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respectively

FIG. 31 illustrates a side-by-side sequence comparison of SEQ ID Nos. 106, 112, and 122. "*" represents complete homology across all sequences at a given sequence position, while ".", " " or spaces refer to conservative, moderate, or very different amino acid mutations across the sequences at a given sequence position respectively

FIG. 32 shows % fasting blood glucose levels from Day 0 to Day 7 for N#1 cat dosed subcutaneously on Day 0 (0.16 mg/kg) with the homodimer of SEQ ID No. 122.

FIG. 33 shows % fasting blood glucose levels from Day 0 to Day 7 for N#1 cat dosed subcutaneously on Day 0 (0.16 mg/kg) with the homodimer of SEQ ID No. 38, in addition to the times that the cat was given food.

FIG. 34 shows the anti-drug antibody titer (µg/mL) for N#1 cat dosed subcutaneously on Day 0 (0.16 mg/kg), Day 14 (0.16 mg/kg), Day 28 (0.11 mg/kg), and Day 42 (0.09 mg/kg) with the homodimer of SEQ ID No. 38.

FIG. 35 shows % fasting blood glucose levels from Day 0 to Day 7 for N#4 cat dosed subcutaneously on Day 0 (0.16 mg/kg) with the homodimer of SEQ ID No. 124.

FIG. 36 shows average % fasting blood glucose levels from Day 0 to Day 7 for N#3 cats dosed subcutaneously on Day 0 (0.16 mg/kg) with the homodimer of SEQ ID No. 40.

FIG. 37 shows average % fasting blood glucose levels from Day 7 to Day 14 for N#9 cats dosed subcutaneously on Day 7 (0.20 mg/kg) with the homodimer of SEQ ID No. 40.

FIG. 38 illustrates the "full aa sequence" of a comparative fusion protein (SEQ ID No. 32) and its corresponding nucleic acid sequence (SEQ ID No. 31).

FIG. 39 illustrates the "full aa sequence" of a comparative fusion protein (SEQ ID No. 34) and its corresponding nucleic acid sequence (SEQ ID No. 33).

FIG. 40 illustrates the "full aa sequence" of a fusion protein (SEQ ID No. 36) and its corresponding nucleic acid sequence (SEQ ID No. 35).

FIG. 41 illustrates the "full aa sequence" of a comparative fusion protein (SEQ ID No. 38) and its corresponding nucleic acid sequence (SEQ ID No. 37).

FIG. 42 illustrates the "full aa sequence" of a comparative fusion protein (SEQ ID No. 40) and its corresponding nucleic acid sequence (SEQ ID No. 39).

DETAILED DESCRIPTION

[0014] An insulin treatment that requires less frequent dosing (e.g., once-weekly injections) would be less burdensome on the owners, leading to better compliance, fewer instances of euthanasia, and better outcomes for the pets. For a given species (e.g., dog or cat), a molecule suitable for an ultra-long acting treatment for diabetes should be manufacturable in mammalian cells, for example human embryonic kidney (HEK, e.g. HEK293) cells, with an acceptable titer of the desired homodimer product (e.g., greater than 50 mg/L homodimer titer from transiently transfected HEK cells, greater than 75 mg/L from transiently transfected from HEK cells, greater than 100 mg/L from transiently transfected HEK cells, etc.). Only candidates with a homodimer titer of greater than 50 mg/L are considered useful in the present invention, because experience has demonstrated that homodimer titers less than this level will not likely result in commercial production homodimer titers in Chinese hamster ovary (CHO) cells that meet the stringent low manufacturing cost requirements for veterinary products. In addition, the molecule must bind the insulin receptor with an appreciable affinity (e.g., IC50 less than 5000 nM, IC50 less than 4000 nM, IC50 less than 3000 nM, etc.) as measured in the 4°C IM-9 insulin receptor binding assay. Based on experience, only molecules exhibiting insulin receptor activity (IC50 values less than 5000 nM are deemed likely to exhibit the requisite bioactivity in the target species. The molecule must also demonstrate sustained bioactivity in vivo (e.g., demonstrate glucose lowering activity greater than about 2 hours, 6 hours, 9 hours, 12 hours, 18 hours, 1 day, 1.5 days, 2 days, 2.5 days, 3 days, 4 days, 5 days, 6 days, 7 days, or longer) to justify less frequent dosing. The molecule must also demonstrate prolonged system residence on time in the target animal (e.g., the serum half-life must be greater than 3 days, or longer). The bioactive potency and duration of the bioactivity may be quantitatively assessed by calculating the area over the percent fasting blood glucose (%FBGL) curve normalized to a given dose in mg/kg (NAOC) with units of %FBGL days/kgmg as described in Example 11. The NAOC increases with a greater drop in %FBGL, which is the case where the molecule demonstrates increased bioactivity, and when the %FBGL takes longer to return to 100%, which is the case where the insulin-Fc fusion protein demonstrates increased duration of action. To be useful as described herein, a molecule must demonstrate a sufficiently high NAOC value (e.g., preferably NAOC greater than 150 %FBGL days/kgmg, more preferably NAOC greater than 200 %FBGL days/kgmg, and even more preferably NAOC greater than 250 %FBGL days/kgmg). Based on experience, at NAOC values greater than 150 %FBGL days/kgmg, the dose requirements in the target species will be sufficiently low so as to reach an acceptable treatment cost. Lastly, to be useful for treating a chronic disease such as diabetes, the molecule must not induce the production of anti-drug antibodies, especially antibodies that neutralize the bioactivity of the molecule after repeated dosing. Therefore, the molecule must demonstrate similar duration and extent of bioactivity (i.e., NAOC after multiple repeated doses in the target animal) (e.g., the ratio of the NAOC after the third weekly subcutaneous injection to the NAOC after the first weekly subcutaneous injection of the molecule (i.e., the NAOC ratio (NAOC₃/NAOC₁) after the third dose) in an order of preference greater than 0.50, greater than 0.60, greater than 0.70, greater than 0.80, or greater than 0.90 or more).

[0015] Proposed ultra-long acting insulin treatments for human clinical use comprise an insulin-Fc fusion protein making use of a human Fc fragment to prolong their action in vivo. As a human Fc fragment is expected to be immunogenic and therefore capable of inducing the production of anti-drug antibodies in companion animals (e.g., dogs or cats), the human Fc fragment must be replaced with a species-specific (e.g., canine or feline) Fc fragment. However, it was found rather unexpectedly that a simple exchange between the human Fc fragment and the species-specific (e.g., canine or feline) Fc fragment did not yield a product with an acceptable homodimer titer (e.g., a homodimer titer greater than 50 mg/L) or a sufficiently high NAOC value (e.g., a NAOC greater than 150 %FBGL days/kgmg). For example, in some cases only a specific isotype (i.e., canine IgG8 or feline IgG18) for the Fc fragment resulted in an insulin-Fc fusion protein with a high enough homodimer titer (e.g., a homodimer titer greater than 50 mg/L) and an acceptably high NAOC value (e.g., a NAOC greater than 150 %FBGL days/kgmg). In other cases, specific amino acids of the insulin polypeptide were found to be immunogenic in the target species thereby requiring site-directed mutations to the relatively small number of embodiments that were both non-immunogenic and bioactive in the target species with acceptably high NAOC values (e.g., NAOC values greater than 150 %FBGL days/kgmg) and NAOC values after the third weekly subcutaneous dose that were greater than 0.5. In further cases, when the Fc fragments were mutated to prevent glycosylation and thereby further reduce the immunogenicity of the insulin-Fc fusion proteins, it was discovered unexpectedly that the desired homodimer titers (e.g., homodimer titers greater than 50 mg/L) and NAOC values (e.g., NAOC greater values than 150 %FBGL days/kgmg). Furthermore, it was discovered that an additional mutation in the insulin component was required to produce these Fc-fused, non-glycosylated insulin-Fc fusion proteins with the desired homodimer titers (e.g., homodimer titers greater than 50 mg/L) and NAOC values (e.g., NAOC greater values than 150 %FBGL days/kgmg), while also achieving NAOC values after the third weekly subcutaneous dose that were greater than 0.5. Provided herein, therefore, are manufacturable, high purity, long-acting, bioactive, non-immunogenic insulin-Fc fusion proteins with acceptably high homodimer titers (e.g., homodimer titers greater than 50 mg/L) and NAOC values (e.g., NAOC values greater than 150 %FBGL days/kgmg), and NAOC values after the third weekly subcutaneous dose greater than 0.5, suitable for the treatment of diabetes in companion animals (e.g., dogs or cats), each of which comprises an insulin polypeptide, an Fc fragment, and a linker between the insulin polypeptide and the Fc fragment.

Definitions

[0016] As used herein, the articles "a" and "an" refer to one or more than one, e.g., to at least one, of the grammatical object of the article. The use of the words "a" or "an" when used in conjunction with the term "comprising" herein may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

[0017] As used herein, "about" and "approximately" generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Exemplary degrees of error are within 20 percent (%), typically, within 10%, and more typically, within 5% of a given range of values.

[0018] As used herein, an amount of a molecule, compound, conjugate, or substance effective to treat a disorder (e.g., a disorder described herein), "therapeutically effective amount," "effective amount" refers to an amount of the molecule, compound, conjugate, or substance which is effective, upon single or multiple dose administrations) to a subject, in treating a subject, or in curing, alleviating, relieving or improving a disorder (e.g., a disorder described herein) beyond that expected in the absence of such treatment.

[0019] As used herein, the term "analog" refers to a compound or conjugate (e.g., a compound or conjugate as described herein, e.g., insulin) having a chemical structure similar to that of another compound or conjugate but differing from it in at least one aspect.

[0020] As used herein, the term "antibody" or "antibody molecule" refers to an immunoglobulin molecule (Ig), immunologically active portions of an immunoglobulin (Ig) molecule, i.e., a molecule that contains an antigen binding site that specifically binds, e.g., immunoreacts with, an antigen. As used herein, the term "antibody domain" refers to a variable or constant region of an immunoglobulin. As used herein, the term "antibody domain" refers to a variable or constant region of an immunoglobulin. It is documented in the art that antibodies comprise several classes, for example IgA, IgM, or IgG in the case of mammals (e.g., humans and felines). Classes of immunoglobulins can be further classified into different isotypes, such as IgA, IgG, IgE, and IgD for canines, or IgG1a, IgG1b, IgG1c, and IgG2 for felines. Those skilled in the art will recognize that immunoglobulin isotypes of a given immunoglobulin class will comprise different amino acid sequences, structures, and functional properties from one another (e.g., different binding affinities to Fcγ receptors). "Specifically binds" or "immunoreacts with" means that the antibody reacts with one or more antigenic determinants of the desired antigen and has a lower affinity for other polypeptides, e.g., does not react with other polypeptides.

[0021] As used herein, the term "area-under-the-curve" or "AUC" refers to the integrated area under the %FBGL vs. time curve for a subject after a given dose of an insulin-Fc fusion protein is administered. As used herein, the term "area-over-the-curve" or "AOC" is used as a measure of the biological potency of an insulin-Fc fusion protein such that the AOC equals the difference between the total possible area under the %FBGL vs. time curve and the AUC value. As used herein, the "normalized AOC" or "NAOC" is the AOC value divided by the actual dose of insulin-Fc fusion protein administered. As used herein, the term "normalized AOC ratio" or "NAOCR" is the ratio of the NAOC resulting from a particular administration of an insulin-Fc fusion protein to the NAOC resulting from the first administration of an insulin-Fc fusion protein in a series of administrations. The NAOCR thus provides a measure of the change in biological activity of an insulin-Fc fusion protein after repeated administrations.

[0022] As used herein, the term "bioactivity," "activity," "biological activity," "potency," "bioactive potency," or "biological potency" refers to the extent to which an insulin-Fc fusion protein activates the insulin receptor and/or exerts a reduction in blood glucose levels in a target subject. As used herein, "in vitro activity" or "in vitro activity" refers to the affinity with which an insulin-Fc fusion protein binds to the insulin receptor and is typically measured by the concentration at which an insulin-Fc fusion protein displaces half of an insulin reference standard from the insulin receptor in a competitive binding assay (i.e., IC50). As used herein, "in vivo activity" refers to the extent and duration of reduction in the target animal's fasting blood glucose level after administration of an insulin-Fc fusion protein.

[0023] As used herein, the term "biosynthesis," "recombinant synthesis," or "recombinantly made" refers to the process by which an insulin-Fc fusion protein is expressed within a host cell by transfecting the cell with a nucleic acid molecule (e.g., vector) encoding the insulin-Fc fusion protein (e.g., where the entire insulin-Fc fusion protein is encoded by a single nucleic acid molecule). Exemplary host cells include mammalian cells, e.g., HEK293 cells or CHO cells. The cells can be cultured using standard methods in the art and the expressed insulin-Fc fusion protein may be harvested and purified from the cell culture using standard methods in the art.

[0024] As used herein, the term "cell surface receptor" refers to a molecule such as a protein, generally found on the external surface of the membrane of a cell and which interacts with soluble molecules, e.g., molecules that circulate in the blood supply. In some embodiments, a cell surface receptor may include a hormone receptor (e.g., an insulin hormone receptor or insulin receptor (IR)) or an Fc receptor which binds to an Fc fragment or the Fc region of an antibody (e.g., an Fcγ receptor), or a Fcγ receptor, or a Fcγ receptor, or an Fc neonatal receptor, or example FcRn). As used herein, "in vitro activity" or "in vitro activity" refers to the affinity with which an insulin-Fc fusion protein binds to the insulin receptor and is typically measured by the concentration at which an insulin-Fc fusion protein displaces half of an insulin reference standard from the insulin receptor in a competitive binding assay (i.e., IC50). As used herein, "in vivo activity" or "in vivo activity" refers to the extent and duration of reduction in the target animal's fasting blood glucose level after administration of an insulin-Fc fusion protein.

[0025] As used herein, the term "fasting blood glucose level" or "FBGL" refers to the average blood glucose level in a target subject at the end of a period during which no food is administered and just prior to the time at which an insulin-Fc fusion protein is administered. As used herein, the term "percent fasting blood glucose level" or "% fasting blood glucose level" or "%FBGL" refers to the ratio of a given blood glucose level to the fasting blood glucose level multiplied by 100.

[0026] As used herein, the term "immunogenic" or "immunogenicity" refers to the capacity for a given molecule (e.g., an insulin-Fc fusion protein of the present invention) to provoke the immune system of a target subject such that after repeated administrations of the molecule, the subject develops antibodies capable of specifically binding the molecule (i.e., antibody antibodies). As used herein, the terms "neutralizing," "neutralizing antibodies," or "neutralizing anti-drug antibodies" refer to the capacity for antibodies to interfere with the compound's biological activity in the target subject. As used herein, the term "immunogenic epitopes," "immunogenic hot spots," or "hot spots" refers to the mutations or epitopes of a given molecule (e.g., an insulin-Fc fusion protein of the present invention) that are responsible for moderate or strong binding of the anti-drug antibodies.

[0027] As used herein, the term "insulin reference standard" is any one of: (i) a naturally occurring insulin from a mammal (e.g., a human, a dog, or a cat); (ii) an insulin polypeptide that does not comprise an Fc fragment; or (iii) a standard of care insulin (e.g., a commercially available insulin).

[0028] As used herein, the term "monomer" refers to a protein or a fusion protein comprising a single polypeptide. In embodiments, the "monomer" is a protein or a fusion protein, e.g., a single polypeptide, comprising an insulin polypeptide and an Fc fragment polypeptide, wherein the insulin and Fc fragment polypeptides are joined by peptide bonds to form the single polypeptide. In embodiments, the monomer is encoded by a single nucleic acid molecule.

[0029] As used herein, "N-terminus" refers to the start of a protein or polypeptide that is initiated by a single amino acid containing a free amino group that is the alpha-amino group of the amino acid (e.g. the free amino that is covalently linked to one carbon atom that is located adjacent to a second carbon atom, wherein the second carbon atom is part of the carbonyl group of the amino acid). As used herein, "C-terminus" refers to the end of a protein or polypeptide that is terminated by an amino acid containing a carboxylic acid group, wherein the carbon atom of the carboxylic acid group is located adjacent to the alpha-amino group of the amino acid.

[0030] As used herein, "pharmacodynamics" or "PD" generally refers to the biological effects of an insulin-Fc fusion protein in a subject. Specifically, herein the PD refers to the measure of the reduction in fasting blood glucose level over time in a subject after the administration of an insulin-Fc fusion protein.

[0031] As used herein, "pharmacokinetics" or "PK" generally refers to the characteristic interactions of an insulin-Fc fusion protein and the body of the subject in terms of its absorption, distribution, metabolism, and excretion. Specifically, herein the PK refers to the concentration of an insulin-Fc fusion protein in the blood or serum of a subject at a given time after the administration of the insulin-Fc fusion protein. As used herein, "half-life" refers to the time taken for the concentration of insulin-Fc fusion protein in the blood or serum of a subject to reach half of its original value as calculated from a first order exponential decay model for drug elimination. Insulin-Fc fusion proteins with greater "half-life" values demonstrate greater duration of action in the target subject.

[0032] The terms "sequence identity," "sequence homology," "homology" or "identical" in amino acid or nucleotide sequences as used herein describes that the same nucleotides or amino acid residues are found within the variant and reference sequences when a specified, contiguous segment of the nucleotide sequence or amino acid sequence of the variant is aligned and compared to the nucleotide sequence or amino acid sequence of the reference sequence. Methods for sequence alignment and for determining identity between sequences are known in the art, including the use of Clustal Omega, which organizes, aligns, and compares sequences for similarity, wherein the software highlights each sequence position and compares across all positions and assigns one of the following scores: a "*" (asterisk) for sequence positions which have a single, fully conserved residue, a "." (colon) indicates a sequence gap, meaning that no local homology exists within a particular set of comparisons within a certain range of the sequences, and an empty space " " indicates little or no sequence homology for that particular position across the compared sequences. See, for example Ausubel et al., eds. (1999) Current Protocols in Molecular Biology, Chapter 19 (Green Publishing and Wiley-Interscience, New York); and the ALIGN program (Dayhoff 1978) in Atlas of Protein Sequence and Structure, 5: Suppl. 3 (National Biomedical Research Foundation, Washington, D.C.). With respect to optimal alignment of two nucleotide sequences, the contiguous segment of the variant nucleotide sequence may have additional nucleotides or deleted nucleotides with respect to the reference nucleotide sequence. Likewise, for purposes of optimal alignment of two amino acid sequences, the contiguous segment of the variant amino acid residues may have additional amino acid residues or deleted amino acid residues with respect to the reference amino acid sequence. In some embodiments, the contiguous segment used for comparison to the reference nucleotide sequence or reference amino acid sequence will comprise at least 6, 10, 15, or 20 contiguous nucleotides, or amino acid residues, and may be 30, 40, 50, 100, or more nucleotides or amino acid residues. Corrections for increased sequence identity associated with inclusion of gaps in the variant's nucleotide sequence or amino acid sequence can be made by assigning gap penalties. Methods of sequence alignment are known in the art.

[0033] In embodiments, the determination of percent identity or "homology" between two sequences is accomplished using a mathematical algorithm. For example, the percent identity of an amino acid sequence is determined using the Smith-Waterman homology search algorithm using an affine g gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix 62. The Smith-Waterman homology search algorithm is described in Smith and Waterman (1981) Adv. Appl. Math. 2:462-491. In embodiments, the percent identity of a nucleotide sequence is determined using the Smith-Waterman homology search algorithm using a gap open penalty of 25 and a gap extension penalty of 5. Such a determination of sequence identity can be performed using, for example, the DeCypher Hardware Accelerator from TimeLogic.

[0034] As used herein, the term "homology" is used to compare two or more proteins by locating common structural characteristics and common spatial distribution of, for instance, beta strands, helices, and motifs. Accordingly, homologous protein structures are defined by spatial analysis. Measuring structural homology involves computing the geometrical-topological features of a space. One approach used to generate and analyze three-dimensional (3D) protein structures is homology modeling (also called comparative modeling or knowledge-based modeling) which works by fitting similar sequences on the basis of the fact that 3D similarity reflects 2D similarity. Homologous structures do not imply sequence similarity as a necessary condition.

[0035] As used herein, the terms "subject" and "patient" are intended to include canine and feline animals. Exemplary canine and feline subjects include dogs and cats having a disease or a disorder, e.g., diabetes or another disease or disorder described herein, or normal subjects.

