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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 January 2011 (06.01.2011)

PCT

(10) International Publication Number
WO 2011/001403 A1

(51) International Patent Classification:
C07K 14/46 (2006.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/IB2010/053018

(22) International Filing Date:
1 July 2010 (01.07.2010)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:
FI2009A000143 2 July 2009 (02.07.2009) IT

(72) Inventors; and
(71) Applicants : **PALMIERI, Angelo, Michele** [IT/IT]; Via Roma, 35, I-71015 Sannicandro Garganico (IT). **PAZZAGLI, Mario** [IT/IT]; Via Pierluigi da Palestrina, 9, I-50144 Firenze (IT). **REFOSCO, Fiorenzo** [IT/IT]; Via Libia, 24/A, I-36078 Valdagno (IT). **DE FILIPPIS, Vincenzo** [IT/IT]; Via Francesco Bonafede, 7, I-35126 Padova (IT). **SQUEO, Maria** [IT/IT]; Via Torre Meleto, 27, I-71015 Sannicandro Garganico (IT). **MONTEMURNO, Concetta** [IT/IT]; Via Romolo Caggese, 2, I-71015 Sannicandro Garganico (IT). **STANISCI, Francesco** [IT/IT]; Via Torre Meleto, 27, I-71015 Sannicandro Garganico (IT). **GRANITO, Mario, Leonardo** [IT/IT]; Via Romolo Caggese, 2, I-71015 Sannicandro Garganico (IT). **ZICHE, Marina** [IT/IT]; Via Castelbonsi, 10, I-50026 San Casciano In Val Di Pesa (IT).

(74) Agents: **GERVASI, Gemma** et al.; Corso di Porta Vittoria, 9, I-20122 Milano (IT).

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2011/001403 A1

(54) Title: PROTEIN WITH APOPTOTIC ACTIVITY ON CANCER CELLS, ITS PREPARATION AND USE

(57) Abstract: There is described a protein capable of performing an apoptotic action on various line of cancer cells.

PROTEIN WITH APOPTOTIC ACTIVITY ON CANCER CELLS, ITS PREPARATION AND USE

FIELD OF THE INVENTION

The present invention relates to the field of products having pharmacological and in particular anti-tumour activity.

BACKGROUND ART

Chemotherapy drugs are classified according to their chemical structure or their biological activity (see "The Pharmacological Basis of Therapeutics" by A. Goodman and M. Gilman, Pergamon Press, New York, 1990). In many cases anti-tumour drugs act on cell life cycle, blocking cell growth.

The classification of chemotherapy drugs also includes natural compounds or derivatives thereof (i.e. taxol or vincristine) capable of binding to tubulin and therefore of inhibiting mitosis.

The patent application WO 2007/085879 (by the same Applicants) describes fractions obtained from python blood (in particular Python Sebae) through centrifugation and collection of the supernatant and indicates some characteristics of a protein contained in said fractions potentially useful as anti-tumour agent.

In particular, a protein isolated from a fraction of suitable pre-treated python blood was identified in the aforesaid application.

Said protein was identified as having an apparent molecular weight of 98±10 kDa and non-covalent tetrameric form.

The protein consisted of four identical units all comprising the sequence His-Lys-Xxx-Glu-Ile-Xxx-His-Gly-Phe-Gly-Asp-Asp [Seq. N° 1], which was therefore considered essential for apoptotic activity.

However, subsequent studies in which the hypothetical protein was artificially reconstructed and tested gave completely negative results (i.e. no apoptotic activity) and it was therefore evident that the protein isolated and described in the preceding application was not the one of interest.

In view of the importance of chemotherapy treatment of tumours and considering that the tests indicated in the aforesaid application confirmed the presence of a substance having apoptotic activity in the fraction isolated from python blood with

the methods indicated therein, studies to attempt to identify said substance were continued.

Summary of the invention

The invention therefore relates to a new protein capable of performing an anti-tumour action on various tumour cell lines.

Detailed Description of the Invention

The present invention makes available two new proteins the apoptotic properties of which have been determined.

In particular, a protein according to the invention consists of an ether-tetramer consisting of four monomers, in two identical pairs.

