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(71) Applicant(s)
Affymax, Inc.

(72) Inventor(s)
Balu, Palani;Lalonde, Guy;Holmes, Christopher;Schatz, Peter J.;Yin, Kevin;Tumelty, David

(74) Agent / Attorney
Phillips Ormonde Fitzpatrick, 367 Collins Street, Melbourne, VIC, 3000

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(71) Applicant *(for all designated States except US)*: **AFFYMAX, INC.** [US/US]; 4001 Miranda Avenue, Palo Alto, CA 94304 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **YIN, Kevin** [—/US]; 747 Coastland Drive, Palo Alto, CA 94303 (US). **HOLMES, Christopher** [—/US]; 13633 Westover Drive, Saratoga, CA 95070 (US). **LALONDE, Guy** [—/US]; 20007 Skyline Boulevard, Woodside, CA 94062 (US). **BALU, Palani** [—/US]; 21856 Dolores Avenue, Cupertino, CA 95014 (US). **SCHATZ, Peter, J.** [—/US]; 10949 Sycamore Drive, Cupertino, CA 95014 (US). **TUMELTY, David** [US/US]; 250 Santa Fe Terrace #106, Sunnyvale, CA 94085 (US).

(74) Agents: **FEHLNER, Ph., D.** et al.; Darby & Darby P.C., P.O. Box 5257, New York, NY 10150-5257 (US).

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(54) Title: PEPTIDES THAT BIND TO THE ERYTHROPOIETIN RECEPTOR

(57) Abstract: The present invention relates to peptide compounds that are agonists of the erythropoietin receptor (EPO-R). The invention also relates to therapeutic methods using such peptide compounds to treat disorders associated with insufficient or defective red blood cell production. Pharmaceutical compositions, which comprise the peptide compounds of the invention, are also provided. The peptides can be in monomeric or dimeric forms and are eventually conjugated to PEG. The peptides have from 1 to 40 residues and comprise the sequence: LYACHX_0GPITX$_1$VCQPLR, wherein X_0 is H or homoserine methylether; X_1 is W or 1-nal or 2-nal.

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NOVEL PEPTIDES THAT BIND TO THE ERYTHROPOIETIN RECEPTOR

CROSS-REFERENCE TO RELATED APPLICATIONS

Priority is claimed to United States provisional patent application Serial No. 60/470,245 filed May 12, 2003. The contents of this priority application are hereby incorporated into this disclosure by reference and in its entirety.

FIELD OF THE INVENTION

The present invention relates to peptide compounds that are agonists of the erythropoietin receptor (EPO-R). The invention also relates to therapeutic methods using such peptide compounds to treat disorders associated with insufficient or defective red blood cell production. Pharmaceutical compositions, which comprise the peptide compounds of the invention, are also provided.

BACKGROUND OF THE INVENTION

Erythropoietin (EPO) is a glycoprotein hormone of 165 amino acids, with a molecular weight of about 34 kilodaltons (kD) and preferred glycosylation sites on amino-acid positions 24, 38, 83, and 126. It is initially produced as a precursor protein with a signal peptide of 23 amino acids. EPO can occur in three forms: α, β, and asialo. The α and β forms differ slightly in their carbohydrate components, but have the same potency, biological activity, and molecular weight. The asialo form is an α or β form with the terminal carbohydrate (sialic acid) removed. The DNA sequences encoding EPO have been reported [U.S. Pat. No. 4,703,008 to Lin].

EPO stimulates mitotic division and differentiation of erythrocyte precursor cells, and thus ensures the production of erythrocytes. It is produced in the kidney when hypoxic conditions prevail. During EPO-induced differentiation of erythrocyte precursor cells, globin synthesis is induced; heme complex synthesis is stimulated; and the number of ferritin receptors increases. These changes allow the cell to take on more iron and synthesize functional hemoglobin, which in mature erythrocytes binds oxygen. Thus, erythrocytes and their hemoglobin play a key role in supplying the body with oxygen. These changes are initiated by the interaction of EPO with an appropriate receptor on the cell surface of the erythrocyte precursor cells [See, *e.g.*, Graber and Krantz (1978) Ann. Rev. Med. 29.51-66].

EPO is present in very low concentrations in plasma when the body is in a healthy state wherein tissues receive sufficient oxygenation from the existing number of erythrocytes. This normal low concentration is sufficient to stimulate replacement of red blood cells which are lost normally through aging.

The amount of EPO in the circulation is increased under conditions of hypoxia when oxygen transport by blood cells in the circulation is reduced. Hypoxia may be caused, for example, by substantial blood loss through hemorrhage, destruction of red blood cells by over-exposure to radiation, reduction in oxygen intake due to high altitude or prolonged unconsciousness, or various forms of anemia. In response to such hypoxic stress, elevated EPO levels increase red blood cell production by stimulating the proliferation of erythroid progenitor cells. When the number of red blood cells in circulation is greater than needed for normal tissue oxygen requirements, EPO levels in circulation are decreased.

Because EPO is essential in the process of red blood cell formation, this hormone has potentially useful applications in both the diagnosis and the treatment of blood disorders characterized by low or defective red blood cell production. Recent studies have provided a basis for the projection of EPO therapy efficacy for a variety of disease states, disorders, and states of hematologic irregularity, including: beta-thalassemia [see, Vedovato, *et al.* (1984) Acta. Haematol. 71:211-213]; cystic fibrosis [see, Vichinsky, *et al.* (1984) J. Pediatric 105:15-21]; pregnancy and menstrual disorders [see, Cotes, *et al.* (193) Brit. J. Ostet. Gyneacol. 90:304-311]; early anemia of prematurity [see, Haga, *et al.* (1983) Acta Pediatr. Scand. 72; 827-831]; spinal cord injury [see, Claus-Walker, *et al.* (1984) Arch. Phys. Med. Rehabil. 65:370-374]; space flight [see, Dunn, *et al.* (1984) Eur. J. Appl. Physiol. 52:178-182]; acute blood loss [see, Miller, *et al.* (1982) Brit. J. Haematol. 52:545-590]; aging [see, Udupa, *et al.* (1984) J. Lab. Clin. Med. 103:574-580 and 581-588 and Lipschitz, *et al.* (1983) Blood 63:502-509]; various neoplastic disease states accompanied by abnormal erythropoiesis [see, Dainiak, *et al.* (1983) Cancer 5:1101-1106 and Schwartz, *et al.* (1983) Otolaryngol. 109:269-272]; and renal insufficiency [see, Eschbach. *et al.* (1987) N. Eng. J. Med. 316:73-78].

Purified, homogeneous EPO has been characterized [U.S. Pat. No. 4,677,195 to Hewick]. A DNA sequence encoding EPO was purified, cloned, and expressed to produce recombinant polypeptides with the same biochemical and immunological properties as natural EPO. A recombinant EPO molecule with oligosaccharides identical to those on natural EPO has also been produced [See, Sasaki, *et al.* (1987) J. Biol. Chem. 262:12059-12076].

The biological effect of EPO appears to be mediated, in part, through interaction with a cell membrane bound receptor. Initial studies, using immature erythroid cells isolated from mouse spleen, suggested that the EPO-binding cell surface proteins comprise two polypeptides having approximate molecular weights of 85,000 Daltons and 100,000 Daltons, respectively [Sawyer, *et al.* (1987) Proc. Nati.

Acad. Sci. USA 84:3690-3694]. The number of EPO-binding sites was calculated to average from 800 to 1000 per cell surface. Of these binding sites, approximately 300 bound EPO with a K_d of approximately 90 pM (picomolar), while the remaining bound EPO with a reduced affinity of approximately 570 pM [Sawyer, *et al.* (1987) J. Biol. Chem. 262:5554-5562]. An independent study suggested that EPO-responsive splenic erythroblasts, prepared from mice injected with the anemic strain (FVA) of the Friend leukemia virus, possess a total of approximately 400 high and low affinity EPO binding sites with K_d values of approximately 100 pM and 800 pM, respectively [Landschulz, *et al.* (1989) Blood 73:1476-1486].

Subsequent work indicated that the two forms of EPO receptor (EPO-R) were encoded by a single gene. This gene has been cloned [See, *e.g.*, Jones, *et al.* (1990) Blood 76, 31-35; Noguchi, *et al.* (1991) Blood 78:2548-2556; Maouche, *et al.* (1991) Blood 78:2557-2563]. For example, the DNA sequences and encoded peptide sequences for murine and human EPO-R proteins are described in PCT Pub. No. WO 90/08822 to D'Andrea, *et al.* Current models suggest that binding of EPO to EPO-R results in the dimerization and activation of two EPO-R molecules, which results in subsequent steps of signal transduction [See, *e.g.*, Watowich, *et al.* (1992) Proc. Natl. Acad. Sci. USA 89:2140-2144].

The availability of cloned genes for EPO-R facilitates the search for agonists and antagonists of this important receptor. The availability of the recombinant receptor protein allows the study of receptor-ligand interaction in a variety of random and semi-random peptide diversity generation systems. These systems include the "peptides on plasmids" system [described in U.S. Pat. No. 6,270,170]; the "peptides on phage" system [described in U.S. Pat. No. 5,432,018 and Cwirla, *et al.* (1990) Proc. Natl. Acad. Sci. USA 87:6378-6382]; the "encoded synthetic library" (ESL) system [described in U.S. patent application Ser. No. 946,239, filed Sep. 16, 1992]; and the "very large scale immobilized polymer synthesis" system [described in U.S. Pat. No. 5,143,854; PCT Pub. No. 90/15070; Fodor, *et al.* (1991) Science 251:767-773; Dower and Fodor (1991) Ann. Rep. Med. Chem. 26:271-180; and U.S. Pat. No. 5,424,186].

Peptides that interact to a least some extent with EPO-R have been identified and are described, for example in U.S. Pat. Nos. 5,773,569; 5,830,851; and 5,986,047 to Wrighton, *et al.*; PCT Pub. No. WO 96/40749 to Wrighton, *et al.*; U.S. Pat. No. 5,767,078 and PCT Pub. No. 96/40772 to Johnson and Zivin; PCT Pub. No. WO 01/38342 to Balu; and WO 01/91780 to Smith-Swintosky, *et al.* In particular, a group of peptides containing a peptide motif has been identified, members of which bind to EPO-R and stimulate EPO-dependent cell proliferation. Yet, peptides identified to date that contain the motif stimulate EPO-dependent cell proliferation *in vitro* with EC50 values between about 20 nanomolar (nM) and 250nM. Thus, peptide concentrations of 20nM to 250nM are required to stimulate 50% of the maximal cell proliferation stimulated by EPO.

2004238868 26 Nov 2009

Given the immense potential of EPO-R agonists, both for studies of the important biological activities mediated by this receptor and for treatment of disease, there remains a need for the identification of peptide EPO-R agonists of enhanced potency and activity. 'The present invention provides such compounds.

The citation and/or discussion of cited references in this section and throughout the specification is provided merely to clarify the description of the present invention and is not an admission that any such reference is "prior art" to the present invention.

A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission or a suggestion that that document or matter was, known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

Throughout the description and claims of the specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

SUMMARY OF THE INVENTION

The present invention provides EPO-R agonist peptides of dramatically enhanced potency an activity. These agonists include monomeric peptide agonists of 17 to about 40 amino acids in length that comprise the core amino acid sequence LYACHX_0GPITX_1VCQPLR (SEQ ID NO: 1), where each amino acid is indicated by standard one letter abbreviation, X_0 is methionine (M) or homoserine methylether (Hsm), and X_I is tryptophan (W), 1-naphthylalanine (1-nal), or 2-naphthylalanine (2-nal); as well as dimeric peptide agonists that comprise two peptide monomers, wherein each peptide monomer is of 17 to about 40 amino acids in length and comprises the core amino acid sequence LYACHX_0GPITX_1VCQPLR (SEQ ID NO: 1), where each amino acid is indicated by standard one letter abbreviation, X_0 is methionine (M) or homioserine methylether (Hsm), and X, is tryptophan (W), 1-naphthylalanine (1-nal), or 2-naphthylalanine (2-nal). The potency of these novel peptide agonists may be further enhanced by one or more modifications, including: acetylation, intramolecular disulfide bond formation, and covalent attachment of one or more polyethylene glycol (PEG) moieties. The invention further provides phannaceutical compositions comprised of such peptide agonists, and methods to treat various medical conditions using such peptide agonists.

2004238868 26 Nov 2009

A further aspect of the present invention relates to a peptide dimer, comprising: a first peptide chain; a second peptide chain; and a linking moiety connecting said first and second peptide chains, wherein at least one of said first peptide chain and said second peptide chain comprises about 17 to about 40 amino acid residues in length and comprises the amino acid sequence:LYACHX_0GPITX_1VCQPLR (SEQ ID NO: 1), wherein X_0 is a residue selected from methionine (M) and homoserine methylether (Hsm), and X_1 is a residue selected from tryptophan (W), 1-naphthylalanine (1-nal), and 2-naphthylalanine (2-nal), wherein said peptide dimer binds to and activates the erythropoietin receptor (EPO-R).

A further aspect of the present invention relates to a method for treating a patient, comprising administering to a patient having a disorder characterized by a deficiency of erythropoietin or a low or defective red blood cell population a therapeutically effective amount of a peptide or peptide dimer as herein described.

DETAILED DESCRIPTION OF THE INVENTION

Definitions:

Amino acid residues in peptides are abbreviated as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G. The unconventional amino acids in peptides are abbreviated as follows: 1-naphthylalanine is 1-nal

or Np; 2-naphthylalanine is 2-nal; N-methylglycine (also known as sarcosine) is MeG or Sc; homoserine methylether is Hsm; and acetylated glycine (N-acetylglycine) is AcG.

As used herein, the term "polypeptide" or "protein" refers to a polymer of amino acid monomers that are alpha amino acids joined together through amide bonds. Polypeptides are therefore at least two amino acid residues in length, and are usually longer. Generally, the term "peptide" refers to a polypeptide that is only a few amino acid residues in length. The novel EPO-R agonist peptides of the present invention are preferably no more than about 50 amino acid residues in length. They are more preferably from about 17 to about 40 amino acid residues in length. A polypeptide, in contrast with a peptide, may comprise any number of amino acid residues. Hence, the term polypeptide included peptides as well as longer sequences of amino acids.

As used herein, the phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are "generally regarded as safe", *e.g.*, that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

As used herein the term "agonist" refers to a biologically active ligand which binds to its complementary biologically active receptor and activates the latter either to cause a biological response in the receptor, or to enhance preexisting biological activity of the receptor.

Novel peptides that are EPO-R agonists

The present invention relates to peptides that are agonists of the EPO-R and show dramatically enhanced potency and activity. These peptide agonists are preferably of 17 to about 40 amino acids in length and comprise the core amino acid sequence LYACHX$_0$GPITX$_1$VCQPLR (SEQ ID NO: 1), where each amino acid is indicated by standard one letter abbreviation, X$_0$ is methionine (M) or homoserine methylether (Hsm), and X$_1$ is tryptophan (W), 1-naphthylalanine (1-nal), or 2-naphthylalanine (2-nal).

The peptides of this invention may be monomers, dimers, or other multimers. The peptide multimers of the invention may be trimers, tetramers, pentamers, or other higher order structures.

Moreover, such dimers and other multimers may be heterodimers or heteromultimers. The peptide monomers of the present invention may be degradation products (e.g., oxidation products of methionine or deamidated glutamine, arganine, and C-terminus amide). Such degradation products may be used in and are therefore considered part of the present invention. In preferred embodiments, the heteromultimers of the invention comprise multiple peptides that are all EPO-R agonist peptides. In highly preferred embodiments, the multimers of the invention are homomultimers: *i.e.*, they comprise multiple EPO-R agonist peptides of the same amino acid sequence.

Accordingly, the present invention also relates to dimeric peptide agonists of EPO-R, which show dramatically enhanced potency and activity. In preferred embodiments, the dimers of the invention comprise two peptides that are both EPO-R agonist peptides. These preferred dimeric peptide agonists comprise two peptide monomers, wherein each peptide monomer is of 17 to about 40 amino acids in length and comprises the core amino acid sequence LYACHX_0GPITX_1VCQPLR (SEQ ID NO: 1), where each amino acid is indicated by standard one letter abbreviation, X_0 is methionine (M) or homoserine methylether (Hsm), and X_1 is tryptophan (W), 1-naphthylalanine (1-nal), or 2-naphthylalanine (2-nal). In particularly preferred embodiments, the dimers of the invention comprise two EPO-R agonist peptides of the same amino acid sequence.

According to some embodiments of the invention, two or more, and preferably from two to six amino acid residues, independently selected from any of the 20 genetically encoded L-amino acids or the stereoisomeric D-amino acids, will be coupled to either or both ends of the core sequence described above. For example, the sequence GG will often be appended to either terminus or both termini of the core sequence.

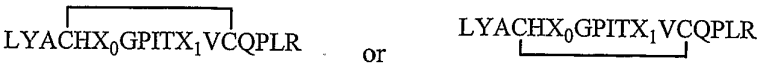
Stereoisomers (*e.g.*, D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as a,a-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for compounds of the present invention. Examples of unconventional amino acids include, but are not limited to: β-alanine, 3-pyridylalanine, 4-hydroxyproline, O-phosphoserine, N-methylglycine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, nor-leucine, and other similar amino acids and imino acids.

Other modifications are also possible, including modification of the amino terminus, modification of the carboxy terminus, replacement of one or more of the naturally occurring genetically encoded amino acids with an unconventional amino acid, modification of the side chain of one or more amino acid residues, peptide phosphorylation, and the like. A preferred amino terminal modification is acetylation (*e.g.*, with acetic acid or a halogen substituted acetic acid). In preferred embodiments an N-terminal glycine is acetylated to N-acetylglycine (AcG). In preferred embodiments, a the C-terminal glycine is N-methylglycine (MeG, also known as sarcosine).

Preferred peptide monomers of the present invention include, but are not limited to:

LYACHMGPITWVCQPLR	(SEQ ID NO: 2);
LYACHMGPIT(1-nal)VCQPLR	(SEQ ID NO: 3);
LYACHMGPIT(2-nal)VCQPLR	(SEQ ID NO: 4);
GGLYACHMGPITWVCQPLRG	(SEQ ID NO: 5);
GGLYACHMGPIT(1-nal)VCQPLRG	(SEQ ID NO: 6);
GGLYACHMGPIT(2-nal)VCQPLRG	(SEQ ID NO: 7);
(AcG)GLYACHMGPITWVCQPLRG	(SEQ ID NO: 8);
(AcG)GLYACHMGPIT(1-nal)VCQPLRG	(SEQ ID NO: 9);
(AcG)GLYACHMGPIT(2-nal)VCQPLRG	(SEQ ID NO: 10);
GGLYACHMGPITWVCQPLR(MeG)	(SEQ ID NO: 11);
GGLYACHMGPIT(1-nal)VCQPLR(MeG)	(SEQ ID NO: 12);
GGLYACHMGPIT(2-nal)VCQPLR(MeG)	(SEQ ID NO: 13);
(AcG)GLYACHMGPITWVCQPLRG(MeG)	(SEQ ID NO: 14);
(AcG)GLYACHMGPIT(1-nal)VCQPLRG(MeG)	(SEQ ID NO: 15);
(AcG)GLYACHMGPIT(2-nal)VCQPLRG(MeG)	(SEQ ID NO: 16);
LYACH(Hsm)GPITWVCQPLR	(SEQ ID NO: 17);
LYACH(Hsm)GPIT(1-nal)VCQPLR	(SEQ ID NO: 18);
LYACH(Hsm)GPIT(2-nal)VCQPLR	(SEQ ID NO: 19);
GGLYACH(Hsm)GPITWVCQPLRG	(SEQ ID NO: 20);
GGLYACH(Hsm)GPIT(1-nal)VCQPLRG	(SEQ ID NO: 21);
GGLYACH(Hsm)GPIT(2-nal)VCQPLRG	(SEQ ID NO: 22);
(AcG)GLYACH(Hsm)GPITWVCQPLRG	(SEQ ID NO: 23);
(AcG)GLYACH(Hsm)GPIT(1-nal)VCQPLRG	(SEQ ID NO: 24);
(AcG)GLYACH(Hsm)GPIT(2-nal)VCQPLRG	(SEQ ID NO: 25);
GGLYACH(Hsm)GPITWVCQPLR(MeG)	(SEQ ID NO: 26);
GGLYACH(Hsm)GPIT(1-nal)VCQPLR(MeG)	(SEQ ID NO: 27);
GGLYACH(Hsm)GPIT(2-nal)VCQPLR(MeG)	(SEQ ID NO: 28);
(AcG)GLYACH(Hsm)GPITWVCQPLRG(MeG)	(SEQ ID NO: 29);
(AcG)GLYACH(Hsm)GPIT(1-nal)VCQPLRG(MeG)	(SEQ ID NO: 30); and.
(AcG)GLYACH(Hsm)GPIT(2-nal)VCQPLRG(MeG)	(SEQ ID NO: 31).

In preferred embodiments, the peptide monomers of the invention contain an intramolecular disulfide bond between the two cysteine residues of the core sequence. Such monomers may be represented schematically as follows:



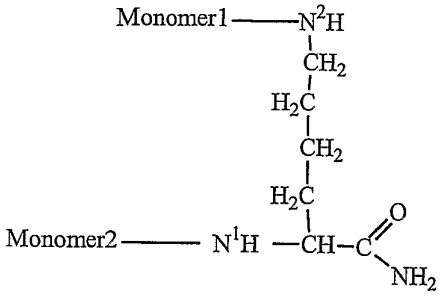
The present invention also provides conjugates of these peptide monomers. Thus, according to a preferred embodiment, the monomeric peptides of the present invention are dimerized or oligomerized, thereby enhancing EPO-R agonist activity.

In one embodiment, the peptide monomers of the invention may be oligomerized using the biotin/streptavidin system. Biotinylated analogs of peptide monomers may be synthesized by standard techniques. For example, the peptide monomers may be C-terminally biotinylated. These biotinylated monomers are then oligomerized by incubation with streptavidin [*e.g.*, at a 4:1 molar ratio at room temperature in phosphate buffered saline (PBS) or HEPES-buffered RPMI medium (Invitrogen) for 1 hour]. In a variation of this embodiment, biotinylated peptide monomers may be oligomerized by incubation with any one of a number of commercially available anti-biotin antibodies [*e.g.*, goat anti-biotin IgG from Kirkegaard & Perry Laboratories, Inc. (Washington, DC)].

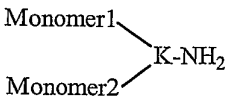
In preferred embodiments, the peptide monomers of the invention are dimerized by covalent attachment to at least one linker moiety. The linker (L_K) moiety is preferably, although not necessarily, a C_{1-12} linking moiety optionally terminated with one or two -NH- linkages and optionally substituted at one or more available carbon atoms with a lower alkyl substituent. Preferably the linker L_K comprises -NH-R-NH- wherein R is a lower (C_{1-6}) alkylene substituted with a functional group such as a carboxyl group or an amino group that enables binding to another molecular moiety (*e.g.*, as may be present on the surface of a solid support). Most preferably the linker is a lysine residue or a lysine amide (a lysine residue wherein the carboxyl group has been converted to an amide moiety -$CONH_2$). In preferred embodiments, the linker bridges the C-termini of two peptide monomers, by simultaneous attachment to the C-terminal amino acid of each monomer.

For example, when the C-terminal linker L_K is a lysine amide the dimer may be illustrated structurally as shown in Formula I, and summarized as shown in Formula II:

Formula I



Formula II



In Formula I and Formula II, N^2 represents the nitrogen atom of lysine's ε-amino group and N^1 represents the nitrogen atom of lysine's α-amino group. The dimeric structure can be written as [peptide]$_2$Lys-amide to denote a peptide bound to both the α and ε amino groups of lysine, or [Ac-peptide]$_2$Lys-amide to denote an N-terminally acetylated peptide bound to both the α and ε amino groups of lysine, or [Ac-peptide, disulfide]$_2$Lys-amide to denote an N-terminally acetylated peptide bound to both the α and ε amino groups of lysine with each peptide containing an intramolecular disulfide loop, or [Ac-peptide, disulfide]$_2$Lys-spacer-PEG to denote an N-terminally acetylated peptide bound to both the α and ε amino groups of lysine with each peptide containing an intramolecular disulfide loop and a spacer molecule forming a covalent linkage between the C-terminus of lysine and a PEG moiety, or [Ac-peptide-Lys*-NH_2]$_2$-Iminodiacetic-*N*-(Boc-βAla) to denote a homodimer of an N-terminally acetylated peptide bearing a C-terminal lysineamide residue where the ε amine of lysine is bound to each of the two carboxyl groups of iminodiacetic acid and where Boc-beta-alanine is covalently bound to the nitrogen atom of iminodiacetic acid via an amide bond.

In an additional embodiment, polyethylene glycol (PEG) may serve as the linker L_K that dimerizes two peptide monomers: for example, a single PEG moiety may be simultaneously attached to the N-termini of both peptide chains of a peptide dimer.

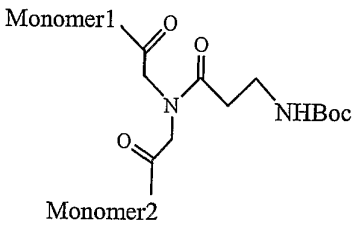
In yet another additional embodiment, the linker (L_K) moiety is preferably, but not necessarily, a molecule containing two carboxylic acids and optionally substituted at one or more available atoms with an additional functional group such as an amine capable of being bound to one or more PEG molecules. Such a molecule can be depicted as:

$$-CO-(CH_2)_n-X-(CH_2)_m-CO-$$

where n is an integer from 0 to 10, m is an integer from 1 to 10, X is selected from O, S, $N(CH_2)_pNR_1$, $NCO(CH_2)_pNR_1$, and $CHNR_1$, R_1 is selected from H, Boc, Cbz, etc., and p is an integer from 1 to 10.

