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(54) Title: GLYCEROL BRANCHED POLYETHYLENE GLYCOL HUMAN GROWTH HORMONE CONJUGATES, PROCESS FOR THEIR PREPARATION, AND METHODS OF USE THEREOF

(57) Abstract: The present invention relates to PEGylation of human Growth Hormone (hGH) using a glycerol branched PEG. The present invention also relates to processes for the PEGylation of hGH. In addition, the present invention relates to pharmaceutical compositions comprising the PEGylated hGH. A further embodiment is the use of the PEGylated hGH for the treatment of growth and development disorders.



WO 2006/024953 A2

**GLYCEROL BRANCHED POLYETHYLENE GLYCOL HUMAN GROWTH HORMONE
CONJUGATES, PROCESS FOR THEIR PREPARATION, AND METHODS OF USE THEREOF**

The present application claims priority under Title 35, United States Code, §119 to United States Provisional application Serial No. 60/605,945, filed August 31, 2004, which is incorporated by reference in its entirety as if written herein.

FIELD OF THE INVENTION

[001] The present invention relates to PEGylation, of human Growth Hormone (hGH) by which the chemical and/or physiological properties of hGH can be changed. The PEGylated hGH conjugate may have an increased plasma residency duration, decreased clearance rate, improved stability, decreased antigenicity, decreased PEGylation heterogeneity or a combination thereof. The present invention also relates to processes for the modification of hGH. In addition, the present invention relates to pharmaceutical compositions comprising the modified hGH. A further embodiment is the use of the modified hGH for the treatment of growth and development disorders.

BACKGROUND OF THE INVENTION

[002] Native human growth hormone (hGH) is a protein comprising a single chain of 191 amino acids cross-linked by two disulphide bridges and the monomeric form has a molecular weight of 22 kDa. Human GH is secreted by the pituitary gland and which also can be produced by recombinant genetic engineering. hGH will cause growth in all bodily tissues that are capable of growth. hGH plays an important role not only in promoting growth in the growing phase in human beings but also in maintaining normal body composition, anabolism, and lipid metabolism (K. Barneis. And U. Keller, Baillieres Clin. Endocrinol. Metab. 10:337 (1996)).

[003] Recombinant hGH has been commercially available for several years. Two types of therapeutically useful recombinant hGH preparations are present on the market: the authentic one, e.g. Genotropin™, or Nutropin™ and an analogue with an additional methionine residue at the N-terminal end, e.g. Somatnorm™. hGH is used to stimulate linear growth in patients with hypopituitary dwarfism also referred to as Growth Hormone Deficiency (GHD) or Turner's syndrome but other indications have also been suggested including long-term treatment of growth failure in children who were born short for gestational age (SGA), for treatment of patients with Prader-Willi syndrome (PWS), chronic renal insufficiency (CRI), AIDS wasting, and Aging. Adult GH deficiency (aGHD) patients have various problems, such as characteristic changes in body composition including increase in fat mass, decrease in lean body mass and extracellular fluid, and reduction of bone mineral density, metabolic abnormalities of lipids, and cardiovascular dysfunction. Many of those problems are improved by hGH replacement therapy (J. Verhelst J and R. Abs. *Drugs*, 62:2399 (2002)).

[004] A major biological effect of growth hormone (GH) is to promote growth in young mammals

and maintenance of tissues in older mammals. The organ systems affected include the skeleton, connective tissue, muscles, and viscera such as liver, intestine, and kidneys. Growth hormones exert their effect through interaction with specific receptors on the target cell's membrane. hGH is a member of a family of homologous hormones that include placental lactogens, prolactins, and other genetic and species variants or growth hormone (Nicoll, C. S., et al. (1986) *Endocrine Reviews* 7: 169). hGH is unusual among these in that it exhibits broad species specificity and binds to either the cloned somatogenic (Leung, D. W., et al. [1987] *Nature* 330: 537) or prolactin receptor (Boutin, J. M., et al. [1988] *Cell*; 53: 69). The cloned gene for hGH has been expressed in a secreted form in *Escherichia coli* (Chang, C. N., et al. [1987] *Gene* 55:189), and its DNA and amino acid sequence has been reported (Goeddel, et al. [1979] *Nature* 281: 544; Gray, et al. [1985] *Gene* 39:247).

[005] Human growth hormone (hGH) participates in much of the regulation of normal human growth and development. This pituitary hormone exhibits a multitude of biological effects including linear growth (somatogenesis), lactation, activation of macrophages, insulin-like and diabetogenic effects among others (Chawla, R. K. (1983) *Ann. Rev. Med.* 34, 519; Edwards, C. K. et al. (1988) *Science* 239, 769; Thomer, M. O., et al. (1988) *J. Clin. Invest.* 81:745). Growth hormone deficiency in children leads to dwarfism, which has been successfully treated for more than a decade by exogenous administration of hGH.

[006] In adults, as well as in children, hGH maintains a normal body composition by increasing nitrogen retention and stimulation of skeletal muscle growth, and by mobilization of body fat. Visceral adipose tissue is particularly responsive to hGH. In addition to enhanced lipolysis, hGH decreases the uptake of triglycerides into body fat stores. Serum concentrations of IGF-I (insulin-like growth factor-I), and IGFBP3 (insulin-like growth factor binding protein 3) are increased by hGH.

[007] hGH is a potent anabolic agent, especially due to retention of nitrogen, phosphorus, potassium, and calcium. Treatment of hypophysectomized rats with GH can restore at least a portion of the growth rate of the rats. Moore et al., *Endocrinology* 122:2920-2926 (1988). Among its most striking effects in hypo pituitary (GH-deficient) subjects is accelerated linear growth of bone-growth-plate-cartilage resulting in increased stature. Kaplan, *Growth Disorders in Children and Adolescents* (Springfield, IL: Charles C. Thomas, 1964).

[008] hGH causes a variety of physiological and metabolic effects in various animal models including linear bone growth, lactation, activation of macrophages, insulin-like and diabetogenic effects, and others (R. K. Chawla et al., *Annu. Rev. Med.* 34:519 (1983); O. G. P. Isaksson et al., *Annu. Rev. Physiol.* 47, 483 (1985); C. K. Edwards et al., *Science* 239, 769 (1988); M. O. Thomer and M. L. Vance, *J. Clin. Invest.* 82:745 (1988); J. P. Hughes and H. G. Friesen, *Ann. Rev. Physiol.* 47:469 (1985)). It has been reported that, especially in women after menopause, GH secretion declines with age. Millard et al., *Neurobiol. Aging*, 11:229-235 (1990); Takahashi et al., *Neuroendocrinology* M, L6- 137-142 (1987). See also Rudman et al., *J. Clin. Invest.*, 67:1361-1369 (1981) and Blackman, *Endocrinology and Aging*, 16:981 (1987). Moreover, a report exists that some of the manifestations of aging, including decreased lean body mass, expansion of adipose-tissue mass, and the thinning of the skin, can be reduced by GH treatment three times a week. See, e.g.,

Rudman et al., *N. Eng. J. Med.*, 323:1-6 (1990) and the accompanying article in the same journal issue by Dr. Vance (pp. 52-54). These biological effects derive from the interaction between hGH and specific cellular receptors. Two different human receptors have been cloned, the hGH liver receptor (D. W. Leung et al., *Nature* 330:537(1987)) and the human prolactin receptor (J. M. Boutin et al., *Mol. Endocrinology*, 3:1455 (1989)). However, there are likely to be others including the human placental lactogen receptor (M. Freemark, M. Comer, G. Komer, and S. Handwerger, *Endocrinol.* 120:1865 (1987)). These homologous receptors contain a glycosylated extracellular hormone binding domain, a single transmembrane domain, and a cytoplasmic domain, which differs considerably in sequence and size. One or more receptors are assumed to play a determining role in the physiological response to hGH.

[0009] It is generally observed that physiologically active proteins administered into a body can show their pharmacological activity only for a short period of time due to their high clearance rate in the body. Furthermore, the relative hydrophobicity of these proteins may limit their stability and/or solubility.

[0010] For the purpose of decreasing the clearance rate, improving stability or abolishing antigenicity of therapeutic proteins, some methods have been proposed wherein the proteins are chemically modified with water-soluble polymers. Chemical modification of this type may block effectively a proteolytic enzyme from physical contact with the protein backbone itself, thus preventing degradation. Chemical attachment of certain water-soluble polymers may effectively reduce renal clearance due to increased hydrodynamic volume of the molecule. Additional advantages include, under certain circumstances, increasing the stability and circulation time of the therapeutic protein, increasing solubility, and decreasing immunogenicity. Poly(alkylene oxide), notably poly(ethylene glycol) (PEG), is one such chemical moiety that has been used in the preparation of therapeutic protein products (the verb "pegylate" meaning to attach at least one PEG molecule). The attachment of poly(ethylene glycol) has been shown to protect against proteolysis, Sada, *et al.*, *J. Fermentation Bioengineering* 71: 137-139 (1991), and methods for attachment of certain poly(ethylene glycol) moieties are available. See U.S. Pat. No. 4,179,337, Davis *et al.*, *Non-Immunogenic Polypeptides*, issued Dec. 18, 1979; and U.S. Pat. No. 4,002,531, Royer, *Modifying Enzymes with Polyethylene Glycol and Product Produced Thereby*, issued Jan. 11, 1977. For a review, see Abuchowski *et al.*, in *Enzymes as Drugs*. (J. S. Holcberg and J. Roberts, eds. pp. 367-383 (1981)).

[0011] Other water-soluble polymers have been used, such as copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, poly(vinyl alcohol), poly(vinyl pyrrolidone), poly(-1,3-dioxolane), poly(-1,3,6-trioxane), ethylene/maleic anhydride copolymer, poly- amino acids (either homopolymers or random copolymers).

[0012] For poly(ethylene glycol), a variety of means have been used to attach the poly(ethylene glycol) molecules to the protein. Generally, poly(ethylene glycol) molecules are connected to the protein via a reactive group found on the protein. Amino groups, such as those on lysine residues or at the N-terminus, are convenient for such attachment. For example, Royer (U.S. Pat. No. 4,002,531, above) states that reductive alkylation was used for attachment of poly(ethylene glycol) molecules to

an enzyme. Chamow *et al.*, *Bioconjugate Chem.* 5: 133-140 (1994) report the modification of CD4 immunoadhesin with monomethoxypoly(ethylene glycol) aldehyde via reductive alkylation. U.S. 5,824,784 demonstrates PEGylating G-CSF including at the N-terminus under reductive alkylation conditions.

