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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.

(54) Title: ACTIVATOR FOR INSULIN DEGRADING ENZYME

(57) Abstract: The present invention relates to the use of Suramin in the manufacture of medicaments for treatment of neurodegenerative disorders, in particular of Alzheimer's Disease.



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Activator for Insulin Degrading Enzyme

The present invention relates to suramin as activator of IDE and the use of Suramin in the treatment of neurodegenerative disorders such as Alzheimer's disease.

The invention relates to suramin as activator of insulin degrading enzyme (IDE).

Furthermore, the invention relates to the use of suramin or pharmaceutically acceptable salts thereof for the manufacture of medicaments for the treatment of IDE-related diseases.

Suramin is known and has been described in McCain et al. (J Biol Chem, 2004, 279:14713-25) having inhibitory and activatory activities on protein-tyrosine phosphatases. US 2005143461 B1 discloses the use of suramin composition as antimicrobial agents and/or sperm-function inhibitors. In Eisenberger et al. (Journal of Clinical Oncology, 1995, 13:2174-86) the authors describe the use of suramin as an anti-cancer agent in the treatment of prostate cancer.

It has been found that suramin enhances IDE activity. Perez et al. (Neurochem Res. 2000; 25(2):247-55) described that the activity of IDE is reduced in Alzheimer's Disease brains. Furthermore, it was shown that neuron-specific overexpression of human IDE in mice transgenic for human Amyloid precursor protein (APP) leads to a reduction of Abeta amyloid (Leissring et al., Neuron. 2003 Dec 18;40(6):1087-93.). Therefore, the invention provides the use of suramin or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for treatment of a neurodegenerative disorder such as Alzheimer's Disease.

The invention also pertains to a pharmaceutical composition comprising suramin, and a compatible pharmaceutically acceptable carrier material.

The carrier material can be an organic or inorganic one suitable for enteral, percutaneous or parenteral administration. Suitable carries include water, gelatine, gum arabic, lactose, starch, magnesium stearate, talc, vegetable oils, polyalkylene-glycols, petroleum jelly and the like. Furthermore, the pharmaceutical preparations may contain other pharmaceutically active agents. Additional additives such as flavouring agents,

preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

The pharmaceutical preparations can be made up in any conventional form including: a) a solid form for oral administration such as tablets, capsules, pills, powders, granules and the like; b) a liquid form for oral administration such as solutions, syrups, suspensions, elixirs and the like; c) preparations for parenteral administration such as sterile solutions, suspensions or emulsions. The pharmaceutical preparations may be sterilized and/or may contain adjuvants such as preservatives, stabilizers, wetting agents, emulsifiers, salts for varying the osmotic pressure and/or buffers.

Parenteral dosage forms may be infusions or injectable solutions which can be injected intravenously or intramuscularly. These preparations can also contain other medicinally active substances. Additional additives such as preservatives, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

15

The term "activator" as used herein refers to a molecule that increases the activity of an enzyme. The term "activity of an enzyme" as used herein refers to the turnover rate of an enzyme for its substrate.

Having the invention now generally described, the same will become better understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified, in connection with the following figures:

20

Figures

Figure 1 shows the assay principle. The substrate, the compound of formula F-P-Q, comprises a fluorophore (grey box), a peptide (circles) with at least one cleavage site of the enzyme (filled circles) and a quencher (framed). The uncleaved substrates shows no or low fluorescence as the quencher reduces the fluorescence of the fluorophore by collisional quenching. When the enzyme E cleaves the peptide the perturbation of the fluorophore stops and the fluorescence increases.

Figure 2 shows a diagram of the formation of Abeta(1-42) fibrils without enzymes, in the presence of NEP (neprilysin) and in presence with recombinant rat IDE (insulin degrading enzyme) evaluated by a thioflavine T assay.

Thioflavine T assay: Abeta1-42 (10 μ M) alone or in the presence of NEP or IDE were incubated at 37°C for 72 h. Thioflavine T fluorescent signal indicates the ability of Abeta1-42 and fragments to form fibrils (A). The degradation of Abeta 1-42 by IDE was followed as a function of time. At various time points (0-30 min) the enzymatic inhibitor, N-ethyl maleimide (NEM from Acros) was added at 1 mM final concentration. Each sample was incubated at 37°C for additional 72 hrs. Thioflavine T fluorescence measurements were performed as described above (B).

Figure 3 shows a diagram of the cell toxicity of the Abeta(1-42) fibrils. When incubated with NEP (neprilysin) or recombinant rat IDE (insulin degrading enzyme) the cell toxicity (MTT assay) is abolished.

MTT assay: Abeta1-42 (1 μ M) alone or with NEP or IDE (10 nM) were incubated on PC12 cells (16 h). Using the MTT kit assay the cell viability was measured as a function of concentration of enzyme (nM). The black squares indicate NEP and Abeta(1-42) (1 μ M), the white circles indicate NEP alone, black circles indicate IDE and Abeta(1-42) (1 μ M), the white squares indicate IDE alone.