[0036] As used herein, the term "titer" or "yield" refers to the amount of a fusion protein product (e.g., an insulin-Fc fusion protein described herein) resulting from the biosynthesis (e.g., in a mammalian cell, e.g., in a HEK293 cell or CHO cell) per volume of the cell culture. The amount of product may be determined at any step of the production process (e.g., before or after purification), but the yield or titer is always stated per volume of the original cell culture. As used herein, the term "product yield" or "total protein yield" refers to the total amount of insulin-Fc fusion protein expressed by cells and purified via at least one affinity chromatography step (e.g., Protein A or Protein G) and includes monomers of insulin-Fc fusion protein, homodimers of insulin-Fc fusion protein, and higher order molecular aggregates of homodimers of insulin-Fc fusion protein. As used herein, the term "percentage homodimer" or "homodimer" refers to the proportion of a fusion protein product (e.g., an insulin-Fc fusion protein described herein) that is the desired homodimer. As used herein, the term "homodimer titer" refers to the product of the %homodimer and the total protein yield after Protein A purification step reported per volume of the cell culture.

[0037] As used herein, the terms "treat" or "treating" includes having a disease or a disorder refer to subjecting the subject to a regimen of the administration of a fusion protein such as a fusion protein described herein, such that at least one symptom of the disease or disorder is cured, healed, alleviated, relieved, altered, remedied, ameliorated, or improved. Treating includes administering an amount effective to alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease or disorder, or the symptoms of the disease or disorder. The treatment may inhibit deterioration of the disease or disorder or a symptom of a disease or disorder.

Insulin-Fc Fusion Protein Components and Structure

[0038] The present disclosure relates to a composition of a fusion protein (i.e., an insulin-Fc fusion protein) comprising an insulin polypeptide linked via a peptide linker to a species-specific Fc fragment, and its use to treat diabetes in companion animals (e.g., dogs or cats). As used herein, the terms "fusion protein" and "insulin-Fc fusion protein" refer to a protein comprising more than one part, for example from different sources (different proteins, polypeptides, cells, etc.) that are covalently linked through peptide bonds. The insulin-Fc fusion proteins are covalently linked by (i) connecting the genes that encode for each part into a single nucleic acid molecule and (ii) expressing in a host cell (e.g., HEK or CHO) the protein for which the nucleic acid molecule encodes for follows (N-terminus)-insulin polypeptide-linker-Fc fragment-(C-terminus). The fully recombinant synthesis approach is preferred over methods in which the insulin polypeptide and Fc fragments are synthesized separately and then chemically conjugated. The chemical conjugation step and subsequent purification process increases the manufacturing complexity, reduce product yield, and increase cost.

[0039] As used herein, the term "dimer" refers to a protein or a fusion protein comprising two polypeptides linked covalently. In embodiments, two identical polypeptides are linked covalently (e.g., via disulfide bonds) forming a "homodimer" (diagrammatically represented in FIG. 1). Disulfide bonds are shown as dotted lines in FIG. 1; total number of disulfide bonds in actuality may be greater or less than the number shown in FIG. 1. In embodiments, the homodimer is made recombinantly inside a cell by first forming insulin-Fc fusion protein monomers and by then assembling two identical insulin-Fc fusion protein monomers into the homodimer upon further processing made the cell.

[0081] The insulin-Fc fusion protein described herein binds to the Fcγ2b receptor with an affinity that is lower than that of an insulin-Fc fusion protein reference standard as measured according to Example 8. In some embodiments, the ratio of the Fcγ2b receptor affinity of the insulin-Fc fusion protein to that of an insulin-Fc fusion protein reference standard is less than 0.50 (e.g., less than 0.40, less than 0.30, less than 0.20).

Treatment and Characteristics of Subject Selection

[0082] Described herein (but not claimed) are methods for treating diabetes (e.g., canine diabetes), comprising the administration of an insulin-Fc fusion protein (e.g., an insulin-Fc fusion protein described herein) to a subject.

[0083] A reference described herein comprises a reference treatment or reference therapy. The reference comprises a standard of care agent for diabetes treatment (e.g., a standard of care agent for canine). The reference standard may be a commercially available insulin or insulin analog. The reference standard may comprise a long-acting insulin, intermediate-acting insulin, short-acting insulin, rapid-acting insulin, short-acting, intermediate-acting, long-acting insulin, such as Vetsulin[®], Prozin[®], insulin NPH, insulin glargine (Lantus[®]), or recombinant human insulin.

[0084] A reference standard used in any method described herein includes an outcome, e.g., an outcome described herein, of a diabetes therapy (e.g., a canine diabetes therapy or a feline diabetes therapy).

[0085] A reference standard may be a level of a marker (e.g., blood glucose or fructosamine) in the subject prior to initiation of a therapy, e.g., an insulin-Fc fusion protein therapy described herein; where the subject has diabetes. The blood glucose level in a dog may be greater than 200 mg/dL (e.g., greater than 250 mg/dL, 300 mg/dL, 350 mg/dL, 400 mg/dL, or more) prior to initiation of therapy. In embodiments, the fructosamine level in a companion animal (e.g., dog or cat) is greater than 250 micromol/L, 350 micromol/L (e.g., greater than 400 micromol/L, 450 micromol/L, 500 micromol/L, 550 micromol/L, 600 micromol/L, 650 micromol/L, 700 micromol/L, 750 micromol/L, or more) prior to initiation of therapy. A reference standard may be a measure of the presence of or the progression of or the severity of the disease, or may measure the presence of or the severity of the disease symptoms prior to initiation of a therapy, e.g., an insulin-Fc fusion protein therapy described herein, e.g., where the subject has diabetes.

Pharmaceutical Compositions and Routes of Administration

[0086] Provided herein (but not claimed) are pharmaceutical compositions containing an insulin-Fc fusion protein as described herein that can be used to lower blood glucose in companion animals (e.g., dogs). The amount and concentration of the insulin-Fc fusion protein in the pharmaceutical compositions, as well as the quantity of the pharmaceutical composition administered to a subject, can be selected based on clinically relevant factors, such as medically relevant characteristics of the subject (e.g., age, sex, weight, gender, other medical conditions, and the like), the solubility of compounds in the pharmaceutical compositions, the potency and activity of the compounds, and the manner of administration of the pharmaceutical compositions. For further information on Routes of Administration and Dosage Regimes the reader is referred to Chapter 25.3 in Volume 5 of Comprehensive Medicinal Chemistry (Corwin Hansdot, Chairman of Editorial Board), Pergamon Press 1990.

[0087] Formulations of the present disclosure include those suitable for parenteral administration. The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by intravenous or subcutaneous injection.

[0088] Examples of suitable aqueous and non-aqueous carriers that may be employed in the pharmaceutical compositions of the disclosure include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required partial pressure in the case of emulsions, and by the use of surfactants, e.g., Tween-like surfactants. In some embodiments, the pharmaceutical composition (e.g., as described herein) comprises a Tween-like surfactant, e.g., polysorbate-20, Tween-20 or Tween-80. In some embodiments, the pharmaceutical composition (e.g., as described herein) comprises a Tween-like surfactant, e.g., Tween-80, at a concentration between about 0.001% and about 2%, or between about 0.005% and about 0.1%, or between about 0.01% and about 0.5%.

[0089] In some embodiments, the concentration of the insulin-Fc fusion protein in the aqueous carrier is about 3 mg/mL. In some embodiments, the concentration of the insulin-Fc fusion protein in the aqueous carrier is about 6 mg/mL. In some embodiments, the concentration of the insulin-Fc fusion protein in the aqueous carrier is about 9 mg/mL, 9 mg/mL, 12 mg/mL, 15 mg/mL, or more.

[0090] In some embodiments, the insulin-Fc fusion protein is administered as a bolus, infusion, or an intravenous push. In some embodiments, the fusion protein is administered through syringe injection, pump, pen, needle, or indwelling catheter. In some embodiments, the insulin-Fc fusion protein is administered by a subcutaneous bolus injection. Methods of introduction may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested in vivo in recent years for the controlled delivery of drugs, including proteinaceous biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-biodegradable polymers, can be used to form an implant for the sustained release of a compound at a particular target site.

Dosages

[0091] Actual dosage levels of the insulin-Fc fusion protein can be varied so as to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular subject (e.g., dog). The selected dosage level will depend upon a variety of factors including the activity of the particular fusion protein employed, the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular fusion protein employed, the age, sex, weight, condition, general health and prior medical history of the subject being treated, and like factors well known in the medical arts.

[0092] In general, a suitable dose of an insulin-Fc fusion protein will be the amount that is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous and subcutaneous doses of the insulin-Fc fusion protein for a dog will range from about 0.001 to about 1 mg per kilogram (e.g., mg/kg) of body weight per day; e.g., about 0.001 to 1 mg/kg/day; about 0.01 to 0.1 mg/kg/day; about 0.1 to 1 mg/kg/day; or about 0.1 to 1 mg/kg/day. In all other embodiments, the fusion protein is administered at a dose between 0.025 and 4 mg per kilogram of body weight per week, e.g., between 0.025 and 0.5 mg/kg/week.

[0093] The present disclosure contemplates formulation of the insulin-Fc fusion protein in any of the aforementioned pharmaceutical compositions and preparations. Furthermore, the present disclosure contemplates administration via any of the foregoing routes of administration. One of skill in the art can select the appropriate formulation and route of administration based on the condition being treated and the overall health, age, and size of the patient being treated.

EXAMPLES

[0094] The present technology is further illustrated by the following Examples, which should not be construed as limiting in any way.

GENERAL METHODS, ASSAYS, AND MATERIALS

Example 1: Synthesis and Methods of Making an Insulin-Fc Fusion Protein in HEK293 Cells

[0095] Insulin-Fc fusion proteins were synthesized as follows. A gene sequence of interest was constructed using proprietary software (LakePharma, Belmont, CA) and was cloned into a high expression mammalian vector. HEK293 cells were seeded in a shake flask 24 hours before transfection and were grown using serum-free chemically defined media. A DNA expression construct that encodes the insulin-Fc fusion protein of interest was transiently transfected into a suspension of HEK293 cells using the (LakePharma, Belmont, CA) standard operating procedure for transient transfection. After 20 hours, the cells were counted to determine the viability and viable cell count, and the titer was measured by FortiBIO[®] Opti[®] (Pall FortiBIO LLC, Fremont, CA). Additional readings were taken throughout the transient transfection production run. The culture was harvested on or after day 5.

Example 2: Synthesis and Methods of Making an Insulin-Fc Fusion Protein in CHO Cells

[0096] A CHO cell line was originally derived from CHO-K1 (LakePharma, Belmont, CA), and the endogenous glutamine synthetase (GS) genes were knocked out by recombinant technology using methods known in the art. Stable expression DNA vectors were designed and optimized for CHO expression and GS selection and incorporated into a high expression mammalian vector (LakePharma, Belmont, CA). The sequence of each completed construct was confirmed prior to initiating scale up experiments. The suspension-adapted CHO cells were cultured in a humidified 5% CO₂ incubator at 37°C in a chemically defined media (CD OptiCHO; Invitrogen, Carlsbad, CA). No serum or other animal-derived products were used in culturing the CHO cells.

[0097] Approximately 80 million suspension-adapted CHO cells, growing in CD OptiCHO media during the exponential growth phase, were transfected by electroporation using MaxCyto[®] 81x[®] system (MaxCyto, Inc., Gaithersburg, MD) with 80 µg DNA to create a stable CHO cell line for each insulin-Fc fusion protein (DNA construct contains the full-length sequence of the insulin-Fc fusion protein). After twenty-four hours, the transfected cells were counted and plated under selection for stable integration of the insulin-Fc fusion genes. The transfected cells were seeded into CD OptiCHO selection media containing between 0-100 µM methionine sulfoxime (MSX) at a cell density of 5 × 10⁶ cells/mL in a shaker flask and incubated at 37°C with 5% CO₂. During a selection process, the cells were spun down and resuspended in fresh selection media every 2-3 days until the CHO stable pool recovered its growth rate and viability. The cell culture was monitored for growth and titer.

[0098] The cells were grown to 2 × 10⁸ cells per mL. At the time of harvest for cell banking, the viability was above 95%. The cells were then centrifuged, and the cell pellet was resuspended in the CD OptiCHO media with 7.5% dimethyl sulfoxide (DMSO) to a cell count of 15 × 10⁶ cells per mL per vial. Vials were cryopreserved for storage in liquid nitrogen.

[0099] A small-scale-up production was performed using the CHO cells as follows. The cells were scaled up for production in CD OptiCHO growth medium containing 100 µM MSX at 37°C and fed every 2-4 days as needed, with CD OptiCHO growth medium supplemented with glucose and additional amino acids as necessary for approximately 14-21 days. The conditioned media supernatant harvested from the stable pool production run was clarified by centrifuge spinning. The protein was run over a Protein A (MabSelect, GE Healthcare, Little Chalfont, United Kingdom) column pre-equilibrated with binding buffer. Washing buffer was then passed through the column until the ODS20 value (NanoDrop, Thermo Scientific) was measured to be at or near background levels. The insulin-Fc fusion protein was eluted using a low pH buffer; elution fractions were collected, and the ODS20 value of each fraction was recorded. Fractions containing the target insulin-Fc fusion protein were pooled and optionally further filtered using a 0.2 µm membrane filter.

[0100] The cell line was optionally further subcloned to monoclonality and optionally further selected for high titer insulin-Fc fusion protein-expressing clones using the method of limiting dilution, a method known to those skilled in the art. After obtaining a high titer, monoclonal insulin-Fc fusion protein-expressing cell line, production of the insulin-Fc fusion protein was accomplished as described above in growth medium without MSX, or optionally in growth medium containing MSX, to obtain a cell culture supernatant containing the recombinant, CHO-made, insulin-Fc fusion protein. The MSX concentration was optionally increased over time to exert additional selectivity for clones capable of yielding higher product titers.

Example 3: Purification of an Insulin-Fc Fusion Protein

[0101] Purification of an insulin-Fc fusion protein was performed as follows. Conditioned media supernatants containing the secreted insulin-Fc fusion protein were harvested from the transiently or stably transfected HEK production runs and were clarified by centrifugation. The supernatant containing the desired insulin-Fc fusion protein was run over a Protein A or a Protein G column and eluted using a low pH gradient. Optionally, recovery of the insulin-Fc fusion proteins could be enhanced by rebinding of the initial Protein A or Protein G column eluent again onto a second Protein A or Protein G column. Afterwards, the eluted fractions containing the desired protein were pooled and buffered into 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer. A final filtration step was performed using a 0.2 µm membrane filter. The final protein concentration was calculated from the solution optical density at 280 nm. Further optional purification by ion-exchange chromatography (e.g., using an anion exchange bead resin or a cation exchange bead resin), gel filtration chromatography, or other methods was performed as necessary.

Example 4: Structure Confirmation by Non-reducing and Reducing CE-SDS

[0102] Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) analysis was performed in a LaSchiP[®] GXII (Perkin Elmer, Waltham, MA) on a solution of a purified insulin-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer, and the electropherogram was plotted. Under non-reducing conditions, the sample was run against known molecular weight (MW) protein standards, and the eluting peak represented the "apparent" MW of the insulin-Fc fusion protein homodimer.

[0103] Under reducing conditions (e.g., using beta-mercaptoethanol to break disulfide bonds of the insulin-Fc fusion homodimer), the apparent MW of the resulting insulin-Fc fusion protein monomer is compared against half the molecular weight of the insulin-Fc fusion protein homodimer as a way of determining that the structural purity of the insulin-Fc fusion protein is likely to be correct.

Example 5: Sequence Identification by LC-MS with Glycan Removal

[0104] To obtain an accurate estimate of the insulin-Fc mass via mass spectrometry (MS), the sample is first treated to remove naturally occurring glycan that might interfere with the MS analysis. 100 µL of a 2.5 mg/mL insulin-Fc fusion protein dissolved in 200 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 7.0 buffer solution is first buffer exchanged into 0.1 M Tris, pH 8.0 buffer containing 5 mM EDTA using a Zeba desalting column (Pierce, ThermoFisher Scientific, Waltham, MA). 1 µL of Phycocyanin F-enzyme (Phycocyanin F-enzyme (Phycocyanin F-enzyme) is added to this solution in order to remove N-linked glycans present in the fusion protein (e.g., glycan linked to the side chain of the asparagine located at the Cys N site), and the mixture is incubated at 37°C overnight in an incubator. The sample is then analyzed via LC-MS (NovaBiosciences, Woburn, MA) resulting in a molecular mass of the molecule which corresponds to the desired homodimer without the glycan. This mass is then further corrected since the enzymatic process used to cleave the glycans from the Cys N asparagine also deaminates the asparagine side chain to form an aspartic acid, and in doing so the enzymatically treated homodimer gains 2 Da overall, corresponding to a mass of 1 Da for each chain present in the homodimer. Therefore, the actual molecular mass is the measured mass minus 2 Da to correct for the enzymatic modification of the insulin-Fc fusion protein structure in the analytical sample.

Example 6: %Homodimer by Size-Exclusion Chromatography

[0105] Size-exclusion chromatography (SEC-HPLC) of insulin-Fc fusion proteins was carried out using a Waters 2795HT HPLC (Waters Corporation, Milford, MA) connected to a 2998 Photodiode array at a wavelength of 280 nm. 100 µL or less of a sample containing an insulin-Fc fusion protein of interest was injected into a MAPac SEC-1, 5 µm, 4 × 300 mm column (ThermoFisher Scientific, Waltham, MA) operating at a flow rate of 0.2 mL/min and with a mobile phase comprising 50 mM sodium phosphate, 300 mM NaCl, and 0.05% w/v sodium azide, pH 6.2. The MAPac SEC-1 column operates on the principle of molecular size separation. Therefore, larger soluble insulin-Fc aggregates (e.g., multimers of insulin-Fc fusion protein homodimers) eluted at earlier retention times, and the non-aggregated homodimers eluted at later retention times. In separating the mixture of homodimers from aggregated multimers/homodimers via analytical SEC-HPLC, the purity of the insulin-Fc fusion protein solution in terms of the percentage of non-aggregated homodimer was ascertained.

Example 7: In vitro IM-9 Insulin Receptor Binding of an Exemplary Insulin-Fc Fusion Protein at 4°C

[0106] Human IM-9 cells (ATCC CCL-159) that express human insulin receptor were cultured and maintained in complete RPMI 5% FBS medium at 70-80% confluency. Cultures of IM-9 cells were centrifuged at 250g (~1000 rpm) for 10 min to pellet the cells. Cells were washed once with HBSS or PBS buffer, resuspended in cold FACS staining medium (HBSS/2mM EDTA/170 Na-azide + 4% horse serum) to a concentration of 8 × 10⁶ cells/mL, and kept on ice at 4°C until test solutions were made. The insulin-Fc protein was diluted in FACS buffer in 1:3 serial dilutions as 2^x concentrations in 1.2 mL tubes (approx. 60 µL volume of each dilution), and the solutions were kept cold on ice until ready for pipetting.

[0107] Biotinylated-RH1 was diluted in FACS staining medium to a concentration of 1.25 µg/mL. 40 µL of the serially diluted test compound and 8 µL of 1.25 µg/mL Biotin-RH1 were added into each well of a V bottom microtiter plate, mixed by slow vortexing, and placed on ice. 40 µL of an IM-9 cell suspension (8 × 10⁵ cells/mL) was then added to each well by multichannel pipette, mixed again gently and incubated on ice for 30 min to allow competitive binding on the insulin receptor in IM-9 cells. Cells were then washed twice with 275 µL of ice-cold FACS wash buffer (HBSS/2mM EDTA/1% Na-azide + 0.5% horse serum) by centrifuging the bottom plate at 3000 rpm for 3 min and aspirating the supernatant. Cells were then resuspended in 40 µL of FACS staining medium containing 1100 diluted Streptavidin-PE (Life Technologies) for 20 min on ice. Cells were then washed once with 275 µL of ice-cold FACS buffer and finally fixed with 3% paraformaldehyde for 10 min at room temp. Cells were then washed once with 275 µL of ice-cold FACS buffer and resuspended in 250 µL of FACS buffer for analysis.

