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DESCRIPTION

FIELD OF INVENTION

[0001] Polypeptides, and polynucleotides encoding same, comprising at least two carboxy-terminal peptides (CTP) of chorionic gonadotropin attached to a peptide-of-interest are disclosed. Pharmaceutical compositions comprising the polypeptides of the invention and uses of the same are also disclosed.

BACKGROUND OF THE INVENTION

[0002] Polypeptides are susceptible to denaturation or enzymatic degradation in the blood, liver or kidney. Accordingly, polypeptides typically have short circulatory half-lives of several hours. Because of their low stability, peptide drugs are usually delivered in a sustained frequency so as to maintain an effective plasma concentration of the active peptide. Moreover, since peptide drugs are usually administrated by infusion, frequent injection of peptide drugs cause considerable discomfort to a subject. Thus, there is a need for technologies that will prolong the half-lives of therapeutic polypeptides while maintaining a high pharmacological efficacy thereof. Such desirous peptide drugs should also meet the requirements of enhanced serum stability, high activity and a low probability of inducing an undesired immune response when injected into a subject.

[0003] Unfavorable pharmacokinetics, such as a short serum half-life, can prevent the pharmaceutical development of many otherwise promising drug candidates. Serum half-life is an empirical characteristic of a molecule, and must be determined experimentally for each new potential drug. For example, with lower molecular weight polypeptide drugs, physiological clearance mechanisms such as renal filtration can make the maintenance of therapeutic levels of a drug unfeasible because of cost or frequency of the required dosing regimen. Conversely, a long serum half-life is undesirable where a drug or its metabolites have toxic side effects.

[0004] US Patent No. 5,759,818 describes partial and complete chorionic gonadotropin carboxy terminal peptide (CTP) units for modifying biologically active proteins and peptides at the amino terminus of these proteins and peptides, and altering their clearance patterns.

SUMMARY OF THE INVENTION

[0005] The present invention relates to treating a growth, weight-related or metabolic condition in a subject using a therapeutically effective amount of CTP-hGH. hGH is used for growth disorders in general and growth hormone deficiency related disorders in particular. In Example 8, we have demonstrated growth gain in hipophysectomized rats (which have no growth

hormone secretion) following injections of CTP-hGH.

[0006] The present invention provides a polypeptide comprising a biological activity, said polypeptide comprising a peptide of interest, a single chorionic gonadotropin carboxy terminal peptide (CTP) attached to the amino terminus of said peptide of interest, and two chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said peptide of interest, wherein said polypeptide is a mature polypeptide wherein the N-terminal signal peptide has been cleaved from the precursor protein, wherein the amino acid sequence of said precursor protein is set forth in SEQ ID NO: 39, and wherein said peptide of interest is hGH.

[0007] The present invention also provides a method of improving a biological half-life of a peptide of interest, comprising the step of attaching a single chorionic gonadotropin carboxy terminal peptide to the amino terminus of said peptide of interest and two chorionic gonadotropin carboxy terminal peptides to the carboxy terminus of said peptide of interest to form a CTP-modified polypeptide, thereby improving said biological half-life of said peptide of interest, wherein said CTP-modified polypeptide is a mature polypeptide wherein the N-terminal signal peptide has been cleaved from the precursor protein, wherein the amino acid sequence of said precursor protein is set forth in SEQ ID NO: 39, and wherein said peptide of interest is hGH.

[0008] In an embodiment, the peptide of interest is glycosylated. In an alternative embodiment, the peptide of interest is not glycosylated.

[0009] In an embodiment, at least one of the chorionic gonadotropin carboxy terminal peptides is glycosylated.

[0010] The present invention also provides a pharmaceutical formulation comprising the polypeptide of the invention. In an embodiment, the pharmaceutical formulation further comprises a buffer, such a citrate or acetate buffer, and a tonicity agent, such as sodium chloride. In an embodiment, the formulation is formulated for injection in a multidose container, with optionally, a preservative, such as benzalkonium chloride or thimerosal. In an embodiment, the formulation is a liquid formulation.

[0011] The present invention also provides the polypeptide or the pharmaceutical formulation of the invention for use as a medicament.

[0012] The present invention also provides the polypeptide or the pharmaceutical formulation of the invention for use in treating a growth, weight-related or metabolic condition.

[0013] The present invention also provides the use of the polypeptide or the pharmaceutical formulation of the invention in the manufacture of a medicament for the treatment of a growth, weight-related or metabolic condition. In an embodiment, the medicament comprises a unit dosage for a once a week administration.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014]

FIGS. 1A-1F are diagrams illustrating six EPO-CTP constructs.

Figure 1A - is a diagram of the polypeptide of SEQ ID NO: 1

Figure 1B is a diagram of the polypeptide of SEQ ID NO: 2

Figure 1C is a diagram of the polypeptide of SEQ ID NO: 3

Figure 1D is a diagram of the polypeptide of SEQ ID NO: 4.

Figure IE is a diagram of the polypeptide of SEQ ID NO: 5.

Figure IF is a diagram of the polypeptide of SEQ ID NO: 6.

FIG. 2 is a photograph illustrating the expression of the EPO-CTP variants from transfected DG44 cells. Final test samples from transfected cells were prepared as described under "sample preparation" and run on SDS/PAGE. Proteins were detected by western blot.

FIG. 3 is a graph illustrating the in vivo bioactivity of recombinant hEPO derivatives and EPO-3 (SEQ ID NO: 3). ICR mice (n=7/group) received a single IV injection/week (15µg/kg) for three weeks of EPO-3, rhEPO-WT (SEQ ID NO: 16), Recormon (Commercial EPO) or Recormon (5µg/kg) 3 times a week. Control animals were injected IV with PBS. Blood samples were collected three times a week and haematocrit levels were detected. Each point represents the group average of haematocrit (%) ± SE.

FIG. 4 is a graph illustrating the in vivo bioactivity of recombinant hEPO derivatives and EPO-1 (SEQ ID NO: 1). ICR mice (n=7/group) received a single IV injection/week (15µg/kg) for three weeks of EPO-1, rhEPO-WT (SEQ ID NO: 16), Recormon or Recormon (5µg/kg) 3 times a week. Control animals were injected IV with PBS. Blood samples were collected three times a week and haematocrit levels were detected. Each point represents the group average of haematocrit (%) ± SE.

FIG. 5 is a graph illustrating the in vivo bioactivity of recombinant hEPO derivatives and EPO-2 (SEQ ID NO: 2). ICR mice (n=7/group) received a single IV injection/week (15µg/kg) for three weeks of EPO-2 (SEQ ID NO: 2), rhEPO-WT (SEQ ID NO: 16), Recormon or Recormon (5µg/kg) 3 times a week. Control animals were injected IV with PBS. Blood samples were collected three times a week and haematocrit levels were detected. Each point represents the group average of haematocrit (%) ± SE.

FIG. 6 is a time graph illustrating the change in reticulocyte level following a single bolus dose of EPO-0 (SEQ ID NO: 16), EPO-3 (SEQ ID NO: 3) and Aranesp.

FIG. 7 is a time graph illustrating the change in hemoglobin level (presented as change from

baseline) following a single bolus dose of EPO-0 (SEQ ID NO: 16), EPO-3 (SEQ ID NO: 3) and Aranesp.

FIG. 8 is a time graph illustrating the change in hematocrit level following a single bolus dose of EPO-0 (SEQ ID NO: 16), EPO-3 (SEQ ID NO: 3) and Aranesp.

FIG. 9 is a graph illustrating the change in serum concentration of EPO -0 (SEQ ID NO: 16), EPO-3 (SEQ ID NO: 3) and Aranesp post i.v. injection.

FIG. 10 is a Western blot illustrating the molecular weight & identity of MOD-4020 (SEQ ID NO: 36), MOD-4021 (SEQ ID NO: 37), MOD-4022 (SEQ ID NO: 38), MOD-4023 (SEQ ID NO: 39) and MOD-4024 (SEQ ID NO: 40). PAGE SDS gel was blotted and stained using monoclonal anti-hGH antibodies. The photograph indicates that like commercial and wild type hGH, MOD-7020-4 variants are recognized by anti hGH antibodies.

FIG. 11 is a bar graph illustrating the weight gain of hypophysectomized rats following administration of the GH-CTP polypeptides of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

[0015] The present application describes long-acting polypeptides and methods of producing and using same. The long-acting polypeptides comprise carboxy terminal peptide (CTP) of human Chorionic Gonadotropin (hCG). In one embodiment, CTP acts as a protectant against degradation of proteins or peptides derived therefrom. In one embodiment, CTP extends circulatory half-lives of proteins or peptides derived therefrom. In some embodiments, CTP enhances the potency of proteins or peptides derived therefrom.

[0016] Thus the present invention provides a polypeptide comprising a biological activity, said polypeptide comprising a peptide of interest, a single chorionic gonadotropin carboxy terminal peptide (CTP) attached to the amino terminus of said peptide of interest, and two chorionic gonadotropin carboxy terminal peptides (CTPs) attached to the carboxy terminus of said peptide of interest, wherein said polypeptide is a mature polypeptide wherein the N-terminal signal peptide has been cleaved from the precursor protein, wherein the amino acid sequence of said precursor protein is set forth in SEQ ID NO: 39, and wherein said peptide of interest is hGH.

[0017] The present invention also provides a method of improving a biological half-life of a peptide of interest, comprising the step of attaching a single chorionic gonadotropin carboxy terminal peptide to the amino terminus of said peptide of interest and two chorionic gonadotropin carboxy terminal peptides to the carboxy terminus of said peptide of interest to form a CTP-modified polypeptide, thereby improving said biological half-life of said peptide of interest, wherein said CTP-modified polypeptide is a mature polypeptide wherein the N-terminal signal peptide has been cleaved from the precursor protein, wherein the amino acid sequence

of said precursor protein is set forth in SEQ ID NO: 39, and wherein said peptide of interest is hGH.

[0018] The terms "CTP peptide," "carboxy terminal peptide," and "CTP sequence" are used interchangeably herein. In an embodiment, the carboxy terminal peptide is a full-length CTP. In another embodiment, the carboxy terminal peptide is a truncated CTP.

[0019] In another embodiment, "signal sequence" and "signal peptide" are used interchangeably herein. In another embodiment, "sequence" when in reference to a polynucleotide can refer to a coding portion.

[0020] In another embodiment, "peptide of interest" and "polypeptide sequence-of-interest" are used interchangeably herein. In another embodiment, the peptide of interest is a full-length protein. In another embodiment, the peptide of interest is a protein fragment.

[0021] In another embodiment, the carboxy-terminal peptide (CTP) is attached to the polypeptide sequence of interest via a linker. In another embodiment, the linker which connects the CTP sequence to the polypeptide sequence of interest is a covalent bond. In another embodiment, the linker which connects the CTP sequence to the polypeptide sequence of interest is a peptide bond. In another embodiment, the linker which connects the CTP sequence to the polypeptide sequence of interest is a substituted peptide bond.

[0022] The phrase "polypeptide sequence of interest" refers to human growth hormone (hGH) polypeptides. In another embodiment, the peptide is glycosylated. In another embodiment, the peptide is non-glycosylated..

[0023] In another embodiment, the carboxy terminal peptide (CTP) of human Chorionic Gonadotropin (hCG) is fused to hGH.

[0024] It is disclosed that CTP sequences at both the amino terminal end of a polypeptide and at the carboxy terminal end of the polypeptide provide enhanced protection against degradation of a protein. In some embodiments, CTP sequences at both the amino terminal end of a polypeptide and at the carboxy terminal end of the polypeptide provide extended half-life of the attached protein.

[0025] In this invention, a CTP sequence at the amino terminal end of a polypeptide, a CTP sequence at the carboxy terminal end of the polypeptide, and an additional CTP sequence attached in tandem to the CTP sequence at the carboxy terminus provide enhanced protection against degradation of a protein. In some embodiments, a CTP sequence at the amino terminal end of a polypeptide, a CTP sequence at the carboxy terminal end of the polypeptide, and an additional CTP sequence attached in tandem to the CTP sequence at the carboxy terminus provide extended half-life of the attached protein. In some embodiments, a CTP sequence at the amino terminal end of a polypeptide, a CTP sequence at the carboxy terminal end of the polypeptide, and an additional CTP sequence attached in tandem to the CTP sequence at the

carboxy terminus provide enhanced activity of the attached protein.

[0026] In another embodiment, the carboxy terminal peptide (CTP) peptide of the present invention comprises the amino acid (AA) sequence from AA 112 to position 145 of human chorionic gonadotropin, as set forth in SEQ ID NO: 17. In another embodiment, the CTP sequence of the present invention comprises the AA sequence from AA 118 to position 145 of human chorionic gonadotropin, as set forth in SEQ ID NO: 18. In another embodiment, the CTP sequence also commences from any position between positions 112-118 and terminates at position 145 of human chorionic gonadotropin. In some embodiments, the CTP sequence peptide is 28, 29, 30, 31, 32, 33 or 34 AAs long and commences at position 112, 113, 114, 115, 116, 117 or 118 of the CTP AA sequence.

[0027] In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 1-5 conservative AA substitutions as described in U.S. Pat. No. 5,712,122. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 1 conservative AA substitution. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 2 conservative AA substitutions. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 3 conservative AA substitutions. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 4 conservative AA substitutions. In another embodiment, the CTP peptide is a variant of chorionic gonadotropin CTP which differs from the native CTP by 5 conservative AA substitutions. In another embodiment, the CTP peptide AA sequence of the present invention is at least 70% homologous to the native CTP AA sequence or a peptide thereof. In another embodiment, the CTP peptide AA sequence of the present invention is at least 80% homologous to the native CTP AA sequence or a peptide thereof. In another embodiment, the CTP peptide AA sequence of the present invention is at least 90% homologous to the native CTP AA sequence or a peptide thereof. In another embodiment, the CTP peptide AA sequence of the present invention is at least 95% homologous to the native CTP AA sequence or a peptide thereof.

[0028] In another embodiment, the CTP peptide DNA sequence of the present invention is at least 70% homologous to the native CTP DNA sequence or a peptide thereof. In another embodiment, the CTP peptide DNA sequence of the present invention is at least 80% homologous to the native CTP DNA sequence or a peptide thereof. In another embodiment, the CTP peptide DNA sequence of the present invention is at least 90% homologous to the native CTP DNA sequence or a peptide thereof. In another embodiment, the CTP peptide DNA sequence of the present invention is at least 95% homologous to the native CTP DNA sequence or a peptide thereof.

[0029] In another embodiment, at least one of the chorionic gonadotropin CTP AA sequences is truncated. In another embodiment, 2 of the chorionic gonadotropin CTP AA sequences are truncated. In another embodiment, 2 or more of the chorionic gonadotropin CTP AA sequences are truncated. In another embodiment, all of the chorionic gonadotropin CTP AA sequences are

truncated. In another embodiment, the truncated CTP comprises the first 10 AA of SEQ ID NO:43, i.e., the amino acid sequence SSSSKAPPPS. In another embodiment, the truncated CTP comprises the first 11 AA of SEQ ID NO:43. In another embodiment, the truncated CTP comprises the first 12 AA of SEQ ID NO:43. In another embodiment, the truncated CTP comprises the first 13 AA of SEQ ID NO:43. In another embodiment, the truncated CTP comprises the first 14 AA of SEQ ID NO:43. In another embodiment, the truncated CTP comprises the first 15 AA of SEQ ID NO:43. In another embodiment, the truncated CTP comprises the first 16 AA of SEQ ID NO:43. In another embodiment, the truncated CTP comprises the last 14 AA of SEQ ID NO:43.

[0030] In another embodiment, at least one of the chorionic gonadotropin CTP AA sequences is glycosylated. In another embodiment, 2 of the chorionic gonadotropin CTP AA sequences are glycosylated. In another embodiment, 2 or more of the chorionic gonadotropin CTP AA sequences are glycosylated. In another embodiment, all of the chorionic gonadotropin CTP AA sequences are glycosylated. In another embodiment, the CTP sequence of the present invention comprises at least one glycosylation site. In another embodiment, the CTP sequence of the present invention comprises 2 glycosylation sites. In another embodiment, the CTP sequence of the present invention comprises 3 glycosylation sites. In another embodiment, the CTP sequence of the present invention comprises 4 glycosylation sites.

[0031] It is disclosed herein that erythropoietin (EPO) may be utilized according to the teachings disclosed herein. In some embodiments, the attachment of CTP sequence to both the amino and carboxy termini of the EPO protein results in increased potency at stimulating erythropoiesis (Figures 3-5) and (Table 5 of Example 4), as compared to recombinant EPO and other combinations of EPO and CTP. In some embodiments, an EPO attached to three CTP sequences does not impair binding to its receptor as evidenced in Table 3 of Example 3 which demonstrates that EPO attached to three CTP sequences is equally effective at stimulating proliferation of TF-1 cells as wild-type EPO. In some embodiments EPO-CTP polypeptides of the present invention are set forth in SEQ ID NO: 3 and SEQ ID NO: 6.

