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(54) Title: PHARMACOLOGICALLY ACTIVE POLYMER-G-CSF CONJUGATES

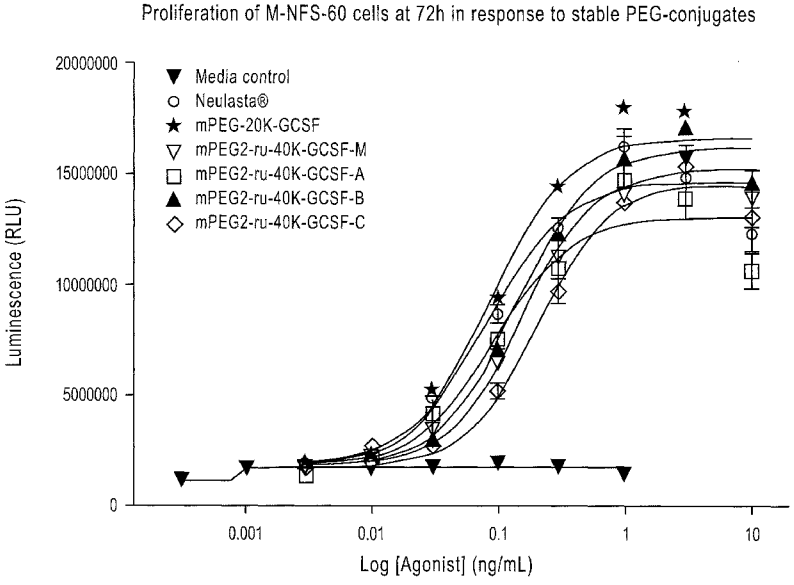

FIG. 2

(57) Abstract: The invention provides a G-CSF moiety chemically modified by covalent attachment of a branched, stably linked water-soluble polymer. A conjugate or composition of the invention, when administered by any of a number of administration routes, exhibits characteristics that are different and advantageous over those characteristics of the G-CSF moiety in unmodified form.

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PHARMACOLOGICALLY ACTIVE POLYMER -G-CSF CONJUGATES

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority of Provisional Patent Application No. 61/411,837 filed November 9, 2010 and Provisional Patent Application No. 61/530,860 filed September 02, 2011, the contents both of which are expressly incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

[0002] Among other things, the present invention relates generally to pharmacologically active G-CSF water-soluble polymer conjugates and compositions comprising the same.

BACKGROUND OF THE INVENTION

[0003] Scientists and clinicians face a number of challenges in their attempts to develop active agents into forms suited for delivery to a patient. Active agents that are polypeptides, for example, are often delivered via injection rather than orally. In this way, the polypeptide is introduced into the systemic circulation without exposure to the proteolytic environment of the stomach. Injection of polypeptides, however, has several drawbacks. For example, many polypeptides have a relatively short half-life, thereby necessitating repeated injections, which are often inconvenient and painful. Moreover, some polypeptides can elicit one or more immune responses with the consequence that the patient's immune system attempts to destroy or otherwise neutralize the immunogenic polypeptide. Of course, once the polypeptide has been destroyed or otherwise neutralized, the polypeptide cannot exert its intended pharmacodynamic activity. Thus, delivery of active agents such as polypeptides is often problematic even when these agents are administered by injection.

[0004] Some success has been achieved in addressing the problems of delivering active agents via injection. For example, conjugating an active agent to a water-soluble polymer has resulted in polymer-active agent conjugates having reduced immunogenicity and antigenicity. In addition, these polymer-active agent conjugates can often have greatly increased half-lives compared to their unconjugated counterparts as a result of decreased clearance through the kidney and/or decreased enzymatic degradation in the systemic circulation. As a result of having a greater half-life, the polymer-active agent conjugate

requires less frequent dosing, which in turn reduces the overall number of painful injections and inconvenient visits with a health care professional. Moreover, active agents that were only marginally soluble demonstrate a significant increase in water solubility when conjugated to a water-soluble polymer.

[0005] Due to its documented safety as well as its approval by the FDA for both topical and internal use, polyethylene glycol has been conjugated to active agents. When an active agent is conjugated to a polymer of polyethylene glycol or "PEG," the conjugated active agent is conventionally referred to as "PEGylated." The commercial success of PEGylated active agents such as PEGASYS® PEGylated interferon alpha-2a (Hoffmann-La Roche, Nutley, NJ), PEG-INTRON® PEGylated interferon alpha-2b (Schering Corp., Kennilworth, NJ), and NEULASTA® PEG-filgrastim (Amgen Inc., Thousand Oaks, CA) demonstrates that administration of a conjugated form of an active agent can have significant advantages over the unconjugated counterpart. Small molecules such as distearoylphosphatidylethanolamine (Zalipsky (1993) *Bioconjug. Chem.* 4(4):296-299) and fluorouracil (Ouchi *et al.* (1992) *Drug Des. Discov.*9(1):93-105) have also been PEGylated. Harris *et al.* have provided a review of the effects of PEGylation on pharmaceuticals. Harris *et al.* (2003) *Nat. Rev. Drug Discov.* 2(3):214-221.

[0006] Despite these successes, conjugation of a polymer to an active agent to provide a commercially relevant drug is often challenging and by no means straightforward. For example, conjugation can result in the polymer being attached at or near a site on the active agent that is necessary for pharmacologic activity (e.g., at or near a binding site). Such conjugates may therefore have unacceptably low activity due to, for example, the steric effects introduced by the polymer. Attempts to remedy issues with conjugates having unacceptably low activity can be frustrated when the active agent has few or no other sites suited for attachment to a polymer. Thus, additional PEGylation alternatives are desirable.

[0007] In the treatment of neutropenia, G-CSF can sometimes be administered to a patient to address or otherwise ameliorate this disorder. Filgrastim and pegfilgratim (available as NEUOPOGEN® and NEULASTA®, respectively, each from Amgen Inc., Thousand Oaks, CA) are drugs used in the treatment of patients suffering from neutropenia. With respect to pegfilgrastim, the compound comprises G-CSF (filgrastim) attached to a linear 20 kilodalton poly(ethylene glycol). Despite the commercial success of this product, additional options for providing compositions having G-CSF activity *in vivo* would provide clinicians with a better ability to customize or tailor treatment to individual patients. Thus,

while G-CSF conjugates have been described (some of which exhibit no or limited *in vitro* binding activity), there remains a need in the art to provide G-CSF conjugates that provide good activity *in vivo*. Moreover, even for G-CSF conjugates that show promise, further improvements or modifications of those conjugates (e.g., by obtaining purified forms of a particular mixture) may provide a better pharmaceutical product. Of course, understanding the important features for further modification, or which conjugates out of a mixture may be most potent, remains a challenge.

[0008] The present invention addresses this and other needs in the art.

SUMMARY OF THE INVENTION

[0009] In a first aspect, provided is a composition comprising G-CSF-polymer conjugates. Each of the conjugates within the composition is comprised of the same G-CSF moiety and the same polymer covalently attached thereto, wherein each conjugate comprises a G-CSF moiety stably covalently attached via an amide linkage to a branched, non-peptidic water-soluble polymer, where intervening between the branch point in the water-soluble polymer and the amide linkage to the G-CSF moiety is a linear spacer having a length of from 3 to 10 atoms. The branched water-soluble polymer has a weight average molecular weight in a range from about 10,000 kiloDaltons to about 100,000 kiloDaltons, and the G-CSF moiety is attached to the branched water-soluble polymer at only one or two of its amino sites selected from internal lysines and the N-terminus.

[0010] In an embodiment related to the first aspect, the linear spacer intervening between the branch point in the water-soluble polymer and the amide linkage has an atom length of one of 3, 4, or 5 atoms.

[0011] In a particular embodiment, the linear spacer corresponds to $-O-(CH_2)_{2-6}-$. In yet another more particular embodiment, the linear spacer is selected from $-O-(CH_2)_2-$, $-O-(CH_2)_3-$, $-O-(CH_2)_4-$, $-O-(CH_2)_5-$, and $-O-(CH_2)_6-$.

[0012] In yet another embodiment, the linear spacer is $-O-(CH_2)_3-$.

[0013] In yet another embodiment related to the first aspect, the composition substantially comprises conjugates having the G-CSF moiety attached to a single branched water-soluble polymer (mono-conjugates) at an internal lysine or N-terminal site.

[0014] In yet a further embodiment related to any one or more of the foregoing, the G-CSF moiety is attached to a single branched water-soluble polymer at its N-terminal site.

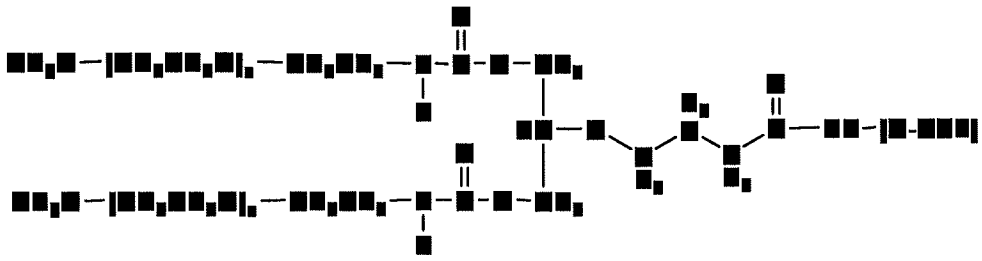
[0015] In an alternative embodiment, the G-CSF moiety is attached to a single branched water-soluble polymer at an ε-amino group of an internal lysine residue of the G-CSF moiety.

[0016] In yet a further embodiment related to the compositions and conjugates provided herein, the weight average molecular weight of the branched water-soluble polymer is in a range of from about 15,000 to 50,000 kiloDaltons.

[0017] In yet another embodiment, the weight average molecular weight of the branched water-soluble polymer ranges from about 20,000 to about 40,000 kiloDaltons.

[0018] In a preferred embodiment, the water-soluble polymer is a polyethylene glycol.

[0019] In yet a further embodiment, the G-CSF-polymer conjugates possess a structure:



wherein each n is from 113 to about 2050, and (G-CSF) represents the G-CSF moiety where NH-G-CSF represents the amino group on the G-CSF moiety to which the branched water-soluble polymer is attached.

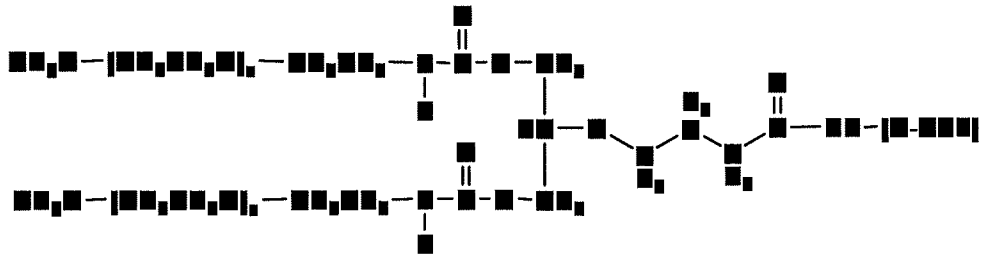
[0020] In yet a further embodiment related to any one or more of the foregoing, the composition substantially comprises conjugates having the G-CSF moiety attached to a single branched water-soluble polymer (mono-conjugates) at an internal lysine or N-terminal site, where the composition substantially comprises a single positional isomer of the G-CSF mono-conjugates.

[0021] In yet another embodiment, the G-CSF moiety is selected from native human G-CSF and recombinant human G-CSF.

[0022] In a particular embodiment, the G-CSF moiety is full length recombinant human G-CSF that is attached to the branched water-soluble polymer at an amino acid site selected from K17, K24, K35, K41 and the N-terminus.

[0023] Also provided herein is a pharmaceutical composition of any one or more of the foregoing compositions, further comprising a pharmaceutically acceptable excipient.

[0024] In a particular and preferred embodiment, provided is a pharmaceutical composition as described above wherein the G-CSF-polymer conjugates substantially comprise a single positional isomer of a mono-conjugate possessing the structure:



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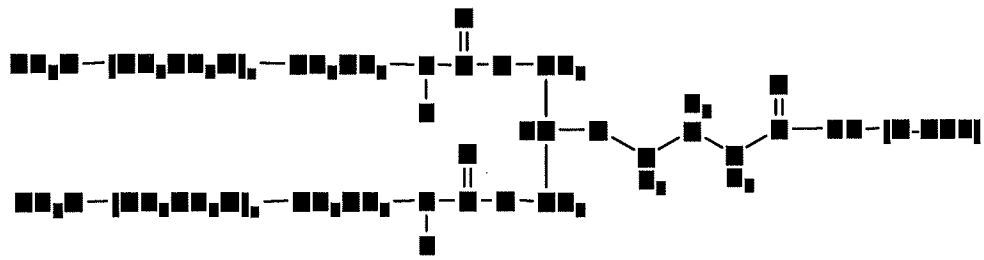
wherein the monoconjugates have a weight average molecular weight of the branched water-soluble polymer of about 40,000 kiloDaltons and are characterized by having an EC50 value in an *in vitro* cell proliferation assay using an M-NFS-60 cell line as described in Example 2 at 72 hours of about 0.09 ng/mL.

[0025] In yet another particular and preferred embodiment, provided is a pharmaceutical composition comprising a dosage amount of G-CSF-polymer conjugates substantially comprising a single positional isomer of a mono-conjugate possessing the structure:



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wherein the monoconjugates have a weight average molecular weight of from about 15,000 to 50,000 kiloDaltons, and the pharmaceutical composition, when administered to a mammal, (i) has a neutrophil stimulatory effect, and (ii) the dosage amount of the G-CSF conjugates (based upon G-CSF moiety content) to achieve the neutrophil stimulatory effect is

less than an amount of the G-CSF moiety in an unconjugated form needed to achieve the same neutrophil stimulatory effect.

[0026] In yet another particular and preferred embodiment, provided is a composition having the features set forth herein for use in administration to a human or animal subject, e.g., for treatment of neutropenia.

[0027] Additional embodiments of the present conjugates, compositions, methods, and the like will be apparent from the following description, examples, and claims. As can be appreciated from the foregoing and following description, each and every feature described herein, and each and every combination of two or more of such features, is included within the scope of the present disclosure provided that the features included in such a combination are not mutually inconsistent. In addition, any feature or combination of features may be specifically excluded from any embodiment of the present invention. Additional aspects and advantages of the present invention are set forth in the following description and claims, particularly when considered in conjunction with the accompanying examples and drawings.

