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- 包括以电子形式分开公布的说明书序列表部分, 应要求可从国际局获得。

(54) Title: Y-TYPE POLYETHYLENE GLYCOL MODIFIED G-CSF AND PREPARATION METHOD AND USE THEREOF

A1

(54) 发明名称: Y型聚乙二醇修饰的G-CSF及其制备方法和应用

WO 2009/086656

(57) Abstract: Provided are mono-modified granulocyte colony-stimulating factor (G-CSF) by Y-type branched polyethylene glycol at the specific lysine position (Lys17) and the preparation method thereof. The pegylated G-CSF can be used for manufacture of pharmaceutical composition for treating neutropenia induced by infection, leukaemia and chemotherapy etc.

(57) 摘要:

提供了 Y 型分支的聚乙二醇在特定的赖氨酸位点 (Lys17) 单位点修饰的粒细胞集落刺激因子 (G-CSF) 及其制备方法。该聚乙二醇化的 G-CSF 可用于制备治疗由感染、白血病、化疗等导致的粒细胞减少症的药物。

**Y-SHAPED POLYETHYLENE GLYCOL MODIFIED G-CSF, THE PREPARATION AND
USE THEREOF**

FIELD OF THE INVENTION

5 The present invention relates to granulocyte colony stimulating factor (G-CSF) modified with Y-shaped polyethylene glycol (YPEG-G-CSF) and the preparation thereof, as well as the pharmaceutical composition comprising the same and use thereof.

BACKGROUND OF THE INVENTION

10 Granulocyte colony stimulating factor (G-CSF) is one of the colony stimulating factors that contribute to the formation of the colonies of bone marrow cells. It can specifically stimulate and regulate the proliferation, differentiation, survival and activation of granulocytes, and is promising in treating granulopenia of various causes.

15 Human G-CSF gene is located in the q21-22 region on chromosome 17 with a full length of 2.5kb. The gene is consisted of 5 exons and 4 introns, and the corresponding mature protein comprises 174 amino acids. G-CSF expressed by *E. coli* has a Met on its N-terminus, and is consisted of 175 amino acids, as shown in Figure 1(SEQ ID NO:1). The molecule comprises 5 cystein residues, wherein Cys37-Cys43 and Cys75-Cys85 form two pairs of disulfide bonds, 20 and comprises 4 lysine residues, located on positions 17, 24, 35 and 41 separately.

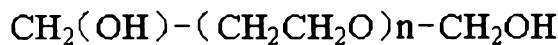
Neutropenia caused by chemotherapy, especially febril neutropenia (FN) is the most common and usually the most serious side effect after chemotherapy of cancer patients, especially in the first cycle of chemotherapy. FN will lead to enforced hospitalization and use of antibiotics, 25 causing extra economic burden and high fatality. Another damaging result is that due to the reduction of neutrophils, the scheme for the treatment of cancer patients has to be changed, for example reducing the dosage for chemotherapy and postponing the next therapeutic cycle, which are directly related to the final therapeutic result. Since the approval of rHuG-CSF by FDA in 1991, millions of cancer patients subjected to chemotherapy have benefited therefrom. 30 The medicament has been listed as one of top 10 sellers of the world's biotech medicament, and is believed to be very promising.

However, recombinant human G-CSF has shortened *in vivo* half life ($t_{1/2}$ of about 1.3-4.2h) and activity, and tends to be hydrolyzed by enzymes and cleared from the kidney, therefore needs to be injected many times, which is very inconvenient for the patient and may cause 5 some undesired responses which will affect the efficacy.

Recently, the development of polyethylene glycol (PEG) modification technique has provided an alternative for solving the above-mentioned technical problem.

10 PEG is a nontoxic and dissolvable neutral polymer. It is biocompatible and hemocompatible, and has been approved by FDA for topical and enteral use and intraveneous injection. PEG modification of a protein is achieved by activating one or both terminal groups on both ends of PEG to creat a functional group, which is reactive with at least one group in the protein to be bound, so as to bind PEG with the N or C terminal of the protein or a specific amino acid, 15 wherein the site for PEG modification is universal.

PEG is the polymer of ethylene glycol and ethylene oxide, also called carbowax, with the structure shown in the following formula:



20 The appearance of conventional PEG changes as its molecular weight increases: it appears from colorless viscous liquid (190-630 Dalton), white paste (950-1050 Dalton) to white waxy or flaky solid (> 1200 Dalton). Usually, PEG for modifying proteins or other drugs has a large molecular weight(Table 1), such as the U-shaped double-stranded PEG with a molecular 25 weight of 40 KD employed in Pegasys (PEGylated interferon α 2a injection, PEGASYS®, Roche, Shanghai), or the linear PEG with a molecular weight of 12 KD employed in Peg-Intron (PEGylated interferon α 2b injection, PEG-INTRON®, Schering-Plough, US), or the linear PEG with a molecular weight of 20 KD employed in Neulasta® (PEGylated 30 granulocyte colony-stimulating factor, Amgen US). The *in vivo* metabolic process of conjugated PEG is quite clear, indicating that PEG is a good, safe drug modifier with no side effects.

Table 1. The parameters for basic physicochemical properties of currently marketed PEGylated protein drugs^[18]

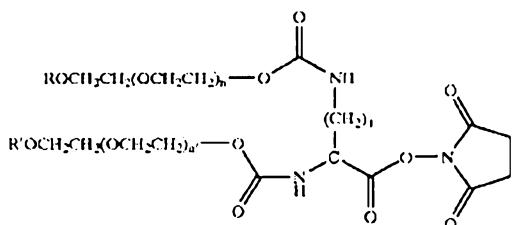
Product (Trade name)	Average MW	MW of PEG	t _{1/2} before pegylation	t _{1/2} after pegylation	Manufacture
Pegylated L-asparaginase (Oncaspar)	143kDa	5kDa	20 h	2 weeks	Enzon
Pegylated IFN α 2b(PEGIntron)	31kDa	12kDa	4 ~ 12 h	40 h (22 ~ 60 h)	Schering
Pegylated IFN α 2a (Pegasys)	60kDa	40kDa	5.1 h (3.7 ~ 8.5 h)	80 h (50 ~ 140 h)	Roche
Pegylated G-CSF(Neulasta)	39kDa	20kDa	3.5 h	15 ~ 80 h	Amgen

5 Proteinaceous drugs after polyethylene-glycolation (PEGylation) will have significantly improved properties, including prolonged pharmacokinetic half-life (Table 1), reduced immunogenicity, improved safety, increased efficacy, decreased frequency of administration, increased solubility, enhanced protease resistance, which facilitate the controlled release of the drugs. For example, as disclosed in U.S. Patent No. 4,179,337, after the conjugation of

10 PEG with enzymes and insulin, the immunogenicity of the proteins is reduced, while a certain percentage of the original activity of the proteins still remains. Another unique effect of pegylation is that the *in vitro* activity of the protein is decreased, but the *in vivo* activity is increased. For example, as disclosed in U.S. Patent No. 4,179,337, after the conjugation of PEG with an enzyme or insulin, the immunogenicity of the protein is reduced, and the

15 activity of the protein is significantly decreased, but the protein still retains a certain percentage of the original activity.

PEGs used to modify drugs are divided into two types according to the structure: the linear type and the branched type. For example, Pegasys® (PEGylated interferon α 2a injection, 20 PEGASYS®, Roche US) employs a U-shaped branched double-stranded PEG derivative, with an average molecular weight ranging from 26KD-66KD (US Pat. 5382657 - Filed Aug 26, 1992 - Hoffmann-La Roche Inc.), which is represented by the following formula:

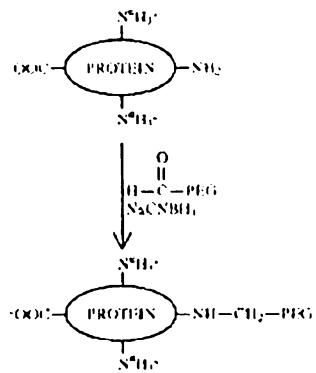


wherein, R and R' are independently low molecular weight alkyl groups, n and n' are from

600 to 1500.

Linear PEG molecule having a molecular weight of 20kD is used in Neulasta® (pegylated granulocyte colony-stimulating factor, Amgen) approved by U.S. FDA in 2002(US Pat.

5 5824784 - Filed Oct 12, 1994 - Amgen Inc.). The reaction for modification is as follows:



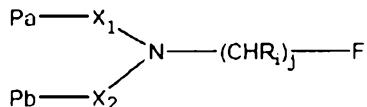
Amgen's Neulasta® employs PEG with aldehyde group on the end. The modification is on the N-terminal amino acid of the protein and PEG-G-CSF modified at a single point is obtained. It is characterized in that PEG is conjugated with G-CSF via C-N bond.

10

PEGs with different configurations are used to modify proteins, resulting in products with apparently different features. Prior art literature (Monfardini C, Schiavon O, Caliceti P, et al. Bioconjugate Chem, 1995, 6 (1) :62-69) reports that comparing to linear PEG, branched PEG improves the protein's pH resistance, thermal stability and resistance to protease digestion.

15

Chinese Patent ZL 03801105.0 reported a new double-stranded Y-shaped PEG derivative, which has the following basic structure:



wherein, Pa and Pb are the same or different hydrophilic polymers, which can be
20 polyethylene glycol, polypropylene glycol, polyvinyl alcohol, polyacrylmorpholine or their
copolymers, preferably is polyethylene glycol and its copolymers:

j is an integer from 1 to 12;

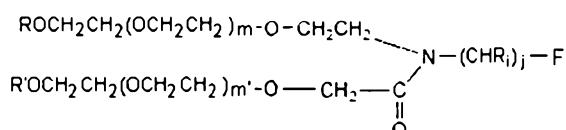
Ri is H, a substituted or unsubstituted C₁₋₁₂ alkyl group, a substituted aryl, an aralkyl or a heteroalkyl;

X_1 and X_2 are independently a linking group, wherein X_1 is $(CH_2)_n$, and X_2 is selected from the group consisting of $(CH_2)_n$, $(CH_2)_nOCO$, $(CH_2)_nNHCO$, and $(CH_2)_nCO$; n is an integer from 1 to 10; and

F is a terminal group selected from the group consisting of a hydroxyl group, a carboxyl group, an ester group, acyl chloride, hydrazide, maleimide, pyridine disulfide, capable of reacting with amino, hydroxyl or mercapto group of a therapeutic agent or a protein to form a covalent bond.

