

A NOVEL CONJUGATE OF GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF) WITH POLYETHYLENE GLYCOL

The present invention is related to pharmaceuticals and medicine, namely, to new physiologically active conjugates of granulocyte colony-stimulating factor (G-CSF), in particular, to the new G-CSF conjugate with polyethylene glycol, suitable for medical use, for example, for the treatment of leukopenia, mainly various types of neutropenia in patients treated with myelosuppressive chemotherapy, patients undergoing hematopoietic stem cell transplantation, patients with chronic neutropenia, patients with AIDS and other infections.

G-CSF is a hematopoietic growth factor that stimulates proliferation, differentiation and maturation of granulocytes (Metcalf, 1992). Introduction of exogenous G-CSF causes rapid, specific and dose-dependent increase of neutrophils in peripheral blood (Welte et al, 1985; Hartung et al, 1995).

Human G-CSF gene was cloned and expressed in bacterial and mammalian cells; as a result, various variants of recombinant human G-CSF (rhG-CSF) have been developed and purified (Nagata et al., 1986; Souza et al., 1986; Komatsu et al., 1987).

There are two variants of rhG-CSF - filgrastim and lenograstim, obtained from E.coli and CHO cells, respectively. Filgrastim consists of 175 amino acids, it is not glycosylated and has the same amino acid sequence as the natural protein, but contains an additional methionine residue at the N-terminus of the molecule. Lenograstim consists of 174 amino acids, it is glycosylated and its amino acid sequence is fully consistent with that of natural human G-CSF.

Various rhG-CSF products which differ in glycosylation and/or protein sequence are available for clinical use for treatment of neutropenia occurring after chemotherapy, idiopathic, congenital and cyclic neutropenia; for the treatment of severe infections in combination with antibiotics and in neutropenic AIDS patients (Molineux, 2004).

However, the clinical use of rhG-CSF has been limited because of rapid absorption from the subcutaneous injection site, proteolytic instability, large volume of distribution, rapid clearance and short serum half-life (Mashkovsky, 2006; de Wit et al., 1996).

As a result, rhG-CSF must be administered to patients by daily injection for optimal effectiveness. And daily monitoring of complete peripheral white blood cell counts must be performed.

The therapeutic efficacy of G-CSF can be improved by using longer-acting medicinal forms, in which the native protein molecule is chemically bound to monomethoxy polyethylene glycol (mPEG). PEGylation of G-CSF leads to improved pharmacokinetics, increased half-life,

reduced clearance, reduced fluctuations of concentration in the blood, reduction of immunogenicity and toxicity, increased activity *in vivo* and increased stability (Molineux, 2003; Molineux, 2004).

Biological properties of PEG-G-CSF conjugates are largely dependent on the molecular weight and type of the used activated mPEG. The reactive functional groups of activated PEGs can then be attached to a specific site on protein, mainly on an amine, a sulfhydryl or other nucleophilic groups. In most cases, the preferred site of modification is the amino group of lysine and the N-terminus of polypeptide chain (Kinstler et al. 1996, Roberts et al., 2002). A wide range of mPEG derivatives has been used for amine PEGylation, for example: PEG-triazine, PEG-succinimidyl carbonate, PEG-succinimidyl succinate, PEG tresylate, PEG-aldehyde, PEG-N-Hydroxysuccinimide-activated esters, etc).

There are several known PEG-G-CSF conjugates.

The patent US 2007/0014762 describes production of PEG-G-CSF conjugates using a variety of activated mPEGs with a molecular weight of 5000 Da, in particular mPEG-succinimidyl butanoate, mPEG-succinimidyl propionate, mPEG-succinimidyl a-methylbutanoate. To obtain the conjugates the rhG-CSF derived from CHO cells was used, containing several amino acid substitutions in different positions. The conjugation reaction was conducted at pH 7-9. The resulting PEG-G-CSF conjugate consisted of several positional isomers; each being conjugated with a PEG 5000 at a different site with epsilon-amino groups of lysine residues and alpha-amino group of the N-terminal amino acid. The PEG-G-CSF conjugate was purified by ion exchange chromatography on a column of SP-Sepharose.

There is no data on activity and purity of the obtained conjugates in the patent.

Disadvantages of the resulting conjugate are as follows:

1. Low molecular weight of the mPEG conjugated to the G-CSF;
2. Formation of several positional isomers, in which PEG was conjugated with G-CSF molecule through the free amino groups of different lysine residues and the N-terminal amino acid.

The patent EP 0401384 describes PEG-G-CSF conjugates, obtained using mPEG of linear and branched structures with a molecular weight of 4,500 - 10,000 Da, activated by different reactive groups (succinimidyl succinate, chloro-s-triazine, polyoxyethylenediamine). These PEG-G-CSF conjugates had a prolonged effect *in vivo*, and the degree of prolongation was dependent on the molecular weight of the conjugated PEG. Half life of PEG-G-CSF conjugate with PEG 10 000 and unmodified G-CSF was 7.05 and 1.79 h, respectively

Disadvantages of the resulting conjugates are:

Succinimidyl succinate -PEG is prepared by reaction of mPEG with succinic anhydride, followed by activation of the carboxylic acid to the succinimidyl ester. The polymer backbone contains a second ester linkage that remains after the conjugation reaction with a protein. This linkage is highly susceptible to hydrolysis after the polymer has been attached to the protein. Not only does this hydrolysis lead to loss of the benefits of PEG attachment, but the succinate tag that remains on the protein after hydrolysis can act as a hapten and lead to immunogenicity of the remaining protein (Roberts, 2002).

