Citation:
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(12) UK Patent (19) GB (11) 2493313 (13) B

(45) Date of B Publication 15.11.2017

(54) Title of the Invention: Kissorphin peptides for use in the treatment of alzheimer's disease, creutzfeldt-jakob disease or diabetes mellitus

(51) INT CL: ***A61K 38/04*** (2006.01) ***A61K 38/07*** (2006.01) ***A61K 38/08*** (2006.01) ***A61K 38/17*** (2006.01) ***A61P 3/10*** (2006.01) ***A61P 25/28*** (2006.01) ***C07K 5/10*** (2006.01) ***C07K 14/47*** (2006.01) ***C07K 16/18*** (2006.01)

(21) Application No: **1220630.6**

(22) Date of Filing: **19.05.2011**

Date Lodged: **16.11.2012**

(30) Priority Data:
(31) **10163479** (32) **20.05.2010** (33) **EP**

(86) International Application Data:
PCT/EP2011/058213 En 19.05.2011

(87) International Publication Data:
WO2011/144714 En 24.11.2011

(43) Date of Reproduction by UK Office **30.01.2013**

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(58) Field of Search:
As for published application 2493313 A viz:
INT CL **A61K, A61P, C07K**
Other: **EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data**
updated as appropriate

(continued on next page)

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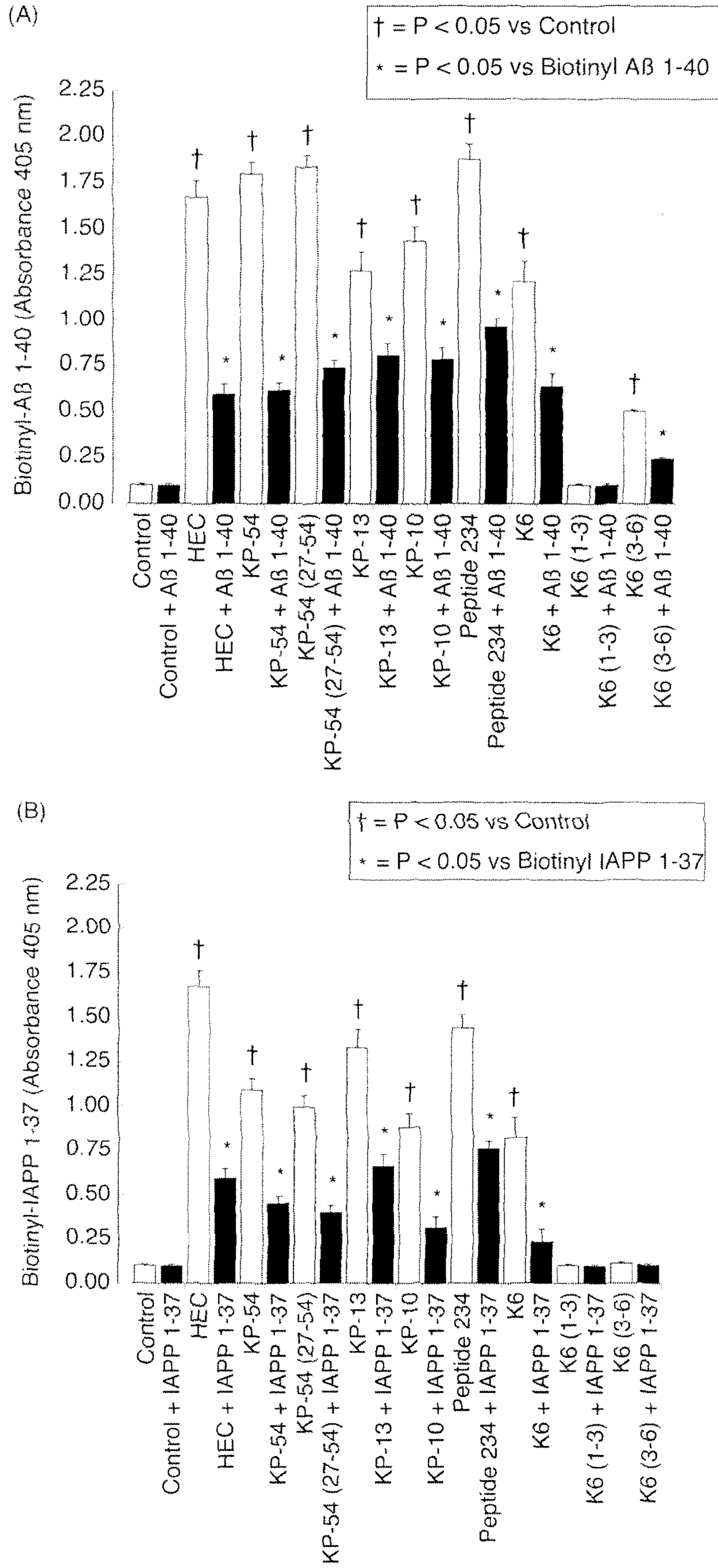

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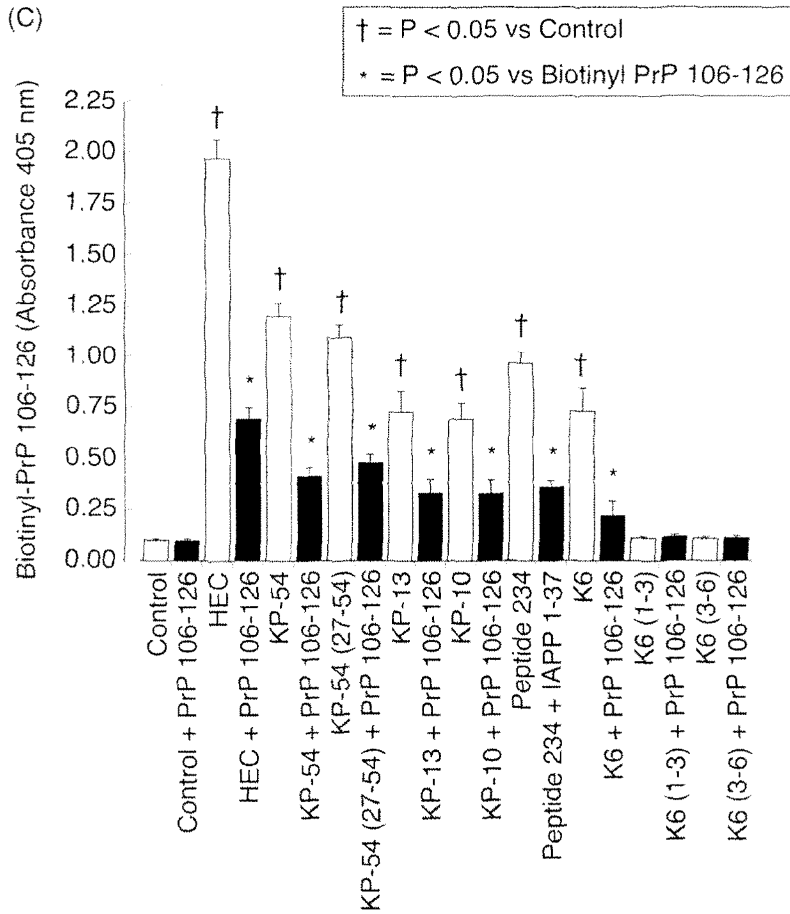

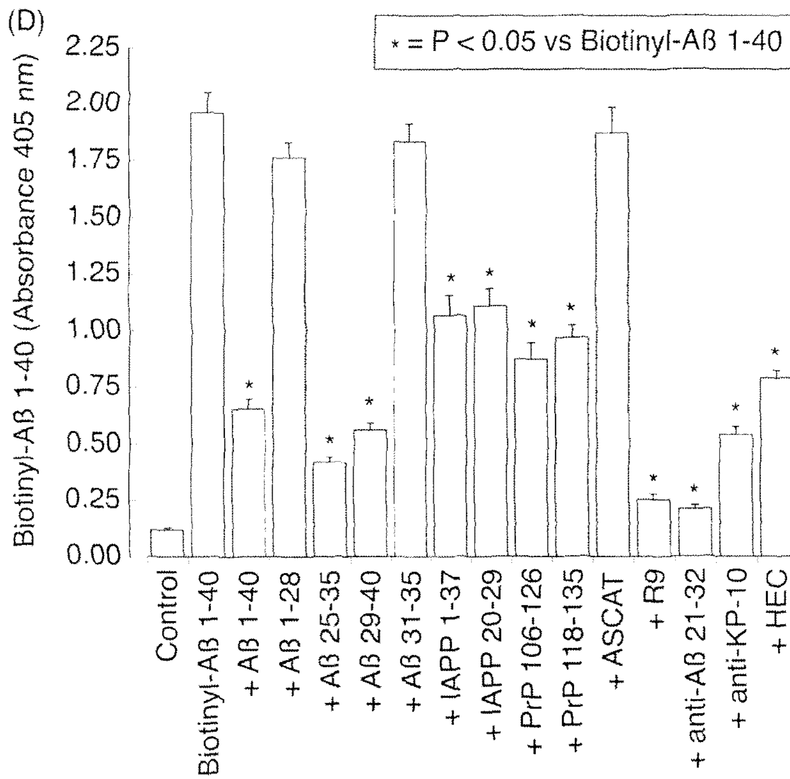

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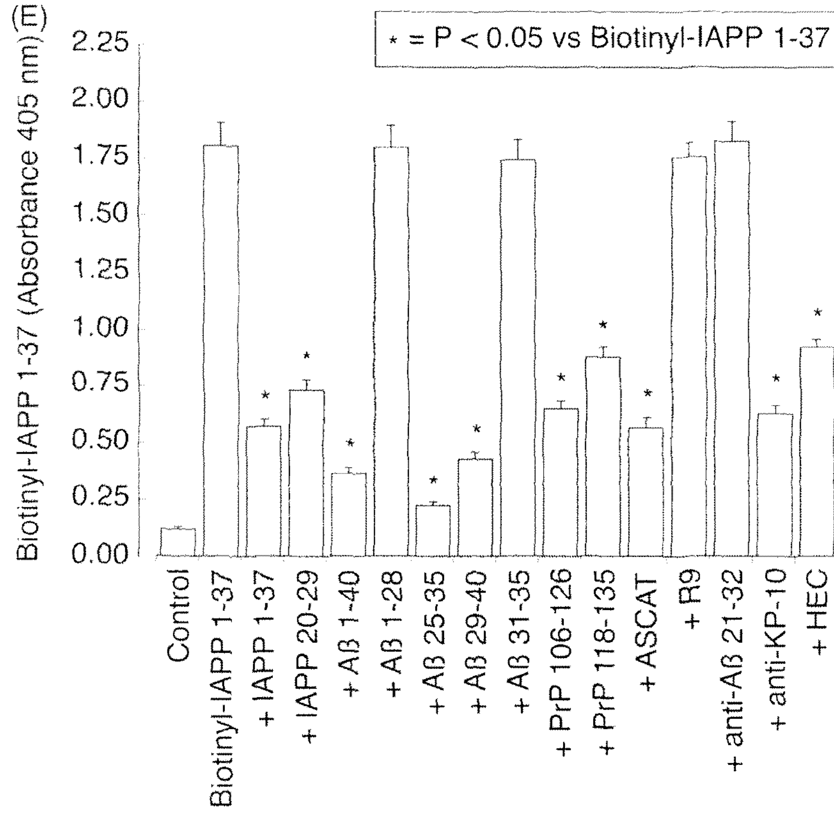

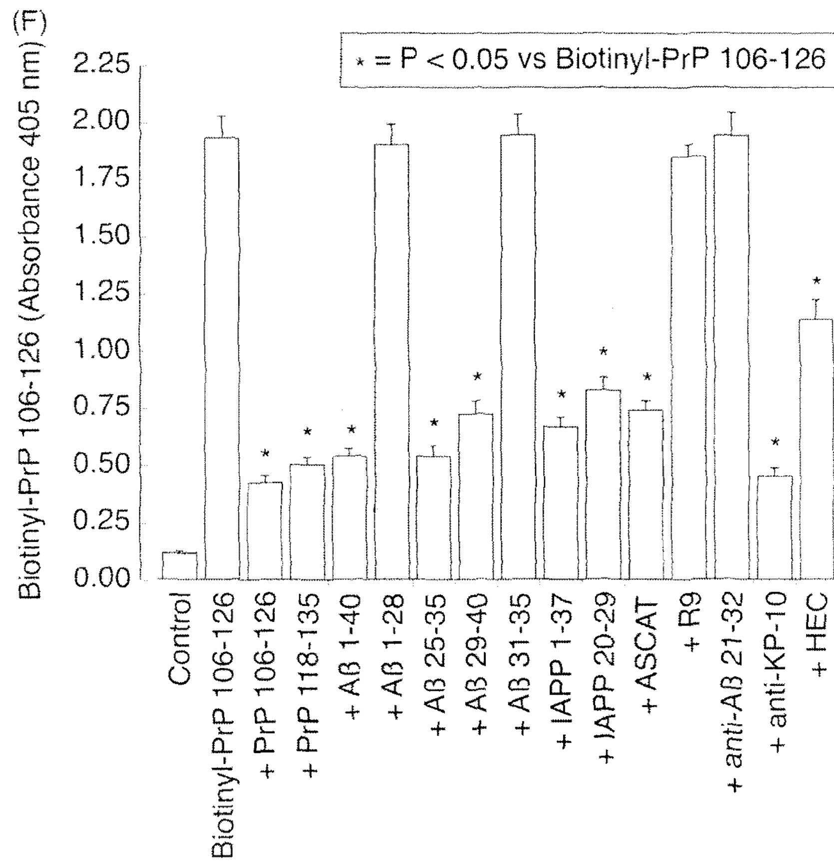

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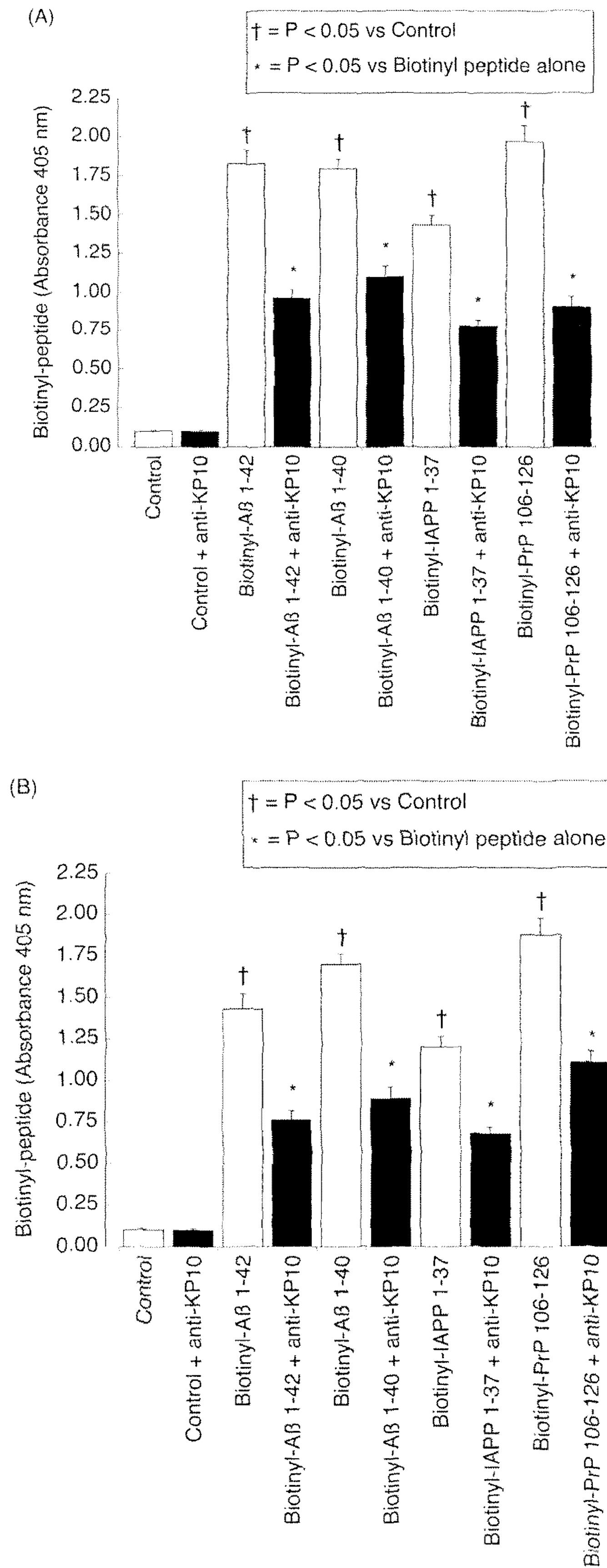

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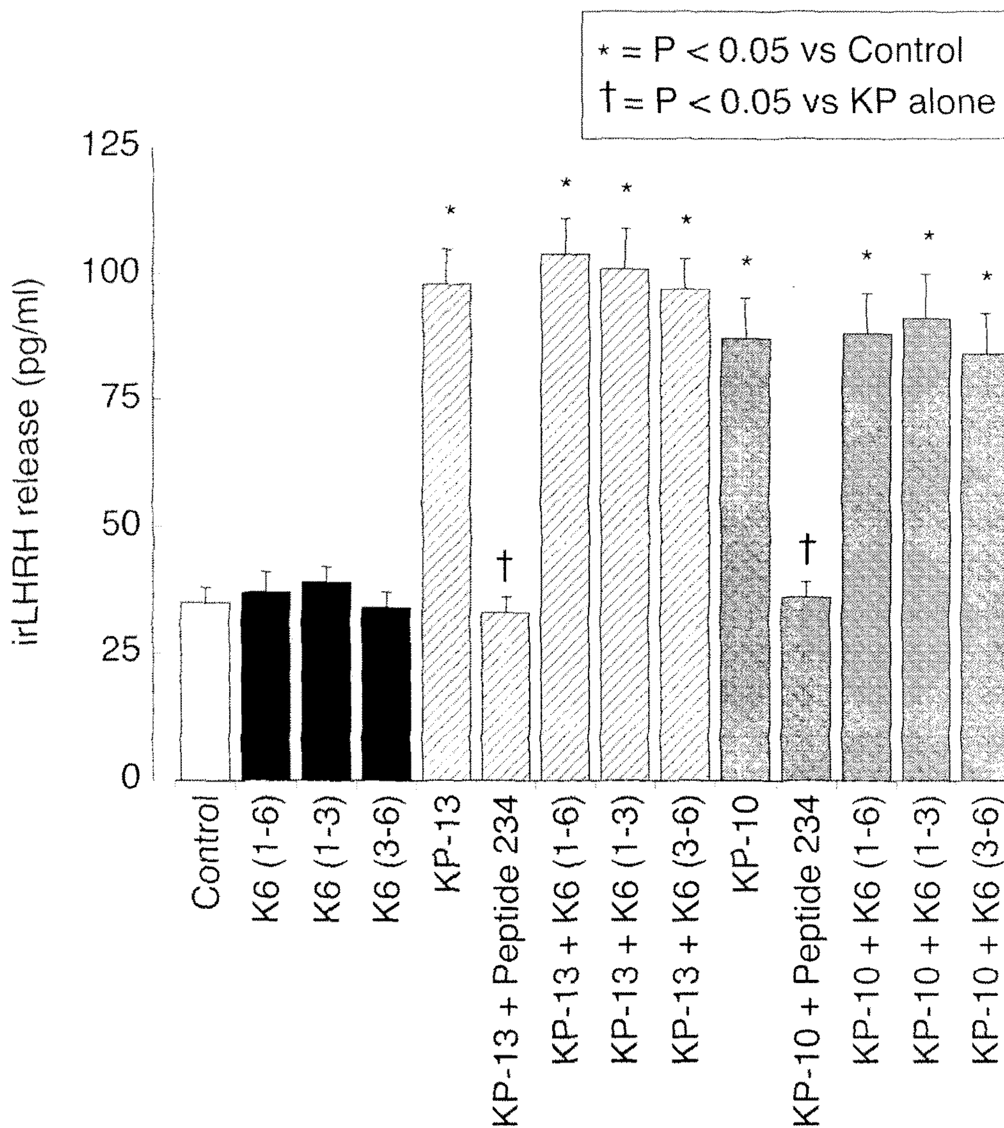

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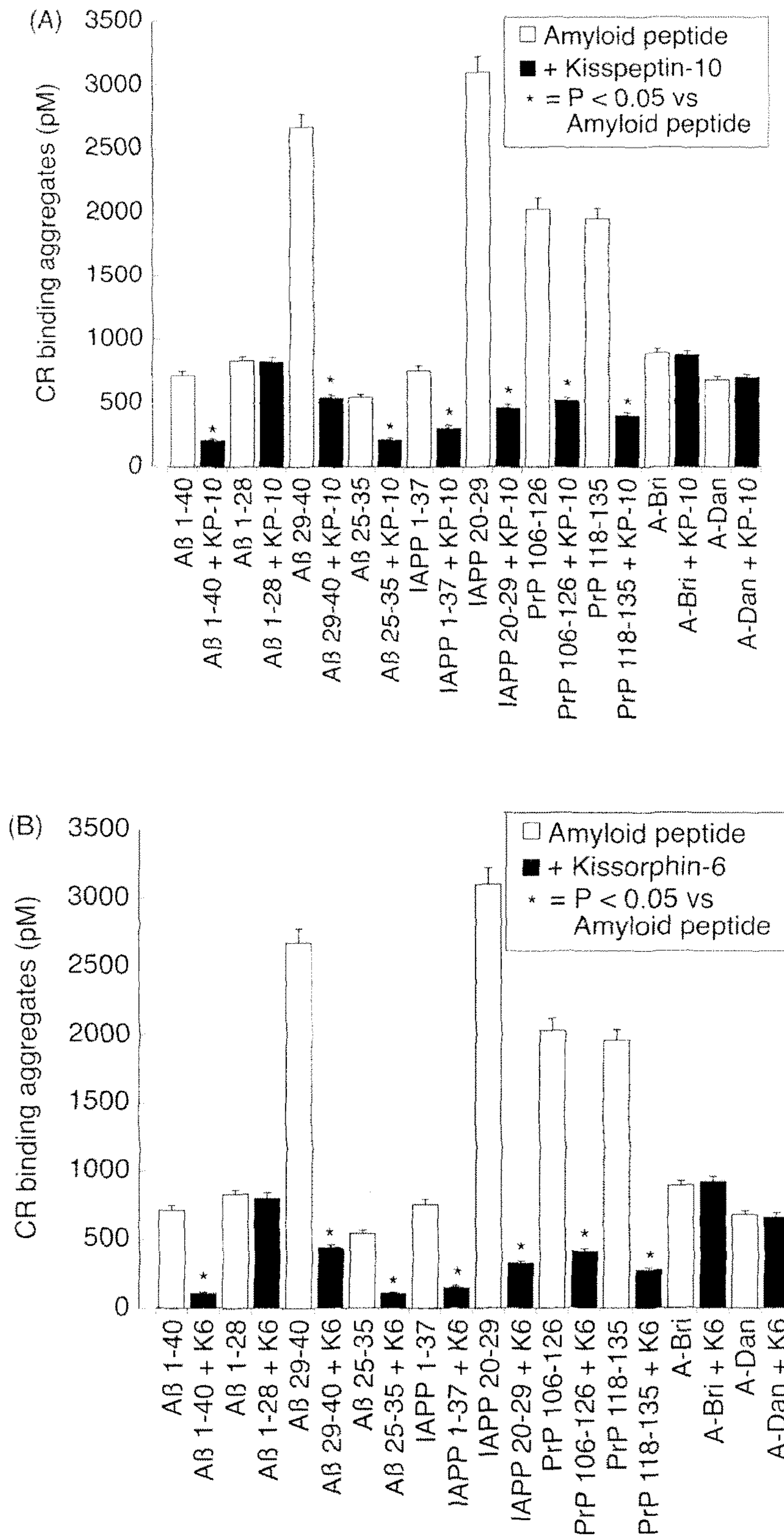

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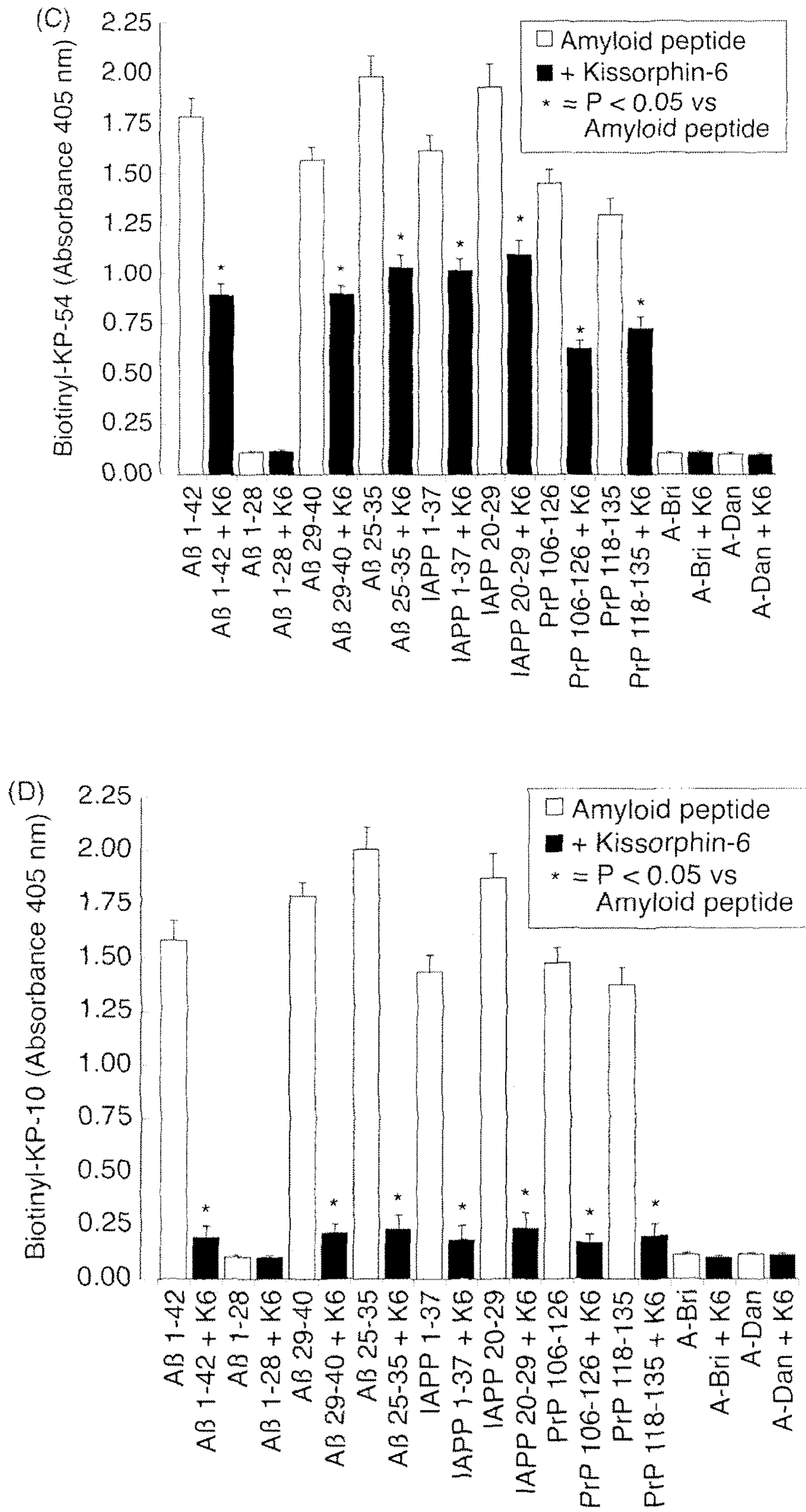

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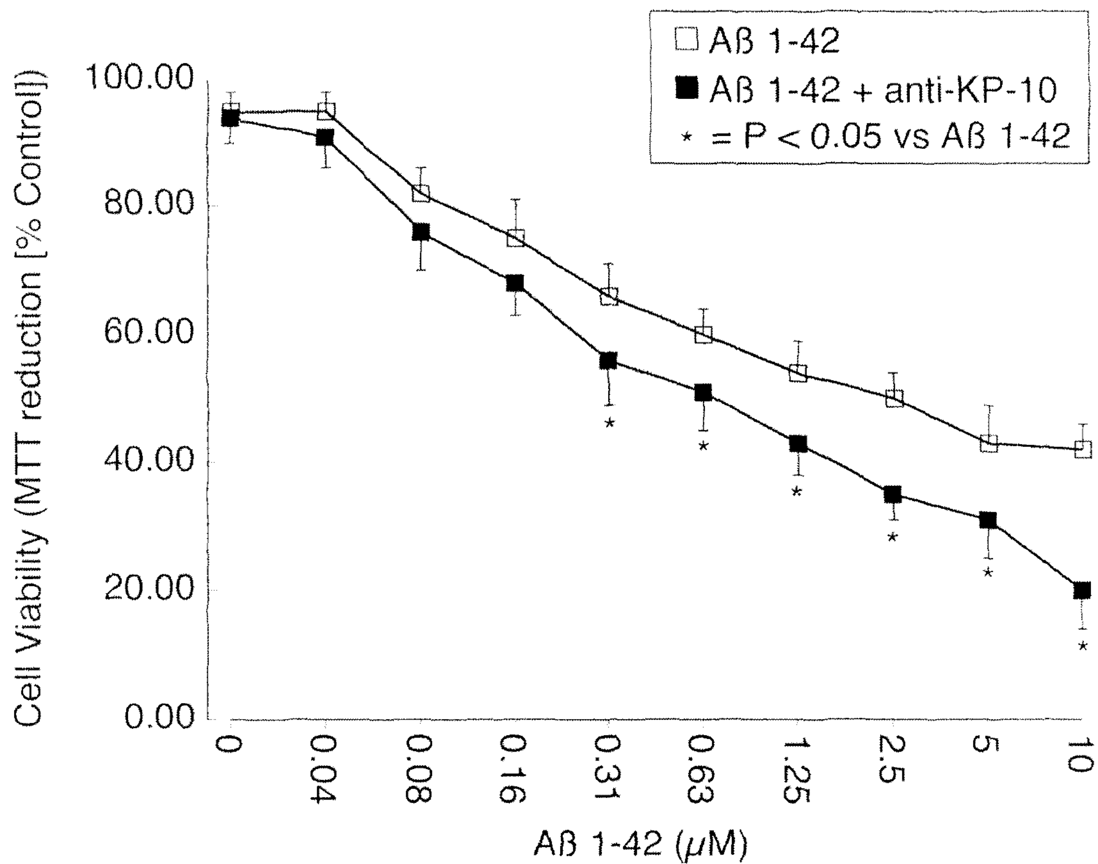
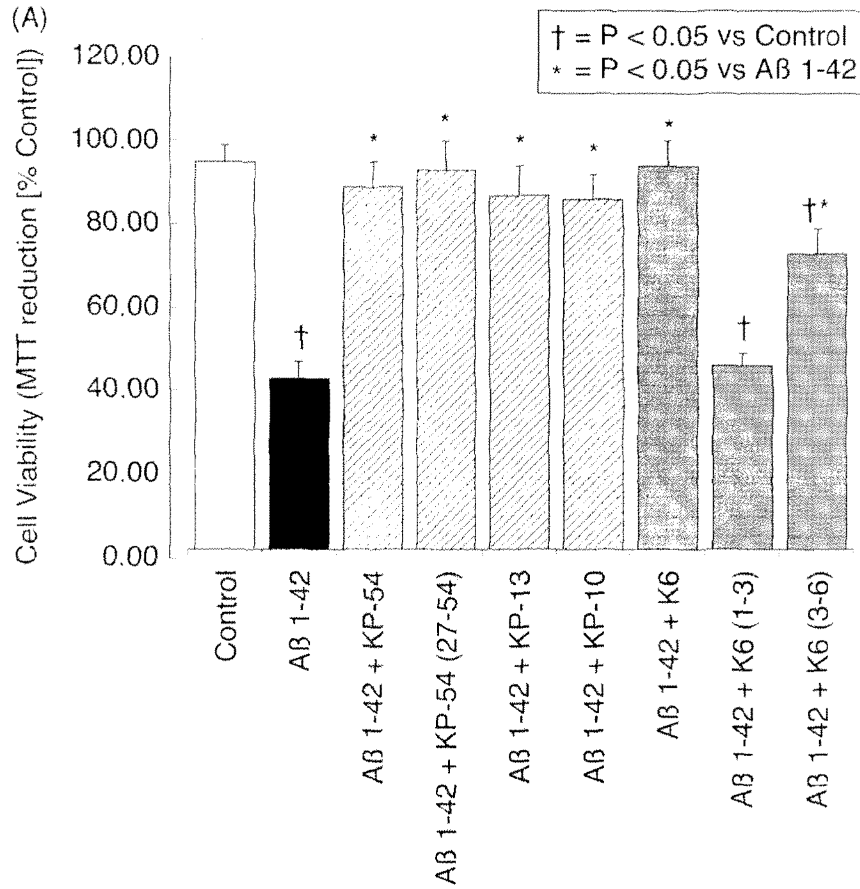
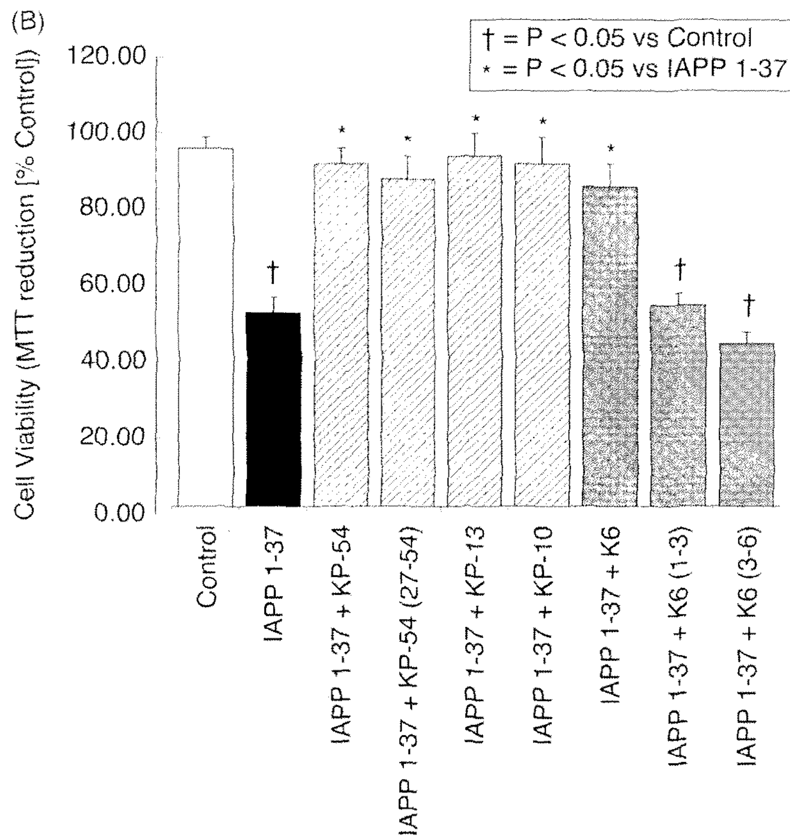


Figure 5





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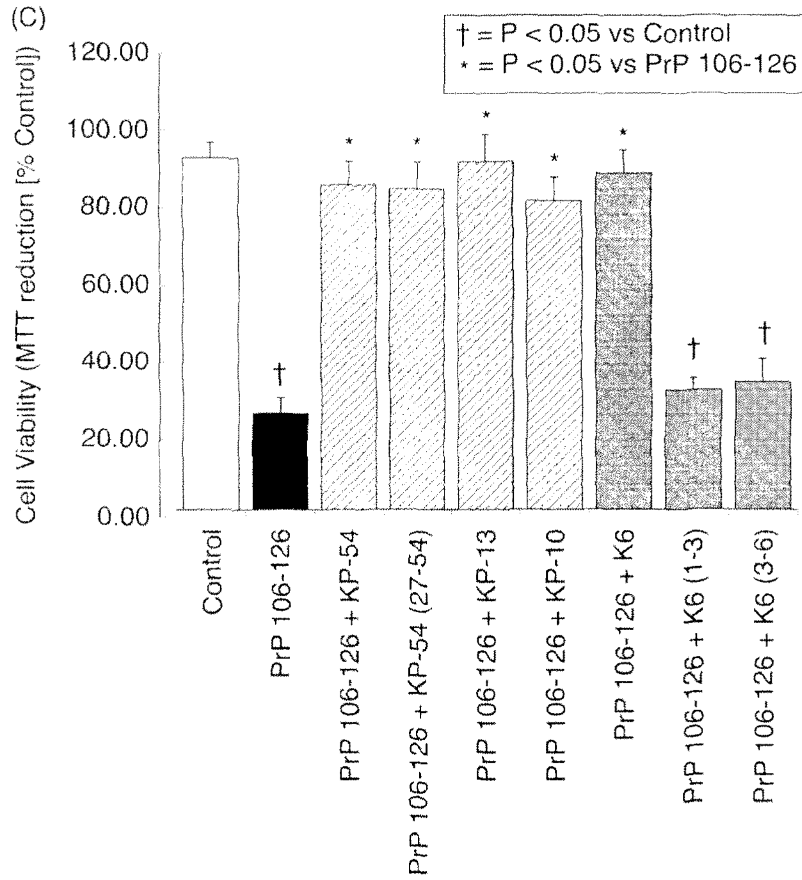

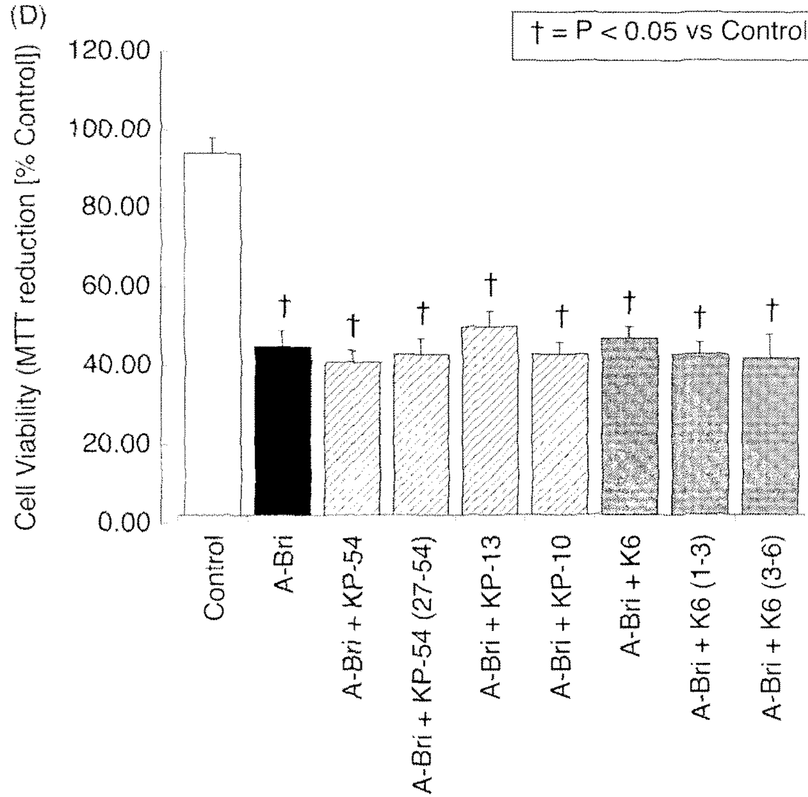

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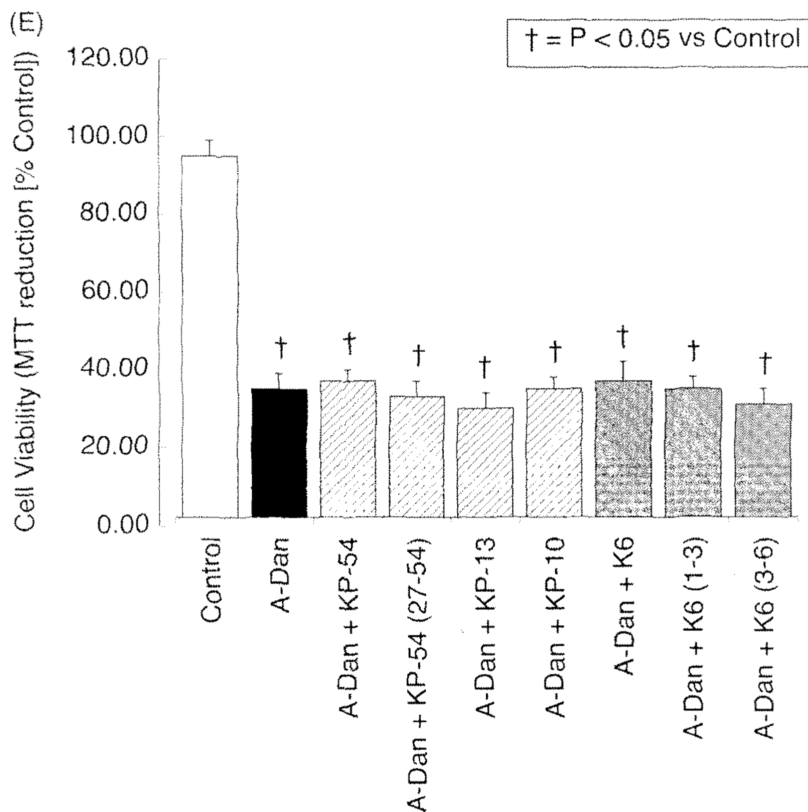

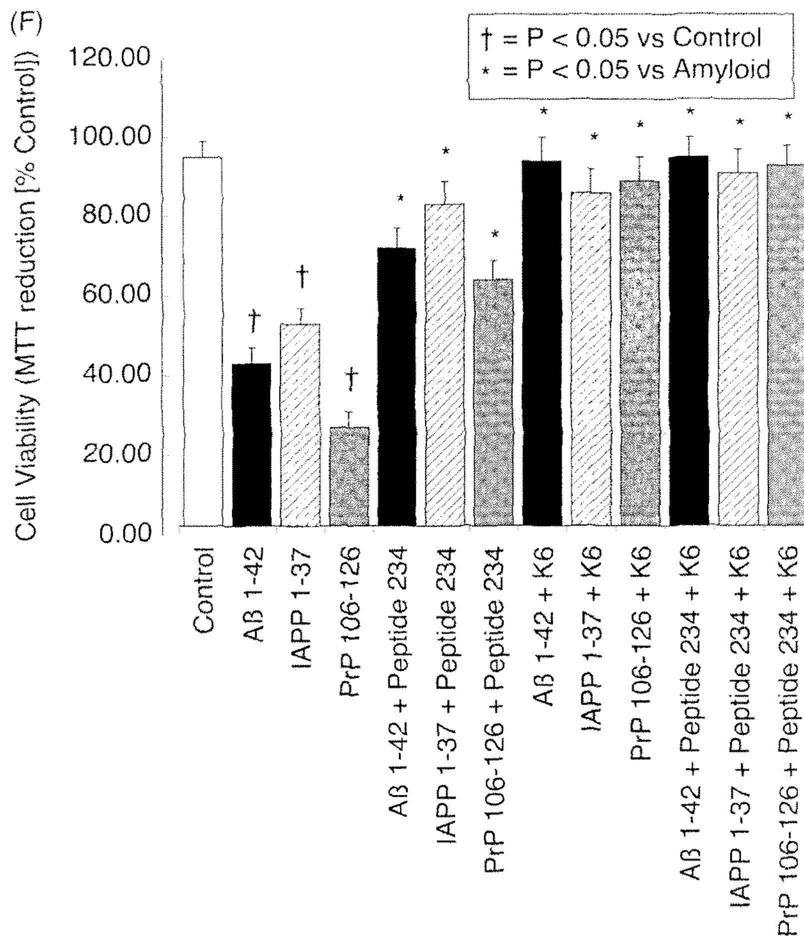

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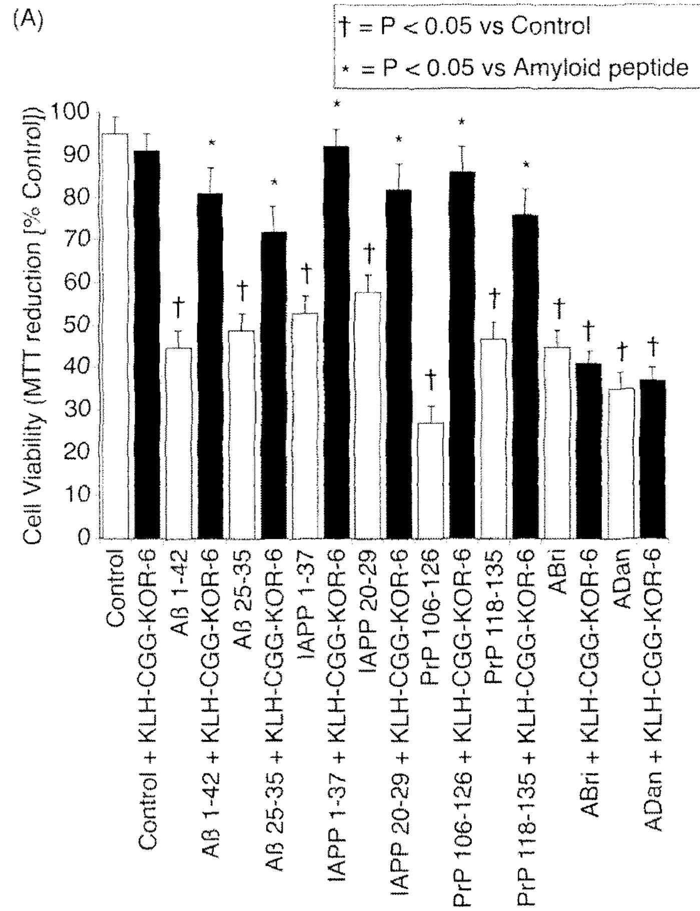


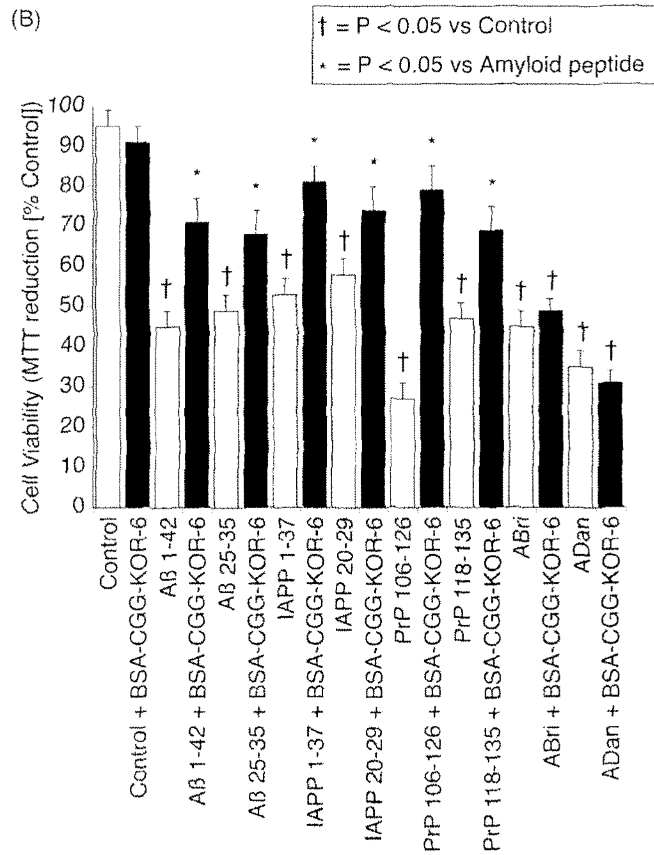
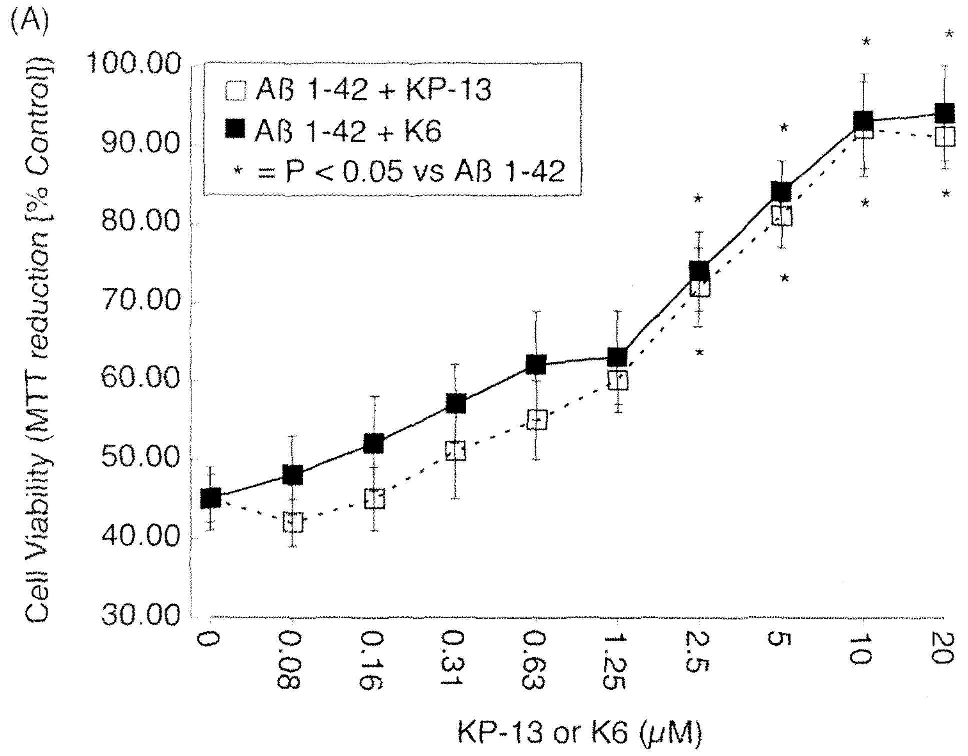
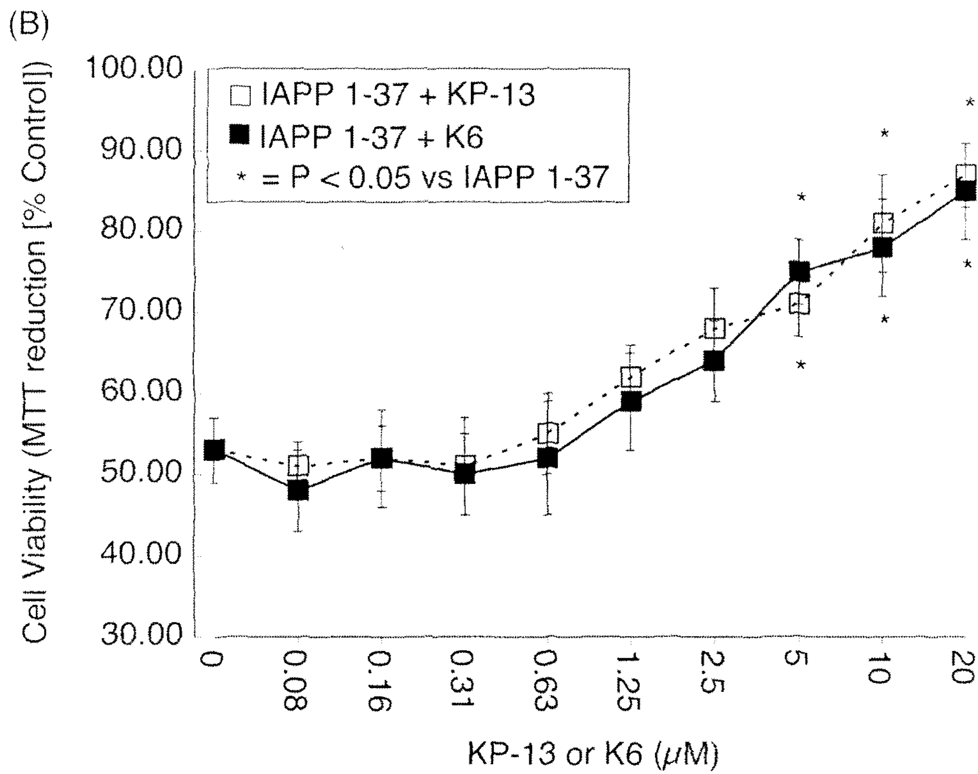


Figure 7





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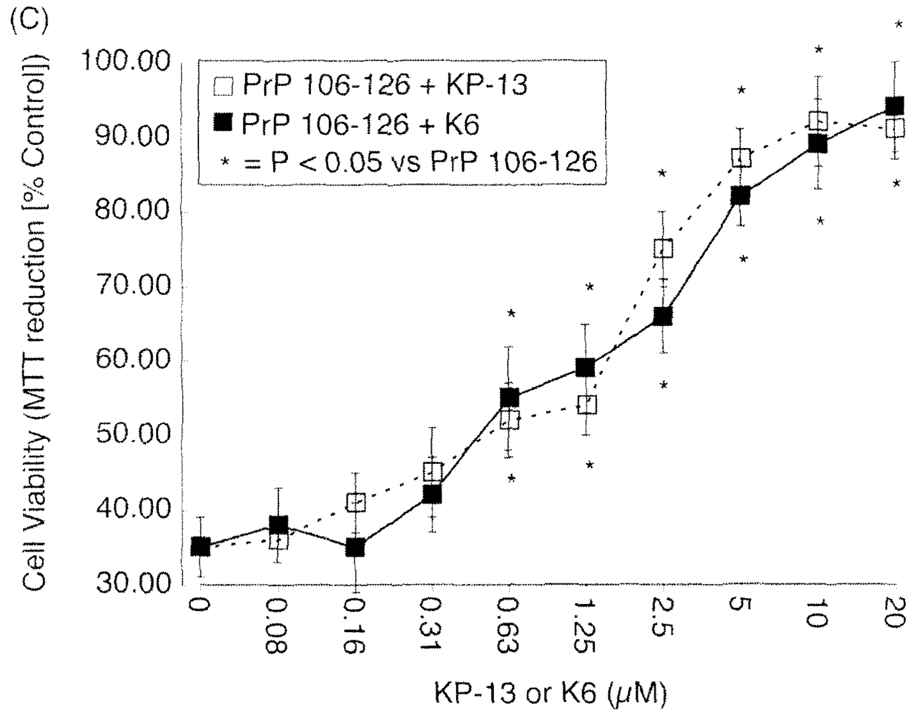

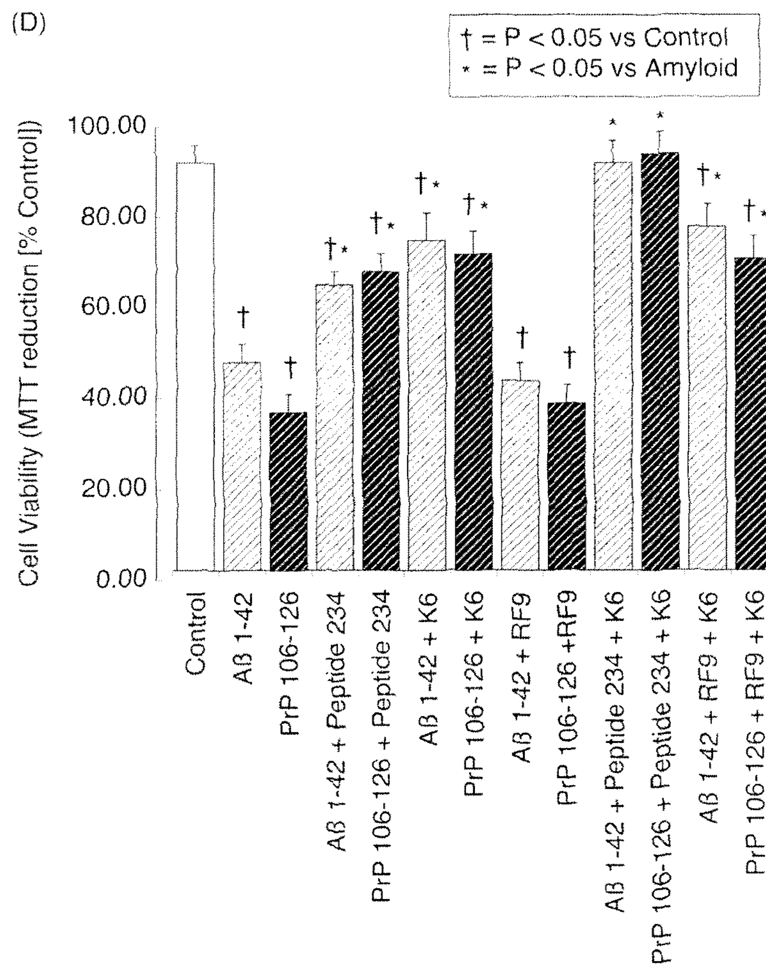

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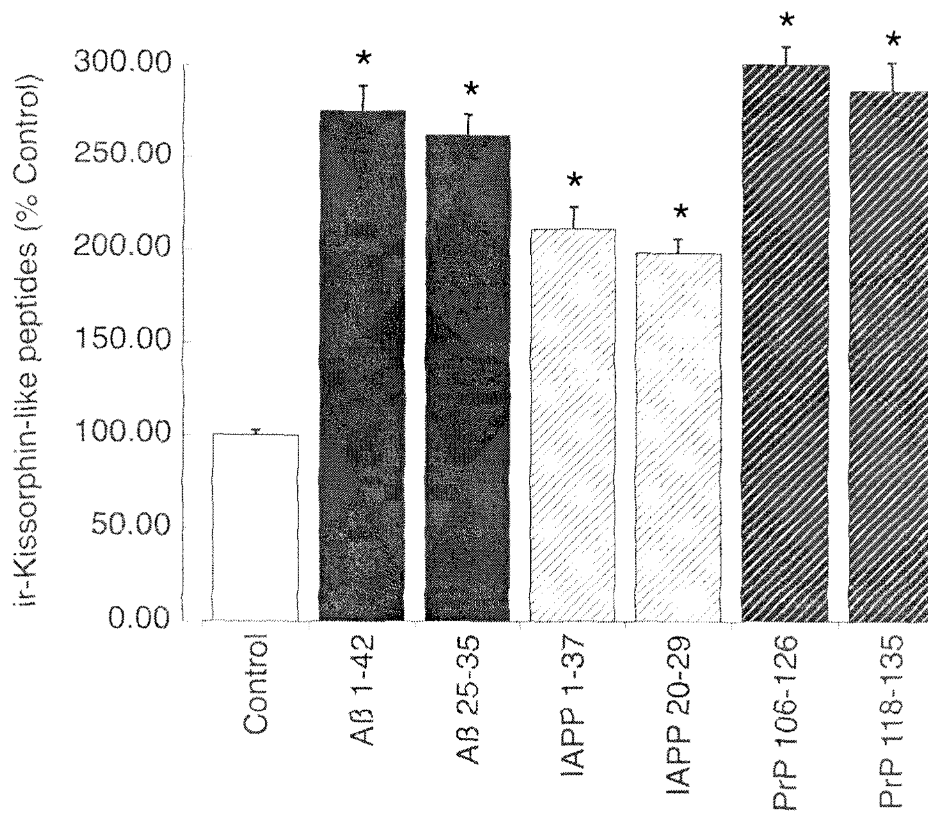

FIGURE 9

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KISSORPHIN PEPTIDES FOR USE IN THE TREATMENT OF ALZHEIMER'S DISEASE, CREUTZFELDT-JAKOB DISEASE OR DIABETES MELLITUS

Field of the Invention

The present invention relates to Kissorphin peptides that target Amyloid fibril-forming peptides and are suitable for use in detection, prevention and therapy of diseases associated with Amyloid fibril-forming peptides, such as Alzheimer's disease, Creutzfeldt-Jakob disease or Diabetes mellitus.

Background of the Invention

Amyloid-fibril forming peptides such as Amyloid-ß (Aß), Islet Amyloid Polypeptide (IAPP) and the Prion Protein (PrP) have been implicated in the pathology of a number of diseases including, but not restricted to, Alzheimer's disease (Panza et al., Aging Clin. Exp. Res. 21: 386-406 (2009)), Type 2 Diabetes mellitus (Höppener & Lips, Int. J. Biochem. Cell Biol. 38, 726-736 (2006)) and Creutzfeldt-Jakob disease (Aguzzi & Calella, Physiol. Rev. 89: 1105-1152, (2009)). The Amyloid-fibril forming peptides have been suggested as therapeutic targets and diagnostic markers for these diseases (Ballard et al., Lancet 377, 1019-31 (2011); Rigter et al., Vaccine 28, 7810-23 (2010); Takeda et al., Mol. BioSyst. 7, 1822-1827 (2011)). The Amyloid-fibril forming peptides Aß, IAPP and PrP share a number of properties and are all capable of forming fibrils with toxic properties mediated by similar mechanisms (Kawahara et al., J. Biol. Chem. 275: 14077-14083 (2000)).

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The progressive loss of cognitive function, characteristic of Alzheimer's disease, is accompanied by two disease-associated pathological changes in the brain. One of the changes involves formation of plaques in the space between neuronal cells. These plaques are composed primarily of Aβ, which is a protein in the brain. The second change involves the deformation of another brain protein, tau, which normally regulates the function of neurons. The deformation of tau results in the formation of neurofibrillary tangles (NFTs), which are insoluble, twisted fibres that build up inside the neuronal cells.

The Aß protein is generated from the Amyloid-ß Precursor Protein (AßPP) with the major forms Aß 1-42 and Aß 1-40 (herein SEQ ID Nos. 3 and 24 respectively) and the N-terminally truncated P3 peptides (Aß 17-40 and Aß 17-42) being generated by alternative enzymatic processing of AβPP. The C-terminally extended forms of

Aß (Aß 1-42 and Aß 17-42) show increased ability to form fibrils and are thought to have a causative action in the neurodegeneration seen in Alzheimer's disease (Tabaton *et al.*, Exp. Neurol. 221: 18-25 (2010): Mattson, Physiol. Rev. 77: 1081-1132 (1997); Rosenblum, J. Neuropath. Exp. Neurol. 58: 575-581 (1999)).

All the major forms of Aß contain a functional neurotoxic domain (Aß 25-35; herein SEQ ID No.26) and mediate their neurotoxicity by binding to the intracellular Aß-binding protein ERAB, an alcohol dehydrogenase (Muirhead *et al.*, Biochem. J. 426: 255-270 (2010): Yan *et al.*, J. Biol. Chem. 274: 2145-2156 (1999); Yanker *et al.*, Science 250: 279-282 (1990)). The major forms of Aß also inhibit hydrogen peroxide breakdown by the antioxidant enzyme catalase, an effect that involves a direct high affinity binding reaction (Milton, Biochem. J. 344: 293-296 (1999)). The Aß 25-35 region shows sequence similarity to the Diabetes-associated Islet Amyloid Polypeptide (IAPP; also known as Amylin) and the Creutzfeldt-Jakob disease associated prion protein (PrP). The Aß peptides can kill both neuronal and non-neuronal cells, a property shared with Islet Amyloid Polypeptide (IAPP) and PrP. Fibril forming fragments of all three peptides are also capable of modifying mitochondrial function; causing oxidative stress; modifying cell cycle processes; forming membrane channels; activating apoptotic cascades; altering intracellular calcium balance; altering glucose metabolism; and modifying metabolic processes (Kawahara *et al.*, J. Biol. Chem. 275: 14077-14083 (2000); Lim et al., FEBS Lett. 582, 2188-2194 (2008); Lim *et al.*, Proteomics 10: 1621-1633 (2010)).

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The Aß 31-35 peptide (herein SEQ ID No.28) is the shortest cytotoxic form of Aß, inhibits catalase and inhibits binding of Aß peptides to catalase (Milton, Biochem. J. 344: 293-296 (1999); Milton & Harris, Micron 40: 800-810 (2009)). Both catalase and antibodies specific to this region prevent Aß cytotoxicity, suggesting that compounds which specifically bind Aß 31-35 may be of therapeutic value in the treatment of Alzheimer's disease.

The Aß 16-20 region has been shown to be responsible for binding to ERAB (Milton *et al.*, Neuroreport 12: 22561-2566 (2001); Oppermann *et al.*, FEBS Lett. 451: 238-242 (1999)). Antibodies which block Aß binding to ERAB prevent Aß 1-42 cytotoxicity, suggesting that compounds which specifically bind Aß 16-20 may also antagonise actions of Aß.

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It has also been proposed that an alteration in the structure of the Aß protein may be an important determinant of cytotoxicity (Selkoe, Nature 399: A23-A31 (1999)). For example the Aß peptide can be phosphorylated at Ser 8 (Kumar et al., EMBO J: doi:10.1038/emboj.2011.138 (2011)) or Ser 26 (Milton, Subcell. Biochem., 38: 381-402 (2005); Milton, Neuroreport 12: 3839-44 (2001)), both of which cause increased toxicity and changes in fibril formation leading to pathology. Chronic inhibition of phosphatases, which would reverse these actions, can cause Alzheimer's-like pathology (Arendt *et al.*, Neurobiol. Aging; 19:3-13 (1998)) suggesting that Alzheimer's pathology may be due to an imbalance of kinase/phosphatase levels and post-translational modification of Aß. The appearance of Aß plaques in such animal models suggests that phosphorylation actions are crucial in the biochemical processes underlying Aß plaque formation. The ability of cyclin-dependent kinase inhibitors to prevent Aß toxicity also suggests a key role for such kinases in the toxic actions of Aß (Giovanni *et al.*, J. Biol. Chem; 274:19011-6 (1999); Alvarez *et al.*, FEBS Lett; 459: 421-6 (1999)). These enzymes phosphorylate serine and threonine residues within substrates and play roles in cell division and apoptosis.

The Aß peptide has been suggested to cause both neurotoxicity (Laurén *et al.*, Nature 457: 1128-1132 (2009)) and memory deficits (Gimbel *et al.*, J. Neurosci. 30(18): 6367-6374 (2010)) via interaction with the cellular PrP (Laurén *et al.*, Nature 457: 1128-1132 (2009)), which is required for PrP toxicity (Radford & Mallucci, Curr. Issues Mol. Biol. 12: 119-128 (2009)) and also Amylin receptors (Jhamandas *et al.*, Am. J. Pathol. 178(1): 140-149 (2011)), which are required for IAPP toxicity (Jhamandas *et al.*, J. Neurophysiol. 89: 2923-2930 (2003)). As such, compounds that prevent these interactions may be neuroprotective. Protective actions of Aß (Plant *et al.*, J. Neurosci. 23: 5531-5535 (2003)), IAPP (Cantarella *et al.*, Pharmacol. Res. 56: 27-34 (2007)) and PrP peptides (Rial *et al.*, Neuroscience 164: 896-907 (2009)) have been suggested. As such, selection of compounds for prevention of toxicity without modifying these beneficial protective actions is preferable.

Kisspeptin (also known as metastin) is a peptide derived from the KiSS-1 metastasis-suppressor gene and is a ligand for the GPR-54 receptor, also known

as the KISS1 or Kisspeptin receptor (Kotani *et al.*, J. Biol. Chem. 276: 34631–34636 (2001); Muir *et al.*, J Biol Chem 276: 28969–28975 (2001); Ohtaki *et al.*, Nature 411: 613–617 (2001)). The Kisspeptin peptides range in size from 10 to 54 amino acids, with the 10 amino acid form the smallest biologically-active derivative and considered the physiologically-active form (Gutiérrez-Pascual *et al.*, Mol. Pharmacol. 76: 58-67 (2009); Bilban *et al.*, J. Cell Sci. 117: 1319-1328 (2004)). The main Kisspeptin forms isolated from tissues and body fluids are the 54 amino acid form Kisspeptin-54 (herein SEQ ID No.1); the 14 amino acid form Kisspeptin-14 (herein SEQ ID No.20); the 13 amino acid form Kisspeptin-13 (herein SEQ ID No.21) and the decapeptide Kisspeptin-10 (herein SEQ ID No.22) (Kotani *et al.*, J. Biol. Chem. 276: 34631–34636 (2001); Bilban *et al.*, J. Cell Sci. 117: 1319-1328 (2004)). The peptides share the same C-terminus and are cleaved from the 145 amino acid Prepro-kisspeptin product of the KiSS-1 gene with Kisspeptin-54 corresponding to residues 68-121, Kisspeptin-14 corresponding to residues 108-121, Kisspeptin-13 corresponding to residues 109-121 and Kisspeptin-10 corresponding to residues 112-121 (Tena-Sempere, Human Reproduction Update, 12, 631–639, (2006)). Full biologically active Kisspeptin peptides require amidation of the C-terminal Phe residue (Kotani et al., J. Biol. Chem. 276: 34631-34636).

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Among the biological activities of Kisspeptins mediated via interaction with the GPR-54 receptor are effects on cell proliferation; prevention of metastasis; activation of luteinizing-hormone-releasing hormone (also known as LHRH, gonadotropin-releasing hormone or GnRH) secretion; effects on cognition; regulation of neurogenesis; a role in the pathogenesis of epilepsy; increased aldosterone production; stimulation of glucose-induced insulin secretion; and actions as potent vasoconstrictors (Oakley *et al.*, Endocrine Reviews 30: 713–743 (2009)).

Kissorphins are post-translationally modified products of the KiSS-1 metastasis-suppressor gene, which have been processed by enzymes such as matrix-metalloprotease (MMP) enzymes to remove the Leu-Arg-Phe motif at the C-terminus (Takino *et al.*, Oncogene, 22: 4617-4626 (2003)) and then alpha-amidated (McDonald et al., Cell Tissue Res., 280: 159-170 (1995)) to give a Phe-NH2 group at the newly created C-terminus. The Kissorphin peptides can be derived from any of the naturally occurring forms of Kisspeptin including, but not restricted to,

Kisspeptin-54, Kisspeptin-14, Kisspeptin-13 and Kisspeptin-10. These sequences have no biological actions mediated by the GPR-54 receptor (also known as Kisspeptin receptor: Takino *et al*., Oncogene, 22: 4617-4626 (2003)).

Summary of the Invention

The present invention is based on the realisation that peptides derived from human Kisspeptin-54 have binding affinity to Amyloid-ß and related proteins. Therefore, such peptides are useful therapeutics in the treatment of conditions associated with Amyloid fibril-forming peptides and can act to prevent fibril formation.

According to a first aspect of the invention, a peptide with the amino acid sequence Tyr-Asn-Trp-Asn-Ser-Phe or a peptide derived from kissorphin comprising said sequence for use in the treatment of a condition selected from Alzheimer’s disease, Creutzfeldt-Jakob disease and type 2 diabetes mellitus associated with IAPP, wherein the peptide does not comprise an amidated C-terminal Arg-Phe group of SEQ ID No. 1.

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According to a second aspect of the invention a composition comprises a peptide according to the first aspect of the invention, in a pharmaceutically-acceptable diluent, for use according to the first aspect of the invention.

According to a third aspect of the invention, an *in vitro* method for determining whether a subject has, or is predisposed to having, a disorder associated with Amyloid formation, comprising treating a sample from the subject with a peptide according to the first aspect of the invention to determine whether the sample comprises Amyloid fibril-forming peptides.

The peptides of the invention are advantageous as they have therapeutic utility and, as they correspond to a natural human sequence, will not stimulate immunorejection. The peptides are modified so that they do not directly activate or inhibit the GPR-54 G-protein coupled receptor (also known as the Kisspeptin receptor), and so do not exhibit a conventional Kisspeptin activity of activating the release of Luteinizing-hormone-releasing hormone (also known as LHRH, Gonadotropin-releasing hormone or GnRH).

Brief Description of the Figures

Figure 1 is a graph illustrating the binding of Kisspeptin-54 (SEQ ID NO. 1), Kisspeptin-54 residues 27-54 (SEQ ID No. 19), Kisspeptin-13 (SEQ ID No. 21), Kisspeptin-10 (SEQ ID NO. 22), Peptide 234 (SEQ ID NO. 14) and Kissorphin-6 (SEQ ID NO. 2) peptides to (A) biotinyl-Aß 1-40 (SEQ ID NO. 33); (B) biotinyl-IAPP 1-37 (SEQ ID No. 34) and (C) biotinyl-PrP 106-126 (SEQ ID No. 35). Inhibition of (D) biotinyl-Aß 1-40 (SEQ ID No. 33); (E) biotinyl-IAPP 1-37 (SEQ ID No. 34) and (F) biotinyl-PrP 106-126 (SEQ ID No. 35) binding to Kissorphin-6 (SEQ ID NO. 2) by amyloid peptides (SEQ ID Nos. 7, 24-31), amyloid antagonist peptides ASCAT and R9 (SEQ ID Nos. 12 and 13 respectively), anti-Aß antibody, anti-Kisspeptin antibody and human erythrocyte catalase is also shown.

Figure 2 is a graph illustrating the binding of biotinyl-Aß 1-42 (SEQ ID No. 32), biotinyl-Aß 1-40 (SEQ ID No. 33), biotinyl-IAPP 1-37 (SEQ ID No. 34) and biotinyl-PrP 106-126 (SEQ ID No. 35) binding to (A) a kissorphin derivative, CGG-KOR6 (SEQ ID No.15) conjugated to keyhole limpet hemocyanin (KLH-CGG-KOR-6) or (B) a kissorphin derivative CGG-KOR6 (SEQ ID No. 15) conjugated to bovine serum albumin (BSA-CGG-KOR-6). Inhibition of biotinyl-Aß 1-42 (SEQ ID No. 32), biotinyl-Aß 1-40 (SEQ ID No. 33), biotinyl-IAPP 1-37 (SEQ ID No. 34) and biotinyl-PrP 106-126 (SEQ ID No. 35) binding to KLH-CGG-KOR-6 conjugate (A) or BSA-CGG-KOR-6 conjugate (B) by anti-Kisspeptin antibody is also shown.

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Figure 3 is a graph illustrating the release of immunoreactive Luteinizing-hormone releasing hormone (LHRH; SEQ ID No's 17 & 18) from the human LA-N-1 cell line in response to Kissorphin-6 (SEQ ID No. 2), Kissorphin-6 residues 1-3 (SEQ ID No. 23), Kissorphin-6 (3-6; SEQ ID No. 11), Peptide 234 (SEQ ID No. 14), Kisspeptin-13 (SEQ ID No. 21) and Kisspeptin-10 (SEQ ID No. 22) plus combinations of Kisspeptin peptides (SEQ ID No's 21 & 22) and either Kissorphin peptides (SEQ ID Nos. 2, 11 & 23) or Peptide 234 (SEQ ID No. 14).

Figure 4 is a graph illustrating the effects of (A) Kisspeptin-10 (SEQ ID No. 22) and (B) Kissorphin-6 (SEQ ID No. 2) peptides on the aggregation of Aß 1-42 (SEQ ID No. 3), IAPP 1-37 (SEQ ID No. 7), PrP 106-126 (SEQ ID No. 30), PrP 118-135 (SEQ ID No. 31), Amyloid-Bri (SEQ ID No. 9) and Amyloid-Dan (SEQ ID No. 10). The binding of (C) biotinyl-Kisspeptin-54 (SEQ ID No. 36) and (D) biotinyl-

Kisspeptin-10 (SEQ ID No. 37) peptides to Aß 1-42 (SEQ ID No. 3), Aß 25-35 (SEQ ID No. 26), IAPP 1-37 (SEQ ID No. 7), IAPP 20-29 (SEQ ID No. 29), PrP 106-126 (SEQ ID No. 30), PrP 118-135 (SEQ ID No. 31), Amyloid-Bri (SEQ ID No. 9) and Amyloid-Dan (SEQ ID No. 10) fibrils are also shown.

Figure 5 is a graph illustrating the effects of an anti-Kisspeptin-10 antibody on Aß 1-42 (SEQ ID No. 3) cytotoxicity in human SH-SY-5Y neuroblastoma cells.

Figure 6 is a graph illustrating the role of Kisspeptin and Kissorphin peptides in amyloid peptide toxicity. The effects of Kisspeptin-54 (SEQ ID No. 1), Kisspeptin-54 (27-54; SEQ ID No. 19), Kisspeptin-13 (SEQ ID No. 21), Kisspeptin-10 (SEQ ID No. 22), Kissorphin-6 (SEQ ID No. 2), Kissorphin-6 (3-6; SEQ ID No. 11) and Kissorphin-6 (1-3; SEQ ID No. 19) peptides on (A) Aß 1-42 (SEQ ID No. 3) cytotoxicity; (B) IAPP 1-37 (SEQ ID No. 7) cytotoxicity; (C) PrP 106-126 (SEQ ID No. 30) cytotoxicity (D) Amyloid-Bri (SEQ ID No. 9) cytotoxicity and (E) Amyloid-Dan (SEQ ID No. 10) cytotoxicity in human SH-SY-5Y neuroblastoma cells. The effects of Peptide 234 (SEQ ID No. 14) on Kissorphin-6 (SEQ ID No. 2) inhibition of Aß 1-42 (SEQ ID No. 3), IAPP 1-37 (SEQ ID No. 7) and PrP 106-126 (SEQ ID No. 30) cytotoxicity (F) human SH-SY-5Y neuroblastoma cells are also shown.

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Figure 7 is a graph illustrating the effects of Kissorphin-protein conjugates on amyloid peptide toxicity. The effects of KLH-CGG-KOR-6 (A) or BSA-CGG-KOR-6 (B) conjugates on Aß 1-42 (SEQ ID No. 3) and Aß 25-35 (SEQ ID No. 26) cytotoxicity, IAPP 1-37 (SEQ ID No. 7) and IAPP 20-29 (SEQ ID No. 29) cytotoxicity, PrP 106-126 (SEQ ID No. 30) and PrP 118-135 (SEQ ID No. 31) cytotoxicity, Amyloid-Bri (SEQ ID No. 9) cytotoxicity and Amyloid-Dan (SEQ ID No. 10) cytotoxicity in human SH-SY-5Y neuroblastoma cells are all shown.

Figure 8 is a graph illustrating the dose response curves of Kisspeptin-13 and Kissorphin-6 peptides in amyloid peptide toxicity using rat cortical neurons. The effects of different concentrations of Kisspeptin-13 (SEQ ID No. 21) and Kissorphin-6 (SEQ ID No. 2) peptides on (A) Aß 1-42 (SEQ ID No. 3), (B) IAPP 1-37 (SEQ ID No. 7) and (C) PrP 106-126 (SEQ ID No. 30) cytotoxicity in rat cortical neurons are all shown. The effects of Peptide 234 (SEQ ID No. 14) and RF9 (SEQ ID No. 16) on Aß 1-42 (SEQ ID No. 3) and PrP 106-126 (SEQ ID No. 30) cytotoxicity plus

Kissorphin-6 (SEQ ID No. 2) inhibition of Aß 1-42 (SEQ ID No. 3) and PrP 106-126 (SEQ ID No. 30) cytotoxicity in rat cortical neurons are also shown (D).

Figure 9 is a graph showing immunoreactive-Kissorphin-like peptide release from human SH-SY-5Y neuroblastoma cells in response to Amyloid-fibril forming peptide stimulation. Results are calculated from a standard curve using Kissorphin-6 (SEQ ID No. 2) as the standard and expressed as % release from control, untreated, cells. The effects of Aß 1-42 (SEQ ID No. 3), Aß 25-35 (SEQ ID No. 26), IAPP 1-37 (SEQ ID No. 7), IAPP 20-29 (SEQ ID No. 29), PrP 106-126 (SEQ ID No. 30) and PrP 118-135 (SEQ ID No. 31) on immunoreactive-Kissorphin-like peptide release are all shown.

Detailed Description of the Invention

The present invention is based on the realisation that peptides derived from Kisspeptin-54 have the ability to bind to Amyloid fibril-forming peptides, and therefore can act to block the biological actions plus the formation of fibrils, thereby preventing the progression of associated disease states.

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The invention is described with reference to various peptide sequences, defined in the sequence listing. Table 1 provides a list and description of the peptides.

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Table 1

SEQ ID No.	Peptide Name	Description of peptide
1	Kisspeptin-54 [KP 54]	Full length biologically active Kisspeptin peptide from the KiSS-1 gene which activates LHRH via the GPR-54 receptor and also binds to Aß, IAPP and PrP peptides
2	Kissorphin-6 [K6]	KiSS-1 derivative which can be generated from Kisspeptin-10 and binds to Aß, IAPP and PrP peptides but has no activity at the GPR-54 receptor
3	Aβ 1-42	Major form of the Amyloid-fibril forming peptide, Amyloid-ß, associated with Alzheimer's disease
4	Kissorphin-9	KiSS-1 derivative which can be generated from Kisspeptin-13 and binds to Aß, IAPP and PrP peptides but has no activity at the GPR-54 receptor
5	Kissorphin-50	KiSS-1 derivative which can be generated from Kisspeptin-54 and binds to Aß, IAPP and PrP peptides but has no activity at the GPR-54 receptor
6	Kissorphin-10	KiSS-1 derivative which can be generated from Kisspeptin-14 and binds to Aß, IAPP and PrP peptides but has no activity at the GPR-54 receptor
7	IAPP 1-37	Major form of the Amyloid-fibril forming peptide, Islet Amyloid Polypeptide, associated with Type 2 Diabetes mellitus
8	PrP 106-135	Fragment of the Amyloid-fibril forming peptide, Prion Protein, associated with Creutzfeldt-Jakob disease
9	Amyloid Bri [A-Bri]	Amyloid-fibril forming peptide associated with the non-Amyloid-ß cerebral amyloidoses familial British dementia
10	Amyloid Dan [A-Dan]	Amyloid-fibril forming peptide associated with the non-Amyloid-ß cerebral amyloidoses familial Danish dementia
11	Kissorphin-6 residues 3-6	Fragment of Kissorphin-6 which binds to Aß but has no activity at the GPR-54 receptor
12	ASCAT	Synthetic peptide that binds to the Aß binding site of Human Catalase
13	R9	Synthetic peptide that binds to Aß
14	Peptide 234	Synthetic Kisspeptin-10 derivative that antagonises the GPR-54 receptor and binds to Aß, IAPP and PrP peptides
15	CGG-KOR6	Synthetic derivative of Kissorphin-6 with an N-terminal linker sequence
16	RF9	Synthetic Arg-Phe-amide peptide derivative that antagonises the Neuropeptide FF (NPFF) receptors GPR-147 and GPR-74
17	LHRH1	Gonadotrophin-releasing hormone isoform which is activated by Kisspeptin via the GPR-54 receptor and itself activates Luteinizing hormone and Follicle-stimulating hormone release from the pituitary
18	LHRH2	Gonadotrophin-releasing hormone isoform which is activated by Kisspeptin via the GPR-54 receptor and itself activates Luteinizing hormone and Follicle-stimulating hormone release from the pituitary
19	Kisspeptin-54 residues 27-54	Biologically active Kisspeptin fragment which activates LHRH and also binds to Aß, IAPP and PrP peptides
20	Kisspeptin-14 [KP-14]	Biologically active Kisspeptin fragment which activates LHRH and also binds to Aß, IAPP and PrP peptides

21	Kisspeptin-13 [KP-13]	Biologically active Kisspeptin fragment which activates LHRH and also binds to Aß, IAPP and PrP peptides
22	Kisspeptin-10 [KP-10]	Biologically active Kisspeptin fragment which activates LHRH and also binds to Aß, IAPP and PrP peptides
23	Kissorphin-6 residues 1-3	Fragment of Kissorphin-6 which does not bind to Aß, IAPP and PrP peptides or have no activity at the GPR-54 receptor
24	Aß 1-40	Major form of the Amyloid-fibril forming peptide, Amyloid-ß, associated with Alzheimer's disease
25	Aß 1-28	Fragment of the Amyloid-ß peptide associated with Alzheimer's disease
26	Aß 25-35	Toxic fragment of the Amyloid-ß peptide associated with Alzheimer's disease
27	Aß 29-40	Toxic fragment of the Amyloid-ß peptide associated with Alzheimer's disease
28	Aß 31-35	Smallest toxic fragment of the Amyloid-ß peptide associated with Alzheimer's disease
29	IAPP 20-29	Fragment of the Islet Amyloid Polypeptide associated with Type 2 Diabetes mellitus
30	PrP 106-126	Fragment of the Amyloid-fibril forming peptide, Prion Protein, associated with Creutzfeldt-Jakob disease
31	PrP 118-135	Fragment of the Amyloid-fibril forming peptide, Prion Protein, associated with Creutzfeldt-Jakob disease
32	Biotinyl-Aß 1-42	Labelled form of Amyloid-ß
33	Biotinyl-Aß 1-40	Labelled form of Amyloid-ß
34	Biotinyl-IAPP 1-37	Labelled form of Islet Amyloid Polypeptide
35	Biotinyl-PrP 106-126	Labelled form of a fragment of the Prion Protein
36	Biotinyl-Kisspeptin-54	Labelled form of Kisspeptin-54
37	Biotinyl-Kisspeptin-10	Labelled form of Kisspeptin-10
38	Biotinyl-Kissorphin-6	Labelled form of Kissorphin-6

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The peptides of the first aspect of the invention will have the sequence Tyr-Asn-Trp-Asn-Ser-Phe. Larger peptides are also envisaged wherein the peptide is derived from kissorphin and comprises the sequence Tyr-Asn-Trp-Asn-Ser-Phe. If the larger peptides also comprise further Kisspeptin amino acid sequences they should be modified so that the sequence does not elicit normal Kisspeptin biological activity; i.e. the peptide will not activate or inhibit the GPR-54 or Kisspeptin receptor. This can be achieved in various ways, including, but not limited to the omission of the Gly-Leu-Arg-Phe C-terminal amino acid segment of the natural Kisspeptin sequence, i.e. amino acid residues 51-54 of SEQ ID No. 1, or, alternatively, modification of the sequence to prevent amidation of the C-terminal Arg-Phe group of SEQ ID No. 1, since amidation of the C-terminal Phe residue is essential for full biologically activity of Kisspeptin peptides.

Polyphenol compounds are known to bind amyloid-fibril forming peptides (Porat *et al.*, Chem. Biol. Drug Des. 67, 27-37 (2006)) and the presence of amino acids with ring structures such as His, Phe, Pro, Trp or Tyr in peptides mimics the polyphenol amyloid-binding feature in Amyloid-binding peptides (Milton et al., Neuroreport 12, 2561-2566 (2001)). The presence of the amino acids His, Phe, Pro, Trp and Tyr with ring structures within the Kisspeptin-54 (SEQ ID No. 1) sequence should therefore be accounted for and replacements based on ring structure are within the scope of this invention to maintain the polyphenol amyloid-binding properties of the peptides.

In preferred embodiments, the peptides for use according to the first aspect of the invention consist of or comprise the following amino acid sequences:

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SEQ ID No. 4: Leu-Pro-Asn-Tyr-Asn-Trp-Asn-Ser-Phe (Residues 42-50 of Kisspeptin-54 (SEQ ID No.1))

SEQ ID No. 5: Gly-Thr-Ser-Leu-Ser-Pro-Pro-Pro-Glu-Ser-Ser-Gly-Ser-Arg-Gln-Gln-Pro-Gly-Leu-Ser-Ala-Pro-His-Ser-Arg-Gln-Ile-Pro-Ala-Pro-Gln-Gly- Ala-Val-Leu-Val -Gln-Arg-Glu-Lys-Asp-Leu-Pro-Asn-Tyr-Asn-Trp-Asn (Residues 1-50 of Kisspeptin-54 (SEQ ID No.1))

SEQ ID No. 6: Asp-Leu-Pro-Asn-Tyr-Asn-Trp-Asn-Ser-Phe (Residues 41-50 of Kisspeptin-54 (SEQ ID No.1))

The peptides according to the first aspect of the invention have the ability to bind to Aß1-42, IAPP 1-37 or PrP 106-135.

The peptides can inhibit the generation of the Aβ peptide from its precursor protein, can inhibit the aggregation of Aβ into neurotoxic aggregates, can block the neurotoxic actions of Aβ peptide forms and can enhance the clearance of the Aβ peptide. The peptides according to the first of the invention have been found to bind to a number of Amyloid fibril-forming peptides. Such peptides include Diabetes-associated Islet Amyloid Polypeptide (IAPP; also known as Amylin), c and

the Alzheimer's-associated Amyloid-ß peptide (Aß), but not the Amyloid-Bri or Amyloid-Dan peptides associated with two non-Aß cerebral amyloidoses familial British and Danish dementias (Ghiso *et al.*, Brain Pathol. 16: 71-79 (2006)). The peptides of the invention do not share the Kisspeptin biological activity of stimulating the release of luteinizing-hormone-releasing hormone (also known as LHRH, gonadotropin-releasing hormone or GnRH) via activation of the GPR-54 G-protein coupled receptor (also known as the Kisspeptin receptor) and are not antagonists of this activity. The peptides of the invention inhibit the aggregation of Aß, IAPP and PrP into toxic aggregates and can prevent the toxicity of Aß, IAPP or PrP.

The Aß peptide is central to Alzheimer's disease (Panza *et al.*, Aging Clin. Exp. Res. 21: 386-406 (2009)) but is also linked to Cerebral amyloid angiopathy (Smith & Greenberg, Stroke 40: 2601-2606 (2009)), Down's syndrome (Lott *et al.*, Curr. Alzheimer Res. 3:521-528 (2006)) plus brain ischemia and brain trauma (Hiltunen *et al.*, J. Alzheimers Dis. 18: 401-412 (2009)). Since Aß can enhance the aggregation of other pathological proteins there is potential for peptides of the invention to also be used in diseases associated with protein aggregates and deposits. For example Aß enhances α-synuclein aggregation plus pathological events, which are involved in the development of Lewy-body diseases (Masliah *et al.*, Proc Natl Acad Sci USA 98: 12245–12250 (2001)). Likewise increased levels or deposits of Aß are also found in a range of Neurodegenerative diseases including Parkinson's (Burack *et al.*, Neurology 74: 77-84 (2010)), Frontotemporal lobar degeneration and amyotrophic lateral sclerosis (Steinacker *et al.*, J. Neural. Transm. 116: 1169-1178 (2009)) plus Huntington's (Mollenhauer *et al.*, J. Neurol. Neurosurg. Psychiatry 77: 1201-1203 (2006)) suggesting the peptides of the invention could be used in these and other conditions in which Aß is implicated.

The IAPP peptide is linked to diabetes mellitus (Höppener & Lips, Int. J. Biochem. Cell Biol. 38, 726-736 (2006)) and also obesity (Roth et al., Arch. Neurol. 66, 306-310 (2009)). The PrP protein is linked to transmissible spongiform encephalopathies (TSEs) such as Creutzfeldt-Jakob disease (Aguzzi & Calella, Physiol. Rev. 89: 1105-1152, (2009)) and Gerstmann-Sträussler-Scheinker disease (Hsiao *et al.*, Nat. Genet. 1: 68-71(1992)).

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Pathology in diseases such as the Creutzfeldt-Jakob disease include Aß deposits alongside PrP deposits in the brain (Paquet *et al.*, Acta Neuropathol 116: 567–573 (2008); and Debatin *et al.*, Neurodegenerative Dis 5: 347–354 (2008)). Likewise, diabetes also includes Aß deposits alongside IAPP in the pancreas (Miklossy *et al.*, Neurobiology of Aging 31: 1503-1515 (2010)), suggesting that drugs targeting combinations of Aß, IAPP and PrP may be more effective in many disorders.

Accordingly, the peptides according to the first aspect of the invention can be used to treat disorders associated with Amyloid peptides. Such disorders include, but are not restricted to: Alzheimer's; Cerebral amyloid angiopathy; Down's syndrome; brain ischemia; brain trauma; Lewy-body diseases; Parkinson's; Frontotemporal lobar degeneration; Amyotrophic lateral sclerosis; Huntington's; diabetes mellitus; obesity; Transmissible spongiform encephalopathies (TSEs); and Creutzfeldt-Jakob disease.

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For the avoidance of doubt, reference to the Aß 1-42 region means the amino acid numbering for the conventional human Aß protein, shown as SEQ ID No. 3. Reference to the IAPP 1-37 region means the amino acid numbering for the conventional human IAPP protein, shown as SEQ ID No. 7. Reference to the PrP 106-135 region means the amino acid numbering for the conventional human PrP 106-135 protein fragment, shown as SEQ ID No. 8. Reference to Amyloid-Bri means the conventional human Amyloid-Bri protein shown in SEQ ID No. 9. Reference to Amyloid-Dan means the conventional human Amyloid-Dan protein shown in SEQ ID No. 10.

The binding of a peptide (or fragment) to the endogenous Aß 1-42, IAPP 1-37, PrP 106-135, Amyloid-Bri or Amyloid-Dan peptides (or fragments thereof) may be determined as shown in the Examples, and using techniques disclosed in Milton, Biochem. J. 344: 293-296 (1999), the content of which is incorporated herein by reference.

The peptides bind with a dissociation constant (Kd) of less than 50 μM, preferably less than 10 μM.

Provided the sequence shown as SEQ ID No. 2 is present, peptides according to the first aspect of the invention may also include sequences that are homologous to sequences comprised within Kisspeptin-54 residues 1-50.

The term "homologue" is used herein in two separate contexts. The peptides of the invention will comprise at least the sequence of SEQ ID No. 2, but may have additional sequences that share the same hydropathy profile as the remaining Kisspeptin sequence. This may be determined by analysing the peptide sequence and evaluating what alternative amino acids could be used as a replacement based on hydropathic character. Table 2 groups together those amino acids with a similar hydropathic character and which can be substituted for an amino acid specified in the peptide sequence.

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Table 2

Amino acid	Acceptable substitutions
Alanine (Ala)	Arg, Gly, Pro, Ser, Thr
Arginine (Arg)	Cys, Gly, Ser, Thr, Trp
Asparagine (Asn)	Asp, Gln, Glu,, His, Lys, Tyr
Aspartic acid (Asp)	Asn, Gln, Glu,, His, Lys, Tyr
Cysteine (Cys)	Arg, Gly, Ser, Trp
Glutamic Acid (Glu)	Asn, Asp, Gln, Lys,
Glutamine (Gln)	Asn, Asp, Glu, His, Lys, Tyr
Glycine (Gly)	Ala, Arg, Cys, Ser, Thr, Trp,
Histidine (His)	Asn, Asp, Gln, Tyr
Isoleucine (Ile)	Leu, Met, Val
Leucine (Leu)	Ile, Met, Phe, Val
Lysine (Lys)	Asn, Asp, Gln, Glu
Methionine (Met)	Ile, Leu, Val
Phenylalanine (Phe)	Leu,
Proline (Pro)	Ala, Ser, Thr
Serine (Ser)	Ala, Arg, Cys, Gly, Pro, Thr, Trp
Threonine (Thr)	Ala, Arg, Gly, Pro, Ser
Tryptophan (Trp)	Arg, Cys, Gly, Ser
Tyrosine (Tyr)	Asn, Asp, Gln, His

Valine (Val)	Ile, Leu, Met

The term "homologue" is also used to refer to peptides that share levels of sequence identity or similarity. Levels of identity or similarity between amino acid sequences can be calculated using known methods. Publicly available computer based methods include BLASTP, BLASTN and FASTA (Atschul *et al.*, Nucleic Acids Res., 25: 3389-3402 (1997)), the BLASTX program available from NCBI, and the GAP program from Genetics Computer Group, Madison WI.

The levels of identity and similarity referred to herein are based on the use of the BLASTP program. All BLAST searches were carried out using the Standard protein-protein BLAST (blastp) on the NCBI web site (www.ncbi.nlm.nih.gov/BLAST) with the BLOSUM62 matrix and Gap Costs of 11 for Existence and 1 for Extension. The statistical significance threshold for reporting matches against database sequences (E) was reset to 100 to account for the use of short peptide sequences in the search.

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It is preferable if there is at least 60% sequence identity or similarity to the specified peptides, preferably 70%, more preferably 80% and most preferably greater than 90%, e.g. at least 95%. The peptides should retain the ability to bind to the Aß protein and will comprise at least SEQ ID No. 2.

Synthetic amino acid derivatives may also be used. For example, the shifting of substitutents within an amino acid residue, from a C atom to a N atom, to produce a peptide having greater resistance to proteolysis, and other modifications, are known and are included within the scope of this invention.

Peptides used according to the first aspect of the invention may also comprise a signal sequence, which aids transport of the peptide *in vivo*. For example, the signal sequence may be provided to aid transport of the peptide across the blood brain barrier. Suitable signal sequences will be apparent to the skilled person.

The peptides may also be modified to prevent their degradation *in vivo*. For example the peptides may be modified to be resistant to peptidases present in the body. Suitable technologies to achieve this will be apparent to the skilled person.

Compounds that bind specifically to the peptide of the first aspect of the invention may also be useful in the diagnosis of a disease associated with Amyloid fibril-forming peptides. Such diseases include, but are not restricted to: Alzheimer's; Cerebral amyloid angiopathy; Down's syndrome; brain ischemia; brain trauma; Lewy-body diseases; Parkinson's; Frontotemporal lobar degeneration; Amyotrophic lateral sclerosis; Huntington's; diabetes mellitus; obesity; Transmissible spongiform encephalopathies (TSEs); and Creutzfeldt-Jakob disease.

Also disclosed herein, it has been found that a shorter sequence, identified herein as SEQ ID No. 11, binds to Aβ and can therefore be used in the treatment of a condition of Aβ toxicity, such as Alzheimer's disease. Therefore, in the context of such disorders, the peptide will comprise at least SEQ ID No. 11.

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SEQ ID No. 11: Xaa1-Asn-Ser-Phe

Residue Xaa1 is selected from His, Pro, Phe, Tyr or Trp.

SEQ ID No. 11 has the following sequence: Trp-Asn-Ser-Phe (Residues 47-50 of Kissspeptin-54 (SEQ ID No. 1))

Peptides of the first aspect of the invention may be synthesised using conventional methods known in the art and can be obtained to order from commercial sources. Peptide synthesis methods are also disclosed in Chan & White, Fmoc Solid Phase Peptide Synthesis: A Practical Approach (2000).

Alternatively, the peptides may be produced using recombinant DNA technology. This can be accomplished using techniques known to those skilled in the art. For example, the DNA sequence to be expressed can be inserted into an appropriate expression vector that contains the necessary regulatory apparatus, e.g. promoters, enhancers etc, to enable expression to occur. The DNA sequence may also be a synthetic polynucleotide. The expression vector can then be inserted into

an appropriate host cell, to enable expression to occur. Suitable methods are disclosed in Sambrook *et al*, Molecular Cloning, A Laboratory Manual (1989), and Ausubel *et al*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc..

Novel antibodies may be raised against the peptides of the first aspect of the invention using known antibody production techniques. For example, said peptides, acting as antigens, may be administered to an animal to produce an antibody-rich serum. This “antiserum” can be purified, to remove unwanted antibody molecules, by, for example, affinity fractionation. Monoclonal antibodies may also be raised by, for example, animal or *in vitro* immunisation techniques and fusion of antigen-exposed spleen cells to a myeloma cell line to produce hybridoma cell lines that secrete antibody. By screening hybridoma cell lines with a peptide of the invention, specific antibody-producing cell lines may be established.

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The term "antibody" includes, but is not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library. Such fragments include fragments of whole antibodies which retain their binding activity for a target substance, Fv, F(ab') and F(ab')2 fragments, as well as single chain antibodies (ScFv), fusion proteins and other synthetic proteins which comprise the antigen-binding site of the antibody.

Also disclosed herein, is a peptide fragment defined herein as SEQ ID No. 2 or SEQ ID NO. 11 used to raise antibodies that are specific for the Kissorphin peptide. The techniques of phage display or ribosome display, both of which are conventional in the art, may be used to select those antibodies with high affinity, preferably greater than 10^{-3} M, more preferably greater than 10^{-5} or 10^{-6} M. The antibodies may be useful in therapy or diagnostic assays.

The peptides or antibodies derived therefrom can be used in diagnostic tests to evaluate the risk of disease, or to provide a prognosis.

It will be evident to the skilled person how the diagnostic test can be carried out. In general, a sample from a subject can be treated with a peptide according to the first

or second aspects of the invention or an antibody thereto, and detection of a binding event can be measured and quantified with reference to a control sample.

The peptide or antibody can be detectably labelled, so that a binding event can be monitored. The label can, for example be a fluorophore or biotin label, covalently bound to the peptide or antibody.

A diagnostic assay where the levels of Kissorphin and Kisspeptin peptides are determined in biological fluids or tissue samples are determined can be used for both diagnosis and therapeutic monitoring of patients being treated with peptides according to the first aspect of the invention and/or other pharmaceutical agents.

According to the present invention, an *in vitro* method for determining whether a subject has, or is pre-disposed to having, a disorder associated with Amyloid fibril formation, comprises treating a sample from the subject with a peptide as defined in the first aspect of the invention, to determine whether the sample comprises Amyloid fibril-forming peptides. The method comprises the steps of: (i) treating a sample obtained from a patient with a peptide as defined in the first aspect of the invention; (ii) detecting binding events between the peptide or antibody and amyloid peptides present in the sample; and (iii) quantifying the binding events with reference to a control sample, wherein an increase in binding events compared to the control is indicative of a disorder associated with Amyloid fibril formation.

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Also disclosed herein, where the disorder associated with Amyloid fibril formation is Aβ toxicity, is an *in vitro* method for determining whether a subject has, or is pre-disposed to having, Aβ toxicity, comprises treating a sample from the subject with a peptide as defined in the second aspect of the invention, or an antibody of the invention, to determine whether the sample comprises Aβ peptides. The method comprises the steps of: (i) treating a sample obtained from a patient with a peptide as defined in the second aspect of the invention, or an antibody of the invention; (ii) detecting binding events between the peptide or antibody and Aβ peptides present in the sample; and (iii) quantifying the binding events with reference to a control sample, wherein an increase in binding events compared to the control is indicative of a disorder associated with Aβ toxicity.

When the method is used for therapeutic monitoring of the treatment of a disorder associated with Amyloid fibril formation, including Aβ toxicity, the patient sample will be obtained from a patient receiving treatment for the disorder.

Therapeutic monitoring is conducted in conjunction with treatment in order to assess whether the treatment is resulting in an improvement in the condition being treated. Therapeutic monitoring may involve monitoring the natural expression of peptides of the first aspect of the invention in patient, or alternatively, administering a composition comprising a peptide according to the invention and monitoring binding to Aβ, IAPP and/or PrP, in order to determine the effectiveness of a treatment.

Techniques such as HPLC or column chromatography can be used to separate different Kissorphin and Kisspeptin forms, which can be detected using standard methods (Lim (ed), HPLC of Small Molecules: a Practical Approach. Oxford: IRL Press; (1986); Hutton & Siddle (eds), Peptide Hormone Secretion: a Practical Approach. Oxford: IRL Press; (1990)). The assay can also use antibodies or other binding agents, capable of specific recognition of the Kissorphin and Kisspeptin peptides for detection and standard immunoassay techniques for level determination (Hutton & Siddle (eds), Peptide Hormone Secretion: a Practical Approach. Oxford: IRL Press; (1990)).

A sample from a patient (blood sample, tissue sample etc.) can be used to detect whether an amyloid peptide, or fragment thereof, that binds the peptide of first aspect of the invention (such as Aß, IAPP or PrP)) is present. The amyloid peptide, or fragment thereof, that binds the peptide according to the first aspect of the invention can be detected, for example, by the use of an antibody that has specificity for a peptide according to the first invention, respectively, or by use of a respective peptide according to the invention.

The peptides of the first aspect of the invention may also be used in assays to identify therapeutic molecules that can target an amyloid peptide, or fragment thereof, that binds the peptide of the invention such as Aß, IAPP or PrP.. Combinatorial chemistry can be used to develop target therapeutic molecules, which are then screened for activity. The target molecules can be brought into

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contact with a peptide of the invention. If the presence of the target molecule results in binding affinity, then it may be a potential therapeutic candidate. Preferably the peptides bind to target molecules with a dissociation constant (Kd) of less than 50 μM, preferably less than 10 μM.

The peptides for use according to the present invention may be formulated, in a therapeutically effective amount, in any suitable pharmaceutically acceptable, diluent or excipient. Acceptable carriers, diluents and excipients are well known to the skilled person and are also described in Remington's Pharmaceutical Sciences, Mack Publishing Co (A.R. Gennaro edit. 1985). The choice of a carrier, diluent or excipient can be selected based on the intended route of administration, etc.

The pharmaceutical composition may be for human or animal usage. The dosage to be administered to a patient depends on the route of administration, the nature of the formulation and the severity of the condition. Intravenous infusion of Kisspeptin-54 over the dose range up to 0.01 mg/kg body weight has been shown to activate endogenous LH in humans (Dhillo *et al.*, J. Clin. Endocrinol. Metab. 90: 6609–6615 (2005)). A Kisspeptin receptor antagonist administered via intracerebroventricular injection in rats at a dose equivalent to 0.03 mg/kg body weight inhibited the actions of Kisspeptin (Roseweir *et al.*, J. Neurosci., 29: 3920 –3929 (2009)). Suitable doses of the peptide of the invention are likely to be in the range of 0.01 –30 mg/kg body weight, preferably from 0.01-10 mg/kg body weight and more preferably 0.01-1 mg/kg body weight.

The route of administration will be dependent on the localization of the Amyloid fibril-forming peptides that are being targeted by the pharmaceutical composition. The possible routes of administration of the composition are not restricted to but will include the enteral, parenteral, epidural, intracerebral, intracerebroventricular, intravitreal and nasal routes. The ability of peripheral clearance of amyloid peptides such as Aβ to reduce central nervous system levels of amyloid (Sutcliffe, J.G. et al., 2011. J. Neurosci. Res, 89(6), pp.808-814 (2011)) will also allow parenteral administration to be used to target central nervous system produced Amyloid fibril-forming peptides. Typically, the composition will be administered parenterally, in which case the composition is in an injectable form, for delivery via the intravenous, intramuscular or subcutaneous route.

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The invention is now further described with reference to the accompanying drawings, in the following non-limiting example.

Example

Amyloid peptide binding to Kisspeptin and Kissorphin peptides

N-terminally Biotinylated Kisspeptin-54 (SEQ ID No. 36), Kisspeptin-10 (SEQ ID No. 37), Aß 1-42 (SEQ ID No. 32), Aß 1-40 (SEQ ID No. 33), PrP 106-126 (SEQ ID No. 35) and IAPP 1-37 (SEQ ID No. 34) were purchased from Bachem or Alpha Diagnostics. ELISA plates were coated with either Kisspeptin-54 (SEQ ID No. 1) Kisspeptin-54 (27-54; SEQ ID No. 19), Kisspeptin-13 (SEQ ID No. 21), Kisspeptin-10 (SEQ ID No. 22), Peptide 234 (SEQ ID No. 14; Roseweir, A.K. et al., J. Neurosci. 29: 3920-20 3929 (2009)), Kissorphin-6 (SEQ ID No. 2), Aß 1-42 (SEQ ID No. 3), Aß 25-35 (SEQ ID No. 26), IAPP 1-37 (SEQ ID No. 7), IAPP 20-29 (SEQ ID No. 29), PrP 106-126 (SEQ ID No. 30), PrP 118-135 (SEQ ID No. 31), Amyloid-Bri (SEQ ID No. 9) or Amyloid-Dan peptides (SEQ ID No. 10), (1 µg/m1) in carbonate buffer and unoccupied sites blocked with 0.2% (w/v) casein. Biotinylated peptides (200 pM) were incubated alone, with control peptides, with Kissorphin peptides, Kisspeptin peptides or with unlabelled forms of the respective amyloid peptides in 50 mM TRIS (containing 0.1% BSA and 0.1% Triton X-100) at 4°C for 16 hours. After washing to remove unbound material an alkaline phosphatase polymer-streptavidin conjugate (Sigma, Dorset, UK) was added and incubated at 24°C for 2 hours. After washing to remove unbound material p-nitrophenylphosphate substrate was added and absorbance at 405 nm determined. Affinity constants were determined by incubating Kissorphin-6 (SEQ ID No. 2) coated plates with biotinylated peptides (200 pM) plus unlabelled peptides over a range of concentrations (0 - 100 nM) and detection of bound peptides by ELISA.

Scatchard analysis (Friguet, B. *et al.*, J. Imm. Meth. 77: 305-319 (1985)) was performed using the following equation: $A0 / (A0 - A) = 1 + KD / a0$ and plotting $v\ [(A0 - A) / A0]$ against v / a, where $A0$ = absorbance in absence of unlabelled peptide, A = absorbance in presence of unlabelled peptide, $a0$ = total concentration of unlabelled peptide and a = concentration of unlabelled peptide added. The KD was equal to $-1 / $ slope of v against v / a.

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The biotinylated Aß 1-42 (SEQ ID No. 32; Figure 1A), IAPP 1-37 (SEQ ID No. 34; Figure 1B) and PrP 106-126 (SEQ ID No. 35; Figure 1C) all showed significant binding to Kisspeptin-54 (SEQ ID No. 1), Kisspeptin-54 (27-54; SEQ ID No. 19), Kisspeptin-13 (SEQ ID No. 21), Kisspeptin-10 (SEQ ID No. 22), Peptide 234 (SEQ ID No. 14) and Kissorphin-6 (SEQ ID No. 2) coated plates. Binding of biotinylated Aß 1-42 (SEQ ID No. 32) to the Kissorphin-6 peptide ((SEQ ID No. 2; Figure 1D) was inhibited by 10µM unlabelled amyloid peptides Aß 1-40 (SEQ ID No. 24), Aß 25-35 (SEQ ID No. 26), Aß 29-40 (SEQ ID No. 27), IAPP 1-37 (SEQ ID No. 7), IAPP 20-29 (SEQ ID No. 29), PrP 106-126 (SEQ ID No. 30) and PrP 118-135 (SEQ ID No. 31), 10µM of the anti-amyloid nonapeptide R9 (SEQ ID No. 13; Milton & Harris, Micron 40: 800-810 (2009)), 10µg/ml of an anti-Aß monoclonal antibody ND1 (Milton & Harris, TSWJ 10: 879-893 (2010)), 10µg/ml of an anti-Kisspeptin-10 antibody, and 10µg/ml of human erythrocyte catalase (HEC: Milton, Biochem. J. 344: 293-296 (1999)). Binding of biotinylated Aß 1-42 (SEQ ID No. 32) to the Kissorphin-6 peptide (SEQ ID No. 2) was not altered by 10µM of a synthetic amyloid antagonist peptide ASCAT (SEQ ID No. 12; Milton et al., Neuroreport 12; 2561-2566 (2001)).

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Binding of biotinylated IAPP 1-37 (SEQ ID No. 34) to the Kissorphin-6 (SEQ ID No. 2) peptide (Figure 1E) was inhibited by 10µM unlabelled amyloid peptides Aß 1-40 (SEQ ID No. 24), Aß 25-35 (SEQ ID No. 26), Aß 29-40 (SEQ ID No. 27), IAPP 1-37 (SEQ ID No. 7), IAPP 20-29 (SEQ ID No. 29), PrP 106-126 (SEQ ID No. 30) and PrP 118-135 (SEQ ID No. 31), 10µM of a synthetic amyloid antagonist peptide ASCAT (SEQ ID No. 12; Milton et al., Neuroreport 12; 2561-2566 (2001)), 10µg/ml of an anti-Kisspeptin-10 antibody and 10µg/ml of human erythrocyte catalase (HEC: Milton, Biochem. J. 344: 293-296 (1999)). The anti-Aß monoclonal antibody ND1 (10µg/ml; Milton & Harris, TSWJ 10: 879-893 (2010)) and 10µM anti-amyloid nonapeptide R9 (SEQ ID No. 13; Milton & Harris, Micron 40: 800-810 (2009)) had no effect on IAPP (SEQ ID No. 34) binding to Kissorphin-6 (SEQ ID No. 2). Binding of biotinylated PrP 106-126 (SEQ ID No. 35) to the Kissorphin-6 (SEQ ID No. 2) peptide (Figure 1F) was inhibited by 10µM unlabelled amyloid peptides Aß 1-40 (SEQ ID No. 24), Aß 25-35 (SEQ ID No. 26), Aß 29-40 (SEQ ID No. 27), IAPP 1-37 (SEQ ID No. 7), IAPP 20-29 (SEQ ID No. 29), PrP 106-126 (SEQ ID No. 30) and PrP 118-135 (SEQ ID No. 31), 10µM of a synthetic amyloid antagonist peptide ASCAT (SEQ ID No. 12; Milton et al., Neuroreport 12; 2561-2566 (2001)), 10µg/ml

of an anti-Kisspeptin-10 antibody and 10µg/ml of human erythrocyte catalase (HEC: Milton, Biochem. J. 344: 293-296 (1999)). The anti-Aß monoclonal antibody ND1 (10µg/ml; Milton & Harris, TSWJ 10: 879-893 (2010)) and 10µM anti-amyloid nonapeptide R9 (SEQ ID No. 13; Milton & Harris, Micron 40: 800-810 (2009)) had no effect of PrP (SEQ ID No. 35) binding to Kissorphin-6 (SEQ ID No. 2).

Addition of 10µM of Aß fragments Aß 1-28 (SEQ ID No. 25) and Aß 31-35 (SEQ ID No. 28) had no effect on binding of biotinylated Aß 1-42 (SEQ ID No. 32), IAPP 1-37 (SEQ ID No. 34) and PrP 106-126 (SEQ ID No. 35) peptides whilst 10µM Aß 25-35 (SEQ ID No. 26) and Aß 29-40 (SEQ ID No. 27) both strongly inhibited binding suggesting that residues 29 and 30 of Aß, plus some of the surrounding residues may be key.

The antibody raised against the Kisspeptin-10 (SEQ ID No. 22) peptide (10µg/ml), which also bound Kissorphin-6 (SEQ ID No. 2), was able to prevent binding of biotinylated Aß 1-42 (SEQ ID No. 32), Aß 1-40 (SEQ ID No. 33), IAPP 1-37 (SEQ ID No. 34) and PrP 106-126 (SEQ ID No. 35) to plates coated with Kissorphin-6 (SEQ ID No. 2). Similar antibodies may be used to monitor Kissorphin levels.

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Affinity constants (KD) for binding to Kissorphin-6 (SEQ ID No. 2) were 0.62 ± 0.07 nM (n=5) for biotinylated Aß 1-42 (SEQ ID No. 32); 0.47 ± 0.04 nM (n=5) for biotinylated Aß 1-40 (SEQ ID No. 33); 11.6 ± 1.2 nM (n=5) for biotinylated IAPP 1-37 (SEQ ID No. 34) and 34.2 ± 3.7 nM (n=5) for biotinylated PrP 106-126 (SEQ ID No. 35) respectively. These constants are similar to those previously determined for Aß 1-42 (SEQ ID No. 32) and IAPP 1-37 (SEQ ID No. 34) binding to human erythrocyte catalase and ERAB (Milton, Biochem. J. 344: 293-296 (1999); Milton et al., Neuroreport 12: 2561-2566 (2001); Milton & Harris, TSWJ 10: 879-893 (2010)).

Binding of Kissorphin-Protein conjugates to Amyloid peptides

A Kissorphin-6 derivative with an N-terminal (Cys-Gly-Gly) linker sequence (CGG-KOR-6; SEQ ID No. 15) was conjugated to either maleimide-activated keyhole limpet hemocyanin KLH) or maleimide-activated bovine serum albumin (BSA). For conjugation of CGG-KOR-6 (SEQ ID No. 15) to KLH a molar ratio in the reaction mixture of 1000:1 (CGG-KOR-6 peptide to maleimide-KLH) was used. For conjugation of CGG-KOR-6 (SEQ ID No. 15) to BSA a molar ratio in the reaction

mixture of 40:1 (CGG-KOR-6 peptide to maleimide-BSA) was used. A 1m1 solution containing 5 mg/ml of maleimide-activated KLH or BSA (Sigma-Aldrich) in 20 mM sodium phosphate buffer with 230 mM NaCl, 2 mM EDTA, and 80 mM sucrose, pH 6.6 was prepared. The CGG-KOR-6 (SEQ ID No. 15) peptide (4 mg) was dissolved in 0.5 ml of 20 mM sodium phosphate buffer with 100 mM EDTA and 80 mM sucrose, pH 6.6. The CGG-KOR-6 (SEQ ID No. 15) peptide solution was immediately mixed with the maleimide-activated KLH or BSA solution in a reaction vial equipped with a stirring bar. The sample was de-gassed, while stirring, under a gentle nitrogen stream for 1–2 minutes. The reaction vial was capped and stirring continued for 2 hours at room temperature. The KLH-CGG-KOR-6 or BSA-CGG-KOR-6 conjugates were purified using Sephadex G-25M gel filtration columns with elution in PBS buffer.

ELISA plates were coated with either KLH-CGG-KOR-6 or BSA-CGG-KOR-6 conjugates (1 µg ml^{-1}), in carbonate buffer and unoccupied sites blocked with 0.2% (w/v) casein. Biotinylated peptides (200 pM) were incubated either alone or with 10 µg ml^{-1} anti-Kisspeptin-10 antibody or with 10 µg ml^{-1} of a control antibody in 50 mM TRIS (containing 0.1% BSA and 0.1% Triton X-100) at 4°C for 16 hours. After washing to remove unbound material an alkaline phosphatase polymer-streptavidin conjugate (Sigma, Dorset, UK) was added and incubated at 24°C for 2 hours. After washing to remove unbound material p-nitrophenylphosphate substrate was added and absorbance at 405 nm determined.

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The biotinylated Aß 1-42 (SEQ ID No. 32), Aß 1-40 (SEQ ID No. 33), IAPP 1-37 (SEQ ID No. 34) and PrP 106-126 (SEQ ID No. 35) all showed significant binding to KLH-CGG-KOR-6 conjugate (Figure 2A) or BSA-CGG-KOR-6 conjugate (Figure 2B) coated plates. An antibody raised against the Kisspeptin-10 peptide (SEQ ID No. 22), which also bound Kissorphin-6 (SEQ ID No. 2), was able to reduce binding of biotinylated Aß 1-42 (SEQ ID No. 32), Aß 1-40 (SEQ ID No. 33), IAPP 1-37 (SEQ ID No. 34) and PrP 106-126 (SEQ ID No. 35) to plates coated with KLH-CGG-KOR-6 or BSA-CGG-KOR-6 conjugates. No significant binding of biotinylated Aß 1-42 (SEQ ID No. 32), Aß 1-40 (SEQ ID No. 33), IAPP 1-37 (SEQ ID No. 34) and PrP 106-126 (SEQ ID No. 35) peptides to either KLH or BSA was detected.

Effects of Kisspeptin and Kissorphin on LHRH release

To determine if the Kisspeptin and Kissorphin sequences tested above had biological activity the release of Luteinizing-hormone-releasing hormone (LHRH) from a Kisspeptin responsive cell line was determined. The human LHRH secreting cell line (LA-N-1) was cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 (ratio 1:1) containing 10% fetal bovine serum (FBS) and sodium bicarbonate 3.7 g/l, in a humidified atmosphere containing 5% CO_2 in air (Chen, A. et al., Endocrinology 142: 830–837 (2001)). Culture media were changed at 48 h intervals. Cells were exposed to Kisspeptin-13 (SEQ ID No. 21), Kisspeptin-10 (SEQ ID No. 22), Peptide 234 (SEQ ID No. 14; Roseweir, A.K. et al., J. Neurosci. 29: 3920-3929(2009)), Kissorphin-6 (SEQ ID No. 2), Kissorphin-6 residues 1-3 (SEQ ID No. 23) or Kissorphin-6 residues 3-6 (SEQ ID No. 11) for 2h and release of LHRH measured by immunoassay (Quaynor et al., Mol. Endocrinol. 21: 3062-3070 (2007)), which detects both LHRH-I (SEQ ID No. 17) and LHRH-II (SEQ ID No. 18).

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Both Kisspeptin-13 (SEQ ID No. 21) and Kisspeptin-10 (SEQ ID No. 22) stimulated a 4-fold increase in LHRH release whilst Peptide 234 (SEQ ID No. 14) and the Kissorphin peptides had no effect. Addition of Kisspeptin-13 (SEQ ID No. 21) or Kisspeptin-10 (SEQ ID No. 22) plus Kissorphin-6 (SEQ ID No. 2), Kissorphin-6 (1-3; SEQ ID No. 23) or Kissorphin-6 (3-6; SEQ ID No. 11) showed that the Kissorphin peptides had no significant effect on Kisspeptin-13 (SEQ ID No. 21) or Kisspeptin-10 (SEQ ID No. 22) stimulated release of immunoreactive LHRH (Figure 3). The Kisspeptin receptor antagonist Peptide 234 (SEQ ID No. 14) significantly inhibited Kisspeptin-13 (SEQ ID No. 21) and Kisspeptin-10 (SEQ ID No. 22) stimulated LHRH release, in agreement with previous studies (Roseweir, A.K. et al., J. Neurosci. 29: 3920-3929(2009)). These results confirmed that the Kissorphin-6 peptide sequences (SEQ ID No's 2, 11 and 23) were neither agonists nor antagonists of LHRH release.

Effects of Kisspeptin and Kissorphin peptides on amyloid peptide aggregation

Amyloid peptides recognised by Kisspeptin and Kissorphin peptides have the ability to aggregate into Congo red staining fibrils and other forms all of which have been linked to pathology and toxicity in Amyloid diseases (Milton & Harris, Micron 40:

800-810 (2009); Milton & Harris, TSWJ 10: 879-893 (2010)). The Kisspeptin and Kissorphin peptides were tested for their ability to inhibit the aggregation process and their interactions with fibrillar aggregates.

Freshly prepared 50 µM solutions of amyloid peptides [Aß 1-42 (SEQ ID No. 3), IAPP 1-37 (SEQ ID No. 7), PrP 106-126 (SEQ ID No. 30), PrP 118-135 (SEQ ID No. 31), Amyloid-Bri (SEQ ID No. 9) and Amyloid-Dan (SEQ ID No. 10)] were incubated either alone or in the presence of either 50µM Kisspeptin-10 (SEQ ID No. 22) or 50µM Kissorphin-6 (SEQ ID No. 2), after dissolving in distilled water, at 37°C for 24h with constant oscillation. Following *in vitro* fibrillogenesis of peptides an aliquot of each test sample was prepared to give a 50 µM concentration of amyloid peptide in phosphate buffered saline (PBS). Congo red, prepared as a 200 µM stock in PBS containing 10% ethanol, was then added to give final concentration 10 µM Congo red to 9.09 µM amyloid peptide and 100 µl aliquots were added to 96 well microtitre plates. After 15 min incubation the absorbance levels at 405 and 540nm were determined. The concentration of amyloid aggregates ([Amyloidagg]) was then calculated, with correction for the pathlength of the reader used, as follows: [Amyloidagg] = 10 x ((540At/4780) – (405At/6830) – (405ACR/8620)), where 540At = absorbance of amyloid peptide + Congo red solution at 540 nm, 405At = absorbance of amyloid peptide + Congo red solution at 405 nm and 405ACR = absorbance of Congo red solution at 405 nm. Absorbance readings at 405 nm and 540 nm were taken for each amyloid peptide and the ratio of 540AAmyloid /405AAmyloid checked, where 405AAmyloid = absorbance of amyloid peptide solution at 405 nm and 540AAmyloid = absorbance of amyloid peptide solution at 540 nm (Milton & Harris, Micron 40: 800-810 (2009)).

The Aß 1-42 (SEQ ID No. 3), IAPP 1-37 (SEQ ID No. 7), PrP 106-126 (SEQ ID No. 30), PrP 118-135 (SEQ ID No. 31), Amyloid-Bri (SEQ ID No. 9) and Amyloid-Dan (SEQ ID No. 10) peptides all showed significant Congo red binding amyloid aggregate formation. The concentrations of Congo red binding amyloid aggregates formed by Aß 1-42 (SEQ ID No. 3), IAPP 1-37 (SEQ ID No. 7), PrP 106-126 (SEQ ID No. 30) and PrP 118-135 (SEQ ID No. 31) were significantly reduced by treatment with Kisspeptin-10 (SEQ ID No. 22; Figure 4A) or Kissorphin-6 (SEQ ID No. 2; Figure 4B). The Kisspeptin-10 (SEQ ID No. 22) and Kissorphin-6 (SEQ ID No. 2) peptides had no significant effect on the Congo red binding amyloid

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aggregate formation by either Amyloid-Bri (SEQ ID No. 9) or Amyloid-Dan (SEQ ID No. 10) peptides. Previous studies have shown that rat Kisspeptin-10 can form aggregates, but that this requires the addition of enhancers such as heparin (Nielsen et al., Biopolymers 93(8), 678-689 (2010)), in the system used here human Kisspeptin-10 (SEQ ID No. 22) and Kissorphin-6 (SEQ ID No. 2) showed no detectable Congo red binding aggregate formation.

Biotinylated Kisspeptin-54 (SEQ ID No. 36; Figure 4C) and Kisspeptin-10 (SEQ ID No. 37; Figure 4D) both bound plates coated with Aß 1-42 (SEQ ID No. 3), Aß 25-35 (SEQ ID No. 26), IAPP 1-37 (SEQ ID No. 7), IAPP 20-29 (SEQ ID No. 29), PrP 106-126 (SEQ ID No. 30) and PrP 118-135 (SEQ ID No. 31) fibrils. No binding to Amyloid-Bri (SEQ ID No. 9) or Amyloid-Dan (SEQ ID No. 10) fibrils was observed. These results confirm that the binding to amyloid peptides could take place in situations where both monomeric, oligomeric and fibrillar forms are present.

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Effects of anti-Kisspeptin antibody, Kisspeptin peptides, Kissorphin peptides and Kissorphin-Protein conjugates on amyloid neurotoxicity

The human neuroblastoma SH-SY5Y cell line was grown in a 1:1 mixture of HAM's F12 and Eagle's minimal essential medium containing 1% non-essential amino acids and supplemented with 10% fetal calf serum and antibiotics (penicillin and streptomycin; 1% each) in a 5% CO_2 humidified incubator at 37°C. The cells were passaged when confluent and medium was replaced every 2-3 days. Cells were used 4–10 days after plating. Cultures were differentiated for 7 days with 10 mM retinoic acid (Milton, Open Enzyme Inhib. J. 1; 34-41 (2008)).

Frozen Primary rat cortical neurons (RCN), prepared from embryonic (day 18) Fisher 344 rat embryos and cryopreserved in a medium containing DMSO, were seeded onto plates coated with poly-D-lysine, in plating media (neurobasal medium with B27 serum-free supplement plus glutamax-1). The medium was replaced every 3 days and cells were used 7–10 days after plating (Molina-Holgado, F. et al., Mol. Cell Neurosci. 28; 189-194 (2005)).

Freshly prepared 50 µM solutions of amyloid peptides (Aß 1-42 (SEQ ID No. 3), Aß 25-35 (SEQ ID No. 26), IAPP 1-37 (SEQ ID No. 7), IAPP 20-29 (SEQ ID No. 29), PrP 106-126 (SEQ ID No. 30), PrP 118-135 (SEQ ID No. 31), Amyloid-Bri (SEQ ID

No. 9) and Amyloid-Dan (SEQ ID No. 10)) were incubated, after dissolving in PBS, at 37°C for 24h with constant oscillation prior to dilution in culture medium for addition to cells and determination of cytotoxicity.

For cytotoxicity experiments 5 x 10^3 cells were incubated in 96 well dishes in 100 µl culture medium and test substances for 24 hours. Cell viability was determined by trypan blue dye exclusion with at least 100 cells counted per well or by MTT reduction (Behl et al., Cell 77; 817-827 (1994)). After incubation with test substances MTT (10 µl: 12mM stock) was added and cells incubated for a further 4 hours. Cell lysis buffer [100 µl/well; 20% (v/v) SDS, 50 % (v/v) N,N-dimethylformamide, pH 4.7] was added and after repeated pipetting to lyse cells the MTT formazan product formation was determined by measurement of absorbance change at 570 nm. Control levels in the absence of test substances were taken as 100% and the absorbance in the presence of cells lysed with Triton X-100 at the start of the incubation period with test substances taken as 0% (Milton, Open Enzyme Inhib. J. 1; 34-41 (2008)). Cell viability was also determined by trypan blue dye exclusion with at least 100 cells counted per well (Milton, Biochem. J. 344: 293-296 (1999)).

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The dose response curve for Aß 1-42 (SEQ ID No. 3) toxicity showed constant enhancement of toxicity by an anti-Kisspeptin-10 antibody (Figure 5) indicating a potential neuroprotective action by an endogenous Kisspeptin-like molecule. These results suggest that an anti-Kisspeptin-10 antibody, which binds Kissorphin-6 (SEQ ID No. 2), could be used to prevent the toxicity of complexes formed between Kisspeptins and amyloid peptides in a similar manner to that reported for antibodies to the amyloid binding Amyloid-P protein (Bodin et al., Nature 468: 93-97 (2010)).

The toxicity of 5 µM Aß 1-42 (SEQ ID No. 3) and 5 µM Aß 25-35 (SEQ ID No. 26) peptides (Milton, Neurotoxicology 22: 767-774 (2001); Milton, Neurosci. Lett. 332: 127-130 (2002)) was significantly inhibited in SH-SY5Y cell cultures by addition of 10 µM Kisspeptin-54 (SEQ ID No. 1), 10 µM Kisspeptin-54 residues 27-54 (SEQ ID No. 19), 10 µM Kisspeptin-13 (SEQ ID No. 21), 10 µM Kisspeptin-10 (SEQ ID No. 22), 10 µM Peptide 234 (SEQ ID No. 14), 10 µM Kissorphin-6 (SEQ ID No. 2) or 10 µM Kissorphin-6 residues 3-6 (SEQ ID No. 11) peptides (Figure 6A). The

Kissorphin-6 residues 1-3 (SEQ ID No. 23) peptide (10 μM) had no affect on the toxicity of Aß 1-42 (SEQ ID No. 3) or Aß 25-35 (SEQ ID No. 26) peptides.

The toxicity of 5 μM IAPP 1-37 (SEQ ID No. 7) and 5 μM IAPP 20-29 (SEQ ID No. 29) peptides (Smith et al., J. Am. Chem. Soc. 131: 4470-4478 (2009); Tenidis et al., J. Mol. Biol. 295: 1055-1071 (2000)) was significantly inhibited in SH-SY5Y cell cultures by addition of 10 μM Kisspeptin-54 (SEQ ID No. 1), 10 μM Kisspeptin-54 residues 27-54 (SEQ ID No. 19), 10 μM Kisspeptin-13 (SEQ ID No. 21), 10 μM Kisspeptin-10 (SEQ ID No. 22), 10 μM Peptide 234 (SEQ ID No. 14) or 10 μM Kissorphin-6 (SEQ ID No. 2) peptides (Figure 6B). The Kissorphin-6 residues 3-6 (SEQ ID No. 11) and Kissorphin-6 residues 1-3 (SEQ ID No. 23) peptides (10 μM) had no affect on the toxicity of IAPP 1-37 (SEQ ID No. 7) or IAPP 20-29 (SEQ ID No. 29) peptides.

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The toxicity of the 5 μM PrP 106-126 (SEQ ID No. 30) and 5 μM PrP 118-135 (SEQ ID No. 31) peptides (Chabry et al., J. Neurosci. 23: 462-469 (2003); Henriques et al., Biochemistry 48: 4198-4208 (2009)) was significantly inhibited in SH-SY5Y cell cultures by addition of 10 μM Kisspeptin-54 (SEQ ID No. 1), 10 μM Kisspeptin-54 residues 27-54 (SEQ ID No. 19), 10 μM Kisspeptin-13 (SEQ ID No. 21), 10 μM Kisspeptin-10 (SEQ ID No. 22), 10 μM Peptide 234 (SEQ ID No. 14) or 10 μM Kissorphin-6 (SEQ ID No. 2) peptides (Figure 6C). The Kissorphin-6 residues 3-6 (SEQ ID No. 11) and Kissorphin-6 residues 1-3 (SEQ ID No. 23) peptides (10 μM) had no affect on the toxicity of PrP 106-126 (SEQ ID No. 30) or PrP 118-135 (SEQ ID No. 31) peptides.

The toxicity of 5 μM Amyloid-Bri (SEQ ID No. 9) peptide (Gibson et al., Biochem Soc Trans. 33: 1111-1112 (2005)) in SH-SY5Y cell cultures was unaffected by the addition of 10 μM Kisspeptin-54 (SEQ ID No. 1), 10 μM Kisspeptin-54 residues 27-54 (SEQ ID No. 19), 10 μM Kisspeptin-13 (SEQ ID No. 21), 10 μM Kisspeptin-10 (SEQ ID No. 22), 10 μM Peptide 234 (SEQ ID No. 14), 10 μM Kissorphin-6 (SEQ ID No. 2), 10 μM Kissorphin-6 residues 3-6 (SEQ ID No. 11) and 10 μM Kissorphin-6 residues 1-3 (SEQ ID No. 23) peptides (Figure 6D).

The toxicity of 5 μM Amyloid-Dan (SEQ ID No. 10) peptide (Gibson et al., Biochem Soc Trans. 33: 1111-1112 (2005)) in SH-SY5Y cell cultures was unaffected by the

addition of 10 µM Kisspeptin-54 (SEQ ID No. 1), 10 µM Kisspeptin-54 residues 27-54 (SEQ ID No. 19), 10 µM Kisspeptin-13 (SEQ ID No. 21), 10 µM Kisspeptin-10 (SEQ ID No. 22), 10 µM Peptide 234 (SEQ ID No. 14), 10 µM Kissorphin-6 (SEQ ID No. 2), 10 µM Kissorphin-6 residues 3-6 (SEQ ID No. 11) and 10 µM Kissorphin-6 residues 1-3 (SEQ ID No. 23) peptides (Figure 6E).

The Kisspeptin receptor (GPR-54) antagonist Peptide 234 (SEQ ID No. 14; 10 µM), which was protective in SH-SY5Y cell cultures against Aß 1-42 (SEQ ID No. 3), IAPP 1-37 (SEQ ID No. 7) or PrP 106-126 (SEQ ID No. 30) peptide (5 µM) toxicity, did not significantly increase protection by 10 µM Kisspeptin-13 (SEQ ID No. 21) or 10 µM Kisspeptin-10 (SEQ ID No. 22; Figure 6F). These results suggest that Kissorphin neuroprotection against Aß, IAPP and PrP peptides is not mediated by a receptor-mediated action on the GPR-54 receptor.

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The toxicity of 5 µM Aß 1-42 (SEQ ID No. 3), Aß 25-35 (SEQ ID No. 26), IAPP 1-37 (SEQ ID No. 7), IAPP 20-29 (SEQ ID No. 29), PrP 106-126 (SEQ ID No. 30) or PrP 118-135 (SEQ ID No. 31) peptides (Milton, Neurotoxicology 22: 767-774 (2001); Milton, Neurosci. Lett. 332: 127-130 (2002); Smith et al., J. Am. Chem. Soc. 131: 4470-4478 (2009); Tenidis et al., J. Mol. Biol. 295: 1055-1071 (2000); Chabry et al., J. Neurosci. 23: 462-469 (2003); Henriques et al., Biochemistry 48: 4198-4208 (2009)) was significantly inhibited by addition of 1 µg/m1 KLH-CGG-KOR-6 conjugate (Figure 7A) or 1 µg/m1 BSA-CGG-KOR-6 conjugate (Figure 7B). The toxicity of the 5 µM Amyloid-Bri (SEQ ID No. 9) or Amyloid-Dan (SEQ ID No. 10) peptides (Gibson et al., Biochem Soc Trans. 33: 1111-1112 (2005)) was unaffected by addition of KLH-CGG-KOR-6 or BSA-CGG-KOR-6 conjugate (1 µg/ml). BSA and KLH (1 µg/ml) had no effects on amyloid peptide toxicity.

In rat cortical neuron primary cell cultures Kisspeptin-13 (SEQ ID No. 21) and Kissorphin-6 (SEQ ID No. 2) showed dose-dependent inhibition of 5 µM Aß 1-42 toxicity, which was significant at concentrations above 2.5 µM (Figure 8A). Kisspeptin-13 (SEQ ID No. 21) and Kissorphin-6 (SEQ ID No. 2) showed dose-dependent inhibition of 5 µM IAPP 1-37 (SEQ ID No. 7) toxicity, which was significant at concentrations above 5 µM (Figure 8B). Kisspeptin-13 (SEQ ID No. 21) and Kissorphin-6 (SEQ ID No. 2) showed dose-dependent inhibition of 5 µM PrP 106-126 (SEQ ID No. 30) toxicity, which was significant at concentrations

above 0.63 pM (Figure8C). At a 10 µM concentration the Kisspeptin receptor antagonist Peptide 234 (SEQ ID No. 14) inhibited the toxicity of 5 µM Aß 1-42 (SEQ ID No. 3) and 5 µM PrP 106-126 (SEQ ID No. 30; Figure 8D). The Peptide 234 (SEQ ID No. 14) antagonist had a significant additive effect on 2.5 µM Kissorphin-6 (SEQ ID No. 2) inhibition of 5 µM Aß 1-42 (SEQ ID No. 3) and 5 µM PrP 106-126 (SEQ ID No. 30) toxicity. The two Neuropeptide FF (NPFF) receptors GPR147 and GPR74 both bind Kisspeptins (Kirby et al., Pharmacol. Rev. 62; 565-78 (2010); Lyubimov et al., Neuroscience 170; 117-22 (2010); Oishi et al., ACS Med. Chem. Lett. 2; 53-57 (2011)) and these actions can be blocked by NPFF receptor antagonists. To determine whether the neuroprotective actions of the Kissorphin-6 peptide (SEQ ID No. 2) were mediated by an action on NPFF receptors we tested the effects of an NPFF receptor antagonist RF9 (SEQ ID No. 16) with actions on both receptor forms (Simonin et al., Proc Natl Acad Sci USA 103: 466-471 (2006)). The NPFF receptor antagonist RF9 (SEQ ID No. 16; 10 pM), which has also been reported to interact with the GPR-54 Kisspeptin receptor (Oishi et al., ACS Med. Chem. Lett. 2; 53-57 (2011)), had no significant effect on its own or in combination with 2.5 µM Kissorphin-6 (SEQ ID No. 2).

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These results confirm that the anti-amyloid actions of Kisspeptin and Kissorphin peptides can be seen in both primary cell cultures of neurons plus neuronal cell lines. They also demonstrate that the actions are restricted to amyloid forms to which Kisspeptin and Kissorphin bind (Aß, IAPP and PrP in this example) and are not mediated by actions of the peptides on the GPR-54 Kisspeptin receptor or the NPFF receptors both of which bind Kisspeptins (Kirby et al., Pharmacol. Rev. 62; 565-78 (2010); Lyubimov et al., Neuroscience 170; 117-22 (2010); Oishi et al., ACS Med. Chem. Lett. 2; 53-57 (2011)).

Effects of Amyloid-fibril forming peptides on Kissorphin release from neuronal cell cultures

Human neuroblastoma SH-SY5Y cells were cultured in 25 ml flasks and differentiated with retinoic acid for 7 days. For kissorphin release experiments 2.5 x 10^5 cells were cultured in 5 ml of medium containing a sub-toxic dose (50 nM) of either Aß 1-42, Aß 25-35, IAPP 1-37, IAPP 20-29, PrP 106-126 or PrP 118-135 for 4h before removing medium for kissorphin determination. Control cells were cultured in medium alone. Using a polyclonal anti-kissorphin-6 antiserum plus

Protein-A agarose the Kissorphin-like peptides were immunoprecipitated. The resultant acidified extracts were further purified using a Sep-Pak C_{18} extraction step. Columns were pre-wetted with Methanol and 0.5M Acetic acid, acidified samples applied and columns washed with 0.5M Acetic acid prior to elution of bound peptide with 70% acetonitrile. After drying under a stream of nitrogen samples were resuspended in PBS buffer.

ELISA plates were coated with anti-kissorphin-6 antiserum and blocked with 5% marvel. Samples or synthetic kissorphin-6 standards were applied in PBS containing 0.1% BSA plus 0.05% Tween 20 and incubated for 24h. After washing to remove unbound material biotinyl-kissorphin-6 [SEQ ID No. 38] was added and incubated for 2h. After washing to remove unbound material immunoreactive (ir) kissorphin-like material was detected using a streptavadin-horse radish peroxidase conjugate and 3,3',5,5'-tetramethylbenzidine substrate. Sample levels were compared to kissorphin-6 standards and ir-kissorphin-like peptide levels expressed as a % of control cell release.

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The amyloid-fibril forming peptides Aß 1-42, Aß 25-35, IAPP 1-37, IAPP 20-29, PrP 106-126 and PrP 118-135 all stimulated a 2-3 fold increase in ir-Kissorphin-like peptide release from SH-SY5Y neurons in culture (Figure 9).

Claims

1. A peptide with the amino acid sequence Tyr-Asn-Trp-Asn-Ser-Phe or a peptide derived from kissorphin comprising said sequence, for use in the treatment of a condition selected from Alzheimer's disease, Creutzfeldt-Jakob disease and type 2 diabetes mellitus associated with IAPP, wherein the peptide does not comprise an amidated C-terminal Arg-Phe group of SEQ ID No. 1.

2. A peptide according to claim 1, wherein the peptide does not comprise the C-terminal amino acid sequence Gly-Leu-Arg-Phe of SEQ ID No.1.

3. A peptide according to any preceding claim, wherein the peptide comprises any of the amino acid sequences defined herein as SEQ ID Nos. 4 to 6.

4. A peptide according to any preceding claim, wherein the peptide further comprises a signal sequence.

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5. A composition comprising a peptide as defined in any of claims 1 to 4, in a pharmaceutically-acceptable diluent, for use according to claim 1.

6. An *in vitro* method for determining whether a subject has, or is predisposed to having, a disorder selected from Alzheimer's disease, Creutzfeldt-Jakob disease and type 2 diabetes mellitus associated with IAPP, comprising treating a sample from the subject with a peptide as defined in any of claims 1 to 4, to determine whether the sample comprises Amyloid fibril-forming peptides.

7. A method according to claim 6, wherein the peptide is detectably-labelled.

8. A method according to claim 7, comprising the steps of:

(i) treating a sample obtained from a patient with the peptide;

(ii) detecting binding events between the peptide and amyloid peptides present in the sample; and

(iii) quantifying the binding events with reference to a control sample, wherein an increase in binding events compared to the control is indicative of a disorder

selected from Alzheimer's disease, Creutzfeldt-Jakob disease and type 2 diabetes mellitus associated with IAPP.

9. A method according to any of claims 6 to 8, wherein the patient sample is obtained from a patient receiving treatment for a disorder selected from Alzheimer's disease, Creutzfeldt-Jakob disease and type 2 diabetes mellitus associated with IAPP and wherein the method is used for therapeutic monitoring of the treatment of the disorder.

10. An *in vitro* method for determining whether a subject has, or is predisposed to having, Alzheimer's disease, comprising treating a sample from the subject with a peptide comprising the amino acid sequence Trp-Asn-Ser-Phe and comprising a detectable label, to determine whether the sample comprises Aβ peptides.

11. A method according to claim 10, comprising the steps of:

(i) treating the sample obtained from the patient with the peptide;

(ii) detecting binding events between the peptide and Aβ peptides present in the sample; and

(iii) quantifying the binding events with reference to a control sample, wherein an increase in binding events compared to the control is indicative of Alzheimer's disease.

12. A method according to any of claims 10 to 11, wherein the patient sample is obtained from a patient receiving treatment for Alzheimer's disease and wherein the method is used for therapeutic monitoring of the treatment of the disorder.

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