(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 September 2006 (28.09.2006)

PCT

(10) International Publication Number
WO 2006/099701 A1

(51) International Patent Classification:
C07K 14/57 (2006.01) *C12N 15/62* (2006.01)
C07K 14/56 (2006.01) *A61K 38/21* (2006.01)

(21) International Application Number:
PCT/BG2005/000013

(22) International Filing Date: 5 October 2005 (05.10.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
109087 21 March 2005 (21.03.2005) BG

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report*
— *with amended claims*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/099701 A1

(54) **Title:** INHIBITOR OF ENDOGENOUS HUMAN INTERFERON - GAMMA

(57) **Abstract:** The invention relates to an inhibitor of endogenous human interferon-gamma (hIFN-γ) in autoimmune diseases, especially in multiple sclerosis. More precisely, the invention relates to inactivated protein derivatives of the hIFN-γ with preserved affinity to the hIFN-γ receptor. The derivatives represent genetically modified variants of hIFN-γ, where the C-terminal part of the molecule is either deleted or replaced with a polypeptide sequence of another human protein and a recombinant hIFN-γ, inactivated by physical or chemical methods.

Inhibitor of endogenous human interferon-gamma

Field of invention

The invention relates to an inhibitor of endogenous human interferon-gamma (hIFN-γ), applicable for treatment autoimmune diseases, especially for multiple sclerosis.

Background of invention

About 2% of the human population is affected by various autoimmune diseases, including multiple sclerosis (MS). MS is neurodegenerative disease affecting the central nervous system (CNS) and leading to a progressive physical disability. Although the exact etiology and pathogenesis of MS is still obscure, it is believed that it might be autoimmune disease [1]. Histopathology of MS is characterized with demyelination of motor neurons in CNS, loss of oligodendrocytes and moderate inflammatory reaction. Affected areas in the brain are usually infiltrated with T-lymphocytes and macrophages. T-lymphocytes belong to the CD+ subtype and are characterized with increased production of Th1 cytokines (IL-2 and IFN-γ) [2]. As a result, the mononuclear cells are induced to produce increased amounts of some destructive substances such as lymphotoxines (LT) and tumor necrosis factor alpha (TNF-α). Many studies show that the abnormal production of IFN-γ plays a key role in the pathogenesis of MS [3-6].

Recombinant DNA technology reveals new approaches for neutralizing the activity of endogenous hIFN-γ to find application for treatment of autoimmune diseases including MS. An inhibitor of the hIFN-γ secretion is hIFN-β, which has already been applied for treatment of MS patients [Patents US082138, WO9530435, CA2361081]. Patents RU2073522, RU2187332, RU02166959 recommend treatment with a mixture of hIFN-α, hIFN-β and hIFN-γ. It is reported, however, that the high daily doses of hIFN-β ($8x10^6$ IU) results in unfavorable consequences related with the following effects of

hIFN-β: a) hIFN-β blocks the T-cells proliferation [7]; b) hIFN-β neutralizes IL-12 thus enhancing the effect of hIFN-γ on dendrite cells [8]; hIFN-β suppresses the activity of T cells, producing hIFN-γ and IL-4, thus lowering the level of CD4+ cells (Th1, Th2) and CD8+ (Tc1) cells without changing the ratio Th1/Th2 [9, 10]; d) after a short-term treatment of MS patients during the acute phase hIFN-β decreases the expression of pro-inflamatory cytokines (such as hIFN-γ and hIFN-α) and increases the expression of anti-inflamatory cytokines (IL-4 and IL-10) [11].

Another approach for healing MS patients consists in neutralizing the endogenous hIFN-γ by specific monoclonal antibodies [12, 13, W00145747]. The long-term treatment with anti-hIFN-γ antibodies, however, results in deterioration of the health conditions, probably because of weakening of the natural defense system.

Patents US0086534 and CA2299361 offer a different approach for suppressing the abnormal production of IFN-γ based on the so called consensus interferons (IFN-con_1, IFN-con_2 and IFN-con_3) belonging to the groups of hIFN-α, hIFN-β and hIFN-τ. These recombinant preparations, however, show side effects, including toxicity.

Proteins with aminoacid sequence partly coinciding with that of the hIFN-γ have been applied as antiviral, antitumor and immunomodulating agents [US4832959, WO0208107, AT393690]. Their effects, however, is hard to be assessed since the descriptions are not supported with experimental data.