When Pa and Pb are preferably PEG or its copolymer, the basic molecular structure thereof is

10



The Y-shaped PEG modifies the protein on the free amino group of the protein, wherein the modification site is not fixed.

15 In prior art, N-hydroxysuccinimide activation can be used to synthesize Y-shaped branched
NHS-PEG which is used for modification. NHS-PEG is characterized in that it can form
amide bond with the free amino group on lysine or the free terminal amino group of rhG-CSF,
and the amide bond can be hydrolyzed *in vivo* slowly, so that the activity of rhG-CSF is
restored. However, the currently used Y-shaped branched NHS-PEG generally have the defect
20 of high activity and poor selectivity, and cannot be used to achieve directional selection of
modification sites, therefore it is difficult to obtain products with modification on a single
fixed-site.

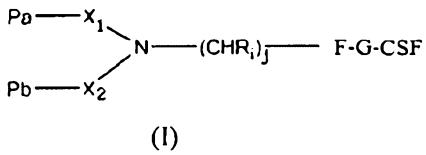
SUMMARY OF THE INVENTION

25 The invention is based on novel Y-shaped PEGylated granulocyte colony stimulating factor (YPEG-G-CSF) with single site modification. Particularly, the invention relates to YPEG modified G-CSF and its preparation method, the pharmaceutical composition comprising YPEG-G-CSF and use thereof. Particularly, YPEG-G-CSF of the present invention is modified on the 17th lysine (K17) as a single site modification. The use of K17 single site
30 modified YPEG-G-CSF achieves good therapeutic effects in animals. In addition, K17 single

site modified YPEG-G-CSF has significantly prolonged pharmacokinetic half-life in serum.

In one aspect, this invention relates to Y-shaped PEG derivatives (also recorded as YPEG) modified G-CSF (also recorded as YPEG-G-CSF or PEG-G-CSF), wherein its molecular

5 composition is as follows



wherein, Pa and Pb are same or different PEGs;

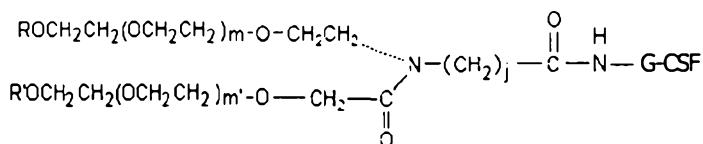
j is an integer from 1 to 12;

10 Ri is H, a substituted or unsubstituted C₁-C₁₂ alkyl group, a substituted aryl, an aralkyl or a heteroalkyl;

X₁ and X₂ are independently a linking group, wherein X₁ is (CH₂)_n, and X₂ is selected from the group consisting of (CH₂)_n, (CH₂)_nOCO, (CH₂)_nNHCO, and (CH₂)_nCO; n is an integer from 1 to 10; and

15 F is a terminal group selected from the group consisting of a hydroxyl group, a carboxyl group, an ester group, acyl chloride, hydrazide, maleimide, pyridine disulfide, capable of reacting with amino, hydroxyl or mercapto group of a therapeutic agent or a substrate to form a covalent bond.

20 In one embodiment, the structural formula of the Y-shaped PEG-G-CSF has the following structure:



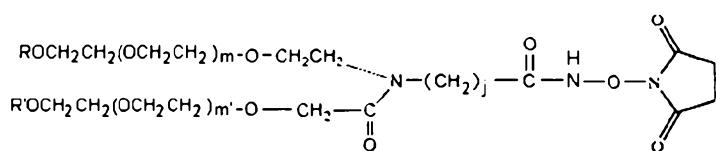
(II)

25 Wherein R and R' are independently a low molecular weight alkyl, preferably methyl; j is an integer of 1-12; m and m' denote the degree of polymerization and can be any integer; preferably m=m' and m+m' is from 600 to 1500. In formula (II), the Y-shaped branched PEG binds to G-CSF through an amide bond at a single site.

In a preferred embodiment, in said Y-shaped PEG-G-CSF, G-CSF binds Y-PEG through the amide bond formed by the ϵ -amino group on the side chain of lysine in G-CSF corresponding to position 17 of SEQ ID NO: 1 and the terminal carboxyl group of Y-PEG.

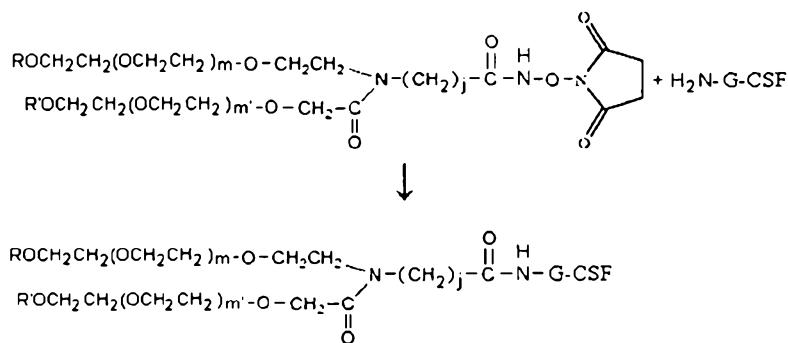
5 Alternatively, the G-CSF of the present invention can be extracted from natural sources or obtained through recombinant biotechnology. Preferably, G-CSF extracted from natural sources or obtained through recombinant biotechnology is human G-CSF (hG-CSF) shown in SEQ ID NO: 1. More preferably, said human G-CSF is recombinant human G-CSF (rhG-CSF). rhG-CSF can be synthesized, expressed by prokaryotic systems such as *E. coli*,
10 expressed by eukaryotic systems such as *Pichia pastoris*, or expressed by other insect cell systems or mammalian cell systems such as CHO cells. The method for preparation of natural or recombinant G-CSF and the method to detect the activities of G-CSF and its YPEG modified products are conventional to one in the art.

15 In another aspect, the present invention relates to a method for preparation of YPEG modified G-CSF. In one embodiment, activated derivatives of YPEG such as polyethylene glycol succinimide (YPEG-NHS) shown in formula III can be used to covalently bind PEG with the ϵ -amino group on lysine corresponding to position 17 in G-CSF through nucleophilic substitution reaction:



Wherein, YPEG-NHS can be prepared as described in EP1496076.

The reaction of G-CSF with YPEG to form YPEG-G-CSF is shown as below:



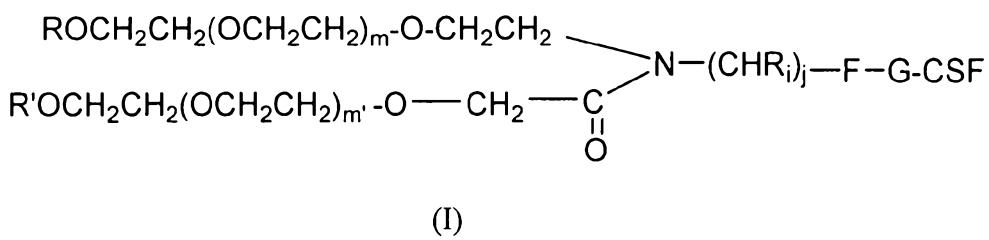
The reaction is performed under mild reaction conditions, pH 6.0-10, preferably 8.0, temperature 0-20°C, mixed by stirring etc.. The modification site of pegylation is determined by performing molecular weight analysis on YPEG-G-CSF combined with molecular 5 fingerprint mapping and trypsin digestion pattern and N-terminal sequencing of the protein. The inventor found that the pegylation site on G-CSF was on lysine of position 17, and the product of the modification was recorded as YPEG-G-CSF (17).

The separation and purification of YPEG-G-CSF (17) can be performed by methods such as 10 ion exchange. In one embodiment, the prepared YPEG-G-CSF is passed through a cationic ion exchange column, the fourth active peak is collected, and the product is further purified by a Sephadex S-400HR column to obtain the purified YPEG-G-CSF (17).

The invention also provides the use of the YPEG-G-CSF of the present invention or the 15 composition comprising the YPEG-G-CSF of the present invention in treating disease in need of G-CSF treatment. The clinical use of the pegylated G-CSF or the composition comprising the pegylated G-CSF of the present invention is the same as that of G-CSF, and they all can be applied in treating granulocytopenia-related diseases such as granulocytopenia induced by 20 severe infection, treatment for leukemia, stem cell transplantation, and malignant solid tumor chemotherapy (Ying Tong et.al, "The clinical application of G-CSF and GM-CSF", Basic & Clinical Medicine 2000 Vol.20 No.2 P. 101-104). *In vivo* results show that compared with G-CSF, the use of YPEG-G-CSF of the present invention achieves unexpected effects—the same or better efficacy is achieved even if the total dosage is reduced, see the working examples. In addition, the pharmacokinetic half life of YPEG-G-CSF of the present invention 25 in serum and other properties are significantly improved. The YPEG-G-CSF of the invention can be administered to patients in the form of composition, wherein the composition contains

a pharmaceutically effective amount of YPEG-G-CSF and a pharmaceutically acceptable carrier or excipient. Preferably, the above composition contains mannitol, amino acids, sodium acetate and acetic acid, wherein the amino acid is selected from aspartic acid, asparagine and glycine. The composition can be prepared into appropriate dosage forms, 5 and administered by experienced physicians in any acceptable suitable manner. The invention also provides a method for treatment of neutropenia, which include the administration of the composition comprising YPEG-G-CSF of the present invention.

In one aspect of the invention there is provided Y-shaped PEGylated G-CSF of the structure as shown in formula (I):



10

wherein R and R' are independently low molecular weight alkyl; m and m' denote the degree of polymerization and can be any integer; R_i is H, substituted or unsubstituted C₁-C₁₂ alkyl group, substituted aryl, aralkyl, or heteroalkyl;

15 j is an integer of 1-12;

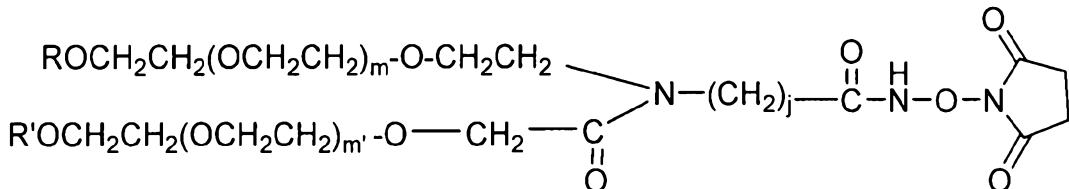
F is a terminal group selected from the group consisting of a hydroxyl group, a carboxyl group, an ester group, acyl chloride, hydrazide, maleimide, pyridine disulfide, capable of reacting with amino, hydroxyl or mercapto group on G-CSF to form a covalent bond.

