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(71) **Applicants and**
(72) **Inventors: COOPER, Garth James Smith** [NZ/NZ]; Auckland University, Level 4, School of Biological Sciences, Symonds Street, Auckland (NZ). XU, **Lance** [NZ/NZ]; University of Auckland, Level 4, School of Biological Sciences, Symonds Street, Auckland (NZ).

(74) **Agents: ADAMS, Matthew, D** et al; A J Park, 6th Floor Huddart Parker Building, PO Box 949, Wellington, 6015 (NZ).

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(54) **Title: MODIFIED ADIPONECTIN PROTEINS**

(57) **Abstract:** Modified adiponectin proteins, isoforms, fragments and conservative variants thereof, and uses therefor, including an adiponectin protein or fragment having at least one modification of its amino acid sequence by substitution or deletion at certain positions compared to a wild-type adiponectin or adiponectin fragment.

MODIFIED ADIPONECTIN PROTEINS

FIELD OF THE INVENTION

The subject invention relates to novel proteins, including the preparation and use of such proteins and nucleic acids encoding the same, and methods for the prevention and treatment of conditions, diseases and disorders that would be improved, eased, or lessened by the administration of, for example, a protein or protein composition of the invention.

BACKGROUND OF THE INVENTION

The following includes information that may be useful in understanding the present inventions. It is not an admission that any of the information provided herein is prior art, or relevant, to the presently described or claimed inventions, or that any publication or document that is specifically or implicitly referenced is prior art. All documents referred to herein are hereby incorporated by reference in their entirety.

Adiponectin (also called ACRP30, adipoQ or GBP28) is a protein secreted from adipocytes. The nucleotide seqence was originally identified by four research groups using different approaches. See, for example, Scherer, P.E., et al., *Journal of Biological Chemistry* 270(45): 26746-26749 (1995); Nakano, Y., et al., *Journal of Biochemistry* 120(4): 803-12 (1996); Hu, E., et al. *Journal of Biological Chemistry* 271(18): 10697-10703 (1996); and Maeda, K., et al., *Biochemical & Biophysical Research Communications,* c221(2):286-9 (1996). The adiponectin gene is located at chromosome 3q27, a susceptibility locus for type 2 diabetes and other metabolic syndromes. Enomoto, N. *et al.,* "Thalidomide prevents alcoholic liver injury in rats through suppression of Kupffer cell sensitization and TNF-alpha production. *Gastroenterology* 123, 291-300 (2002); Berg, A.H., *et al.,* "ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism," *Trends Endocrinol. Metab.* 13, 84-89 (2002); Shaprio, L. & Scherer, P.E., "The crystal structure of a complement-Iq family protein suggests an evolutionary link to tumor necrosis factor," *Curr. Biol.* 8, 335-338 (1998). Several recent studies have been said to support the idea that adiponectin may be a hormone that could link obesity, insulin. resistance and type 2 diabetes. Jarvelainen, H.A. et al., "Kupffer cell inactivation alleviates ethanol-induced steatosis and CYP2E1 induction but not inflammatory responses in rat liver," J. Hepatol. 32, 900-910 (2000); Iimuro, Y., *et al,* "Antibodies to tumor necrosis

factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat," *Hepatology 26,* 1530-1537 (1997); Yin, M. *et al,* "Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice," *Gastroenterology* 177, 942-952 (1999). See also Cooper, Garth J.S *et al,* "The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice," *J. of Clinical Investigation* 112:91-99 (2003); Cooper *et al.* US Patent Application 20040023854.

BRIEF DESCRIPTION OF THE INVENTION

The inventions described and claimed herein have many attributes and embodiments including, but not limited to, those set forth or described or referenced in this Brief Summary. The Brief Summary is not intended to be all-inclusive and the inventions described and claimed herein are not limited to or by the features or embodiments identified in this Brief Description, which is included for purposes of illustration only and not restriction.

In one aspect the present invention is a modified adiponectin protein wherein the adiponectin protein is glycosylated and wherein one or more of the threonine amino acid corresponding to the residues at positions 20, 21 and 22 of human adiponectin have been deleted or substituted with another naturally occurring or non-naturally occurring amino acid. .

In another aspect the present invention is a modified adiponectin protein fragment that is bioactive, and one or more of the threonine amino acid in the fragment corresponding to the residues at positions 20, 21 and 22 of human adiponectin have been deleted or substituted with another naturally occurring or non-naturally occurring amino acid.

Additional modified adiponectins, or modified adiponectin protein fragments, are those in which at least one amino acid residue in a modified adiponectin or modified adiponectin protein fragment other than one or more of the threonine amino acid corresponding to the residues at positions 20, 21 and 22 of human adiponectin has been removed and a different residue inserted in its place, and include modified adiponectins with one or more conservative substitutions.

Preferably the modified adiponectin, or modified adiponectin protein fragment, is of human origin. Other modified adiponectin species and modified adiponectin protein fragments species are within the scope of the invention, including but not limited to those of mouse, rat, bovine, ovine, monkey, and dog origin.

Preferably, the naturally occurring or non-naturally occurring amino acid substituted for one or more of the threonine amino acid residues corresponding to positions 20, 21 and 22 of human adiponectin is not an amino acid subject to glycosylation in a recombinant, transgenic, or other production system capable of glycosylating proteins.

Preferably the amino acid substituted for one or more of the threonine residues corresponding to positions 20, 21 and 22 of human adiponectin is alanine or glycine.

In another embodiment, the modified adiponectin is recombinant or synthesized. Preferably the modified adiponectin is recombinant. Most preferably the modified adiponectin is a recombinant modified adiponectin.

Preferably the modified adiponectin is isolated or purified. Preferably but not necessarily the modified adiponectin is at least about 50% pure; more preferably is at least about 80% pure; still more preferably is at least about 90% pure; still even more preferably is at least about 95% pure; and most preferably is at least about 99% pure.

The prolyl residue corresponding to proline residue 91 of human adiponectin is or is not hydroxylated in the modified adiponectin. In one range of embodiments the prolyl residue is hydroxylated. In another range of embodiments it is not. Other residues may be substituted for hydroxyproline corresponding to the amino acid position 91 of human adiponectin wherein the substitution does not have an undesired effect on the activity of the modified adiponectin.

Preferably at least one of the lysine residues corresponding to lysine residues 65, 68, 77, and 101 of human adiponectin in the modified adiponectin (including but not limited to human adiponectin) is glycosylated.

Preferably but not necessarily the glycosylation of the modified adiponectin at one or more sites of glycosylation within the molecule is with any one or more of a glucosylgalactosyl moiety, a glucosylglucosyl moiety, a galactosylglucosyl moiety, or a galactosylgalactosyl moiety.

Irrespective of the lysine or other residues glycosylated in some embodiments two or more of the lysine residues corresponding to lysine residues 65, 68, 77, and 101 or other glycosylation sites of human adiponectin in the modified adiponectin (including but not limited to human adiponectin) are glycosylated. In others three or more of the lysine residues corresponding to lysine residues 65, 68, 77, and 101 or other glycosylation sites of human adiponectin in the modified adiponectin (including but not limited to human adiponectin) are glycosylated. In still others all four of the lysine residues corresponding to lysine residues 65, 68, 77, and 101 or other glycosylation sites of human adiponectin in the modified adiponectin (including but not limited to human adiponectin) are glycosylated, i.e., In some forms of the present invention the modified adiponectin has at least one sugar moiety at each of lysine residues 65, 68, 77, and 101 corresponding to human adiponectin. Preferably the glycosylation is with a single sugar moiety. In other embodiments glycosylation is with multiple sugar moieties. Preferably the glycosylation is with any one or more of a glucosylgalactosyl moiety, a glucosylglucosyl moiety, a galactosylglucosyl moiety, or a

Preferably the modified adiponectin has one or more of an α-1-2-glucosylgalactosyl-O-hydroxylysine residue at the position corresponding to lysine residue 65 of human adiponectin, an α-l-2-glucosylgalactosyl-O-hydroxylysine residue at the position corresponding to lysine residue 68 of human adiponectin, an α-1-2-glucosylgalactosyl-O-hydroxylysine residue at the position corresponding to lysine residue 77 of human adiponectin, and/or an α-l-2-glucosylgalactosyl-O-hydroxylysine residue at the position corresponding to lysine residue 101 of human adiponectin (i.e., all fifteen possibilities). Preferably each of the residues of the modified adiponectin corresponding to lysine residues 65, 68, 77 and 101 of human adiponectin is α-l-2-glucosylgalactosyl-O-hydroxylysine. The prolyl residue of the modified adiponectin polypeptide corresponding to residue 91 of human adiponectin need not necessarily be hydroxyproline but preferably is hydroxylated.

The modified adiponectin may be selected from one or more of the following, for example;

i) a modified adiponectin wherein one, two, three or four of the residues corresponding to lysine residues 65, 68, 77, and 101 of human adiponectin is glycosylated,

ii) a modified adiponectin as defined in i) wherein glycosylation is with, for example, any one or more of a glucosylgalactosyl moiety, a glucosylglucosyl--moiety, a galactosylglucosyl moiety, or a galactosylgalactosyl moiety,

iii) a modified adiponectin wherein one, two, three or four of the residues corresponding to lysine residues 65, 68, 77 and 101 of human adiponectin is, for example, α-l-2-glucosylgalactosyl-O-hydroxylysine and wherein the residue Corresponding to proline residue 91 of human adiponectin is hydroxyproline,

iv) a modified adiponectin wherein one, two, three or four of the residues corresponding to lysine residues 65, 68, 77 and 101 of human adiponectin is, for example, α-l-2-glucosylgalactosyl-O-hydroxylysine and wherein the residue corresponding to proline residue 91 of human adiponectin is not hydroxyproline, and

.v) a glycosylated modified adiponectin having a desired level of adiponectin activity as compared against a naturally occurring adiponectin.

The invention also provides for nucleic acid sequences and nucleic acid constructs (including, for example, vectors, such as cloning vectors and expression vectors) coding for a modified adiponectin as provided herein.

The invention also includes host cells having such sequences and constructs, as well as other expression systems for the production of the modified adiponectins, including transgenic animals.

The invention also provides antibodies against one or more modified adiponectins.

In another aspect the invention provides a product produced by the process comprising insertion of a polynucleotide sequence encoding an modified adiponectin in a suitable expression vector, introduction of the expression vector incorporating the polynucleotide sequence in an appropriate eukaryotic host cell capable of expressing, and/or processing, and/or glycosylating said modified adiponectin to yield a desired biologically active product.

In one embodiment the polynucleotide sequence encodes a full length modified adiponectin that is the pro- or prepro- form or, for example, a modified adiponectin encoding nucleotide sequence containing a signal or other sequence sufficient to yield a glycosylated molecule.

The present invention also includes a modified adiponectin formulated with one or more of the group consisting of pharmaceutically acceptable carriers, buffers, tonicifiers, excipients, co-actives or diluents so as to be suitable for administration to a mammalian patient. Preferably the predominant modified adiponectin species in a pharmaceutical composition or other preparation is fully glycosylated. Preferably the amino acids corresponding to Lys-65, 68, 77, and 101 of human adiponectin are all glycosylated.

In another aspect the invention provides for the use of a modified adiponectin in the preparation of a pharmaceutical composition or medicament or dosage unit.

In yet another aspect the present invention provides a pharmaceutical composition or pharmaceutical dosage unit comprising a modified adiponectin wherein each of the residues corresponding to lysine residues 65, 68, 77 and 101 of human adiponectin, and/or other natural or synthetic glycosylation sites, is α-l-2-glucosylgalactosyl-O-hydroxylysine and wherein the residue corresponding to proline residue 91 of human adiponectin is hydroxyproline. Preferably the composition or dosage unit is formulated with other pharmaceutically acceptable excipients, co-actives, diluents or the like so as to be suitable for administration to mammalian patients.