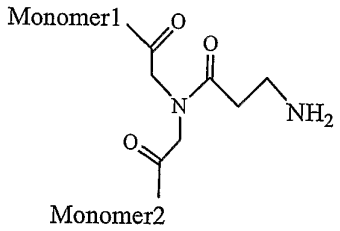
In preferred embodiments, one amino group of each of the peptides form an amide bond with the linker L_K. In particularly preferred embodiments, the amino group of the peptide bound to the linker L_K is the epsilon amine of a lysine residue or the alpha amine of the N-terminal residue, or an amino group of the optional spacer molecule. In particularly preferred embodiments, both n and m are one, X is $NCO(CH_2)_pNR_1$, p is two, and R_1 is Boc. A dimeric EPO peptide containing such a preferred linker may be structurally illustrated as shown in Formula III.

Formula III



Optionally, the Boc group can be removed to liberate a reactive amine group capable of forming a covalent bond with a suitably activated water soluble polymer species, for example, a PEG species such as mPEG-para-nitrophenylcarbonate (mPEG-NPC), mPEG-succinimidyl propionate (mPEG-SPA), and N-hydroxysuccinimide-PEG (NHS-PEG) (see, e.g., US Patent No. 5,672,662). A dimeric EPO peptide containing such a preferred linker may be structurally illustrated as shown in Formula IV.

Formula IV



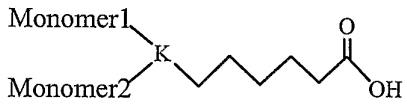
Generally, although not necessarily, peptide dimers will also contain one or more intramolecular disulfide bonds between cysteine residues of the peptide monomers. Preferably, the two monomers contain at least one intramolecular disulfide bond. Most preferably, both monomers of a peptide dimer contain an intramolecular disulfide bond, such that each monomer contains a cyclic group.

A peptide monomer or dimer may further comprise one or more spacer moieties. Such spacer moieties may be attached to a peptide monomer or to a peptide dimer. Preferably, such spacer moieties are attached to the linker L_K moiety that connects the monomers of a peptide dimer. For example, such spacer moieties may be attached to a peptide dimer via the carbonyl carbon of a lysine linker, or via the nitrogen atom of an iminodiacetic acid linker. For example, such a spacer may connect the linker of a

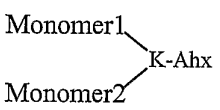
peptide dimer to an attached water soluble polymer moiety or a protecting group. In another example, such a spacer may connect a peptide monomer to an attached water soluble polymer moiety.

In one embodiment, the spacer moiety is a C_{1-12} linking moiety optionally terminated with -NH- linkages or carboxyl (-COOH) groups, and optionally substituted at one or more available carbon atoms with a lower alkyl substituent. In one embodiment, the spacer is R-COOH wherein R is a lower (C_{1-6}) alkylene optionally substituted with a functional group such as a carboxyl group or an amino group that enables binding to another molecular moiety. For example, the spacer may be a glycine (G) residue, or an amino hexanoic acid. In preferred embodiments the amino hexanoic acid is 6-amino hexanoic acid (Ahx). For example, where the spacer 6-amino hexanoic acid (Ahx) is bound to the N-terminus of a peptide, the peptide terminal amine group may be linked to the carboxyl group of Ahx via a standard amide coupling. In another example, where Ahx is bound to the C-terminus of a peptide, the amine of Ahx may be linked to the carboxyl group of the terminal amino acid via a standard amide coupling. The structure of such a peptide may be depicted as shown in Formula V, and summarized as shown in Formula VI.

Formula V



Formula VI



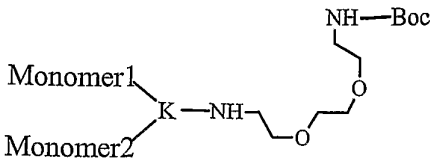
In other embodiments, the spacer is -NH-R-NH- wherein R is a lower (C_{1-6}) alkylene substituted with a functional group such as a carboxyl group or an amino group that enables binding to another molecular moiety. For example, the spacer may be a lysine (K) residue or a lysine amide (K-NH_2, a lysine residue wherein the carboxyl group has been converted to an amide moiety -$CONH_2$).

In preferred embodiments, the spacer moiety has the following structure:

$$-NH-(CH_2)_\alpha-[O-(CH_2)_\beta]_\gamma-O_\delta-(CH_2)_\varepsilon-Y-$$

where α, β, γ, δ, and ε are each integers whose values are independently selected. In preferred embodiments, α, β, and ε are each integers whose values are independently selected from one to about six, δ is zero or one, γ is an integer selected from zero to about ten, except that when γ is greater than one, β is two, and Y is selected from NH or CO. In particularly preferred embodiments α, β, and ε are each equal to two, both γ and δ are equal to 1, and Y is NH. For example, a peptide dimer containing such a spacer is illustrated schematically in Formula VII, where the linker is a lysine and the spacer joins the linker to a Boc protecting group.

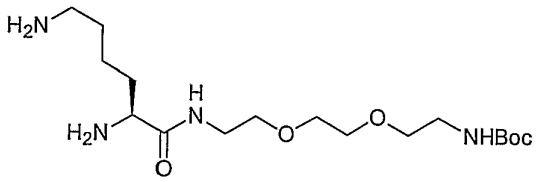
Formula VII



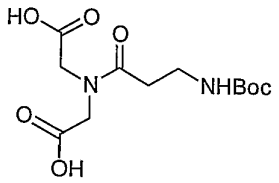
In another particularly preferred embodiment γ and δ are zero, α and ε together equal five, and Y is CO.

In particularly preferred embodiments, the linker plus spacer moiety has the structure shown in Formula VIII or Formula IX.

Formula VIII



Formula IX



The peptide monomers, dimers, or multimers of the invention may further comprise one or more water soluble polymer moieties. Preferably, these polymers are covalently attached to the peptide compounds of the invention. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the art will be able to select the desired polymer based on such considerations as whether the polymer-peptide conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. The water soluble polymer may be, for example, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, polypropylene oxide/ethylene oxide copolymers, and polyoxyethylated polyols. A preferred water soluble polymer is PEG.

The polymer may be of any molecular weight, and may be branched or unbranched. A preferred PEG for use in the present invention comprises linear, unbranched PEG having a molecular weight that is greater than 10 kilodaltons (kD) and is more preferably between about 20 and 60 kD in molecular weight. Still more preferably, the linear unbranched PEG moiety should have a molecular weight of between about 20 and 40 kD, with 20 kD PEG being particularly preferred. It is understood that in a given preparation of PEG, the molecular weights will typically vary among individual molecules. Some

molecules will weight more, and some less, than the stated molecular weight. Such variation is generally reflect by use of the word "about" to describe molecular weights of the PEG molecules.

The number of polymer molecules attached may vary; for example, one, two, three, or more water soluble polymers may be attached to an EPO-R agonist peptide of the invention. The multiple attached polymers may be the same or different chemical moieties (*e.g.*, PEGs of different molecular weight). Thus, in a preferred embodiment the invention contemplates EPO-R agonist peptides having two or more PEG moieities attached thereto. Preferably, both of the PEG moietieis are linear, unbranched PEG each preferably having a molecular weight of between about 10 and about 60 kD. More preferably, each linear unbranched PEG moiety has a molecular weight that is between about 20 and 40 kD, and still more preferably between about 20 and 30 kD with a molecular weight of about 20 kD for each linear PEG moiety being particularly preferred. However, other molecular weights for PEG are also contemplated in such embodiments. For example, the invention contemplates and encompasses EPO-R agonist peptides having two or more linear unbranched PEG moieties attached thereto, at least one or both of which has a molecular weight between about 20 and 40 kD or between about 20 and 30 kD. In other embodiments the invention contemplates and encompasses EPO-R agonist peptides having two or more linear unbranched PEG moieties attached thereto, at least one of which has a molecular weight between about 40 and 60 kD.

In one embodiment, PEG may serve as a linker that dimerizes two peptide monomers. In one embodiment, PEG is attached to at least one terminus (N-terminus or C-terminus) of a peptide monomer or dimer. In another embodiment, PEG is attached to a spacer moiety of a peptide monomer or dimer. In a preferred embodiment PEG is attached to the linker moiety of a peptide dimer. In a highly preferred embodiment, PEG is attached to a spacer moiety, where said spacer moiety is attached to the linker L_K moiety that connects the monomers of a peptide dimer. In particularly preferred embodiments, PEG is attached to a spacer moiety, where said spacer moiety is attached to a peptide dimer via the carbonyl carbon of a lysine linker, or the amide nitrogen of a lysine amide linker.

Preferred peptide dimers of the present invention include, but are not limited to:

Compound designation	Peptide dimer
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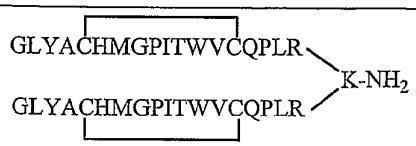
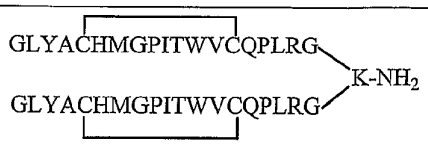
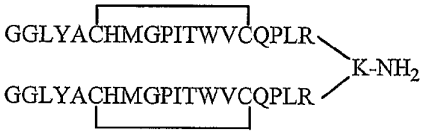
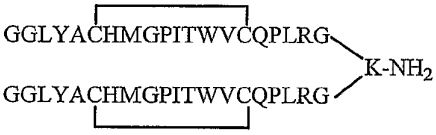
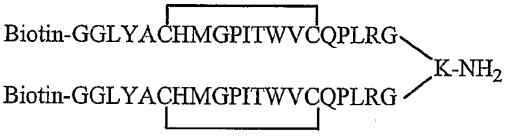
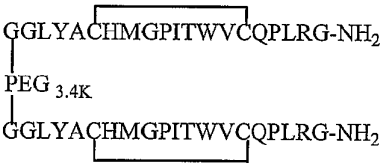
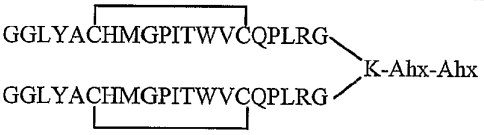
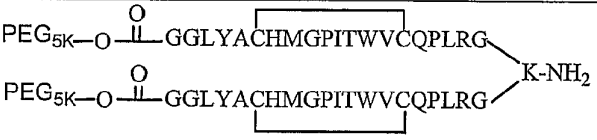
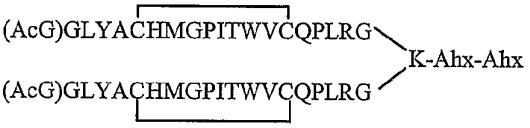
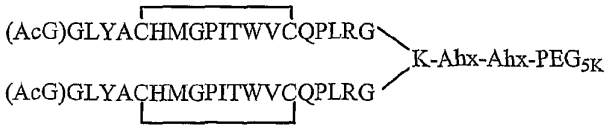
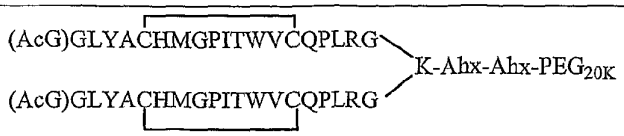
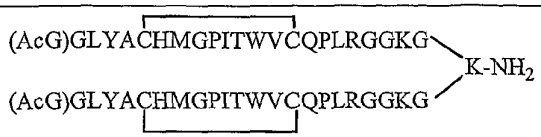
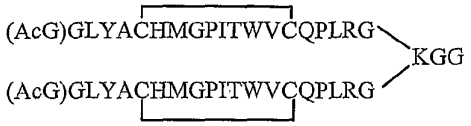
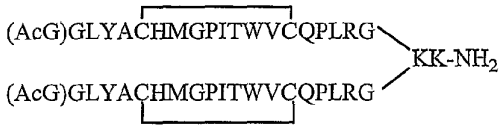
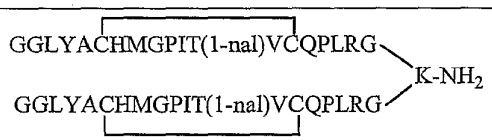
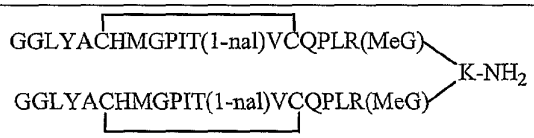
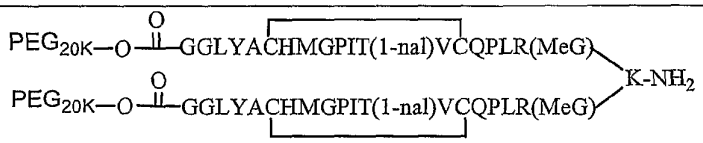
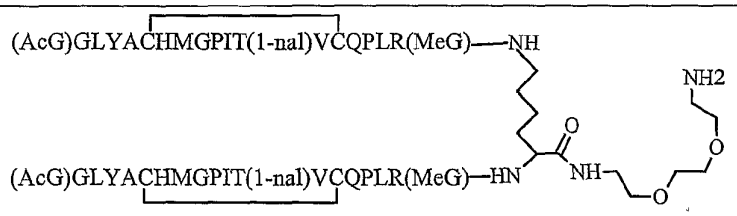
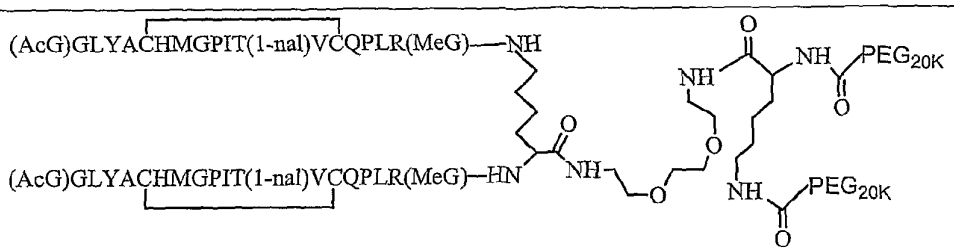
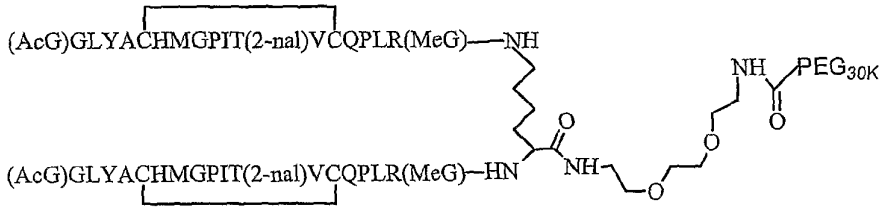
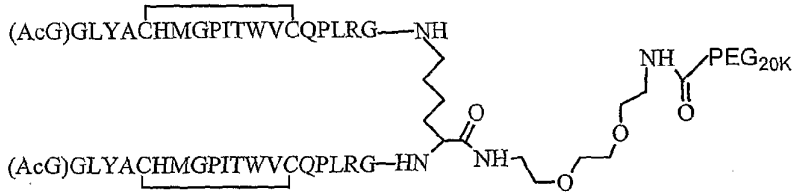
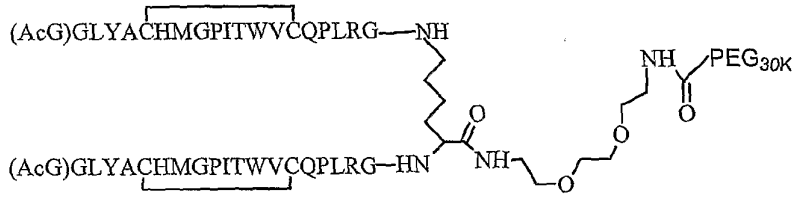
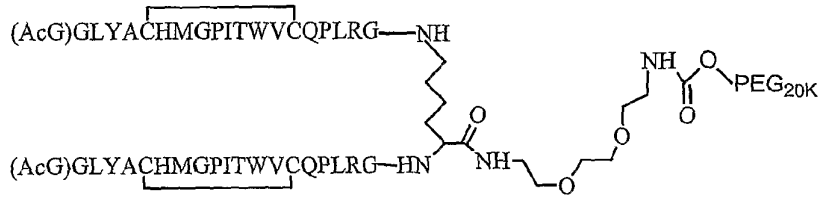
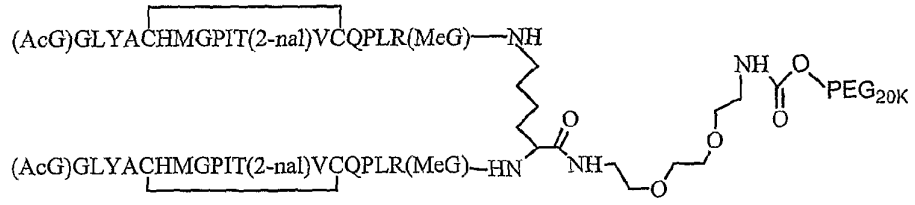
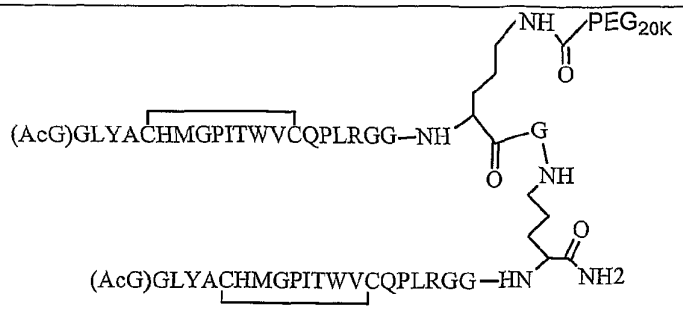
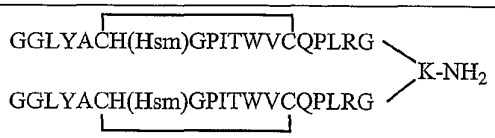
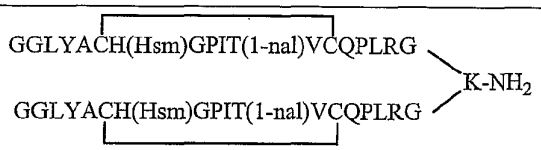
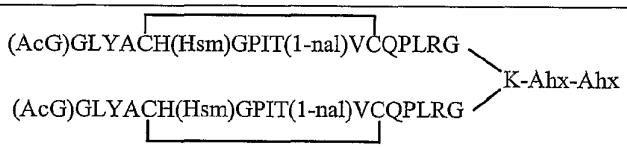
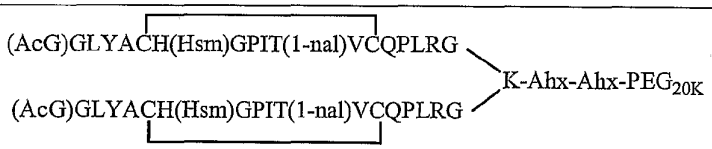
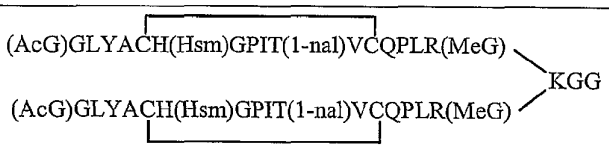
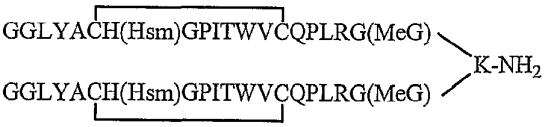
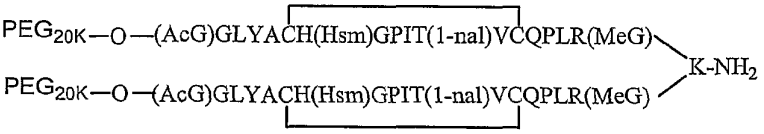
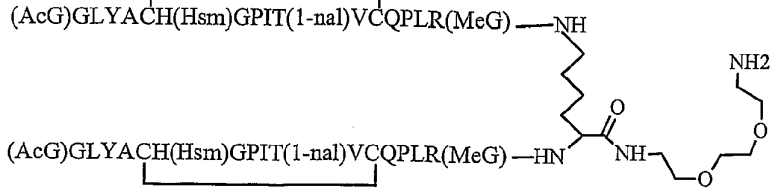
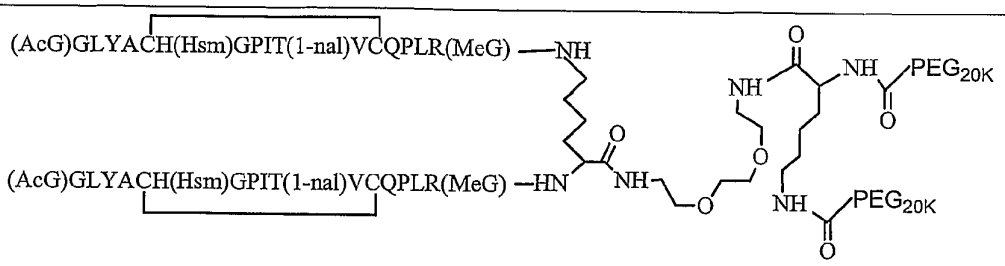
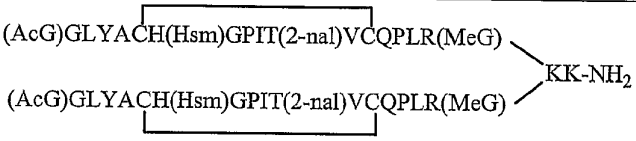
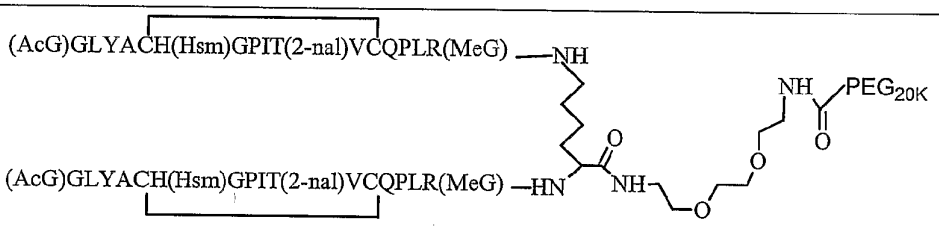
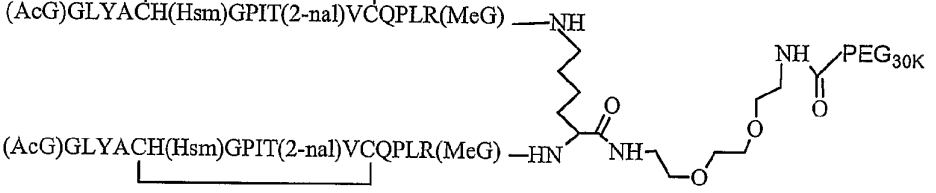
AF34602	
AF34395	
AF34601	
AF32579	
AF33068	
AF33131	
AF34351	
AF34350	
AF34753	

AF34757	
AF35062	
AF35218	
AF35462	
AF35464	
AF33197	
AF34994	
AF35083	
AF35525	

AF35526	
AF35563	
AF35575	
AF35592	
AF35593	
AF35594	

AF35219	
AF32876	
AF32881	
AF35179	
AF35180	
AF35463	
AF35090	
AF35148	

AF35149	
AF35168	
AF35361	
AF35595	
AF35564	

Still other peptides of the present invention, including peptide monomers and dimers, are as follows:

SEQ ID NO:	PEG size																															Linker	
34						Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2					Monomer
35					[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	H2 Dimer
36					[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA + Boc	Boc on IDA
37	40 KDa				[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	PEG
38																																	
39					[	Ac	G	G	L	Y	A	C	H	M(O2	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Bis sulfone
40	40 KDa				[	Ac	G	G	L	Y	A	C	H	M(O2	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Bis sulfone
41					[	Ac	G	G	L	Y	A	C	H	M(O)	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Bis sulfoxide, asymmetric
42					[	Ac	G	G	L	Y	A	C	H	M(O)	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Bis sulfoxide, asymmetric
43					[	Ac	G	G	L	Y	A	C	H	MXX	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Mono sulfoxide, asymmetric
44					[	Ac	G	G	L	Y	A	C	H	MXX	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Mono sulfoxide, asymmetric
45					[	Ac	G	G	L	Y	A	C	H	MXX	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Mono sulfone, asymmetric
46						[	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	NH2	]2				IDA	N-terminal dimer
47					[	Ahx	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	NH2	]2				IDA	N-terminal dimer
48					[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Parallel disulfide
49					[	Ac	G	G	L	Y	A	C	H	M(O)	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Bis sulfoxide
50					[	Ac	G	G	L	Y	A	C	H	M	G	P	O	T	1Nal	V	C	Q	P	L	R	Sar	K	NH2	]2			IDA	Crisscross disulfide
51				[	Ac	S	R	T	R	Y	R	C	E	M	G	P	L	T	W	V	C	R	R	W	K	NH2	]2					IDA + Boc	
52				[	Ac	L	T	R	L	Y	S	C	H	M	G	P	S	T	W	V	C	S	T	A	L	R	K	NH2	]2			IDA + Boc	
53				[	Ac	R	G	Q	L	Y	A	C	H	F	G	P	V	T	W	V	C	R	R	R	R	R	V	K	NH2	]2		IDA + Boc	
54				[	Ac	S	G	I	L	Y	E	C	H	M	G	P	L	T	W	V	C	T	P	S	R	R	R	K	NH2	]2		IDA + Boc	
55				[	Ac	L	G	R	R	Y	S	C	H	F	G	A	L	T	W	V	C	Q	P	A	R	R	D	K	NH2	]2		IDA + Boc	
56				[	Ac	G	S	R	T	Y	S	C	Q	L	G	P	V	D	W	V	C	G	R	R	R	K	NH2	]2				IDA + Boc	
57					Ac	A	R	G	R	Y	Q	C	Q	F	G	P	L	T	W	E	C	A	R	I	R	P	R	K	NH2				Monomer
58					Ac	V	T	R	M	Y	R	C	R	M	G	P	L	T	W	V	C	E	R	K	NH2								Monomer
59					Ac	R	P	S	L	Y	E	C	H	L	G	P	L	T	W	E	C	R	P	R	R	R	E	K	NH2				Monomer
60					Ac	R	G	H	M	Y	S	C	Q	L	G	P	V	T	W	V	C	R	P	L	S	G	R	K	NH2				Monomer
61					Ac	I	T	P	T	Y	H	C	R	F	G	P	Q	T	W	V	C	A	P	R	R	S	A	L	T	K	NH2		Monomer
62					Ac	G	N	R	M	Y	Q	C	H	M	G	P	L	T	W	V	C	Q	P	T	R	I	H	K	NH2				Monomer
63				[	Ac	A	R	G	R	Y	Q	C	Q	F	G	P	L	T	W	E	C	L	P	I	R	P	R	K	NH2	]2		IDA + Boc	
64				[	Ac	V	T	R	M	Y	R	C	R	M	G	P	L	T	W	V	C	E	R	K	NH2	]2						IDA + Boc	
65				[	Ac	R	P	S	L	Y	E	C	H	L	G	P	L	T	W	E	C	R	P	R	R	R	E	K	NH2	]2		IDA + Boc	
66				[	Ac	R	G	H	M	Y	S	C	Q	L	G	P	V	T	W	V	C	R	P	L	S	G	R	K	NH2	]2		IDA + Boc	
67				[	Ac	I	T	P	T	Y	H	C	R	F	G	P	Q	T	W	V	C	A	P	R	R	S	A	L	T	K	NH2]2	IDA + Boc	
68				[	Ac	G	N	R	M	Y	Q	C	H	M	G	P	L	T	W	V	C	Q	P	T	R	I	H	K	NH2	]2		IDA + Boc	
69				[	Ac	R	N	H	L	Y	G	C	R	M	G	P	L	T	W	V	C	S	S	R	G	T	Q	K	NH2	]2		IDA + Boc	
70				[	Ac	P	D	L	A	Y	S	C	R	M	G	P	L	T	W	V	C	A	P	N	R	K	NH2	]2				IDA + Boc	
71				[	Ac	L	G	R	R	Y	S	C	H	F	G	P	L	T	W	V	C	Q	P	A	R	R	D	K	NH2	]2		IDA + Boc	
72				[	Ac	L	L	R	G	Y	E	C	Y	M	G	P	L	T	W	V	C	R	S	S	R	P	R	K	NH2	]2		IDA + Boc	
73				[	Ac	M	R	T	R	Y	R	C	Y	M	G	P	L	T	W	V	C	E	G	S	R	L	K	NH2	]2			IDA + Boc	
74				[	Ac	H	L	G	R	Y	D	C	S	F	G	P	Q	T	W	V	C	R	P	R	R	S	L	K	NH2	]2		IDA + Boc	
75				[	Ac	I	R	G	R	N	R	C	R	F	G	P	Q	T	W	V	C	P	D	S	Y	E	F	K	NH2	]2		IDA + Boc	
76		Ac	Q	R	R	H	V	F	L	S	D	G	A	A	Y	V	G	L	W	V	E	C	D	D	I	S	K	NH2					Monomer
77				[	Ac	V	L	P	L	Y	R	C	R	M	G	R	E	T	W	E	C	M	R	A	A	G	V	T	K	NH2	]2	IDA + Boc	
78				[	Ac	P	G	N	S	Y	R	C	H	M	G	P	L	T	W	V	C	G	R	D	R	H	L	K	NH2	]2		IDA + Boc	

79					Ac	R	N	H	L	Y	G	C	R	M	G	P	L	T	W	V	C	S	S	R	G	T	Q	K	NH_2					Monomer				
80					Ac	P	D	L	A	Y	S	C	R	M	G	P	L	T	W	V	C	A	P	N	R	K	NH_2						Monomer				
81					Ac	L	G	R	R	Y	S	C	H	F	G	P	L	T	W	V	C	Q	P	A	R	R	D	K	NH_2					Monomer				
82					Ac	L	L	R	G	Y	E	C	Y	M	G	P	L	T	W	V	C	R	S	S	R	P	R	K	NH_2					Monomer				
83					Ac	M	R	T	R	Y	R	C	Y	M	G	P	L	T	W	V	C	E	G	S	R	L	K	NH_2						Monomer				
84					Ac	H	L	G	R	Y	D	C	S	F	G	P	Q	T	W	V	C	R	P	R	R	S	L	K	NH_2					Monomer				
85					Ac	I	R	G	R	N	R	C	R	F	G	P	Q	T	W	V	C	P	D	S	Y	E	F	K	NH_2					Monomer				
86					Ac	R	P	R	P	Y	S	C	T	M	G	P	R	T	W	V	C	G	G	V	R	A	G	K	NH_2					Monomer				
87					Ac	V	L	P	L	Y	R	C	R	M	G	R	E	T	W	E	C	M	R	A	A	G	V	T	K	NH_2				Monomer				
88					Ac	P	G	N	S	Y	R	C	M	G	P	L	T	W	V	C	G	R	D	R	H	L	K	NH_2					Monomer				
89	40 KDa				[	Ac	G	G	L	Y	A	C	H	M(x)	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	Monosulfoxide				
90	40 KDa				[	Ac	G	G	L	Y	A	C	H	M(x)	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	Monosulfoxide				
91					[	Ac	G	G	I	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	D Leu				
92					[	Ac	G	G	L	Y	A	C	H	m	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	D Met				
93					[	Ac	G	G	L	Y	A	c	H	M	G	P	I	T	1Nal	V	c	Q	P	L	R	Sar	K	NH_2	]2				IDA	2 D cys				
94					[	Ac	G	G	L	Y	A	c	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	D Cys				
95					[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	c	Q	P	L	R	Sar	K	NH_2	]2				IDA	D Cys				
96					[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	p	L	R	Sar	K	NH_2	]2				IDA	D Pro				
97							[	Ac	E	Y	L	C	R	M	G	P	I	T	W	V	C	E	R	Y	K	NH_2	]2					IDA + Boc				
98							[	Ac	T	Y	S	C	H	F	G	P	L	T	W	V	C	R	P	Q	K	NH_2	]2					IDA + Boc				
99							[	Ac	D	Y	H	C	R	M	G	P	L	T	W	V	C	R	P	L	K	NH_2	]2					IDA + Boc				
100							[	Ac	L	Y	E	C	R	M	G	P	M	T	W	V	C	R	P	G	K	NH_2	]2					IDA + Boc				
101							[	Ac	L	Y	L	C	R	M	G	P	V	T	W	E	C	Q	P	R	K	NH_2	]2					IDA + Boc				
102							[	Ac	D	Y	N	C	R	F	G	P	L	T	W	V	C	R	P	S	K	NH_2	]2					IDA + Boc				
103							[	Ac	S	Y	L	C	R	R	G	P	T	T	W	L	C	T	A	Q	K	NH_2	]2					IDA				
104							[	Ac	E	Y	S	C	R	M	G	P	M	T	W	V	C	S	P	T	K	NH_2	]2					IDA				
105							[	Ac	L	Y	L	C	R	F	G	P	V	T	W	D	C	G	Y	K	NH_2	]2						IDA				
106							[	Ac	I	Y	R	C	L	M	G	P	L	T	W	V	C	T	P	D	K	NH_2	]2					IDA				
107					[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	D Leu				
108	40 KDa				[	Ahx	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	NH_2	]2					IDA	N-terminal dimer				
109	40 KDa					[	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	NH_2	]2					IDA	Parallel disulfide				
110					[	Ac	G	G	L	Y	A	C	H	M	G	p	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	D Pro				
111					[	Ac	G	G	L	Y	A	C	h	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	D His				
112					[	Ac	G	G	L	Y	a	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	D Ala				
113					[	Ac	G	G	L	Y	A	Cxx	H	M	G	P	I	T	1Nal	V	Cxx	Q	P	L	R	Sar	K	NH_2	]2				IDA	1/2 Crisscross disulfide				
114					[	Ac	G	G	L	Y	A	C(Acm)	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	1/2 Parallel disulfide				
115	40 KDa					[	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	NH_2	]2					IDA	N-Terminal dimer				
116	40 KDa				[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2				IDA	Crisscross disulfide				
117					[	Ac	G	G	L	Y	A	CSH	H	M	G	P	I	T	1Nal	V	CSH	Q	P	L	R	Sar	K	NH_2	]2				IDA	Reduced disulfide				
118					[	Ac	G	G	L	Y	A	C(Ace)	H	M	G	P	I	T	1Nal	V	C(Ace)	Q	P	L	R	Sar	K	NH_2	]2				IDA	Capped Cysteine, no SS				
119				[	Ac	R	T	R	E	Y	S	C	Q	M	G	P	L	T	W	T	C	V	P	R	S	K	NH_2	]2					IDA + Boc				
120				[	Ac	S	R	A	R	Y	M	C	H	M	G	P	L	T	W	V	C	R	P	E	V	K	NH_2	]2					IDA + Boc				
121				[	Ac	G	G	R	A	Y	M	C	R	L	G	P	V	T	W	V	C	S	P	R	I	R	I	K	NH_2	]2			IDA + Boc				
122				[	Ac	T	I	A	Q	Y	I	C	Y	M	G	P	E	T	W	E	C	R	P	S	P	R	A	K	NH_2	]2			IDA + Boc				
123				[	Ac	N	G	R	T	Y	S	C	Q	L	G	P	V	T	W	V	C	S	R	G	V	R	R	K	NH_2	]2			IDA + Boc				

122	[	Ac	T	I	A	Q	Y	I	C	Y	M	G	P	E	T	W	E	C	R	P	S	P	R	A	K	NH_2	]2	IDA + Boc	
123	[	Ac	N	G	R	T	Y	S	C	Q	L	G	P	V	T	W	V	C	S	R	G	V	R	R	K	NH_2	]2	IDA + Boc	
124	[	Ac	M	R	T	R	Y	R	C	Y	M	G	P	L	T	W	V	C	E	G	S	R	L	K	NH_2	]2		IDA + Boc	
125	[	Ac	S	R	T	R	Y	R	C	E	M	G	P	L	T	W	V	C	E	R	W	K	NH_2	]2				IDA + Boc	
126	[	Ac	G	S	R	T	Y	S	C	Q	L	G	P	V	T	W	V	C	G	R	R	R	K	NH_2	]2			IDA + Boc	
127	[	Ac	R	P	R	P	Y	S	C	T	M	G	P	R	T	W	V	C	G	G	V	R	A	G	K	NH_2	]2	IDA + Boc	
128	[	Ac	G	G	T	Y	S	C	H	F	G	P	L	T	W	V	C	R	P	Q	G	G	K	NH_2	]2			IDA + Boc	
129	[	Ac	G	G	D	Y	H	C	R	M	G	P	L	T	W	V	C	R	P	L	G	G	K	NH_2	]2			IDA + Boc	
130	[	Ac	G	G	V	Y	A	C	R	M	G	P	I	T	W	V	C	S	P	L	G	G	K	NH_2	]2			IDA	
131	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	Y	1Nal	V	C	E	P	L	R	Sar	K	NH_2	]2			IDA	Deamidated
132	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	E	P	L	R	Sar	K	OH	]2			IDA	Bis-Deamidated
133	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	k	NH2	]2			IDA	D Lys
134	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	t	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2			IDA	D Thr
135	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	q	P	L	R	Sar	K	NH_2	]2			IDA	D Gln
136	[	Ac	G	G	L	y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2			IDA	D Tyr
137	[	Ac	G	G	L	Y	A	C	H	M	G	P	i	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2			IDA	D Ile
138	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	v	C	Q	P	L	R	Sar	K	NH_2	]2			IDA	D Val
139	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1nal	V	C	Q	P	L	R	Sar	K	NH_2	]2			IDA	D 1Nal
140	[	Ac	G	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	Gly Insertion
141	[	Ac	G	G	L	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	Leu Insertion
142	[	Ac	G	G	L	Y	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	Tyr Insertion
143	[	Ac	G	G	L	Y	A	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	Ala Insertion
144	[	Ac	G	G	L	Y	A	C	H	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	His Insertion
145	[	Ac	G	G	L	Y	A	C	H	M	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	Met Insertion
146	[	Ac	N	Y	T	C	R	F	G	P	L	T	W	E	C	T	P	Q	K	NH_2	]2							IDA-Boc	
147	[	Ac	S	W	D	C	R	I	G	P	I	T	W	V	C	R	W	S	K	NH_2	]2							IDA-Boc	
148	[	Ac	N	Y	M	C	H	F	G	P	I	T	W	V	C	R	P	G	K	NH_2	]2							IDA-Boc	
149	[	Ac	L	Y	L	C	R	M	G	P	Q	T	W	M	C	Q	P	G	K	NH_2	]2							IDA-Boc	
150	[	Ac	W	Y	S	C	L	M	G	P	M	T	W	V	C	R	A	H	K	NH_2	]2							IDA-Boc	
151	[	Ac	E	Y	F	C	R	M	G	P	I	T	W	V	C	Q	R	S	K	NH_2	]2							IDA-Boc	
152	[	Ac	G	G	L	Y	A	C	H	M	G	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	Gly Insertion
153	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	I	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	Ile Insertion
154	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	T	1Nal	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	Thr Insertion
155	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	V	C	Q	P	L	R	Sar	K	NH_2	]2		IDA	Val Insertion
156	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	Q	P	L	R	Sar	K	NH_2	]2		IDA	Gln Insertion
157	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	P	L	R	Sar	K	NH_2	]2		IDA	Pro Insertion
158	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	L	R	Sar	K	NH_2	]2		IDA	Leu Insertion
159	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	R	Sar	K	NH_2	]2		IDA	Arg Insertion
160	[	Ac	G	G	L	Y	A	C	H	M	G	P	I	T	1Nal	V	C	Q	P	L	R	Sar	Sar	K	NH_2	]2		IDA	Sar Insertion

Branching amino acid to linker shown shaded

IDA is imino diacetic acid (BetaAla conjugate)

The peptide sequences of the present invention can be present alone or in conjunction with N-terminal and/or C-terminal extensions of the peptide chain. Such extensions may be naturally encoded peptide sequences optionally with or substantially without non-naturally occurring sequences; the extensions may include any additions, deletions, point mutations, or other sequence modifications or combinations as desired by those skilled in the art. For example and not limitation, naturally-occurring sequences may be full-length or partial length and may include amino acid substitutions to provide a site for attachment of carbohydrate, PEG, other polymer, or the like via side chain conjugation. In a variation, the amino acid substitution results in humanization of a sequence to make in compatible with the human immune system. Fusion proteins of all types are provided, including immunoglobulin sequences adjacent to or in near proximity to the EPO-R activating sequences of the present invention with or without a non-immunoglobulin spacer sequence. One type of embodiment is an immunoglobulin chain having the EPO-R activating sequence in place of the variable (V) region of the heavy and/or light chain.

Preparation of the peptide compounds of the invention:

Peptide synthesis

The peptides of the invention may be prepared by classical methods known in the art. These standard methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis, and recombinant DNA technology [See, *e.g.*, Merrifield J. Am. Chem. Soc. 1963 85:2149].

In one embodiment, the peptide monomers of a peptide dimer are synthesized individually and dimerized subsequent to synthesis. In preferred embodiments the peptide monomers of a dimer have the same amino acid sequence.

In particularly preferred embodiments, the peptide monomers of a dimer are linked via their C-termini by a linker L_K moiety having two functional groups capable of serving as initiation sites for peptide synthesis and a third functional group (*e.g.*, a carboxyl group or an amino group) that enables binding to another molecular moiety (*e.g.*, as may be present on the surface of a solid support). In this case, the two peptide monomers may be synthesized directly onto two reactive nitrogen groups of the linker L_K moiety in a variation of the solid phase synthesis technique. Such synthesis may be sequential or simultaneous.

Where sequential synthesis of the peptide chains of a dimer onto a linker is to be performed, two amine functional groups on the linker molecule are protected with two different orthogonally removable amine protecting groups. In preferred embodiments, the protected diamine is a protected lysine. The protected linker is coupled to a solid support via the linker's third functional group. The first amine

protecting group is removed, and the first peptide of the dimer is synthesized on the first deprotected amine moiety. Then the second amine protecting group is removed, and the second peptide of the dimer is synthesized on the second deprotected amine moiety. For example, the first amino moiety of the linker may be protected with Alloc, and the second with Fmoc. In this case, the Fmoc group (but not the Alloc group) may be removed by treatment with a mild base [*e.g.*, 20% piperidine in dimethyl formamide (DMF)], and the first peptide chain synthesized. Thereafter the Alloc group may be removed with a suitable reagent [*e.g.*, $Pd(PPh_3)$/4-methyl morpholine and chloroform], and the second peptide chain synthesized. This technique may be used to generate dimers wherein the sequences of the two peptide chains are identical or different. Note that where different thiol-protecting groups for cysteine are to be used to control disulfide bond formation (as discussed below) this technique must be used even where the final amino acid sequences of the peptide chains of a dimer are identical.

Where simultaneous synthesis of the peptide chains of a dimer onto a linker is to be performed, two amine functional groups of the linker molecule are protected with the same removable amine protecting group. In preferred embodiments, the protected diamine is a protected lysine. The protected linker is coupled to a solid support via the linker's third functional group. In this case the two protected functional groups of the linker molecule are simultaneously deprotected, and the two peptide chains simultaneously synthesized on the deprotected amines. Note that using this technique, the sequences of the peptide chains of the dimer will be identical, and the thiol-protecting groups for the cysteine residues are all the same.

A preferred method for peptide synthesis is solid phase synthesis. Solid phase peptide synthesis procedures are well-known in the art [see, *e.g.*, Stewart Solid Phase Peptide Syntheses (Freeman and Co.: San Francisco) 1969; 2002/2003 General Catalog from Novabiochem Corp, San Diego, USA; Goodman Synthesis of Peptides and Peptidomimetics (Houben-Weyl, Stuttgart) 2002]. In solid phase synthesis, synthesis is typically commenced from the C-terminal end of the peptide using an α-amino protected resin. A suitable starting material can be prepared, for instance, by attaching the required α-amino acid to a chloromethylated resin, a hydroxymethyl resin, a polystyrene resin, a benzhydrylamine resin, or the like. One such chloromethylated resin is sold under the trade name BIO-BEADS SX-1 by Bio Rad Laboratories (Richmond, CA). The preparation of the hydroxymethyl resin has been described [Bodonszky, *et al.* (1966) Chem. Ind. London 38:1597]. The benzhydrylamine (BHA) resin has been described [Pietta and Marshall (1970) Chem. Commun. 650], and the hydrochloride form is commercially available from Beckman Instruments, Inc. (Palo Alto, CA). For example, an α-amino protected amino acid may be coupled to a chloromethylated resin with the aid of a cesium bicarbonate catalyst, according to the method described by Gisin (1973) Helv. Chim. Acta 56:1467.

After initial coupling, the α-amino protecting group is removed, for example, using trifluoroacetic acid (TFA) or hydrochloric acid (HCl) solutions in organic solvents at room temperature. Thereafter, α-amino protected amino acids are successively coupled to a growing support-bound peptide chain. The α-amino protecting groups are those known to be useful in the art of stepwise synthesis of peptides, including: acyl-type protecting groups (*e.g.*, formyl, trifluoroacetyl, acetyl), aromatic urethane-type protecting groups [*e.g.*, benzyloxycarboyl (Cbz) and substituted Cbz], aliphatic urethane protecting groups [*e.g.*, t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, cyclohexyloxycarbonyl], and alkyl type protecting groups (*e.g.*, benzyl, triphenylmethyl), fluorenylmethyl oxycarbonyl (Fmoc), allyloxycarbonyl (Alloc), and 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde).

The side chain protecting groups (typically ethers, esters, trityl, PMC, and the like) remain intact during coupling and is not split off during the deprotection of the amino-terminus protecting group or during coupling. The side chain protecting group must be removable upon the completion of the synthesis of the final peptide and under reaction conditions that will not alter the target peptide. The side chain protecting groups for Tyr include tetrahydropyranyl, tert-butyl, trityl, benzyl, Cbz, Z-Br-Cbz, and 2,5-dichlorobenzyl. The side chain protecting groups for Asp include benzyl, 2,6-dichlorobenzyl, methyl, ethyl, and cyclohexyl. The side chain protecting groups for Thr and Ser include acetyl, benzoyl, trityl, tetrahydropyranyl, benzyl, 2,6-dichlorobenzyl, and Cbz. The side chain protecting groups for Arg include nitro, Tosyl (Tos), Cbz, adamantyloxycarbonyl mesitoylsulfonyl (Mts), 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf), 4-mthoxy-2,3,6-trimethyl-benzenesulfonyl (Mtr), or Boc. The side chain protecting groups for Lys include Cbz, 2-chlorobenzyloxycarbonyl (2-Cl-Cbz), 2-bromobenzyloxycarbonyl (2-Br-Cbz), Tos, or Boc.

After removal of the α-amino protecting group, the remaining protected amino acids are coupled stepwise in the desired order. Each protected amino acid is generally reacted in about a 3-fold excess using an appropriate carboxyl group activator such as 2-(1H-benzotriazol-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate (HBTU) or dicyclohexylcarbodimide (DCC) in solution, for example, in methylene chloride (CH_2Cl_2), N-methyl pyrrolidone, dimethyl formamide (DMF), or mixtures thereof.

After the desired amino acid sequence has been completed, the desired peptide is decoupled from the resin support by treatment with a reagent, such as trifluoroacetic acid (TFA) or hydrogen fluoride (HF), which not only cleaves the peptide from the resin, but also cleaves all remaining side chain protecting groups. When a chloromethylated resin is used, hydrogen fluoride treatment results in the formation of the free peptide acids. When the benzhydrylamine resin is used, hydrogen fluoride treatment results directly in the free peptide amide. Alternatively, when the chloromethylated resin is employed, the side chain protected peptide can be decoupled by treatment of the peptide resin with ammonia to give the

desired side chain protected amide or with an alkylamine to give a side chain protected alkylamide or dialkylamide. Side chain protection is then removed in the usual fashion by treatment with hydrogen fluoride to give the free amides, alkylamides, or dialkylamides. In preparing the esters of the invention, the resins used to prepare the peptide acids are employed, and the side chain protected peptide is cleaved with base and the appropriate alcohol (*e.g.*, methanol). Side chain protecting groups are then removed in the usual fashion by treatment with hydrogen fluoride to obtain the desired ester.

These procedures can also be used to synthesize peptides in which amino acids other than the 20 naturally occurring, genetically encoded amino acids are substituted at one, two, or more positions of any of the compounds of the invention. Synthetic amino acids that can be substituted into the peptides of the present invention include, but are not limited to, N-methyl, L-hydroxypropyl, L-3, 4-dihydroxyphenylalanyl, δ amino acids such as L- δ-hydroxylysyl and D- δ-methylalanyl, L-α-methylalanyl, β amino acids, and isoquinolyl. D-amino acids and non-naturally occurring synthetic amino acids can also be incorporated into the peptides of the present invention.

Peptide modifications

One can also modify the amino and/or carboxy termini of the peptide compounds of the invention to produce other compounds of the invention. Amino terminus modifications include methylation (*e.g.*, --$NHCH_3$ or --$N(CH_3)_2$), acetylation (*e.g.*, with acetic acid or a halogenated derivative thereof such as α-chloroacetic acid, α-bromoacetic acid, or α-iodoacetic acid), adding a benzyloxycarbonyl (Cbz) group, or blocking the amino terminus with any blocking group containing a carboxylate functionality defined by RCOO-- or sulfonyl functionality defined by R--SO_2 --, where R is selected from alkyl, aryl, heteroaryl, alkyl aryl, and the like, and similar groups. One can also incorporate a desamino acid at the N-terminus (so that there is no N-terminal amino group) to decrease susceptibility to proteases or to restrict the conformation of the peptide compound. In preferred embodiments, the N-terminus is acetylated. In particularly preferred embodiments an N-terminal glycine is acetylated to yield N-acetylglycine (AcG).

Carboxy terminus modifications include replacing the free acid with a carboxamide group or forming a cyclic lactam at the carboxy terminus to introduce structural constraints. One can also cyclize the peptides of the invention, or incorporate a desamino or descarboxy residue at the termini of the peptide, so that there is no terminal amino or carboxyl group, to decrease susceptibility to proteases or to restrict the conformation of the peptide. C-terminal functional groups of the compounds of the present invention include amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, and carboxy, and the lower ester derivatives thereof, and the pharmaceutically acceptable salts thereof.

One can replace the naturally occurring side chains of the 20 genetically encoded amino acids (or the stereoisomeric D amino acids) with other side chains, for instance with groups such as alkyl, lower

alkyl, cyclic 4-, 5-, 6-, to 7-membered alkyl, amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, carboxy and the lower ester derivatives thereof, and with 4-, 5-, 6-, to 7-membered heterocyclic. In particular, proline analogues in which the ring size of the proline residue is changed from 5 members to 4, 6, or 7 members can be employed. Cyclic groups can be saturated or unsaturated, and if unsaturated, can be aromatic or non-aromatic. Heterocyclic groups preferably contain one or more nitrogen, oxygen, and/or sulfur heteroatoms. Examples of such groups include the furazanyl, furyl, imidazolidinyl, imidazolyl, imidazolinyl, isothiazolyl, isoxazolyl, morpholinyl (*e.g.* morpholino), oxazolyl, piperazinyl (*e.g.*, 1-piperazinyl), piperidyl (*e.g.*, 1-piperidyl, piperidino), pyranyl, pyrazinyl, pyrazolidinyl, pyrazolinyl, pyrazolyl, pyridazinyl, pyridyl, pyrimidinyl, pyrrolidinyl (*e.g.*, 1-pyrrolidinyl), pyrrolinyl, pyrrolyl, thiadiazolyl, thiazolyl, thienyl, thiomorpholinyl (*e.g.*, thiomorpholino), and triazolyl. These heterocyclic groups can be substituted or unsubstituted. Where a group is substituted, the substituent can be alkyl, alkoxy, halogen, oxygen, or substituted or unsubstituted phenyl.