The invention also provides use of the Y-shaped PEGylated G-CSF as referred to above in 20 the manufacture of a medicament for treating diseases in need of G-CSF treatment, wherein the diseases in need of G-CSF treatment is for example granulocytopenia induced by severe infection, leukemia, stem cell transplantation, and chemotherapy for malignant solid tumor.

The invention further provides a method for preparing and purifying the Y-shaped PEGylated G-CSF as referred to above, comprising the following steps:

(a) mixing the Y-shaped branched PEG of the following formula (III) with G-CSF in the ratio of protein:PEG=1:6 (mass ratio), and allowing them to react at 4°C to obtain

5 YPEGylated G-CSF;



wherein both R and R' are methyl; j is an integer between 1-12; m and m' denote the degree of polymerization and can be any integer;

(b) diluting the reacting mixture with 10mM NaAc/HAc pH4.0, and loading the
10 diluted mixture onto a cationic ion exchange column equilibrated with 10mM NaAc/HAc pH 4.0; eluting with 10mM NaAc/HAc +NaCl 160mM pH4.0 buffer gradient, collecting the fourth peak, removing macromolecular polymers by a Sephadex S-400HR column (GE Healthcare) equilibrated with 10mM sodium phosphate buffer (pH7.0), and collecting the eluent from the active peak to obtain YPEG-G-CSF modified at position K17.

15 There is also provided a method of treating granulocytopenia, comprising the step of administering the YPEGylated G-CSF as referred to above.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps
20 but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of
25 endeavour to which this specification relates.

2007363326 09 Jan 2014

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequence of G-CSF (SEQ ID NO:1), MW18801.79, PI 5.62, wherein the underlined amino acids represent five theoretical sites for modification by YPEG in the amino acid sequence, including N-terminal amino group and the free amino group on Lys.

Figure 2 shows the peaks obtained from separation of YPEG-G-CSF by a cationic ion exchange column, wherein the fourth (4#) peak is the target peak of YPEG-G-CSF (17).

Figure 3 shows the peak obtained from purification of YPEG-G-CSF by use of Sephadex 10 S-400HR column.

Figure 4 shows the result obtained by using MALDI-TOF to determine the molecular weight of YPEG-G-CSF.

Figure 5 shows the result obtained by using Maldi-Tof to perform peptide mass fingerprint mapping on peptide fragments of G-CSF.

15 Figure 6 shows the result obtained by using Maldi-Tof to perform peptide mass fingerprint mapping on peptide fragments of PEG-G-CSF.

Figure 7 shows separation of trypsin-digested G-CSF by RP-HPLC C18 column.

Figure 8 shows separation of trypsin-digested PEG-G-CSF by RP-HPLC C18 column.

Figure 9 shows the sequencing of N-terminal amino acid sequence of YPEG-G-CSF.

5 Figure 10 shows the result of the assay for the specific biological activity of YPEG-G-CSF.

Figure 11 shows the effect of YPEG-rHuG-CSF on the logarithm value of the number of neutrophils in ^{60}Co irradiated monkey.

10 Figure 12 shows the comparison of pharmacokinetic curves of PEG-GCSF and G-CSF.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be further illustrated by the following examples. The advantages of the present invention compared to prior art are:

15

1. YPEG-G-CSF of the present invention is obtained by using YPEG to modify G-CSF and purifying the reaction mixture to obtain products with single site modification on K¹⁷, so as to solve the following technical problem to facilitate quality control and to ensure batch-to-batch stability in mass production: since NHS-PEG has high activity, poor selectivity, 20 and cannot be used for directional selecting of the modification site, it is difficult to obtain products with a single site modified.

25

2. The *in vivo* circulating half-life of YPEG-G-CSF of the present invention is significantly prolonged compared to G-CSF.

30

3. YPEG-G-CSF of the present invention significantly improves in its pharmacodynamical features compared with non-pegylated G-CSF. Particularly, when using the same amount or even smaller amount of YPEG-G-CSF compared to conventional G-CSF, the former can achieve the same or even more obvious therapeutic effects.

30

One in the art should understand that any examples or their combination should not be

understood as limiting the scope of the present invention. The scope of the present invention is defined by the accompanying claims, and a person of ordinary skill can clearly understand the scope defined by the claims combined with the teaching of the description and common sense.

5

Examples

Example 1 Preparation of YPEG-G-CSF

The stock buffer system containing 400mg G-CSF at a concentration of 9.58mg/ml was replaced by 50mM sodium borate buffer (pH8.0), and 2mM HCl was used to dissolve 3.2g

10 40KD NHS-PEG (purchased from Beijing JianKai Technology Co., Ltd.). The protein and PEG were mixed at the ratio of protein: PEG = 1:6 (mass ratio). The mixture was maintained at 4°C for 3 hours, and then diluted 15 times with 10mM NaAc pH4.0 before loaded on a cationic ion exchange column (purchased from GE Healthcare) equilibrated with 10mM NaAc/HAc pH4.0. 10mM NaAc/HAc + NaCl 160mM pH4.0 gradient elution buffer was used 15 to elute the diluted mixture, and the fourth active peak was collected (Figure 2). Sephadex S-400HR column (purchased from GE Corporation) equilibrated with 10mM sodium phosphate buffer (pH7.0) was used to remove the macromolecular polymers, and the active peak was collected (Figure 3). 10mM NaAc pH4.0 buffer system was used for ultrafiltration, and 73 mg sample was obtained.

20

Example 2 Determination of the molecular weight of PEG-G-CSF

MALDI-TOF was used to determine the molecular weight of the PEG-G-CSF obtained in

Example 1. Particularly autoflex TOF / TOF mass spectrometry (Bruker Daltonics, Germany)

and the TOF / TOF MS method were used to determine the molecular weight of

25 YPEG-rHuG-CSF and rHuG-CSF. Matrix used is sinapinic acid (SA, C₁₁H₁₂O₅, MW 224.22), and the analysis software is the flexAnalysis Ver.3.0.54.0..

Test results: The MS molecular weight of YPEG-rHuG-CSF was about 59kD, consistent with the theoretical molecular weight 58801.8Dalton. The result was shown in Figure 4.

30

YPEG-rHuG-CSF was obtained by modifying rHuG-CSF with YPEG. Modifier (YPEG) was

the mixture of a series YPEGs whose molecular weights were normally distributed, with an average molecular weight of 40kD \pm 10%. Due to the normal distribution of the molecular weight of YPEG, the molecular weights of YPEG modified products were also normally distributed (\pm 10%), i.e. the molecular weight of YPEG-rHuG-CSF should be 58802.0 Dalton

5 \pm 10%.

Example 3 Determination of the pegylation site

The pegylation site in PEG-G-CSF obtained from Example 1 was analyzed.

10 The buffer containing G-CSF and PEG-G-CSF was replaced by 50mM (NH₄)HCO₃, pH8.0. Endoproteinase Glu-C was added in the ratio of 1:20 to digest YPEG-G-CSF, and the Maldi-Tof method was used to perform peptide mass fingerprint mapping on the obtained peptide fragments. The results were shown in Figure 5 and Figure 6.

15 The above results indicated that, the following peptide fragments could be obtained from G-CSF/Glu-C: peptide fragments of 1-20 amino acids, which contained the YPEG reaction sites M¹ and K¹⁷ (MTPLGPASSLPQSFLLKCLE, molecular weight 2132.6D), peptide fragments of 21-34 amino acids, which contained YPEG reaction site K²⁴ (QVRKIQGDGAALQE, molecular weight 1512.7D), peptide fragments of 35-47 amino acids, which contained YPEG reaction sites K³⁵ and K⁴¹ (KLCATYKLCHPEE, molecular weight 1534.7D). Peptide fragment of 1-20 amino acids could not be obtained from YPEG-G-CSF/Glu-C, but peptide fragments of 21-34 amino acids (QVRKIQGDGAALQE) and of 35-47 amino acids (KLCATYKLCHPEE) were obtained, indicating that the modification did not occur at K²⁴, K³⁵ and K⁴¹, but in the N-terminal amino acid or lysine at

20 position 17, resulting in the change of the molecular weight of the peptide fragment compared with the unmodified peptides due to the conjugation with YPEG of 40kD.

25

To confirm this conclusion, the inventor compared the mapping of PEG-G-CSF with that of G-CSF. Sequencing grade trypsin (Promega, swine, seq. grade modified,> 5000 U / mg) was diluted with a buffer solution to the concentration of 1 μ g/ul. G-CSF-PEG was subjected to ultra-filtration, and the buffer was replaced with 50mM NH₄HCO₃ pH8.0 so that sample

solution with the concentration of PEG-G-CSF of 1mg/ml was obtained. Adding 1 μ l trypsin into 50 μ l YPEG-G-CSF sample, and incubating the mixture at 37°C for 24 hours. The unmodified G-CSF sample was treated in the same way as control.

5 Digested G-CSF and PEG-G-CSF samples were separated by C18 RP-HPLC column filled with octadecylsilyl (Φ4.6mm × 150mm, particle diameter 5 μ m, pore size 300Å). Gradient elution was performed under the following conditions: mobile phase A 0.1% TFA/H₂O(V/V), mobile phase B 0.1% TFA/90% ACN (V/V); flow rate 1.0ml/min, as shown in Table 2. The result was shown in Figure 7 and Figure 8.

10

Table 2 Elution gradients on HPLC-RPC C18 for mapping of digested YPEG-rHuG-CSF

	time (min)	A%	B%
1	0	100	0
2	1	100	0
3	71	30	70
4	81	0	100
5	91	100	0
6	100	100	0

15 Compared with the unmodified G-CSF, the peptide mapping of PEG-G-CSF showed an additional peak at 52.479min but the peak at 39.172min disappeared. The peptide fragments were collected and the five N-terminal amino acids were subjected to Edman degradation. The result showed that all the peptide fragments had the same N-terminal sequence MTPLG. The retention time of the peptide fragments changed after conjugation with YPEG, indicating that the YPEGylation site indeed occurred at the N-terminal segment.

20 The PEG-G-CSF was further subjected to N terminal sequencing, and was found to have the N terminal 15 amino acid sequence MTPLGPASSLPQSFL. Analysis showed that the first amino acid was the Met, and compared to the second amino acid Thr, its peak area was not significantly reduced (Figure 9), indicating the existence of free amino group on the N terminus, which was not modified. That is to say PEG modification did not occur on the N terminal M1, but on K17. The result proved that the YPEG-G-CSF prepared in Example 1 was modified on the single site of K17.