[0108] The bottom plates containing cells were then analyzed on a Guava B-4 flow cytometer (Millipore). Biotinylated-RH1 binding to insulin receptor was quantified by the median fluorescence intensity (MFI) of the cells on the FACS FL-2 channel for each concentration of the test compound. Control wells were labeled only with biotinylated-RH1 and were used to calculate the percent (%) inhibition resulting from each test compound concentration. The % inhibition by test compounds of biotinylated-RH1 binding on IM-9 cells was plotted against log concentrations of the test compound, and the resulting IC50 values were calculated using GraphPad Prism (GraphPad Software, La Jolla, CA) for the test compounds. Lower IC50 values of the test compound therefore indicate greater levels of biotinylated-RH1 inhibition at lower concentrations indicating stronger binding of the insulin-Fc fusion protein to the insulin receptor. A control compound, such as unlabeled recombinant human insulin (RH1) was also used as an internal standard to generate an RH1 IC50 against which a given compound IC50 could be related (IC50(compound)/IC50(RH1)). Lower IC50 ratios have more similar binding to RH1 (stronger binding to insulin receptor), while higher IC50 ratios have weaker binding to the insulin receptor relative to RH1.

Example 8: In vitro Fcγ2b Receptor Binding Affinity Assay

[1215] The binding of insulin-Fc fusion proteins to the FcγRIIIa receptor 1 at pH 7.4 was conducted using an ELISA assay as follows. Since neither canine nor feline FcγRIIIa receptor 1 was not commercially available, human FcγRIIIa receptor 1 (i.e., hFcγRIIIa receptor 1) was used as a surrogate mammalian receptor. Insulin-Fc compounds were diluted to 10 μg/mL in sodium bicarbonate buffer at pH 8.6 and coated on MaxiSorp (Nunc) microtiter plates overnight at 4°C. After which the microplate strips were washed 5 times with PBST (PBS/0.05% Tween-20) buffer and blocked with SuperBlock blocking reagent (ThermoFisher). Serial dilutions of biotinylated hFcγRIIIa receptor 1 (recombinant human FcγRIIIa)R1 (R&D Systems) were prepared in PBST/1% SuperBlock buffer from 6000 ng/mL to 8.2 ng/mL, and loaded at 100 μL/well onto the microplate strips coated with insulin-Fc fusion protein. The microtiter plate was incubated for 1 hour at room temperature after which the microplate strips were washed 5 times with PBST and then loaded with 100 μL/well streptavidin-HRP diluted 1:1000 in PBST/1% SuperBlock buffer. After incubating for 45 min, the microplate strips were washed again 5 times with PBST. TMB was added to reveal the bound hFcγRIIIa receptor 1 protein and stopped with ELISA stop reagent (Bioss Bioproducts). The plate was read in an ELISA plate reader at 450 nm, and the OD values (proportional to the binding of hFcγRIIIa receptor 1 to insulin-Fc fusion protein) were plotted against log concentrations of FcγRIIIa receptor 1 to insulin-Fc fusion protein. I added to each well to generate binding curves using GraphPad Prism software.

Example 9: In vivo Measurement of Insulin-Fc Fusion Protein Affinity for the Canine FcγRIIIa Receptor

[1216] In vitro binding affinity of insulin-Fc fusion proteins containing Fc fragments of canine or feline IgG1 origin to the canine FcγRIIIa receptor was measured via an ELISA technique conducted as a solution pH of 5.5. The slightly acidic pH is the preferred binding environment for Fc fragment-containing molecules to bind to the FcγRIIIa receptor. In vivo, cells express FcγRIIIa on their surfaces and internally in the endosomes. As molecules containing Fc fragments are brought into the cell through natural processes (e.g. pinocytosis or endocytosis), the pH changes to a lower pH in the endosomes, where the FcγRIIIa receptor binds to Fc fragment-containing molecules that would otherwise be degraded in the endosomal-lysosomal compartments, thereby allowing these molecules to escape back to the cellular surface where the cell is closer to neutral (e.g., pH 7.0-7.4). Neutral pH disfavors binding to the FcγRIIIa receptor and allows release of the Fc fragment-containing molecules back into circulation. This is a primary mechanism by which Fc fragment-containing molecules exhibit prolonged circulatory pharmacokinetic half-lives in vivo.

[1217] Insulin-Fc fusion proteins comprising Fc fragments of canine or feline origin were diluted to 10 μg/mL in sodium bicarbonate pH 8.6 buffer and coated in duplicate on MaxiSorp ELISA plate strips for 1-2 hours at RT. The strips were then washed 4 times with PBST (PBS/0.1% Tween-20) buffer and blocked with SuperBlock blocking reagent (ThermoFisher). Strips for FcγRIIIa binding were then washed again twice with pH 5.5 MES/NaCl/Tween (50mM MES/150mM NaCl/0.1% Tween-20) buffer before addition of the Fc reagent (biotinylated canine FcγRIIIa; Immunotrack). Since no feline FcγRIIIa reagent was to be commercially available, insulin-Fc fusion protein containing either a canine Fc fragment or a feline Fc fragment (Nunc) were assayed for binding to the canine FcγRIIIa. Serial dilutions (1:3X dilution) of biotinylated FcγRIIIa reagent were prepared in pH 5.5 MES/NaCl/Tween/10% SuperBlock buffer at concentrations from 1000 ng/mL to 0.45 ng/mL and loaded at 100 μL/well using a multichannel pipettor onto the strips coated with the insulin-Fc fusion protein compounds. The assay plate was then incubated for 1 hour at room temperature. FcγRIIIa binding strips were washed 4 times with pH 5.5 MES/NaCl/Tween buffer and then loaded with 100 μL/well streptavidin-HRP diluted 1:1000 in pH 5.5 MES/NaCl/Tween/10% SuperBlock buffer. After incubating for 45 minutes, strips were washed again 4 times with pH 5.5 MES/NaCl/Tween buffer. TMB was freshly added to reveal the bound biotinylated-canine FcγRIIIa reagent, and the color development was stopped with the ELISA stop reagent. The plate was read in an ELISA plate reader at a wavelength of 450 nm. The OD values (proportional to the binding of canine FcγRIIIa to the insulin-Fc fusion protein test compounds) were plotted against log concentrations of FcγRIIIa reagent to each well to generate binding curves using GraphPad Prism software. EC50 values for each binding curve were calculated to compare between different compounds.

Example 10: Generalized Procedure for Determination of In vivo Pharmacokinetics (PK) After Single Administration of Insulin-Fc Fusion Proteins in Dogs or Cats (comparative).

[1218] Insulin-Fc fusion proteins were assessed for their effects on fasting blood glucose levels as follows. N=1, 2, 3 or more healthy, antibody-naïve, dogs weighing approximately 10-15 kg or cats weighing approximately 5 kg were used, one for each insulin-Fc fusion protein. Animals were also observed twice daily for any anaphylaxis, lethargy, distress, pain, etc., and, optionally for some compounds, treatment was continued for an additional three weekly subcutaneous injections or more to observe if the glucose lowering capability of the compounds lessened over time, a key sign of potential induction of neutralizing anti-drug antibodies. On day 0, the animals received a single intramuscular administration of a pharmaceutical composition containing an insulin-Fc fusion protein homodimer at a concentration between 1 and 10 mg/mL in a solution of between 10-50 mM sodium hydrogen phosphate, 0.005-0.05% v/v Tween-80, and optionally a bacteriostat (e.g. phenol, m-cresol, or metylparaben) at a concentration of between 0.02-0.100 mg/mL, at a solution pH of between 7.0-8.0, at a dose of 0.08-0.80 mg insulin-Fc fusion protein/kg (or approximately equivalent to 1.2-12.3 mmoles/kg or approximately equivalent to 0.4-4.0 U/kg insulin equivalent on molar basis). On day 0, blood was collected from a suitable vein immediately prior to injection and on day 1, 30, 45, 60, 120, 240, 360, and 480 min and at 1, 2, 3, 4, 5, 6, and 7 days post injection.

[1219] For each time point, a minimum of 1 mL of whole blood was collected. A glucose level reading was immediately determined using a glucose meter (ACCU-CHEK® Aviva Plus), which required approximately one drop of blood. Average % fasting blood glucose levels (% FBGL) from day 0 to day 7 were plotted to assess the bioactivity of a given insulin-Fc fusion protein.

Example 11: Generalized Procedure for Determination of In vivo Pharmacokinetics (PK) After Repeated Administration of Insulin-Fc Fusion Proteins in Canines or Comparative Felinae.

[1214] Insulin-Fc fusion proteins were assessed for their effects on blood glucose levels over repeated injections as follows. Healthy, antibody-naïve, dogs or cats weighing approximately between 5 and 20 kg were used, and each animal was administered doses of an insulin-Fc fusion protein. Animals were observed twice daily for signs of anaphylaxis, lethargy, distress, pain, and other negative side effects, and optionally for some compounds, treatment was continued for up to an additional two to five subcutaneous injections to observe if the glucose lowering capability of the compounds decreased over time, indicating the presence of neutralizing anti-drug antibodies in vivo. On day 0, the animals received a single subcutaneous injection of a pharmaceutical composition containing an insulin-Fc fusion protein in a solution of 10-50 mM sodium hydrogen phosphate, 0.005-0.05% v/v Tween-80, and optionally a bacteriostat (e.g. phenol, m-cresol, or metylparaben) at a concentration of between 0.02-0.100 mg/mL, at a solution pH of between 7.0-8.0, at a dose of 0.08-0.80 mg insulin-Fc fusion protein/kg (or approximately equivalent to 1.2-12.3 mmoles/kg or approximately equivalent to 0.4-4.0 U/kg insulin equivalent on molar basis). On day 0, blood was collected from a suitable vein immediately prior to injection and on day 1, 30, 45, 60, 120, 240, 360, and 480 min and at 1, 2, 3, 4, 5, 6, and 7 days post injection.

[1215] Subsequent subcutaneous injections were given no more frequently than once-weekly, and in some cases the injections were given at different intervals based on the pharmacokinetics of a given insulin-Fc fusion protein formulation. Subsequent injections for each insulin-Fc fusion protein were adjusted to higher or lower doses, depending on the demonstrated pharmacokinetics of the insulin-Fc fusion protein. For instance, if the dose of a first injection on day 0 was found to be ineffective at lowering blood glucose, the subsequent dose levels of injected insulin-Fc fusion protein were adjusted upward. In a similar manner, if the dose of a first injection on day 1 was found to lower blood glucose 100% in a manner, then subsequent dose levels of injected insulin-Fc fusion protein were adjusted downward. It was also found that interim doses or final doses could be adjusted in a similar manner as needed. For each dose, blood was collected from a suitable vein just immediately prior to injection and at 30, 45, 60, 120, 240, 360, and 480 min and at 1, 2, 3, 4, 5, 6, 7 days (and optionally 14 days) post injection. For each time point, a minimum of 1 mL of whole blood was collected. Average % fasting blood glucose levels (% FBGL) during the study were plotted against time which allowed the bioactivity of a fusion protein to be determined.

[1216] To determine the bioactivity of each dose, an area-over-the-curve (AOC) analysis was conducted as follows. After constructing the %FBGL versus time data, the data was then entered into data analysis software (GraphPad Prism, GraphPad Software, San Diego CA). The software was used to first conduct an area-under-the-curve analysis (AUC) to integrate the area under the %FBGL vs. time curve for each dose. To convert the AUC data into the desired AOC data, the following equation was used: AOC = TRP - AUC, where TRP is the total possible area obtained by multiplying a given dose lifetime (e.g., 7 days, 14 days, etc.) by 100% (where 100% represents the %FBGL of the test cases). For example, given a dose lifetime of 7 days and a calculated AUC of 50% %FBGL days, the following for AOC: AOC = 100% %FBGL - 7 days) = (100% %FBGL days) = 200 %FBGL days. The analysis can be performed for each injected dose to obtain the AOC values for injection 1, injection 2, injection 3, etc.

[1217] As the doses of insulin-Fc fusion protein may vary as previously discussed, it is often more convenient to normalize all calculated AOC values for a given insulin-Fc fusion protein to a particular dose of that insulin-Fc fusion protein. Doing so allows for convenient comparison of the glucose-lowering potency of an insulin-Fc fusion protein across multiple injections, even if the dose levels change across the injections of a given study. Normalized AOC (NAOC) for a given dose is calculated as follows: NAOC = AOC / D with units of %FBGL days (where D is the actual dose injected into the animal in mg/kg). NAOC values may be calculated for each injection in a series of injections for a given animal and may be averaged across a group of animals receiving the same insulin-Fc fusion protein formulation.

[1218] The NAOC ratio (NAOCR) may also be calculated for each injection in a series of injections for a given animal by taking the NAOC values for each injection (e.g. injections 1, 2, 3, ... N) and dividing each NAOC for a given injection by the NAOC from injection 1 as follows: NAOCR = (NAOC(N) injection) / (NAOC(injection 1)). By evaluating the NAOCR of a given insulin-Fc homodimer fusion protein formulation for the Nth injection in a series of injections, it is possible to determine whether the in vivo glucose lowering activity of a given insulin-Fc fusion protein was substantially related to previous injections over a series of N doses (e.g., NAOCR for the Nth dose of greater than 0.5) or whether the in vivo glucose lowering activity of a given insulin-Fc fusion protein has lost a substantial portion of its potency (e.g., NAOCR of the Nth dose is less than 0.5) over a course of N doses, indicating the potential formation of neutralizing anti-drug antibodies in vivo. In preferred embodiments, the ratio of NAOC following the third subcutaneous injection to the NAOC following the first subcutaneous injection is greater than 0.5 (i.e., the NAOCR of the third subcutaneous injection is greater than 0.5).

Example 12: Generalized Procedure for the Determination of In vivo Pharmacokinetics (PK) in Canine and Comparative Felinae Serum

[1219] An assay was conducted for measuring the concentrations of insulin-Fc fusion proteins comprising Fc fragments of a canine epitope in canine serum as follows. The assay comprises a sandwich ELISA format in which therapeutic compounds in serum samples are captured by an anti-insulin/insulin monoclonal antibody (mAb) coated on the ELISA plates and then detected by a HRP-conjugated anti-canine IgG1 Fc specific antibody followed by use of a TMB substrate system for color development. MaxiSorp ELISA Plates (Nunc) are coated with the anti-insulin mAb done D6C4 (Biorad) in coating buffer (pH9.6 sodium carbonate-sodium bicarbonate buffer) at 5 μg/mL overnight at 4°C. Plates are then washed 5x with PBST (PBS + 0.05% Tween 20) and blocked for a minimum of one hour at room temperature (or overnight at 4°C) with SuperBlock blocking solution (ThermoFisher). Test serum samples are diluted to 1:20 in PBST/BS/20%HS sample dilution buffer (PBS/0.1% Tween 20/10% SuperBlock/20% horse serum). For making a standard curve, the insulin-Fc fusion protein of interest is diluted in sample dilution buffer (PBST/BS/20%HS + 5% of pooled heparin serum (Bio/Tr)) from a concentration range of 200 ng/mL to 0.82 ng/mL in 1:2.5 serial dilutions. Standards and diluted serum samples are added to the blocked plates at 100 μL/well in duplicate and are incubated for 1 hour at room temperature. Following incubation, samples and standards are washed 5x with PBST. HRP-conjugated goat anti-canine IgG1 (Sigma) detection antibody is diluted to about 1:15,000 in PBST/BS/20%HS buffer and 100 μL is added to all the wells and incubated for 45 minutes at room temperature in the dark. Plates are washed 5x with PBST and once with deionized water and developed by the addition of 100 μL/well TMB (Invitrogen) for 8-10 minutes at room temperature. Color development is then stopped by the addition of 100 μL/well ELISA Stop Solution (Bioss Bioproducts) and the absorbance is read at 450 nm using a SpectraMax plate reader (Molecular Devices) within 30 minutes. Concentrations of insulin-Fc fusion protein compounds in the samples are calculated by interpolation on a 4-PL curve using SoftMaxPro software.

[1220] Similarly, an assay was constructed for measuring the concentrations of insulin-Fc fusion proteins comprising Fc fragments of a feline epitope in feline serum as follows. The assay comprises a sandwich ELISA format in which therapeutic compounds in serum samples are captured by an anti-insulin/insulin mAb coated on the ELISA plates and then detected by a HRP-conjugated goat anti-feline IgG1 Fc specific antibody followed by use of a TMB substrate system for color development. MaxiSorp ELISA Plates (Nunc) are coated with the anti-insulin mAb done D6C4 (Biorad) in coating buffer (pH9.6 sodium carbonate-sodium bicarbonate buffer) at 5 μg/mL overnight at 4°C. Plates are then washed 5x with PBST (PBS + 0.05% Tween 20) and blocked for a minimum of one hour at room temperature (or overnight at 4°C) with SuperBlock blocking solution (ThermoFisher). Test serum samples are diluted to 1:20 in PBST/BS/20%HS sample dilution buffer (PBS/0.1% Tween 20/10% SuperBlock/20% horse serum). For making a standard curve, the insulin-Fc fusion protein of interest is diluted in sample dilution buffer (PBST/BS/20%HS + 5% of normal cat serum (Jackson ImmunoResearch)) from a concentration range of 200 ng/mL to 0.82 ng/mL in 1:2.5 serial dilutions. Standards and diluted serum samples are added to the blocked plates at 100 μL/well in duplicate and are incubated for 1 hour at room temperature. Following incubation, samples and standards are washed 5x with PBST. HRP-conjugated goat anti-feline IgG1 Fc (Bethyl Lab) detection antibody is diluted to about 1:20,000 in PBST/BS/20%HS buffer and 100 μL is added to all the wells and incubated for 45 minutes at room temperature in the dark. Plates are washed 5x with PBST and once with deionized water and developed by the addition of 100 μL/well TMB (Invitrogen) for 8-10 minutes at room temperature. Color development is then stopped by the addition of 100 μL/well ELISA Stop Solution (Bioss Bioproducts) and the absorbance is read at 450 nm using a SpectraMax plate reader and then processed by interpolation on a 4-PL curve using SoftMaxPro software.

Example 13: Assay Protocol for Measuring Anti-Drug Antibodies in Canine Serum

[1221] MaxiSorp ELISA Plates (Nunc) are coated with the insulin-Fc fusion protein of interest diluted in coating buffer (pH9.6 Carbonate-Bicarbonate buffer) at 10 μg/mL overnight at 4°C for measuring ADAs against the test compound. For measuring ADAs against the insulin portion of the insulin-Fc fusion protein containing an Fc fragment of canine IgG1 origin, plates are coated with purified insulin at 30 μg/mL in coating buffer. Plates are then washed 5x with PBST (PBS + 0.05% Tween 20) and blocked for at least 1 hour (or overnight) with SuperBlock blocking solution (ThermoFisher, Waltham MA). For calculating the ADAs in canine IgG1 units, strips are directly coated with 1:2 serial dilutions of canine IgG1 (Jackson ImmunoResearch Laboratories, West Grove PA) in pH9.6 Carb-Bicarb coating buffer at concentrations between 300-4.69 ng/mL overnight at 4°C and used to create a 7-point pseudo-standard curve. The standards strip plates are also washed and blocked with SuperBlock blocking solution for at least 1 hour (or overnight).

[1222] Test serum samples are diluted to greater than or equal to 1:100 (typically tested as 1:200) in PBST/BS/20%HS sample dilution buffer (PBS/0.1% Tween 20/10% SuperBlock/20% horse serum) and added to the insulin-Fc fusion protein coated (or RH coated) strips at 100 μL/well in duplicate. Duplicate strips of canine IgG1 coated standard strips are also added to each plate and filled with PBST/BS (PBS/0.1% Tween 20/10% SuperBlock) buffer at 100 μL/well. Plates are incubated for 1 hour at room temperature. For detection of ADAs, HRP-conjugated goat anti-feline IgG1 Fc (Biorad) anti-feline IgG1 Fc (Biorad) anti-feline IgG1 Fc (Biorad) reagent is cross-reactive to canine antibodies. Jackson ImmunoResearch Laboratories, West Grove PA) which is diluted in PBST/BS by a factor of 1:1000 and added at 100 μL/well to both sample and standard wells and incubated for 45 minutes at room temperature in the dark. Plates are washed 5x with PBST and then one time with deionized water and then developed by adding 100 μL/well TMB substrate (Invitrogen, ThermoFisher Scientific, Waltham MA) for 15-20 minutes at room temperature in the dark. Color development is then stopped by addition of 100 μL/well ELISA Stop Solution (Bioss Bioproducts) and the absorbance is read at 450 nm using a SpectraMax plate reader and then processed by interpolation on a 4-PL pseudo-standard curve using SoftMax Pro Software (Molecular Devices, San Jose CA).