[0032] In this invention, human growth hormone (hGH) is utilized as the peptide of interest according to the teachings of the present invention. In some embodiments, the attachment of CTP sequence to both the amino and carboxy termini of the hGH protein results in increased potency (Figures 11). In some embodiments, the attachment of CTP sequence to both the amino and carboxy termini of the hGH protein results in prolonged in-vivo activity. In one embodiment, CTP-hGH polypeptides are set forth in SEQ ID NO: 39- 41.

[0033] In one embodiment, as disclosed herein, the phrase "human growth hormone" (hGH) refers to a polypeptide, such as set forth in Genbank Accession No. P01241 (SEQ ID NO: 47), exhibiting hGH activity (i.e. stimulation of growth).

[0034] It is disclosed that "human growth hormone" (hGH) refers to a polypeptide, such as set forth in Genbank Accession No. P01241, exhibiting hGH activity (i.e. stimulation of growth). In one embodiment, hGH of the present invention also refers to homologs. In one embodiment,

hGH AA sequence of the present invention is at least 50% homologous to an hGH sequence set forth in GenBank Accession No. P01241 as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters). In one embodiment, hGH AA sequence of the present invention is at least 60% homologous to an hGH sequence set forth in GenBank Accession No. P01241 as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters). In one embodiment, hGH AA sequence of the present invention is at least 70% homologous to an hGH sequence set forth in GenBank Accession No. P01241 as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters). In one embodiment, hGH AA sequence of the present invention is at least 80% homologous to an hGH sequence set forth in GenBank Accession No. P01241 as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters). In one embodiment, hGH AA sequence of the present invention is at least 90% homologous to an hGH sequence set forth in GenBank Accession No. P01241 as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters). In one embodiment, hGH AA sequence of the present invention is at least 95% homologous to an hGH sequence set forth in GenBank Accession No. P01241 as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters).

[0035] The hGH polypeptide in the present invention is a mature hGH protein of SEQ ID NO: 39 from which the signal peptide has been cleaved.

[0036] Other CTP-hGH polypeptides are set forth in SEQ ID NO: 40 and SEQ ID NO: 41.

[0037] Disclosed herein is an hGh peptide having additionally at least one CTP AA peptide on the N-terminus and at least one CTP AA peptide on the C-terminus for stimulating muscle growth, for stimulating bone growth, for treating a wasting disease, for increasing cardiac function, for increasing lipolysis, for improving fluid balance, for treating osteoporosis, for inhibiting osteoporosis, for improving lung function, for improving immunity, for regrowing vital organs or for restoring REM sleep in a subject.

[0038] The present invention provides the hGh peptide having additionally one CTP AA peptide on the N-terminus and two CTP AA peptides on the C-terminus for use in stimulating muscle growth, stimulating bone growth, treating a wasting disease, increasing cardiac function, increasing lipolysis, improving fluid balance, treating osteoporosis, inhibiting osteoporosis, improving lung function, improving immunity, regrowing vital organs or restoring REM sleep in a subject..

[0039] The present invention provides the CTP-modified hGH peptide of SEQ ID NO: 39, from which the signal peptide has been cleaved, for use in stimulating muscle growth, stimulating bone growth, treating a wasting disease, increasing cardiac function, increasing lipolysis, improving fluid balance, treating osteoporosis, inhibiting osteoporosis, improving lung function, improving immunity, regrowing vital organs or restoring REM sleep in a subject.

[0040] Also disclosed herein is an hGH peptide set forth in SEQ ID NO: 23 having additionally at least one CTP AA peptide on the N-terminus and at least one CTP AA peptide on the C-terminus, an hGH peptide set forth in SEQ ID NO: 36 having additionally at least one CTP AA peptide on the N-terminus and at least one CTP AA peptide on the C-terminus, an hGH peptide set forth in SEQ ID NO: 37 having additionally at least one CTP AA peptide on the N-terminus, an hGH peptide set forth in SEQ ID NO: 38 having additionally at least one CTP AA peptide on the N-terminus, an hGH peptide set forth in SEQ ID NO: 40, an hGH peptide set forth in SEQ ID NO: 41, an hGH peptide set forth in SEQ ID NO: 42 having additionally at least one CTP AA peptide on the N-terminus for stimulating muscle growth, or an hGH peptide set forth in SEQ ID NO: 44 for stimulating muscle growth, for stimulating bone growth, for treating a wasting disease, for increasing cardiac function, for increasing lipolysis, for improving fluid balance, for treating osteoporosis, for inhibiting osteoporosis, for improving lung function, for improving immunity, for regrowing vital organs or for restoring REM sleep in a subject.

[0041] Also disclosed herein is a nucleic acid sequence encoding an hGH peptide having additionally at least one CTP AA peptide on the N-terminus and at least one CTP AA peptide on the C-terminus, a nucleic acid sequence encoding an hGH peptide having additionally one CTP AA peptide on the N-terminus and two CTP AA peptides on the C-terminus, a nucleic acid of SEQ ID NO: 45 encoding an hGH peptide comprising one CTP AA peptide on the N-terminus and two CTP AA peptides on the C-terminus, and a nucleic acid of SEQ ID NO: 46 encoding an hGH peptide and one CTP AA peptide on the N-terminus and two CTP AA peptides on the C-terminus for stimulating muscle growth, for stimulating bone growth, for treating a wasting disease, for increasing cardiac function, for increasing lipolysis, for improving fluid balance, for treating osteoporosis, for inhibiting osteoporosis, for improving lung function, for improving immunity, for regrowing vital organs or for restoring REM sleep in a subject.

[0042] In another embodiment, the hGh peptide of the present invention may also be for use in maintaining muscle quality or bone quality or for use in improving exercise capacity.

[0043] In another embodiment, the hGh peptide of the present invention may be used for increasing sense of well-being .

[0044] In some embodiments, homology according to the present invention also encompasses deletions, insertions, or substitution variants, including an AA substitution, thereof and biologically active polypeptide fragments thereof. In one embodiment the substitution variant is one, in which the glutamine in position 65 of hGH is substituted by a valine (SEQ ID NO: 23) [Gellerfors et al., J Pharm Biomed Anal 1989, 7:173-83].

[0045] In one embodiment, polypeptides, antibodies, or polynucleotides of the present invention are administered to a human. In

[0046] In some embodiments, the CTP sequences modification is advantageous in permitting lower dosages to be used.

[0047] In some embodiments, "polypeptide" as used herein encompasses native polypeptides (either degradation products, synthetically synthesized polypeptides or recombinant polypeptides) and peptidomimetics (typically, synthetically synthesized polypeptides), as well as peptoids and semipeptoids which are polypeptide analogs, which have, in some embodiments, modifications rendering the polypeptides even more stable while in a body or more capable of penetrating into cells.

[0048] In some embodiments, modifications include, but are not limited to N terminus modification, C terminus modification, polypeptide bond modification, including, but not limited to, CH2-NH, CH2-S, CH2-S=O, O=C-NH, CH2-O, CH2-CH2, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992). Further details in this respect are provided hereinunder.

[0049] In some embodiments, polypeptide bonds (-CO-NH-) within the polypeptide are substituted. In some embodiments, the polypeptide bonds are substituted by N-methylated bonds (-N(CH3)-CO-). In some embodiments, the polypeptide bonds are substituted by ester bonds (-C(R)H-C-O-O-C(R)-N-). In some embodiments, the polypeptide bonds are substituted by ketomethylen bonds (-CO-CH2-). In some embodiments, the polypeptide bonds are substituted by α-aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH2-NH-). In some embodiments, the polypeptide bonds are substituted by hydroxyethylene bonds (-CH(OH)-CH2-). In some embodiments, the polypeptide bonds are substituted by thioamide bonds (-CS-NH-). In some embodiments, the polypeptide bonds are substituted by olefinic double bonds (-CH=CH-). In some embodiments, the polypeptide bonds are substituted by retro amide bonds (-NH-CO-). In some embodiments, the polypeptide bonds are substituted by polypeptide derivatives (-N(R)-CH2-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom. In some embodiments, these modifications occur at any of the bonds along the polypeptide chain and even at several (2-3 bonds) at the same time.

[0050] In some embodiments, natural aromatic AA of the polypeptide such as Trp, Tyr and Phe, be substituted for synthetic non-natural acid such as Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr. In some embodiments, the polypeptides of the present invention include one or more modified AA or one or more non-AA monomers (e.g. fatty acid, complex carbohydrates etc).

[0051] In one embodiment, "AA" or "AA" is understood to include the 20 naturally occurring AA; those AA often modified post-translationally *in vivo,* including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual AA including, but not limited to, 2-aminoadipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. In one embodiment, "AA" includes both D- and L-AA.

[0052] In some embodiments, the polypeptides of the present invention are utilized in therapeutics which require the polypeptides to be in a soluble form. In some embodiments, the

polypeptides of the present invention include one or more non-natural or natural polar AA, including but not limited to serine and threonine which are capable of increasing polypeptide solubility due to their hydroxyl-containing side chain.

[0053] In some embodiments, the polypeptides of the present invention are utilized in a linear form, although it will be appreciated by one skilled in the art that in cases where cyclicization does not severely interfere with polypeptide characteristics, cyclic forms of the polypeptide can also be utilized.

[0054] In some embodiments, the polypeptides of present invention are biochemically synthesized such as by using standard solid phase techniques. In some embodiments, these biochemical methods include exclusive solid phase synthesis, partial solid phase synthesis, fragment condensation, or classical solution synthesis. In some embodiments, these methods are used when the polypeptide is relatively short (about 5-15kDa) and/or when it cannot be produced by recombinant techniques (i.e., not encoded by a nucleic acid sequence) and therefore involves different chemistry.

[0055] In some embodiments, solid phase polypeptide synthesis procedures are well known to one skilled in the art and further described by John Morrow Stewart and Janis Dillaha Young, Solid Phase Polypeptide Syntheses (2nd Ed., Pierce Chemical Company, 1984). In some embodiments, synthetic polypeptides are purified by preparative high performance liquid chromatography [Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] and the composition of which can be confirmed via AA sequencing by methods known to one skilled in the art.

[0056] In some embodiments, recombinant protein techniques are used to generate the polypeptides of the present invention. In some embodiments, recombinant protein techniques are used for generation of relatively long polypeptides (e.g., longer than 18-25 AAs). In some embodiments, recombinant protein techniques are used for the generation of large amounts of the polypeptide of the present invention. In some embodiments, recombinant techniques are described by Bitter et al., (1987) Methods in Enzymol. 153:516-544, Studier et al. (1990) Methods in Enzymol. 185:60-89, Brisson et al. (1984) Nature 310:511-514, Takamatsu et al. (1987) EMBO J. 6:307-311, Coruzzi et al. (1984) EMBO J. 3:1671-1680 and Brogli et al., (1984) Science 224:838-843, Gurley et al. (1986) Mol. Cell. Biol. 6:559-565 and Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463.

[0057] In one embodiment, a polypeptide of the present invention is synthesized using a polynucleotide encoding a polypeptide of the present invention. In some embodiments, the polynucleotide encoding a polypeptide of the present invention is ligated into an expression vector, comprising a transcriptional control of a cis-regulatory sequence (e.g., promoter sequence). In some embodiments, the cis-regulatory sequence is suitable for directing constitutive expression of the polypeptide of the present invention. In some embodiments, the cis-regulatory sequence is suitable for directing tissue specific expression of the polypeptide of

the present invention. In some embodiments, the cis-regulatory sequence is suitable for directing inducible expression of the polypeptide of the present invention.

[0058] In some embodiments, polynucleotides which express the polypeptides of the present invention are as set forth in SEQ ID NOs: 44, 45 and 46.

[0059] Suitable tissue-specific promoters include sequences which are functional in specific cell population, example include, but are not limited to promoters such as albumin that is liver specific [Pinkert et al., (1987) Genes Dev. 1:268-277], lymphoid specific promoters [Calame et al., (1988) Adv. Immunol. 43:235-275]; in particular promoters of T-cell receptors [Winoto et al., (1989) EMBO J. 8:729-733] and immunoglobulins; [Banerji et al. (1983) Cell 33729-740], neuron-specific promoters such as the neurofilament promoter [Byrne et al. (1989) Proc. Natl. Acad. Sci. USA 86:5473-5477], pancreas-specific promoters [Edlunch et al. (1985) Science 230:912-916] or mammary gland-specific promoters such as the milk whey promoter (U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Inducible promoters suitable for use with the present invention include for example the tetracycline-inducible promoter (Srour, M.A., et al., 2003. Thromb. Haemost. 90: 398-405).

[0060] In one embodiment, the phrase "a polynucleotide" refers to a single or double stranded nucleic acid sequence which be isolated and provided in the form of an RNA sequence, a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

[0061] In one embodiment, "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. In one embodiment, the sequence can be subsequently amplified *in vivo* or *in vitro* using a DNA polymerase.

[0062] In one embodiment, "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

[0063] In one embodiment, "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. In one embodiment, a composite sequence can include some exonal sequences required to encode the polypeptide of the present invention, as well as some intronic sequences interposing therebetween. In one embodiment, the intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. In one embodiment, intronic sequences include cis acting expression regulatory elements. In one embodiment, the polynucleotides further comprise a signal sequence encoding a signal peptide for the secretion of the polypeptides of the present invention. In some embodiments, signal sequences include, but are not limited to the endogenous signal sequence for EPO as set forth in SEQ ID NO: 19 or the endogenous signal sequence for IFN-beta 1 as set forth in SEQ ID NO: 26. The signal sequence may be N-terminal to the CTP sequence that is in turn N-terminal to the polypeptide sequence of interest; e.g. the sequence is (a) signal sequence- (b) CTP- (c) sequence-of-

interest- (d) two additional CTP sequences. In one embodiment, following expression and secretion, the signal peptides are cleaved from the precursor proteins resulting in the mature proteins.

[0064] In some embodiments, the polynucleotides may be prepared using PCR techniques as described in Example 1, or any other method or procedure known to one skilled in the art. In some embodiments, the procedure involves the ligation of two different DNA sequences (See, for example, "Current Protocols in Molecular Biology", eds. Ausubel et al., John Wiley & Sons, 1992).

[0065] In one embodiment, the polynucleotides are inserted into expression vectors (i.e., a nucleic acid construct) to enable expression of the recombinant polypeptide of the present invention. In one embodiment, the expression vector includes additional sequences which render this vector suitable for replication and integration in prokaryotes. In one embodiment, the expression vector includes additional sequences which render this vector suitable for replication and integration in eukaryotes. In one embodiment, the expression vector includes a shuttle vector which renders this vector suitable for replication and integration in both prokaryotes and eukaryotes. In some embodiments, cloning vectors comprise transcription and translation initiation sequences (e.g., promoters, enhances) and transcription and translation terminators (e.g., polyadenylation signals).

[0066] A variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the polypeptides of the present invention. In some embodiments, these include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the polypeptide coding sequence; yeast transformed with recombinant yeast expression vectors containing the polypeptide coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the polypeptide coding sequence.

[0067] In some embodiments, non-bacterial expression systems are used (e.g. mammalian expression systems such as CHO cells) to express the polypeptide of the present invention. In one embodiment, the expression vector used to express polynucleotides of the present invention in mammalian cells is pCI-DHFR vector comprising a CMV promoter and a neomycin resistance gene. Construction of the pCI-dhfr vector is described, according to one embodiment, in Example 1.

[0068] In some embodiments, in bacterial systems, a number of expression vectors can be advantageously selected depending upon the use intended for the polypeptide expressed. In one embodiment, large quantities of polypeptide are desired. In one embodiment, vectors that direct the expression of high levels of the protein product, possibly as a fusion with a hydrophobic signal sequence, which directs the expressed product into the periplasm of the bacteria or the culture medium where the protein product is readily purified are desired. In one

embodiment, certain fusion protein engineered with a specific cleavage site to aid in recovery of the polypeptide. In one embodiment, vectors adaptable to such manipulation include, but are not limited to, the pET series of *E. coli* expression vectors [Studier et al., Methods in Enzymol. 185:60-89 (1990)].

[0069] In one embodiment, yeast expression systems are used. In one embodiment, a number of vectors containing constitutive or inducible promoters can be used in yeast as disclosed in U.S. Pat. Application No: 5,932,447. In another embodiment, vectors which promote integration of foreign DNA sequences into the yeast chromosome are used.

[0070] In one embodiment, the expression vector can further include additional polynucleotide sequences that allow, for example, the translation of several proteins from a single mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide.