BRIEF DESCRIPTION OF DRAWINGS

[0028] **FIG. 1** shows a chromatogram produced during cation-exchange chromatography as further described in connection with Example 1.

[0029] **FIG. 2** shows a plot of the proliferation of M-NFS cells at 72 hours in response to exemplary G-CSF water-soluble polymer conjugates as further described in Example 2.

[0030] **FIG. 3** shows a plot of the mean neutrophil counts for various time points following administration of test articles as further described in Example 3.

DETAILED DESCRIPTION

[0031] As used in this specification and the intended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polymer" includes a single polymer as well as two or more of the same or different polymers; reference to "an optional excipient" or to "a pharmaceutically

acceptable excipient" refers to a single optional excipient as well as two or more of the same or different optional excipients, and the like.

[0032] In describing and claiming one or more embodiments of the present invention, the following terminology will be used in accordance with the definitions described below.

[0033] As used herein, the terms "peptide," "polypeptide," and "protein," refer to polymers comprised of amino acid monomers linked by amide bonds. For use herein, each of "peptide," "polypeptide" and "protein" will be referred to as "peptide." Peptides may include the standard 20 α-amino acids that are used in protein synthesis by cells (*i.e.* natural amino acids), as well as non-natural amino acids (non-natural amino acids may be found in nature, but not used in protein synthesis by cells, *e.g.*, ornithine, citrulline, and sarcosine, or may be chemically synthesized), amino acid analogs, and peptidomimetics. The amino acids may be D- or L-optical isomers. Peptides may be formed by a condensation or coupling reaction between the α-carbon carboxyl group of one amino acid and the amino group of another amino acid. The terminal amino acid at one end of the chain (amino terminal) therefore has a free amino group, while the terminal amino acid at the other end of the chain (carboxy terminal) has a free carboxyl group. Alternatively, the peptides may be non-linear, branched peptides or cyclic peptides. Moreover, the peptides may optionally be modified or protected with a variety of functional groups or protecting groups, including on the amino and/or carboxy terminus.

[0034] 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.

[0035] The terms "therapeutic peptide fragment" or "fragments of therapeutic peptides" refer to a peptide that comprises a truncation at the amino-terminus and/or a truncation at the carboxyl-terminus of a therapeutic peptide as defined herein. The terms "therapeutic peptide fragment" or "fragments of therapeutic peptides" also encompasses amino-terminal and/or carboxyl-terminal truncations of therapeutic peptide variants and therapeutic peptide derivatives. Therapeutic peptide fragments may be produced by synthetic techniques known in the art or may arise from *in vivo* protease activity on longer peptide

sequences. It will be understood that therapeutic peptide fragments retain some or all of the therapeutic activities of the therapeutic peptides.

[0036] As used herein, the terms "therapeutic peptide variants" or "variants of therapeutic peptides" refer to therapeutic peptides such as G-CSF having one or more amino acid substitutions, including conservative substitutions and non-conservative substitutions, amino acid deletions (either internal deletions and/or *C*- and/or *N*- terminal truncations), amino acid additions (either internal additions and/or *C*- and/or *N*- terminal additions, *e.g.*, fusion peptides), or any combination thereof. Variants may be naturally occurring (*e.g.* homologs or orthologs), or non-natural in origin. The term "therapeutic peptide variants" may also be used to refer to therapeutic peptides incorporating one or more non-natural amino acids, amino acid analogs, and peptidomimetics. It will be understood that, in accordance with the invention, therapeutic peptide fragments retain some or all of the therapeutic activities of the therapeutic peptides.

[0037] "PEG," "polyethylene glycol" and "poly(ethylene glycol)" as used herein, are interchangeable and encompass any non-peptidic water-soluble poly(ethylene oxide). Typically, PEGs for use in accordance with the invention comprise the following structure "-$(OCH_2CH_2)_n$-" where (n) is 2 to 4000. As used herein, PEG also includes "-CH_2CH_2-$O(CH_2CH_2O)_n$-CH_2CH_2-" and "-$(OCH_2CH_2)_nO$-," depending upon whether or not the terminal oxygens have been displaced. Throughout the specification and claims, it should be remembered that the term "PEG" includes structures having various terminal or "end capping" groups and so forth. The term "PEG" also means a polymer that contains a majority, that is to say, greater than 50%, of -OCH_2CH_2- repeating subunits. With respect to specific forms, the PEG can take any number of a variety of molecular weights, as well as structures or geometries such as "branched," "linear," "forked," "multifunctional," and the like, to be described in greater detail below.

[0038] The terms "end-capped" and "terminally capped" are interchangeably used herein to refer to a terminal or endpoint of a polymer having an end-capping moiety. Typically, although not necessarily, the end-capping moiety comprises a hydroxy or C_{1-20} alkoxy group, more preferably a C_{1-10} alkoxy group, and still more preferably a C_{1-5} alkoxy group. Thus, examples of end-capping moieties include alkoxy (*e.g.*, methoxy, ethoxy and benzyloxy), as well as aryl, heteroaryl, cyclo, heterocyclo, and the like. It must be remembered that the end-capping moiety may include one or more atoms of the terminal monomer in the polymer [*e.g.*, the end-capping moiety "methoxy" in $CH_3O(CH_2CH_2O)_n$- and

$CH_3(OCH_2CH_2)_n$-]. In addition, saturated, unsaturated, substituted and unsubstituted forms of each of the foregoing are envisioned. Moreover, the end-capping group can also be a silane. The end-capping group can also advantageously comprise a detectable label. When the polymer has an end-capping group comprising a detectable label, the amount or location of the polymer and/or the moiety (*e.g.*, active agent) to which the polymer is coupled can be determined by using a suitable detector. Such labels include, without limitation, fluorescers, chemiluminescers, moieties used in enzyme labeling, colorimetric (*e.g.*, dyes), metal ions, radioactive moieties, gold particles, quantum dots, and the like. Suitable detectors include photometers, films, spectrometers, and the like. The end-capping group can also advantageously comprise a phospholipid. When the polymer has an end-capping group comprising a phospholipid, unique properties are imparted to the polymer and the resulting conjugate. Exemplary phospholipids include, without limitation, those selected from the class of phospholipids called phosphatidylcholines. Specific phospholipids include, without limitation, those selected from the group consisting of dilauroylphosphatidylcholine, dioleylphosphatidylcholine, dipalmitoylphosphatidylcholine, disteroylphosphatidylcholine, behenoylphosphatidylcholine, arachidoylphosphatidylcholine, and lecithin.

[0039] "Non-naturally occurring" with respect to a polymer as described herein, means a polymer that in its entirety is not found in nature. A non-naturally occurring polymer, however, may contain one or more monomers or segments of monomers that are naturally occurring, so long as the overall polymer structure is not found in nature.

[0040] The term "water-soluble" as in a "water-soluble polymer" is any polymer that is soluble in water at room temperature. Typically, a water-soluble polymer will transmit at least about 75%, more preferably at least about 95%, of light transmitted by the same solution after filtering. On a weight basis, a water-soluble polymer will preferably be at least about 35% (by weight) soluble in water, more preferably at least about 50% (by weight) soluble in water, still more preferably about 70% (by weight) soluble in water, and still more preferably about 85% (by weight) soluble in water. It is most preferred, however, that the water-soluble polymer is about 95% (by weight) soluble in water or completely soluble in water.

[0041] Molecular weight in the context of a water-soluble polymer, such as PEG, can be expressed as either a number average molecular weight or a weight average molecular weight. Unless otherwise indicated, all references to molecular weight herein refer to the weight average molecular weight. Both molecular weight determinations, number average and weight average, can be measured using gel permeation chromatography or other liquid

chromatography techniques. Other methods for measuring molecular weight values can also be used, such as the use of end-group analysis or the measurement of colligative properties (*e.g.*, freezing-point depression, boiling-point elevation, and osmotic pressure) to determine number average molecular weight, or the use of light scattering techniques, ultracentrifugation or viscometry to determine weight average molecular weight. The polymers of the invention are typically polydisperse (*i.e.*, number average molecular weight and weight average molecular weight of the polymers are not equal), possessing low polydispersity values of preferably less than about 1.2, more preferably less than about 1.15, still more preferably less than about 1.10, yet still more preferably less than about 1.05, and most preferably less than about 1.03.

[0042] The term "active" or "activated" when used in conjunction with a particular functional group refers to a reactive functional group that reacts readily with an electrophile or a nucleophile on another molecule. This is in contrast to those groups that require strong catalysts or highly impractical reaction conditions in order to react (*i.e.*, a "non-reactive" or "inert" group).

[0043] As used herein, the term "functional group" or any synonym thereof is meant to encompass protected forms thereof as well as unprotected forms.

[0044] The terms "spacer moiety," "linkage" and "linker" are used herein to refer to an atom or a collection of atoms optionally used to link interconnecting moieties such as a terminus of a polymer segment and a therapeutic peptide or an electrophile or nucleophile of a therapeutic peptide. The spacer moiety may be hydrolytically stable or may include a physiologically hydrolyzable or enzymatically degradable linkage. Unless the context clearly dictates otherwise, a spacer moiety optionally exists between any two elements of a compound (e.g., the provided conjugates comprising a residue of a therapeutic peptide and a water-soluble polymer that can be attached directly or indirectly through a spacer moiety). Atom length in reference to a linear spacer as referred to herein refers to the number of atoms making up the linear chain, e.g., -O-$CH_2CH_2CH_2$- refers to a chain length of 4 atoms, not counting the hydrogens on the carbon atoms.

[0045] "Alkyl" refers to a hydrocarbon, typically ranging from about 1 to 15 atoms in length. Such hydrocarbons are preferably but not necessarily saturated and may be branched or straight chain, although typically straight chain is preferred. Exemplary alkyl groups include methyl, ethyl, propyl, butyl, pentyl, 2-methylbutyl, 2-ethylpropyl, 3-methylpentyl,

and the like. As used herein, "alkyl" includes cycloalkyl as well as cycloalkylene-containing alkyl.

[0046] "Lower alkyl" refers to an alkyl group containing from 1 to 6 carbon atoms, and may be straight chain or branched, as exemplified by methyl, ethyl, *n*-butyl, *i*-butyl, and *t*-butyl.

[0047] "Cycloalkyl" refers to a saturated or unsaturated cyclic hydrocarbon chain, including bridged, fused, or spiro cyclic compounds, preferably made up of 3 to about 12 carbon atoms, more preferably 3 to about 8 carbon atoms. "Cycloalkylene" refers to a cycloalkyl group that is inserted into an alkyl chain by bonding of the chain at any two carbons in the cyclic ring system.

[0048] "Alkoxy" refers to an -O-R group, wherein R is alkyl or substituted alkyl, preferably C_{1-6} alkyl (*e.g.*, methoxy, ethoxy, propyloxy, and so forth).

[0049] The term "substituted" as in, for example, "substituted alkyl," refers to a moiety (*e.g.*, an alkyl group) substituted with one or more noninterfering substituents, such as, but not limited to: alkyl; C_{3-8} cycloalkyl, *e.g.*, cyclopropyl, cyclobutyl, and the like; halo, *e.g.*, fluoro, chloro, bromo, and iodo; cyano; alkoxy, lower phenyl; substituted phenyl; and the like. "Substituted aryl" is aryl having one or more noninterfering groups as a substituent. For substitutions on a phenyl ring, the substituents may be in any orientation (*i.e.*, ortho, meta, or para).

[0050] "Noninterfering substituents" are those groups that, when present in a molecule, are typically nonreactive with other functional groups contained within the molecule.

[0051] "Aryl" means one or more aromatic rings, each of 5 or 6 core carbon atoms. Aryl includes multiple aryl rings that may be fused, as in naphthyl or unfused, as in biphenyl. Aryl rings may also be fused or unfused with one or more cyclic hydrocarbon, heteroaryl, or heterocyclic rings. As used herein, "aryl" includes heteroaryl.

[0052] "Heteroaryl" is an aryl group containing from one to four heteroatoms, preferably sulfur, oxygen, or nitrogen, or a combination thereof. Heteroaryl rings may also be fused with one or more cyclic hydrocarbon, heterocyclic, aryl, or heteroaryl rings.

[0053] "Heterocycle" or "heterocyclic" means one or more rings of 5-12 atoms, preferably 5-7 atoms, with or without unsaturation or aromatic character and having at least

one ring atom that is not a carbon. Preferred heteroatoms include sulfur, oxygen, and nitrogen.

[0054] "Substituted heteroaryl" is heteroaryl having one or more noninterfering groups as substituents.

[0055] "Substituted heterocycle" is a heterocycle having one or more side chains formed from noninterfering substituents.

[0056] An "organic radical" as used herein shall include alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl, and substituted aryl.

[0057] "Electrophile" and "electrophilic group" refer to an ion or atom or collection of atoms, which may be ionic, having an electrophilic center, *i.e.*, a center that is electron seeking, capable of reacting with a nucleophile.

[0058] "Nucleophile" and "nucleophilic group" refers to an ion or atom or collection of atoms that may be ionic having a nucleophilic center, *i.e.*, a center that is seeking an electrophilic center or with an electrophile.

[0059] A "physiologically cleavable" or "hydrolyzable" or "degradable" bond is a bond that reacts with water (*i.e.*, is hydrolyzed) under physiological conditions. The tendency of a bond to hydrolyze in water will depend not only on the general type of linkage connecting two central atoms but also on the substituents attached to these central atoms. Appropriate hydrolytically unstable or weak linkages include but are not limited to carboxylate ester, phosphate ester, anhydrides, acetals, ketals, acyloxyalkyl ether, imines, orthoesters, peptides and oligonucleotides.

[0060] "Releasably attached," *e.g.*, in reference to a therapeutic peptide releasably attached to a water-soluble polymer, refers to a therapeutic peptide that is covalently attached via a linker that includes a degradable linkage as disclosed herein, wherein upon degradation (*e.g.*, hydrolysis), the therapeutic peptide is released. The therapeutic peptide thus released will typically correspond to the unmodified parent or native therapeutic peptide, or may be slightly altered, *e.g.*, possessing a short organic tag. Preferably, the unmodified parent therapeutic peptide is released.

