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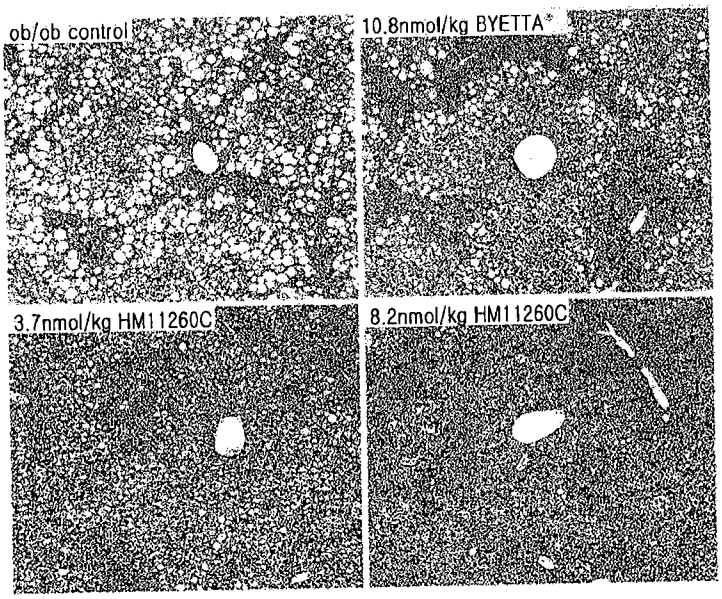
(54) Title: PHARMACEUTICAL COMPOSITION FOR THE PREVENTION OR TREATMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE



(57) Abstract: The present invention relates to a pharmaceutical composition for the prevention and treatment of non-alcoholic fatty liver disease (NAFLD), including a conjugate prepared by covalently linking an insulinotropic peptide, a non-peptidyl polymer and an immunoglobulin Fc region. The composition of the present invention maintains the in-vivo activity of the peptide at a relatively high level, and remarkably increases the blood half-life, thereby preventing triglyceride accumulation which is a typical feature of non-alcoholic fatty liver disease. Ultimately, it can be desirably employed for the prevention and treatment of non-alcoholic fatty liver disease.

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Description

Title of Invention: PHARMACEUTICAL COMPOSITION FOR THE PREVENTION OR TREATMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE

Technical Field

[1] The present invention relates to a pharmaceutical composition including a long-acting insulinotropic peptide conjugate which can be used for the prevention or treatment of non-alcoholic fatty liver disease. In particular, the present invention relates to an insulinotropic peptide conjugate in which an insulinotropic peptide, a non-peptidyl polymer, and an immunoglobulin Fc region are covalently linked to each other so as to remarkably increase blood half-life, to effectively prevent triglyceride accumulation, and to a use thereof in the prevention or treatment of non-alcoholic fatty liver disease.

[2]

Background Art

[3] Non-alcoholic fatty liver disease refers to a broad spectrum of diseases ranging from simple steatosis, which is not accompanied by an inflammatory response in a patient with no excessive intake of alcohol, to liver fibrosis and liver cirrhosis, which result from the progression of simple steatosis and exhibit hepatocellular inflammation.

[4]

[5] Non-alcoholic fatty liver disease may be categorized into primary and secondary non-alcoholic fatty liver diseases depending on the pathological cause. The primary one is caused by hyperlipidemia, diabetes, obesity or the like which is a characteristic of metabolic syndrome. The secondary one is a result of nutritional causes (sudden body weight loss, starvation, intestinal bypass surgery), various drugs, toxic substances (poisonous mushrooms, bacterial toxins), metabolic causes and other factors.

[6]

[7] It is known that the incidence of primary non-alcoholic fatty liver disease in which diabetes and obesity, which are important characteristics of metabolic syndrome, are a primary factor is in about 50% of diabetic patients, about 76% of obesity patients, and most obese diabetic patients (Gupte P et al., 2004). Further, when a liver biopsy is performed on diabetic and obesity patients with an increased level of alanine amino-transferase (ALT), the incidence of steatohepatitis is in the range of 18 to 36% (Braillon A et al., 1985).

[8]

[9] Currently, there is no established method for determining the cause of non-alcoholic

fatty liver disease. This is because the incidence of non-alcoholic fatty liver disease is associated with a variety of factors such as diabetes, obesity, coronary artery diseases, and lifestyle habits. There are some reports about effects of anti-diabetic or obesity drugs on fatty liver disease. Orlistat, which is used as an oral anti-obesity drug, exhibited histological improvements of the liver in patients with steatohepatitis (Hussein et al., 2007), and metformin exhibited decreases in blood levels of hepatic enzymes and hepatic necrotic inflammation and fibrosis in non-alcoholic fatty liver disease patients with no exhibition of diabetes (Bugianesi et al., 2005). Further, thiazolidinedione (TZD) class drugs, which are PPAR (peroxisome proliferator-activated receptor) agonists, inhibit the accumulation of fat in the liver and muscles, and exhibit direct anti-fibrotic actions on the liver in animal models of non-alcoholic fatty liver diseases (Galli A et al., 2002).

[10]

[11] Meanwhile, Glucagon-Like Peptide-1 (GLP-1) is an endogenous peptide present in the body and is a hormone secreted from the intestinal L cells in response to stimulation by nutrients or blood glucose level in the intestine. GLP-1 has a variety of physiological activities including regulation of blood glucose level by stimulating insulin secretion, pancreatic β cell proliferation, inhibition of upper gastrointestinal tract motility, and inhibition of appetite. Recently, GLP-1 receptor expression was found in hepatocytes and GLP-1 shows good effects on the treatment of non-alcoholic fatty liver disease by activation of phosphoinositide-dependent kinase-1 (PDK-1) and protein kinase C-(PKC-) which are major proteins in the insulin signaling pathway via the GLP-1 receptor of hepatocytes (Gupta NA et al., 2010). GLP-1 also functions to reduce fatty acid accumulation or protect hepatocytes from death caused by endoplasmic reticulum stress through activation of both chaperone-mediated autophagy (CMA) and macroautophagy (Sharma S et al., 2011). A recent study reported that GLP-1 promotes hepatic lipid oxidation to prevent hepatic fat accumulation and promotes insulin actions (Svegliati-Baroni G et al., 2011). These many reports suggest that GLP-1 derivative can be an important candidate for the development of a prophylactic and therapeutic agent for non-alcoholic fatty liver disease.

[12]

[13] However, the primary obstacle for the use of GLP-1 as a therapeutic agent for non-alcoholic fatty liver is its short blood half-life (maximum half-life: 2 minutes). It is attributed to the loss of the titers of GLP-1 through the cleavage between the 8th amino acid (Ala) and the 9th amino acid (Asp) by a dipeptidyl pepdidase IV (DPP IV) in the body. Therefore, various investigations have been made on a GLP-1 analog having resistance to DPP IV and trials have been made for substitution of Ala^{8} with Gly (Deacon et al., 1998; Burcelin et al., 1999), or with Leu or D-Ala (Xiao et al., 2001),

thereby increasing the resistance to DPP IV, while maintaining the activity. The N-terminal amino acid His^7 of GLP-1 is critical for the GLP-1 activity and serves as a target of DPP IV. Accordingly, US Patent No. 5,545,618 describes that the N-terminus is modified with an alkyl or acyl group and Gallwitz, et al. describes that 7th His was subject to N-methylation, or alpha-methylation, or the entire His is substituted with imidazole to increase the resistance to DPP IV and to maintain physiological activity.

[14]

[15] In addition to these modifications, an exendin-4, which is a GLP-1 analog purified from the salivary gland of a gila monster (US Patent No. 5,424,686), has resistance to DPP IV and higher physiological activity than GLP-1. As a result, it had an in-vivo half-life of 2 to 4 hours, a time period that was longer than that of GLP-1. However, with only the method for increasing the resistance to DPP IV, the physiological activity is not sufficiently sustained and, for example, in the case of a commercially available exendin-4 (exenatide) it needs to be injected into a patient twice a day. This frequency is still difficult for patients. The peptide prepared to improve the problem is exendin-4 which is resistant to DPP IV, which has a blood half-life of 2 to 4 hours. Although its blood half-life is longer than that of GLP-1, it also needs to be injected every day.

[16]

Disclosure of Invention

Technical Problem

[17] Accordingly, the present inventors used a method of site-specifically linking an immunoglobulin Fc region, a non-peptidyl polymer, and an insulinotropic peptide by a covalent bond so as to maximize the effects of increasing the blood half-life of the insulinotropic peptide and maintaining the in-vivo activity. As a result, the present inventors found that the method remarkably increased the blood half-life of the peptide conjugate and provided much longer blood half-life than the known in-frame fusion method. The present inventors also found that the conjugate prepared by site-specific linkage of the immunoglobulin Fc to an amine group or a thiol group present at an amino acid residue other than the N-terminus of the insulinotropic peptide maintains higher titers than a conjugate prepared by linkage at the N-terminus of the insulinotropic peptide. Consequently, it was confirmed that the conjugate shows excellent therapeutic effects on non-alcoholic fatty liver disease even though it is less frequently administered than the known exendin-4 formulations, thereby completing the present invention.

[18]

Solution to Problem

[19] An object of the present invention is to provide a long-acting insulinotropic peptide

conjugate which maintains a prolonged in-vivo half-life and effectively prevents triglyceride accumulation and thus is useful for the prevention or treatment of non-alcoholic fatty liver disease.

[20]

Advantageous Effects of Invention

[21] The insulinotropic peptide conjugate, according to the present invention, maintains in-vivo activity of the peptide at a relatively high level, has a remarkably increased blood half-life, and effectively activates major proteins involved in lipolysis to prevent triglyceride accumulation, thereby being useful for the prevention and treatment of non-alcoholic fatty liver disease.

[22]

Brief Description of Drawings

[23] FIG. 1 shows images of the liver tissue of ob/ob mouse which was administered with the long-acting exendin-4 conjugate according to one embodiment of the present invention (Hematoxylin & Eosin staining, H&E staining, area stained in purple: normal liver tissue, area stained in white: lipid droplet); and

[24] FIG. 2 shows a graph of intrahepatic triglyceride accumulation in high fat induced-obese mice which were administered with the long-acting exendin-4 conjugate according to one embodiment of the present invention (#: a significant increase at 99% confidence, compared to a normal diet group ($p<0.01$), *: a significant decrease at 99% confidence, compared to a high fat diet group ($p<0.01$)).

[25]

Best Mode for Carrying out the Invention

[26] In one aspect, to achieve the above objects, one embodiment relates to a pharmaceutical composition for the prevention or treatment of non-alcoholic fatty liver disease including an insulinotropic peptide drug conjugate, which is prepared by covalently linking an insulinotropic peptide and an immunoglobulin Fc region via a non-peptidyl polymer, as an active ingredient.

[27] In the pharmaceutical composition of the present invention, the insulinotropic peptide is selected from the group consisting of exendin-4, an exendin-4 derivative prepared by deleting the N-terminal amine group of exendin-4, an exendin-4 derivative prepared by substituting the N-terminal amine group of exendin-4 with a hydroxyl group, an exendin-4 derivative prepared by modifying the N-terminal amine group of exendin-4 with a dimethyl group, and an exendin-4 derivative prepared by deleting alpha-carbon of the N-terminal histidine residue of exendin-4 and the N-terminal amine group linked to the alpha-carbon.

[28] The non-peptidyl polymer is selected from the group consisting of polyethylene

glycol, polypropylene glycol, copolymers of ethylene glycol-propylene glycol, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, dextran, polyvinyl ethyl ether, biodegradable polymers, lipid polymers, chitins, hyaluronic acid, and combinations thereof.

[29] The insulinotropic peptide of the present invention is a peptide possessing an insulinotropic function to promote the synthesis and the expression of insulin in a pancreatic beta cell. These peptides include precursors, derivatives, fragments, variants or the like, and preferably GLP (glucagon like peptide)-1, exendin-3, exendin-4 or the like.

[30] GLP-1 is a hormone that is secreted by the small intestine. In general, it promotes the biosynthesis and secretion of insulin, inhibits the secretion of glucagon, and promotes glucose absorption in the cells. In the small intestine, a glucagon precursor is decomposed into three peptides, that is, glucagon, GLP-1, and GLP-2. Here, the GLP-1 means GLP-1 (1-37), which is originally in the form having no insulinotropic function. But it is then processed and converted into the activated GLP-1 (7-37) form. The amino acid sequence of GLP-1 (7-37) is as follows:

[31]

[32] GLP-1(7-37)(SEQ ID NO:1)

[33] HAEGT FTSDV SSYLE GQAAK EPIAW LVKGR G

[34]

[35] The GLP-1 derivative means a peptide which exhibits an amino acid sequence homology of at least 80% with that of GLP-1, may be in the chemically modified form, and exhibits an insulinotropic function of at least equivalent to or more than that of GLP-1.