Example 4 Assay of the biological activity of PEG-G-CSF

1 Formulation of agents and cell cultivation

1.1 1640 medium: RPMI1640 liquid medium, which was incubated at 4°C; or which was formulated according to the instructions of the manufacture. Penicillin and streptomycin were

5 added into the medium to 105IU/L before use.

1.2. Minimal culture medium: 1640 solution with 2.5% fetal bovine serum (FBS, v/v) and 12.5% horse serum (ES, v/v), incubated at 4°C.

1.3 Complete culture medium: rhG-CSF was added into minimal culture medium to a final concentration of 20ng (2000U)/ml, 4°C.

10 1.4 NFS-60 cell line: The cells were cultivated in complete culture medium at 37°C, 5% CO₂ and passaged every 48 to 72 hours. The concentration of the cells was controlled between 2.0×10⁵-10.0×10⁵/ml. The titer of rhG-CSF was determined 24-36 hours after the last passage.

15 1.5 Phosphate buffer (Hyclone): 8g NaCl, 0.2g KCl, 1.44g disodium hydrogen phosphate, 0.24g of potassium dihydrogen phosphate were formulated into 1000ml solution with ultra-pure water. After 121°C, 15 min of incubation the solution was autoclaved.

1.6 MTT solution: MTT powder (Sigma) was formulated with phosphate buffer solution into 5.0mg/ml, and sterilized by 0.22μm membrane filter, stored at 4°C in dark.

1.7 lysis buffer

20 Lysis buffer 1: Isopropyl alcohol solution containing 1% concentrated HCL, 5% TritonX-100, stored at room temperature in dark.

Or lysis buffer 2: Isopropyl alcohol solution containing 2.8% concentrated HCL, 10% TritonX-100, stored at room temperature in dark.

25 2. Assay of the biological activity of PEG-G-CSF

2.1 Preparation of cell suspension

Enough amount of NFS-60 cell culture was centrifugated to collect NFS-60 cells. The collected cells were washed with PBS 3 times, and then resuspended in minimal culture medium. The cell concentration was adjusted to about 2.0×10⁵/ml, preserved at 37°C.

30 2.2 G-CSF standard (working standard, or national standard provided by NICPBP, with the international standard used as a reference for check) and the samples to be tested were

prediluted to 2ng/ml by minimal culture medium according to the amount of protein, and the dilution factor for each step was no more than 10.

2.3 Minimal culture medium was added into the 96 cell plate at 50 μ l/well. The reference materials and samples to be tested obtained in 2.2 were serially diluted at 1:2 to eight different concentrations of 1ng/ml, 0.5ng/ml, 0.25ng/ml..., and each well contained 50 μ l. Negative control (without rhG-CSF) and positive control (with rhG-CSF 2ng/ml) were set, each in at least triplicate.

2.4 50 μ l cell suspension per well was added, and incubated at 37 °C, 5% CO₂ for 40-48 hours. After a substantive number of the negative cells (> 95%) were disrupted, MTT solution was added at 20 μ l per well, incubated at 37°C, with 5% CO₂ for 4-6 hours.

2.5 180 μ l lysis buffer 1 or 100 μ l lysis buffer 2 was added into each well, mixed and assayed by colorimetric method at wavelength of 570nm, and the reference wavelength was 630nm.

3. Result

The dose-effect relationship of the standard and the sample to be tested was plotted with OD570nm-630nm value as Y axis and the logarithmic values of the dilution gradient on the plate as X-axis. Test data were processed by four factor fitting method. The ED₅₀ (50% effective dose) value at OD570nm-630nm was calculated as the mean value of the maximum drug concentration and the minimum drug concentration on the curve of the standard at OD570nm-630nm. The logarithm value of the dilution factor corresponding to ED50 at OD570nm-630nm on the sample curve was defined as the C value of the sample. The calculation was performed according to the following equation:

Titer of Sample to be tested (IU/vial) = titer of the standard \times C1/C2 \times D1/D2 \times V

Wherein: C1 is the dilution factor of the sample corresponding to ED50 of the standard;

C2 is the dilution factor of the standard at ED50;

25 D1 is the pre-dilution factor of sample to be tested;

D2 is the pre-dilution factor of the standard;

V is labelled volume of the vial (expressed in ml).

It was determined that the biological activity of YPEG-G-CSF prepared in the working

30 Examples was 2.96×10^7 IU/mg (Figure 10).

Example 5 Pharmacodynamic assay of YPEG-G-CSF

(A) Effect of the pegylated granulocyte colony stimulating factor (YPEG-G-CSF) on 5-fluorouracil induced granulocytopenia

1. Materials and Methods

5 The test sample: YPEG-G-CSF standard (Biosteel Gene Expression Tech Co. Ltd.), : 1mg/vial, stored at 2-8 °C.

Positive control: Filgrastim (rhG-CSF) 300μg/vial (KKPHARM Co.Ltd), stored at 2 ~ 8°C in dark.

10 Animals: 140 male mice (Kunming, SPF grade, Staidson Pharmaceutical Co., Ltd. Beijing, license number SCXK-(Beijing) -2006-0004). The mice were randomly divided into 7 groups: the normal control group, model control group, low dose group 1, low dose Group 2, medium dose group and high dose group and the positive control group.

15 Modelling approach: Except for the normal control group, the model control group and each treatment group received a dose of 150mg/kg of 5-FU, and were subjected to therapy with the test sample or control the next day.

Dosage and frequency:

YPEG-rHuG-CSF was administered at a dosage of 15, 50, 150, 500 μg/kg, every 4 days, for 3 times. The dosage of the positive control was 50μg/kg, administered every 1-11 days. The schedule was shown in Table 3.

20 Table 3 YPEG-rHuG-CSF dose design

Group	Dosage μg/kg	Modelling	Frequency
Normal control	0	-	-
Model control	0	5-FU, iv, 150mg/kg	-
Low dose 1	YPEG-rHuG-CSF-1	15	5-FU, iv, 150mg/kg
Low dose2	YPEG-rHuG-CSF -2	50	5-FU, iv, 150mg/kg
Medium dose	YPEG-rHuG-CSF -3	150	5-FU, iv, 150mg/kg
High dose	YPEG-rHuG-CSF -4	500	5-FU, iv, 150mg/kg
Positive control	G-CSF	50	5-FU, iv, 150mg/kg
			d1-d11

Administration:

Except for the normal control group and the model control group, other groups were administered subcutaneously with the test sample and positive control 24 hours after administration for modeling at the given frequency.

5

2. Result

The number of neutrophils in mice was reduced in response to 5-FU, and after 3-9 days the number of neutrophils in most animals was too low to be detected. The percentages of neutrophils before test, and 3 and 9 days after the test were determined, and the absolute neutrophil count (ANC) was calculated. The results were shown in Table 4. The results showed that after the administration of 5-FU, ANCs of model control group and the 15 μ g/kg group were significantly reduced on day 3 (p <0.05 and p <0.01 compared to the normal control group), while the ANCs of the 50-500 μ g/kg group and the positive control group were not reduced compared to the number of WBC in control groups. The results indicated that the test sample of 50-500 μ g/kg could slow down the reduction of ANC. To day 9, ANC of the model group was still lower than that of normal control, while the ANC of the 150 and 500 μ g/kg group was higher than the level of the normal control group, and the ANC of the positive control group also reached that of the normal control group. The ANC level of the model control group at 11 day reached the level of the normal control group, indicating that the test sample or positive control could increase animal neutrophils to normal levels sooner.

Table 4 Effect of YPEG-rHuG-CSF on 5-FU mice ANC ($\times 10^9/L$)

Group	time	D0 (n=20)	D3 (n=10)	D9 (n=10)
Normal control		3.73 \pm 1.23	5.73 \pm 1.88	3.81 \pm 1.26
Model control		3.13 \pm 0.97	3.25 \pm 1.43 [▲]	1.78 \pm 0.68 [▲]
Low dose 1		4.53 \pm 2.14	2.74 \pm 1.06 ^{▲▲}	2.59 \pm 1.30
Low dose 2		4.72 \pm 3.87	3.90 \pm 1.82	3.00 \pm 0.96
Medium dose		3.96 \pm 1.21	4.63 \pm 1.70	7.80 \pm 2.42 ^{▲▲}
High dose		4.34 \pm 1.59	4.87 \pm 2.13	12.81 \pm 6.15 ^{▲▲}

Positive control	4.23±1.79	4.97±1.20 ^{**}	4.84±3.10
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The values shown in the table is $\bar{x} \pm$ SD. n represents for the number of animals, but the actual number of animals in which the number of neutrophils was measurable is less than n. Compared with the model group, **: P <0.01; *: P <0.05; with the normal control group, ▲: ▲: P <0.01; ▲: P <0.05.

5 Continued Table 4 YPEG-rHuG-CSF on 5-FU in mice ANC ($\times 10^9$ / L)

Group \ Time	D10 (n=10)	D11 (n=10)	D12 (n=10)
Normal control	4.99±2.13	5.26±1.19	5.23±1.22
Model control	2.24±0.92 [▲]	5.48±3.17	5.73±2.23
Low dose 1	10.35±2.87 ^{**▲▲}	7.92±2.91 ^{**▲▲}	5.62±2.31 ^{▲▲}
Low dose 2	15.71±7.15 ^{**▲▲}	23.60±7.39 ^{**▲▲}	8.47±3.45 ^{**▲▲}
Medium dose	33.07±11.42 ^{**▲▲}	44.41±16.28 ^{**▲▲}	21.02±7.90 ^{**▲▲}
High dose	32.80±16.74 ^{**▲▲}	52.67±16.17 ^{**▲▲}	41.08±23.71 ^{**▲▲}
Positive control	9.72±3.66 ^{**▲▲}	9.56±6.40	24.21±7.20 ^{**▲▲}

The values shown in the table is $\bar{x} \pm$ SD. n represents for the number of animals, but the actual number of animals in which the number of neutrophils was measurable is less than n. Compared with the model group, **: P <0.01; *: P <0.05; with the normal control group, ▲: P <0.01; ▲: P <0.05.