[0061] An "enzymatically degradable linkage" means a linkage that is subject to degradation by one or more enzymes.

[0062] A "hydrolytically stable" linkage or bond refers to a chemical bond, typically a covalent bond, which is substantially stable in water, that is to say, does not undergo hydrolysis under physiological conditions to any appreciable extent over an extended period of time. Examples of hydrolytically stable linkages include, but are not limited to, the following: carbon-carbon bonds (*e.g.*, in aliphatic chains), ethers, amides, urethanes, and the like. Generally, a hydrolytically stable linkage is one that exhibits a rate of hydrolysis of less than about 1-2% per day under physiological conditions. Hydrolysis rates of representative chemical bonds can be found in most standard chemistry textbooks. It must be pointed out that some linkages can be hydrolytically stable or hydrolyzable, depending upon (for example) adjacent and neighboring atoms and ambient conditions. One of ordinary skill in the art can determine whether a given linkage or bond is hydrolytically stable or hydrolyzable in a given context by, for example, placing a linkage-containing molecule of interest under conditions of interest and testing for evidence of hydrolysis (*e.g.*, the presence and amount of two molecules resulting from the cleavage of a single molecule). Other approaches known to those of ordinary skill in the art for determining whether a given linkage or bond is hydrolytically stable or hydrolyzable can also be used.

[0063] The terms "pharmaceutically acceptable excipient" and "pharmaceutically acceptable carrier" refer to an excipient that may optionally be included in the compositions of the invention and that causes no significant adverse toxicological effects to the patient.

[0064] "Pharmacologically effective amount," "physiologically effective amount," and "therapeutically effective amount" are used interchangeably herein to mean the amount of a polymer-(therapeutic peptide) conjugate that is needed to provide a desired level of the conjugate (or corresponding unconjugated therapeutic peptide) in the bloodstream or in the target tissue. The precise amount will depend upon numerous factors, *e.g.*, the particular therapeutic peptide, the components and physical characteristics of the therapeutic composition, intended patient population, individual patient considerations, and the like, and can readily be determined by one skilled in the art, based upon the information provided herein.

[0065] "Multi-functional" means a polymer having three or more functional groups contained therein, where the functional groups may be the same or different. Multi-functional polymeric reagents of the invention will typically contain from about 3-100 functional groups, or from 3-50 functional groups, or from 3-25 functional groups, or from 3-15 functional groups, or from 3 to 10 functional groups, or will contain 3, 4, 5, 6, 7, 8, 9 or 10 functional groups within the polymer backbone. A "difunctional" polymer means a polymer

having two functional groups contained therein, either the same (*i.e.*, homodifunctional) or different (*i.e.*, heterodifunctional).

[0066] The terms "subject," "individual," or "patient" are used interchangeably herein and refer to a vertebrate, preferably a mammal. Mammals include, but are not limited to, murines, rodents, simians, humans, farm animals, sport animals, and pets.

[0067] "Optional" or "optionally" means that the subsequently described circumstance may or may not occur, so that the description includes instances where the circumstance occurs and instances where it does not.

[0068] "Substantially" (unless specifically defined for a particular context elsewhere or the context clearly dictates otherwise) means nearly totally or completely, for instance, 95% or greater of some given quantity or condition.

[0069] A "minor" amount refers to 5% or less of some given quantity. A minor amount may, e.g., range from less than 5% to 0.1% of some given quantity.

[0070] A "majority" of a particular item refers to greater than 50% of such item out of the population under consideration. For example, a composition that contains a majority of a single positional isomer of a G-CSF mono-conjugate is one that contains greater than 50% of such mono-conjugate out of a population that includes the other possible positional isomers of the mono-conjugate. Thus, a majority is necessarily greater than 50% of a given population, but may, in actuality be present in an amount greater than 60%, greater than 70%, greater than 80%, or even greater than 90%.

[0071] Unless the context clearly dictates otherwise, when the term "about" precedes a numerical value, the numerical value is understood to mean the stated numerical value and also ± 10% of the stated numerical value.

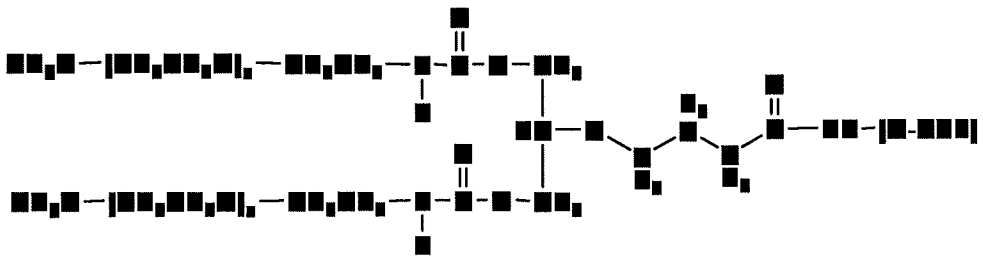
Overview

[0072] The present disclosure provides water-soluble polymer conjugates of G-CSF and related compositions. The conjugate comprises a G-CSF moiety (to be described more fully below) stably covalently attached via an amide linkage to a branched, non-peptidic water-soluble polymer, where intervening between the branch point in the water-soluble polymer and the amide linkage to the G-CSF moiety is a linear spacer having a length of from 3 to 10 atoms. The branched water-soluble polymer has a weight average molecular

weight in a range from about 10,000 kiloDaltons to about 100,000 kiloDaltons, and the G-CSF moiety is attached to the branched water-soluble polymer at only one (mono-conjugate) or two (di-conjugate) of its amino sites selected from internal lysines and the N-terminus.

[0073] Generally, stable covalent attachment of large, water-soluble polymers such as PEG to a polypeptide significantly decreases the bioactivity of the polypeptide. In general, the bioactivity of a protein conjugate in comparison to unmodified protein decreases as the molecular weight of the polymer increases. However, in many cases, an accompanying increase in circulating half-life can make a drug conjugate having an extremely low bioactivity an attractive and efficacious drug candidate due to the overall advantages brought about by altered pharmacokinetics and pharmacodynamics. For instance, PEGASYS® (PEGylated interferon α-2a covalently attached to a "lysine-branched" 40 kiloDalton polyethylene glycol), manufactured by Genentech/Roche) for treatment of hepatitis B, despite being a highly successful product, possesses a bioactivity that is only 1% of native interferon α-2a. Grace, M., *et al., Antiviral Chemistry and Chemotherapy*,15:287-297 (2004). In preparing the conjugates described herein, it was discovered that contrary to expectations, high molecular weight, branched, stably linked conjugates of G-CSF unexpectedly retained significant bioactivity. The details surrounding such conjugates, compositions, and related methods will now be described more fully.

[0074] Illustrative conjugates in accordance with the invention can be characterized by the following formula:



wherein each n is from 113 to about 2050 and (G-CSF) is a residue of a G-CSF moiety. The above conjugate is branched, with two polymer "arms" extending from a "CH" branch point, and having a linear spacer intervening between the branch point and the amide linkage to G-CSF with an atom length of 4 atoms (-O-$CH_2CH_2CH_2$-). Preferably, a spacer intervening between the polymer branch or bifurcation point and the amide linkage will possess a length of from 3-10 atoms, i.e., 3, 4, 5, 6, 7, 8, 9, or 10. Even more preferably, the spacer will possess a length of 3-5 atoms, or even more preferably 4 atoms. Without being bound by

theory, it may be that the length of the spacer when considered with the geometric, charge, and spatial considerations of the branched polymer, enables the G-CSF moiety to maintain, to a significant degree, its bioactivity.

G-CSF Moiety

[0075] With respect to the G-CSF moiety, the term "G-CSF moiety," as used herein, refers to those peptides, polypeptides and proteins having G-CSF activity (and includes G-CSF activity-containing peptides derived through site-directed mutagenesis or other mutations), including (for example) G-CSF. Prior to conjugation, the G-CSF moiety has at least one electrophilic group or nucleophilic group suitable for reaction with a water-soluble polymer. In addition, the term "G-CSF moiety" encompasses both the G-CSF moiety prior to conjugation as well as the G-CSF moiety residue following conjugation. It will be understood, however, that when the G-CSF moiety is covalently attached to a water-soluble polymer, the G-CSF moiety is slightly altered due to the presence of one or more covalent bonds associated with linkage to the polymer (or linker that is attached to the polymer), due to reaction of one of more reactive groups of the G-CSF moiety (*e.g.*, an amino, carboxyl, etc.), with the water-soluble polymer. In some instances, this slightly altered form of the G-CSF moiety attached to another molecule, such as a water-soluble polymer, is referred to as a "residue" of the G-CSF moiety. As will be explained in further detail below, one of ordinary skill in the art can determine whether any given moiety has G-CSF activity.

[0076] The term "G-CSF moiety," as used herein, refers to a moiety having G-CSF activity, and, unless the context clearly dictates otherwise, also refers to a G-CSF precursor moiety (an exemplary sequence of which is provided in SEQ ID NO:3). The G-CSF moiety will also have at least one electrophilic group or nucleophilic group suitable for reaction with a polymeric reagent. Preferably, such a group is an amino group. In addition, the term "G-CSF moiety" encompasses both the G-CSF moiety prior to conjugation as well as the G-CSF moiety residue following conjugation. As will be explained in further detail below, one of ordinary skill in the art can determine whether any given moiety has G-CSF activity. Proteins comprising an amino acid sequence corresponding to any one of SEQ ID NOS:1 through 2 correspond to a G-CSF moiety, as well as any protein or polypeptide substantially homologous thereto, whose biological properties result in the stimulation of growth and/or number of neutrophils and/or an activity similar to G-CSF. As used herein, the term "G-CSF

moiety" includes such proteins modified deliberately, as for example, by site directed mutagenesis or accidentally through mutations. These terms also include analogs having from 1 to 6 additional glycosylation sites, analogs having at least one additional amino acid at the carboxy terminal end of the protein wherein the additional amino acid(s) includes at least one glycosylation site, and analogs having an amino acid sequence which includes at least one glycosylation site. These terms include both natural and recombinantly produced G-CSF.

[0077] The G-CSF moiety can be produced non-recombinantly. For example, as described in U.S. Patent No. 4,810,643, it is possible to collect G-CSF from the culture medium of a human carcinoma cell line denominated 5637 and deposited under restrictive conditions with the American Type Culture Collection, Rockville MD as A.T.C.C. Deposit No. HTB-9.

[0078] The G-CSF moiety can be derived from recombinant methods and can be expressed in bacterial (*e.g.*, *E. coli*), mammalian (*e.g.*, Chinese hamster ovary cells), and yeast (*e.g.*, *Saccharomyces cerevisiae*) expression systems. The expression can occur via exogeneous expression or via endogenous expression. For example, Nagata *et al.* (1986) *Nature* 319:415 provides the cDNA for human G-CSF ("hG-CSF") isolated from human squamous cell carcinoma cell line CHU-II and also describes a process for expressing of the protein in COS cells (African Green Monkey cells). Souza et al. describes a process for expressing G-CSF in *E. coli* cells. U.S. Patent No. 4,810,643 describes recombinant-based methods for preparing methionyl G-CSF (*i.e.*, G-CSF to which the N-terminus has the amino acid methionine attached). In addition, U.S. Patent No. 5,633,352 describes recombinant methods for preparing G-CSF.

[0079] The amino acid sequence for human native G-CSF is provided in SEQ ID NO:1. As provided therein, a methionine residue-containing form is also contemplated for this, and all other sequences, described herein. SEQ ID NO:2 corresponds to a G-CSF moiety having a different sequence than SEQ ID NO 1.

[0080] Although recombinant-based methods for preparing proteins can differ, recombinant methods typically involve constructing the nucleic acid encoding the desired polypeptide or fragment, cloning the nucleic acid into an expression vector, transforming a host cell (*e.g.*, plant, bacteria, yeast, transgenic animal cell, or mammalian cell such as Chinese hamster ovary cell or baby hamster kidney cell), and expressing the nucleic acid to produce the desired polypeptide or fragment. Methods for producing and expressing

recombinant polypeptides *in vitro* and in prokaryotic and eukaryotic host cells are known to those of ordinary skill in the art.

[0081] To facilitate identification and purification of the recombinant polypeptide, nucleic acid sequences that encode for an epitope tag or other affinity binding sequence can be inserted or added in-frame with the coding sequence, thereby producing a fusion protein comprised of the desired polypeptide and a polypeptide suited for binding. Fusion proteins can be identified and purified by first running a mixture containing the fusion protein through an affinity column bearing binding moieties (*e.g.*, antibodies) directed against the epitope tag or other binding sequence in the fusion proteins, thereby binding the fusion protein within the column. Thereafter, the fusion protein can be recovered by washing the column with the appropriate solution (e.g., acid) to release the bound fusion protein. The recombinant polypeptide can also be identified and purified by lysing the host cells, separating the polypeptide, e.g., by size exclusion chromatography, and collecting the polypeptide. These and other methods for identifying and purifying recombinant polypeptides are known to those of ordinary skill in the art. In one or more embodiments of the invention, however, it is preferred that the G-CSF moiety is not in the form of a fusion protein.

[0082] Depending on the system used to express proteins having G-CSF activity, the G-CSF moiety can be unglycosylated or glycosylated and either may be used. That is, the G-CSF moiety can be unglycosylated or the G-CSF moiety can be glycosylated. In one or more embodiments of the invention, it is preferred that the G-CSF moiety is not glycosylated.

[0083] The G-CSF moiety can also advantageously be modified to include one or more amino acid residues such as, for example, lysine, cysteine and/or arginine, in order to provide facile attachment of a polymer to an atom within the side chain of the amino acid. In addition, the G-CSF moiety can be modified to include a non-naturally occurring amino acid residue. Techniques for adding amino acid residues and non-naturally occurring amino acid residues are well known to those of ordinary skill in the art. Reference is made to J. March, *Advanced Organic Chemistry: Reactions Mechanisms and Structure*, 4th Ed. (New York: Wiley-Interscience, 1992). In one or more embodiments of the invention, it is preferred that the G-CSF moiety is not modified to include one or more amino acid residues. Exemplary G-CSF moieties having at least one substitution relative to hG-CSF are provided in U.S. Patent No. 6,646,110, and are suited for use as a G-CSF moiety herein. Further, exemplary G-CSF moieties having at least one substitution relative to hG-CSF are provided in U.S. Patent Nos. 6,004,548 and 5,580,755, and are suited for use as a G-CSF moiety herein.