In particular, two of said monomers, identified as P1, have sequence (SEQ ID N° 2), while the other two, identified as P2, have sequence (SEQ. ID N° 3).

Surprisingly, the protein identified above, and forming the subject-matter of the present application, notwithstanding its apparent similarity with the one identified in the preceding application and discussed above, has the desired apoptotic activity which was totally absent in the previous protein.

With regard to the apparent similarity between the two proteins, the following differences are in any case noted, which in their entirety undoubtedly justify the difference in behaviour. In particular, P1 has the following mutations with respect to the proteins identified previously: Gln17Glu, Arg26Lys, Pro 38 methylProline and Ile69Met, while P2 contains the following mutations: Asp65Glu, Pro178Tyr and Ala179Glu. Moreover, unlike the proteins indicated in the previous patent, P1 and P2 have a carbohydrate chain linked to the Asn138 residue.

The protein according to the invention was isolated from the active fraction of python blood identified in the aforesaid application as SX fraction, which represents the supernatant fraction obtained by simple centrifugation of the snake blood as prepared in the previous application PCT/EP01/14727 to which reference is made for the complete description of the process.

Briefly, aliquots of snake blood are collected by venipunture and centrifuged, preferably at about 3000 rpm for 5 minutes, collecting the supernatant. Alternatively, as described in the aforesaid patent, the supernatant collected can be only partially purified by means of ethanol fractional precipitation.

centrifuged for two minutes at 13,000 rpm and filtered with Millipore 0.45 μm filter, and subsequently loaded on the column, which was equilibrated in 20 mM Tris-HCl pH 7.5, containing 0.15 M NaCl at a flow rate of 0.5 ml/min, and eluted with a linear gradient of NaCl from 0.15 to 1.0 M. Absorbance of the effluent was determined at 280 nm. The protein material, eluted at the chromatographic peaks corresponding to the fractions from F1 to F10, was collected in fractions of 0.5 ml and the protein content was determined by means of the Bradford and Bicinchoninic Acid (BCA) procedures. Aliquots of these fractions were tested for cytotoxic and anti-tumour activity as described above.

Determination of the Molecular Weight by means of analytical gel-filtration chromatography

The apparent molecular weight of the protein material present in the fraction F5 and coming from ion exchange chromatography, was determined by means of analytical gel-filtration chromatography. Aliquots (0.1-0.2 ml) of the fraction F5 were loaded on a Superdose-12 (1 x 30 cm) column (GE-Healthcare), eluted with Tris-HCl buffer (20 mM, pH 7.5) at a flow rate of 0.3 mL/min. The column was calibrated with a standard mixture of proteins containing bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa). The interstitial volume (V_i) and the void volume (V_0) of the column were determined using the tripeptide Gly-Tyr-Gly and dextran blue (2000 kDa), respectively. The value of the distribution constant (K_D) was calculated by means of the equation $K_D = (V_e - V_0)/(V_i - V_e)$, where V_e is the elution volume of the various standard proteins loaded in the column. The chromatographic profile (G1) indicates the presence of a main peak at retention times compatible with the apparent molecular weight of the protein material contained in F5 equal to 87 kDa.

Reverse Phase Chromatography (RP-HPLC).

An aliquot (about 50 μg) of the protein material eluted at the main chromatographic peak obtained by gel-filtration chromatography (fraction G1) was acidified to pH 2.5 with 0.5 ml of trifluoroacetic acid (0.1% TFA in water) and fractioned by means of reverse phase HPLC (RP-HPLC), on a Vydac C4 analytical column (The Separation Group, Hesperia, CA) (4.6 x 150 mm, particle size 5 μm). The column was equilibrated with TFA 0.1% and the sample was eluted with a linear gradient

The supernatant (SX) is then collected and if necessary frozen.

The supernatant was firstly fractioned by means of ion exchange chromatography, obtaining fractions from F1 to F10. These fractions were all inactive, except for the fraction F5, which showed significant anti-tumour activity. The fraction F5 eluted from the ion exchange column was subsequently purified by means of gel-filtration chromatography, obtaining a main component (G1) that is eluted to retention times compatible with an apparent molecular weight of around 87 kDa. The protein material eluted from the gel-filtration column at the main chromatographic peak was collected and anti-tumour activity assays were carried out on this material, which confirmed that the protein species G1 is responsible for apoptotic activity. The fraction G1 was reduced to a small volume (0.5 ml) by means of slow evaporation under nitrogen flow and loaded on a reverse phase HPLC column. The chromatographic profile showed the presence of two main peaks named P1 and P2.