1. Chloro-s-triazine mPEG derivatives are able to conjugate with the functional groups of lysine and cysteine residues, which leads to the emergence of many isomers, some of which are characterized by an unstable bond. Moreover, the triazine mPEG derivatives are currently not used because of their high toxicity (Veronese & Pasut, 2005).

The patent EP 0335423 describes G-CSF conjugates with triazine mPEG derivatives with a molecular weight of 300 - 30 000 Da. These PEG-G-CSF conjugates had a prolonged action, their specific activity ranged from 11 to 60% of the activity of unmodified G-G-CSF.

Disadvantages of the resulting conjugates are:

1. Low specific activity.

2. Use of the mPEG-triazine derivatives, that resulted in a number of positional isomers, some of which are characterized by an unstable bond. Moreover, the mPEG-triazine derivatives are currently not used because of their high toxicity (Veronese & Pasut, 2005);

As a prototype of the present invention, a PEG-G-CSF conjugate as described in the patent US 5,824,784 (Example 2) has been chosen. The patent describes the reaction of reductive alkylation of E. coli derived rhG-CSF with linear mPEG with a molecular weight of 6,000 to 25 000, with an activated propionaldehyde group (ALD-mPEG). The PEGylation reaction was conducted at pH 5. Under acidic conditions aldehyde is largely selective for the N-terminal α -amine because of the lower pK of the α -amine compared to other nucleophiles. Coupling of aldehyde proceeds through a Schiff base that is reduced *in situ* to give a stable secondary amine linkage (Kinstler et al., 1996).

N-terminal PEGylation of rhG-CSF prepared in this manner with PEG 20 kDa was employed in the development of pegfilgrastim ("Neulasta") used in the treatment and prevention of different neutropenic states.

The mono-mPEG-G-CSF conjugate was isolated by ion-exchange chromatography using a SP Sepharose HP column. Biological activity of the resulting purified PEG-G-CSF conjugate was 68% of the native G-CSF activity. The content of unmodified G-CSF in the resulting conjugate was no more than 5%. Pharmacokinetic parameters of the conjugate were

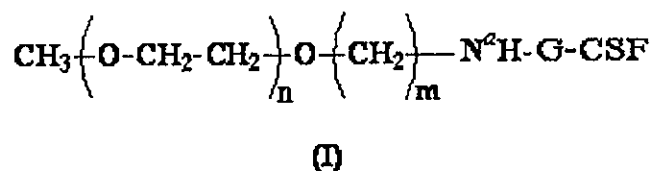
better than those of unmodified G-CSF. Animal experiments have shown that the introduction of PEG-G-CSF conjugate led to increased white blood cell count, and the PEG-G-CSF conjugate had more prolonged effect compared to unmodified G-CSF. When using a single dose of G-CSF PEG-conjugate, the content of white blood cells of animals reached a maximum after 1 day after injection, this level was maintained during the day and then the level of white blood cells decreased to baseline level 4 days after injection. With the introduction of unmodified G-CSF, the maximum level of leukocytes in blood of animals was observed after 1 day after injection, then the content of leukocytes rapidly decreased.

Disadvantages of the resulting conjugate are:

1. Low specific activity of the obtained conjugate (68% of the activity of unmodified G-CSF);
2. The presence of unmodified G-CSF in the conjugate.

The aim of the present invention was to obtain a new stable highly purified PEGylated G-CSF conjugate with high activity, with more prolonged effect, improved stability, improved pharmacokinetic parameters, with optimal combination of parameters of molecular weight of PEG and specific activity, with high degree of purity and suitable for medical use, as well as pharmaceutical compositions based on the claimed conjugate.

The problem is solved by creation of a new functionally active molecule of the PEG-G-CSF conjugate with the activity of G-CSF, in which linear PEG molecule with a molecular mass of 30 000 Da is conjugated with the G-CSF molecule by a stable bond strictly through the alpha amino group of the N-terminus amino acid (methionine) resulting in a compound with the following formula (I):



where:

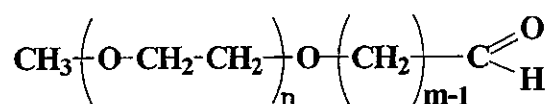
n – integers from 681 to 1 000;

m – integer ≥ 4 ;

$\text{N}^a\text{H}-\text{G-CSF}$ – natural or recombinant polypeptide having the activity of G-CSF.

In the derived conjugate a linear PEG with molecular weight 30 000 - 40 000 Da is conjugated with the alpha amino group of the N-terminus amino acid of G-CSF molecule.

Selectivity of G-CSF modification via alpha-amino N-terminus amino acid is achieved through the use of aldehyde-activated mPEG with general formula (II) and the PEGylation reaction at $\text{pH} \leq 5$:



(II)

n – integers from 681 to 1 000; the molecular weight of PEG is about 30 000 - 40 000 Da;

m – integer ≥ 4 ;

The optimal ratio of PEG molecular weight and specific G-CSF activity is achieved through the use of activated mPEG (II) with a value of $m \geq 4$, which leads to more effective interaction of the claimed conjugate with receptors resulting in the higher biological activity. Reduced immunogenicity, toxicity and improved pharmacokinetic parameters are achieved by increasing the molecular weight of the attached PEG. New features of the invention in comparison with the prototype are:

1. Aldehyde derivatives of the activated mPEG (formula II) are used for production of the claimed PEG-G-CSF;
2. Structural formula of the PEG-G-CSF conjugate is new;
3. The molecular weight of the PEG-IFN conjugate is increased due to the weight of the PEG;
4. The level of the specific activity is higher;
5. Pharmacokinetic parameters are improved.