[0013] WO 93/00109 relates to a method for stimulating a mammal's or avian's GH responsive tissues comprising, maintaining a continuous, effective plasma GH concentration for a period of 3 or more days. One way of achieving such plasma concentration is stated to be by use of GH coupled to a macromolecular substance such as PEG (polyethylene glycol). The coupling to a macromolecular substance is stated to result in improved half-life. PEGylated human growth hormone has been reported in WO 93/00109 using mPEG aldehyde-5000 and mPEG N-hydroxysuccinimidyl ester (mPEG-NHS-5000) to achieve a hydrodynamic volume greater than the 70K molecular weight cut-off of the kidney filtration as described (Knauf, M.J. et al, *J. Biol. Chem.* 263:15064-15070, 1988). The use of mPEG-NHS resulted in heterogeneous mixtures of multiply PEGylated forms of hGH. WO 93/00109 also discloses the use of mPEG-maleimide to PEGylate cysteine hGH variants.

[0014] WO 99/03887 discloses a cysteine variant growth hormone that is PEGylated. Designated as BT-005, this conjugate is purported to be more effective at stimulating weight gain in growth hormone deficient rats and to have a longer half-life than hGH.

[0015] PEGylated human growth hormone has also been reported in Clark et al. using succinimidyl ester of carboxymethylated PEG (*Journal of Biological Chemistry* 271:21969-21977, 1996). Clark et al. describes derivatives of hGH of increasing size using mPEG-NHS-5000, which selectively conjugates to primary amines. Increasing levels of PEG modification reduced the affinity for its receptor and increased the EC₅₀ in a cell-based assay up to 1500 fold. Olson et al., *Polymer Preprints* 38:568-569, 1997 discloses the use of N-hydroxysuccinimide (NHS)PEG and succinimidyl propionate (SPA)PEG to achieve multiply PEGylated hGH species.

[0016] WO 94/20069 prophetically discloses PEGylated hGH as part of a formulation for pulmonary delivery.

[0017] US 4,179,337 discloses methods of PEGylating enzymes and hormones to obtain physiologically active non-immunogenic, water-soluble polypeptide conjugates. GH is mentioned as one example of a hormone to be PEGylated.

[0018] EP 458064 A2 discloses PEGylation of introduced or naturally present cysteine residues in somatotropin. EP 458064 A2 further mentions the incorporation of two cysteine residues in a loop termed the omega loop stated to be located at residues 102-112 in wild type bovine somatotropin, more specifically EP 458064 A2 discloses the substitution of residues numbered 102 and 112 of bovine somatotropin from Ser to Cys and Tyr to Cys, respectively.

[0019] WO 95/11987 suggests attachment of PEG to the thio group of a cysteine residue being either present in the parent molecule or introduced by site directed mutagenesis. WO 95/11987 relates to PEGylation of protease nexin-1, however PEGylation in general of hGH and other proteins is suggested as well.

[0020] WO 99/03887 discloses, e.g., growth hormone modified by replacement serine at position 25 with a cysteine residue and attachment of PEG to the introduced cysteine residue.

[0021] WO 00/42175 relates to a method for making proteins containing free cysteine residues for attachment of PEG. WO 00/42175 discloses the following muteins of hGH: T3C, S144C and T148C and the cysteine PEGylation thereof.

[0022] WO 97/11178 (as well as US 5849535, US 6004931, and US 6022711) relates to the use of GH variants as agonists or antagonists of hGH. WO 97/11178 also discloses PEGylation of hGH, including lysine PEGylation and the introduction or replacement of lysine (e.g. K168A and K172R). WO 97/11178 also discloses the substitution G120K.

[0023] WO 03/044056 discloses a variety of PEGylated hGH species including a lysine branched 40K PEG aldehyde hGH conjugate.

[0024] US 2004/0127417 discloses lysine branched PEG butyraldehyde hGH conjugates.

[0025] WO 04/46222, US 2005/0058620, JP 08-059818, JP 11-228685, and JP 2000-001541 disclose polyalkylene glycol derivatives having a reactive group at the primary carbon at the 1-position of a glycerol skeleton and having polyalkylene glycol chains at the 2- and 3-positions.

[0026] Currently administration of rhGH is daily for a long period of time, and therefore a less frequent administration would be highly desirable. An hGH molecule with a longer circulation half-life would decrease the number of necessary administrations and potentially provide more optimal therapeutic hGH levels with concomitant enhanced therapeutic effect.

[0027] Despite a number of attempts to develop a long lasting form of hGH, including PEGylating hGH, there is still an unmet need for a PEGylated hGH molecule with the appropriate properties to be a viable commercial product. The present invention provides PEG-hGH conjugates having a single PEG attached predominately at the N-terminal phenylalanine of hGH, which provides advantages over other PEG-hGH conjugates. The attachment of multiple low molecular weight (5Kd) PEGs at α - or ϵ -amino sites (N-terminus and nine lysines in hGH) using mPEG aldehyde-5000 or mPEG N-hydroxysuccinimidyl ester (mPEG-NHS-5000) has been described in WO 93/00109, Clark et al. (*Journal of Biological Chemistry* 271:21969-21977, 1996, and Olson et al. (*Polymer Preprints* 38:568-569, 1997)). This results in a heterogeneous population. As an illustration hGH with nine lysines may have some molecules having ten PEGs attached, some with nine, some with eight, some with seven, some with six, some with five, some with four, some with three, some with two, some with one and some with zero. And, among the molecules with several, the PEG may not be attached at the same location on different molecules. This resulting heterogeneity is disadvantageous when developing a therapeutic product making conjugation, purification, and characterization difficult, costly, and highly irreproducible. Another approach (WO 00/42175) has been to use hGH variants containing free cysteine residues for attachment of PEG. However, this approach results in an unnatural hGH variant and can also lead to incorrectly folded protein having incorrectly paired disulfide bonds resulting in a heterogeneous PEGylated product that has the PEG attached at some or all of the cysteines. Having multiple PEGs attached to multiple sites may lead to molecules that have less stable bonds between the PEG and the various sites, which can become dissociated at different rates. This makes it difficult

to accurately predict the pharmacokinetics of the product resulting in inaccurate dosing. A heterogeneous product also poses unwanted problems in obtaining regulatory approval for the therapeutic product.

Therefore, it would be desirable to have a PEGylated hGH molecule that has a single PEG attached at a single site. The present invention addresses this need in a number of ways.

SUMMARY OF THE INVENTION

[0028] The present invention relates to PEGylated hGH using a glycerol branched poly(ethylene glycol) moiety which may have at least one improved chemical or physiological property selected from but not limited to; decreased clearance rate, increased plasma residency duration, increased stability, improved solubility, and decreased antigenicity. Thus, as described below in more detail, the present invention has a number of aspects relating to chemically modifying hGH using a glycerol branched poly(ethylene glycol) moiety.

[0029] The present invention may also have one or more improved properties compared to lysine based branched PEG human growth hormone conjugates including but not limited to: a) increased stability of the glycerol skeleton, b) increased receptor binding, c) decreased cost, d) increased N-terminal selectivity of attachment, e) increased solubility, f) decreased immunogenicity, g) increased stability of the conjugate, h) increased manufacturability, and i) decreased proteolysis.

[0030] The present invention also relates to methods of producing the PEGylated hGH. Particularly, the present invention relates to a method of producing a PEGylated hGH using a glycerol branched PEG.

[0031] The present invention also relates to compositions comprising the PEGylated hGH alone or in combination with another therapeutic agent. The present invention also relates to the use of the PEGylated hGH of the present invention, alone or in combination with another therapeutic agent, in the prevention and/or treatment of disorders and/or diseases in which GH treatment is useful.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] Figure 1 is a size exclusion HPLC tracing showing the elution profile of the purified monoPEGylated glycerol branched 43K PEG aldehyde hGH reaction product on a TSK G4000PWXL column.

[0033] Figure 2 is a HPLC tracing of tryptic map analysis of hGH and glycerol branched 43K PEG aldehyde hGH. The top panel is the tryptic map of hGH. The lower panel is the tryptic map of glycerol branched 43K PEG aldehyde hGH. T1 is the N-terminal tryptic fragment.

[0034] Figure 3 shows the amino acid sequence of human growth hormone (SEQ ID NO:1).

[0035] Figure 4 shows the glycerol branched 43K PEG aldehyde hGH efficacy in an eleven-day Rat Weight Gain Assay. Hypophysectomized female Sprague-Dawley rats were purchased at the age of 4-5 weeks (85-110g) from Harlan Labs. Upon entering animal facilities, the animals were

maintained at a constant room temperature of 80°F. After 3 days' acclimation, the rats were weighed daily for 4-10 days in order to establish basal growth rates. Starting at day 0, rats (~100g) in control groups then received one *daily* subcutaneous injection of ~0.3 mg/kg hGH (▲), or PBS vehicle (●) for eleven consecutive days. The glycerol branched 43K PEG aldehyde hGH test group (■) received single doses of 1.8 mg/kg of glycerol branched 43K PEG aldehyde hGH on days 0 and 6. There were 6 animals per group. Plotted values represent average weight gain $\pm$ SEM.

[0036] Figure 5 shows eleven-day tibia growth in response to glycerol branched 43K PEG aldehyde hGH. Animals were those treated in Figure 4. Animals were sacrificed after taking day 11 weights, the left tibias were X-rayed, and the bone length measured using a caliper. Average length $\pm$ SEM is plotted. Asterisks denote significant differences from control group ($P < 0.05$). There were 6 animals per group.

[0037] Figure 6 shows eleven-day blood urea nitrogen levels in response to glycerol branched 43K PEG aldehyde hGH. Blood samples were taken from animals treated in Figure 4. Serum was prepared and urea nitrogen levels were measured. Average $\pm$ SEM is plotted (6 animals per group). Asterisks denote significant differences from control group ($P < 0.05$).

[0038] Figure 7 shows a six-day dose escalation efficacy study for glycerol branched 43K PEG aldehyde hGH. This growth study was performed in a similar manner to that described in Figure 4 except that varied single doses of glycerol branched 43K PEG aldehyde hGH were administered only on day 0 and the study was run for 6 days. Control groups received once-daily subcutaneous injections of either 0.3 mg/kg hGH (◆) or PBS vehicle (○) for six consecutive days. The glycerol branched 43K PEG aldehyde hGH test groups received a single dose of glycerol branched 43K PEG aldehyde hGH on day 0. The glycerol branched 43K PEG aldehyde hGH doses were 1.8 mg/kg (■), 0.6 mg/kg (X), 0.2 mg/kg, (+), 0.067 mg/kg (▲). There were 6 animals per group.

[0039] Figure 8 shows serum IGF-1 levels for six-day efficacy study. Animals were treated as described in Figure 7. Blood samples were taken at the various times plotted and the serum IGF-1 levels determined by ELISA. Group (n=6) means were used to calculate the IGF-1 response using one-way analysis of variance on the measured values and AUC d0-6 (ng/mL*24h) values of 37,839, 28,1292, 22,958, and 20,040 were determined for the 1.8, 0.6, 0.2, and 0.067 mg/kg dosing cohorts, respectively.

