THERAPEUTIC USE OF PROTEIN-POLYMER CONJUGATES

CROSS-REFERENCE TO RELATED APPLICATION PARAGRAPH

This application claims the benefit of U.S. Provisional Application No. 61/285,411 filed on December 10, 2009, the content of which is hereby incorporated by reference in its entirety.

BACKGROUND

Advance in cell biology and recombinant protein technologies has led to the development of protein therapeutics.

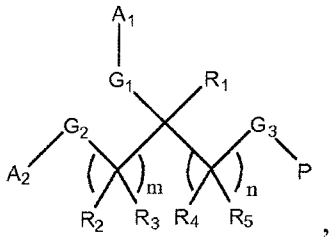
Yet, major hurdles still exist. Most proteins are susceptible to proteolytic degradation and therefore have a short half-life in the circulating system. Other disadvantages include low water solubility and inducement of neutralizing antibodies.

Attachment of a polymer, e.g., polyethylene glycol (PEG), to a protein hinders access of proteolytic enzymes to the protein backbone, resulting in enhanced protein stability. In addition, it also improves water solubility and minimizes immuogenicity. There is a need for effective methods of attaching a polymer to a protein.

SUMMARY

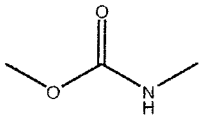
An aspect of this invention relates to use a protein-polymer conjugate to treat various diseases. The conjugate contains at least one polymer moiety, an interferon-α moiety, and a linker. In the conjugate, the total molecular weight is 2-200 kD (preferably 40 kD) and the number of polymer moieties in the conjugate is not more than 10. The polymer moiety or moieties are attached to the linker; the nitrogen atom of the N-terminus of the interferon-α moiety is bonded to the linker; and the linker is a covalent bond, C_{1-10} alkylene, C_{2-10} alkenylene, or C_{2-10} alkynylene. Preferably, the conjugate is substantially pure, e.g., having a purity of more than 70%, 80%, or 90%. The diseases that can be treated by the conjugate include multiple sclerosis, chronic viral infection (such as hepatitis B virus infection, hepatitis C virus infection, and human papilloma virus infection), cancer, idiopathic myelofibrosis, polycythaemia vera, and essential thromobocythaemia.

Another aspect of the present invention relates to use of a protein-polymer conjugate of formula I shown below to treat the above-mentioned diseases:



formula I

in which each of R_1, R_2, R_3, R_4, and R_5, independently, is H, C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, aryl, heteraryl, C_{3-8} cycloalkyl, or C_{3-8} heterocycloalkyl; each of A_1 and A_2, independently, is a polymer moiety; each of G_1, G_2, and G_3, independently, is a bond or a linking functional group; P is an interferon-α moiety; m is 0 or an integer of 1-10; and n is an integer of 1-10.

Referring to the above formula, the protein-polymer conjugate may have one or more of the following features: G_3 is a bond and P is an interferon-α moiety in which the amino group at the N-terminus is attached to G_3; A_1 and A_2 are polyalkylene oxide moieties having a molecular weight of 2-100 kD (preferably 10-30 kD), each of G_1 and G_2 is  (in which O is attached to A_1 or A_2, and NH is attached to a carbon atom as shown in formula I), or each of G_1 and G_2 is urea, sulfonamide, or amide, (in which N is attached to a carbon atom as shown in formula I); m is 4, n is 2, and each of R_1, R_2, R_3, R_4, and R_5 is H; and P is a modified interferon-α moiety containing 1-4 additional amino acid residues.

The term "alkyl" refers to a mono-valent straight-chained or branched hydrocarbon radical. Examples of alkyl groups include methyl, ethyl, n-propyl, isopropyl, *tert*-butyl, and n-pentyl. Similarly, the term "alkenyl" or "alkynyl" refers to a mono-valent straight-chained or branched hydrocarbon radical containing one or more C=C double bonds or one or more C≡C triple bonds.

The term "alkylene" refers to a bi-valent straight-chained or branched hydrocarbon radical. Similarly, the term "alkenylene" or "alkynylene" refers to a bi-valent straight-chained or branched hydrocarbon radical containing one or more C=C double bonds or one or more C≡C triple bonds.

The term "aryl" refers to a hydrocarbon ring system (mono-cyclic or bi-cyclic) having at least one aromatic ring. Examples of aryl moieties include, but are not limited to, phenyl, naphthyl, and pyrenyl.