Description

The invention relates to an inhibitor of endogenous human interferon-gamma (hIFN-γ) in autoimmune diseases, especially in multiple sclerosis. More precisely, the invention relates to inactivated protein derivatives of the hIFN-γ with preserved affinity to the hIFN-γ receptor. These inactivated protein derivatives of the hIFN-γ represent genetically modified variants of

hIFN-γ, where the C-terminal part of the molecule is either deleted or replaced with a polypeptide sequence of another human protein (e.g. hIFN-α) and a recombinant hIFN-γ, inactivated by physical or chemical methods.

The inactivated protein derivatives of the hIFN-γ according to the invention are constructed on the basis of both the spatial structure and functional map of hIFN-γ. Since the receptor binding sites are located in the N-terminal region, the primary structures of the inactivated protein derivatives according to the invention coincides with that part of the hIFN-γ molecule.

1. Genetically modified variants of hIFN-γ where the C-terminal part of the molecule is deleted (Truncated hIFN-γ)

To construct a genetically modified variant where the C-terminal part of hIFN-γ is deleted, two oligonucleotides are synthesized and used as a primers for polymerase chain reaction (PCR). Nucleotide sequence of the forward primer (SEQ ID No:1) coincides with that of the 5' coding sequence of the hIFN-γ gene and is designed to introduce a *Hind*III cloning site. The reverse primer (SEQ ID No: 2) covers the cutting site at the 3' terminus of hIFN-γ gene (27 codons upstream from the stop codon) and introduces a *Bam*HI cloning site. The truncated hIFN-γ gene (coding for 116 aminoacid residues) is prepared by a two step PCR using a full size synthetic human hIFN-γ gene (BG75781) as a template and the two above mentioned synthetic primers and cloned in the expression vector pJP_1R_3 (Fig. 1). *E. coli* LE392 are transformed and the yield of recombinant product is determined by ELISA. The truncated hIFN-γ is purified by two step (hydrophobic/cationic) chromatography as it is already described [EP0446582]. The activity of the truncated IFN-γ is determined by its antiviral activity (protecting effect of hIFN-γ on WISH cells against the cytopatic action of the vesicular stomatitis virus (VSV) [14]. The obtained results show that the truncated hIFN-γ is deprived of antiviral activity and is capable of competing with the full size protein for the hIFN-γ receptor.

2. Genetically modified variants of hIFN-γ where the C-terminal part of the molecule is replaced with a polypeptide sequence of another human protein (Hybrid hIFN-γ/ hIFN-α protein)

The genetically modified variants of hIFN-γ where the C-terminal part of the molecule is substituted, represent a hybrid molecule where 27 aminoacids originating from a human proteins such as IFN-α, IFN-β, IL-2, etc. are substituted at the C-terminal part of the human IFN-γ. The size of the hybrid protein is 143 aminoacid residues (equal to that of the human IFN-γ). The hybrid IFN-γ/IFN-α gene is constructed by ligation of two DNA molecules one of which (containing 116 codons) originates from the 5'-terminal part of the hIFN-γ gene and the other (containing 27 in frame codons) comes from the 3'-terminal part of the IFN-α gene. The two gene fragments are prepared by PCR using full size hIFN-γ and hIFN-α genes as templates and a set of four synthetic primers. The forward primer for the hIFN-γ gene (SEQ ID No: 3) is designed to introduce a *Hind*III site at the 5'-terminus and the reverse primer (SEQ ID No:) to introduce a *Eco*RI site and also to eliminate the last 27 codons from the 3'-terminus of the hIFN-γ gene. The forward primer designed for modification of the IFN-α gene (SEQ ID No: 5) introduces an *Eco*RI site at the 5'-terminus of the IFN-α gene fragment and also to remove all but the last 27 codons from the IFN-α gene. The reverse primer (SEQ ID No: 6) introduces a stop-codon (TAA) and a *Bam*H1 cloning site at the 3'-end of the IFN-α gene fragment. The two gene fragments are amplified by PCR, purified by agarose gel electrophoresis and ligated to each other and then to the expression vector pJP_1R_3. The expression plasmid thus obtained (containing the hybrid hIFN-γ/hIFN-α gene) is transformed into *E. coli* LE392 cells. Bacteria are cultivated and the hybrid protein is purified as described above. The antiviral test shows that the hybrid hIFN-γ/hIFN-α protein is devoid of antiviral activity on WISH cells and competes successfully with the intact hIFN-γ for the hIFN-γ receptor.

