyanin (KLH), ovalbumin, casein, hemocyanin, thyroglobulin, fibrinogen, cholera toxin, purified protein derivative (PPD), diphtheria toxin or a derivative thereof such as CRM197, tetanus toxin or a derivative thereof, Haemophilus protein D (PD), outer membrane protein complex of serogroup B meningococcus (OMPC), recombinant non-toxic form of Pseudomonas aeruginosa exotoxin A (rEPA), Shigella O-antigen, Staphylococcus aureus type 5 and 8 capsular polysaccharide, S. Typhi Vi antigen, liposomes, polysaccharides (e.g., AECM-Ficoll, dextran, agar, carboxymethyl cellulose), synthetic polypeptides (e.g., poly-L-lysine and poly-L-glutamic acid), inorganic gold particles, dendrimers, and nanodiscs, outer membrane vesicles, generalized modules for membrane antigens (GMMA), glycoengineered proteins, virus-like particles, and/or protein nanocages.

[0018] 6. The binding reagent of any of clauses 1-5, wherein the carrier comprises KLH.

[0019] 7. The binding reagent of any of clauses 1-6, linked to a surface.

[0020] 8. The binding reagent of any of clauses 1-7, linked to a surface for use in an immunoassay.

[0021] 9. The binding reagent of any of clauses 1-8, linked to a nitrocellulose membrane, a cotton linter membrane, a glass fiber membrane, a silicon chip, a magnetic bead, a gold bead, a microelectrode, a microfluidic device, a flow cytometry bead, and/or a multi-well plate.

[0022] 10. The binding reagent of any of clauses 1-9, linked to a bead or particle having a diameter of from about 10 nm to about 200 µm.

[0023] 11. The binding reagent of any of clauses 1-10, wherein the bead or particle comprises a polystyrene bead, a magnetic bead, or a gold particle.

[0024] 12. A composition comprising the binding reagent of any of clauses 1-11 and a pharmaceutically acceptable adjuvant or excipient.

[0025] 13. The composition of clause 12, contained within a vial that optionally comprises a pierceable septum, such as a pierceable cap.

[0026] 14. An immunoassay device comprising the binding reagent of any of clauses 1-11.

[0027] 15. The immunoassay device of clause 14, in the form of a lateral flow device.

[0028] 16. A method of detecting an antibody to ZIKV in serum obtained from a patient, comprising: incubating the binding reagent of any of clauses 1-11 with a sample obtained from the patient; and subjecting the binding reagent and serum to a binding assay.

[0029] 17. The method of clause 16, wherein the binding reagent further comprises a tag.

[0030] 18. The method of clause 16 or clause 17, wherein the tag is a fluorescent tag.

[0031] 19. The method of any of clauses 16-18, wherein the binding assay is one or more of an enzyme-linked immunosorbent assay (ELISA), a fluorescence activated cell sorting assay (FACS), an antibody-binding assay, a competitive binding assay, and/or immunoprecipitation.

[0032] 20. A kit comprising the binding reagent of any of clauses 1-11 and one or more reagents for a binding assay.

[0033] 21. A method of eliciting an immune response to ZIKV in a patient, comprising administering the binding reagent of any of clauses 1-11 to the patient in one or more doses such that an immune response to ZIKV is raised in the patient.

[0034] 22. A method of identifying an antibody fragment specific to ZIKV, comprising screening a display library, such as a phage display library, with the binding reagent of any of clauses 1-11 and identifying one or more antibody fragment clones from the display library that specifically bind to ZIKV.

[0035] 23. A method of isolating an antibody to ZIKV in serum obtained from a patient, comprising: linking the binding agent of any of clauses 1-11 to a surface; incubating the binding reagent with a solution containing antibodies; and washing the surface-linked binding agent to dissociate bound antibodies from the binding reagent.

[0036] 24. A composition comprising antibodies isolated by clause 23, and a pharmaceutically acceptable excipient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] Figure 1 shows exemplary structures of binding reagents according to non-limiting embodiments described herein.

[0038] Figure 2 provides an elevated (*top*) and a top (*bottom*) schematic view of a typical lateral flow device.

[0039] Figure 3 shows a schematic representation of the synthesis of the OBOC DEL. Split and pool solid-phase synthesis was employed at each step. 10μm TentaGel beads (grey oval) were first acylated with one of two chloroacids (red). The chloride was then displaced by one of six primary amines. The resultant secondary amine was acylated with one of six azide-containing, Fmoc-protected amino acids (blue). After reduction of the azide with TCEP, the resultant amine was reductively alkylated with one of 85 aldehydes and NaCNBH3. The Fmoc group was removed, and the two amines were then acylated with one of 7 haloacids (red). Finally, the halide was displaced by a secondary amine to provide the final DEL. In the box, the DNA tag (present on ≈ 0.4% of the available sites) and the linker are shown.

[0040] Figure 4 shows chemical structures of the building blocks used at the R1, R2, R3, R5 and R6 positions (related to Figure 1).

[0041] Figure 5 shows chemical structures of the aldehydes used to install the R4 unit (related to Figure 1).

[0042] Figure 6 shows a flowchart illustrating the down selection of Zika-specific reactive PICCOs identified during the screening procedure.

[0043] Figure 7 shows a distribution of PICCOs in terms of the number of sample hits.

[0044] Figure 8 shows a number of distinct PICCO compounds detected once, twice, or multiple times across Zika discovery runs.

[0045] Figure 9 shows a heatmap showing the chemical sequence similarity clustering of the screening candidate PICCOs. Five Zika-specific clusters encompassing 40 unique PICCO candidates were identified.

[0046] Figure 10 shows a comparison of antibody specificity profiles of Zika-specific PICCOs from within the same chemical sequence cluster versus distinct clusters.

[0047] Figure 11 shows the antibody binding profile against CZV1-1 and a “des-aldehyde” side product CZV1-1a.

[0048] Figure 12 shows a binding profile of Zika-specific antibodies to CZV1-1 derivatives.

[0049] Figure 13 shows validation of CZV1-1 as a Zika diagnostic marker. The receiver operating characteristics (ROC) curve for all samples and for the DENV-immune patients is shown. The calculated area under the curve (AUC) and 95% confidence intervals (95% CI) are depicted.

[0050] Figures 14A-14B show a binding profile of the purified IgG specific to CZV1-1 against (Panel A) ZIKV envelope protein (full-length and domain III) and (Panel B) DENV1-4 envelope proteins. (Panel C) Blockage of binding experiments of CZV1-1 interaction with ZIKV envelope by competition with known mouse monoclonal ZIKV antibodies. (Panel D) Reverse blocking experiments to assess the competition of the mAbs for binding of IgG specific to CZV1-1 to the ZIKV envelope. (Panel E) Neutralization of Zika viruses (French Polynesia and Brazilian strains) but not DENV-3 by the purified IgG specific to CZV1-1.

[0051] Figure 15 shows binding of purified IgG against CZV1-1 against ZIKV and DENV structural and nonstructural proteins. The dotted line along y-axis represents blank average for the binding assay. ZIKV FL: Full-length envelope protein; ZIKV DIII: domain III of ZIKV envelope protein.

[0052] Figure 16 shows binding of purified IgG against CZV1-1 and several Zika-specific (ZV2, ZV48, ZV64, ZV67) or dengue-specific (DV10 and 2H2) monoclonal antibodies against DENV (Panel A) and ZIKV (Panel B) envelope proteins.

[0053] Figure 17 shows the binding free energy minima for the mAb/CZV1-1 complexes. The black arrow indicates the compound that obtained the most negative binding free energy, and thus the strongest association.

[0054] Figures 18A-C show that anti-Zika human sera reacts with BSA-CZV1-1 and CZV1-1-Cys in an ELISA format.

[0055] Figure 19 shows that mice immunized with CZV1-1-KLH and CZV1-1-Cys induce very high levels of antibodies anti-CZV1-1.

[0056] Figures 20A-C show that CZV1-1 immunization elicited very high titers of anti-CZV1-1 antibodies.

[0057] Figure 21 shows that mice immunized with CZV1-1-KLH induce anti-Zika envelope protein.

DESCRIPTION OF THE INVENTION

[0058] The use of numerical values in the various ranges specified in this application, unless expressly indicated otherwise, are stated as approximations as though the minimum and maximum values within the stated ranges are both preceded by the word "about". In this manner, slight variations above and below the stated ranges can be used to achieve substantially the same results as values within the ranges. Also, unless indicated otherwise, the disclosure of ranges is intended as a continuous range including every value between the minimum and maximum values. As used herein, "a" and "an" refer to one or more.

[0059] As used herein, the term "comprising" is open-ended and may be synonymous with 'including', 'containing', or 'characterized by'. The term "consisting essentially of" limits the scope of a claim to the specified materials or steps, and those that do not materially affect basic and novel characteristic(s). The term "consisting of" excludes any element, step, or ingredient not specified in the claim. As used herein, embodiments "comprising" one or more stated elements or steps also include but are not limited to embodiments "consisting essentially of" and "consisting of" these stated elements or steps.

[0060] As used herein, the term "patient" or "subject" refers to members of the animal kingdom including but not limited to human beings, and "mammal" refers to all mammals, including, but not limited to human beings.

[0061] A "binding reagent" is a reagent, compound, or composition, e.g., a ligand, able to specifically bind a target compound, such as, for example and without limitation: anti-Zika virus (ZIKV) antibodies, such as anti-Zika virus (ZIKV) antibody paratope-containing molecules, for example to the exclusion of substantial binding to other antibodies, such as anti-dengue virus antibodies. Binding reagents include, without limitation, antibodies (polyclonal, monoclonal, humanized, etc.), antibody fragments (e.g., a recombinant scFv), antibody mimetics such as affibodies, affilins, affimers, affitins, alphabodies, anticalins, avimers, DARPins, fynomers, monobodies, nucleic acid ligands (e.g., aptamers), engineered proteins, antigens, epitopes, epitope surrogates, haptens, or any target-specific binding reagent. In aspects, binding reagents includes as a class: monoclonal antibodies, or derivatives or analogs thereof, including without limitation: Fv fragments, single chain Fv (scFv) fragments, Fab' fragments, F(ab')2 fragments, single domain antibodies, camelized antibodies and antibody fragments, humanized antibodies and antibody fragments, multivalent versions of the foregoing, and any paratope-containing compound or composition; multivalent activators including without limitation: monospecific or bispecific antibodies, such as disulfide stabilized Fv fragments, scFv tandems ((scFv)2 fragments),

diabodies, tribodies or tetrabodies, which typically are covalently linked or otherwise stabilized (e.g., leucine zipper or helix stabilized) scFv fragments; nucleic acids and analogs thereof that bind a target compound; or receptor molecules which naturally interact with a desired target molecule.

[0062] As used herein, a “non-peptide” molecule is a molecule that does not comprise a polypeptide moiety of two or more contiguous amino acids linked in a chain by conventional peptide bonds. For example, CZV1-1 is a non-peptide molecule. Note that herein, “CZV” and “CZ” are used interchangeably (e.g., CVZ1-1 is the same as CZ1-1). A non-protein molecule may be linked to a peptide-containing molecule, such as, without limitation, a protein or polypeptide chain or glycoprotein, such as in a hapten-carrier complex with a non-peptide hapten moiety, or in the case of a peptide-containing surface to which the non-peptide molecule is linked. Methods are known, such as multiplexed methods, and are described herein for testing large numbers of molecules, such as molecules of a chemical library, for their ability to act as an epitope surrogate to an antigen, such as for example as a zikavirus antibody or a zikavirus neutralizing antibody surrogate epitope that optionally does not bind an anti-denguevirus antibody. Such methods often employ multiplexed screening, or adsorption of candidate molecules by binding to an antibody molecule, and optionally excluding significant binding to another antibody, such as binding to an anti-zikavirus antibody but not to an anti-denguevirus antibody.

[0063] As used herein, the term “epitope surrogate” refers to an artificial molecule or composition that replicates naturally occurring epitopes. An epitope surrogate may bind anti-virus antibodies, for example, anti-zika virus antibodies, and in examples, does not bind anti-dengue virus antibodies to any significant extent. An epitope surrogate may be a peptide molecule or composition that that specifically bind antibodies, e.g., antibodies relevant to a specific disease or condition. An epitope surrogate may be a non-peptide molecule or composition that bind antibodies relevant to a specific disease or condition. An epitope surrogate may be used as an immunogen or hapten for use in generating antibodies or an antibody response to a specific target antigen, such as, for example and without limitation, to a flavivirus or a zikavirus antigen as described herein.

[0064] As used herein, the term “hapten” refers to any molecule that does not generate an immunogenic reaction on its own but can be made to if it is conjugated to a suitable carrier. Carriers for haptens may be immunogenic on their own, or may produce no immune response without conjugation to a hapten. Carriers for haptens (also called hapten carriers or hapten-carriers) may include, but are not limited to, gelatin, serum globulins, albumin, keyhole

limpet hemocyanin (KLH), ovalbumin, casein, hemocyanin, thyroglobulin, fibrinogen, cholera toxin, purified protein derivative (PPD), diphtheria toxin or a derivative thereof, tetanus toxin or a derivative thereof such as CRM197 (a non-toxic mutant of diphtheria toxin having a single amino acid substitution of glutamic acid for glycine, e.g., PeliCRM197®, Primrose Bio), Haemophilus protein D (PD), outer membrane protein complex of serogroup B meningococcus (OMPC), recombinant non-toxic form of Pseudomonas aeruginosa exotoxin A (rEPA), Shigella O-antigens, Staphylococcus aureus type 5 and 8 capsular polysaccharide (PS), S. Typhi Vi antigen, liposomes, polysaccharides (e.g., AECM-Ficoll, dextran, agar, carboxymethyl cellulose), synthetic polypeptides (e.g., poly-L-lysine and poly-L-glutamic acid), inorganic gold particles, dendrimers, and nanodiscs, outer membrane vesicles, generalized modules for membrane antigens (GMMA), glycoengineered proteins, virus-like particles, protein nanocages, and peptides (see, e.g., Lemus R, Karol MH. Conjugation of haptens. Methods Mol Med. 2008;138:167-82 and Van der Put RMF, Metz B, Pieters RJ. Carriers and Antigens: New Developments in Glycoconjugate Vaccines. Vaccines (Basel). 2023 Jan 19;11(2):219). Linking of the ethynyl amino acetamide moiety may be accomplished readily by any useful synthesis method reacting a reactive group in the carrier with, for example, either the terminal acetamide group or the terminal ethynyl group. Suitable linkers and linking chemistries may be used to link the carrier to the hapten as are broadly-known in the organic synthesis field.

[0065] An immune response is a response of a cell of the immune system, such as a B-cell, T-cell, macrophage or polymorphonucleocyte, to a stimulus, such as an antigen. An immune response may include any cell of the body involved in a host defense response for example, an epithelial cell that secretes an interferon or a cytokine. An immunogen refers to a compound, composition, or substance which is capable, under appropriate conditions, of eliciting, stimulating, or raising an immune response, such as the production of antibodies, such as neutralizing antibodies, or a T-cell response in a patient, including compositions that are injected or absorbed into a patient.

[0066] As used herein, “to immunize” and/or “immunizing” refers to rendering a subject protected from an infectious disease, such as by vaccination. A vaccine refers to a preparation of immunogenic material capable of stimulating or eliciting an immune response, administered for the prevention, inhibition, amelioration, or treatment of infectious, such as ZIKV infections, or other types of disease. The immunogenic material may include antigenic proteins, peptides, non-peptides, or DNA derived from them. Vaccines may elicit both prophylactic (preventative or protective) and therapeutic responses. Methods of

administration vary according to the vaccine, but may include inoculation by topical, intramuscular, intravenous, subcutaneous, intradermal, subdermal, ingestion, inhalation, intraocular, intraperitoneal, and/or other routes of administration as known to those of skill in the art. Vaccines may be administered with an adjuvant to boost the immune response raised in the patient.

[0067] A “moiety” refers to a portion of a larger molecule and may comprise a group that has a chemical or biological function, such as a reactive group such as an ethynyl group or an acetamide group, or a group that results in the binding of a surrogate epitope to an antibody, paratope, or binding partner.

[0068] As used herein, the term “antibody fragment” refers to any derivative of an antibody which is less than full-length. In exemplary embodiments, the antibody fragment retains at least a significant portion of the full-length antibody's specific binding ability. Examples of antibody fragments, but are not limited to, Fab, Fab′, F(ab′)2, Fv, Fd, dsFv, scFv, diabody, triabody, tetrabody, di-scFv (dimeric single-chain variable fragment), bi-specific T-cell engager (BiTE), single-domain antibody (sdAb), or antibody binding domain fragments. The antibody fragment may be produced by any means. For instance, the antibody fragment may be enzymatically or chemically produced by fragmentation of an intact antibody, or it may be recombinantly or synthetically produced. The antibody fragment may optionally be a single chain antibody fragment. Alternatively, the fragment may comprise multiple chains which are linked together, for instance, by disulfide linkages. The fragment may also optionally be a multi-molecular complex. A functional antibody fragment may consist of at least about 50 amino acids or at least about 200 amino acids. Antibody fragments also include miniaturized antibodies or other engineered binding reagents, such as scFvs, that exploit the modular nature of antibody structure, comprising, often as a single chain, one or more antigen-binding or epitope-binding (e.g., paratope) sequences and, at a minimum, any other amino acid sequences needed to ensure appropriate specificity, delivery, and stability of the composition.

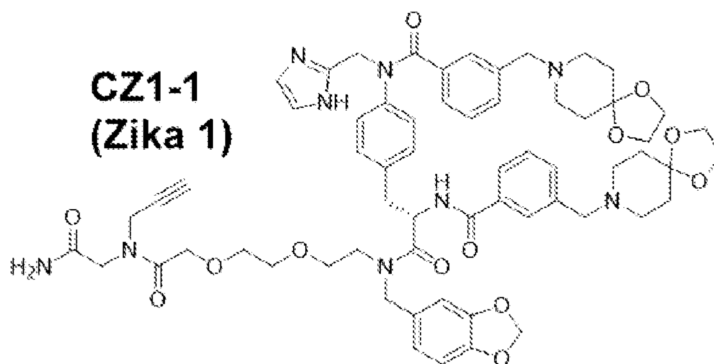
[0069] For methods disclosed herein, according to common practice, a binding reagent may be conjugated (covalently linked) or otherwise attached to a surface such as beads, e.g., magnetic beads or agarose beads (e.g., SEPHAROSE beads), or a membrane, e.g., a nitrocellulose membrane. In non-limiting embodiments, the binding reagent may be conjugated to a semi-conductor polymer such as polyaniline, poly-(3-amino-4-hydroxybenzoic acid)-modified pencil carbon graphite, and/or Electrocatalytic Prussian Blue Nanostructured Film. A binding reagent may also be attached to another mobile compound,

including but not limited to a carrier, e.g., a carrier protein. Attachment may be accomplished, among other methods, by adding a terminal cysteine to the binding reagent and conjugating it to the surface by forming a thioether bond. Following use, the antibodies bound to the surface-conjugated binding reagent can be reconstituted by dissociating the target antibodies from the binding reagent by washing according to common practice. The binding reagent may be an epitope surrogate, or any other suitable binding reagent. The surface may be a magnetic bead, and the bound target may then be removed by magnetic removal. The surface may be particles or beads retained in a column. The surface may be a membrane that is part of, for example and without limitation, a lateral flow device. In non-limiting embodiments, methods of using binding reagents as described herein may include neutralization and/or purification, and a person of ordinary skill can readily envision a large variety of ways neutralization/affinity purification may be used to remove and/or neutralize a target.