In another aspect the invention is an article of manufacture comprising or including a vessel or delivery unit containing a modified adiponectin or formulation thereof and instructions for use, including for use in treatment of a subject.

In another aspect the invention provides a composition additionally including an insulin or insulin analog. Preferably the insulin or insulin analog is present in an amount or concentration sufficient to elicit a blood insulin or analog concentration of between about 5OpM and about 400Pm, between about lOOpM and about 30OpM, or about 200 pM.

Preferably modified adiponectins and modified adiponectin preparations are substantially free of non-glycosylated species or isoforms.

Compositions of the invention may comprise more than one isoform of an modified adiponectin. Such isoforms include glycosylation variants of modified adiponectins.

In another aspect the invention includes a mixture of isoforms of a modified adiponectin by virtue of enrichment or removal, conversion or synthesis of isoforms in which at least one or more of the residues corresponding to lysine residues 65, 68, 77, and

101 of human adiponectin is glyscosylated and wherein the prolyl residue corresponding to proline residue 91 of human adiponectin is (or is not) hydroxylated.

Modified adiponectins may be formulated in a manner suitable for administration to a human, preferably, for example, in a form for parenteral administration via routes such as subcutaneous (s.c), intradermal (i.d.), intravenous (i.v.), intraperitoneal (i.p.) or transdermal. Other preparations are also envisaged in which said adiponectin is administered via the oral, buccal, rectal, vaginal, intravesical, intrathecal, intraventricular, intracerebral or other routes known or desired to those skilled in the art.

The preferred routes of administration are parenteral. A modified adiponectin suitable for parenteral administration is formulated, for example, in aqueous solution containing buffers for stabilization, is preferably at or near isotonic strength, and with suitable antiseptic, antifoaming, anti-precipitation and other stabilizing agents known to those skilled in the art to be suitable for pharmaceutical formulation of proteins suitable for administration to mammals, particularly humans for example, and particularly those suitable for stabilization in solution of therapeutic proteins for administration to mammals including humans.

The administration of protein or protein composition of the invention to a mammal may be used to enhance the effect of insulin. The protein or protein composition may also be used to allow a subphysiological blood insulin concentration to elicit the biological effect of a normal physiological blood insulin concentration.

Proteins and compositions of the invention may be used to inhibit gluconeogenesis when administered to an individual.

Proteins and compositions of the invention may be used, for example, to elicit a plasma modified adiponectin concentration of between about 1 microgram/mL and about 20 microgram/mL (more preferably, for example, to elicit a plasma adiponectin polypeptide concentration of between about 1.9 microg/niL and about 17 microg/niL).

Preferably but not necessarily the individual is a human.

In yet another aspect the invention is a method for treating a disease state associated with, for example, adiponectin polypeptide regulation or aberrant insulin sensitivity comprising administering with or without pharmaceutically acceptable excipients, co-actives, diluents or the like an effective amount of a modified adiponectin.

The invention provides for use of modified adiponectins, modified adiponectin fragment, conservative variants of either, and compositions including any of them to treat one or more of, for example, hyperglycemia, insulin resistance, metabolic syndromes associated with insulin resistance, Type 2 diabetes mellitus, or obesity (e.g. including weight gain, reduction or control or weight gain prevention), metabolic syndromes including hypertension, atherosclerosis including artherosclerosis, coronary heart disease, ischemic heart disease, or polycystic ovary syndrome.

In another aspect, the invention provides a method for treating a mammalian patient deficient in adiponectin or who would otherwise benefit from such treatment comprising administering with or without pharmaceutically accepted excipients, co-actives, diluents or the like an effective amount of a modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

Accordingly to another aspect the present invention includes a method of treating a mammalian patient subject to or for, for example, liver disease and/or having any of the characteristics of liver disease which comprises or includes administering to that patient an effective amount of a modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

The liver disease may be, for example, any one or more of acute liver disease; chronic liver disease, inflammation of the liver, dysfunction of the liver, fatty liver (hepatic steatosis), fibrosis of the liver, cirrhosis of the liver, necrosis of the liver, hepatocellular necrosis, alcoholic liver disease, alcoholic hepatic steatosis, alcoholic hepatitis, alcoholic hepatic necrosis, alcoholic hepatic cirrhosis, hepatic necrosis, hepatic steatosis, hepatic steatosis associated with diabetes, hepatic steatosis associated with a diet rich in lipids, hepatic steatosis associated with abnormalities of lipid metabolism, hepatitis caused by any condition, hepatic necrosis caused by any condition, acute hepatitis, chronic hepatitis, chronic active hepatitis, hepatitis secondary to viral infection or inflammation of the liver, hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, hepatitis G, hepatitis secondary

to the action of any drug or toxin, hepatitis or hepatic dysfunction consequent upon cholestasis, primary biliary cirrhosis, hepatic granulomatosis, and/or conditions in which elevated tissue or blood concentrations of tumor necrosis factor a play a pathogenic role.

In a further aspect the present invention consists in a method of treating a mammalian patient subject to or for alcoholic liver disease and/or having any of the characteristics of alcoholic liver disease which comprises or includes administering to that patient an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

In a further aspect the present invention consists in a method of treating a mammalian patient to prevent and/or reverse liver disease and/or any of the characteristics of liver disease which comprises or includes administering to that patient an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

In a further aspect the present invention consists in a method of treating a mammalian patient to prevent and/or reverse alcoholic liver disease and/or any of the characteristics of alcoholic liver disease which comprises or includes administering to that patient an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

In still a further aspect the present invention consists in a method of treating a human being subject to alcoholic liver disease and/or having any of the characteristics of alcoholic liver disease which comprises administering to that patient an effective amount of an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

In a yet further aspect the present invention consists in a method of treating a mammalian patient subject to any one or more of hepatic steatosis (fatty infiltration), hepatic inflammation, hepatic necrosis, hepatic fibrosis, hepatic cirrhosis, and/or hepatic dysfunction which comprises or includes administering to that patient an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

As will be described hereinafter in its most simplistic forms and not dependant upon any vessel (whether a capsule or otherwise) an modified adiponectin or modified adiponectin fragment of the invention, or a conservative variant of either of them, can also be administered by means of a surgically implanted delivery device such as an osmotic pump, as is well know in the are alternative dosage forms suitable for administration of therapeutic protein may also be used.

Accordingly, in another aspect of the invention there is provided a method of treating a mammalian patient itself still able to encode for adiponectin comprising administering to the patient a modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them in a sufficient amount(s) to suppress TNF-α levels below those that would have been or likely would have been present without such administration. For example, such administration may be to treat, ameliorate, prevent and/or reverse a TNF-α disease or disorder and/or any of the characteristics of a TNF-α disease or disorder.

Preferably said TNF-α disease or disorder is, for example, any one or more of the following:- inflammatory disease, circulatory disease, portal hypertension, pulmonary hypertension, allegic diseases, Crohn's disease, autoimmune haemolytic anemia, psoriasis, hepatic disease, pancreatic disease, neurodegenerative disease, central nerve failure, toxaemia, climacteric failure, gestosis, adiposis, hyperlipidemia, hypercholesteremia, abnormal glucose tolerance, solid tumor, tumor cancer and accompanying cachexia, endocrine disease, Creutzfeldt-Jakob disease, viral infection, post-percutaneous coronary arterioplasty, vascular hypertrophy or occlusion, post-PTCA/stenting /bypass surgery

vascular reocclusion/restenosis, post-intervention vascular hypertrophy or occlusion, suppression of implantation-induced vascular failure and rejection, rejection episodes following organ or tissue transplant and automimmune disease, side effects associated with TNF generation duringneoplastic therapy and also to eliminate or ameliorate shock related symptoms associated with the treatment or prevention of graft rejection, dialytic hypotension, glaucoma, high ocular tension, myasthenia gravis, chronic defatigation, bone disease, neurological disorders, TNF-D induced insulin resistance, aberrant apoptqsis, complications of diabetes mellitus or stress hyperglycemia, chronic obstructive pulmonary disease, chronic bronchitis and emphysema.

Preferably said inflammatory response is, for example, any one of the following: diabetic complications such as retinopathy, nephropathy, neuropathy, major vascular and microvascular disorders; arthritis such as chronic rheumatoid arthritis, osteoarthritis, rheumatoid myelitis and periosteosis; postooperative/posttraumatic inflammation; remedy of swelling; pharyngitis; cystitis; pneumonia; myocarditis; cardiomyopathy; atopic dermatitis; inflammatory intestinal disease such as Crohn's disease and ulcerative colitis;- meningitis; inflammatory ophthalmic disease; inflammatory pulmonary disease such as pneumonia, silicotuberculosis, pulmonary sarcoidosis, inflammatory bone disorders and pulmonary tuberculosis

Preferably said circulatory disease includes, for example, any one of the following: chronic heart failure including arrhythmia, angina pectris, myocardial infarction, cardiac insufficiency and congestive heart failure, arteriosclerosis including atherosclerosis, hypertension, deep vein thrombosis, occlusive peripheral circulation failure, ischemic cerebral circulation failure, disseminated intravascular coagulation syndrome, Raynaud's disease, Buerger disease.

.Preferably said allergic disease includes, for example, any one of the following: asthma, allergic rhinitis, conjunctivitis, digestive tract allergy, pollinosis and anaphylaxis, chronic occlusive pulmonary disease, collagenosis.

Preferably said neurodegenerative disease includes, for example, any one of the following: Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, AIDS, encephalopathy.

Preferably said central nerve failure includes, for example, any one of the following: cerebrovascular failure such as cerebral hemorrhage and cerebral infarction and its sequela, cranial trauma, spinal damage, cerebral edema, dementia, memory failure, consciousness failure, multiple sclerosis.

.Preferably said toxemia includes, for example, any one of the following: sepsis, septic shock, endotoxic shock, gram negative sepsis, toxin shock syndrome.

Preferably said cancerous tumor includes, for example, any one of the following: malignant melanoma, malignant lymphoma and cancer of the digestive organ.

Preferably said endocrine disease includes, for example, any one of the following: Addison disease, Cushing's syndrome, melanocytoma and primary aldosteronism.

Preferably said autoimmune disease includes, for example, any one of the following: organ specific diseases such as thyroiditis or non-specific organ diseases such as rheumatoid and osteo-arthritis.

Preferably said bone disease includes, for example, any one of the following: fracture, re-fracture, osteoporosis, osteomalacia, bone Behcet disease, ankylosing spondylitis, chronic rheumatoid arthritis and osteogonarthritis as well as articular tissue destruction in disease related thereto.

Preferably said neurological disorders include, for example, trauma, injury, compression to individual nerves, nerve roots, spinal cord and/or the brain, acute spinal cord and brain injure, demyelinating diseases, such as multiple sclerosis, spinal cord compression due to metastatic cancer, primary or metastatic brain tumors, chronic pain syndromes due to metastatic tumor, inflammatory CNS diseases, such as subacute sclerosing panenencephalitis, Huntington's disease, Guillain-Barre syndrome, Bell's palsy, diabetic neuropathy, optic neuritis, macular degeneration, retinitis pigmentosa, diabetic retinopathy, muscular dystrophy, and polymyositis-dermatomyositis.

Preferably said aberrant apoptosis includes, for example, any virally-induced inhibition of apoptosis.

Preferably said complications of diabetes mellitus or stress hyperglycemia include, for example, any one or more of the following: myocardia infarction, congestive heart failure and cardiogenic shock.

The term "comprising" as used in this specification means "consisting at least in part of. When interpreting each statement in this specification that includes the term "comprising", features other than that or those prefaced by the term may also be present. Related terms such as "comprise" and "comprises" are to be interpreted in the same manner.