3. hIFN-γ inactivated by irradiation with UV light (Photoinactivated hIFN-γ)

hIFN-γ contains single tryptophan (Trp) residue, which is indispensable for its biological activity. This residue is destroyed as follows: Recombinant IFN-γ is irradiated with UV light at 290 nm for 15 min. The results show that the biological activity of the photoinactivated hIFN-γ decreases drastically and the inactivated protein competes successfully with the intact hIFN-γ for its receptor.

Biological tests with the three derivative compounds of the hIFN-γ according to the invention show undoubtedly that they all have their basic biological activities (antiviral and antiproliferative) lost or drastically decreased and also that they all compete with hIFN-γ for the hIFN-γ receptor. Due to these properties, the inactive hIFN-γ derivative compounds can be used for suppression of the endogenous hIFN-γ activity. Since this effect is dose dependent, the activity of the endogenous hIFN-γ can be modulated by varying blood concentration of the hIFN-γ derivative proteins. This approach is applicable in the cases when the overproduction of endogenous hIFN-γ causes health problems as in the case of autoimmune diseases, including MS.

Brief description of the Figures

Fig. 1 represents vector for expression of the hIFN-γ derivative, where:
P_1 is a synthetic phage promoter
R_3 is a synthetic ribosome binding site.

The following examples illustrate the present invention without limiting its scope and spirit :

Example 1: Truncated human hIFN-γ

Truncated human hIFN-γ protein composed of 116 aminoacid residues is obtained by expressing of a truncated hIFN-γ gene in *E. coli* LE392 cells. The latter is prepared by PCR using a synthetic full size hIFN-γ gene as a template and two synthetic forward and reverse primers (SEQ ID No: 1 and SEQ ID No: 2). The two primers are synthesized on a Cyclon Plus (MilliGene) gene synthesizer by the phosphoramidite method (0.2 μmole scale) and purified by electrophoresis in 15 % urea-polyacrylamide gel.

The truncated IFN-γ gene is prepared by two-step PCR amplification under the following conditions:

Table 1: Conditions for PCR

Programme	Number of cycles	Time	Temperature
I	1	5 min	92°C
II	5	1min	92°C
		1min	60°C
		1min	72°C
III	35	1min	92°C
		1min	65°C
		1min	72°C
IV	1	10 min	72°C

Table 2: Composition of the reaction mixture

Substances	Quantity
Tamplate DNA (50pg/μl)	1μl
Reverse primer (20pmol/μl)	1μl
Forward primer (20pmol/μl)	1μl
Taq-polymeraze (3 U/μl)	1μl
10 x PCR buffer	2μl
2 mM dNTP`s	2μl
dH_2O	12μl
Total	20μl

The amplified DNA is digested with *Hind*III and *Bam*HI, purified by agarose gel electrophoresis and cloned in the expression vector pJP_1R_3 (Fig. 1). To this end 20 μg plasmid DNA is dissolved in 150 μl *Hind*III buffer and digested with 20 U *Hind*III for 3 h at 37 °C. Reaction mixture is extracted consecutively with phenol and chloroform and the DNA is precipitated with ethanol. DNA is dissolved in 150 μl *Bam*HI buffer containing 20 U *Bam*HI for 3 h at 37 °C. The latter enzyme is inactivated by heating at 65°C for 10 min and the vector DNA is dephosphorylated with 1 μl (1 U/μl) calf intestinal alkaline phosphatase (Boehringer Mannhein) for 30 min at 37 °C. Reaction is stopped by adding 1/10 v/v 10xSTE buffer (100 mM Tris, 1 M NaCl, 10 mM EDTA, 10% SDS) followed by deproteinization with phenol and chloroform. DNA is then precipitated with ethanol and purified by agarose gel electrophoresis.

Ligation reaction is carried out overnight at 4°C at a molar ratio of vector to fragment DNA 3:1 and the ligation mixture is used for transformation of *E. coli* LE392 cells. The recombinant bacteria thus obtained are cultivated in LB medium (1% bacto-trypton, 0.5% yeast extract and 1% NaCl). LB agar is prepared by dissolving 1.5% bacto-agar in LB.

Primary transformants are selected in LB containing 50 μg/ml ampicillin following by cultivation on LB agar supplemented with 10 μg/ml tetracycline. The level of expression of the truncated IFN-γ gene is evaluated by ELISA using IFN-γ specific monoclonal antibodies. The truncated IFN-γ is purified by two step chromatography on C8-Sepharose and CM-Sepharose as described in EP0446582 B1. Antiviral activity (in international units) is determined by the protective effect of IFN-γ on WISH cells against the cytopatic action of stomatitis vesicular virus (VSV) as recommended by Forti et al. [14]. Analyses show that the truncated IFN-γ is devoid of any antiviral activity.

Example 2: Construction of a hybrid IFN-γ/IFN-α protein

The hybrid protein hIFN-γ/hIFN-α comprising 143 aminoacid residues consists of two N- and C-terminal parts: hIFN-γ (composed of 116 aminoacids) and hIFN-α (composed of 27 aminoacids). This protein is product of a hybrid hIFN-γ/hIFN-α gene prepared by ligation of two DNA fragments containing 116 (5' terminal) hIFN-γ and 27 (3' terminal) hIFN-α codons respectively. The two DNA molecules are obtained by PCR using full size hIFN-γ and hIFN-α genes as templates and two sets of primers (SEQ ID No 3– 6). The forward primer for modification of the hIFN-γ gene (SEQ ID No: 3) is designed to introduce a *Hind*III site at the 5' terminus (for ligation to the expression vector) and the reverse primer (SEQ ID No: 4) introduces *Eco*RI site at the 3' terminus (for ligation to the hIFN-α gene). The latter is designed also to eliminate the last 27 codons from the hIFN-γ gene. The forward primer for the hIFN-α gene (SEQ ID No: 5) carries a *Eco*RI site at the 5' terminus (for ligation to the hIFN-γ gene) and also to removes all but the last 27 codons from the hIFN-α gene. The reverse primer (SEQ ID No: 6) is designed to introduce a stop-codon (TAA) and a *Bam*H1 site (for ligation to the expression vector) at the 3' end of the hIFN-α gene fragment.

PCR is carried out under conditions described in Tables 1 and 2 and the amplified DNA fragments are digested with *Hind*III and *Eco*RI for hIFN-γ and *Eco*RI and *Bam*HI for hIFN-α respectively. The DNA fragments are further purified by agarose gel electrophoresis and ligated first to each other and then to the expression vector. The expression plasmid carrying the hybrid hIFN-γ/hIFN-α gene is transformed into *E. coli* LE392 cells. Bacteria are cultivated and the hybrid protein is purified as described in Example 1. The antiviral test shows that the hybrid protein is devoid of any antiviral activity.

Example 3: Inactivation of hIFN-γ by UV irradiation

Recombinant human hIFN-γ (purity higher than 99 %) is dissolved in 0.14 M NaCl, 10 mM Tris, pH 7.4 and exposed in a quartz cuvette to UV light at 290 nm for 15 min. This treatment leads to photolysis of the unique tryptophan residue and to 100 fold decrease in the hIFN-γ antiviral activity.

Example 4: Inhibitory effect of inactive hIFN-γ derivative proteins on the biological activity of intact hIFN-γ

Inhibitory effect of inactive hIFN-γ derivative proteins on the biological activity of intact hIFN-γ is investigated using an amniotic cell line WISH (known to be rich of hIFN-γ receptors). To saturate the hIFN-γ receptors, WISH cells are pre-incubated with inactive hIFN-γ derivative proteins for 1 h. The proteins are washed out, the cells are treated with different concentrations of intact hIFN-γ and infected with VSV according to [14]. The obtained results show a strongest inhibitory effect for the truncated (116 aminoacids) hIFN-γ, followed by the hybrid hIFN-γ/hIFN-α protein and the UV-inactivated hIFN-γ. Since all hIFN-γ inactive derivative proteins preserve their affinity to the hIFN-γ receptor, they a capable of suppressing biological activity of endogenous (native) hIFN-γ.

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CLAIMS

1. Inhibitor of endogenous human interferon-gamma (hIFN-γ), characterized in that they represent inactivated protein derivatives of the hIFN-γ with preserved affinity to the hIFN-γ receptor.

2. Inhibitor of endogenous hIFN-γ according to claim 1, characterized in that the inactivated protein derivatives of the hIFN-γ represent genetically modified variants of the hIFN-γ or recombinant hIFN-γ inactivated by physical or chemical methods.