10

3. Conclusion

The inventor found that besides the significant long-lasting effect, YPEG-G-CSF demonstrated significant protection against 5-fluorouracil induced neutropenia in mice. YPEG-G-CSF could shorten the course of neutropenia in neutropenia mouse model, so that 15 the number of peripheral blood neutrophils could be recovered at an accelerated rate. Comparing G-CSF administered once a day at 50 μ g/kg for 11 time (Filgrastim 50 μ g/kg \times 11) to YPEG-G-CSF 50 μ g/kg administered every 4 days for 3 times (low dose YPEG-G-CSF 50 μ g/kg \times 3, medium dose CSF 50 μ g/kg \times 3), it was apparent that although the total dosage of YPEG-G-CSF of the present invention was significantly reduced, its effect on increasing the 20 number of neutrophils was equivalent or much better.

(B) The therapeutic effect of Y-shaped PEG modified recombinant human granulocyte colony-stimulating factor (YPEG-rHuG-CSF) on ^{60}Co 3.0Gy radiation-induced neutropenia in the monkey

1. Materials and Methods

5 Test sample: YPEG-G-CSF (Biosteed Gene Expression Tech Co. Ltd.): 1mg/vial, stored at 2-8°C.

Positive control: Filgrastim (rhG-CSF) 300 μg /vial (KKPHARM Co.Ltd), stored at 2 ~ 8°C, dark.

10 Experimental animals: Cynomolgus monkeys, male and female, 3-4 years old, weight 2.5-4.5kg (Xishanzhongke Laboratory Animal Co., Ltd., Su Zhou, License No.: SCXK (Su) 2002-0032). Model control group comprised 5 animals, the other groups comprised 4 animals each.

15 Modelling and administration: After 30 days of quarantine and adaptative feeding, animals were irradiated with ^{60}Co at 3.0Gy on whole body, radiation dose rate 1.8Gy/min. Animals were administered on the day of exposure (in 5 hours) subcutaneously on the inside of hind limbs. See Table 5 below.

Table 5 Dosage regimen design

Group	Dosage ($\mu\text{g}/\text{kg}$)	Number of animals	Frequency
Normal control	—	6	—
Model control	—	5	—
Test sample	25	4	Once every 6 days, d0, d6, d12, d18, d24
Positive control	10	4	Once per day, d0-d24

20 2. Analysis of the results

During the test, blood parameters were measured every day, and the number of neutrophils was counted. The results showed that, in low, medium and high dose groups and the positive control group, the mean ANCs were higher than that in model control group, and comparison between dose groups and model control group at different time points showed significant

differences ($p < 0.05$ or $p < 0.01$). The results were shown in Table 6, Figure 11.

Table 6 YPEG-rHuG-CSF on ANC of ^{60}Co irradiated monkey ($\times 10^9/\text{L}$)

group \ time	D0	D3	D6	D9
Model control	5.94 ± 1.88	2.40 ± 0.95	1.67 ± 0.57	2.30 ± 0.87
Positive control	5.62 ± 2.68	$8.37 \pm 3.78^*$	$7.44 \pm 2.67^{**}$	$7.71 \pm 1.40^{**}$
Test sample	4.52 ± 0.85	$7.15 \pm 2.24^{**}$	$6.04 \pm 1.28^{**}$	$12.31 \pm 5.33^{**}$

The values shown in the table is $\bar{x} \pm \text{SD}$. Model control group included 5 mice, and other groups comprised 4 mice.

Compared to model control group, **: $P < 0.01$; *: $P < 0.05$.

Continued Table 6-1 YPEG-rHuG-CSF on ANC of ^{60}Co irradiated monkey ($\times 10^9/\text{L}$)

group \ time	D12	D15	D18	D21
Model control	1.78 ± 1.13	1.58 ± 0.71	1.41 ± 0.91	1.01 ± 1.03
Positive control	$6.40 \pm 2.43^*$	$3.44 \pm 1.12^*$	$4.26 \pm 1.36^*$	$5.24 \pm 3.77^*$
Test sample	$6.60 \pm 2.59^*$	$7.52 \pm 4.91^*$	$3.00 \pm 0.87^*$	$4.36 \pm 2.11^*$

The values shown in the table is $\bar{x} \pm \text{SD}$. Model control group included 5 mice, and other groups comprised 4 mice.

Compared to model control group, **: $P < 0.01$; *: $P < 0.05$.

Continued Table 6-2 YPEG-rHuG-CSF on ANC of ^{60}Co irradiated monkey ($\times 10^9/\text{L}$)

group \ time	D25	D28	D30	D34
Model control	0.46 ± 0.36	0.59 ± 0.28	0.69 ± 0.25	1.96 ± 1.35
Positive control	$22.58 \pm 20.07^*$	8.76 ± 7.91	$6.19 \pm 4.15^*$	$6.50 \pm 3.33^*$
Test sample	$24.68 \pm 13.79^*$	$7.30 \pm 2.98^{**}$	$6.28 \pm 3.80^*$	$6.98 \pm 3.79^*$

The values shown in the table was $\bar{x} \pm \text{SD}$. Model control group included 5 mice, and other groups comprised 4 mice.

Compared to model control group, **: P<0.01; *: P<0.05.

Continued Table 6-3 YPEG-rHuG-CSF on ANC of ^{60}Co irradiated monkey ($\times 10^9/\text{L}$)

group \ time	D38	D42	D46	D50
Model control	2.05 ± 1.16	2.90 ± 0.92	3.00 ± 0.91	3.22 ± 0.51
Positive control	$6.84 \pm 2.80^*$	4.56 ± 1.52	4.32 ± 1.34	5.94 ± 3.12
Test sample	5.29 ± 3.03	$10.19 \pm 5.65^*$	$8.62 \pm 3.61^*$	10.14 ± 9.80

The values shown in the table was $\bar{x} \pm \text{SD}$. Model control group included 5 mice, and other groups comprised 4 mice.

Compared to model control group, **: P<0.01; *: P<0.05.

3. Conclusion

Y-PEGylated recombinant human granulocyte colony-stimulating factor (YPEG-rHuG-CSF) exerted therapeutic effects on radiation-induced neutropenia of cynomolgus monkey.

YPEG-rHuG-CSF had a prolonged effect *in vivo*. The above results showed that, compared with injection of 10 $\mu\text{g}/\text{kg}$ G-CSF once per day for 25 times, injection of 25 $\mu\text{g}/\text{kg}$ YPEG-G-CSF of the invention once every 6 days for 5 times achieved equivalent stimulating effects on neutrophils with less amount of drug.

Example 6. *In vivo* pharmacokinetic assay for YPEG-G-CSF

1. Methods and procedures

1.1 Drugs and reagents

Test sample: YPEG-G-CSF standard (Biosteed Gene Expression Tech Co. Ltd.) : 1mg/ per injection, stored at 2~8 °C.

Positive control: Filgrastim(rhG-CSF) 300 $\mu\text{g}/\text{vial}$ (KKPHARM Co.Ltd), stored at 2 ~ 8°C in dark.

1.2 Animals

6 Cynomolgus monkeys, 3 males and 3 females. (Monkey Forest Management and

Development Center of Guangxi feeding base production (certificate number: SCXK (Gui) 2005-0005)), weight 3.11 ~ 5.62 kg, subjected to sub-caged feeding with standard monkey feed. Water was provided ad lib. and fresh fruit was provided twice a day.

1.3 Experiment Design

5 The experiment included 2 groups, YPEG-G-CSF of 300 μ g/kg subcutaneously, G-CSF(Filgrastim) 300 μ g/kg.

2 Experimental Methods

2.1 Blood sampling

YPEG-rHuG-CSF was injected subcutaneously once before administration, or 0.25h, 0.5h, 1 10 h, 2h, 4 h, 8h, 24 h, 48h, 96h, 168h, 240h, 312h, 384h and 480h after administration and 1mLvenous blood was taken from the opposite hind limb. For the Filgrastim (G-CSF) group, 1 mL venous blood was taken before administration or 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12h, 24h after administration. The blood samples were incubated 4 °C for 30 min, then subjected to 3000 rpm low speed centrifugation for 10 min, and serum was immediately 15 separated and stored at -20°C until analysis.

2.2 Assay of serum drug concentration

Immunohistochemical Assay Kit (ELISA) was used for determining serum G-CSF or YPEG-rHuG-CSF concentrations in cynomolgus monkeys. Human G-CSF ELISA kit (Ray Biotech Inc.) was used in ELISA for the determining serum G-CSF and YPEG-rHuG-CSF 20 concentrations.

2.2.1 Principle of the assay

The analysis was based on quantitative sandwich technique. Monoclonal antibodies against recombinant human G-CSF were pre-coated on the microplate. The standard and the samples were transferred into the wells, wherein G-CSF or YPEG-G-CSF would bind the immobilized 25 antibodies. All unconjugated materials were washed off, and anti-human G-CSF IgG conjugated with horseradish peroxidase (HRP) was added into the wells. After washing off all the unconjugated antibody-enzyme reagents, the color produced after addition of the substrate for HRP was proportional to the amount of the conjugated G-CSF or YPEG-G-CSF. Stop the reaction to determine the intensity of color. The concentration of G-CSF or YPEG-G-CSF in 30 the sample increased with the OD value.

2.2.2 Procedures of the assay

The assay was operated according to the instructions in the kit. 100 μ L standard or serum samples were added into each well and mixed gently. According to the expected concentration of the samples, the mixture is diluted into the range of standard calibration curve. Standard calibration curve for recombinant G-CSF or YPEG-G-CSF were set for each 5 plate to calculate the concentration of the unknown samples. The mixture was incubated at room temperature for 1h. The plate was washed 3 times, and 100 μ L second antibody was added into each well, and incubated at room temperature for 1h. The plate was washed for 3 times. 100 μ L HRP was added into each well and the reaction was held at room temperature for 1h. 100 μ L TMB substrate was added into each well and kept in dark at room temperature 10 for 15min. 100 μ L stop solution was added to each well, mixed gently to stop the reaction. OD values at 450 nm were read in 5 mins.

2.2.3 Results

Origin® software was used to plot the logarithm values of different concentrations against log OD values. According to the standard curve for determining the concentration of the 15 samples in the 96-well plate, the concentration of recombinant G-CSF or YPEG-G-CSF in the samples was calculated by linear regression, and the serum concentration was obtained after calibration by the dilution factors.

2.3 Statistical methods and estimation of pharmacokinetic parameters

The pharmacokinetic parameters were calculated by Rosenblueth method of non 20 compartment model, and the software used was version 3P97. Comparison of the data of the same monkey was performed by Student's paired t-test, and comparison of the data of different monkeys was performed by t-test, and in both comparisons the calculation was performed by the statistical software provided by Microsoft Office Excel (version XP). The experimental data were processed by Origin® software to obtain the regression equation and 25 relevant statistical parameters. The comparison of pharmacokinetic curves was shown in Figure 12.