The claimed PEG-G-CSF conjugate is highly purified, with a prolonged effect and is characterized by a high specific biological activity (at least 84-94% of the unmodified G-CSF), the purity of the protein $\geq 97\%$, high thermal stability, increased resistance to the action of protein-degrading enzymes, improved pharmacokinetic parameters.

Also the present invention includes pharmaceutical compositions containing claimed PEG-G-CSF conjugate in an effective quantity as the active ingredient. These compositions may also include pharmaceutically acceptable carriers, buffer agents (organic and inorganic acids and their salts, such as citrate, succinate, tartrate, fumarate, gluconate, oxalate, lactate, acetate buffers), stabilizers (sugar alcohols, amino acids, organic sugar or sugar alcohol, inositol, polyethylene glycol, polymers of amino acids, sulfur-reducing agent, such as urea, glutathione, thioglycerol etc., polypeptides with low molecular weight proteins such as human serum albumin, immunoglobulin, hydrophilic polymers such as polyvinylpyrrolidone, monosaccharides such as mannose, xylose, fructose, glucose, disaccharides such as lactose, maltose, sucrose, trisaccharides such as raffinose, and polysaccharides such as dextran),

preservatives (phenol, benzyl alcohol, metacresol, methyl paraben, propyl paraben, octadecyl dimethyl benzyl ammonium chloride, benzalkan, halogenides, alkyl parabens), antioxidants (methionine, vitamin E, ascorbic acid), isotonic agents (sugar alcohols (sodium chloride, sorbitol, mannitol, arabitol, xylitol, etc.), nonionic surfactants and detergents (polysorbate, polyols, twin), co-solvents, fillers (starch), chelating agents (eg, EDTA). These pharmaceutical compositions can be applied in various forms, for example, lyophilizate or liquid formulation (injections, sprays, drops, etc.).

The present invention also includes medicines on the basis of the claimed conjugate, in particular, for treatment of neutropenia.

The claimed PEGylated G-CSF, as well as pharmaceutical compositions and medicinal products based on it can be used for the treatment of neutropenia occurring after chemotherapy, to improve tolerability of immunosuppressive medical drugs in bone marrow transplantation, to improve immune status patients suffering from AIDS and other infections, in particular systemic or invasive candidiasis.

Conjugate of the formula (I) with an optional pharmaceutically acceptable filler, diluents and / or pharmaceutically acceptable excipients is administered intravenously, subcutaneously, intramuscularly or by any other suitable means. Route of administration vary depending, for example, on symptoms and age, frequency of administration and the interval between injections. Depending on the disease and its severity or the aim of administration (therapeutic or prophylactic use), an effective amount of the PEG-G-CSF agent is selected according to the abovementioned factors.

To obtain the claimed conjugate PEG-G-CSF a highly purified recombinant human G-CSF (filgrastim, produced by CJSC "Biocad") was used as well as butyraldehyde mPEG derivatives of formula (II) with a molecular mass of 30 000 - 40 000 Da.

The conjugation reaction was conducted at a pH below 5.0 in the presence of a reducing agent at a temperature at or below 20°C. The molar ratio of PEG/protein was 2,5-5/1.

The reaction progress was monitored by SDS-PAGE in reducing conditions and by RP-HPLC. MonoPEG-G-CSF (G-CSF with a single PEG chain per molecule) was purified from the reaction products (unmodified G-CSF and undesirable forms of PEG-G-CSF, containing two or more chains of PEG per protein molecule) by cation-exchange chromatography. MonoPEG-IFN conjugate elution was performed by linear gradient of concentration of sodium chloride from 0.05 to 0.2 M in buffer solution with pH below 5. The purified monoPEG-G-CSF was dialyzed against 10-50 mM buffer solution at pH 4-5, added salt, or polysaccharides, or alcohols, or polyvinylpyrrolidone, or monosaccharides, and amino sugars, or proteins, or amino

acids, and nonionic detergents and stored in plastic or glass bottles with siliconized surface at 4 ± 2 °C.

To characterize the resulting monoPEG-G-CSF conjugate its purity, homogeneity, physical-chemical, biological and pharmacokinetic parameters were investigated in comparison with the unmodified G-CSF.

The proposed invention is illustrated by following figures:
Fig. 1. Kinetics of G-CSF PEGylation.

Fig. 2. Reverse phase high performance liquid chromatogram (RP-HPLC) of the PEG-G-CSF conjugate.

Fig. 3. Size exclusion high performance liquid chromatogram (SEC-HPLC) of the PEG-G-CSF conjugate.

Fig. 4. SDS-PAGE analysis of the PEG-G-CSF conjugate (line 1) and unmodified G-CSF (line 2).

Fig. 5. MALDI mass spectra of unmodified G-CSF (A) and PEG-G-CSF conjugate (B).

Fig. 6. Comparison of mass spectra of tryptic peptides of G-CSF (A) and PEG-G-CSF conjugate (B).

Fig. 7. Thermal stability of PEG-G-CSF conjugate (green line) and unmodified G-CSF (red line) at $+(50 \pm 2)$ °C

Fig. 8. Proteolytic stability of PEG-G-CSF conjugate (green line) and unmodified G-CSF (red line).

Fig. 9. Immunoreactivity of PEG-G-CSF conjugate (green line) and unmodified G-CSF (red line).

Fig. 10. Pharmacokinetics of the PEG-G-CSF conjugate (green line) and unmodified G-CSF (red line).

Examples of specific performance of PEG-G-CSF production method and study of its properties are given below.

EXAMPLE 1

Preparation of recombinant human G-CSF(rhG-CSF, filgrastim)

Isolation and purification of rhG-CSF was performed as described in patent RU 2278870.