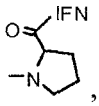
The term "heteroaryl" refers to a hydrocarbon ring system (mono-cyclic or bi-cyclic) having at least one aromatic ring which contains at least one heteroatom such as O, N, or S as part of the ring system and the reminder being carbon. Examples of heteroaryl moieties include, but are not limited to, furyl, pyrrolyl, thienyl, oxazolyl, imidazolyl, thiazolyl, pyridinyl, pyrimidinyl, quinazolinyl, and indolyl.

The term "cycloalkyl" refers to a partially or fully saturated mono-cyclic or bi-cyclic ring system having only carbon ring atoms. Examples include, but are not limited to, cyclopropanyl, cyclopentanyl, and cyclohexanyl.

The term "heterocycloalkyl" refers to a partially or fully saturated mono-cyclic or bi-cyclic ring system having, in addition to carbon, one or more heteroatoms (e.g., O, N, or S), as ring atoms. Examples include, but are not limited to, piperidine, piperazine, morpholine, thiomorpholine, and 1,4-oxazepane.

Alkyl, alkenyl, alkynyl, aryl, heteroaryl, cycloalkyl, and heterocycloalkyl mentioned herein include both substituted and unsubstituted moieties. Examples of substituents include C_1–C_{10} alkyl, C_2–C_{10} alkenyl, C_2–C_{10} alkynyl, C_3–C_8 cycloalkyl, C_5–C_8 cycloalkenyl, C_1–C_{10} alkoxy, aryl, aryloxy, heteroaryl, heteroaryloxy, amino, C_1–C_{10} alkylamino, C_1–C_{20} dialkylamino, arylamino, diarylamino, hydroxyamino, alkoxyamino, C_1–C_{10} alkylsulfonamide, arylsulfonamide, hydroxy, halogen, thio, C_1–C_{10} alkylthio, arylthio, cyano, nitro, acyl, acyloxy, carboxyl, and carboxylic ester.

The term "polyalkylene oxide moiety" refers to a mono-valent radical derived from linear, branched, or star–shaped polyalkylene oxide. The molecular weight of a polyalkylene oxide moiety may be 2-100 kD. The polyalkylene oxide moiety is either saturated or unsaturated. Examples of a polyalkylene oxide moiety include, but are not limited to, polyethylene oxide, polyethylene glycol, polyisopropylene oxide, polybutenylene oxide, and copolymers thereof. Other polymers such as dextran, polyvinyl alcohols, polyacrylamides, or carbohydrate-based polymers can also be used to replace the polyalkylene oxide moiety, as long as they are not antigenic, toxic, or eliciting immune response. The polyalkylene oxide moiety is either substituted or unsubstituted. For example, it can be methoxy-capped polyethylene glycol (mPEG).

The term "interferon-α moiety" refers to a mono-valent radical derived from either interferon-α. "Interferon-α" refers to a family of highly homologous species-specific proteins that inhibit viral replication and cellular proliferation and modulate immune response. See Bonnem et al., J. Biol. Response Mod., 1984, 3(6):580-598; and Finter, J. Hepatol., 1986, 3 Suppl 2:S157-160. It can be in a naturally occurring or a modified form. The modified interferon-α can be, e.g., a protein containing interferon-α and 1-4 additional amino acid residues at the N–terminus of the interferon. An example of such a modified interferon is , IFN representing an interferon-α_{2b} moiety, the amino group at the N-terminus of which is bonded to the carbonyl group.

Many types of interferon-α proteins are commercially available, including Intron-A interferon provided by Schering Corporation, Kenilworth, N.J., Roferon interferon provided by Hoffmann-La Roche, Nutley, N.J., Berofor alpha 2 interferon provided by Boehringer Ingelheim Pharmaceutical, Inc., Ridgefield, Conn., Sumiferon provided by Sumitomo, Japan, and Wellferon interferon alpha-n1 (INS) provided by Glaxo-Wellcome Ltd., London, Great Britain.