[0084] In addition, the G-CSF moiety can advantageously be modified to include attachment of a functional group (other than through addition of a functional group-containing amino acid residue). For example, the G-CSF moiety can be modified to include a thiol group. In addition, the G-CSF moiety can be modified to include an N-terminal alpha carbon. In addition, the G-CSF moiety can be modified to include one or more carbohydrate moieties. In some embodiments of the invention, it is preferred that the G-CSF moiety is not modified to include a thiol group and/or an N-terminal alpha carbon. G-CSF moieties containing an aminoxy, aldehyde or some other functional group can be used.

[0085] A preferred G-CSF moiety has an amino acid sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2. Unless specifically noted, all assignments of a numeric location of an amino acid residue as provided herein are based on SEQ ID NO:1 (ignoring any leading methionyl residue). Sequences that are useful to serve as G-CSF moieties include those sequences of the proteins found in commercially available versions G-CSF-containing formulations such as NEUPOGEN® G-CSF (Amgen, Thousand Oaks, CA) and GRASTIM® G-CSF (Dr. Reddy's, Hyderabad, India).

[0086] A hG-CSF moiety (as provided in SEQ ID NO: 1) can be used as well as truncated versions, hybrid variants, and peptide mimetics of the sequence. Biologically active fragments, deletion variants, substitution variants or addition variants of any of the foregoing that maintain at least some degree of G-CSF activity can also serve as a G-CSF moiety.

[0087] For any given peptide or protein moiety, it is possible to determine whether that moiety has G-CSF activity. For example, as described in U.S. Patent No. 5,580,755, it is possible to administer a G-CSF moiety of interest with buffer into the blood stream of a hamster and count the granulocytes. The G-CSF moiety of interest can serve as an G-CSF moiety in accordance with the present invention if the hamster injected with the proposed G-CSF moiety exhibits a statistically significant increase in granulocytes when compared to a control hamster not injected with the proposed G-CSF moiety (*e.g.*, simply buffer).

Water-Soluble Polymer

[0088] With respect to the water-soluble, non-peptidic polymer used in the polymer-G-CSF conjugates in connection with the present invention, the water-soluble, non-peptidic polymer is hydrophilic, non-peptidic, and biocompatible. A substance is considered biocompatible if the beneficial effects associated with use of the substance alone or with another substance (*e.g.*, an active agent such a therapeutic peptide) in connection with living tissues (*e.g.*, administration to a patient) outweighs any deleterious effects as evaluated by a clinician, *e.g.*, a physician. A substance is considered non-immunogenic if the intended use of the substance *in vivo* does not produce an undesired immune response (*e.g.*, the formation of antibodies) or, if an immune response is produced, that such a response is not deemed clinically significant or important as evaluated by a clinician. Typically, the water-soluble polymer is hydrophilic, biocompatible and non-immunogenic.

[0089] Further the water-soluble polymer is typically characterized as having from 2 to about 300 termini, preferably from 2 to 100 termini, and more preferably from about 2 to 50 termini. Examples of such polymers include, but are not limited to, poly(alkylene glycols) such as polyethylene glycol (PEG), poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α-hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), and combinations of any of the foregoing, including copolymers and terpolymers thereof.

[0090] The water-soluble polymer is not limited to a particular structure and may possess a linear architecture (*e.g.*, alkoxy PEG or bifunctional PEG), or a non-linear architecture, such as branched, forked, multi-armed (*e.g.*, PEGs attached to a polyol core), or dendritic (*i.e.* having a densely branched structure with numerous end groups). Moreover, the polymer subunits can be organized in any number of different patterns and can be selected, *e.g.*, from homopolymer, alternating copolymer, random copolymer, block copolymer, alternating tripolymer, random tripolymer, and block tripolymer. Preferred in connection with the present invention is a water-soluble, non-peptidic polymer that is branched and/or a homopolymer.

[0091] One particularly preferred type of water-soluble, non-peptidic polymer is a polyalkylene oxide, and in particular, polyethylene glycol (or PEG). Generally, a PEG used to prepare a therapeutic peptide polymer conjugate of the invention is "activated" or reactive. That is to say, the activated PEG (and other activated water-soluble polymers collectively

referred to herein as "polymeric reagents") used to form a conjugate comprises an activated functional group suitable for coupling to a desired site or sites on the therapeutic peptide. Thus, a polymeric reagent for use in preparing a conjugate includes a functional group for reaction with the therapeutic peptide.

[0092] Representative polymeric reagents and methods for conjugating such polymers to an active moiety are known in the art, and are, *e.g.*, described in Harris, J.M. and Zalipsky, S., eds, *Poly(ethylene glycol), Chemistry and Biological Applications*, ACS, Washington, 1997; Veronese, F., and J.M Harris, eds., *Peptide and Protein PEGylation*, Advanced Drug Delivery Reviews, 54(4); 453-609 (2002); Zalipsky, S., *et al.*, "*Use of Functionalized Poly(Ethylene Glycols) for Modification of Polypeptides*" in *Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications*, J. M. Harris, ed., Plenus Press, New York (1992); Zalipsky (1995) *Advanced Drug Reviews*16:157-182, and in Roberts, *et al., Adv. Drug Delivery Reviews,* 54, 459-476 (2002).

[0093] PEG reagents suitable for use in the present invention are available from commercial sources and can be prepared synthetically. Descriptions of polymeric reagents, as well as methods for making polymeric reagents, can be found in, for example, U.S. Patent Nos. 5,252,714, 5,650,234, 5,739,208, 5,932,462, 5,629,384, 5,672,662, 5,990,237, 6,448,369, 6,362,254, 6,495,659, 6,413,507, 6,376,604, 6,348,558, 6,602,498, 7,026,440, 7,157,546 and in U.S. Patent Application Publication No. 2005/0009988.

[0094] Typically, for the G-CSF conjugates provided herein, the weight-average molecular weight of the water-soluble polymer in the conjugate is from about 5,000 Daltons to about 150,000 Daltons, more preferably from 10,000 Daltons to 100,000 Daltons. Exemplary ranges include weight-average molecular weights in the range of from about 5,000 Daltons to about 80,000 Daltons, from 10,000 Daltons to about 80,000 Daltons, from about 10,000 Daltons to about 65,000 Daltons, from about 10,000 Daltons to about 50,000 Daltons, from 15,000 Daltons to about 50,000 Daltons, from greater than 5,000 Daltons to about 80,000 Daltons, from about 15,000 Daltons to about 45,000 Daltons, from about 20,000 Daltons to about 45,000 Daltons, from about 20,000 Daltons to about 40,000 Daltons, from about 30,000 Daltons to about 50,000 Daltons, and from about 35,000 Daltons to about 45,000 Daltons.

[0095] Exemplary weight-average molecular weights for the water-soluble polymer include about 5,000 Daltons, about 5,500 Daltons, about 6,000 Daltons, about 7,000 Daltons,

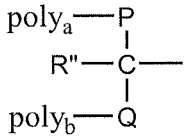
about 7,500 Daltons, about 8,000 Daltons, about 9,000 Daltons, about 10,000 Daltons, about 11,000 Daltons, about 12,000 Daltons, about 13,000 Daltons, about 14,000 Daltons, about 15,000 Daltons, about 20,000 Daltons, about 22,500 Daltons, about 25,000 Daltons, about 30,000 Daltons, about 35,000 Daltons, about 40,000 Daltons, about 45,000 Daltons, about 50,000 Daltons, about 55,000 Daltons, about 60,000 Daltons, about 65,000 Daltons, about 70,000 Daltons, and about 75,000 Daltons.

[0096] Branched versions of the water-soluble polymer (*e.g.*, a branched 40,000 Dalton water-soluble polymer comprised of two 20,000 Dalton polymers or the like) having a total molecular weight of any of the foregoing can also be used. In one or more particular embodiments, depending upon the other features of the subject therapeutic peptide polymer conjugate, the conjugate is one that does not have one or more attached PEG moieties having a weight-average molecular weight of less than about 6,000 Daltons.

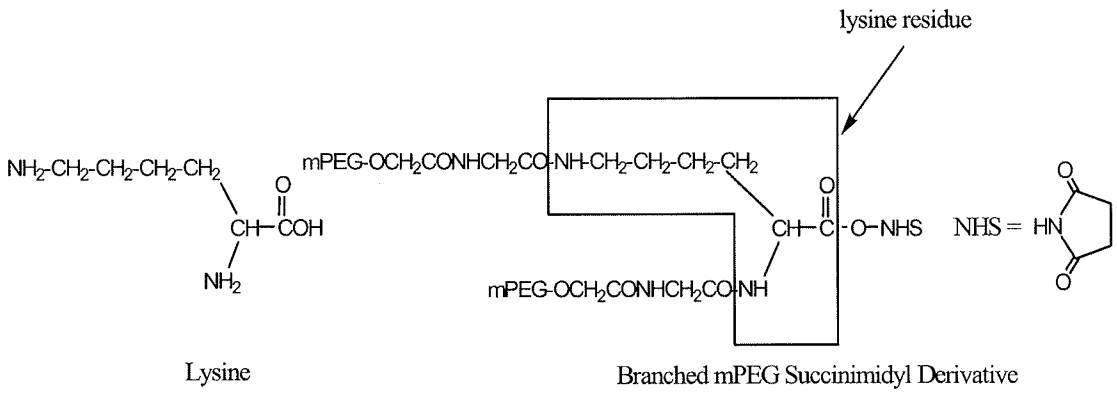
[0097] In instances in which the water-soluble polymer is a PEG, the PEG will typically comprise a number of (OCH_2CH_2) monomers. As used herein, the number of repeat units is typically identified by the subscript "*n*" in, for example, "$(OCH_2CH_2)_n$." Thus, the value of (n) typically falls within one or more of the following ranges: from 113 to about 2050; from about 136 to about 2050, from about 225 to about 1930, from about 450 to about 1930, from about 1200 to about 1930, from about 568 to about 2727, from about 660 to about 2730, from about 795 to about 2730, from about 795 to about 2730, from about 909 to about 2730, and from about 1,200 to about 1,900. For any given polymer in which the molecular weight is known, it is possible to determine the number of repeating units (*i.e.*, "*n*") by dividing the total weight-average molecular weight of the polymer by the molecular weight of the repeating monomer.

[0098] A polymer for use in the invention may be end-capped, that is, a polymer having at least one terminus capped with a relatively inert group, such as a lower alkoxy group (*i.e.*, a C_{1-6} alkoxy group) or a hydroxyl group. One frequently employed end-capped polymer is methoxy-PEG (commonly referred to as mPEG), wherein one terminus of the polymer is a methoxy ($-OCH_3$) group. The -PEG- symbol used in the foregoing generally represents the following structural unit: $-CH_2CH_2O-(CH_2CH_2O)_n-CH_2CH_2-$, where (n) generally ranges from about zero to about 4,000.

[0099] Multi-armed or branched PEG molecules, such as those described in U.S. Patent No. 5,932,462, are particularly suitable for use in the present invention. For example, the PEG may be described generally according to the structure:

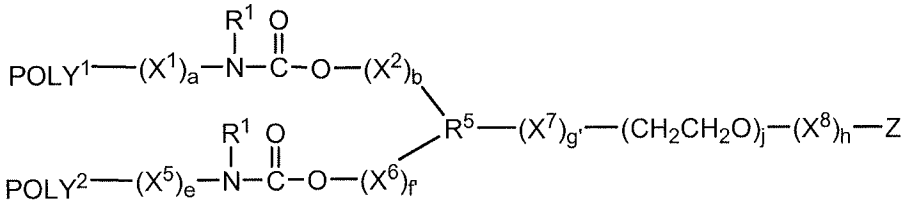


where $poly_a$ and $poly_b$ are PEG backbones (either the same or different), such as methoxy poly(ethylene glycol); R" is a non-reactive moiety, such as H, methyl or a PEG backbone; and P and Q are non-reactive linkages. In one embodiment, the branched PEG molecule is one that includes a lysine residue, such as the following reactive PEG suitable for use in forming a therapeutic peptide conjugate. Although the branched PEG below is shown with a reactive succinimidyl group, this represents only one of a myriad of reactive functional groups suitable for reacting with a therapeutic peptide.



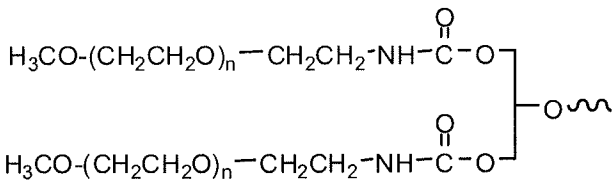
[0100] In some instances, the polymeric reagent (as well as the corresponding conjugate prepared from the polymeric reagent) may lack a lysine residue in which the polymeric portions are connected to amine groups of the lysine via a "-$OCH_2CONHCH_2CO$-" group. In still other instances, the polymeric reagent (as well as the corresponding conjugate prepared from the polymeric reagent) may lack a branched water-soluble polymer that includes a lysine residue (wherein the lysine residue is used to effect branching).

[0101] Additional branched PEGs for use as polymeric reagents to prepare the polymer-G-CSF conjugates include those polymer reagents described in U.S. Patent Application Publication No. 2005/0009988. Representative branched polymers described therein include those having the following generalized structure:

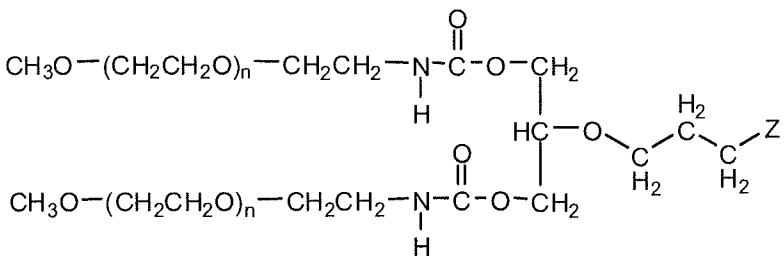


wherein: $POLY^1$ is a water-soluble polymer; $POLY^2$ is a water-soluble polymer; (a) is 0, 1, 2 or 3; (b) is 0, 1, 2 or 3; (e) is 0, 1, 2 or 3; (f') is 0, 1, 2 or 3; (g') is 0, 1, 2 or 3; (h) is 0, 1, 2 or 3; (j) is 0 to 20; each R^1 is independently H or an organic radical selected from alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, substituted alkynyl, aryl and substituted aryl; X^1, when present, is a spacer moiety; X^2, when present, is a spacer moiety; X^5, when present, is a spacer moiety; X^6, when present, is a spacer moiety; X^7, when present, is a spacer moiety; X^8, when present, is a spacer moiety; R^5 is a branching moiety; and Z is a reactive group for coupling to a therapeutic peptide, optionally via an intervening spacer. $POLY^1$ and $POLY^2$ in the preceding branched polymer structure may be different or identical, *i.e.*, are of the same polymer type (structure) and molecular weight.