EXAMPLE 2

Preparation of pegylated rhG-CSF.

50 ml of 1 M solution of sodium cyanoborohydride were added to 2300 ml buffer (50 mM sodium acetate, pH 5.0 ± 0.2), containing 2500 mg of purified rhG-CSF, prepared in accordance with example 1. The mixture was stirred, then 10 grams of solid mPEG-ButyrALD (Methoxy-PEG-Butyraldehyde) with an average molecular weight, 30 kDa were added. The conjugation reaction mixture was stirred for 22 hours at $(20 \pm 2) ^\circ\text{C}$. After various time intervals 50 microliters samples were taken from the reaction mixture and the kinetics of PEG-G-CSF conjugate formation was analyzed using SDS-PAGE on 12,5% gel in reducing conditions. For that, to the selected sample 17 microliters of a buffer containing 125 mM Tris-HCl, pH 6.8, 20% glycerol, 3% SDS, 15% 2-mercaptoethanol, 0,005% bromphenol blue was added, and it was heated for 3 min on a boiling water bath. Samples in the volume of 5 microliters were loaded into each wells. SDS-PAGE was performed using mini Protean System (BioRad) The gel was stained with Coomassie R-250. Kinetics of G-CSF PEGylation is shown in Fig. 1. At the time when the content of PEG-G-CSF was more than 70%, the reaction mixture was diluted 3 times in 10 mM sodium acetate buffer, pH $4,8 \pm 0.2$.

EXAMPLE 3

MonoPEG-G-CSF conjugate purification

The diluted reaction mixture prepared in accordance with Example 2 was loaded onto a column packed with 300 mL of CM-sepharose equilibrated with 10 mM sodium acetate buffer, pH 4.8 (buffer A) at flow rate of 10 ml / min. After loading of the material the column was washed to remove unbound material with 1500 ml of buffer A. A linear gradient 0-0,2 M of NaCl in buffer A (6000 mL) was used to elute the monoPEG-G-CSF from the column at flow rate of 5 ml / min. Fractions (50 mL) were collected and the absorbance at 280 and 260 nm was determined. Fractions containing protein were analyzed by RP-HPLC and SDS-PAGE as

described in Example 2. Fractions containing monoPEGylated G-CSF with a purity of over 95%, were pooled, dialyzed against 10 volumes of 1.6 mM sodium acetate buffer, pH 4.0 ± 0.2 . Then sterile filtration was performed and samples were stored at 4 ± 2 °C.

EXAMPLE 4

Purity analysis of PEG-G-CSF conjugate by reverse-phase high performance liquid chromatography (RP-HPLC).

The purified PEG-G-CSF conjugate, prepared in accordance with Example 3, was diluted with 20 mM sodium acetate buffer pH 5.0 to a protein concentration of 0.3 mg / ml. Analytical RP HPLC was performed employing a gradient Waters 'Breeze' chromatograph on a C4 Symmetry column (4.6x150 mm) with UV detection at 214 nm. 100 microliters of the obtained sample was injected into the column. The mono PEG-G-CSF is shown by RP_ HPLC (Fig. 2) eluted from the column as a single, symmetrical peak and to be highly pure, as judged by the presence of only minor impurities eluting before and after the main UV-absorbing peak (FIG. 2). The impurities amount to less than 1,15% of the material eluting in the main peak. Thus, from the data presented it can be concluded that according to RP-HPLC the purity of the claimed PEG-G-CSF conjugate is $\geq 98\%$.

EXAMPLE 5

Purity analysis of PEG-G-CSF conjugate by high performance size-exclusion chromatography (SEC-HPLC).

The purified PEG-G-CSF conjugate, prepared in accordance with Example 3, was diluted with 20 mM sodium acetate buffer pH 5.0 to a protein concentration of 0.3 mg / ml. Analytical SEC HPLC was performed employing a Waters 'Breeze' chromatograph on a TSK G3000SWX column (30cm x 7.8mm). with UV detection at 214 nm. 100 microliters of the obtained sample was injected into the column. The results of SEC-HPLC are presented on Fig. 3. It is seen that the sample of PEG-G-CSF eluted from the column as one distinct symmetrical peak, constituting 98.47%. From the results presented in Fig. 3, it follows that the claimed PEG-G-CSF conjugate does not contain free modified G-CSF. The content of high-molecular weight aggregates in the claimed conjugate does not exceed 1.53%. Purity of the claimed PEG-G-CSF conjugate according to the SEC HPLC is more than 98%.

EXAMPLE 6

Determination of the endotoxin level in the PEG-G-CSF conjugate

The content of bacterial endotoxins (BE) in the PEG-G-CSF conjugate sample, prepared in accordance with Example 3, was determined in vitro using the Limulus Amebocyte Lysate (LAL) method (modification of the gel clot test) in accordance with the requirements of the FFS 42-0002-00. A multi component diagnostic kit «Associates of CAPE COD, Inc.», LAL-reagent PYROTELL ® with sensitivity of 0.03 EU / ml, Endotoxin Reference Standard (CSE, 0.5 mg vial) and water for LAL-test were used. The results presented in Table 1 show that the BE content in the PEG-G-CSF conjugate sample is less than 3 EU (endotoxin units) per mg of protein, which is much lower than BE level allowable for drugs based on recombinant proteins.

Table 1. The content of bacterial endotoxins in the PEG-G-CSF conjugate.