Listed below are amino acid sequences of five exemplary human interferon-α proteins, either in precursor form or in mature form:

maltfallva llvlsckssc svgcdlpqth slgsrrtlml laqmrrislf sclkdrhdfg fpqeefgnqf qkaetipvlh emiqqifnlf stkdssaawd etlldkfyte lyqqlndlea cviqgvgvte tplmkedsil avrkyfqrit lylkekkysp cawevvraei mrsfslstnl qeslrske SEQ ID NO.: 1

(See Krasagakis et al., Cancer Invest. 26 (6), 562-568, 2008)

cdlpqthslg srrtlmllaq mrkislfscl kdrhdfgfpq eefgnqfqka etipvlhemi qqifnlfstk dssaawdetl ldkfytelyq qlndleacvi qgvgvtetpl mkedsilavr kyfqritlyl kekkyspcaw evvraeimrs fslstnlqes lrske SEQ ID NO.: 2

(See Klaus, et al., J. Mol. Biol. 274 (4), 661-675, 1997)

mcdlpqthsl gsrrtlmlla qmrrislfsc lkdrhdfgfp qeefgnqfqk aetipvlhem iqqifnlfst kdssaawdet lldkfytely

qqlndleacv iqgvgvtetp lmkedsilav rkyfqritly lkekkyspca wevvraeimr sfslstnlqe slrske SEQ ID NO.: 3

(See GenBank Accession Number AAP20099, the 30-APR-2003 version.)

mallfpllaa lvmtsyspvg slgcdlpqnh gllsrntlvl lhqmrrispf lclkdrrdfr fpqemvkgsq lqkahvmsvl hemlqqifsl fhterssaaw nmtlldqlht elhqqlqhle tcllqvvgeg esagaisspa ltlrryfqgi rvylkekkys dcawevvrme imkslflstn mqerlrskdr dlgss SEQ ID NO.: 4

(See Capon et al., J. Mol. Cell. Biol. 5 (4):768-779, 1985)

lsyksicslg cdlpqthslg nrralillaq mgrispfscl kdrhdfglpq eefdgnqfqk tqaisvlhem iqqtfnlfst edssaaweqs llekfstely qqlnnleacv iqevgmeetp lmnedsilav rkyfqritly ltekkyspca wevvraeimr slsfstnlqk rlrrkd SEQ ID NO.: 5

(See Lund et al., J. Interferon Res. 5 (2), 229-238, 1985)

In one example, the interferon-α protein used for making the conjugate of this invention has an amino acid sequence at least 80% (e.g., 85%, 90%, 95%, or 99%) identical to one of the above listed amino acid sequences, or to the fragment thereof that corresponds to a mature interferon alpha.

The "percent identity" of two amino acid sequences is determined using the algorithm of Karlin and Altschul *Proc. Natl. Acad. Sci.* USA 87:2264-68, 1990, modified as in Karlin and Altschul *Proc. Natl. Acad. Sci.* USA 90:5873-77, 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al. J. Mol. Biol.* 215:403-10, 1990. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the protein molecules of the invention. Where gaps exist between two sequences, Gapped BLAST can be utilized as described in Altschul *et al., Nucleic Acids Res.* 25(17):3389-3402, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used.

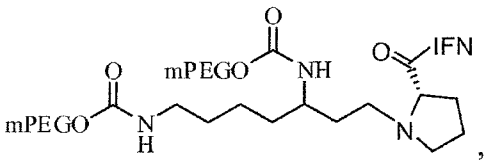
The term "linking functional group" refers to a bi-valent functional group, one end being connected to the polymer moiety and the other end being connected to the protein moiety. Examples include, but are not limited to, -O-, -S-, carboxylic ester,

carbonyl, carbonate, amide, carbamate, urea, sulfonyl, sulfinyl, amino, imino, hydroxyamino, phosphonate, or phosphate group.

The protein-polymer conjugate described above can be in the free form or in the form of salt, if applicable. A salt, for example, can be formed between an anion and a positively charged group (e.g., amino) on a protein-polymer conjugate of this invention. Suitable anions include chloride, bromide, iodide, sulfate, nitrate, phosphate, citrate, methanesulfonate, trifluoroacetate, and acetate. Likewise, a salt can also be formed between a cation and a negatively charged group (e.g., carboxylate) on a protein-polymer conjugate of this invention. Suitable cations include sodium ion, potassium ion, magnesium ion, calcium ion, and an ammonium cation such as tetramethylammonium ion.

In addition, the protein-polymer conjugate may have one or more double bonds, or one or more asymmetric centers. Such a conjugate can occur as racemates, racemic mixtures, single enantiomers, individual diastereomers, diastereomeric mixtures, and cis- or trans- or *E*- or *Z*- double bond isomeric forms.