In this specification where reference has been made to patent specifications, other external documents, or other sources of information, this is generally for the purpose of providing a context for discussing the features of the invention. Unless specifically stated otherwise, reference to such external documents is not to be construed as an admission that such documents, or such sources of information, in any jurisdiction, are prior art, or form part of the common general knowledge in the art.The invention also includes all of the subject matter as defined by and within the scope of the appended claims.

BRIEF DESCRIPTION **OF THE DRAWINGS**

Figure 1 shows in lane 1 the molecular weight marker (Invitrogen #LC 5925) and in lane 2 the purified sample of the expressed modified human adiponectin from CHO-S cells identified at 3OkDa.

Figure 2 is a Western blot detection of the SDS-PAGE pattern that compares wild type adiponectin to modified adiponectin under native conditions and at different loadings. Lanes 1, 3 and 5 represent the wild type adiponectin at loadings of 10 ng, 4ng and 2ng respectively. Lanes 2, 4 and 6 represent the modified adiponectin at the same loadings as for the wild type. Lane 7 represents the reduced and denatured wild type adiponectin.

Figure 3 is a full view of a 2-Dimensional Gel of the wild type human adiponectin as expressed in CHO-S cells.

Figure 4 is a close up view of Figure 3.

Figure 5 is the same as for Figure 3 except this is of the modified form of human adiponectin expressed in the CHO-S cells.

Figure 6 is a close up of the box in Figure 5.

DETAILED DESCRIPTION OF THE INVENTION

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, nucleic acid chemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook *et ah*, 1989) and *Molecular Cloning: A Laboratory Manual*, third edition (Sambrook and Russel, 2001), (jointly referred to herein as "Sambrook"); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987, including supplements through 2001); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, and Harlow and Lane (1999) *Using Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (jointly referred to herein as "Harlow and Lane"), Beaucage et al. eds., *Current Protocols in Nucleic Acid Chemistiy* John Wiley & Sons, Inc., New York, 2000).

Definitions

The term "antibody" is used in the broadest sense and specifically covers, without limitation, intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e. g., bispecific antibodies) formed from at least two intact antibodies, single chain antibodies, diabodies, triabodies, tetrabodies, and antibody fragments so long as they exhibit the desired biological activity, as well as chimeric antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, Nature, 256: 495 (1975), and other subsequent publications and according to methods known in the art.

As used herein, "biologically active" means having the ability to or capable of exerting one or more of natural activities of adiponectin, preferably human adiponectin, in structural, regulatory, biochemical or biophysical events.

As used herein, "modified adiponectin" refers to an adiponectin protein that includes at least one modification of its amino acid sequence by the substitution or deletion

of one or more of the amino acids corresponding to positions 20, 21 and/or 22 of human adiponectin compared to a wild-type adiponectin.

An exemplary modified adiponectin is presented in the attached sequence listing as Sequence ID No. 1, in which the amino acids corresponding to positions 20, 21, and 22 of human adiponectin are identified as "variant".

The terms "substantially pure" or "isolated," when referring to proteins that are separated as desired from other proteins or molecules with which they are naturally associated, or associated in a manufacturing process. A protein or polypeptide is considered substantially pure when that protein makes up greater than about 50% of the total protein content of the composition containing that protein, and typically, greater than about 60% of the total protein content. More typically, a substantially pure or isolated protein or polypeptide will make up at least about 75%, more preferably, at least about 90%, of the total protein. Preferably, the protein will make up greater than about 90%, and more preferably, greater than about 95% of the total protein in the composition.

An "effective amount" is an amount sufficient to effect beneficial or desired results including beneficial or desired clinical results. Beneficial results can include but are not limited to an improvement in an individual's ability to be sensitized to insulin, decrease in insulin resistance, reduction in hyperglycemia, and an improvement in an individual's weight or obesity or other disease state or condition, including those noted herein. An effective amount can be administered in one or more administrations by various routes of administration.

When adiponectin deficiency is present, ideally a sufficient amount of an modified adiponectin is administered to the human or other mammal under treatment to restore the circulating, blood or tissue levels of adiponectin activity to normal, or within ± 5% of normal; or within ± 10% of normal, or within ± 25% of normal, or within ± 50% of normal. Therapy can also be effective when apparently normal circulating concentrations of adiponectin are present, however, and the presence of normal adiponectin levels is thus not necessarily a contraindication to adiponectin or adiponectin agonist therapy.

As used herein, "treatment" is an approach for obtaining beneficial or desired results including and preferably clinical results. Beneficial or desired clinical results include but are not limited to an improvement in an individual's ability to be sensitized to insulin,

decrease in insulin resistance, reduction in hyperglycemia, and an improvement in an individual's weight or obesity or other disease state or condition. A treatment plan may occur over a period of time and may involve multiple dosages, multiple administrations, and/or different routes of administration. Generally, an effective amount of a composition comprising glycosylated adiponectin or adiponectin agonist, including a modified human adiponectin agonist, is administered for treatment purposes.

An "individual" is a subject, for example a vertebrate, preferably a mammal, and more preferably a human, for example. Mammals include, but are not limited to, farm animals, sport animals, pets, primates, mice and rats.

The terms "polynucleotide" and "nucleic acid" are used interchangeably herein and refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. It is understood that the double stranded polynucleotide sequences described herein also include the modifications described herein. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate (P-NH2) or a mixed phosphoramidate-phosphodiester oligomer. A phosphorothioate linkage can be used in place of a phosphodiester linkage. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer. The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

A "vector" is a self-replicating nucleic acid molecule that transfers an inserted nucleic acid molecule into and/or between host cells. The term includes vectors that

function primarily for insertion of a nucleic acid molecule into a cell, replication of vectors that function primarily for the replication of nucleic acid, and expression vectors that function for transcription and/or translation of the DNA or RNA. Also included are vectors that provide more than one of the above functions. The vector may be capable of replication in at least one additional host system, such as *E. coli.*

The term "expression construct" refers to a genetic construct that includes the necessary elements that permit transcribing the insert polynucleotide molecule, and, optionally, translating the transcript into a polypeptide. An "expression vector" is an example of an expression construct. An expression construct typically comprises in a 5' to 3' direction: (a) promoter functional in the host cell into which the construct will be transformed, (b) the polynucleotide to be expressed, and (c) terminator functional in the host cell into which the construct will be transformed. The term "coding region" or "open reading frame" (ORF) refers to the sense strand of a genomic DNA sequence or a cDNA sequence that is capable of producing a transcription product and/or a polypeptide under the control of appropriate regulatory sequences. The coding sequence is identified by the presence of a 5' translation start codon and a 3' translation stop codon. When inserted into a genetic construct, a "coding sequence" is capable of being expressed when it is operably linked to promoter and terminator sequences. "Operably-linked" means that the sequence to be expressed is placed under the control of regulatory elements that include promoters, tissue-specific regulatory elements, temporal regulatory elements, enhancers, repressors and terminators. The term "noncoding region" refers to untranslated sequences that are upstream of the translational start site and downstream of the translational stop site. These sequences are also referred to respectively as the 5' UTR and the 3' UTR. These regions include elements required for transcription initiation and termination and for regulation of translation efficiency. Terminators are sequences, which terminate transcription, and are found in the 3' untranslated ends of genes downstream of the translated sequence. Terminators are important determinants of mRNA stability and in some cases have been found to have spatial regulatory functions. The term "promoter" refers to nontranscribed cis-regulatory elements upstream of the coding region that regulate gene transcription. Promoters comprise cis-initiator elements which specify the transcription initiation site and conserved boxes such as the TATA box, and motifs that are bound by transcription factors.

Additional modified adiponectins are those in which at least one amino acid residue in a modified adiponectin has been removed and a different residue inserted in its place. Such substitutions, sometimes referred to as "conservative substitutions," may be made in accordance with those shown indicated below, for example. Modified adiponectins can also comprise one or more unnatural amino acids.

A "conservative amino acid substitution" is one in which an amino acid residue is replaced with another residue having a chemically similar or derivitized side chain. Families of amino acid residues having similar side chains, for example, have been defined in the art. These families include, for example, amino acids with basic side chains *(for example,* lysine, arginine, histidine), acidic side chains *(for example,* aspartic acid, glutamic acid), uncharged polar side chains *(for example,* glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains *(for example,* alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains *(for example,* threonine, valine, isoleucine) and aromatic side chains *(for example,* tyrosine, phenylalanine, tryptophan, histidine). Amino acid analogs *(for example,* phosphorylated amino acids) are also contemplated in the present invention, as are peptides substituted with non-naturally occurring amino acids, including but not limited to D-amino acids, β amino acids, and γ amino acids. A "non-naturally occurring amino acid" refers to a residue, other than those naturally occurring amino acid residues listed above, which is able to covalently bind adjacent amino acid residues(s) in a polypeptide chain. Examples of non-naturally occurring amino acid residues include, e.g., norleucine, ornithine, norvaline, homoserine and other amino acid residue analogues such as those described in Ellman *et al. Meth. Enzym.* 202:301-336 (1991) and U.S. Pat. Application publications 20030108885 and 20030082575.

Variants of modified adiponectins may be made by substituting amino acids which do not substantially alter the bioactivity of the modified adiponectins (e.g., conservative substitutions). Selection of amino acids for substitution can depend on the size, structure, charge, and can be either an amino acid found in nature or synthetic amino acid. Generally, amino acids which have a similar charge (i.e., hydrophobic for hydrophobic) or similar size (i.e., isoleucine for leucine) can be selected for substitution. One or more substitutions can be made in a stepwise fashion or concurrently. Variations in the residues included in the

peptide are also both possible and contemplated. For example, it is possible to substitute amino acids in a sequence with equivalent amino acids using conventional techniques. Groups of amino acids known normally to be equivalent are:

(a) Ala Ser Thr Pro GIy;

(b) Asn Asp GIu GIn;

(c) His Arg Lys;

(d) Met GIu He VaI; and

(e) Phe Tyr TYp.

The substitution of one amino acid for another in the same group as shown above is an example of a conservative amino acid substitution as used herein. Modified adiponectins variants produced by one or more conservative amino acid substitutions are herein termed "conservative variants".

Conservative variants can be tested for biological function, such as for example, to stimulate glucose incorporation into glycogen, whether *in vivo* or *in vitro*. The biological activity of a modified adiponectin variant is at least about 25% of a modified adiponectin, preferably at least about 35%, preferably at least about 50%, preferably at least about 60%, preferably at least about 75%, preferably at least about 85%, and more preferably at least about 95%.

The invention also encompasses active fragments with modified adiponectin bioactive functionality. Such active fragments may be obtained by deletion of one or more amino acid residues of full-length modified adiponectins. Active fragments or portions of modified adiponectins may be ascertained by stepwise deletions of amino acid residues, from the N-terminal end or the C-terminal end or from within the modified adiponectin. If an amino acid is deleted and the bioactivity of a modified adiponectin is not substantially reduced, then the amino acid may not comprise a portion of the active fragment. Further, polypeptides comprising an active fragment of modified adiponectins or modified adiponectin variant(s) are also encompassed in the invention. For example, active fragments of modified adiponectins may comprise about 95% contiguous amino acids of a modified adiponectin, more preferably about 90% contiguous amino acids, more preferably about 85% contiguous amino acids, more preferably about 80% contiguous amino acids, more preferably about 75% contiguous amino acids, about contiguous amino acids, about

70% contiguous amino acids, about 65% contiguous amino acids, or more preferably 60% contiguous amino acids, 55% contiguous amino acids, or 50% contiguous amino acids.

Additions and/or deletions of amino acids may also be made as long as the resulting modified adiponectin is immunologically cross-reactive with and/or has substantially the same function as the parent modified adiponectin. The invention also encompasses polynucleotides which code for modified adiponectins or variants or active fragments thereof.