[0102] A exemplary branched construct used in a branched polymer corresponds to the following structure:



wherein each n is from 113 to about 2050. Within this exemplary branched construct, exemplary branched polymeric reagents can have the following structure, or those with any suitable spacer as described previously:



wherein each n is from 113 to about 2050 and Z is an electrophile-containing reactive group

[0103] Branched polymers suitable for preparing conjugates useful in connection with the present invention also include those represented more generally by the formula

$R(POLY)_y$, where R is a central or core molecule from which extends 2 or more POLY arms such as PEG. The variable y represents the number of POLY arms, where each of the polymer arms can independently be end-capped or alternatively, possess a reactive functional group at its terminus. A more explicit structure in accordance with this embodiment of the invention possesses the structure, $R(POLY\text{-}Z)_y$, where each Z is independently an end-capping group or a reactive group, *e.g.*, suitable for reaction with a therapeutic peptide. In yet a further embodiment when Z is a reactive group, upon reaction with a therapeutic peptide, the resulting linkage can be hydrolytically stable, or alternatively, may be degradable, *i.e.*, hydrolyzable. Typically, at least one polymer arm possesses a terminal functional group suitable for reaction with, *e.g.*, a therapeutic peptide. Branched PEGs such as those represented generally by the formula, $R(PEG)_y$ above possess 2 polymer arms to about 300 polymer arms (*i.e.*, n ranges from 2 to about 300). Preferably, such branched PEGs typically possess from 2 to about 25 polymer arms, such as from 2 to about 20 polymer arms, from 2 to about 15 polymer arms, or from 3 to about 15 polymer arms. Multi-armed polymers include those having 3, 4, 5, 6, 7 or 8 arms.

[0104] Core molecules in branched PEGs as described above include polyols, which are then further functionalized. Such polyols include aliphatic polyols having from 1 to 10 carbon atoms and from 1 to 10 hydroxyl groups, including ethylene glycol, alkane diols, alkyl glycols, alkylidene alkyl diols, alkyl cycloalkane diols, 1,5-decalindiol, 4,8-bis(hydroxymethyl)tricyclodecane, cycloalkylidene diols, dihydroxyalkanes, trihydroxyalkanes, and the like. Cycloaliphatic polyols may also be employed, including straight chained or closed-ring sugars and sugar alcohols, such as mannitol, sorbitol, inositol, xylitol, quebrachitol, threitol, arabitol, erythritol, adonitol, ducitol, facose, ribose, arabinose, xylose, lyxose, rhamnose, galactose, glucose, fructose, sorbose, mannose, pyranose, altrose, talose, tagitose, pyranosides, sucrose, lactose, maltose, and the like. Additional aliphatic polyols include derivatives of glyceraldehyde, glucose, ribose, mannose, galactose, and related stereoisomers. Other core polyols that may be used include crown ether, cyclodextrins, dextrins and other carbohydrates such as starches and amylose. Typical polyols include glycerol, pentaerythritol, sorbitol, and trimethylolpropane.

[0105] Alternatively, the polymer may possess an overall forked structure as described in U.S. Patent No. 6,362,254. This type of polymer is useful for reaction with two therapeutic peptide moieties, where the two therapeutic peptide moieties are positioned a precise or predetermined distance apart.

[0106] In any of the representative structures provided herein, one or more degradable linkages may additionally be contained in the polymer, POLY, to allow generation *in vivo* of a conjugate having a smaller PEG chain than in the initially administered conjugate. Appropriate physiologically cleavable (*i.e.*, releasable) linkages include but are not limited to ester, carbonate ester, carbamate, sulfate, phosphate, acyloxyalkyl ether, acetal, and ketal. Such linkages when contained in a given polymer segment will often be stable upon storage and upon initial administration.

[0107] The PEG polymer used to prepare a conjugate may comprise a pendant PEG molecule having reactive groups, such as carboxyl or amino, covalently attached along the length of the PEG rather than at the end of the PEG chain(s). The pendant reactive groups can be attached to the PEG directly or through a spacer moiety, such as an alkylene group.

[0108] One of ordinary skill in the art can determine the proper molecular size of the water-soluble, non-peptidic polymer. For example, one of ordinary skill in the art, using routine experimentation, can determine a proper molecular size by first preparing a variety of conjugates with different weight-average molecular weights of the polymer and then obtaining the clearance profile for each conjugate by administering the conjugate to a patient and taking periodic blood and/or urine samples. Once a series of clearance profiles has been obtained for each tested conjugate, a conjugate or mixture of conjugates having the desired clearance profile(s) can be determined.

[0109] Those of ordinary skill in the art will recognize that the foregoing discussion describing water-soluble polymers for use in forming a conjugate is by no means exhaustive and is merely illustrative, and that all polymeric materials having the qualities described above are contemplated. As used herein, the term "polymeric reagent" generally refers to an entire molecule, which can comprise a water-soluble polymer segment, as well as additional spacers and functional groups.

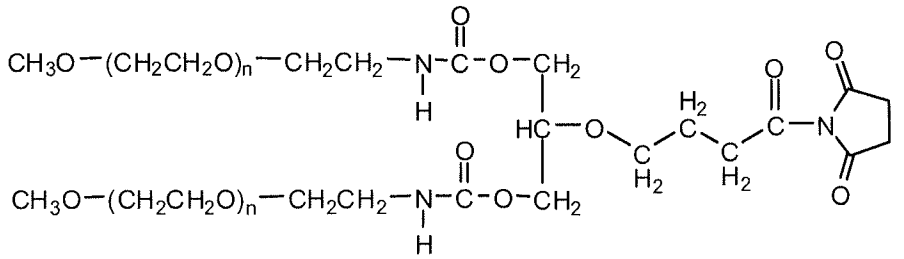
G-CSF Conjugates

[0110] Each conjugate in a composition as provided herein has the water-soluble, non-peptidic, branched polymer covalently attached via an amide-containing linkage to an amino group of the G-CSF moiety. Thus, although the overall linkage between the residue of the G-CSF moiety and the water-soluble, non-peptidic polymer will depend on a number of factors, the linkage will nevertheless contain a amide (*i.e.*, a -NHC(O)- or -C(O)NH-) group.

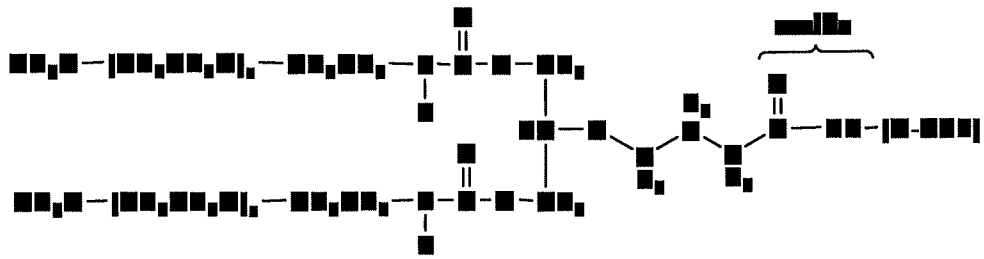
Such factors include, for example, the particular linkage chemistry employed, the particular atoms (if any) surrounding the functional groups effecting the linkage, and so forth. The amide-containing linkage is preferably relatively stable.

[0111] Typically, the nitrogen atom within the amide-containing linkage is contributed by an amine group associated with the G-CSF moiety. When an amine group associated with the G-CSF moiety contributes the nitrogen for the amide-containing, usually it is the amine acting as a nucleophile for an electrophilically activated polymeric reagent (*e.g.*, a water-soluble, non-peptidic polymer bearing an electrophile). In this way, this nitrogen atom effectively becomes the point of attachment for the water-soluble, non-peptidic polymer.

[0112] Exemplary electrophilically activated polymeric reagents include water-soluble, non-peptidic polymers bearing an electrophile selected from the group consisting of acetals, esters (such as succinimidyl esters of carboxylic acids) and carbonates. For example, an electrophilically activated polymeric reagent useful in connection with the present invention is encompassed by the following structure:



wherein each n is from 113 to about 2050. Use of this polymeric reagent, produces the exemplary polymer-G-CSF conjugate of the formula (shown with the location of the amide-containing linkage):



wherein each n is from 113 to about 2050 and (G-CSF) is a residue of a G-CSF moiety.

[0113] Conjugation of a polymeric reagent to a nitrogen atom within a G-CSF moiety can be accomplished by a variety of techniques. In one approach, the G-CSF moiety is

conjugated to a polymeric reagent functionalized with an active ester such as a succinimidyl derivative (*e.g.*, an N-hydroxysuccinimide ester). In this approach, the polymeric reagent bearing the reactive ester is reacted with the G-CSF moiety in aqueous media under appropriate pH conditions, *e.g.*, from pHs ranging from about 3 to about 8, about 3 to about 7, or about 4 to about 6.5. Most polymer active esters can couple to a target peptide such as G-CSF moiety at physiological pH, *e.g.*, at 7.0. However, less reactive derivatives may require a different pH. Typically, activated PEGs can be attached to a peptide such as therapeutic peptide at pHs from about 7.0 to about 10.0 for covalent attachment to an internal lysine. Typically, lower pHs are used, *e.g.*, 4 to about 5.75, for preferential covalent attachment to the N-terminus. Conjugation reactions can often be carried out at room temperature, although lower temperatures may also be used. Reaction times are typically on the order of minutes, *e.g.*, 30 minutes, to hours, *e.g.*, from about 1 to about 36 hours), depending upon the pH and temperature of the reaction. Varying ratios of polymeric reagent to the G-CSF moiety may be employed, *e.g.*, from an equimolar ratio up to a 10-fold molar excess of polymeric reagent. Typically, up to a 5-fold molar excess of polymeric reagent will suffice.

[0114] Progress of the reaction can be monitored by withdrawing aliquots from the reaction mixture at various time points and analyzing the reaction mixture by SDS-PAGE or MALDI-TOF mass spectrometry or any other suitable analytical method. Once a plateau is reached with respect to the amount of conjugate formed or the amount of unconjugated polymer remaining, the reaction is assumed to be complete. The resulting product mixture is preferably, but not necessarily purified, to separate out excess reagents, unconjugated reactants (*e.g.*, G-CSF in unconjugated form) undesired multi-conjugated species, and free or unreacted polymeric reagent. The resulting conjugates can then be further characterized using analytical methods such as MALDI, capillary electrophoresis, gel electrophoresis, and/or chromatography.

[0115] With respect to the specific nitrogen atom contributed by the G-CSF moiety for forming an amide-containing linkage (and which represents the point of attachment for the water-soluble, non-peptidic polymer), the nitrogen atom can be associated with the N-terminal amine of the G-CSF moiety or be associated with the epsilon amine of a lysine residue within the G-CSF moiety.

[0116] It is possible to influence at which nitrogen atom within the G-CSF moiety the polymeric reagent will attach. Techniques to do so include changing pH (to render amines

more or less available for attachment based on pKa), using blocking chemistries (wherein one or more amines can be selectively blocked prior to conjugation with a polymeric reagent, followed by a de-blocking step) and incorporating the water-soluble, non-peptidic polymer during peptide synthesis (see, for example, WO 95/00162). In this regard, reference is made to the Examples section for a description of influencing the location of polymeric reagent attachment.

[0117] Even with the ability to influence the location of attachment, it is seldom the case that the attachment occurs only at an intended location. As a consequence, compositions are formed that result in positional isomers (wherein, among conjugates in the composition, location(s) of attachment change although the number of polymers does not) and numeric isomers (wherein, among conjugates within the composition, the number of polymers may change).

[0118] For polymer-G-CSF conjugates in which it is intended that the N-terminal amine is the most represented location of polymer attachment within a composition, it is preferred that at least 60%, more preferably at least 70%, still more preferably at least 80%, and yet still more preferably at least 90% of all polymer-G-CSF conjugates in the composition have only a single attachment of a water-soluble, non-peptidic polymer attached at the N-terminal amine.

