THE NEW STABLE POLYETHYLENE GLYCOL CONJUGATE OF INTERFERON ALPHA, REPRESENTED BY ONE POSITIONAL ISOMER.

DESCRIPTION

The present invention is related to pharmaceuticals, namely, to physiologically active conjugates of interferon, in particular, to new conjugates of interferon with polyethylene glycol (PEG), that can be used in medicine, for example, for treatment of viral, immunological and oncological diseases.

Interferons (IFN) are a group of biologically active proteins or glycoproteins that are produced by various cells in response to viral infection or as a result of impact of certain chemical and biological substances on cells (Isaacs & Lindeman, 1957; Pestka et al., 2007). Conjugation of IFN with cell receptors leads to induction of a number of intracellular proteins that mediate antiviral, immunomodulatory and antiproliferative IFN effects (Pestka et al., 2004; Bekisz, 2004).

Genes of different human IFNs were cloned and expressed in bacterial and animal cells, as a result many recombinant IFNs have been developed (Pestka et al., 2004; Pestka, 2007). Medications containing recombinant IFN are used in clinical practice to treat a range of viral, oncological and immunological diseases (Pestka et al., 2004; Chevaliez & Pawlotsky, 2009).

At present day, medications based on interferon are used in clinical practice to treat a range of viral, oncological and immunological diseases (Pestka et al., 2004). IFN medications are most widely used for treatment of viral hepatitis (Chevaliez & Pawlotsky, 2009), which is one of the most serious social problems. Today there are about 350 million chronically infected with hepatitis B virus and 170 million patients with hepatitis C (Marcellin, 2009) all over the world. Hepatitis C in most cases assumes a chronic course leading to severe outcomes - liver cirrhosis and hepatocellular carcinoma (Modi & Liang, 2008). According to the World Health Organization, since 1961 chronic hepatitis and liver cirrhosis as the cause of death in the U.S. and Western European countries moved from 10th to 5th position (Marcellin, 2009).

Usage of medications containing IFN is common for the treatment of leukemias and solid tumors, including recurrent melanoma (Bukowski et al., 2002; Decantris et al., 2002; Qintas-Cardama et al., 2006).

The effectiveness of medications containing native IFN is limited by rapid absorption from the subcutaneous tissue, large volume of distribution, relatively low stability, short half-life, high immunogenicity and toxicity (Wills, 1990). As a result, within a few hours after the

administration, rapid drop of the IFN concentration in the blood plasma is observed, and the interferon could not be detected in plasma already after 24 h since injection (Chatelut et al, 1999). Rapid decline of interferon concentration leads to resumption of viral replication and increase of viral particles concentration (Lam et al., 1997). Therefore, there is a need for frequent IFN injections to achieve effective therapeutic concentrations in blood plasma, resulting in dose-dependent side effects. Because of this monotherapy of chronic hepatitis C (CHC) with IFN-α2b during 12 months led to a sustained virological response in only 15 - 20% of patients, while in patients with genotype Ib and high virus load the efficacy of medication was not observed at all (McHutchinson et al., 1998).

The therapeutic efficacy of IFN can be enhanced when using sustained release drugs, in particular PEGylated IFNs (Manns et al, 2001; Hadziyannis et al, 2004; Zeuzem et al., 2001). PEGylated IFNs result from chemical conjugation of interferon molecule with the polymer – for example, monomethoxypolyethylene glycol (MPEG), consisting of repeating residues of ethylene oxide with a methoxy group at one terminus and a hydroxyl group - at another. MPEG molecule may have different molecular weight and the stereochemical structure (linear or branched). For the PEGylation reaction hydroxyl group at the end of MPEG is activated by various reactive functional groups. Activated MPEG can be covalently conjugated with a protein in one or several locations, depending on the nature of the activated group and the reaction conditions (Zalipsky & Hurris, 1997; Roberts et al, 2002).

IFN PEGylation leads to better pharmacokinetics, increased half-life time, reduced fluctuations of concentration in the blood, reduced immunogenicity and toxicity, increased activity *in vivo* (with decrease in activity *in vitro*); increased stability (Glue et al, 2000; Reddy et al, 2001).

Reduced activity in the *in vitro* system depends on the PEG molecular weight. Conjugation of a small PEG molecule with molecular weight $\leq$ 5000 Da causes slight changes in activity *in vitro* (Grace et al, 2005). Pharmacodynamic and pharmacokinetic properties of PEGylated proteins containing PEG with such mass and of unmodified proteins hardly differ. Conjugation of PEG with higher molecular weight leads to a significant reduction in biological activity *in vitro* as a result of inefficient conjugation with the receptor, but in this case a significant improvement of pharmacokinetic and pharmacodynamic properties of PEGylated proteins can be seen (Delgado et al., 1992), resulting in increased biological activity in vivo (Bailon & Berthold, 1998). PEG structure also affects the biological activity and pharmacokinetic properties - conjugate with linear PEG has a larger distribution volume than conjugate with branched PEG structure (Caliceti et al, 2003).

Antiviral activity and biological properties of PEG-IFN conjugates depend also on the usage of different activated MPEGs, since their functional groups vary by their ability to modify different protein amino acid residues and by the type of chemical bonds formed with the protein (Roberts et al, 2002). For protein pegylation activated MPEGs that are able to conjugate with free amino groups of proteins are most widely used (succinimide carbonate, succinimide succinate, trisilate, triazine, hydroxysuccinimide esters, aldehyde acetal, etc.).

Following PEG-IFN conjugates are known at the moment.

A PEG-IFN-α2a conjugate with linear PEG with molecular weight 1-5 000 Da is known (Patent US No. 5,382,657, 1995). For the conjugation MPEG ethanediol derivatives were used, the reaction was carried out at pH = 10 at a room temperature for 0,5-4 hours. For the IFN PEGylation reaction 3-fold excess of PEG was used. In the conditions used, the binding of PEG to IFN occurred via steric accessibility of free amino groups of different lysines (Lys-31, Lys133, Lys134, Lys23, Lys131, Lys 121, Lys70, Lys83, Lys49 and Lys112) with the formation of carbamide bonds (Monkarsh et al., 1997). Thus, PEG-IFN-α2a consisted of a mixture of the resulting positional isomers of PEG-IFN-α2a conjugate, where each isomer contained a single PEG. In comparison with the unmodified IFN specific antiviral activity of isomers ranged from 6% (for Lys112) to 40% (for Lys133). The total specific activity of the obtained conjugate that was composed of 11 isomers, was 34% of the unmodified IFN. Activity of these conjugates in vitro also depended on the molecular weight of PEG and ranged from 45% (for 2500 Da PEG) to 25% (in the case of 10 000 Da PEG). The disadvantages of the resulting conjugate are as follows:

1. Usage of the ethanediol-activated MPEG with low molecular weight (≤ 5000 Da). As a result pharmacokinetic parameters of the PEG-IFN conjugate and unmodified IFN had only a slight difference. Due to this such a conjugate has not proceeded to clinical trials;

2. Formation of a large number of positional isomers with different specific activity, due to PEG conjugation with an IFN molecule through free amino groups of different lysines.

There are known PEG-IFN-α2b conjugates, obtained by conjugation of native IFN-α2b with linear (patent RU 2311930, 2004) or branched (patent RU 2382048, 2008) MPEG derivatives with molecular weight 13 000 - 17 000 Da. The reaction was conducted at pH 9.5 for 60 hours (for the linear MPEG) or at pH 7.5 for 12 hours (for the branched MPEG), using a 50-100-fold molar excess of activated PEG. As a result PEG-IFN conjugates have been obtained, the total antiviral activity of which varied from 29 to 38% of the activity of unmodified IFN. Data about conjugate stability, the number of positional isomers and their specific antiviral

activity are absent. The disadvantages of these conjugates are as follows:

1. Usage of tresylate - derivatives of activated MPEG with the half-life time in aqueous solutions less than 20 minutes. Previously it has been shown that in the interaction of tresylate derivatives of PEG with proteins, in addition to the stable amide bonds through amino groups unstable sulfamate bonds are also formed (Gais & Ruppert, 1995);

2. Bonding of tresylate activated PEG with proteins is made through all sterically accessible free amino groups of lysine (Roberts et al., 2002), which leads to the formation of several positional isomers;

3. Conducting the IFN PEGylation reaction at high pH values (pH = 10) for a long time (60 hours) can alter the IFN structure;

4. Significant excess of activated PEG during the IFN PEGylation reaction (molar ratio of PEG / protein was 50-100/1) leads to the high cost of the obtained product;

There is a known PEG-IFN-α2b conjugate obtained by conjugation of native IFN-α2b with branched triazine derivative MPEG with molecular weight 7500 - 35 000 Da (Patent RU 2298560, 2004), the total antiviral activity of which amounted to 6,4% of the activity of unmodified IFN. Data about conjugation stability, the number of positional isomers and their specific antiviral activity are absent.

The disadvantages of the obtained conjugate are the following:

1. The use of triazine-chloride derivatives of activated MPEG, which are able to conjugate with functional groups of other amino acids - serine, tyrosine, threonine, and histidine in addition to free amino groups, which leads to the emergence of many isomers, some of which are characterized by an unstable bond. Moreover, triazine derivatives are currently not used because of their high toxicity (Veronese & Pasut, 2005);

2. Low specific activity of the PEG-IFN conjugate.

There is a known PEG-IFN-α2a conjugate, obtained by conjugation of IFN-α2a with branched PEG with molecular weight 40 kDa (patent RU 2180595, 1997). For conjugation a N-hydroxysuccinimide-ester derivative of MPEG was used, which selectively interacts with the available free amino groups of protein to form stable amide bond. The reaction was conducted at pH 9,4°C for 2 hours at the 3:1 ratio of MPEG / protein. The reaction has been stopped and obtained conjugate has been purified by the sorbent Fractogel EMD CM 650 (M). Yield of the purified conjugate was 40-45%. In this case, the obtained IFN-PEG conjugate consisted of six positional isomers, each of which was conjugated with one PEG molecule by a stable bond through free amino groups of lysines - Lys31, Lys121, Lys 131, Lys 134, Lys 70 and Lys 83 (Vailon et al, 2001; Foser et al, 2003). Isomers conjugated with PEG through lysines in positions

31, 121, 131 and 134 formed 94% of the total conjugate. The activity of the total conjugate consisting of 6 positional isomers, was 1-7% of the native IFN-α2a (Bailon et al, 2001; Boulestin et al, 2006). Despite the low antiviral activity in vitro, this conjugate had improved pharmacokinetic properties in comparison with unmodified IFN (Silva et al., 2006, Boulestin et al, 2006) and is currently on the market under the trade name "Pegasys" (produced by "Hoffmann-La Roche").

The disadvantages of the obtained conjugate are the following:

1. The use of N-hydroxysuccinimide-ester activated PEG leads to conjugation with all sterically accessible amino groups, as a result the obtained conjugate is a mixture of 6 isomers, differing in specific activity.

2. Low antiviral activity of the obtained conjugate *in vitro*.

The prototype of this invention is the PEG-IFN-α2b conjugate described in the patent US 5,951,974, 1999. For PEGylation reaction a linear MPEG activated by succinimide carbonate group (SC-MPEG) with molecular weight from 5 000 to 12 000 Da was used. According to the described method an IFN conjugated with PEG with molecular mass of 12 kDa was obtained, which is currently on the market for treatment of viral hepatitis under the trade name "PegIntron" ("Schering-Plough", USA).

It was found that this PEG-IFN-α2b conjugate (medication "PegIntron") consists of 13 position isomers, each of which contains one PEG molecule conjugated with various parts of the protein molecule (Wang et al. 2000; Grace et al., 2001; Youngster et al, 2002). It was shown that PEG molecule was conjugated with interferon not only through free amino lysines (Lys31, Lys 49, Lys 83, Lys 121, Lys 131, Lys 133, Lys 134 и Lys 164) and (Cys1), but also through histidine imidazole ring (His7, His34), tyrosine (Tyr129) and serine OH-group (Ser163). Content of individual isomers varied from 0,8 (Tyr129) to 47 % (His34). Antiviral activity of PegIntron consisting of 13 position isomers is 28 % of the native protein. Specific activity of individual isomers varied from 11 to 37 % of the native IFN-α2b (Youngster et al, 2002). The main isomer conjugated with PEG through His34 has the highest specific activity (37 % of the native protein). The free amino group of IFN has been conjugated with PEG by a stable bond, while in the main isomer, comprising about 47%, PEG was conjugated with the histidine imidazole ring (His34) through the unstable (in aqueous solutions) carbamate bond (Roberts et al., 2002).

Pharmacokinetic parameters of the conjugate were better than that of unmodified IFN (Silva et al., 2006).

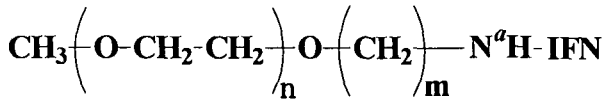
The disadvantages of the obtained conjugate are the following:

1. The use of succinimide carbonate activated PEG conjugating not only with the free amino groups of IFN, but also with the functional groups of other amino acids. As a result, obtained conjugate consisted of a mixture of 13 positional isomers, differing in antiviral specific activity;

2. Formation of an unstable imidazole-carbonate (carbamate) bond in the main (His-34) isomer of the PEG-IFN conjugate leads to the fact that after "Pegintron" administration the conjugate is hydrolyzed with release of the native IFN, which exerts its biological effect. Thus, the drug "PegIntron" is best regarded as a pro-drug which after injection is hydrolyzed with the formation of active interferon (Foster, 2004). Due to the instability of the PEG-IFN conjugate in solution "PegIntron" dosage form is stored only in the form of lyophilized powder.

The aim of the present invention was to obtain a new stable PEGylated IFN with the activity of IFN-alpha, consisting of one positional isomer, with improved stability, with reduced immunogenicity, improved pharmacokinetic parameters, with the optimal combination of PEG molecular weight and antiviral activity of the conjugate, suitable for medical use, as well as pharmaceutical compositions based on these PEG-IFN conjugate.

The problem is solved by creation of a new functionally active PEG-IFN conjugate with interferon-alpha activity, in which the linear molecule of PEG with molecular mass from 10 000 to 40 000 Da is bound to IFN-α molecule by a stable bond strictly through alpha amino group of N-terminal cysteine resulting in a compound with following formula (I):



(I)

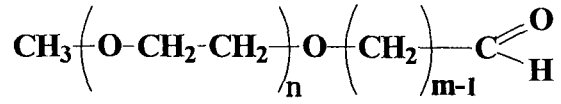
where: n - integral values from 227 to 10 000;

m – integer ≥ 4;

IFN - natural or recombinant polypeptide having the activity of IFN-alpha.

In the obtained conjugate linear PEG with the molecular weight 10 000 - 40 000 Da is bound with the alpha amino group of the N-terminus amino acid of IFNa.

Selectivity of IFN modification strictly via alpha-amino group of N-terminal amino acid is achieved by the use of aldehyde-activated mPEGs of general formula (II) via reaction of PEGylation at pH ≤ 6:



(II)

where: n - integral values from 227 to 10 000, so that molecular mass of PEG is approximately 10 000 – 40 000 Da;

m – integer ≥ 4;

The optimum ratio of PEG molecular weight and antiviral activity is achieved through the use of activated MPEG (II) with value of m ≥ 4.

Reduction of immunogenicity and toxicity as well as improved pharmacokinetic parameters are achieved by increasing the molecular weight of the conjugated PEG.

New features in comparison with the prototype are:

In comparison with prior art the novelty is as follows:

1. Aldehyde derivatives of activated mPEGs were used for production of PEG-IFN conjugate;
2. Structural formula of PEG-IFN conjugate is novel;
3. Produced PEG-IFN conjugate is represented by one position isomer only;
4. A bond between PEG molecule and IFN molecule is stable;
5. Molecular mass of PEG-IFN conjugate is increased because of the size of the bound PEG;
6. Pharmacokinetic parameters were improved.

For production of claimed PEG-IFN conjugate the recombinant human IFN-α2b (produced by ZAO "Biocad") and butyr- or pentaldehyde derivatives of mPEG corresponding to formula (II) with molecular mass of 20 000 Da were used.

The conjugation reaction was performed at pH below 6.0 with a reducing agent at a temperature at or below 20 ^{0}C. The molar ratio of PEG / protein was 2,5-5 : 1. Control of PEG-IFN conjugate formation was performed using polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) in reducing conditions and reversed-phase high pressure liquid chromatography (RP-HPLC). Purification of monoPEG-IFN from the reaction products that included unmodified IFN and undesirable forms of PEG-IFN, containing two or more PEG molecules per protein molecule, was carried out by chromatography on cation-exchange sorbents. MonoPEG-IFN elution was performed by gradient concentration of sodium

chloride from 0,05 to 0,2 M in buffer solution with a pH below 6. The purified monoPEG-IFN product was dialyzed against 10-50 mM buffer solution at pH 4-5, adding 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 the temperature of 4±2 ^{0}C.

To characterize obtained monoPEG-IFN-α2b conjugate studies of its purity, homogeneity, physical-chemical, biological, and pharmacokinetic characteristics were performed in comparison with unmodified IFN.

The proposed invention is illustrated by following figures:

Fig. 1. Kinetics of PEG-IFN-α2b conjugate formation during the reaction with butyraldehyde MPEG derivative.

Fig. 2. Reverse-phase HPLC analysis of purified PEG-IFN-α2b conjugate on "Symmetry C18 " (4,6 x 150 mm) column.

Fig. 3. SDS-PAGE analysis of the purified PEG-IFN-α2b conjugate compared to unmodified IFN-α2b.

Fig. 4. MALDI-MS analysis of unmodified IFN-α2b and PEG-IFN-α2b conjugate.

Fig. 5. Location of PEG attachment site in the PEG-IFN-α2b conjugate. Comparison of mass spectra of tryptic peptides of unmodified IFN-α2b (A) and PEG-IFN-α2b (B) conjugate in the range m / z 1200-1400.

Fig. 6. Thermal stability of the PEG-IFN-α2b conjugate and unmodified IFN-α2b.

Fig. 7. Proteolytic stability of the PEG-IFN-α2b conjugate and unmodified IFN-α2b.

Fig. 8. The immunogenicity of the PEG-IFN-α2b conjugate compared with unmodified IFN-α2b.

Fig. 9. Pharmacokinetics of the PEG-IFN-α2b conjugate compared with unmodified IFN-α2b.

Examples of specific PEG-IFN-α2b production methods and results of the study of its properties are given below.

PEG-IFN-α2 conjugates of the formula (I), which are the object of the present invention can be used to produce medicines for preventing and / or treatment of viral diseases, because in addition to high stability and reduced immunogenicity, they have high antiviral activity. Also, pharmaceutical compositions containing as an active ingredient an effective amount of conjugate

of formula (I) produced by well-known pharmaceutical methods, can be used separately or in conjunction with other therapeutic agents (e.g., ribavirin) for the prevention and / or treatment of viral infections (e.g. chronic active hepatitis (particularly hepatitis B and C) (Jay et al, 2006; McHutchison et al, 2009). The object of the present invention is also a way to prevent and / or treat viral diseases such as hepatitis C and hepatitis B, including introduction of a therapeutically effective amount of the claimed conjugate of formula (I).

It is known that IFN-α and PEGylated interferon-α can be used as immunomodulating agents for treatment of cancer, in particular, leukemic reticuloendotheliosis, laryngeal papillomatosis, melanoma, renal cell carcinoma, myeloid leukemia, Kaposi's sarcoma (Decatris et al, 2002; Bukowski et al. , 2002; Qintas-Cardama et al., 2006; Loquai et al., 2008; Kaehler et al, 2010). On the basis of the well-known pathogenesis of these diseases and the experience of IFN-α as well as conjugates use for treatment of these diseases, the objects of the present invention are also medicines and pharmaceutical compositions containing the declared conjugate PEG-IFN-α in an effective quantity, possessing antiproliferative and immunomodulatory effects, which can be used to prevent and / or treat cancer and diseases associated with primary or secondary immunodeficiency, as well as the object of the present invention is a method of prevention and / or treatment of cancer and diseases associated with primary or secondary immunodeficiency, including introduction of therapeutically effective amount of conjugate PEG-IFN-α of the formula (I).

Conjugate of the formula (I) with an optional pharmaceutically acceptable filler, diluents and / or pharmaceutically acceptable excipients is administered intravenously, subcutaneously, intramuscularly or via any other suitable routes, for example, in the form of capsules, syrups, sprays, drops, injections or suppositories. Route of administration vary depending, for example, on symptoms and age. Frequency of administration and the interval between the injections vary depending on the disease and its severity or the aim of administration (therapeutic or prophylactic use). An effective amount of PEG-IFN-α is selected according to the abovementioned factors.

Pharmaceutically acceptable excipients may be included into a pharmaceutical composition with PEG-IFN-α: buffer salts (e.g. acetate, citrate, hydrocarbonate, phosphate buffers), stabilizers (e.g., polysorbate, EDTA, polyvinyl pyrollidone, dextranes, human serum albumin, ethers paraoxybenzoic acid, alcohols (e.g. benzyl alcohol), phenols, sorbic acid), antiseptic components (e.g., benzyl alcohol), a regulator of osmotic pressure (for example, sodium chloride, potassium chloride, polyols, for example, glycerol, arabitol, sorbitol, mannitol,

lactose, dextran), surfactants (e.g., HSA, polyvinylpyrrolidone, lecithin, polysorbate 80, polyoxyethylene-polyoxypropylene copolymer, casein), surface-active substances (eg, block copolymers of ethylene oxide and propylene oxide, propylene oxide and ethylene oxide, sorbitan monolaurate, sorbitol ester, polyglycerol fatty acid ester, cocamide DEA lauryl sulfate, alkanolamide, stearyl polyoxyethylene propylene glycol, lauric ether polyoxyethylene, polyoxyethylene cetyl ether, polysorbate, glycerol monostearate, glycerol distearate, sorbitol monopalmitate, sorbitan monooleate polyoxyethylene, sorbitan monolaurate polyoxyethylene monostearate and propylene glycol), antioxidants (eg, Trilon B, L-cysteine, sodium sulfite, sodium ascorbate, glutathione, 2-mercaptoethanol, dithiothreitol, cysteine hydrochloride, monohydrate, ascorbic acid) and diluents (e.g., water).

Example 1

Production of PEG-IFN-α2b conjugate using butyraldehyde MPEG derivative

16 ml of 1M sodium cyanoborohydride solution were added to 785 ml of buffer solution (100 mM sodium acetate, 150 mM sodium chloride, pH 5,0), containing recombinant IFN-α2 with the concentration of 1,7 mg per ml, then the solution was stirred and 5 g of dry butyraldehyde PEG derivative with molecular weight of 20 000 Dalton was added. The mixture was thoroughly stirred and incubated for 22 hours at the temperature of 17±3 ^{0}C. 50 µl aliquots were sampled from the mixture after different time intervals, and the kinetics of production of the PEG-IFN-α2 conjugate was analyzed using the electrophoresis method in polyacrylamide gel in the presence of dodecylsulfate. For this purpose, buffer with pH 6,8 in the amount of 1/3 of the total volume, containing 125 mM of Tris-HCl, 20 % glycerin, 3% dodecylsulfate, 0,005% bromphenol blue, was added to the mixture and heated for 3 min on a boiling water bath. Samples of 5 µl were placed into wells of prepared 12,5 % polyacrylamide gel slabs, then electrophoresis was performed in the presence of dodecylsulfate. After completion of the electrophoresis, the gel was colored with Coomassie R-250. The kinetics of production of the PEG-IFN-α2 conjugate is represented on Fig. 1. At the point when PEG-IFN content comprised over 70%, the reaction mixture was diluted 10 times with 5 mM of sodium acetate buffer, pH 5,0.

Example 2

Production of PEG-IFN-α2b conjugate using pentaldehyde MPEG derivative

2,05 ml of 1M sodium cyanoborohydride solution were added to 100 ml of buffer solution (100 mM sodium acetate, 150 mM sodium chloride, pH 5,5), containing recombinant IFN-α2b with the concentration of 2,2 mg per ml, then stirred; 0,9 g of dry penthalaldehyde PEG derivative with molecular weight of 20 000 Dalton was added. The mixture was thoroughly stirred and incubated for 24 hours at the temperature of 6±2 ^{0}C. 50 µl aliquots were sampled from the mixture after different time intervals, and the kinetics of production of the PEG-IFN conjugate was analyzed using the electrophoresis method in polyacrylamide gel as described in example 1. At the point when PEG-IFN content comprised over 70%, the reaction mixture was diluted 10 times with 5 mM of sodium acetate buffer, pH 5,0.

Example 3

Purification of the monoPEG-IFN-α2b conjugate by ion-exchange chromatography on a cation-exchange adsorbent

The diluted solution produced in example 1 was placed on a column with a cation-exchange adsorbent (CM sepharose, 300 ml), equilibrated with 5 mM sodium acetate buffer, pH 5.0, (buffer A) at the rate of 5 ml per min. The adsorbent column was washed consecutively with buffer A and buffer A containing NaCl concentration gradient from 0,05 to 0,2 M. Aliquots were sampled from each fraction for the purpose of sample analysis using the electrophoresis method in polyacrylamide gel as described in example 1. The fractions that contained the monoPEGylated PEG-IFN-α2 conjugate were combined, dialyzed with 10 volumes of 20 mM sodium acetate buffer, containing 150 mM sodium chloride, then sterile filtration was performed and the resulting solution was stored at 4±2 ^{0}C.

Example 4

Purification of the monoPEG-IFN-α2b conjugate by ion-exchange chromatography on a cation-exchange adsorbent

The diluted solution produced in example 2 was placed on a column with a cation-exchange adsorbent (SP sepharose, 50 ml), equilibrated with 5 mM sodium acetate buffer, pH 5.0, (buffer A) at the rate of 5 ml per min. The adsorbent column was washed consecutively with buffer A and buffer A containing NaCl concentration gradient from 0,03 to 0,3 M. Aliquots were sampled from each fraction for the purpose of sample analysis using the electrophoresis method in polyacrylamide gel as described in example 1. The fractions that contained the monoPEGylated PEG-IFN-α2 conjugate were combined, dialyzed with 10 volumes of 20 mM sodium acetate buffer, containing 150 mM sodium chloride, then sterile filtration was performed and the resulting solution was stored at 4±2 ^{0}C.

Example 5

Reversed phase–HPLC analysis of PEG-IFN conjugate

The PEG-IFN-α2 conjugate produced in example 3 was diluted with 20 mM sodium acetate buffer, pH 5.0, down to the concentration of 0,1 mg per ml and 100 μl of this sample were placed on the Symmetry C18 column (4.6x150 mm). The assay was performed at 214 nm using the "Breeze" chromatograph manufactured by the Waters Company. Based on results

represented on Fig. 2 we may conclude that, according to RP-HPLC findings of, the purity of the claimed PEG-IFN-α2 conjugate is over 99%.

Example 6

Determination of endotoxin level

The concentration of bacterial endotoxins (BE) in samples of the PEG-IFN-α2 conjugate produced in accordance with examples 3 and 4 was determined in vitro by means of a LAL test (gel-clot method version) in accordance with requirements of European Pharmacopoeia 6.0 Article 2.6.14. A diagnostic kit manufactured by Associates of CAPE COD, Inc., LAL reagent with sensitivity of 0,03 EU per ml, Endotoxin Reference Standard (0,5 μg in a vial) and water for the LAL test were used in the assay. Based on results represented in table 1 we may conclude that BE content in conjugate sample is less than 1,5 endotoxin units (EU) per mg of protein, which is much lower than the BE level that is allowed for drugs based on recombinant proteins.

Table 1. Concentration of bacterial endotoxins in the PEG-IFN-α2 conjugate

PEG-IFN-α2 conjugate preparation	Endotoxin content (EU per mg of protein)
PEG-IFN-α2 produced in accordance with example 3	≤1,5
PEG-IFN-α2 produced in accordance with example 4	≤1,4

Example 7

Electrophoretic analysis of the PEG-IFN–α2b conjugate as compared to unmodified IFN–α2b.

A sample of the PEG-IFN conjugate produced in accordance with example 3 was analyzed using the electrophoresis method in polyacrylamide gel as described in example 1. The electrophoresis was performed with load of 40 μg of protein per well under non-reducing conditions. Protein coloring in the gel was performed with Coomassie R-250 dye. Simultaneously, electrophoresis of unmodified IFN-α2 was performed. The PEG-IFN conjugate was represented with one band (Fig. 3, track 1). Based on electrophoregrams of the PEG-IFN-α2 conjugate and of unmodified IFN-α2, as represented on Fig. 3, we may see that the PEG-IFN-α conjugate has a higher molecular weight, than unmodified IFN-α (Fig. 3, tracks 1 and 2). It ought to be noted that it is impossible to determine the exact molecular weight of PEGylated

proteins using the electrophoresis method, as addition of the hydrophilic PEG molecule to the protein considerably increases the Stokes radius of the resulting compound. As a result, movement of the PEG-protein compound in the gel is slowed down, and its molecular weight is seems to be considerably higher than the sum of molecular weights of the protein and the PEG.

Example 8

Determination of molecular weight of the PEG-IFN–α2b conjugate as compared to unmodified IFN –α2b using the mass spectrometry method

1 μl sample of the PEG-IFN-α2b conjugate produced in accordance with example 3, and 0,3 μl of solution of the 2,5-dihydroxybenzoic acid (Aldrich,10 mg×ml^{-1} in 20 % acetonitrile in water with 0.5 % uranium tetrafluoride) were mixed together and air-dried. A sample of unmodified IFN-α2 was prepared using a similar method.

The mass spectra were obtained on Ultraflex II BRUKER (Germany) MALDI-TOF mass spectrometer equipped with a UV laser (Nd). The mass spectra were obtained in the linear positive ion mode; the average weight measurement error does not exceed 10-15 Dalton.

The results of mass spectrometric analysis of unmodified rhIFN-α2 and the PEG-IFN-α2 conjugate in the m/z range of 10 000 to 50 000 are presented on Fig. 4. Based on results presented on Fig. 4A we may conclude that the mass spectrum of rhIFN-α2 contains a base peak corresponding to the [M]+ monovalent ion with molecular weight of 19 295 Dalton.

In the mass spectrum of the PEG-IFN-α2 conjugate as shown on Fig. 4B we may see a diffused main peak corresponding to the [M]+ monovalent ion with its molecular weight of 40 498 Dalton. The diffusion of the peak is caused by the heterogeneity of monomethoxyPEG preparations. The measured m/z value for the PEG-IFN conjugate in the peak maximum (40 498 Dalton) coincides with calculated sum of molecular weights of IFN-α2 (19 295 Dalton) and the attached PEG (20 000 Dalton).

Example 9

Localization of the PEGylation site in the PEG-IFN-α2b conjugate

5 μl of modified trypsin solution (Promega) in 0.05 M NH_4HCO_3 with concentration of 15 μg×ml^{-1} were added to 5 μl of the PEG-IFN conjugate sample produced in accordance with example 3. Hydrolytic decomposition was performed for 16 h at 37 °C, then 10 μl of solution of 0.5 % uranium tetrafluoride in 10 % solution of acetonitrile in water was added and thoroughly

stirred. A sample of unmodified IFN was prepared using a similar method. The resulting solutions were used to obtain MALDI mass spectra.

The localization of the site of PEG bond with the IFN molecule was determined by comparison of mass spectra of the PEG-IFN-α2 conjugate and unmodified IFN-α2 after tryptic digestion. PEG-IFN-α2 conjugate tryptic digest should lack the peptide corresponding to the part of the protein, in which modification occurred. In table 2 you may see mass spectra of experimental tryptic digests of the unmodified IFN-α and the PEG-IFN-α2 conjugate. Based on results presented in the table we may conclude that mass spectra of tryptic digests of the unmodified IFN-α2b virtually coincide with mass spectra of tryptic digests of the PEG-IFN-α2 conjugate (table 2), however the tryptic digest of the PEG-IFN-α2 conjugate lacks the peptide with the molecular weight of 1313.649, which is present in the tryptic digest of the unmodified IFN-α2 (see also Fig. 5). According to the analysis of theoretical weights of the IFN-α2 tryptic digest, the peak with m/z 1313,649 corresponds to an N-terminal protein peptide (table 2). Its absence in the tryptic digest of the PEG-IFN-α2 conjugate testifies to modification of the N-terminal peptide, which, becoming heavier by the weight of the PEG, falls outside of the spectrum area. PEG binding to the IFN molecule occurred in the only free amino group present in this peptide, the N-terminal cysteine amino group.

Table 2. Experimental mass spectra of tryptic digests of the PEG-IFN-2 conjugate and of the unmodified IFN-2 as compared to theoretical values.

Experimentally observed peptides $[M+H^+]^+$		*Theoretical peptides*		
IFN-a2b	***PEG-IFN conjugate***	Mass $[M+H^+]^+$	Peptide	Sequence
1313.649	-	1313.6266	1-12	CDLPQTHSLGSR
1232.720	1232.673	1232.6966	13-22	RTLMLLAQMR
1076.598	1076.558	1076.5955	14-22	TLMLLAQMR
910.463	910.463	910.5066	24-31	ISLFSCLK
2225.976	2225.928	2225.9999	32-49	DRHDFGFPQEEFGNQ
2459.264	2459.239	2459.3002	50-70	AETIPVLHEMIQQIFN
772.447	772.421	772.456	99-106	VIQGVGVT
902.471	902.443	902.4941	113-120	EDSILAVR
1030.590	1030.546	1030.5891	113-121	EDSILAVRK
741.431	741.424	741.4042	121-125	KYFQR
750.492	750.476	750.4760	126-131	ITLYLK
1337.695	1337.651	1337.6670	134-144	KYSPCAWEVVR

1209.565	1209.539	1209.5721	135-144	YSPCAWEVVR
1481.783	1481.735	1481.7594	150-162	SFSLSTNLQESLR

Example 10

Determination of the specific antiviral activity of the PEG-IFN-α2b conjugate

The samples produced in accordance with example 3 and example 4 were tested for specific antiviral activity in accordance with the process described in the European Pharmacopoeia using a culture of passaged MBDK cells sensitive to alpha-type interferon and a culture of vesicular stomatitis virus (VSV). Table 3 presents the results of study of the antiviral activity. International reference standard was used as the reference level. Proceeding from table 3 we may conclude that, despite of the conjugation with the PEG molecule with its molecular weight of 20 000 Dalton, the antiviral activity of the produced conjugates comprises 42-45% of the activity of unmodified IFN-α2. It is necessary to note that the PEG-IFN conjugates described in the prototype method, containing PEG with molecular weight of 12 000 Dalton, possessed lower activity (28-30%).

Table 3. Specific antiviral activity of the PEG-IFN-α2 conjugate as compared to unmodified IFN-α2.

Preparation	Specific antiviral activity	
	(IU per mg)	% of the activity of unmodified IFN-a2b
IFN-α2	$2,1 \times 10^8$	100 %
PEG-IFN-α2 conjugate produced in accordance with example 3	$0,7 \times 10^8$	40%
PEG-IFN-α2 conjugate produced in accordance with example 4	$0,9 \times 10^8$	45%

Example 11

Study of the PEG-IFN-α2b conjugate thermal stability

5 ml of the PEG-IFN conjugate sample produced in accordance with example 3 were

placed into a water bath at the temperature of (50 ± 2) °C, and occurrence of turbidity in the protein solution was tested after different time intervals, measuring optical density at 340 nm. Study of thermal stability of unmodified IFN-α2 was performed in a similar manner. Creation of insoluble protein compounds led to occurrence of turbidity and to increase of optical density at 340 nm. Results of the study are presented on Fig. 6. During the period of observation (28 hours) the PEG-IFN-α2 conjugate remained stable, whereas unmodified IFN-α2b precipitated almost completely after 28 h.

Thus, the data obtained in this study allows to draw the conclusion on substantial increase of the PEG-IFN-α2 conjugate thermal stability as compared to the unmodified protein.

Example 12

Study of the PEG-IFN-α2b conjugate proteolytic stability when treated with trypsin

150 μl of M Tris-HCl buffer, pH 8,5, 2 μl of 0,5 M $CaCl_2$ solution and 15 μl of trypsin (Promega) with concentration of 20 μg per ml were added to 1 ml of the PEG-IFN conjugate produced in accordance with example 3. The samples were incubated at the temperature of 37°C. 100 μl aliquots were sampled from the mixture after different time intervals, and 150 μl of trifluoroacetic acid solution were added to stop the reaction. Samples containing unmodified IFN-α2 were prepared using a similar method. Thereafter, the samples were analyzed with reversed-phase HPLC with determination of the area of the main peak. The results of the study as shown on Fig. 7, demonstrate that in case of treatment with trypsin the proteolytic stability of the PEG-IFN-α2 conjugate is higher than that of the unmodified IFN-α2b.

Example 13

Determination of the PEG-IFN-α2b conjugate stability during storage

Aliquots were taken from samples produced in accordance with example 3 into sterile Eppendorf test tubes with caps. Protein concentration in the PEG-IFN-α2 conjugate was 1 mg per ml. The samples were placed into a refrigerator at the temperature of (6 ± 2) °C. Samples were taken after predefined time intervals and analyzed for specific antiviral activity and homogeneity of the preparation by means of RP-HPLC and electrophoresis (Eph) in polyacrylamide gel in the presence of dodecylsulfate at non-reducing conditions as described in example 1. Based on results presented in table 4 we may conclude that during storage at the temperature of (6 ± 2) °C the PEG-IFN-α2 conjugate is stable for at least 24 months.

Table 4. PEG-IFN-α2 conjugate stability at the temperature of (6 ± 2) °C

Parameters	Storage life (months)						
	0	3	6	9	12	18	24
Activity (IU per ml)	$0,7x10^8$	$0,75x10^8$	$0,7x10^8$	$0,75x10^8$	$0.72x10^8$	$0.7x10^8$	$0,75 x10^8$
Homogeneity, % (RP-HPLC)	99,67	99,28	99,61	99,30	99,6	99,21	99,35
Homogeneity, % (SDS-PAGE)	≥ 98	≥ 98	≥ 98	≥ 98	≥ 98	≥ 98	≥ 98

Example 14

Study of the PEG-IFN-α2b conjugate immunogenicity

PEG-IFN conjugates produced in accordance with example 3 and example 4 were diluted down to the concentration of 5 mln. IU per ml and injected intramuscularly in the dose of 200 μl (1 mln IU per a mouse) into ICR mice with body weight of 20-22 g once a week for 5 weeks (5 groups with 5 mice in each group). Simultaneously, samples of unmodified IFN were prepared similarly and injected into mice at the doze of 1 mln. IU. At the end of the fifth week blood tests were taken from the mice by means of retroorbital paracentesis. Blood serum was received using the standard method. Antibody titer in sera obtained after of immunization of mice with unmodified IFN and PEG-IFN conjugates was determined by means of direct ELISA technique. For this purpose 100 μl of the unmodified IFN-α2 solution with concentration of 200 ng per 100 μl were placed into wells of microplates. The antisera obtained from different groups of animals were placed into the wells of the plate in series from consecutive dilutions in duplicates. For detection of the resulting immune complex, antimouse antibodies conjugated with peroxidase were used. Antibody titer after administration of unmodified IFN was 1/1024. Antibody titer after administration of PEG-IFN conjugates was 1/128. Results of the study are presented on Fig. 8.

Thus, based on the results obtained, we may conclude that the immunogenicity of the claimed PEG-IFN-α2 conjugates is 8 times lower than that of unmodified IFN.

Example 15

Study of the PEG-IFN-α2b conjugate pharmacokinetics

A sample of the PEG-IFN conjugate produced in accordance with example 3 in the amount of 1 mln IU was injected intraperitoneally to male mice. Simultaneously, unmodified IFN-α2 was injected to another group of mice. Blood samples were taken from animals after predetermined time intervals by means of retroorbital paracentesis. Blood serum was obtained using the standard method. Presence of interferon in the serum was detected based on antiviral activity. Results of the pharmacokinetic study are represented on Fig. 9. Based on these results we may conclude that the claimed PEG-IFN-α2 conjugate possesses significantly prolonged effect. After injection of native IFN-α2, its concentration in the blood of the animals reached a maximum after 1 h after injection, and then decreased very quickly. After injection of the PEG-IFN conjugate the maximal IFN concentration in the blood of the animals was observed after 12 hours (Fig. 9), and this level was preserved for 50 hours, and only then a slow decrease in IFN-α2 concentration was observed for 350 hours.

The area under the curve (AUC) for the claimed PEG-IFN conjugate exceeded the respective values for unmodified IFN by more than 50 times. Notably, in case of the prototype PEG-IFN-α2b conjugate coupled with PEG with molecular weight of 12 000 Dalton, the value of the AUC exceeded the IFN AUC by 3-10 times only.

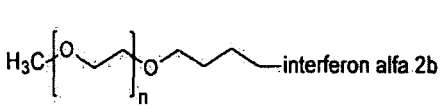
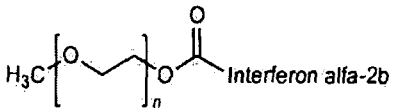
Based on the obtained results (Fig. 9) the primary pharmacokinetic parameters were calculated for the claimed PEG-IFN-α2 conjugate. The results have shown that intake from the site of injection, volume of distribution, the clearance of the PEG-IFN conjugate are considerably slowed down as compared to unmodified IFN, which ensured prolonged circulation of the claimed PEG-IFN conjugate in blood (over 14 days).

Example 16

Comparative characteristics of the claimed PEG-IFN-α2b conjugate as compared to the PEG-IFN-a2b conjugate described in the prototype method

Comparison of the structure, basic physical, chemical and pharmacokinetic parameters of the claimed PEG-IFN-α2b conjugate as compared to the PEG-IFN-α2b conjugate described in the prototype method was performed (table 5). The data represented in table 5 testify to significant improvement of the properties of the claimed PEG-IFN conjugate as compared to the conjugate described in the prototype method.

Table 5. Basic physical, chemical and pharmacokinetic parameters of the claimed PEG-IFN-α2b conjugate as compared to the PEG-IFN-α2b conjugate described in the prototype method (US 5,951,974).

Parameters	PEG-IFN conjugate	
	Claimed PEG-IFN conjugate	Conjugate described in the prototype method (US 5,951,974)
IFN	**IFN-α2b**	**IFN-α2b**
PEG structure	Linear	Linear
Molecular weight of the PEG (kDalton)	20	12
Structural formula of the PEG-IFN* conjugate		
Specific activity, % of native IFN	40	28
Number of positional isomers	1	13
Type of PEG connection to the protein	Stable	Unstable (for the main isomer linked to PEG via His34)
Stability in water solutions	Stable	Unstable
Pharmacokinetics parameters		
Ratio AUC PEG-IFN/IFN ratio	50	3-10

* - The structural formula of the PEG-IFN conjugate produced using the prototype method is given under the reference "WHO Drug Information", 2001, v. 15, N. 3-4, p.206.

Example 17

Examples of pharmaceutical compositions based on the claimed PEG-IFN-α2b conjugate

(A)

PEG-IFN-α2b in accordance with example 3	10-500 μg
Sodium acetate trihydrate	1 – 5 mg
Glacial acetic acid	up to pH 5,0
Sodium chloride	8 -9 mg
Polysorbate 80	0,01-0,1 mg

Ethylenediamine tetraacetate disodium salt	0,01- 0,1 mg
Water for injection	1,0 ml

(B)

PEG-IFN-α2b in accordance with example 4	10-500 μg
Sodium acetate	0,4 – 2,0 mg
Acetic acid	up to pH 5,5
Mannitol	50 mg
Dextran	15-30 mg
Polysorbate 80	0,01-0,1 mg
Ethylenediamine tetraacetate disodium salt	0,01- 0,1
Water for injection	1,0 ml

(C)

PEG-IFN-α2b in accordance with example 4	10-500 μg
Sodium acetate	0,4 – 2,0 mg
Acetic acid	Up to pH 5,5
Mannitol	50 mg
Human serum albumin	10-12,5 mg
Polysorbate 80	0,01-0,1 mg
Ethylenediamine tetraacetate disodium salt	0,01- 0,1
Water for injection	1,0 ml

Example 18

Containerization of a medicinal agent containing PEG-IFN-α2b

A medical agent containing an effective amount of the claimed PEG-IFN-α2b (Example 17) is to be dispensed in sterile conditions into syringes from neutral glass of the 1st hydrolytic class with brazed-in needles covered with elastic or hard protective caps, sealed with nozzles on butyl rubber plungers laminated with a fluoropolymer with the volume of 0,5, 0,8, 1,0 and 1,2 ml (for 100 μg per ml dosage), 0,5, 0,6, 0,75 and 0,9 ml (for 200 μg per ml dosage), 0,5, 0,6, 0,8, 1,0 and 1,2 ml (for 300 μg per ml dosage).

Example 19

Containerization of a medicinal agent containing PEG-IFN-α2b

A medical agent containing an effective amount of PEG- interferon alpha-2b (Example 17) is to be dispensed in sterile conditions into vials from neutral glass of the 1st hydrolytic class sealed with fluoric rubber or nozzles on butyl rubber covers with teflon coating tightened with aluminium caps with the volume of 0,5, 0,8, 1,0 and 1,2 ml (for 100 μg per ml dosage), 0,5, 0,6, 0,75 and 0,9 ml (for 200 μg per ml dosage), 0,5, 0,6, 0,8, 1,0 and 1,2 ml (for 300 μg per ml dosage).

Example 20

A kit including a containerized medicinal agent containing PEG-IFN-α2b

The kit contains 1 or 4 syringes together with plungers (1 or 4 accordingly) / or vials in an blister made of polymeric film together with a prescribing information, placed into a cardboard pack.

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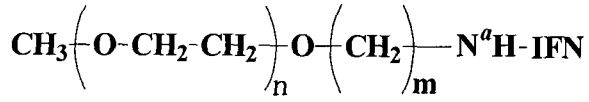
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CLAIMS

1. Stable conjugate of PEGylated interferon-α of formula (I), constituting one position isomer



(I)

where:

n - integral values from 227 to 10 000;

m – integer ≥ 4;

$N^{\alpha}H$-IFN – interferon-α

2. Conjugate according to example 1, in which m is an integer equal to 4.

3. Conjugate according to example 1, in which interferon-α is a natural or recombinant interferon-α-2b.

4. Conjugate according to example 1, in which average molecular mass of polyethylene glycol is from 10 to 40 kDa.

5. Conjugate according to example 1, in which N-terminal group is represented by cysteine residue (Cys).

6. Pharmaceutical composition, having antiviral, antiproliferative and immunomodulating activity, containing conjugate of general formula (I) in effective quantity and pharmaceutically acceptable excipients.

7. Pharmaceutical composition according to example 6 for application as a drug for treatment of viral and oncologic diseases and diseases associated with primary or secondary immunodeficiency.

8. Pharmaceutical composition according to example 7, where viral disease is hepatitis C or hepatitis B.

9. Pharmaceutical composition according to example 7, where oncologic disease is a myeloid leukemia.

10. Pharmaceutical composition according to example 7, where oncologic disease is a melanoma.

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11. Medicinal preparation, including conjugate of formula (I), having antiviral, antiproliferative and immunomodulating activity.

12. Medicinal preparation, according to example 11, including buffer, isotonic agent, stabilizer and water.

13. Medicinal preparation, according to example 12, including sodium acetate trihydrate, acetic acid, sodium chloride, polysorbate 80, EDTA, water for injection.

14. Use of conjugate of formula (I) for production of medicinal preparation, having antiviral, antiproliferative and immunomodulating activity.

15. Use according to example 14 for production of medicinal preparation, having antiviral activity with regard to hepatitis C or hepatitis B.

16. Approach to prophylaxis and/or treatment of viral diseases, including introduction of therapeutically effective quantity of conjugate of formula (I).

17. Use according to example 16, where viral disease is hepatitis C or hepatitis B.

18. Use according to example 16, including additional introduction of therapeutically effective quantity of ribavirin.

19. Approach to prophylaxis and/or treatment of diseases associated with primary or secondary immunodeficiency, including introduction of therapeutically effective quantity of conjugate of formula (I).

20. Approach to prophylaxis and/or treatment of oncologic diseases, including introduction of therapeutically effective quantity of conjugate of formula (I).

21. Use according to example 20, where oncologic disease is a myeloid leukemia.

22. Use according to example 20, where oncologic disease is a melanoma.

23. Container, hermetically and aseptically sealed and compliant to storage condition requirements, including pharmaceutical composition according to example 6.

24. Container according to example 23, where specified container is a prefilled syringe, vial or autoinjector.

25. Kit, including container according to example 24 and prescribing information.

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