[0071] In some embodiments, mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

[0072] In some embodiments, expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses may be used. SV40 vectors include pSVT7 and pMT2. In some embodiments, vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein Bar virus include pHEBO, and p2O5. Other exemplary vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0073] It is disclosed herein that recombinant viral vectors may be useful for *in vivo* expression of the polypeptides of the present invention since they offer advantages such as lateral infection and targeting specificity. In one embodiment, lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. In one embodiment, the result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. In one embodiment, viral vectors are produced that are unable to spread laterally. In one embodiment, this characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

[0074] Various methods can be used to introduce the expression vector of the present invention into cells. Such methods are generally described in Sambrook et al., Molecular Cloning: A

Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

[0075] In some embodiments, introduction of nucleic acid by viral infection offers several advantages over other methods such as lipofection and electroporation, since higher transfection efficiency can be obtained due to the infectious nature of viruses.

[0076] As disclosed herein, it will be appreciated that the polypeptides of the present invention can also be expressed from a nucleic acid construct administered to the individual employing any suitable mode of administration, described hereinabove (i.e., in-vivo gene therapy). In one embodiment, the nucleic acid construct is introduced into a suitable cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the individual (i.e., ex-vivo gene therapy).

[0077] In vivo gene therapy using EPO has been attempted in animal models such as rodents [Bohl et al., Blood. 2000; 95:2793-2798], primates [Gao et al., Blood, 2004, Volume 103, Number 9] and has proven successful in human clinical trials for patients with chronic renal failure [Lippin et al Blood 2005, 106, Number 7].

[0078] In one embodiment, plant expression vectors are used. In one embodiment, the expression of a polypeptide coding sequence is driven by a number of promoters. In some embodiments, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV [Brisson et al., Nature 310:511-514 (1984)], or the coat protein promoter to TMV [Takamatsu et al., EMBO J. 6:307-311 (1987)] are used. In another embodiment,, plant promoters are used such as, for example, the small subunit of RUBISCO [Coruzzi et al., EMBO J. 3:1671-1680 (1984); and Brogli et al., Science 224:838-843 (1984)] or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B [Gurley et al., Mol. Cell. Biol. 6:559-565 (1986)]. In one embodiment, constructs are introduced into plant cells using Ti plasmid, Ri plasmid, plant viral vectors, direct DNA transformation, microinjection, electroporation and other techniques well known to the skilled artisan. See, for example, Weissbach & Weissbach [Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463 (1988)]. Other expression systems such as insects and mammalian host cell systems, which are well known in the art, can also be used.

[0079] It will be appreciated that other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the polypeptide), the expression constructs can also include sequences engineered to optimize stability, production, purification, yield or activity of the expressed polypeptide.

[0080] Various methods, in some embodiments, can be used to introduce the expression vector into the host cell system. In some embodiments, such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

[0081] In some embodiments, transformed cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant polypeptide. In some embodiments, effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. In one embodiment, an effective medium refers to any medium in which a cell is cultured to produce the recombinant polypeptide of the present invention. In some embodiments, a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. In some embodiments, cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes and petri plates. In some embodiments, culturing is carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. In some embodiments, culturing conditions are within the expertise of one of ordinary skill in the art.

[0082] In some embodiments, depending on the vector and host system used for production, resultant polypeptides of the present invention either remain within the recombinant cell, secreted into the fermentation medium, secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or retained on the outer surface of a cell or viral membrane.

[0083] In one embodiment, following a predetermined time in culture, recovery of the recombinant polypeptide is effected.

[0084] In one embodiment, the phrase "recovering the recombinant polypeptide" used herein refers to collecting the whole fermentation medium containing the polypeptide and need not imply additional steps of separation or purification.

[0085] In one embodiment, polypeptides of the present invention are purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

[0086] In one embodiment, to facilitate recovery, the expressed coding sequence can be

engineered to encode the polypeptide of the present invention and fused cleavable moiety. In one embodiment, a fusion protein can be designed so that the polypeptide can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the cleavable moiety. In one embodiment, a cleavage site is engineered between the polypeptide and the cleavable moiety and the polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that specifically cleaves the fusion protein at this site [e.g., see Booth et al., Immunol. Lett. 19:65-70 (1988); and Gardella et al., J. Biol. Chem. 265:15854-15859 (1990)].

[0087] In one embodiment, the polypeptide of the present invention is retrieved in "substantially pure" form.

[0088] In one embodiment, the phrase "substantially pure" refers to a purity that allows for the effective use of the protein in the applications described herein.

[0089] In one embodiment, the polypeptide of the present invention can also be synthesized using *in vitro* expression systems. In one embodiment, *in vitro* synthesis methods are well known in the art and the components of the system are commercially available.

[0090] Production of CTP-EPO-CTP polypeptides using recombinant DNA technology is illustrated in Example 1.

[0091] In some embodiments, the recombinant polypeptides are synthesized and purified; their therapeutic efficacy can be assayed either in vivo or in vitro. The binding activities of recombinant EPO polypeptides can be ascertained using various assays as described in Examples 2-6 and 8-9. In one embodiment, in vitro binding activity is ascertained by measuring the ability of the polypeptide to stimulate proliferation of TF-1 cells. In one embodiment, in vivo activity is deduced by analyzing heamatocrit levels (Figures 3-5) and/or as a percentage of reticulocytes.

[0092] In one embodiment, the present invention comprises CTP-hGH-CTP polypeptides. In one embodiment, recombinant DNA technology methods are used for the production of CTP-hGH-CTP polypeptides as illustrated in Example 7. In one embodiment, the therapeutic efficacy of the CTP-hGH-CTP polypeptides of the present invention is assayed in vivo. In one embodiment, the therapeutic efficacy of the CTP-hGH-CTP polypeptides of the present invention is assayed in vitro. In one embodiment, the binding activities of the recombinant hGH polypeptides of the present invention are measured using Nb2 (a prolactin-dependent rat lymphoma cell line (ECACC Cell Bank)) or a FCD-P1 murine cell line, previously transfected with human growth hormone receptor. In one embodiment, binding of hGH to these receptors induces cell proliferation which in one embodiment is measured by the levels of MTT cellular stain as a function of hGH activity. In one embodiment, in vivo activity is deduced by measuring weight gain over time in treated growth hormone deficient animals.

[0093] In some embodiment, human growth hormone polypeptides of the present invention are

for use in treating a subject, with conditions related to growth and weight, such as a growth deficiency disorder, AIDS wasting, aging, impaired immune function of HIV-infected subjects, a catabolic illness, surgical recovery, a congestive cardiomyopathy, liver transplantation, liver regeneration after hepatectomy, chronic renal failure, renal osteodystrophy, osteoporosis, achondroplasia/hypochondroplasia, skeletal dysplasia, a chronic inflammatory or nutritional disorder such as Crohn's disease, short bowel syndrome, juvenile chronic arthritis, cystic fibrosis, male infertility, X-linked hypophosphatemic rickets, Down's syndrome, Spina bifida, Noonan Syndrome, obesity, impaired muscle strength and fibromyalgia.

[0094] In one embodiment, the polypeptides of the present invention can be provided to the individual *per se.* In one embodiment, the polypeptides of the present invention can be provided to the individual as part of a pharmaceutical composition where it is mixed with a pharmaceutically acceptable carrier.

[0095] In one embodiment, a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

[0096] In one embodiment, "active ingredient" refers to the polypeptide sequence of interest, which is accountable for the biological effect.

[0097] In one embodiment, the present invention provides combined preparations. In one embodiment, "a combined preparation" defines especially a "kit of parts" in the sense that the combination partners as defined above can be dosed independently or by use of different fixed combinations with distinguished amounts of the combination partners i.e., simultaneously, concurrently, separately or sequentially. In some embodiments, the parts of the kit of parts can then, e.g., be administered simultaneously or chronologically staggered, that is at different time points and with equal or different time intervals for any part of the kit of parts. The ratio of the total amounts of the combination partners, in some embodiments, can be administered in the combined preparation. In one embodiment, the combined preparation can be varied, e.g., in order to cope with the needs of a patient subpopulation to be treated or the needs of the single patient which different needs can be due to a particular disease, severity of a disease, age, sex, or body weight as can be readily made by a person skilled in the art.

[0098] In one embodiment, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases. In one embodiment, one of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media (Mutter et al. (1979).

[0099] In one embodiment, "excipient" refers to an inert substance added to a pharmaceutical

composition to further facilitate administration of an active ingredient. In one embodiment, excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

[0100] Techniques for formulation and administration of drugs are found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA.

[0101] In one embodiment, suitable routes of administration, for example, include oral, rectal, transmucosal, transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, inrtaperitoneal, intranasal, or intraocular injections.

[0102] In one embodiment, the preparation is to be administered in a local rather than systemic manner, for example, via injection of the preparation directly into a specific region of a patient's body.

[0103] Various embodiments of dosage ranges are contemplated by this invention. The dosage of the polypeptide of the present invention, in one embodiment, is in the range of 0.05-80 mg/day. In another embodiment, the dosage is in the range of 0.05-50 mg/day. In another embodiment, the dosage is in the range of 0.1-20 mg/day. In another embodiment, the dosage is in the range of 0.1-10 mg/day. In another embodiment, the dosage is in the range of 0.1-5 mg/day. In another embodiment, the dosage is in the range of 0.5-5 mg/day. In another embodiment, the dosage is in the range of 0.5-50 mg/day. In another embodiment, the dosage is in the range of 5-80 mg/day. In another embodiment, the dosage is in the range of 35-65 mg/day. In another embodiment, the dosage is in the range of 35-65 mg/day. In another embodiment, the dosage is in the range of 20-60 mg/day. In another embodiment, the dosage is in the range of 40-60 mg/day. In another embodiment, the dosage is in a range of 45-60 mg/day. In another embodiment, the dosage is in the range of 40-60 mg/day. In another embodiment, the dosage is in a range of 60-120 mg/day. In another embodiment, the dosage is in the range of 120-240 mg/day. In another embodiment, the dosage is in the range of 40-60 mg/day. In another embodiment, the dosage is in a range of 240-400 mg/day. In another embodiment, the dosage is in a range of 45-60 mg/day. In another embodiment, the dosage is in the range of 15-25 mg/day. In another embodiment, the dosage is in the range of 5-10 mg/day. In another embodiment, the dosage is in the range of 55-65 mg/day.

[0104] In one embodiment, the dosage is 20 mg/day. In another embodiment, the dosage is 30 mg/day. In another embodiment, the dosage is 40 mg/day. In another embodiment, the dosage is 50 mg/day. In another embodiment, the dosage is 60 mg/day. In another embodiment, the dosage is 70 mg/day. In another embodiment, the dosage is 80 mg/day. In another embodiment, the dosage is 90 mg/day. In another embodiment, the dosage is 100 mg/day.

[0105] Oral administration, in one embodiment, comprises a unit dosage form comprising tablets, capsules, lozenges, chewable tablets, suspensions, emulsions and the like. Such unit dosage forms comprise a safe and effective amount of the desired compound, or compounds,

each of which is in one embodiment, from about 0.7 or 3.5 mg to about 280 mg/70 kg, or in another embodiment, about 0.5 or 10 mg to about 210 mg/70 kg. The pharmaceutically-acceptable carriers suitable for the preparation of unit dosage forms for peroral administration are well-known in the art. In some embodiments, tablets typically comprise conventional pharmaceutically-compatible adjuvants as inert diluents, such as calcium carbonate, sodium carbonate, mannitol, lactose and cellulose; binders such as starch, gelatin and sucrose; disintegrants such as starch, alginic acid and croscarmelose; lubricants such as magnesium stearate, stearic acid and talc. In one embodiment, glidants such as silicon dioxide can be used to improve flow characteristics of the powder-mixture. In one embodiment, coloring agents, such as the FD&C dyes, can be added for appearance. Sweeteners and flavoring agents, such as aspartame, saccharin, menthol, peppermint, and fruit flavors, are useful adjuvants for chewable tablets. Capsules typically comprise one or more solid diluents disclosed above. In some embodiments, the selection of carrier components depends on secondary considerations like taste, cost, and shelf stability, which are not critical for the purposes of this invention, and can be readily made by a person skilled in the art.

[0106] In one embodiment, the oral dosage form comprises predefined release profile. In one embodiment, the oral dosage form of the present invention comprises an extended release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form of the present invention comprises a slow release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form of the present invention comprises an immediate release tablets, capsules, lozenges or chewable tablets. In one embodiment, the oral dosage form is formulated according to the desired release profile of the pharmaceutical active ingredient as known to one skilled in the art.

[0107] Peroral compositions, in some embodiments, comprise liquid solutions, emulsions, suspensions, and the like. In some embodiments, pharmaceutically-acceptable carriers suitable for preparation of such compositions are well known in the art. In some embodiments, liquid oral compositions comprise from about 0.012% to about 0.933% of the desired compound or compounds, or in another embodiment, from about 0.033% to about 0.7%.

[0108] In some embodiments, compositions for use in this invention comprise solutions or emulsions, which in some embodiments are aqueous solutions or emulsions comprising a safe and effective amount of the compounds of the present invention and optionally, other compounds, intended for topical intranasal administration. In some embodiments, compositions comprise from about 0.01% to about 10.0% w/v of a subject compound, more preferably from about 0.1% to about 2.0, which is used for systemic delivery of the compounds by the intranasal route.

[0109] In another embodiment, the pharmaceutical compositions are to be administered by intravenous, intra-arterial, or intramuscular injection of a liquid preparation. In some embodiments, liquid formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In one embodiment, the pharmaceutical compositions are to be administered intravenously, and are thus formulated in a form suitable for intravenous administration. In

another embodiment, the pharmaceutical compositions are to be administered intra-arterially, and are thus formulated in a form suitable for intra-arterial administration. In another embodiment, the pharmaceutical compositions are to be administered intramuscularly, and are thus formulated in a form suitable for intramuscular administration.

[0110] Further, in another embodiment, the pharmaceutical compositions are to be administered topically to body surfaces, and are thus formulated in a form suitable for topical administration. Suitable topical formulations include gels, ointments, creams, lotions, drops and the like. For topical administration, the compounds of the present invention are combined with an additional appropriate therapeutic agent or agents, prepared and applied as solutions, suspensions, or emulsions in a physiologically acceptable diluent with or without a pharmaceutical carrier.

[0111] In one embodiment, pharmaceutical compositions of the present invention are manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

[0112] In one embodiment, pharmaceutical compositions for use in accordance with the present invention are formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which can be used pharmaceutically. In one embodiment, formulation is dependent upon the route of administration chosen.

[0113] In one embodiment, injectables of the invention are formulated in aqueous solutions. In one embodiment, injectables of the invention are formulated in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. In some embodiments, for transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0114] In one embodiment, the preparations described herein are formulated for parenteral administration, e.g., by bolus injection or continuous infusion. In some embodiments, formulations for injection are presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. In some embodiments, compositions are suspensions, solutions or emulsions in oily or aqueous vehicles, and contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

[0115] The compositions also comprise, in some embodiments, preservatives, such as benzalkonium chloride and thimerosal and the like; chelating agents, such as edetate sodium and others; buffers such as phosphate, citrate and acetate; tonicity agents such as sodium chloride, potassium chloride, glycerin, mannitol and others; antioxidants such as ascorbic acid, acetylcystine, sodium metabisulfote and others; aromatic agents; viscosity adjustors, such as polymers, including cellulose and derivatives thereof; and polyvinyl alcohol and acid and bases to adjust the pH of these aqueous compositions as needed. The compositions also comprise, in

some embodiments, local anesthetics or other actives. The compositions can be used as sprays, mists, drops, and the like.

[0116] In some embodiments, pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients, in some embodiments, are prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include, in some embodiments, fatty oils such as sesame oil, or synthetic fatty acid esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions contain, in some embodiments, substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. In another embodiment, the suspension also contains suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

[0117] In another embodiment, the active compound can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez- Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid).

[0118] In another embodiment, the pharmaceutical composition to be delivered in a controlled release system is formulated for intravenous infusion, implantable osmotic pump, transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump is used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989). In another embodiment, polymeric materials can be used. In yet another embodiment, a controlled release system can be placed in proximity to the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984). Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990).

[0119] In some embodiments, the active ingredient is in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use. Compositions are formulated, in some embodiments, for atomization and inhalation administration. In another embodiment, compositions are contained in a container with attached atomizing means.

[0120] In one embodiment, the preparation of the present invention is formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

[0121] In some embodiments, pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. In some embodiments, a therapeutically effective amount means an amount of active ingredients effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

[0122] In one embodiment, determination of a therapeutically effective amount is well within the capability of those skilled in the art.

[0123] The compositions also comprise preservatives, such as benzalkonium chloride and thimerosal and the like; chelating agents, such as edetate sodium and others; buffers such as phosphate, citrate and acetate; tonicity agents such as sodium chloride, potassium chloride, glycerin, mannitol and others; antioxidants such as ascorbic acid, acetylcystine, sodium metabisulfote and others; aromatic agents; viscosity adjustors, such as polymers, including cellulose and derivatives thereof; and polyvinyl alcohol and acid and bases to adjust the pH of these aqueous compositions as needed. The compositions also comprise local anesthetics or other actives. The compositions can be used as sprays, mists, drops, and the like.