One can also readily modify peptides by phosphorylation, and other methods [*e.g.*, as described in Hruby, *et al.* (1990) Biochem J. 268:249-262].

The peptide compounds of the invention also serve as structural models for non-peptidic compounds with similar biological activity. Those of skill in the art recognize that a variety of techniques are available for constructing compounds with the same or similar desired biological activity as the lead peptide compound, but with more favorable activity than the lead with respect to solubility, stability, and susceptibility to hydrolysis and proteolysis [See, Morgan and Gainor (1989) Ann. Rep. Med. Chem. 24:243-252]. These techniques include replacing the peptide backbone with a backbone composed of phosphonates, amidates, carbamates, sulfonamides, secondary amines, and N-methylamino acids.

Formation of disulfide bonds

The compounds of the present invention may contain one or more intramolecular disulfide bonds. In one embodiment, embodiment, a peptide monomer or dimer comprises at least one intramolecular disulfide bond. In preferred embodiments, a peptide dimer comprises two intramolecular disulfide bonds.

Such disulfide bonds may be formed by oxidation of the cysteine residues of the peptide core sequence. In one embodiment the control of cysteine bond formation is exercised by choosing an oxidizing agent of the type and concentration effective to optimize formation of the desired isomer. For example, oxidation of a peptide dimer to form two intramolecular disulfide bonds (one on each peptide chain) is preferentially achieved (over formation of intermolecular disulfide bonds) when the oxidizing agent is DMSO.

In preferred embodiments, the formation of cysteine bonds is controlled by the selective use of thiol-protecting groups during peptide synthesis. For example, where a dimer with two intramolecular

disulfide bonds is desired, the first monomer peptide chain is synthesized with the two cysteine residues of the core sequence protected with a first thiol protecting group [*e.g.*, trityl(Trt), allyloxycarbonyl (Alloc), and 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde)or the like], then the second monomer peptide is synthesized the two cysteine residues of the core sequence protected with a second thiol protecting group different from the first thiol protecting group [*e.g.*, acetamidomethyl (Acm), t-butyl (tBu), or the like]. Thereafter, the first thiol protecting groups are removed effecting bisulfide cyclization of the first monomer, and then the second thiol protecting groups are removed effecting bisulfide cyclization of the second monomer.

Other embodiments of this invention provide for analogues of these disulfide derivatives in which one of the sulfurs has been replaced by a CH_2 group or other isotere for sulfur. These analogues can be prepared from the compounds of the present invention, wherein each core sequence contains at least one C or homocysteine residue and an α-amino-γ-butyric acid in place of the second C residue, via an intramolecular or intermolecular displacement, using methods known in the art [See, *e.g.*, Barker, *et al.* (1992) J. Med. Chem. 35:2040-2048 and Or, *et al.* (1991) J. Org. Chem. 56:3146-3149]. One of skill in the art will readily appreciate that this displacement can also occur using other homologs of α-amino-γ-butyric acid and homocysteine.

In addition to the foregoing cyclization strategies, other non-disulfide peptide cyclization strategies can be employed. Such alternative cyclization strategies include, for example, amide-cyclization strategies as well as those involving the formation of thio-ether bonds. Thus, the compounds of the present invention can exist in a cyclized form with either an intramolecular amide bond or an intramolecular thio-ether bond. For example, a peptide may be synthesized wherein one cysteine of the core sequence is replaced with lysine and the second cysteine is replaced with glutamic acid. Thereafter a cyclic monomer may be formed through an amide bond between the side chains of these two residues. Alternatively, a peptide may be synthesized wherein one cysteine of the core sequence is replaced with lysine. A cyclic monomer may then be formed through a thio-ether linkage between the side chains of the lysine residue and the second cysteine residue of the core sequence. As such, in addition to disulfide cyclization strategies, amide-cyclization strategies and thio-ether cyclization strategies can both be readily used to cyclize the compounds of the present invention. Alternatively, the amino-terminus of the peptide can be capped with an α-substituted acetic acid, wherein the α-substituent is a leaving group, such as an α-haloacetic acid, for example, α.-chloroacetic acid, α -bromoacetic acid, or α-iodoacetic acid.

Addition of linkers

In embodiments where a peptide dimer is dimerized by a linker L_K moiety, said linker may be incorporated into the peptide during peptide synthesis. For example, where a linker L_K moiety contains

two functional groups capable of serving as initiation sites for peptide synthesis and a third functional group (*e.g.*, a carboxyl group or an amino group) that enables binding to another molecular moiety, the linker may be conjugated to a solid support. Thereafter, two peptide monomers may be synthesized directly onto the two reactive nitrogen groups of the linker L_K moiety in a variation of the solid phase synthesis technique.

In alternate embodiments where a peptide dimer is dimerized by a linker L_K moiety, said linker may be conjugated to the two peptide monomers of a peptide dimer after peptide synthesis. Such conjugation may be achieved by methods well established in the art. In one embodiment, the linker contains at least two functional groups suitable for attachment to the target functional groups of the synthesized peptide monomers. For example, a linker with two free amine groups may be reacted with the C-terminal carboxyl groups of each of two peptide monomers. In another example, linkers containing two carboxyl groups, either preactivated or in the presence of a suitable coupling reagent, may be reacted with the N-terminal or side chain amine groups, or C-terminal lysine amides, of each of two peptide monomers.

Addition of spacers

In embodiments where the peptide compounds contain a spacer moiety, said spacer may be incorporated into the peptide during peptide synthesis. For example, where a spacer contains a free amino group and a second functional group (*e.g.*, a carboxyl group or an amino group) that enables binding to another molecular moiety, the spacer may be conjugated to the solid support. Thereafter, the peptide may be synthesized directly onto the spacer's free amino group by standard solid phase techniques.

In a preferred embodiment, a spacer containing two functional groups is first coupled to the solid support via a first functional group. Next a linker L_K moiety having two functional groups capable of serving as initiation sites for peptide synthesis and a third functional group (*e.g.*, a carboxyl group or an amino group) that enables binding to another molecular moiety is conjugated to the spacer via the spacer's second functional group and the linker's third functional group. Thereafter, two peptide monomers may be synthesized directly onto the two reactive nitrogen groups of the linker L_K moiety in a variation of the solid phase synthesis technique. For example, a solid support coupled spacer with a free amine group may be reacted with a lysine linker via the linker's free carboxyl group.

In alternate embodiments where the peptide compounds contain a spacer moiety, said spacer may be conjugated to the peptide after peptide synthesis. Such conjugation may be achieved by methods well established in the art. In one embodiment, the linker contains at least one functional group suitable for attachment to the target functional group of the synthesized peptide. For example, a spacer with a free amine group may be reacted with a peptide's C-terminal carboxyl group. In another example, a linker

with a free carboxyl group may be reacted with the free amine group of a peptide's N-terminus or of a lysine residue. In yet another example, a spacer containing a free sulfhydryl group may be conjugated to a cysteine residue of a peptide by oxidation to form a disulfide bond.

Attachment of water soluble polymers

In recent years, water-soluble polymers, such as polyethylene glycol (PEG), have been used for the covalent modification of peptides of therapeutic and diagnostic importance. Attachment of such polymers is thought to enhance biological activity, prolong blood circulation time, reduce immunogenicity, increase aqueous solubility, and enhance resistance to protease digestion. For example, covalent attachment of PEG to therapeutic polypeptides such as interleukins [Knauf, *et al.* (1988) J. Biol. Chem. 263;15064; Tsutsumi, *et al.* (1995) J. Controlled Release 33:447), interferons (Kita, *et al.* (1990) Drug Des. Delivery 6:157), catalase (Abuchowski, *et al.* (1977) J. Biol. Chem. 252:582), superoxide dismutase (Beauchamp, *et al.* (1983) Anal. Biochem. 131:25), and adenosine deaminase (Chen, *et al.* (1981) Biochim. Biophy. Acta 660:293), has been reported to extend their half life *in vivo*, and/or reduce their immunogenicity and antigenicity.

The peptide compounds of the invention may further comprise one or more water soluble polymer moieties. Preferably, these polymers are covalently attached to the peptide compounds. The water soluble polymer may be, for example, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, polypropylene oxide/ethylene oxide copolymers, and polyoxyethylated polyols. A preferred water soluble polymer is PEG.

Peptides, peptide dimers and other peptide-based molecules of the invention can be attached to water-soluble polymers (e.g., PEG) using any of a variety of chemistries to link the water-soluble polymer(s) to the receptor-binding portion of the molecule (e.g., peptide + spacer). A typical embodiment employs a single attachment junction for covalent attachment of the water soluble polymer(s) to the receptor-binding portion, however in alternative embodiments multiple attachment junctions may be used, including further variations wherein different species of water-soluble polymer are attached to the receptor-binding portion at distinct attachment junctions, which may include covalent attachment junction(s) to the spacer and/or to one or both peptide chains. In some embodiments, the dimer or higher order multimer will comprise distinct species of peptide chain (i.e., a heterodimer or other heteromultimer). By way of example and not limitation, a dimer may comprise a first peptide chain having a PEG attachment junction and the second peptide chain may either lack a PEG attachment

junction or utilize a different linkage chemistry than the first peptide chain and in some variations the spacer may contain or lack a PEG attachment junction and said spacer, if PEGylated, may utilize a linkage chemistry different than that of the first and/or second peptide chains. An alternative embodiment employs a PEG attached to the spacer portion of the receptor-binding portion and a different water-soluble polymer (e.g., a carbohydrate) conjugated to a side chain of one of the amino acids of the peptide portion of the molecule.

A wide variety of polyethylene glycol (PEG) species may be used for PEGylation of the receptor-binding portion (peptides + spacer). Substantially any suitable reactive PEG reagent can be used. In preferred embodiments, the reactive PEG reagent will result in formation of a carbamate or amide bond upon conjugation to the receptor-binding portion. Suitable reactive PEG species include, but are not limited to, those which are available for sale in the Drug Delivery Systems catalog (2003) of NOF Corporation (Yebisu Garden Place Tower, 20-3 Ebisu 4-chome, Shibuya-ku, Tokyo 150-6019) and the Molecular Engineering catalog (2003) of Nektar Therapeutics (490 Discovery Drive, Huntsville, Alabama 35806). For example and not limitation, the following PEG reagents are often preferred in various embodiments: mPEG2-NHS, mPEG2-ALD, multi-Arm PEG, mPEG(MAL)2, mPEG2(MAL), mPEG-NH2, mPEG-SPA, mPEG-SBA, mPEG-thioesters, mPEG-Double Esters, mPEG-BTC, mPEG-ButyrALD, mPEG-ACET, heterofunctional PEGs (NH2-PEG-COOH, Boc-PEG-NHS, Fmoc-PEG-NHS, NHS-PEG-VS, NHS-PEG-MAL), PEG acrylates (ACRL-PEG-NHS), PEG-phospholipids (e.g., mPEG-DSPE), multiarmed PEGs of the SUNBRITE series including the GL series of glycerine-based PEGs activated by a chemistry chosen by those skilled in the art, any of the SUNBRITE activated PEGs (including but not limited to carboxyl-PEGs, p-NP-PEGs, Tresyl-PEGs, aldehyde PEGs, acetal-PEGs, amino-PEGs, thiol-PEGs, maleimido-PEGs, hydroxyl-PEG-amine, amino-PEG-COOH, hydroxyl-PEG-aldehyde, carboxylic anhydride type-PEG, functionalized PEG-phospholipid, and other similar and/or suitable reactive PEGs as selected by those skilled in the art for their particular application and usage.

The polymer may be of any molecular weight, and may be branched or unbranched. A preferred PEG for use in the present invention comprises linear, unbranched PEG having a molecular weight of from about 20 kilodaltons (kD) to about 40kD (the term "about" indicating that in preparations of PEG, some molecules will weigh more, some less, than the stated molecular weight). Most preferably, the PEG has a molecular weight of from about 30kD to about 40kD. Other sizes may be used, depending on the desired therapeutic profile (*e.g.*, duration of sustained release desired; effects, if any, on biological activity; ease in handling; degree or lack of antigenicity; and other known effects of PEG on a therapeutic peptide).

The number of polymer molecules attached may vary; for example, one, two, three, or more water soluble polymers may be attached to an EPO-R agonist peptide of the invention. The multiple attached polymers may be the same or different chemical moieties (*e.g.*, PEGs of different molecular weight). In some cases, the degree of polymer attachment (the number of polymer moieties attached to a peptide and/or the total number of peptides to which a polymer is attached) may be influenced by the proportion of polymer molecules versus peptide molecules in an attachment reaction, as well as by the total concentration of each in the reaction mixture. In general, the optimum polymer versus peptide ratio (in terms of reaction efficiency to provide for no excess unreacted peptides and/or polymer moieties) will be determined by factors such as the desired degree of polymer attachment (*e.g.*, mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions for a particular attachment method.

In preferred embodiments, the covalently attached water soluble polymer is PEG. For illustrative purposes, examples of methods for covalent attachment of PEG (PEGylation) are described below. These illustrative descriptions are not intended to be limiting. One of ordinary skill in the art will appreciate that a variety of methods for covalent attachment of a broad range of water soluble polymers is well established in the art. As such, peptide compounds to which any of a number of water soluble polymers known in the art have been attached by any of a number of attachment methods known in the art are encompassed by the present invention.

In one embodiment, PEG may serve as a linker that dimerizes two peptide monomers. In one embodiment, PEG is attached to at least one terminus (N-terminus or C-terminus) of a peptide monomer or dimer. In another embodiment, PEG is attached to a spacer moiety of a peptide monomer or dimer. In a preferred embodiment PEG is attached to the linker moiety of a peptide dimer. In a highly preferred embodiment, PEG is attached to a spacer moiety, where said spacer moiety is attached to the linker L_K moiety that connects the monomers of a peptide dimer. Most preferably, PEG is attached to a spacer moiety, where said spacer moiety is attached to a peptide dimer via the carbonyl carbon of a lysine linker, or the amide nitrogen of a lysine amide linker.

There are a number of PEG attachment methods available to those skilled in the art [see, *e.g.*, Goodson, *et al.* (1990) Bio/Technology 8:343 (PEGylation of interleukin-2 at its glycosylation site after site-directed mutagenesis); EP 0 401 384 (coupling PEG to G-CSF); Malik, *et al.*, (1992) Exp. Hematol. 20:1028-1035 (PEGylation of GM-CSF using tresyl chloride); PCT Pub. No. WO 90/12874 (PEGylation of erythropoietin containing a recombinantly introduced cysteine residue using a cysteine-specific mPEG derivative); U.S. Pat. No. 5,757,078 (PEGylation of EPO peptides); and U.S. Pat. No. 6,077,939 (PEGylation of an N-terminal α-carbon of a peptide)].

For example, PEG may be covalently bound to amino acid residues via a reactive group. Reactive groups are those to which an activated PEG molecule may be bound (*e.g.*, a free amino or carboxyl group). For example, N-terminal amino acid residues and lysine (K) residues have a free amino group; and C-terminal amino acid residues have a free carboxyl group. Sulfhydryl groups (*e.g.*, as found on cysteine residues) may also be used as a reactive group for attaching PEG. In addition, enzyme-assisted methods for introducing activated groups (*e.g.*, hydrazide, aldehyde, and aromatic-amino groups) specifically at the C-terminus of a polypeptide have been described [Schwarz, *et al.* (1990) Methods Enzymol. 184:160; Rose, *et al.* (1991) Bioconjugate Chem. 2:154; Gaertner, *et al.* (1994) J. Biol. Chem. 269:7224].

For example, PEG molecules may be attached to peptide amino groups using methoxylated PEG ("mPEG") having different reactive moieties. Such polymers include mPEG-succinimidyl succinate, mPEG-succinimidyl carbonate, mPEG-imidate, mPEG-4-nitrophenyl carbonate, and mPEG-cyanuric chloride. Similarly, PEG molecules may be attached to peptide carboxyl groups using methoxylated PEG with a free amine group (mPEG-NH_2).

Where attachment of the PEG is non-specific and a peptide containing a specific PEG attachment is desired, the desired PEGylated compound may be purified from the mixture of PEGylated compounds. For example, if an N-terminally PEGylated peptide is desired, the N-terminally PEGylated form may be purified from a population of randomly PEGylated peptides (*i.e.*, separating this moiety from other monoPEGylated moieties).

In preferred embodiments, PEG is attached site-specifically to a peptide. Site-specific PEGylation at the N-terminus, side chain, and C-terminus of a potent analog of growth hormone-releasing factor has been performed through solid-phase synthesis [Felix, *et al.* (1995) Int. J. Peptide Protein Res. 46:253]. Another site-specific method involves attaching a peptide to extremities of liposomal surface-grafted PEG chains in a site-specific manner through a reactive aldehyde group at the N-terminus generated by sodium periodate oxidation of N-terminal threonine [Zalipsky, *et al.* (1995) Bioconj. Chem. 6:705]. However, this method is limited to polypeptides with N-terminal serine or threonine residues. Another site-specific method for N-terminal PEGylation of a peptide via a hydrazone, reduced hydrazone, oxime, or reduced oxime bond is described in U.S. Pat. No. 6,077,939 to Wei, *et al.*

In one method, selective N-terminal PEGylation may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, a carbonyl group containing PEG is selective attached to the N-terminus of a peptide. For example, one may selectively N-terminally PEGylate the protein by performing the reaction at a pH which exploits the pK_a differences between the ε-amino groups of a lysine residue and the α- amino group of the N-terminal

residue of the peptide. By such selective attachment, PEGylation takes place predominantly at the N-terminus of the protein, with no significant modification of other reactive groups (*e.g.*, lysine side chain amino groups). Using reductive alkylation, the PEG should have a single reactive aldehyde for coupling to the protein (*e.g.*, PEG proprionaldehyde may be used).

Site-specific mutagenesis is a further approach which may be used to prepare peptides for site-specific polymer attachment. By this method, the amino acid sequence of a peptide is designed to incorporate an appropriate reactive group at the desired position within the peptide. For example, WO 90/12874 describes the site-directed PEGylation of proteins modified by the insertion of cysteine residues or the substitution of other residues for cysteine residues. This publication also describes the preparation of mPEG-erythropoietin ("mPEG-EPO") by reacting a cysteine-specific mPEG derivative with a recombinantly introduced cysteine residue on EPO.

Where PEG is attached to a spacer or linker moiety, similar attachment methods may be used. In this case, the linker or spacer contains a reactive group and an activated PEG molecule containing the appropriate complementary reactive group is used to effect covalent attachment. In preferred embodiments the linker or spacer reactive group contains a terminal amino group (*i.e.*, positioned at the terminus of the linker or spacer) which is reacted with a suitably activated PEG molecule to make a stable covalent bond such as an amide or a carbamate. Suitable activated PEG species include, but are not limited to, mPEG-para-nitrophenylcarbonate (mPEG-NPC), mPEG-succinimidyl carbonate (mPEG-SC), and mPEG-succinimidyl propionate (mPEG-SPA). In other preferred embodiments, the linker or spacer reactive group contains a carboxyl group capable of being activated to form a covalent bond with an amine-containing PEG molecule under suitable reaction conditions. Suitable PEG molecules include mPEG-NH_2 and suitable reaction conditions include carbodiimide-mediated amide formation or the like.

EPO-R agonist activity assays:

In vitro *functional assays*

In vitro competitive binding assays quantitate the ability of a test peptide to compete with EPO for binding to EPO-R. For example (see, *e.g.*, as described in U.S. Patent 5,773,569), the extracellular domain of the human EPO-R (EPO binding protein, EBP) may be recombinantly produced in *E. coli* and the recombinant protein coupled to a solid support, such as a microtitre dish or a synthetic bead [*e.g.*, Sulfolink beads from Pierce Chemical Co. (Rockford, IL)]. Immobilized EBP is then incubated with labeled recombinant EPO, or with labeled recombinant EPO and a test peptide. Serial dilutions of test peptide are employed for such experiments. Assay points with no added test peptide define total EPO binding to EBP. For reactions containing test peptide, the amount of bound EPO is quantitated and expressed as a percentage of the control (total=100%) binding. These values are plotted versus peptide

concentration. The IC50 value is defined as the concentration of test peptide which reduces the binding of EPO to EBP by 50% (*i.e.*, 50% inhibition of EPO binding).

A different *in vitro* competitive binding assay measures the light signal generated as a function of the proximity of two beads: an EPO-conjugated bead and an EPO-R-conjugated bead. Bead proximity is generated by the binding of EPO to EPO-R. A test peptide that competes with EPO for binding to EPO-R will prevent this binding, causing a decrease in light emission. The concentration of test peptide that results in a 50% decrease in light emission is defined as the IC50 value.

The peptides of the present invention compete very efficiently with EPO for binding to the EPO-R. This enhanced function is represented by their ability to inhibit the binding of EPO at substantially lower concentrations of peptide (*i.e.*, they have very low IC50 values).

The biological activity and potency of monomeric and dimeric peptide EPO-R agonists of the invention, which bind specifically to the EPO-receptor, may be measured using *in vitro* cell-based functional assays.

One assay is based upon a murine pre-B-cell line expressing human EPO-R and further transfected with a fos promoter-driven luciferase reporter gene construct. Upon exposure to EPO or another EPO-R agonist, such cells respond by synthesizing luciferase. Luciferase causes the emission of light upon addition of its substrate luciferin. Thus, the level of EPO-R activation in such cells may be quantitated via measurement of luciferase activity. The activity of a test peptide is measured by adding serial dilutions of the test peptide to the cells, which are then incubated for 4 hours. After incubation, luciferin substrate is added to the cells, and light emission is measured. The concentration of test peptide that results in a half-maximal emission of light is recorded as the EC50.

The peptides of the present invention show dramatically enhanced ability to promote EPO-R signaling-dependent luciferase expression in this assay. This enhanced function is represented by their ability to yield half of the maximal luciferase activity at substantially lower concentrations of peptide (*i.e.*, they have very low EC50 values). This assay is a preferred method for estimating the potency and activity of an EPO-R agonist peptide of the invention.

Another assay may be performed using FDC-P1/ER cells [Dexter, *et al.* (1980) J. Exp. Med. 152:1036-1047], a well characterized nontransformed murine bone marrow derived cell line into which EPO-R has been stably transfected. These cells exhibit EPO-dependent proliferation.

In one such assay, the cells are grown to half stationary density in the presence of the necessary growth factors (see, *e.g.*, as described in U.S. Patent 5,773,569). The cells are then washed in PBS and starved for 16-24 hours in whole media without the growth factors. After determining the viability of the cells (*e.g.*, by trypan blue staining), stock solutions (in whole media without the growth factors) are made to give about 10^5 cells per 50μL. Serial dilutions of the peptide EPO-R agonist compounds (typically the

free, solution phase peptide as opposed to a phage-bound or other bound or immobilized peptide) to be tested are made in 96-well tissue culture plates for a final volume of 50μL per well. Cells (50μL) are added to each well and the cells are incubated 24-48 hours, at which point the negative controls should die or be quiescent. Cell proliferation is then measured by techniques known in the art, such as an MTT assay which measures H^3-thymidine incorporation as an indication of cell proliferation [see, Mosmann (1983) J. Immunol. Methods 65:55-63]. Peptides are evaluated on both the EPO-R-expressing cell line and a parental non-expressing cell line. The concentration of test peptide necessary to yield one half of the maximal cell proliferation is recorded as the EC50.

The peptides of the present invention show dramatically enhanced ability to promote EPO-dependent cell growth in this assay. This enhanced function is represented by their ability to yield half of the maximal cell proliferation stimulation activity at substantially lower concentrations of peptide (*i.e.*, they have very low EC50 values). This assay is a preferred method for estimating the potency and activity of an EPO-R agonist peptide of the invention.

In another assay, the cells are grown to stationary phase in EPO-supplemented medium, collected, and then cultured for an additional 18 hr in medium without EPO. The cells are divided into three groups of equal cell density: one group with no added factor (negative control), a group with EPO (positive control), and an experimental group with the test peptide. The cultured cells are then collected at various time points, fixed, and stained with a DNA-binding fluorescent dye (*e.g.*, propidium iodide or Hoechst dye, both available from Sigma). Fluorescence is then measured, for example, using a FACS Scan Flow cytometer. The percentage of cells in each phase of the cell cycle may then be determined, for example, using the SOBR model of CellFIT software (Becton Dickinson). Cells treated with EPO or an active peptide will show a greater proportion of cells in S phase (as determined by increased fluorescence as an indicator of increased DNA content) relative to the negative control group.

Similar assays may be performed using FDCP-1 [see, *e.g.*, Dexter *et al.* (1980) J. Exp. Med. 152:1036-1047] or TF-1 [Kitamura, *et al.* (1989) Blood 73:375-380] cell lines. FDCP-1 is a growth factor dependent murine multi-potential primitive hematopoietic progenitor cell line that can proliferate, but not differentiate, when supplemented with WEHI-3-conditioned media (a medium that contains IL-3, ATCC number TIB-68). For such experiments, the FDCP-1 cell line is transfected with the human or murine EPO-R to produce FDCP-1-hEPO-R or FDCP-1-mEPO-R cell lines, respectively, that can proliferate, but not differentiate, in the presence of EPO. TF-1, an EPO-dependent cell line, may also be used to measure the effects of peptide EPO-R agonists on cellular proliferation.

In yet another assay, the procedure set forth in Krystal (1983) Exp. Hematol 11:649-660 for a microassay based on H^3-thymidine incorporation into spleen cells may be employed to ascertain the ability of the compounds of the present invention to serve as EPO agonists. In brief, $B6C3F_1$ mice are

injected daily for two days with phenylhydrazine (60 mg/kg). On the third day, spleen cells are removed and their ability to proliferate over a 24 hour period ascertained using an MTT assay.