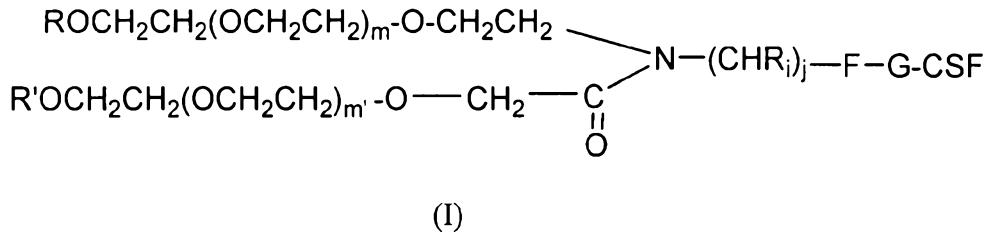
After injecting 300 μ g/kg of YPEG-rHuG-CSF subcutaneously into cynomolgus monkeys, the serum YPEG-rHuG-CSF concentration reached its peak after 8 ~ 24h, and C_{max} was 4.53 30 \pm 0.86. Terminal half-life was 77.55 ± 0.34 h, MRT was 95.03 ± 14.51 h. $AUC_{(0-480h)}$ was $534.75 \pm 155.28 \mu\text{g}\cdot\text{h}\cdot\text{mL}^{-1}$; $AUC_{(0-\infty)}$ was $539.27 \pm 158.32 \mu\text{g}\cdot\text{h}\cdot\text{mL}^{-1}$. The average clearance

rate was $0.60 \pm 0.20 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, substantially exhibited the characteristics of linear pharmacokinetics.

After single subcutaneous injection of 300 $\mu\text{g}/\text{kg}$ G-CSF, C_{\max} was $2.49 \pm 0.20 \mu\text{g} \cdot \text{h} \cdot \text{mL}^{-1}$, 5 $AUC_{(0-24h)}$ was $23.07 \pm 2.93 \mu\text{g} \cdot \text{h} \cdot \text{mL}^{-1}$, clearance rate was $12.95 \pm 1.95 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, terminal half-life was $4.00 \pm 1.44 \text{ h}$, MRT was $6.48 \pm 1.35 \text{ h}$, and V_{SS} was $72.53 \pm 18.86 \text{ mL} \cdot \text{kg}^{-1}$. The result indicated that the pharmacokinetic characteristics of YPEG-G-CSF were greatly different than that of G-CSF in cynomolgus monkeys, and the YPEG modification of G-CSF resulted in the obvious reduction of the elimination rate constant, clearance rate and apparent 10 volume of distribution and in prolonged MRT and terminal half-life ($77.55 \pm 0.34 \text{ h}$ vs $4.00 \pm 1.44 \text{ h}$)

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Y-shaped PEGylated G-CSF of the structure as shown in formula (I):



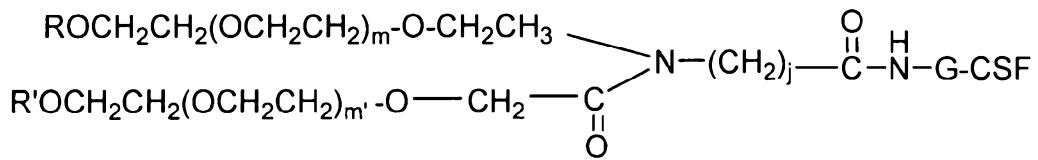
5 wherein R and R' are independently low molecular weight alkyl;
m and m' denote the degree of polymerization and can be any integer;
R_i is H, substituted or unsubstituted C₁-C₁₂ alkyl group, substituted aryl, aralkyl, or heteroalkyl;
j is an integer of 1-12;

10 F is a terminal group selected from the group consisting of a hydroxyl group, a carboxyl group, an ester group, acyl chloride, hydrazide, maleimide, pyridine disulfide, capable of reacting with amino, hydroxyl or mercapto group on G-CSF to form a covalent bond.

2. The Y-shaped PEGylated G-CSF according to claim 1, wherein R and R' are methyl.

15

3. The Y-shaped PEGylated G-CSF of claim 1 or claim 2, wherein the Y-shaped PEG is conjugated with the amino group on G-CSF through its terminal carboxyl group, as shown in formula (II):



20 wherein both R and R' are methyl; j is an integer between 1-12; m=m' and m+m' is from 600 to 1500.

4. The Y-shaped PEGylated G-CSF of claim 3, wherein the amino group is the side chain ε -amino group of a Lys residue in G-CSF corresponding to position 17 of SEQ ID NO: 1.

5. The Y-shaped PEGylated G-CSF of any one of claims 1-4, wherein the average total molecular weight of the PEG is from about 10000 to about 60000 Dalton.

5 6. The Y-shaped PEGylated G-CSF according to claim 5, wherein the average total molecular weight of the PEG is about 40000 ± 4000 (10%) Dalton.

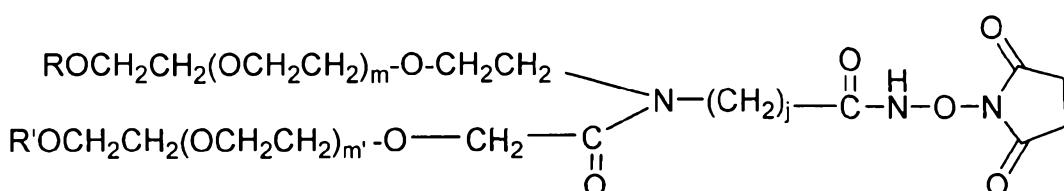
7. The Y-shaped PEGylated G-CSF of any one of claims 1-6, wherein the G-CSF is extracted from a natural source or obtained through recombinant biotechnology.

10 8. The Y-shaped PEGylated G-CSF according to claim 7, wherein the G-CSF has the sequence as shown in SEQ ID No.1.

9. Use of the Y-shaped PEGylated G-CSF of any one of claims 1-8 in the manufacture 15 of a medicament for treating diseases in need of G-CSF treatment, wherein the diseases in need of G-CSF treatment is for example granulocytopenia induced by severe infection, leukemia, stem cell transplantation, and chemotherapy for malignant solid tumor.

10. A method for preparing and purifying the Y-shaped PEGylated G-CSF of claim 4, 20 comprising the following steps:

(a) mixing the Y-shaped branched PEG of the following formula (III) with G-CSF in the ratio of protein:PEG=1:6 (mass ratio), and allowing them to react at 4°C to obtain YPEGylated G-CSF;



25 wherein both R and R' are methyl; j is an integer between 1-12; m and m' denote the degree of polymerization and can be any integer;

(b) diluting the reacting mixture with 10mM NaAc/HAc pH4.0, and loading the diluted mixture onto a cationic ion exchange column equilibrated with 10mm NaAc/HAc

pH 4.0; eluting with 10mM NaAc/HAc +NaCl 160mM pH4.0 buffer gradient, collecting the fourth peak, removing macromolecular polymers by a Sephadex S-400HR column (GE Healthcare) equilibrated with 10mM sodium phosphate buffer (pH7.0), and collecting the eluent from the active peak to obtain YPEG-G-CSF modified at position K17.

5

11. A method for preparing and purifying the Y-shaped PEGylated G-CSF according to claim 10, wherein $m=m'$ and $m+m'$ is from 600 to 1500.
12. A method of treating granulocytopenia, comprising the step of administering the YPEGylated G-CSF of claim 4.
13. A Y-shaped PEGylated G-CSF as claimed in claim 1, substantially as hereinbefore described.

FIGURE 1

The amino acid sequence of G-CSF (SEQ ID NO:1)

MTPLGPASSL PQSFLLKCLE QVRKIQGDGA ALQEKLCATY
KLCHPEELVL LGHSLGIPWA PLSSCPSQAL QLAGCLSQLH
SGLFLYQGLL QALEGISPEL GPTLDTLQLD VADFATTIWQ
QMEELGMAPA LQPTQGAMPA FASAFQRRAG GVLVASHLQS
FLEVSYRVLR HLAQP