PEG-G-CSF conjugate	The content of bacterial endotoxins (EU / mg protein)
PEG-G-CSF, obtained according to the Example 3	$\leq 3,0$

EXAMPLE 7

Purity analysis of the PEG-G-CSF conjugate by SDS-PAGE

The purified PEG-G-CSF conjugate sample, prepared in accordance with the Example 3, was analyzed by, SDS-PAGE as described in Example 2. Samples containing 40 micrograms both PEG-G-CSF and unmodified G-CSF were loaded into each wells. The gel was stained with Coomassie R-250. Figure 4 illustrates the SDS-PAGE profile of PEG-G-CSF (line 1) and unmodified G-CSF (line 2).. The purified PEG-G-CSF appeared as single diffuse band with an apparent molecular weight higher than that in unmodified G-CSF. It has to be noted that the exact value of molecular weight for PEGylated proteins could not be determined by SDS-PAGE, since conjugation of hydrophilic PEG molecules to the protein increases the Stokes radius of the PEGylated protein. As a result, the electrophoretic mobility of the PEG-protein complex in the gel is slowed down and the value of its molecular weight is much higher than the sum of molecular masses of unmodified protein and PEG.

EXAMPLE 8

Mass spectrometric molecular-weight determination of PEG-G-CSF conjugate in comparison with the unmodified G-CSF

Matrix assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) was used to determine of the true molecular weight of PEG-G-CSF. MALDI TOF MS was performed with an Ultraflex II (Bruker Daltonics) mass spectrometer equipped with a UV laser (Nd). 1 microliters of PEG-G-CSF conjugate sample, prepared in accordance with the Example 3, and 0.3 microliters of the 2,5- dihydroxybenzoic acid solution (Aldrich, 10 mg x ml⁻¹ in 20% acetonitrile in water with 0.5% TFA) were mixed and dried in the air. In the same way unmodified G-CSF sample was prepared.

Mass spectra were obtained in the positive mode MS spectra, the average mass errors does not exceed 10-15 Da.

MALDI-TOF mass spectra of unmodified G-CSF and PEG-conjugated G-CSF in the range m / z 10 000 - 50 000 are shown in Fig. 5.

Fig. 5A shows that the mass spectrum of G-CSF contains the main peak corresponding to a singly charged ion [M]⁺ with a molecular weight of 18,816 Da.

In the mass spectrum of the PEG-G-CSF conjugate that is shown on Fig. 5B, a broad spectral peak with a center of mass around 49 600 Da is present. The broad spectrum is due to the heterogeneous population of the PEG polymer species.

Considering the molecular weights of G-CSF (19 295 Da) and PEG (30 000 Da), the obtained mass of the conjugates (49 600) was very consistent with the calculated mass sum of individual molecules.

EXAMPLE 9

PEGylation site determination in the PEG-G-CSF conjugate

5 microliters of the solution of the modified trypsin (Promega) in 0.05 M NH₄HCO₃ with a concentration of 15 mcg×ml⁻¹ were added to 5 microliters of the PEG-G-CSF conjugate probe, prepared according to Example 3. Hydrolysis was carried out during 16 h at the temperature 37 °C, then 10 microliters 0.5 % TFA in 10% acetonitrile solution in water were added to the solution and thoroughly mixed. In the same way the unmodified G-CSF sample was prepared.. These tryptic digests were analysed by MALDI-MS.

The site of PEG conjugation with a G-CSF molecule was determined by comparing the mass spectra of tryptic peptides of the PEG-G-CSF conjugate and unmodified G-CSF. In the tryptic digest of the PEG-G-CSF conjugate there should be no peptide corresponding to the portion of the protein where a modification occurred. In Table 2 shows the mass spectra of experimental tryptic peptides for unmodified G-CSF and PEG-G-CSF conjugate. The table shows that the mass spectra of tryptic peptides of the unmodified G-CSF almost coincide with those of the PEG-G-CSF conjugate. (Table 2), but in the tryptic digest of the PEG-G-CSF conjugate there are no peptides with a molecular mass 1432.7, present in the tryptic digest of the unmodified G-CSF. The experimentally obtained mass spectra of tryptic peptides for unmodified G-CSF and PEG-G-CSF conjugate are presented in Fig. 6, which shows that there is no peptide with a molecular weight of 1432.7 in the tryptic digest of the PEG-G-CSF conjugate. According to the analysis of theoretical masses of tryptic peptides of G-CSF, the peak at m/z 1432.7 corresponds to the N-terminus peptide of protein (Table 2). Its absence in tryptic digest of the PEG-G-CSF conjugate matches modification of the N-terminus peptide, which became heavier by the weight of PEG, which is outside the studied spectral area. The conjugation of PEG with a G-CSF molecule occurred at the only free amino group present in this peptide - amino group of the N-terminus methionine.

Tab. 2. Experimental mass spectra of the tryptic peptides of the PEG-G-CSF conjugate and unmodified G-CSF compared with theoretical values.

experimental peptide's m/z		Theoretical peptides		
G-CSF	PEG-G-CSF	<i>Mass</i>	<i>Peptides</i>	<i>Sequence</i>
1432.7	-	1432.75	1-14	MTPLGPASSLPQSF
747.4	747.4	747.36	18-23	CLEQVR
1129.6	1129.6	1129.61	20-29	EQVRKIQGDG
1359.7	1359.7	1359.75	42-53	LCHPEELVLLGH
2527.3	2527.3	2527.30	54-78	SLGIPWAPLSSCPSQ
953.6	953.6	952.56	55-63	LGIPWAPLS
818.4	818.4	817.41	61-68	PLSSCPSQ
2033.1	2033.1	2033.10	72-90	LAGCLS QLHSG LFL
2104.1	2104.1	2104.14	75-93	CLS QLHSG LFL YQG
1241.7	1241.7	1241.68	158-167	LQSFLEVSYR