[0124] Some examples of substances which can serve as pharmaceutically-acceptable carriers or components thereof are sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, and methyl cellulose; powdered tragacanth; malt; gelatin; talc; solid lubricants, such as stearic acid and magnesium stearate; calcium sulfate; vegetable oils, such as peanut oil, cottonseed oil, sesame oil, olive oil, corn oil and oil of theobroma; polyols such as propylene glycol, glycerine, sorbitol, mannitol, and polyethylene glycol; alginic acid; emulsifiers, such as the Tween™ brand emulsifiers; wetting agents, such sodium lauryl sulfate; coloring agents; flavoring agents; tableting agents, stabilizers; antioxidants; preservatives; pyrogen-free water; isotonic saline; and phosphate buffer solutions. The choice of a pharmaceutically-acceptable carrier to be used in conjunction with the compound is basically determined by the way the compound is to be administered. If the subject compound is to be injected, in one embodiment, the pharmaceutically-acceptable carrier is sterile, physiological saline, with a blood-compatible suspending agent, the pH of which has been adjusted to about 7.4.

[0125] In addition, the compositions further comprise binders (e.g. acacia, cornstarch, gelatin, carbomer, ethyl cellulose, guar gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, povidone), disintegrating agents (e.g. cornstarch, potato starch, alginic acid, silicon dioxide, croscarmelose sodium, crospovidone, guar gum, sodium starch glycolate), buffers (e.g., Tris-HCl., acetate, phosphate) of various pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), protease inhibitors, surfactants (e.g. sodium lauryl sulfate), permeation enhancers, solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite, butylated hydroxyanisole), stabilizers (e.g. hydroxypropyl cellulose, hyroxypropylmethyl cellulose), viscosity increasing agents(e.g. carbomer, colloidal silicon dioxide, ethyl cellulose, guar gum), sweeteners (e.g. aspartame, citric acid), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), lubricants (e.g. stearic acid, magnesium stearate, polyethylene glycol, sodium lauryl sulfate), flow-aids (e.g. colloidal silicon dioxide), plasticizers (e.g. diethyl phthalate, triethyl citrate), emulsifiers (e.g. carbomer, hydroxypropyl cellulose, sodium lauryl sulfate), polymer coatings (e.g., poloxamers or poloxamines), coating and film forming agents (e.g. ethyl cellulose, acrylates, polymethacrylates) and/or adjuvants.

[0126] Typical components of carriers for syrups, elixirs, emulsions and suspensions include ethanol, glycerol, propylene glycol, polyethylene glycol, liquid sucrose, sorbitol and water. For a suspension, typical suspending agents include methyl cellulose, sodium carboxymethyl cellulose, cellulose (e.g. Avicel™, RC-591), tragacanth and sodium alginate; typical wetting agents include lecithin and polyethylene oxide sorbitan (e.g. polysorbate 80). Typical preservatives include methyl paraben and sodium benzoate. In another embodiment, peroral liquid compositions also contain one or more components such as sweeteners, flavoring agents and colorants disclosed above.

[0127] The compositions also include incorporation of the active material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts.) Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance.

[0128] Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors.

[0129] In some embodiments, compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline. In another embodiment, the modified compounds exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds. In one embodiment, modifications also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. In another embodiment, the desired *in vivo* biological activity is achieved by the administration of such polymer-compound abducts less frequently or in lower doses than with the unmodified compound.

[0130] In some embodiments, preparation of effective amount or dose can be estimated initially from in vitro assays. In one embodiment, a dose can be formulated in animal models and such information can be used to more accurately determine useful doses in humans.

[0131] In one embodiment, toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro,* in cell cultures or experimental animals. In one embodiment, the data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. In one embodiment, the dosages vary depending upon the dosage form employed and the route of administration utilized. In one embodiment, the exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. [See e.g., Fingl, et al., (1975) "The Pharmacological Basis of Therapeutics", Ch. 1 p.1].

[0132] In one embodiment, depending on the severity and responsiveness of the condition to

be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

[0133] In one embodiment, the amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

[0134] In one embodiment, compositions including the preparation of the present invention formulated in a compatible pharmaceutical carrier are also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

[0135] In one embodiment, compositions of the present invention are presented in a pack or dispenser device, such as an FDA approved kit, which contain one or more unit dosage forms containing the active ingredient. In one embodiment, the pack, for example, comprises metal or plastic foil, such as a blister pack. In one embodiment, the pack or dispenser device is accompanied by instructions for administration. In one embodiment, the pack or dispenser is accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, in one embodiment, is labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

[0136] In one embodiment, it will be appreciated that the polypeptides of the present invention can be provided to the individual with additional active agents to achieve an improved therapeutic effect as compared to treatment with each agent by itself. In another embodiment, measures (e.g., dosing and selection of the complementary agent) are taken to adverse side effects which are associated with combination therapies.

[0137] Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

[0138] Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical

Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996). Other general references are provided throughout this document.

[0139] In the following, Examples 7 and 8 are to be considered as a part of the invention to the extent that they refer to CTP-modified polypeptides consisting of an hGH and three CTPs, wherein a first chorionic gonadotropin CTP is attached to the amino terminus of the hGH and second and third chorionic gonadotropin CTPs are attached to the carboxy terminus of the hGH, as claimed. Examples 1 to 6 are comparative.

EXAMPLE 1

Generation of EPO constructs

MATERIALS AND METHODS:

[0140] ***Construction of expression vector pCI-dhfr:*** pCI-neo mammalian expression vector was purchased from Promega (catalog no.E1841). The vector contains a CMV IE enhancer/promoter and neomycin phosphotransferase gene. The pSV2-dhfr clone was purchased from ATCC (Catalog No.37146). The plasmid contains the murine dhfr gene. The construction of pCI-dhfr vector was performed as follows:

1. a. The pSV2-dhfr plasmid was digested with restriction enzyme BglII (3' end of the dhfr

gene). DNA polymerase I, Large (Klenow) Fragment was used to fill-in the 5' overhangs to form blunt ends. The DNA was then digested with restriction enzyme AvrII (5' end of the dhfr gene). The dhfr gene (AvrII -blunt end) fragment was isolated.

2. b. The pCI-neo vector was digested with restriction enzyme BstXI (3' end of the neo gene). DNA polymerase I, Large (Klenow) Fragment was used to remove the 3' overhangs to form blunt ends. The DNA was then digested with restriction enzyme AvrII (5' end of the neo gene). The expression vector (AvrII -blunt end) was isolated.
3. c. The dhfr gene was ligated into pCI vector to form an expression vector containing the dhfr gene (pCI-dhfr).

[0141] ***Construction of hEPO-CTP variants:*** A cassette gene containing the C-Terminal peptide (CTP) of the beta subunit of hCG was fused to the coding sequence of human EPO (NP_000790.2) at different locations. Four EPO-CTP variants were constructed as illustrated in Figures 1A-D. The proEPO signal peptide was used for the construction of the secreted EPO-CTP variants. XbaI - NotI fragments containing Epo sequences were ligated into the pCI-dhfr expression vector of the present invention.

[0142] Table 1 hereinbelow summarizes the primer sequences used for constructing the CTP - containing polypeptides of the present invention.

Table 1

Primer number	SEQ ID NO	sequence	Restriction site (underlined in sequence)
1	7	5' AATCTAGAGGTCATCATGGGGGTGC 3'	XbaI
2	8	5'ATTGCGGCCGCGGATCCAGAAGACCTTTATTG 3'	NotI
17^R	9	5' TAAATATTGGGGTGTCCGAGGGCCC 3'	SspI
10	10	5' CCAATATTACCACAAGCCCCACCACGCCTCAT 3'	SspI
11^R	11	5'TGCGGCCGCGGATCCTTATCTGTCCCCTGTCCTGC 3'	NotI
15	12	5' GCCCTGCTGTCGGAAGC 3'	
2^R	13	5' ATTGCGGCCGCGGATCCAGAAGACCTTTATTG	NotI
23^R	14	5'CTTTGAGGAAGAGGAGCCCAGGACTGGGAGGC3'	
24	15	5' CCTGGGCTCCTCTTCCTCAAAGGC 3'	
38^R	16	5' GCTTCCGACAGCAGGGC 3'	

[0143] ***EPO-1 701-1-p17-6 (Epo-1 - SEQ ID NO: 1)*****:** The XbaI-NotI 702 bp fragment was constructed by PCR using the above primers (SEQ ID NOs: 7-16). Then the XbaI-NotI PCR

fragment containing Epo-ctp sequence was ligated into pCI-dhfr expression vector.

[0144] ***EPO-2 701-2-p24-2 (Epo-2- SEQ ID NO: 2):*** The XbaI/ApaI fragment (hGH-ctp) of pCI-dhfr-401-2-p21-2 (hGH-ctpx2) was replaced by the XbaI/ApaI fragment (EPO-ctp) of 701-1-p17-6 to create an Epo-ctpx2.

[0145] ***EPO-4-701-4-p42-1(Epo-4- SEQ ID NO: 4)****:* Firstly, a fragment from pCI-dhfr- EPO-ctp (701-1-p17-6) was constructed by PCR using primers 1 and 17 followed by XbaI/SspI digestion. This resulted in a fragment containing EPO and partial 5' CTP.

[0146] Secondly, a new fragment was constructed by overlapping PCR, on pGT123-hEpo as a template, using primer 10 and primer 11. SspI/NotI digestion resulted in fragment containing 3' partial CTP and Epo.

[0147] The two fragments were ligated into pCI-dhfr to construct the p701-4-p42-1 clone.

[0148] ***EPO-3-p56-6 (Epo-3 SEQ ID NO; 3):*** Primers were purchased from Sigma-Genosys. PCR reaction was performed using primer 15 (SEQ ID NO: 12) and primer 2^R (SEQ ID NO: 13) and plasmid DNA of pCI-dhfr- EPO-ctp x2 (701-2-p24-2) as a template. As a result of the PCR amplification, a 486 bp product was formed and ligated into TA cloning vector (Invitrogen, catalog K2000-01). Stu I -NotI fragment containing *Epo-ctp x2 sequence was isolated (209 bp).

[0149] Three sequential PCR reactions were performed. The first reaction was conducted with primer 1 (SEQ ID NO: 7) and primer 23^R (SEQ ID NO: 14) and plasmid DNA of pGT123-epo-ctp as a template; as a result of the PCR amplification, an 80 bp product was formed (signal peptide).

[0150] The second reaction was conducted with primer 24 (SEQ ID NO: 15) and primer 11^R (SEQ ID NO: 11) and plasmid DNA of 701-4-p42-1 as a template; as a result of the PCR amplification, a 610 bp product was formed.

[0151] The last reaction was conducted with primers 1 (SEQ ID NO: 7) and 11^R (SEQ ID NO: 11) and a mixture of the products of the previous two reactions as a template; as a result of the PCR amplification, a 700 bp product was formed and the XbaI-StuI fragment was isolated.

[0152] The two fragments (XbaI-StuI and StuI -NotI) were inserted into the eukaryotic expression vector pCI-dhfr (triple ligation) to yield the 701-3-p56-6 clone.

[0153] ***EPO-5-p91-4 (Epo-5 SEQ ID NO; 5 - (ctp- Epo):*** Primers were ordered from Sigma-Genosys. A PCR reaction was performed using primer 1 (SEQ ID NO: 7) and primer 11^R (SEQ ID NO: 11) and plasmid DNA of pCI-dhfr- ctp-EPO-ctp x2 (701-3-p56-6) as a template; as a result of the PCR amplification, a 670 bp product was formed and ligated into TA cloning vector (Invitrogen, catalog K2000-01) . XbaI -NotI fragment containing ctp-Epo sequence was ligated

into our eukaryotic expression vector pCI-dhfr to yield the 701-5-p91-4 clone.

[0154] ***EPO-6-p90-1 (Epo-6 SEQ ID NO: 6 - (ctp- Epo-ctp):*** Three PCR reactions were performed. The first reaction was conducted with primer 1 (SEQ ID NO: 7) and primer 38^R (SEQ ID NO: 16) and plasmid DNA of 701-3-p56-6 as a template; as a result of the PCR amplification, a 400 bp product was formed.

[0155] The second reaction was conducted with primer 15 (SEQ ID NO: 12) and primer 2^R (SEQ ID NO: 13) and plasmid DNA of 701-1-p17-6 as a template; as a result of the PCR amplification, a 390 bp product was formed.

[0156] The last reaction was conducted with primers 1 (SEQ ID NO: 7) and 2^R (SEQ ID NO: 13) and a mixture of the products of the previous two reactions as a template; as a result of the PCR amplification, a 787 bp product was formed and ligated into TA cloning vector (Invitrogen, catalog K2000-01). The XbaI -NotI fragment containing ctp-Epo-ctp sequence was ligated into the eukaryotic expression vector pCI-dhfr to yield the 701-6-p90-1 clone.

EXAMPLE 2

Expression and Isolation of EPO-CTP polypeptides

MATERIALS AND METHODS

[0157] ***DNA transfection and clone selection:*** DG44 cells were transfected with pCI-DHFR expression vectors containing EPO-CTP variants using FuGENE6 Reagent (FuGENE Transfection Reagent - Roche Cat.11 815 091 001). 48 hr following transfection, cells were diluted and seeded at 50-200 cells per well in a selective medium (CD DG44 Medium w/o HT (Gibco: Scotland part: #07990111A) Sku num.:ME060027 supplemented with 8 mM L-Glutamine Biological Industries: Cat: 03-020-1A) and 18 mL/L of 10 % Pluronic F-68 solution (Gibco: Cat: 240040-032). Selected clones were screened for highest protein production using commercial ELISA. 3-5 producing clones per each variant were frozen for a backup cell bank. A selected clone for each variant was adapted to growth in larger scale cultures up to 1L flasks on an orbital shaker platform. Supernatants were collected and analyzed by ELISA, SDS-PAGE and western blot. Following the withdrawal of aliquots, the protein-containing supernatants were kept frozen until further use.

[0158] ***Cell culture:*** DG44 cells were maintained in DG44 medium with HT (cat# 12610-010, Gibco) supplemented with 8 mM L-Glutamine (Biological Industries: Cat: 03-020-1A) and 18 mL/L of 10 % Pluronic F-68 solution (Gibco: Cat: 240040-032), at 37 °C in humidified 8 % CO_2 incubator. Transfected clones were maintained in DG44 basal medium without HT supplement,

hypoxanthine and thymidine, with pluronic acid and L-glutamine.

[0159] ***Sample preparation:*** Supernatants were collected, filtrated and analyzed by ELISA to determine protein concentration. SDS-PAGE and western blot were used to determine purity and identity. Following ELISA, sample concentrations were defined and the solution was dialyzed against PBS. Following the withdrawal of aliquots, the protein-contained supernatants were kept frozen at -20 °C until further use.

[0160] ***Western Blotting:*** Samples were electrophoresed on nondenaturing 15 % SDS-polyacrylamide gels. Gels were allowed to equilibrate for 10 min in 25 mM Tris and 192 mM glycine in 20 % (vol/vol) methanol). Proteins were transferred to a 0.2 µm pore size nitrocellulose membrane (Sigma, Saint Louis, MO) at 250 mA for 3h, using a Mini Trans-Blot electrophoresis cell (Biorad Laboratories, Richmond, CA). The nitrocellulose membrane was incubated in 5 % non-fat dry milk for 2 h at room temperature. The membrane was incubated with EPO anti-serum (1:1000 titer) overnight at 4 °C followed by three consecutive washes in PBS containing 0.1 % Tween (10 min/wash). The membrane was incubated with secondary antibody conjugated to Horse Radish Peroxidase (HRP) (Zymed, San Francisco, CA) for 2 h at room temperature, followed by three washes. Finally, the nitrocellulose paper was reacted with enhanced chemiluminescent substrate (ECL) (Pierce, Rockford, IL) for 5 min, dried with a Whatman sheet, and exposed to X-ray film.

RESULTS

[0161] Table 2 hereinbelow shows the concentrations of the various CTP-modified EPO forms obtained from 5 selected clones and their preparation for further testing.

Table 2

#Version	*# Clone*	*Stock Titer IU/ml[1]*	*Post dilution in Mock sup. according to Epo3 titer IU/ml[2]*	*Post ultrafiltration IU/ml[3]*
Epo0	17	3093	102	335
SEQ ID NO: 16				
Epo1	47	1049	104	291
SEQ ID NO: 1				
Epo2	67	2160	110	303
SEQ ID NO: 2				
Epo3	85	105	119	392
SEQ ID NO: 3				

#Version	# Clone	Stock Titer IU/ml[1]	Post dilution in Mock sup. according to Epo3 titer IU/ml[2]	Post ultrafiltration IU/ml[3]
Epo4	112	6100	ND	342
SEQ ID NO: 4				
1. EPO variants stock concentration were determined by ELISA (Quantikine IVD Epo ELISA, DEP00, R&D Systems) 2. Samples EPO-0, 1, 2 and 4 were diluted to 105 IU/ml in mock sup (Adjusted to Epo3 titer). Epo0 = wild type EPO expressed in the same system as the CTP modified EPOs 3. All samples were concentrated and dialyzed by ultrafiltration against PBS to a final concentration of 180 IU/ml.				