[36] The GLP-1 fragment means the form in which one or more amino acids are added or deleted at the N-terminus or C-terminus of the native GLP-1, and the added amino acid is possibly a non-naturally occurring amino acid (e.g., D-type amino acid).

[37] The GLP-1 variant means a peptide possessing an insulinotropic function which has one or more amino acid sequences different from those of the native GLP-1.

[38]

[39] The exendin-3 and the exendin-4 are insulinotropic peptides consisting of 39 amino acids which have a 53% amino acid sequence homology with GLP-1. The amino acid sequences of the exendin-3 and the exendin-4 are as follows:

[40]

[41] Exendin-3 (SEQ ID NO:2)

[42] HSDGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPS

[43] Exendin-4 (SEQ ID NO:3)

[44] HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPS

[45]

[46] The exendin derivative means a peptide having at least 80% amino acid sequence homology with the native exendin, which may have some groups on the amino acid residue chemically substituted, and exhibits an insulinotropic function of at least equivalent to or more than that of the native exendin.

[47] The exendin fragment means a fragment having one or more amino acids added or deleted at the N-terminus or the C-terminus of the native exendin, and the added amino acid is possibly a non-naturally occurring amino acid (e.g., D-type amino acid).

[48] The exendin variant means a peptide possessing an insulinotropic function which has one or more amino acid sequences different from those of the native exendin.

[49] In a specific embodiment, the native insulinotropic peptide used in the present invention and the modified insulinotropic peptide may be synthesized using a solid phase synthesis method and most of the native peptides, including the native insulinotropic peptide, may be produced by a recombination technology.

[50] Further, the insulinotropic peptide used in the present invention may bind to the non-peptidyl polymer on various sites.

[51] The conjugate prepared in the present invention may have activity which varies depending on the binding sites of the insulinotropic peptide.

[52] For example, it may be coupled with the N-terminus, and other terminus including the C-terminus, respectively, which indicates difference in the in-vitro activity. The aldehyde reactive group selectively binds to the N-terminus at a low pH and may bind to a lysine residue to form a covalent bond at a high pH, for example, pH 9.0. A pegylation reaction is allowed to proceed with varying pH and an ion exchange column may then be used to separate a positional isomer from the reaction mixture.

[53] If the insulinotropic peptide is to be coupled at a site other than the N-terminus, which is an important site for the in-vivo activity, a reactive thiol group can be introduced to the site of amino acid residue to be modified in the native amino acid sequence so as to form a covalent bond using a maleimide linker at the non-peptidyl polymer.

[54] If the insulinotropic peptide is to be coupled at a site other than the N-terminus, which is an important site for the in-vivo activity, a reactive amine group can be introduced to the site of amino acid residue to be modified in the native amino acid sequence so as to form a covalent bond using an aldehyde linker at the non-peptidyl polymer.

[55] When the aldehyde linker at the non-peptidyl polymer is used, it is reacted with an amine group at the N-terminus and the lysine residue, and a modified form of the insulinotropic peptide may be used to selectively increase the reaction yield. For example, only one amine group to be reacted may be retained on a desired site, using

an N-terminus blocking method, a lysine residue substituting method, a method for introducing an amine group at a carboxyl terminus, or the like, thereby increasing the yield of pegylation and coupling reactions. The methods for protecting the N-terminus include dimethylation, as well as methylation, deamination, acetylation, etc., but are not limited to such alkylation methods.

[56] In one preferred embodiment, the insulinotropic peptide conjugate of the present invention is an insulinotropic peptide conjugate in which an immunoglobulin Fc region specifically binds to an amine group other than ones at the N-terminus of the insulinotropic peptide.

[57] In one specific embodiment, the present inventors induced a pegylation of a native exendin-4 at pH 9.0 to selectively couple the PEG to the lysine residue of the insulinotropic peptide. Alternatively, the exendin-4 derivatives having the N-terminus deleted or protected may be synthesized to be coupled. The pegylation at the N-terminus can be blocked either by deleting the alpha amine group of the N-terminal histidine or by modifying the N-terminal histidine with two methyl groups. Such N-terminal modification does not influence in-vitro activity (Table 1).

[58] Unlike the N-terminal coupling of exendin-4, coupling at the lysine residue maintained the in-vitro activity at approximately 6% (Table 1). Further, the exendin-4-PEG-immunoglobulin Fc conjugate prepared in the present invention exhibited a remarkably increased blood half-life of 60~70 hours, indicating an unexpectedly high duration of efficacy. Therefore, the titer reduction was also minimized by coupling to the lysine residue which does not affect the activity, and thus a new long-acting exendin-4 formulation capable of maintaining its in-vivo activity could be prepared.

[59] The immunoglobulin Fc region is safe for use as a drug carrier because it is a biodegradable polypeptide that is metabolized in vivo. Also, the immunoglobulin Fc region has a relatively low molecular weight as compared to the whole immunoglobulin molecules and thus it is advantageous in the preparation, purification, and yield of the conjugate. Since the immunoglobulin Fc region does not contain a Fab fragment whose amino acid sequence differs according to the antibody subclasses and which thus is highly non-homogenous, it can be expected that the immunoglobulin Fc region may greatly increase the homogeneity of substances and be less antigenic.

[60] The term "immunoglobulin Fc region" as used herein refers to the heavy-chain constant region 2 (C_H2) and the heavy-chain constant region 3 (C_H3) and excludes the variable regions of the heavy and light chains, the heavy-chain constant region 1 (C_H1), and the light-chain constant region 1 (C_L1) of the immunoglobulin. It may further include a hinge region at the heavy-chain constant region. Also, the immunoglobulin Fc region of the present invention may contain a part or all of the Fc region including the heavy-chain constant region 1 (C_H1) and/or the light-chain constant region 1 (C_L1),

except for the variable regions of the heavy and light chains, as long as it has effects substantially similar to or better than the native protein. Also, the immunoglobulin Fc region may be a fragment having a deletion in a relatively long portion of the amino acid sequence of C_H2 and/or C_H3. That is, the immunoglobulin Fc region of the present invention may include 1) a C_H1 domain, a C_H2 domain, a C_H3 domain and a C_H4 domain, 2) a C_H1 domain and a C_H2 domain, 3) a C_H1 domain and a C_H3 domain, 4) a C_H2 domain and a C_H3 domain, 5) a combination of one or more domains and an immunoglobulin hinge region (or a portion of the hinge region), and 6) a dimer of each domain of the heavy-chain constant regions and the light-chain constant region.

[61] The immunoglobulin Fc region of the present invention includes a native amino acid sequence and a sequence derivative (mutant) thereof. An amino acid sequence derivative is a sequence that is different from the native amino acid sequence due to a deletion, an insertion, a non-conservative or conservative substitution, or combinations thereof of one or more amino acid residues. For example, in an IgG Fc, amino acid residues known to be important in binding at positions 214 to 238, 297 to 299, 318 to 322, or 327 to 331 may be used as a suitable target for modification. Also, other various derivatives are possible, including one in which a region capable of forming a disulfide bond is deleted or certain amino acid residues are eliminated at the N-terminus of a native Fc form, or a methionine residue is added thereto. Further, to remove effector functions, a deletion may occur in a complement-binding site, such as a C1q-binding site and an ADCC site. Techniques of preparing such sequence derivatives of the immunoglobulin Fc region are disclosed in International Patent Publication Nos. WO 97/34631 and WO 96/32478.

[62] Amino acid exchanges in proteins and peptides, which do not generally alter the activity of molecules, are known in the art (H.Neurath, R.L.Hill, The Proteins, Academic Press, New York, 197 9). The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Thy/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly in both directions.

[63] The Fc region, if desired, may be modified by phosphorylation, sulfation, acrylation, glycosylation, methylation, farnesylation, acetylation, amidation, and the like.

[64] The aforementioned Fc derivatives are derivatives that have a biological activity identical to the Fc region of the present invention or improved structural stability against heat, pH, or the like.

[65] In addition, these Fc regions may be obtained from native forms isolated from humans and other animals including cows, goats, swine, mice, rabbits, hamsters, rats and guinea pigs, or may be recombinants or derivatives thereof, obtained from transformed animal cells or microorganisms. Herein, they may be obtained from a native immunoglobulin by isolating whole immunoglobulins from human or animal

organisms and treating them with a proteolytic enzyme. Papain digests the native immunoglobulin into Fab and Fc regions, and pepsin treatment results in the production of pF'c and F(ab)2 fragments. These fragments may be subjected to size exclusion chromatography to isolate Fc or pF'c.

[66] Preferably, a human-derived Fc region is a recombinant immunoglobulin Fc region that is obtained from a microorganism.

[67] In addition, the immunoglobulin Fc region may be in the form of having native sugar chains, increased sugar chains compared to a native form, or decreased sugar chains compared to the native form, or may be in a deglycosylated form. The increase, decrease or removal of the immunoglobulin Fc sugar chains may be achieved by methods common in the art, such as a chemical method, an enzymatic method and a genetic engineering method using a microorganism. The removal of sugar chains from an Fc region results in a sharp decrease in binding affinity to the complement (c1q) and a decrease or loss in antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity, thereby not inducing unnecessary immune responses in-vivo. In this regard, an immunoglobulin Fc region in a deglycosylated or aglycosylated form may be more suitable to the object of the present invention as a drug carrier.

[68] As used herein, the term "deglycosylation" refers to enzymatically removed sugar moieties from an Fc region and the term "aglycosylation" means that an Fc region is produced in an unglycosylated form by a prokaryote, preferably E. coli.

[69] While the immunoglobulin Fc region may preferably be derived from humans it may also be derived from other animals including cows, goats, swine, mice, rabbits, hamsters, rats and guinea pigs. In addition, the immunoglobulin Fc region may be an Fc region that is derived from IgG, IgA, IgD, IgE and IgM, or that is made by combinations thereof or hybrids thereof. Preferably, it is derived from IgG or IgM, which is among the most abundant proteins in human blood, and most preferably derived from IgG, which is known to enhance the half-lives of ligand-binding proteins.

[70] On the other hand, the term "combination", as used herein, means that polypeptides encoding single-chain immunoglobulin Fc regions of the same origin are linked to a single-chain polypeptide of a different origin to form a dimer or multimer. That is, a dimer or multimer may be formed from two or more fragments selected from the group consisting of IgG Fc, IgA Fc, IgM Fc, IgD Fc, and IgE Fc fragments.

[71] The term "hybrid", as used herein, means that sequences encoding two or more immunoglobulin Fc regions of different origin are present in a single-chain immunoglobulin Fc region. In the present invention, various types of hybrids are possible. That is, domain hybrids may be composed of one to four domains selected from the group consisting of CH1, CH2, CH3 and CH4 of IgG Fc, IgM Fc, IgA Fc, IgE Fc and IgD Fc, and may include the hinge region.

[72] On the other hand, IgG may be divided into IgG1, IgG2, IgG3 and IgG4 subclasses, and the present invention may include combinations and hybrids thereof. Preferred are IgG2 and IgG4 subclasses, and most preferred is the Fc region of IgG4 rarely having effector functions such as CDC (complement dependent cytotoxicity).

[73] That is, as the drug carrier of the present invention, the most preferable immunoglobulin Fc region is a human IgG4-derived non-glycosylated Fc region. The human-derived Fc region is more preferable than a non-human derived Fc region which may act as an antigen in the human body and cause undesirable immune responses such as the production of a new antibody against the antigen.

[74] The term "non-peptidyl polymer", as used herein, refers to a biocompatible polymer including two or more repeating units linked to each other by any covalent bond excluding a peptide bond.

[75] The non-peptidyl polymer which can be used in the present invention may be selected from the group consisting of polyethylene glycol, polypropylene glycol, copolymers of ethylene glycol and propylene glycol, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, dextran, polyvinyl ethyl ether, biodegradable polymers such as PLA (polylactic acid) and PLGA (polylactic-glycolic acid), lipid polymers, chitins, hyaluronic acid, and combinations thereof, the preferred of which is polyethylene glycol. Also, derivatives thereof well known in the art and being easily prepared within the skill of the art are included in the scope of the present invention.

[76] The peptide linker which is used in the fusion protein obtained by a conventional in-frame fusion method has drawbacks in that it is easily in-vivo cleaved by a proteolytic enzyme and thus a sufficient effect of increasing the blood half-life of the active drug by a carrier cannot be obtained as expected. However, in the present invention, a polymer having resistance to the proteolytic enzyme can be used to maintain the blood half-life of the peptide to be similar to that of the carrier. Therefore, any non-peptidyl polymer to be used in the present invention can be used without any limitation as long as it is a polymer having the aforementioned function, that is, a polymer having resistance to the in-vivo proteolytic enzyme. The non-peptidyl polymer preferably has a molecular weight in the range of 1 to 100 kDa, and preferably of 1 to 20 kDa. Also, the non-peptidyl polymer of the present invention, linked to the immunoglobulin Fc region, may be one polymer or a combination of different types of polymers.