The material corresponding to the peaks P1 and P2 was then collected and treated separately with bovine trypsin. The proteolytic fragments were separated by means of reverse phase chromatography and analysed by means of high resolution mass spectrometry. The data obtained with mass spectrometry and by means of sequencing of the N-terminal end allowed us to obtain the amino acid sequence of P1 and P2 indicated below. Our results indicate that the active component of F5 is a tetrameric protein consisting of two molecules of P1 and two molecules of P2. Both P1 and P2 are glycoproteins and both contain 16 cysteine residues, which probably form eight disulphide bonds.

P1 and P2 have a similar sequence, but with significant differences in the amino acid composition and sequence. Anti-tumour activity is associated with the presence of the heterotetramer of P1 and P2. Neither the homotetramer of P1 or the homotetramer of P2 show anti-tumour activity.

EXAMPLE 1

Preparation of the sample and ion exchange chromatography

The python serum (200 µl), diluted 1:5 in Tris-HCl 20 mM, pH 7.5, 0.15 M NaCl, was fractioned by means of ion exchange chromatography using a MonoQ column (0.6 x 6 cm) (GE Healthcare). The serum diluted in Tris-HCl buffer was first

of acetonitrile-0.1% TFA from 35 to 60% in 35 min and at a flow rate of 0.8 ml/min. Absorbance of the effluent was determined at 280 nm. In these conditions, the chromatographic profile shows the presence of two main peaks, named P1 and P2, which were then lyophilised and subjected to subsequent chemical testing.

Determination of the Molecular weight of P1 and P2 by means of electrophoresis (SDS-PAGE) and mass spectrometry

The protein fractions P1 and P2 (50 µl) were lyophilised and analysed by means of electrophoresis on 12% polyacrylamide gel (SDS-PAGE). The protein bands were stained with a 0.1% solution of Coomassie Brilliant blue R-250 in water/methanol/ acetic acid (5:4:1) for 30 min. Destaining of the polyacrylamide gel was carried out by successive washes with a solution of 40% methanol and 10% acetic acid in water. The fractions P1 and P2 not utilised and their S-alkylated forms (P1-CM, P2-CM, P1-PE and P2-PE) were lyophylised, solubilised in 20 µl of a H_2O-acetonitrile solution (1:1 v/v), containing 1 % formic acid, and analysed by means of mass spectrometry, using a Mariner ESI-TOF Spectrometer (Perseptive Biosystems, Stafford, TX, USA). The power applied was 3.0 kV, the power of the capillary and the nozzle temperature 200 Volts and 140.0°C, respectively. The test results by means of SDS-PAGE indicate for both proteins a molecular weight slightly greater than 20 kDa, while analysis by means of high resolution mass spectrometry provides a value of 23135.2 ± 0.9 amu for P1 and of 23152.1 ± 0.8 amu for P2.

Identification of carbohydrate chains in P1 and P2

Identification of carbohydrate chains in P1 and P2 was carried out using a specific stain for glycoproteins, GelCode, by Pierce. P1 and P2 were first subjected to electrophoretic analysis by means of SDS-PAGE. Subsequently, the polyacrylamide gel was stained with the glycoprotein gel staining kit, obtaining a violet stain both for P1 and for P2, confirming their glycoprotein nature.

Determination of the Number of Cys Residues in the fractions P1 and P2.