An example of the protein-polymer conjugate of this invention is shown below:



in which mPEG has a molecular weight of 20 kD and IFN is an interferon-α_{2b} moiety.

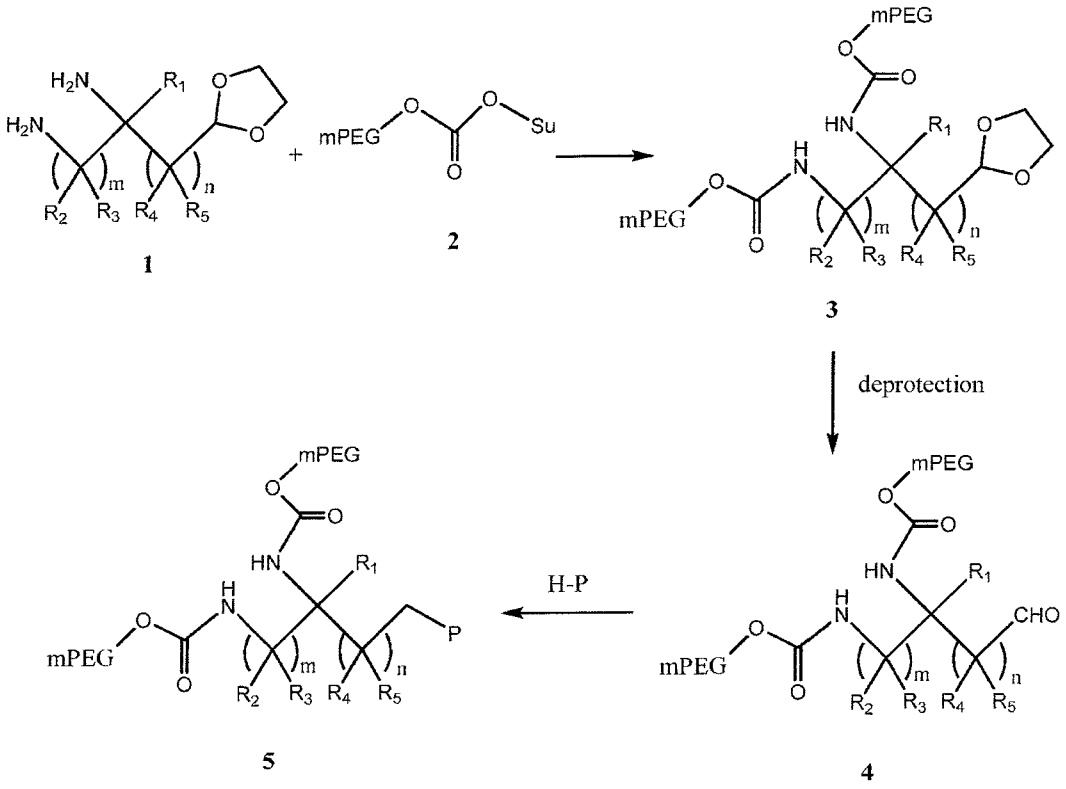
Also within the scope of this invention is the use of the conjugate for the manufacture of a medicament for treating one of the above-mentioned disorders.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

DETAILED DESCRIPTION

Protein-polymer conjugates used to practice the present invention can be prepared by synthetic methods well known in the chemical art, e.g., the methods described in U.S. Serial No. 12/192,485. Scheme 1 shows an example of preparing protein-polymer conjugates of this invention. Diamine compound **1**, which contains an acetal group, is reacted with N-hydroxysuccinimidyl carbonate mPEG (i.e.,

compound **2**) to form di-PEGylated compound **3**, which is subsequently converted to aldehyde **4**. This aldehyde compound is reacted with protein having a free amino group via reductive alkylation to afford a protein-polymer conjugate of this invention.



Scheme 1

A protein-polymer conjugate thus synthesized can be further purified by a method such as ion exchange chromatography, gel filtration chromatography, electrophoresis, dialysis, ultrafiltration, or ultracentrifugation.