As used herein the term "dosage forms" includes any appropriate dosage form well known in the art to be suitable for pharmaceutical formulation of proteins suitable for administration to mammals, and in particular to humans, particularly (although not solely) those suitable for stabilization in solution of therapeutic proteins for administration to mammals preferably humans. All this is irrespective of whether or not the adiponectin is in the form of a composition.

One example is oral delivery forms of tablet, capsule, lozenge, or the like form, or any liquid form such as syrups, aqueous solutions, emulsion and the like, capable of protecting the therapeutic protein from degradation prior to eliciting an effect, e.g.; in the alimentary canal if an oral dosage form. Examples of dosage forms for transdermal delivery include transdermal patches, transdermal bandages, and the like. Included within the topical dosage forms are any lotion, stick, spray, ointment, paste, cream, gel, etc. whether applied directly to the skin or via an intermediary such as a pad, patch or the like. Examples of dosage forms for suppository delivery include any solid or other dosage form to be inserted into a bodily orifice (particularly those inserted rectally, vaginally and urethrally). Examples of dosage units for transmucosal delivery include depositories, solutions for enemas, pessaries, tampons, creams, gels, pastes, foams, nebulised solutions, powders and similar formulations containing in addition to the active ingredients such carriers as are known in the art to be appropriate. Examples of dosage units for depot administration include pellets or small cylinders of active agent or solid forms wherein the active agent is entrapped in a matrix of biodegradable polymers, microemulsions, liposomes or is microencapsulated. Examples of implantable infusion devices include any solid form in which the active agent is encapsulated within or dispersed throughout a biodegradable polymer or synthetic, polymer such as silicone, silicone rubber, silastic or

similar polymer. Alternatively dosage forms for infusion devices may employ liposome delivery systems. Examples of dosage units for delivery via bolus include single or multiple administrations by intravenous injection, subcutaneous, subdermal, and intramuscular administration or oral administration. Examples of dosage units for inhalation or insufflation include compositions comprising solutions and/or suspensions in pharmaceutically acceptable, aqueous, or organic solvents, or mixture thereof and/or powders.

Modified Adiponectins and Modified Adiponectin Compositions

The invention relates generally to biologically active modified adiponectins, and active fragments thereof, and conservative variants of either, and compositions including one or more of the foregoing. It has been surprisingly discovered that active modified adiponectins can be made by altering one or more of the residues corresponding to threonine residues at positions 20, 21, and/or 22 of human adiponectin.

In one aspect the present invention is a modified human adiponectin species (or active modified human adiponectin fragment thereof, or conservative variant of either) wherein the adiponectin species (or active modified human adiponectin fragment thereof, or conservative variant of either) is glycosylated and wherein one or more of the threonine amino acid residues at positions 20, 21 and 22 have been deleted or substituted with another naturally occurring or non-naturally occurring amino acid.

Preferably, the naturally occurring or non-naturally occurring amino acid substituted for one or more of the threonine amino acid residues at positions 20, 21 and 22 (where the molecule is based on human adiponectin) is not an amino acid that could or would be glycosylated in a recombinant production system.

Preferably the amino acid substituted for one or more of the threonine residues at positions 20, 21 and 22 (where the molecule is based on human adiponectin) is alanine or glycine.

The modified adiponectins and related compositions of the invention, conservative variants thereof, and active fragments of any of them, are useful, *inter alia*, for therapeutic and other uses.

In one aspect, the invention provides a modified adiponectin protein or modified adiponectin protein fragment, or conservative variant of either, that is glycosylated and is

recombinant or synthesized. Preferably, the modified adiponectin or modified adiponectin protein fragment, or conservative variant of either, is a human adiponectin or fragment and, for example, at least one and preferably more that one of the residues corresponding to human adiponectin lysine residues 65, 68, 77 and 101 (residues numbered according to the human peptide) is glycosylated.

Adiponectin polypeptides that differ from one another by the glycosylation (or lack thereof) at lysine residues 65, 68, 77, and 101 are sometimes referred to herein as "glycoisoforms".

As used herein, a modified adiponectin or modified adiponectin fragment, or conservative variant of either, can be recombinant or synthetic. In one embodiment, the modified adiponectin or modified adiponectin fragment or conservative variant is of animal origin, *e.g.*, a mammal, such as human, non-human primate, mouse, rat, dog, or bovine. In another embodiment, the modified adiponectin or modified adiponectin fragment or conservative variant is the glycosylated mature form lacking the signal sequence of the pre-pro form. In another embodiment, the modified adiponectin or modified adiponectin fragment or conservative variant also differs from a naturally occurring adiponectin or adiponectin fragment by additions to or truncations (natural or recombinant) of the protein and/or conservative substitutions, as well as by derivitazation. In one embodiment, the modified adiponectin or modified adiponectin fragment or conservative variant has a sequence that is substantially similar (i.e., substantially identical) to that of a naturally occurring adiponectin. Truncated modified adiponectins and modified adiponectin fragments and conservative variants may be substantially homologous to native adiponectin or native adiponectin fragments and still retain biological activity. Other useful molecules may be obtained by deletion or truncation of a modified adiponectin or a modified adiponectin fragment or conservative variant. Active fragments or portions of adiponectin may be ascertained by stepwise deletions of amino acid residues, from the N-terminal end or the C-terminal end or from within the modified adiponectin or modified adiponectin fragment. If an amino acid is deleted and the biological activity of a modified adiponectin or a modified adiponectin fragment is not substantially reduced, then the amino acid may not comprise a portion (or may comprise an unneeded portion) of the active fragment.

Such active fragments or portions of adiponectin may be fused with other polypeptides by methods well known in the art to yield a chimeric polypeptide. Any such chimeric polypeptides that retain biological activity are also considered to within the scope of the invention.

Modified adiponectins, fragments and conservative variants can be characterized by biological function, wherein they serve as is an agonist of the site of action of adiponectin capable of eliciting one or more biological activities of adiponectin.

It will also be apparent that whilst reference is made herein specifically to α-1-2-glucosyl-gylactosyl-O-hydroxylysine the term includes within its compass alternative nomenclatures, including for example, glucosyl-α-l-2-galactosyl-O-lysine.

Whilst reference is made herein specifically to glycosylation by a sugar or mix of sugars or more specifically to various entities such as glucosylglactosyl moieties the term includes within its scope any expansion or variation of that glycosylating moiety that elicits a similar biological activity to that more specifically identified.

Whilst reference is made herein specifically to hydroxylation the term includes within its compass any expansion or variation of that hydroxylating moiety including other modifications that elicits a similar biological activity to that more specifically identified.

Whilst reference is made herein specifically to hydroxyproline the term includes within its scope any amino acid including modified amino acids that elicits a similar biological activity to that more specifically identified.

Molecules of the invention may be formulated in a manner suitable for administration to a human, preferably in a form for parenteral administration via routes such as subcutaneous (s.α), intradermal (i.d.), intravenous (i.v.), intraperitoneal (i.p.) or transdermal. Other preparations are also envisaged in which said adiponectin is administered via the oral, rectal, vaginal, intravesical, intrathecal, intraventricular, intracerebral or other routes known to those skilled in the art.

The preferred routes of administration are parenteral. Molecules of the invention for parenteral administration are formulated in aqueous solution containing buffers for stabilization, preferably at or near isotonic strength, and optionally with suitable antiseptic, antifoaming, anti-precipitation and other stabilizing agents known to or learned by those skilled in the art to be suitable for pharmaceutical formulation of proteins suitable for

administration to mammals particularly humans, particularly those suitable for stabilization in solution of therapeutic proteins for administration to mammals preferably humans.

In one embodiment of the invention, the composition contains a modified adiponectin or modified adiponectin fragment, or conservative variant of either, for example, with a biological activity detectable in an *in vitro* assay, for example, measuring the ability of hepatocytes to respond to insulin. In another embodiment, a modified adiponectin or modified adiponectin fragment, or conservative variant of either has at least biological activity that is enhancement of the effect of insulin, decrease in insulin resistance in an individual, inhibition of gluconeogenesis, reduction in hyperglycemia, or improvement in the health of an individual subject to obesity, for example. In various embodiments, the biological activity of a modified adiponectin or modified adiponectin fragment, or conservative variant of either of a composition of the invention is at least about 50%, and often at least about 95% of human adiponectin.

B. Isoforms

In one aspect, the invention relates to compositions containing one or more isoforms of a modified adiponectin or modified adiponectin fragment, or conservative variant of either. As used herein, "isoforms" are forms of a modified adiponectin or modified adiponectin fragment or conservative variant of either which are distinguished on the basis of pi and apparent molecular weight. The different isoforms can be identified by standard methods such as electrophoresis. Some isoforms are glycosylated and others are not.

C. Glycoisoforms

Proteins made in mammalian cells can undergo post-translational modifications such as glycosylation. Human adiponectin lysine residues 65, 68, 77, and 101 (and corresponding lysine amino acids in non-human adiponectins), are targets for glycosylation. In one aspect, the invention provides compositions containing, for example, modified human adiponectin, modified adiponectin from non-human species, and fragments and conservative variants thereof, glycosylated at one or more of the residues corresponding to lysine residues 65, 68, 77, and 101 of human adiponectin. It wili be appreciated when referring to a modified adiponectin of non-human species, for example, or a conservative variant, or a fragment or truncated version, the residues of the molecule can be referred to using the numbering of the corresponding original sequence, as

determined by optimally aligning the two sequences. For example, in naturally occurring mouse adiponectin, the corresponding lysine residues are 68, 71, 80, and 104. It will be appreciated that, when discussing numbering in one species *(e.g.*, human or mouse), the discussion is intended to refer also to the equivalent numbering in other species.

In one aspect, the invention provides a modified adiponectin or modified adiponectin fragment, or conservative variant of either which is glycosylated and wherein it is recombinant or synthesized. In another embodiment, the modified adiponectin or modified adiponectin fragment, or conservative variant of either is based on human adiponectin. In another embodiment, at least one of the lysine residues corresponding to lysine residues 68, 71, 80, and 104 (mouse) or residues 65, 68, 77, and 101 (human) is glycosylated. In one embodiment, the modified adiponectin or modified adiponectin fragment, or conservative variant of either is fully glycosylated. "Fully glycosylated" refers to a state of glycosylation wherein all lysine residues are glycosylated with at least one sugar moiety (e.g. all four of the lysine residues corresponding to lysine residues 68, 71, 80, and 104 (mouse) or residues 65, 68, 77, and 101 (human) are glycosylated). Additional glycosylation may be added and/or existing glycosylation sites moved as desired for biological activity.

The glycosylation at lysine residues is typically O-linked and can result in one or more sugar moieties being added to each lysine residues. In one aspect of the invention, the sugar moieties which are added to the lysine residues are a glucosylgalactosyl moiety or galactosylglucosyl moiety. In another aspect, the adiponectin polypeptide has at least one glucosylgalactosyl moiety or galactosylglucosyl moiety at each of lysine residues 68, 71, 80, and 104 (mouse) or residues 65, 68, 77, and 101 (human), for example. In another embodiment, the adiponectin polypeptide has a structure X1 at at least one of lysine residues 68, 71, 80, and 104 (mouse) or residues 65, 68, 77, and 101 (human) or at all of Lys-68, 71, 80, and 104 (mouse) or Lys-65, 68, 77, and 101 (human) wherein each X1 is independently selected from one or more of a glucosylgalactosyl moiety, a glucosylglucosyl moiety, a galactosylgalactosyl moiety, and galactosylglucosyl moiety. In one embodiment, all lysines in adiponectin polypeptides are fully glycosylated. In further embodiments, the modified adiponectin or modified adiponectin fragment or conservative variant of either may be an isoform.