[0162] All proteins were detected by Western blot as illustrated in Figure 2.

EXAMPLE 3

Biological activity of the EPO-CTP polypeptides of the present invention

[0163] The TF-1 bioactivity test represents the ability of the EPO-CTP variants to bind its receptor and then stimulate activity which results in cell proliferation. Therefore, this test was used as a first step in evaluating the biological potency of the EPO-CTP polypeptides of the present invention.

MATERIALS AND METHODS

[0164] ***Cell Proliferation Analysis:*** Proliferation assay was performed with the cell line TF-1, measuring levels of MTT cellular stain as a function of EPO activity (Kitamura *et al.,* Kitamura, T. *et al.* (1989) *Establishment and characterization of a unique human cell line that proliferates*; Hammerling U. et al. In vitro bioassay for human erythropoietin based on proliferative stimulation of an erythroid cell line and analysis of carbohydrate-dependent microheterogeneity. Journal of Pharm. Biomed. Analysis 14(11): 1455-1469 (1996). Exponentially growing TF-1 cells were washed twice, plated at about 10^4 cells/well in microtiter plates, and incubated in basal medium with a titrated dilution series of EPO (Recormon), EPO standard (NIBSC standard), rhEPO (MOD-7010), MOD-701 variants (EPO-1, EPO-2, EPO-3 and EPO-4) for 48 hours. 4 hours prior to assaying for cell proliferation, MTT reagent was added to the wells, and absorbance was measured by ELISA reader. A calculated protein concentration value for each variant protein was obtained from Eprex's (Epoetin (EPO)-man-made form of the human

hormone) dose-response standard curve.

RESULTS

[0165] The *in vitro* biological activity of EPO polypeptides was determined with an Epo-dependent cell line, human erythroleukemia TF-1 (DSMZ Cell Bank) [Dong et al., Biochemical and Biophysical Research Communications, Volume 339, Issue 1, 6 January 2006, Pages 380-385]. The MTT assay was performed [Hammerling U. et al. In vitro bioassay for human erythropoietin based on proliferative stimulation of an erythroid cell line and analysis of carbohydrate-dependent microheterogeneity. Journal of Pharm. Biomed. Analysis 14(11): 1455-1469 (1996);], and the laboratory standard of EPO used to generate the standard curve was calibrated against the International Standard (Epo ampoule code 87/684 of NIBSC).

[0166] The results are summarized in Table 3 hereinbelow. The results indicate that the highest potency was achieved by using EPO 3 and EPO 0 in both 2 and 0.5 IU/ml concentrations.

Table 3

Eprex STD	***TF-1 Bioactivity IU/ml***						
IU/ml	EPO 0 SEQ ID NO: 16	EPO 1 SEQ ID NO: 1	EPO 2 SEQ ID NO: 2	EPO 3 SEQ ID NO: 3	EPO 4 SEQ ID NO: 4	Recormon	EPO st
2	4.93	2.32	2.13	6.91	3.55	3.44	7.40
0.5	1.60	0.76	0.53	1.34	0.84	0.87	1.53

CONCLUSION

[0167] As summarized in Table 3 hereinabove, different levels of potency were exerted by EPO-CTP polypeptides, indicating differences in receptor binding. EPO-CTP polypeptides differ by the number of CTP cassettes and the location to which they are fused. EPO-1 and EPO-2 contain 1 CTP sequence or 2 CTP sequences at the C-terminal of EPO, while EPO-3 contains 1 CTP at N-terminal and 2 CTP sequences at C-terminal. EPO-4 is a dimer of two EPO molecules linked by CTP sequence. EPO-3 demonstrated potency level quite similar to WT-EPO, while EPO-1 and EPO-4 were about 50 % less potent than WT-EPO, and EPO-2 potency was even less than 50 %.

EXAMPLE 4

Evaluation of the EPO-CTP polypeptides of the present invention in a mouse model

[0168] The following experiment was performed in order to compare the bio-activity of the EPO-CTP polypeptides of the present invention and commercial EPO

MATERIALS AND METHODS

Animals:

[0169] Species/Strain: ICR or CD-1 Mice of either sex about 20-25g
Group Size: n=7
No. Groups: 9
Total No. Animals: n=63

[0170] ***Experimental design of the study:*** The experiment was set up as summarized in Table 4 hereinbelow.

Table 4

Group No.	***No. Mice per Group***	***TREATMENT***		
		Compound	***Dose Level***	***Dosing Regimen***
1	*n*=7	Vehicle (Control)	0	1x weekly
2		MOCK		
3		MOD-7010	15 µg/kg	
4		MOD-7011		
5		MOD-7012		
6		MOD-7013		
7		MOD-7014		
8		Commercial rhEPO	15 µg/kg	
9			5 µg/kg	3 x weekly

[0171] ***Animal treatment:*** All animals were administered with either control or the test EPO polypeptides of the present invention by bolus injection. The injection volume did not exceed 10 ml/kg. The length of the experiment was 22 days. A morbidity and mortality check was performed daily.

[0172] ***Reticulocyte count and hematocrit (hct) examination:*** Reticulocyte count was carried out in all test animals at day 2 and 14 hrs following the 1st respective vehicle or treatment injection. HCT was determined in all animals once prior to initial treatment ("0" Baseline control) and at 24 hrs after the 1st respective vehicle or treatment injection, and

thereafter twice weekly until study termination (Day-22).

RESULTS

[0173] The hematocrit results which are illustrated in Figures 3-5 show that EPO 3 has the highest hematocrit percentage change from baseline compared to EPO 1, EPO 2, Recormon 1, Recormon 3, rhEPO, and vehicle. The results demonstrating the percentage of reticulocytes in mice treated with the EPO-CTP polypeptides are summarized in Table 5 hereinbelow. These results show that EPO-3 is the most potent stimulator of erythropoiesis.

Table 5

	% reticulocytes	
Days	***2***	***14***
Control	3.72	3.46
	1.08	0.8
Mock	3.5	3.64
	0.6	1.13
7010 SEQ ID NO: 16	3.5	3.9
	0.6	1.54
7011 SEQ ID NO: 1	3.52	1.94
	1.38	1.08
7012 SEQ ID NO: 2	3.82	3.0
	1.02	0.88
7013 SEQ ID NO: 3	2.66	5.20
	0.97	2.96
7014 SEQ ID NO: 4	3.48	3.82
	0.71	0.90
Recormon 1/W	3.23	3.27
	0.73	0.59
Recormon 3/w	4.13	4.24
	1.21	1.14

CONCLUSION

[0174] The *in vivo* experiment was designed to measure two parameters; the first was to measure erythropoiesis parameters such as percentage of reticulocytes and increase of hemoglobin, RBC and hematocrit levels. The second was to measure the durability of the

biological activity of each variant by injecting once weekly doses.

[0175] A superior performance of EPO-3 in its ability to stimulate erythropoiesis was observed in normal mice.

EXAMPLE 5

Comparison of the EPO-CTP polypeptides of the present invention to Aranesp

[0176] The following experiment was performed in order to compare the biological activity of a single bolus dose of some EPO-CTP polypeptides of the present invention, commercial EPO and Aranesp. Aranesp is a commercial long-acting recombinant erythropoietin in which two site mutations were introduced, resulting in two additional N-glycosylation sites and an increase in the number of incorporated sialic acid residues.

MATERIALS AND METHODS

Animals:

[0177] Species/Strain: Female CD-1 Mice of either sex about 20-25g
Group Size: n=3

[0178] ***Experimental design of the study:*** The experiment was set up as summarized in Table 6 hereinbelow.

Table 6

Group #	Test Article	animals/ group/ time-point	Dose Solution Conc. (µg/mL)	Dose Volume (mL/kg)	Time-Points * (hours post-administration)
1	MOD-7010 SEQ ID NO: 11	3	1.5	10	0 (Pre-dose), 0.25, 0.5, 1, 2, 6, 24, 48, 96, 168, 216, 264 and 336 hr post-dose administration
2	MOD-7013 SEQ ID NO: 3	3	1.5	10	0.25, 0.5, 1, 2, 6, 24, 48, 96, 168, 216, 264 and 336 hr post-dose administration
3	Aranesp	3	1.5	10	0.25, 0.5, 1, 2, 6, 24, 48, 96, 168, 216, 264 and 336 hr post-dose administration

[0179] ***Animal treatment:*** All animals were administered with either control or the test EPO polypeptides of the present invention by bolus injection. The injection volume did not exceed 10 ml/kg. The length of the experiment was 14 days. A morbidity and mortality check was performed daily.

[0180] ***Reticulocyte count and hematocrit (hct) examination:*** Reticulocyte count and hematocrit examination were performed as described above.

RESULTS

[0181] The results are illustrated in Figures 6-9. Following a single I.V. injection of 15 µg/kg of EPO 3, all three blood parameters associated with erythropoietin i.e. number of reticulocytes, hemoglobin level and hematocrit, were improved relative to those obtained with similar injected dose of rhEPO or Aranesp.

EXAMPLE 6

Comparison of the pharmacokinetics of EPO-CTP polypeptides of the present invention to Aranesp

[0182] The following experiment was performed in order to compare the pharmacokinetics of EPO-CTP polypeptide of the present invention, commercial EPO and Aranesp.

MATERIALS AND METHODS

[0183] Serum samples were analyzed in order to determine specific concentration levels for each sample. Concentration and time-point data were processed using WinNonLin noncompartmental analysis. Parameters determined included: AUC, CL, Ke, t1/2, Cmax, Tmax, and Vdz.

[0184] Serum concentrations were determined using two ELISA kits in parallel. EPO-3 serum concentration was measured using StemCell ELISA kit in comparison to EPO-0 and Aranesp serum concentration which were determined using R&D system ELISA kit.

RESULTS

[0185] The results of the pharmacokinetic analysis are summarized in Table 7, hereinbelow. These results show that EPO 3 exhibited favorable pharmacokinetic measures as indicated for example in AUC measures, t1/2, and Cmax. Tmax measures were equal to EPO-0, EPO-3, and Aranesp.

Table 7

Parameters	Units	EPO-0	EPO-3	Aranesp
AUClast	hr*mIU/mL	31739	306072	178661
CL^	mL/hr/kg	1.1152	0.2188	0.1207
Ke	1/hr	0.157	0.0529	0.0639
t1/2	hr	4.4139	13.1141	10.84
Cmax	mIU/mL	10766	16466	13266
Tmax	Hr	0.25	0.25	0.25
Vdz	mL/kg	7.1017	4.1394	1.8877

[0186] The results of the serum concentration analysis are illustrated in Figure 9. These results show that EPO-3 was still detectable in the serum after about 190 hours. Both EPO-0 and Aranesp were not detectable in the serum after about 140 hours and 50 hours, respectively.

CONCLUSION

[0187] Clearance of EPO-3 (MOD-7013) from the blood of CD-1 mice was significantly slower than that for rhEPO or Aranesp. The corresponding calculated half life times were: rhEPO - 4.41 h; Aranesp - 0.84 h; and MOD-7013 - 13.11 h.

EXAMPLE 7

Generation of hGH constructs

MATERIALS AND METHODS

[0188] Four hGH clones (variants of 20kD protein) were synthesized. Xba I -Not I fragments containing hGH sequences from the four variants were ligated into the eukaryotic expression vector pCI-dhfr previously digested with XbaI -NotI. DNA from the 4 clones (401-0, 1, 2, 3 and 4) was prepared. Another partial hGH clone (1-242 bp) from 22kD protein was also synthesized (0606114). Primers were ordered from Sigma-Genosys. The primer sequences used to generate the hGH-CTP polypeptides of the present invention are summarized in Table 8,

hereinbelow.

Table 8

Primer number	SEQ ID NO	Sequence	Restriction site (underlined in sequence)
25	27	5' CTCTAGAGGACATGGCCAC 3'	XbaI
32^R	28	5' ACAGGGAGGTCTGGGGGTTCTGCA 3'	
33	29	5' TGCAGAACCCCCAGACCTCCCTGTGC 3'	
4^R	30	5' CCAAACTCATCAATGTATCTTA 3'	
25	31	5' CTCTAGAGGACATGGCCAC 3'	XbaI
35^R	32	5' CGAACTCCTGGTAGGTGTCAAAGGC 3'	
34	33	5' GCCTTTGACACCTACCAGGAGTTCG 3'	
37^R	34	5'ACGCGGCCGCATCCAGACCTTCATCACTGAGGC 3'	NotI
39^R	35	5' GCGGCCGCGGACTCATCAGAAGCCGCAGCTGCCC 3'	

[0189] ***Construction of 402-0-p69-1 (hGH) SEQ ID NO:*** **36:** MOD-4020 is the wild type recombinant human growth hormone (without CTP) which was prepared for use as control in the below described experiments.

[0190] Three PCR reactions were performed. The first reaction was conducted with primer 25 and primer 32^R and plasmid DNA of 0606114 (partial clone of hGH 1-242 bp) as a template; as a result of the PCR amplification, a 245 bp product was formed.

[0191] The second reaction was conducted with primer 33 and primer 4^R and plasmid DNA of 401-0-p57-2 as a template; as a result of the PCR amplification, a 542 bp product was formed.

[0192] The last reaction was conducted with primers 25 and 4^R and a mixture of the products of the previous two reactions as a template; as a result of the PCR amplification, a 705 bp product was formed and ligated into the TA cloning vector (Invitrogen, catalog K2000-01). The XbaI - NotI fragment containing hGH-0 sequence was ligated into the eukaryotic expression vector pCI-dhfr. The vector was transfected into DG-44 CHO cells. Cells were grown in protein-free medium.

[0193] ***Construction of 402-1-p83-5 (hGH-CTP) - SEQ ID NO: 37 and 402-2-p72-3(hGH-CTPx2) - SEQ ID NO: 38:*** MOD-4021 is a recombinant human growth hormone which was fused to 1 copy of the C-terminal peptide of the beta chain of human Chorionic Gonadotropin

(CTP). The CTP cassette of MOD-4021 was attached to the C-terminus (one cassette). MOD-4022 is a recombinant human growth hormone which was fused to 2 copies of the C-terminal peptide of the beta chain of human Chorionic Gonadotropin (CTP). The two CTP cassettes of MOD-4022 were attached to the C-terminus (two cassettes).

[0194] Construction of hGH-CTP and hGH-CTP-CTP was performed in the same way as the construction of hGH-0. pCI-dhfr-401-1-p20-1 (hGH*-ctp) and pCI-dhfr-401-2-p21-2 (hGH*-ctp x2) were used as templates in the second PCR reaction.

[0195] MOD-4021 and MOD-4022 were expressed in DG-44 CHO cells. Cells were grown in protein-free medium. The molecular weight of MOD-4021 is ~30.5Kd since hGH has a MW of 22 Kd while each "CTP cassette" contributes 8.5 Kd to the overall molecular weight (see Figure 10). The molecular weight of MOD-4022 is -39 Kd (see Figure 10).

[0196] ***Construction of 402-3-p81-4 (CTP-hGH-CTP-CTP) - SEQ ID NO: 39 and 402-4-p82-9(CTP*hGH-CTP-CTP) - SEQ ID NO: 40:*** MOD-4023 is a recombinant human growth hormone which was fused to 3 copies of the C-terminal peptide of the beta chain of human Chorionic Gonadotropin (CTP). The three CTP cassettes of MOD-4023 were attached to both N-terminus (one cassette) and the C-terminus (two cassettes). MOD-4024 is a recombinant human growth hormone which is fused to 1 truncated and 2 complete copies of the C-terminal peptide of the beta chain of human Chorionic Gonadotropin (CTP). The truncated CTP cassette of MOD-4024 was attached to the N-terminus and two CTP cassettes were attached to the C-terminus (two cassettes).

[0197] Three PCR reactions were performed. The first reaction was conducted with primer 25 and primer 35^R and plasmid DNA of p401-3-p12-5 or 401-4-p22-1as a template; as a result of the PCR amplification, a 265 or 220 bp product was formed. The second reaction was conducted with primer 34 and primer 37^R and plasmid DNA of TA-hGH-2-q65-1 as a template; as a result of the PCR amplification, a 695 bp product was formed. The last reaction was conducted with primers 25 and 37^R and a mixture of the products of the previous two reactions as a template; as a result of the PCR amplification, a 938 or 891bp product was formed and ligated into TA cloning vector (Invitrogen, catalog K2000-01). Xba I -Not I fragment containing hGH sequence was ligated into our eukaryotic expression vector pCI-dhfr.