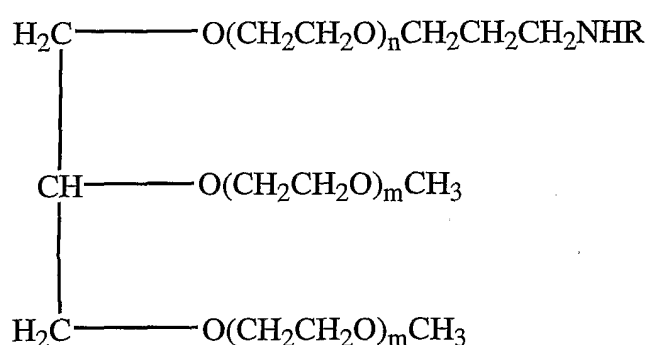
[0040] Figure 9 shows the PK/PD assessment following single dose administration of glycerol branched 43K PEG aldehyde hGH to hypophysectomized female rats. The effect of single 1.8 mg/kg SC dose administration of glycerol branched 43K PEG aldehyde hGH upon plasma drug levels (a) or plasma IGF-1 response (b).

DETAILED DESCRIPTION OF THE INVENTION

[0041] The present invention relates to glycerol branched polyethylene glycol-human growth hormone conjugates. In a specific embodiment the glycerol branched polyethylene glycol derivative has an aldehyde reactive group and optionally a linker between the polyethylene glycol and the

reactive functional group at the primary carbon at the 1-position of a glycerol skeleton and having polyalkylene glycol chains at the 2- and 3-positions as described in WO 04/46222 or US 2005/0058620 (incorporated by reference) to create hGH conjugates. The linker is not particularly limited as far as it is a covalent bond, but preferably includes an alkylene group and an alkylene group containing an ester bond, a urethane bond, an amide bond, an ether bond, a carbonate bond, or a secondary amino group. Preferable alkylene group includes a methylene group, an ethylene group, a trimethylene group, a propylene group, an isopropylene group, a tetramethylene group, a butylene group, an isobutylene group, a pentamethylene group, and a hexamethylene group.

[0042] A specific embodiment of the present invention is a human growth hormone-PEG conjugate having the structure of the Formula:



wherein

n is an integer between 60 and 75;

m is an integer between 450 and 460; and

R is a human growth hormone polypeptide.

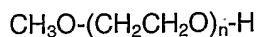
[0043] In a particular embodiment n is between about 64 and about 72.

[0044] In a particular embodiment the $(\text{CH}_2\text{CH}_2\text{O})_n$ moiety has an average molecular weight between about 2.6 and about 3.5Kd, and particularly the average molecular weight is about 3Kd,

[0045] In a particular embodiment each $(\text{CH}_2\text{CH}_2\text{O})_m$ moiety has an average molecular weight between about 17.6 and about 22Kd, and particularly the average molecular weight is about 20Kd.

[0046] In a specific embodiment the $(\text{CH}_2\text{CH}_2\text{O})_n$ moiety has an average molecular weight of about 3Kd and each $(\text{CH}_2\text{CH}_2\text{O})_m$ moiety has an average molecular weight of about 20Kd.

[0047] The term "about" when used in connection with the molecular weight of a PEG moiety means that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight and the stated molecular weight refers to the average molecular weight. It is understood that there is some degree of polydispersity associated with polymers such as poly(ethylene glycol). It is preferable to use PEGs with low polydispersity. In a specific embodiment one of the terminal polymer hydroxyl end-groups is converted or capped with a methyl group. As used herein, the term "mPEG" refers to a PEG, which is capped at one end with a methyl group. The mPEG can be represented structurally as



[0048] The term "human growth hormone polypeptide", "hGH polypeptide" or "hGH protein", when used herein, encompasses all hGH polypeptides, characterized by promoting growth in the growing phase and in maintaining normal body composition, anabolism, and lipid metabolism. Preferably, the term "hGH polypeptide" refers to the hGH polypeptide of SEQ ID NO:1

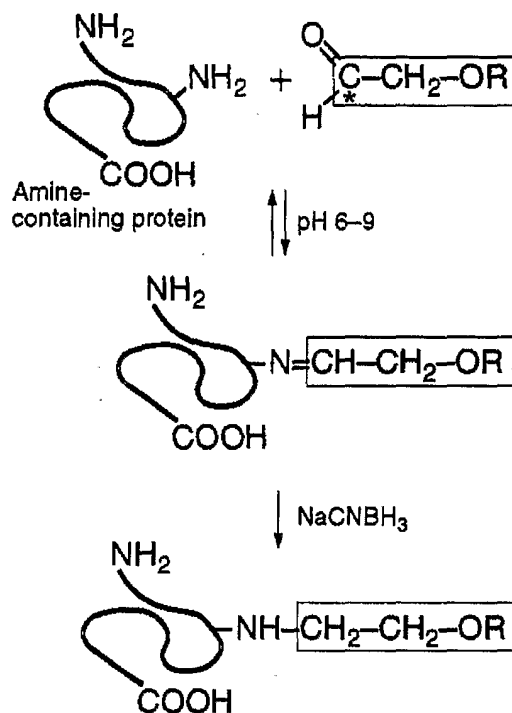
[0049] The hGH polypeptides of the present invention can be prepared in any suitable manner. Such hGH polypeptides and fragments thereof may be purified from natural sources, chemically synthesized, produced by recombinant techniques including *in vitro* translation techniques or expression in a recombinant cell able to express hGH cDNA, or a combination of these methods, using techniques known to those skilled in the art (See, for example, "Methods in Enzymology, Academic Press, 1993" for a variety of methods for purifying proteins; Creighton, (1983) *Proteins: Structures and Molecular Principles*, W.H. Freeman & Co. 2nd Ed., T. E., New York; and Hunkapiller *et al.*, (1984) *Nature*. 310(5973): 105-11 for chemical synthesis of proteins and Davis *et al.* (1986) *Basic Methods in Molecular Biology*, ed., Elsevier Press, NY for recombinant techniques, which disclosures are incorporated by reference in their entireties). The polypeptides of the present invention are preferably provided in an isolated form, and may be partially or preferably substantially purified.

[0050] A specific embodiment of the present invention is a human growth hormone-PEG conjugate wherein greater than 80%, more preferably 81%, more preferably 82%, more preferably 83%, more preferably 84%, more preferably 85%, more preferably 86%, more preferably 87%, more preferably 88%, more preferably 89%, more preferably 90%, more preferably 91%, more preferably 92%, more preferably 93%, more preferably 94%, more preferably 95%, more preferably 96%, more preferably 97, and more preferably 98% of the polyethylene glycol is conjugated to the amino-terminal phenylalanine of the human growth hormone of SEQ ID NO:1.

[0051] Another embodiment of the present invention is a substantially homogenous preparation of N-terminally PEGylated hGH optionally in a pharmaceutically acceptable diluent, carrier or adjuvant, said preparation being essentially free of hGH PEGylated at sites other than the N-terminus. The term "substantially homogenous preparation" means a preparation where greater than 80%, more preferably 81%, more preferably 82%, more preferably 83%, more preferably 84%, more preferably 85%, more preferably 86%, more preferably 87%, more preferably 88%, more preferably 89%, more preferably 90%, more preferably 91%, more preferably 92%, more preferably 93%, more preferably 94%, more preferably 95%, more preferably 96%, more preferably 97, and more preferably 98% is monoPEGylated.

[0052] In one embodiment of the invention secondary amine linkages are formed between the N-terminal primary α - amino group of a hGH polypeptide and a glycerol branched chain PEG aldehyde by reductive alkylation as described in Chamow *et al.*, *Bioconjugate Chem.* 5: 133-140 (1994), US Pat No. 4,002,531, WO 90/05534, and US Pat. No 5,824,784 with a suitable reducing agent such as

NaCNBH₃, NaBH₃, Pyridine Borane etc. The glycerol branched PEG aldehyde is incubated with an hGH polypeptide resulting in the addition of the PEG moiety to amino groups via Schiff's base formation. These linkages are converted to stable secondary amines by reduction with a reducing agent. The reductive alkylation process is depicted in the scheme below (from Chamow et al.).



[0053] Conjugation reactions, referred to as "PEGylation reactions", were historically carried out in solution with molar excess of polymer and without regard to where the polymer will attach to the protein. Such general techniques, however, have typically been proven inadequate for conjugating bioactive proteins to non-antigenic polymers while retaining sufficient bioactivity. One way to maintain the hGH bioactivity is to substantially avoid the conjugation of those hGH reactive groups associated with the receptor binding site(s) in the polymer coupling process. Another aspect of the present invention is to provide a process of conjugating poly(ethylene glycol) to hGH maintaining high levels of retained activity.

[0054] The chemical modification through a covalent bond may be performed under any suitable condition generally adopted in a reaction of a biologically active substance with the activated poly(ethylene glycol). The conjugation reaction is carried out under relatively mild conditions to avoid inactivating the hGH. Mild conditions include maintaining the pH of the reaction solution in the range of 3 to 10 and the reaction temperatures within the range of from about 0° - 37°C . In the cases where the reactive amino acid residues in hGH have free amino groups, the above modification is preferably carried out in a non-limiting list of suitable buffers (pH 4 to 10), including phosphate, MES, citrate, acetate, succinate or HEPES, for 1-48 hrs at 4° - 37°C . In targeting N-terminal amino groups with

reagents such as PEG aldehydes pH 4-8 is preferably maintained. The activated poly(ethylene glycol) may be used in about 0.01-100 times, preferably about 0.01-2.5 times, the molar amount of the number of free amino groups of hGH.

[0055] Although the reaction conditions described herein can result in significant amounts of unmodified hGH, the unmodified hGH can be readily recycled into future batches for additional conjugation reactions. The processes of the present invention generate surprisingly very little, *i.e.* less than about 20% and more preferably, less than about 10%, of high molecular weight species and species containing more than one polymer strand per hGH. These reaction conditions are to be contrasted with those typically used for polymeric conjugation reactions wherein the activated polymer is present in several-fold molar excesses with respect to the target.

[0056] The conjugation reactions of the present invention initially provide a reaction mixture or pool containing mono- PEG-hGH conjugates, unreacted hGH, unreacted polymer, and less than about 20% high molecular weight species. The high molecular weight species include conjugates containing more than one polymer strand and/or polymerized PEG-hGH species. After the unreacted species and high molecular weight species have been removed, compositions containing primarily mono- PEGylated-hGH conjugates are recovered. Given the fact that the conjugates for the most part include a single polymer strand, the conjugates are substantially homogeneous. These modified hGH have at least about 0.1% of the *in vitro* biological activity associated with the native or unmodified hGH as measured using standard FDC-P1 cell proliferation assays, (Clark et al. *Journal of Biological Chemistry* 271:21969-21977, 1996), receptor binding assay (US 5,057,417), or hypophysectomized rat growth (Clark et al. *Journal of Biological Chemistry* 271:21969-21977, 1996). In preferred aspects of the invention, however, the modified hGH have about 25% of the *in vitro* biological activity, more preferably, the modified hGH have about 50% of the *in vitro* biological activity, more preferably, the modified hGH have about 75% of the *in vitro* biological activity, and most preferably the modified hGH have equivalent or improved *in vitro* biological activity.