FIGURE 2

Separation of YPEG-G-CSF with a cationic ion exchange column

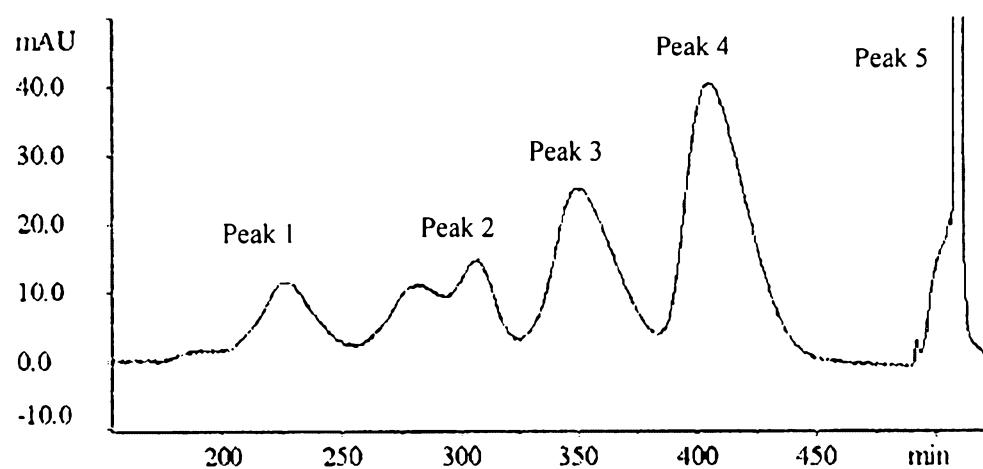


FIGURE 3

Purification of G-CSF by Sephacryl S-400HRcolumn

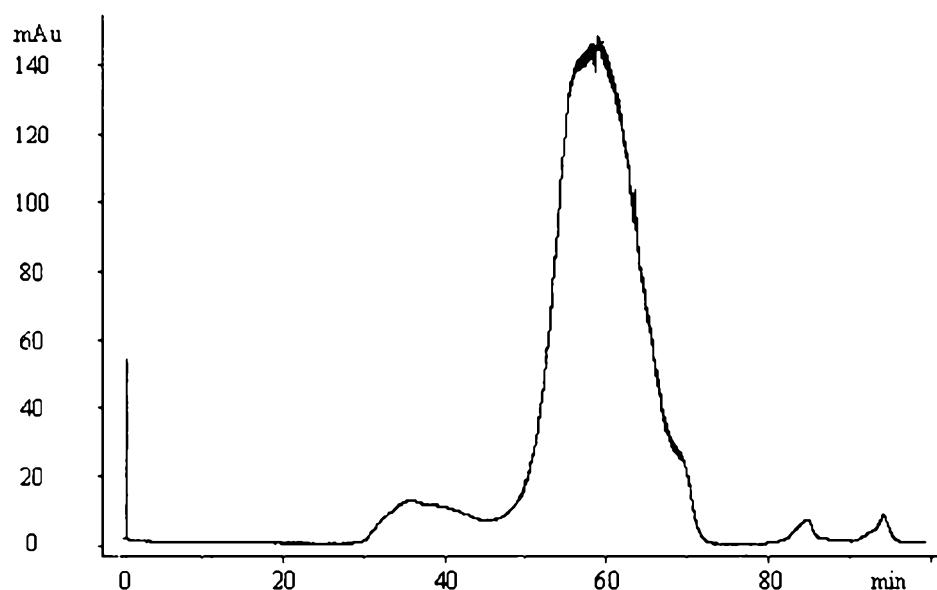


FIGURE 4

Using MALDI-TOF to determine the molecular weight of YPEG-G-CSF

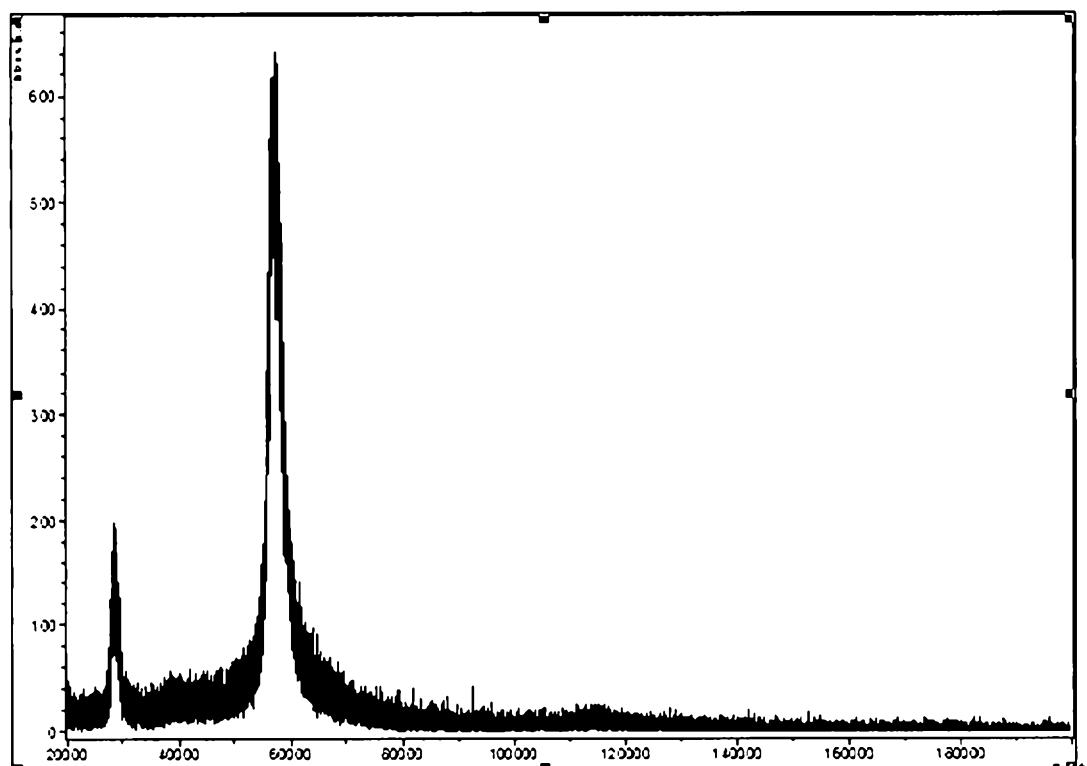


FIGURE 5

Peptide mass fingerprint mapping on peptide fragments of G-CSF by Maldi-Tof

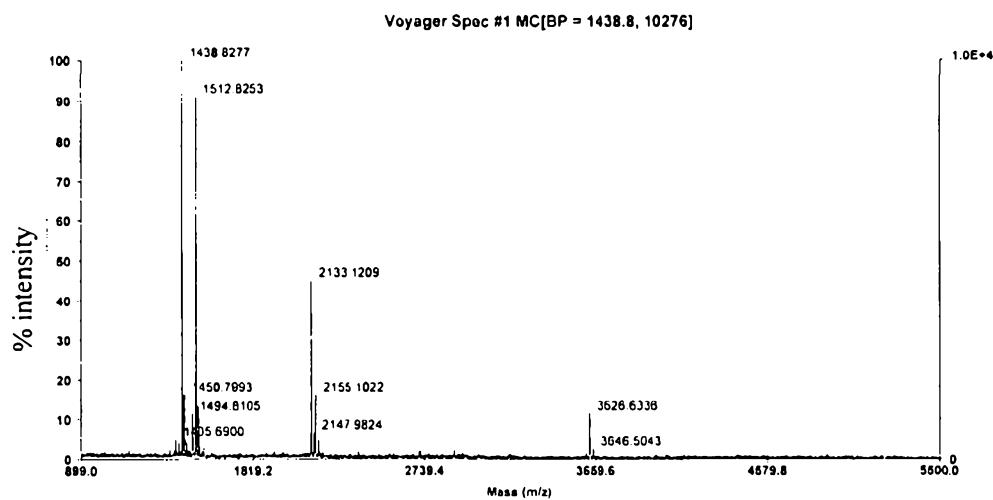


FIGURE 6

Peptide mass fingerprint mapping on peptide fragments of PEG-G-CSF by Maldi-Tof

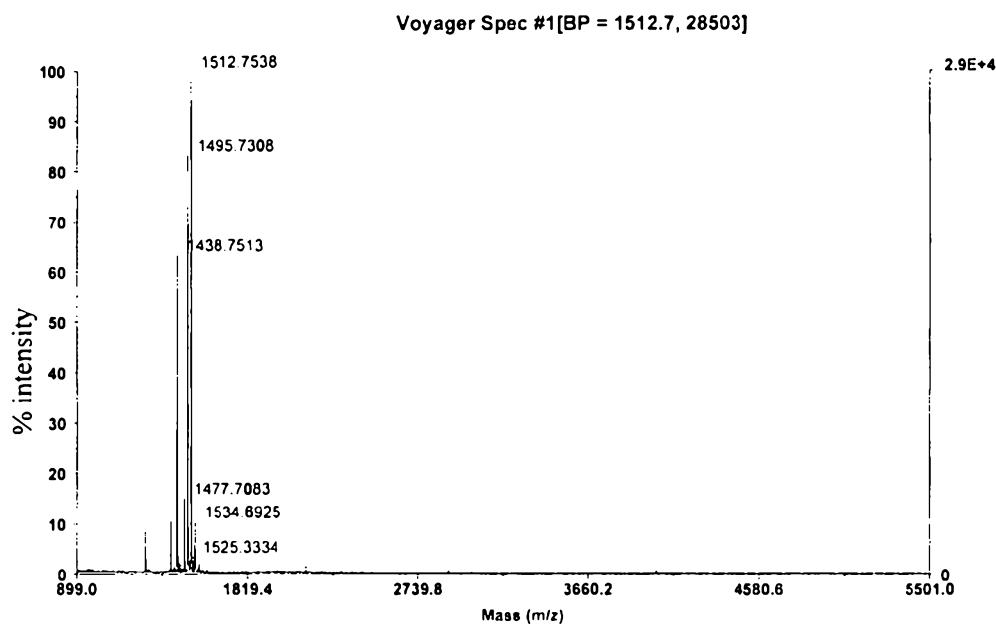


FIGURE 7

Separation of trypsin-digested G-CSF by RP-HPLC C18 column

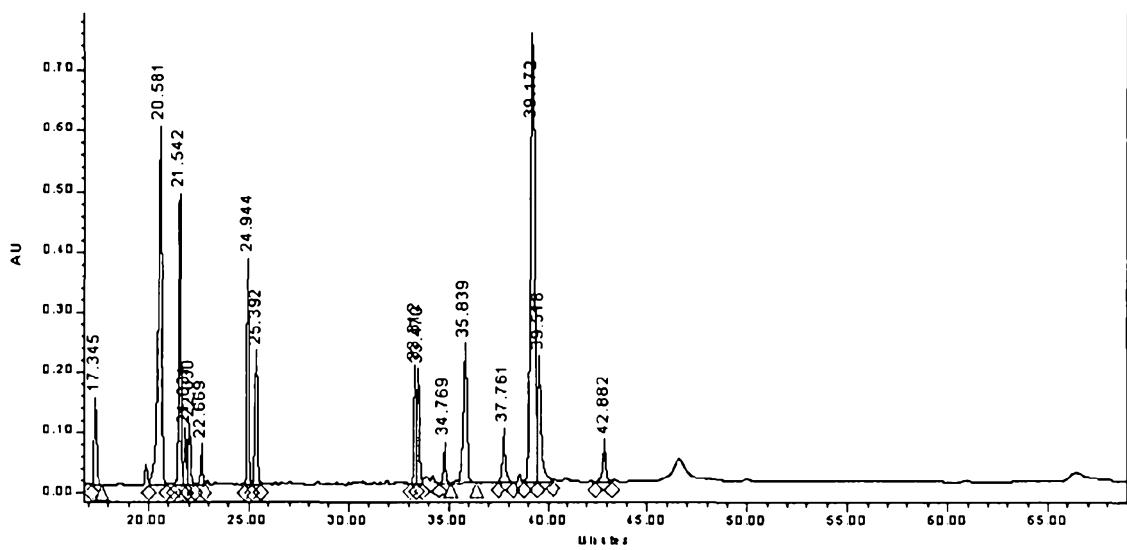
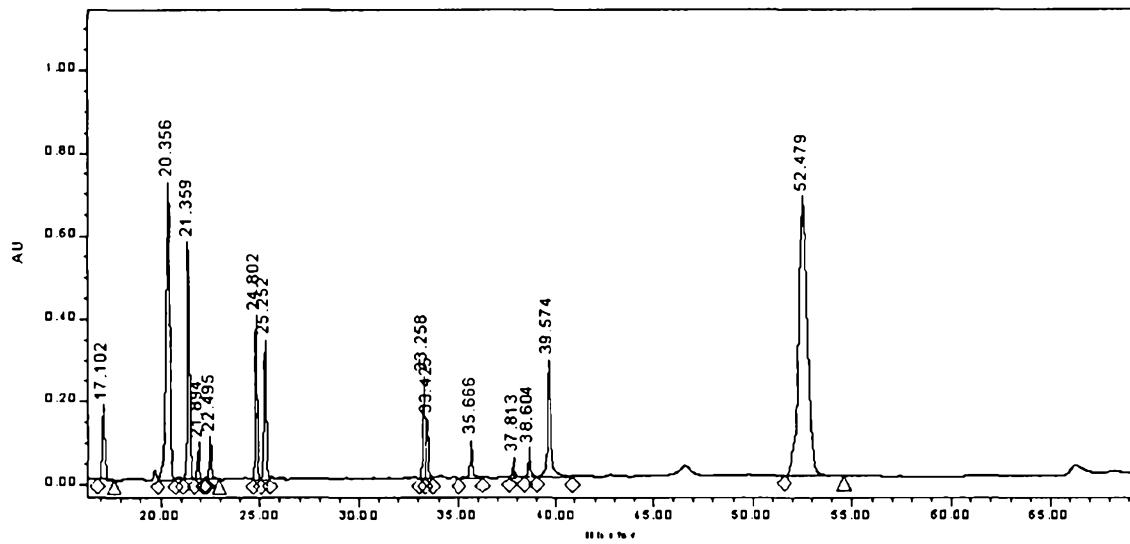