[0119] The amide-containing linkage that serves to link the G-CSF moiety to the water-soluble, non-peptidic polymer can include one or more additional atoms in addition to the amide. The one or more additional atoms making up the amide-containing linkage can include one or more of carbon atoms, nitrogen atoms, sulfur atoms, oxygen atoms, and combinations thereof. Nonlimiting examples of amide-containing linkages or spacers include those selected from the group consisting of -C(O)-NH-, -NH-C(O)-, -NH-C(O)-NH-, -O-C(O)-NH-, -NH-C(O)-O-, -C(O)-NH-CH_2-, -C(O)-NH-CH_2-CH_2-, -CH_2-C(O)-NH-CH_2-, -CH_2-CH_2-C(O)-NH-, -C(O)-NH-CH_2-CH_2-CH_2-, -CH_2-C(O)-NH-CH_2-CH_2-, -CH_2-CH_2-C(O)-NH-CH_2-, -CH_2-CH_2-CH_2-C(O)-NH-, -C(O)-NH-CH_2-CH_2-CH_2-CH_2-, -CH_2-C(O)-NH-CH_2-CH_2-CH_2-, -CH_2-CH_2-C(O)-NH-CH_2-CH_2-, -CH_2-CH_2-CH_2-C(O)-NH-CH_2-, -CH_2-CH_2-CH_2-C(O)-NH-CH_2-CH_2-, -CH_2-CH_2-CH_2-CH_2-C(O)-NH-, -NH-C(O)-CH_2-, -CH_2-NH-C(O)-CH_2-, -CH_2-CH_2-NH-C(O)-CH_2-, -NH-C(O)-CH_2-CH_2-, -CH_2-NH-C(O)-CH_2-CH_2-, -CH_2-CH_2-NH-C(O)-CH_2-CH_2-, -C(O)-NH-CH_2-, -C(O)-NH-CH_2-CH_2-, -O-C(O)-NH-CH_2-, -O-C(O)-NH-CH_2-CH_2-, -CH_2-CH_2-CH_2-C(O)-NH-CH_2-CH_2-NH-,

$-CH_2-CH_2-CH_2-C(O)-NH-CH_2-CH_2-NH-C(O)-$,
$-CH_2-CH_2-CH_2-C(O)-NH-CH_2-CH_2-NH-C(O)-CH_2-$,
$-CH_2-CH_2-CH_2-C(O)-NH-CH_2-CH_2-NH-C(O)-CH_2-CH_2-$,
$-O-C(O)-NH-[CH_2]_h-(OCH_2CH_2)_j-$, (h) is zero to six, and (j) is zero to 20. Other amide-containing linkages have the following structures: $-C(O)-NH-(CH_2)_{1-6}-NH-C(O)-$, $-NH-C(O)-NH-(CH_2)_{1-6}-NH-C(O)-$, and $-O-C(O)-NH-(CH_2)_{1-6}-NH-C(O)-$, wherein the subscript values following each methylene indicate the number of methylenes contained in the structure, *e.g.*, $(CH_2)_{1-6}$ means that the linkage can contain 1, 2, 3, 4, 5 or 6 methylenes. Additionally, any of the above amide-containing linkages may further include an ethylene oxide oligomer chain comprising 1 to 20 ethylene oxide monomer units [*i.e.*, $-(CH_2CH_2O)_{1-20}$]. That is, the ethylene oxide oligomer chain can occur before or after the amide-containing linkage. Also, the oligomer chain would not be considered part of the amide-containing linkage if the oligomer is adjacent to a water-soluble, non-peptidic polymer and merely represents an extension of the polymer.

The polymer-G-CSF conjugates associated with the present invention can be purified to obtain/isolate different conjugate species. Specifically, a product mixture can be purified to obtain the desired numeric isomer. In one embodiment of the invention, the G-CSF conjugates that make up a composition or dosage form of water-soluble polymer-G-CSF conjugates are mono-conjugates. The strategy for purification of a conjugate reaction mixture will depend upon a number of factors, including, for example, the molecular weight of the polymeric reagent employed, the particular G-CSF moiety, and the desired characteristics of the product – *e.g.*, monomer, dimer, particular positional isomers, and so forth.

[0120] If desired, conjugates having different molecular weights can be isolated using gel filtration chromatography and/or ion exchange chromatography. Gel filtration chromatography may be used to fractionate different conjugates (*e.g.*, 1-mer, 2-mer, 3-mer, and so forth, wherein "1-mer" indicates one polymer molecule per G-CSF moeity, "2-mer" indicates two polymers attached to the G-CSF moiety, and so on) on the basis of their differing molecular weights (where the difference corresponds essentially to the average molecular weight of the water-soluble, non-peptidic polymer). While this approach can be used to separate PEG and other water-soluble, non-peptidic polymer conjugates having different molecular weights, this approach is generally ineffective for separating positional isomers having different polymer attachment sites within the G-CSF moiety. For example,

gel filtration chromatography can be used to separate from each other mixtures of PEG 1-mers, 2-mers, 3-mers, and so forth, although each of the recovered PEG-mer compositions may contain PEGs attached to different reactive amino groups (*e.g.*, lysine residues) or other functional groups of the therapeutic peptide.

[0121] Gel filtration columns suitable for carrying out this type of separation include Superdex™ and Sephadex™ columns available from Amersham Biosciences (Piscataway, NJ). Selection of a particular column will depend upon the desired fractionation range desired. Elution is generally carried out using a suitable buffer, such as phosphate, acetate, or the like. The collected fractions may be analyzed by a number of different methods, for example, (i) optical density (OD) at 280 nm for protein content, (ii) bovine serum albumin (BSA) protein analysis, (iii) iodine testing for PEG content (Sims *et al.* (1980) *Anal. Biochem*, 107:60-63), and (iv) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), followed by staining with barium iodide.

[0122] Separation of positional isomers is typically carried out by reverse phase chromatography using a reverse phase-high performance liquid chromatography (RP-HPLC) C18 column (Amersham Biosciences or Vydac) or by ion exchange chromatography using an ion exchange column, *e.g.*, a DEAE- or CM-Sepharose™ ion exchange column available from Amersham Biosciences. Either approach can be used to separate polymer-therapeutic peptide isomers having the same molecular weight (positional isomers).

[0123] The resulting purified compositions are preferably substantially free of the non-conjugated G-CSF moiety. In addition, the compositions preferably are substantially free of all other non-covalently attached water-soluble, non-peptidic polymers. Further, the compositions are preferably substantially free of albumin.

[0124] The preparation of exemplary branched water-soluble polymer conjugates of G-CSF is provided in Example 1. G-CSF conjugate compositions comprising conjugates designated "mPEG2-ru-40K-GCSF" are described therein, where the compositions substantially comprise mono-G-CSF conjugates. Illustrative compositions were prepared such that each composition comprised substantially a single mono-conjugate positional isomer (compositions A, B, and C) or an approximately equal mixture of each of the three positional isomers.

[0125] The illustrative G-CSF conjugate compositions were then evaluated in an *in-vitro* cell proliferation assay using an M-NFS cell line as described in detail in Example 2.

As can be seen, each of the mono-conjugate compositions retained significant bioactivity in the in-vitro bioactivity model, with bioactivities ranging from about 40% to nearly 90% relative to pegfilgrastim (Neulasta®). Such bioactivities were completely unexpected, based upon the large molecular weight of the polymer, its overall branched architecture, and electronic configuration.

[0126] Moreover, in an *in-vivo* study in neutropenic mice as described in Example 3, illustrative G-CSF conjugate compositions were found to possess greater potency (bioactivity) and a different bioactivity profile in the hours post-dosing than the commercial filgrastim product, Neulasta®. A striking difference in bioactivity is particularly notable at 96 hours post-dosing. See FIG. 3. These results were particularly unexpected, considering the differences between Neulasta® (linear 20 kD PEG) and the representative conjugate, mPEG2-ru-40K-GCSF (branched 40 kD PEG). Such differences would generally lead one skilled in the art to expect a significantly lower bioactivity for the mPEG2-ru-40K-GCSF conjugate relative to pegfilgrastim, however, just the opposite was observed.

Pharmaceutical Compositions

[0127] With respect to pharmaceutical compositions, the pharmaceutical composition will typically satisfy one or more of the following characteristics: at least about 85% of the conjugates in the composition will have one branched polymer attached to the G-CSF moiety; at least about 95% of the conjugates in the composition will possess one branched polymer attached to the G-CSF moiety; and at least about 99% of the conjugates in the composition will have one branched polymer attached to the G-CSF moiety.

[0128] Moreover, with respect to pharmaceutical excipients, the pharmaceutical composition of the invention may contain only one pharmaceutical excipient or the pharmaceutical composition may contain more than one pharmaceutical excipient. The specific pharmaceutical excipient(s) included in the composition can vary and is influenced by the particular needs of the formulation and route of administration.

[0129] The pharmaceutical compositions of the invention encompass all types of formulations and in particular those that are suited for injection, *e.g.*, powders or lyophilates that can be reconstituted as well as liquids, as well as for inhalation. Examples of suitable diluents for reconstituting solid compositions prior to injection include bacteriostatic endotoxin-free water for injection, dextrose 5% in water, phosphate-buffered saline, Ringer's

solution, saline, sterile water, deionized water, and combinations thereof. With respect to liquid pharmaceutical compositions, solutions and suspensions are envisioned.

[0130] Exemplary pharmaceutically acceptable excipients include, without limitation, carbohydrates, inorganic salts, antimicrobial agents, antioxidants, surfactants, buffers, acids, bases, and combinations thereof.

[0131] Representative carbohydrates for use in the compositions of the present invention include sugars, derivatized sugars such as alditols, aldonic acids, esterified sugars, and sugar polymers. Exemplary carbohydrate excipients suitable for use in the present invention include, for example, monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol), pyranosyl sorbitol, myoinositol and the like.

[0132] Exemplary protein excipients include albumins such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, hemoglobin, and the like. The compositions may also include a buffer or a pH-adjusting agent, typically but not necessarily a salt prepared from an organic acid or base. Representative buffers include organic acid salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid, succinic acid, acetic acid, or phthalic acid. Other suitable buffers include Tris, tromethamine hydrochloride, borate, glycerol phosphate, and phosphate. Amino acids such as glycine are also suitable.

[0133] The pharmaceutical compositions of the present invention may also include one or more additional polymeric excipients/additives, *e.g.*, polyvinylpyrrolidones, derivatized celluloses such as hydroxymethylcellulose, hydroxyethylcellulose, and hydroxypropylmethylcellulose, FICOLLs (a polymeric sugar), hydroxyethylstarch (HES), dextrates (*e.g.*, cyclodextrins, such as 2-hydroxypropyl-β-cyclodextrin and sulfobutylether-β-cyclodextrin), polyethylene glycols, and pectin.

[0134] The pharmaceutical compositions may further include flavoring agents, taste-masking agents, inorganic salts (*e.g.*, sodium chloride), antimicrobial agents (*e.g.*, benzalkonium chloride), sweeteners, antioxidants, antistatic agents, surfactants (*e.g.*, polysorbates such as "TWEEN 20" and "TWEEN 80," and pluronics such as F68 and F88, available from BASF), sorbitan esters, lipids (*e.g.*, phospholipids such as lecithin and other phosphatidylcholines, phosphatidylethanolamines, although preferably not in liposomal

form), fatty acids and fatty esters, steroids (*e.g.*, cholesterol), and chelating agents (*e.g.*, zinc and other such suitable cations). The use of certain di-substituted phosphatidylcholines for producing perforated microstructures (*i.e.*, hollow, porous microspheres) may also be employed.

[0135] Other pharmaceutical excipients and/or additives suitable for use in the compositions according to the present invention are listed in "Remington: The Science & Practice of Pharmacy," 21st ed., Williams & Williams, (2005), and in the "Physician's Desk Reference," 60th ed., Medical Economics, Montvale, N.J. (2006).

[0136] The dose of the polymer-G-CSF conjugate in a pharmaceutical composition (typically present as a pharmaceutical composition having a single dose) is an amount that achieves a neutrophil stimulatory effect, which dose is less (in terms of amount based upon the G-CSF moiety) than the dose of the G-CSF moiety in unconjugated form that would be required to achieve the same neutrophil-lowering effect. In addition, a pharmaceutical preparation, if in solution form, can be housed in a syringe.

[0137] The amount of any individual excipient in the composition will vary depending on the activity of the excipient and particular needs of the composition. Typically, the optimal amount of any individual excipient is determined through routine experimentation, *i.e.*, by preparing compositions containing varying amounts of the excipient (ranging from low to high), examining the stability and other parameters, and then determining the range at which optimal performance is attained with no significant adverse effects.

[0138] Generally, however, the excipient or excipients will be present in the composition in an amount of about 1% to about 99% by weight, from about 5% to about 98% by weight, from about 15 to about 95% by weight of the excipient, or with concentrations less than 30% by weight. In general, a high concentration of the therapeutic peptide is desired in the final pharmaceutical formulation.

Administration

[0139] The pharmaceutical compositions described herein can be administered by any of a number of routes including without limitation, oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal), intrathecal, and pulmonary. A preferred forms

of administration is parenteral administration. Suitable formulation types for parenteral administration include ready-for-injection solutions, dry powders for combination with a solvent prior to use, suspensions ready for injection, dry insoluble compositions for combination with a vehicle prior to use, and emulsions and liquid concentrates for dilution prior to administration, among others.

[0140] In one or more embodiments of the invention, a method is provided, the method comprising delivering a G-CSF water-soluble polymer conjugate as provided herein to a patient, the method comprising the step of administering to the patient a pharmaceutical composition as provided herein. Administration can be effected by any of the routes herein described. The method may be used to treat a mammal suffering from a low white blood cell count.

[0141] Also provided herein is a method for administering a G-CSF conjugate as provided herein to a patient suffering from a condition that is responsive to treatment with the conjugate such as neutropenia. Generally, a G-CSF conjugate composition as described herein may be administered to a patient undergoing certain types of cancer treatment, to boost the patient's white blood cell count, i.e., for the treatment of neutropenia. A G-CSF conjugate composition as provided herein may be administered to treat neutropenia resulting from any of a number of causes, such as bone marrow transplant, HIV, drug-induced neutropenia, and the like. The method comprises administering, generally via injection, a therapeutically effective amount of the G-CSF conjugate (preferably provided as part of a pharmaceutical preparation). The method of administering may be used to treat any condition that can be remedied or prevented by administration of the G-CSF moiety. For example, the G-CSF conjugates provided herein can suitably be administered to patients with most types of cancer undergoing moderately myelosuppressive chemotherapy to help protect the patients from infection which can result form low white blood cell counts.

[0142] The actual dose of the conjugate to be administered will vary depending upon the age, weight, and general condition of the subject as well as the severity of the condition being treated, the judgment of the health care professional, and conjugate being administered. On a weight basis, a therapeutically effective dosage amount of a therapeutic peptide conjugate as described herein will range from about 0.001 mg per day to about 1000 mg per day for an adult. For example, dosages may range from about 0.1 mg per day to about 100 mg per day, or from about 1.0 mg per day to about 10 mg/day. On an activity basis,

corresponding doses based on international units of activity can be calculated by one of ordinary skill in the art.

[0143] Generally, a therapeutically effective amount will range from about 0.001 mg to 100 mg, preferably in doses from 0.01 mg/day to 75 mg/day, and more preferably in doses from 0.10 mg/day to 50 mg/day. For instance, a therapeutically effective amount of a G-CSF conjugate composition as provided herein may range from an amount that averages to about 0.50 microgram/kg/day to about 20 microgram/kg/day, e.g., may be about 0.50 microgram/kg/day, 1.0 microgram/kg/day, 2.0 microgram/kg/day, 3.0 microgram/kg/day, 4.0 microgram/kg/day, 5.0 microgram/kg/day, 6.0 microgram/kg/day, 7.0 microgram/kg/day, 8.0 microgram/kg/day, 9.0 microgram/kg/day, 10.0 microgram/kg/day, 11.0 microgram/kg/day, 12 microgram/kg/day, 13 microgram/kg/day, 14 microgram/kg/day, 15 microgram/kg/day, 16 microgram/kg/day, 17 microgram/kg/day, 18 microgram/kg/day, 19 microgram/kg/day, or 20 microgram/kg/day, depending upon the factors noted above, although the actual dosing regimen is not necessarily daily.