[0198] MOD-4023 and MOD-4024 were expressed in DG-44 CHO cells. Cells were grown in protein-free medium. The molecular weight of MOD-4023 is ~47.5Kd (see Figure 10) and the molecular weight of MOD-4024 is ~43.25Kd (see Figure 10).

[0199] ***Construction of 402-6-p95a-8 (CTP-hGH-CTP) - SEQ ID NO: 41:*** Construction of hGH-6 was performed in the same way as the construction of hGH-3. pCI-dhfr-402-1-p83-5 (hGH-ctp) was used as a template in the second PCR reaction.

[0200] ***Construction of 402-5-p96-4 (CTP-hGH) - SEQ ID NO:*** **42**: PCR reaction was

performed using primer 25 and primer 39^R and plasmid DNA of pCI-dhfr- ctp-EPO-ctp (402-6-p95a-8) as a template; as a result of the PCR amplification , a 763 bp product was formed and ligated into TA cloning vector (Invitrogen, catalog K2000-01) . Xba I -Not I fragment containing ctp-hGH sequence was ligated into our eukaryotic expression vector pCI-dhfr to yield 402-5-p96-4 clone.

EXAMPLE 8

In vivo bioactivity tests of hGH-CTP polypeptides of the present invention

[0201] The following experiment was performed in order to test the potential long acting biological activity of hGH-CTP polypeptides in comparison with commercial recombinant human GH and MOD-4020.

MATERIALS AND METHODS

[0202] Female hypophysectomized rats (60 -100 g) received a weekly S.C. injection of 21.7 µg hGH-CTP polypeptides or a once daily 5 µg S.C. injection of control commercial rhGH.

[0203] Weight was measured in all animals before treatment, 24 hours following first injection and then every other day until the end of the study on day 21. Each point represents the group's average weight gain percentage ((Weight day 0-weight last day)/Weight day 0). Average weight gain was normalized against once-daily injection of commercial hGH. The treatment schedule is summarized in Table 9.

RESULTS

[0204] Results are summarized in Figure 11. These results show that MOD-4023 (SEQ ID NO: 39) and MOD-4024 (SEQ ID NO: 40) induced over 120% weight gain compared to commercial rhGH which induced 100% weight gain.

Table 9

No.	***Drug***	***N***	***Route***	***Treatment Schedule***	***Equimolar Dose (µg/rat)***	***Accumulate Dosage (µg/rat)***	***Dose Vol.(ml)***
1	Vehicle	7	s.c.	days 1, 7 and 13; 1/W	NA	NA	0.25
2	Mock	7	s.c	days 1, 7 and 13;1/W	NA	NA	0.25

No.	*Drug*	*N*	*Route*	*Treatment Schedule*	*Equimolar Dose (µg/rat)*	*Accumulate Dosage (µg/rat)*	*Dose Vol.(ml)*
3	MOD-4020 SEQ ID NO: 36	7	s.c	days 1, 7 and 13; 1/W	21.7	65	0.25
4	MOD-4021 SEQ ID NO: 37	7	s.c.	days 1, 7 and 13; 1/W	21.7	65	0.25
5	MOD-4022 SEQ ID NO: 38	7	s.c.	days 1, 7 and 13; 1/W	21.7	65	0.25
6	MOD-4023 SEQ ID NO: 39	7	s.c.	days 1, 7 and 13; 1/W	21.7	65	0.25
7	MOD-4024 SEQ ID NO: 40	7	s.c.	days 1, 7 and 13; 1/W	21.7	65	0.25
8	Commercial hGH v.1	7	s.c.	days 1, 7 and 13; 1/W	21.7	65	0.25
9	Commercial hGH v.1	7	s.c.	days 1-13; d/W	5	65	0.25

CONCLUSION

[0205] 3 weekly doses (Days of injections;1, 7, and 13) of 21.7µg of MOD-4023 (SEQ ID NO: 39) and MOD-4024 (SEQ ID NO: 40) induced a 30 % greater weight increase in hypophysectomised rats compared to commercial rhGH injected at the same accumulated dose which was administered once per day at a dose of 5 µg for 13 days.

SEQUENCE LISTING

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Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
    50                  55                  60

Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
65                  70                  75                  80

Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu
                85                  90                  95

Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser
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Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
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Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu
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Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
        35                  40                  45

Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
    50                  55                  60

Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
65                  70                  75                  80

Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu
                85                  90                  95

Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser
            100                 105                 110

Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
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Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu
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Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu
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Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp
            180                 185                 190

Arg Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser
        195                 200                 205

Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln Ser Ser Ser
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            20                  25                  30

Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
        35                  40                  45

Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
    50                  55                  60

Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
65                  70                  75                  80

Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu
                85                  90                  95

Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser
            100                 105                 110

Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
        115                 120                 125

Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu
    130                 135                 140

Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile
145                 150                 155                 160

Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu
                165                 170                 175

Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp
            180                 185                 190

Arg Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser
        195                 200                 205

Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln Ala Pro Pro
    210                 215                 220
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Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp
            260                 265                 270

Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu
        275                 280                 285

Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn
    290                 295                 300

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        35                  40                  45

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    50                  55                  60

Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu Ala Glu Asn Ile
65                  70                  75                  80

Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu Asn Ile Thr Val
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                85                  90                  95

Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg Met Glu Val Gly
            100                 105                 110

Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala
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Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser Gln Pro Trp Glu
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Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile Thr Ala Asp Thr
            180                 185                 190

Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu Arg Gly Lys Leu
        195                 200                 205

Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp Arg Ser Ser Ser
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Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
1               5                  10                  15

Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
            20                  25                  30

Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
        35                  40                  45

Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
    50                  55                  60

Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
65                  70                  75                  80

Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu
                85                  90                  95

Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser
            100                 105                 110

Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
        115                 120                 125
```

```
Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu
    130                 135                 140

Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile

145                 150                 155                 160

Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu
                165                 170                 175

Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp
            180                 185                 190

Arg
```

<210> 17

<211> 34

<212> PRT

<213> Artificial

<220>

<223> Artificial short sequence

<400> 17

```
Asp Pro Arg Phe Gln Asp Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser
1               5                   10                  15

Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu
            20                  25                  30

Pro Gln
```

<210> 18

<211> 28

<212> PRT

<213> Artificial

<220>

<223> Artificial short sequence

<400> 18

```
Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg
1               5                   10                  15

Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln
            20                  25
```

<210> 19

<211> 27

<212> PRT

<213> Homo sapiens

<400> 19

Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu

1 5 10 15

Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly
20 25

<210> 20
<211> 786
<212> DNA
<213> Homo sapiens

<400> 20
tctagaggtc atcatggggg tgcacgaatg tcctgcctgg ctgtggcttc tcctgtccct 60
tctgtcgctc cctctgggcc tcccagtcct gggctcctct tcctcaaagg cccctccccc 120
gagccttcca agtccatccc gactcccggg gccctcggac accccaatat taccacaagc 180
cccaccacgc ctcatctgtg acagccgagt cctggagagg tacctcttgg aggccaagga 240
ggccgagaat atcacgacgg gctgtgctga acactgcagc ttgaatgaga atatcactgt 300
cccagacacc aaagttaatt tctatgcctg gaagaggatg gaggtcgggc agcaggccgt 360
agaagtctgg cagggcctgg ccctgctgtc ggaagctgtc ctgcggggcc aggccctgtt 420
ggtcaactct tcccagccgt gggagccccc gcagctgcat gtggataaag ccgtcagtgg 480
ccttcgcagc ctcaccactc tgcttcgggc tctgggagcc cagaaggaag ccatctcccc 540
tccagatgcg gcctcagctg ctccactccg aacaatcact gctgacactt tccgcaaact 600
cttccgagtc tactccaatt tcctccgggg aaagctgaag ctgtacacag gggaggcctg 660
caggacaggg gacagatcct cttcctcaaa ggcccctccc ccgagccttc caagtccatc 720
ccgactcccg gggccctcgg acaccccgat cctcccacaa taaaggtctt ctggatccgc 780
ggccgc 786

<210> 21
<211> 873
<212> DNA
<213> Homo sapiens

<400> 21
tctagaggtc atcatggggg tgcacgaatg tcctgcctgg ctgtggcttc tcctgtccct 60
tctgtcgctc cctctgggcc tcccagtcct gggctcctct tcctcaaagg cccctccccc 120
gagccttcca agtccatccc gactcccggg gccctcggac accccaatat taccacaagc 180
cccaccacgc ctcatctgtg acagccgagt cctggagagg tacctcttgg aggccaagga 240
ggccgagaat atcacgacgg gctgtgctga acactgcagc ttgaatgaga atatcactgt 300
cccagacacc aaagttaatt tctatgcctg gaagaggatg gaggtcgggc agcaggccgt 360
agaagtctgg cagggcctgg ccctgctgtc ggaagctgtc ctgcggggcc aggccctgtt 420
ggtcaactct tcccagccgt gggagccccc gcagctgcat gtggataaag ccgtcagtgg 480
ccttcgcagc ctcaccactc tgcttcgggc tctgggagcc cagaaggaag ccatctcccc 540

```
tccagatgcg gcctcagctg ctccactccg aacaatcact gctgacactt tccgcaaact    600

cttccgagtc tactccaatt tcctccgggg aaagctgaag ctgtacacag gggaggcctg    660

caggacaggg gacagatcct cttcctcaaa ggcccctccc ccgagccttc caagtccatc    720

ccgactcccg gggccctccg acacaccaat cctgccacag agcagctcct ctaaggcccc    780

tcctccatcc ctgccatccc cctcccggct gcctggcccc tctgacaccc ctatcctgcc    840

tcagtgatga aggtcttctg gatccgcggc cgc                                 873
```

<210> 22

<211> 221

<212> PRT

<213> Homo sapiens

<400> 22

```
Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser Leu
1               5                  10                  15

Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Pro Pro Arg Leu
            20                  25                  30

Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu Ala Lys Glu
        35                  40                  45

Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys Ser Leu Asn Glu
    50                  55                  60

Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
65                  70                  75                  80

Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu
                85                  90                  95

Leu Ser Glu Ala Val Leu Arg Ser Gln Ala Leu Leu Val Asn Ser Ser
            100                 105                 110

Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
        115                 120                 125

Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu
    130                 135                 140

Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile
145                 150                 155                 160

Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu
                165                 170                 175

Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp
            180                 185                 190

Arg Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser
        195                 200                 205
```

```
Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln
    210                 215                 220
```

<210> 23

<211> 217

<212> PRT

<213> Homo sapiens

<400> 23

```
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1               5                  10                  15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu
            20                  25                  30

Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln
        35                  40                  45

Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys
    50                  55                  60

Val Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe
65                  70                  75                  80

Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys
                85                  90                  95

Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp
            100                 105                 110

Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val
        115                 120                 125

Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu
    130                 135                 140

Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg
145                 150                 155                 160

Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
                165                 170                 175

His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe
            180                 185                 190

Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys
        195                 200                 205

Arg Ser Val Glu Gly Ser Cys Gly Phe
    210                 215
```

<210> 24

<211> 166

<212> PRT

<213> Homo sapiens

<400> 24

```
Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln
1               5                  10                  15

Ser Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cys Leu
            20                  25                  30

Lys Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys Gln Leu Gln
        35                  40                  45

Gln Phe Gln Lys Glu Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln
    50                  55                  60

Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn
65                  70                  75                  80

Glu Thr Ile Val Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ile Asn
                85                  90                  95

His Leu Lys Thr Val Leu Glu Glu Lys Leu Glu Lys Glu Asp Phe Thr
            100                 105                 110

Arg Gly Lys Leu Met Ser Ser Leu His Leu Lys Arg Tyr Tyr Gly Arg
        115                 120                 125

Ile Leu His Tyr Leu Lys Ala Lys Glu Tyr Ser His Cys Ala Trp Thr
    130                 135                 140

Ile Val Arg Val Glu Ile Leu Arg Asn Phe Tyr Phe Ile Asn Arg Leu
145                 150                 155                 160

Thr Gly Tyr Leu Arg Asn
                165
```

<210> 25

<211> 30

<212> PRT

<213> Homo sapiens

<400> 25

```
His Ala Glu Gly Thr Phe Thr Ser Asp Val Ser Ser Tyr Leu Glu Gly
1               5                  10                  15

Gln Ala Ala Lys Glu Phe Ile Ala Trp Leu Val Lys Gly Arg
            20                  25                  30
```

<210> 26

<211> 21

<212> PRT

<213> Homo sapiens

<400> 26

```
Met Thr Asn Lys Cys Leu Leu Gln Ile Ala Leu Leu Leu Cys Phe Ser
1               5                  10                  15
```

Thr Thr Ala Leu Ser
20

<210> 27
<211> 19
<212> DNA
<213> Artificial

<220>
<223> Artificial short sequence

<400> 27
ctctagagga catggccac 19

<210> 28
<211> 24
<212> DNA
<213> Artificial

<220>
<223> Artificial short sequence

<400> 28
acagggaggt ctgggggttc tgca 24

<210> 29
<211> 26
<212> DNA
<213> Artificial

<220>
<223> Artificial short sequence

<400> 29
tgcagaaccc ccagacctcc ctgtgc 26

<210> 30
<211> 22
<212> DNA
<213> Artificial

<220>
<223> Artificial short sequence

<400> 30
ccaaactcat caatgtatct ta 22

<210> 31
<211> 19

<212> DNA
<213> Artificial

<220>
<223> Artificial short sequence

<400> 31
ctctagagga catggccac 19

<210> 32
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Artificial short sequence

<400> 32
cgaactcctg gtaggtgtca aaggc 25

<210> 33
<211> 25
<212> DNA
<213> Artificial

<220>
<223> Artificial short sequence

<400> 33
gcctttgaca cctaccagga gttcg 25

<210> 34
<211> 33
<212> DNA
<213> Artificial

<220>
<223> Artificial short sequence

<400> 34
acgcggccgc atccagacct tcatcactga ggc 33

<210> 35
<211> 34
<212> DNA
<213> Artificial

<220>
<223> Artificial short sequence

<400> 35

gcggccgcgg actcatcaga agccgcagct gccc 34

<210> 36

<211> 217

<212> PRT

<213> Homo sapiens

<400> 36

```
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1               5                  10                  15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu
            20                  25                  30

Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln
        35                  40                  45

Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys
    50                  55                  60

Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe
65                  70                  75                  80

Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys
                85                  90                  95

Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp
            100                 105                 110

Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val
        115                 120                 125

Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu
    130                 135                 140

Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg
145                 150                 155                 160

Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
                165                 170                 175

His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe
            180                 185                 190

Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys
        195                 200                 205

Arg Ser Val Glu Gly Ser Cys Gly Phe
    210                 215
```

<210> 37

<211> 245

<212> PRT

<213> Homo sapiens

<400> 37

```
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1               5                  10                  15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu
            20                  25                  30

Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln
        35                  40                  45

Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys
    50                  55                  60

Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe
65                  70                  75                  80

Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys
                85                  90                  95

Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp
            100                 105                 110

Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val
        115                 120                 125

Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu
    130                 135                 140

Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg
145                 150                 155                 160

Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
                165                 170                 175

His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe
            180                 185                 190

Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys
        195                 200                 205

Arg Ser Val Glu Gly Ser Cys Gly Phe Ser Ser Ser Ser Lys Ala Pro
    210                 215                 220

Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr
225                 230                 235                 240

Pro Ile Leu Pro Gln
                245
```

<210> 38

<211> 273

<212> PRT

<213> Homo sapiens

<400> 38

```
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1               5                  10                  15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Phe Pro Thr Ile Pro Leu
            20                  25                  30

Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln
        35                  40                  45

Leu Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys
    50                  55                  60

Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe
65                  70                  75                  80

Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys
                85                  90                  95

Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp
            100                 105                 110

Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val
        115                 120                 125

Tyr Gly Ala Ser Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu
    130                 135                 140

Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg
145                 150                 155                 160

Thr Gly Gln Ile Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser
                165                 170                 175

His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe
            180                 185                 190

Arg Lys Asp Met Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys
        195                 200                 205

Arg Ser Val Glu Gly Ser Cys Gly Phe Ser Ser Ser Ser Lys Ala Pro
    210                 215                 220

Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr
225                 230                 235                 240

Pro Ile Leu Pro Gln Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu
                245                 250                 255

Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro
            260                 265                 270

Gln
```

<210> 39

<211> 301

<212> PRT

<213> Homo sapiens

<400> 39

```
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1               5                  10                  15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Ser Ser Ser Ser Lys Ala
            20                  25                  30

Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp
        35                  40                  45

Thr Pro Ile Leu Pro Gln Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe
    50                  55                  60

Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln Leu Ala Phe Asp
65                  70                  75                  80

Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr
                85                  90                  95

Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile
            100                 105                 110

Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu
        115                 120                 125

Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val
    130                 135                 140

Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser
145                 150                 155                 160

Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln
                165                 170                 175

Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile
            180                 185                 190

Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser His Asn Asp Asp
        195                 200                 205

Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met
    210                 215                 220

Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys Arg Ser Val Glu
225                 230                 235                 240

Gly Ser Cys Gly Phe Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu
                245                 250                 255

Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro
            260                 265                 270
```