[1223] To demonstrate the specificity of the detected ADAs, an "inhibition" assay is carried out. In the drug inhibition ADA assay, serum samples are diluted 1:100 in PBST/BS/20%HS buffer and mixed with an equal volume of 300 μg/mL of the relevant therapeutic compound (final sample dilution at 1:200 and final inhibitory compound at 150 μg/mL) and incubated for 30-40 minutes at room temperature to allow inhibitory antibodies to bind the free inhibitor (i.e., the therapeutic compound). After pre-incubation, the samples are added to insulin-Fc fusion protein coated (or RH coated) strips at 100 μL/well in duplicate. Samples diluted 1:200 in PBST/BS/20%HS buffer without the inhibitory compound are also tested in the sample plates along with duplicate strips of canine IgG1 coated standards. Remaining steps of the assay procedure are carried out as described above. The ADAs measured in the drug-inhibited wells are matched with the non-inhibited ADA concentrations to assess the specificity of the ADAs. If significant inhibition of ADA signals is observed in the drug-inhibited wells, this means the ADAs are specific to the therapeutic compound.

Comparative Example 14: Assay Protocol for Measuring Anti-Drug Antibodies in Feline Serum

[1224] MaxiSorp ELISA Plates (Nunc) are coated with the insulin-Fc fusion protein of interest diluted in coating buffer (pH9.6 Carbonate-Bicarbonate buffer) at 10 μg/mL overnight at 4°C for measuring ADAs against the insulin-Fc fusion protein containing an Fc fragment of feline IgG1 origin. For measuring ADAs against the insulin portion of the insulin-Fc fusion protein, plates are coated with purified insulin at 30 μg/mL in coating buffer. Plates are then washed 5x with PBST (PBS + 0.05% Tween 20) and blocked for at least 1 hour (or overnight) with SuperBlock blocking solution (ThermoFisher, Waltham MA). For calculating the ADAs in feline IgG1 units, strips are directly coated with 1:2 serial dilutions of feline IgG1 (Jackson ImmunoResearch Laboratories, West Grove PA) in pH9.6 sodium carbonate-sodium bicarbonate coating buffer at concentrations between 300-4.69 ng/mL overnight at 4°C and used to create a 7-point pseudo-standard curve. The standards strip plates are also washed and blocked with SuperBlock blocking solution for at least 1 hour (or overnight).

[1225] Test serum samples are diluted to greater than or equal to 1:100 (typically tested as 1:200) in PBST/BS/20%HS sample dilution buffer (PBS/0.1% Tween 20/10% SuperBlock/20% horse serum) and added to the insulin-Fc fusion protein coated (or RH coated) strips at 100 μL/well in duplicate. Duplicate strips of feline IgG1 coated standard strips are also added to each plate and filled with PBST/BS (PBS/0.1% Tween 20/10% SuperBlock) buffer at 100 μL/well. Plates are incubated for 1 hour at room temperature and then washed 5x with PBST. For detection of ADAs, HRP-conjugated goat anti-feline IgG1 Fc (Biorad) anti-feline IgG1 Fc (Biorad) reagent is cross-reactive to canine antibodies. Jackson ImmunoResearch Laboratories, West Grove PA) which is diluted in PBST/BS by a factor of 1:1000 and added at 100 μL/well to both sample and standard wells and incubated for 45 minutes at room temperature in the dark. Plates are washed 5x with PBST and then one time with deionized water and developed by adding 100 μL/well TMB substrate (Invitrogen, ThermoFisher Scientific, Waltham MA) for 15-20 minutes at room temperature in the dark. Color development is then stopped by addition of 100 μL/well ELISA Stop Solution (Bioss Bioproducts, Ashland MA) and the absorbance is read at 450 nm using a SpectraMax plate reader and then processed by interpolation on a 4-PL pseudo-standard curve using SoftMax Pro Software (Molecular Devices, San Jose CA).

Example 15: Assay Protocol for Immunogenic Epitope Identification

[1226] MaxiSorp ELISA microplates (Nunc) are coated with a library of insulin-Fc fusion protein homodimer compounds with known amino acid sequences, and the coated plates are blocked in a similar manner as described in the anti-drug antibody ELISA assay Examples 13 and 14, except that each compound in the library is coated on a separate individual strip of ELISA microplate wells. The compounds in the library comprise a range of insulin-Fc fusion proteins with different insulin polypeptide amino acid compositions, including various B-chain, C-chain, and A-chain amino acid mutations, different linker compositions, and different Fc fragment compositions, including some of human origin. Separately, some plate strip wells are directly coated with 1:2 serial dilutions of canine or feline IgG1 (Jackson ImmunoResearch Laboratories, West Grove PA) for calculating the anti-drug antibodies (ADA) in canine or feline IgG1 units, respectively, as described in Examples 13 and 14.

[1227] Serum obtained from individual dogs or cats receiving repeated doses of an insulin-Fc fusion protein is first screened on the anti-drug antibody ELISA assay (Example 13 for dogs and Example 14 for cats). Serum samples demonstrating moderate or high positivity (e.g. moderate or high titers of antibodies) on the assay of Example 13 or Example 14 are serially diluted (1:200 to 1:6000) in PBST/BS/20%HS sample dilution buffer (PBS/0.1% Tween 20/10% SuperBlock/20% horse serum) and added to the plates coated with the library of insulin-Fc fusion protein compounds for 1 hour at RT. Following incubation, the plates are washed 5 times with PBST. For detection of canine or feline antibodies capable of cross-reacting to the coated compound library, HRP-conjugated goat anti-feline IgG1 Fc (Biorad) Jackson ImmunoResearch Laboratories, West Grove PA), which is cross-reactive to both canine and feline IgG1s, is diluted in PBST/BS to 1:1000 and added to 100 μL/well to both sample and standard wells and incubated for 45 minutes at room temperature in the dark. Color development is then stopped by addition of 100 μL/well ELISA Stop Solution (Bioss Bioproducts, Ashland MA) and the absorbance is read at 450 nm using a SpectraMax plate reader and then processed by interpolation on a 4-PL pseudo-standard curve against the directly coated canine or feline IgG1 antibody controls using SoftMax Pro Software (Molecular Devices, San Jose CA).

[1228] By correlating the resulting antibody concentrations from the assay with the known amino acid compositions of the coated insulin-Fc fusion protein library, one can determine whether particular amino acid mutations or epitopes are responsible for causing none, some, most, or all of the total antibody signal on the assay, indicating non-binding, weak binding, or strong binding to various insulin-Fc fusion protein homodimers. The mutations or epitopes responsible for moderate or strong binding are herein referred to as immunogenic "hot spots".

Example 16: Design Process for Obtaining Insulin-Fc Fusion Proteins with High Homodimer Titers and Acceptable Levels of Acute and Repeated Dose Bioactivity in the Target Species

[1229] The process for meeting the design goals described in the Detailed Description of the Invention comprised the following steps. First, the insulin polypeptide of SEQ ID NO: 4 or SEQ ID NO: 5 was combined with a species-specific Fc fragment of a particular IgG isotype and a linker such that the resulting insulin-Fc fusion protein was most likely to yield a long acting bioactivity product with minimal immunogenicity (e.g., a species-specific IgG1 epitope was chosen with minimal FcγRIIIa epitope binding). The DNA sequence coding for the desired fusion protein was prepared, cloned into a vector (Lac/ExPharma, San Carlos, CA), and the vector was then used to transfect the HEK293 cells according to the procedure described in Example 1. The insulin-Fc fusion protein was then purified according to Examples 2 and 3 and the overall protein yield and homodimer measured according to Example 6. Only compounds with a homodimer titer of more than 50 ng/mL were considered acceptable, because titers less than this level are not likely to result in commercial production titers that meet the stringently low manufacturing cost requirements for veterinary products. Selected insulin-Fc fusion proteins were then screened for indicators of bioactivity through in vitro insulin receptor binding studies as described in Example 7. Based on experience, only compounds that exhibited IR activity (C50 values less than 5000 nM) were deemed likely to exhibit bioactivity in the target species. Although the in vitro IR C50 value is a useful qualitative screening tool, it utilizes human IM-9 cells which express the human insulin receptor and therefore it may not capture some of the small differences in affinity between the canine or feline IR and the human IR. Furthermore, factors other than insulin receptor bioactivity may influence a compound's bioactivity in vivo (e.g., affinity for canine or feline FcγRII to allow for extended pharmacokinetics; elimination half-lives in vivo). Therefore, selected insulin-Fc fusion proteins that were acceptable from a manufacturing and IR activity (C50 value standpoint) were further screened for bioactivity in the animal of interest (e.g., dog or cat) to screen out any materials with less than the desired potency and/or duration of bioactivity (e.g., NAOCR of less than

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SEQ ID NO: 31 atggaatggagctgggtctttctcttctctcctgtcagtaacgactgggtgtccactccttc
 SEQ ID NO: 32 M E W S W V F L F F L S V T T G V H S F
 gtgaaccagcacctgtgctggctcccacctgggtggaagctctggaactcgtgtgctggcgag
 V N Q H L C G S H L V E A L E L V C G E
 cggggcttccactacgggggtggcggaggaggttctgggtggcggcggaggcatcgtggaa
 R G F H Y G G G G G S G G G G I V E
 cagtgtgcacctccacctgctccctggaccagctggaaaactactgcggtggcggaggt
 Q C C T S T C S L D Q L E N Y C G G G G
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 G Q G G G G Q G G G G Q G G G G D C P
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 K C P A P E M L G G P S V F I F P P K P
 aaggacacactgctgatcgccaggacccccggaggtgacctgcgtgggtgggtggacctggat
 K D T L L I A R T P E V T C V V V D L D
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 P E D P E V Q I S W F V D G K Q M Q T A
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 K T Q P R E E Q F N G T Y R V V S V L P
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 I G H Q D W L K G K Q F T C K V N N K A
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 L P S P I E R T I S K A R G Q A H Q P S
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 V Y V L P P S R E E L S K N T V S L T C
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 L I K D F F P P D I D V E W Q S N G Q Q
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 E P E S K Y R T T P P Q L D E D G S Y F
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 L Y S K L S V D K S R W Q R G D T F I C
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 A V M H E A L H N H Y T Q E S L S H S P
 ggatag
 G -

FIG. 38

SEQ ID NO: 33 atggaatggagctgggtctttctcttcttctcctgtcagtaacgactgggtgtccactccttc
 SEQ ID NO: 34 M E W S W V F L F F L S V T T G V H S F
 gtgaaccagcacctgtgctggctcccacctgggtggaagctctggaactcgtgtgctggcgag
 V N Q H L C G S H L V E A L E L V C G E
 cggggcttccactacgggggtggcggaggaggttctggtggcggcggaggcatcgtggaa
 R G F H Y G G G G G S G G G G G I V E
 cagtgtgtcacctccacctgctccctggaccagctggaaaactactgcaacgggtggcgga
 Q C C T S T C S L D Q L E N Y C N G G G
 ggtggtcaaggaggcgggtggacagggtggaggtgggcagggaggaggcgggggagactgc
 G G Q G G G G Q G G G G Q G G G G G D C
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 P K C P A P E M L G G P S V F I F P P K
 cccaaggacacactgctgatcgccaggaccccggaggtgacctgcgtggtgggtggacctg
 P K D T L L I A R T P E V T C V V V D L
 gatcccgaaagaccccaggtgcagatcagctggttcgtggatggaaaagcagatgcagacc
 D P E D P E V Q I S W F V D G K Q M Q T
 gccaaagaccaaccccgggaagagcagttcaacggcacctacaggggtggtgagtgtgttg
 A K T Q P R E E Q F N G T Y R V V S V L
 cccatcggccaccaggactggctgaagggaagcaattcacatgcaaggtaataacaag
 P I G H Q D W L K G K Q F T C K V N N K
 gccctgccagccccatcgagaggaccatcagcaaggccaggggccaggcccaccagcca
 A L P S P I E R T I S K A R G Q A H Q P
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 S V Y V L P P S R E E L S K N T V S L T
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 C L I K D F F P P D I D V E W Q S N G Q
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 Q E P E S K Y R T T P P Q L D E D G S Y
 ttcctctacagcaaattgagcgttgacaaaagcaggtggcagcggagcgacaccttcac
 F L Y S K L S V D K S R W Q R G D T F I
 tgcccgctgatgcacgaggctttgcataaccactacaccagagagcctgtcccacagc
 C A V M H E A L H N H Y T Q E S L S H S
 cccgatag
 F G -

FIG. 39

SEQ ID NO: 35 atggaatggagctgggtctttctcttcttctcctgtcagtaacgactgggtgccactccttc
 SEQ ID NO: 36 M E W S W V F L F F L S V T T G V H S F
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 R G F H Y G G G G G G S G G G G G I V E
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 Q C C T S T C S L D Q L E N Y C G G G G
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 K C P A P E M L G G P S V F I F P P K P
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 K D T L L I A R T P E V T C V V V D L D
 cccgaagacccccgaggtgcagatcagctggttctggtgatggaaagcagatgcagaccgcc
 P E D P E V Q I S W F V D G K Q M Q T A
 aagaccaacccccggaagagcagttctcaggcacctacaggggtggtgagtgtgttgc
 K T Q P R E E Q F S G T Y R V V S V L P
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 I G H Q D W L K G K Q F T C K V N N K A
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 L P S P I E R T I S K A R G Q A H Q P S
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 V Y V L P P S R E E L S K N T V S L T C
 ctgatcaaggacttcttcccaccggacatagacgtggagtggcagagtaacggccagcag
 L I K D F F P P D I D V E W Q S N G Q Q
 gagcccagagcaagtataggaccacaccgcccccaactggacgaggacggaagctacttc
 E P E S K Y R T T P P Q L D E D G S Y F
 ctctacagcaaattgagcgttgacaaaagcaggtggcagcagggcgacaccttcatctgc
 L Y S K L S V D K S R W Q R G D T F I C
 gccgtgatgcacgaggctttgcataaccactacaccaggagagcctgtcccacagcccc
 A V M H E A L H N H Y T Q E S L S H S P
 ggatag
 G -

FIG. 40

SEQ ID NO: 37 atggaatggagctgggtctttctcttcttctcctgtcagtaacgactgggtgtccactccttc
 SEQ ID NO: 38 M E W S W V F L F F L S V T T G V H S F
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 R G F H Y G G G G G S G G G G G I V E
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 Q C C T S T C S L D Q L E N Y C G G G G
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 K C P P P E M L G G P S I F I F P P K P
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 K D T L S I S R T P E V T C L V V D L G
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 P D D S D V Q I T W F V D N T Q V Y T A
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 K T S P R E E Q F N S T Y R V V S V L P
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 I L H Q D W L K G K E F K C K V N S K S
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 L P S P I E R T I S K D K G Q P H E P Q
 gtctactgtgctgccccagcacaggaagagctgagcaggaacaaggttagcgtgacatgc
 V Y V L P P A Q E E L S R N K V S V T C
 ctgatcaggggtttctaccccagcgcacatcgccgtggagtgggaaatcaccggccaaccc
 L I E G F Y P S D I A V E W E I T G Q P
 gagcccgagaacaactacaggaccactccgcccgaactggacagcgcagggacactcttc
 E P E N N Y R T T P P Q L D S D G T Y F
 ttgtatagcaggctgagcgtggaccggagcaggtggcagaggggcaacacctacacttgc
 L Y S R L S V D R S R W Q R G N T Y T C
 agcgtgagccacgaggccttgcacagccaccacactcagaagagtctgaccagagccccg
 S V S H E A L H S H H T Q K S L T Q S P
 ggatag
 G -

FIG. 41

SEQ ID NO: 39 atggaatggagctgggtctttctcttcttctctgtcagtaacgactgggtgtccactccttc
 SEQ ID NO: 40 M E W S W V F L F F L S V T T G V H S F
 gtgaaccagcacctgtgcggtctccacctgggtggaagctctggcaactcgtgtgcgggcgag
 V N Q H L C G S H L V E A L A L V C G E
 cggggcttccactacgggggtggcggaggaggttctgggtggcggcgaggcatcgtggaa
 R G F H Y G G G G G S G G G G G I V E
 cagtgtgcacctccactgctccctggaccagctggaaaactactgcggtggcggaggt
 Q C C T S T C S L D Q L E N Y C G G G G
 ggtcaaggaggcgggtggacagggtggaggtgggcaggaggaggcgggggagactgcccc
 G Q G G G G Q G G G G Q G G G G G D C P
 aaatgtctccgctgagatgctgggtggccctagcatcttcatcttcccgcccaagccc
 K C P P P E M L G G P S I F I F P P K P
 aaggatactctgtccattagcaggacccccgaggtgacctgctgggtggacctgggg
 K D T L S I S R T P E V T C L V V D L G
 ccagacgactctgacgtgcagatcacctggttcgttagacaacaccaggtttacactgcc
 P D D S D V Q I T W F V D N T Q V Y T A
 aagaccagtcccaggaggagcagttcagcagcacatacagggtgggtgagcgttctgccc
 K T S P R E E Q F S S T Y R V V S V L P
 atcctgcaccaggactggctgaaaggcaaagagttcaagtgttaaggtgaacagcaagagc
 I L H Q D W L K G K E F K C K V N S K S
 ctgcccagccccattgaaaggaccatcagcaaggacaagggccagccgacgagccccaa
 L P S P I E R T I S K D K G Q P H E P Q
 gtctacgtgctgccccagcacaggaagagctgagcaggaacaaggtttagcgtgacatgc
 V Y V L P P A Q E E L S R N K V S V T C
 ctgatcgagggtttctaccccagcgacatcgccgtggagtgggaaatcaccggccaaccc
 L I E G F Y P S D I A V E W E I T G Q P
 gagccccgagaacaactacaggaccactccgcccgaactggacagcgacgggacctacttc
 E P E N N Y R T T P P Q L D S D G T Y F
 ttgtatagcaggctgagcgtggaccggagcaggtggcagaggggcaacacctacacttgc
 L Y S R L S V D R S R W Q R G N T Y T C
 agcgtgagccacgaggccttgacagccaccacactcagaagagtctgaccagagccccg
 S V S H E A L H S H H T Q K S L T Q S P
 ggatag
 G -

FIG. 42

PATENTKRAV

1. Fremgangsmåde til fremstilling af en rekombinant celle, som omfatter en nukleinsyre, der koder for et fusionsprotein, hvilket fusionsprotein omfatter et insulinpolypeptid og et Fc-fragment,
5 hvor insulinpolypeptidet og Fc-fragmentet er forbundet via en linker, hvor Fc-fragmentet omfatter følgende sekvens:
DCPKCPAPEMLGGPSVFIFPPKPKDTHLLIARTPEVTCVVVDLDPEDPEVQISWVFDGK
QMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISKAR
10 GQAHQPSVYVLPSSREELSKNTVSLTCLIKDFFPPDIDVEWQSNQQEPESKYRTTPP
QLDEGGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG (SEQ ID NO: 22),
og hvor insulinpolypeptidet omfatter følgende sekvens:
FVNQHLCGSHLVEALALVCGERGFHYGGGGGGSGGGGGIVEQCCTSTCSLDQLEN
15 YC (SEQ ID NO: 10),
og hvor X_1 ikke er D, og X_2 ikke er H,
hvilken fremgangsmåde omfatter:
transfektion af en værtscelle med en nukleinsyre, der koder for fusionsproteinet, hvor fusionsproteinet udtrykkes i den rekombinante celle efter transfektionstrinet.
20
2. Fremgangsmåde ifølge krav 1, som yderligere omfatter:
dyrkning af den rekombinante celle i celledyrkningsmedie; og
høst af cellekultursupernatanten fra celledyrkningsmediet, hvilken
cellekultursupernatant omfatter det udtrykte fusionsprotein.
25
3. Fremgangsmåde ifølge krav 2, som yderligere omfatter:
oprensning eller isolering fra celledyrkningsmediet.
4. Fremgangsmåde ifølge krav 3, hvor oprensnings- eller isoleringstrinene omfatter
30 centrifugering, filtrering og/eller kromatografi.
5. Fremgangsmåde ifølge krav 1, hvor transfektionstrinet omfatter stabil transfektion af værtscellen.
- 35 6. Fremgangsmåde ifølge krav 1, hvor nukleinsyren omfatter cDNA, der koder for fusionsproteinet, hvilket fusionsprotein omfatter følgende sekvens:
FVNQHLCGSHLVEALALVCGERGFHYGGGGGGSGGGGGIVEQCCTSTCSLDQLENY
CGGGGGQGGGGQGGGGQGGGGDCPKCPAPEMLGGPSVFIFPPKPKDTHLLIARTP

EVTCVVLDLPEDPEVQISWFVDGKQMQTAKTQPREEQFSGTYRWSVLPIGHQDWL
KGKQFTCKVNNKALPSPIERTISKARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFF
PPDIDVEWQSNQEQEPESKYRTTPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVM
HEALHNHYTQESLSHSPG (SEQ ID NO: 36).