FIGURE 8

Separation of trypsin-digested PEG-G-CSF by RP-HPLC C18 column

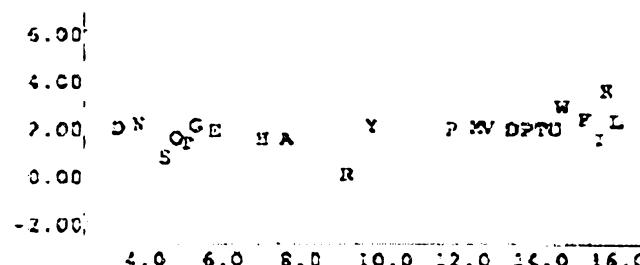


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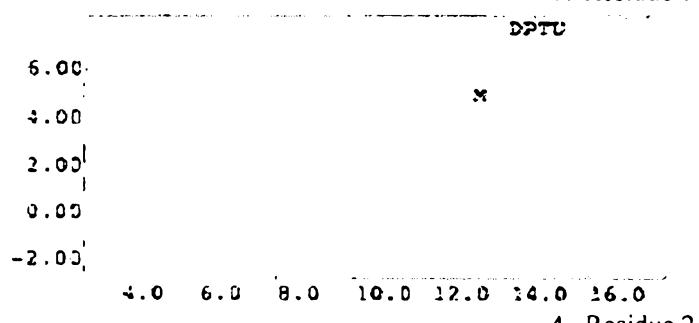
FIGURE 9

The sequencing of the N-terminal amino acid sequence

2: Standard 1



3: Residue 1



4: Residue 2

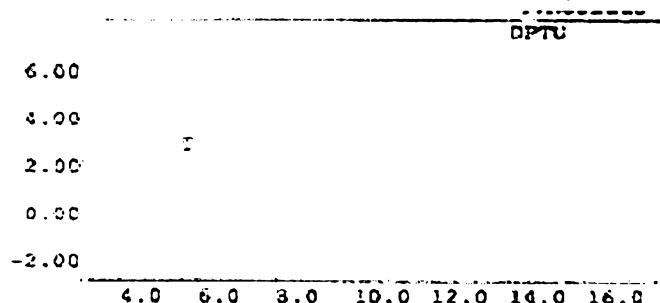


FIGURE 10

Assay for the specific biological activity of YPEG-G-CSF

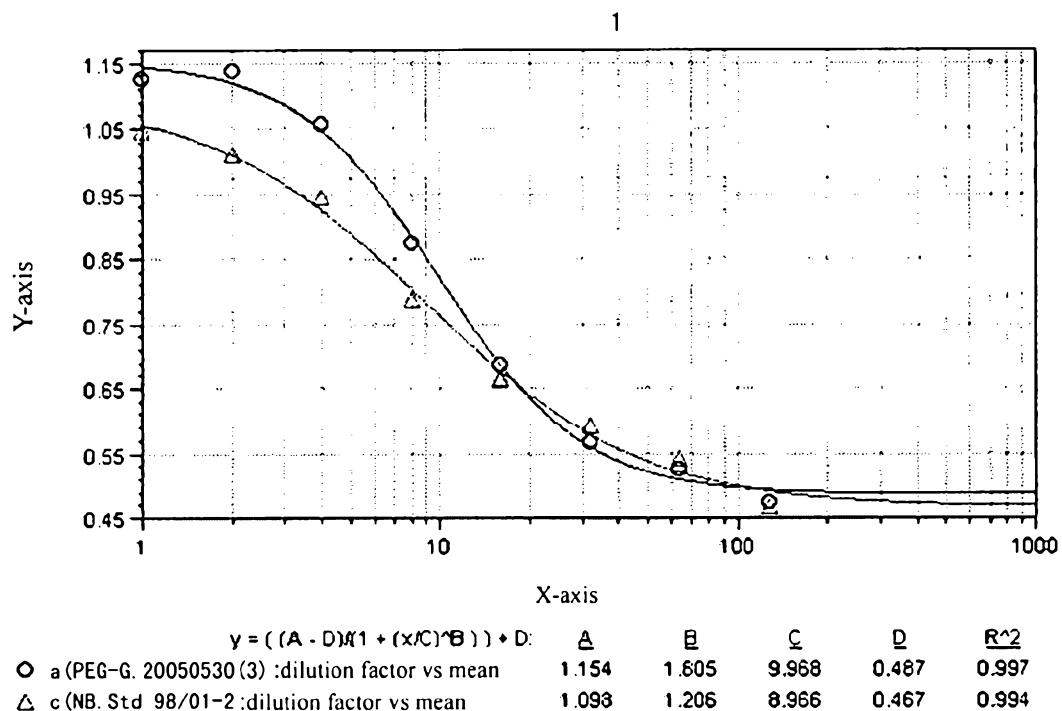


FIGURE 11

Effect of YPEG-rHuG-CSF on the logarithm value of the number of neutrophils in ^{60}Co irradiated monkey

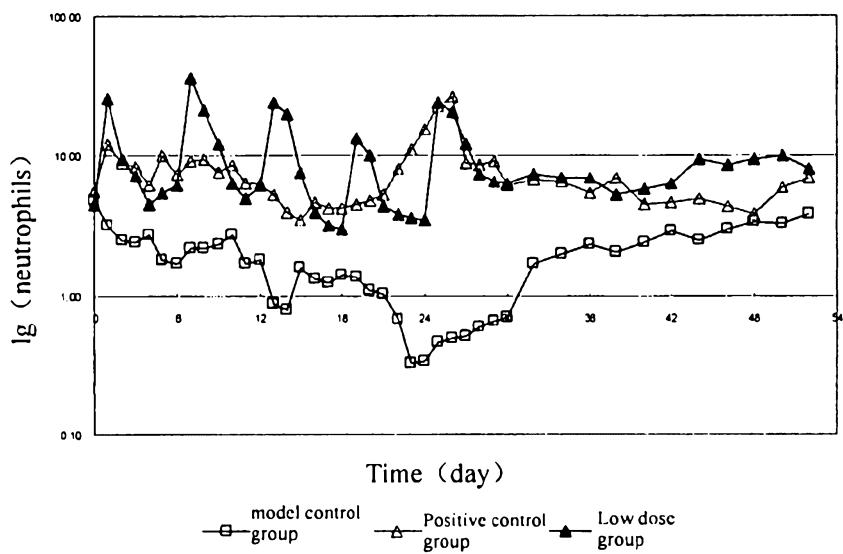
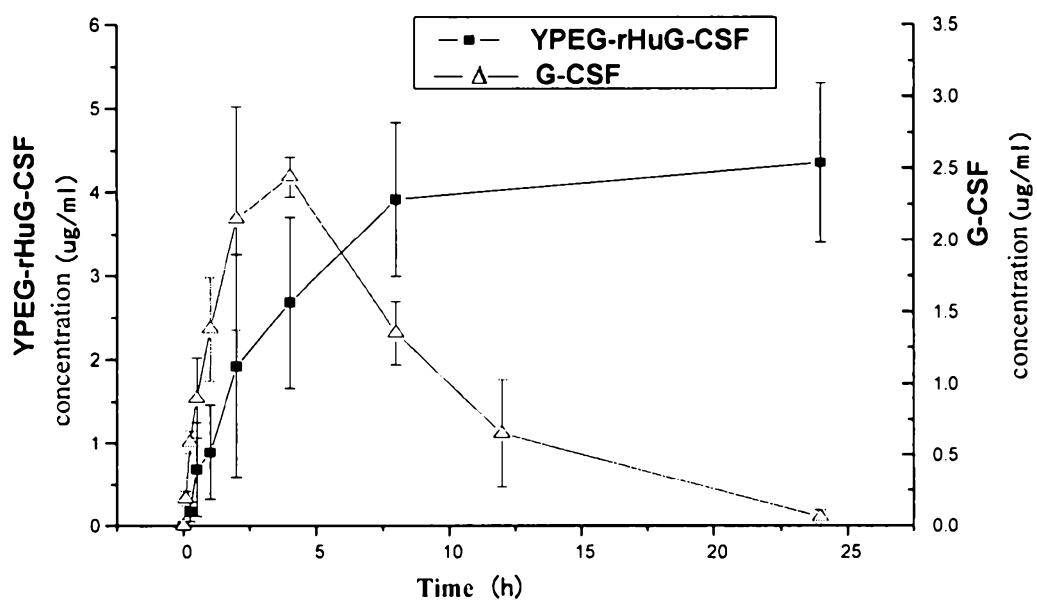


FIGURE 12

Comparison of pharmacokinetic curves of PEG-GCSF and G-CSF



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sequence listing.txt
SEQUENCE LISTING

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Gln Glu Lys Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu
35 40 45

Val Leu Leu Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser
50 55 60

Cys Pro Ser Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His
65 70 75 80

Ser Gly Leu Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile
85 90 95

Ser Pro Glu Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala
100 105 110

Asp Phe Ala Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala
115 120 125

Pro Ala Leu Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala
130 135 140

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

Phe Leu Glu Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
165 170 175

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