The chemical reactions described above include using solvents, reagents, catalysts, protecting group and deprotecting group reagents, and certain reaction conditions. They may additionally include steps, either before or after the steps described specifically herein, to add or remove suitable protecting groups in order to ultimately allow for synthesis of a protein-polymer conjugate. In addition, various synthetic steps may be performed in an alternate sequence or order to give the desired protein-polymer conjugates. Synthetic chemistry transformations and protecting group methodologies (protection and deprotection) useful in synthesizing applicable protein-polymer conjugates are known in the art and include, for example, those

described in R. Larock, *Comprehensive Organic Transformations*, VCH Publishers (1989); T.W. Greene and P.G.M. Wuts, *Protective Groups in Organic Synthesis*, 2d. Ed., John Wiley and Sons (1991); L. Fieser and M. Fieser, *Fieser and Fieser's Reagents for Organic Synthesis*, John Wiley and Sons (1994); and L. Paquette, ed., *Encyclopedia of Reagents for Organic Synthesis*, John Wiley and Sons (1995) and subsequent editions thereof.

The conjugate of the invention can have a very high purity. Namely, 60% or more (e.g., 70%, 80%, or 90%) of the conjugate molecules are identical in all aspects, including the sequence of the protein moiety and its bonding position to the polymer moiety.

The conjugate of this invention may be pharmaceutically active in the conjugate form. Alternatively, it can release a pharmaceutically active interferon-α *in vivo* (e.g., through hydrolysis) by enzymatically cleaving the linkage between the protein moiety and the polymer moiety. Examples of enzymes involved in *in vivo* cleaving linkages include oxidative enzymes (e.g., peroxidases, amine oxidases, or dehydrogenases), reductive enzymes (e.g., keto reductases), and hydrolytic enzymes (e.g., proteases, esterases, sulfatases, or phosphatases).

The conjugate of this invention can be used to treat multiple sclerosis, chronic viral infection (such as hepatitis B virus infection, hepatitis C virus infection, and human papilloma virus infection), cancer, idiopaic myelofibrosis, polycythaemia vera, and essentia thromobocythaemia. It has an unexpectedly long *in vivo* half life, a reduced drug dose, and/or a prolonged dosing interval.

As used herein, the term "treating" or "treatment" is defined as the application or administration of a composition including a protein-polymer conjugate to a subject (human or animal), who has a disorder, a symptom of the disorder, a disease or disorder secondary to the disorder, or a predisposition toward the disorder, with the purpose to cure, alleviate, relieve, remedy, or ameliorate the disorder, the symptom of the disorder, the disease or disorder secondary to the disorder, or the predisposition toward the disorder. "An effective amount" refers to an amount of a protein-polymer conjugate which confers a therapeutic effect on the treated subject. The therapeutic effect may be objective (i.e., measurably by some tests or markers) or subjective (i.e., a subject gives an indication of or feels an effect).

Also within the scope of this invention is a pharmaceutical composition contains an effective amount of at least one of the protein-polymer conjugates described above and a pharmaceutical acceptable carrier. Further, this invention includes a method of administering an effective amount of one or more of the protein-polymer conjugates to a patient with one or more diseases. Effective doses will vary, as recognized by those skilled in the art, depending on, e.g., the rate of hydrolysis of a protein-polymer conjugate, the types of diseases to be treated, the route of administration, the excipient usage, and the possibility of co-usage with other therapeutic treatment.

To practice the method of the present invention, a composition having one or more of the above–mentioned compounds can be administered parenterally, orally, nasally, rectally, topically, or buccally. The term "parenteral" as used herein refers to subcutaneous, intracutaneous, intravenous, intramuscular, intraarticular, intraarterial, intrasynovial, intrasternal, intrathecal, intralesional, intraperitoneal, intratracheal or intracranial injection, as well as any suitable infusion technique.

A sterile injectable composition can be a solution or suspension in a non–toxic parenterally acceptable diluent or solvent, such as a solution in 1,3–butanediol. Among the acceptable vehicles and solvents that can be employed are mannitol, water, Ringer's solution, and isotonic sodium chloride solution. In addition, fixed oils are conventionally employed as a solvent or suspending medium (e.g., synthetic mono- or di-glycerides). Fatty acid, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions can also contain a long chain alcohol diluent or dispersant, or carboxymethyl cellulose or similar dispersing agents. Other commonly used surfactants such as Tweens or Spans or other similar emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms can also be used for the purpose of formulation.

A composition for oral administration can be any orally acceptable dosage form including capsules, tablets, emulsions, and aqueous suspensions, dispersions, and solutions. In the case of tablets, commonly used carriers include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn

starch. When aqueous suspensions or emulsions are administered orally, the active ingredient can be suspended or dissolved in an oily phase combined with emulsifying or suspending agents. If desired, certain sweetening, flavoring, or coloring agents can be added.