[77] The non-peptidyl polymer used in the present invention has a reactive group capable of binding to the immunoglobulin Fc region and the protein drug.

[78] The non-peptidyl polymer has a reactive group at both ends which is preferably selected from the group consisting of a reactive aldehyde group, a propionaldehyde group, a butyraldehyde group, a maleimide group and a succinimide derivative. The succinimide derivative may be succinimidyl propionate, hydroxy succinimidyl, suc-

cinimidyl carboxymethyl, or succinimidyl carbonate. In particular, when the non-peptidyl polymer has a reactive aldehyde group at both ends, it is effective in linking at both ends with a physiologically active polypeptide and an immunoglobulin with minimal non-specific reactions. A final product generated by reductive alkylation via an aldehyde bond is much more stable than when linked by an amide bond. The aldehyde reactive group selectively binds to the N-terminus at a low pH and can bind to a lysine residue to form a covalent bond at a high pH, for example, at pH 9.0.

[79] The reactive groups at both ends of the non-peptidyl polymer may be the same or different. For example, the non-peptide polymer may possess a maleimide group at one end and at the other end it may possess an aldehyde group, a propionaldehyde group or a butyraldehyde group. When a polyethylene glycol having a reactive hydroxy group at both ends thereof is used as the non-peptidyl polymer, the hydroxy group may be activated to various reactive groups by known chemical reactions, or a polyethylene glycol having a commercially available modified reactive group may be used so as to prepare the insulinotropic peptide conjugate of the present invention.

[80] In another embodiment, the present invention provides a method for preparing an insulinotropic peptide conjugate including the steps of:

[81] (1) covalently linking a non-peptidyl polymer having a reactive group of aldehyde, maleimide, or succinimide derivative at both ends thereof, with an amine group or thiol group of an insulinotropic peptide;

[82] (2) isolating a conjugate including the insulinotropic peptide from the reaction mixture of (1), in which the non-peptidyl polymer is covalently linked to a site other than the amino terminus; and

[83] (3) covalently linking an immunoglobulin Fc region to the other end of the non-peptidyl polymer of the isolated conjugate so as to produce a peptide conjugate having the immunoglobulin Fc region and the insulinotropic peptide, which are linked to each end of the non-peptidyl polymer.

[84] The term "conjugate", as used herein, refers to an intermediate prepared by covalently linking the non-peptidyl polymer with the insulinotropic peptide and subsequently the immunoglobulin Fc region is linked to the other end of the non-peptidyl polymer in the conjugate.

[85] In one preferred embodiment, the present invention provides a preparation method including the steps of:

[86] (1) covalently linking a non-peptidyl polymer having an aldehyde reactive group at both ends thereof with the lysine residue of exendin-4;

[87] (2) isolating a conjugate including exendin-4 from the reaction mixture of (1), in which the non-peptidyl polymer is covalently linked to the lysine residue; and

[88] (3) covalently linking an immunoglobulin Fc region to the other end of the non-

peptidyl polymer of the isolated conjugate so as to produce a protein conjugate including the immunoglobulin Fc region and exendin-4, which are linked to each end of the non-peptidyl polymer. More preferably, the non-peptidyl polymer and the lysine residue of exendin-4 in (1) are linked at pH 9.0 or higher.

[89] The insulinotropic peptide conjugate of the present invention activates major proteins of the insulin signaling pathway via the GLP-1 receptor, and thus can be used for the prevention or treatment of non-alcoholic fatty liver disease. In particular, the insulinotropic peptide conjugate of the present invention increases the activity of PKC-ζ (Protein Kinase C-ζ) which regulates enzymatic activity involved in lipolysis and maintains in-vivo activity of the known insulinotropic peptide which increases Glut2 (Glucose transporter protein-2) expression, and increases the blood half-life of insulinotropic peptide, thereby remarkably increasing duration of in-vivo efficacy. Accordingly, excellent therapeutic effects on non-alcoholic fatty liver disease can be obtained with less administration frequency than the known formulations.

[90] In the present invention, non-alcoholic fatty liver disease (NAFLD) includes primary and secondary non-alcoholic fatty liver diseases, and more specifically means non-alcoholic fatty liver disease caused by primary hyperlipidemia, diabetes, or obesity. For example, non-alcoholic fatty liver disease includes simple steatosis, fatty liver diseases caused by malnutrition, starvation, obesity and diabetes, steatohepatitis, and liver fibrosis and liver cirrhosis occurring due to the progression of these diseases.

[91] The pharmaceutical composition including the insulinotropic peptide conjugate of the present invention may further include a pharmaceutically acceptable carrier. For oral administration, the pharmaceutically acceptable carrier may include a binder, a lubricant, a disintegrator, an excipient, a solubilizer, a dispersing agent, a stabilizer, a suspending agent, a coloring agent, and a perfume. For injectable preparations, the pharmaceutically acceptable carrier may include a buffering agent, a preserving agent, an analgesic, a solubilizer, an isotonic agent, and a stabilizer. For preparations for topical administration, the pharmaceutically acceptable carrier may include a base, an excipient, a lubricant, and a preserving agent. The pharmaceutical composition of the present invention may be formulated into a variety of dosage forms in combination with the aforementioned pharmaceutically acceptable carriers. For example, for oral administration, the pharmaceutical composition may be formulated into tablets, troches, capsules, elixirs, suspensions, syrups or wafers. For injectable preparations, the pharmaceutical composition may be formulated into an ampule as a single-dose dosage form or a unit dosage form, such as a multidose container. The pharmaceutical composition may be also formulated into solutions, suspensions, tablets, pills, capsules and long-acting preparations.

[92] On the other hand, examples of the carrier, the excipient, and the diluent suitable for

the pharmaceutical formulations include lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starch, acacia, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, talc, magnesium stearate and mineral oils. In addition, the pharmaceutical formulations may further include fillers, anti-coagulating agents, lubricants, humectants, perfumes, and antiseptics.

[93] The conjugate according to the present invention is useful to prevent or treat non-alcoholic fatty liver disease. Accordingly, a pharmaceutical composition including the conjugate may be administered for the treatment of the disease.

[94] The term "administration", as used herein, means introduction of a predetermined substance into a patient by a certain suitable method. The conjugate of the present invention may be administered via any of the common routes as long as it is able to reach a desired tissue. A variety of modes of administration are contemplated, including intraperitoneally, intravenously, intramuscularly, subcutaneously, intradermally, orally, topically, intranasally, intrapulmonarily and intrarectally, but the present invention is not limited to these exemplified modes of administration. However, since peptides are digested upon oral administration, active ingredients of a composition for oral administration should be coated or formulated for protection against degradation in the stomach. Preferably, the present composition may be administered in an injectable form. In addition, the pharmaceutical composition may be administered using a certain apparatus capable of transporting the active ingredients into a target cell.

[95] The pharmaceutical composition of the present invention can be determined by several related factors including the types of diseases to be treated, administration routes, the patient's age, gender, weight and severity of the illness, as well as by the types of the drug as an active component. Since the pharmaceutical composition of the present invention has excellent duration of in-vivo efficacy and titer, it can remarkably reduce the administration frequency and dose of pharmaceutical drugs of the present invention.

[96] Further, the pharmaceutical composition of the present invention may be used singly or in combination with surgical operation, hormone therapy, drug therapy and biological response regulators in order to prevent and treat non-alcoholic fatty liver disease.

[97]

[98] In one aspect of the present invention relates to a use of the pharmaceutical composition in the preparation of drugs for the prevention or treatment of non-alcoholic liver disease.

[99]

Mode for the Invention

[100] Hereinafter, the present invention will be described in more detail with reference to the following Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

[101]

[102] **Example 1. Test of in-vitro activity of long-acting exendin-4**

[103] A variety of long-acting exendin-4 derivatives used in this experiment were prepared in the same manner as in Korean Patent No. 10-1058315 of the present inventors.

[104] A method for measuring the in-vitro cell activity was used so as to measure the efficacy of long acting preparation of exendin-4. In the in-vitro activity measurement, RIN-m5F was used, which is known as a rat insulinoma cell. Because this cell has a GLP-1 receptor, it is commonly used in the methods for measuring the in-vitro activity of the GLP-1 family. RIN-m5F was treated with GLP-1, exendin-4, and test materials at varying concentrations. EC50 values were determined by measuring the occurrence of cAMP's, which are signaling molecules in the cells, caused by the test materials, and compared to each other. The results are summarized in Table 1.

[105]

[106] Table 1

[Table 1]

Test material	Blood half-life (hr)	In-vitro titer (%)
Exendin-4	0.7	100
Exendin-4(N)-PEG-Fc	61.5	< 0.2
Exendin-4(Lys27)-PEG-Fc	70.5	6.3

[107] Exendin-4(N)-PEG-Fc: conjugate prepared by linking the N-terminus of exendin-4 and Fc region via PEG.

[108] Exendin-4(Lys27)-PEG-Fc: conjugate prepared by linking the 27th lysine residue of exendin-4 and Fc region via PEG.

[109]

[110] As shown in Table 1, when the non-peptidyl polymer was linked to the lysine residue other than the N-terminus of the native exendin-4, the in-vitro titer was maintained at 6.3%, and the blood half-life was remarkably increased to approximately 70 hours.

[111]

[112] **Example 2. Effects on fatty liver formation in obese animal model ob/ob mouse**

[113] **<2-1> Division of experimental animals**

[114] Female 5-week-old ob/ob mice (C57BL/6JHamSlc-ob/ob, 24-34 g) were purchased from Slc, Japan. The ob/ob mouse is an animal model commonly used in the efficacy

tests of anti-obesity and anti-diabetic formulations. They were freely fed with solid feed for experimental animals, which was sterilized by radiation (manufacturer: Picolab Rodent Diet, product name: 5053), and had free access to filtered, UV ir-radiation-sterilized tap water in a water bottle. They were maintained in a casing system meeting the GLP Standard requirements on a 12 hr dark-light cycle (light switched on at 6:00 am and off at 6:00 pm) in accordance with animal care standard guidelines. Thereafter, healthy ob/ob mice were selected and acclimated to the laboratory conditions for 1 week. Then, drug administration was performed, and mice were divided into 4 groups and administered as follows.

[115] Group 1 (negative control): subcutaneous injection of DULBECCO'S PHOSPHATE BUFFERED SALINE (Sigma) once or more a week at an administration volume of 5 ml/kg

[116] Group 2 (positive control): subcutaneous injection of 10.8 nmol/kg of BYETTA every day at an administration volume of 5 ml/kg

[117] Group 3 (3.7 nmol/kg of long-acting exendin-4 derivative-treated group): subcutaneous injection of 3.7 nmol/kg of long-acting exendin-4 derivative (HM11260C) once a week at an administration volume of 5 ml/kg

[118] Group 4 (8.2 nmol/kg of long-acting exendin-4 derivative-treated group): subcutaneous injection of 8.2 nmol/kg of long-acting exendin-4 derivative (HM11260C) once a week at an administration volume of 5 ml/kg

[119]

[120] BYETTA (Eli Lilly) is the native exendin-4, and the long-acting exendin-4 derivative (HM11260C) is a CA exendin-4-PEG-Fc conjugate prepared by linking imidazoacetyl-exendin-4 with removal of alpha carbon of the first amino acid histidine to Fc region via PEG, described in Korean Patent No. 10-1058315.

[121]

[122] Each group was administered with a saline solution or drugs for 7 weeks, and their effects on fatty liver formation were analyzed.

[123]

[124] **<2-2> Effects of long-acting exendin-4 derivative on fatty liver formation**

[125] In order to examine the effects of the long-acting exendin-4 derivatives according to the present invention on fatty liver formation in ob/ob mouse, the following experiment was performed. Drugs were administered into the groups divided in Example <2-1>, and the livers were taken from the ob/ob mice, and a part thereof was fixed in 4% formaldehyde and embedded in paraffin, followed by H&E staining. The results are shown in FIG. 1.

[126] As shown in FIG. 1, pathological features of fatty liver were clearly observed in the negative control group treated with a vehicle, whereas a remarkable dose-dependent

reduction in pathological features of fatty liver was observed in the experimental group treated with the long-acting exendin-4 derivative of the present invention. It was also found that the long-acting exendin-4 derivative of the present invention showed excellent therapeutic effects on fatty liver even with a lower dose, compared to the positive control BYETTA.