EXAMPLE 10

Determination of specific activity of PEG-G-CSF specific conjugate compared with unmodified rhG-CSF

PEG-G-CSF conjugate, prepared according to Example 3, diluted to a concentration of 0.2 ng / ml of RPMI medium and the serial dilutions of the samples to be tested were prepared in assay media. Serial dilutions of the unmodified G-CSF and international reference standard of G-CSF activity (1-st International Standard 88/502, granulocyte colony stimulating factor, human, rDNA-derived, 10000 IU/amp) were analyzed in parallel. 100 microliters of the diluted protein samples were added to the test wells of a flat-bottom 96-well tissue culture plate. For bioassays the M-NFS cells were washed and resuspended at a concentration of $1,5 \times 10^5$ /ml in RPMI media. 100 microliters of the cell suspension were added to each well and the plates incubated at 37 °C in a 5% CO₂ tissue culture incubator for 50 hrs. Then, in each well 20 microliters of dye Alamar Blue (or similar, for example, MTT) were added and plates incubated under the same conditions for 14 hours.

The increase in the number of cells was determined using a microplate reader by an increase of optical density at a wavelength of 545 nm and 630 nm. The specific biological activity of the tested samples (B_0) in IU / mg was calculated using the formula:

$$B_0 = \frac{A_0}{C},$$

where, B_0 – specific biological activity of PEG-G-CSF or G-CSF (IU / mg);

A_0 - biological activity of PEG-G-CSF or G-CSF (IU / ml);

C – protein content in the solution of PEG-G-CSF or G-CSF (mg / ml).

Table 3. Specific activity of PEG-G-CSF and unmodified G-CSF

Preparation	Specific activity (IU / mg)
Conjugate PEG-G-CSF, prepared according to Example 3	$(0,845 \pm 0,091) \times 10^8$
Unmodified G-CSF	$(1,00 \pm 0,081) \times 10^8$

EXAMPLE 11

Thermal stability analysis of PEG-G-CSF in comparison with the unmodified G-CSF

A sample of PEG-G-CSF conjugate, prepared in accordance with example 3, diluted with 5 mM sodium acetate buffer, pH 4.0, containing 140 mM NaCl, the protein concentration up to 0.28 mg / ml, was placed in a water bath at a temperature $(50 \pm 2)^\circ\text{C}$ and at various intervals of time the appearance of turbidity in the solution of the protein was studied by measuring the absorbance at 340 nm. Similarly, the study of thermal stability of unmodified G-CSF was conducted. Incubation of proteins at a temperature $(50 \pm 2)^\circ\text{C}$ leads to the formation of insoluble aggregates, resulting in the increased turbidity of protein solution, which can be measured by determining the optical density of the solution at 340 nm. Fig. 7 presents the results of the study, which show that the turbidity of the preparation of unmodified G-CSF increases with time of incubation, whereas the preparation of PEG-G-CSF remained stable during the whole period of observation. These data indicate that the thermal stability of the claimed PEG-G-CSF conjugate is much higher than that of unmodified G-CSF.

EXAMPLE 12

Proteolytic stability of PEG-G-CSF in comparison with the unmodified G-CSF

A sample of PEG-G-CSF conjugate, prepared in accordance with example 3, was diluted to a concentration of 0.6 mg / ml, then 150 microliters of 1M Tris-HCl, pH 8.5 was added to 850 microliters of resulting sample, so that the final pH of the sample was 7.4. Then 2 microliters 0,5 M CaCl_2 were added to a final concentration of 1 mM, stirred, then 15 microliters of trypsin (Promega, Gold) were added to a final concentration of 0.02 micrograms/ml. Similarly sample containing unmodified G-CSF was prepared. The samples were incubated at 37°C . Subsamples (100 microliters) were taken every 60 min and 20 microliters 10% SDS was added to stop the tryptic reaction and SDS-PAGE was performed, as described in Example 1.. After electrophoresis gels were stained with Coomassie-R-250 and densitometric analysis of stained gels was performed. The area of the main band was determined by a computer program GelPro Analyser. The area of the main band in the initial (untreated with trypsin) sample was taken as 100%. Fig. 8 presents data on the sensitivity of the PEG-G-CSF conjugate and G-CSF to the treatment by trypsin. It is evident that with increasing time of trypsin treatment the

content of PEG-G-CSF and G-CSF reduces, but claimed PEG-G-CSF conjugate is more resistant to the action of trypsin in comparison with the unmodified G-CSF.

EXAMPLE 13

Immunoreactivity of PEG-G-CSF conjugate in comparison to unmodified G-CSF

Immunoreactivity of the claimed PEG-G-CSF conjugate was determined by antibody binding activity using a modification of the ELISA assay with the use of a set of reagents ProCon G-CSF ("Protein contour" LLC). PEG-G-CSF conjugate, prepared in accordance with example 3, was diluted in 10 mM Tris-HCl buffer, pH 7.2, containing 140 mM NaCl and 1% bovine serum albumin to a concentration of 1 mkg/ ml. Then 100 microliters of the obtained samples were added into the wells of the microtiter plate previously coated with monoclonal antibodies against G-CSF. After incubation at 37 °C (60 min) monoclonal antibodies to independent epitope, labeled by biotin were added into the wells. The resulting immune complex was detected using streptavidin, labeled by horseradish peroxidase. For the development of color reaction in the wells 100 microliters of substrate solution (200 micrograms / ml tetramethylbenzidine («Sigma») and 0,1% H₂O₂ in 0.05 M TB, pH 7,2 were added and incubated until the development of blue color. The reaction was stopped by introducing 50 microliters 2 M sulfuric acid solution into the wells. The results were taken into account, measuring the absorbance at a wavelength of 405 nm on a microplate reader «Multiscan» EX. Similarly the immunoreactivity of the unmodified G-CSF was studied. Interaction of unmodified G-CSF with immunoglobulins was taken as 100%. From the results presented in Fig. 9, it is seen that the immunoreactivity of the claimed PEG-G-CSF conjugate is 25% of the immunoreactivity of unmodified G-CSF.