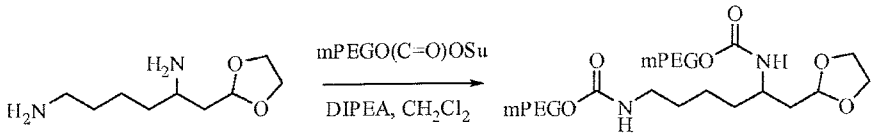
A nasal aerosol or inhalation composition can be prepared according to techniques well known in the art of pharmaceutical formulation. For example, such a composition can be prepared as a solution in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art. A composition having one or more of the above–described compounds can also be administered in the form of suppositories for rectal administration.

A pharmaceutically acceptable carrier is routinely used with one or more active above–mentioned compounds. The carrier in the pharmaceutical composition must be "acceptable" in the sense that it is compatible with the active ingredient of the composition (and preferably, capable of stabilizing the active ingredient) and not deleterious to the subject to be treated. One or more solubilizing agents can be utilized as pharmaceutical excipients for delivery of an above–mentioned compound. Examples of other carriers include colloidal silicon oxide, magnesium stearate, cellulose, sodium lauryl sulfate, and D&C Yellow # 10.

Suitable assays can be used to preliminarily evaluate the efficacy of the above-described conjugates in treating various diseases. For example, one can assess the effectiveness of the conjugate in treating polycythemia vera and essential thromobocythaemia following the methods described in Kiladjian et al., Blood 2008; 112(8): 3065-72 and Langer et al., Haetatologica 2005; 90: 1333-1338, respectively.

The example below is to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent. All publications cited herein are hereby incorporated by reference in their entirety.

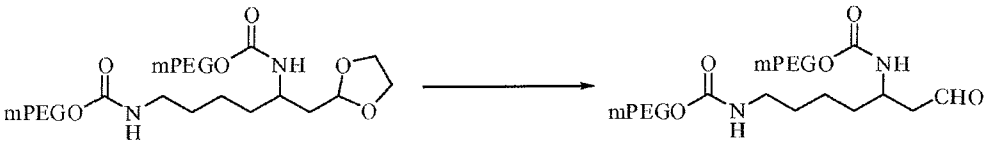
Preparation of di-PEG aldehyde



20 kD PEGO(C=O)OSu was prepared from 20 kD mPEGOH purchased from (SunBio Inc., CA, USA) according to the method described in Bioconjugate Chem. 1993, 4, 568-569.

A solution of 6-(1,3-dioxolan-2-yl)hexane-1,5-diamine in dichloromethane (11.97 g of the solution containing 9.03 mg of diamine, 47.8 μmol) was added to a flask containing 20 kD PEGO(C=O)OSu (1.72 g, 86.0 μmol). After PEGO(C=O)OSu was completely dissolved, N, N-diisopropylethylamine (79 μL, 478 μmol) was added. The reaction mixture was stirred at room temperature for 24 h, and then methyl t-butyl ether (200 mL) was added dropwise with stirring. The resulting precipitate was collected and dried under vacuum to give di-PEG acetal (1.69 g, 98%) as a white solid.

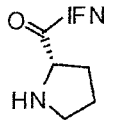
^{1}H NMR (400 MHz, d_6-DMSO) δ 7.16 (t, J = 5.2 Hz, 1 H), 7.06 (d, J = 8.8 Hz, 1 H), 4.76 (t, J = 4.8 Hz, 1 H), 4.10-3.95 (m, 4 H), 1.80-1.65 (m, 1 H), 1.65-1.50 (m, 1 H), 1.48-1.10 (m, 6 H).



Di-PEG acetal (4.0 g, 0.2 mmol) was suspended in pH 2.0 buffer (critic acid, 40 mL). The reaction mixture was stirred at 35°C for 24 h and then extracted with dichloromethane (3 x 50 mL). The combined organic layers were dried over magnesium sulfate, concentrated, and then re-dissolved in dichloromethane (20 mL). The solution was added dropwisely to methyl t-butyl ether (400 mL) with stirring. The resulting precipitate was collected and dried at reduced pressure to give di-PEG aldehyde (3.8 g, 95%) as a white solid.