[0057] The processes of the present invention preferably include rather limited ratios of polymer to hGH. Thus, the hGH conjugates have been found to be predominantly limited to species containing only one strand of polymer. Furthermore, the attachment of the polymer to the hGH reactive groups is substantially less random than when higher molar excesses of polymer linker are used. The unmodified hGH present in the reaction pool, after the conjugation reaction has been quenched, can be recycled into future reactions using ion exchange or size exclusion chromatography or similar separation techniques.

[0058] A poly(ethylene glycol)-modified hGH may be purified from a reaction mixture by conventional methods which are used for purification of proteins, such as dialysis, salting-out, ultrafiltration, ion-exchange chromatography, hydrophobic interaction chromatography (HIC), gel chromatography and electrophoresis. Ion-exchange chromatography is particularly effective in removing unreacted poly(ethylene glycol) and hGH. In a further embodiment of the invention, the mono PEGylated-hGH species is isolated from the reaction mixture to remove high molecular weight species, and unmodified hGH. Separation is effected by placing the mixed species in a buffer solution

containing from about 0.5-10 mg/mL of the hGH-polymer conjugates. Suitable solutions have a pH from about 4 to about 10. The solutions preferably contain one or more buffer salts selected from KCl, NaCl, K_2HPO_4 , KH_2PO_4 , Na_2HPO_4 , NaH_2PO_4 , $NaHCO_3$, $NaBO_4$, CH_3CO_2H , and NaOH.

[0059] Depending upon the reaction buffer, the hGH polymer conjugate solution may first have to undergo buffer exchange/ultrafiltration to remove any unreacted polymer. For example, the PEG-hGH conjugate solution can be ultrafiltered across a low molecular weight cut-off (10,000 to 30,000 Dalton) membrane to remove most unwanted materials such as unreacted polymer, surfactants, if present, or the like.

[0060] The fractionation of the conjugates into a pool containing the desired species is preferably carried out using an ion exchange chromatography medium. Such media are capable of selectively binding PEG-hGH conjugates via differences in charge, which vary in a somewhat predictable fashion. For example, the surface charge of hGH is determined by the number of available charged groups on the surface of the protein. These charged groups typically serve as the point of potential attachment of poly(alkylene oxide) polymers. Therefore, hGH conjugates will have a different charge from the other species to allow selective isolation.

[0061] Strongly polar anion or cation exchange resins such as quaternary amine or sulfopropyl resins, respectively, are used for the method of the present invention. Anion exchange resins are especially preferred. A non-limiting list of included commercially available cation exchange resins suitable for use with the present invention are SP-hitrap®, SP Sepharose HP® and SP Sepharose® fast flow. Other suitable cation exchange resins e.g. S and CM resins can also be used. A non-limiting list of anion exchange resins, including commercially available anion exchange resins, suitable for use with the present invention are Q-hitrap®, Q Sepharose HP®, and Q sepharose® fast flow. Other suitable anion exchange resins, e.g. DEAE resins, can also be used.

[0062] For example, the anion or cation exchange resin is preferably packed in a column and equilibrated by conventional means. A buffer having the same pH and osmolality as the polymer conjugated hGH solution is used. The elution buffer preferably contains one or more salts selected from KCl, NaCl, K_2HPO_4 , KH_2PO_4 , Na_2HPO_4 , NaH_2PO_4 , $NaHCO_3$, $NaBO_4$, and $(NH_4)_2CO_3$. The conjugate-containing solution is then adsorbed onto the column with unreacted polymer and some high molecular weight species not being retained. At the completion of the loading, a gradient flow of an elution buffer with increasing salt concentrations is applied to the column to elute the desired fraction of polyalkylene oxide-conjugated hGH. The eluted pooled fractions are preferably limited to uniform polymer conjugates after the cation or anion exchange separation step. Any unconjugated hGH species can then be back washed from the column by conventional techniques. If desired, mono and multiply pegylated hGH species can be further separated from each other via additional ion exchange chromatography or size exclusion chromatography.

[0063] Techniques utilizing multiple isocratic steps of increasing concentration of salt or pH can also be used. Multiple isocratic elution steps of increasing concentration will result in the sequential elution of di- and then mono-hGH-polymer conjugates.

[0064] The temperature range for elution is between about 4°C and about 25°C. Preferably, elution is carried out at a temperature of from about 4°C to about 22°C. For example, the elution of the PEG-hGH fraction is detected by UV absorbance at 280 nm. Fraction collection may be achieved through simple time elution profiles.

[0065] A surfactant can be used in the processes of conjugating the poly(ethylene glycol) polymer with the hGH moiety. Suitable surfactants include ionic-type agents such as sodium dodecyl sulfate (SDS). Other ionic surfactants such as lithium dodecyl sulfate, quaternary ammonium compounds, taurocholic acid, caprylic acid, decane sulfonic acid, etc. can also be used. Non-ionic surfactants can also be used. For example, materials such as poly(oxyethylene) sorbitans (Tweens), poly(oxyethylene) ethers (Tritons) can be used. See also Neugebauer, A Guide to the Properties and Uses of Detergents in Biology and Biochemistry (1992) Calbiochem Corp. The only limitations on the surfactants used in the processes of the invention are that they are used under conditions and at concentrations that do not cause substantial irreversible denaturation of the hGH and do not completely inhibit polymer conjugation. The surfactants are present in the reaction mixtures in amounts from about 0.01-0.5%; preferably from 0.05-0.5%; and most preferably from about 0.075-0.25%. Mixtures of the surfactants are also contemplated.

[0066] It is thought that the surfactants provide a temporary, reversible protecting system during the polymer conjugation process. Surfactants have been shown to be effective in selectively discouraging polymer conjugation while allowing lysine-based or amino terminal-based conjugation to proceed.

[0067] The present poly(ethylene glycol)-modified hGH has a more enduring pharmacological effect, which may be possibly attributed to its prolonged half-life *in vivo*.

[0068] Another embodiment of the invention relates to methods for the prevention and/or treatment of a disease or disorder in which use of GH, preferably hGH is beneficial, comprising administering to a patient in need thereof a therapeutically effective amount of a poly(ethylene glycol)-modified hGH of the invention or agonist variant thereof, alone or in combination with another therapeutic agent. The invention also relate to the use of a poly(ethylene glycol)-modified hGH of the invention or agonist variant thereof in the manufacture of a medicament for the prevention and/or treatment of a disease or disorder in which use of GH, preferably hGH is beneficial. In addition, the invention also relates to a pharmaceutical composition comprising a poly(ethylene glycol)-modified hGH of the invention or agonist variant thereof for the prevention and/or treatment of a disease or disorder in which use of GH, preferably hGH is beneficial.

[0069] Diseases or disorders in which the use of GH is beneficial include, but are limited to, growth hormone deficiency (GHD), adult growth hormone deficiency (aGHD), Turner's syndrome, growth failure in children who were born short for gestational age (SGA), Prader-Willi syndrome (PWS), chronic renal insufficiency (CRI), Aids wasting, Aging, end-stage Renal Failure, Cystic Fibrosis, Erectile dysfunction, HIV lipodystrophy, Fibromyalgia, Osteoporosis, Memory disorders, Depression, Crohn's disease, Skeletal dysplasias, Traumatic brain injury, Subarachnoid haemorrhage, Noonan's syndrome, Down's syndrome, Idiopathic short stature (ISS), End stage renal

disease (ESRD), Very low birth weight (VLBW), Bone marrow stem cell rescue, Metabolic syndrome, Glucocorticoid myopathy, Short stature due to glucocorticoid treatment in children, and Failure of growth catching for short premature children.

[0070] In a more specific embodiment of the invention, the poly(ethylene glycol)-modified hGH of the invention or agonist variants thereof are used in the prevention and/or treatment of a disorders or diseases selected from the group consisting of GHD, aGHD, SGA, PWS, Turner's syndrome and CRI.

[0071] In another more specific embodiment of the invention, the poly(ethylene glycol)-modified hGH of the invention or agonist variants thereof are used in the prevention and/or treatment of a disorders or diseases selected from the group consisting of idiopathic short stature, very low birth weight, traumatic brain injury, metabolic syndrome, and Noonan's syndrome.

[0072] Another embodiment of the invention relate to pharmaceutical compositions comprising a poly(ethylene glycol)-modified hGH of the invention alone or in combination with another therapeutic agent, and at least one pharmaceutically acceptable excipient or carrier. The present poly(ethylene glycol)-modified hGH may then be formulated into pharmaceuticals containing also a pharmaceutically acceptable diluent, an agent for preparing an isotonic solution, a pH-conditioner and the like in order to administer them into a patient.

[0073] The above pharmaceuticals may be administered subcutaneously, intramuscularly, intravenously, pulmonary, intradermally, or orally, depending on a purpose of treatment. A dose may be also based on the kind and condition of the disorder of a patient to be treated, being normally between 0.1 mg and 5 mg by injection and between 0.1 mg and 50 mg in an oral administration for an adult.

[0074] As used herein, the poly(ethylene glycol)-modified hGH or agonist variants thereof of the present invention may be used in combination with another therapeutic agent. As used herein, the terms "co-administration", "co-administered" and "in combination with", referring to the compounds A and one or more other therapeutic agents, is intended to mean, and does refer to and include the following :

- simultaneous administration of such combination of A and therapeutic agent(s) to a patient in need of treatment, when such components are formulated together into a single dosage form which releases said components at substantially the same time to said patient;
- substantially simultaneous administration of such combination of A and therapeutic agent(s) to a patient in need of treatment, when such components are formulated apart from each other into separate dosage forms which are taken at substantially the same time by said patient, whereupon said components are released at substantially the same time to said patient;
- sequential administration of such combination of A and therapeutic agent(s) to a patient in need of treatment, when such components are formulated apart from each other into separate dosage forms which are taken at consecutive times by said patient with a significant time interval between each administration,

whereupon said components are released at substantially different times to said patient; and

- o sequential administration of such combination of A and therapeutic agent(s) to a patient in need of treatment, when such components are formulated together into a single dosage form which releases said components in a controlled manner whereupon they are concurrently, consecutively, and/or overlappingly administered at the same and/or different times by said patient.