[0070] In non-limiting embodiments, a bead or particle to which the binding reagents described herein may be linked may be selected from a polystyrene bead, a magnetic bead, and/or a gold particle. Such beads and particles are known, and may be of any suitable size. In non-limiting embodiments, a bead and/or particle useful with the binding reagents described herein may have a diameter ranging from 10 nanometers (nm) to 200 micrometers (μm), all values and subranges therebetween inclusive.

[0071] Provided herein are compositions including a binding reagent or any pharmaceutically-acceptable salt thereof for a Zika virus (ZIKV) antibody paratope, pharmaceutical compositions including such binding reagents or any pharmaceutically-acceptable salt thereof, and methods of detecting ZIKV antibodies in a sample obtained from a patient, eliciting an immune response to ZIKV, identifying antibodies to ZIKV, and neutralizing ZIKV in patients. As used herein, the term "paratope" means a portion of an antibody that recognizes and binds to an epitope of an antigen, for example, an epitope of ZIKV. In non-limiting embodiments, the epitope of ZIKV is an envelope protein. In non-limiting embodiments, the envelope protein is in the Domain III (DIII) of the ZIKV envelope protein, more specifically a cryptic epitope in the C-C' loop arranged within the 'sandwich' core of DIII. Suitable compositions may have a structure according to one or more of the following depicted in **FIG. 1**.

[0072] In non-limiting embodiments, a binding reagent as described herein has the following structure:



[0073] Certain compounds described here may have asymmetric centers and therefore exist in different enantiomeric and diastereomeric forms. A compound can be in the form of an optical isomer or a diastereomer. Accordingly, compounds described herein include their optical isomers, diastereoisomers and mixtures thereof, including a racemic mixture unless otherwise specified. Optical isomers of the compounds of the invention can be obtained by known techniques such as asymmetric synthesis, chiral chromatography, simulated moving bed technology or via chemical separation of stereoisomers through the employment of optically active resolving agents.

[0074] Unless otherwise indicated, "stereoisomer" means one stereoisomer of a compound that is substantially free of other stereoisomers of that compound. Thus, a stereomerically pure compound having one chiral center will be substantially free of the opposite enantiomer of the compound. A stereomerically pure compound having two chiral centers will be substantially free of other diastereomers of the compound. A typical stereomerically pure compound comprises greater than about 80% by weight of one stereoisomer of the compound and less than about 20% by weight of other stereoisomers of the compound, for example greater than about 90% by weight of one stereoisomer of the compound and less than about 10% by weight of the other stereoisomers of the compound, or greater than about 95% by weight of one stereoisomer of the compound and less than about 5% by weight of the other stereoisomers of the compound, or greater than about 97% by weight of one stereoisomer of the compound and less than about 3% by weight of the other stereoisomers of the compound.

[0075] As used herein, unless indicated otherwise, for instance in a structure, all compounds and/or structures described herein comprise all possible stereoisomers, individually or mixtures thereof. The compound and/or structure may be an enantiopure preparation consisting essentially of an (-) or (+) enantiomer of the compound, or may be a mixture of enantiomers in either equal (racemic) or unequal proportions.

[0076] Binding reagents such as the compounds disclosed herein may include a fluorescent tag or other colorimetric indicator tag, such as a non-fluorescent dye or particle or enzyme, as are broadly-known, to allow for detection in binding assays and the like. Target antibodies isolated using binding reagents such as the compounds disclosed herein may include a fluorescent tag or other colorimetric indicator tag, such as a non-fluorescent dye or particle or enzyme, as are broadly-known, to allow for detection in binding assays and the like. Examples of suitable tags include, without limitation: fluorescent proteins (e.g. green fluorescent protein, GFP), cyanine based fluorescent dyes (e.g. cyanine-3, Cy3), triarylmethane dyes such as fluorescein based fluorescent dyes (e.g. fluorescein isothiocyanate, FITC) and rhodamine based fluorescent dyes (e.g. tetramethylrhodamine isothiocyanate, TRITC), coumarin based fluorescent dyes, phthalocyanine, colloidal gold, latex beads, quantum dots, magnetic beads, horseradish peroxidase, alkaline phosphatase, β-galactosidase, acetylcholinesterase, catalase, and glucose oxidase.

[0077] Binding reagents or any pharmaceutically-acceptable salt thereof, such as the compounds disclosed herein, may also be formulated as pharmaceutical compositions for administration to a patient, for example to elicit an immune response and/or to neutralize ZIKV and/or to treat one or more conditions associated with infection with ZIKV, including at least Guillain-Barré syndrome, neuropathy, and/or myelitis.

[0078] Binding assays are assays that utilize at least one binding reagent to determine the presence or concentration of analyte(s) and/or the interactions between two molecules. Immunoassays are highly selective bioanalytical methods that measure the presence or concentration of analytes in a solution using an antibody or an antigen as a biorecognition agent. They are based on the antibody-antigen immunoreaction and can achieve high specificity and sensitivity through signal transduction and amplification. Immunoassays may include, but are not limited, to enzyme-linked immunosorbent assays (ELISAs), lateral flow tests, magnetic immunoassays, western blots, dot blots, lateral flow tests, fluorescence activated cell sorting assays (FACS), antibody-binding assays, competitive binding assays, and immunoprecipitation. The definitions of binding assays and immunoassays may overlap, and some assays may be considered both binding assay and immunoassays.

[0079] ELISAs are a plate-based assay technique designed for detecting and quantifying soluble substances such as peptides, proteins, antibodies, and hormones. Other names, such as enzyme immunoassay (EIA), are also used to describe the same technology. In an ELISA, the antigen (target macromolecule) is immobilized on a solid surface (microplate) and then complexed with an antibody that is linked to a reporter enzyme. Detection is accomplished by

measuring the activity of the reporter enzyme via incubation with the appropriate substrate to produce a measurable product.

[0080] Immunoassay devices are devices designed to implement highly selective bioanalytical methods that measure the presence or concentration of analytes in a solution using an antibody or an antigen as a biorecognition agent, including, but not limited to, lateral flow devices. Lateral flow devices are devices in which binding reagents are immobilized on a strip of nitrocellulose or other suitable membrane, and a sample is applied at one end of the strip and any antibodies present diffuse along it until they reach the position where the appropriate binding reagent is immobilized. Antibodies specific for the binding reagent bind to and remain at the site of the binding reagent and are visualized using labeled detection antibody. A lateral flow device may have a positive control binding reagent placed on the strip so that it is encountered after the test binding reagent to confirm that the sample has diffused a sufficient distance to interact with the test binding reagent.

[0081] Therapeutic compositions, including those containing the binding reagents or any pharmaceutically-acceptable salt thereof disclosed herein, may comprise a pharmaceutically acceptable carrier, or excipient. An excipient is an inactive substance used as a carrier for the active ingredients of a medication. Although "inactive," excipients may facilitate and aid in increasing the delivery or bioavailability of an active ingredient in a drug product. Non-limiting examples of useful excipients include: adjuvants, antiadherents, binders, rheology modifiers, carriers, coatings, disintegrants, emulsifiers, oils, buffers, salts, acids, bases, fillers, diluents, solvents, flavors, colorants, glidants, lubricants, preservatives, antioxidants, sorbents, vitamins, sweeteners, etc., as are available in the pharmaceutical/compounding arts.

[0082] The compositions, methods, and dosage forms disclosed herein may include one or more adjuvants or molecules with immune stimulant or adjuvant effect. In other examples, an adjuvant is not included in the composition, but is separately administered to a subject (for example, in combination with a composition disclosed herein) before, after, or substantially simultaneously with administration of one or more of the immunogen-containing compositions disclosed herein. Adjuvants are agents that increase or enhance an immune response in a subject administered an antigen, compared to administration of the antigen in the absence of an adjuvant. One example of an adjuvant is an aluminum salt, such as aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate, or aluminum hydroxyphosphate. Other adjuvants include biological adjuvants, such as cytokines (for example, IL-2, IL-6, IL-12, RANTES, GM-CSF, TNF-α, or IFN-γ), growth factors (for example, GM-CSF or G-CSF), one or more molecules such as OX-40L or 4-1 BBL,

immunostimulatory oligonucleotides (for example, CpG oligonucleotides, for example, see U.S. Pat. Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199, each of which are incorporated herein by reference in their entirety), Toll-like receptor agonists (for example, TLR2, TLR4, TLR7/8, or TLR9 agonists), and bacterial lipopolysaccharides or their derivatives (such as 3D-MPL). Additional adjuvants include oil and water emulsions, squalene, or other agents. An adjuvant may be a water-in-oil emulsion in which antigen solution is emulsified in mineral oil (for example, Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity. In one example, the adjuvant is a mixture of stabilizing detergents, micelle-forming agent, and oil available under the name PROVAX® (IDEC Pharmaceuticals, San Diego, Calif.). One of skill in the art can select a suitable adjuvant or combination of adjuvants to be included in the compositions disclosed herein or administered to a subject in combination with the compositions disclosed herein. Molecules with immune stimulant or adjuvant effects, include, without limitation: TLR3 agonists such as Poly(I:C) or Poly-ICLC; TLR 4 agonists such as LPS or monophosphoryl lipid derivatives; TLR 5 agonists such as flagellin derivatives; TLR 7/8 agonists such as imiquimod or R848; TLR 9 agonists such as CpG sequences; Stimulator of Interferon Genes (STING) pathway agonists such as ADU-S100; stimulatory neuroimmune mediators such as calcitonin gene-related peptide (CGRP); neurokinin 1 (NK1) receptor agonists such as Hemokinin 1 and Substance P; saponin related adjuvants such as QS-21 (Quillaja saponaria); purinoergic receptor agonists such as ATP; or oil-in-water emulsion adjuvants such as MF59.

[0083] Useful dosage forms for the binding reagents or any pharmaceutically-acceptable salt thereof disclosed herein include, for example and without limitation: parenteral, intravenous, intramuscular, intraocular, or intraperitoneal solutions, oral tablets or liquids, topical drops, ointments, or creams, and transdermal devices (e.g., patches). The compound may be a sterile solution comprising the active ingredient (drug or compound), and a solvent, such as water, saline, lactated Ringer's solution, or phosphate-buffered saline (PBS). Additional excipients, such as polyethylene glycol, emulsifiers, salts and buffers may be included in the solution.

[0084] Suitable dosage forms may include single-dose, or multiple-dose vials or other containers, such as medical syringes or droppers, e.g., eye droppers.

[0085] Pharmaceutical formulations adapted for administration include aqueous and non-aqueous sterile solutions which may contain, in addition to the active pharmaceutical ingredient or drug, for example and without limitation, adjuvants, anti-oxidants, buffers,

bacteriostats, lipids, liposomes, lipid nanoparticles, emulsifiers, suspending agents, and rheology modifiers. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous solutions and suspensions may be prepared from sterile powders, granules and tablets.

[0086] Therapeutic/pharmaceutical compositions as described herein may be prepared in accordance with acceptable pharmaceutical procedures, such as described in Remington: The Science and Practice of Pharmacy, 21st edition, ed. Paul Beringer *et al.*, Lippincott, Williams & Wilkins, Baltimore, MD Easton, Pa. (2005) (see, *e.g.*, Chapters 37, 39, 41, 42 and 45 for examples of powder, liquid, parenteral, intravenous and oral solid formulations and methods of making such formulations).

[0087] Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. For example, sterile injectable solutions can be prepared by incorporating the active agent in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, typical methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

[0088] Also provided herein are methods of using binding reagents and any pharmaceutically-acceptable salt thereof and/or pharmaceutical compositions including the same as disclosed herein for treatment of individuals at risk of being infected with, or who are infected with, ZIKV. Those of skill in the art will appreciate that various dosage forms, dosage routes, and/or dosage regimens may be designed consistent with the present description. In non-limiting embodiments, a pharmaceutical composition including binding reagent and any pharmaceutically-acceptable salt thereof as disclosed herein may be administered to a patient at risk of being infected with ZIKV, thereby eliciting an immune

response to ZIKV. In non-limiting embodiments, a binding reagent and any pharmaceutically-acceptable salt thereof as disclosed herein may be used to screen a phage display library for an antibody fragment that binds to the binding reagent, and thus to a ZIKV epitope that is mimicked by the binding reagent. Phage display libraries and systems, their construction and screening methods are described in detail in, among others, United States Patent Nos. 5,702,892, 5,750,373, 5,821,047 and 6,127,132, each of which are incorporated herein by reference in their entirety.

[0089] **FIG. 2** provides an elevated (*top*) and a top (*bottom*) schematic view of a typical lateral flow device 10, with which binding reagents as disclosed herein may be used. Lateral flow device 10 may include a housing 12 comprising a first hole 14 into which a sample is placed according to common usage, and a second hole 16, permitting visualization of a membrane 18, such as a nitrocellulose membrane, internal to the housing 12 and spanning the first 14 and second 16 holes, the extent of which is shown in phantom in the bottom schematic. Also shown in the bottom of the schematic is a test line 20, and a control line 21 (e.g., positive control), where, for example, the test line comprises, affixed to the membrane 18, a bound binding reagent, such as membrane-bound CZV1-1, and the control line 21 comprises a surface-bound reagent that provides confirmation that an indicator used in the assay is functioning. The indicator, such as a colorimetric indicator, is typically absorbed into the membrane 18 between the first 14 and second 16 holes, and when liquid is placed in the first hole 14, it may migrate along the membrane 18 past all testing lines, carrying the indicator past all test lines. Test line 20 is not visible unless any test sample placed in the first hole comprises the analyte to be tested, such as an anti-zikavirus antibody. Additional test lines may be added for binding different antibodies or other analytes. **FIG. 2** is illustrative of one example of a lateral flow device. As would be recognized by one of ordinary skill in the art, choice of analytes, including molecules or compositions bound at the respective lines, and detection reagents, as well as the overall size, shape, and organization of the device, including choice of suitable materials, is a matter of routine optimization based on the teachings of the present disclosure when combined with common knowledge regarding lateral flow devices and designs therefor.

Examples

Example 1

[0090] Reported here is an innovative approach using “epitope surrogate” technology, which allows the unbiased discovery of IgG antibodies that distinguish two patient populations. The method involves labeling IgGs in serum samples collected from case and

control patient populations with different colored fluorescent dyes by pre-incubation with dye-conjugated single-chain anti-IgG antibodies. The serum samples are then mixed and incubated with a one-bead-one-compound (OBOC) DNA-encoded library (DEL) of synthetic, nonpeptidic molecules. After washing, the beads are passed through a fluorescence-activated cell sorter (FACS) gated to collect beads that display a high level of fluorescence in the "case channel" but a low level of fluorescence in the "control channel". The assumption is that these beads display a ligand for the antigen-binding site of antibodies that are present at much higher levels in the case sera than the control sera. These ligands are called epitope surrogates. In addition to providing an unbiased method for the discovery of antibodies that differentiate two patient populations, epitope surrogate technology has the additional advantage of providing a pathway to identify the native antigen recognized by the differentiating antibodies. This involves using an immobilized epitope surrogate as an affinity reagent to enrich from the serum the IgGs to which it binds. These antibodies can then be used to identify the native antigen they recognize by either testing purified candidate antigens in ELISA assays or by carrying out immunoprecipitation from extracts that contain the native antigen, then identifying the retained proteins by mass spectrometry.

[0091] An OBOC DEL of peptoid-inspired conformationally constrained oligomers (PICCOs) was screened against differentially labeled serum samples collected from individuals infected with either DENV or ZIKV. We performed next-generation sequencing (NGS) of the encoding tags on beads that retained high levels of antibodies from the "ZIKV only" patients and low levels of antibodies from the "DENV only" patients. Clustering analysis of structurally homologous hits that reacted with "Zika only" sera identified 40 PICCOs that were candidate epitope surrogates engaging ZIKV-specific antibodies. In this study, we report the detailed characterization of one of these screening hits, which we call "CZV1-1". FACS-based screening of a larger panel of acute and convalescent serum samples from patients with a repertoire of flavivirus immune reactivity revealed that CZV1-1 classified patients as Zika immune with a sensitivity and specificity of 85.3% (95% CI: 69.8-93.5) and 98.4% (95% CI: 95.5-99.6), respectively. Affinity chromatography using immobilized CZV1-1 resulted in a ≈ 600-fold enrichment of the antibodies from the serum that recognizes this synthetic molecule. These enriched IgGs were then tested for binding to various candidate antigens, which revealed they recognize domain III (DIII) of the ZIKV envelope protein. These CZV1-1 binding antibodies show minimal reactivity to DIII of DENV1-4 envelope proteins. Competitive binding assays using CZV1-1 specific IgG and several ZIKV-specific monoclonal antibodies identified previously demonstrated that CZV1-

1 binding antibodies recognize an epitope in the ZIKV envelope DIII C-C' loop region. Finally, CZV1-1 specific IgG neutralized infection of distinct strains of ZIKV (Brazil and French Polynesia) with a per milligram potency comparable to that of highly specific ZIKV monoclonal antibodies. These results demonstrate that systematic mining of 'antigenically agnostic' libraries of non-biological small molecules (that is, molecular libraries constructed with no reference to known virus sequences, structures, or antigens) can nonetheless be used to discover biomarker correlates of virus neutralization.

Methods

Library synthesis and screening, and next-generation sequencing (NGS)

[0092] The DNA-encoded library (DEL) employed for this study was constructed by encoded split and pool solid-phase synthesis on 10 μm TentaGel beads carrying the invariant linker (see **FIG. 3**). A total of 2.5 million beads per tube were used for FACS-based library screening to account for a 5-fold redundancy of the library. Pooled sera were prepared at a concentration of 50mg/ml of total protein and labeled using PE-conjugated or Alexa fluor 647-conjugated goat-anti human IgG. Labeled sera were then collected and incubated with pre-blocked DEL beads. Following overnight incubation, plates were washed and beads were resuspended in PBS and sonicated before FACS acquisition and sorting in a FACS Aria III. Sorted beads were transferred to PCR tubes for onbead PCR amplification of the DNA string that corresponds to the chemical on the bead. Amplified DNA products were analyzed using NGS, and FASTQ files were received from the IonTorrent IonProton instrument.

[0093] IonTorrent barcodes were used to separate information from individual screening campaigns as different files. DNA sequence deconvolution allowed analysis of the chemical structure on the beads.

Serum sample panels

[0094] For PICCOs library screening, the serum panel from Zika-infected patients consisted of PCR-confirmed Zika infections of dengue naïve individuals collected from several clinical-epidemiological studies carried out in Recife, Northeast Brazil. These samples were serologically characterized as Zika reactive only by binding IgM and IgG assays for dengue, Zika, and Chikungunya and by neutralization assays for dengue and Zika (**Table 1**).

Table 1

Patient ID	Year of sample collection	Age	Gender	Binding assays (ELISA)			Neutralization assays, antibody titer				
				ZIKV IgM	Dengue IgM	Dengue IgG	ZIKV	DENV-1	DENV-2	DENV-3	DENV-4
01-007-2-2	2016	19	Female	NEG	NEG	NEG	140.0	<20	<20	<20	<20
01-008-2-2	2016	25	Female	NEG	NEG	NEG	276.6	<20	<20	<20	<20
01-009-2-2	2016	27	Female	NEG	NEG	NEG	529.1	<20	<20	<20	<20
03-006-2-2	2016	28	Female	NEG	NEG	NEG	672.6	<20	<20	<20	<20
62-0005	2015	36	Female	POS	NEG	NEG	980.4	<20	<20	<20	<20

[0095] The dengue panel consisted of serial samples from patients with confirmed (by PCR, IgM, and IgG and neutralization assays) primary dengue infections and divided into two groups: a) acute samples collected no more than 7 days from the start of the symptoms and confirmed as dengue-IgG negative by ELISA, and b) convalescent samples collected after 23 days from the start of the symptoms and confirmed as anti-dengue IgG positive. Dengue samples were from two natural history cohorts carried out in Northeast Brazil before Zika introduction in the area (2004-2009) (**Table 2**).