Aliquots (100 µg) of the fractions P1 and P2, obtained after RP-HPLC, were subjected to reaction for reduction of the disulphide bonds and a carboxyamidomethylation reaction of the cysteine residues, which may be present along the amino acid sequence of the two proteins, resulting in the formation of S-

carboxyamidomethylated derivatives (S-CM). The reduction reaction was carried out at 37°C in Tris/HCl 0.1 M buffer, pH 7.8, containing 1 mM EDTA and 0.125 M dithiothreitol (DTT). After 2 h of reaction, iodoacetamide was added up to a final concentration of 0.25 M continuing the reaction for 90 minutes at 37°C and maintaining the pH constant by adding Tris base. The reaction mixture was subsequently fractioned by means of RP-HPLC on a C4 analytical column (4.6 x 150 mm, particle size 5 μm), equilibrated with 0.1% TFA in water and eluted with linear gradient of acetonitrile-0.1% TFA from 30 to 60% in 35 min and at a flow rate of 0.8 ml/min. Alternatively, the material eluted at the fractions P1 and P2 (about 100 μg), coming from RP-HPLC, after reduction reaction of the disulphide bridges, were treated for 90 min at 37°C with 4-vinylpyridine (4VP) (0.25 M), obtaining the corresponding S-pyridylethyl derivatives (S-PE). The reaction mixture was subsequently fractioned by means of RP-HPLC using a C3 Zorbax analytical column (4.6 x 150 mm, particle size 5 μm) (Agilent Technologies), eluted with linear gradient of acetonitrile-0.1% TFA from 10 to 30% in 5 min, and at a flow rate of 0.8 ml/min. Absorbance of the effluent was determined at 280 nm. The results of mass analysis indicate that the derivatisation of P1 and P2 with iodoacetamide determines in both cases an increase in mass equal to 912.9±0.5 amu, with respect to the protein with the reduced cysteines. The derivatisation of P1 and P2 with 4-vinylpyridine instead determines an increase in mass equal to 1696±0.7 amu. These results clearly indicate that 16 cysteine residues are contained both in P1 and in P2.

Determination of the N-terminal sequence by means of Edman chemistry.

The first 12 amino acids of the purified fractions of P1 and P2 were determined by means of Edman degradation carried out by the firm PRIMM (Biotech Products and Services, Milan, Italy) using an automated protein sequencer mod. 477-A (Applied Biosystems, Foster City, CA). Our data indicate that both in P1 and in P2, the N-terminal sequence is the same and corresponds to: HKCEICHGFGDD [SEQ. ID N° 4).

Determination of the amino acid sequence of P1 and P2 by means of enzymatic fingerprinting coupled with mass spectrometry techniques.

Digestion of S-CM-P1 and S-CM-P2 was carried out at 37°C for 12 h adding sequencing grade trypsin (by Promega, Madison, WI, USA) until obtaining a protease:protein ratio of 1:50 w/w. Tryptic digestion was blocked by adding 5 µl of trifluoroacetic acid at 5%. The tryptic digest of S-CM-P1 (1 µl) was placed on an AnchorChip™ target plate (Bruker Daltonics, Bremen, Germany) and dried; 0.35 µl of (α-cyano-4-hydroxycinnamic acid 5g/l in 50/50 acetonitrile/0.1% TFA) matrix was subsequently added. After having left the solvent to evaporate, mass analysis was carried out on an Ultraflex MALDI-TOF-TOF spectrometer (Bruker Daltonics) using Flex Control™ 2.4 software for data acquisition. The mass spectra were acquired in reflectron mode in the interval 800-3500 m/z. The instrument parameters are the following: the ion source power was set to 25kV, the reflectron to 26.30 kV, the delay time to 20 nsec. The instrument was calibrated prior to analysis using the Bruker peptide calibrant kit (1000-3000 Da) and the sample spectra were recalibrated internally with the trypsin autolysis signals. To acquire the MS data, a total of 400 shots were collected. The peptide masses present in each mass spectrum were used for their identification using the integrated software Biotools™ 3.0 in the NCBInr databank. The results obtained from enzymatic fingerprinting combined with mass spectrometry indicate that the sequence of P1 and P2 correspond respectively to:

HKCEICHGFG DDCDGYEEEC PSPEDKCGKI LIDIALAP*VS FRATHKNCFS
SSICKLGRVD IHVWDGVYMR GRTNCCDNDQ CEDQPLPGLP LSLQNGLYCP
GAFGIFTEDS TEHEVKCRGT ETMCLDLVGY RQESYAGNIT YNIKGCVSSC
PLVTLSERGH EGRKNDLKKV ECREALKPAS SD

and

HKCEICHGFG DDCDGYQEEC PSPEDRCGKI LIDIALAPVS FRATHKNCFS
SSICKLGRVD IHVWEGVYIR GRTNCCDNDQ CEDQPLPGLP LSLQNGLYCP
GAFGIFTEDS TEHEVKCRGT ETMCLDLVGY RQESYAGNIT YNIKGCVSSC
PLVTLSERGH EGRKNDLKKV ECREALKYES SD

Wherein P* is methylproline.