The modified adiponectin or modified adiponectin fragment or conservative variant of either can also be characterized by its biological effects. In one embodiment, the administration of the modified adiponectin or modified adiponectin fragment, or conservative variant of either to a mammal is useful to treat a disease state as herein described associated with adiponectin regulation. In another embodiment, the administration of a modified adiponectin or modified adiponectin fragment, or conservative variant of either to a mammal enhances the effect of insulin as described, for example, in Cooper, Garth J.S *et ah,* "The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice," *J. Clinical Investigation* 112:91-99 (2003), Cooper *et al.* US Patent Application 20040023854. In another embodiment, the modified adiponectin or modified adiponectin fragment or conservative variant of either inhibits gluconeogenesis when administered to an animal.

In another aspect, the invention provides a composition comprising a modified adiponectin or modified adiponectin fragment or conservative variant of either including recombinant molecules that are glycosylated formulated with or without other pharmaceutically acceptable excipients, co-actives, diluents or the like so as to be suitable for administration to mammalian patients. In another embodiment, the composition additionally comprises an insulin or an insulin analog. Preferably, the insulin or analog is at a concentration or amount sufficient to elicit a blood insulin or analog concentration of between about 5OpM and about 40OpM. Preferably, the insulin or analog is at a concentration or amount sufficient to elicit a blood insulin or analog concentration of between about lOOpM and about 30OpM. More preferably, the insulin or analog is at a concentration or amount sufficient to elicit a blood insulin concentration of about 20OpM.

In another aspect, the invention provides a composition of a modified adiponectin or modified adiponectin fragment or conservative variant of either wherein at least one of the lysine residues corresponding to lysine residues 68, 71, 80, and 104 (mouse) or residues 65, 68, 77, and 101 (human) is glycosylated. In one embodiment, the modified adiponectin or modified adiponectin fragment or conservative variant of either is fully glycosylated.

The composition of a glycosylated modified adiponectin or modified adiponectin fragment or conservative variant of either can also be characterized by its biological effects, as previously described.

In one aspect, the invention provides a composition of a modified adiponectin or modified adiponectin fragment or conservative variant of either that is substantially free of at least one non-glycosylated adiponectin isoform. In one aspect, the composition is substantially free of any non-glycosylated adiponectin isoform. As used herein, a composition is "substantially free" from an isoform when that form is less than about 20%, preferably less than about 10%, preferably less than about 5%, most preferably less than about 1% or about 0.1% by weight of the adiponectin protein in the composition. Methods for obtaining such compositions include those well known in the protein purification- and chromatography arts.

In yet another aspect, the invention provides a composition containing a modified adiponectin or modified adiponectin fragment or conservative variant of either wherein the only or predominant species is fully glycosylated. In one embodiment, for example, the composition contains more than one isoform of adiponectin and/or adiponectin in more than one glycosylation state. The composition can be such that any one of its isoforms is the predominant adiponectin in the composition. In this context, "predominant" refers to the composition in which at least about 50% of the adiponectin polypeptide in the composition is in the specified glycosylation state or of the specified isoform, preferably at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, and at least about 98% modified human adiponectin.

Method of obtaining glycosylated adiponectin

Several methods can be used to obtain a composition comprising a modified adiponectin or modified adiponectin fragment or conservative variant of either. It will be appreciated that a modified adiponectin or modified adiponectin fragment or conservative variant of either can be produced by recombinant or synthetic means.

In one embodiment, one or more isoforms or glycoisoforms is prepared by recombinant methods. A modified adiponectin or modified adiponectin fragment or conservative variant of either may be produced recombinantly by inserting a polynucleotide (usually DNA) sequence that encodes the protein into an expression vector and expressing the peptide in an appropriate host. A polynucleotide encoding the desired polypeptide, whether in fused or mature form, and whether or not containing a signal sequence to permit secretion, may be ligated into expression vectors suitable for any convenient host. Any of a

variety of expression vectors known to those of ordinary skill in the art may be employed, although eukaryotic expression systems are recommended because of the ability of eukaryotic cells to perform post-translational modifications, such as glycosylation. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule which encodes the recombinant peptides. Examples of eukaryotic host cells are known in the art and include yeast, avian, insect, plant, and animal cells such as COS7, HeLa, CHO and other mammalian cells. Standard techniques for recombinant production are described- for example, in Sambrook *supra.* A modified adiponectin or modified adiponectin fragment or conservative variant of either can be obtained by expression of a recombinant polynucleotide encoding a modified adiponectin or modified adiponectin fragment or conservative variant of either in mammalian cells.

A modified adiponectin or modified adiponectin fragment or conservative variant of either can be separated on the basis of molecular weight, pi, and/or the amount of glycosylation present by routine methods, *e.g.,* electrophoresis or chromatography as disclosed in the Examples. In one aspect of the invention, compositions containing isoforms or glycoisoforms of a modified adiponectin or modified adiponectin fragment or conservative variant of either, *e.g.,* as described above, are prepared by differential purification. For example, according to a method of the invention, this involves obtaining a first composition containing at least two forms of adiponectin that differ in their degree or type of glycosylation and then separating the adiponectin forms based on the degree or type of glycosylation. This method produces a second composition that differs from the first composition in the adiponectin profile.

A modified adiponectin or modified adiponectin fragment or conservative variant of either can be separated from other proteins and polypeptides, etc., by several methods known in the protein purification art. In one embodiment, the separation is effected by two-dimensional electrophoresis and subsequent excision and elution of the protein from the gel. In another embodiment, the separation is effected by using an affinity column which selects on the basis of electrical charge. In another embodiment, the separation is effected by using an affinity column loaded with lectins. In other embodiments, alternate protein purification methods are used, *e.g.,* immunoaffmity column, size-exclusion column,

lectin affinity, hydrophobic interaction, reversed phase, anion and cation exchange chromatography, and the like (see, generally, R. Scopes, *Protein Purification*, Springer-Verlag, N.Y. (1982) and Deutscher, *Methods in Enzymology* Vol. 182: Guide to Protein Purification, Academic Press, Inc. N.Y. (1990)) .

In one embodiment, compositions containing predominantly a modified adiponectin or modified adiponectin fragment or conservative variant of either are obtained by using a lectin column, for example, a concanavalin A or wheat germ agglutinin column, to bind modified human adiponectins. Non-glycosylated proteins will not bind to the column and thus, will flow through the column. The target proteins are then eluted from the column to obtain a composition containing predominantly a modified adiponectin or modified adiponectin fragment or conservative variant of either.

In another embodiment, various isoforms and/or glycoisoforms are obtained by running adiponectin, either obtained recombinantly or from animal tissues, on a two-dimensional gel, identifying the glycosylated species by an antibody, excising the spot or band of the glycosylated adiponectin isoform, and eluting the glycosylated adiponectin isoform from the band to obtain a substantially pure composition of one glycosylated adiponectin isoform. It will be recognized that compositions of the invention can be made by routine techniques, such as those described above, and including separating and recombining specific isoforms and/or glycoisoforms to prepare desired embodiments.

Methods of treatment and preparation of medicaments

The invention also provides a treatment, for example, of a disease state associated with adiponectin or adiponectin regulation in an individual. A treatment plan generally includes the administration of an effective amount of a composition of a modified adiponectin or modified adiponectin fragment or conservative variant of either to the individual being treated. An effective amount can be determined by assessing biological activity, for example, for insulin sensitization. A skilled artisan may determine the amount of a composition by stepwise increments of dosage and assessing biological function at each step.

The administration of protein or protein composition of the invention to a mammal may be used to enhance the effect of insulin. The protein or protein composition may also

be used to allow a subphysiological blood insulin concentration to elicit the biological effect of a normal physiological blood insulin concentration.

Proteins and compositions of the invention may be used to inhibit gluconeogenesis when administered to an individual.

Proteins and compositions of the invention may be used, for example, to elicit a plasma modified adiponectin concentration of between about 1 microgram/mL and about 20 microgram/mL (more preferably, for example, to elicit a plasma adiponectin polypeptide-concentration of between about 1.9 microg/mL and about 17 microg/mL).

Preferably but not necessarily the individual is a human.

In yet another aspect the invention is a method for treating a disease state associated with, for example, adiponectin polypeptide regulation or aberrant insulin sensitivity comprising administering with or without pharmaceutically acceptable excipients, co-actives, diluents or the like an effective amount of a modified adiponectin.

The invention provides for use of modified adiponectins, modified adiponectin fragment, conservative variants of either, and compositions including any of them to treat one or more of, for example, hyperglycemia, insulin resistance, metabolic syndromes associated with insulin resistance, Type 2 diabetes mellitus, or obesity (e.g. including weight gain, reduction or control or weight gain prevention), metabolic syndromes including hypertension, atherosclerosis, coronary heart disease, ischemic heart disease, or polycystic ovary syndrome.

In another aspect, the invention provides a method for treating a mammalian patient deficient in adiponectin or who would otherwise benefit from such treatment comprising administering with or without pharmaceutically accepted excipients, co-actives, diluents or the like an effective amount of a modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

Accordingly to another aspect the present invention includes a method of treating a mammalian patient subject to or for, for example, liver disease and/or having any of the characteristics of liver disease which comprises or includes administering to that patient an effective amount of a modified adiponectin or modified adiponectin composition, a

modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

The liver disease may be, for example, any one or more of acute liver disease, chronic liver disease, inflammation of the liver, dysfunction of the liver, fatty liver (hepatic steatosis), fibrosis of the liver, cirrhosis of the liver, necrosis of the liver, hepatocellular necrosis, alcoholic liver disease, alcoholic hepatic steatosis, alcoholic hepatitis, alcoholic hepatic necrosis, alcoholic hepatic cirrhosis, hepatic necrosis, hepatic steatosis, hepatic steatosis associated with diabetes, hepatic steatosis associated with a diet rich in lipids, hepatic steatosis associated with abnormalities of lipid metabolism, hepatitis caused by any condition, hepatic necrosis caused by any condition, acute hepatitis, chronic hepatitis, chronic active hepatitis, hepatitis secondary to viral infection or inflammation of the liver, hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, hepatitis G, hepatitis secondary to the action of any drug or toxin, hepatitis or hepatic dysfunction consequent upon cholestasis, primary biliary cirrhosis, hepatic granulomatosis, and/or conditions in which elevated tissue or blood concentrations of tumor necrosis factor a play a pathogenic role.

In a further aspect the present invention consists in a method of treating a mammalian patient subject to or for alcoholic liver disease and/or having any of the characteristics of alcoholic liver disease which comprises or includes administering to that patient an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

In a further aspect the present invention consists in a method of treating a mammalian patient to prevent and/or reverse liver disease and/or any of the characteristics of liver disease which comprises or includes administering to that patient an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

In a further aspect the present invention consists in a method of treating a mammalian patient to prevent and/or reverse alcoholic liver disease and/or any of the

characteristics of alcoholic liver disease which comprises or includes administering to that patient an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

In still a further aspect the present invention consists in a method of treating a human being subject to alcoholic liver disease and/or having any of the characteristics of alcoholic liver disease which comprises administering to that patient an effective amount of an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

In a yet further aspect the present invention consists in a method of treating a mammalian patient subject to any one or more of hepatic steatosis (fatty infiltration), hepatic inflammation, hepatic necrosis, hepatic fibrosis, hepatic cirrhosis, and/or hepatic dysfunction which comprises or includes administering to that patient an modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them.

As will be described hereinafter in its most simplistic forms and not dependant upon any vessel (whether a capsule or otherwise) an modified adiponectin or modified adiponectin fragment of the invention, or a conservative variant of either of them, can also be administered by means of a surgically implanted delivery device such as an osmotic pump, as is well know in the are alternative dosage forms suitable for administration of therapeutic protein may also be used.