Table 2

Patient ID	Days from start of symptoms	Year of sample collection	Age	Sex	IgM DENV ENV	IgG DENV ENV	IgG NS1	Group category
287	3	2005	16	F	NEG	NEG	49	Dengue 1, Acute
287	5	2005	16	F	NEG	NEG	49	
287	31	2005	16	F	POS	POS	17074	Dengue 1, convalescent
287	324	2006	16	F		POS	4479	
263	2	2005	25	F	NEG	NEG	49	Dengue 1, Acute
263	5	2005	25	F	NEG	NEG	49	
263	21	2005	25	F	POS	POS	2235	Dengue 1, convalescent
263	324	2006	25	F		POS	5090	
295	5	2005	18	M	POS	NEG	49	Dengue 2, Acute
295	26	2005	18	M	POS	POS	245013	Dengue 2, convalescent
295	34	2005	18	M	POS	POS	3220	
295	46	2005	18	M	POS	POS	2749	
249	5	2005	43	M	NEG	NEG	49	Dengue 2, Acute
249	7	2005	43	M	POS	NEG	86	
249	9	2005	43	M	POS	NEG	98	Dengue 2, convalescent
249	33	2005	43	M	POS	POS	20264	
299	7	2005	56	M	POS	NEG	49	Dengue 2, Acute
299	16	2005	56	M	POS	POS	4706	Dengue 2, convalescent
299	297	2006	56	M	POS	POS	2169	
574	4	2006	30	F	POS	NEG	88	Dengue 3, Acute
574	27	2006	30	F	POS	POS	18558	Dengue 3, convalescent
574	48	2006	30	F	POS	POS	24840	
576	5	2006	66	F	NEG	NEG	89	Dengue 3, Acute
576	33	2006	66	F	POS	POS	5368	Dengue 3, convalescent
576	383	2007	66	F		POS	2670	
279	3	2005	17	F	NEG	NEG	49	Dengue 3, Acute
279	36	2005	17	F	POS	POS		Dengue 3, convalescent
279	304	2006	17	F		POS		
396	2	2006	10	M	NEG	NEG	49	Dengue 4, Acute
396	7	2006	10	M	NEG	NEG	64.5	
396	29	2006	10	M	POS	POS	14235	Dengue 4, convalescent
577	4	2006	8	F	POS	NEG	49	Dengue 4, Acute

577	29	2006	8	F	POS	POS	28350	Dengue 4, convalescent
393	5	2006	6	F	POS	NEG	49	Dengue 4, Acute
393	26	2006	6	F	POS	POS	5942	Dengue4, convalescent
394	8	2006	8	M	POS	NEG	49	Dengue 4, Acute
394	39	2006	8	M	NEG	POS	1859	Dengue 4, convalescent
327	2	2005	35	M	NEG	NEG	49	Dengue 5, Acute
327	4	2005	35	M	NEG	NEG	49	
327	31	2005	35	M	POS	POS	1216	Dengue 5, convalescent
527	2	2006	38	M	NEG	NEG	49	Dengue 5, Acute
527	6	2006	38	M	NEG	NEG	62.3	
527	31	2006	38	M	POS	POS	4919	Dengue 5, convalescent
658	4	2006	27	F	NEG	NEG	49	Dengue 5, Acute
658	6	2006	27	F	POS	NEG	49	
658	33	2006	27	F	POS	POS	6296	Dengue 5, convalescent
658	437	2007	27	F		POS	674	
644	4	2006	48	F	NEG	NEG	49	Dengue 5, Acute
644	6	2006	48	F	POS	NEG	49	
644	33	2006	48	F	POS	POS	1423	Dengue 5, convalescent
644	403	2007	48	F		POS	1505	

[0096] For hit validation, evaluation of the diagnostic performance of the selected PICCO candidate, and antigen surrogate discovery, a larger set of well-characterized serum samples (n=226) was included. Zika immune patients included 34 DENV naïve and 29 DENV immune samples. Among the ZIKV immune patients, eight were virologically confirmed ZIKV cases (all previously DENV immune), and nine were ZIKV cases confirmed by IgM and IgG seroconversion between acute and convalescent samples. The remaining ZIKV immune samples were positive for IgG and PRNT for 10 ZIKV. Zika naïve samples included DENV naïve (n=61), DENV naïve, YFV vaccinees (n=5), DENV naïve, CHIKV immune (n=5), DENV immune, multiple serotype exposures (n=55), and DENV immune, CHIKV immune (n=66) patients.

Hierarchical clustering analysis

[0097] This analysis was carried out to determine if two or more highhit PICCOs were similar in structure. We assigned a probability of similarity for each of the six chemical components based on the number of alternative components at that site. For positions one through six, the number of chemical component synthesis options at each position were respectively 2, 6, 6, 81, 7, and 13. We calculated the empirical probability of observing the identical chemical component at each for each of the six positions using the frequency distributions in the unselected DNA of the same chemical at each position if we randomly

picked two PICCOs from among 54,853 PICCOs. This generated empirical values for the probability of identify of 0.501, 0.169, 0.203, 0.0141, 0.192, 0.102 at each of the six positions, respectively. The distance was defined with log10 of the probability with the range 0 (completely different) to of -5.32 (identical). For example, the probability that two PICCOs would have an identical chemical structure at positions two, three, four, five, and six is roughly $\log^{10}$ (0.169*0.203*0.0141*0.192*0.102) = -5.02. The hierarchical clustering method used was Ward's minimum variance method, to generate compact, spherical clusters. The R code function “hclust()” with method=”ward.D2” was used in generating the heatmap. Chemical structure clusters were defined as contiguous groups with similarity probability scores of -4 or lower.

Affinity-purification of IgG antibody from serum

[0098] Antibodies that bind CZV1-1 were enriched from a pool of ZIKV immune sera by affinity purification. CZV1-1 was covalently immobilized to a SulfoLinkTM affinity column (ThermoFisher) following the manufacturer’s protocol. Undiluted, pooled ZIKV immune sera was incubated to the column overnight at 4°C. The column was then washed, and the bound IgG was eluted using Gentle Ag/Ab elution buffer pH 6.6 (ThermoFisher). The IgG was dialyzed overnight, and the sample was concentrated using centrifugal concentrators. Total IgG levels were quantified using a sandwich ELISA.

Binding IgG ELISA Assays

[0099] To assess the binding of the purified IgG-CZV1 to different ZIKV and DENV antigens, high-binding, half-area 96-well flat-bottom polystyrene plates (Corning) were coated with the following recombinant proteins (at 2μg/ml) or cell extracts in carbonate/bicarbonate buffer overnight at 4°C: ZIKV or DENV envelope protein (Sino Biologicals), ZIKV or DENV1-4 NS1 proteins (Native Antigen), ZIKV or DENV1-4 cell extracts, and ZIKV or DENV1-4 cell lysates. Plates were blocked with non-fat dry milk (NFDM; Bio-Rad) at 5% (w/v) in PBS for 1 hour at room temperature. Then, purified IgG-CZV1 diluted in 5% (w/v) of NFDM in PBS were added to the plate at different concentrations and incubated for 1 hour at room temperature. Plates were washed six times with PBS-T and incubated for 1 hour with horseradish peroxidase (HRP)-linked goat antihuman IgG (Jackson ImmunoResearch). After five washes with PBS-T, plates were incubated at room temperature for 20 minutes with HRP substrate, 3, 3′, 5, 5″-tetramethylbenzidine (SeraCare), and the developing reaction was stopped by adding 1N hydrochloric acid (Sigma). Optical densities at a wavelength of 450nm (OD450nm) were measured using SpectraMax Plus PC380 microplate spectrophotometer using SoftMax Pro

software version 6.4 (Molecular Devices, USA). Optical densities from blank wells were subtracted from all measurements before analysis.

Blockade-of-binding (BOB) ELISA Assays

[00100] BOB assay measures the level of purified IgG specific to CZV1-1 that blocks the binding of highly specific monoclonal antibodies (mAbs) to the ZIKV envelope. Briefly, high-binding, half-area 96-well flat-bottom polystyrene plates (Corning) were coated with 2μg/ml of ZIKV or DENV recombinant envelope protein (Sino Biologicals) in carbonate/bicarbonate buffer overnight at 4°C. Plates were blocked with non-fat dry milk (NFDM; Bio-Rad) at 5% (w/v) in PBS for 1 hour at room temperature. Then, purified IgG-CZV1 diluted in 5% (w/v) of NFDM in PBS were added to the plate at different concentrations (600, 300, 100, and 33ng/mL). Plates were then incubated with IgG-CZV1 for 30, 45, and 55 minutes. A solution containing 200ng/mL of ZIKV or DENV-specific mouse mAbs (ZV-2, ZV-67, ZV-64, ZV-48, DV-10, Absolute antibody) was immediately pipetted on top of the IgG-CZV1 sample and incubated for 30, 15, and 5 minutes for a total incubation period of 60 minutes. Plates were washed with PBS-T and incubated with horseradish peroxidase (HRP)-linked goat anti-human IgG (Jackson ImmunoResearch). For the reverse blocking experiments, mAbs diluted in 5% (w/v) of NFDM in PBS were added to the plate at different concentrations (800, 400, 200, 100, and 50ng/mL) and were incubated for 30 minutes. A solution containing 200ng/mL of purified IgG-CZV1 was immediately pipetted on top of the mAbs and incubated for additional 30 minutes. Plates were washed with PBS-T and incubated with horseradish peroxidase (HRP)-linked goat anti-mouse IgG (Jackson ImmunoResearch). After washing, plates were incubated with HRP substrate, 3, 3′, 5, 5″-tetramethylbenzidine (SeraCare), and the developing reaction was stopped by adding 1N hydrochloric acid (Sigma). Optical densities at a wavelength of 450nm (OD450nm) were measured using SpectraMax Plus PC380 microplate spectrophotometer using SoftMax Pro software version 6.4 (Molecular Devices, USA). Optical densities from blank wells were subtracted from all measurements before analysis.

Neutralization Assays

[00101] The plaque reduction neutralization test (PRNT) was used to assess the neutralization activity of the purified IgG-CZV1 following a protocol described in detail elsewhere. The assay was carried out using the following virus strains: ZIKV strain H. sapiens/PE243/2015 (Brazil 2015), ZIKV strain H. sapiens/PF/2013 (French Polynesia, 2013), and DENV-3 H. sapiens/PE/02-95016 (Brazil 2002). The PRNT positivity was

defined based on a 50% reduction in plaque counts (PRNT50) and neutralizing antibody titers were estimated using a four-parameter-non-linear regression.

Molecular and metadynamic simulations

[00102] The binding pose prediction of the CZV1-1 adducts was performed using a semi-flexible approach coupled to the genetic algorithm of the GOLD software (34), considering the following x-ray structures as receptors: 5KVE, 5KVG, and 5VIC. The x-ray structures 5KVE and 5KVG correspond to the ZIKV envelope protein complexed to the mAbs ZV48 and ZV67, respectively; and 5VIC corresponds to the envelope protein of DENV-1 complexed to neutralizing mAb Z004. The final structures from the molecular dynamics simulations were used as a starting point for metadynamics calculations using Plumed version 2.0.1 within NAMD version 2.13.

Results

Discovery of epitope surrogates that bind ZIKV-specific antibodies

[00103] We screened an OBOC DEL of PICCOs to identify ligands that recognize IgG antibodies from ZIKV immune individuals. The library consisted of 508,032 unique molecular structures that were constructed by encoded split and pool solid-phase synthesis on 10μm TentaGel beads carrying an invariant linker using published protocols. The synthetic workflow that was employed to construct the library is shown schematically in **FIG. 3**. Each PICCO featured chemical diversity at six positions (R1 to R6). Positions R1 and R5 were derived from diverse chloroacids. Positions R2 and R6 were derived from amine building blocks. Position R3 was derived from Fmoc-protected amino acids that also contained an azide functionality and position R4 was derived from diverse aldehydes (**FIGS. 4 and 5**). To identify ligands that bind specifically to anti-ZIKV IgG antibodies but not to IgG antibodies induced by infection with the closely related dengue viruses, the PICCOs library was screened in a stepwise manner using two well-characterized serum panels from documented ZIKV infected patients (Table 1, above) and DENV-infected patients (Table 2, above). For detection and exclusion of dengue cross-reactive IgG antibodies, matched pre- and post-infection sera were available from 16 dengue-infected patients. Matched pre- and post-infection sera were pooled to form five matched pools of 2-4 patients each for flow cytometry testing. For Zika-specific IgG antibody detection, no pre-infection sera were available, so highly characterized ZIKV-specific sera from five patients were tested against flavivirus-negative sera (negative for ZIKV and DENV antibodies) (**Table 3**).

Table 3

Group	Patient ID	Year of sample collection	Age	Sex	Binding assays (ELISA)		Neutralization assays, antibody titer			
					Dengue IgM	Dengue IgG	DENV-1	DENV-2	DENV-3	DENV-4
Naïve 1	B021	2011	24	Female	NEG	NEG	<20	<20	<20	<20
	B036	2011	16	Female	NEG	NEG	<20	<20	<20	<20
	B235	2011	16	Female	NEG	NEG	<20	<20	<20	<20
Naïve 2	B266	2011	27	Female	NEG	NEG	<20	<20	<20	<20
	B282	2011	14	Female	NEG	NEG	<20	<20	<20	<20
	B313	2012	16	Female	NEG	NEG	<20	<20	<20	<20
Naïve 3	B357	2012	18	Female	NEG	NEG	<20	<20	<20	<20
	B084	2011	21	Female	NEG	NEG	<20	<20	<20	<20
	B215	2011	21	Female	NEG	NEG	<20	<20	<20	<20

[00104] For the ZIKV screens, ten pools of two ZIKV-infected patients each were tested against three pools of flavivirus-negative sera. Positive PICCO-beads were identified and sorted out by two-color screen strategies (Zika immune sera versus flavivirus-naïve sera, and dengue pre-infection versus dengue post-infection sera). Because screening of bead-displayed libraries can have a significant false positive rate, we screened several copies of the library and only carried forward those structures that were selected as positive on multiple beads. We have shown previously that these "redundant hits" are usually bona fide ligands to the target antibody while "singletons" are usually false positives. Six replicate flow cytometric runs were performed for each of the five matched pre- and post-dengue infected serum pools, and two replicates were performed for each of the 30 combinations of the 10 unmatched Zika pools versus 3 naïve serum pools. Five-fold redundancy of each PICCO, for a total of 2.5 million beads, was mixed with the labeled sera to allow for the detection of repeated PICCO molecular "hits" on each run. Each DNA-encoding Zika hit identified was then amplified, sequenced, and decoded using NGS to generate a list of putative PICCO ligands for anti-Zika IgG antibodies. We initially selected 37,291 unique PICCOs from the Zika antibody screens (**FIG. 6**). PICCOs that were positive on a single run across multiple replicate runs were considered false positives (n=24,586).

[00105] After excluding PICCOS that were positive on both Zika and dengue antibody screens (n=9,603), a total of 3,102 unique PICCOs (0.6% of the starting library of 508K PICCOs) were considered legitimate reactive Zika PICCO candidates. Because we are seeking truly Zika-specific PICCOs for use in serological assays, we focused on Zika-candidate PICCOs that reacted in at least seven Zika screen runs but zero dengue screen runs (n=50). As expected, there was a considerable overlap of dengue-reactive and Zika-reactive

PICCOs (**FIG. 7**). This finding of PICCOs that bound with anti-flavivirus serum in two completely different experiments (dengue, and Zika) is evidence that these PICCOs are presumed to react with Zika/ dengue cross-reactive IgG antibodies and are likely to be surrogates for antigens with broad cross-reactivity across the flaviviruses.

Hierarchical clustering reveals clusters of ZIKV-specific PICCOs as high-priority chemotype candidates

[00106] NGS analysis of the PICCO-displaying beads selected by FACS sorting allowed for the generation of lists of the corresponding chemical structures. From the starting library of over 500,000 possible chemical structures, we selected 150 PICCOs of special interest based on their antibody binding patterns (**FIG. 6**). “Zika only” were those 50 PICCOs with multiple hits (more than 7 runs) with Zika-positive sera but no hits with dengue positive sera; “dengue only” were those 50 PICCOS with multiple hits with dengue positive sera (more than 5 runs) but no hits with Zika positive sera; and “flavivirus cross-reactive” were those 50 PICCOs with multiple hits with both Zika positive sera and dengue positive sera (**Table 4**).

Table 4

	Beads Screened by FACS	Beads Positive by FACS	Total Unique PICCOs detected	Total Unique Specific PICCOs detected*	High Hit Specific PICCOs Selected for Clustering**	PICCOs Specific Clusters Identified	Unique PICCO selected for Detailed Study
Zika	150 million (60 runs)	171,309	37,291	3,102	50	5	CZV1-1
Dengue	75 million (30 runs)	128,942	30,789	2,516	50	3	-
Flavivirus	-	-	-	-	50	6	-

* Zika Specific: Positive ≥ 2 runs, zero dengue runs; Dengue Specific: Positive ≥ 2 runs, zero Zika runs

** Zika Specific: Positive ≥ 7 runs, zero dengue runs; Dengue Specific: Positive ≥ 5 runs, zero Zika runs; Flavivirus cross-reactive: Positive ≥ 26 dengue runs and ≥ 38 Zika runs

[00107] These structures were then subjected to a clustering analysis based on their degree of structural conservation at positions R1 through R6. Nearest neighbor hierarchical clustering was used to generate tree relationships and structure-based identity score heatmaps. A PICCO cluster was defined as multiple PICCOs that shared identical sub-units at four or five of the six variable positions (see **FIG. 3**). Five PICCO clusters were identified that encompassed 40 of the 50 PICCOs of interest (**FIG. 6**). Over 4,000 distinct Zika and dengue cross-reactive PICCOs were detected in more than 7 runs, while 2,220 distinct Zika and dengue cross-reactive PICCOs were detected in more than 15 runs (**FIG. 8**). For the top 50 Zika-specific PICCO candidates, only one unique PICCO compound was detected in a

maximum of 15 runs (CZV1-1). We found five clusters of chemically similar "Zika-only" PICCOs, three clusters of "dengue-only" PICCOs, and eight clusters of "flavivirus cross-reactive" PICCOs (**FIG. 9**). Of these five Zika only clusters, the average cluster size was 8 PICCOs. All five PICCO unique compound's identity clusters were internally homogeneous regarding their antibody binding properties, even though this was not a criterion for clustering and clustered exclusively with other chemically similar "Zika only" PICCOs. The five Zika clusters were named to reflect the mean number of runs in which each distinct PICCO was detected (**Table 5**).