The binding of EPO to EPO-R in an erythropoietin-responsive cell line induces tyrosine phosphorylation of both the receptor and numerous intracellular proteins, including Shc, vav and JAK2 kinase. Therefore, another *in vitro* assay measures the ability of peptides of the invention to induce tyrosine phosphorylation of EPO-R and downstream intracellular signal transducer proteins. Active peptides, as identified by binding and proliferation assays described above, elicit a phosphorylation pattern nearly identical to that of EPO in erythropoietin-responsive cells. For this assay, FDC-P1/ER cells [Dexter, *et al.* (1980) J Exp Med 152:1036-47] are maintained in EPO-supplemented medium and grown to stationary phase. These cells are then cultured in medium without EPO for 24 hr. A defined number of such cells is then incubated with a test peptide for approximately 10 min at 37°C. A control sample of cells with EPO is also run with each assay. The treated cells are then collected by centrifugation, resuspended in SDS lysis buffer, and subjected to SDS polyacrylamide gel electrophoresis. The electrophoresed proteins in the gel are transferred to nitrocellulose, and the phosphotyrosine containing proteins on the blot visualized by standard immunological techniques. For example, the blot may be probed with an anti-phosphotyrosine antibody (*e.g.*, mouse anti-phosphotyrosine IgG from Upstate Biotechnology, Inc.), washed, and then probed with a secondary antibody [*e.g.*, peroxidase labeled goat anti-mouse IgG from Kirkegaard & Perry Laboratories, Inc. (Washington, DC)]. Thereafter, phosphotyrosine-containing proteins may be visualized by standard techniques including colorimetric, chemiluminescent, or fluorescent assays. For example, a chemiluminescent assay may be performed using the ECL Western Blotting System from Amersham.

Another cell-based *in vitro* assay that may be used to assess the activity of the peptides of the present invention comprises a colony assay, using murine bone marrow or human peripheral blood cells. Murine bone marrow may be obtained from the femurs of mice, while a sample of human peripheral blood may obtained from a healthy donor. In the case of peripheral blood, mononuclear cells are first isolated from the blood, for example, by centrifugation through a Ficoll-Hypaque gradient [Stem Cell Technologies, Inc. (Vancouver, Canada)]. For this assay a nucleated cell count is performed to establish the number and concentration of nucleated cells in the original sample. A defined number of cells is plated on methyl cellulose as per manufacturer's instructions [Stem Cell Technologies, Inc. (Vancouver, Canada)]. An experimental group is treated with a test peptide, a positive control group is treated with EPO, and a negative control group receives no treatment. The number of growing colonies for each group is then scored after defined periods of incubation, generally 10 days and 18 days. An active peptide will promote colony formation.

Other *in vitro* biological assays that can be used to demonstrate the activity of the compounds of the present invention are disclosed in Greenberger, *et al.* (1983) Proc. Natl. Acad. Sci. USA 80:2931-2935 (EPO-dependent hematopoietic progenitor cell line); Quelle and Wojchowski (1991) J. Biol. Chem. 266:609-614 (protein tyrosine phosphorylation in B6SUt.EP cells); Dusanter-Fourt, *et al.* (1992) J. Biol. Chem. 287:10670-10678 (tyrosine phosphorylation of EPO-receptor in human EPO-responsive cells); Quelle, *et al.* (1992) J. Biol. Chem. 267:17055-17060 (tyrosine phosphorylation of a cytosolic protein, pp 100, in FDC-ER cells); Worthington, *et al.* (1987) Exp. Hematol. 15:85-92 (colorimetric assay for hemoglobin); Kaiho and Miuno (1985) Anal. Biochem. 149:117-120 (detection of hemoglobin with 2,7-diaminofluorene); Patel, *et al.* (1992) J. Biol. Chem. 267:21300-21302 (expression of c-myb); Witthuhn, *et al.* (1993) Cell 74:227-236 (association and tyrosine phosphorylation of JAK2); Leonard, *et al.* (1993) Blood 82:1071-1079 (expression of GATA transcription factors); and Ando, *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:9571-9575 (regulation of G_1 transition by cycling D2 and D3).

An instrument designed by Molecular Devices Corp., known as a microphysiometer, has been reported to be successfully used for measurement of the effect of agonists and antagonists on various receptors. The basis for this apparatus is the measurement of the alterations in the acidification rate of the extracellular media in response to receptor activation.

In vivo *functional assays*

One *in vivo* functional assay that may be used to assess the potency of a test peptide is the polycythemic exhypoxic mouse bioassay. For this assay, mice are subjected to an alternating conditioning cycle for several days. In this cycle, the mice alternate between periods of hypobaric conditions and ambient pressure conditions. Thereafter, the mice are maintained at ambient pressure for 2-3 days prior to administration of test samples. Test peptide samples, or EPO standard in the case positive control mice, are injected subcutaneously into the conditioned mice. Radiolabeled iron (*e.g.*, Fe^{59}) is administered 2 days later, and blood samples taken two days after administration of radiolabeled iron. Hematocrits and radioactivity measurements are then determined for each blood sample by standard techniques. Blood samples from mice injected with active test peptides will show greater radioactivity (due to binding of Fe^{59} by erythrocyte hemoglobin) than mice that did not receive test peptides or EPO.

Another *in vivo* functional assay that may be used to assess the potency of a test peptide is the reticulocyte assay. For this assay, normal untreated mice are subcutaneously injected on three consecutive days with either EPO or test peptide. On the third day, the mice are also intraperitoneally injected with iron dextran. At day five, blood samples are collected from the mice. The percent (%) of reticulocytes in the blood is determined by thiazole orange staining and flow cytometer analysis (retic-

count program). In addition, hematocrits are manually determined. The percent of corrected reticulocytes is determined using the following formula:

$$\%\ RETIC_{CORRECTED} = \%\ RETIC_{OBSERVED} \times (Hematocrit_{INDIVIDUAL} / Hematocrit_{NORMAL})$$

Active test compounds will show an increased % $RETIC_{CORRECTED}$ level relative to mice that did not receive test peptides or EPO.

Use of EPO-R agonist peptides of the invention

The peptide compounds of the invention are useful *in vitro* as tools for understanding the biological role of EPO, including the evaluation of the many factors thought to influence, and be influenced by, the production of EPO and the binding of EPO to the EPO-R (*e.g.*, the mechanism of EPO / EPO-R signal transduction/receptor activation). The present peptides are also useful in the development of other compounds that bind to the EPO-R, because the present compounds provide important structure-activity-relationship information that facilitate that development.

Moreover, based on their ability to bind to EPO-R, the peptides of the present invention can be used as reagents for detecting EPO-R on living cells; fixed cells; in biological fluids; in tissue homogenates; in purified, natural biological materials; etc. For example, by labeling such peptides, one can identify cells having EPO-R on their surfaces. In addition, based on their ability to bind EPO-R, the peptides of the present invention can be used in *in situ* staining, FACS (fluorescence-activated cell sorting) analysis, Western blotting, ELISA (enzyme-linked immunosorbent assay), etc. In addition, based on their ability to bind to EPO-R, the peptides of the present invention can be used in receptor purification, or in purifying cells expressing EPO-R on the cell surface (or inside permeabilized cells).

The peptides of the invention can also be utilized as commercial reagents for various medical research and diagnostic purposes. Such uses can include but are not limited to: (1) use as a calibration standard for quantitating the activities of candidate EPO-R agonists in a variety of functional assays; (2) use as blocking reagents in random peptide screening, *i.e.*, in looking for new families of EPO-R peptide ligands, the peptides can be used to block recovery of EPO peptides of the present invention; (3) use in co-crystallization with EPO-R, *i.e.*, crystals of the peptides of the present invention bound to the EPO-R may be formed, enabling determination of receptor/peptide structure by X-ray crystallography; (4) use to measure the capacity of erythrocyte precursor cells induce globin synthesis and heme complex synthesis, and to increase the number of ferritin receptors, by initiating differentiation; (5) use to maintain the proliferation and growth of EPO-dependent cell lines, such as the FDCP-1-mEPO-R and the TF-1 cell lines; and (6) other research and diagnostic applications wherein the EPO-R is preferably activated or such activation is conveniently calibrated against a known quantity of an EPO-R agonist, and the like.

In yet another aspect of the present invention, methods of treatment and manufacture of a medicament are provided. The peptide compounds of the invention may be administered to warm blooded animals, including humans, to simulate the binding of EPO to the EPO-R *in vivo*. Thus, the present invention encompasses methods for therapeutic treatment of disorders associated with a deficiency of EPO, which methods comprise administering a peptide of the invention in amounts sufficient to stimulate the EPO-R and thus, alleviate the symptoms associated with a deficiency of EPO *in vivo*. For example, the peptides of this invention will find use in the treatment of renal insufficiency and/or end-stage renal failure/dialysis; anemia associated with AIDS; anemia associated with chronic inflammatory diseases (for example, rheumatoid arthritis and chronic bowel inflammation) and autoimmune disease; and for boosting the red blood count of a patient prior to surgery. Other disease states, disorders, and states of hematologic irregularity that may be treated by administration of the peptides of this invention include: beta-thalassemia; cystic fibrosis; pregnancy and menstrual disorders; early anemia of prematurity; spinal cord injury; space flight; acute blood loss; aging; and various neoplastic disease states accompanied by abnormal erythropoiesis.

In other embodiments, the peptide compounds of the invention may be used for the treatment of disorders which are not characterized by low or deficient red blood cells, for example as a pretreatment prior to transfusions. In addition, administration of the compounds of this invention can result in a decrease in bleeding time and thus, will find use in the administration to patients prior to surgery or for indications wherein bleeding is expected to occur. In addition, the compounds of this invention will find use in the activation of megakaryoctes.

Since EPO has been shown to have a mitogenic and chemotactic effect on vascular endothelial cells as well as an effect on central cholinergic neurons [see, *e.g.*, Amagnostou, *et al.* (1990) Proc. Natl. Acad. Sci. USA 87:5978-5982 and Konishi, *et al.* (1993) Brain Res. 609:29-35], the compounds of this invention will also find use for the treatment of a variety of vascular disorders, such as: promoting wound healing; promoting growth of collateral coronary blood vessels (such as those that may occur after myocardial infarction); trauma treatment; and post-vascular graft treatment. The compounds of this invention will also find use for the treatment of a variety of neurological disorders, generally characterized by low absolute levels of acetyl choline or low relative levels of acetyl choline as compared to other neuroactive substances *e.g.*, neurotransmitters.

Pharmaceutical compositions

In yet another aspect of the present invention, pharmaceutical compositions of the above EPO-R agonist peptide compounds are provided. Conditions alleviated or modulated by the administration of such compositions include those indicated above. Such pharmaceutical compositions may be for

administration by oral, parenteral (intramuscular, intraperitoneal, intravenous (IV) or subcutaneous injection), transdermal (either passively or using iontophoresis or electroporation), transmucosal (nasal, vaginal, rectal, or sublingual) routes of administration or using bioerodible inserts and can be formulated in dosage forms appropriate for each route of administration. In general, comprehended by the invention are pharmaceutical compositions comprising effective amounts of an EPO-R agonist peptide, or derivative products, of the invention together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (*e.g.*, Tween 20, Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g.*, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by reference. The compositions may be prepared in liquid form, or may be in dried powder (*e.g.*, lyophilized) form.

Oral Delivery

Contemplated for use herein are oral solid dosage forms, which are described generally in Remington's Pharmaceutical Sciences, 18th Ed. 1990 (Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets, pellets, powders, or granules. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatized with various polymers (*e.g.*, U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, K. In: *Modern Pharmaceutics* Edited by G.S. Banker and C.T. Rhodes Chapter 10, 1979, herein incorporated by reference. In general, the formulation will include the EPO-R agonist peptides (or chemically modified forms thereof) and inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Also contemplated for use herein are liquid dosage forms for oral administration, including pharmaceutically acceptable emulsions, solutions, suspensions, and syrups, which may contain other

components including inert diluents; adjuvants such as wetting agents, emulsifying and suspending agents; and sweetening, flavoring, and perfuming agents.

The peptides may be chemically modified so that oral delivery of the derivative is efficacious. Generally, the chemical modification contemplated is the attachment of at least one moiety to the component molecule itself, where said moiety permits (a) inhibition of proteolysis; and (b) uptake into the blood stream from the stomach or intestine. Also desired is the increase in overall stability of the component or components and increase in circulation time in the body. As discussed above, PEGylation is a preferred chemical modification for pharmaceutical usage. Other moieties that may be used include: propylene glycol, copolymers of ethylene glycol and propylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, polyproline, poly-1,3-dioxolane and poly-1,3,6-tioxocane [see, *e.g.*, Abuchowski and Davis (1981) "Soluble Polymer-Enzyme Adducts," in *Enzymes as Drugs*. Hocenberg and Roberts, eds. (Wiley-Interscience: New York, NY) pp. 367-383; and Newmark, *et al.* (1982) J. Appl. Biochem. 4:185-189].

For oral formulations, the location of release may be the stomach, the small intestine (the duodenum, the jejunem, or the ileum), or the large intestine. One skilled in the art has available formulations which will not dissolve in the stomach, yet will release the material in the duodenum or elsewhere in the intestine. Preferably, the release will avoid the deleterious effects of the stomach environment, either by protection of the peptide (or derivative) or by release of the peptide (or derivative) beyond the stomach environment, such as in the intestine.

To ensure full gastric resistance a coating impermeable to at least pH 5.0 is essential. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), HPMCP 50, HPMCP 55, polyvinyl acetate phthalate (PVAP), Eudragit L30D, Aquateric, cellulose acetate phthalate (CAP), Eudragit L, Eudragit S, and Shellac. These coatings may be used as mixed films.

A coating or mixture of coatings can also be used on tablets, which are not intended for protection against the stomach. This can include sugar coatings, or coatings which make the tablet easier to swallow. Capsules may consist of a hard shell (such as gelatin) for delivery of dry therapeutic (*i.e.* powder), for liquid forms a soft gelatin shell may be used. The shell material of cachets could be thick starch or other edible paper. For pills, lozenges, molded tablets or tablet triturates, moist massing techniques can be used.

The peptide (or derivative) can be included in the formulation as fine multiparticulates in the form of granules or pellets of particle size about 1mm. The formulation of the material for capsule administration could also be as a powder, lightly compressed plugs, or even as tablets. These therapeutics could be prepared by compression.

Colorants and/or flavoring agents may also be included. For example, the peptide (or derivative) may be formulated (such as by liposome or microsphere encapsulation) and then further contained within an edible product, such as a refrigerated beverage containing colorants and flavoring agents.

One may dilute or increase the volume of the peptide (or derivative) with an inert material. These diluents could include carbohydrates, especially mannitol, α-lactose, anhydrous lactose, cellulose, sucrose, modified dextrans and starch. Certain inorganic salts may be also be used as fillers including calcium triphosphate, magnesium carbonate and sodium chloride. Some commercially available diluents are Fast-Flo, Emdex, STA-Rx 1500, Emcompress and Avicell.

Disintegrants may be included in the formulation of the therapeutic into a solid dosage form. Materials used as disintegrates include but are not limited to starch, including the commercial disintegrant based on starch, Explotab. Sodium starch glycolate, Amberlite, sodium carboxymethylcellulose, ultramylopectin, sodium alginate, gelatin, orange peel, acid carboxymethyl cellulose, natural sponge and bentonite may all be used. The disintegrants may also be insoluble cationic exchange resins. Powdered gums may be used as disintegrants and as binders. and can include powdered gums such as agar, Karaya or tragacanth. Alginic acid and its sodium salt are also useful as disintegrants.

Binders may be used to hold the peptide (or derivative) agent together to form a hard tablet and include materials from natural products such as acacia, tragacanth, starch and gelatin. Others include methyl cellulose (MC), ethyl cellulose (EC) and carboxymethyl cellulose (CMC). Polyvinyl pyrrolidone (PVP) and hydroxypropylmethyl cellulose (HPMC) could both be used in alcoholic solutions to granulate the peptide (or derivative).

An antifrictional agent may be included in the formulation of the peptide (or derivative) to prevent sticking during the formulation process. Lubricants may be used as a layer between the peptide (or derivative) and the die wall, and these can include but are not limited to; stearic acid including its magnesium and calcium salts, polytetrafluoroethylene (PTFE), liquid paraffin, vegetable oils and waxes. Soluble lubricants may also be used such as sodium lauryl sulfate, magnesium lauryl sulfate, polyethylene glycol of various molecular weights, Carbowax 4000 and 6000.

Glidants that might improve the flow properties of the drug during formulation and to aid rearrangement during compression might be added. The glidants may include starch, talc, pyrogenic silica and hydrated silicoaluminate.

To aid dissolution of the peptide (or derivative) into the aqueous environment a surfactant might be added as a wetting agent. Surfactants may include anionic detergents such as sodium lauryl sulfate, dioctyl sodium sulfosuccinate and dioctyl sodium sulfonate. Cationic detergents might be used and could include benzalkonium chloride or benzethomium chloride. The list of potential nonionic detergents that could be included in the formulation as surfactants are lauromacrogol 400, polyoxyl 40 stearate,

polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 20, 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose and carboxymethyl cellulose. These surfactants could be present in the formulation of the protein or derivative either alone or as a mixture in different ratios.

Additives which potentially enhance uptake of the peptide (or derivative) are for instance the fatty acids oleic acid, linoleic acid and linolenic acid.

Controlled release oral formulations may be desirable. The peptide (or derivative) could be incorporated into an inert matrix which permits release by either diffusion or leaching mechanisms, *e.g.*, gums. Slowly degenerating matrices may also be incorporated into the formulation. Some enteric coatings also have a delayed release effect. Another form of a controlled release is by a method based on the Oros therapeutic system (Alza Corp.), *i.e.* the drug is enclosed in a semipermeable membrane which allows water to enter and push drug out through a single small opening due to osmotic effects.

Other coatings may be used for the formulation. These include a variety of sugars which could be applied in a coating pan. The peptide (or derivative) could also be given in a film coated tablet and the materials used in this instance are divided into 2 groups. The first are the nonenteric materials and include methyl cellulose, ethyl cellulose, hydroxyethyl cellulose, methylhydroxy-ethyl cellulose, hydroxypropyl cellulose, hydroxypropyl-methyl cellulose, sodium carboxy-methyl cellulose, providone and the polyethylene glycols. The second group consists of the enteric materials that are commonly esters of phthalic acid.

A mix of materials might be used to provide the optimum film coating. Film coating may be carried out in a pan coater or in a fluidized bed or by compression coating.

Parenteral delivery

Preparations according to this invention for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They may be sterilized by, for example, filtration through a bacteria retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured using sterile water, or some other sterile injectable medium, immediately before use.

Rectal or vaginal delivery

Compositions for rectal or vaginal administration are preferably suppositories which may contain, in addition to the active substance, excipients such as cocoa butter or a suppository wax. Compositions for nasal or sublingual administration are also prepared with standard excipients well known in the art.

Pulmonary Delivery

Also contemplated herein is pulmonary delivery of the EPO-R agonist peptides (or derivatives thereof). The peptide (or derivative) is delivered to the lungs of a mammal while inhaling and traverses across the lung epithelial lining to the blood stream [see, *e.g.*, Adjei, *et al.* (1990) Pharmaceutical Research 7:565-569; Adjei, *et al.* (1990) Int. J. Pharmaceutics 63:135-144 (leuprolide acetate); Braquet, *et al.* (1989) J. Cardiovascular Pharmacology 13(sup5):143-146 (endothelin-1); Hubbard, *et al.* (1989) Annals of Internal Medicine, Vol. III, pp. 206-212 (α1-antitrypsin); Smith, *et al.* (1989) J. Clin. Invest. 84:1145-1146 (α-1-proteinase); Oswein, *et al.* (1990) "Aerosolization of Proteins", Proceedings of Symposium on Respiratory Drug Delivery II Keystone, Colorado (recombinant human growth hormone); Debs, *et al.* (1988) J. Immunol. 140:3482-3488 (interferon-γ and tumor necrosis factor α); and U.S. Pat. No. 5,284,656 to Platz, *et al.* (granulocyte colony stimulating factor). A method and composition for pulmonary delivery of drugs for systemic effect is described in U.S. Pat. No. 5,451,569 to Wong, *et al.*

Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products, including but not limited to nebulizers, metered dose inhalers, and powder inhalers, all of which are familiar to those skilled in the art. Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer (Mallinckrodt Inc., St. Louis, MO); the Acorn II nebulizer (Marquest Medical Products, Englewood, CO); the Ventolin metered dose inhaler (Glaxo Inc., Research Triangle Park, NC); and the Spinhaler powder inhaler (Fisons Corp., Bedford, MA).

All such devices require the use of formulations suitable for the dispensing of peptide (or derivative). Typically, each formulation is specific to the type of device employed and may involve the use of an appropriate propellant material, in addition to the usual diluents, adjuvants and/or carriers useful in therapy. Also, the use of liposomes, microcapsules or microspheres, inclusion complexes, or other types of carriers is contemplated. Chemically modified peptides may also be prepared in different formulations depending on the type of chemical modification or the type of device employed.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise peptide (or derivative) dissolved in water at a concentration of about 0.1 to 25 mg of biologically active protein per mL of solution. The formulation may also include a buffer and a simple sugar (*e.g.*, for protein stabilization and regulation of osmotic pressure). The nebulizer formulation may also contain a

surfactant, to reduce or prevent surface induced aggregation of the peptide (or derivative) caused by atomization of the solution in forming the aerosol.

Formulations for use with a metered-dose inhaler device will generally comprise a finely divided powder containing the peptide (or derivative) suspended in a propellant with the aid of a surfactant. The propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol, and 1,1,1,2-tetrafluoroethane, or combinations thereof. Suitable surfactants include sorbitan trioleate and soya lecithin. Oleic acid may also be useful as a surfactant.

Formulations for dispensing from a powder inhaler device will comprise a finely divided dry powder containing peptide (or derivative) and may also include a bulking agent, such as lactose, sorbitol, sucrose, or mannitol in amounts which facilitate dispersal of the powder from the device, *e.g.*, 50 to 90% by weight of the formulation. The peptide (or derivative) should most advantageously be prepared in particulate form with an average particle size of less than 10 mm (or microns), most preferably 0.5 to 5 mm, for most effective delivery to the distal lung.

Nasal Delivery

Nasal delivery of the EPO-R agonist peptides (or derivatives) is also contemplated. Nasal delivery allows the passage of the peptide to the blood stream directly after administering the therapeutic product to the nose, without the necessity for deposition of the product in the lung. Formulations for nasal delivery include those with dextran or cyclodextran.

Dosages

For all of the peptide compounds, as further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age, and general health of the recipient, will be able to ascertain proper dosing. The selected dosage depends upon the desired therapeutic effect, on the route of administration, and on the duration of the treatment desired. Generally dosage levels of 0.001 to 10 mg/kg of body weight daily are administered to mammals. Generally, for intravenous injection or infusion dosage may be lower. The dosing schedule may vary, depending on the circulation half-life, and the formulation used.

The peptides of the present invention (or their derivatives) may be administered in conjunction with one or more additional active ingredients or pharmaceutical compositions.

Examples

The present invention is next described by means of the following examples. However, the use of these and other examples anywhere in the specification is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified form. Likewise, the invention is not limited to any particular preferred embodiments described herein. Indeed, many modifications and variations of the invention may be apparent to those skilled in the art upon reading this specification, and can be made without departing from its spirit and scope. The invention is therefore to be limited only by the terms of the appended claims, along with the full scope of equivalents to which the claims are entitled.

Example 1: Synthesis of EPO-R agonist peptides

1. Peptide Monomer Synthesis

Various peptide monomers of the invention were synthesized using the Merrifield solid phase synthesis technique [see, Stewart and Young. Solid Phase Peptide Synthesis, 2nd edition (Pierce Chemical, Rockford, IL) 1984] on an Applied Biosystems 433A automated instrument. The resin used was PAL (Milligen/Biosearch), which is cross-linked polystyrene with 5-(4'-Fmoc-aminomethyl-3,5'-dimethoxyphenoxy) valeric acid. Use of PAL resin results in a carboxyl terminal amide function upon cleavage of the peptide from the resin. Primary amine protection on amino acids was achieved with Fmoc, and side chain protection groups were t-butyl for serine, threonine, and tyrosine hydroxyls; trityl for glutamine and asparagine amides; Trt or Acm for cysteine; and Pmc (2,2,5,7,8-pentamethylchroman sulfonate) for the arginine guanidino group. Each coupling was performed for either 1 hr or 2 hr with BOP (benzotriazolyl N-oxtrisdimethylaminophosphonium hexafluorophosphate) and HOBt (1 - hydroxybenztriazole).

For the synthesis of peptides with an amidated carboxy terminus, the fully assembled peptide was cleaved with a mixture of 90% trifluoroacetic acid, 5% ethanedithiol, and 5% water, initially at 4°C and gradually increasing to room temperature over 1.5 hr. The deprotected product was filtered from the resin and precipitated with diethyl ether. After thorough drying the product was purified by C18 reverse phase high performance liquid chromatography with a gradient of acetonitrile/water in 0.1% trifluoroacetic acid.

2. Peptide Dimer Synthesis

Various peptide dimers of the invention were synthesized directly onto a lysine linker in a variation of the solid phase technique.

For simultaneous synthesis of the two peptide chains, Fmoc-Lys-Fmoc was coupled to a PAL resin (Milligen/Biosearch), thereby providing an initial lysine residue to serve as the linker between the two chains to be synthesized. The Fmoc protecting groups were removed with mild base (20% piperidine

in DMF), and the peptide chains were synthesized using the resulting free amino groups as starting points. Peptide chain synthesis was performed using the solid phase synthesis technique described above. Trt was used to protect all cysteine residues. Following dimer deprotection, cleavage from the resin, and purification, oxidation of the cysteine residues was performed by incubating the deprotected dimer in 100% DMSO for 2-3 days at 5°C to 25°C. This oxidation reaction yielded predominantly (>75%) dimers with two intramolecular disulfide bonds.