5

7. Fremgangsmåde ifølge krav 6, hvor cDNA'et omfatter følgende nukleinsyresekvens:

atggaatggagctgggtcttctcttctcctgtcagtaacgactgggtccactcctcgtgaaccagcacctgtgctggctccc
acctggtggaagctctggcactcgtgtgctggcgagcggggctccactacgggggtggcggaggaggttctggtggcgg
cggaggcatcgtggaacagtgtgtgacacctccacctgtccctggaccagctggaaaactactgctgggtggcggagggtgt
10 caaggaggcgggtggacaggggtggaggtgggcagggaggaggcgggggagactgcccccaagtgccccgctcccga
gatgctggcgccgaccagcgtgttcatcttccctccaagcccaaggacacactgctgatcgccaggacccccggagggtg
acctgctgggtggacctggatcccgaagacccccgaggtgcagatcagctggtcgtggatggaaagcagatgcag
accgccaagacccaacccccggaagagcagttctcaggcacctacaggggtggtgagtggttggccatcggccaccag
gactggctgaaggggaagcaattcacatgcaaggttaataacaaggccctgccagccccatcgagaggacatcag
15 caaggccaggggcccaggcccaccagccatctgtgtacgtgctgccccatctaggagggaactgagcaagaacaca
gtcagccttacttgctgatcaaggacttctcccaccggacatagacgtggagtgccagagtaacggccagcaggagc
ccgagagcaagtataggaccacacggccccactggacgaggacggaagctacttctctacagcaaatgagcgttg
acaaaagcaggtggcagcggagggcagacacctcatctgcgcccgtgatgcacgaggccttgataaccactacaccagg
agagcctgtcccacagccccgatag (SEQ ID NO: 35).

DRAWINGS

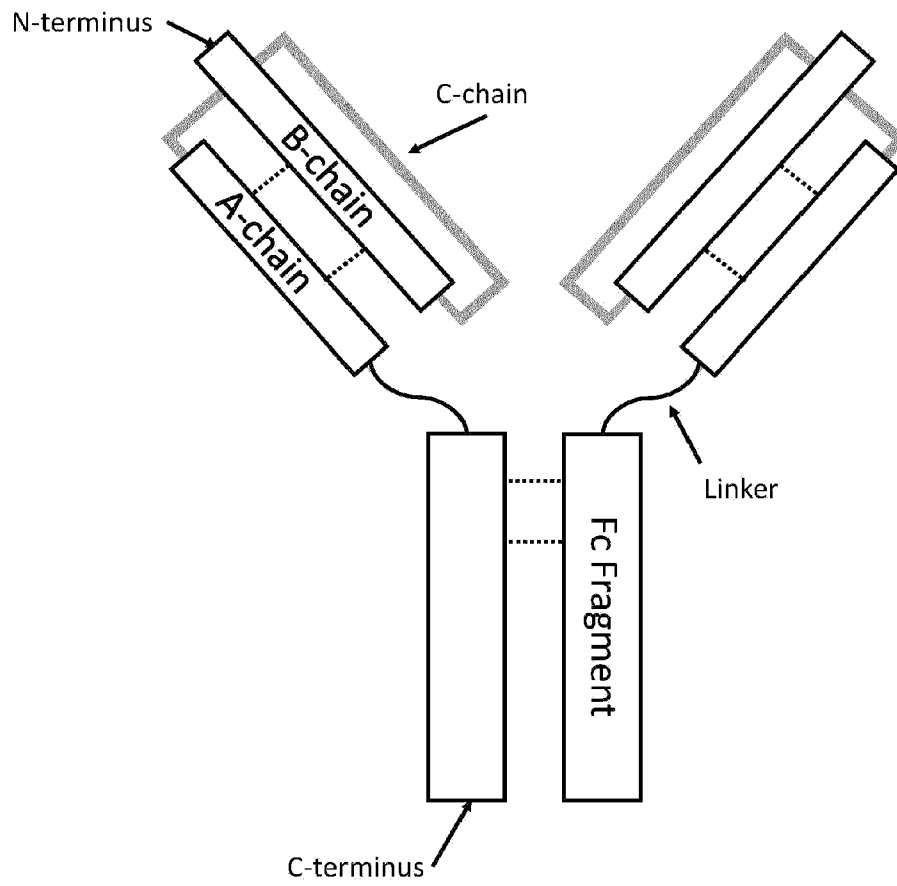


FIG. 1

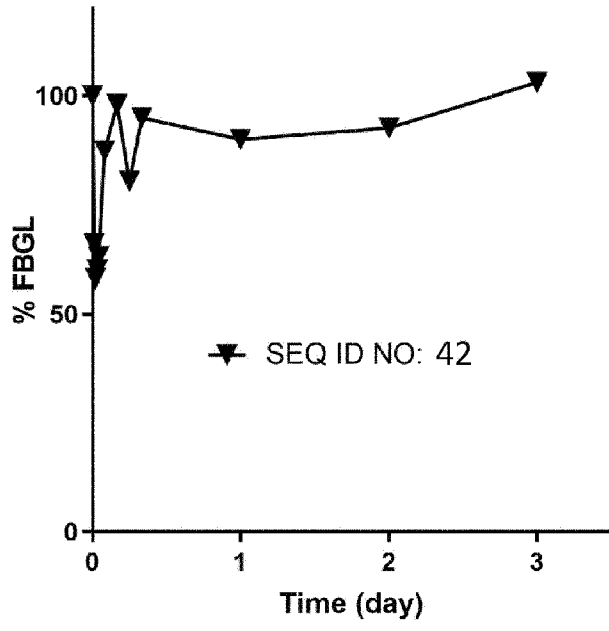


FIG. 2

```

SEQ ID NO: 44      FVNQHLCGSDLVEALALVCGERGFYTDPTGGGFRRGIVEQCCHSICSLYQLENYCNGGG      60
SEQ ID NO: 46      FVNQHLCGSDLVEALALVCGERGFYTDPTGGGFRRGIVEQCCHSICSLYQLENYCNGGG      60
SEQ ID NO: 48      FVNQHLCGSDLVEALALVCGERGFYTDPTGGGFRRGIVEQCCHSICSLYQLENYCNGGG      60
SEQ ID NO: 42      FVNQHLCGSDLVEALALVCGERGFYTDPTGGGFRRGIVEQCCHSICSLYQLENYCNGGG      60
SEQ ID NO: 50      FVNQHLCGSDLVEALALVCGERGFYTDPTGGGFRRGIVEQCCHSICSLYQLENYCNGGG      60
*****

SEQ ID NO: 44      GAGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRI TRTPEVTCVVLDLGREDPEVQI      120
SEQ ID NO: 46      GAGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRI TRTPEVTCVVLDLGREDPEVQI      120
SEQ ID NO: 48      GAGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRI TRTPEVTCVVLDLGREDPEVQI      120
SEQ ID NO: 42      GAGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRI TRTPEVTCVVLDLGREDPEVQI      120
SEQ ID NO: 50      GAGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRI TRTPEVTCVVLDLGREDPEVQI      120
*****

SEQ ID NO: 44      SWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEQDWTGKEFKCRVNHIDLPSPIERT      180
SEQ ID NO: 46      SWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEQDWTGKEFKCRVNHIDLPSPIERT      180
SEQ ID NO: 48      SWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEQDWTGKEFKCRVNHIDLPSPIERT      180
SEQ ID NO: 42      SWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEQDWTGKEFKCRVNHIDLPSPIERT      180
SEQ ID NO: 50      SWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEQDWTGKEFKCRVNHIDLPSPIERT      180
*****

SEQ ID NO: 44      ISKARGRAHKPSVYVLPSPKELSSDTSITCLIKDFYPPDIDVEWQSNQGQEPERKHR      240
SEQ ID NO: 46      ISKARGRAHKPSVYVLPSPKELSSDTSITCLIKDFYPPDIDVEWQSNQGQEPERKHR      240
SEQ ID NO: 48      ISKARGRAHKPSVYVLPSPKELSSDTSITCLIKDFYPPDIDVEWQSNQGQEPERKHR      240
SEQ ID NO: 42      ISKARGRAHKPSVYVLPSPKELSSDTSITCLIKDFYPPDIDVEWQSNQGQEPERKHR      240
SEQ ID NO: 50      ISKARGRAHKPSVYVLPSPKELSSDTSITCLIKDFYPPDIDVEWQSNQGQEPERKHR      240
*****

SEQ ID NO: 44      MTPPQLDEDGSYFLYSKLSVDKSRWQQGDPFTCAVLHEALHSHYTQKSLSLSPG      294
SEQ ID NO: 46      MTPPQLDEDGSYFLYSKLSVDKSRWQQGDPFTCAVLHETLQSHYTDLSLSHSPG      294
SEQ ID NO: 48      MTPPQLDEDGSYFLYSKLSVDKSRWQQGDPFTCAVMHETLQSHYTDLSLSHSPG      294
SEQ ID NO: 42      MTPPQLDEDGSYFLYSKLSVDKSRWQQGDPFTCAVMHETLQNHYTDLNHYTDLSLSHSPG      294
SEQ ID NO: 50      MTPPQLDEDGSYFLYSKLSVDKSRWQQGDPFTCAVLHETLQNHYTDLNHYTDLSLSHSPG      294
*****;*:*.***: *** **

```

FIG. 3

```

SEQ ID NO: 42      FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG      60
SEQ ID NO: 56      FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG      60
SEQ ID NO: 52      FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG      60
SEQ ID NO: 54      FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG      60
*****

SEQ ID NO: 42      GAGGGGRCTDTPPCPVPEPLGGPSVLIFFPKPKDILRITRTPPEVTCVVLDLGREDEPVEVQI      120
SEQ ID NO: 56      GAGGGGC---ISPCVPVESLGGPSVFIFFPKPKDILRITRTPPEITCVVLDLGREDEPVEVQI      117
SEQ ID NO: 52      GAGGGGDCPK---CPAPEMLGGPSVFIFFPKPKDILLIARTPEVTCVVVLDLDPEDPVEVQI      117
SEQ ID NO: 54      GAGGGG-CNN-CPCPGCGLLGGPSVFIFFPKPKDILVTARTPTVTCVVVLDLDPENVEVQI      118
*****          **          *****:***** * :*** :****:*. *.*****

SEQ ID NO: 42      SWFVDGKEVHTAKTQSREQQFNGTYRVVSVLPIEHQDWTGKEFKCRVNHIDLPSPIERT      180
SEQ ID NO: 56      SWFVDGKEVHTAKTQPREQQFNSTYRVVSVLPIEHQDWTGKEFKCRVNHIGLPSPIERT      177
SEQ ID NO: 52      SWFVDGKQMKTAKTQPREEQFNGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERT      177
SEQ ID NO: 54      SWFVDSKQVQTANTQPREEQSNGTYRVVSVLPIGHQDWLSGKQFKCKVNNKALPSPIEEI      178
*****.*:;:*:* **:* * ,***** *****.*:*:*:*: *****.

SEQ ID NO: 42      ISKARGRAHKPSVYVLPSPKELSSSDTVSITCLIKDFYPPDIDVEWQSNQGQEPERKHR      240
SEQ ID NO: 56      ISKARGQAHQPSVYVLPSPKELSSSDTVTLTCLIKDFPPEIDVEWQSNQGQEPESKYH      237
SEQ ID NO: 52      ISKARGQAHQPSVYVLPSPREELS-KNTVSLTCLIKDFPDPIDVEWQSNQGQEPESKYR      236
SEQ ID NO: 54      ISKTPGQAHQPNVYVLPSPRDEMS-KNTVTLTCLVKDFPPEIDVEWQSNQGQEPESKYR      237
***: *:*:*.* ,***** .*:* .:*:*:***:***:*.***** *** *;:

SEQ ID NO: 42      MTPPQLDEDGGSYFLYSKLSVDKSRWQQGDPFTCAVMHETLQNHYTDLSSLHSPG      294
SEQ ID NO: 56      TTAPQLDEDGGSYFLYSKLSVDKSRWQQGDTFTCAVMHEALQNHYTDLSSLHSPG      291
SEQ ID NO: 52      TTPPQLDEDGGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG      290
SEQ ID NO: 54      MTPPQLDEDGGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQISLSHSPG      291
* *****:***:***:*****

```

FIG. 4

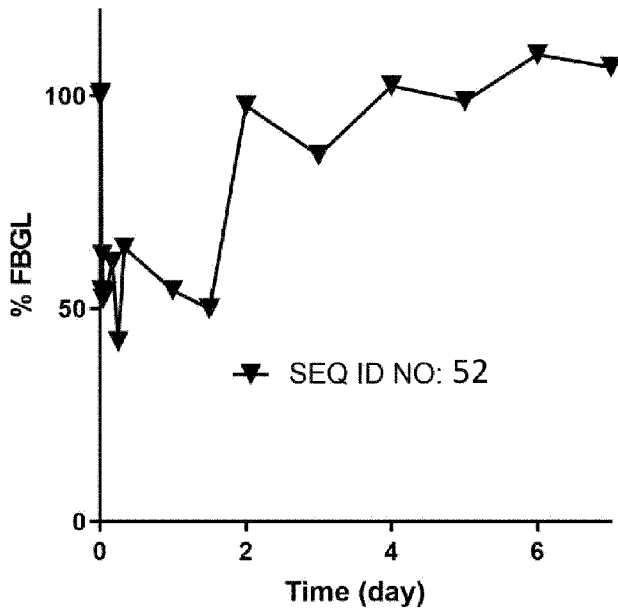


FIG. 5

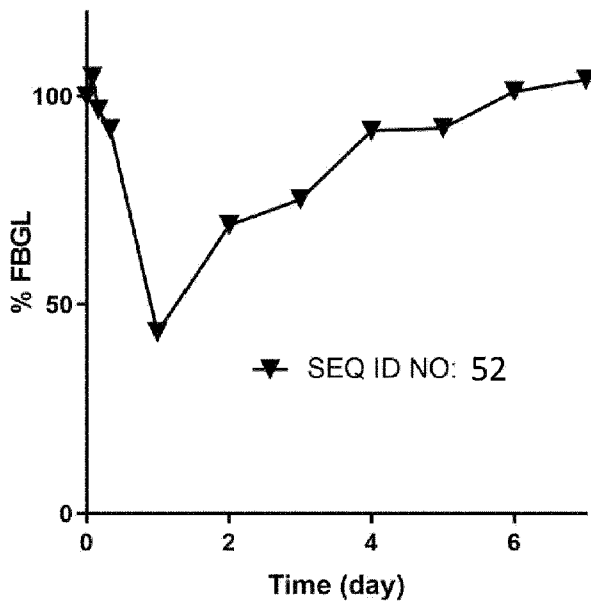


FIG. 6

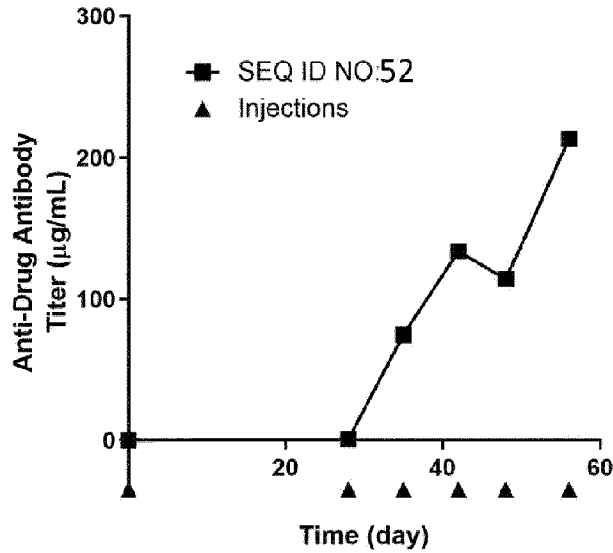


FIG. 7

| | | |
|---------------|---|-----|
| SEQ ID NO: 58 | FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG | 60 |
| SEQ ID NO: 60 | FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG | 60 |
| SEQ ID NO: 62 | FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG | 60 |
| SEQ ID NO: 64 | FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG | 60 |
| ***** | | |
| SEQ ID NO: 58 | GAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF | 120 |
| SEQ ID NO: 60 | GAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF | 120 |
| SEQ ID NO: 62 | GAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF | 120 |
| SEQ ID NO: 64 | GAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF | 120 |
| ***** | | |
| SEQ ID NO: 58 | VDGKQMOTAKTQPREEQFGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK | 180 |
| SEQ ID NO: 60 | VDGKQMOTAKTQPREEQFGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK | 180 |
| SEQ ID NO: 62 | VDGKQMOTAKTQPREEQFGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK | 180 |
| SEQ ID NO: 64 | VDGKQMOTAKTQPREEQFGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK | 180 |
| ***** | | |
| SEQ ID NO: 58 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQQEPESKYRTTPP | 240 |
| SEQ ID NO: 60 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQQEPESKYRTTPP | 240 |
| SEQ ID NO: 62 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQQEPESKYRTTPP | 240 |
| SEQ ID NO: 64 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQQEPESKYRTTPP | 240 |
| ***** | | |
| SEQ ID NO: 58 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG | 290 |
| SEQ ID NO: 60 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG | 290 |
| SEQ ID NO: 62 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG | 290 |
| SEQ ID NO: 64 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG | 290 |
| ***** | | |

FIG. 8

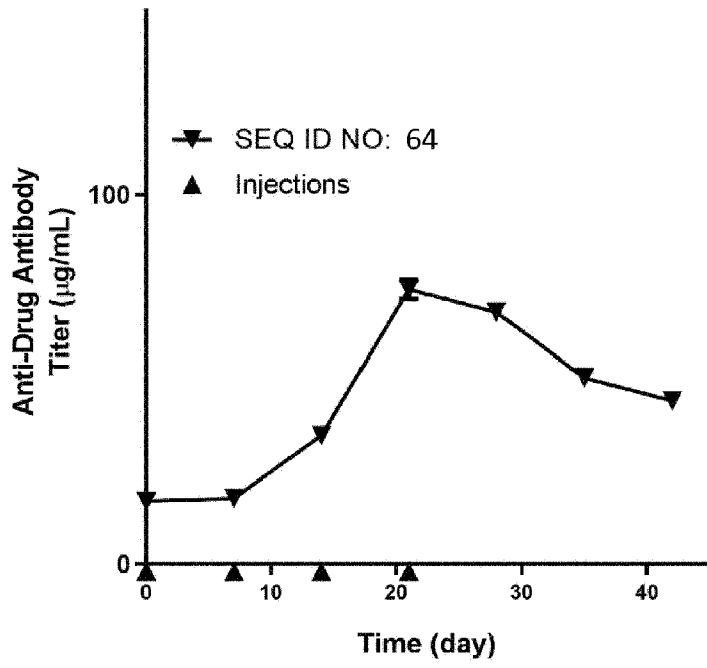


FIG. 9

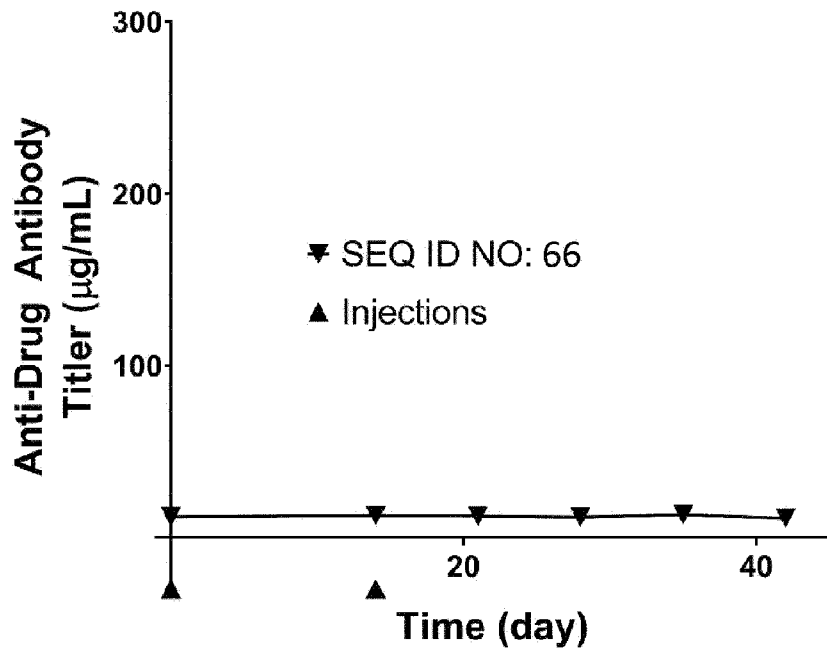


FIG. 10

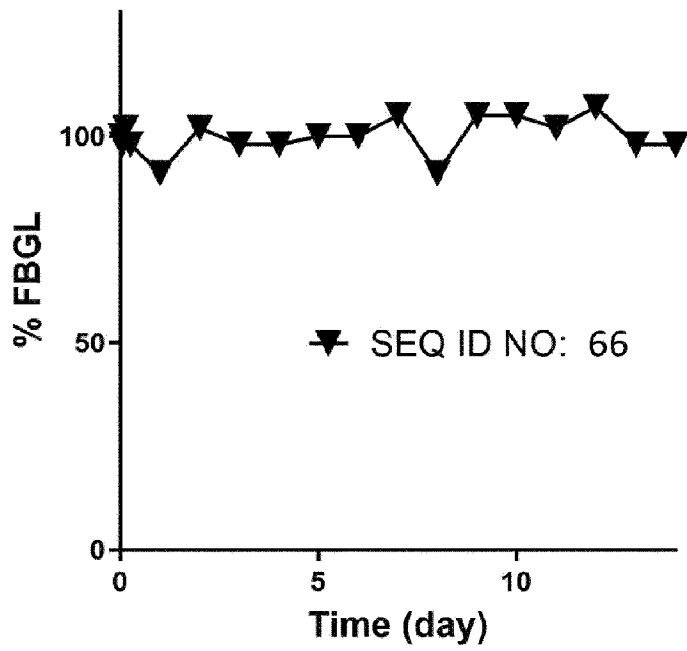


FIG. 11

| | | |
|---------------|---|-----|
| SEQ ID NO: 66 | FVNQHLCGSHLVEALALVCGERGFYTDPTGGGPRRGIVEQCCTSICSLYQLENYCNGG | 60 |
| SEQ ID NO: 68 | FVNQHLCGSHLVQALYLVCGERGFYTDPTGGGPRRGIVEQCCTSICSLYQLENYCGG-G | 59 |
| SEQ ID NO: 70 | FVNQHLCGSELVEALALVCGERGFYTDPTGGGPRRGIVEQCCTSICSLYQLENYCGG-G | 59 |
| SEQ ID NO: 72 | FVNQHLCGSHLVEALALVCGEAGFFYTDPTGGGPRRGIVEQCCTSICSLYQLENYCGG-G | 59 |
| SEQ ID NO: 74 | FVNQHLCGSHLVEALALVCGERGFYTDPTGGGPRRGIVEQCCTSICSLYQLENYCGG-G | 59 |
| SEQ ID NO: 76 | FVNQHLCGSHLVEALALVCGERGFYTDPTGGGPRRGIVEQCCTSICSLYQLENYCGG-G *****. **: * | 59 |
| SEQ ID NO: 66 | GAGGGGDCPKCPAPEMLGGPSVFIFFPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF | 120 |
| SEQ ID NO: 68 | GAGGGGDCPKCPAPEMLGGPSVFIFFPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF | 119 |
| SEQ ID NO: 70 | GAGGGGDCPKCPAPEMLGGPSVFIFFPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF | 119 |
| SEQ ID NO: 72 | GAGGGGDCPKCPAPEMLGGPSVFIFFPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF | 119 |
| SEQ ID NO: 74 | GAGGGGDCPKCPAPEMLGGPSVFIFFPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF | 119 |
| SEQ ID NO: 76 | GAGGGGDCPKCPAPEMLGGPSVFIFFPKPKDTLLIARTPEVTCVVVDLDPEDPEVQISWF ***** | 119 |
| SEQ ID NO: 66 | VDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK | 180 |
| SEQ ID NO: 68 | VDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK | 179 |
| SEQ ID NO: 70 | VDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK | 179 |
| SEQ ID NO: 72 | VDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK | 179 |
| SEQ ID NO: 74 | VDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK | 179 |
| SEQ ID NO: 76 | VDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTISK *****. ***** | 179 |
| SEQ ID NO: 66 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFFPPDIDVEWQSNQQEPESKYRTTPP | 240 |
| SEQ ID NO: 68 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFFPPDIDVEWQSNQQEPESKYRTTPP | 239 |
| SEQ ID NO: 70 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFFPPDIDVEWQSNQQEPESKYRTTPP | 239 |
| SEQ ID NO: 72 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFFPPDIDVEWQSNQQEPESKYRTTPP | 239 |
| SEQ ID NO: 74 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFFPPDIDVEWQSNQQEPESKYRTTPP | 239 |
| SEQ ID NO: 76 | ARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFFPPDIDVEWQSNQQEPESKYRTTPP ***** | 239 |
| SEQ ID NO: 66 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG | 290 |
| SEQ ID NO: 68 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG | 289 |
| SEQ ID NO: 70 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG | 289 |
| SEQ ID NO: 72 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG | 289 |
| SEQ ID NO: 74 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG | 289 |
| SEQ ID NO: 76 | QLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG ***** | 289 |

FIG. 12

```

SEQ ID NO: 66      FVNQHLCGSHLVEALALVCGERGFYTDPTGG-----GPRRGIVEQCCTSICSLYQLENY 55
SEQ ID NO: 78      FVNQHLCGSHLVQALYLVCGERGFYTDPTQRGGG--GGQRGIVEQCCTSICSLYQLENY 58
SEQ ID NO: 80      FVNQHLCGSHLVEALALVCGERGFYTDPTGGGGGGGGGGGIVEQCCTSICSLYQLENY 60
SEQ ID NO: 82      FVNQHLCGSHLVEALALVCGERGFYTDPGGGG---GGGGGIVEQCCTSICSLYQLENY 56
SEQ ID NO: 84      FVNQHLCGSHLVEALALVCGERGFYTDPGGGG---GGGGGIVEQCCTSICSLYQLENY 55
                    *****;* ***** * * *****

SEQ ID NO: 66      CNGGGGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLDPEDPEV 115
SEQ ID NO: 78      CGG-GGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLDPEDPEV 117
SEQ ID NO: 80      CGG-GGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLDPEDPEV 119
SEQ ID NO: 82      CGG-GGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLDPEDPEV 115
SEQ ID NO: 84      CGG-GGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLDPEDPEV 114
                    *. *****

SEQ ID NO: 66      QISWFVDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIE 175
SEQ ID NO: 78      QISWFVDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIE 177
SEQ ID NO: 80      QISWFVDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIE 179
SEQ ID NO: 82      QISWFVDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIE 175
SEQ ID NO: 84      QISWFVDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIE 174
                    *****.*****

SEQ ID NO: 66      RTISKARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPPIDVEWQSNQQQEPESKY 235
SEQ ID NO: 78      RTISKARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPPIDVEWQSNQQQEPESKY 237
SEQ ID NO: 80      RTISKARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPPIDVEWQSNQQQEPESKY 239
SEQ ID NO: 82      RTISKARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPPIDVEWQSNQQQEPESKY 235
SEQ ID NO: 84      RTISKARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPPIDVEWQSNQQQEPESKY 234
                    *****

SEQ ID NO: 66      RTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG 290
SEQ ID NO: 78      RTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG 292
SEQ ID NO: 80      RTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG 294
SEQ ID NO: 82      RTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG 290
SEQ ID NO: 84      RTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG 289
                    *****

```

FIG. 13

```

SEQ ID NO: 86      FVNQHLCGSHLVEALALVCGERGFYTDPTGGGPRRGIVEQCCTSICSLYQLENYCGGG 60
SEQ ID NO: 66      FVNQHLCGSHLVEALALVCGERGFYTDPTGGGPRRGIVEQCCTSICSLYQLENYCNGGG 60
SEQ ID NO: 76      FVNQHLCGSHLVEALALVCGERGFYTDPTGGGPRRGIVEQCCTSICSLYQLENYCGGG 60
                    *****.***

SEQ ID NO: 86      GQGGGGQGGGGQGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLD 120
SEQ ID NO: 66      GA-----GGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLD 109
SEQ ID NO: 76      A-----GGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVDLD 108
                    .
                    *****

SEQ ID NO: 86      PEDPEVQISWFVDGKQMOTAKTQPREEQFSGYRVVSVLPIGHQDWLKGKQFTCKVNNKA 180
SEQ ID NO: 66      PEDPEVQISWFVDGKQMOTAKTQPREEQFQGYRVVSVLPIGHQDWLKGKQFTCKVNNKA 169
SEQ ID NO: 76      PEDPEVQISWFVDGKQMOTAKTQPREEQFSGYRVVSVLPIGHQDWLKGKQFTCKVNNKA 168
                    *****.*****

SEQ ID NO: 86      LPSPIERTISKARGQAHQPSVYVLPSSREELSKNTVSLTCLIKDFPPDIDVEWQSNQQ 240
SEQ ID NO: 66      LPSPIERTISKARGQAHQPSVYVLPSSREELSKNTVSLTCLIKDFPPDIDVEWQSNQQ 229
SEQ ID NO: 76      LPSPIERTISKARGQAHQPSVYVLPSSREELSKNTVSLTCLIKDFPPDIDVEWQSNQQ 228
                    *****

SEQ ID NO: 86      EPESKYRTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSP 300
SEQ ID NO: 66      EPESKYRTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSP 289
SEQ ID NO: 76      EPESKYRTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSP 288
                    *****

SEQ ID NO: 86      G      301
SEQ ID NO: 66      G      290
SEQ ID NO: 76      G      289
                    *

```

FIG. 14

```

SEQ ID NO: 66      FVNQHLCGSHLVEALALVCGERGFYTDPTGGG-PRRGIVEQCCTSICSLYQLENYCGG 59
SEQ ID NO: 82      FVNQHLCGSHLVEALALVCGERGFYTDPGGGGGGGGIVEQCCTSICSLYQLENYCGG- 59
SEQ ID NO: 88      FVNQHLCGSHLVEALALVCGERGFYTDG-GGGGGGGGIVEQCCTSICSLYQLENYCGG- 58
SEQ ID NO: 84      FVNQHLCGSHLVEALALVCGERGFYTPG-GGGGGGGGIVEQCCTSICSLYQLENYCGG- 58
                    ***** **
SEQ ID NO: 66      GGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVLDLDPEDPEVQISW 119
SEQ ID NO: 82      GGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVLDLDPEDPEVQISW 119
SEQ ID NO: 88      GGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVLDLDPEDPEVQISW 118
SEQ ID NO: 84      GGAGGGGDCPKCPAPEMLGGPSVFI FPPKPKDTLLIARTPEVTCVVVLDLDPEDPEVQISW 118
                    *****
SEQ ID NO: 66      FVDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTIS 179
SEQ ID NO: 82      FVDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTIS 179
SEQ ID NO: 88      FVDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTIS 178
SEQ ID NO: 84      FVDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNKALPSPIERTIS 178
                    *****
SEQ ID NO: 66      KARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQQEPEPE SKYRTTP 239
SEQ ID NO: 82      KARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQQEPEPE SKYRTTP 239
SEQ ID NO: 88      KARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQQEPEPE SKYRTTP 238
SEQ ID NO: 84      KARGQAHQPSVYVLPSPREELSKNTVSLTCLIKDFPPDIDVEWQSNQQEPEPE SKYRTTP 238
                    *****
SEQ ID NO: 66      PQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG 290
SEQ ID NO: 82      PQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG 290
SEQ ID NO: 88      PQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG 289
SEQ ID NO: 84      PQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHSPG 289
                    *****

```

FIG. 15

```

SEQ ID NO: 66      FVNQHLCGSHLVEALALVCGERGFYTDPTGG-GPRRGIVEQCCTSIICSLYQLENYCNGG 59
SEQ ID NO: 90      FVNQHLCGSHLVEALELVCGERGFYTPKTBGGSGGGGGIVEQCCTSTCSLDQLENYCGG- 59
SEQ ID NO: 92      FVNQHLCGSHLVEALELVCGERGFHYGGGGGGSGGGGGIVEQCCTSTCSLDQLENYCNHG 60
SEQ ID NO: 34      FVNQHLCGSHLVEALELVCGERGFHYGGGGGGSGGGGGIVEQCCTSTCSLDQLENYCNHG 60
SEQ ID NO: 32      FVNQHLCGSHLVEALELVCGERGFHYGGGGGGSGGGGGIVEQCCTSTCSLDQLENYCGG- 59
SEQ ID NO: 94      FVNQHLCGSHLVEALELVCGERGFYGGGGGGSGGGGGIVEQCCTSTCSLDQLENYCGG- 59
                    ***** , * * * ***** **

```

```

SEQ ID NO: 66      GG-----AGGGDCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVTCVVVD 107
SEQ ID NO: 90      GGGQGGGGQGGGGQGGGGDCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVTCVVVD 119
SEQ ID NO: 92      GGGQGGGGQGGGGQGGGGDCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVTCVVVD 120
SEQ ID NO: 34      GGGQGGGGQGGGGQGGGGDCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVTCVVVD 120
SEQ ID NO: 32      GGGQGGGGQGGGGQGGGGDCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVTCVVVD 119
SEQ ID NO: 94      GGGQGGGGQGGGGQGGGGDCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVTCVVVD 119
                    ** .*****

```

```

SEQ ID NO: 66      LDPEDPEVQISWFVDGKQMCTAKTQPREEQFNGTYRVVSVLPIGHQDWLKGKQFTCKVNN 167
SEQ ID NO: 90      LDPEDPEVQISWFVDGKQMCTAKTQPREEQFNGTYRVVSVLPIGHQDWLKGKQFTCKVNN 179
SEQ ID NO: 92      LDPEDPEVQISWFVDGKQMCTAKTQPREEQFNGTYRVVSVLPIGHQDWLKGKQFTCKVNN 180
SEQ ID NO: 34      LDPEDPEVQISWFVDGKQMCTAKTQPREEQFNGTYRVVSVLPIGHQDWLKGKQFTCKVNN 180
SEQ ID NO: 32      LDPEDPEVQISWFVDGKQMCTAKTQPREEQFNGTYRVVSVLPIGHQDWLKGKQFTCKVNN 179
SEQ ID NO: 94      LDPEDPEVQISWFVDGKQMCTAKTQPREEQFNGTYRVVSVLPIGHQDWLKGKQFTCKVNN 179
                    *****;*****

```

```

SEQ ID NO: 66      KALPSPIERTISKARGQAHQPSVYVLPSSREELSKNTVSLTCLIKDFFPPDIDVEWQSNG 227
SEQ ID NO: 90      KALPSPIERTISKARGQAHQPSVYVLPSSREELSKNTVSLTCLIKDFFPPDIDVEWQSNG 239
SEQ ID NO: 92      KALPSPIERTISKARGQAHQPSVYVLPSSREELSKNTVSLTCLIKDFFPPDIDVEWQSNG 240
SEQ ID NO: 34      KALPSPIERTISKARGQAHQPSVYVLPSSREELSKNTVSLTCLIKDFFPPDIDVEWQSNG 240
SEQ ID NO: 32      KALPSPIERTISKARGQAHQPSVYVLPSSREELSKNTVSLTCLIKDFFPPDIDVEWQSNG 239
SEQ ID NO: 94      KALPSPIERTISKARGQAHQPSVYVLPSSREELSKNTVSLTCLIKDFFPPDIDVEWQSNG 239
                    *****

```

```

SEQ ID NO: 66      QQEPESKYRTTPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSH 287
SEQ ID NO: 90      QQEPESKYRTTPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSH 299
SEQ ID NO: 92      QQEPESKYRTTPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSH 300
SEQ ID NO: 34      QQEPESKYRTTPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSH 300
SEQ ID NO: 32      QQEPESKYRTTPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSH 299
SEQ ID NO: 94      QQEPESKYRTTPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSH 299
                    *****

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```

SEQ ID NO: 66      SPG 290
SEQ ID NO: 90      SPG 302
SEQ ID NO: 92      SPG 303
SEQ ID NO: 34      SPG 303
SEQ ID NO: 32      SPG 302
SEQ ID NO: 94      SPG 302
                    ***

```

FIG. 16

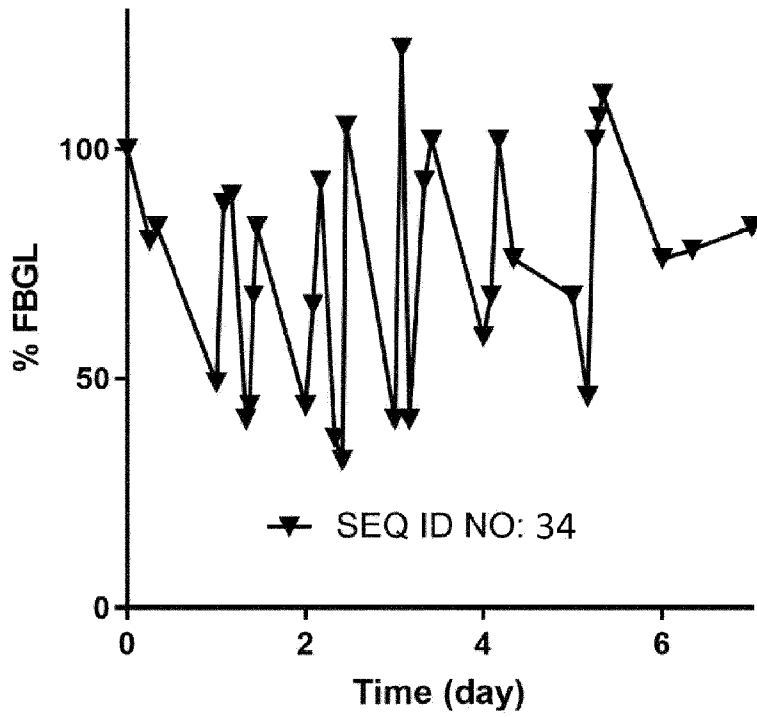


FIG. 17

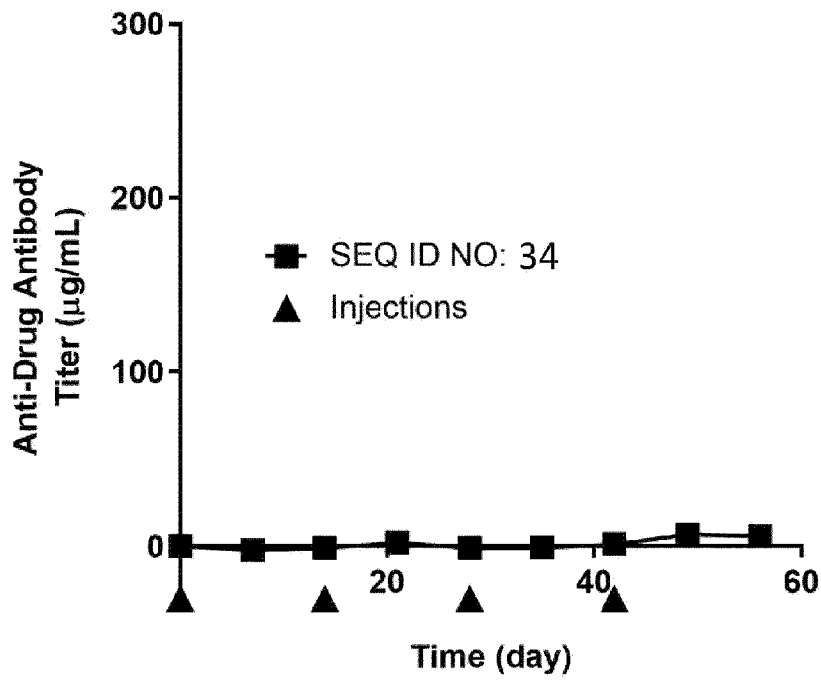


FIG. 18

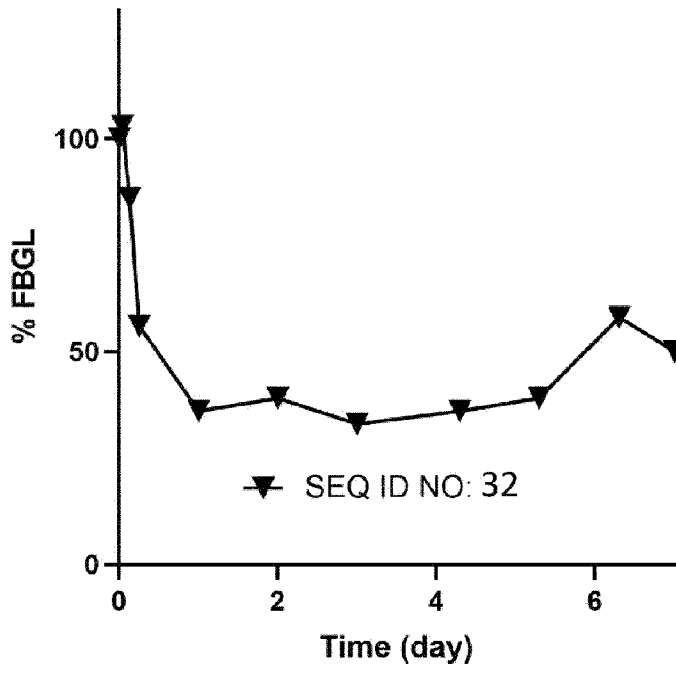


FIG. 19

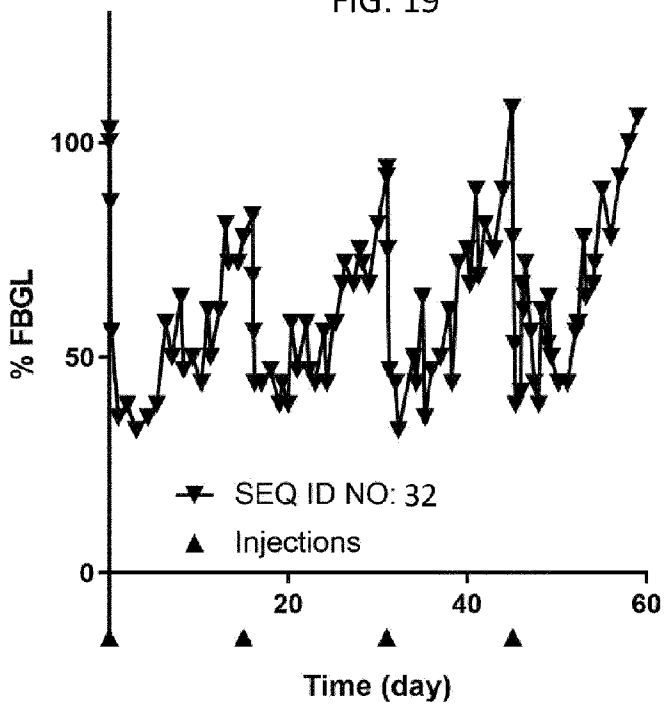


FIG. 20

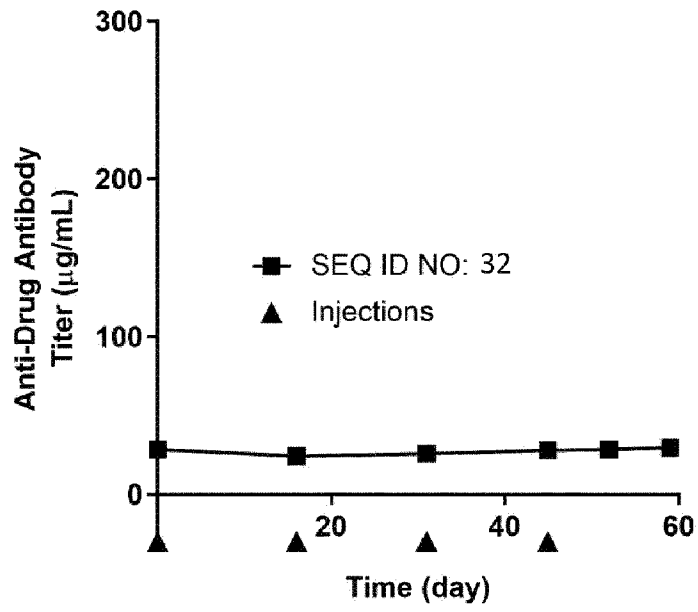


FIG. 21

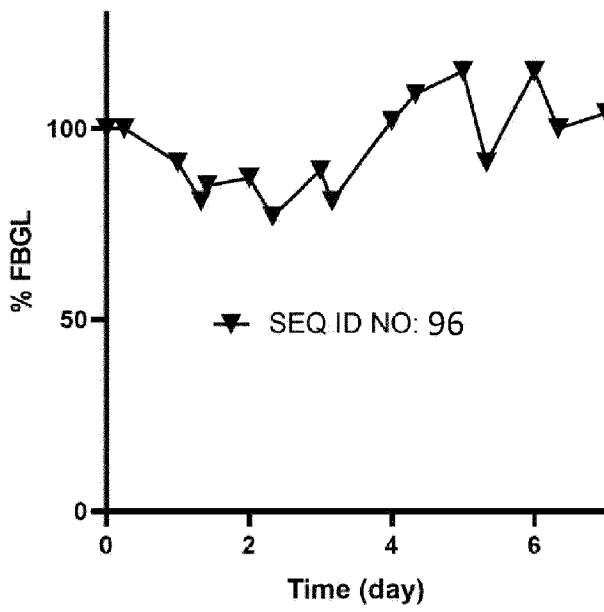


FIG. 22

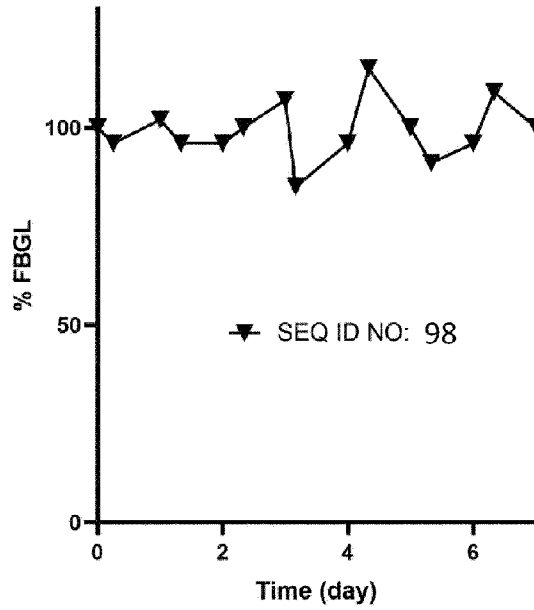


FIG. 23

```

SEQ ID NO: 102      FVNQHLCGSHLVEALELVCGERGFIHYGGGGGGGGGGGIVEQCCTSTCSLDQLENYCGGG 60
SEQ ID NO: 104      FVNQHLCGSHLVEALELVCGERGFIHYGGGGGGGGGGGIVEQCCTSTCSLDQLENYCGGG 60
*****

SEQ ID NO: 102      GGQGGGGQGGGGQGGGGGDCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVTCVVVAL 120
SEQ ID NO: 104      GGQGGGGQGGGGQGGGGGDCPKCPAPEMLGGPSVFI FPPKPKDILLIARTPEVTCVVVDL 120
***** *

SEQ ID NO: 102      DPEDPEVQISWFDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNK 180
SEQ ID NO: 104      DPEDPEVQISWFDGKQMQTAKTQPREEQFSGTYRVVSVLPIGHQDWLKGKQFTCKVNNK 180
*****

SEQ ID NO: 102      ALPSPIERTISKARGQAHQPSVYVLPSPREELSKNVSLTCLIKDFPPDIDVEWQSNQG 240
SEQ ID NO: 104      ALPSPIERTISKARGQAHQPSVYVLPSPREELSKNVSLTCLIKDFPPDIDVEWQSNQG 240
*****

SEQ ID NO: 102      QEPESKYRTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHS 300
SEQ ID NO: 104      QEPESKYRTTPPQLDEDGSYFLYSKLSVDKSRWQRGDTFICAVMHEALHNHYTQESLSHS 300
*****

SEQ ID NO: 102      PG 302
SEQ ID NO: 104      PG 302
**
    
```

FIG. 24

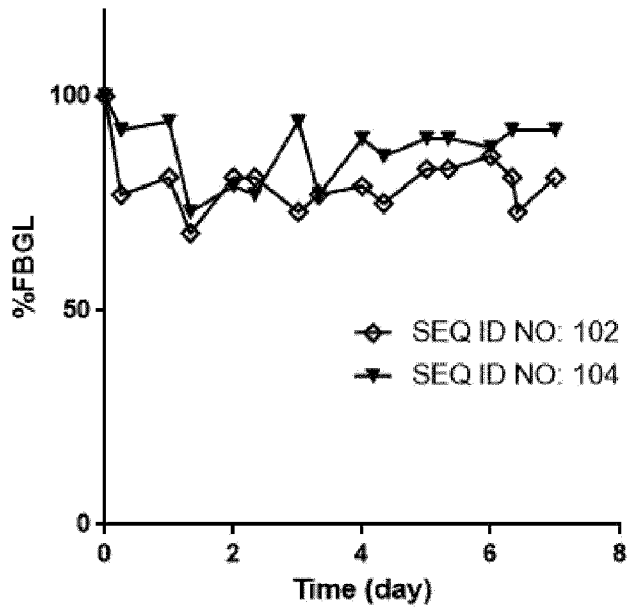


FIG. 25

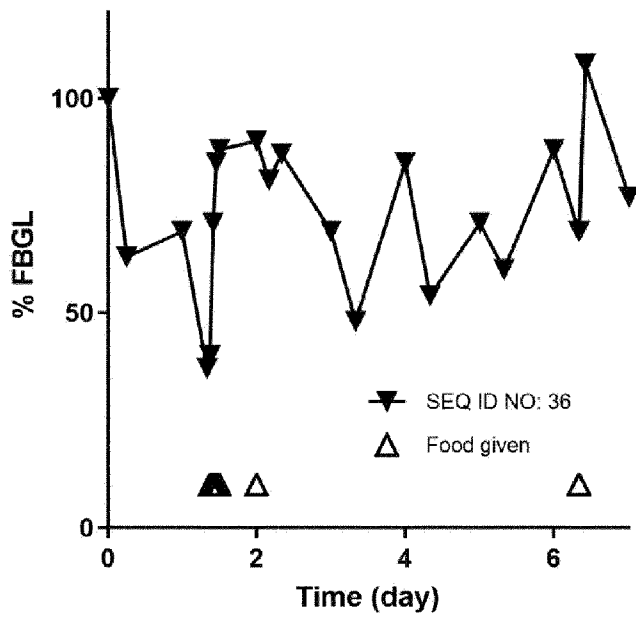


FIG. 26

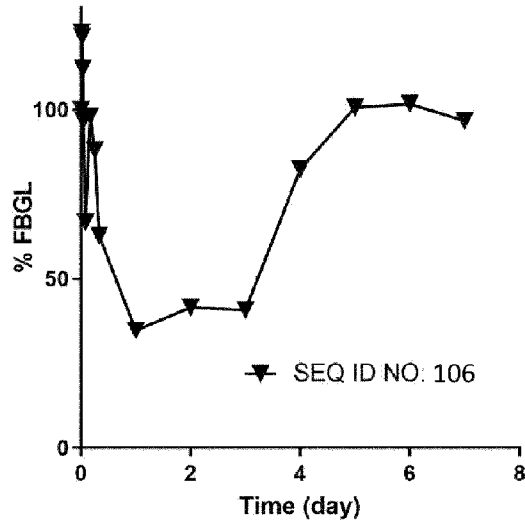


FIG. 27

| | | |
|---|--|-----|
| SEQ ID NO: 108 | FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG | 60 |
| SEQ ID NO: 110 | FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG | 60 |
| SEQ ID NO: 106 | FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG | 60 |
| SEQ ID NO: 112 | FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCHSICSLYQLENYCNGGG | 60 |
| ***** | | |
| SEQ ID NO: 108 | GSGG-GGDCPKCPPPEMLGGPSIFIFPPKPKDLSISRTPVETCLVVDLGPDDSDVQITW | 119 |
| SEQ ID NO: 110 | GAGGGGGEGPKCPVPEIPGAPSVFIFPPKPKDLSISRTPVETCLVVDLGPDDSNVQITW | 120 |
| SEQ ID NO: 106 | GSGGGGGEGPKCPVPEIPGAPSVFIFPPKPKDLSISRTPVETCLVVDLGPDDSDVQITW | 120 |
| SEQ ID NO: 112 | GSGGGGGEGPKCPVPEIPGAPSVFIFPPKPKDLSISRTPVETCLVVDLGPDDSNVQITW | 120 |
| *:** **: **** **: *.**:*:*****:*****:***** | | |
| SEQ ID NO: 108 | FVDNTQVYTAKTSPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSPIERTIS | 179 |
| SEQ ID NO: 110 | FVDNTEMHTAKTRPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSAMERTIS | 180 |
| SEQ ID NO: 106 | FVDNTEMHTAKTRPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSAMERTIS | 180 |
| SEQ ID NO: 112 | FVDNTEMHTAKTRPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSAMERTIS | 180 |
| *****:;:**** *****:*****:*****:*****:***** | | |
| SEQ ID NO: 108 | KDKGQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEPENNYQTTP | 239 |
| SEQ ID NO: 110 | KAKGQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEPENNYQTTP | 240 |
| SEQ ID NO: 106 | KAKGQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEPENNYQTTP | 240 |
| SEQ ID NO: 112 | KAKGQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEPENNYQTTP | 240 |
| * *****:*****:*****:*****:*****:*****:*****:***** | | |
| SEQ ID NO: 108 | PQLSDGTYFLYSRLSVDRSRWQRGNTYTCSVSHEALSHHTQKSLTQSPG | 290 |
| SEQ ID NO: 110 | PQLSDGTYFLYSRLSVDRSHWQRGNTYTCSVSHEALSHHTQKSLTQSPG | 291 |
| SEQ ID NO: 106 | PQLSDGTYFLYSRLSVDRSHWQRGNTYTCSVSHEALSHHTQKSLTQSPG | 291 |
| SEQ ID NO: 112 | PQLSDGTYFLYSRLSVDRSHWQRGNTYTCSVSHEALSHHTQKSLTQSPG | 291 |
| *****:*****:*****:*****:*****:*****:***** | | |

FIG. 28

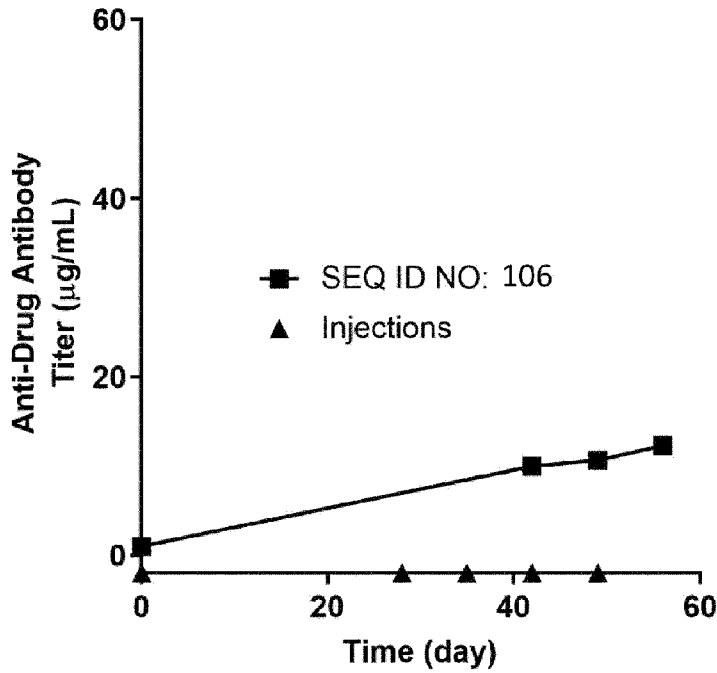


FIG. 29

| | | |
|----------------|---|-----|
| SEQ ID NO: 114 | FVNQHLCGSHLVEALALVCGERGFYTDPAAGGPRRGIVEQCCASVCSLYQLEHYCGGGG | 60 |
| SEQ ID NO: 116 | FVNQHLCGSHLVEALALVCGERGFYTDPAAGGPRRGIVEQCCASVCSLYQLEHYCGGGG | 60 |
| SEQ ID NO: 108 | FVNQHLCGSDLVEALALVCGERGFYTDPTGGGPRRGIVEQCCSHICSLSLYQLENYCNGGG | 60 |
| SEQ ID NO: 118 | FVNQHLCGSHLVEALALVCGERGFYTDPAAGGPRRGIVEQCCASVCSLYQLEHYCGG-G | 59 |
| | *****,*****;*****;***** *;*****;**,* * | |
| SEQ ID NO: 114 | AGGGGGEGPKCFVPEIPGAPSVFI FPPKPKDLSISRTPEVTCLVVDLGPDDSNVQITWF | 120 |
| SEQ ID NO: 116 | AGGGGGEGPKCFVPEIPGAPSVFI FPPKPKDLSISRTPEVTCLVVDLGPDDSNVQITWF | 120 |
| SEQ ID NO: 108 | GGGGGDCPKCFPEMLGGPSIFIFPPKPKDLSISRTPEVTCLVVDLGPDDSDVQITWF | 120 |
| SEQ ID NO: 118 | GAGGGDCPKCFPEMLGGPSIFIFPPKPKDLSISRTPEVTCLVVALGPDDSDVQITWF | 119 |
| | ..****; **** **; *,**;*****;***** *****;***** | |
| SEQ ID NO: 114 | VDNTEMHTAKTRPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSAMERTISK | 180 |
| SEQ ID NO: 116 | VDNTEMHTAKTRPREEQFSSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSAMERTISK | 180 |
| SEQ ID NO: 108 | VDNTQVYTAKTSPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSPIERTISK | 180 |
| SEQ ID NO: 118 | VDNTQVYTAKTSPREEQFSSTYRVVSVLPILHQDWLKGKEFKCKVNSKSLPSPIERTISK | 179 |
| | ****;.:**** *****.*****;***** *****;***** | |
| SEQ ID NO: 114 | AKGQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEFENNYQTTPP | 240 |
| SEQ ID NO: 116 | AKGQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITGQPEFENNYQTTPP | 240 |
| SEQ ID NO: 108 | DKGQPHEPQVYVLPPEQEELSRNKVSVTCLIEGFYPSDIAVEWEITGQPEFENNYRTTPP | 240 |
| SEQ ID NO: 118 | DRGQPHEPQVYVLPPEQEELSRNKVSVTCLIEGFYPSDIAVEWEITGQPEFENNYRTTPP | 239 |
| | *****;*****;*****;*****;*****;*****;*****;*****;***** | |
| SEQ ID NO: 114 | QLSDSGTYFLYSRLSVDRSHWQRGNTYTCVSHALSHHTQKSLTQSP- | 289 |
| SEQ ID NO: 116 | QLSDSGTYFLYSRLSVDRSHWQRGNTYTCVSHALSHHTQKSLTQSPG | 290 |
| SEQ ID NO: 108 | QLSDSGTYFLYSRLSVDRSRWQRGNTYTCVSHALSHHTQKSLTQSPG | 290 |
| SEQ ID NO: 118 | QLSDSGTYFLYSRLSVDRSRWQRGNTYTCVSHALSHHTQKSLTQSPG | 289 |
| | *****;*****;*****;*****;*****;*****;*****;*****;***** | |