3. Inhibitor of endogenous hIFN-γ according to claim 1 and 2, characterized in that its N-terminal primary structure coincides with that of the human hIFN-γ.

4. Inhibitor of endogenous hIFN-γ according to claim 1 to 3, characterized in that the genetically modified variants of hIFN-γ are derivatives of the hIFN-γ where the C-terminal part of the molecule is either deleted or replaced with a C-terminal fragment of another human protein.

5. Inhibitor of endogenous hIFN-γ according to claim 1 to 4, characterized in that the genetically modified variant of hIFN-γ is a hybrid protein hIFN-γ/ hIFN-α where the C-terminal part corresponds to that of the hIFN-α.

6. Inhibitor of endogenous hIFN-γ according to claim 1 and 2, characterized in that the inactivated hIFN-γ is obtained by UV irradiation of a recombinant human hIFN-γ at 290 nm.

7. Use of the inhibitor of endogenous hIFN-γ in the manufacture of a medicament for the treatment of autoimmune diseases.

8. Use of the inhibitor of endogenous hIFN-γ in the manufacture of a medicament for the treatment of multiple sclerosis.

AMENDED CLAIMS

Received by the International Bureau on 07 June 2006 (07.06.2006)

1. Inhibitor of endogenous human interferon-gamma (hIFN-γ) on the basis of inactivated protein derivatives of recombinant hIFN-γ characterised in that it represents genetically modified or physically or chemically treated variants of hIFN-γ with preserved affinity to the hIFN-γ receptor.

2. Inhibitor of endogenous hIFN-γ according to claim 1, characterised in that its N-terminal primary structure coincides with that of the human hIFN-γ.

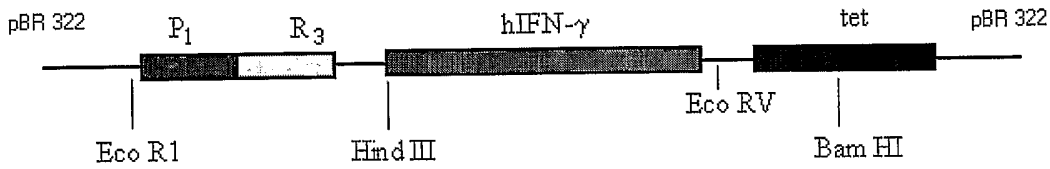
3. Inhibitor of endogenous hIFN-γ according to claims 1 and 2, characterised in that the genetically modified variants of hIFN-γ are derivatives of the hIFN-γ where the C-terminal part of the molecule is either truncated by 27 amino acids or replaced with a C-terminal fragment of another human protein.

4. Inhibitor of endogenous hIFN-γ according to claims 1-3, characterised in that the genetically modified variant of hIFN-γ is a hybrid protein hIFN-γ/hIFN-α where the C-terminal part corresponds to that of the hIFN-α.

5. Inhibitor of endogenous hIFN-γ according to claims 1 and 2, characterized in that the inactivated hIFN-γ is obtained by UV irradiation of a recombinant human hIFN-γ at 290 nm.

6. Use of the inhibitor of endogenous hIFN-γ according to Claims 1-5, in the manufacture of a medicament for the treatment of autoimmune diseases.

7. Use of the inhibitor of endogenous hIFN-γ according to Claims 1-6, in the manufacture of a medicament for the treatment of multiple sclerosis.



Фиг. 1

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INTERNATIONAL SEARCH REPORT

International application No
PCT/BG2005/000013

A. CLASSIFICATION OF SUBJECT MATTER
C07K14/57 C07K14/56 C12N15/62 A61K38/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NACHEVA GENOVEVA ET AL: "Human interferon gamma: Significance of the C-terminal flexible domain for its biological activity." ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 413, no. 1, 1 May 2003 (2003-05-01), pages 91-98, XP002373770 ISSN: 0003-9861 figure 2; table 3	1-4
X	WO 00/43033 A (THE UNIVERSITY OF QUEENSLAND; MORTON, HALLE; CAVANAGH, ALICE) 27 July 2000 (2000-07-27) the whole document	7,8
X	WO 94/12531 A (SCHERING CORPORATION; SEELIG, GAIL, F) 9 June 1994 (1994-06-09) the whole document	7,8

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