[127]

[128] **Example 3. Effects on intrahepatic triglyceride accumulation in high fat induced-obese mice**

[129] **<3-1> Division of experimental animals**

[130] 6-week-old C57BL/6 mice were stabilized and divided into two groups, and received a normal diet containing 10% fat and a high-fat diet containing 60% fat for 12 weeks, (manufacturer: Research diets Inc., product name: D12492). Thus, normal mice and high fat induced-obese mice were prepared and used for experiments. They were maintained in a casing system meeting the GLP Standard requirements on a 12 hr dark-light cycle (light switched on at 6:00am and off at 6:00pm) in accordance with animal care standard guidelines. Thereafter, healthy high fat induced-obese mice were selected and acclimated to the laboratory conditions for 1 week. Then, drug administration was performed, and mice were divided into 4 groups and administered as follows.

[131] Group 1 (normal diet group): subcutaneous injection of DULBECCO'S PHOSPHATE BUFFERED SALINE (Sigma) once or more a week at an administration volume of 5 ml/kg

[132] Group 2 (high fat diet group): subcutaneous injection of DULBECCO'S PHOSPHATE BUFFERED SALINE (Sigma) once or more a week at an administration volume of 5 ml/kg

[133] Group 3 (high fat diet group treated with 3 nmol/kg of long-acting exendin-4 derivative): subcutaneous injection of 3 nmol/kg of long-acting exendin-4 derivative (HM11260C) once a week at an administration volume of 5 ml/kg

[134] Group 4 (high fat diet group treated with 10 nmol/kg of long-acting exendin-4 derivative): subcutaneous injection of 10 nmol/kg of long-acting exendin-4 derivative (HM11260C) once a week at an administration volume of 5 ml/kg

[135]

[136] Each group was administered with a saline solution or drugs for 2 weeks, and the amount of triglyceride accumulated in the liver tissue was analyzed.

[137]

[138] **<3-2> Measurement of intrahepatic triglyceride accumulation in high fat induced-obese mice**

[139] The livers were taken from the groups divided in Example <3-1>, which were high fat induced-obese mice administered with or without the long-acting exendin-4

derivative, and intrahepatic triglyceride concentrations were determined. As shown in FIG. 2, intrahepatic triglyceride concentration of the high fat diet group was 172.3 mg/g, which was higher than that of the low fat diet group (114.0 mg/g), but the high fat diet group treated with 3 nmol/kg of the long-acting exendin-4 derivative showed 93 mg/g of triglyceride level, showing a 46% reduction, compared to the high fat diet group. These results suggest that the long-acting exendin-4 derivative of the present invention has therapeutic effects on non-alcoholic fatty liver disease.

Claims

[Claim 1] A pharmaceutical composition for the prevention or treatment of non-alcoholic fatty liver disease comprising an insulinotropic peptide drug conjugate prepared by covalently linking an insulinotropic peptide and an immunoglobulin Fc region via a non-peptidyl polymer as an active ingredient, wherein the insulinotropic peptide is selected from the group consisting of exendin-4, an exendin-4 derivative prepared by deleting the N-terminal amine group of exendin-4, an exendin-4 derivative prepared by substituting the N-terminal amine group of exendin-4 with a hydroxyl group, an exendin-4 derivative prepared by modifying the N-terminal amine group of exendin-4 with a dimethyl group, and an exendin-4 derivative prepared by deleting alpha-carbon of the N-terminal histidine residue of exendin-4 and the N-terminal amine group linked to the alpha-carbon, and the non-peptidyl polymer is selected from the group consisting of polyethylene glycol, polypropylene glycol, copolymers of ethylene glycol-propylene glycol, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, dextran, polyvinyl ethyl ether, biodegradable polymers, lipid polymers, chitins, hyaluronic acid, and combinations thereof.

[Claim 2] The pharmaceutical composition according to claim 1, wherein the non-peptidyl polymer is linked to the amino acid residue other than the N-terminus of the insulinotropic peptide.

[Claim 3] The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc region and an amine group or a thiol group of the insulinotropic peptide are linked at both ends of the non-peptidyl polymer, respectively.

[Claim 4] The pharmaceutical composition according to claim 1, wherein the non-peptidyl polymer is linked to the lysine residue of the insulinotropic peptide.

[Claim 5] The pharmaceutical composition according to claim 1, wherein the non-peptidyl polymer is polyethylene glycol.

[Claim 6] The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc region is aglycosylated.

[Claim 7] The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc region is composed of one to four domains selected from the group consisting of CH1, CH2, CH3 and CH4 domains.

[Claim 8] The pharmaceutical composition according to claim 7, wherein the im-

munoglobulin Fc region further include a hinge region.

[Claim 9] The pharmaceutical composition according to claim 1, wherein the immunoglobulin Fc region is an Fc region that is derived from an immunoglobulin selected from the group consisting of IgG, IgA, IgD, IgE and IgM.

[Claim 10] The pharmaceutical composition according to claim 9, wherein the immunoglobulin Fc region is an IgG4 Fc region.

[Claim 11] The pharmaceutical composition according to claim 10, wherein the immunoglobulin Fc region is a human non-glycosylated IgG4 Fc region.

[Claim 12] The pharmaceutical composition according to claim 1, wherein the reactive group of the non-peptidyl polymer is selected from the group consisting of an aldehyde group, a propionaldehyde group, a butyraldehyde group, a maleimide group and a succinimide derivative.

[Claim 13] The pharmaceutical composition according to claim 12, wherein the succinimide derivative is selected from the group consisting of succinimidyl propionate, succinimidyl carboxymethyl, hydroxy succinimidyl, and succinimidyl carbonate.

[Claim 14] The pharmaceutical composition according to claim 1, wherein the non-peptidyl polymer has reactive aldehyde groups at both ends thereof.

[Claim 15] The pharmaceutical composition according to claim 1, wherein the insulinotropic peptide drug conjugate increases the activity of PKC-ζ (Protein Kinase C-ζ) regulating the enzymatic activity involved in lipolysis.

[Claim 16] The pharmaceutical composition according to claim 1, wherein the insulinotropic peptide drug conjugate increases expression of Glut2 (Glucose transporter protein-2) involved in lipolysis.

[Claim 17] The pharmaceutical composition according to claim 1, wherein the non-alcoholic fatty liver disease is selected from the group consisting of simple steatosis, fatty liver diseases caused by malnutrition, starvation, obesity and diabetes, steatohepatitis, liver fibrosis and liver cirrhosis.

[Claim 18] A method for preventing or treating non-alcoholic liver disease, comprising the step of administering to a subject the pharmaceutical composition of any one of claims 1 to 17.

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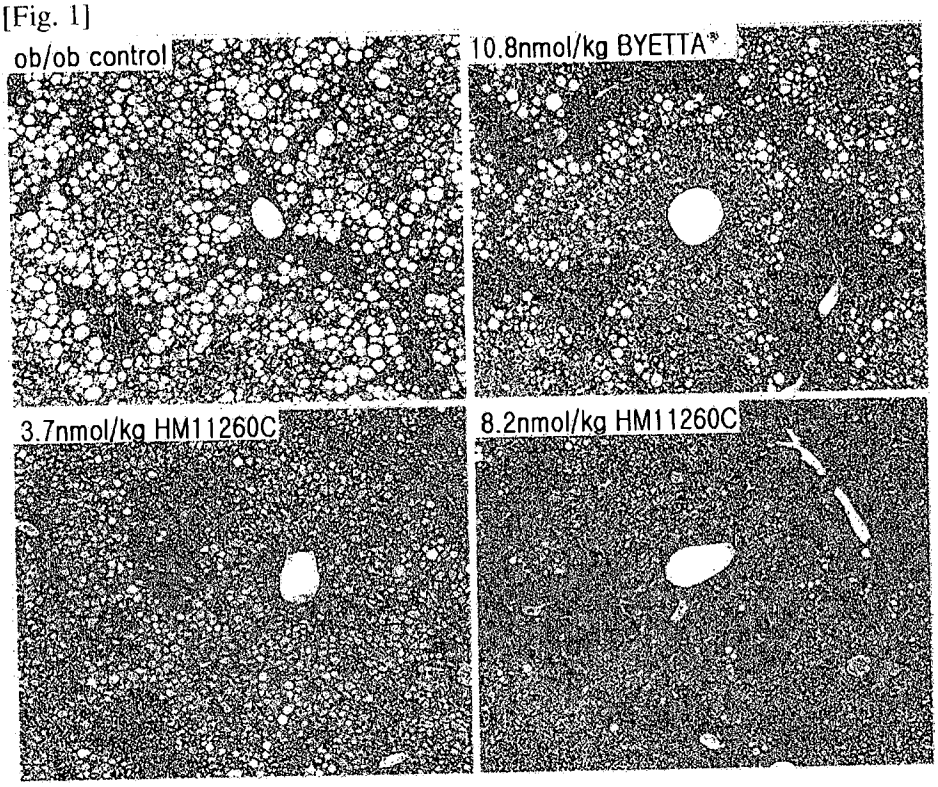
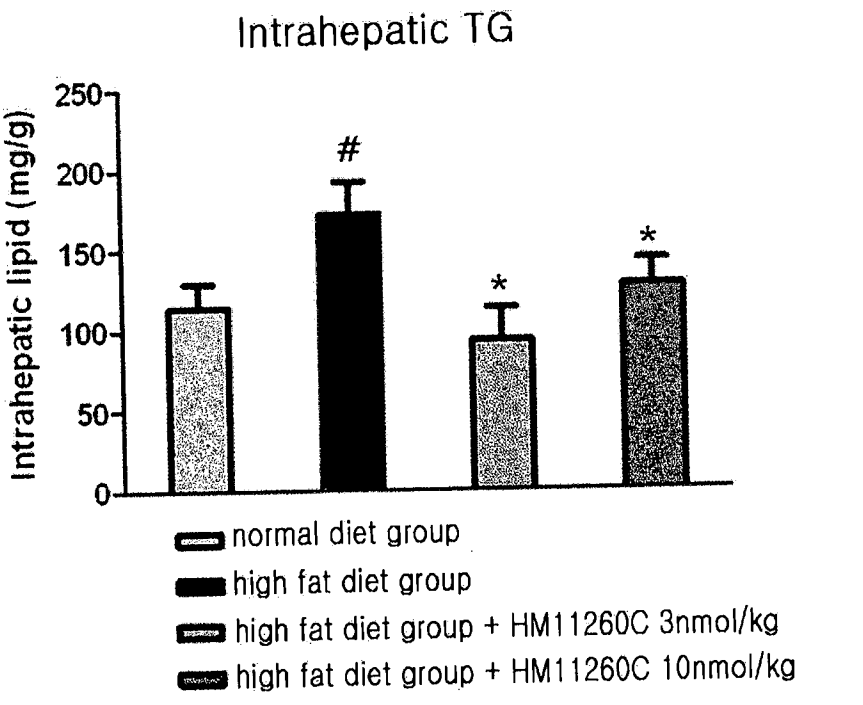
[Fig. 1]



[Fig. 2]



#; significant increase compared to normal diet group ($p<0.01$)

*; significant decrease compared to high fat diet group ($p<0.01$)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2013/001897

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/17(2006.01)i, A61K 38/16(2006.01)i, A61P 1/16(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 39/00, A61P 30/06, 3/10, 9/00, C07K 16/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: exendin-4, immunoglobulin fc, non peptidyl polymer, nonalcoholic fatty liver disease

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010-0330108 A1 (SONG, DAE HAE et al.) 30 December 2010 See paragraphs [0014],[0018]-[0019] and claims 1-4.	1,2,4,5,7-17
Y		3,6
Y	US 2010-0105877 A1 (SONG, DAE HAE et al.) 29 April 2010 See claims 1,2,5,6,14.	3
Y	US 2011-0200623 A1 (SONG, DAE HAE et al.) 18 August 2011 See claims 1-3, 11, 16-18.	6
A	WO 2012-011752 A2 (HANMI HOLDINGS CO., LTD. et al.) 26 January 2012 See claims 1,6,8-19,32-37.	1-17
A	WO 2011-109787 A1 (CONJUCHEM, LLC et al.) 09 September 2011 See paragraphs [0040],[0050],[0370].	1-17

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

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of 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
12 June 2013 (12.06.2013)	**13 June 2013 (13.06.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
KIM, Seung Beom
Telephone No. 82-42-481-3371

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2013/001897

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

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

1. ☒ Claims Nos.: 18
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 18 pertains to methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

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

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

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

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

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2013/001897

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2013/001897

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INTERNATIONAL SEARCH REPORT

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Ser Gly Ala Pro Pro Pro Ser
35

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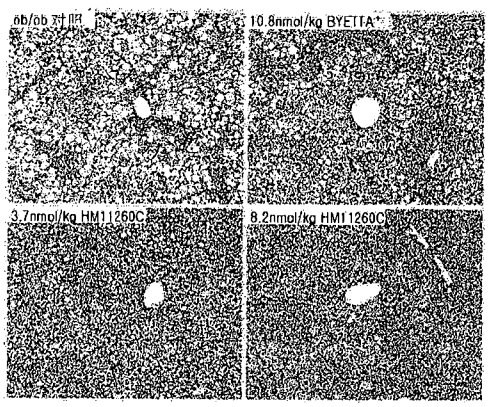
序列表2页 附图1页