Table 5

Clusters Zika Specific	Number of Unique PICCOs in Cluster	Number of Runs in which Each Unique PICCO was Detected	Mean (STDV) of Number Runs Detected
CZV1	11	15,14,11,10,9,8,8,8,8,8,7	9.6 (2.7)
CZV2	8	12,10,10,9,9,8,7,7	9.2 (1.5)
CZV3	8	12,10,9,9,9,8,8,7	9.0 (1.5)
CZV4	10	10,8,8,8,8,8,8,7,7,7	7.9 (0.9)
CZV5	3	8,8,8	8
No Cluster	10	12,9,9,8,8,8,8,8,7,7	8.4 (1.4)

[00108] In the heat map (**FIG. 9**), reading from the upper left down toward the lower right, the five Zika clusters in order are designated as clusters Zika 3 (CZV-3), Zika 1 (CZV-1), Zika 2 (CZV-2), Zika 4 (CZV-4), and Zika 5 (CZV-5). This clear demonstration that PICCOs with chemical structure similarities also showed antibody binding similarities provides internal validation for our selection process. This clustering pattern suggests that some variability of PICCO chemical structure is permitted around an antibody-binding target without losing binding specificity. Altogether, 40 of the 50 PICCOs of special interest clearly clustered with other similar PICCOs. The remaining 10 PICCOs, despite having been selected for multi-hit binding specificity, did not chemically cluster with other PICCOs and were deemed of lower priority in these studies.

Hit validation and characterization of the antibody-binding "pharmacophore"

[00109] Following the clustering analysis, sixteen Zika candidate diagnostic PICCOs from clear "Zika only" clusters were selected for further patient-specific binding validation experiments. In addition to the Zika PICCOs, seven random, non-reactive PICCOs were included as negative controls. Each Zika candidate PICCO was resynthesized in bulk on-resin without the DNA encoding tag using 10μm TentaGel beads for further serum binding validation experiments. The validation serum panels included: (1) two pools of 3-4 serum each from Zika IgG positive, dengue IgG negative patients; (2) four pools of 3 serum each

from dengue IgG positive, Zika IgG negative patients; and (3) two pools of 3 serum each from patients positive for both Zika and dengue IgG antibodies (**Table 6**).

Table 6

Groups	Patient ID	Year of sample collection	Age	Binding assays (ELISA)			Neutralization assays, antibody titer				
				Dengue IgM	Dengue IgG	ZIKV IgM	DENV-1	DENV-2	DENV-3	DENV-4	ZIKV
Zika 1	62-0020	14		NEG	NEG	POS	<20	<20	<20	<20	1227
	1	-		NEG	NEG	NEG	<20	<20	<20	<20	2560
	5	-		NEG	NEG	NEG	<20	<20	<20	<20	12960
Zika 2	62-0035	16		NEG	NEG	POS	<20	<20	<20	<20	11569
	13	-		NEG	NEG	EQUIV	<20	<20	<20	<20	1575
	1	-		NEG	NEG	NEG	<20	<20	<20	<20	2560
	41	-		NEG	NEG	EQUIV	<20	<20	<20	<20	343
Dengue 1	514 S4	2010	12	POS	POS	NEG					<20
	273 S2	2010	58	POS	POS	NEG					<20
	426 S3	2010	15	POS	POS	NEG					<20
Dengue 2	270 S2	2010	27	POS	POS	NEG					<20
	304 S3	2010	71	POS	POS	NEG					<20
	139 S4	2010	75	POS	POS	NEG					<20
Dengue 3	235 S3	2010	70	POS	POS	NEG					<20
	197 S3	2010	25	POS	POS	NEG					<20
	502 S4	2010	15	POS	POS	NEG					<20
Dengue 4	515 S3	2010	80	POS	POS	NEG					<20
	257 S2	2010	8	POS	POS	NEG					<20
	183 S4	2010	26	POS	POS	NEG					<20
Flavivirus 1	62-0071	18		NEG	POS	POS	<20	92	1004	3251	4174
	1	-		NEG	NEG	NEG	<20	<20	<20	<20	2560
	7	-		NEG	POS	POS	<20	<20	80	<20	2560
Flavivirus 2	23	-		NEG	NEG	NEG	<20	<20	<20	<20	573
	41	-		NEG	NEG	EQUIV	<20	<20	<20	<20	43
	62-0026	11		POS	POS	POS	<20	<20	1262	1387	14092

[00110] The three pools of 3 samples each from Flavivirus naïve patients (IgG negative for Zika and dengue) were the same used for the screening step (**Table 4**, above). Single-color FACS-based validation experiments were performed by incubating each pooled sera group with the selected Zika candidate PICCOs. PICCOs and pooled sera were tested randomly to blind operator from ligand and sera specificity. Histograms were plotted and PICCOs were confirmed as positive by a shift to the right of distributions of the serum pools in comparison to blank control (bead without sera) and the percentage of positive beads was then calculated. All sixteen Zika PICCOs candidates were seroreactive: six showed strong Zika specificity and no reactivity with dengue samples, five showed marginal reactivity, and five showed some level of unwanted cross-reactivity with dengue samples. The comparison of the antibody specificity profiles of PICCOs from within the same chemical sequence cluster versus different clusters reveal that PICCOs with similar chemical sequence structures have

similar binding specificity profiles (**FIG. 10**). Sera groups responded minimally to the set of negative controls PICCOs analyzed.

[00111] Based on these validation studies, we selected PICCO "CZV1-1" as the most promising ligand for ZIKV-specific antibodies. CZV1-1 was re-synthesized on-resin without the DNA encoding tag on TentaGel beads containing a RAM linker, allowing the compound to be released from the resin and characterized by liquid chromatography/mass spectrometry (LC-MS). This is important because in the high-throughput library synthesis, unexpected events sometimes occur, and it is possible that a "side product" may be the active compound rather than the structure predicted by the DNA sequence. Indeed, using the same conditions that had been employed to construct the library, we found that the reductive amination reaction used to add the formyl imidazole unit (R4 in CZV1-1) to the resin was inefficient and the anticipated structure (CZV1-1) was only a minor product. This observation strongly suggested that the library beads presumed to display CZV1-1 displayed mostly a "des-aldehyde" analog, along with a small amount of CZV1-1. This called into question whether the imidazole unit was indeed important for binding the Zika antibodies. We developed improved conditions for this reductive amination that allowed the synthesis of pure CZV1-1 on resin. When 10 μm TentaGel beads displaying bona fide CZV1-1 were employed in an antibody-binding assay using serum from Zika-infected patients, they robustly retained IgG from the sample. In contrast, beads displaying the "des-aldehyde" side product CZV1-1a displayed no significant IgG binding (**FIG. 11**). Samples from Zika only patients (Zika positive, dengue negative) were not included in this analysis due to limited serum availability. These data show that the imidazole group derived from the reductive amination at step R4 is essential for antibody recognition. Several other derivatives of CZV1-1 were synthesized and assessed for their ability to bind Zika-specific antibodies to acquire additional information on how the ligand interacts with the antibody. We found that when an electron-poor aromatic ring was substituted for the electron-rich piperonyl ring at position R2 (CZV1-1b), binding was decreased by about 10-fold. Replacement of either one of the chloroacids + amine units at positions R5 (CZV1-1c) and R6 (CZV1-1d) with an acetyl group resulted in a complete and a 10-fold reduction in binding, respectively (**FIG. 12**). To determine if this was largely a requirement of the chloroacid-derived R5 unit or the amine-derived R6 unit, we also synthesized compound CZV1-1e, in which a benzoic acid was placed at each position. In other words, the R5 unit was present but not the R6 unit. Strikingly, only a slight reduction in antibody retention was observed, arguing that the aromatic rings at each of the R5 positions are important for binding, but the amine-derived

units at R6 are not. These data, which show a clear structure-activity relationship pattern, strongly support the idea that CZV1-1 is a selective ligand for the ZIKV-specific antibodies.

CZV1-1 discriminates between Zika and other flaviviruses infections

[00112] Next, the antibody binding properties of CZV1-1 were further examined using a larger set of serum samples with a broad repertoire of flavivirus immune reactivity. Binding to CZV1-1 was assessed for each serum individually using a single-color FACS-based assay and each serum was tested blindly without previous knowledge of the patient serostatus. The percentage of positive CZV1-1 beads was used for discriminating between Zika-positive and negative samples. The receiver operating characteristic (ROC) curve showed that a threshold of 11.3% positive beads correctly classified patients as Zika immune or not among all samples tested with a sensitivity and specificity of 85.3% (95% CI: 69.8-93.5) and 98.4% (95% CI: 95.5-99.6), respectively (**Table 7 and FIG. 13**). For the DENV immune, ZIKV naive individuals, only 1.6% of the samples showed reactivity with CZV1-1 beads with a percentage of positive beads above the cutoff. Among the DENV immune individuals, the sensitivity and specificity of assays were 82.7% (95% CI: 65.4-92.4) and 98.3% (95% CI: 94.2-99.7), respectively (**Table 7 and FIG. 13**). For this study, we tested only one PICCO, CZV1-1, in detail for its diagnostic properties.

Table 7

Results with conventional assays				Binding to CZV1-1		
ZIKV	*DENV*	YFV*	CHIKV	*Sera tested*	*Sera Positive*	%Sera Positive
-	-	-	-	61	1	1.6%
-	+	-	-	55	2	3.6%
-	-	+	-	5	0	0%
-	-	-	+	5	0	0%
-	+	-	+	66	0	0%
		Total ZIKV negatives		192	3	1.6%
+	-	-	-	5	5	100%
+	+	-	-	29	24	82.7%
		Total ZIKV Positives		34	29	85.3%

ZIKV: Zika virus, DENV: Dengue viruses, CHIKV: Chikungunya virus, YFV: Yellow fever virus. *Samples from YFV vaccinees.

Discovery of the native antigen recognized by CZV1-1-binding antibodies

[00113] We next focused on identifying the native antigen recognized by the IgG antibodies that bind specifically to CZV1-1. First, CZV1-1 was covalently attached to an agarose column, and pooled ZIKV immune sera were used to enrich for IgG that binds specifically to CZV1-1. Two rounds of purification were performed using a pool of serum

samples from ZIKV immune individuals that had shown strong reactivity to CZV1-1 in the FACS-based assay. Pooled serum was passed over the column with immobilized CZV1-1 and, following washing, bound IgG was released from the resin using a gentle antigen/antibody elution buffer (pH 6.6). The concentration of IgG following buffer exchange was determined by ELISA. The fraction of IgG specific to CZV1-1 isolated corresponded to ~0.3% of the total IgG present in the initial pooled sera used for affinity purification. FACS-based assay confirmed the binding of this purified CZV1-1-specific IgG to CZV1-1 PICCO beads and revealed that the enriched IgG had an ~600-fold increase in specific binding when compared to the initial pooled ZIKV immune sera. We then assessed the binding of CZV1-1-specific IgG against an array of ZIKV and DENV structural and non-structural antigens. We found that CZV1-1-specific IgG binds strongly to full-length ZIKV envelope protein as well as to domain three (DIII) of the ZIKV envelope, with 50% binding concentrations of 100ng/ml and 126ng/ml, respectively (**FIGS. 14A-14B, panel A**). CZV1-1-specific IgG showed no or weak reactivity to the full-length envelope of DENV1-4 (**FIGS. 14A-B, panel B**), and no reactivity to ZIKV and DENV1-4 NS1 (**FIG. 15**).

[00114] To gain further insights into the natural epitope target corresponding to CZV1-1, we performed antibody competition assays against several monoclonal antibodies (mAbs) known to bind spatially distinct epitopes on DIII of ZIKV envelope (mouse monoclonal antibodies ZV2, ZV48, ZV64, ZV67). Two mAbs that bind to DENV envelope DIII (DV10) and prM (2H2) were also included as controls. IgG from flavivirus-naïve human subjects was included as a source of negative control sera. We first assessed the binding of CZV1-1-specific IgG and the mAbs directly to the ZIKV envelope DIII protein. We found that CZV1-1-specific IgG, and two of the four ZIKV mAbs (ZV48 and ZV67), were able to bind with high potency to the ZIKV envelope DIII protein used in our assays (**FIG. 16**). We next compared the abilities of these different mAbs to block the binding of CZV1-1-specific IgG to the ZIKV envelope DIII captured on an ELISA plate. We found that CZV1-1-specific IgG exhibited substantial competition for binding with ZV48 and ZV67, as 100ng of these mAbs blocked 50% binding of 100 ng/ml of the IgG specific to CZV1-1 (**FIGS. 14A-14B, panel C**). We next performed reverse blocking experiments to assess the competition of the mAbs for binding of IgG specific to CZV1-1. We found that CZV1-1-specific IgG competes for binding with ZV48 but not ZV67 suggesting that CZV1-1 is a surrogate for the C- C' loop epitope which projects away from the 'sandwich' core of ZIKV envelope DIII (**FIGS. 14A-14B, panel D**).

[00115] Because the ZIKV-specific mAbs used in these experiments not only recognize antigen determinants in the DIII of the ZIKV envelope but are also known to have neutralization properties, we next performed neutralization assays using multiple ZIKV strains. One dengue strain isolated in Brazil (DENV-3, 95016/BR-PE/02) was included as a control for the assay. Remarkably, we found that CZV1-1-specific IgG neutralizes the Brazilian ZIKV strain (PE243) with a potency similar to that of monoclonal antibody ZV67 (IC50: 2.05 versus 1.27 ug/ml) and better than ZV48 (IC50: 2.05 versus 3.8 ug/ml). CZV1-1-specific IgG also neutralized the ZIKV French Polynesia strain (H/PF2013) but with lower potency than ZV48 and ZV67 (**FIGS. 14A-14B, panel E**). We speculate that because the CZV1-1-specific IgG antibodies used in these studies were collected from Brazilians who were infected with ZIKV, this may be the reason why they neutralized the Brazilian strain better than the French Polynesian strain. The number of Zika strains that could be tested here was limited by the quantity of our purified human CZV1-1-specific IgG.

[00116] Molecular docking simulations suggested that CZV1-1 binds to mAb ZV48 with higher affinity (**Table 8**).

Table 8

Neutralizing mAb	Ligand	GOLD score
ZV48	E-DIII	N/A*
	CZV1-1	74.10
	CZV1-1c	61.23
ZV67	E-DIII	N/A*
	CZV1-1	65.19
	CZV1-1c	60.63
Z004	CZV1-1	47.57
	CZV1-1c	46.08

*Gold software is not suitable for protein-protein docking.

[00117] To further evaluate the flexibility and intermolecular interactions of the mAbs and CZV1-1 complexes, molecular dynamics (MD) simulations were performed. MD trajectory analyses showed that CZV1-1 exhibits a high rate of native contacts and hydrogen bonds with mAb ZV48 than other mAbs (data not shown). Furthermore, CZV1-1 shares a greater number of common interactions with the ZIKV DIII envelope subunit when bound to mAb ZV48 (data not shown), which includes ZV48 residues Y31, Y38, W56, Y97, Y100, Y102, W161, M178, and M231 (data not shown). It is worth noting a network of p-stacking interactions between residues Y101, Y39, and W57 and the aromatic rings of CZV1-1; a hydrogen bond with the side chain of the amino acid S100, and a salt bridge with Y98 (data not shown). In

contrast, molecular docking showed a low affinity of CZV1-1c (negative control) to mAb ZV48 (**Table 8**, above). These results are confirmed by metadynamics simulations of the CZV1-1 and CZV1-1c adducts and the mAbs ZV48 and ZV67 (**FIG. 17**).

Discussion

[00118] In this study, we extended our prior experience in screening combinatorial libraries of small synthetic molecules to efficiently identify a small synthetic molecule “CZV1-1” that binds specifically to IgG present in serum from Zika-immune persons, but not in serum from dengue-immune persons. Our PICCO library design was “antigenically agnostic” - one that does not require foreknowledge of the pathogen structure or sequences - in that there was nothing about the half million small molecules that should have preferentially presented ZIKV antigenic mimics.

[00119] Nonetheless, we were able to identify and resynthesize a ZIKV molecular mimic that has real potential as a diagnostic reagent to distinguish between natural infections caused by the two closely related Zika and dengue viruses. The diagnostic sensitivity of CZV1-1 alone was above 80%. This sensitivity could be further improved by additional structure-activity research to optimize the antibody binding properties of CZV1-1. It is also possible that further development of PICCOs from other structural clusters that are distinct from CZV1-1, could lead to development of a multi-PICCO assay format. Further characterizations of the binding properties against a wide array of other flaviviruses should also be done. If CZV1-1-based assays continue to show favorable diagnostic properties, they could have considerable value in high-risk situations like ZIKV infections in pregnancy. This approach for the discovery of specific small molecule biomarkers of viral infection and immunity, shown here in the specific case of Zika, can potentially be applied to any virus. Indeed, the general strategy of discovery of antibody biomarkers by comparing the PICCO binding profile of IgG in serum specimens from patients with, versus those without, a disease might also be applicable to non-infectious diseases such as cancers, autoimmune diseases, and other chronic conditions.

[00120] A critical component of this study was our ability to construct “gold-plated” panels of Zika-immune and dengue-immune human sera that were carefully characterized by conventional ELISA and neutralization assays. Although we were unable to determine the timing of sample collection after infection for the Zika-immune individuals used for our initial screens, all serum samples were confirmed to show no reactivity with dengue antigens in ELISA and neutralization assays, confirming that samples were highly ZIKV immune specific. Then, in our validation and evaluation experiments, we showed that samples from

individuals with immunity to both Zika and dengue infections still retained IgG that bound specifically to CZV1-1. This is important since most individuals living in ZIKV endemic areas have background exposure to multiple flaviviruses.

[00121] DNA-encoding of the PICCO-displaying beads enabled NGS-based analysis which provided rapid elucidation of the chemical structures identified during the screening steps. Our clustering analysis provided insights into the structural similarities and features important for Zika-specific IgG binding to these molecules. Our data showing similar IgG binding profiles within hit candidates selected from the ZIKV-specific clusters revealed that a larger set of potential, analogous small molecule candidates could also be used for improving diagnostic accuracy. Affinity purification experiments yielded concentrated CZV1-1-specific IgG antibodies. Subsequent binding competition experiments revealed that the purified CZV1-1-specific IgG competed with monoclonal antibodies directed to the DIII of ZIKV envelope protein, indicating that CZV1-1 epitope mimetic was within or overlapped with DIII. More specifically, CZV1-1-specific IgG competed strongly with monoclonal antibody ZV48 suggesting that CZV1-1 mimics a cryptic epitope in the C-C' loop arranged within the 'sandwich' core of DIII. The fact that IgG against an epitope that is not predicted to be accessible on the mature ZIKV virion can be detected in samples from immune individuals further indicates that viral breathing likely allows the exposure of the C-C' loop during ZIKV infections *in vivo*. Purified CZV1-1-specific IgG has neutralization activity against at least two ZIKV strains, which suggests the potential for CZV1-1 to be used as a vaccine immunogen. Although CZV1-1 is not a high-affinity ligand for IgG interaction, this limitation might be circumvented by further structural optimizations of the PICCO molecule, synthesis of vaccines with multiple copies, conjugation of CZV1-1 to carriers, or use of adjuvants. Regardless, our results further indicate that the tools and approaches used here might be useful in the rational design of small molecule vaccines to stimulate high potency Zika IgG neutralizing antibodies.

[00122] In summary, by comparing the binding profiles of Zika and dengue human antibodies to a large random library of small molecules, we identified, produced, and characterized a small nonbiological synthetic molecular mimic (CZV1-1) of a dominant Zika envelope protein neutralizing epitope. Importantly, the small molecule CZV1-1 corresponds to a conformational epitope that would be difficult to synthesize from knowledge of the envelope protein structure. We have shown here that the CZV1-1 PICCO molecule can serve as a biomarker on which to base diagnostic assays for evidence of prior Zika infection. Because it is a mimic of a neutralizing epitope, it may also serve as a biomarker of immunity

to Zika. The general strategy we employed here to identify and characterize a small molecule mimic of a virus-neutralizing epitope for Zika can probably be used to find small molecule mimics of important epitopes for a wide range of other infectious and non-infectious diseases. Finally, our approach has potential impact in vaccinology as these molecules can potentially be used as a fully synthetic, non-biological, small molecule vaccine.