[0144] The unit dosage of any given conjugate (again, preferably provided as part of a pharmaceutical preparation) can be administered in a variety of dosing schedules depending on the judgment of the clinician, needs of the patient, and so forth. The specific dosing schedule will be known by those of ordinary skill in the art or can be determined experimentally using routine methods. Exemplary dosing schedules include, without limitation, administration five times a day, four times a day, three times a day, twice daily, once daily, three times weekly, twice weekly, once weekly, twice monthly, once monthly, and any combination thereof. For example, dosing may commence at a certain time point following a round of chemotherapy, e.g., within 24 hours, within 48 hours, within 36 hours, and so on. Once the clinical endpoint has been achieved, dosing of the composition is halted.

[0145] It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, the foregoing description as well as the examples that follow are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

[0146] All articles, books, patents and other publications referenced herein are hereby incorporated by reference in their entireties.

EXAMPLES

[0147] The practice of the invention will employ, unless otherwise indicated, conventional techniques of organic synthesis, biochemistry, protein purification and the like, which are within the skill of the art. Such techniques are fully explained in the literature. See, for example, J. March, Advanced Organic Chemistry: Reactions Mechanisms and Structure, 4th Ed. (New York: Wiley-Interscience, 1992), *supra*.

[0148] In the following examples, efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.) but some experimental error and deviation should be taken into account. Unless indicated otherwise, temperature is in degrees °C and pressure is at or near atmospheric pressure at sea level. Each of the following examples is considered to be instructive to one of ordinary skill in the art for carrying out one or more of the embodiments described herein.

[0149] Recombinant human G-CSF ("rhG-CSF") used in the examples was obtained from a commercial source.

[0150] SEC-HPLC Analysis: Size exclusion chromatography (SEC-HPLC) analysis was performed on an Agilent 1100 HPLC system (Agilent). Samples were analyzed using a TSK-GEL G3000SWxl column (7.8 x 300 mm, Phenomenex), and a mobile phase consisting of 100 mM phosphoric acid, pH 2.5. The flow rate for the column was 0.5 ml/min. Eluted protein and PEG-protein conjugates were detected using UV at 227 nm and 280 nm.

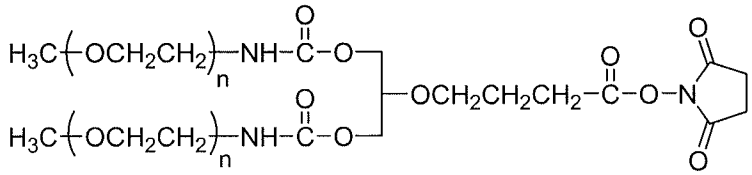
[0151] RP-HPLC Analysis: Reversed phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1100 HPLC system (Agilent). Samples were analyzed using a Zorbax 300SB-C3 column (3.5 μm particle size, 150 mm x 3.0 mm, Agilent), and mobile phases consisting of 0.1% trifluoroacetic acid in water (buffer A) and 0.1% trifluoroacetic acid in acetonitrile (buffer B). The flow rate for the column was 0.3 ml/min. The protein and PEG-protein conjugates were eluted with a linear gradient over 25 minutes, and were detected using UV at 280nm.

[0152] Cation Exchange Chromatography: A HiTrap SP Sepharose HP cation exchange column (GE Healthcare, Piscataway NJ) was used with the AKTA purification system (GE Healthcare, Piscatway NJ) to purify the PEG-G-CSF conjugates prepared. For each conjugate solution prepared, the conjugate solution was loaded on a column that was pre-equilibrated in 20 mM NaOAc buffer, pH 4.0 (buffer A) and then washed with a few column volumes of buffer A to remove any unreacted PEG reagent. Subsequently, a gradient

of buffer A with 0-100% buffer B (20mM MaOAc with 0.5 M NaCl buffer, pH 4.0) was raised. The eluent was monitored by UV detector at 280 nm. The di-PEG-G-CSF conjugates were eluted first, followed by mono-PEG-G-CSF positional isomers, and finally the unconjugated G-CSF. The fractions were pooled according to the chromatogram, and the purity of the individual conjugate was determined by HPLC or SDS-PAGE.

Example 1

PEGylation of G-CSF with Branched mPEG-N-Hydroxysuccinimide Derivative, 40kDa



Branched mPEG-N-Hydroxysuccinimide Derivative, 40kDa, ("mPEG2-ru-40K-NHS")

[0153] mPEG2-ru-40K-NHS, stored at -20°C under argon, was warmed to ambient temperature. Excess PEG reagent (relative to the amount of G-CSF in a measured aliquot of the stock G-CSF solution) of the warmed mPEG2-ru-40K-NHS was dissolved in 2mM HCl to form a 10% reagent solution. The 10% reagent solution was quickly added to the aliquot of stock GCSF solution (4 mg/mL buffer solution, pH 7-9) and mixed well. To allow for coupling of the mPEG2-ru-40K-NHS to G-CSF via an amide-containing linkage, the reaction solution was kept at room temperature first, and then 4°C. The reaction was quenched with acetic acid to lower the pH to 4.0.

[0154] A cation-exchange chromatography method using SP Sepharose High Performance and NaOAc buffer was used to purify the conjugate solution such that mono-conjugates (*i.e.*, a branched polymer attached a single location with the G-CSF) were collected (and substantially lacking mPEG2-ru-40K-NHS, di-conjugates and unconjugated G-CSF). In this regard, **FIG. 1** shows the chromatogram produced during cation-exchange chromatography. Collection of individual fractions occurred such that four compositions resulted: a "mono-a" composition, a "mono-b" composition, a "mono-c" compositions, and a composition of each of "mona-a," mono-b, and "mono-c" (corresponding to "mPEG2-ru-40K-GCSF-A," "mPEG2-ru-40K-GCSF-B," "mPEG2-ru-40K-GCSF-C" and "mPEG2-ru-40K-GCSF-M," respectively). These compositions were then subjected to further analyses.

Example 2

In vitro Cell Proliferation Assays

[0155] *In vitro* cell proliferation assays in an M-NFS-60 cell line were performed to determine relative activities between the three positional isomers of the conjugates of Example 1. Briefly, the M-NFS-60 (mouse myeloid leukemia) cell line responds to growth factors wherein activity is based on the stimulatory effect of a test article via presence of ATP, which signals the presence of metabolically active cells (Promega's CEllTiter-Glo® kit). At the initiation of the study, about 4,000 cells (~90 μL) and test article (in ~10 μL) at eight different concentrations are each incubated at 37° C with 5% CO_2 conditions for 72 hours. At 72 hours, CellTiter-Glo® reagent (100 μL) was added to each sample followed by mixing for two minutes. Following incubation for ten minutes at room temperature, proliferation was read with a luminescence counter for determination of EC50 values.

[0156] Table 1 lists the potency at 72 hours of the individual positional isomers and mixture prepared in connection with Example 1 compared to commercial pegfilgrastim (sold under the NEUPOGEN® brand from Amgen Inc., Thousand Oaks, CA)

Table 1

Relative Potency of Tested G-CSF Preparations

Compound	EC50 (ng/mL) @72 h	Relative potency (%) @72 h
filgrastim (rhGCSF)	0.0148	-
pegfilgrastim	0.07732	100
mPEG2-ru-40K-GCSF-M	0.1491	52
mPEG2-ru-40K-GCSF-A	0.0888	87
mPEG2-ru-40K-GCSF-B	0.1366	57
mPEG2-ru-40K-GCSF-C	0.1996	39

[0157] Graphical representation of the same data is shown in **FIG. 2**.

Example 3

Neutrophil Recovery in Neutropenic Mice

[0158] The ability of test articles to stimulate the production of neutrophils was measured by administering the test article to neutropenic mice and counting the number of neutrophils at various time points.

[0159] Mice (Charles River, CD1, females) were provided a standard diet and allowed to acclimatize for one week prior to initiation of the study. Animals are separately grouped based on the test article being administered, wherein animals were further grouped based on whether they received 100, 300 or 1000 μg/kg of test article.

[0160] Animals were made neutropenic by intraperitoneal administration of cyclophosphamide on days -4 and -1 (150 mg/kg and 100mg/kg, respectively). At time 0 on day 1 (prior to dosing with test article) and at times 4, 12, 24, 36, 48, 60, 72, 96 and 144 hours, up to six animals from each group were sacrificed and the hematology panel run on the sample and the mean neutrophil values from sacrified animals for each time point reported.

[0161] Tables 2A and 2B show the mean neutrophil counts of various doses of Neulasta® pegfilgrastim and mPEG2-ru-40K-GCSF-A, respectively. **FIG. 3** provides the mean neutrophil counts for at time points 48, 60, 72, 96 and 144 hours of Neulasta® pegfilgrastim and mPEG-2-ru-40K-GCSF-A, each at the 1000 μg/kg dose.

Table 2A

Mean Neutrophil Counts of Various Doses of Neulasta® pegfilgrastim

Time (hr)	**Mean Neutrophil Count (in 10^3/μL) of 100 μg/kg of Neulasta® pegfilgrastim**	**Mean Neutrophil Count (in 10^3/μL) of 300 μg/kg of Neulasta® pegfilgrastim**	**Mean Neutrophil Count (in 10^3/μL) of 1000 μg/kg of Neulasta® pegfilgrastim**
0	0.143	0.19	0.087
4	0.233	0.228	0.268
12	0.404	0.136	0.052
24	0.183	0.162	0.243
36	0.118	0.532	1.082
48	2.063	1.624	2.205
60	6.825	16.56	NA
72	NA	18.253	15.25
96	13.007	8.95	9.56
144	1.67	1.258	3.42

Table 2B

Mean Neutrophil Counts of Various Doses of mPEG-2-ru-40K-GCSF-A

Time (hr)	**Mean Neutrophil Count (in $10^3/\mu L$) of 100 µg/kg of** mPEG-2-ru-40K-GCSF-A	**Mean Neutrophil Count (in $10^3/\mu L$) of 300 µg/kg of** mPEG-2-ru-40K-GCSF-A	**Mean Neutrophil Count (in $10^3/\mu L$) of 1000 µg/kg of** mPEG-2-ru-40K-GCSF-A
0	0.117	0.052	0.082
4	0.19	0.202	0.275
12	0.158	0.097	0.095
24	0.192	0.148	0.157
36	0.317	0.388	1.674
48	0.96	0.957	1.527
60	4.86	8.205	8.567
72	9.258	13.167	15.8
96	7.928	8.937	25.59
144	4.026	4.818	7.406

The results provided above and shown in FIG. 3 further illustrate the surprising and advantageous nature of the G-CSF conjugates and conjugate mixtures provided herein.

WHAT IS CLAIMED IS:

1. A composition comprising G-CSF-polymer conjugates, each comprised of the same G-CSF moiety and the same polymer covalently attached thereto, wherein each conjugate comprises a G-CSF moiety stably covalently attached via an amide linkage to a branched, non-peptidic water-soluble polymer having a weight average molecular weight in a range from about 10,000 kiloDaltons to about 100,000 kiloDaltons, where (i) the G-CSF moiety is attached to the branched water-soluble polymer at only one or two of its amino sites selected from internal lysines and the N-terminus, and (ii) intervening between the branch point in the water-soluble polymer and the amide linkage to the G-CSF moiety is a linear spacer comprising from 3 to 10 atoms.

2. The composition of claim 1, wherein the linear spacer comprises 3, 4, or 5 atoms intervening between the branch point of the water-soluble polymer and the amide linkage.

3. The composition of claim 1, wherein the linear spacer corresponds to $-O-(CH_2)_{2-6}-$.

4. The composition of any one of claims 1-3, substantially comprising conjugates having the G-CSF moiety attached to a single branched water-soluble polymer (mono-conjugates) at an internal lysine or N-terminal site.

5. The composition of claim 4, wherein the G-CSF moiety is attached to the single branched water-soluble polymer at its N-terminal site.

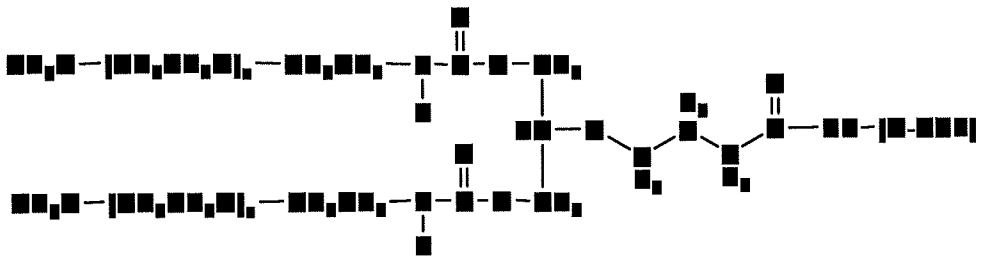
6. The composition of claim 4, wherein the G-CSF moiety is attached to the single branched water-soluble polymer at an ε-amino group of an internal lysine site.

7. The composition of any one of claims 1 to 6, wherein the weight average molecular weight of the branched water-soluble polymer is in a range of from about 15,000 to 50,000 kiloDaltons.

8. The composition of claim 7, wherein the weight average molecular weight of the branched water-soluble polymer is about 20,000 to about 40,000 kiloDaltons.

9. The composition of any one of claims 1-8, wherein the water-soluble polymer is a polyethylene glycol.

10. The composition of any one of claims 1 to 9, wherein the G-CSF-polymer conjugates possess a structure:



wherein each n is from 113 to about 2050, and

(G-CSF) represents the G-CSF moiety where NH-G-CSF represents the amino group on the G-CSF moiety to which the branched water-soluble polymer is attached.