For sequential synthesis of the two peptide chains, Fmoc-Lys-Alloc was coupled to a PAL resin (Milligen/Biosearch), thereby providing an initial lysine residue to serve as the linker between the two chains to be synthesized. The Fmoc protecting group was removed with mild base (20% piperidine in DMF). The first peptide chain was then syntheszed using the resulting free amino group as a starting point. Peptide synthesis was performed using the solid phase technique described above. The two cysteine residues of the first chain were protected with Trt. Following synthesis of the first peptide chain, the Alloc group was removed from the support-bound lysine linker with $Pd[P(C_6H_5)_3]_4$, 4-methyl morpholine, and chloroform. The second peptide chain was then synthesized on this second free amino group. The two cysteine residues of the second chain were protected with Acm. An intramolecular disulfide bond was formed in the first peptide chain by removing the Trt protecting groups using trifluoroacetic acid, followed by oxidation by stirring in 20% DMSO overnight. An intramolecular disulfide bond was then formed in the second peptide chain by simultaneously removing the Acm protecting groups and oxidizing the deprotected cysteine residues using iodine, methanol, and thalium trifluoroacetate.

3. Attachment of spacers

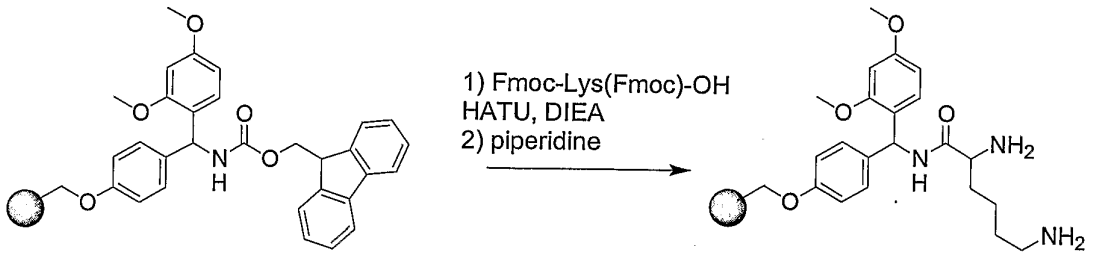
Where the spacer was an amino acid (e.g., glycine or lysine as in AF35462 and AF35464, respectively), the spacer was incorporated into the peptide during solid phase peptide synthesis. In this case, the spacer amino acid was coupled to the PAL resin, and its free amino group served as the basis for the attachment of another spacer amino acid, or of the lysine linker. Following the attachment of the lysine linker, dimeric peptides were synthesized as described above.

4. Synthesis of exemplary peptide dimers

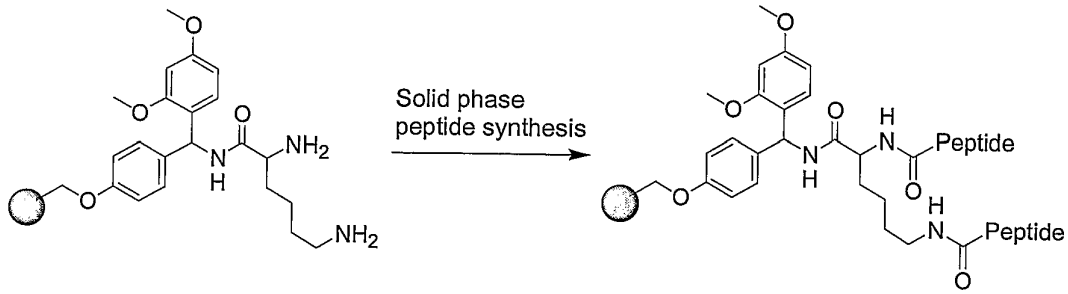
Exemplary embodiments of these synthesis techniques are outlined below. In one example, the synthesis of a peptide dimer linked via a C-terminal lysine amide is described. In another example, the synthesis of a peptide dimer linked via a C-terminal lysine, and containing a spacer molecule attached to the linking lysine, is described.

Synthesis of a peptide dimer linked via a C-terminal lysine amide:

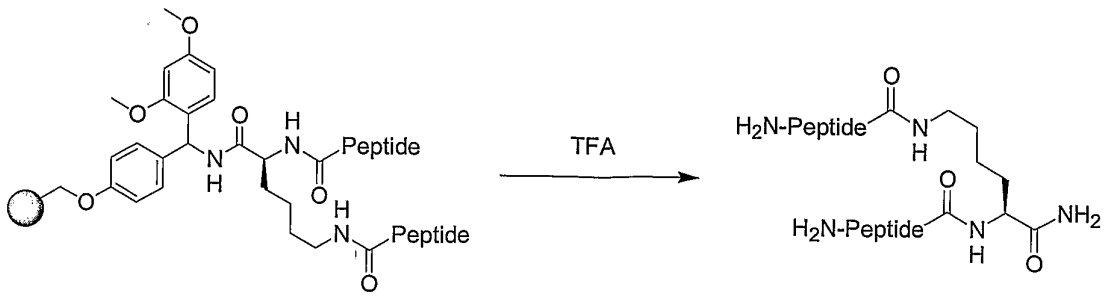
Step 1 - Formation of TentaGel-Rink-Lys: TentaGel-Rink resin (0.18 mml/g from Rapp Polymere, Germany) was treated with a activated solution of Fmoc-Lys(Fmoc)-OH (prepared from 5 eq. of amino acid and 5 eq.of HATU dissolved at 0.5 M in DMF, followed by the addition of 10 eq. of DIEA) and allowed to gently shake 14h. The resin was washed (DMF, THF, DCM, MeOH) and dried to yield the protected resin. Residual amine groups were capped by treating the resin with a solution of 10% acetic anhydride, 20% pyridine in DCM for 20 minutes, followed by washing as above. The Fmoc groups were removed by gently shaking the resin in 30% piperideine in DMF for 20 minutes, followed by washing (DMF, THF, DCM, MeOH) and drying.



Step 2 - Formation of TentaGel-Rink-Lys(Peptide)$_2$: The resin from Step 1 was subjected to repeated cycles of Fmoc-amino acid couplings with HBTU/HOBt activation and Fmoc removal with piperidine to build both peptide chains simultaneously. This was convenently carried out on a ABI 433 automated peptide synthesizer available from Applied Biosystems, Inc. After the final Fmoc removal, the terminal amine groups were acylated with acetic anhydride (10 eq.) and DIEA (20 eq.) in DMF for 20 minutes, follwed by washing as above.



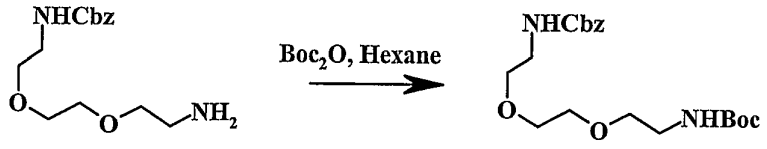
Step 3 - Cleavage from resin: The resin from above is suspended in to a solution of TFA (82.5%), phenol (5%), ethanedithiol (2.5%), water (5%), and thioanisole (5%) for 3h at room temperature. Alternative cleavage cocktails such as TFA (95%), water (2.5%), and triisopropylsilane (2.5%) can also be used. The TFA solution is cooled to 5 °C and poured into Et_2O to percipatate the peptide. Filtration and drying under reduced pressure gave the desired peptide. Purification via preparative HPLC with a C18 column affords the pure peptide.



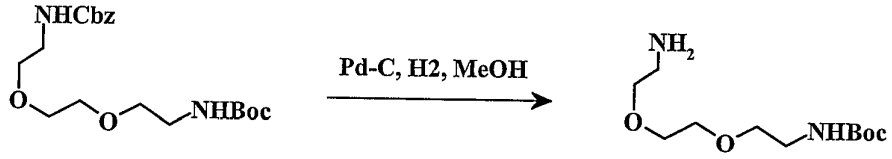
Synthesis of a peptide dimer linked via a C-terminal lysine amide and containing a spacer molecule:

Step 1 - Synthesis of Cbz-TAP: A solution containing the commercially available diamine ("TAP" from Aldrich Chemical Co.) (10g, 67.47mmol) in anhydrous DCM (100 ml) was cooled to 0^0C. A solution of benzyl chloroformate (4.82ml, 33.7mmol) in anhydrous DCM (50ml) was added slowly through a dropping funnel over a period of 6-7 h, maintaining the temperature of the reaction mixture at 0^0C throughout, then allowed to warm to room temperature (~25^0C). After a further 16h, the DCM was removed under vacuum and the residue partitioned between 3N HCl and ether. The aqueous layers were collected and neutralized with 50% aq. NaOH to pH8-9 and extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous Na_2SO_4, then concentrated under vacuum to provide the crude mono-Cbz-TAP (5g, about 50% yield). This compound was used for the next reaction without any further purification.

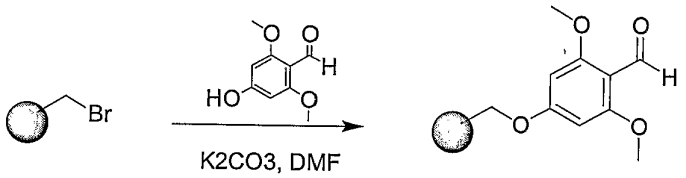
Step 2 - Synthesis of Cbz-TAP-Boc: To a vigorously stirred suspension of the Cbz-TAP (5g, 17.7mmol) in hexane (25ml) was added Boc_2O (3.86g, 17.7mmol) and stirring continued at RT overnight. The reaction mixture was diluted with DCM (25ml) and washed with 10% aq. citric acid (2X), water (2X) and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum. The crude product (yield 5g) was used directly in the next reaction.



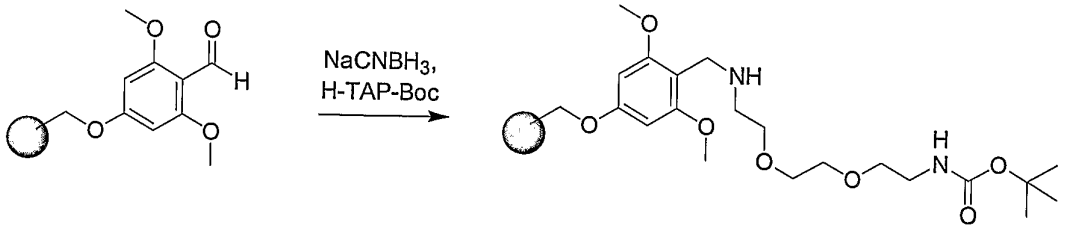
Step3 - Synthesis of Boc-TAP: The crude product from the previous reaction was dissolved in methanol (25ml) and hydrogenated in presence of 5% Pd on Carbon (5% w/W) under balloon pressure for 16 hrs. The mixture was filtered, washed with methanol and the filtrate concentrated *in vacuo* to provide the crude H-TAP-Boc product (yield 3.7g). The overall approximate yield of Boc-TAP after Steps 1-3 was 44% (calculated based on the amount of Cbz-Cl used.)



Step 4 - Synthesis of TentaGel-Linker: TentaGel bromide (2.5 g, 0.48 mmol/g, from Rapp Polymere, Germany), phenolic linker (5 equivalent, and K_2CO_3 (5 equivalent) were heated in 20 mL of DMFto 70°C for 14h. After cooling to room temperature, the resin was washed (0.1 N HCl, water, ACN, DMF, MeOH) and dried to give an amber-colored resin.

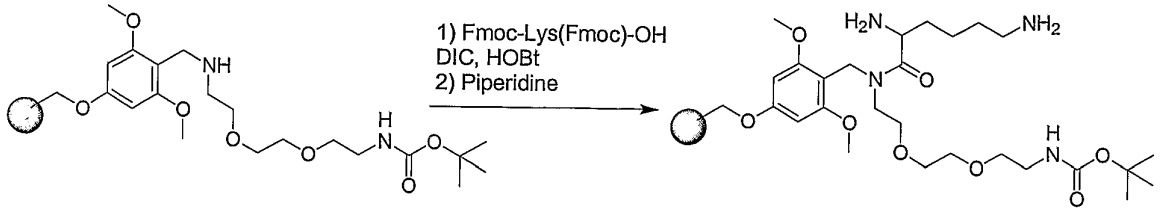


Step 5 - Synthesis of TentaGel-linker-TAP(Boc): 2.5 gms of the resin from above and H-TAP-Boc (1.5gms, 5eq.) and glacial AcOH (34 µl, 5 eq.) was taken in a mixture of 1:1 MeOH-THF and shaken overnight. A 1M solution of sodium cyanoborohydride (5eq) in THF was added to this and shaken for another 7hrs. The resin was filtered washed (DMF, THF, 0.1 N HCl, water, MeOH) and dried. A small amount of the resin was benzoylated with Bz-Cl and DIEA in DCM and cleaved with 70% TFA-DCM and checked by LCMS and HPLC.

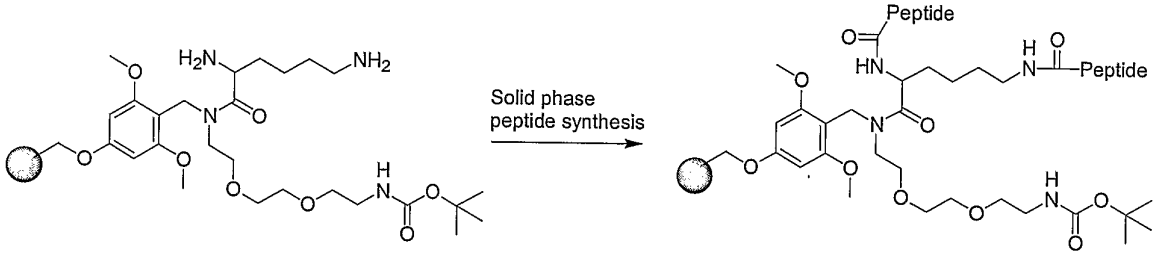


Step 6 - Synthesis of TentaGel-linker-TAP-Lys: The resin from above was treated with a activated solution of Fmoc-Lys(Fmoc)-OH (prepared from 5 eq. of amino acid and 5 eq.of HATU dissolved at 0.5 M in DMF, followed by the addition of 10 eq. of DIEA) and allowed to gently shake 14h.

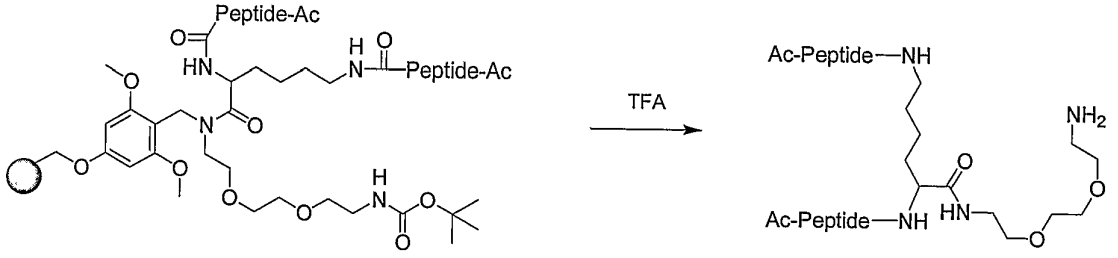
The resin was washed (DMF, THF, DCM, MeOH) and dried to yield the protected resin. Residual amine groups were capped by treating the resin with a solution of 10% acetic anhydride, 20% pyridine in DCM for 20 minutes, followed by washing as above. The Fmoc groups are removed by gently shaking the resin in 30% piperideine in DMF for 20 minutes, followed by washing (DMF, THF, DCM, MeOH) and drying.



Step 7 - Synthesis of TentaGel-Linker-TAP-Lys(Peptide)$_2$: The resin from above was subjected to repeated cycles of Fmoc-amino acid couplings with HBTU/HOBt activation and Fmoc removal with piperidine to build both peptide chains simultaneously. This was convenently carried out on a ABI 433 automated peptide synthesizer available from Applied Biosystems, Inc. After the final Fmoc removal, the terminal amine groups were acylated with acetic anhydride (10 eq.) and DIEA (20 eq.) in DMF for 20 minutes, follwed by washing as above.

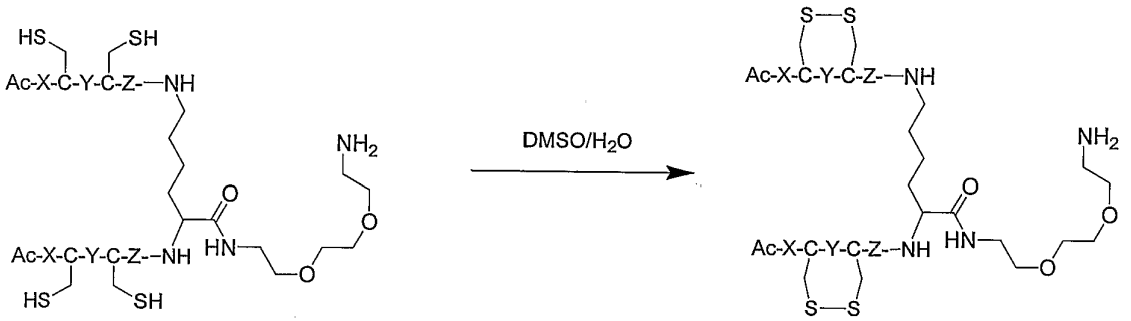


Step 8 - Cleavage from resin: The resin from above was suspended in a solution of TFA (82.5%), phenol (5%), ethanedithiol (2.5%), water (5%), and thioanisole (5%) for 3h at room temperature. Alternative cleavage cocktails such as TFA (95%), water (2.5%), and triisopropylsilane (2.5%) can also be used. The TFA solution was cooled to 5 °C and poured into Et_2O to percipatate the peptide. Filtration and drying under reduced pressure gave the desired peptide. Purification via preparative HPLC with a C18 column afforded the pure peptide.



5. Oxidation of peptides to form intramolecular disulfide bonds

The peptide dimer was dissolved in 20% DMSO/water (1 mg dry weight peptide/mL) and allowed to stand at room temperature for 36h. The peptide was purified by loading the reaction mixture onto a C18 HPLC column (Waters Delta-Pak C18, 15 micron particle size, 300 angstrom pore size, 40 mm x 200 mm length), followed by a linear ACN/water/0.01% TFA gradiant from 5 to 95% ACN over 40 minutes. Lyopholization of the fractions containing the desired peptide affords the product as a fluffy white solid. For example, in the case of AF35525, this reaction may be illustrated schematically as follows:



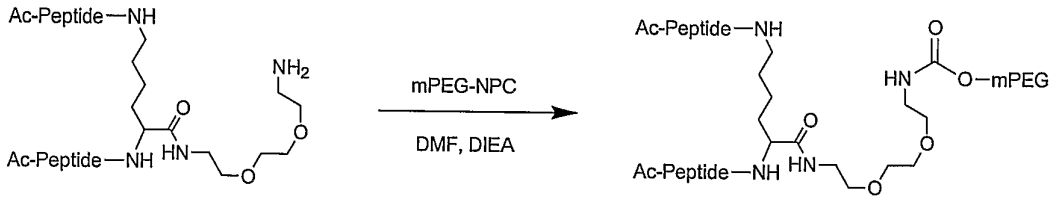
Dimeric peptide (XYZ) containing reduced cysteine residues

Dimeric peptide (XYZ) containing oxidized disulfide bonds

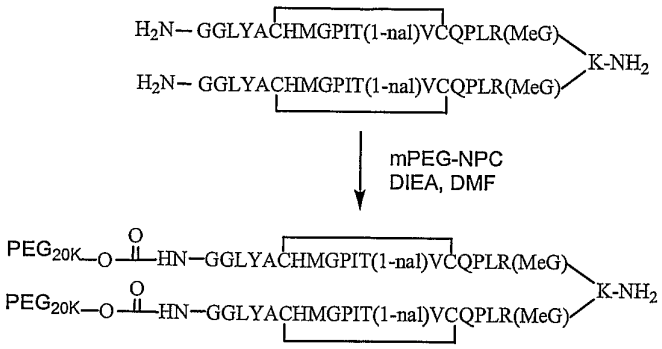
6. PEGylation of peptides

PEGylation of the peptides of the invention was carried out using several different techniques.

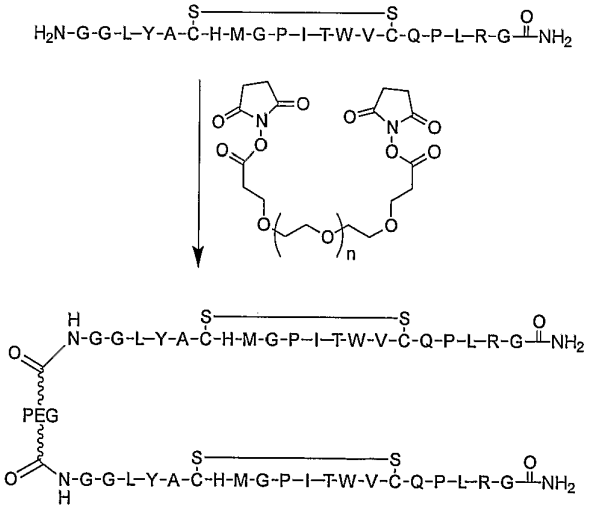
PEGylation of a terminal -NH_2 group: The peptide dimer was mixed with 1.5 eq. (mole basis) of activated PEG species (mPEG-NPC from NOF Corp. Japan) in dry DMF to afford a clear solution. After 5 minutes 4eq of DIEA was added to above solution. The mixture was stirred at ambient temperature 14h, followed by purification with C18 reverse phase HPLC. The structure of PEGylated peptide was confirmed by MALDI mass. The purified peptide was also subjected to purification via cation ion exchange chromatography as outlined below. For example, in the case of AF35593, the monoPEGylation of the terminal -NH_2 group of the spacer moiety may be illustrated schematically as follows:



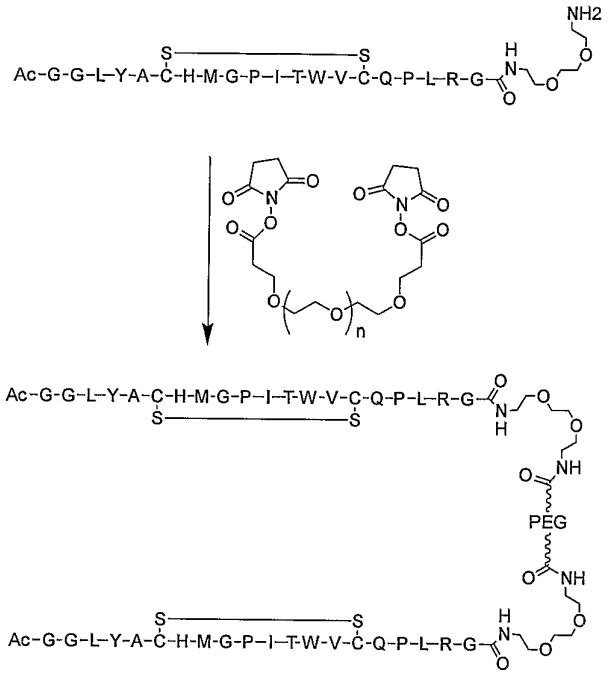
DiPEGylation of the N-termini of a peptide dimer: The peptide dimer is mixed with 2.5 eq. (mole basis) of activated PEG species (mPEG-NPC from NOF Corp. Japan) in dry DMF to afford a clear solution. After 5 minutes 4eq of DIEA is added to above solution. The mixture is stirred at ambient temperature 14h, followed by purification with C18 reverse phase HPLC. The purified peptide is also subjected to purification via cation ion exchange chromatography as outlined below. For example, in the case of AF35083, this reaction may be illustrated schematically as follows:



Peptide dimerization via PEGylation of N-termini: The peptide (2.5 eq.) and PEG-(SPA-NHS)$_2$ (1 eq. from Shearwater Corp, USA.) were dissolved in dry DMF at 0.25M to afford a clear solution. After 5 minutes 10eq of DIEA is added to above solution. The mixture is stirred at ambient temperature 2h, followed by purification with C18 reverse phase HPLC. The purified peptide is also subjected to purification via cation ion exchange chromatography as outlined below. For example, in the case of AF33131, this reaction may be illustrated schematically as follows:



Peptide dimerization via PEGylation of C-termini: The peptide (2.5 eq.) and PEG-(SPA-NHS)$_2$ (1 eq. from Shearwater Corp, USA.) were dissolved in dry DMF at 0.25M to afford a clear solution. After 5 minutes 10eq of DIEA is added to above solution. The mixture is stirred at ambient temperature 2h, followed by purification with C18 reverse phase HPLC. The purified peptide is also subjected to purification via cation ion exchange chromatography as outlined below. For example, this reaction may be summarized as follows:



7. Ion exchange purification of peptides.

Several exchange supports were surveyed for their ability to separate the above peptide-PEG conjugate from unreacted (or hydrolyzed) PEG, in addition to their ability to retain the starting dimeric peptides. The ion exchange resin (2-3g) was loaded into a 1 cm column, followed by conversion to the sodium form (0.2 N NaOH loaded onto column until elutant was pH 14, ca. 5 column volumes), and than to the hydrogen form (eluted with either 0.1 N HCl or 0.1 M HOAc until elutant matched load pH, ca. 5 column volumes), followed by washing with 25% ACN/water until pH 6. Either the peptide prior to conjugation or the peptide-PEG conjugate was dissolved in 25% ACN/water (10 mg/mL) and the pH adjusted to <3 with TFA, then loaded on the column. After washing with 2-3 column volumes of 25% ACN/water and collecting 5 mL fractions, the peptide was released from the column by elution with 0.1 M NH_4OAc in 25% ACN/water, again collecting 5 mL fractions. Analysis via HPLC revealed which fractions contained the desired peptide. Analysis with an Evaporative Light-Scattering Detector (ELSD) indicated that when the peptide was retained on the column and was eluted with the NH_4OAc solution

(generally between fractions 4 and 10), no non-conjugated PEG was observed as a contaminant. When the peptide eluted in the initial wash buffer (generally the first 2 fractions), no separation of desired PEG-conjugate and excess PEG was observed.