[0075] Suitable examples of other therapeutic agents which may be used in combination with A, their pharmaceutically acceptable salts and/or their derived forms include, but are by no mean limited to: aromatase inhibitors such as exemestane, formestane, atamestane, fadrozole, letrozole, vorozole and anastrozole; free fatty acid regulators including fibric acid derivatives (such as fenofibrate, clofibrate, gemfibrozil, bezafibrate and ciprofibrate) and nicotinic acid derivatives such as acipimox; insulin sensitizing agents including but not limited to biguanides such as metformin, PPAR gamma insulin sensitizing agents and thiazolidenediones such as troglitazone and rosiglitazone Troglitazone, 5-[[4-[3,4-Dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-]-benzopyran-2-yl) methoxy]phenyl]methyl-3,2,4-thiazolidinedione V411 (DIABII, Glaucanin) Pioglitazone (ACTOS, AD 4833, U 72107, U 72107A, U 72107E, ZACTOS) Chemical Name: 2,4-Thiazolidinedione, 5-[[4-[2-(5-ethyl-2-pyridinyl)ethoxy]phenyl]methyl]-, monohydrochloride, (a/-); Rosiglitazone (Avandia, BRL 49653, BRL 49653C) Chemical Name: 2,4 Thiazolidinedione, 5-[[4-[2- (methyl-2-pyridinylamino)ethoxy]phenyl]methyl]; 25 Bexarotene-oral (LGD 1069 oral, Targretin oral, Targretin, Targretyn oral Targrexin oral) Chemical Name: 4-[1-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-2-naphthyl) ethenyl]benzoic acid; ZD 2079, (ICI D 2079) (Chemical Name: R)-N-[2-4- (Carboxymethyl) 30 phenoxy]ethyl)-N-(2-hydroxy-2-phenethyl) ammonium chloride: Netoglitazone, (Isaglitazone, MCC 555, RWJ 241947) (Chemical Name: 5-[6-(2-Fluorobenzyloxy)naphthalen-2-ylmethyl]thiazolidine-2,4-dione) ; INS (D-chiro-inositol) (Chemical Name: D-1,2,3,4,5,6- Hexahydroxycyclohexane), ON 2344 (DRF 2593); Dextipotam, Chemical Name: 5(R)-(1,2-Dithiolan-3-yl) pentaloic 35 acid; HQL 975, Chemical Name: 3-[4- [2-(5-Methyl-2-phenyloxazol-4-yl) ethoxy]phenyl]-2(S)-(propylamino) propionic acid; YM 268, Chemical Name: 5,5'-Methylene-bis(1,4-phenylene)bismethylenebis (thiazolidine-2,4-dione). I PPAR agonists under development include: Reglitazar (JTT 501, PNU 182716, PNU 716) (Chemical Name: Isoxazolidien-3,5-dione, i 4-[[4-(2-phenyl-5-methyl)-1,3-oxazolyl]ethoxyphenyl-4] methyl-, (4RS)); I(RP 297, Chemical Name: 10 5-(2,4-DioXothiazolidin-5-ylmethyl)-2-methoxy-N-[4-(trifluoromethyl) benzyl]benzamide; R 119702 (CI 1037, CS 011) ChemicalName: (-)-5-[4-(5-Methoxy- 1H benzimidazol-2-ylmethoxy)benzyl] thiazolin-2,4-dione; hydrochloride; 15 DRF 2189, Chemical Name: 5-[[4-[2-(1-Indolyl)ethoxy]phenyl]methyl] thiazolidine-2,4-dione; cortisol synthesis inhibitors such as Ketoconazole, econazole or miconazole; growth hormones such as somatropin or somatorm and their derivatives such as human growth hormone fusion proteins such as ALBUTROPIN; polyethylene glycol growth hormones such as the cysteine-pegylated growth hormone, BT 005 (Bolder BioTechnology Inc.); growth hormone secretagogues such as, for example, SM 130686 (Sumitomo) capromorelin (Pfizer), mecasermin (Fujisawa), sermorelin (Salk Institute, Bio-Technology General),

somatrem, somatomedin (C Llorente; Pharmacia Corporation) examorelin, tabimorelin; CP 464709 (Pfizer), LY 426410 and LY 444711 (Lilly); 8-(aminoalkoxyimino)-8H-dibenzo[a,e]triazolo[4,5-c]cycloheptenes as disclosed in WO2002057241, 2-substituted dibenzo[a,e]1,2,3-triazolo[4,5-c][7]annulen-8-ones as described in WO2002056873 growth hormone releasing peptides GHRP-6 and GHRP-1 as described in U.S. Patent No. 4,411,890, and publications WO 89/07110, WO 89/07111, B-HT920, hexarelin and GHRP-2 as described in WO 93/04081 or growth hormone releasing hormone (GHRH, also designated GRF) and its analogs, somatomedins including IGF-1 and IGF-2 and their derivatives such as SomatoKine - a recombinant fusion of insulin-like growth factor-1 and its binding protein, BP-3, alpha-2-adrenergic agonists such as clonidine, xylazine, detomidine and medetomidine or serotonin 5HT_{1D} agonists such as sumatriptan or agents which inhibit somatostatin or its release such as physostigmine and pyridostigmine, ThGRF 1-44 (Theratechnologies); L 165166 (Merck & Company); dipeptide derivatives as described in WO9858947, Inhibitors of dipeptidyl peptidase IV such as amino-acylpyrrolidine nitrile as described in US6521644, WO95/15309 and WO98/19998; Beta-amino heterocyclic dipeptidyl peptidase inhibitors such as those described in US20030100563 and WO2003082817; growth hormone releasing compounds as described in US20030055261, US20030040483, EP 18 072, EP 83 864, WO 89/07110, WO 89/01711, WO 89/10933, WO 88/9780, WO 83/02272, WO 91/18016, WO 92/01711, WO 93/04081, WO 9514666, EP0923539, U.S. Patent Nos. 5,206,235, 5,283,241, 5,284,841, 5,310,737, 5,317,017, 5,374,721, 5,430,144, 5,434,261, 5,438,136, 5,494,919, 5,494,920, 5,492,916, 5,536,716 and 5,578,593, WO 94/13696, WO 94/19367, WO 95/03289, WO 95/03290, WO 95/09633, WO 95/11029, WO 95/12598, WO 95/13069, WO 95/14666, WO 95/16675, WO 95/16692, WO 95/17422, WO 95/17423, WO 95/34311, and WO 96/02530, Piperidines, pyrrolidines and hexahydro-1H-azepines as described in US5804578, US5783582, WO2004007468, AMIDO SPIROPIPERIDINES such as those described in WO0104119, 2-amino-5-pyrimidine acetic acid compounds including 2-[(5,6-Dimethyl-2-benzimidazolyl)amino]-4-hydroxy-6-methyl-5-pyrimidine acetic acid (2) and 2-[(5,6-Dimethyl-2-benzimidazolyl)amino]-4-hydroxy-6-methyl-5-pyrimidine acetic acid, ethyl ester as described in US6329383, benzimidazoles as described in EP1155014, analogous peptidyl compounds related to GRF and the peptides of U.S. Patent 4,411,890, antagonists of gonadotropin releasing hormone such as those described in WO0170228, WO0170227, WO0170228, WO0069433, WO0004013, WO995156, WO9951595, WO9951231-4, WO9941251-2, WO9921557, WO9921553 and 6-AZAINDOLE COMPOUNDS as described in WO0053602, WO0053185, WO0053181, WO0053180, WO0053179, WO0053178, US6288078; IGF-1 secretagogues; insulin-like growth factor-2 (IGF-2 or somatomedin A) and IGF-2 secretagogues; myostatin antagonists and compounds which inhibit fibroblast growth factor receptor-3 (FGFR-3) tyrosine kinase.

[0076] The polymeric substances included are also preferably water-soluble at room temperature. A non-limiting list of such polymers include poly(alkylene oxide) homopolymers such as poly(ethylene glycol) or poly(propylene glycols), poly(oxyethylenated polyols), copolymers thereof and block copolymers thereof, provided that the water solubility of the block copolymers is maintained.

[0077] As an alternative to PEG-based polymers, effectively non-antigenic materials such as dextran, poly(vinyl pyrrolidones), poly(acrylamides), poly(vinyl alcohols), carbohydrate-based polymers, and the like can be used. Indeed, the activation of α - and ε -terminal groups of these polymeric substances can be effected in fashions similar to that used to convert poly(alkylene oxides) and thus will be apparent to those of ordinary skill. Those of ordinary skill in the art will realize that the foregoing list is merely illustrative and that all polymer materials having the qualities described herein are contemplated. For purposes of the present invention, "effectively non-antigenic" means all materials understood in the art as being nontoxic and not eliciting an appreciable immunogenic response in mammals.

Definitions

[0078] The following is a list of abbreviations and the corresponding meanings as used interchangeably herein:

-	g	gram(s)
-	mg	milligram(s)
-	ml or mL	milliliter(s)
-	RT	room temperature
-	PEG	poly (ethylene glycol)

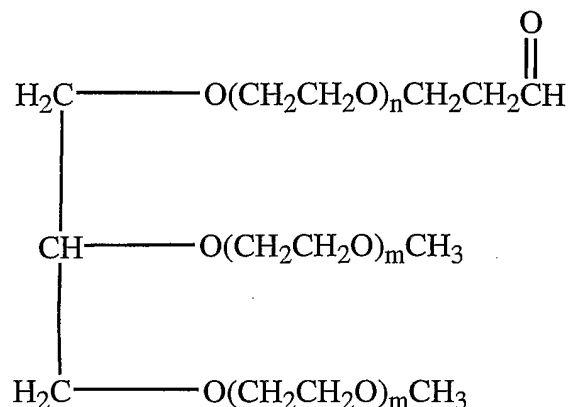
[0079] The complete content of all publications, patents, and patent applications cited in this disclosure are herein incorporated by reference as if each individual publication, patent, or patent application were specifically and individually indicated to be incorporated by reference.

[0080] Although the foregoing invention has been described in some detail by way of illustration and example for the purposes of clarity of understanding, it will be readily apparent to one skilled in the art in light of the teachings of this invention that changes and modifications can be made without departing from the spirit and scope of the present invention. The following examples are provided for exemplification purposes only and are not intended to limit the scope of the invention, which has been described in broad terms above.

[0081] In the following examples, the hGH is that of SEQ ID NO:1. It is understood that other hGH polypeptides could also be PEGylated in a similar manner as exemplified in the subsequent examples.

EXAMPLES

EXAMPLE 1

Glycerol branched 43K PEG aldehyde hGH

wherein

$(\text{CH}_2\text{CH}_2\text{O})_n$ has an average molecular weight of about 3Kd and each $(\text{CH}_2\text{CH}_2\text{O})_m$ has an average molecular weight of about 20Kd

(GL3-400AL2)

[0082] This example demonstrates generation of N-terminally monoPEGylated hGH by reductive alkylation. The glycerol branched PEG aldehyde reagent of approximately 43,000 MW (GL3-400AL2 NOF corporation) was coupled via reductive alkylation to the N-terminus of hGH by taking advantage of the difference in the relative pK_a value of the primary amine at the N-terminus versus pK_a values of primary amines at the ϵ -amino position of lysine residues. hGH protein dissolved at 4, 7, or 10 mg/mL in 25 mM MES (Sigma Chemical, St. Louis, MO) pH 5.8 or 20mM HEPES pH 7.0 was reacted with glycerol branched 43K PEG aldehyde by addition of the reagent to yield a relative PEG:hGH molar ratio of 1.5:1, 2:1, 3.4:1, 4:1, or 5:1. Reactions were catalyzed by addition of Pyridine Borane (Sigma Chemical, St. Louis, MO), to a final concentration of 10 mM. Reactions were carried out in the dark at 4 degrees C. for 16-87 hours. Reactions were stopped by dilution into appropriate buffer for purification.