EXAMPLE 14

Determination of the PEG-G-CSF conjugate stability during storage

Sample, prepared according to the Example 3, was diluted to 1 mg / ml in 1.6 mM sodium acetate buffer, pH 4.0 ± 0.2. From the diluted sample aliquots were taken and placed into sterile tubes with lids, type "Eppendorf". These were stored at a temperature (6 ± 2) ° C. Specific activity and homogeneity of PEG-G-CSF conjugate were determined at different time intervals of storage. Homogeneity of the PEG-G-CSF was analyzed using SDS-PAGE and

SEC-HPLC as described in Example 1 and Example 5, respectively. Table. 4 shows that PEG-G-CSF conjugate is stable for at least 24 months when stored at a temperature $(6 \pm 2)^\circ \text{C}$

Tabel. 4. The stability of the claimed PEG-G-CSF conjugate at $(6 \pm 2)^\circ \text{C}$.

Parameters	Shelf life (months)				
	0	6	12	18	24
Activity (IU / mg)	$(0,84 \pm 0,09) \times 10^8$	$(0,75 \pm 0,15) \times 10^8$	$(0,9 \pm 0,10) \times 10^8$	$(0,80 \pm 0,15) \times 10^8$	$(0,86 \pm 0,07) \times 10^8$
Homogeneity by HPLC GF (%)	>95	>95	>95	>95	>95
Homogeneity by EF in PAGE (%)	> 98	> 98	>98	> 98	> 98

EXAMPLE 15

Pharmacokinetic analysis of PEG-G-CSF conjugate

A 5 micrograms sample of PEG-IFN conjugate, prepared according to the Example 3, was injected intraperitoneally to 20 g male mice of the ICR line. In parallel, another group of mice was injected by unmodified G-CSF. Blood samples were taken by a retro-orbital puncture at different time after injection. Blood serum was obtained by the standard method.

The serum samples for measuring either native or monoPEGylated G-CSF were used in the ELISA assay with mouse monoclonal immunoglobulins to different epitopes of human G-CSF.

A wells of the microtiter plate were coated with monoclonal antibodies against G-CSF (250 ng/well in 100 microliters of 20 mM Tris-HCl buffer (TB), pH 9.0. After an overnight incubation at 4°C , 200 microliters of TB, pH 7.4, containing 1% bovine serum albumin (BSA) and 0.05% Tween-20 were added into the wells to block of nonspecific binding sites.

Then 100 microliters of the analyzed serum, diluted by TB, containing 1% BSA and 0.05% Tween-20, at a ratio of 1:2, 1:4 and 1:8 were added into the wells. In parallel for the

construction of the standard calibration curve, known concentrations of PEG-G-CSF sample in the range of 25 ng / ml to 600 ng / ml in buffer TB containing 1% BSA and 0.05% Tween-20 were added to the wells. After incubation for 1 h at 37°C, a biotinylated anti human G-CSF monoclonal antibodies and streptavidin-peroxidase conjugate were added into the wells sequentially. After incubation and washing to remove all unbound enzyme, 100 microliters of the substrate solution (100 mM sodium acetate buffer, pH 5.0, containing 0.015% hydrogen peroxide and 0,2 mg / ml tetramethylbenzidine) were added to induce a coloured reaction product. The reaction was stopped by the addition of 2 M 2M H₂SO₄. The optical density measured at 450 nm in the microplate reader. The concentration of G-CSF in serum was determined using calibration curve taking into account the dilution of the studied serum.

Calibration curve created with a known native G-CSF concentration (in the range from 6.25 ng / ml to 200 ng / ml in buffer TB containing 1% BSA and 0.05% Tween-20) was used in the analysis of unmodified G-CSF pharmacokinetics.

Results of the claimed PEG-G-CSF pharmacokinetics compared with the unmodified G-CSF are presented in Fig. 10. From the results it follows that the claimed PEG-G-CSF conjugate has a significantly prolonged effect. With the administration of non-modified G-CSF, its concentration in the blood of animals reached a maximum at 1 h after injection and then rapidly decreased. With the administration of PEG-IFN conjugate, a maximum content of G-CSF in blood of animals was observed after 6 hours (Figure 10) and then slow decrease of G-CSF in blood was observed for 140 hours. Based on the results (Fig. 10) main pharmacokinetic parameters of the claimed PEG-G-CSF conjugate were calculated. The results showed that absorption from the site of injection, volume of distribution, clearance of PEG-G-CSF conjugate were significantly slow in comparison to the unmodified G-CSF, which led to long-term circulation of the claimed PEG-G-CSF conjugate in the blood (more than 140 hours).

EXAMPLE 16

Comparative characteristics of the claimed PEG-G-CSF conjugate and PEG-G-CSF conjugates as described in the prototype method.

The comparison of the structure, basic physicochemical and pharmacokinetic parameters of the claimed PEG-G-GSF conjugate, obtained according to the Example 3 with PEG-G-CSF conjugates as described in the prototype method was performed (Table 5). The data presented in Table 5 show a significant improvement of properties of the claimed PEG- G-CSF conjugate in comparison with conjugates from the prototype method.

Table 5. The main physicochemical and pharmacokinetic parameters of the claimed PEG-G-CSF conjugate compared with PEG-G-CSF conjugates as described in the prototype method (patent US 5,824,784)

Parameters	PEG-G-CSF conjugates	
	Claimed PEG-G-CSF conjugates	Conjugate described in the prototype method (Patent US 5,824,784)
G-CSF	filgrastim	filgrastim
PEG structure	Linear	Linear
PEG molecular weight (kDa)	30-40	6-25
PEG-G-CSF conjugate structural formula	$\text{H}_3\text{C} \left[\text{O} \text{---} \text{CH}_2 \text{---} \text{CH}_2 \right]_n \text{O} \text{---} \text{CH}_2 \text{---} \text{CH}_2 \text{---} \text{CH}_2 \text{---} \text{Met-G-CSF}$	$\text{H}_3\text{C} \left[\text{O} \text{---} \text{CH}_2 \text{---} \text{CH}_2 \right]_n \text{O} \text{---} \text{CH}_2 \text{---} \text{CH}_2 \text{---} \text{CH}_2 \text{---} \text{Met-G-CSF}$
Specific activity,% of unmodified G-CSF	84-94	68
Purity according to the PAGE (%)	100	**
Purity results by HPLC (%)	>98	**
Purity results by the GF HPLC (%)	>98	**
The content of bacterial endotoxins (EU / mg)	< 3	**
Pharmacokinetic parameters		
The ratio of AUC PEG-G-CSF/G-CSF	18	**

*- PEG-G-CSF conjugate structural formula, obtained by the prototype method given in «WHO Drug Information», 2,002, v. 16, N. 1, 102, List 47.

** - no data.

EXAMPLE 17

Solution for subcutaneous injection 0.6 mg / ml, 1.0 mg / ml, 2.0 mg / ml, 3.0 mg / ml

Solution for subcutaneous injection used as a medicine containing PEGylated recombinant granulocyte colony-stimulating human factor (PEG-G-CSF) as an active ingredient and the additional pharmaceutically acceptable components in the following ratio:

Active ingredient: PEGylated recombinant granulocyte colony-stimulating human factor (PEG-G-CSF), - 0,6 - 3,0 mg

Excipients:

Polysorbate 20 - 0,04 mg

Mannitol - 50 mg

Sodium acetate trihydrate - 0,23 mg

Glacial Acetic acid up to pH 4,0

Water for injection - up to 1 ml.

EXAMPLE 18

Packaging of the finished product, for example, an aqueous solution containing PEG-G-CSF, obtained according to Example 3

In syringes from a neutral glass of the hydrolytic class I, with soldered needles, protective caps, covered with protective caps, corked with syringe plunger.

Or

In vials of neutral glass of the hydrolytic class I, corked by Teflon-coated fluororubber or butylrubber plugs covered with aluminum caps.

EXAMPLE 19

A set containing the medical drug, for example, an aqueous solution containing PEG-G-CSF, obtained according to the Example 3 as an active ingredient.

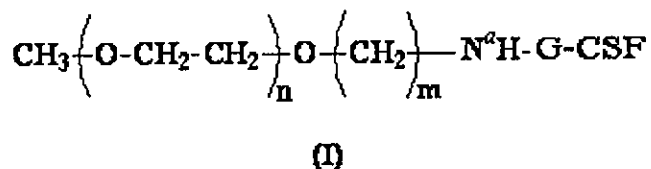
Set containing 1 syringe complete with the piston in the contour cellular packaging made of the polymer film, along with instructions for use that are placed in a cardboard package.

Or

Set containing 1 vial in the contour blister made of the polymer film, together with instructions for use, which is placed in a cardboard package

CLAIMS

1. A human granulocyte colony stimulating factor conjugated with monomethoxypolyethylene glycol of formula (I).



where:

n – integers from 681 to 1 000;

m – integer ≥ 4 ;

$\text{N}^a\text{H}-\text{G}-\text{CSF}$ – natural or recombinant polypeptide, having the activity of G-CSF.

2. A conjugate according to claim 1, wherein the average molecular weight of polyethylene glycol is from 30 to 40 kDa.
3. A conjugate according to claim 2, wherein the molecular weight of polyethylene glycol is 30 kDa.
4. A conjugate according to claim 1, wherein m is integer 4 or 6.
5. A conjugate according to claim 1, wherein the N-terminal group is a methionine residue (Met).
6. A pharmaceutical composition, having the activity corresponding to that of granulocyte colony stimulating factor, containing the conjugate of general formula (I) in the effective amount and pharmaceutically acceptable excipients.
7. A pharmaceutical composition according to claim 6, for medicinal use in treatment of neutropenia of various origins.
8. A pharmaceutical composition according to claim 6, containing the conjugate of general formula (I) in the effective amount, polysorbate, mannitol, sodium acetate trihydrate, acetic acid and water for injection to 1 ml.
9. A pharmaceutical composition according to claim 8, wherein the pH value is from 3 to 5.
10. A pharmaceutical composition according to claim 8, wherein the pH value is mainly 4.
11. A medicinal agent, having the activity corresponding to that of granulocyte colony stimulating factor, including the conjugate of general formula (I).
12. A medicinal agent according to claim 11 for treatment of neutropenia of various origin.

13. A use of conjugate of formula (I) for preparation of medicinal agent having the activity, corresponding to that of granulocyte colony stimulating factor.
14. A method of prophylaxis or treatment of neutropenia, involving administration of effective amount of the conjugate of formula (I).