Moreover, the activity of the proteins as described above was assessed, comparing it with that of the known protein [SEQ. ID 1], the experimental data are given in the Table below.

	Capsase-3 activity (ADU)	PS IH % stained cells over basal
Ctr	0.4 ± 0.03	0 %
SEQ ID N° 1	0.5 ± 0.05	5%
SEQ ID N° 2-3	0.9 ± 0.02	60%

Tumour cells (A431) were subjected to treatment (50 μg/ml) for 18 h using as control a culture medium with 1% serum.

Capsase-3 activity was measured with Western blotting.

The data of three determinations are given as arbitrary density unit (ADU).

Phosphatidylserine (PS) expression was evaluated by immunohistochemical analysis and the data obtained are given as % of cells expressing PS positive staining with respect to the control.

Capsase-3 is a key mediator of apoptosis in mammal cells.

The data given in the Table clearly indicate how the sequences SEQ N 2 and 3 significantly promote capsase-3 activation.

When the cells undergo apoptotic death, PS becomes exposed on the cell surface. Coherently with capsase-3 activation, the sequences SEQ N 2 and 3 significantly promote the expression of PS on tumour cells showing a clear pro-apoptotic effect. On the contrary, SEQ N 1 has no effect either on capsase-3 activity or on PS expression.

Claims

1. Protein consisting of two identical pairs of monomers the N-terminal ends of said monomers having sequence: HKCEICHGFGDD (SEQ. HKCEICHGFGDD (SEQ. ID N° 4).

2. Protein according to Claim 1 wherein a monomer in each pair of monomers has sequence (SEQ ID N° 2) while the other has sequence (SEQ. ID N° 3):

SEQ ID N° 2

HKCEICHGFG DDCDGYEEEC PSPEDKCGKI LIDIALAP*VS FRATHKNCFS
SSICKLGRVD IHVWDGVYMR GRTNCCDNDQ CEDQPLPGLP LSLQNGLYCP
GAFGIFTEDS TEHEVKCRGT ETMCLDLVGY RQESYAGNIT YNIKGCVSSC
PLVTLSERGH EGRKNDLKKV ECREALKPAS SD

and

SEQ. ID N° 3

HKCEICHGFG DDCDGYQEEC PSPEDRCGKI LIDIALAPVS FRATHKNCFS
SSICKLGRVD IHVWEGVYIR GRTNCCDNDQ CEDQPLPGLP LSLQNGLYCP
GAFGIFTEDS TEHEVKCRGT ETMCLDLVGY RQESYAGNIT YNIKGCVSSC
PLVTLSERGH EGRKNDLKKV ECREALKYES SD

Wherein P* is methylproline.

3. Use of the protein according to Claims 1 or 2 for the preparation of pharmaceutical compositions having apoptotic activity on cancer cells.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/053018

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/46 A61P35/00
ADD.

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 A61P

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	WO 2007/085879 A1 (ZICHE MARINA [IT]; PAZZAGLI MARIO [IT]; PALMIERI ANGELO MICHELE [IT];) 2 August 2007 (2007-08-02) page 5, line 9 - line 10; claims 15, 16 page 10, line 21 - page 11, line 8	1-3
A	THWIN MM ET AL: "Recombinant antitoxic and antiinflammatory factor from the nonvenomous snake Python reticulatus: phospholipase A2 inhibition and venom neutralizing potential" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY, EASTON, PA.; US, vol. 39, no. 31, 8 August 2000 (2000-08-08), pages 9604-9611, XP002179017 ISSN: 0006-2960 * abstract	1-3

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 November 2010	17/11/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vreugde, Sarah

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2010/053018

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007085879 A1	02-08-2007	NONE	