Accordingly, in another aspect of the invention there is provided a method of treating a mammalian patient itself still able to encode for adiponectin comprising administering to the patient a modified adiponectin or modified adiponectin composition, a modified adiponectin fragment or modified adiponectin fragment composition, a conservative variant of a modified adiponectin or a modified adiponectin fragment or a composition including either of them in a sufficient amount(s) to suppress TNF-α levels

below those that would have been or likely would have been present without such administration. For example, such administration may be to treat, ameliorate, prevent and/or reverse a TNF-α disease or disorder and/or any of the characteristics of a TNF-α disease or disorder.

Preferably said TNF-α disease or disorder is, for example, any one or more of the following:- inflammatory disease, circulatory disease, portal hypertension, pulmonary hypertension, allegic diseases, Crohn's disease, autoimmune haemolytic anemia, psoriasis, hepatic disease, pancreatic disease, neurodegenerative disease, central nerve failure, toxaemia, climacteric failure, gestosis, adiposis, hyperlipidemia, hypercholesteremia, abnormal glucose tolerance, solid tumor, tumor cancer and accompanying cachexia, endocrine disease, Creutzfeldt-Jakob disease, viral infection, post-percutaneous coronary arterioplasty, vascular hypertrophy or occlusion, post-PTCA/stenting /bypass surgery vascular reocclusion/restenosis, post-intervention vascular hypertrophy or occlusion, suppression of implantation-induced vascular failure and rejection, rejection episodes following organ or tissue transplant and automimmune disease, side effects associated with TNF generation duringneoplastic therapy and also to eliminate or ameliorate shock related symptoms associated with the treatment or prevention of graft rejection, dialytic hypotension, glaucoma, high ocular tension, myasthenia gravis, chronic defatigation, bone disease, neurological disorders, TNF-α induced insulin resistance, aberrant apoptosis, complications of diabetes mellitus or stress hyperglycemia, chronic obstructive pulmonary disease, chronic bronchitis and emphysema.

Preferably said inflammatory response is, for example, any one of the following: diabetic complications such as retinopathy, nephropathy, neuropathy, major vascular and microvascular disorders; arthritis such as chronic rheumatoid arthritis, osteoarthritis, rheumatoid myelitis and periosteosis; postooperative/posttraumatic inflammation; remedy of swelling; pharyngitis; cystitis; pneumonia; myocarditis; cardiomyopathy; atopic dermatitis; inflammatory intestinal disease such as Crohn's disease and ulcerative colitis; meningitis; inflammatory ophthalmic disease; inflammatory pulmonary disease such as pneumonia, silicotuberculosis, pulmonary sarcoidosis, inflammatory bone disorders and pulmonary tuberculosis

Preferably said circulatory disease includes, for example, any one of the following: chronic heart failure including arrhythmia, angina pectris, myocardial infarction, cardiac insufficiency and congestive heart failure, arteriosclerosis including atherosclerosis, hypertension, deep vein thrombosis, occlusive peripheral circulation failure, ischemic cerebral circulation failure, disseminated intravascular coagulation syndrome, Raynaud's disease, Buerger disease.

Preferably said allergic disease includes, for example, any one of the following: asthma, allergic rhinitis, conjunctivitis, digestive tract allergy, pollinosis and anaphylaxis, chronic occlusive pulmonary disease, collagenosis.

Preferably said neurodegenerative disease includes, for example, any one of the following: Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, AIDS, encephalopathy.

Preferably said central nerve failure includes, for example, any one of the following: cerebrovascular failure such as cerebral hemorrhage and cerebral infarction and its sequela, cranial trauma, spinal damage, cerebral edema, dementia, memory failure, consciousness failure, multiple sclerosis.

Preferably said toxemia includes, for example, any one of the following: sepsis, septic shock, endotoxic shock, gram negative sepsis, toxin shock syndrome.

Preferably said cancerous tumor includes, for example, any one of the following: malignant melanoma, malignant lymphoma and cancer of the digestive organ.

Preferably said endocrine disease includes, for example, any one of the following: Addison disease, Cushing's syndrome, melanocytoma and primary aldosteronism.

Preferably said autoimmune disease includes, for example, any one of the following: organ specific diseases such as thyroiditis or non-specific organ diseases such as rheumatoid and osteo-arthritis.

Preferably said bone disease includes, for example, any one of the following: fracture, re-fracture, osteoporosis, osteomalacia, bone Behcet disease, ankylosing spondylitis, chronic rheumatoid arthritis and osteogonarthritis as well as articular tissue destruction in disease related thereto.

Preferably said neurological disorders include, for example, trauma, injury, compression to individual nerves, nerve roots, spinal cord and/or the brain, acute spinal

cord and brain injure, demyelinating diseases, such as multiple sclerosis, spinal .cord compression due to metastatic cancer, primary or metastatic brain tumors, chronic pain syndromes due to metastatic tumor, inflammatory CNS diseases, such as subacute sclerosing panenencephalitis, Huntington's disease, Guillain-Barre syndrome, Bell's palsy, diabetic neuropathy, optic neuritis, macular degeneration, retinitis pigmentosa, diabetic retinopathy, muscular dystrophy, and polymyositis-dermatomyositis.

Preferably said aberrant apoptosis includes, for example, any virally-induced inhibition of apoptosis.

Preferably said complications of diabetes mellitus or stress hyperglycemia include, for example, any one or more of the following: myocardia infarction, congestive heart failure and cardiogenic shock.

The composition of a modified adiponectin or modified adiponectin fragment or conservative variant of either may be administered in a pharmaceutically accepted excipient. Pharmaceutically acceptable excipients are known in the art, and are relatively inert substances that facilitate administration of a pharmacologically effective substance. In some embodiments, the compositions of the invention comprising a modified adiponectin or modified adiponectin fragment or conservative variant of either are formulated for administration by injection *(e.g.,* intraperitoneal^, intravenously, subcutaneously, intramuscularly, etc.).

Accordingly, a composition of a modified adiponectin or modified adiponectin fragment or conservative variant of either can be combined, for example, with pharmaceutically acceptable vehicles such as saline, Ringer's solution, dextrose solution, and the like. The particular dosage regimen, *i.e.*, dose, timing and repetition, will depend on the particular individual and that individual's medical history. The treatment may include multiple administrations over a period of time. The treatment can be assessed for biological function using routine clinical measurements including but not limited to glucose level, glucose fasting test, and *in vitro* insulin sensitization test.

Antimicrobial agents in bacteriostatic or fungistatic concentrations may also be added to comply with the United States Pharmacopeia (USP). These agents must be added to preparations contained in multiple dose containers. There must be an adequate concentration at the time of use to prevent the multiplication of microorganisms

inadvertently introduced into the preparation while withdrawing a portion of the contents for example with a hypodermic needle. Examples of suitable antimicrobial agents, include for example, benzyl alcohol, benzalkonium chloride, phenol, m-cresol, methyl p-hydroxybenzoate, benzoic acid, phenoxyethanol, methyl paraben, and propyl paraben and combinations of any of the above.

Sodium chloride or other salt may be added to adjust the tonicity of the composition, especially for parenteral formulations that must be isotonic or substantially isotonic otherwise significant irritation and pain will occur at the site of administration.

It will be appreciated the invention also provides the use of the adiponectin and adiponectin agonist compositions disclosed herein in preparation of pharmaceutical compositions.

In yet a further aspect, the invention consists in the use of a modified adiponectin or modified adiponectin fragment or conservative variant of either in the preparation of a dosage unit or pharmaceutical composition or medicament useful in the treatment of a disease state associated with adiponectin regulation, or useful to enhance the effects of insulin, or useful to inhibit gluconeogenesis, in a mammalian patient. In one embodiment, the modified adiponectin or modified adiponectin fragment or conservative variant of either is recombinant or synthesized. In another embodiment, the modified adiponectin or modified adiponectin fragment or conservative variant of either is a based on human adiponectin or an active human adiponectin fragment. In another embodiment, at least one of the residues, for example, lysine residues, corresponding to lysine residues corresponding to 68, 71, 80, and 104 (mouse) or 65, 68, 77, and 101 (human) is glycosylated. In another embodiment, the dosage unit or pharmaceutical composition or medicament may additionally comprise an insulin or an insulin analog. Preferably, the insulin or analog is present in an amount sufficient to elicit a blood insulin concentration of between about 5OpM and about 40OpM. Preferably, the insulin or analog is at a concentration or amount sufficient to elicit a blood insulin or analog concentration of between about lOOpM and about 30OpM. More preferably, the insulin or analog is present in an amount sufficient to elicit a blood insulin or analog concentration of about 20OpM.

Suitable routes of administration of a parenteral formulation of the present invention include intramuscular, intravenous, subcutaneous, intradermal, intraarticular, intrathecal

and the like. The subcutaneous route of administration is preferred. Mucosal delivery is also permissible.

It is envisaged the present invention can be co-administered or serially administered and/or mixed with an insulin or an insulin analog as a composition and/or formulation. This will depend on the situation and the patient. A suitable treatment regime may be best determined by a doctor or medical practitioner for each patient. Many insulins are available from a number of companies and include Eli Lilly & Company and Novo Nordisk. Types of insulin available are fast-, intermediate- and long- acting insulins. There are also various types of insulins within these categories. The ratio of insulin or analog and a modified adiponectin or modified adiponectin fragment or conservative variant of either will depend upon the individual needs of a particular patient. A suitable treatment regime may be best determined by a doctor or medical practitioner for each patient.

Compositions useful in the invention are prepared by mixing the ingredients following generally accepted procedures. For example, the selected components may be mixed in a blender or other standard device to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water or thickening agent and possibly a buffer to control pH or an additional solute to control tonicity.

The following Examples are provided to illustrate but not to limit the invention in any manner.

EXAMPLE 1

EXPRESSION AND CONFIRMATION OF A RECOMBINANT MODIFIED HUMAN ADIPONECTIN

Site-directed mutagenesis was used to generate the recombinant modified human adiponectin production vector. The vector used as the template was created by cloning the full length human adiponectin gene that was tagged with a FLAG epitope at its -COOH terminus into the commercial vector pcDNA3.1 (+) (Invitrogen #V790-20). Mutagenesis was then carried out using this vector, where the three threonines (T) at amino acid residues 20, 21 and 22 were substituted with alanines. Stratagene's QuikChange site directed mutagenesis kit was used to accomplish this in conjunction with oligonucleotides

5'cccggtcatgaccaggaagccgcggctcaagggcccggagtcc - 3' [SEQ.ID.No.2] and 5' - ggactccgggcccttgagccgcggcttcctggtcatgaccggg - 3' [SEQ.ID:No.3] as mutagenic primers. Amplification of the mutated DNA was achieved using PCR. To confirm the success of mutagenesis, XLl -Blue cell colonies were transformed with the mutated DNA and then grown on Amp^+-agar plate for DNA amplification. Isolated DNA was sequenced, and the fidelity of the DNA verified whereby the DNA sequence corresponding to Thr20, Thr21, and Thr22 (accacgact) where changed to Ala20, Ala21, and ala22 (gccgcggct).

EXAMPLE 2

STABLE MODIFIED ADIPONECTIN EXPRESSION CLONE

The expression vector containing cDNA encoding the modified adiponectin was transiently transfected into adherently cultured CHO-S cells using Lipofectamine LTX Reagent (Invitrogen #15338-019). Stable monoclonal expression clones were selected using Geneticin (1000 μg/ml) and routinely maintained by exposure to a dose of 300 μg/ml. Adherent clone cultures were prepared as freezer cultures and stored in liquid nitrogen. Western blotting data revealed that four stable clones (X_6, X_8, X_3 and X_7) produced modified adiponectin proteins at high levels.

EXAMPLE 3

METHOD OF PURIFICATION

Purification of the culture media containing the modified adiponectin was carried out using the ANTI-FLAG® M2 affinity gel and the Batch Absorption method as described herein.