```
Gln Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser
        275                 280                 285

Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln
    290                 295                 300
```

<210> 40

<211> 285

<212> PRT

<213> Homo sapiens

<400> 40

```
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1               5                   10                  15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Ser Ser Ser Ser Lys Ala
            20                  25                  30

Pro Pro Pro Ser Leu Pro Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe
        35                  40                  45

Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln Leu Ala Phe Asp
    50                  55                  60

Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr
65                  70                  75                  80

Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile
                85                  90                  95

Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu
            100                 105                 110

Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val
        115                 120                 125

Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser
    130                 135                 140

Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln
145                 150                 155                 160

Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile
                165                 170                 175

Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser His Asn Asp Asp
            180                 185                 190

Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met
        195                 200                 205

Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys Arg Ser Val Glu
    210                 215                 220

Gly Ser Cys Gly Phe Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu
```

```
225                 230                 235                 240

Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro
                245                 250                 255

Gln Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser
            260                 265                 270

Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln
        275                 280                 285
```

<210> 41

<211> 273

<212> PRT

<213> Homo sapiens

<400> 41

```
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1               5                   10                  15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Ser Ser Ser Ser Lys Ala
            20                  25                  30

Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp
        35                  40                  45

Thr Pro Ile Leu Pro Gln Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe
    50                  55                  60

Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln Leu Ala Phe Asp
65                  70                  75                  80

Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr
                85                  90                  95

Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile
            100                 105                 110

Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu
        115                 120                 125

Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val
    130                 135                 140

Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser
145                 150                 155                 160

Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln
                165                 170                 175

Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile
            180                 185                 190

Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser His Asn Asp Asp
        195                 200                 205
```

```
Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met
    210                 215                 220

Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys Arg Ser Val Glu
225                 230                 235                 240

Gly Ser Cys Gly Phe Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu
                245                 250                 255

Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro
            260                 265                 270

Gln
```

<210> 42

<211> 245

<212> PRT

<213> Homo sapiens

<400> 42

```
Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
1               5                   10                  15

Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Ser Ser Ser Ser Lys Ala
            20                  25                  30

Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp
        35                  40                  45

Thr Pro Ile Leu Pro Gln Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe
    50                  55                  60

Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln Leu Ala Phe Asp
65                  70                  75                  80

Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr
                85                  90                  95

Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe Ser Glu Ser Ile
            100                 105                 110

Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu
        115                 120                 125

Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp Leu Glu Pro Val
    130                 135                 140

Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser
145                 150                 155                 160

Asp Ser Asn Val Tyr Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln
                165                 170                 175

Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile
            180                 185                 190
```

```
Phe Lys Gln Thr Tyr Ser Lys Phe Asp Thr Asn Ser His Asn Asp Asp
        195                 200                 205

Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met
    210                 215                 220

Asp Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys Arg Ser Val Glu
225                 230                 235                 240

Gly Ser Cys Gly Phe
                245
```

<210> 43

<211> 12

<212> PRT

<213> Artificial

<220>

<223> Artificial short protein

<400> 43

```
Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro
1               5                   10
```

<210> 44

<211> 853

<212> DNA

<213> Homo sapiens

<400> 44

```
tctagaggac atggccaccg gcagcaggac cagcctgctg ctggccttcg gcctgctgtg     60

cctgccatgg ctgcaggagg gcagcgccag ctcttcttct aaggctccac ccccatctct    120

gcccagcccc agcagactgc cgggccccag cgacacaccc attctgcccc agttccccac    180

catccccctg agcaggctgt tcgacaacgc catgctgagg gctcacaggc tgcaccagct    240

ggcctttgac acctaccagg agttcgagga agcctacatc cccaaggagc agaagtacag    300

cttcctgcag aacccccaga cctccctgtg cttcagcgag agcatcccca cccccagcaa    360

cagagaggag acccagcaga agagcaacct ggagctgctg aggatctccc tgctgctgat    420

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Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser Leu Cys Phe
65                  70                  75                  80

Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys
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Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu Leu Ile Gln Ser Trp
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REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- US5759818A [0004]
- US5712122A [0027]
- US4873316A [0059]
- EP264166A [0059]
- US5932447A [0069]
- US5464764A [0074] [0080]
- US5487992A [0074] [0080]
- US4666828A [0138]
- US4683202A [0138]
- US4801531A [0138]
- US5192659A [0138]
- US5272057A [0138]
- US3791932A [0138]
- US3839153A [0138]
- US3850752A [0138]
- US3850578A [0138]
- US3853987A [0138]
- US3867517A [0138]
- US3879262A [0138]
- US3901654A [0138]
- US3935074A [0138]
- US3984533A [0138]
- US3996345A [0138]
- US4034074A [0138]
- US4098876A [0138]
- US4879219A [0138]
- US5011771A [0138]
- US5281521A [0138]
- EP14197286 [0206]
- EP12179805 [0206]
- EP07749922 [0206]

- US60764761B [0206]

Non-patent literature cited in the description

- **GELLERFORS et al.**J Pharm Biomed Anal, 1989, vol. 7, 173-83 [0044]
- **C.A. RAMSDEN GD.**Quantitative Drug DesignF. Choplin Pergamon Press19920000 [0048]
- **JOHN MORROW STEWARTJANIS DILLAHA YOUNG**Solid Phase Polypeptide SynthesesPierce Chemical Company19840000 [0055]
- **CREIGHTON T.**Proteins, structures and molecular principlesWH Freeman and Co.19830000 [0055]
- **BITTER et al.**Methods in Enzymol., 1987, vol. 153, 516-544 [0056]
- **STUDIER et al.**Methods in Enzymol., 1990, vol. 185, 60-89 [0056] [0068]
- **BRISSON et al.**Nature, 1984, vol. 310, 511-514 [0056] [0078]
- **TAKAMATSU et al.**EMBO J., 1987, vol. 6, 307-311 [0056] [0078]
- **CORUZZI et al.**EMBO J., 1984, vol. 3, 1671-1680 [0056] [0078]
- **BROGLI et al.**Science, 1984, vol. 224, 838-843 [0056] [0078]
- **GURLEY et al.**Mol. Cell. Biol.19860000vol. 6, 559-565 [0056]
- **WEISSBACHWEISSBACH**Methods for Plant Molecular BiologyAcademic Press19880000421-463 [0056] [0078]
- **PINKERT et al.**Genes Dev., 1987, vol. 1, 268-277 [0059]
- **CALAME et al.**Adv. Immunol., 1988, vol. 43, 235-275 [0059]
- **WINOTO et al.**EMBO J., 1989, vol. 8, 729-733 [0059]
- **BANERJI et al.**Cell, 1983, 33729-740 [0059]
- **BYRNE et al.**Proc. Natl. Acad. Sci. USA, 1989, vol. 86, 5473-5477 [0059]
- **EDLUNCH et al.**Science, 1985, vol. 230, 912-916 [0059]
- **SROUR, M.A. et al.**Thromb. Haemost., 2003, vol. 90, 398-405 [0059]
- Current Protocols in Molecular BiologyJohn Wiley & Sons19920000 [0064]
- **SAMBROOK et al.**Molecular Cloning: A Laboratory ManualCold Springs Harbor Laboratory19890000 [0074] [0080]
- **AUSUBEL et al.**Current Protocols in Molecular BiologyJohn Wiley and Sons19890000 [0074] [0080] [0138]
- **CHANG et al.**Somatic Gene TherapyCRC Press19950000 [0074] [0080]
- **VEGA et al.**Gene TargetingCRC Press19950000 [0074] [0080]
- Vectors: A Survey of Molecular Cloning Vectors and Their UsesButterworths19880000 [0074]
- **GILBOA**Biotechniques, 1986, vol. 4, 6504-512 [0074] [0080]
- **BOHL et al.**Blood, 2000, vol. 95, 2793-2798 [0077]
- **GAO et al.**Blood, 2004, vol. 103, 9 [0077]
- **LIPPIN et al.**Blood, 2005, vol. 106, 7 [0077]
- **GURLEY et al.**Mol. Cell. Biol., 1986, vol. 6, 559-565 [0078]

- **VECTORS**A Survey of Molecular Cloning Vectors and Their UsesButterworths19880000 [0080]
- **BOOTH et al.**Immunol. Lett., 1988, vol. 19, 65-70 [0086]
- **GARDELLA et al.**J. Biol. Chem., 1990, vol. 265, 15854-15859 [0086]
- Remington's Pharmaceutical SciencesMack Publishing Co. [0100]
- **LANGER**Science, 1990, vol. 249, 1527-1533 [0117] [0118]
- **TREAT et al.**Liposomes in the Therapy of Infectious Disease and CancerLiss19890000353-365 [0117]
- LIPOSOMES IN THE THERAPY OF INFECTIOUS DISEASE AND CANCER317-327 [0117]
- **SEFTON**CRC Crit. Ref. Biomed. Eng., 1987, vol. 14, 201- [0118]
- **BUCHWALD et al.**Surgery, 1980, vol. 88, 507- [0118]
- **SAUDEK et al.**N. Engl. J. Med., 1989, vol. 321, 574- [0118]
- **GOODSON**Medical Applications of Controlled Release, 1984, vol. 2, 115-138 [0118]
- **FINGL et al.**The Pharmacological Basis of Therapeutics197500001- [0131]
- **SAMBROOK et al.**Molecular Cloning: A laboratory Manual19890000 [0138]
- Current Protocols in Molecular Biology19940000vol. I-III, [0138]
- **PERBAL**A Practical Guide to Molecular CloningJohn Wiley & Sons19880000 [0138]
- Recombinant DNA**WATSON et al.**Scientific American Books [0138]
- Genome Analysis: A Laboratory Manual SeriesCold Spring Harbor Laboratory Press19980000vol. 1-4, [0138]
- Cell Biology: A Laboratory Handbook19940000vol. I-III, [0138]
- **FRESHNEY**Culture of Animal Cells - A Manual of Basic TechniqueWiley-Liss19940000 [0138]
- Current Protocols in Immunology19940000vol. I-III, [0138]
- Basic and Clinical ImmunologyAppleton & Lange19940000 [0138]
- Selected Methods in Cellular ImmunologyW. H. Freeman and Co.19800000 [0138]
- Oligonucleotide Synthesis19840000 [0138]
- Nucleic Acid Hybridization19850000 [0138]
- Transcription and Translation19840000 [0138]
- Animal Cell Culture19860000 [0138]
- Immobilized Cells and EnzymesIRL Press19860000 [0138]
- **PERBAL, B.**A Practical Guide to Molecular Cloning19840000 [0138]
- Methods in EnzymologyAcademic Pressvol. 1-317, [0138]
- PCR Protocols: A Guide To Methods And ApplicationsAcademic Press19900000 [0138]
- **MARSHAK et al.**Strategies for Protein Purification and Characterization - A Laboratory Course ManualCSHL Press19960000 [0138]
- **HAMMERLING U. et al.**In vitro bioassay for human erythropoietin based on proliferative stimulation of an erythroid cell line and analysis of carbohydrate-dependent microheterogeneityJournal of Pharm. Biomed. Analysis, 1996, vol. 14, 111455-1469 [0164] [0165]
- **DONG et al.**Biochemical and Biophysical Research Communications, 2006, vol. 339, 1380-385 [0165]

Patentkrav

1. Polypeptid omfattende en biologisk aktivitet, hvilket polypeptid omfatter et peptid af interesse, et enkelt choriongonadotropin-carboxyterminalpeptid (CTP) forbundet med amino-terminalen af peptidet af interesse, og to choriongonadotropin-carboxyterminalpeptider (CTPs) forbundet med carboxy-terminalen af peptidet af interesse, hvor polypeptidet er et modent polypeptid, hvor N-terminalsignalpeptidet er blevet spaltet fra precursorproteinet, hvor aminosyresekvensen af precursorproteinet er angivet i SEQ ID NO: 39, og hvor peptidet af interesse er hGH.

2. Polypeptid ifølge krav 1, hvor peptidet af interesse er glycosyleret.

3. Polypeptid ifølge krav 1, hvor peptidet af interesse ikke er glycosyleret.

4. Polypeptid ifølge et hvilket som helst af kravene 1-3, hvor mindst et af choriongonadotropin-carboxyterminalpeptiderne er glycosyleret.

5. Farmaceutisk formulering omfattende polypeptidet ifølge et hvilket som helst af kravene 1-4.

6. Farmaceutisk formulering ifølge krav 5, hvor formuleringen yderligere omfatter en buffer, såsom en citrat- eller acetatbuffer, og et tonicitetsmiddel, såsom natriumchlorid.

7. Farmaceutisk formulering ifølge krav 5 eller 6, hvor formuleringen er formuleret til injektion i en multidosisbeholder, med eventuelt, et konserveringsmiddel, såsom benzalkoniumchlorid eller thimerosal.

8. Farmaceutisk formulering ifølge et hvilket som helst af kravene 5-7, hvor formuleringen er en flydende formulering.

9. Polypeptid ifølge et hvilket som helst af kravene 1-4 eller den farmaceutiske formulering ifølge et hvilket som helst af kravene 5-8, til anvendelse som et

medikament.

10. Polypeptid ifølge et hvilket som helst af kravene 1-4 eller den farmaceutiske formulering ifølge et hvilket som helst af kravene 5-8, til anvendelse til behandling af en vækst-, vægtrelateret eller stofskiftesygdom.

11. Fremgangsmåde til forbedring af en biologisk halveringstid for et peptid af interesse, omfattende trinnet at forbinde et enkelt choriongonadotropin-carboxyterminalpeptid med aminoterminalen af peptidet af interesse og to choriongonadotropin-carboxyterminalpeptider til carboxyterminalen af peptidet af interesse for at danne et CTP-modificeret polypeptid, for derved at forbedre den biologiske halveringstid for peptidet af interesse, hvor det CTP-modificerede polypeptid er et modent polypeptid, hvor N-terminalsignalpeptidet er blevet spaltet fra precursorproteinet, hvor aminosyresekvensen af precursorproteinet er angivet i SEQ ID NO: 39, og hvor peptidet af interesse er hGH.

12. Fremgangsmåde ifølge krav 11, hvor peptidet af interesse er glycosyleret.

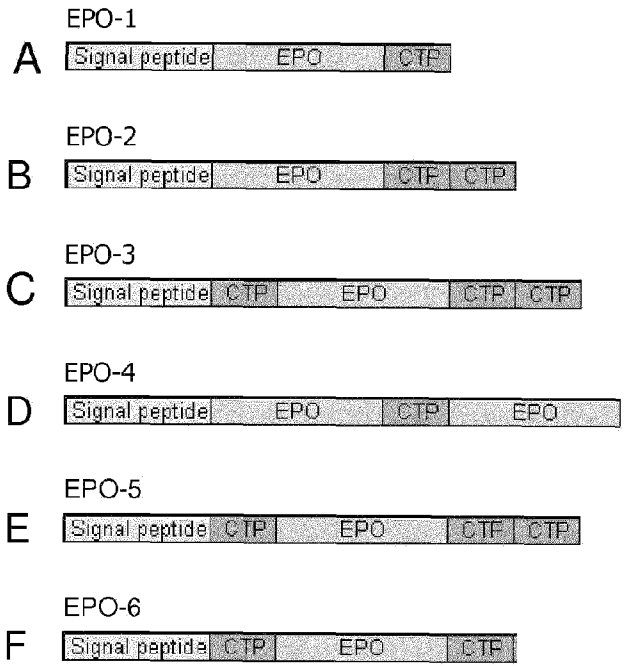
13. Fremgangsmåde ifølge krav 11, hvor peptidet af interesse ikke er glycosyleret.

14. Fremgangsmåde ifølge et hvilket som helst af kravene 11-13, hvor mindst et af choriongonadotropin-carboxyterminalpeptiderne er glycosyleret.

15. Anvendelse af polypeptidet ifølge et hvilket som helst af kravene 1-4 eller den farmaceutiske formulering ifølge et hvilket som helst af kravene 5-8, til fremstillingen af et medikament til behandling af en vækst-, vægtrelateret eller stofskiftesygdom.