^{1}H NMR (400 MHz, d_6-DMSO) δ 9.60 (s, 1 H), 7.24 (d, J = 8.4 Hz, 1 H), 7.16 (t, J = 5.2 Hz, 1 H), 4.10-3.95 (m, 4 H), 3.95-3.80 (m, 1 H), 3.00-2.85 (m, 2 H), 2.58-2.36 (m, 2 H), 1.46-1.15 (m, 6 H).

Preparation of modified interferon



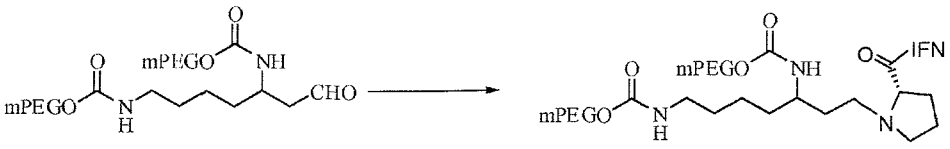
A modified recombinant human interferon–α_{2b} was cloned by a PCR method using human genomic DNA as a template. The oligonucleotides were synthesized based on the flanking sequences of human interferon–α_{2b} (GenBank Accession # J00207, January 8, 2008). The derived PCR products were subcloned into pGEM–T vector (Promega). The IFN variant was PCR amplified again through the pGEM–T clones and subsequently subcloned into protein expression vector pET–24a (Novagen), a T7 RNA polymerase promoter driven vector, using NdeI/BamHI as the cloning sites. Vector pET–24a was then transformed into *E. coli* BL21–CodonPlus (DE 3)-RIL (Stratagene) strain. The high–expression clones were selected by maintaining the transformed *E. coli* BL21–CodonPlus (DE 3)–RIL in the presence of karamycin (50 μg/mL) and chloramphenical (50 μg/mL).

Terrific broth medium (BD, 200 mL) was employed for the propagation of BL21–CodonPlus (DE 3)–RIL with Pro–IFN gene in a 1000 mL flask. The flask was shaken at 37°C at 230 rpm for 16 hr. Batch and fed–batch fermentations were performed in a 5–liter jar fermentor (Bioflo 3000; New Brunswick Scientific Co., Edison, NJ). The batch fermentation used 150 mL of an overnight preculture inoculum and 3 L of the Terrific broth medium with karamycin (50 μg/mL), chloramphenical (50 ug/mL), 0.4% glycerol, and 0.5% (v/v) trace elements (10 g/L of $FeSO_4 \cdot 7H_2O$, 2.25 g/L of $ZnSO_4 \cdot 7H_2O$, 1 g/L of $CuSO_4 \cdot 5H_2O$, 0.5 g/L of $MnSO_4 \cdot H_2O$, 0.3 g/L of H_3BO_3, 2 g/L of $CaCl_2 \cdot 2H_2O$, 0.1 g/L of $(NH_4)_6Mo_7O_{24}$, 0.84 g/L EDTA, 50 ml/L HCl). The dissolved oxygen concentration was controlled at 35% and the pH was kept at 7.2 by adding a 5 N NaOH aqueous solution. A feeding solution containing 600 g/L of glucose and 20 g/L of $MgSO_4 \cdot 7H_2O$ was prepared. When the pH rose to a value greater than the set point, an appropriate volume of the feeding solution was added to increase the glucose concentration in the culture broth. Expression of the Pro-IFN gene was induced by adding IPTG to a final concentration of 1 mM and the culture broth was harvested after incubating for 3 hr.

The collected cell pellet was resuspended with TEN buffer (50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl) in an approximate ratio of 1:10 (wet weight g/mL) and disrupted by a microfluidizer, and then centrifuged at 10,000 rpm for 20 min. The pellet containing inclusion body (IB) was washed twice with TEN buffer and centrifuged as described above. The pellet containing IB was then suspended in 150 mL of a 4 M guanidium HCl (GuHCl) aqueous solution and centrifuged at 20,000 rpm for 15 min. The IB was then solubilized in 50 mL of 6 M GuHCl solution. The GuHCl solubilized material was centrifuged at 20,000 rpm for 20 min. Refolding was initiated by dilution of denatured IB in 1.5 L of a freshly prepared refolding buffer (100 mM Tris–HCl (pH 8.0), 0.5 M L–Arginine, 2 mM EDTA) that was stirred only during the addition. The refolding reaction mixture was allowed to incubate for 48 hr without stirring. The refolded recombinant human interferon–α_{2b} (i.e., Pro–IFN) was dialyzed against 20 mM Tris buffer (with 2 mM EDTA and 0.1M urea, pH 7.0) for further purification by Q–Sepharose column chromatography.