Example 2

[00123] Additional experiments have been performed to determine the utility of epitope surrogates, like CZV1-1, in applications including the detection of antibodies for ZIKV and stimulating an immune response to ZIKV. The results of these experiments showed that CZV1-1 can be used in an ELISA as a specific marker for Zika, that CZV1-1 is immunogenic in mice, and that immunized mouse serum has anti-CZV1-1 that reacts specifically with Zika and does not cross-react with dengue.

[00124] In these experiments, CZV1-1 cysteine was conjugated to a carrier protein BSA using commercially available kits (Imject™ Maleimide-Activated BSA, ThermoFisher Scientific, Catalog number 77116) and used for ELISA assays. For the ELISAs, plates were coated overnight with 1ug/mL of CZV1-1 conjugated to mcBSA (CZV1-1:mcBSA), CZV1-1 cysteine (unconjugated), or BSA (FIGS. 18A-C). The next day, plates were then blocked with 5% goat sera in PBS. Pooled human sera with varying immune profiles to ZIKV were diluted (1:20 to 1:540) in 1% goat sera in PBS and added to the plate. Following 1 hour of incubation, plates were washed and incubated with goat anti-human IgG conjugated to HRP as a secondary antibody. No reactivity of human sera and BSA was detected as expected (**FIG. 18C**). CZV1-1:mcBSA ELISA shows reactivity of human sera immune to ZIKV and no reactivity of human sera negative for ZIKV with the conjugated CZV1-1 (**FIG. 18A**). CZV1-1 cysteine (unconjugated) does not discriminate between ZIKV+DENV+ and ZIKV-DENV+ (**FIG. 18B**) likely due to unspecific binding to the CZV1-1 cysteine groups. For the immunizations experiments, CZV1-1 cysteine was conjugated to a carrier protein KLH using commercially available kits (Imject™ Maleimide-Activated mcKLH, ThermoFisher Scientific, Catalog number 77605). The mouse immunization scheme included the following groups of CD1 mouse: mice immunized with CZV1-1:mcKLH (n=3), mice immunized with CZV1-1 cysteine (unconjugated, n=3), and mice immunized with mcKLH (n=3). Each mouse was immunized with 50 ug of immunogen. Complete Freund's adjuvant used for the first injection, incomplete Freund's adjuvant for subsequent injections. Mice were immunized on day 0, day 21, day 42, day 49, and day 63. Mice were bled at pre-injection, day 42, day 70, and final exsanguination at day 111. **FIG. 19** shows the results of the CZV1-1 flow

cytometry-based assay (logMFI and % positive beads) using mice sera collected at day 70. Human ZIKV positive and negative serum were included as positive and negative controls for the assay. We observed strong reactions with CZV1-1 beads with both the CZV1-1:mcKLH and the CZV1-1 (unconjugated) mouse sera. CZV1-1:mcKLH and the CZV1-1 (unconjugated) mouse sera also reacted with the (CZV1-1:mcBSA) and CZV1-1 cysteine (unconjugated) ELISAs (**FIGS. 20A-C**), showing that immunized mice sera react with CZV1-1 in both the ELISA-based and flow-cytometry based assay formats. Mouse sera immunized with CZV1-1:mcKLH also reacted with ZIKV envelope protein coated on an ELISA plate, but with varying degrees (**FIG. 21**).

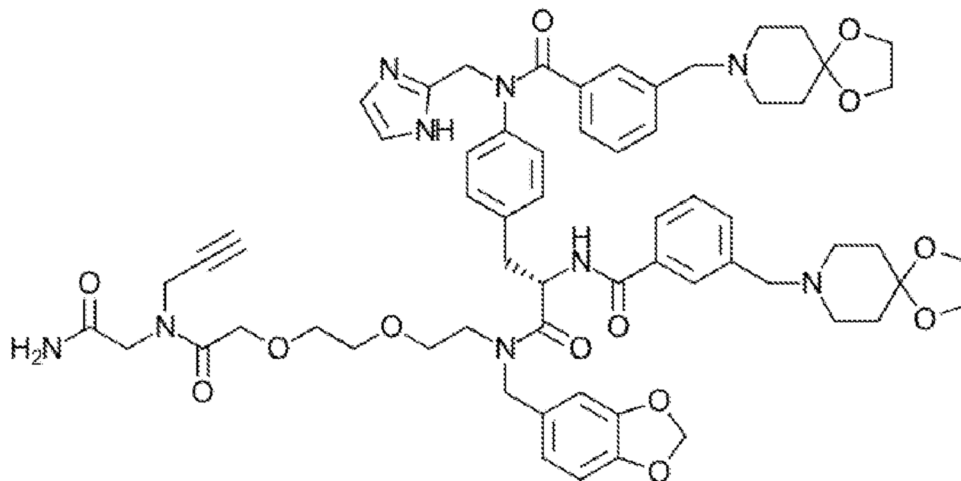
[00125] The present invention has been described with reference to certain exemplary embodiments, dispersible compositions and uses thereof. However, it will be recognized by those of ordinary skill in the art that various substitutions, modifications or combinations of any of the exemplary embodiments may be made without departing from the spirit and scope of the invention. Thus, the invention is not limited by the description of the exemplary embodiments, but rather by the appended claims as originally filed.

THE INVENTION CLAIMED IS

1. A non-peptide binding reagent for a Zika virus (ZIKV) antibody paratope.

2. The binding reagent of claim 1, wherein the binding reagent binds a paratope of an antibody to a protein in the ZIKV envelope DIII C-C' loop region.

3. The binding reagent of claim 1, having the following structure:



,

or a pharmaceutically-acceptable salt thereof, including stereoisomers and enantiomeric mixtures thereof.

4. The binding reagent of any one of claims 1-3, linked to a hapten carrier to produce an immunogen for use in eliciting antibodies directed to the binding reagent.

5. The binding reagent of claim 4, wherein the hapten carrier comprises: ZIKV HLA restricted T-cell epitopes, gelatin, serum globulin, albumin, keyhole limpet hemocyanin (KLH), ovalbumin, casein, hemocyanin, thyroglobulin, fibrinogen, cholera toxin, purified protein derivative (PPD), diphtheria toxin or a derivative thereof such as CRM197, tetanus toxin or a derivative thereof, Haemophilus protein D (PD), outer membrane protein complex of serogroup B meningococcus (OMPC), recombinant non-toxic form of Pseudomonas aeruginosa exotoxin A (rEPA), Shigella O-antigen, Staphylococcus aureus type 5 and 8 capsular polysaccharide, S. Typhi Vi antigen, liposomes, polysaccharides (*e.g.*, AECM-Ficoll, dextran, agar, carboxymethyl cellulose), synthetic polypeptides (*e.g.*, poly-L-lysine and poly-L-glutamic acid), inorganic gold particles, dendrimers, and nanodiscs, outer membrane vesicles, generalized modules for membrane antigens (GMMA), glycoengineered proteins, virus-like particles, and/or protein nanocages.

6. The binding reagent of claim 5, wherein the carrier comprises KLH.

7. The binding reagent of any one of claims 1-3, linked to a surface.

8. The binding reagent of claim 7, linked to a surface for use in an immunoassay.

9. The binding reagent of claim 8, linked to a semi-conductor polymer such as polyaniline, poly-(3-amino-4-hydroxybenzoic acid)-modified pencil carbon graphite, Electrocatalytic Prussian Blue Nanostructured Film, nitrocellulose membrane, a cotton linter membrane, a glass fiber membrane, a silicon chip, a magnetic bead, a gold bead, a microelectrode, a microfluidic device, a flow cytometry bead, and/or a multi-well plate.

10. The binding reagent of claim 8, linked to a bead or particle having a diameter of from about 10 nm to about 200 μm.

11. The binding reagent of claim 10, wherein the bead or particle comprises a polystyrene bead, a magnetic bead, or a gold particle.

12. A composition comprising the binding reagent of any one of claims 1-11 and a pharmaceutically acceptable adjuvant or excipient.

13. The composition of claim 12, contained within a vial that optionally comprises a pierceable septum, such as a pierceable cap.

14. An immunoassay device comprising the binding reagent of any one of claims 7-11.

15. The immunoassay device of claim 14, in the form of a lateral flow device.

16. A method of detecting an antibody to ZIKV in serum obtained from a patient, comprising:

incubating the binding reagent of any of one claims 1-6 with a sample obtained from the patient; and

subjecting the binding reagent and serum to a binding assay.

17. The method of claim 16, wherein the binding reagent further comprises a tag.

18. The method of claim 17, wherein the tag is a fluorescent tag.

19. The method of any one of claims 16-18, wherein the binding assay is one or more of an enzyme-linked immunosorbent assay (ELISA), a fluorescence activated cell sorting assay (FACS), an antibody-binding assay, a competitive binding assay, and/or immunoprecipitation.

20. A kit comprising the binding reagent of any one of claims 1-6 and one or more reagents for a binding assay.

21. A method of eliciting an immune response to ZIKV in a patient, comprising administering the binding reagent of any one of claims 1-6 to the patient in one or more doses such that an immune response to ZIKV is raised in the patient.

22. A method of identifying an antibody fragment specific to ZIKV, comprising screening a display library, such as a phage display library, with the binding reagent of any one of claims 1-6 and identifying one or more antibody fragment clones from the display library that specifically bind to ZIKV.

23. A method of isolating an antibody to ZIKV in serum obtained from a patient, comprising:

linking the binding agent of any of one claims 1-3 to a surface;

incubating the binding reagent with a solution containing antibodies; and

washing the surface-linked binding agent to dissociate bound antibodies from the binding reagent.