16. Anvendelse ifølge krav 15, hvor medikamentet omfatter en enhedsdosis til indgivelse en gang om ugen.

DRAWINGS



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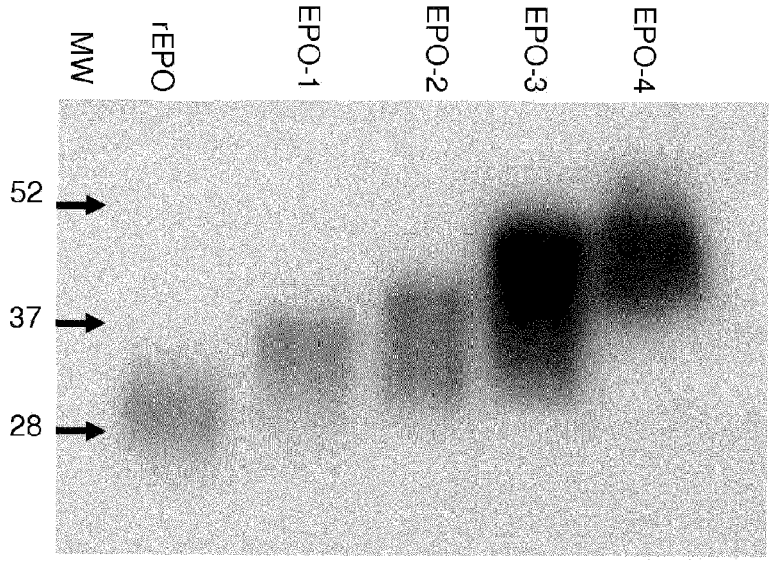

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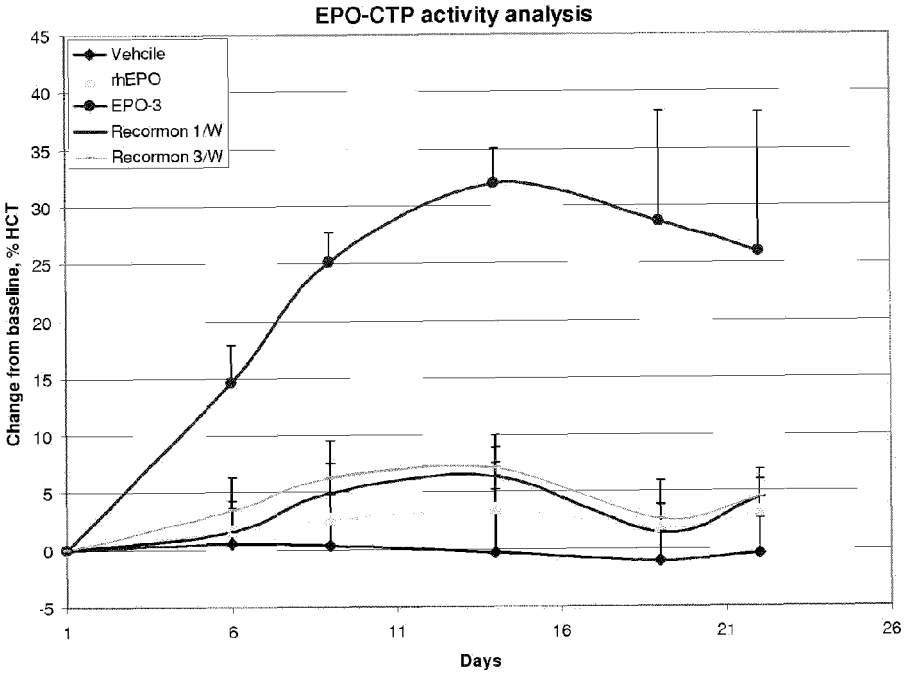

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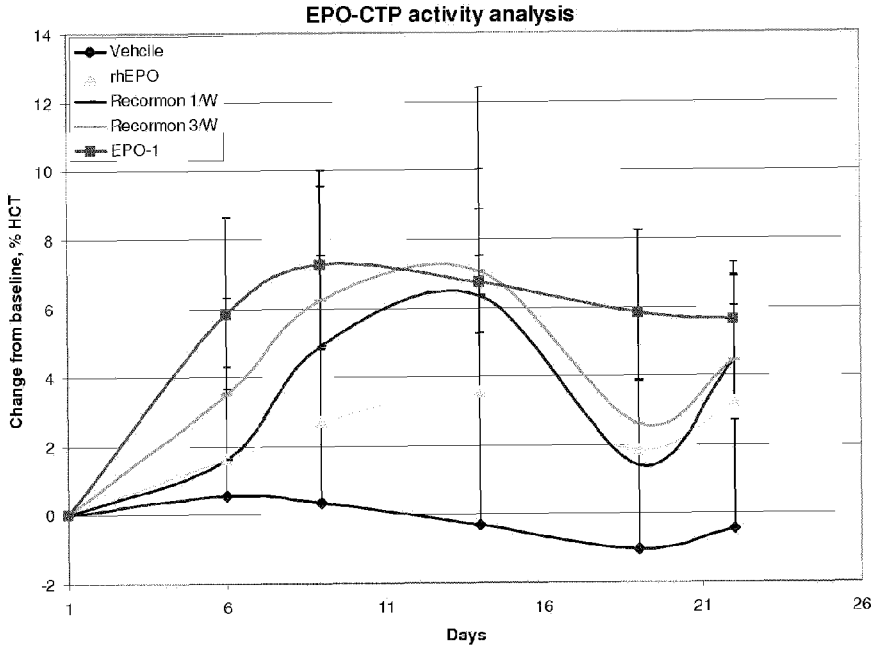

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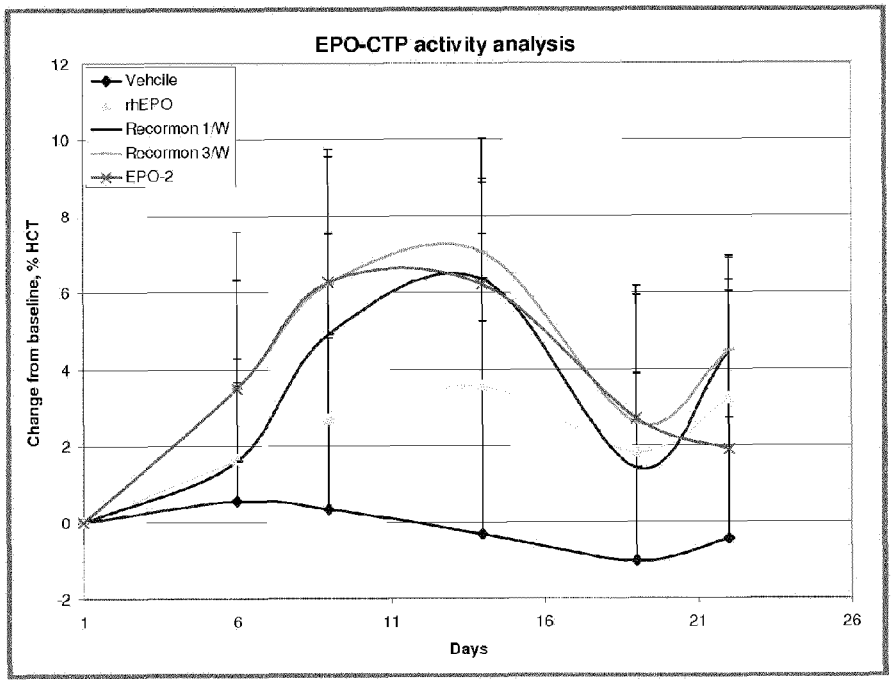

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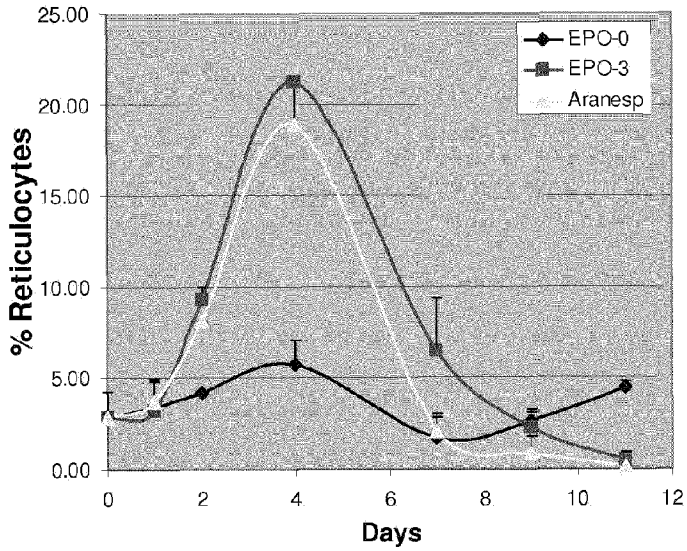

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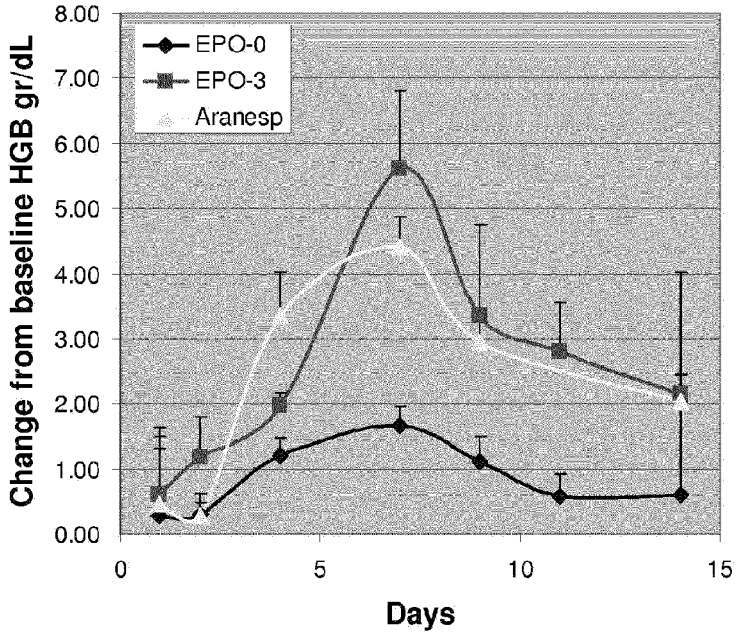

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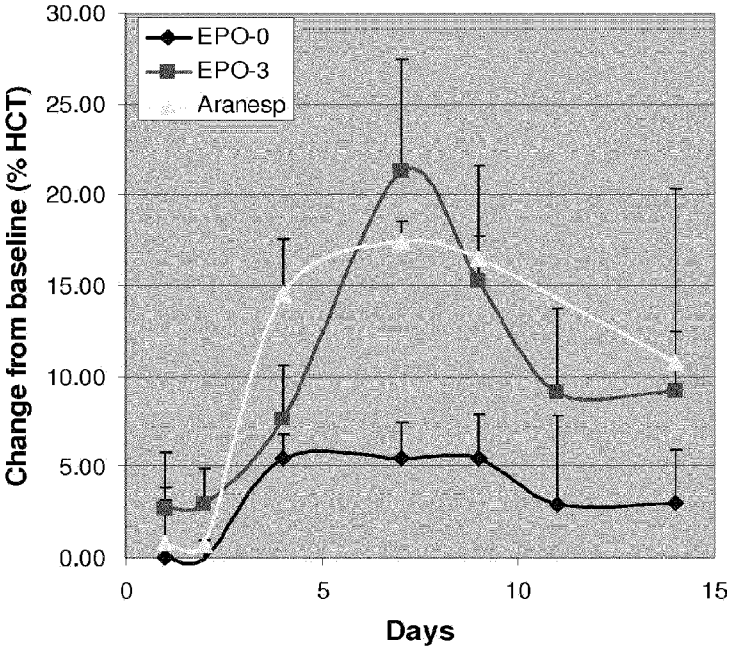

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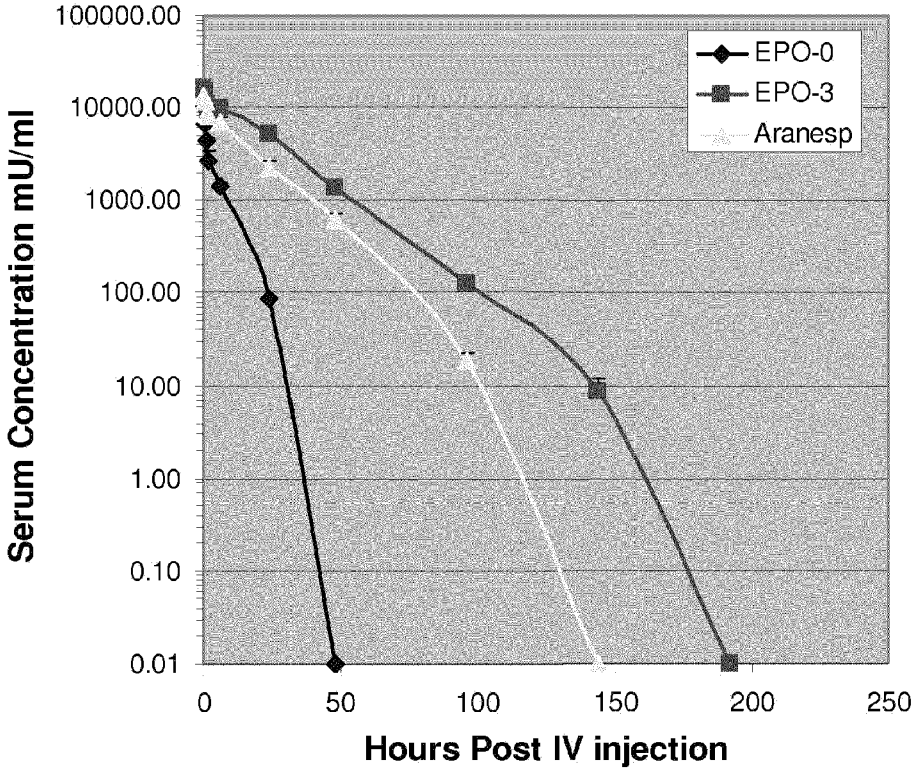

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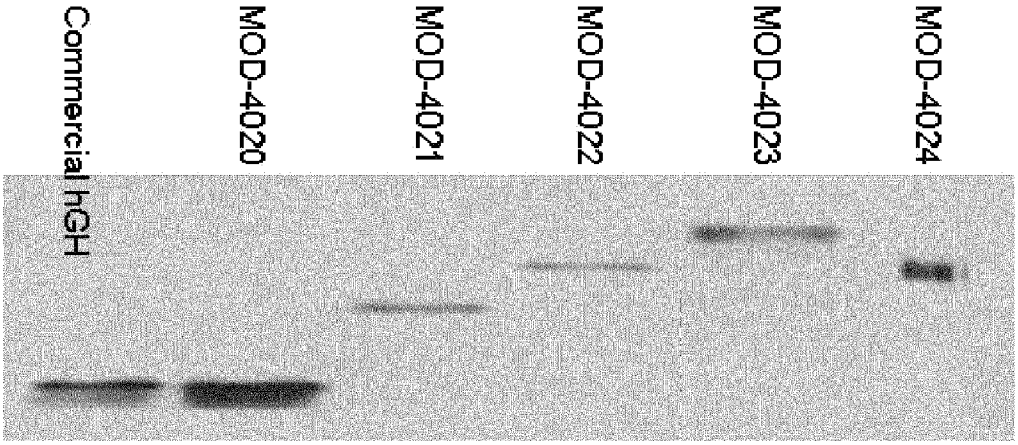

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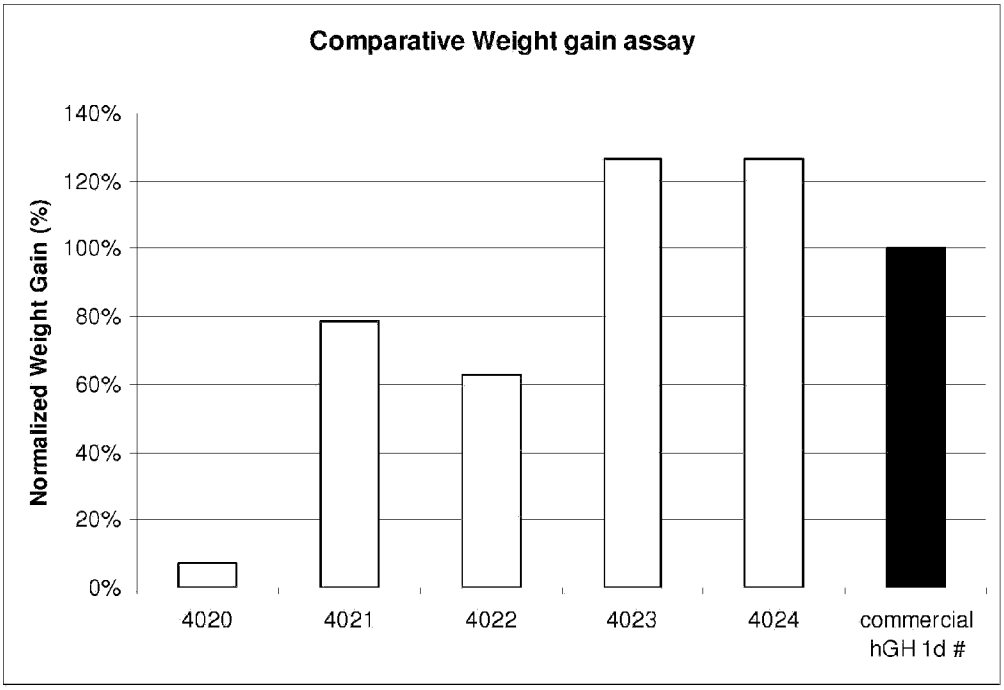
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FIGURE 11