(54)发明名称

用于预防或治疗非酒精性脂肪肝疾病的药学组合物

(57)摘要

本发明涉及用于预防和治疗非酒精性脂肪肝疾病(NAFLD)的药学组合物，其包括通过共价连接促胰岛素释放肽、非肽基聚合物和免疫球蛋白Fc区制备的缀合物。本发明的组合物维持肽的体内活性在相对高的水平，并且显著增加血液半衰期，从而预防是非酒精性脂肪肝疾病的典型特征的甘油三酯积聚。最后，其可期望用于预防和治疗非酒精性脂肪肝疾病。



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1. 用于预防或治疗非酒精性脂肪肝疾病的药学组合物，其包括作为活性成分的促胰岛素释放肽药物缀合物，所述缀合物通过经非肽基聚合物共价连接促胰岛素释放肽和免疫球蛋白 Fc 区制备，其中所述促胰岛素释放肽选自毒蜥外泌肽 -4、通过缺失毒蜥外泌肽 -4 的 N- 末端胺基团制备的毒蜥外泌肽 -4 衍生物、通过用羟基基团取代毒蜥外泌肽 -4 的 N- 末端胺基团制备的毒蜥外泌肽 -4 衍生物、通过用二甲基修饰毒蜥外泌肽 -4 的 N- 末端胺基团制备的毒蜥外泌肽 -4 衍生物，和通过缺失毒蜥外泌肽 -4 的 N- 末端组氨酸残基的 α - 碳和与所述 α - 碳连接的 N- 末端胺基团制备的毒蜥外泌肽 -4 衍生物，并且所述非肽基聚合物选自聚乙二醇、聚丙二醇、乙二醇 - 丙二醇的共聚物、聚氧乙烯化多元醇、聚乙烯醇、多糖、葡聚糖、聚乙烯基乙醚、生物可降解聚合物、脂质聚合物、几丁质、透明质酸和其组合。

2. 根据权利要求 1 所述的药学组合物，其中所述非肽基聚合物连接至所述促胰岛素释放肽的 N- 末端之外的氨基酸残基。

3. 根据权利要求 1 所述的药学组合物，其中所述免疫球蛋白 Fc 区和所述促胰岛素释放肽的胺基团或硫羟基团分别连接至所述非肽基聚合物的两个末端。

4. 根据权利要求 1 所述的药学组合物，其中所述非肽基聚合物连接至所述促胰岛素释放肽的赖氨酸残基。

5. 根据权利要求 1 所述的药学组合物，其中所述非肽基聚合物是聚乙二醇。

6. 根据权利要求 1 所述的药学组合物，其中所述免疫球蛋白 Fc 区是无糖基化的。

7. 根据权利要求 1 所述的药学组合物，其中所述免疫球蛋白 Fc 区由选自 CH1、CH2、CH3 和 CH4 结构域的一至四个结构域组成。

8. 根据权利要求 7 所述的药学组合物，其中所述免疫球蛋白 Fc 区进一步包括铰链区。

9. 根据权利要求 1 所述的药学组合物，其中所述免疫球蛋白 Fc 区是源自选自 IgG、IgA、IgD、IgE 和 IgM 的免疫球蛋白的 Fc 区。

10. 根据权利要求 9 所述的药学组合物，其中所述免疫球蛋白 Fc 区是 IgG4Fc 区。

11. 根据权利要求 10 所述的药学组合物，其中所述免疫球蛋白 Fc 区是人非糖基化的 IgG4Fc 区。

12. 根据权利要求 1 所述的药学组合物，其中所述非肽基聚合物的反应基团选自乙醛基、丙醛基、丁醛基、马来酰亚胺基团和琥珀酰亚胺衍生物。

13. 根据权利要求 12 所述的药学组合物，其中所述琥珀酰亚胺衍生物选自琥珀酰亚胺基丙酸、琥珀酰亚胺基羧甲基、羟基琥珀酰亚胺基、和琥珀酰亚胺基碳酸酯。

14. 根据权利要求 1 所述的药学组合物，其中所述非肽基聚合物在其两个末端具有反应性醛基。

15. 根据权利要求 1 所述的药学组合物，其中所述促胰岛素释放肽药物缀合物增加调节参与脂分解的酶活性的 PKC- ζ（蛋白质激酶 C- ζ）的活性。

16. 根据权利要求 1 所述的药学组合物，其中所述促胰岛素释放肽药物缀合物增加参与脂分解的 Glut2（葡萄糖载体蛋白质 -2）的表达。

17. 根据权利要求 1 所述的药学组合物，其中所述非酒精性脂肪肝疾病选自单纯脂肪变性；营养不良、饥饿、肥胖和糖尿病造成的脂肪肝疾病；脂肪性肝炎；肝纤维化和肝硬化。

18. 用于预防或治疗非酒精性肝疾病的方法，其包括向对象施用根据权利要求 1 至 17 任一项所述的药学组合物的步骤。

用于预防或治疗非酒精性脂肪肝疾病的药学组合物

技术领域

[0001] 本发明涉及包括长效促胰岛素释放肽缀合物的药学组合物，其可用于预防或治疗非酒精性脂肪肝疾病。尤其，本发明涉及促胰岛素释放肽缀合物，其中促胰岛素释放肽、非肽基聚合物和免疫球蛋白 Fc 区彼此共价连接，以便显著增加血液半衰期，有效防止甘油三酯积聚，并且涉及其在预防或治疗非酒精性脂肪肝疾病中的用途。

背景技术

[0002] 非酒精性脂肪肝疾病指范围从单纯脂肪变性到肝纤维化和肝硬化的宽范围疾病，所述单纯脂肪变性在没有过度摄入酒精的患者中不伴随炎症反应，所述肝纤维化和肝硬化由单纯脂肪变性的进展导致并且显示肝细胞炎症。

[0003] 非酒精性脂肪肝疾病取决于病理学原因可划分为原发性和继发性非酒精性脂肪肝疾病。原发性脂肪肝疾病由是代谢综合症特征的高血脂症、糖尿病、肥胖等造成。继发性脂肪肝疾病是营养原因（突然体重下降、饥饿、肠改道手术）、各种药物、毒性物质（有毒蘑菇、细菌毒素）、代谢原因和其他因素的结果。

[0004] 已知其中是代谢综合症重要特征的糖尿病和肥胖症是原发性因素的原发性非酒精性脂肪肝疾病的发病率为大约 50％的糖尿病患者、大约 76％的肥胖患者，并且大部分是肥胖糖尿病患者 (Gupte P 等，2004)。此外，当对具有增加水平的丙氨酸转氨酶 (ALT) 的糖尿病和肥胖患者进行肝活组织检查时，脂肪性肝炎的发病率范围为 18％至 36％ (Braillon A 等，1985)。

[0005] 目前，没有成熟的方法用于确定非酒精性脂肪肝疾病的原因。这是因为非酒精性脂肪肝疾病的发病率与各种因素比如糖尿病、肥胖、冠状动脉疾病和生活方式习惯相关。有关于抗糖尿病或肥胖药物对脂肪肝疾病的影响的一些报道。用作口服抗肥胖药物的奥尔利司他 (orlistat) 在具有脂肪性肝炎 (steatohepatitis) 的患者中展示肝的组织学改进 (Hussein 等，2007)，并且二甲双胍在未展现糖尿病的非酒精性脂肪肝疾病患者中展示肝酶血液水平的下降和肝坏死性炎症和纤维化的下降 (Bugianesi 等，2005)。此外，噻唑烷二酮 (TZD) 类药物——其是 PPAR（过氧化物酶体增殖体 - 激活的受体）显效剂——抑制在肝和肌肉中脂肪的积聚，并且对非酒精性脂肪肝疾病的动物模型中的肝展示直接抗纤维作用 (Galli A 等，2002)。

[0006] 同时，类胰高血糖素肽 -1(GLP-1) 是身体中存在的内源肽并且是响应肠中养分或血糖水平的刺激从肠道 L 细胞分泌的激素。GLP-1 具有各种生理学活性，包括通过刺激胰岛素分泌、胰腺 β 细胞增殖、抑制上胃肠道运动和抑制食欲来调节血糖水平。最近，GLP-1 受体表达出现在肝细胞中，并且 GLP-1 经肝细胞的 GLP-1 受体通过活化是胰岛素信号传导途径中的主要蛋白质的磷酸肌醇 - 依赖性激酶 -1(PDK-1) 和蛋白质激酶 C-(PKC-)，对治疗非酒精性脂肪肝疾病显示出好的效果 (GuptaNA 等，2010)。GLP-1 也用于通过活化分子伴侣蛋白介导的自噬 (chaperone-mediated autophagy)(CMA) 和巨自噬二者减少脂肪酸积聚或防止肝细胞由内质网应力造成的死亡 (Sharma S 等，2011)。最近研究报道 GLP-1 促进肝脂质

氧化，以防止肝脂肪积聚和促进胰岛素作用（Svegliati-Baroni G等，2011）。这些许多报道提出GLP-1衍生物可能是用于开发非酒精性脂肪肝疾病的预防剂和治疗剂的重要候选物。

[0007] 但是，使用GLP-1作为非酒精性脂肪肝的治疗剂的主要障碍是其短的血液半衰期（最大半衰期：2分钟）。这归因于经由身体中第8位氨基酸（Ala）和第9位氨基酸（Asp）之间被二肽基肽酶IV（dipeptidylpepdidase IV）（DPP IV）切割，GLP-1滴度的丢失。所以，已经对具有DPP IV抗性的GLP-1类似物进行了各种研究，并且已经进行了试验用于用Gly取代Ala^8（Deacon等，1998；Burcelin等，1999）取代Ala^8，或用Leu或D-Ala取代Ala^8（Xiao等，2001），从而增加对DPP IV的抗性，同时保持活性。GLP-1的N-末端氨基酸His^7对于GLP-1活性是至关重要的并且用作DPP IV的靶标。因此，美国专利号5,545,618描述N-末端被烷基或酰基修饰，并且Gallwitz等描述第7位His进行N-甲基化，或α-甲基化，或整个His用咪唑取代，以增加对DPP IV的抗性并且维持生理学活性。

[0008] 除了这些修饰，毒蜥外泌肽（exendin）-4——其是从希拉毒蜥（gila monster）的唾液腺纯化的GLP-1类似物（美国专利号5,424,686）——具有对DPP IV的抗性和比GLP-1更高的生理学活性。结果，其体内半衰期是2至4个小时，是比GLP-1更长的时间周期。但是，利用仅仅用于增加对DPP IV抗性的方法，不足以维持生理学活性，并且例如，在商业上可获得的毒蜥外泌肽-4（艾塞那肽（exenatide））的情况下，其需要每天两次注入患者。该频率对于患者仍是困难的。为改善该问题制备的肽是抗DPP IV的毒蜥外泌肽-4，其具有2至4个小时的血液半衰期。尽管其血液半衰期比GLP-1的更长，但是其也需要每天注入。

发明内容

[0009] 技术问题

[0010] 因此，本发明人使用通过共价键位点特异性连接免疫球蛋白Fc区、非肽基聚合物和促胰岛素释放肽的方法，以便最大化增加促胰岛素释放肽的血液半衰期并且保持体内活性的作用。结果，本发明人发现，该方法显著增加肽缀合物的血液半衰期，并且提供比已知的框内融合方法长得多的血液半衰期。本发明人也发现，通过位点特异性连接免疫球蛋白Fc至促胰岛素释放肽的氨基酸残基处而不是N-末端处出现的氨基或硫羟基团制备的缀合物比通过在促胰岛素释放肽的N-末端处连接制备的缀合物保持更高的滴度。因此，确认缀合物对非酒精性脂肪肝疾病显示卓越的疗效，即使其比已知的毒蜥外泌肽-4剂型较不频繁地施用，从而完成本发明。

[0011] 技术方案

[0012] 本发明的目的是提供长效促胰岛素释放肽缀合物，其维持延长的体内半衰期和有效防止甘油三酯积聚，并且因此用于预防或治疗非酒精性脂肪肝疾病。

[0013] 有益效果

[0014] 根据本发明的促胰岛素释放肽缀合物维持肽的体内活性在相对高的水平，具有显著增加的血液半衰期，和有效活化参与脂分解的主要蛋白质，以防止甘油三酯积聚，从而用于预防和治疗非酒精性脂肪肝疾病。