24. A composition comprising antibodies isolated by claim 23, and a pharmaceutically acceptable excipient.

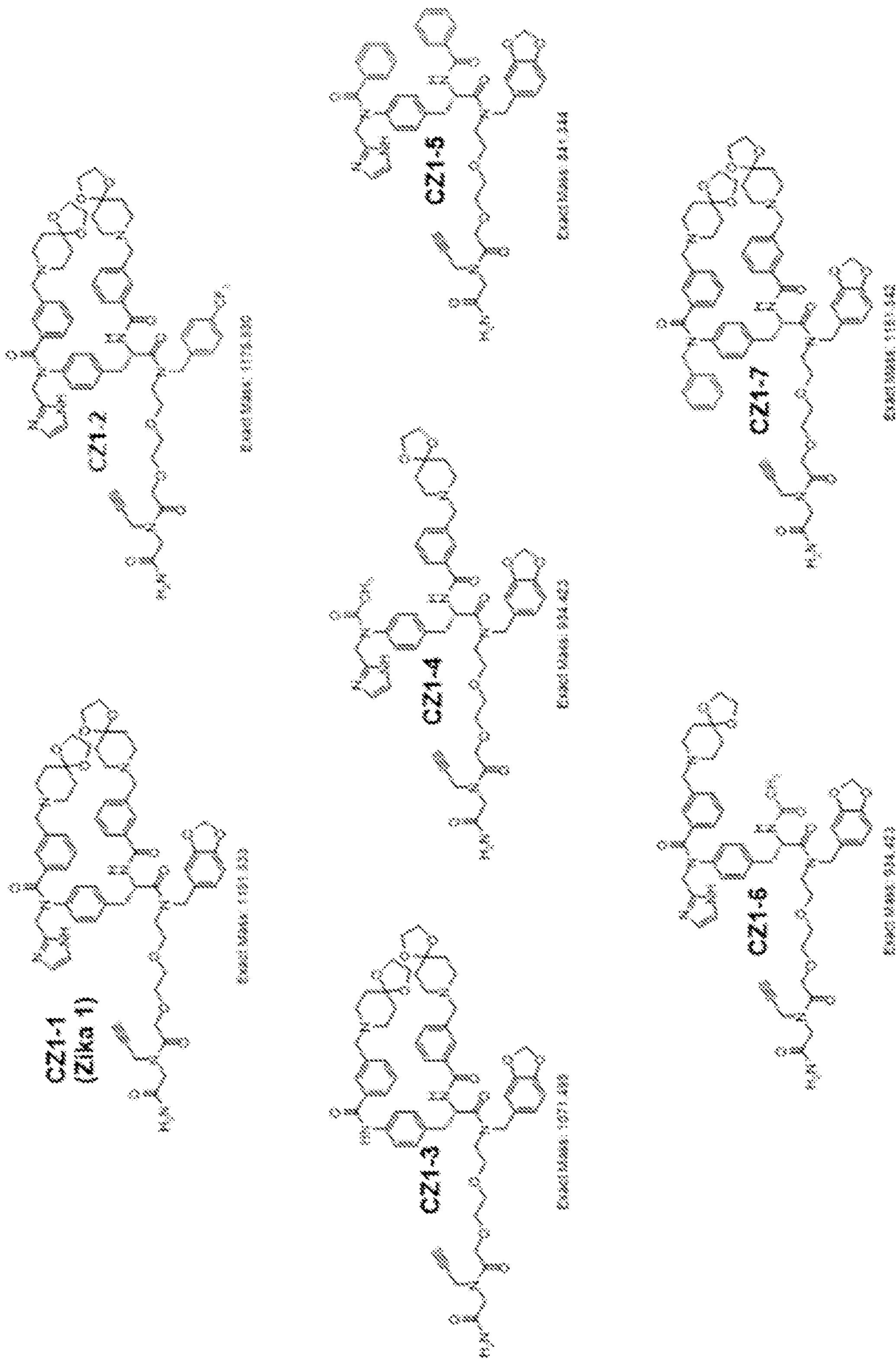


FIG. 1

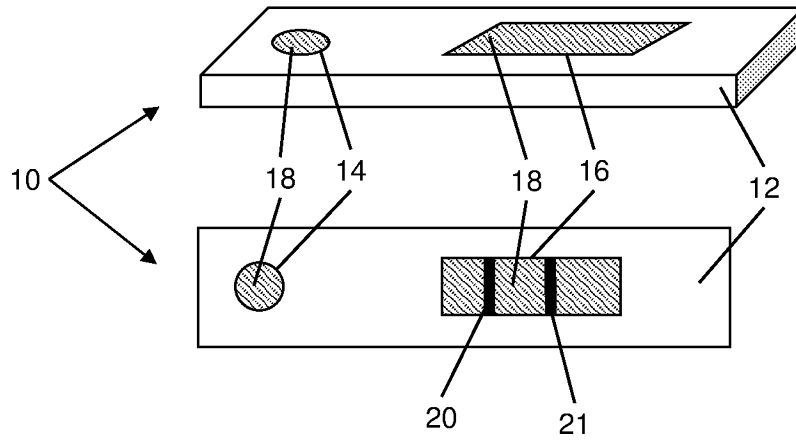


FIG. 2

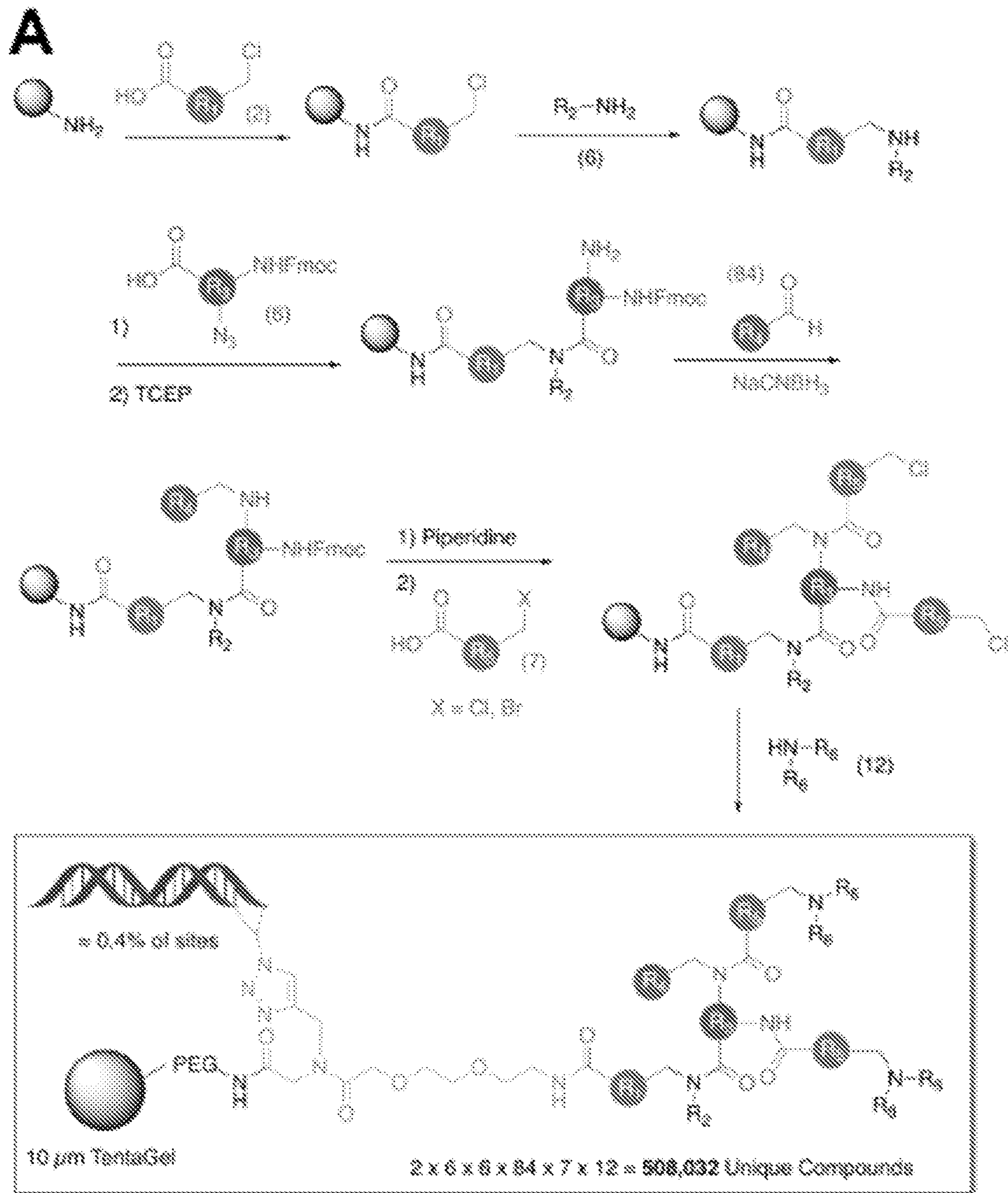


FIG. 3

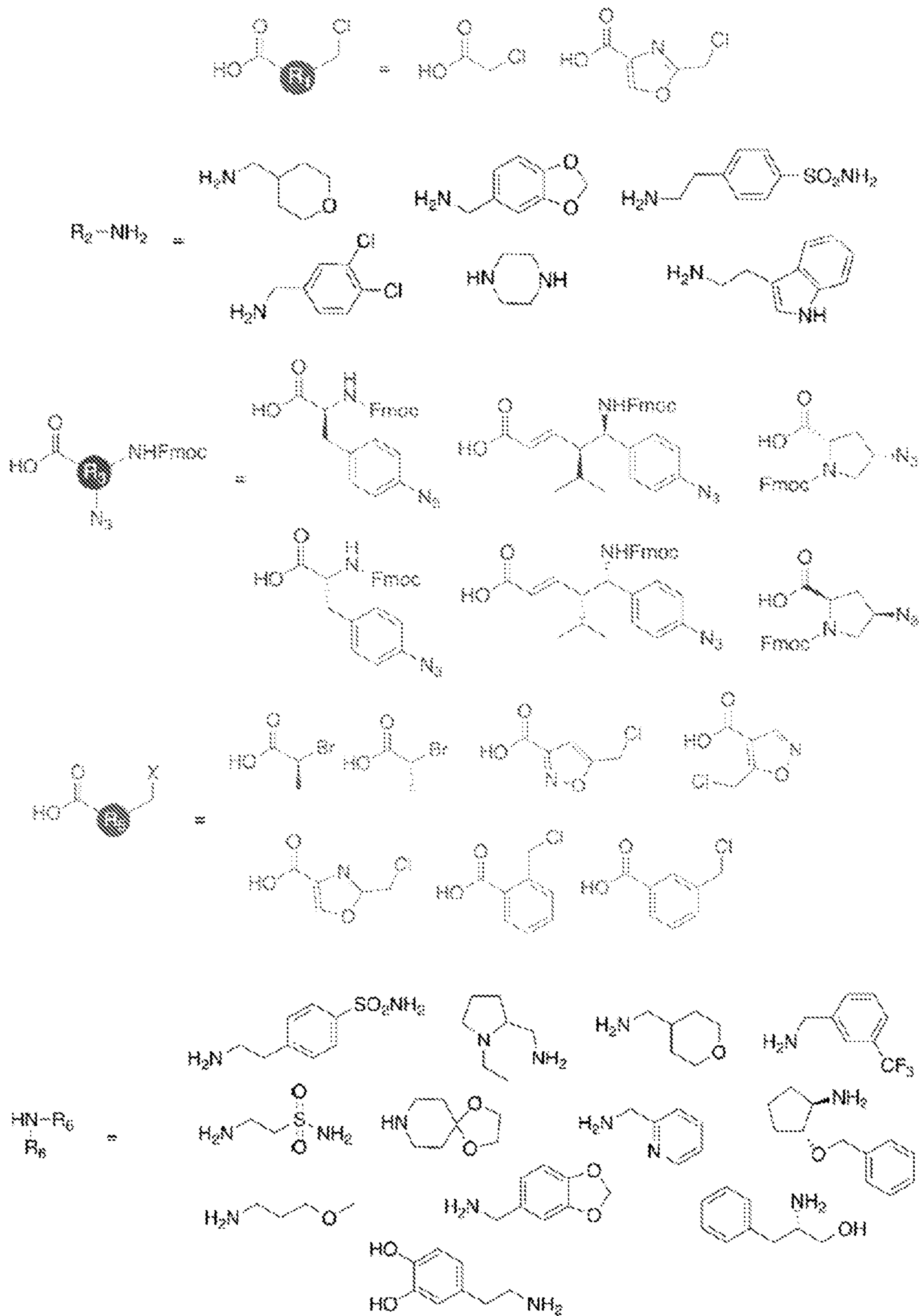


FIG. 4

FIG. 5

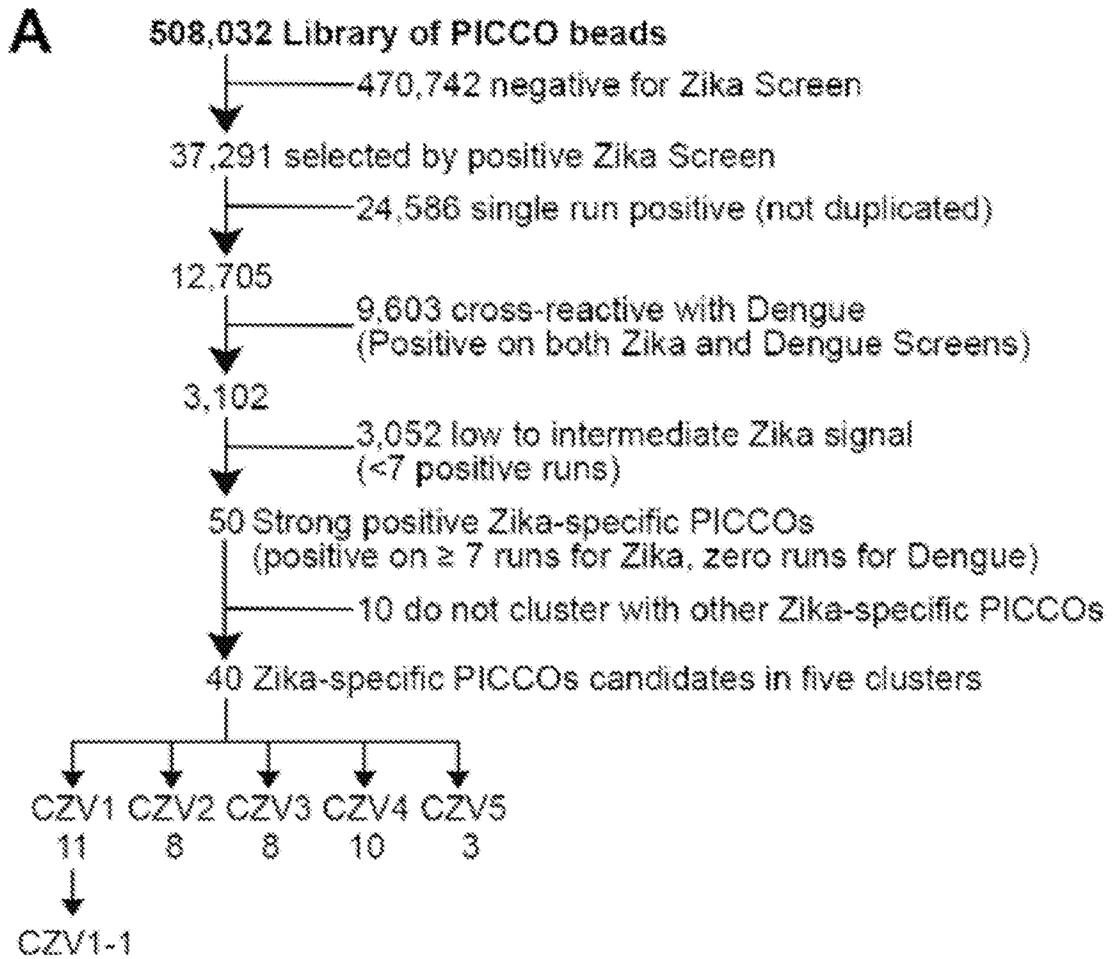


FIG. 6

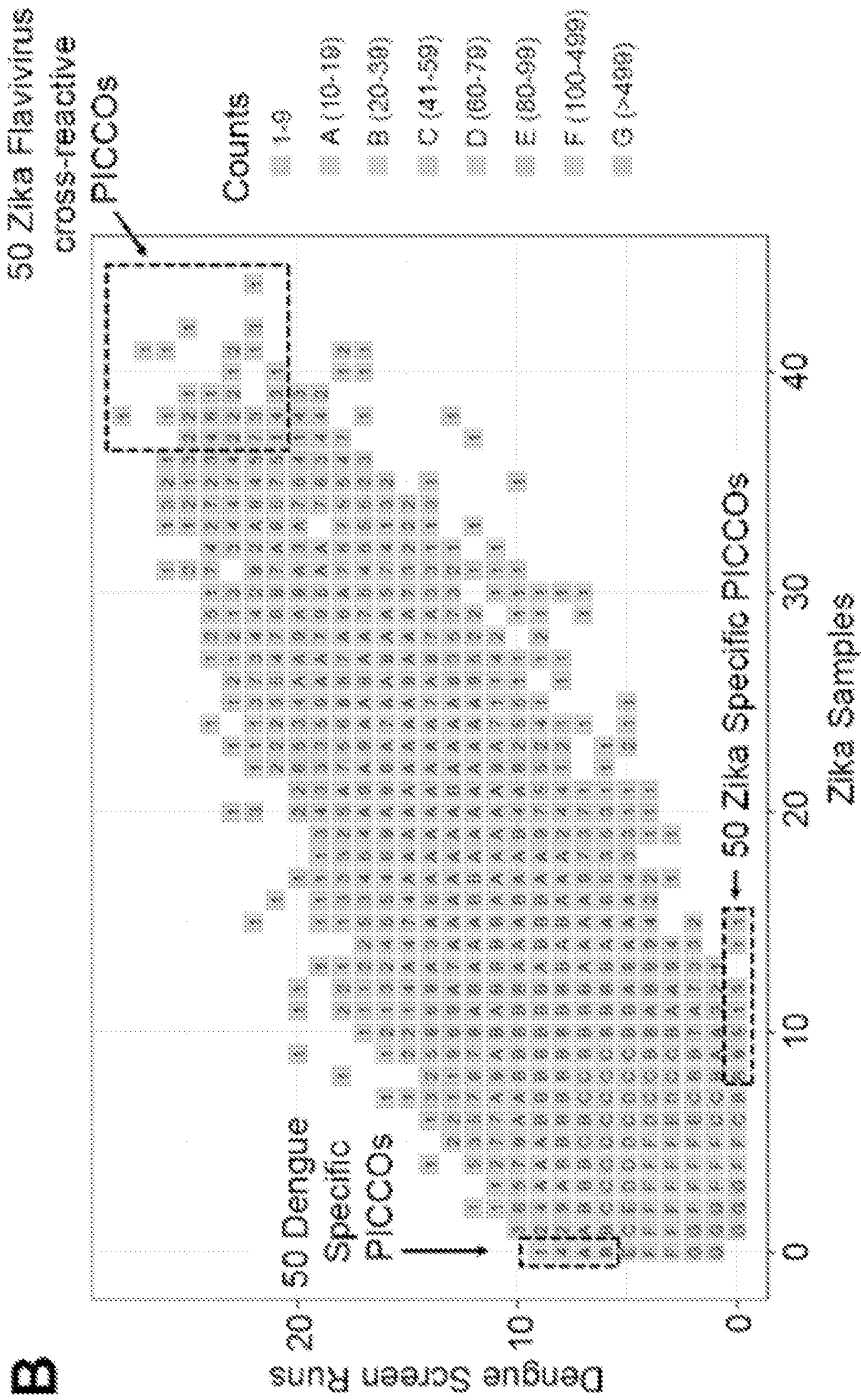


FIG. 7

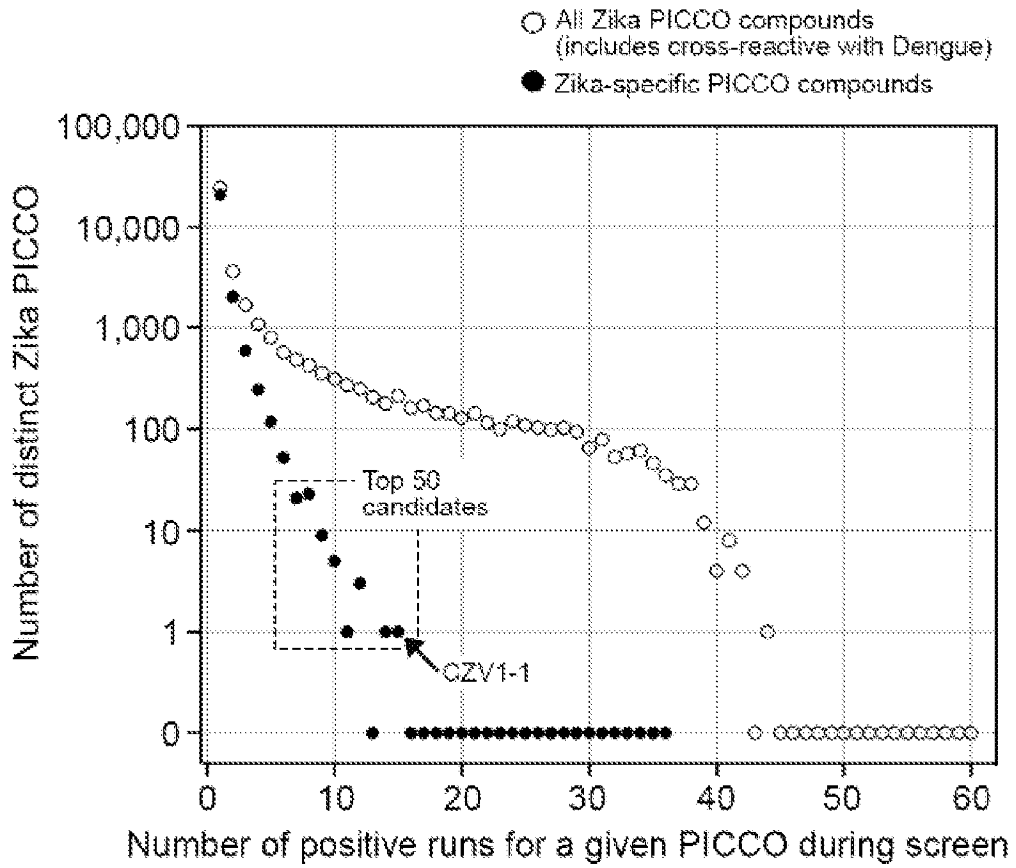


FIG. 8

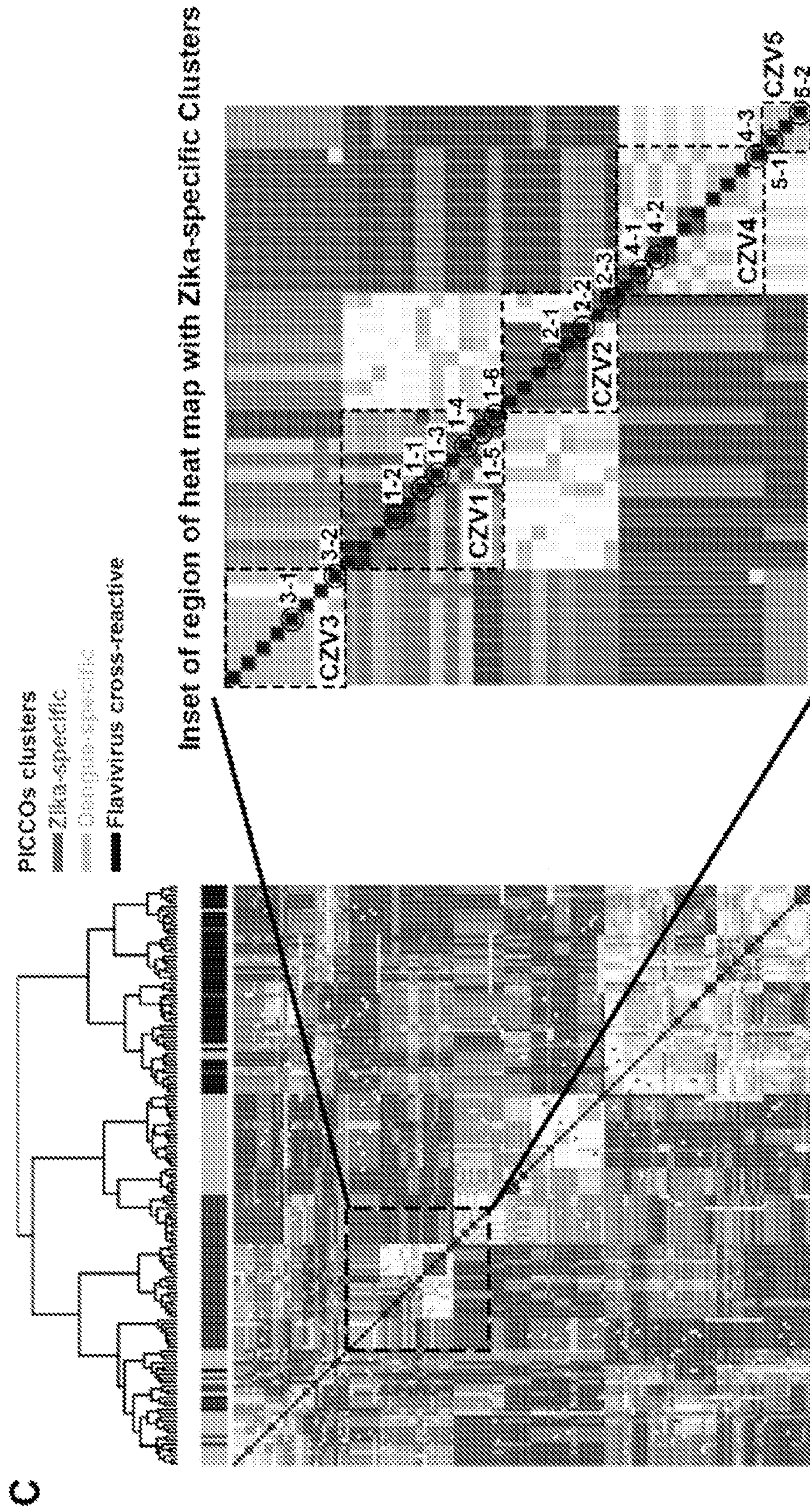


FIG. 9

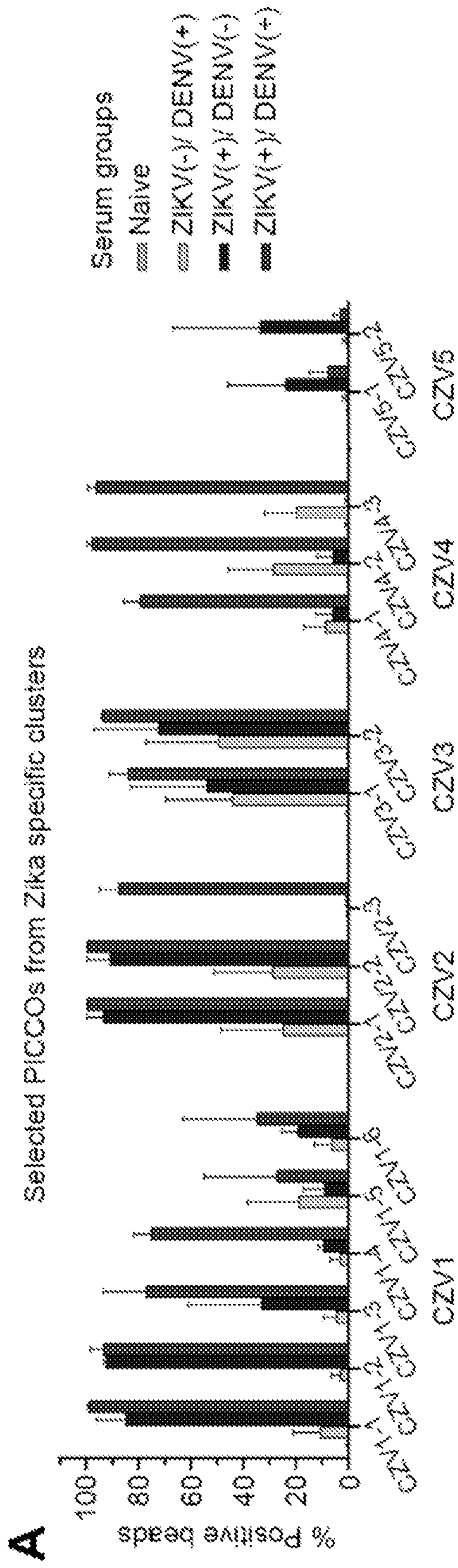


FIG. 10

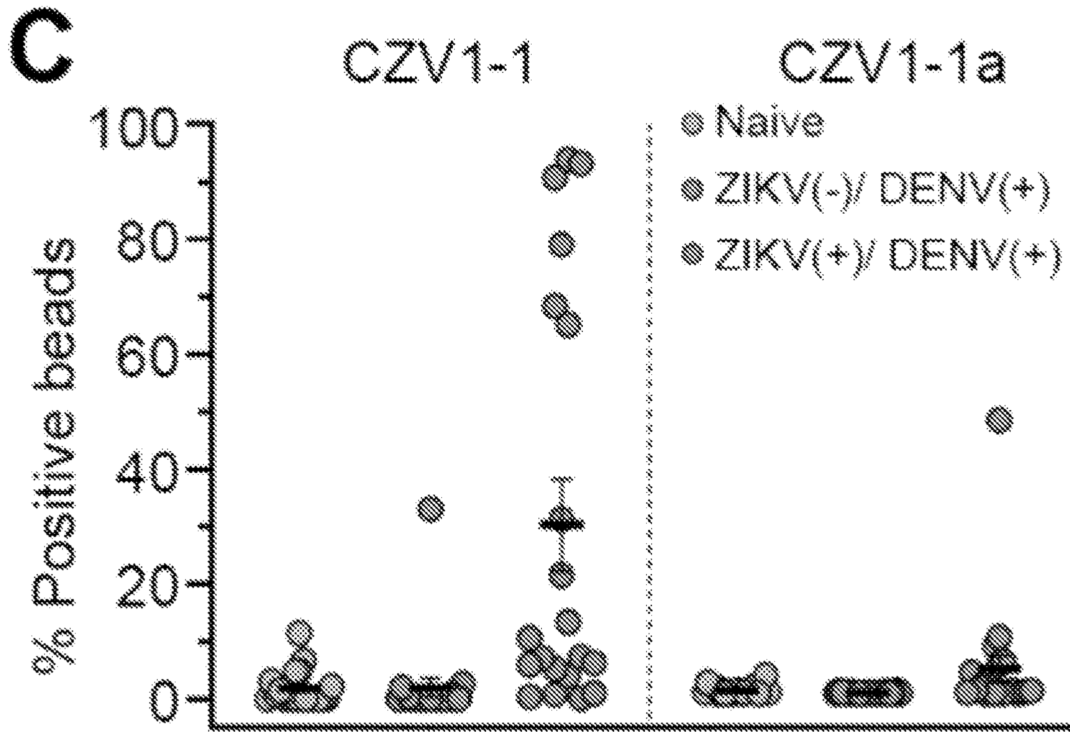


FIG. 11

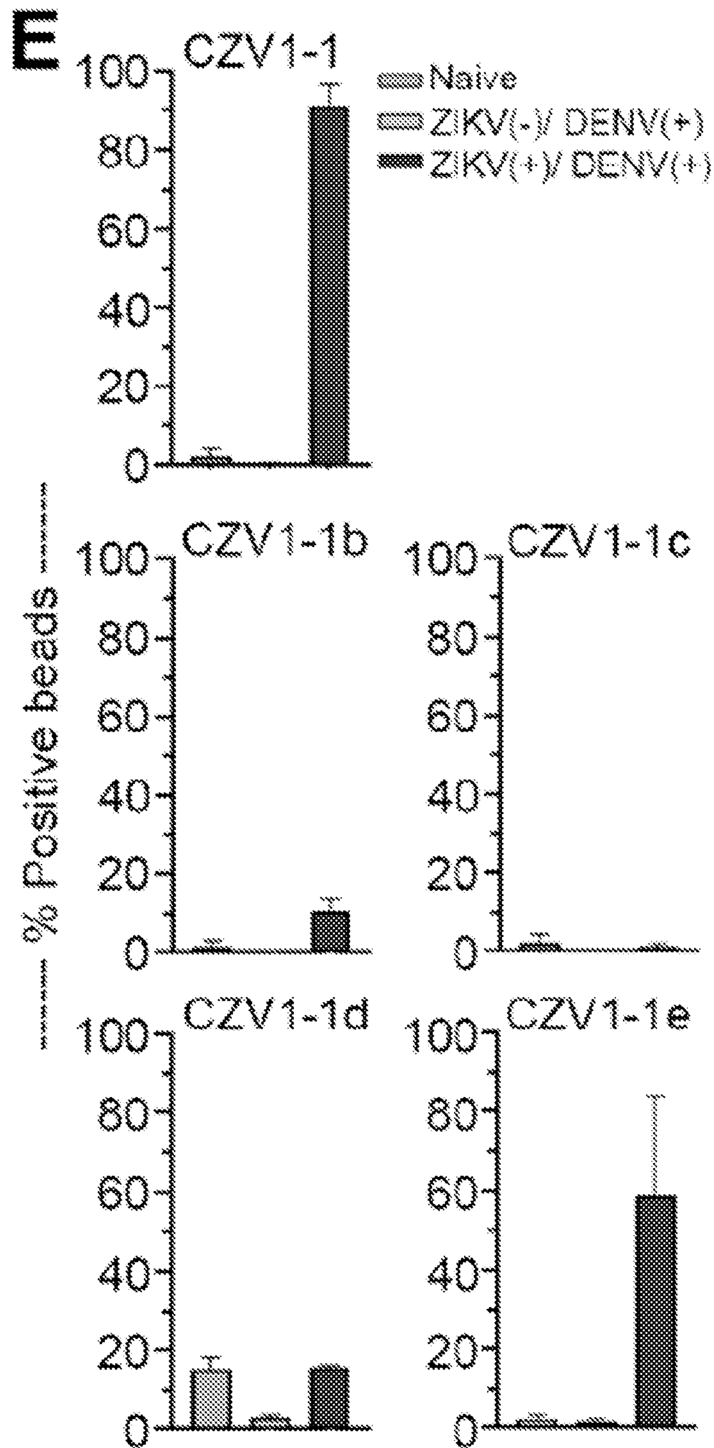


FIG. 12

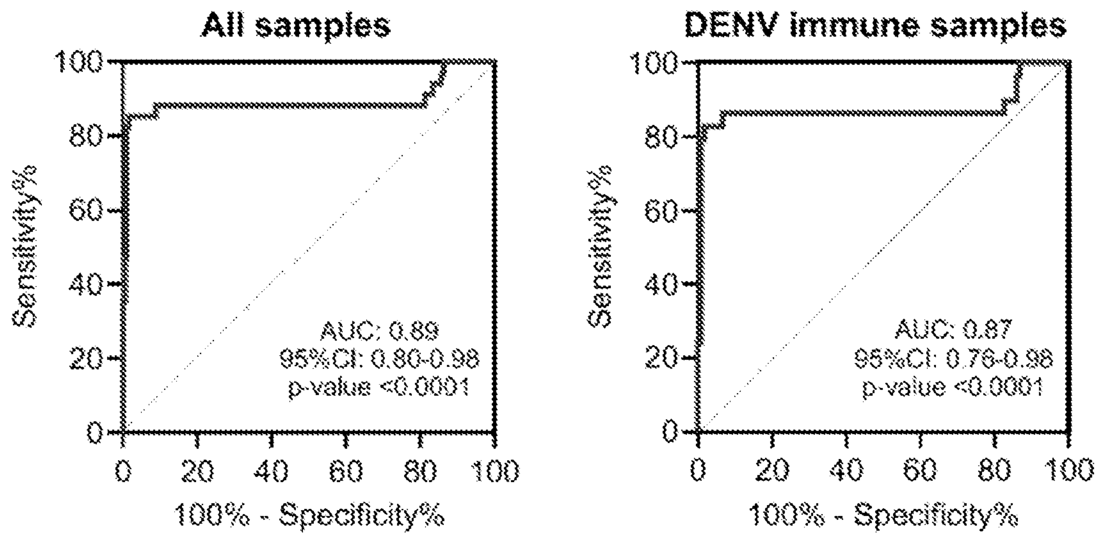


FIG. 13

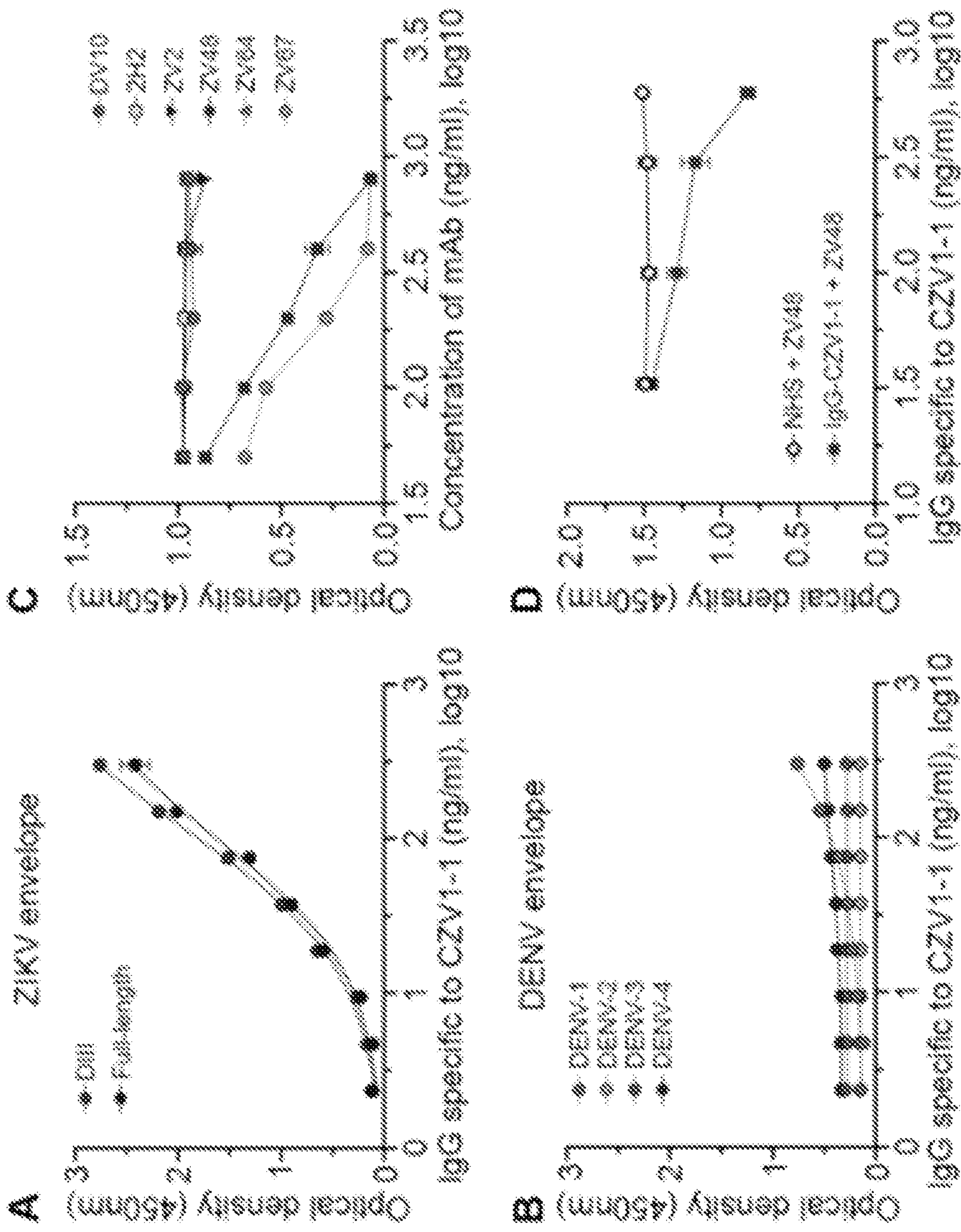


FIG. 14A

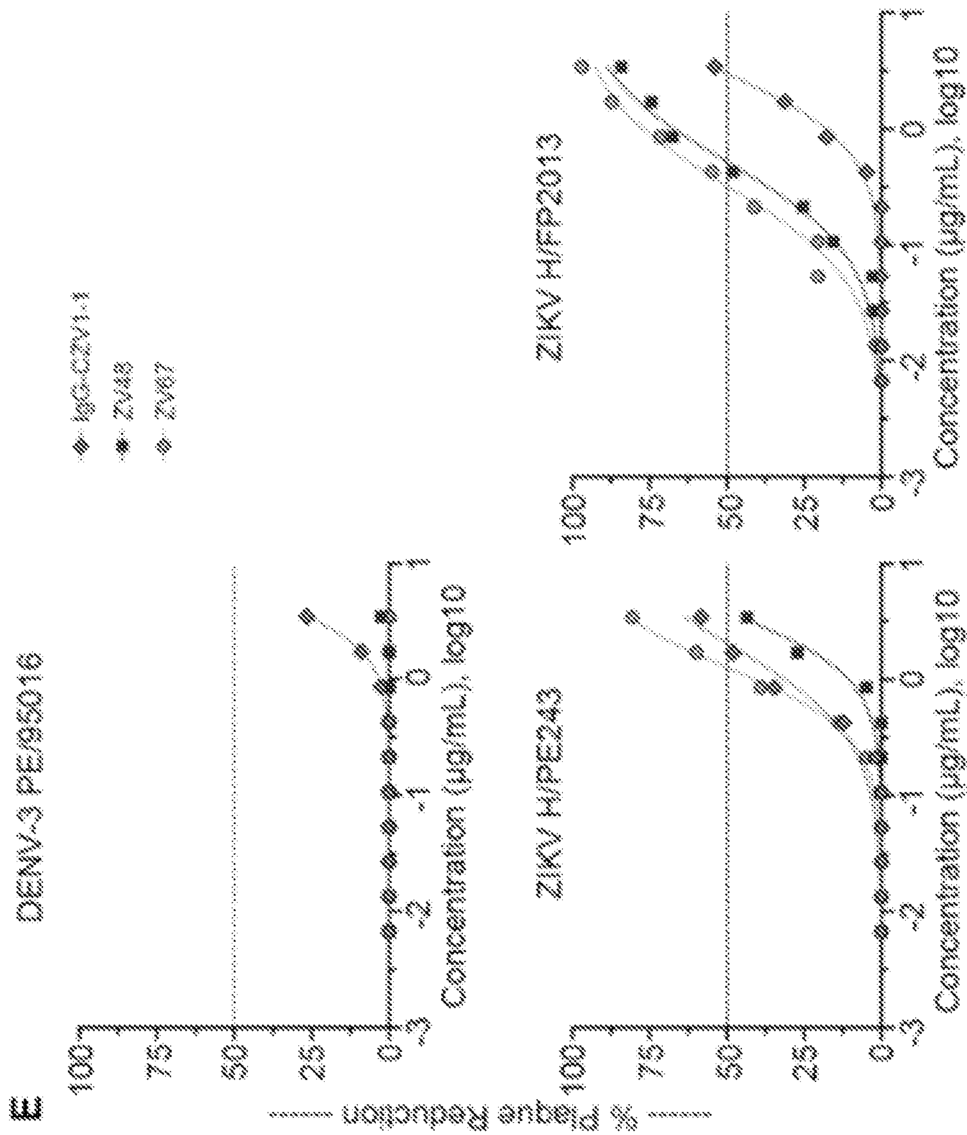


FIG. 14B

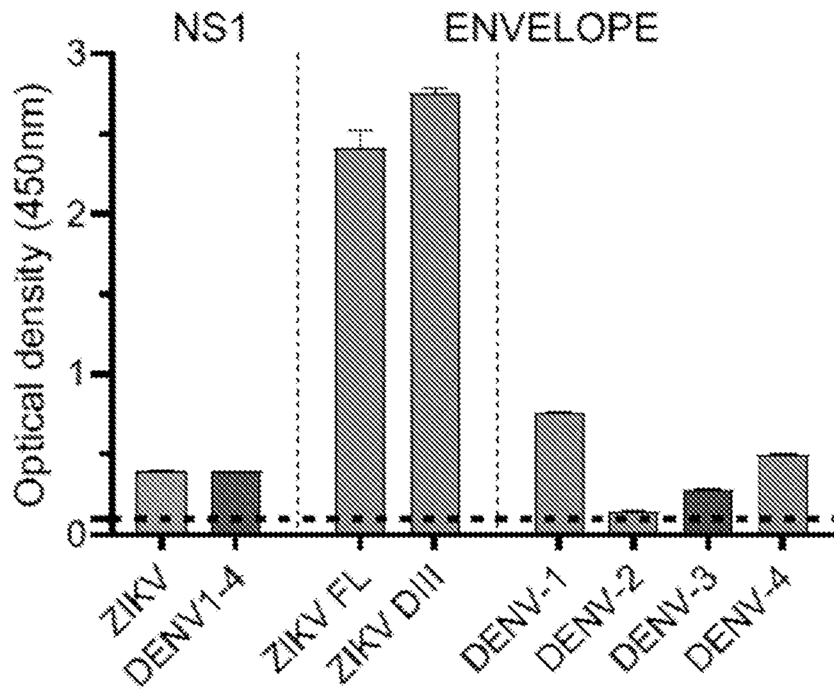


FIG. 15

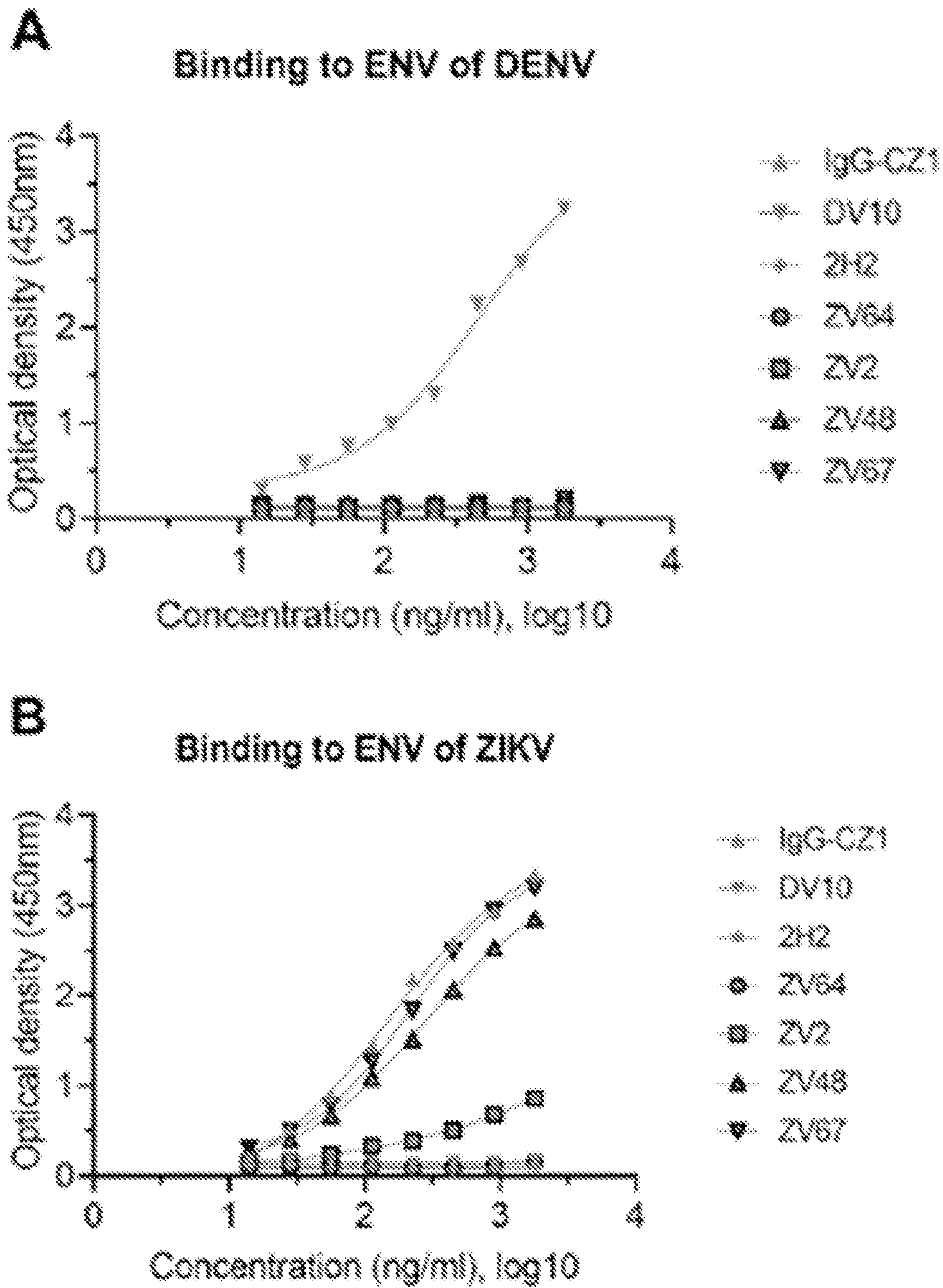


FIG. 16

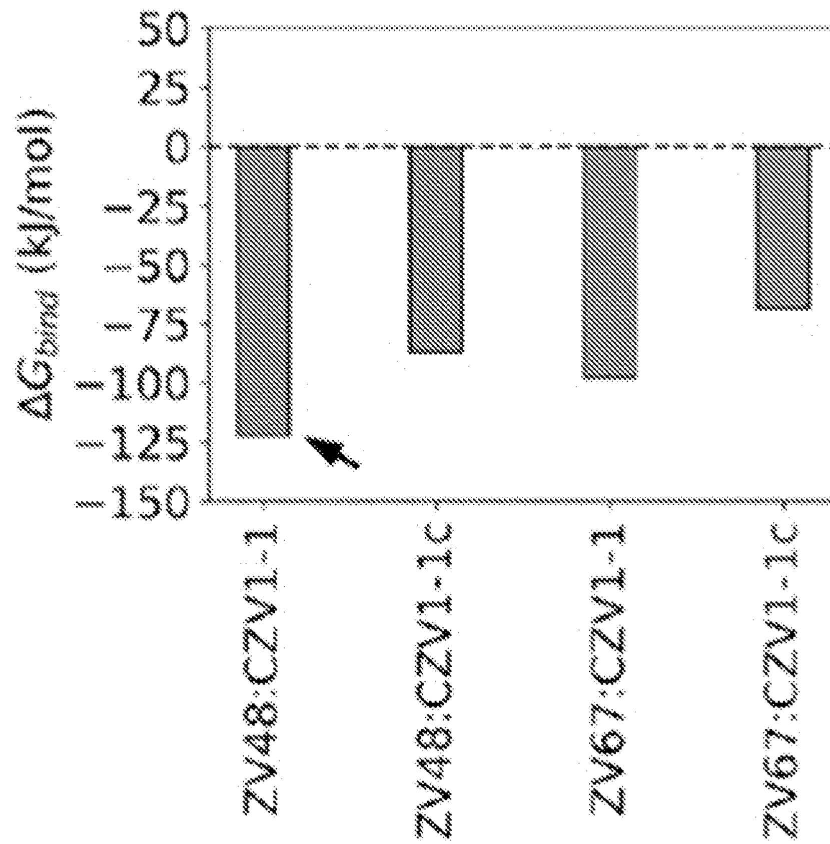


FIG. 17

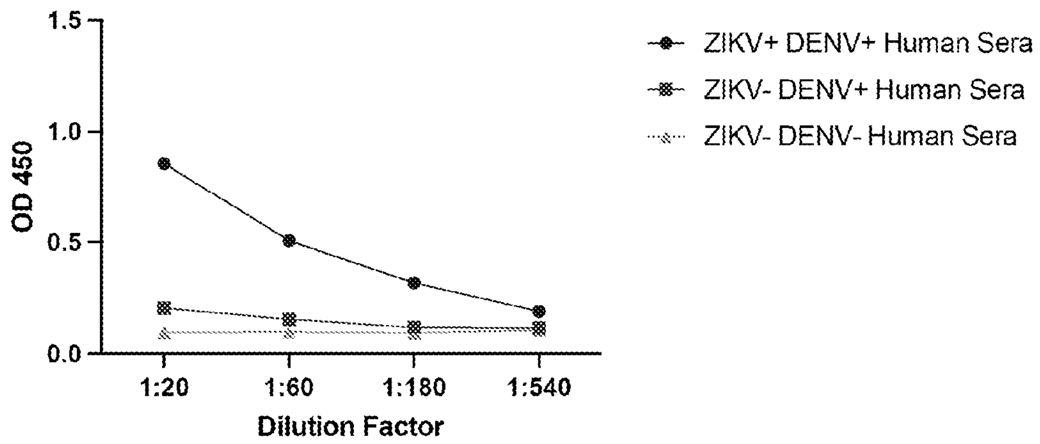


FIG. 18A

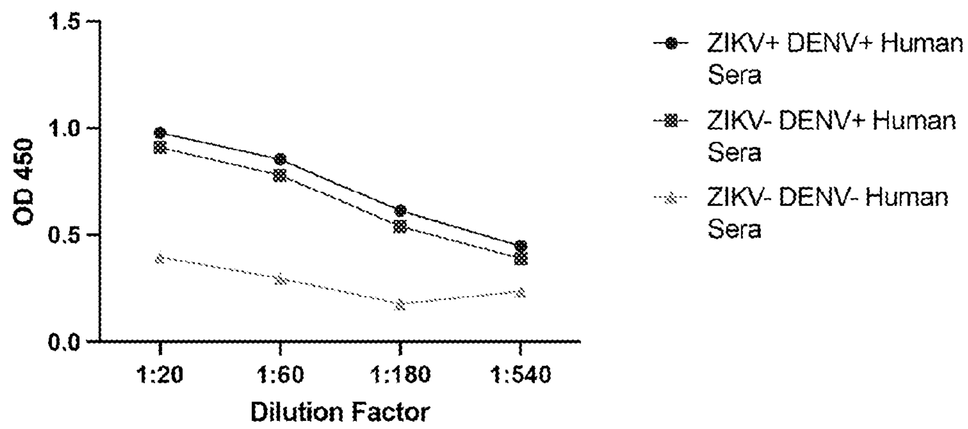


FIG. 18B

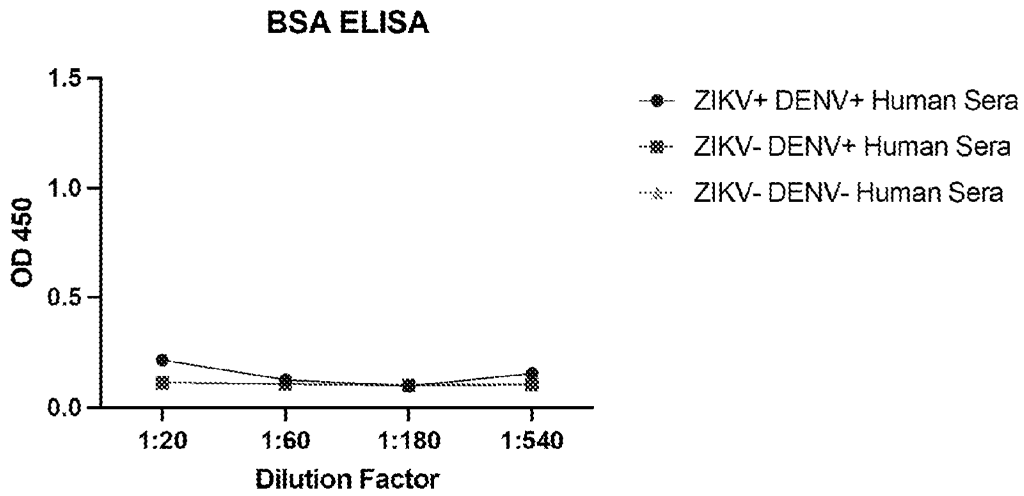


FIG. 18C

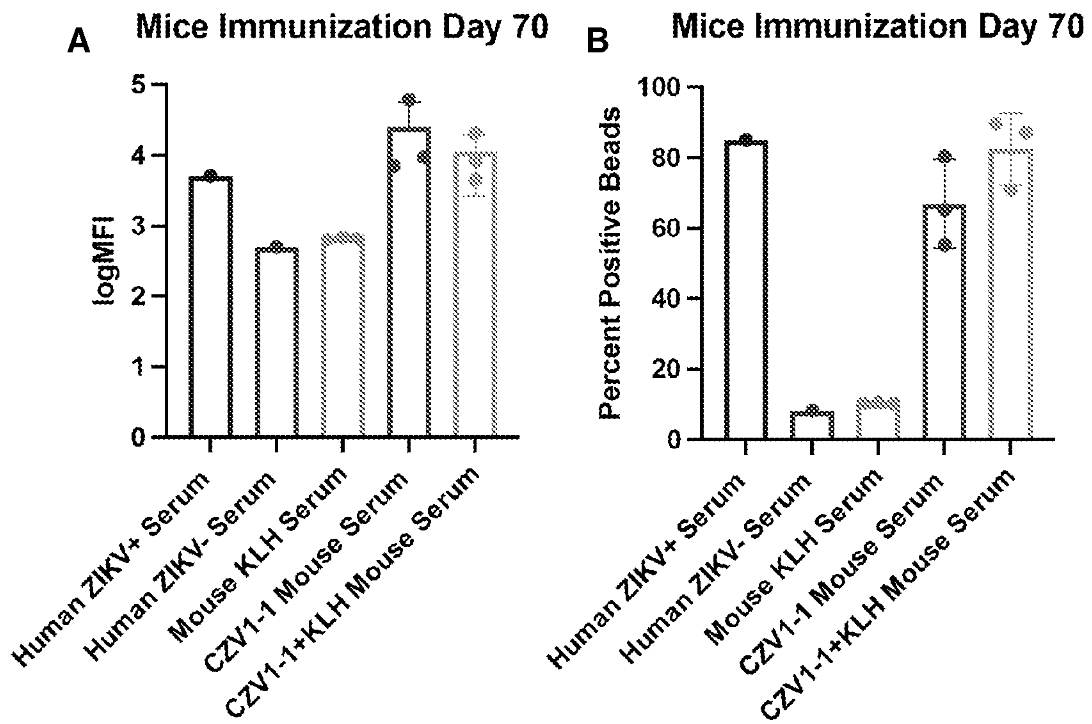


FIG. 19

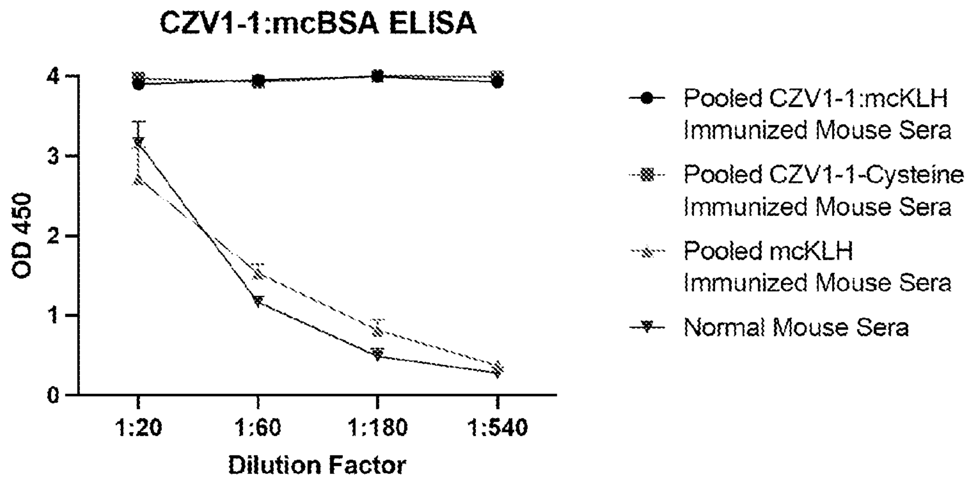


FIG. 20A

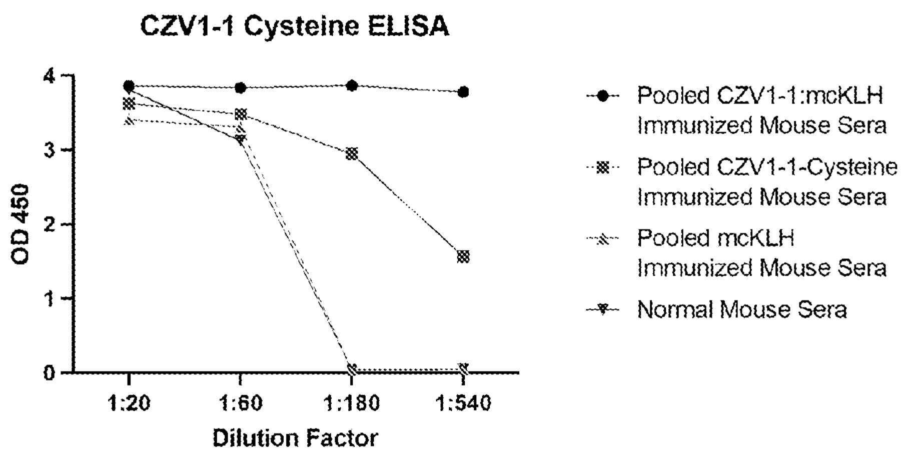


FIG. 20B

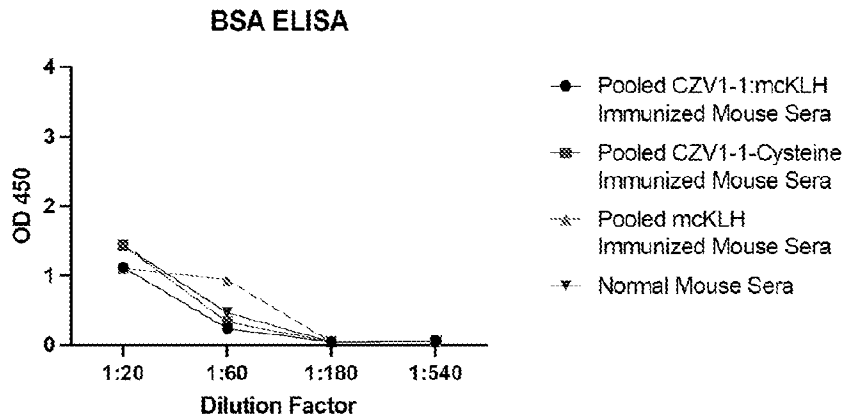


FIG. 20C

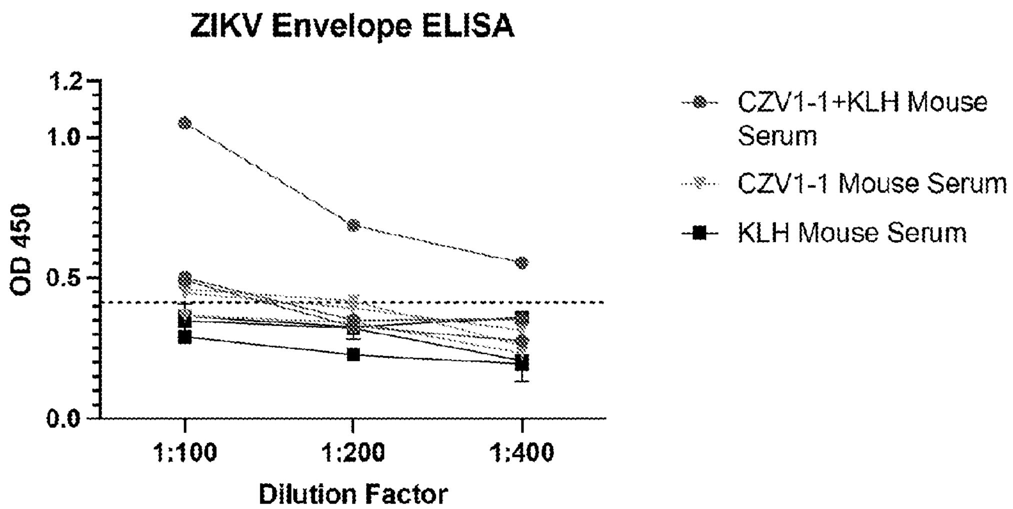


FIG. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/061012

A. CLASSIFICATION OF SUBJECT MATTER

C07D 491/113(2025.01)i; ***A61K 31/438***(2025.01)i; ***A61P 31/14***(2025.01)i; ***G01N 33/569***(2025.01)i
CPC:C07D 491/113; A61K 31/438; A61P 31/14; G01N 33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07D 491/113; A61K 31/438; A61P 31/14; G01N 33/569
CPC:C07D 491/113; A61K 31/438; A61P 31/14; G01N 33/569

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases consulted: Google Patents, CAPLUS, REGISTRY, PubMed, Google Scholar, Orbit, AI based search, SciFinder Search terms used: Zika, ZIKV, epitope, epitope surrogate, paratope, antibody, antibody formation, antigen-binding site, non-peptide, small-molecule, small-compound, antibody-binding compound, Flaviviridae, Dengue, DENV, virus, viral.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Mendes, Kimberly R., et al. "High-throughput identification of DNA-encoded IgG ligands that distinguish active and latent mycobacterium tuberculosis infections." ACS chemical biology 12.1 (2017): 234-243.# DOI: <10.1021/acschembio.6b00855>. Published: 29 November 2016. (2016/11/28)	1,4-24
Y	Particularly abstract, introduction, figure 1 and references 11-14.	2
X	Kodadek, T. and McEnaney, P.J., "Towards Vast Libraries of Scaffold-Diverse, Conformationally Constrained Oligomers." Chemical Communications 52.36 (2016): 6038-6059. DOI: <10.1039/C6CC00617E>. Published: 21 March 2016. Retrieved from URL: <https://pmc.ncbi.nlm.nih.gov/articles/PMC4846527/pdf/nihms771758.pdf>. (2016/03/21)	1,4-24
Y	Abstract and figures 13, 15 and 16.	2
A	WO 2022/224035 A2 (POLYNEURON PHARMACEUTICALS AG [CH])27 October 2022 (2022-10-27)	1-24

☑ Further documents are listed in the continuation of Box C.   ☑ See patent family annex.

* Special categories of cited documents:
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"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 2025	**27 March 2025**

Name and mailing address of the ISA/IL

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Authorized officer

LAJCHER Leah Rachel

Telephone No.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Zhao, H., et al. "Structural basis of Zika virus-specific antibody protection." Cell 166.4 (2016): 1016-1027. DOI: <10.1016/j.cell.2016.07.020>. Published online: 27 July 2016. Retrieved from URL: <https://www.cell.com/cell/pdfExtended/S0092-8674(16)30927-8>. (2016/07/27) Abstract.	2
A	Nguyen, A. T. V., et al. "Development of a peptide aptamer pair-linked rapid fluorescent diagnostic system for Zika virus detection." Biosensors and Bioelectronics 197 (2022): 113768. DOI: <10.1016/j.bios.2021.113768>. Published online: 02 November 2021. Retrieved from URL: <https://www.sciencedirect.com/science/article/pii/S095 6566321008058>. (2021/11/02) Abstract.	1-24
P,X	Castanha, P.M.S. et al., "Identification and characterization of a nonbiological small-mol ecular mimic of a Zika virus conformational neutralizing epitope." Proceedings of the National Academy of Sciences 121.21 (2024): e2312755121. DOI: <10.1073/pnas.231 2755121>. Published: 14 May 2024. Retrieved from URL: <https://www.pnas.org/doi/epub/ 10.1073/pnas.2312755121>. (2024/05/14) The whole document.	1-24

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2024/061012

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
WO 2022/224035 A2	27 October 2022	WO 2022224035 A2	27 October 2022
		WO 2022224035 A3	01 December 2022