[0083] Table 1 shows the percent of multi-PEGylated species, mono-PEGylated conjugate, un-reacted hGH, and final purification yield for glycerol branched 43K PEG aldehyde hGH reacted for 63 hrs at a pH of 5.8 and a 1.5:1:1 molar ratio.

Table 1**Glycerol branched 43K PEG aldehyde hGH conjugate Synthesis and Purification Process**

Synthesis and Purification yields for glycerol branched 43K PEG aldehyde-hGH	
Species in the reaction mix:	
	glycerol branched 43K PEG aldehyde-hGH
multi-PEG product	4%
mono-PEG product	68%
un-reacted hGH	28%
Purification yield	35%

EXAMPLE 2*Purification of PEGylated hGH*

[0084] PEGylated hGH species were purified from the reaction mixture to >95% (SEC analysis Figure 1) using a single ion exchange chromatography step.

Anion exchange chromatography

[0085] The PEG hGH species were purified from the reaction mixture to >95% (SEC analysis Figure 1) using a single anion exchange chromatography step. Mono-PEGylated hGH was purified from unmodified hGH and multi-PEGylated hGH species using anion exchange chromatography. A typical glycerol branched 43K PEG aldehyde hGH reaction mixture (80 or 1500 mg protein), as described above, was purified on a Q-Sepharose Hitrap column (5 mL)(Amersham Pharmacia Biotech, Piscataway, NJ) or Q-Sepharose column (26/20, 70 mL bed volume)(Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in 25 mM HEPES, pH 7.3 (Buffer A). The reaction mixture was diluted 7X with buffer A and loaded onto the column at a flow rate of 2.5 mL/min. The column was washed with 3-10 column volumes of buffer A. Subsequently, the various hGH species were eluted from the column in 20 column volumes of Buffer A and a linear NaCl gradient of 0-100 mM. The eluant was monitored by absorbance at 280 nm (A_{280}) and appropriate size fractions were collected. Fractions were pooled as to extent of PEGylation, e.g., mono, di, tri etc. (as assessed in example 3). The pool was then concentrated to 0.5-5 mg/mL in a Centriprep YM10 concentrator (Amicon, Technology Corporation, Northborough, MA) or by diafiltration. Protein concentration of pool was determined by A_{280} using an extinction coefficient of 0.78.

EXAMPLE 3*Biochemical Characterization*

[0086] The purified PEGylated hGH pools were characterized by non-reducing SDS-PAGE, non-denaturing Size Exclusion Chromatography, and peptide mapping.

Size Exclusion High Performance Liquid Chromatography (SEC-HPLC)

[0087] The reaction mixture of glycerol branched 43K PEG aldehyde with hGH, anion exchange purification pools and final purified products were assessed using non-denaturing SEC-HPLC. Analytical non-denaturing SEC-HPLC was carried out using a column, TSK G4000PWXL (Tosohaas) or Shodex KW-804 (Waters Corp) in 20 mM Phosphate pH 7.2, 150 mM NaCl at a flow rate of 0.5 mL/minute (optionally Superdex 200 7.8 mm X 30 cm, Amersham Bioscience, Piscataway, NJ). PEGylation greatly increases the hydrodynamic volume of the protein resulting in a shift to an earlier retention time. New species were observed in the PEG aldehyde hGH reaction mixtures along with unmodified hGH. These PEGylated and non-PEGylated species were separated on Q-Sepharose chromatography, and the resultant purified mono PEG-Aldehyde hGH species were subsequently shown to elute as a single peak on non-denaturing SEC (> 95% purity, Figure 1). The Q-Sepharose chromatography step effectively removed free PEG, hGH, and multi PEGylated hGH species from the mono-PEGylated hGH.

SDS-PAGE

[0088] SDS-PAGE was used to assess the reaction of glycerol branched 43K PEG aldehyde with hGH and the purified final products. SDS-PAGE was carried out on 1 mm thick 10-NuPAGE gels (Invitrogen, Carlsbad, CA) under reducing and non-reducing conditions and stained using a Novex Colloidal CoomassieTM G-250 staining kit (Invitrogen, Carlsbad, CA).

N-terminal Sequence

[0089] Automated Edman degradation chemistry is used to determine the NH₂-terminal protein sequence. An Applied Biosystems Model 494 Procise sequencer (Perkin Elmer, Wellesley, MA) is employed for the degradation. The respective PTH-AA derivatives are identified by RP-HPLC analysis in an on-line fashion employing an Applied Biosystems Model 140C PTH analyzer fitted with a Perkin Elmer/Brownlee 2.1 mm i.d. PTH-C18 column.

Peptide Mapping

[0090] Tryptic digests were performed at a concentration of 1 mg/mL and typically 25 ug of material was used per digest. Trypsin was added such that the trypsin to PEG-hGH ratio was 1:30 (w/w). Tris buffer was present at 30 mM, pH 7.5. Samples were incubated at room temperature for 16 ± 0.5 hours. Reactions were quenched by the addition of 50 µL of 1N HCl per mL of digestion solution. Samples were diluted, prior to placing the samples in the autosampler, to a final concentration of 0.25 mg/ml in 6.25 % acetonitrile. Acetonitrile was added first (to 19.8% acetonitrile), mixed gently, and then water was added to final volume (four times the starting volume). Extra digestion solution may be removed and stored for up to 1 week at -20°C.

[0091] A Waters Alliance 2695 HPLC system was used for analysis, but other systems should produce similar results. An Astec C-4 polymeric 25 cm x 4.6 mm column with 5 µm particles was

used. Experiments were conducted at ambient temperature on a typical load of 50 µg of protein per sample. Buffer A is 0.1% trifluoroacetic acid in water; buffer B was 0.085% trifluoroacetic acid in acetonitrile. Samples were eluted with a linear gradient of 0-45% B over 90 minutes

[0092] Peaks were detected using a Waters 996 PDA detector collecting data between 210 and 300 nm. The extracted chromatogram at 214 nm was used for sample analysis.

[0093] Tryptic maps were performed for hGH, and glycerol branched 43K PEG aldehyde reacted at a molar ratio of 2:1 (PEG:hGH), (Figure 2). The N-terminal tryptic fragment was referred to as T-1. The percent of T-1 present compared to unPEGylated hGH suggests that greater than 99% of the PEG modification is at the N-terminus with remainder apparently linked to one of several possible lysine residues.

Table 2
T-1 present comparison

	% T-1 present	% T-1 present compared to unPEGylated hGH
hGH	28.0%	100%
glycerol branched 43K PEG aldehyde/hGH	Less than 1%	Less than 1%

Example 4

In vitro biology

The ability of the glycerol branched 43K PEG aldehyde hGH to recognize the human receptor was tested using a Biacore 3000 instrument in an assay configured to assess the specific interactions between the conjugate and the human growth hormone receptor (hGHR) (28kDa extracellular domain). Results from the surface plasmon resonance (SPR) experiments are shown below in Table 3.

Table 3. Biacore assay results.

Sample.	Avg. $k_a \times 10^5$ ($M^{-1}s^{-1} \pm \text{stdev}$) ^a	Avg. k_d ($s^{-1} \pm \text{stdev}$) ^a	$K_D = k_d/k_a$, mM	K_D relative to hGH
hGH (n=9)	3.07 ± 8.20	38.8 ± 3.6	0.13 ± 0.03	1.000
43K PEG-hGH (n=3)	0.28 ± 0.03	90.0 ± 31.0	3.22 ± 0.10	0.041

^a k_a (on-rate) and k_d (off-rate) were determined at a flow rate of 50 µL/min at 37°C in HEP-BES buffer (0.01 M HEPES, pH 7.4, plus 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20), using human growth hormone binding protein (28kDa, extracellular domain) labeled on a CM5 chip through amine coupling chemistry at $\Delta RU \approx 3000$ -5000. k_a , expressed as per M per second, k_d expressed as per second, both are an average value of at least 3 measurements on 1 chip $\pm$ standard deviation. The data assumes to measure hGH binding to the high affinity site 1 on GHBP at 1:1 ratio.

Example 5

Pharmacodynamic Studies

*In vivo potency - Efficacy in 11-day Rat Assays (weight gain, tibia growth, serum BUN depression)**Rat weight gain*

[0094] Female Sprague Dawley rats, hypophysectomized at Harlan Labs, were prescreened for growth rate for a period of 4 to 10 days. Rats were divided into groups of six. Starting at day 0, control rats received one daily subcutaneous injection of either 0.3 mg/kg hGH, or vehicle, for eleven consecutive days. The test group received single (once/weekly) doses of 1.8 mg/kg of glycerol branched 43K PEG aldehyde hGH on days 0 and 6. The animals were weighed daily. Figure 4 shows the effects of hGH and glycerol branched 43K PEG aldehyde hGH on body weight gain in a representative study.

[0095] Combining the data represented by the study depicted in Figure 4 with data from historical 11-day growth studies for hGH (control) treated rats plus an additional growth study using the 43K glycerol branched PEG aldehyde hGH conjugate, the average incremental weight gain for animals treated once weekly with 1.8 mg/kg conjugate was 109% of that achieved following daily hGH administration (cumulative 3.3 mg/kg).

Rat tibia length

[0096] Animals in 11-Day weight gain studies at day 11 were sacrificed, left tibias were removed and X-rayed and bone lengths were measured using a caliper. Figure 5 shows tibia length measurements for Glycerol branched 43K PEG aldehyde hGH treated animals.

Rat BUN levels

[0097] As a biomarker for the metabolic effects following hGH-treatment, blood urea nitrogen levels were determined from day-11 blood samples. Figure 6 shows that both daily hGH and once/weekly glycerol branched 43K PEG aldehyde hGH treatment results in significant reduction of blood urea nitrogen.

Rat Weight Gain 6-Day Dose Escalation Study

[0098] Hypophysectomized rats were treated with various single doses of glycerol branched 43K PEG aldehyde hGH or else treated daily with hGH, and weight gain was monitored for 6 days. Figure 7 shows the weight gain that was obtained for the various treatment groups. Blood samples were taken at the indicated times and the serum IGF-1 levels determined by ELISA. Plotted are averages $\pm$ SEM. Group (n=6) means were used to calculate the IGF-1 response using one-way analysis of variance on the measured values and AUC_{d0-6} (ng-hr/mL) values of 20,040, 22,958, 28,129, and 37,839 were determined for the 0.067, 0.2, 0.6, and 1.8 mg/kg dosing cohorts, respectively.