附图说明

[0015] 图1显示施用根据本发明一种实施方式的长效毒蜥外泌肽-4缀合物的ob/ob小

鼠的肝组织的图像（苏木精 & 伊红染色，H&E 染色，紫色染色的区域：正常肝组织，白色染色的区域：脂质滴）；和

[0016] 图 2 显示施用根据本发明一种实施方式的长效毒蜥外泌肽 -4 缀合物的高脂肪诱导的 - 肥大小鼠中的肝内甘油三酯积聚的图（#：与正常饮食组相比，在 99％置信度显著增加（$p<0.01$），*：与高脂肪饮食组相比，在 99％置信度显著下降（$p<0.01$））。

具体实施方式

[0017] 在一个方面中，为实现上述目标，一种实施方式涉及用于预防或治疗非酒精性脂肪肝疾病的药学组合物，其包括作为活性成分的促胰岛素释放肽药物缀合物，其通过经非肽基聚合物共价连接促胰岛素释放肽和免疫球蛋白 Fc 区制备。

[0018] 在本发明的药学组合物中，促胰岛素释放肽选自毒蜥外泌肽 -4、通过缺失毒蜥外泌肽 -4 的 N- 末端氨基制备的毒蜥外泌肽 -4 衍生物、通过用羟基基团取代毒蜥外泌肽 -4 的 N- 末端氨基制备的毒蜥外泌肽 -4 衍生物、通过用二甲基修饰毒蜥外泌肽 -4 的 N- 末端氨基制备的毒蜥外泌肽 -4 衍生物，和通过缺失毒蜥外泌肽 -4 的 N- 末端组氨酸残基的 α - 碳和与 α - 碳连接的 N- 末端氨基制备的毒蜥外泌肽 -4 衍生物。

[0019] 非肽基聚合物选自聚乙二醇、聚丙二醇、乙二醇 - 丙二醇的共聚物、聚氧乙烯化多元醇、聚乙烯醇、多糖、葡聚糖、聚乙烯基乙醚、生物可降解聚合物、脂质聚合物、几丁质、透明质酸和其组合。

[0020] 本发明的促胰岛素释放肽是具有促胰岛素释放功能以促进胰腺 β 细胞中胰岛素的合成和表达的肽。这些肽包括前体、衍生物、片段、变体或类似物，并且优选 GLP（类胰高血糖素肽）-1、毒蜥外泌肽 -3、毒蜥外泌肽 -4 或类似物。

[0021] GLP-1 是由小肠分泌的激素。一般而言，其促进胰岛素的生物合成和分泌、抑制胰高血糖素的分泌，并且促进细胞中的葡萄糖吸收。在小肠中，胰高血糖素前体分解成三种肽，即，胰高血糖素、GLP-1 和 GLP-2。这里，GLP-1 意思是 GLP-1(1-37)，其起初是以没有促胰岛素释放功能的形式。但是其然后被处理并且转化成活化的 GLP-1(7-37) 形式。GLP-1(7-37) 的氨基酸序列如下：

[0022] GLP-1(7-37)(SEQ ID NO:1)

[0023] HAEGT FTSDV SSYLE GQAAK EPIAW LVKGR G

[0024] GLP-1 衍生物意思是展示与 GLP-1 至少 80％氨基酸序列同源性的肽，其可以是化学修饰的形式，并且展示至少等于或大于 GLP-1 的促胰岛素释放功能。

[0025] GLP-1 片段意思是其中在天然 GLP-1 的 N- 末端或 C- 末端添加或缺失一个或多个氨基酸并且添加的氨基酸可能是非天然存在的氨基酸（例如，D- 型氨基酸）的形式。

[0026] GLP-1 变体意思是具备促胰岛素释放功能的肽，其具有不同于天然 GLP-1 的一个或多个氨基酸序列。

[0027] 毒蜥外泌肽 -3 和毒蜥外泌肽 -4 是由与 GLP-1 具有 53％氨基酸序列同源性的 39 个氨基酸组成的促胰岛素释放肽。毒蜥外泌肽 -3 和毒蜥外泌肽 -4 的氨基酸序列如下：

[0028] 毒蜥外泌肽 -3(SEQ ID NO:2)

[0029] HSDGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPS

[0030] 毒蜥外泌肽 -4(SEQ ID NO:3)

[0031] HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPPS

[0032] 毒蜥外泌肽衍生物意思是与天然毒蜥外泌肽具有至少 80％氨基酸序列同源性的肽，其在氨基酸残基上可具有一些基团被化学取代，并且展示至少等于或大于天然毒蜥外泌肽的促胰岛素释放功能。

[0033] 毒蜥外泌肽片段意思是在天然毒蜥外泌肽的 N- 末端或 C- 末端具有一个或多个添加的或缺失的氨基酸的片段，并且添加的氨基酸可能是非天然存在的氨基酸（例如，D- 型氨基酸）。

[0034] 毒蜥外泌肽变体意思是具备促胰岛素释放功能的肽，其具有不同于天然毒蜥外泌肽的一个或多个氨基酸序列。

[0035] 在具体的实施方式中，本发明使用的天然促胰岛素释放肽和修饰的促胰岛素释放肽可使用固相合成方法合成并且大部分天然肽，包括天然促胰岛素释放肽，可通过重组技术产生。

[0036] 此外，本发明使用的促胰岛素释放肽可在不同的位点上结合至非肽基聚合物。

[0037] 本发明中制备的缀合物可具有取决于促胰岛素释放肽的结合位点而改变的活性。

[0038] 例如，其可分别与 N- 末端，和其他末端包括 C- 末端偶联，其显示不同的体外活性。醛反应基团选择性在低 pH 结合至 N- 末端并且可结合至赖氨酸残基以在高 pH 例如 pH9.0 形成共价键。允许以不同的 pH 进行聚乙二醇化反应并且离子交换柱可然后用于从反应混合物分离位置异构体。

[0039] 如果促胰岛素释放肽在 N- 末端——其是对体内活性重要的位点——之外的位点被偶联，那么反应性硫羟基团可引入至天然氨基酸序列中待修饰的氨基酸残基的位点，以便使用在非肽基聚合物处的马来酰亚胺连接体形成共价键。

[0040] 如果促胰岛素释放肽在 N- 末端——对于体内活性重要的位点——之外的位点偶联，反应性胺基团可引入至天然氨基酸序列中待修饰的氨基酸残基的位点，以便使用在非肽基聚合物处的醛连接体形成共价键。

[0041] 当使用在非肽基聚合物处的醛连接体时，其与在 N- 末端的胺基团和赖氨酸残基反应，并且修饰形式的促胰岛素释放肽可用于选择性增加反应产率。例如，使用 N- 末端封闭方法、赖氨酸残基取代方法、用于在羧基末端引入胺基团的方法等仅仅一个待反应的胺基团可保留在期望的位点上，从而增加聚乙二醇化反应和偶联反应的产率。用于保护 N- 末端的方法包括二甲基化，以及甲基化、脱氨基、乙酰化等，但不限于此类烷基化方法。

[0042] 在一种优选的实施方式中，本发明的促胰岛素释放肽缀合物是其中免疫球蛋白 Fc 区特异性结合至促胰岛素释放肽的 N- 末端处之外的胺基团的促胰岛素释放肽缀合物。

[0043] 在一种具体的实施方式中，本发明人在 pH9.0 引起天然毒蜥外泌肽 -4 的聚乙二醇化，以选择性地将 PEG 偶联至促胰岛素释放肽的赖氨酸残基。可选地，可合成使 N- 末端缺失或受保护的毒蜥外泌肽 -4 衍生物以被偶联。在 N- 末端的聚乙二醇化可通过缺失 N- 末端组氨酸的 α 胺基团或通过用两个甲基修饰氮末端组氨酸而封闭。这种 N- 末端修饰不影响体外活性（表 1）。

[0044] 不像毒蜥外泌肽 -4 的 N- 末端偶联，在赖氨酸残基处的偶联保持体外活性在大约 6％（表 1）。此外，本发明中制备的毒蜥外泌肽 -4-PEG- 免疫球蛋白 Fc 缀合物展示 60 ～ 70 小时的显著增加的血液半衰期，指示出人意料的高持久性效力。所以，通过偶联至不影响

活性的赖氨酸残基也使滴度下降被最小化，并且因此可制备能够保持其体内活性的新型长效毒蜥外泌肽 -4 制剂。

[0045] 免疫球蛋白 Fc 区用作药物载体是安全的，因为其是体内代谢的生物可降解的多肽。而且，免疫球蛋白 Fc 区与整个免疫球蛋白分子相比具有相对低的分子量，并且因此其对于缀合物的制备、纯化和产率是有利的。因为免疫球蛋白 Fc 区不包含其氨基酸序列根据抗体亚类不同并且因此是高度非同源性的 Fab 片段，可预测免疫球蛋白 Fc 区可大大增加物质的同种性并且是较小抗原的。

[0046] 如本文所使用，术语“免疫球蛋白 Fc 区”指免疫球蛋白的重链恒定区 2(C_H2) 和重链恒定区 3(C_H3) 并且不包括重链和轻链的可变区、重链恒定区 1(C_H1)、和轻链恒定区 1(C_L1)。其可进一步包括在重链恒定区的铰链区。而且，除了重链和轻链的可变区，本发明的免疫球蛋白 Fc 区可包含部分或所有的 Fc 区，包括重链恒定区 1(C_H1) 和 / 或轻链恒定区 1(C_L1)，只要其具有基本上与天然蛋白质类似或更好的效果。而且，免疫球蛋白 Fc 区可以是在 C_H2 和 / 或 C_H3 的氨基酸序列的相对长的部分具有缺失的片段。即，本发明的免疫球蛋白 Fc 区可包括 1)C_H1 结构域、C_H2 结构域、C_H3 结构域和 C_H4 结构域，2)C_H1 结构域和 C_H2 结构域，3)C_H1 结构域和 C_H3 结构域，4)C_H2 结构域和 C_H3 结构域，5) 一个或多个结构域和免疫球蛋白铰链区（或一部分铰链区）的组合，和 6) 重链恒定区和轻链恒定区的每个结构域的二聚体。

[0047] 本发明的免疫球蛋白 Fc 区包括天然氨基酸序列和其序列衍生物（突变体）。氨基酸序列衍生物是由于一个或多个氨基酸残基的缺失、插入、非保守或保守取代或其组合而与天然氨基酸序列不同的序列。例如，在 IgG Fc 中，已知对于在位置 214 至 238、297 至 299、318 至 322，或 327 至 331 的结合重要的氨基酸残基可用作适当的用于修饰的靶标。而且，其他各种衍生物是可能的，包括其中能够形成二硫键的区域被缺失或某些氨基酸残基在天然 Fc 形式的 N- 末端被去除，或向其添加甲硫氨酸残基的衍生物。此外，为了去除效应器功能，缺失可发生在补体 - 结合位点，比如 Clq- 结合位点和 ADCC 位点。国际专利公开号 WO97/34631 和 WO 96/32478 公开了制备免疫球蛋白 Fc 区的此类序列衍生物的技术。

[0048] 本领域已知在蛋白质和肽中氨基酸交换，其一般不改变分子活性 (H. Neurath, R. L. Hill, The Proteins, Academic Press, New York, 1979)。最常发生的交换是双方向的 Ala/Ser、Val/Ile、Asp/Glu、Thr/Ser、Ala/Gly、Ala/Thr、Ser/Asn、Ala/Val、Ser/Gly、Thy/Phe、Ala/Pro、Lys/Arg、Asp/Asn、Leu/Ile、Leu/Val、Ala/Glu、Asp/Gly。

[0049] 如果期望，Fc 区可通过磷酸化、硫酸盐化、丙烯酸化 (acrylation)、糖基化、甲基化、法呢基化 (farnesylation)、乙酰化、酰胺化等等修饰。

[0050] 前面提到的 Fc 衍生物是具有与本发明 Fc 区相同的生物活性或针对热、pH 等改善的结构稳定性的衍生物。

[0051] 另外，这些 Fc 区可从由人和其他动物包括母牛、山羊、猪、小鼠、兔子、仓鼠、大鼠和豚鼠分离的天然形式获得，或可以是获得自转化动物细胞或微生物的重组体或其衍生物。本文，它们可通过从人或动物有机体分离整个免疫球蛋白并且用蛋白水解酶处理它们，获得自天然免疫球蛋白。木瓜蛋白酶将天然免疫球蛋白消化成 Fab 和 Fc 区，并且胃蛋白酶处理引起 pF' c 和 F(ab)2 片段的产生。这些片段可进行尺寸排阻色谱以分离 Fc 或 pF' c。

[0052] 优选地，人源的 Fc 区是获得自微生物的重组体免疫球蛋白 Fc 区。

[0053] 另外，免疫球蛋白 Fc 区可以是具有天然糖链、与天然形式相比增加的糖链，或与天然形式相比减少的糖链的形式，或可以是去糖基化形式。免疫球蛋白 Fc 糖链的增加、减少或去除可通过本领域常见方法实现，比如化学方法、酶方法和使用微生物的基因工程方法。从 Fc 区去除糖链导致对补体 (c1q) 的结合亲和力急剧下降并且抗体 - 依赖性细胞介导的细胞毒性或补体 - 依赖性细胞毒性下降或损失，从而在体内不诱导不必要的免疫应答。在这点上，以去糖基化或无糖基化形式的免疫球蛋白 Fc 区可作为药物载体更适于本发明的目的。

[0054] 如本文所使用，术语“去糖基化”指从 Fc 区酶学上去除糖部分，术语“无糖基化”意思是通过原核生物，优选地大肠杆菌以非糖基化的形式产生 Fc 区。

[0055] 尽管免疫球蛋白 Fc 区可优选地源自人，但是其也可源自其他动物包括母牛、山羊、猪、小鼠、兔子、仓鼠、大鼠和豚鼠。另外，免疫球蛋白 Fc 区可以是源自 IgG、IgA、IgD、IgE 和 IgM 的 Fc 区，或通过其组合物或其混合体制造的 Fc 区。优选地，其源自 IgG 或 IgM——其是人血液中最丰富的蛋白质之一，并且最优选地源自 IgG——其已知增强配体 - 结合蛋白的半衰期。

[0056] 另一方面，术语“组合”，如本文所使用，意思是编码相同起源的单链免疫球蛋白 Fc 区的多肽连接至不同起源的单链多肽，以形成二聚体或多聚体。即，二聚体或多聚体可由选自 IgG Fc、IgA Fc、IgM Fc、IgD Fc 和 IgE Fc 片段的两个或更多个片段形成。

[0057] 如本文所使用，术语“混合体”意思是编码不同起源的两个或更多个免疫球蛋白 Fc 区的序列出现在单链免疫球蛋白 Fc 区中。在本发明中，各种类型的混合体是可能的。即，结构域混合体可由选自 IgG Fc、IgM Fc、IgA Fc、IgE Fc 和 IgD Fc 的 CH1、CH2、CH3 和 CH4 的一至四个结构域组成，并且可包括铰链区。

[0058] 另一方面，IgG 可分成 IgG1、IgG2、IgG3 和 IgG4 亚类，并且本发明可包括其组合物和混合体。优选的是 IgG2 和 IgG4 亚类，并且最优选的是 IgG4 的 Fc 区，其很少有效应器功能，比如 CDC(补体依赖性细胞毒性)。

[0059] 即，作为本发明的药物载体，最优选的免疫球蛋白 Fc 区是源自人 IgG4 的非糖基化的 Fc 区。人源的 Fc 区比非人源的 Fc 区更优选，所述非人源的 Fc 区可作为人体中的抗原并且造成非期望的免疫应答，比如针对该抗原产生新的抗体。

[0060] 如本文所使用，术语“非肽基聚合物”指包括通过除了肽键的任何共价键彼此连接的两个或更多个重复单元的生物相容性聚合物。

[0061] 可用于本发明的非肽基聚合物可选自聚乙二醇、聚丙二醇、乙二醇和丙二醇的共聚物、聚氧乙烯化多元醇、聚乙烯醇、多糖、葡聚糖、聚乙烯基乙醚、生物可降解聚合物，比如 PLA(聚乳酸) 和 PLGA(聚乳酸 - 甘醇酸)、脂质聚合物、几丁质、透明质酸，和其组合，其优选的是聚乙二醇。而且，本领域熟知的并且本领域技术人员容易制备的其衍生物包括在本发明的范围内。

[0062] 用于通过常规的框内融合方法获得的融合蛋白的肽连接体具有的缺点是，其容易在体内被蛋白水解酶切割，并且因此不能如期望的获得足够的通过载体增加活性药物血液半衰期的效果。但是，在本发明中，具有对蛋白水解酶抗性的聚合物可用于维持肽的血液半衰期与载体的类似。所以，可以使用本发明使用的任何非肽基聚合物而没有任何限制，只要其是具有前面提到的功能的聚合物，即，具有对体内蛋白水解酶抗性的聚合物。优选地，非

肽基聚合物具有 1 至 100kDa 范围的分子量，和优选地 1 至 20kDa。而且，与免疫球蛋白 Fc 区连接的本发明的非肽基聚合物可以是一种聚合物或不同类型聚合物的组合。

[0063] 本发明使用的非肽基聚合物具有能够结合至免疫球蛋白 Fc 区和蛋白质药物的反应基团。

[0064] 非肽基聚合物在两个末端具有反应基团，其优选地选自反应性乙醛基 (aldehyde group)、丙醛基、丁醛基、马来酰亚胺基团和琥珀酰亚胺衍生物。琥珀酰亚胺衍生物可以是琥珀酰亚胺基丙酸酯、羟基琥珀酰亚胺基、琥珀酰亚胺基羧甲基、或琥珀酰亚胺基碳酸酯。尤其，当非肽基聚合物在两个末端具有反应性乙醛基时，其在两个末端与生理活性多肽和具有最小非特异性反应的免疫球蛋白连接是有效的。经醛键通过还原性烷基化产生的终产物比当通过酰胺键连接时稳定得多。醛反应基团选择性地在低 pH 下结合至 N- 末端并且在高 pH 例如在 pH9.0 下可结合至赖氨酸残基以形成共价键。

[0065] 在非肽基聚合物两个末端的反应基团可以相同或不同。例如，非肽聚合物可在一端具有马来酰亚胺基团并且在另一端其可具有乙醛基、丙醛基或丁醛基。当在其两个末端具有反应性羟基基团的聚乙二醇用作非肽基聚合物时，羟基基团可通过已知的化学反应被活化成各种反应基团，或可使用具有商业上可获得的修饰的反应基团的聚乙二醇，以便制备本发明的促胰岛素释放肽缀合物。

[0066] 在另一实施方式中，本发明提供制备促胰岛素释放肽缀合物的方法，其包括下述步骤：

[0067] (1) 共价连接在其两个末端具有醛、马来酰亚胺或琥珀酰亚胺衍生物的反应基团的非肽基聚合物与促胰岛素释放肽的胺基团或硫羟基团；

[0068] (2) 从 (1) 的反应混合物分离包括促胰岛素释放肽的缀合物，其中非肽基聚合物共价连接至氨基末端之外的位点；和

[0069] (3) 共价连接免疫球蛋白 Fc 区至分离的缀合物的非肽基聚合物的另一末端，从而产生具有连接至非肽基聚合物的各自末端的免疫球蛋白 Fc 区和促胰岛素释放肽的肽缀合物。

[0070] 如本文所使用，术语“缀合物”指通过共价连接非肽基聚合物与促胰岛素释放肽制备的中间体，并且随后免疫球蛋白 Fc 区连接至缀合物中非肽基聚合物的另一末端。

[0071] 在一种优选的实施方式中，本发明提供了制备方法，其包括下述步骤：

[0072] (1) 共价连接在其两个末端具有醛反应基团的非肽基聚合物与毒蜥外泌肽 -4 的赖氨酸残基；

[0073] (2) 从 (1) 的反应混合物分离包括毒蜥外泌肽 -4 的缀合物，其中非肽基聚合物共价连接至赖氨酸残基；和

[0074] (3) 共价连接免疫球蛋白 Fc 区至分离的缀合物的非肽基聚合物的另一末端，从而生产蛋白质缀合物，其包括连接至非肽基聚合物的各自末端的免疫球蛋白 Fc 区和毒蜥外泌肽 -4。更优选地，(1) 中的非肽基聚合物和毒蜥外泌肽 -4 的赖氨酸残基在 pH9.0 或更高 pH 下被连接。

[0075] 本发明的促胰岛素释放肽缀合物经 GLP-1 受体活化胰岛素信号传导途径的主要蛋白质，并且因此可用于预防或治疗非酒精性脂肪肝疾病。尤其，本发明的促胰岛素释放肽缀合物增加 PKC-ζ（蛋白质激酶 C-ζ）的活性，其调节参与脂分解的酶活性并且维持已知

增加Glut2(葡萄糖载体蛋白质-2)表达的促胰岛素释放肽的体内活性,并且增加促胰岛素释放肽的血液半衰期,从而显著增加体内效力的持续时间。因此,可用比已知剂型较低的施用频率获得对非酒精性脂肪肝疾病的卓越治疗效果。

[0076] 在本发明中,非酒精性脂肪肝疾病(NAFLD)包括原发性和继发性非酒精性脂肪肝疾病,并且更具体地意思是由原发性高血脂症、糖尿病或肥胖造成的非酒精性脂肪肝疾病。例如,非酒精性脂肪肝疾病包括单纯脂肪变性;营养不良、饥饿、肥胖和糖尿病造成的脂肪肝疾病;脂肪性肝炎;以及由于这些疾病的进展发生的肝纤维化和肝硬化。

[0077] 包括本发明的促胰岛素释放肽缀合物的药学组合物可进一步包括药学上可接受的载体。为了口服施用,药学上可接受的载体可包括粘合剂、润滑剂、崩解剂、赋形剂、增溶剂、分散剂、稳定剂、悬浮剂、着色剂和香料。为了可注射的制剂,药学上可接受的载体可包括缓冲剂、防腐剂、镇痛药、增溶剂、等渗剂和稳定剂。对于局部施用的制剂,药学上可接受的载体可包括碱、赋形剂、润滑剂和防腐剂。本发明的药学组合物可配制成与前述提到的药学上可接受的载体组合的各种药物剂型。例如,对于口服施用,药学组合物可配制成片剂、含锭、胶囊、酏剂、悬液、糖浆剂或糯米纸囊剂。对于可注射的制剂,药学组合物可配制成作为单次剂量给药形式或单位给药形式的安瓿,比如多剂量容器。药学组合物也可配制成溶液、悬液、片剂、丸剂、胶囊和长效制剂。

[0078] 另一方面,适于药物制剂的载体、赋形剂和稀释剂的例子包括乳糖、右旋糖、蔗糖、山梨糖醇、甘露醇、木糖醇、赤藓糖醇、麦芽糖醇、淀粉、阿拉伯胶、藻酸盐、明胶、磷酸钙、硅酸钙、纤维素、甲基纤维素、微晶纤维素、聚乙烯吡咯烷酮、水、羟基苯甲酸甲酯、羟基苯甲酸丙酯、滑石、硬脂酸镁和矿物油。另外,药物制剂可进一步包括填充剂、抗凝结剂、润滑剂、致湿物、香料和抗菌剂。

[0079] 根据本发明的缀合物用于预防或治疗非酒精性脂肪肝疾病。因此,包括缀合物的药学组合物可被施用用于治疗该疾病。

[0080] 如本文所使用,术语"施用"意思是通过某些适当的方法将预定的物质引入至患者体内。本发明的缀合物可经任何常见的路径施用,只要其能够到达期望的组织。考虑各种施用的模式,包括腹膜内、静脉内、肌内、皮下、皮内、口服、局部、鼻内、肺内和直肠内,但是本发明不限于这些示例性的施用模式。然而,因为当口服施用时肽被消化,用于口服施用的组合物的活性成分应被加以包覆或配制为防止在胃中的降解。优选地,本组合物可以以可注射的形式施用。另外,药学组合物可使用能够将活性成分运输至靶细胞的某些装置施用。

[0081] 本发明的药学组合物可通过数个相关的因素确定,所述因素包括待治疗疾病的类型、施用路径、患者的年龄、性别、体重和疾病的严重性、以及作为活性组分的药物的类型。因为本发明的药学组合物具有卓越的体内效力的持续时间和滴度,其可显著降低本发明药学药物的施用频率和剂量。

[0082] 此外,本发明的药学组合物可单独或与外科手术操作、激素疗法、药物疗法和生物应答调节剂组合使用,以便预防和治疗非酒精性脂肪肝疾病。

[0083] 在本发明的一个方面中涉及药学组合物在制备用于预防或治疗非酒精性肝疾病的药物中的用途。

[0084] 实施例

[0085] 下文,参考下述实施例更详细描述本发明。但是,这些实施例仅仅是用于示意性目

的，并且本发明不打算被这些实施例限制。

[0086] 实施例 1. 测试长效毒蜥外泌肽 -4 的体外活性

[0087] 以与本发明人韩国专利号 10-1058315 中相同的方式制备该实验使用的各种长效毒蜥外泌肽 -4 衍生物。

[0088] 使用用于测量体外细胞活性的方法，以便测量毒蜥外泌肽 -4 的长效制剂的效力。在体外活性测量中，使用称为大鼠胰岛素瘤细胞的 RIN-m5F。因为该细胞具有 GLP-1 受体，其通常在测量 GLP-1 家族的体外活性的方法中使用。用不同浓度的 GLP-1、毒蜥外泌肽 -4 和测试材料处理 RIN-m5F。通过测量由测试材料引起的细胞中的信号传导分子 cAMP 的发生测定 EC50 值，并且彼此比较。结果总结在表 1 中。

[0089] 表 1

[0090]

测试材料	血液半衰期 (hr)	体外滴度 (%)
毒蜥外泌肽 -4	0.7	100
毒蜥外泌肽 -4(N)-PEG-Fc	61.5	<0.2
毒蜥外泌肽 -4(Lys27)-PEG-Fc	70.5	6.3

[0091]

[0092] 毒蜥外泌肽 -4(N)-PEG-Fc：通过经 PEG 连接毒蜥外泌肽 -4 的 N- 末端和 Fc 区制备的缀合物。

[0093] 毒蜥外泌肽 -4(Lys27)-PEG-Fc：通过经 PEG 连接毒蜥外泌肽 -4 的第 27 位赖氨酸残基和 Fc 区制备的缀合物。

[0094] 如表 1 中所显示，当非肽基聚合物连接至天然毒蜥外泌肽 -4 的 N- 末端之外的赖氨酸残基时，体外滴度保持在 6.3%，并且血液半衰期显著增加至约 70 小时。

[0095] 实施例 2. 对肥大动物模型 ob/ob 小鼠中脂肪肝形成的作用

[0096] <2-1> 实验动物的分组

[0097] 雌性 5 周大 ob/ob 小鼠 (C57BL/6JHamSlc-ob/ob，24-34g) 购买自日本的 Slc。ob/ob 小鼠是通常用于抗肥胖和抗糖尿病剂型的效力测试中的动物模型。它们自由进食通过辐射灭菌的用于实验动物的固体饲料 (制造商 :Picolab Rodent Diet，产品名 :5053)，并且自由饮食水瓶中过滤的、UV 照射 - 灭菌的自来水。根据动物护理标准指南 (animal care standard guidelines)，它们保持在符合 GLP 标准要求以 12 小时暗 - 亮循环 (在上午 6:00 打开灯并且在下午 6:00 关闭) 的箱子系统中。其后，选择健康的 ob/ob 小鼠并且使之适应实验室条件 1 周。然后，进行药物施用，并且小鼠分成 4 组和如下给药。

[0098] 组 1(阴性对照)：以 5ml/kg 的施用体积皮下注射 DULBECCO 的 PHOSPHATE BUFFERED SALINE(Sigma) 一周一次或更多次

[0099] 组 2(阳性对照)：以 5ml/kg 的施用体积每天皮下注射 10.8nmol/kg 的 BYETTA

[0100] 组 3(3.7nmol/kg 的长效毒蜥外泌肽 -4 衍生物 - 处理组)：以 5ml/kg 的施用体积皮下注射 3.7nmol/kg 的长效毒蜥外泌肽 -4 衍生物 (HM11260C) 每周一次

[0101] 组 4(8.2nmol/kg 的长效毒蜥外泌肽 -4 衍生物 - 处理组)：以 5ml/kg 的施用体积

皮下注射 8.2nmol/kg 的长效毒蜥外泌肽 -4 衍生物 (HM11260C) 每周一次

[0102] BYETTA(Eli Lilly) 是天然毒蜥外泌肽 -4，并且长效毒蜥外泌肽 -4 衍生物 (HM11260C) 是通过经 PEG 将去除第一个氨基酸组氨酸的 α 碳的咪唑乙酰基 - 毒蜥外泌肽 -4 连接至 Fc 区制备的 CA 毒蜥外泌肽 -4-PEG-Fc 缀合物，如韩国专利号 10-1058315 中描述。

[0103] 每个组施用盐水溶液或药物 7 周，并且分析它们对脂肪肝形成的作用。

[0104] <2-2> 长效毒蜥外泌肽 -4 衍生物对脂肪肝形成的作用

[0105] 为了检查根据本发明的长效毒蜥外泌肽 -4 衍生物对 ob/ob 小鼠中脂肪肝形成的作用，进行下述实验。将药物施用至实施例 <2-1> 划分的组中，和从 ob/ob 小鼠中取出肝，并且其一部分固定在 4% 甲醛中并且嵌入石蜡内，随后 H&E 染色。结果显示在图 1 中。

[0106] 如图 1 中所显示，在用媒介物处理的阴性对照组中清楚地观察到脂肪肝的病理学特征，而在用本发明的长效毒蜥外泌肽 -4 衍生物处理的实验组中观察到显著的脂肪肝病理性特征的剂量依赖性降低。也发现，与阳性对照 BYETTA 比较，本发明的长效毒蜥外泌肽 -4 衍生物显示甚至用更低的剂量对脂肪肝卓越的疗效。

[0107] 实施例 3. 对高脂肪诱导的 - 肥大小鼠中肝内甘油三酯积聚的作用

[0108] <3-1> 实验动物的分组

[0109] 使 6 周大 C57BL/6 小鼠稳定并且分成两个组，并且接收包含 10% 脂肪的正常饮食和包含 60% 脂肪的高脂肪饮食 12 周 (制造商 :Research diets Inc.，产品名 :D12492)。因此，准备正常小鼠和高脂肪诱导的 - 肥大小鼠，并且用于实验。根据动物护理标准指南，它们保持在符合 GLP 标准要求以 12 小时暗 - 亮循环 (在上午 6:00 打开灯并且在下午 6:00 关闭) 的箱子系统中。其后，选择健康的高脂肪诱导的 - 肥大小鼠并且使之适应实验室条件 1 周。然后，进行药物施用，并且小鼠分成 4 组和如下施用。

[0110] 组 1(正常饮食组) :以 5ml/kg 的施用体积皮下注射 DULBECCO 的 PHOSPHATE BUFFERED SALINE(Sigma) 一周一次或更多次

[0111] 组 2(高脂肪饮食组) :以 5ml/kg 的施用体积皮下注射 DULBECCO 的 PHOSPHATE BUFFERED SALINE(Sigma) 一周一次或更多次

[0112] 组 3(用 3nmol/kg 的长效毒蜥外泌肽 -4 衍生物处理的高脂肪饮食组) :以 5ml/kg 的施用体积皮下注射 3nmol/kg 的长效毒蜥外泌肽 -4 衍生物 (HM11260C) 每周一次

[0113] 组 4(用 10nmol/kg 的长效毒蜥外泌肽 -4 衍生物处理的高脂肪饮食组) :以 5ml/kg 的施用体积皮下注射 10nmol/kg 的长效毒蜥外泌肽 -4 衍生物 (HM11260C) 每周一次

[0114] 每个组施用盐水溶液或药物 2 周，并且分析肝组织中积聚的甘油三酯的量。

[0115] <3-2> 测量高脂肪诱导的 - 肥大小鼠中的肝内甘油三酯积聚

[0116] 从实施例 <3-1> 划分的组中取出肝，它们是施用或不施用长效毒蜥外泌肽 -4 衍生物的高脂肪诱导的 - 肥大小鼠，并且测定肝内甘油三酯浓度。如图 2 中所显示，高脂肪饮食组的肝内甘油三酯浓度是 172.3mg/g，其比低脂肪饮食组 (114.0mg/g) 更高，但是用 3nmol/kg 的长效毒蜥外泌肽 -4 衍生物处理的高脂肪饮食组显示 93mg/g 的甘油三酯水平，与高脂肪饮食组相比显示 46% 的下降。这些结果提示本发明的长效毒蜥外泌肽 -4 衍生物对非酒精性脂肪肝疾病的具有疗效。

[0001]

OPA13019_序列表.txt

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<120>用于预防或治疗非酒精性脂肪肝疾病的药学组合物

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[0002]

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Ser Gly Ala Pro Pro Pro Ser
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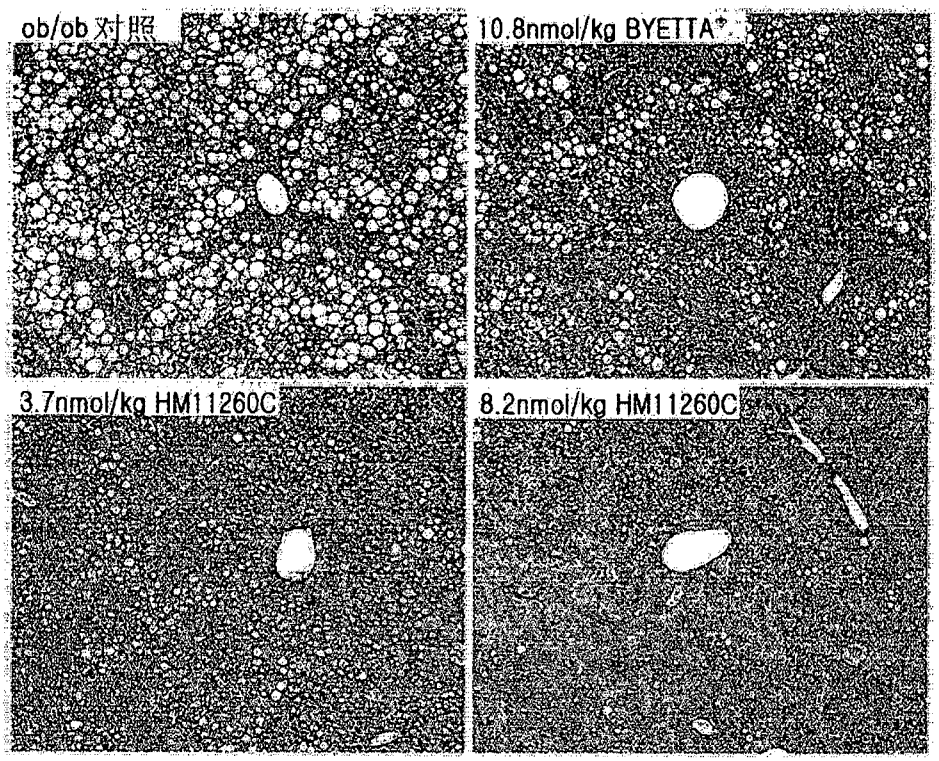
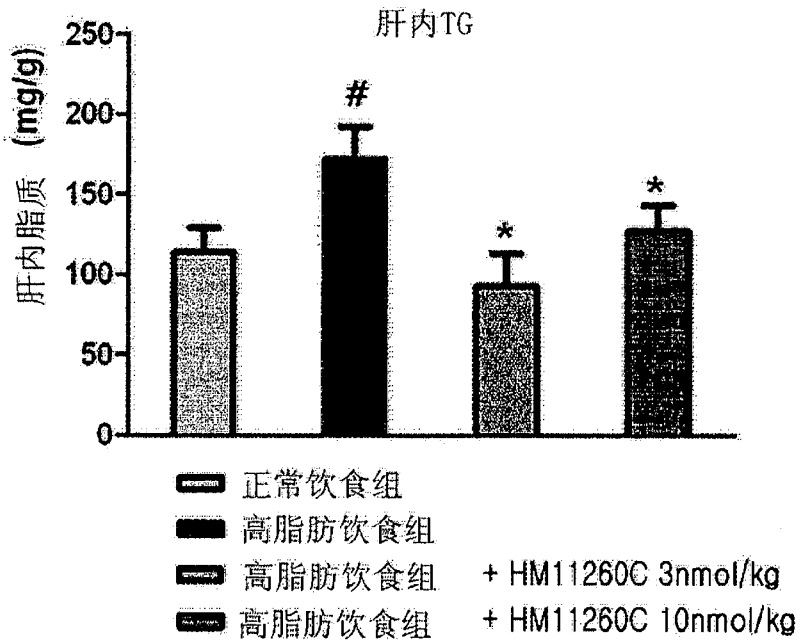


图 1



#; 与正常饮食组相比显著增加 ($p<0.01$)

*; 与高脂肪饮食组相比显著减少 ($p<0.01$)

图 2

Pharmaceutical Composition for the Prevention or Treatment of Non-Alcoholic Fatty Liver Disease

ABSTRACT

The present invention relates to a pharmaceutical composition for the prevention and treatment of non-alcoholic fatty liver disease (NAFLD), including a conjugate prepared by covalently linking an insulinotropic peptide, a non-peptidyl polymer and an immunoglobulin Fc region. The composition of the present invention maintains the in-vivo activity of the peptide at a relatively high level, and remarkably increases the blood half-life, thereby preventing triglyceride accumulation which is a typical feature of non-alcoholic fatty liver disease. Ultimately, it can be desirably employed for the prevention and treatment of non-alcoholic fatty liver disease.

用于预防或治疗非酒精性脂肪肝疾病的药学组合物

摘要

本发明涉及用于预防和治疗非酒精性脂肪肝疾病(NAFLD)的药学组合物，其包括通过共价连接促胰岛素释放肽、非肽基聚合物和免疫球蛋白 Fc 区制备的缀合物。本发明的组合物维持肽的体内活性在相对高的水平，并且显著增加血液半衰期，从而预防是非酒精性脂肪肝疾病的典型特征的甘油三酯积聚。最后，其可期望用于预防和治疗非酒精性脂肪肝疾病。