The following columns successfully retained both the peptide and the peptide-PEG conjugate, and successfully purified the peptide-PEG conjugate from the unconjugates peptide:

Table 1: Ion Exhange Resins

Support	Source
Mono S HR 5/5 strong cation exchange pre-loaded column	Amersham Biosciences
SE53 Cellulose, microgranular strong cation exchange support	Whatman
SP Sepharose Fast Flow strong cation exchange support	Amersham Biosciences

Example 2: *In vitro* activity assays

This example describes various *in vitro* assays that are useful in evaluating the activity and potency of EPO-R agonist peptides of the invention. The results for these assays demonstrate that the novel peptides of this invention bind to EPO-R and activate EPO-R signaling. Moreover, the results for these assays show that the novel peptide compositions exhibit a surprising increase in EPO-R binding affinity and biological activity compared to EPO mimetic peptides that have been previously described.

EPO-R agonist peptide monomers and dimers are prepared according to the methods provided in Example 1. The potency of these peptide monomers and dimers is evaluated using a series of *in vitro* activity assays, including: a reporter assay, a proliferation assay, a competitive binding assay, and a C/BFU-e assay. These four assays are described in further detail below.

The results of these *in vitro* activity assays are summarized in Table 2 (for peptide monomers) and Table 3 (for peptide dimers). These tables provide the compound designation and structure for each tested peptide, as well as the experimental results for each of these four assays. These results demonstrate the dramatically enhanced potency of the novel peptides of the invention.

1. Reporter assay

This assay is based upon a on a murine pre-B-cell line derived reporter cell, Baf3/EpoR/GCSFR fos/lux. This reporter cell line expresses a chimeric receptor comprising the extra-cellular portion of the human EPO receptor to the intra-cellular portion of the human GCSF receptor. This cell line is further transfected with a fos promoter-driven luciferase reporter gene construct. Activation of this chimeric receptor through addition of erythropoietic agent results in the expression of the luciferase reporter gene,

and therefore the production of light upon addition of the luciferase substrate luciferin. Thus, the level of EPO-R activation in such cells may be quantitated via measurement of luciferase activity.

The Baf3/EpoR/GCSFR fos/lux cells are cultured in DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone), 10% WEHI-3 supernatant (the supernatant from a culture of WEHI-3 cells, ATCC # TIB-68), and penicillin/streptomycin. Approximately 18 h before the assay, cells are starved by transferring them to DMEM/F12 medium supplemented with 10% FBS and 0.1% WEHI-3 supernatant. On the day of assay, cells are washed once with DMEM/F12 medium supplemented with 10% FBS (no WEHI-3 supernatant), then 1 X 10^6 cells/mL are cultured in the presence of a known concentration of test peptide, or with EPO (R & D Systems Inc., Minneapolis, MN) as a positive control, in DMEM/F12 medium supplemented with 10% FBS (no WEHI-3 supernatant). Serial dilutions of the test peptide are concurrently tested in this assay. Assay plates are incubated for 4h at 37°C in a 5% CO_2 atmosphere, after which luciferin (Steady-Glo; Promega, Madison, Wi) is added to each well. Following a 5-minute incubation, light emission is measured on a Packard Topcount Luminometer (Packard Instrument Co., Downers Grove, Ill.). Light counts are plotted relative to test peptide concentration and analysed using Graph Pad software. The concentration of test peptide that results in a half-maximal emission of light is recorded as the EC50 [See Tables 2 and 3: Reporter EC50].

2. Proliferation assay

This assay is based upon a murine pre-B-cell line, Baf3, transfected to express human EPO-R. Proliferation of the resulting cell line, BaF3/Gal4/Elk/EPOR, is dependent on EPO-R activation. The degree of cell proliferation is quantitated using MTT, where the signal in the MTT assay is proportional to the number of viable cells.

The BaF3/Gal4/Elk/EPOR cells are cultured in spinner flasks in DMEM/F12 medium (Gibco) supplemented with 10% FBS (Hyclone) and 2% WEHI-3 supernatant (ATCC # TIB-68). Cultured cells are starved overnight, in a spinner flask at a cell density of $1x10^6$ cells/ml, in DMEM/F12 medium supplemented with 10% FBS and 0.1% WEHI-3 supernatant. The starved cells are then washed twice with Dulbecco's PBS (Gibco), and resuspended to a density of $1x10^6$ cells/ml in DMEM/F12 supplemented with 10% FBS (no WEHI-3 supernatant). 50µL aliquots (~50,000 cells) of the cell suspension are then plated, in triplicate, in 96 well assay plates. 50µL aliquots of dilution series of test EPO mimetic peptides, or 50µL EPO (R & D Systems Inc., Minneapolis, MN) or Aranesp™ (darbepoeitin alpha, an ERO-R agonist commerically available from Amgen) in DMEM/F12 media supplemented with 10% FBS (no WEHI-3 supernatant I) are added to the 96 well assay plates (final well volume of 100µL). For example, 12 different dilutions may be tested where the final concentration of test peptide (or control EPO peptide) ranges from 810pM to 0.0045pM. The plated cells are then incubated

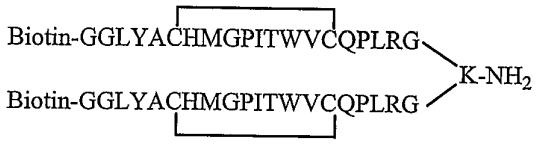
for 48h at 37°C. Next, 10μL of MTT (Roche Diagnostics) is added to each culture dish well, and then allowed to incubate for 4h. The reaction is then stopped by adding 10% SDS + 0.01N HCl. The plates are then incubated overnight at 37°C. Absorbance of each well at a wavelenght of 595nm is then measured by spectrophotometry. Plots of the absorbance readings versus test peptide concentration are constructed and the EC50 calculated using Graph Pad software. The concentration of test peptide that results in a half-maximal absorbance is recorded as the EC50 [See Table 3: Proliferation EC50].

3. Competitive Binding Assay

Competitive binding calculations are made using an assay in which a light signal is generated as a function of the proximity of two beads: a streptavidin donor bead bearing a biotinylated EPO-R-binding peptide tracer and an acceptor bead to which is bound EPO-R. Light is generated by non-radiative energy transfer, during which a singlet oxygen is released from a first bead upon illumination, and contact with the released singlet oxygen causes the second bead to emit light. These bead sets are commercially available (Packard). Bead proximity is generated by the binding of the EPO-R-binding peptide tracer to the EPO-R. A test peptide that competes with the EPO-R-binding peptide tracer for binding to EPO-R will prevent this binding, causing a decrease in light emission.

In more detail the method is as follows: Add 4μL of serial dilutions of the test EPO-R agonist peptide, or positive or negative controls, to wells of a 384 well plate. Thereafter, add 2μL / well of receptor/bead cocktail. Receptor bead cocktail consists of: 15μL of 5mg/ml streptavidin donor beads (Packard), 15μL of 5mg/ml monoclonal antibody ab179 (this antibody recognizes the portion of the human placental alkaline phosphatase protein contained in the recombinant EPO-R), protein A-coated acceptor beads (protein A will bind to the ab179 antibody; Packard), 112.5μL of a 1:6.6 dilution of recombinant EPO-R (produced in Chinese Hamster Ovary cells as a fusion protein to a portion of the human placental alkaline phosphatase protein which contains the ab179 target epitope) and 607.5μL of Alphaquest buffer (40mM HEPES, pH 7.4; 1mM $MgCl_2$; 0.1% BSA, 0.05% Tween 20). Tap to mix. Add 2μL/well of the biotinylated EPO-R-binding peptide tracer, AF33068 (30nM final concentration). AF33068, an EPO-R binding peptide (see Table 3 "Reporter EC50 (pM)"), is made according to the methods described in Example 1.

AF33068



Centrifuge 1 min to mix. Seal plate with Packard Top Seal and wrap in foil. Incubate overnight at room temperature. After 18 hours read light emission using an AlphaQuest reader (Packard). Plot light emission vs concentration of peptide and analyse with Graph Pad or Excel.

The concentration of test peptide that results in a 50% decrease in light emission, relative to that observed without test peptide, is recorded as the IC50 [See Tables 2 and 3: AQ IC50].

4. C/BFU-e Assay

EPO-R signaling stimulates the differentiation of bone marrow stem cells into proliferating red blood cell presursors. This assay measures the ability of test peptides to stimulate the proliferation and differentiation of red blood cell precursors from primary human bone marrow pluripotent stem cells.

For this assay, serial dilutions of test peptide are made in IMDM medium (Gibco) supplemented with 10% FBS (Hyclone). These serial dilutions, or positive control EPO peptide, are then added to methylcellulose to give a final volume of 1.5mL. The methylcellulose and peptide mixture is then vortexed thoroughly. Aliquots (100,000 cells/mL) of human, bone marrow derived CD34+ cells (Poietics/Cambrex) are thawed. The thawed cells are gently added to 0.1 mL of 1mg/ml DNAse (Stem Cells) in a 50mL tube. Next, 40-50 mL IMDM medium is added gently to cells: the medium is added drop by drop along the side of the 50mL tube for the first 10mL, and then the remaining volume of medium is slowly dispensed along the side of the tube. The cells are then spun at 900rpm for 20 min, and the media removed carefully by gentle aspiration. The cells are resuspended in 1ml of IMDM medium and the cell density per mL is counted on hemacytometer slide (10μL aliquot of cell suspension on slide, and cell density is the average count X 10,000 cells/ml). The cells are then diluted in IMDM medium to a cell density of 15,000 cells/mL. A 100μL of diluted cells is then added to each 1.5 mL methyl cellulose plus peptide sample (final cell concentration in assay media is 1000 cells/ mL), and the mixture is vortexed. Allow the bubbles in the mixture to disappear, and then aspirate 1mL using blunt-end needle. Add 0.25 mL aspirated mixture from each sample into each of 4 wells of a 24-well plate (Falcon brand). Incubate the plated mixtures at 37^{0}C under 5% CO_2 in a humid incubator for 14 days. Score for the presence of erythroid colonies using a phase microscope (5X-10X objective, final magnification of 100X). The concentration of test peptide at which the numer of formed colonies is 90% of maximum, relative to that observed with the EPO positive control, is recorded as the EC90 [See Table 3: C/BFU-e EC90].

5. Radioligand Competitive Binding Assay

An alternative radioligand competition binding assay can also be used to measure IC50 values for peptides of the present invention.. This assay measures binding of ^{125}I-EPO to EPOr. The assay may be performed according to the following exemplary protocol:

A. Materials

Recombinant Human EPO R/Fc Chimera	• Identification: Recombinant Human EPO R/Fc Chimera • Supplier:R&D Systems (Minneapolis, MN, US) • Catalog number: 963-ER • Lot number: EOK033071 • Storage: 4 °C
Iodinated recombinant human Erythropoietin	• Identification: (3[^{125}I]iodotyrosyl)Erythropoietin, human recombinant, high specific activity, 370 kBq, 10 µCi • Supplier:Amersham Biosciences (Piscataway, NJ, US) • Catalog number: IM219-10µCi • Lot number: • Storage: 4 °C
Protein-G Sepharose	• Identification: Protein-G Sepharose 4 Fast Flow • Supplier:Amersham Biosciences (Piscataway, NJ, US) • Catalog number 17-0618-01 • Lot number: • Storage: 4 °C
Assay Buffer	• Phosphate Buffered Saline (PBS), pH 7.4, containing 0.1% Bovine Serum Albumin and 0.1% Sodium Azide • Storage: 4 °C

B. Determination of appropriate receptor concentration.

One 50 µg vial of lyophilized recombinant EPOr extracellular domain fused to the Fc portion of human IgG1 is reconstituted in 1 mL of assay buffer. To determine the correct amount of receptor to use in the assay, 100 µL serial dilutions of this receptor preparation are combined with approximately 20,000 cpm in 200 µL of iodinated recombinant human Erythropoietin (^{125}I-EPO) in 12 x 75 mm polypropylene test tubes. Tubes are capped and mixed gently at 4 °C overnight on a LabQuake rotating shaker.

The next day, 50 µL of a 50 % slurry of Protein-G Sepharose is added to each tube. Tubes are then incubated for 2 hours at 4 °C, mixing gently. The tubes are then centrifuged for 15 min at 4000 RPM (3297 x G) to pellet the protein-G sepharose. The supernatants are carefully removed and discarded. After washing 3 times with 1 mL of 4 °C assay buffer, the pellets are counted in a Wallac Wizard gamma counter. Results were then analyzed and the dilution required to reach 50 % of the maximum binding value was calculated.

C. IC_{50} Determination for peptide

To determine the IC_{50} of a peptide of the present invention, 100 µL serial dilutions of the peptide are combined with 100 µL of recombinant erythropoietin receptor (100 pg/tube) in 12 x 75 mm

polypropylene test tubes. Then 100 µL of iodinated recombinant human Erythropoietin (^{125}I-EPO) is added to each tube and the tubes were capped and mixed gently at 4°C overnight.

The next day, bound ^{125}I-EPO is quantitated as described above. The results are analyzed and the IC_{50} value calculated using Graphpad Prism version 4.0, from GraphPad Software, Inc. (San Diego, CA) The assay is preferably repeated 2 or more times for each peptide whose IC_{50} value is measured by this procedure, for a total of 3 replicate IC_{50} determinations.

6. Discussion

The *in vitro* reporter assay results for peptide monomers of the present invention were directly compared with those for related peptide sequences previously disclosed (see AF31552 and AF31748 in Table 2): namely,

GGLYACHMGPMTVCQPLRG SEQ ID NO: 32 and

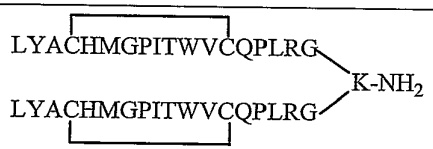
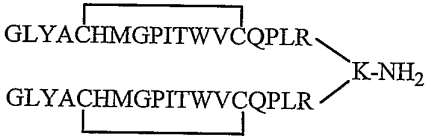
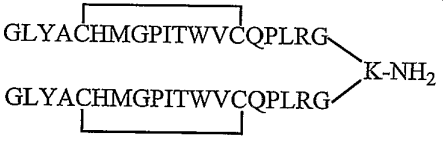
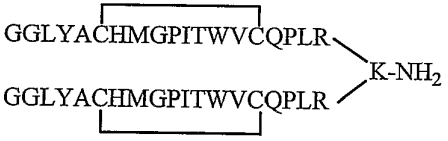
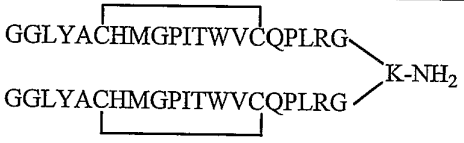
GGLYACHMGPMT(1-nal)VCQPLRG SEQ ID NO: 33.

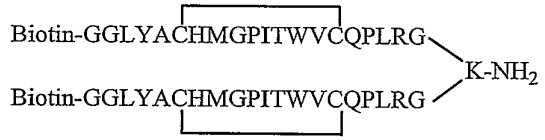
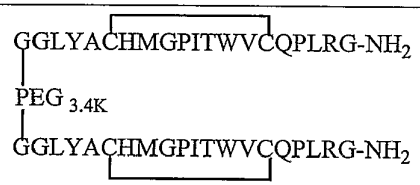
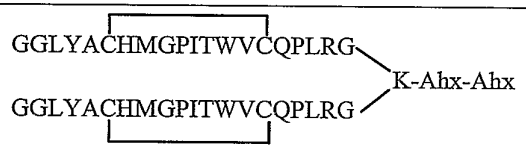
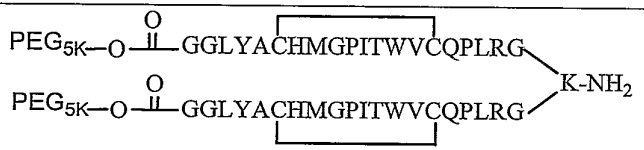
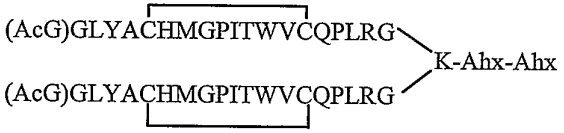
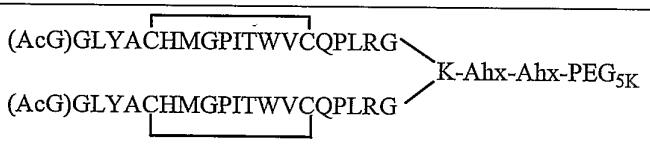
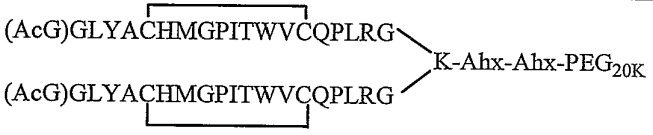
These results demonstrate the dramatically improved potency of the novel peptide monomers of the invention, as the novel peptide dimers were 3 to 7.5 times as potent as the previously disclosed peptide monomers in the reporter assay. These novel peptide monomers were then used to prepare novel peptide dimers of even greater potency and activity.

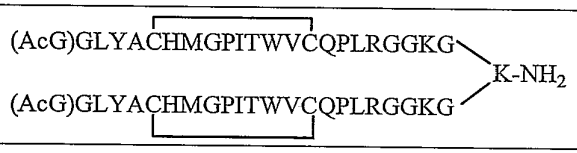
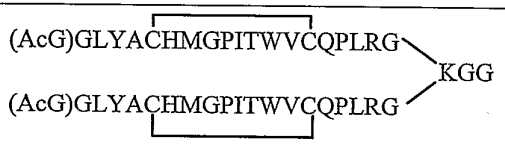
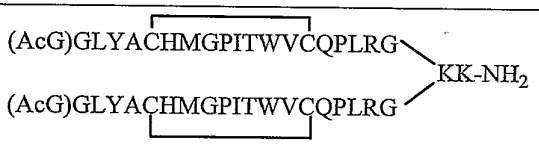
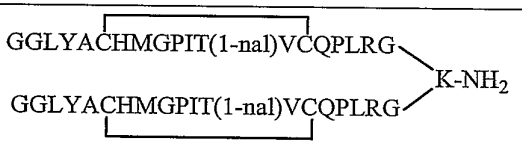
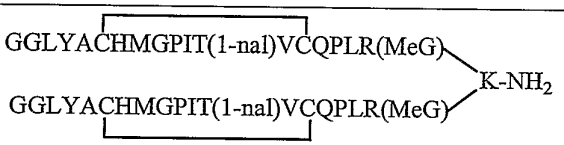
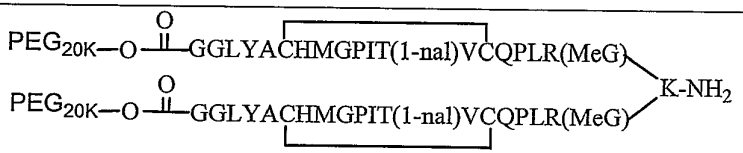
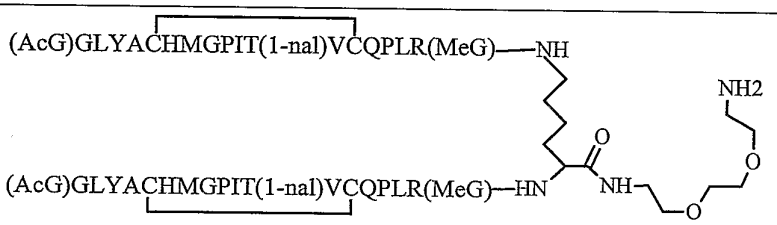
Table 2: *In vitro* reporter assay for peptide monomers

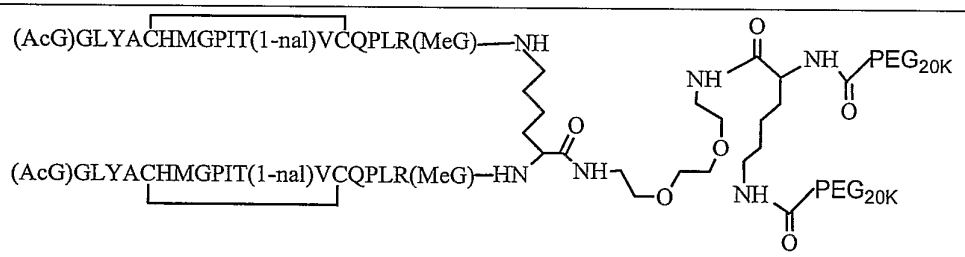
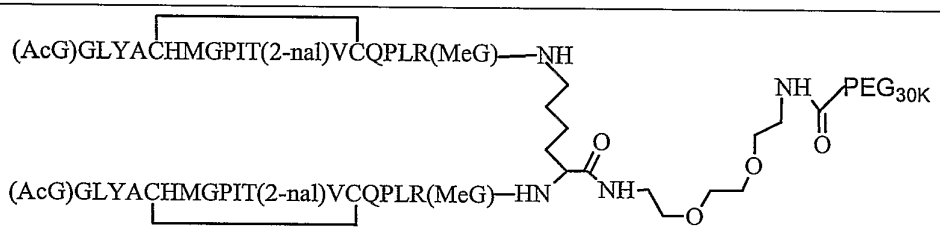
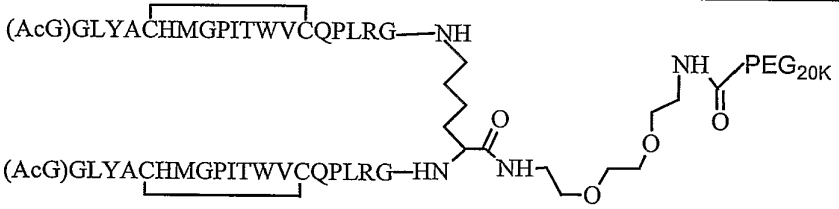
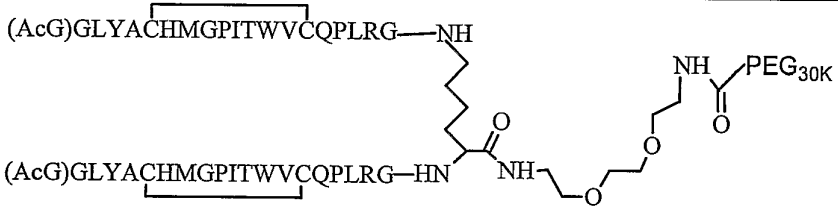
Compound designation	Peptide monomer	Reporter EC50 (nM)
AF31552	GGLYACHMGPMTWVCQPLRG-NH_2	100
AF31748	GGLYACHMGPMT(1-nal)VCQPLRG-NH_2	40
AF33128	GGLYACHMGPITWVCQPLRG-NH_2	13
AF36729	(AcG)GLYACHMGPIT(1-nal)VCQPLRK-NH2	13.3

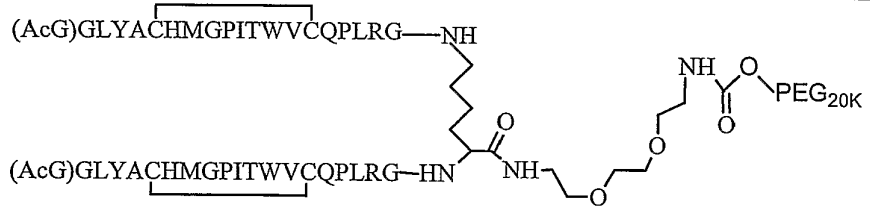
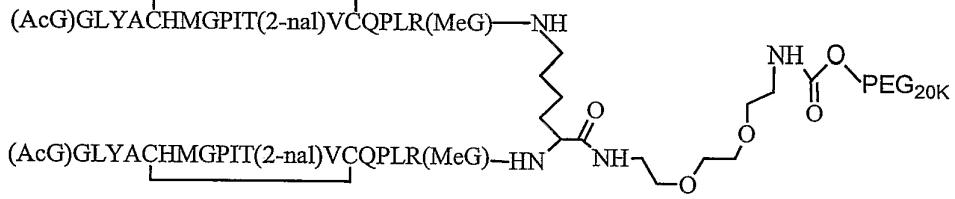
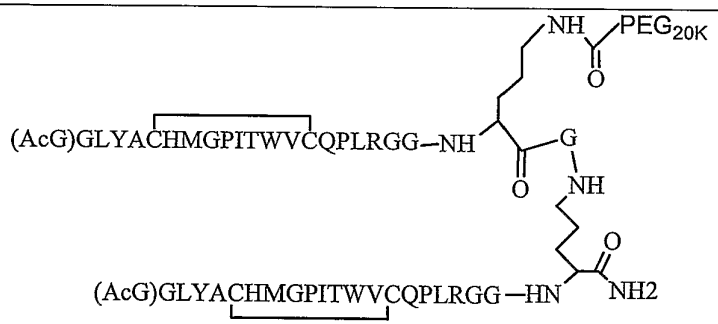
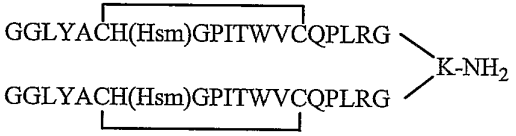
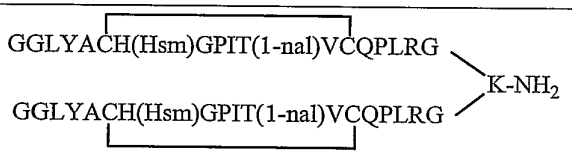
Table 3: *In vitro* activity assays for peptide dimers