Rat Weight Gain 11-Day Dose Escalation Study

[0099] In a second study, animals were treated with either 1.8 mg/kg or with a higher dose, i.e., 5.1 mg/kg of the 43K glycerol branched PEG aldehyde hGH conjugate, on day 0 and again on day 6. Table 4 shows the dose-related weight gain at 6 days and 11 days compared to that achieved following the daily (QD 11) administration of either vehicle or daily hGH (at 0.3 mg/kg/d).

Table 4. Dose-related weight gain at day 6 and 11.

	Day 0 BW (g)	Day 6 BW (g)	Day 11 BW (g)
Vehicle (QD11)	106.2±2.3	106.2±2.8 (0.07±0.7)	106.7±2.8 (0.51±0.6)
hGH (0.3 mg/kg) (QD11)	109.2±1.3	123.1±1.4* (13.9±0.5)	136.7±1.6 (27.5±0.7)*
Glycerol branched 43K PEG aldehyde hGH (1.8 mg/kg)(D0,6)	106.8±1.3	120.6±1.1* (13.8±0.7)	133.6±1.0 (26.7±0.8)*
Glycerol branched 43K PEG aldehyde hGH (5.1 mg/kg)(D0,6)	106.1±1.4	127.1±1.3*† (21.0±1.0)	142.5±1.4 (36.4±1.2)*†

* p < 0.05 vs. Vehicle, † p < 0.05 vs. hGH; mean incremental weight gain (g) as measured from day 0 BW, body weight; hGH, human growth hormone.

Values represent mean ± SEM (standard error of the mean).

Values in parenthesis represent change from Day 0 in mean ± SEM.

IGF-1 Studies

[00100] Animals from six-day weight gain studies were used. Blood samples were taken at the various times during the study and the serum IGF-1 levels determined by ELISA as shown in Figure 8. Rat IGF-1 levels were monitored by immunoassay kit (Diagnostic System Laboratories).

Example 6

Pharmacokinetic Studies

[00101] Pharmacokinetic studies were conducted in normal, cannulated Sprague-Dawley male rats. Injections were made as a single intravenous dose of 1.0 mg/kg or a single subcutaneous bolus of 1.8 mg/kg hGH or glycerol branched 43K PEG aldehyde hGH using six rats per group. Blood samples are taken over one to five days as appropriate for assessment of relevant PK parameters. hGH and glycerol branched 43K PEG aldehyde hGH blood levels are monitored at each sampling using immuno-assay. Table 4 shows the rat PK parameters for glycerol branched 43K PEG aldehyde hGH. The effect of PEGylation is evident in the observed half-life for elimination as this parameter exceeded 6 hours for the conjugate while data reported for hGH from similar studies is reported as 1.35±0.2 (Clark *ibid.*) 0.77-1.7 (Jorgensen *et.al.*, "Polyethylene glycol-conjugated proteins", PSTT 1 (8), November 1998), or 1 hr (Genotropin® (PNU-180307) Investigator Brochure).

hGH Immunoassay

[00102] hGH and glycerol branched 43K PEG aldehyde hGH protein concentration levels in rat plasma were determined using the hGH AutoDELFIA kit fluorescence immunoassay (Perkin-Elmer).

Table 5.

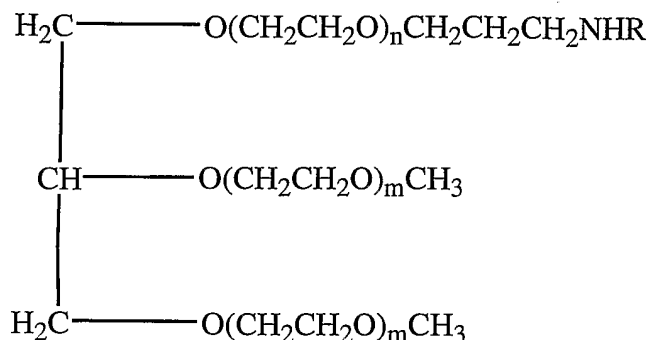
		glycerol branched 43K PEG aldehyde hGH
Route		iv/sc
Dose	mg/kg	1.0/1.8
T _{1/2}	h	6.44±2.38
AUC _{0-∞} , iv	μg•h/mL	489±16
AUC _{0-∞} , sc	μg•h/mL	97.7±2.1
CL _{total}	mL/hr/kg.	2.05±0.07
V _{ss}	mL/kg	27.2±2.8
T _{max}	h	12±0
C _{max}	μg/ml	1.96±0.05
F	%	11.1±0.2
* AUC _{0-∞} , sc normalize for 1.8 mpk		

The relationship between plasma drug levels and the IGF-1 response was also determined directly in a comprehensive study design following the subcutaneous administration of a single dose of the glycerol branched 43K PEG aldehyde hGH (1.8 mg/kg) to hypophysectomized, female rodents.

WHAT IS CLAIMED IS:

1. A polyethylene glycol-human growth hormone (PEG-hGH) conjugate having the structure;

5



wherein

n is an integer between 60 and 75;

10 m is an integer between 450 and 460; and

R is a human growth hormone.

2. The PEG-hGH conjugate of claim 1 wherein said $(\text{CH}_2\text{CH}_2\text{O})_n$ moiety has an average molecular weight of about 3Kd and each $(\text{CH}_2\text{CH}_2\text{O})_m$ moiety has an average molecular weight of about 20Kd.
3. The PEG-hGH conjugate of claim 1 or 2 wherein said human growth hormone comprises an amino acid sequence of SEQ ID NO:1.
4. The PEG-hGH conjugate of claim 3 wherein the PEG is conjugated to the N-terminal phenylalanine of SEQ ID NO:1.
5. The PEG-hGH conjugate of claim 4 wherein said conjugate is mono-PEGylated.
6. The PEG-hGH conjugate of claim 4 wherein at least 80% of the PEG is conjugated to the alpha amino group of the N-terminal phenylalanine of SEQ ID NO:1.
7. The PEG-hGH conjugate of claim 5 wherein at least 90% of the PEG is conjugated to the alpha amino group of the N-terminal phenylalanine of SEQ ID NO:1.
8. The PEG-hGH conjugate of claim 5 wherein at least 95% of the PEG is conjugated to the alpha amino group of the N-terminal phenylalanine of SEQ ID NO:1.

9. The PEG-hGH conjugate of claim 5 wherein at least 98% of the PEG is conjugated to the N-terminal phenylalanine of SEQ ID NO:1.
10. A method of treating a patient having a growth or development disorder comprising administering to said patient a therapeutically effective amount of the human growth hormone-PEG conjugate of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9.
11. The method of claim 10 wherein said growth or development disorder is selected from the group consisting of Growth Hormone Deficiency (GHD), Turner's syndrome, Chronic Renal Insufficiency, and short for gestational age (SGA).
12. The method of claim 11 wherein said growth or development disorder is selected from the group consisting of Erectile dysfunction, HIV lipodystrophy, Fibromyalgia, Osteoporosis, Memory disorders, Depression, Crohn's disease, Skeletal dysplasias, Traumatic brain injury, Subarachnoid haemorrhage, Noonan's syndrome, Down's syndrome, Idiopathic short stature (ISS), End stage renal disease (ESRD), Very low birth weight (VLBW), Bone marrow stem cell rescue, Metabolic syndrome, Glucocorticoid myopathy, Short stature due to glucocorticoid treatment in children, and Failure of growth catching for short premature children.

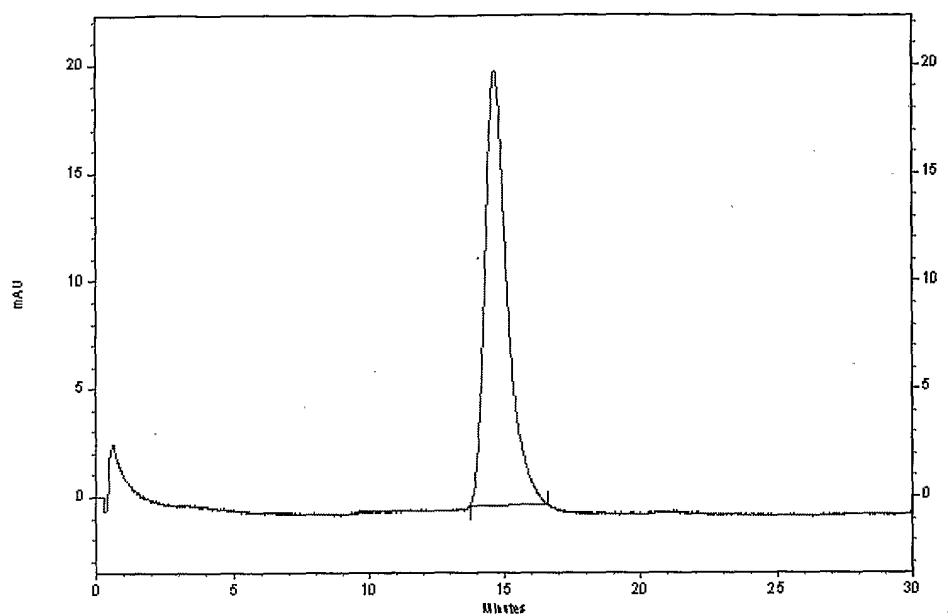
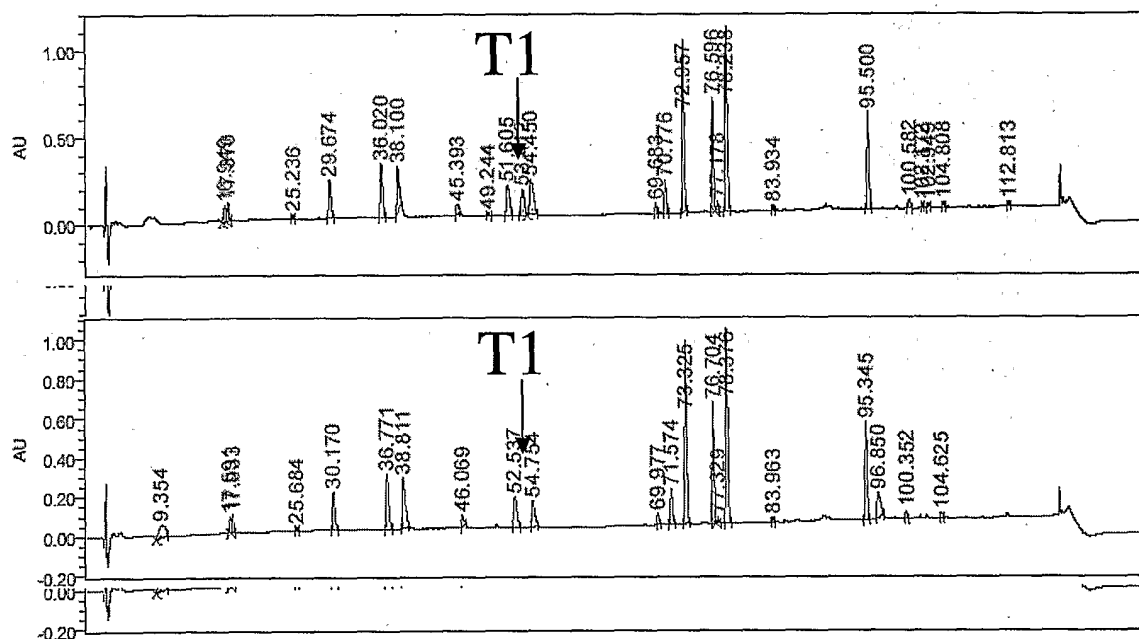
Figure 1**Figure 2**