Figure 4 shows schematically cleavage sites of Abeta(1-42) for NEP (neprilysin) and recombinant human IDE (insulin degrading enzyme). The position of the major cleavage sites of Abeta(1-42), Cys-Abeta 16-23-Trp and MR121-Cys-Abeta 16-23-Trp were identified by MS following IDE (empty arrowheads) or NEP (filled arrowheads) digestion.

Figure 5 shows a diagram of the kinetic measurements with (a) 10 nM recombinant human IDE and 10 μ M substrate (ratio labeled to unlabeled substrate: 1:1000), (b) 1 nM

NEP and 3 μ M substrate (ratio labeled to unlabeled substrate: 1:1000), crosses: substrate and enzyme, circles: only substrate.

Figure 6 shows a diagram of the K_m and V_{max} determination for recombinant human IDE. In the diagram the slopes of the linear part of kinetic measurements were
5 plotted against the total substrate concentration.

Figure 7 shows a screen of a small library using recombinant human IDE. The dashed lines indicate the three standard deviation from the average signal expressed in the percent activation of the enzyme. One positive was identified: Suramin.

Figure 8 shows the K_m and V_{max} dependency of recombinant human IDE on
10 Suramin.

Examples

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated.

5 Example 1: activators for degradation of Abeta (1-42)

In this example the described method is used to identify enhancers that selectively activate IDE (Insulin-degrading enzyme) or NEP (neprilysin). By enhancement of these amyloid degrading enzymes the degradation of Abeta shall be increased and thereby prevent the formation of amyloid plaques or reduce the already formed ones.

10 A) Substrate

Materials

Synthetic Amyloid beta peptide 1-42 (Abeta1-42) was purchased from Bachem (Bubendorf, Switzerland);

Hexafluoro-2-Propanol was purchased from Sigma (HFIP; Sigma H-8508).

15 Buffer 1 (50 mM Hepes buffer pH 7.4, 100 mM NaCl, 0.05% bovine serum albumin) (Leissring MA, Lu A, Condron MM, Teplow DB, Stein RL, Farris W, Selkoe DJ Kinetics of amyloid beta-protein degradation determined by novel fluorescence- and fluorescence polarization-based assays. *J Biol Chem.* (2003), 278(39):37314-20).

Recombinant rat IDE, was purchased from Calbiochem, (Germany, His tag
20 recombinant, Spodoptera).Ref: Song, E-S., et al(2001) *J.Biol.Chem.* 276, 1152-115

Recombinant Human IDE was produced in sf9 cells as previously described in Song, E-S., et al. (*J.Biol.Chem.*, 2001. 276, 1152-115) and Chésneau V and Rosner M.R, (Protein Expression and Purification 19, 91-98 (2000)

NEP was produced in *Pischia pastoris* as previously described in Dale G.E et al,
25 (*Acta Cryst.* (2000). D56, 894-897).

Methods

Identification of proteolytic cleavage site of Abeta1-42

Abeta synthetic peptide was pre-solubilized in HFIP as previously described in Dahlgren, K. N., Manelli, A. M., Stine, W. B., Jr., Baker, L. K., Krafft, G. A., and LaDu, M. J. (2002) Oligomeric and fibrillar species of amyloid-beta peptides differentially affect cell viability. *J Biol Chem*, 277: 32046-32053). HFIP pre-treated Abeta was solubilized to 10µM final concentration in Buffer 1 and incubated at room temperature either with 10nM NEP or IDE. At various time points (after 0, 2 and 5 min) the reaction mixture was removed and analyzed by HPLC and MS.

For MS analysis the solution was mixed with a saturated alpha-cyano cinnamic acid (Sigma) and applied to MALDI-target (Bruker, Bremen, Germany). The samples were analyzed with a Bruker Daltonics Ultraflex TOF/TOF mass spectrometer (Bruker, Bremen, Germany).

High performance liquid chromatography (HPLC) analysis was performed on a 1110 Agilent system using reverse phase column (Poroshell 300SB-C8 available at Agilent, 395 Page Mill Road, Palo Alto, CA 94306) and a linear gradient (20-60 % acetonitril, 01% TFA in water) over 60min. Peptides were monitored by UV light absorbance (at 205 nm and 280 nm) and peaks collected for further MS analysis. The UV area of peaks was correlated to the relative amount of each proteolytic fragment.

Cell toxicity assay

Abeta1-42 prepared at 50 µM in a 10 mM Tris buffer, pH 8 was added to the medium of PC12 cells (ATTC: CRL1721) (HAM's F-12K medium from Biosources International, Napa, California 94558, United States) containing 10% HS, 5% FCS) at 1 µM alone, or with NEP or IDE. The cells were incubated 16 h; the cellular viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation kit (MTT, Roche Molecular Biochemicals, Switzerland).

Thioflavin T assay

Amyloid formation was quantitatively evaluated by the fluorescence emission of thioflavin T bound to amyloid fibrils. Abeta1-42 at 10 µM was incubated alone, or with NEP (200 nM) or IDE (100 nM) at 37°C for 72 h. After incubation 50 mM glycine buffer (pH9.2) and 2 µM Thioflavin T were added and fluorescence measured (ex: 435 nm, em: 485 nm) in a 96 well plate reader (Polarstar galaxy BMG Fluostar OPTIMA Labtechnologies, In der Spöck 10, Offenburg, DE).

The degradation of Abeta(1-42) by IDE was stopped at various time points with the inhibitor N-ethyl maleimide (NEM from Acros Organics B.V.B.A., Janssen Pharmaceuticallaan 3A, Geel, Belgium). Abeta1-42 at 10 μ M was incubated alone, or with IDE (100 nM) at 37°C. At various time points (0-30 min) the enzymatic inhibitor, N-ethyl maleimide was added at 1 mM final concentration. Each sample was incubated at 37°C for additional 72 hrs. Thioflavine T fluorescence measurements were performed as described above.

10 Results

Identification of major proteolytic cleavage sites of Abeta1-42.

The major cleavage site of Abeta1-42 by NEP and IDE were determined by mass spectrometry and HPLC analysis (Table1). Both enzymes degraded Abeta1-42 at various cleavage sites located mainly within the central hydrophobic region. IDE and NEP
15 primarily initiated proteolysis from residues 18 to 21 and 16 to 20 respectively. Both enzymes shared a common cleavage site between the Phe19-Phe20 residues.

NEP or IDE endopeptidase activity prevents Abeta fibril formation

The central region of Abeta is highly hydrophobic and is essential for the
20 amyloidogenic property of the peptide. Interestingly both enzymes degrade Abeta within this central region. Both enzymes were able to abolish formation of Abeta1-42 fibrils as shown in the thioflavine T assay (figure 2). Co-incubation of Abeta1-42 with NEP or IDE abolished toxicity of PC12 cells induced by Abeta1-42 fibrils (figure 3). These results suggested that the proteolytic activity of both enzymes was sufficient to prevent Abeta1-
25 42 aggregation and Abeta 1-42 induced cytotoxicity.

MR121-substrate design

The substrate for the assay for identifying activators of IDE and NEP contains the main proteolytic cleavage sites identified in the central hydrophobic region of Abeta1-42,
30 namely the sequence of Abeta16-23 (Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp). A cysteine was

added at the N-terminus of the peptide to link the MR121 fluorophore and a tryptophan residue at the C-terminus of the peptide (see Materials and Methods).

The proteolytic fragments of MR121-C-Aβ₁₆₋₂₃-W and unlabeled substrate (C-Aβ₁₆₋₂₃-W) following NEP and IDE digestion were identified by mass spectrometry MS and HPLC as previously described. Results confirmed that both substrates labelled and unlabeled share identical cleavage sites than those identified with Aβ₁₋₄₂ (Figure 4).

B) Assay

10 Material and Methods

IDE/NEP

The 10 amino acid peptide Cys- Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Trp contained in the substrated was synthesized with a purity of 95% by Biosyntan GmbH, Berlin, DE (For improved yield of synthesis the carboxyl group of the tryptophan was replaced by an amide group (CO-NH₂)). The reactive form of the fluorophore, MR121-maleimide, is described in EP 747 447. MR121-maleimide was attached to the sulfhydryl group of the cysteine residue of the substrate peptide by covalent coupling (Derek G. Smyth, Atsuo Nagamatsu, Joseph S. Fruton; Some Reactions of N-Ethylmaleimide; *J. Am. Chem. Soc.* (1960); 82(17): 4600-4604) and purified on an analytical HPLC (Merck Hitachi D-6000, Merck USA) using a C18 column (Marchery-Nagel, cc125/4, Nucleosil 100-5, protect 1). IDE was the commercially available recombinant rat insulin degrading enzyme from Calbiochem (Merck Biosciences, San Diego, California, USA). The extracellular domain of human neutral endopeptidase (neprilysin, huNEP) was expressed in *Pichia pastoris* and purified as previously described (Dale, G. E.; D'Arcy, B.; Yuvaniyama, C.; Wipf, B.; Oefner, C.; D'Arcy, A. *Acta Crystallogr D Biol Crystallorg* (2000), 56: 894-897). All experiments were carried out in assay buffer (50 mM Hepes, 100 mM NaCl, 0.05% BSA, pH 7.4) with a total assay volume of 40 µl.

All measurements were performed in 384 well microtiter plates (Costar ref. 3640, clear, non-binding surface). For IDE the total assay volume was 40 µl. For the kinetic readout a Zeiss platevision reader was used with excitation at 630 nm (bandpass filter: 630(50) nm) and emission at 695 nm (bandpass filter: 695(55) nm) and with excitation at 560 nm (bandpass filter: 560(55) nm) and emission at 645 nm (bandpass filter: 645(75) nm) for Atto 590 at a constant temperature of 30°C.

Results

Figure 5 shows kinetic measurements of the enzymatic reactions with 10 nM IDE (a) and 1 nM NEP (b). The concentrations of the respective substrates were 10 μ M (IDE) and 3 μ M (NEP). The readout was started immediately after initiation of the reaction by the addition of the enzyme to the substrate. The MR121 fluorescence was measured for approximately three hours every five minutes (for IDE and NEP).

To determine K_m and V_{max} kinetic measurements with 10 nM IDE and different substrate concentrations ranging from 300 nM to 15 μ M were carried out. Because of light absorption at high concentrations of MR121 only 0.1% of the total substrate was labelled with the fluorophore. The readout was started immediately after the reaction was initiated by the addition of 20 μ l enzyme to 20 μ l substrate and measured for approximately three hours every five minutes. K_m and V_{max} were calculated from the linear slope of the enzyme kinetic as a function of the total substrate concentration (Fig. 6a). The measurement resulted in $K_m = 1.54 \mu$ M and $V_{max} = 38'537$ rfu/min. A similar measurement was done for NEP (data not shown) giving $K_m = 1.4 \mu$ M and $V_{max} = 78'000$ rfu/min.

A screen of a small subset of our library was carried out at 20 μ M candidate compound, 10 nM IDE and 100 nM substrate (100% MR121 labelled). The compounds were diluted by adding 62.5 μ l assay buffer to 2 μ l of a 5mM DMSO stock solution. 5 μ l of the resulting compound solution were transferred to the assay plate containing 20 μ l substrate. With the addition of 15 μ l IDE the reaction was started and measurements of the MR121 fluorescence were done every two minutes for one hour. Figure 7 shows the activation of IDE by the test compounds where 100% is the enzyme activity without compound. One clearly positive compound was identified, activating IDE approximately by a factor of four. No false positives due to interference of background fluorescence of the compounds were detected proving this assay as robust and sensitive.

For different concentrations of the one activator found in the screened subset of the library K_m and V_{max} were determined. Figure 8 shows that this activator has no influence on K_m but increases V_{max} substantially. This experiment was done with 10 nM IDE and substrate concentrations ranging from 100 nM to 15 μ M with 0.1% of the substrate labelled with MR121.

A)

Order	Fragment	IDE	
		mass calculated	mass observed
1	1-42	4512.3	4512.6
2	1-19	2314.1	2314.0
3	1-20	2461.2	2460.9
4	1-18	2167.0	2166.8
	1-13	1561.7	1561.5
5	1-14	1698.7	1698.6
6	15-28	1581.8	1581.9

B)

Order	Fragment	NEP	
		mass calculated	mass observed
1	1-42	4512.3	4512.6
2	1-17	2068.0	2067.9
	1-19	2314.1	2314.1
3	1-16	1954.9	1954.9
4	1-9	1033.4	1033.4

- 5 Table 2: Proteolytic fragments of Abeta1-42, following IDE (A) and NEP (B) digestion identified by mass spectrometry. All masses are determined as single charged monoisotopic masses (MH^+). Order of appearance of the fragments was determined by relative quantitative HPLC analysis.

Claims

1. Use of Suramin in the manufacture of medicaments for treatment of neurodegenerative disorders.
2. Use of Suramin according to claim 1, wherein the neurodegenerative disorder is the
5 Alzheimer's Disease.

Figure 1:

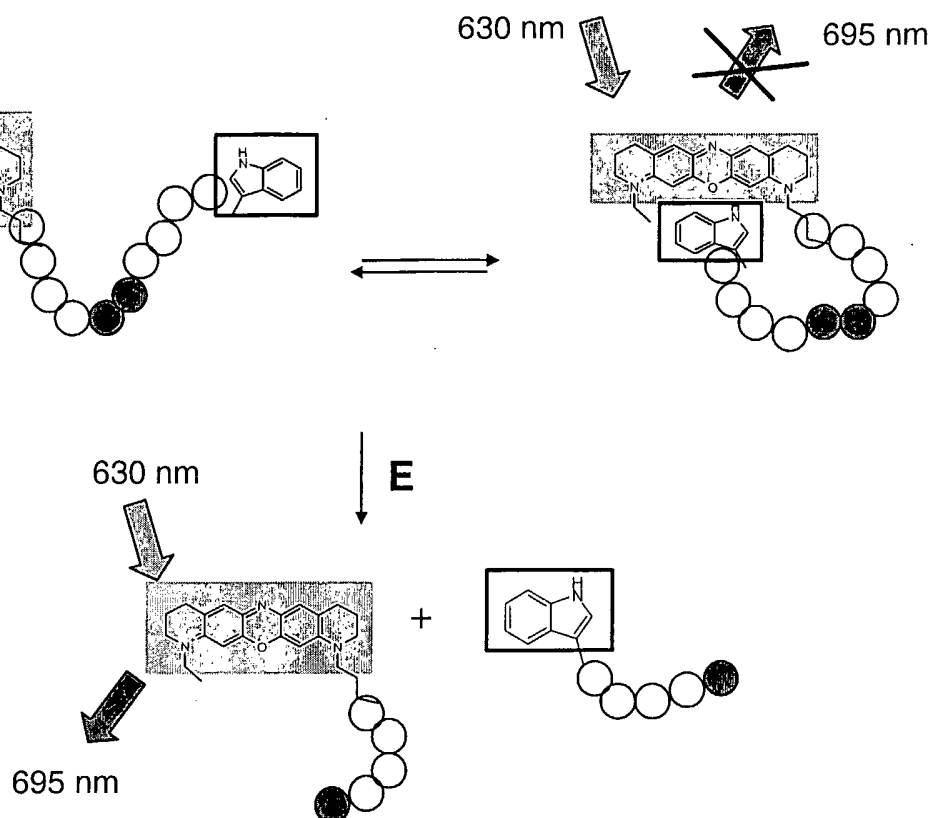


Figure 2:

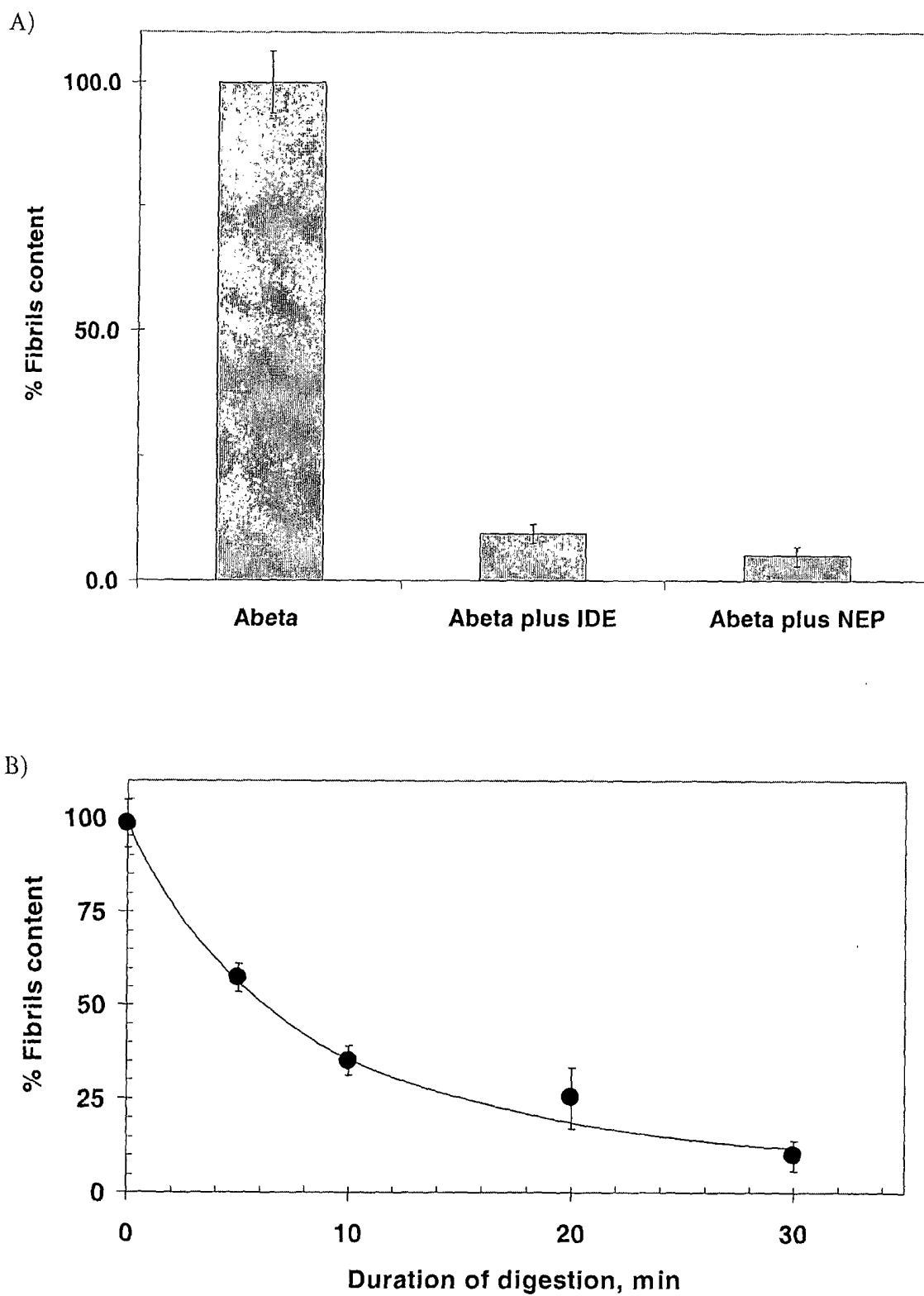
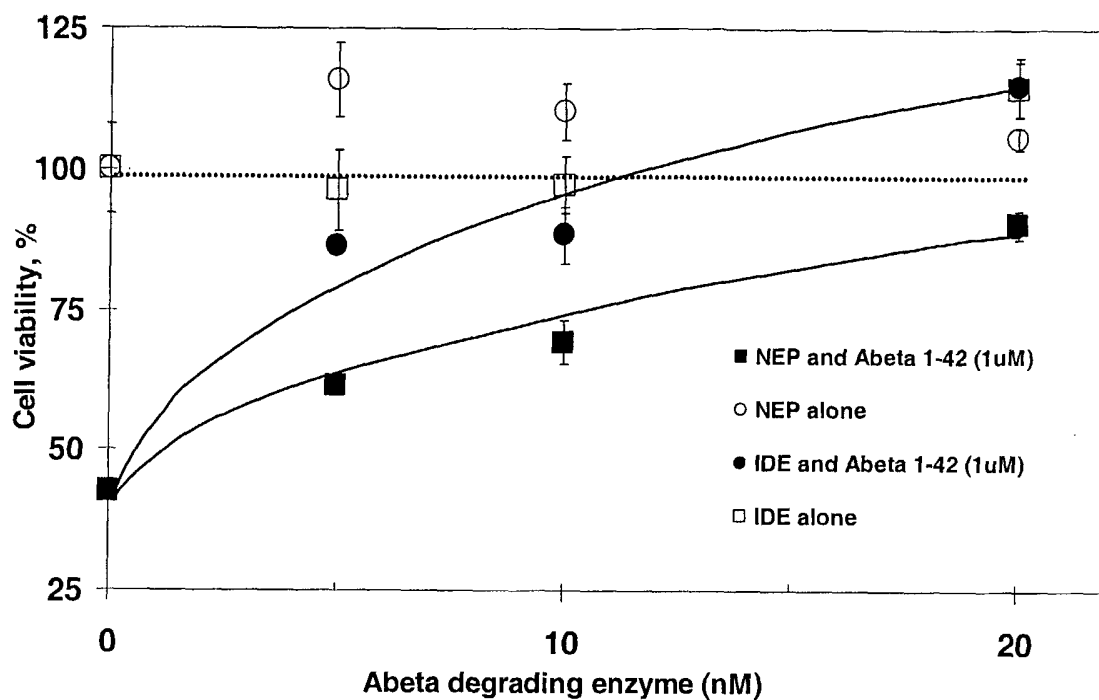


Figure 3:



5 Figure 4:

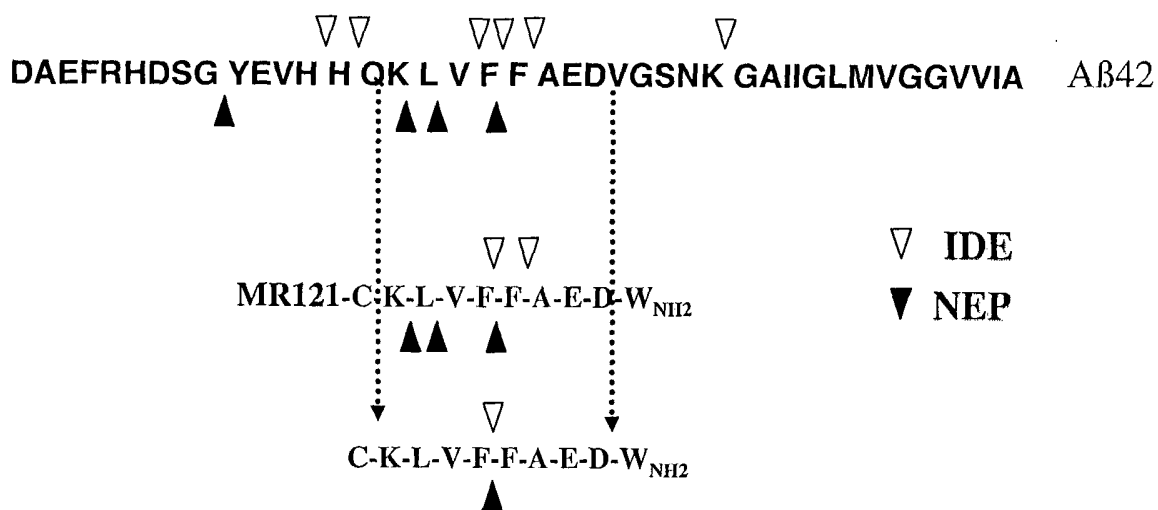


Figure 5:

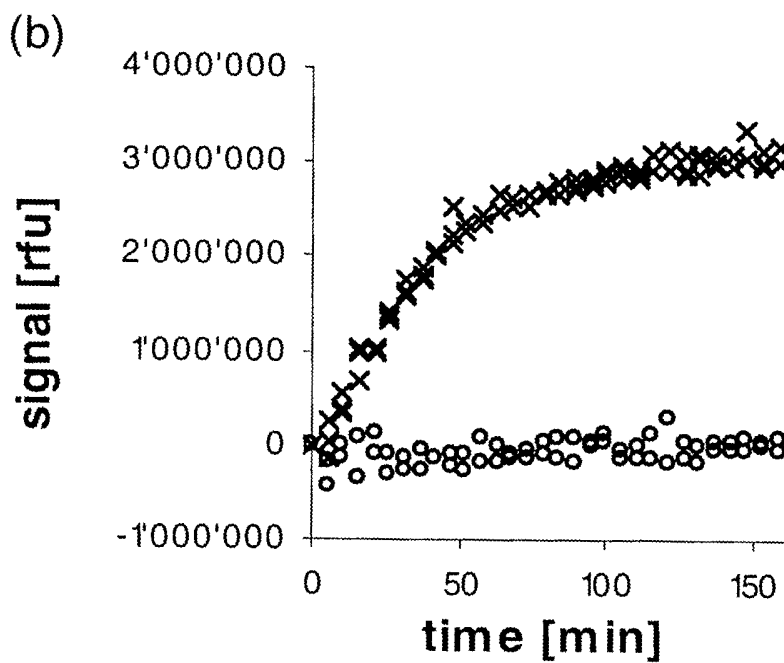
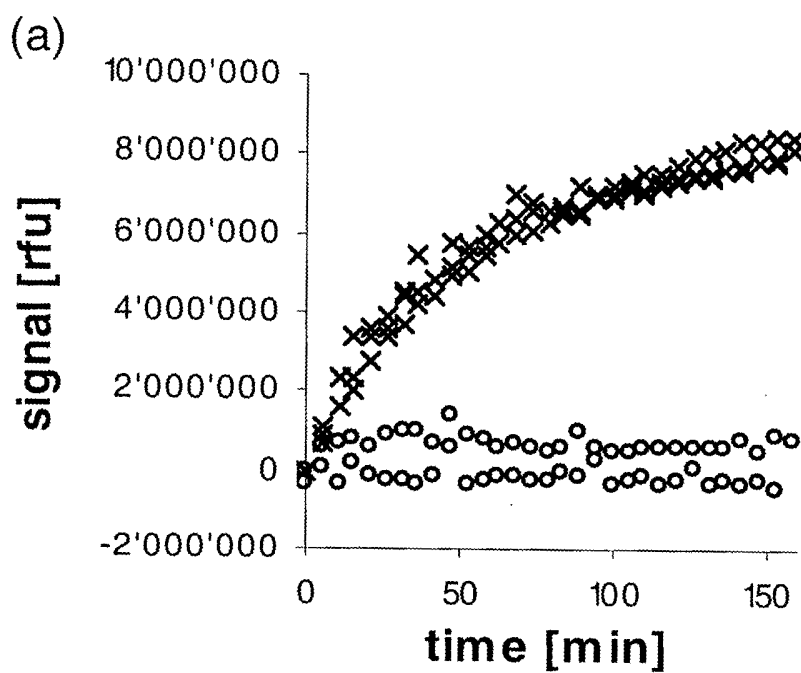


Figure 6:

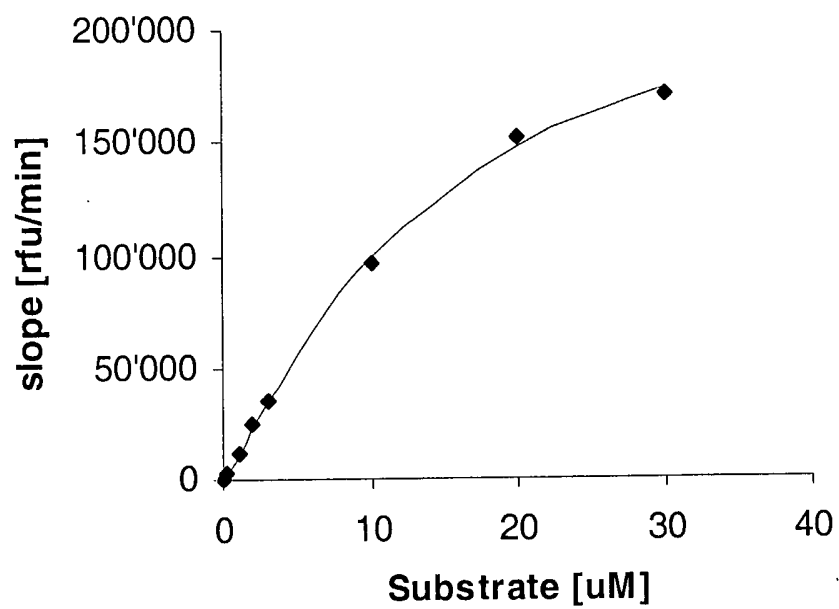
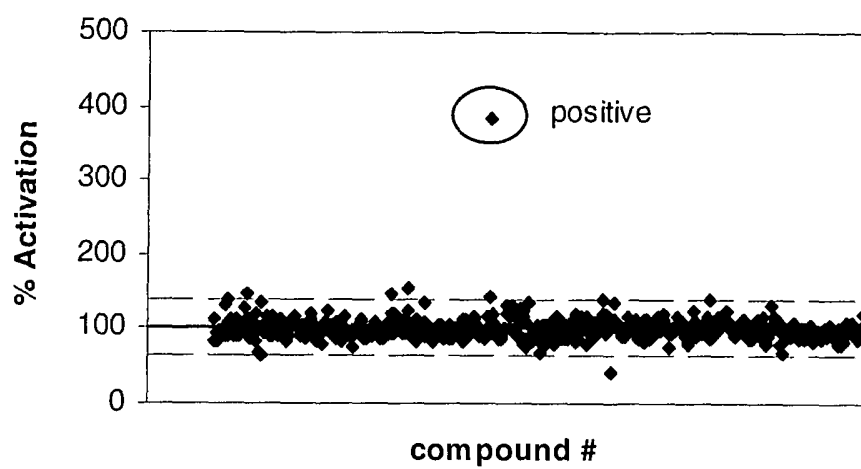
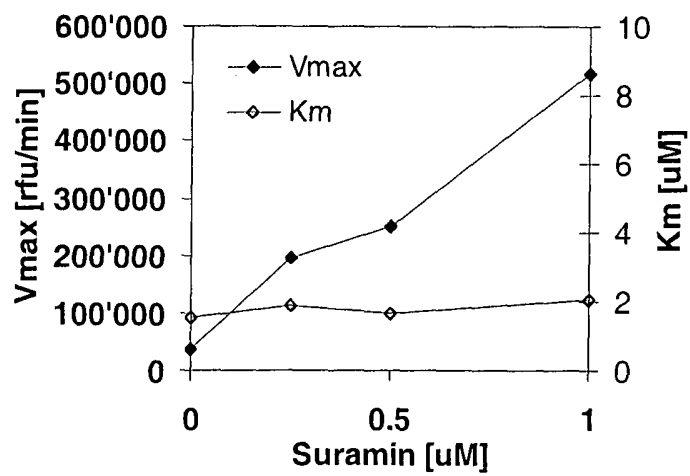


Figure 7:



5 Figure 8:



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/013653

A. CLASSIFICATION OF SUBJECT MATTER
A61K31/00

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)
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, EMBASE, 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	WINKLHOFFER K F ET AL: "SURAMIN PREVENTS PROPAGATION OF INFECTIOUS PRION PROTEIN AND DELAYS ONSET OF PRION DISEASE" SOCIETY FOR NEUROSCIENCE ABSTRACTS, SOCIETY FOR NEUROSCIENCE, US, vol. 27, no. 2, 2001, page 1996, XP001098832 ISSN: 0190-5295 last two sentences	1
X	WO 98/03178 A (SMITHKLINE BEECHAM PLC; BROWN, FRANK; MITCHELL, DAVINA, ELIZABETH; RAH) 29 January 1998 (1998-01-29) page 3, lines 2-16; page 12 - page 14, tables 6 and 7; claim 4 ----- -/--	1,2

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

☒ See patent family annex.

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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

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Date of the actual completion of the international search

13 March 2006

Date of mailing of the international search report

30/03/2006

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Hennard, C

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/013653

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EISENBERGER MARIO A ET AL: "Phase I and clinical evaluation of a pharmacologically guided regimen of suramin in patients with hormone-refractory prostate cancer" JOURNAL OF CLINICAL ONCOLOGY, GRUNE AND STRATTON, NEW YORK, NY, US, vol. 13, no. 9, September 1995 (1995-09), pages 2174-2186, XP002332944 ISSN: 0732-183X cited in the application page 2175, right-hand column -----	1,2
Y	GILCH SABINE ET AL: "Recognition of luminal prion protein aggregates by post-ER quality control mechanisms is mediated by the preoctarepeat region of PrP." TRAFFIC (COPENHAGEN, DENMARK) APR 2004, vol. 5, no. 4, April 2004 (2004-04), pages 300-313, XP002371726 ISSN: 1398-9219 page 301, "Results"; page 303, figure 3 -----	1,2
Y	CHAN CHIN-FENG ET AL: "Suramin prevents cerebellar granule cell-death induced by dequalinium" NEUROCHEMISTRY INTERNATIONAL, vol. 38, no. 2, February 2001 (2001-02), pages 135-143, XP002371727 ISSN: 0197-0186 page 138, last paragraph -----	1,2
A	US 6 348 464 B1 (JACKSON PAUL F ET AL) 19 February 2002 (2002-02-19) column 23, lins 34-47; column 24, lines 33-41; column 25, lines 8-10 and 34-36; claims 9 and 15 -----	1,2

INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/EP2005/013653

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9803178	A	29-01-1998	NONE	
US 6348464	B1	19-02-2002	AU 1597201 A	06-06-2001
			WO 0134596 A2	17-05-2001