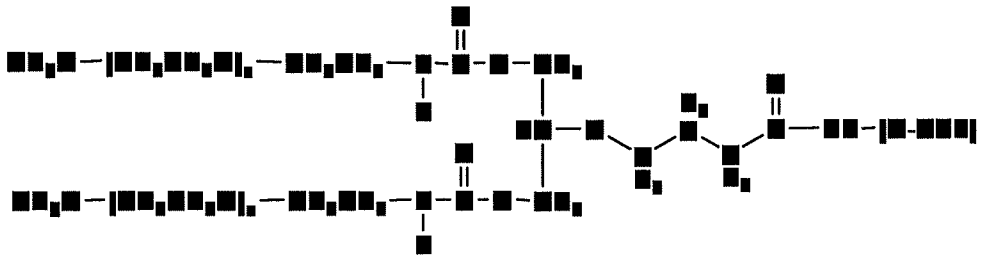
11. The composition of claim 10, substantially comprising conjugates having the G-CSF moiety attached to a single branched water-soluble polymer (mono-conjugates) at an internal lysine or N-terminal site, the composition substantially comprising a single positional isomer of the G-CSF mono-conjugates.

12. The composition of claim 10 or claim 11, wherein the G-CSF moiety is selected from native human G-CSF and recombinant human G-CSF.

13. The composition of claim 12, wherein the G-CSF moiety is full length recombinant human G-CSF that is attached to the branched water-soluble polymer at an amino acid site selected from K17, K24, K35, K41 and the N-terminus.

14. A pharmaceutical composition of any one of claims 1-13, further comprising a pharmaceutically acceptable excipient.

15. A pharmaceutical composition of claim 14, wherein the G-CSF-polymer conjugates substantially comprise a single positional isomer of a mono-conjugate possessing the structure:



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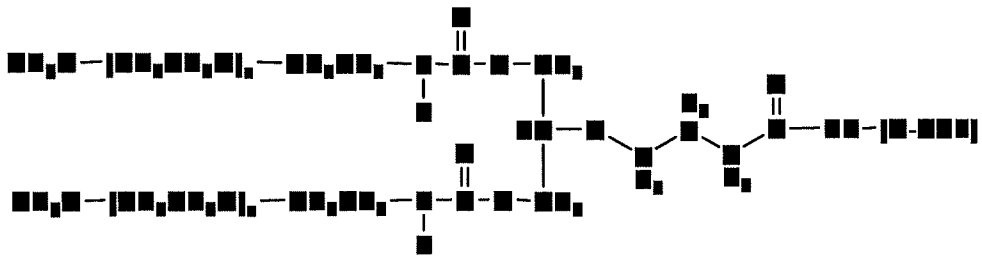
wherein the monoconjugates have a weight average molecular weight of the branched water-soluble polymer of about 40,000 kiloDaltons and are characterized by having an EC50 value in an *in vitro* cell proliferation assay using an M-NFS-60 cell line as described in Example 2 at 72 hours of about 0.09 ng/mL.

16. A pharmaceutical composition of claim 14 comprising a dosage amount of G-CSF-polymer conjugates substantially comprising a single positional isomer of a mono-conjugate possessing the structure:



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wherein the monoconjugates have a weight average molecular weight of from about 15,000 to 50,000 kiloDaltons, and the pharmaceutical composition, when administered to a mammal, (i) has a neutrophil stimulatory effect, and (ii) the dosage amount of the G-CSF conjugates (based upon G-CSF moiety content) to achieve the neutrophil stimulatory effect is less than an amount of the G-CSF moiety in an unconjugated form needed to achieve the same neutrophil stimulatory effect.

17. A composition of any one of claims 1 to 16 for use in administration to a human or animal subject.

18. The composition of claim 17 for use in administration to a human subject for treatment of neutropenia.

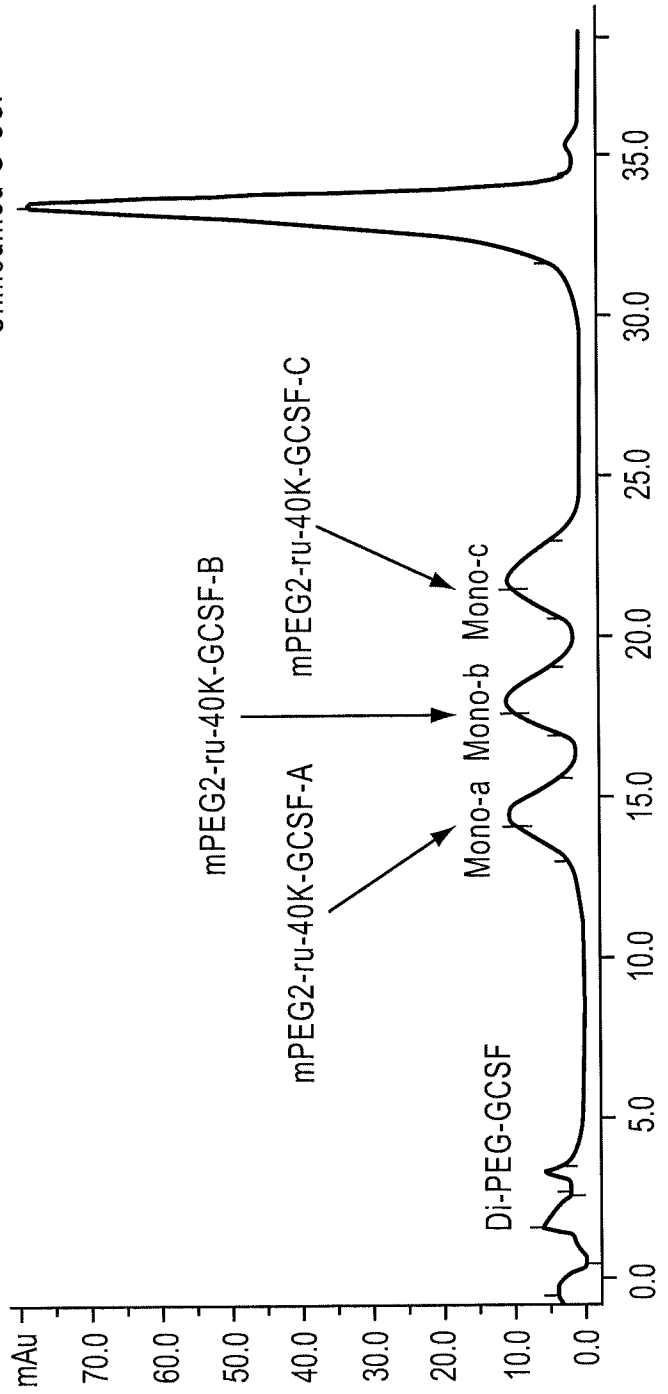
Unmodified G-CSF
mPEG2-ru-40K-GCSF-B
mPEG2-ru-40K-GCSF-C
mPEG2-ru-40K-GCSF-A
Mono-a Mono-b Mono-c
Di-PEG-GCSF
mAu
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0

FIG. 1

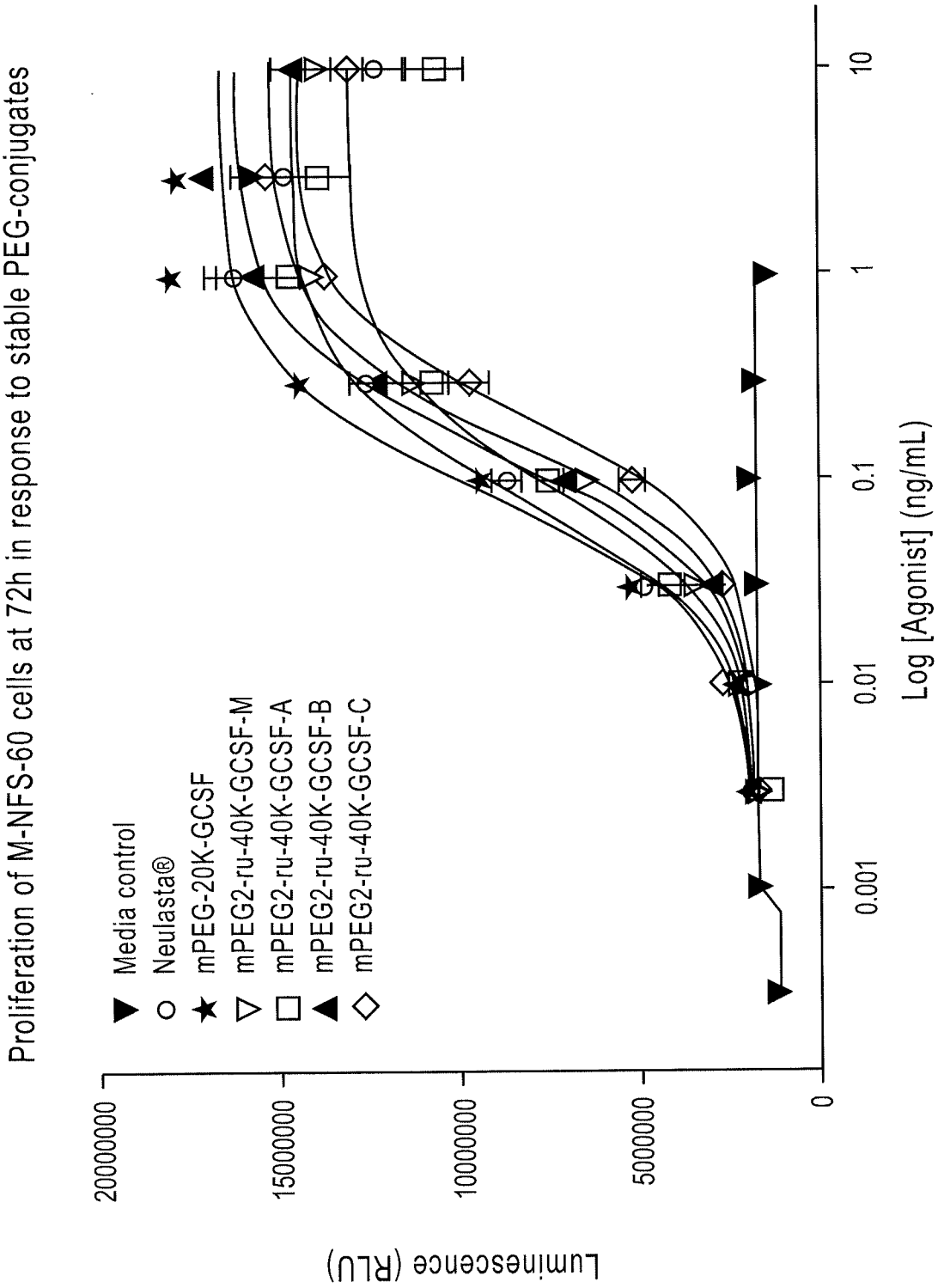
Proliferation of M-NFS-60 cells at 72h in response to stable PEG-conjugates
Media control
Neulasta®
mPEG-20K-GCSF
mPEG2-ru-40K-GCSF-M
mPEG2-ru-40K-GCSF-A
mPEG2-ru-40K-GCSF-B
mPEG2-ru-40K-GCSF-C
Luminescence (RLU)
20000000
15000000
10000000
5000000
0
0.001
0.01
0.1
1
10
Log [Agonist] (ng/mL)

FIG. 2

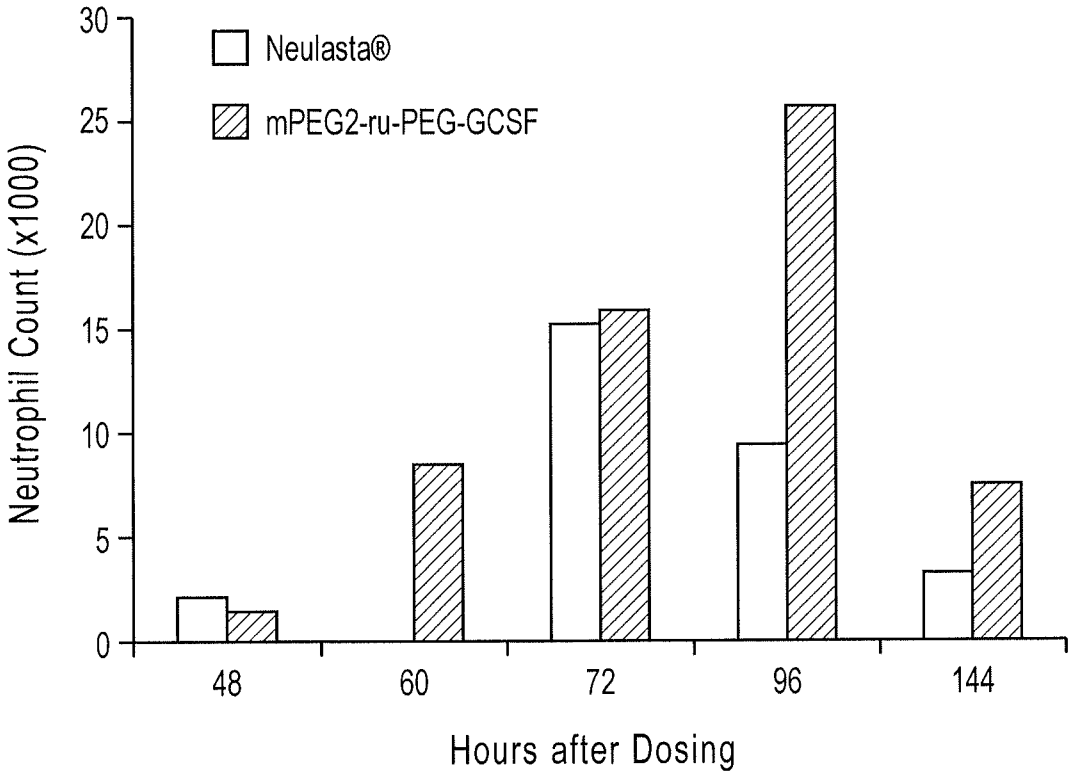
30
25
20
15
10
5
0
Neutrophil Count (x1000)
Neulasta®
mPEG2-ru-PEG-GCSF
48
60
72
96
144
Hours after Dosing

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/060016

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 14/53 (2012.01)
USPC - 435/68.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C07K 14/53; C12P 21/06 (2012.01)
USPC - 435/68.1, 69.5, 69.7

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent, Google Scholar, Google Patents

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2007/019331 A2 (BOSSARD et al) 15 February 2007 (15.02.2007) entire document	1-6
Y	US 7,306,931 B2 (ROSENDAHL et al) 11 December 2007 (11.12.2007) entire document	1-6

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 February 2012	15 MAR 2012

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2011/060016

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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

3. ☒ Claims Nos.: 7-18
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.