Figure 3

Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe Asp Asn	12
Ala Met Leu Arg Ala His Arg Leu His Gln Leu Ala	24
Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile	36
Pro Lys Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro	48
Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile Pro Thr	60
Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser Asn	72
Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln	84
Ser Trp Leu Glu Pro Val Gln Ser Leu Arg Ser Val	96
Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser	108
Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly	120
Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser	132
Pro Arg Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser	144
Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu	156
Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg Lys	168
Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val	180
Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe	191

SEQ ID NO:1

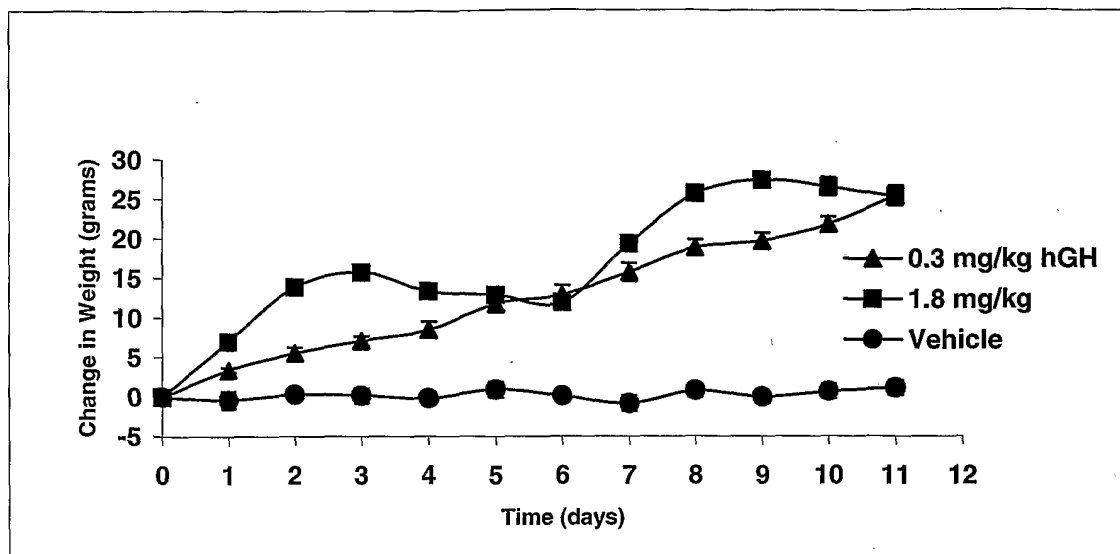
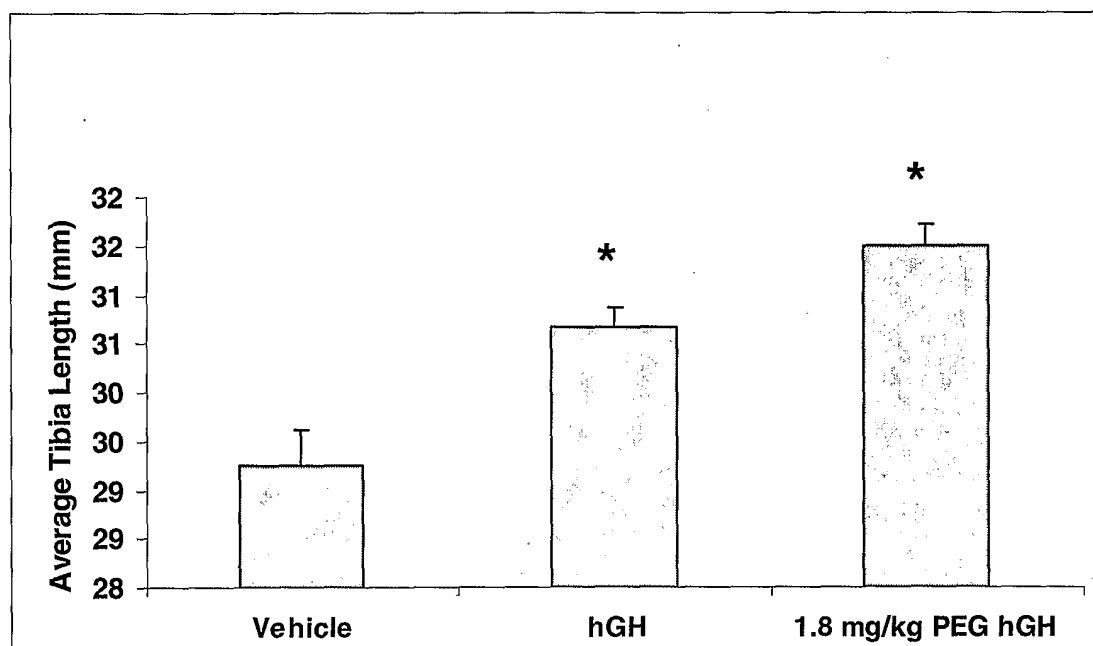
Figure 4**Figure 5**

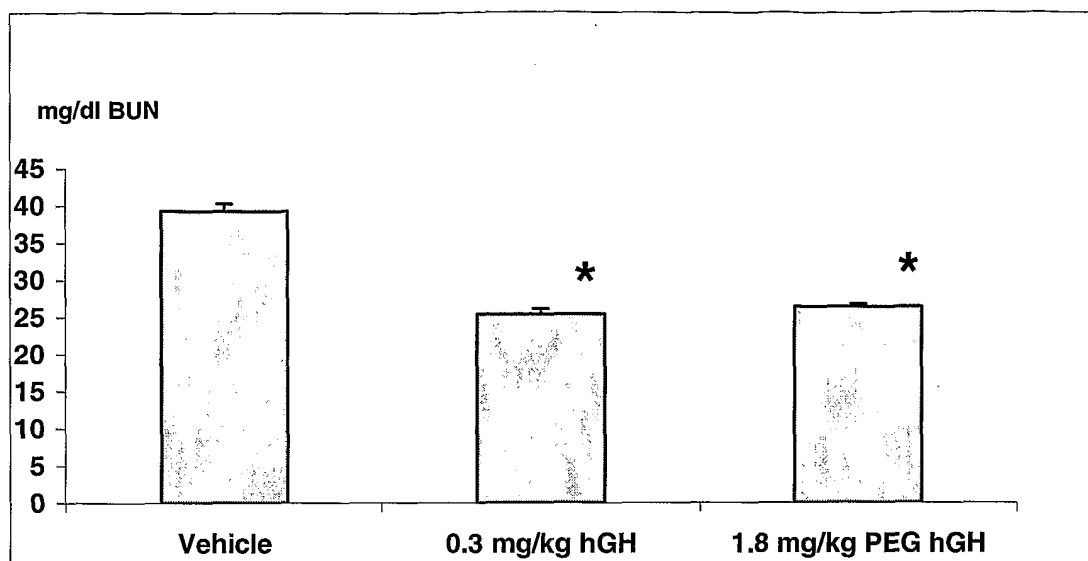
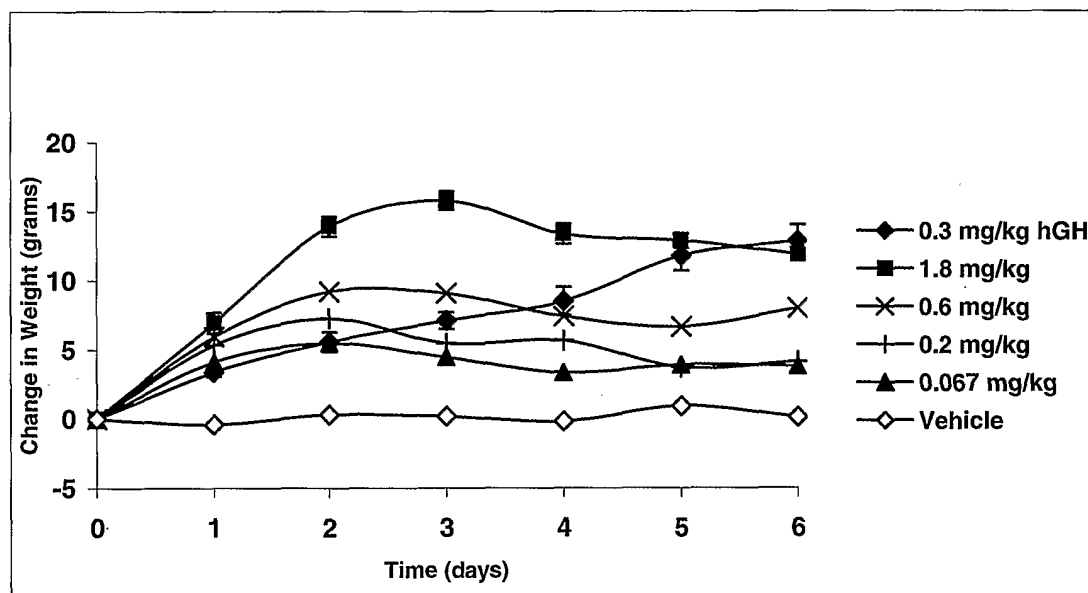
Figure 6**Figure 7**

Figure 8

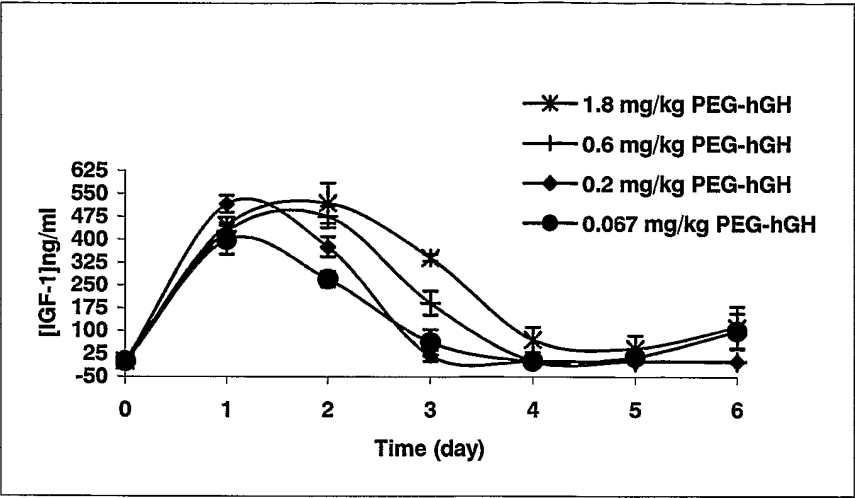


Figure 9