Firstly, the culture media was collected and then spun and filtered (0.2 μm pore size) to remove any suspended particles. The resultant media then underwent binding incubation with the M2 affinity gel to purify the modified adiponectin extracted from the culture media. The materials utilized for the batch absorption method included the following: TBS buffer (comprising 50 mM Tris-HCl, 150 mM NaCl, pH 7), an elution buffer (comprising 100 mM Glycine-HCl, pH 3.5 (at room temperature)), a wash buffer (comprising TBS Buffer containing 0.02 % sodium azide, 1 M Tris-HCl, pH 8.0), a bead storage buffer (comprising 50 % Glycerol in TBS Buffer containing 0.02 % Sodium azide), a chromatography column (5 ml plastic disposable columns, PIERCE #29922), M2 beads

(comprising ANTI-FLAG® M2-Agarose from mouse #A2220, Sigma), a water bath at 37 ^{0}C, an orbital shaker, T175 flasks, and a refrigerated centrifuge at 8 °C.

The following batch absorption method was then used:

1. 1000 ml of the culture media was mixed with 7 ml of the M2 beads. This was then split into 4 x 250 ml aliquots in large glass flasks, and placed on the orbital shaker and incubated overnight in a cold room.

2. After incubation, the beads were collected into 2 x 50 ml tubes by centrifugation at 1500 x g, 8 °C for 5 min. The supernatant was frozen for later use.

3. The resultant gel pellets were resuspended in TBS buffer and transferred into empty columns and rinsed twice with TBS buffer.

4. The columns were then washed 20 times with 3.5 ml TBS buffer aliquots and then transferred into 6 different vials containing 100µl 1 M Tris-HCl (pH 8.0). The vials containing the modified adiponectin were then eluted through the columns using 0.1 M Glycine (pH 3.5).

5. The column was then equilibrated till neutral with TBS Buffer, washed with a storage buffer, and then frozen for later use.

6. 48 ml of the resultant eluent was then concentrated to ~100 µl at 8 ^{0}C using a Vivaspin 6 ml concentrator with MWCO 5000 (Vivascience #VS0612), and then washed with PBS to remove the elution buffer, re-concentrating after each wash. The washed aliquot was then concentrated down to ~175 µl.

7. 175 µl of purified sample was quantitated by Nanodrop measurement as 0.8 µg/µl. To assay the purity of the resultant sample, 4 µg of the purified protein was reduced using β-mercaptoethanol, then heat-denatured (99 ^{0}C for 6 mins), followed by SDS-PAGE on a 12 % gel. The gel was stained with 0.1 % (w/v) Coomassie Bright Blue R250 (SERVA #35051) in 45 % (v/v) methanol and 10% (v/v) acetic acid overnight, with gentle shaking. The stained gel was then destained with several changes of destaining solution (30 % methanol: 10 % acetic acid) over several hours. The molecular weight marker used was Invitrogen #LC 5925 (see lane 1 of Figure 1). Figure 1 shows the modified adiponectin in lane 2 where the gel was visually checked and a heavily stained band was found at 30 kDa, indicating expression of modified adiponectin by the CHO-S cells.

EXAMPLE 4

CHARACTERIZATION OF PURIFIED OF MODIFIED ADIPONECTIN

To investigate the mutagenesis effect on adiponectin multimerization, a sample of the modified adiponectin (purified) and wild type adiponectin were both electrophorised under non-reducing conditions and then visualized by Western blotting. See Figure 2. Samples used for lanes 1-6 were treated for 30 minutes with a non-reducing loading buffer (% SDS and 10% glycerol in 50 mM Tris-HCl (pH 6.8)) at room temperature. The sample used for lane was denatured by applying heat at 99^{0}C.

Further characterization was then carried out using two-dimensional gel electrophoresis (2-DE) using the following procedure: The samples were prepared by taking 60 µg of wild type adiponectin or the modified adiponectin and then mixing the respective samples with a solubilization buffer (160 mg Urea [7 M]; 57 mg Thiourea [2 M]; 7.2 mg CHAPS [2%]; 3.6 mg DTT [65 mM]; 3.6 µl Pharmalyte 3-10 [1%]; 1 µl Bromophenol blue [trace]). The samples were then made up to a final volume of 360 µl with water. This was then gently agitated for one hour in an incubator at room temperature. The immobiline DryStrip was rehydrated overnight and then transferred to the strip alignment in the Multiphor cooling plate for the 1st dimensional isoelectric focus. Electrode wicks and bars were positioned to each ends of the gel strip, covered with Ondina Oil and connected to the power supply. Electrophoresis was performed in GRADIENT running mode at 20 ^{0}C for 22 hours using the following programme:

Step	Voltage (V)	Current (mA)	Time (h)	Power (W)
1	500	1	0.01	5
2	500	1	5	5
3	3500	1	5	5
4	3500	1	12	5

For the 2nd Dimensional SDS-PAGE an equilibration buffer was prepared with 6 M Urea, 1% SDS, 30% Glycerol and 100 mM Tris-HCl (pH 6.8) solution, and mixed with 15 ml 65 mM DTT (buffer I), or 240 mM IAA (buffer II).

After isoelectric focusing, the gel strips were equilibrated in buffer I and then buffer II at room temperature with gentle agitation for 10 min respectively. During gel equilibration, anode and cathode buffer strips were placed respectively at the top and bottom of the ExcelGel which had been placed on the cooling plate surface. The

equilibrated gel strip was rinsed with water, carefully blotted on damp filter paper and positioned face down onto the SDS gel, and then loaded with the samples. The SDS-PAGE was performed in STEP running mode following the programme below:

Step	time	Voltage (V)	Current (mA)	Power (W)
1	45 min	1000	20	20
2	5 min	1000	40	40
3	2 h 45 min	1000	40	40

Following 2-dimensional electrophoresis, the protein sample was stained with GBB. See Figures 3-6.

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All patents, publications, scientific articles, web sites, and other documents and materials referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced document and material is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such patents, publications, scientific articles, web sites, electronically available information, and other referenced materials or documents.

The written description portion of this patent includes all claims. Furthermore, all claims, including all original claims as well as all claims from any and all priority documents, are hereby incorporated by reference in their entirety into the written description portion of the specification, and Applicants reserve the right to physically incorporate into the written description or any other portion of the application, any and all such claims. Thus, for example, under no circumstances may the patent be interpreted as allegedly not providing a written description for a claim on the assertion that the precise wording of the claim is not set forth *in haec verba* in written description portion of the patent.

The claims will be interpreted according to law. However, and notwithstanding the alleged or perceived ease or difficulty of interpreting any claim or portion thereof, under no circumstances may any adjustment or amendment of a claim or any portion thereof during

prosecution of the application or applications leading to this patent be interpreted as having forfeited any right to any and all equivalents thereof that do not form a part of the prior art.

All of the features disclosed in this specification may be combined in any combination. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Thus, from the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Other aspects, advantages, and modifications are within the scope of the following claims and the present invention is not limited except as by the appended claims.

The specific methods and compositions described herein are representative of preferred embodiments and are exemplary and not intended as limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. Thus, for example, in each instance herein, in embodiments or examples of the present invention, the terms "comprising", "including", "containing", *etc.* are to be read expansively and without limitation. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the

invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by various embodiments and/or preferred embodiments and optional features, any and all modifications and variations of the concepts herein disclosed that may be resorted to by those skilled in the art are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

It is also to be understood that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise, the term "X and/or Y" means "X" or "Y" or both "X" and "Y", and the letter "s" following a noun designates both the plural and singular forms of that noun. In addition, where features or aspects of the invention are described in terms of Markush groups, it is intended, and those skilled in the art will recognize, that the invention embraces and is also thereby described in terms of any individual member or subgroup of members of the Markush group.

Other embodiments are within the following claims. The patent may not be interpreted to be limited to the specific examples or embodiments or methods specifically and/or expressly disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

CLAIMS

1. Isolated modified adiponectin.
2. The modified adiponectin of claim 1 which is selected from the group consisting of an adiponectin having an amino acid substitution at the position corresponding to position 20 of human adiponectin; an adiponectin having an amino acid substitution at the position corresponding to position 21 of human adiponectin; an adiponectin having an amino acid substitution at the position corresponding to position 22 of human adiponectin; an adiponectin having an amino acid substitution at the positions corresponding to positions 20 and 21 of human adiponectin; an adiponectin having an amino acid substitution at the positions corresponding to positions 20 and 22 of human adiponectin; an adiponectin having an amino acid substitution at the position corresponding to positions 21 and 22 of human adiponectin; and, an adiponectin having an amino acid substitution at the position corresponding to positions 20, 21 and 22 of human adiponectin.
3. The modified adiponectin of claim 2 wherein the substitution is an alanine or a glycine.
4. The modified adiponectin of claim 1 wherein one or more of the amino acids at the positions corresponding to positions of 20, 21 and/or 22 of human adiponectin has been deleted.
5. The modified adiponectin of claim 1 wherein one or more of the amino acids at the positions corresponding to positions of 20, 21 and/or 22 of human adiponectin has been deleted or substituted with another amino acid.
6. The modified adiponectin of claim 1 having one or more of the biological activities of wild-type adiponectin.
7. The modified adiponectin of claim 1 wherein the modified adiponectin is of human origin.
8. A modified adiponectin of claim 1 wherein each of the residues corresponding to lysine residues 65, 68, 77 and 101 of human adiponectin is α-1-2-glucosylgalactosyl-O-hydroxylysine, or the residue corresponding to proline residue 91 of human adiponectin is hydroxyproline, or both.

9. A composition comprising a modified adiponectin of any of claims 1-7 or 8 and a pharmaceutically acceptable carrier.

10. A polynucleotide comprising a modified adiponectin of claim 1.

11. A vector comprising a polynucleotide of claim 10.

12. A host cell comprising a polynucleotide of claim 10.

13. A host cell comprising a vector of claim 11.

14. A host cell of claim 12, wherein the cell is eukaryotic.

15. A host cell of claim 13, wherein the cell is eukaryotic.

16. A method of expressing modified adiponectin comprising culturing a host cell of claim 14 under conditions which express the encoded protein.

17. A method of expressing modified adiponectin comprising culturing a host cell of claim 15 under conditions which express the encoded protein.

18. A transgenic animal comprising a polynucleotide of claim 10.

19. A method of producing a modified adiponectin comprising isolating a modified adiponectin from a transgenic animal of claim 18.

20. A method of claim 19, wherein the modified adiponectin is isolated from a biological fluid from the transgenic animal.

21. A method of claim 20, wherein the fluid is serum or milk.

22. A method of treating a disease or disease symptom in a patient, comprising the step of administering a modified adiponectin of claim 1.

23. A modified adiponectin of claim 4, wherein the modified adiponectin has one or more other amino acid substitutions or deletions in addition to an amino acid substitution or deletion at one or more of positions 20, 21 and/or 22.

24. A composition comprising a modified adiponectin of claim 23 and a pharmaceutically acceptable carrier.

25. A method of treating a subject comprising administering to the subject a therapeutically effective amount of a modified adiponectin of claim 24.

26. A method of claim 22 or 25, wherein the subject is suffering from hyperglycemia, insulin resistance, metabolic syndromes associated with insulin resistance, Type 2 diabetes mellitus, or obesity, metabolic syndromes including hypertension,

artherosclerosis, coronary heart disease, ischemic heart disease, or polycystic ovary syndrome.

27. A method of claim 22 or 25, wherein the subject is suffering elevated level of glucose as compared to a healthy subject.

28. A method of claim 27, wherein the elevated level of glucose is associated with diabetes, insulin resistance or metabolic syndrome.

29. A method of claim 28, wherein the diabetes is type 2 diabetes.

30. A method of claim 22 or 25 wherein the subject is suffering from a liver disease or condition.

31. A method of claim 30 wherein the liver disease or condition is alcoholic liver disease, hepatic steatosis (fatty infiltration), hepatic inflammation, hepatic necrosis, hepatic fibrosis, hepatic cirrhosis, and/or hepatic dysfunction which comprises or includes administering to that patient adiponectin and/or an agonist thereof.