The refolded recombinant human protein Pro–IFN was loaded onto a Q–Sepharose column (GE Amersham Pharmacia, Pittsburgh, PA). The column was pre–equilibrated and washed with a 20 mM Tris–HCl buffer (pH 7.0). The product was eluted with a mixture of 20 mM Tris–HCl buffer (pH 7.0) and 200 mM NaCl. Fractions containing Pro–IFN was collected based on its absorbance at 280 nm. The concentration of Pro–IFN was determined by a protein assay kit using the Bradford method (Pierce, Rockford, IL).

Prepare Protein-polymer Conjugate



To a solution of di-PEG aldehyde prepared above (1.2 g, 0.03 mmol) in water (72 mL) was added 2 M sodium phosphate buffer (pH 4.0, 5 mL) and Pro–IFN (200 mg in 22.2 mL of pH 7.0 buffer containing 20 mM Tris-HCl and 0.2M NaCl, 0.01 mmol). The reaction mixture was stirred at room temperature for 10 min; then sodium cyanoborohydride aqueous solution (400 mM, 1.25 mL, 0.5 mmol) was added. The reaction mixture was stirred in the dark for 16 h and purified by SP XL Sepharose chromatography. Fractions containing the desired polymer–protein conjugate were collected based on their retention time and absorbance at 280 nm. The concentration of the conjugate was determined by a protein assay kit using the Bradford method (Pierce, Rockford, IL). The isolated yield of the conjugate was roughly 40% or higher.

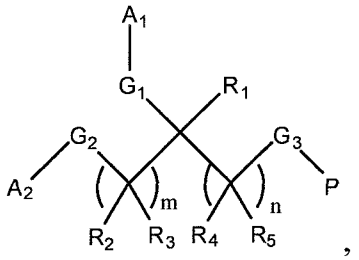
OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the scope of the following claims.

WHAT IS CLAIMED IS:

1. The use of an effective amount of a conjugate of formula I:



formula I

wherein

each of R_1, R_2, R_3, R_4, and R_5, independently, is H, C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, aryl, heteraryl, C_{3-8} cycloalkyl, or C_{3-8} heterocycloalkyl;

each of A_1 and A_2, independently, is a polymer moiety;

each of G_1 and G_2, independently, is a bond or a linking functional group;

G_3 is a bond;

P is an interferon-α moiety in which the amino group at the N-terminus is bonded to G_3;

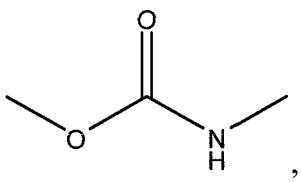
m is 0 or an integer of 1-10; and

n is an integer of 1-10;

in the manufacture of a medicament for the treatment of idiopathic myelofibrosis, polycythaemia vera, or essential thromobocythaemia.

2. The use of claim 1, wherein each of A_1 and A_2 is an mPEG moiety having a molecular weight of 10-30 kD.

3. The use of claim 1 or claim 2, wherein each of G_1 and G_2 is

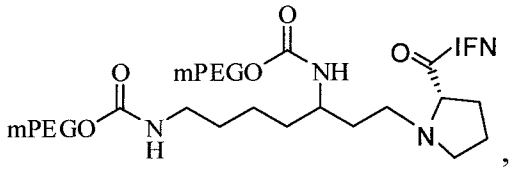


wherein O is attached to A_1 or A_2, and NH is attached to a carbon atom as shown in formula I.

4. The use of any one of claims 1 to 3, wherein P is a modified interferon moiety containing 1-4 additional amino acid residues at the N-terminus.

5. The use of any one of claims 1 to 4, wherein n is 2.

6. The use of any one of claims 1 to 5, wherein the conjugate is



in which mPEG has a molecular weight of 20 kD and IFN is an interferon-α_{2b} moiety.

7. The use of any one of claims 1 to 6, wherein the disease is idiopathic myelofibrosis.

8. The use of any one of claims 1 to 7, wherein the disease is polycythaemia vera.

9. The use of any one of claims 1 to 8, wherein the disease is essential thromobocythaemia.