FIG. 30

| | | |
|----------------|--|-----|
| SEQ ID NO: 106 | FVNQHLCGSDLVEALYLVCGERGFFYTDPTGG-GPRRGIVEQCCHSICSLYQLENYCNGG | 59 |
| SEQ ID NO: 112 | FVNQHLCGSDLVEALALVCGERGFFYTDPTGG-GPRRGIVEQCCHSICSLYQLENYCNGG | 59 |
| SEQ ID NO: 122 | FVNQHLCGSHLVEALELVCGERGFHYGGGGGGGGGGIVEQCCTSTCSLDQLENYCNGG *****.***** *****.* . ** * ***** * *** *****.* | 60 |
| SEQ ID NO: 106 | GGSG-----GGGEGPKCPVPEIPGAPSVFIFPPKPKDTLSISRTPEVTCLVVD | 108 |
| SEQ ID NO: 112 | GGSG-----GGGEGPKCPVPEIPGAPSVFIFPPKPKDTLSISRTPEVTCLVVD | 108 |
| SEQ ID NO: 122 | GGQGGGGQGGGGGGGGGGEGPKCPVPEIPGAPSVFIFPPKPKDTLSISRTPEVTCLVVD **.* ***** | 120 |
| SEQ ID NO: 106 | LGPDDSNVQITWFDNTEMHTAKTRPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNS | 168 |
| SEQ ID NO: 112 | LGPDDSNVQITWFDNTEMHTAKTRPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNS | 168 |
| SEQ ID NO: 122 | LGPDDSNVQITWFDNTEMHTAKTRPREEQFNSTYRVVSVLPILHQDWLKGKEFKCKVNS ***** | 180 |
| SEQ ID NO: 106 | KSLPSAMERTISKAKGQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITG | 228 |
| SEQ ID NO: 112 | KSLPSAMERTISKAKGQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITG | 228 |
| SEQ ID NO: 122 | KSLPSAMERTISKAKGQPHEPQVYVLPPTQEELSENKVSVTCLIKGFHPPDIAVEWEITG ***** | 240 |
| SEQ ID NO: 106 | QPEPENNYQTTPPQLSDGTYFLYSRLSVD RSHWQRGNTYTCSVSHEALSHHTQKSLTQ | 288 |
| SEQ ID NO: 112 | QPEPENNYQTTPPQLSDGTYFLYSRLSVD RSHWQRGNTYTCSVSHEALSHHTQKSLTQ | 288 |
| SEQ ID NO: 122 | QPEPENNYQTTPPQLSDGTYFLYSRLSVD RSHWQRGNTYTCSVSHEALSHHTQKSLTQ ***** | 300 |
| SEQ ID NO: 106 | SPG 291 | |
| SEQ ID NO: 112 | SPG 291 | |
| SEQ ID NO: 122 | SPG 303 *** | |

FIG. 31

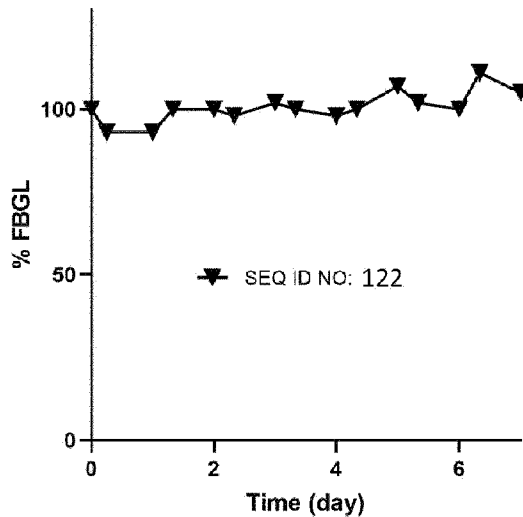


FIG. 32

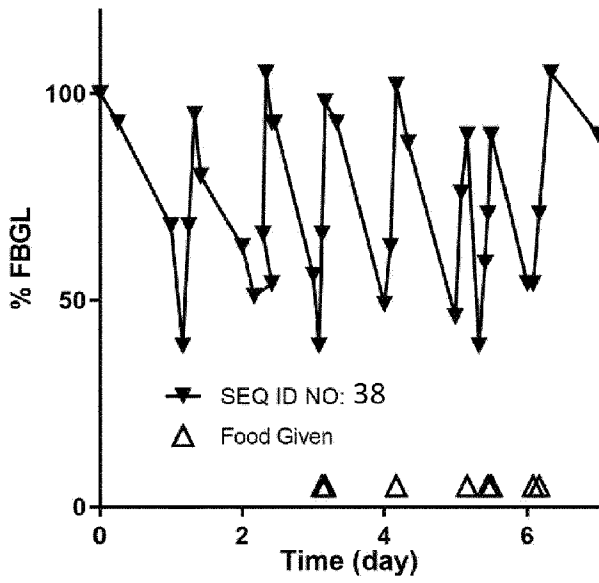


FIG. 33

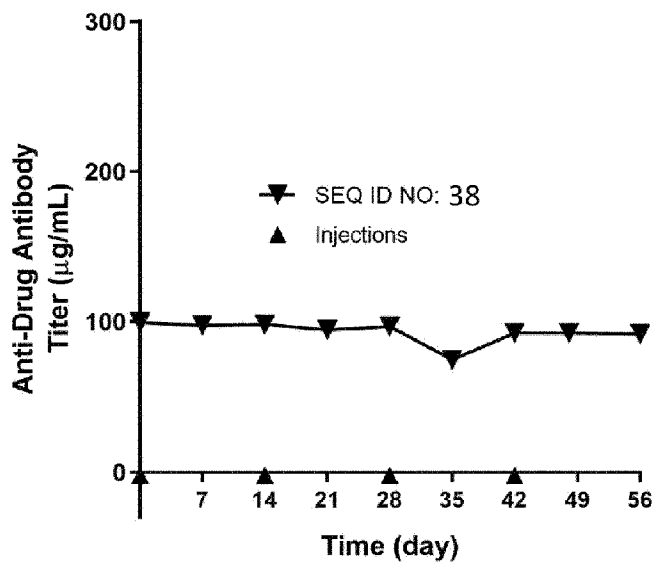


FIG. 34

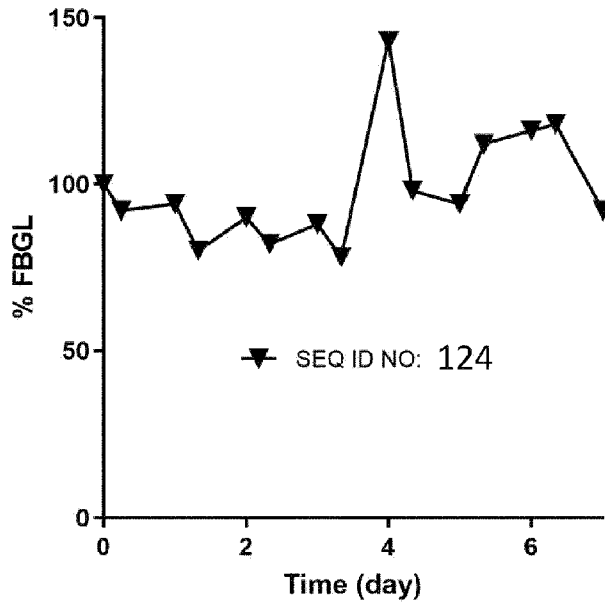


FIG. 35

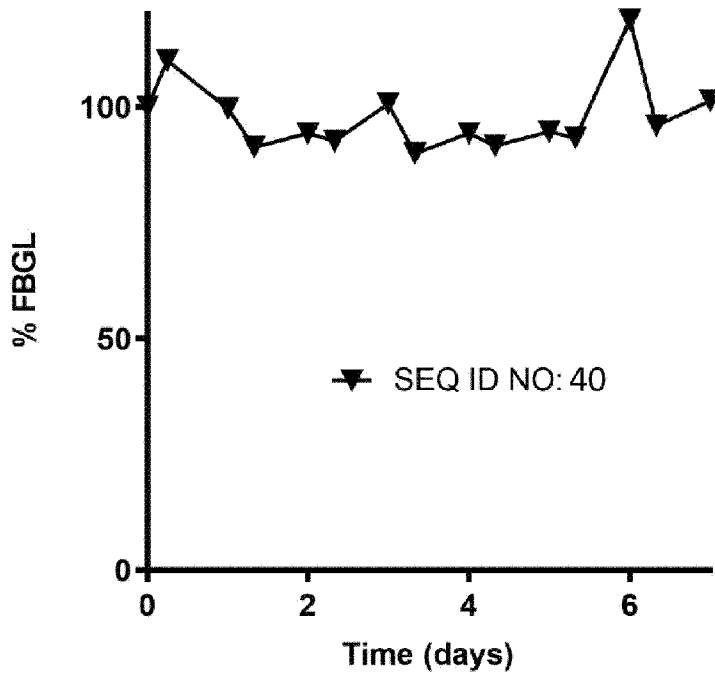


FIG. 36

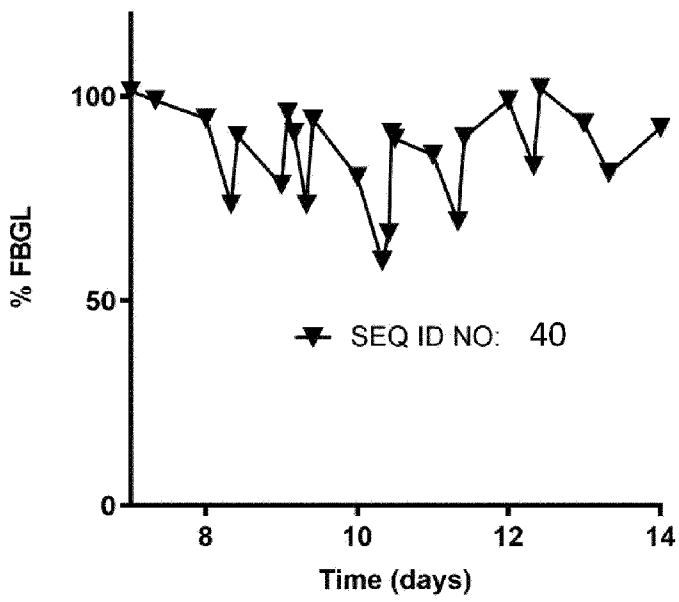


FIG. 37

SEQ ID NO: 31 atggaatggagctgggtctttctctctctctctctctctctctcagtaacgactgggtgtccactccttc
 SEQ ID NO: 32 M E W S W V F L F F L S V T T G V H S F
 gtgaaccagcacctgtgoggctcccacctgggtggaagctctggaactcgtgtgoggcgag
 V N Q H L C G S H L V E A L E L V C G E
 cgggcttccactacgggggtggcggaggaggttctggtggcggcggaggcatcgtggaa
 R G F H Y G G G G G S G G G G G I V E
 cagtgtgcacctccacctgctcctggaccagctggaaaactactgcggtggcggaggt
 Q C C T S T C S L D Q L E N Y C G G G G
 ggtcaaggaggcgggtggacagggtggaggtgggcaggaggaggcgggggagactgcccc
 G Q G G G G Q G G G G Q G G G G G D C P
 aagtgccccgctcccagatgctggcggaccacagcgtgttcactcttcccctcccaggccc
 K C P A P E M L G G P S V F I F P P K P
 aaggacacactgtgatcgccaggaccccgagggtgacctgctggtgggtggacctggat
 K D T L L I A R T P E V T C V V V D L D
 ccccgaagccccgaggtgcagatcagctggttcgtggatggaaagcagatgcagaccgcc
 P E D P E V Q I S W F V D G K Q M Q T A
 aagccaacccccggaagagcagttcaacggcacctacagggtggtgagtggtgtgccc
 K T Q P R E E Q F N G T Y R V V S V L P
 atcgccaccaggactggctgaagggaagcaattcacatgcaaggtaataacaagccc
 I G H Q D W L K G K Q F T C K V N N K A
 ctgcccagccccatcgagaggaccatcagcaaggccaggggccaggcccaccagccatct
 L P S P I E R T I S K A R G Q A H Q P S
 gtgtacgtgctgccccatctagggaggaactgagcaagaacacagtcagccttacttgc
 V Y V L P P S R E E L S K N T V S L T C
 ctgatcaaggacttcttcccaccggacatagacgtggagtggcagagtaacggccagcag
 L I K D F F P P D I D V E W Q S N G Q Q
 gagcccgagagcaagtataggaccacacggcccccaactggacgaggacggaagctacttc
 E P E S K Y R T T P P Q L D E D G S Y F
 ctctacagcaaattgagcgttgacaaaagcaggtggcagcggaggcgacaccttcatctgc
 L Y S K L S V D K S R W Q R G D T F I C
 gccgtgatgcacgaggctttgcataaccactacaccaggagagcctgtcccacagcccc
 A V M H E A L H N H Y T Q E S L S H S P
 ggatag
 G -

FIG. 38

SEQ ID NO: 33 atggaatggagctgggtctttctcttcttctctgtcagtaacgactggtgtccactccttc
 SEQ ID NO: 34 M E W S W V F L F F L S V T T G V H S F
 gtgaaccagcacctgtgctgggtcccacctggtggaagctctggaactcgtgtgctggcgag
 V N Q H L C G S H L V E A L E L V C G E
 cggggcttccactacgggggtggcggaggaggttctggtggcggcggaggcatcgtggaa
 R G F H Y G G G G G S G G G G G I V E
 cagtgtgcacctccacctgctccctggaccagctggaaaactactgcaacggtggcgga
 Q C C T S T C S L D Q L E N Y C N G G G
 ggtggtcaaggaggcgtggacaggggtggaggtgggcagggaggaggcgggggagactgc
 G G Q G G G G Q G G G Q G G G G D C
 cccaagtgcctccctcccagagatgctggcggaccagcgtgttcatcttccctccaag
 P K C P A P E M L G G P S V F I F P P K
 cccaagacacactgctgatcgccaggaccccggaggtgacctgctggtggtggctg
 P K D T L L I A R T P E V T C V V D L
 gatcccgaagaccccagggtgcagatcagctggttctggtgatgaaagcagatgcagacc
 D P E D P E V Q I S W F V D G K Q M Q T
 gccaaagacccaaccccgggaagagcagttcaacggcacctacagggtggtgagtgtgtg
 A K T Q P R E E Q F N G T Y R V V S V L
 cccatcgccaccaggactggctgaagggaagcaattcacatgcaaggtaataacaag
 P I G H Q D W L K G K Q F T C K V N N K
 gccctgccagccccatcgagaggaccatcagcaaggccaggggcccagggcccaccagcca
 A L P S P I E R T I S K A R G Q A H Q P
 tctgtgtacgtgctgccccatctaggagggaactgagcaagaacacagtcagccttact
 S V Y V L P P S R E E L S K N T V S L T
 tgcctgatcaaggacttcttcccaccggacatagacgtggagtgccagagtaacggccag
 C L I K D F F P P D I D V E W Q S N G Q
 caggagcccagagcaagtataggaccacaccgcccccaactggacgaggacggaagctac
 Q E P E S K Y R T T P P Q L D E D G S Y
 ttctctacagcaaattgagcgttgacaaaagcaggtggcagcgaggcgacaccttcatc
 F L Y S K L S V D K S R W Q R G D T F I
 tgcgccgtgatgcacgaggctttgcataaccactaccccaggagagcctgtcccacagc
 C A V M H E A L H N H Y T Q E S L S H S
 cccggatag
 P G -

FIG. 39

SEQ ID NO: 37 atggaatggagctgggtctttctcttctctcctgtcagtaacgactgggtgtocactccttc
 SEQ ID NO: 38 M E W S W V F L F F L S V T T G V H S F
 gtgaaccagcacctgtgcggtcccacctggtggaagctctggaactcgtgtgcggcgag
 V N Q H L C G S H L V E A L E L V C G E
 cggggcttccactacgggggtggcgaggaggttctggtggcgggcggaggcatcgtggaa
 R G F H Y G G G G G G S G G G G G I V E
 cagtgtgcacctccacctgctccctggaccagctggaaaactactgcggtggcgagggt
 Q C C T S T C S L D Q L E N Y C G G G G
 ggtcaaggaggcgggtggacaggggtggaggtgggcaggaggaggcgggggagactgcccc
 G Q G G G G Q G G G G Q G G G G G D C P
 aaatgtcctccgcctgagatgctgggtggccctagcatcttcatcttcccgcccaagccc
 K C P P P E M L G G P S I F I F P P K P
 aaggatactctgtccattagcaggacccccgaggtgacctgcctggtggtggacctgggg
 K D T L S I S R T P E V T C L V V D L G
 ccagcagctctgacgtgcagatcacctggttcgtagacaacaccaggtttacactgcc
 P D D S D V Q I T W F V D N T Q V Y T A
 aagaccagtcccaggaggagcagttcaacagcacatacagggtggtgagcgttctgccc
 K T S P R E E Q F N S T Y R V V S V L P
 atcctgcaccaggactggctgaaaggcaaagagttcaagtgttaaggtgaacagcaagagc
 I L H Q D W L K G K E F K C K V N S K S
 ctgcccagccccattgaaaggaccatcagcaaggacaagggccagccgcacgagccccaa
 L P S P I E R T I S K D K G Q P H E P Q
 gtctacgtgctgccccagcacaggaagagctgagcaggaacaaggttagcgtgacatgc
 V Y V L P P A Q E E L S R N K V S V T C
 ctgatcaggggttctaccccagcgacatcgccgtggagtgggaaatcaccggccaaccc
 L I E G F Y P S D I A V E W E I T G Q P
 gagcccgagaacaactacaggaccactccgcccgaactggacagcgcaggggacactcttc
 E P E N N Y R T T P P Q L D S D G T Y F
 ttgtatagcaggctgagcgtggaccggagcaggtggcagaggggcaacacctacacttgc
 L Y S R L S V D R S R W Q R G N T Y T C
 agcgtgagccaagggccttgcacagccaccacactcagaagagtctgaccagagcccg
 S V S H E A L H S H H T Q K S L T Q S P
 ggatag
 G -

FIG. 41

SEQ ID NO: 39 atggaatggagctgggtctttctcttcttctcctgtcagtaacgactgggtgtccactccttc
 SEQ ID NO: 40 M E W S W V F L F F L S V T T G V H S F
 gtgaaccagcacctgtgctggctcccacctgggtggaagctctggcactcgtgtgctggcgag
 V N Q H L C G S H L V E A L A L V C G E
 cggggcttccactacgggggtggcggaggaggttctgggtggcggcgaggcatcgtggaa
 R G F H Y G G G G G S G G G G G G I V E
 cagtgtgcacctccacctgctccctggaccagctggaaaactactgcggtggcggagggt
 Q C C T S T C S L D Q L E N Y C G G G G
 ggtcaaggaggcgtggacagggtggaggtgggcaggaggaggcgggggagactgcccc
 G Q G G G G Q G G G G Q G G G G G D C P
 aaatgtcctccgctgagatgctgggtggccctagcatcttcatcttcccgcccaagccc
 K C P P P E M L G G P S I F I F P P K P
 aaggatactctgtccattagcaggacccccgaggtgacctgcctgggtgggtggacctgggg
 K D T L S I S R T P E V T C L V V D L G
 ccagacgactctgactgcagatcacctggttcgtagacaacacccaggtttacactgcc
 P D D S D V Q I T W F V D N T Q V Y T A
 aagaccagtcccaggaggaggcagttcagcagcacatacagggtgggtgagcgttctgccc
 K T S P R E E Q F S S T Y R V V S V L P
 atcctgcaccaggactggctgaaaggcaaagagttcaagtgtagggtgaacagcaagagc
 I L H Q D W L K G K E F K C K V N S K S
 ctgcccagccccattgaaaggaccatcagcaaggacaagggccagccgacagagccccaa
 L P S P I E R T I S K D K G Q P H E P Q
 gtctactgtgctgccccagcacaggaagagctgagcaggaacaaggttagcgtgacatgc
 V Y V L P P A Q E E L S R N K V S V T C
 ctgatcgagggtttctaccccagcgacatcgccgtggagtgggaaatcacccggccaaccc
 L I E G F Y P S D I A V E W E I T G Q P
 gagcccgagaacaactacaggaccactccgcccgaactggacagcgacgggacctacttc
 E P E N N Y R T T P P Q L D S D G T Y F
 ttgtatagcaggctgagcgtggaccgggagcaggtggcagaggggcaacacctacacttgc
 L Y S R L S V D R S R W Q R G N T Y T C
 agcgtgagccacgaggccttgcacagccaccacactcagaagagtcctgaccagagcccg
 S V S H E A L H S H H T Q K S L T Q S P
 ggatag
 G -

FIG. 42