32. A method of claim 30 wherein the liver disease is selected from acute liver disease, chronic liver disease, inflammation of the liver, dysfunction of the liver, fatty liver (hepatic steatosis), fibrosis of the liver, cirrhosis of the liver, necrosis of the liver, hepatocellular necrosis, alcoholic liver disease, alcoholic hepatic steatosis, alcoholic hepatitis, alcoholic hepatic necrosis, alcoholic hepatic cirrhosis, hepatic necrosis, hepatic steatosis, hepatic steatosis associated with diabetes, hepatic steatosis associated with a diet rich in lipids, hepatic steatosis associated with abnormalities of lipid metabolism, hepatitis caused by any condition, hepatic necrosis caused by any condition, acute hepatitis, chronic hepatitis, chronic active hepatitis, hepatitis secondary to viral infection or inflammation of the liver, hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, hepatitis G, hepatitis secondary to the action of any drug or toxin, hepatitis or hepatic dysfunction consequent upon cholestasis, primary biliary cirrhosis, hepatic granulomatosis, and/or conditions in which elevated tissue or blood concentrations of tumour necrosis factor a play a pathogenic role, which comprises or includes administering to that patient adiponectin and/or an agonist thereof.

33. A method of claim 22 or 25, wherein the subject is suffering from a disease caused in whole or in part by undesired levels of TNFα.

34. A method of preventing or treating a disease or condition associated with TNFα comprising administering to the subject a therapeutically effective amount of a modified adiponectin.

35. A method of regulating TNFα in a subject comprising administering to the subject a therapeutically effective amount of a modified adiponectin.

36. A modified adiponectin of claim 1 or claim 2 wherein the prolyl residue corresponding to proline residue 91 of human adiponectin is not hydroxylated.

37. A modified adiponectin of claim 1 or claim 2 wherein the prolyl residue corresponding to proline residue 91 of human adiponectin is hydroxylated.

38. A modified adiponectin of claim 1 or claim 2 wherein at least one of the lysine residues corresponding to lysine residues 65, 68, 77, and 101 of human adiponectin is glycosylated.

39. A modified adiponectin of claim 37 wherein the glycosylation is with any one or more of a glucosylgalactosyl moiety, a glucosylglucosyl moiety, a galactosylglucosyl moiety, or a galactosylgalactosyl moiety.

40. A modified adiponectin of claim 37 wherein one or more of the lysine residues corresponding to lysine residues 65, 68, 77, and 101 of human adiponectin are glycosylated.

41. A modified adiponectin of claim 39 wherein all four of the lysine residues corresponding to lysine residues 65, 68, 77, and 101 of human adiponectin are glycosylated.

42. A modified adiponectin of claim 1 or 2 wherein the modified adiponectin has an α-1-2-glucosylgalactosyl-O-hydroxylysine residue at one or more of the positions corresponding to lysine residues 65, 68, 77 and/or 101 of human adiponectin.

43. A modified adiponectin of claim 38 wherein the glycosylation is with any one or more of a glucosylgalactosyl moiety, a glucosylglucosyl moiety, a galactosylglucosyl moiety, or a galactosylgalactosyl moiety.

44. A modified adiponectin claim 1 which is at least about 50% pure.

45. A modified adiponectin claim 1 which is at least about 80% pure.

46. A modified adiponectin claim 1 which is at least about 90% pure.

47. A modified adiponectin claim 1 which is at least about 95% pure.

48. A modified adiponectin claim 1 which is at least about 99% pure.

49. A modified adiponectin claim 2 which is at least about 50% pure.

50. A modified adiponectin claim 2 which is at least about 80% pure.

51. A modified adiponectin claim 2 which is at least about 90% pure.

52. A modified adiponectin claim 2 which is at least about 95% pure.

53. A modified adiponectin claim 2 which is at least about 99% pure.

54. A method comprising preparation of a pharmaceutical composition comprising the step of bringing together a modified adiponectin of claim 1 and a pharmaceutically acceptable carrier.

55. An article of manufacture comprising or including a vessel or delivery unit containing at least a modified adiponectin and instructions for use of the modified adiponectin effective for use in a mammalian patient.

56. A pharmaceutical composition comprising a modified adiponectin of claim 1 wherein (i) at least one of the lysine residues corresponding to lysine residues 65, 68, 77, and 101 of human adiponectin is glycosylated; (ii) glycosylation is with any one or more of a glucosylgalactosyl moiety, a glucosylglucosyl moiety, a galactosylglucosyl moiety, or a galactosylgalactosyl moiety; and, (iii) the residue corresponding to proline residue 91 of human adiponectin is or is not hydroxyproline.

57. A composition comprising a modified adiponectin of claim 1 wherein the modified adiponectin is formulated in a manner suitable for parenteral administration.

Figure 1

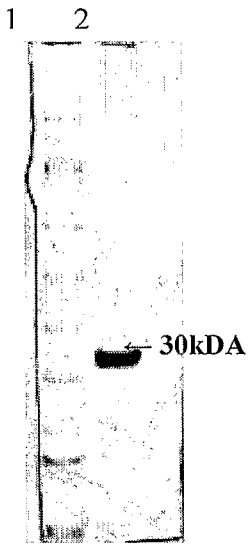

Figure 2

1 2 3 4 5 6 7

Figure 3

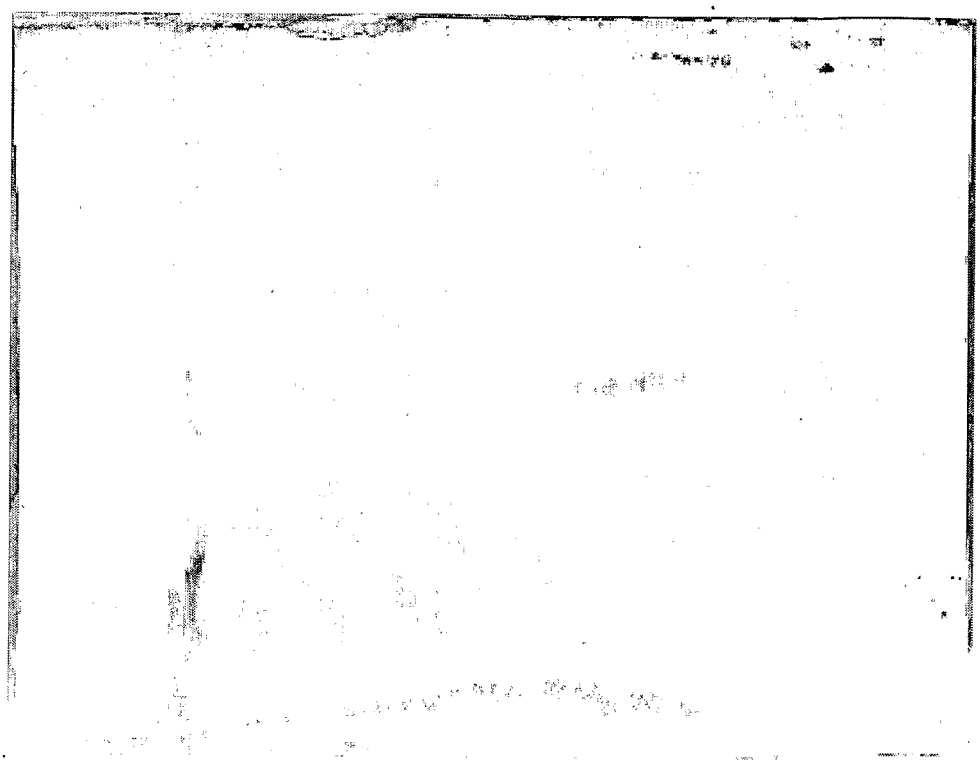

Figure 4

Figure 5

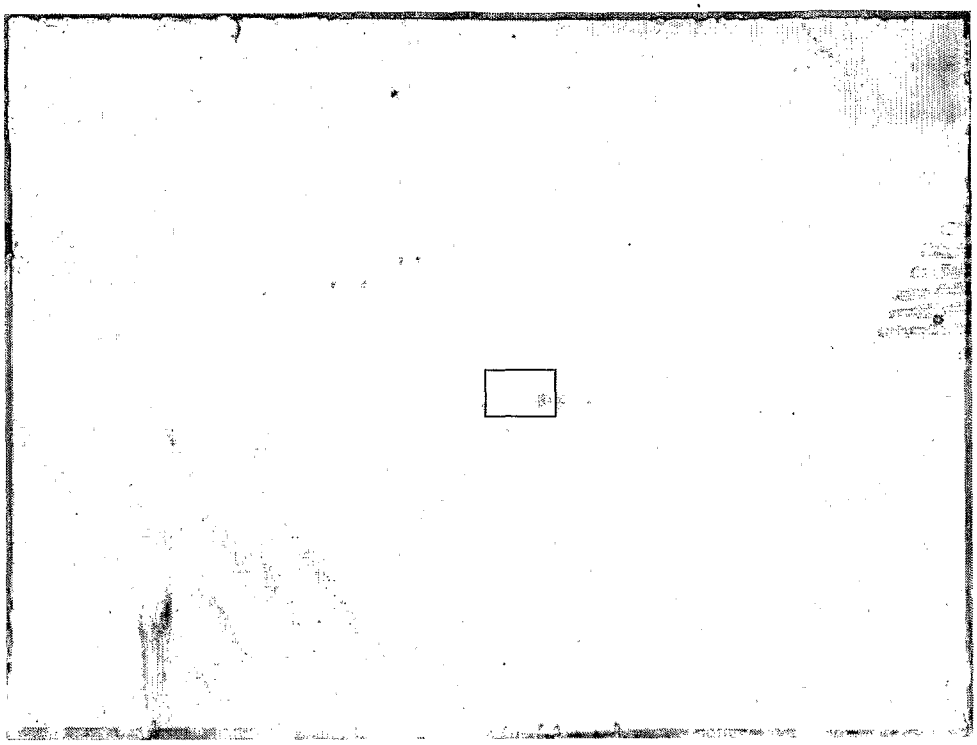

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2008/000073

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07K 14/575 (2006.01) A61K 38/22 (2006.0\)A61P 3/04 (2006.0I)ZKJiP *3/10* (2006.01) *C07H 21/04* (2006.01) *C12N 15/64* (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA: Subsequence Search of a modified SEQ ID 1

C DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
x	WO2003/062275 A1 (PROTEMLX CORPORATION LIMITED) 31 July 2003 See whole document esp. page 10-3nd Paragraph , page 11-3rd and 4th paragraphs, page 12-4th-7th paragraphs page 18-4*-6th paragraph; page 21-5th paragraph, and page 34-last paragraph, pages 43-46 and claims	1-57
X	WO2006/062422 A1 (COOPER. G. J. S) 15 June 2006 See whole document esp. abstract, page 13-16, 17, 18 and 30 and claims	1-57

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 August 2008	12 AUG 2008
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address pct@ipaustralia gov au Facsimile No. +61 2 6283 7999	Authorized officer RICKY FUNG AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No (02) 6222 3648

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2008/000073

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/074432 A2 (XENCOR) 13 July 2006 See whole document esp. paragraphs 9, 11, 17, 96, 99, 101 and claims	1-57

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NZ2008/000073

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2003/062275	AU	2003285686	CA	2473781	EP	1474445
		JP	2005535561	US	2004023854		
WO	2006/062422	AU	2005312435	CA	2590177	EP	1830873
		US	2006128610				
WO	2006/074432	AU	33164/01	CA	2383968	CN	1386220
		EP	1299780	KR	20070110319	MX	PA02002445
		US	2002026514	WO	0157823		

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX