Title: PEPTIDES INHIBITING SPECIFIC CLEAVING ACTIVITIES OF PRESENLINS

Abstract: The present invention relates to the identification of peptides that specifically inhibit the cleavage activity of presenlins. More specifically, the present invention discloses peptides derived from amyloid precursor protein that specifically inhibit cleavage of amyloid precursor protein by gamma-secretase without affecting the cleavage of notch by the same enzyme. Consequently, these peptides can be efficiently used to prevent plaque deposition in the brain of Alzheimer’s disease patients.
Peptides inhibiting specific cleaving activities of presenilins

Field of the invention
The present invention relates to the identification of peptides that specifically inhibit the cleavage activity of presenilins. More specifically, the present invention discloses peptides derived from amyloid precursor protein that specifically inhibit cleavage of amyloid precursor protein by gamma-secretase without affecting the cleavage of other substrates such as Notch, Syndecans or Erb-B4 by the same enzyme. Consequently, these peptides can be efficiently used to prevent plaque deposition in the brain of Alzheimer’s disease patients.

Background of the invention
Presenilin 1 and 2 (PS1 and PS2) are highly homologous proteins implicated in familial Alzheimer’s disease (FAD) (Rogaev et al., 1995; Sherrington et al., 1995). All FAD-mutations in the presenilins (PSs) increase the secretion of the highly amyloidogenic Aβ42 peptide, a major constituent of the plaques in the brains of AD patients (Citron et al., 1997; Duff et al., 1996; Scheuner et al., 1996). Aβ42- and the more abundant Aβ40-peptide are generated from the larger amyloid precursor protein (APP) by the consecutive action of two enzymes, β- and γ-secretes (De Strooper and Annaert, 2000; Haass and Selkoe, 1993; Selkoe, 1998). Several lines of evidence imply the PSs in γ-secretase activity. (i) Aβ secretion in cell lines and neurons derived from PS1-/- or PS1-/-PS2-/- embryos is strongly inhibited while the α- and β-cleaved APP C-terminal stubs, the immediate substrates for γ-secretase, accumulate in these cells (De Strooper et al., 1998; Herreman et al., 2000; Naruse et al., 1998; Zhang et al., 2000). (ii) PSs are part of a multiprotein complex that exhibits γ-secretase activity in detergent extracts (Li et al., 2000). (iii) Indirect evidence implies two conserved aspartic acid residues of the transmembrane domains 6 and 7 in the catalytic activity of the γ-secretase (Wolfe et al., 1999). This putative active site displays remote similarity with the catalytic site of the bacterial type-4 prepeptin peptidases(Steiner et al., 2000). (iv) Potent γ-secretase inhibitors designed to act as transition state analogues bind PSs (Esler et al., 2000; Li et al., 2000).

PSs are also required for the regulated intramembrane proteolysis of the Notch proteins (De Strooper et al., 1999; Struhl and Greenwald, 1999), thereby acting as molecular switches between proteolysis and cell signaling (Annaert and De Strooper, 1999; Brown et al., 2000). While the absolute requirement of PS for γ-secretase processing is thus clearly established, several observations indicate that a “PS is γ-secretase” hypothesis is probably too simplistic. PSs are for instance integrated into a multiprotein complex (Capell et al., 1998; Verdile et al., 2000; Yu et al., 1998) and one of its components, nicastrin, is apparently involved in the regulation of its proteolytic activity (Yu et al., 2000). Other observations also indicate that the
exact role of PS in γ-secretase activity needs further scrutiny. For instance the mutation of Asp257, one of the two aspartates of the putative catalytic site of PS, as well as certain other missense mutations in PS, inhibit Notch but not APP cleavage (Capell et al., 2000; Kulic et al., 2000). This is difficult to conciliate with the idea that the two aspartates constitute the active site of a single protease. Another paradox that needs further work is the discrepancy between the subcellular distribution of PSs and the sites where γ-secretase cleavage of APP or Notch is supposed to occur ("the spatial paradox"; Annaert and De Strooper, 1999; Annaert et al., 1999). The complexity of the issues involved is illustrated by studies that demonstrate the role of PS in the Wnt/ β-catenin signaling pathway. Several authors found that PS can bind proteins of the armadillo family. PS1 indirectly modulates Wnt signaling by stabilizing β-catenin (De Strooper and Annaert, 2001; Kang et al., 1999; Nishimura et al., 1999; Soriano et al., 2001; Zhang et al., 1998). As β-catenin binding to PS1 is independent of γ-secretase function (Saura et al., 2000) it follows that PS1 contains several functional domains, and regulates at least more than one signaling pathway. The exact molecular domains involved in the interaction between presenilin and its substrates are not known. It has been shown in the art that presenilins are endoproteolysed yielding saturable and stable complexes of N-terminal and C-terminal fragments (NTF and CTF)(Thinakaran et al., 1996) (Thinakaran et al., 1997) and that the integrity of PS including intramolecular interactions between both fragments, is required for its normal biological function (Saura et al., 1999; Tomita et al., 1998). It has also been shown that co-immunoprecipitation of Notch with the PS1-NTF as well as with the PS1-CTF can occur (Ray et al., 1999), although the exact binding sites were not identified. Furthermore, mutational analysis has demonstrated that the C-terminus of PS is needed for the stabilization, endoproteolysis and Aβ42 overproduction caused by FAD-linked mutations(Thinakaran et al., 1997; Tomita et al., 1999).

The present invention discloses peptides that specifically inhibit the cleavage activity of presenilins. More specifically, the present invention discloses peptides mainly derived from amyloid precursor protein that specifically inhibits cleavage of amyloid precursor protein by gamma-secretase without affecting the cleavage of Notch, Erb-B4 or syndecans by the same enzyme and vice-versa.

**Brief description of figures**

**Fig. 1:** Peptides that mimic the binding domains in PS1 or APP inhibit the in vitro production of amyloid peptides (Aβ)

The PS1-APP binding is mediated by the C-terminus of PS1 (and the first transmembrane domain of PS1) and part of the APP transmembrane region downstream of the γ-secretase cleavage site (and bearing the FAD-associated mutations affecting the γ-secretase cleavage).
Peptides mimicking these domains were synthesized as such or with a transport peptide (TP) or palmitoyl group (palm) attached to it. The amino acid sequence of said transport peptide is GRQLRIAGRRLGRSR and the origin of said TP is fully described in WO 02/00882. In this regard, it should be clear that other known transport peptides, such as the peptide having sequence GRKKRRQRRPPQ, can be used. CHAPS extracts of HeLa cell membranes were incubated with the APP-C99flag-tagged substrate in the presence of DMSO (control) or the different peptides at the concentrations indicated (peptide). γ-secretase inhibitor (10μM) was included as a positive control for Aβ inhibition. After the reaction, samples were analyzed by SDS-PAGE followed by western blotting using an antibody recognizing Aβ. The binding domains as such only inhibited Aβ production at the highest concentration. However, inhibition was strongly enhanced when the respective peptides were attached to a conserved transmembrane region. The highest inhibition was obtained with peptides comprising the APP binding domain fused to TP having the sequence acetyl-VVIATVIVITLVMLK-TP-NH2 and acetyl-ATVIVITLVMLK-TP-NH2.

**Fig. 2:** More synthetic peptides mimicking the binding domain of presenilin 1 within the transmembrane region of APP were tested using a cell-free in vitro assay to monitor de novo Aβ production. Briefly, CHAPS-extracts of HeLa cells are incubated with the recombinant substrate APP-C99 (the direct substrate for γ-secretase cleavage by presenilin 1) in the absence or presence of peptide inhibitors. As controls, DMSO (dilution medium of the peptides), reversed or scrambled peptides are used. The newly produced Aβ is detected using SDS-PAGE followed by westernblotting using primary antibody WO-2 (that recognizes Aβ peptides) and chemiluminiscence detection.

**Fig. 3:** Dose-dependent inhibition of γ-secretase activity by the peptide D7 having the amino acid sequence VVIATVIVITLVMLK-TP (mimicking the PS1 binding domain within the transmembrane region of APP) in a cell-based assay. As a control, the transport peptide (Con) without the binding domain is used.

**Fig. 4:** An identical assay as for Figure 3 has been performed, but the cells were cotransfected with a NotchΔE construct fused to GAL4-VP16. This figure demonstrates that the peptide D7 specifically inhibits gamma-secretase cleavage of the substrate APP (fused with Gal4-VP16) without affecting the cleavage of NotchΔE (fused with Gal4-VP16).

**Fig. 5:** Dose-dependent pull-down of APP, but not syndecan, via a biotinylated inhibitory peptide.
**Fig. 5A:** Pull-down with biotinylated S44-1B12 peptide from wild-type and PS1&2 dKO MEF extracts. In the bound fraction (left) full-length APP is recovered in a dose-dependent way in the presence or absence of presenilins.

**Fig. 5B:** Syndecan3, another gamma-secretase substrate is not pulled down from MEF stably overexpressing syndecan3. This figure demonstrates that the inhibitory peptides mimicking the binding domain within APP may interfere with gamma-secretase cleavage through a direct interaction with the substrate APP itself.

**Aims and detailed description of the invention**

The present invention relates to the usage of a type I transmembrane domain of a protein selected from APP, Notch and Erb-B4 to specifically inhibit cleavage of APP Notch and Erb-B4, respectively, by presenilin.

The term 'type I transmembrane domain of a protein selected from APP, Notch and Erb-B4' refers in fact to all type I transmembrane domains or peptides able to locate themselves partly or completely in biological membranes and which are derived from proteins that are well known in the art such as amyloid precursor protein (APP), Notch, cadherins such as E-cadherin, Nicastrin, Ire1p, Erb-B4, alpha-secretase, beta-secretase, syndecans such as syndecan3 and members of the ICAM-protein family such as telencephalin (TLN). The term specifically refers to domains or peptides derived from APP, Notch, Syndecans and Erb-B4.

The terms 'specifically inhibit cleavage of APP, Notch, Syndecans and Erb-B4, respectively, by presenilin' indicates that the peptides (domains) of the present invention inhibit the cleavage of only one substrate, for example APP, without affecting the cleavage of the other substrates, for example of Notch or Syndecans or Erb-B4, by presenilins. The peptides of the present invention may thus also specifically inhibit the cleavage of Notch by presenilins without affecting the cleavage of APP and/or Erb-B4 and/or Syndecans for example. The present invention thus relates to the surprising finding that peptides derived from a particular protein which is cleaved by presenilins are capable of inhibiting 'specifically' the cleavage of that particular protein by presenilins without affecting the cleavage of other substrates of presenilins.

The present invention specifically relates to gamma-secretase. Gamma-secretase or presenilin 1 mediates the transmembrane cleavage of APP. This type of proteolytic processing has been recently called "regulated intramembrane proteolysis" (rip) (Brown et al. (2000) Cell 100, 391). Presenilin 1 is also involved in the proteolytic processing of the transmembrane domain of other proteins like Notch, a signaling protein involved in cell fate decisions (De Strooper et al., 1999, Nature 398, 518), Syndecans such as syndecan3, Erb-B4 and Ire1p, a protein involved in the control of the unfolded protein response (Niwa et al., 1999, Cell 99, 691). Syndecans are
described in WO98/49290 to Grootjans et al. and Yoneda & Couchman (Matrix Biol 2003:25-33), which are hereby incorporated by reference.

The present invention thus relates to specific peptides which can be used to inhibit presenilin cleavage of a specific substrate without affecting the cleavage of other substrates of presenilin. More specifically, the present invention discloses the usage of type I transmembrane domains/peptides comprising the amino acid sequence TVIVITLVMLK (one letter code) or Thr Val Ileu Val Ileu Thr Leu Val Met Leu Lys (three letter code) as given by SEQ ID No 1.

More specifically, the invention relates to the same usage as described above with regard to peptides having the amino acid sequence VVIATVIVITLVMLKGRQLRIAGRRLLGRSR (SEQ ID No 2; also denominated as D7) or fragments or variants of the latter sequence which, on their turn, also inhibit cleavage of APP by presenilin.

The present invention further relates to specific variants comprising any amino acid substitutions on the positions 714, 715, 716, 717 and 722 as is in detail disclosed in WO 98/03643. The latter position or codon numbering corresponds with the codon numbering of the APP770 isoform (Wirak et al. (1991) Science 253:323). Thus, position 714 corresponds with the 714th amino acid position in APP770, which refers to the 770 amino acid residue long polypeptide encoded by the human APP gene. Alzheimer disease mutations which are also included among the specific variants of the present invention are also disclosed in detail on http://molgen-www.uia.ac.be/ADMutations/.

The present invention further discloses specific fragments as described above having the following amino acid sequence:

- ATVIVITLVMLKGRQLRIAGRRLLGRSR (SEQ ID No 3), or
- VVIATVIVITLVMLKGRQLRIAGRRLLGRSR (SEQ ID No 4; also denominated as BB7), or
- VVIATVIVITLVMLKGRQLRIAGRRLLGRSR (SEQ ID No 5; also denominated as BB10), or

The present invention also describes specific variants comprising one of the following amino acid sequences:

- VVIATVIVITLVMLKKGQRQLRIAGRRLLGRSR (SEQ ID No 6; also denominated as AA4)
- VVIATVIVITLVMLKKGQRQLRIAGRRLLGRSR (SEQ ID No 7; also denominated as FF12)
- VVIATVIVITLVMLKKGQRQLRIAGRRLLGRSR (SEQ ID No 8; also denominated as EE12)
- VVIATVIVITLVMLKKGQRQLRIAGRRLLGRSR (SEQ ID No 9; also denominated as BB11)
- VIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 10; also denominated as GG8)
- TVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 11; also denominated as BB6)
- GGVVIATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 12)
- MVGGVVIATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 13; also denominated as DD3)
- VVIAVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 14)
- VVIATMIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 15; also denominated as AA7)
- VVIATAVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 16; also denominated as AA8)
- VVIATVVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 17; also denominated as AA9)
- VVIATVIIITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 18; also denominated as AA10)
- VVIATRRVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 19; also denominated as BB1)
- VVIAVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 20)
- VVIATMIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 21)
- VVIATAVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 22)
- VVIATVVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 23)
- VVIATVIIITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 24)
- VVIATVIIITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 25)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 26)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 27)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 28)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 29)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 30)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 31)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 32)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 33)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 34)
- ATVIVITLVMLKKQGRQLRIAGRRLGRS (SEQ ID N° 35)
GRQLRIAGRRLLGRSRVLQPFDQLAFHQFYI-COOH (SEQ ID N° 36; also denominated as A4)
- GRQLRIAGRRLLGRSRVFMDQLAFHQFYI-COOH (SEQ ID N° 37; also denominated as A5)
- GRQLRIAGRRLLGRSRVFMDQLAFHQFYI-COOH (SEQ ID N° 38; also denominated as A8)

It should hereby be noted that sequences given by SEQ IN N° 36-38 are peptides derived from
presenilin also inhibiting APP cleavage. It should also be noted that the N-terminus of these
peptides can be, but is not limited to, -NH₂ or an acetyl group and that the C-terminus of these
peptides can be, but is not limited to, -COOH or an amide group.

The present invention not only relates to the above described fragments and variants but also
encompasses peptido mimetics of SEQ ID N° 1 and 2, and of the latter fragments and variants.
The term ‘fragments’ relates to peptides containing less amino acids than SEQ ID N° 2. The
term ‘variants’ relates to variations in the amino acid composition set forth by SEQ ID N° 2 so
that variants may also relate to sequences having a higher number of amino acids compared
to the number of amino acids given by SEQ ID N° 2.

The term ‘variant’ further relates to peptides that differ only in conservative substitutions and/or
modifications, or, encompasses post-expression modification of the (poly)peptide, for example,
glycosylation, acetylations, phosphorylations and the like. Included within the definitions are
(poly)peptides containing one or more analogues of an amino acid (including, for example,
unnatural amino acids etc), polypeptides with substituted linkages as well as other
modifications known in the art.

The term ‘peptido mimetic’ means a molecule able to mimic the biological activity of a peptide
but is no longer peptidic in chemical nature. By strict definition, a peptido mimetic is a molecule
that no longer contains any peptide bonds (that is, amide bonds between amino acids).
However, the term peptido mimetic is sometimes used to describe molecules that are no
longer completely peptidic in nature, such as pseudo-peptides, semi-peptides and peptoids.
Whether completely or partially non-peptide, peptido mimetics according to this invention
provide a spatial arrangement of reactive chemical moieties that closely resembles the three-
dimensional arrangement of active groups in the peptide on which the peptido mimetic is
based. As a result of this similar active-site geometry, the peptido mimetic has effects on
biological systems, which are similar to the biological activity of the peptide. The peptido
mimetic of this invention are preferably substantially similar in both three-dimensional shape
and biological activity to the peptides set forth above. Substantial similarity means that the
geometric relationship of groups in the peptide that react with for example a type I
transmembrane protein is preserved. There are clear advantages for using a mimetic of a
given peptide rather than the peptide itself, because peptides commonly exhibit two undesirable properties: (1) poor bioavailability; and (2) short duration of action. Peptido mimetics offer an obvious route around these two major obstacles, since the molecules concerned are small enough to be both orally active and have a long duration of action. There are also considerable cost savings and improved patient compliance associated with peptido mimetics, since they can be administered orally compared with parenteral administration for peptides. Furthermore, peptido mimetics are much cheaper to produce than peptides. Finally, there are problems associated with stability, storage and immunoreactivity for peptides that are not experienced with peptido mimetics. The peptides described in the present invention have utility in the development of such small chemical compounds with similar biological activities and therefore with similar therapeutic utilities. The techniques of developing peptido mimetics are conventional. Thus, peptide bonds can be replaced by non-peptide bonds that allow the peptido mimetic to adopt a similar structure, and therefore biological activity, to the original peptide. Further modifications can also be made by replacing chemical groups of the amino acids with other chemical groups of similar structure. The development of peptido mimetics can be aided by determining the tertiary structure of the original peptide, either free or bound to a substrate, e.g. presenilin or a transmembrane part of a type I transmembrane protein, by NMR spectroscopy, crystallography and/or computer-aided molecular modelling. These techniques aid in the development of novel compositions of higher potency and/or greater bioavailability and/or greater stability than the original peptide (Dean (1994), BioEssays, 16: 683-687; Cohen and Shatzmiller (1993), J. Mol. Graph., 11: 166-173; Wiley and Rich (1993), Med. Res. Rev., 13: 327-384; Moore (1994), Trends Pharmacol. Sci., 15: 124-129; Hruby (1993), Biopolymers, 33: 1073-1082; Bugg et al. (1993), Sci. Am., 269: 92-98, all incorporated herein by reference]. Once a potential peptido mimetic compound is identified, it may be synthesized and assayed using the method described herein to assess its activity. Thus, through use of the methods described above, the present invention provides compounds exhibiting enhanced therapeutic activity in comparison to the peptides described above. The peptido mimetic compounds obtained by the above methods, having the biological activity of the above named peptides and similar three-dimensional structure, are encompassed by this invention. It will be readily apparent to one skilled in the art that a peptido mimetic can be generated from any of the modified peptides described in the previous section or from a peptide bearing more than one of the modifications described from the previous section. It will furthermore be apparent that the peptido mimetics of this invention can be further used for the development of even more potent non-peptidic compounds, in addition to their utility as therapeutic compounds.

It is clear that the peptides of the present invention can be used as a medicament to treat Alzheimer's disease. It is thus a further embodiment of this invention to provide a method for
the production of a pharmaceutical composition comprising the peptides of the present invention and mixing said peptides with a pharmaceutically acceptable carrier. The administration of a compound or a pharmaceutically acceptable salt thereof may be by way of oral, inhaled or parenteral administration. The active compound may be administered alone or preferably formulated as a pharmaceutical composition. A unit dose will normally contain 0.01 to 50 mg for example 0.01 to 10 mg, or 0.05 to 2 mg of compound or a pharmaceutically acceptable salt thereof. Unit doses will normally be administered once or more than once a day, for example 2, 3, or 4 times a day, more usually 1 to 3 times a day, such that the total daily dose is normally in the range of 0.0001 to 1 mg/kg; thus a suitable total daily dose for a 70 kg adult is 0.01 to 50 mg, for example 0.01 to 10 mg or more usually 0.05 to 10 mg. It is greatly preferred that the compound or a pharmaceutically acceptable salt thereof is administered in the form of a unit-dose composition, such as a unit dose oral, parenteral, or inhaled composition. Such compositions are prepared by admixture and are suitably adapted for oral, inhaled or parenteral administration, and as such may be in the form of tablets, capsules, oral liquid preparations, powders, granules, lozenges, reconstitutable powders, injectable and infusible solutions or suspensions or suppositories or aerosols. Tablets and capsules for oral administration are usually presented in a unit dose, and contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, colourants, flavourings, and wetting agents. The tablets may be coated according to well-known methods in the art. Suitable fillers for use include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include starch, polyvinylpyrrolidone and starch derivatives such as sodium starch glycollate. Suitable lubricants include, for example, magnesium stearate. Suitable pharmaceutically acceptable wetting agents include sodium lauryl sulphate. These solid oral compositions may be prepared by conventional methods of blending, filling, tableting or the like. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example, almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and if desired conventional flavouring or colouring agents. Oral formulations also include conventional sustained release formulations, such as tablets or
granules having an enteric coating. Preferably, compositions for inhalation are presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns, for example between 1 and 5 microns, such as between 2 and 5 microns. A favored inhaled dose will be in the range of 0.05 to 2 mg, for example 0.05 to 0.5 mg, 0.1 to 1 mg or 0.5 to 2 mg. For parenteral administration, fluid unit dose forms are prepared containing a compound of the present invention and a sterile vehicle. The active compound, depending on the vehicle and the concentration, can be either suspended or dissolved. Parenteral solutions are normally prepared by dissolving the compound in a vehicle and filter sterilising before filling into a suitable vial or ampoule and sealing. Advantageously, adjuvants such as a local anaesthetic, preservatives and buffering agents are also dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the active compound. Where appropriate, small amounts of bronchodilators for example sympathomimetic amines such as isoprenaline, isethionate, salbutamol, phenylephrine and ephedrine; xanthine derivatives such as theophylline and aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included. As is common practice, the compositions will usually be accompanied by written or printed directions for use in the medical treatment concerned.

Examples

1. Peptides such as D7 inhibit γ-secretase cleavage of APP

Peptides mimicking the binding domains in the amyloid precursor protein (APP) and in presenilin 1 (PS1) are tested on their potential to inhibit γ-secretase cleavage of APP and to modulate γ-secretase activity through interfering with the binding of the substrate (APP) to the catalytic core of the enzymatic protein complex. Two binding domains in PS1, i.e. comprising the first transmembrane region (TMR) and the final 39 C-terminal amino acids, are assumed to form a binding pocket with the binding domain in APP, i.e. the 11 amino acids downstream of the gamma-secretase cleavage site and comprised within the TMR of APP. These binding domains predict a ring structure topology for PS as is described in WO 02/074804 to De Strooper and Annaert. As it is equally assumed that the catalytic domain of PS is constituted
by the two aspartate residues in TMR 6 and 7, there is a spatial separation between substrate binding and the catalytic site. This further implies that substrate binding precedes intramembrane proteolytic cleavage of APP C-terminal fragment. A second assumption is that binding characteristics and domains may differ between PS1, APP and other substrates of γ-secretase activity as the peptide inhibitors mimicking these binding domains display, to our surprise, specificity towards their substrates. As such, these binding domains are suitable targets for selective gamma-secretase inhibition.

The efficacy of peptide inhibition of γ-secretase activity is assessed by three assays, one cell-free and two cell-based assays, described herewith:

**Cell-free assay to monitor γ-secretase activity.**

-Step 1: Preparation of membrane extracts.

Cells (for instance HeLa, but also other lines) are harvested, homogenized using a ball-bearing cell cracker and a postnuclear supernatant is prepared by low speed centrifugation (10 min, 2000 rpm). This fraction is subjected to high speed centrifugation (1hrs, 60K rpm) to obtain a crude membrane pellet. Membrane pellets are washed twice in TBS containing 0.5% saponin and finally extracted in 1% CHAPS. After 1hr at 4°C, extracts are cleared by ultracentrifugation, protein content is measured and extracts are aliquoted and stored at −70°C until use.

-Step 2: Preparation of APP C-terminal fragment tagged with a FLAG epitope.

E. coli are transformed with a plasmid containing the sequence encoding the β-cleaved APP C-terminal fragment tagged at its C-terminal end with a FLAG-tag sequence and induced to express the recombinant protein. Recombinant FLAG-tagged APP CTF is isolated from E. coli cell extracts by a one-step procedure using immunoaffinity chromatography with anti-FLAG antibodies covalently coupled to sepharose beads.

Recombinant protein is eluted from the column by lowering the pH. After dialysis and readjustment to pH 7.3, recombinant protein is snap-frozen and stored at −70°C until use.

-Step 3:

15 μl of cell extract (between 7 and 10 μg protein) is incubated with 1μl APP CTF-FLAG substrate in the absence (control) or presence of peptide inhibitors overnight at 37°C. The next day, the reaction is stopped by adding a 4 times concentrated stock solution of sample buffer. Newly formed Aβ peptides are analyzed by SDS-PAGE followed by western blotting using monoclonal antibody WO-2 as primary antibody and HRP-conjugated goat anti-mouse polyclonal antibody as secondary antibody. Detection is done by chemiluminiscence.

**Cell-based assay to monitor γ-secretase activity.**

One day before the experiment, HeLa cells are plated out in 12 well plates and grown to 60-70% confluence. The next day, cells are double transfected with plasmids encoding the reporter gene (luciferase) and the substrate. Plasmids encoding substrates encompass full-length APP
fused within its cytoplasmic tail domain with GAL4-VP16. Other substrates include NotchΔE or Erb-B4 fused to GAL4-VP16.

As a control, only the reporter gene (negative) or GAL4-VP16 lacking the substrate sequence (positive) is used. Twentyfour hours later, transfected cells are incubated without (control) or with different concentrations of the respective peptide inhibitors. Eighteen hours later, γ-secretase activity is measured by lysing and incubating the cells with enzyme substrate. The emitted light is correlated with γ-secretase activity and is captured by fluorospectrophotometry. γ-Secretase activity is expressed as relative light units and is normalized to toxicity (as judged from the activity of the GAL4-VP16 construct in the presence of inhibitor) and to control (substrate-GAL4-VP16 in the presence of DMSO).

**Cell-based assay to monitor APP processing.**

Primary cultures of cortical neurons (or, alternatively, neuronal and nonneuronal cell lines stably expressing APPswe) are transfected with full-length APP using the semiliki forest virus for overexpression. Three hours after the start of transduction, neurons are metabolically labeled with [35S]-methionine in the absence (DMSO control) or presence of the indicated concentrations of peptide inhibitor. After 3 hrs of labeling, the conditioned media are collected and neuronal cell lysates are prepared. Both media and cleared cell extracts are subjected to immunoprecipitation using specific antibodies directed against different regions of APP. SDS-PAGE followed by phosphorimaging allows us to analyze quantitatively the effects of peptide inhibitors on the proteolytic processing of APP by β- and γ-secretase. Similar experiments using overexpression of Notch- or Erb-B4ΔE fragments are included to establish the specificity of γ-secretase inhibition.

Synthetic peptides corresponding to the carboxyterminal binding domain in PS1 or to the binding domain within the transmembrane region of APP and following the γ-secretase cleavage site at amino acid 40 (of the Aβsequence) are being tested for their inhibitory properties on γ-secretase activity. The targets for the synthetic peptides (or peptide antagonists) are intracellular and close to, or inside, intracellular membranes. Therefore, the peptide antagonists are coupled to a transport peptide which is able to bind to membranes and translocate over membranes. This will allow the peptides to become closely associated with the proteolytic complex. Another advantage of the cationic transport peptide is that it increases the solubility of the hydrophobic APP-part of the antagonistic peptide.

Peptides have thus been synthesized in which the APP-part and the Etna-derived-part (i.e. the transport peptide or TP) are combined in one peptide and tested for their ability to inhibit γ-secretase activity in the assays described above.

These peptides are first tested in a cell-free assay, and strong candidate inhibitors are further analyzed using the cell-based assays.
In the cell-free assay, peptides mimicking the carboxyterminus of PS1 or the binding domain within the APP transmembrane domain inhibit de novo production of Aβ peptides from FLAG-tagged APP C-terminal fragment. PS1-based peptides are however less potent as compared to APP-based peptides, giving maximum inhibition at 100 μM and 10 μM, respectively. The control peptide, i.e. APP peptide on its own, did not give any inhibition of γ-secretase activity indicating that the effect are contributed by the combination of the APP-part and the TP-part.

A potent inhibitor (tentatively called D7 and comprising the PS1 binding domain within the APP transmembrane region) was further tested using the cell-based assay. After correction for toxicity and normalization, D7 was found to inhibit γ-secretase activity up to 60-40% at a concentration of 1,5 μM (see Fig. 3). Although known γ-secretase inhibitors (e.g. Elan) inhibit cleavage fully in the nmolar range, they do not show substrate selectivity. Indeed in contrast to this inhibitor, the D7 peptide did not affect S3-cleavage within the transmembrane domain of Notch as assessed by the cell-based assay (see Fig 4).

**Pull-down assays to monitor binding of inhibitory peptides.**

Inhibitory peptides are chemically modified by adding a biotin molecule. Biotinylated peptides are then used in pull-down assays using streptavidin immobilized on sepharose beads. Detergent extracts of cell lines or neurons are incubated with increasing amounts of inhibitory peptides (range 0.1 to 8μM) together with streptavidin-sepharose for time periods ranging from 1 hr to overnight. After incubation, streptavidin beads are washed and analyzed for the presence of biotinylated peptide together with putative interacting protein targets including components of the gamma-secretase complex and APP. Pull-down experiments are performed in detergent extracts derived from wild-type cells or PS1&2 deficient mouse embryonic fibroblasts. This allows to distinguish indirect and direct interactions with APP.

**2. More peptides inhibit γ-secretase cleavage of APP.**

Synthetic peptides mimicking the binding domain of presenilin 1 within the transmembrane region of APP were tested using a cell-free in vitro assay to monitor de novo Aβ production as described above. Briefly, CHAPS-extracts of HeLa cells are incubated with the recombinant substrate APP-C99 (the direct substrate for γ-secretase cleavage by presenilin 1) in the absence or presence of peptide inhibitors. As controls, DMSO (dilution medium of the peptides), reversed or scrambled peptides are used. The newly produced Aβ is detected using SDS-PAGE followed by westernblotting using primary antibody WO-2 (that recognizes Aβ peptides) and chemiluminiscence detection.

The following peptides are tested:
Experiment 1:

Peptides

<table>
<thead>
<tr>
<th>D7</th>
<th>VVIATVIVITLVMLKGRQLRIAGRLGRSRR</th>
<th>Wild-type</th>
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<tbody>
<tr>
<td>AA4</td>
<td>VVIATVIVITLVMLKQKQGRQLRIAGRLGRSRR</td>
<td>Wild-type (longer form)</td>
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<td>AA7</td>
<td>VVIATMIVITLVMLKQKQGRQLRIAGRLGRSRR</td>
<td>715 French mutation</td>
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<td>AA8</td>
<td>VVIATAVVITLVMLKQKQGRQLRIAGRLGRSRR</td>
<td>715+716 German+Florida</td>
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<td>AA9</td>
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<td>717 London mutation</td>
</tr>
<tr>
<td>AA10</td>
<td>VVIATVILITLVMLKQKQGRQLRIAGRLGRSRR</td>
<td>717 Indiana mutation</td>
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<tr>
<td>AA11</td>
<td>GRQLRIAGRLGRSRRVVIATVIVITLVMLKQKQ</td>
<td>Reversed peptide</td>
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<td>VVIATEEIVITLVMLKQKQGRQLRIAGRLGRSRR</td>
<td>EE mutation</td>
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<td>BB1</td>
<td>VVIATRRVITLVMLKQKQGRQLRIAGRLGRSRR</td>
<td>RR mutation</td>
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<td>BB2</td>
<td>KLTVQKVMIIVKALTVGRQLRIAGRLGRSRR</td>
<td>Scrambled</td>
</tr>
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<td>BB3</td>
<td>VLTKVQKVIIIVTAVMKLGRQLRIAGRLGRSRR</td>
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<td>BB4</td>
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<tr>
<td>HH10</td>
<td>PDMGLAHQFQYIGRQLRIAGRLGRSRR</td>
<td>C-terminus of PS1</td>
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<tr>
<td>99 + A</td>
<td>C-Term + TMR 1</td>
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</tr>
<tr>
<td>100 + B</td>
<td>C-Term + TMR 1</td>
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</table>

It can be seen that including FAD-linked mutation at 717 position in the APP sequence (either the London or Indiana mutation) increases the inhibitory effect of the AA4 peptide on Aβ production (see Fig. 2A).

Experiment 2:

Peptides

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See Fig. 2 B
### Experiment 3

**Peptides**

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<td>GG8</td>
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</table>

Increasing the length at the C-terminus of the binding domain in APP only has moderate effects. Using a peptide extended on both sides of the binding domain (e.g. peptide DD3) significantly increases the inhibitory activity (see Fig 2C).
References


and characterization of its interaction with the leukocyte integrin CD11a/CD18 [In Process Citation]. Eur J Immunol 30, 810-8.


Claims

1. Use of a type I transmembrane domain of a protein selected from APP, Notch, Syndecans and Erb-B4 to specifically inhibit cleavage of APP, Notch, Syndecans and Erb-B4, respectively, by presenilin.
2. Use according to claim 1 wherein said presenilin is gamma-secretase.
3. Use according to claims 1 and 2 wherein said syndecan is syndecan3.
4. Use according to claims 1 to 3 wherein said type I transmembrane domain comprises the amino acid sequence as given by SEQ ID N° 1.
5. Use according to claims 1 to 3 wherein said type I transmembrane domain comprises the amino acid sequence as given by the SEQ ID N° 2 or fragments or variants thereof which inhibit cleavage of APP by presenilin.
6. Fragments according to claim 5 comprising the amino acid sequence as given by SEQ ID N° 3, 4 or 5.
7. Variants according to claim 5 comprising the amino acid sequences as given by SEQ ID N° 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37 or 38.
8. Fragments and variants according to claims 6 and 7, respectively, for use as a medicament.
9. Use of fragments and variants according to claims 6 and 7 for the preparation of a medicament to treat Alzheimer's disease.
10. Use of type I transmembrane domains as defined in claims 1 to 5 for the preparation of a medicament to treat Alzheimer's disease.
Fig. 1:  

γ-inhibitor (μM) control  

Peptide (μM)  

Aβ  

A1 LVQPFMDQLAFHQFYI  
A2 FMDQLAFHQFYI  
A3 LAFHQFYI  
A4 GRQLRIGRLRGRGRSR LVQPFMDQLAFHQFYI  
A5 GRQLRIGRLRGRGRSR FMDQLAFHQFYI  
A8 GRQLRIGRLRGRGRSR LAFHQFYI  
A11 Palm-LVQPFMDQLAFHQFYI  
A12 Palm-FMDQLAFHQFYI  
B2 Palm-LAFHQFYI  
D1 VVIATVITLVMLKKQ  
D5 ATVIVITLVMLKKQ  
D7 VVIATVITLVMLKKQ GRQLRIGRLRGRGRSR  
D9 ATVIVITLVMLKKQ GRQLRIGRLRGRGRSR  

PEPTIDE  

LVQPFMDQLAFHQFYI  
GRQLRIGRLRGRGRSR LVQPFMDQLAFHQFYI  
\( \text{Palm}^-\text{LVQPFMDQLAFHQFYI} \)  
VVIATVITLVMLKKQ  
VVIATVITLVMLKKQ GRQLRIGRLRGRGRSR  

PS1-C-terminal binding domain  
APP binding domain
Fig. 2:

A

Concentration: 1μM

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Concentration: 1μM

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Concentration: 1μM

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C

Concentration: 1μM

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Fig. 5:

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APP

PS1-NTF

PS1-CTF

B.

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Syndecan3
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

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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)

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

Electronic database consulted during the international search (name of database and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE, EMBASE, CHEM ABS Data, SEQUENCE SEARCH

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<td>WO 02 074804 A (VLAAMS INTERUNIVERSITAIR INST ; ANNAERT WIM (BE); DE STROOPER BART) 26 September 2002 (2002-09-26)</td>
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<td>* see claims, fig.6, pages 6-7 and page 14 lines 9-13 *</td>
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<td>WO 98 03643 A (MASTERS COLIN LOUIS; SMITHKLINE BEECHAM AUSTRALIA P (AU); BEYREUTH) 29 January 1998 (1998-01-29)</td>
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<td>WO 98 49290 A (VLAAMS INTERUNIV INST BIOTECH ; DAVID GUIDO (BE); GROOTJANS JAN (BE) 5 November 1998 (1998-11-05)</td>
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Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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Date of the actual completion of the international search

16 January 2004

Date of mailing of the international search report

23/01/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL – 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016

Authorized officer

Merckling-Ruiz, V
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<td>WO 01 77144 A (MERCK SHARP &amp; DOHME; STEVENSON GRAEME IRVINE (GB); NADIN ALAN JOHN) 18 October 2001 (2001-10-18) * see claim 1, page 2 lines 1-21 *</td>
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<td>WO 02 04644 A (BOEHRINGER INGELHEIM PHARMA; KOSTKA MARCUS (DE); FECHTELER KATJA () 17 January 2002 (2002-01-17) * see claims 24-27, pages 1-2 and 10-11 *</td>
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<td>LICHTENTHALER S. ET AL.: &quot;Mechanism of the cleavage specificity of Alzheimer's disease gamma-secretase identified by phenylalanine-scanning mutagenesis of the transmembrane domain of amyloid precursor protein.&quot; PROC. NAT. ACAD. SCI. USA, vol. 96, March 1999 (1999-03), pages 3053-3058, XP002242752 * see abstract, fig.1 and page 3058 *</td>
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