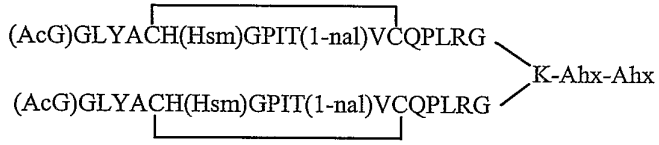
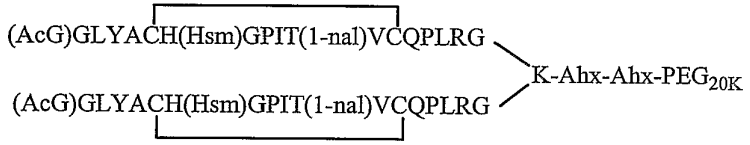
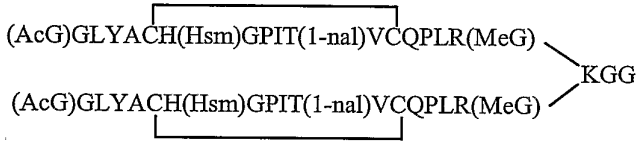
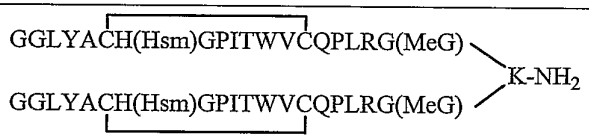
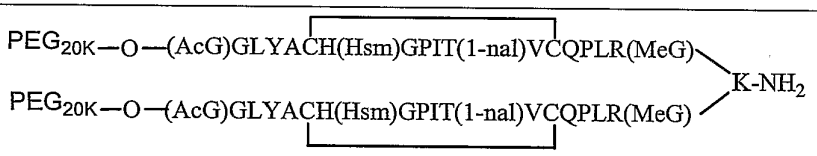
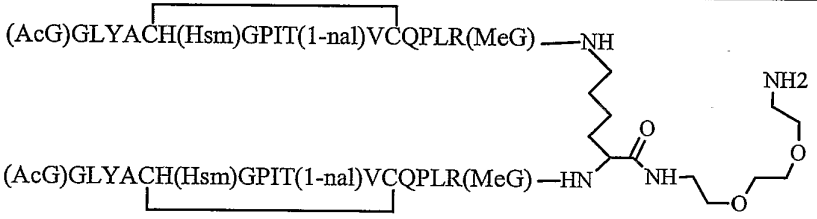
Compound designation	Peptide dimer	Reporter EC50 (pM)	Proliferation EC50 (pM)	AQ IC50 (nM)	C/BFU-e EC90 (nM)
AF33065		300	---	---	---
AF34602		727	---	---	---
AF34395		100	---	---	---
AF34601		100	---	---	---
AF32579		47	142	1.7	3

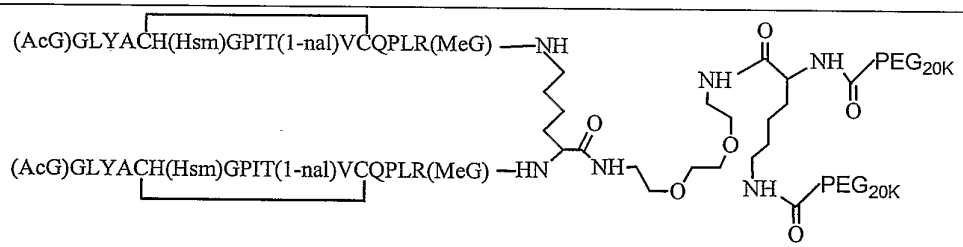
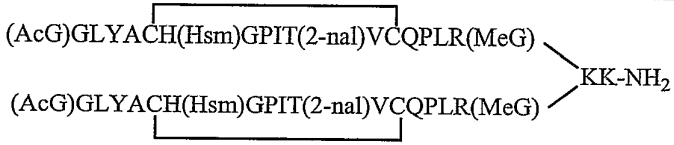
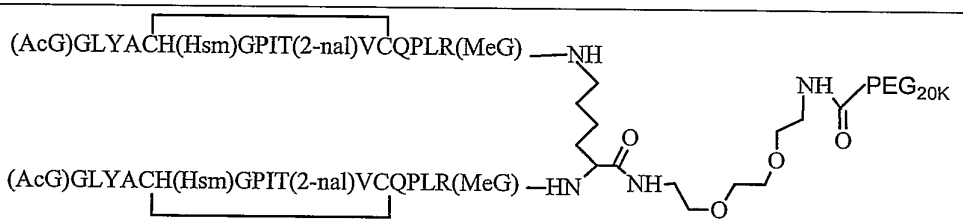
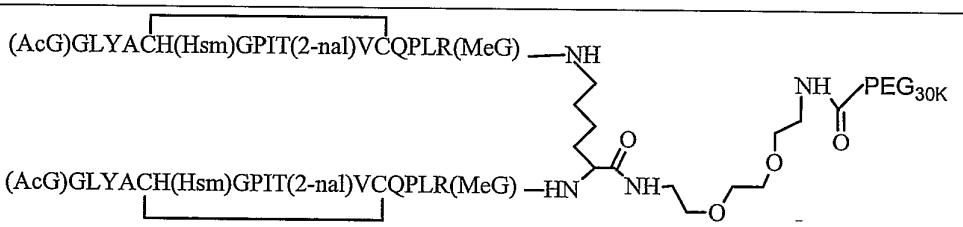
AF33068		170	---	---	---
AF33131		158	---	18	---
AF34351		50	---	---	---
AF34350		267	---	16	---
AF34753		73	---	---	1.2
AF34757		110	---	---	2.7
AF35062		91	---	---	1.6

AF35218		27	---	---	---
AF35462		194	---	---	---
AF35464		181	---	---	---
AF33197		80	---	---	---
AF34994		27	170	---	3.7
AF35083		92	---	---	---
AF35525		57	57	---	3

AF35526		900	800	---	13
AF35563		135	47	---	3
AF35575		127	57,000	---	1.1
AF35592		67	40	---	1.3

AF35593		76	32	---	1.5
AF35594		54	40	---	2.2
AF35219		403	---	---	---
AF32876		100	---	---	---
AF32881		100	---	---	11

AF35179		57	---	---	3.7
AF35180		303	---	---	---
AF35463		125	---	---	---
AF35090		35	190	---	3.7
AF35148		3600	---	---	---
AF35149		43	57	---	2

AF35168		1800	---	---	---
AF35361		77	---	---	---
AF35595		65	42	---	1.5
AF35564		887	270	---	4.5

Example 3: *In vivo* activity assays

This example describes various *in vivo* assays that are useful in evaluating the activity and potency of EPO-R agonist peptides of the invention. EPO-R agonist peptide monomers and dimers are prepared according to the methods provided in Example 1. The *in vivo* activity of these peptide monomers and dimers is evaluated using a series assays, including a polycythemic exhypoxic mouse bioassay and a reticulocyte assay. These two assays are described in further detail below.

1. Polycythemic Exhypoxic Mouse Bioassay

Test peptides are assayed for *in vivo* activity in the polycythemic exhypoxic mouse bioassay adapted from the method described by Cotes and Bangham (1961), Nature 191: 1065-1067. This assay examines the ability of a test peptide to function as an EPO mimetic: *i.e.*, to activate EPO-R and induce new red blood cell synthesis. Red blood cell synthesis is quantitated based upon incorporation of radiolabeled iron into hemoglobin of the synthesized red blood cells.

BDF1 mice are allowed to acclimate to ambient conditions for 7-10 days. Body weights are determined for all animals, and low weight animals (<15 grams) are not used. Mice are subjected to successive conditioning cycles in a hypobaric chamber for a total of 14 days. Each 24 hour cycle consista of 18 hr at 0.40±0.02% atmospheric pressure and 6 hr at ambient pressure. After conditioning the mice are maintained at ambient pressure for an additional 72 hr prior to dosing.

Test peptides, or recombinant human EPO standards, are diluted in PBS + 0.1% BSA vehicle (PBS/BSA). Peptide monomer stock solutions are first solubilized in dimethyl sulfoxide (DMSO). Negative control groups include one group of mice injected with PBS/BSA alone, and one group injected with 1% DMSO. Each dose group containa 10 mice. Mice are injected subcutaneously (scruff of neck) with 0.5 mL of the appropriate sample.

Forty eight hours following sample injection, the mice are administered an intraperitoneal injection of 0.2 ml of Fe^{59} (Dupont, NEN), for a dose of approximately 0.75 μCuries/mouse. Mouse body weights are determined 24hr after Fe^{59} administration, and the mice are sacrificed 48hr after Fe^{59} administration. Blood is collected from each animal by cardiac puncture and hematocrits are determined (heparin was used as the anticoagulant). Each blood sample (0.2 ml) is analyzed for Fe^{59} incorporation using a Packard gamma counter. Non-responder mice (*i.e.*, those mice with radioactive incorporation less than the negative control group) are eliminated from the appropriate data set. Mice that have hematocrit values less than 53% of the negative control group are also eliminated.

Results are derived from sets of 10 animals for each experimental dose. The average amount of radioactivity incorporated [counts per minute (CPM)] into blood samples from each group is calculated.

2. Reticulocyte Assay

Normal BDF1 mice are dosed (0.5 mL, injected subcutaneously) on three consecutive days with either EPO control or test peptide. At day three, mice are also dosed (0.1 mL, injected intraperitoneally) with iron dextran (100 mg/ml). At day five, mice are anesthetized with CO_2 and bled by cardiac puncture. The percent (%) reticulocytes for each blood sample is determined by thiazole orange staining and flow cytometer analysis (retic-count program). Hematocrits are manually determined. The corrected percent of reticulocytes is determined using the following formula:

$$\%\ RETIC_{CORRECTED} = \%\ RETIC_{OBSERVED} \times (Hematocrit_{INDIVIDUAL} / Hematocrit_{NORMAL})$$

3. Hematological Assay

Normal CD1 mice are dosed with four weekly bolus intravenous injections of either EPO positive control, test peptide, or vehicle. A range of positive control and test peptide doses, expressed as mg/kg, are tested by varying the active compound concentration in the formulation. Volumes injected are 5ml/kg. The vehicle control group is comprised twelve animals, while 8 animals are in each of the remaining dose groups. Daily viability and weekly body weights are recorded.

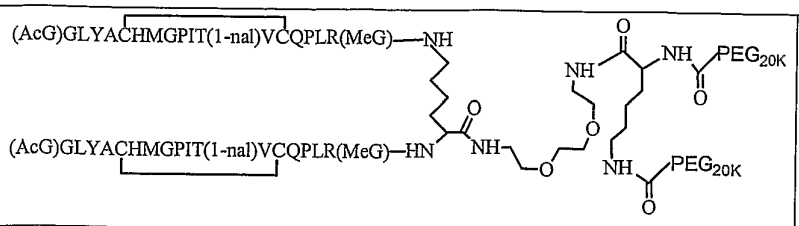
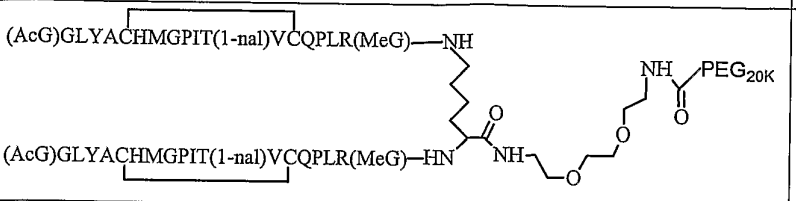
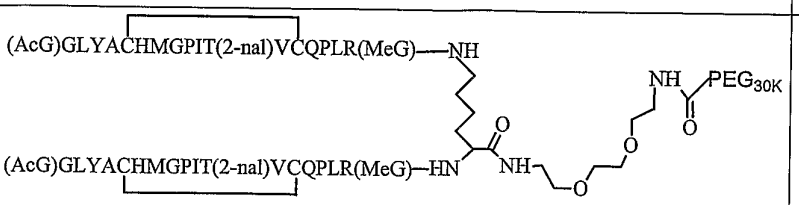
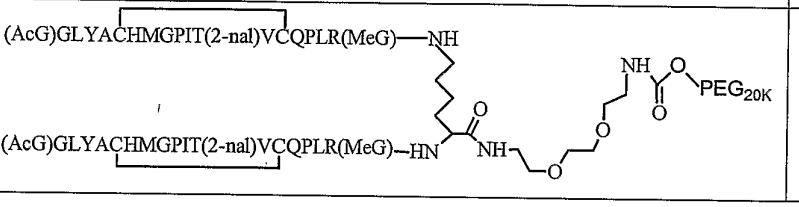
The dosed mice are mice are fasted and then anesthetized with inhaled isoflurane and terminal blood samples are collected via cardiac or abdominal aorta puncture on Day 1 (for vehicle control mice) and on Days 15 and 29 (4 mice/group/day). The blood is transferred to Vacutainer® brand tubes. Preferred anticoagulant is ethylenediaminetetraacetic acid (EDTA).

Blood samples are evaluated for endpoints measuring red blood synthesis and physiology such as hematocrit (Hct), hemoglobin (Hgb) and total erythrocyte count (RBC) using automated clinical analysers well known in the art (*e.g.*, those made by Coulter, Inc.).

Data for representative EPO-R agonist peptides in this assay are given in Table 4. Results are given as increase in percent (%) hematocrit (Ht), relative to vehicle injected control mice, at day 15 and at day 29. The indicated peptide compounds were administered to test mice at a dose of 1 mg/kg.

Table 4: *In vivo* hematological assay for peptide dimers

Compound designation	Peptide dimer	Increase in Ht (%) at day 15	Increase in Ht (%) at day 29

AF35526		22.1	28.6
AF35527		6.8	15.9
AF35563		18.3	23.8
AF35594		7.9	12.8

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Numerous references, including patents, patent applications, and various publications are cited and discussed in the description of this invention. The citation and/or discussion of such references is provided merely to clarify the description of the present invention and is not an admission that any such reference is "prior art" to the present invention. All references cited and discussed in this specification are incorporated herein by reference in their entirety and to the same extent as if each reference was individually incorporated by reference.

2004238868 26 Nov 2009

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A peptide, comprising about 17 to about 40 amino acid residues in length and comprising the amino acid sequence:

$LYACHX_0GPITX_1VCQPLR$ (SEQ ID NO: 1),

wherein X_0 is a residue selected from methionine (M) and homoserine methylether (Hsm), and X_1 is a residue selected from tryptophan (W), 1-naphthylalanine (1-nal), and 2-naphthylalanine (2-nal),

wherein said peptide binds to and activates the erythropoietin receptor (EPO-R).

2. A peptide according to claim 1, wherein the N-terminal of said peptide is acetylated.

3. A peptide according to claim 1 or 2, said peptide comprising an amino acid sequence selected from:

LYACHMGPITWVCQPLR	(SEQ ID NO: 2);
LYACHMGPIT(1-nal)VCQPLR	(SEQ ID NO: 3);
LYACHMGPIT(2-nal)VCQPLR	(SEQ ID NO: 4);
GGLYACHMGPITWVCQPLRG	(SEQ ID NO: 5);
GGLYACHMGPIT(1-nal)VCQPLRG	(SEQ ID NO: 6);
GGLYACHMGPIT(2-nal)VCQPLRG	(SEQ ID NO: 7);
(AcG)GLYACHMGPITWVCQPLRG	(SEQ ID NO: 8);
(AcG)GLYACHMGPIT(1-nal)VCQPLRG	(SEQ ID NO: 9);
(AcG)GLYACHMGPIT(2-nal)VCQPLRG	(SEQ ID NO: 10);
GGLYACHMGPITWVCQPLR(MeG)	(SEQ ID NO: 11);
GGLYACHMGPIT(1-nal)VCQPLR(MeG)	(SEQ ID NO: 12);
GGLYACHMGPIT(2-nal)VCQPLR(MeG)	(SEQ ID NO: 13);
(AcG)GLYACHMGPITWVCQPLRG(MeG)	(SEQ ID NO: 14);
(AcG)GLYACHMGPIT(1-nal)VCQPLRG(MeG)	(SEQ ID NO: 15);
(AcG)GLYACHMGPIT(2-nal)VCQPLRG(MeG)	(SEQ ID NO: 16);
LYACH(Hsm)GPITWVCQPLR	(SEQ ID NO: 17);
LYACH(Hsm)GPIT(1-nal)VCQPLR	(SEQ ID NO: 18);
LYACH(Hsm)GPIT(2-nal)VCQPLR	(SEQ ID NO: 19);
GGLYACH(Hsm)GPITWVCQPLRG	(SEQ ID NO: 20);
GGLYACH(Hsm)GPIT(1-nal)VCQPLRG	(SEQ ID NO: 21);
GGLYACH(Hsm)GPIT(2-nal)VCQPLRG	(SEQ ID NO: 22);

2004238868 26 Nov 2009

(AcG)GLYACH(Hsm)GPITWVCQPLRG	(SEQ ID NO: 23);
(AcG)GLYACH(Hsm)GPIT(1-nal)VCQPLRG	(SEQ ID NO: 24);
(AcG)GLYACH(Hsm)GPIT(2-nal)VCQPLRG	(SEQ ID NO: 25);
GGLYACH(Hsm)GPITWVCQPLR(MeG)	(SEQ ID NO: 26);
GGLYACH(Hsm)GPIT(1-nal)VCQPLR(MeG)	(SEQ ID NO: 27);
GGLYACH(Hsm)GPIT(2-nal)VCQPLR(MeG)	(SEQ ID NO: 28);
(AcG)GLYACH(Hsm)GPITWVCQPLRG(MeG)	(SEQ ID NO: 29);
(AcG)GLYACH(Hsm)GPIT(1-nal)VCQPLRG(MeG)	(SEQ ID NO: 30); and
(AcG)GLYACH(Hsm)GPIT(2-nal)VCQPLRG(MeG)	(SEQ ID NO: 31).

4. A peptide according to any of the preceding claims, wherein the peptide is a monomer.

5. A peptide according to any of the preceding claims, wherein the peptide is a dimer.

6. A peptide according to claim 5, wherein the peptide is a homodimer.

7. A peptide dimer, comprising:

(a) a first peptide chain;

(b) a second peptide chain; and

(c) a linking moiety connecting said first and second peptide chains,

wherein at least one of said first peptide chain and said second peptide chain comprises about 17 to about 40 amino acid residues in length and comprises the amino acid sequence:

$LYACHX_0GPITX_1VCQPLR$ (SEQ ID NO: 1),

wherein X_0 is a residue selected from methionine (M) and homoserine methylether (Hsm), and X_1 is a residue selected from tryptophan (W), 1-naphthylalanine (1-nal), and 2-naphthylalanine (2-nal),

wherein said peptide dimer binds to and activates the erythropoietin receptor (EPO-R).

8. A peptide dimer according to claim 7, wherein at least one of said first peptide chain and said second peptide chain comprises an amino acid sequence selected from:

LYACHMGPITWVCQPLR	(SEQ ID NO: 2);
LYACHMGPIT(1-nal)VCQPLR	(SEQ ID NO: 3);

2004238868 26 Nov 2009

LYACHMGPIT(2-nal)VCQPLR	(SEQ ID NO: 4);
GGLYACHMGPITWVCQPLRG	(SEQ ID NO: 5);
GGLYACHMGPIT(1-nal)VCQPLRG	(SEQ ID NO: 6);
GGLYACHMGPIT(2-nal)VCQPLRG	(SEQ ID NO: 7);
(AcG)GLYACHMGPITWVCQPLRG	(SEQ ID NO: 8);
(AcG)GLYACHMGPIT(1-nal)VCQPLRG	(SEQ, ID NO: 9);
(AcG)GLYACHMGPIT(2-nal)VCQPLRG	(SEQ ID NO: 10);
GGLYACHMGPITWVCQPLR(MeG)	(SEQ ID NO: 11);
GGLYACHMGPIT(1-nal)VCQPLR(MeG)	(SEQ ID NO: 12);
GGLYACHMGPIT(2-nal)VCQPLR(MeG)	(SEQ ID NO: 13);
(AcG)GLYACHMGPITWVCQPLRG(MeG)	(SEQ ID NO: 14);
(AcG)GLYACHMGPIT(1-nal)VCQPLRG(MeG)	(SEQ ID NO: 15);
(AcG)GLYACHMGPIT(2-nal)VCQPLRG(MeG)	(SEQ ID NO: 16);
LYACH(Hsm)GPITWVCQPLR	(SEQ ID NO: 17);
LYACH(Hsm)GPIT(1-nal)VCQPLR	(SEQ ID NO: 18);
LYACH(Hsm)GPIT(2-nal)VCQPLR	(SEQ ID NO: 19);
GGLYACH(Hsm)GPITWVCQPLRG	(SEQ ID NO: 20);
GGLYACH(Hsm)GPIT(1-nal)VCQPLRG	(SEQ ID NO: 21);
GGLYACH(Hsm)GPIT(2-nal)VCQPLRG	(SEQ ID NO: 22);
(AcG)GLYACH(Hsm)GPITWVCQPLRG	(SEQ ID NO: 23);
(AcG)GLYACH(Hsm)GPIT(1-nal)VCQPLRG	(SEQ ID NO: 24);
(AcG)GLYACH(Hsm)GPIT(2-nal)VCQPLRG	(SEQ ID NO: 25);
GGLYACH(Hsm)GPITWVCQPLR(MeG)	(SEQ ID NO: 26);
GGLYACH(Hsm)GPIT(1-nal)VCQPLR(MeG)	(SEQ ID NO: 27);
GGLYACH(Hsm)GPIT(2-nal)VCQPLR(MeG)	(SEQ ID NO: 28);
(AcG)GLYACH(Hsm)GPITWVCQPLRG(MeG)	(SEQ ID NO: 29);
(AcG)GLYACH(Hsm)GPIT(I-nal)VCQPLRG(MeG)	(SEQ ID NO: 30), and
(AcG)GLYACH(Hsm)GPIT(2-nal)VCQPLRG(MeG)	(SEQ ID NO: 31

9. A peptide dimer according to claim 7 or 8, wherein the linking moiety comprises the formula:

$$-NH-R_3-NH-$$

wherein R_3 is a lower (C_{1-6}) alkylene.

2004238868 26 Nov 2009

10. A peptide dimer according to claim 9, wherein the linking moiety is a lysine residue.

11. A peptide dimer according to claims 7 or 8, wherein the linking moiety comprises the formula:

$$-CO-(CH_2)_n-X-(CH_2)_m-CO-$$

wherein n is an integer from 0 to 10, m is an integer from 1 to 10, X is selected from O, S, $N(CH_2)_pNR_1$, $NCO(CH_2)_pNR_1$, and $CHNR_I$, R_1 is selected from H, Boc, and Cbz, and p is an integer from 1 to 10.

12. A peptide dimer according to claim 11, wherein n and m are each 1, X is $NCO(CH_2)_pNR_1$, p is 2, and R_1 is H.

13. A peptide dimer according to any of the preceding claims, further comprising a spacer moiety.

14. A peptide dimer according to claim 13, wherein the spacer moiety comprises the formula:

$$-NH-(CH_2)_\alpha-[O-(CH_2)_\beta]_\gamma-O_\delta-(CH_2)_\varepsilon-Y-$$

wherein a, β, and ε, are each integers whose values are independently selected from 1 to 6, δ is 0 or 1, γ is an integer selected from 0 to 10, and Y is selected from NH or CO, provided that β is 2 when γ is greater than 1.

15. A peptide dimer according to claim 14 wherein each of α, β, and ε is 2, each of γ and δ is 1, and Y is NH.

16. A peptide or peptide dimer according to any of the preceding claims, further comprising one or more water soluble polymers.

17. A peptide dimer according to any of claims 7-15, further comprising a water soluble polymer covalently bound to the linker moiety.

18. A peptide dimer according to any of claims 13-15, further comprising one or more water soluble polymers covalently bound to the spacer moiety.

2004238868 26 Nov 2009

19. A peptide or peptide dimer according any of claims 16-18, wherein the water soluble polymer is polyethylene glycol (PEG).

20. A peptide or peptide dimer according to claim 19, wherein the PEG is a linear unbranched PEG having a molecular weight of about 500 to about 60,000 Daltons.

21. A peptide or peptide dimer according to claim 20, wherein the PEG has a molecular weight of about 500 to less than about 20,000 Daltons.

22. A peptide or peptide dimer according to claim 20, wherein the PEG has a molecular weight of about 20,000 to 60,000 Daltons.

23. A peptide or peptide dimer according to claim 22, wherein the PEG has a molecular weight of about 20,000 to about 40,000 Daltons.

24. A peptide or peptide according to claim 19, wherein two PEG moieties are covalently bound to the peptide, each of said PEG comprising a linear unbranched molecule.

25. A peptide or peptide according to claim 24, wherein each of said PEG has a molecular weight of about 20,000 to about 30,000 Daltons.

26. A method for treating a patient, comprising administering to a patient having a disorder characterized by a deficiency of erythropoietin or a low or defective red blood cell population a therapeutically effective amount of a peptide or peptide dimer according to any of the preceding claims.

27. A method according to claim 26, wherein the disorder is selected from:
end stage renal failure or dialysis; anemia associated with AIDS, auto immune disease or a malignancy; beta-thalassemia; cystic fibrosis; early anemia of prematurity; anemia associated with chronic inflammatory disease; spinal cord injury; acute blood loss; aging; and neoplastic disease states accompanied by abnormal erythropoiesis.

2004238868 26 Nov 2009

28. A pharmaceutical composition comprising:

(i) a peptide or peptide dimer according to any of claims 1-25; and

(ii) a pharmaceutically acceptable carrier.

29. The peptide of claim 1 or 7, substantially as hereinbefore described with reference to any of the examples.

30. The method of claim 26, substantially as hereinbefore described with reference to any of the examples.