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(54) Title: APPLICATION OF PEPTIDE CONJUGATES TO ALZHEIMER'S DISEASE

(57) Abstract: A chemical compound and a method for its use in the diagnosis and treatment of Alzheimer's disease in which the at least the sequence of the D-isomers of the amino acids (phenylalanine-phenylalanine-valine-leucine-lysine) is capable of crossing the blood brain barrier, recognizing the formation of plaques characteristic of the pathogenesis of Alzheimer's disease, and interfering with the formation of fibril from beta amyloid peptide effecting an inhibition of the disease process.



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APPLICATION OF PEPTIDE CONJUGATES TO ALZHEIMER'S DISEASE

FIELD OF THE INVENTION

[001] The invention relates to chemical compounds for the diagnosis and treatment of Alzheimer's disease in humans.

BACKGROUND OF THE INVENTION

[002] In 1906, a Bavarian psychiatrist, Alois Alzheimer, first reported the clinical syndrome now known as Alzheimer's Disease (AD) (common senile dementia). As life expectancy increased dramatically in the 20th century, it has become a common disease among aged people with a prevalence rate of 13% after age 65 and increasing to 47% after 85 years old (Wood, 2001).

[003] In the AD literature, AD was divided into two types, Familial Alzheimer's Disease (FAD) and non-familial Alzheimer's Disease. The latter one is also called sporadic AD. FAD refers to the early onset of Alzheimer's Disease while the sporadic AD usually refers to the AD that happens in the later age. The degree that genetic factors contribute to both types of disease is not clear yet. But research has showed that there is no phenotypic difference between these two types of disease (Selkoe 2001).

[004] The diagnosis of AD, is still based on the cognitive behavior of the patient. Definitive AD is only confirmed by the examination of brain tissue obtained at autopsy. AD is a progressive neurodegenerative disease characterized by memory loss, language deterioration, impaired visuospatial skill, poor judgement and indifferent attitude, but preserved motor function.

[005] Genetic research has revealed that there are three genes associated with AD, namely Amyloid Precursor Protein (APP) gene on chromosome 21 (Goate et al., 1995), Presenilin-1 (PS-1) gene on chromosome 14 (Sherrington et al., 1995; Van Broeckhoven et al., 1995) and Presenilin-2 (PS-2) gene on chromosome 1 (Rogaev et al., 1995; Levy-Lahad et al., 1995). Early onset Familial Alzheimer's Disease (FAD) in various kindreds is associated with point mutations in these genes.

[006] The gross pathology of the brain in AD is characterized by diffuse atrophy, especially of the cortex and hippocampus. The presence of Senile Plaques is the hallmark of AD. The SP of AD is a complex extracellular lesion composed of a central deposition of β -Amyloid peptide. More and more genetic, neuropathological and biochemical evidence have shown that these deposits of β -amyloid peptide play an important role in the pathogenesis of AD (Glennner et al., 1984; Master et al., 1985; Selkoe, 1994). β -amyloid peptide is a 39-43 amino acid peptide derived from the Amyloid Precursor Protein (APP) by proteolytical processing.

[007] APP is a functional neuronal receptor which couples to intracellular signaling pathway through the GTP-binding protein G(O). It has 5 isoforms: APP395, APP563, APP695, APP751 and APP770, produced by alternative splicing. APP695 is the predominant form in neuronal tissue, APP(751) is the predominant form elsewhere. The full sequence of APP770 is shown in Figure 2. The cleavage by alpha-secretase or beta-secretase leads to generation and extracellular release of soluble APP fragments, APP¹⁸⁻⁶⁸⁷ (sAPP α) and APP¹⁸⁻⁶⁷¹ (sAPP β), and retention of corresponding membrane-anchored carboxy-terminal fragments which are later released by gamma-secretase. Alpha-secretase gives rise to C83 (APP⁶⁸⁸⁻⁷⁷⁰) which

is subsequently processed to yield p3 peptides (APP⁶⁸⁸⁻⁷¹¹ or APP⁶⁸⁸⁻⁷¹³). This pathway is thought to be nonamyloidogenic. Beta-secretase gives rise to C99 (APP⁶⁷²⁻⁷⁷⁰) which is processed to yield beta-amyloid peptide A β 40 and A β 42. Deposition of fibrillar amyloid proteins as intraneuronal neurofibrillary tangles, extracellular amyloid plaques and vascular amyloid deposits, is characteristic of both Alzheimer's Disease and aged Down's syndrome.

[008] It is noteworthy to state here that while both A β 40 and A β 42 are major components in the deposit of amyloid fibrils, A β 42 is more amyloidogenic and believed to play a more important role in the early stage of fibril formation and have the seeding effect. The fibril formation process is proposed to have two phases, first nucleation and then extension phase. The nucleation phase requires a series of association steps from monomers that are thermodynamically unfavorable and therefore rate-limiting steps. Once the nucleus have been formed, further addition of monomers becomes thermodynamically favorable, resulting in rapid extension of amyloid fibrils (Harper & Lansbury, 1997).

[009] PS-1 and PS-2 are also related to AD. Both proteins have their normal physiological functions. They may play a role in intracellular signaling and gene expression or in linking chromatin to the nuclear membrane. It may also function in the cytoplasmic partitioning of proteins. Defects in PS1 and PS2 result in an overproduction of A β 42. Point mutations of these proteins have shown an increase in the production of β -amyloid peptides or an increase in the ratio of A β 42/A β 40 (Cruts et al., 1998; Kovacs et al., 1996; Annaert et al., 2000). Evidence showed that presenilins played required roles in the γ -secretase activity (De Strooper, 1998).

[010] The exact mechanism on how the deposition of A β causes the AD is not clear yet. But it is now widely accepted that the deposition is a key step in AD. Some researchers believe that it causes radical production. Yan et al (Yan et al., 1997) found that it binds an intracellular polypeptide known as ERAB, thought to be a hydroxysteroid dehydrogenase enzyme, which is expressed in normal tissues, but is overexpressed in neurons affected in AD. ERAB immunoprecipitates with amyloid-beta, and when cell cultures are exposed to amyloid-beta, ERAB inside the cell is rapidly redistributed to the plasma membrane. The toxic effect of amyloid-beta on these cells is prevented by blocking ERAB and is enhanced by overexpression of ERAB. By interacting with intracellular amyloid-beta, ERAB may therefore contribute to the neuronal dysfunction associated with AD.

[011] To briefly summarize this section, AD is directly related to β -amyloid peptide (A β), which is a proteolytical fragment from APP by β , γ -secretase cleavage. A β 42 is more amyloidogenic and played more important roles in AD. APP, PS-1 and PS-2 are all related to AD indirectly. Point mutation of them can either increase the A β production or the ratio of A β 42/A β 40.

[012] Many drugs for AD are currently under clinical trial. They include Donepezil Hydrochloride, ENA713, Galatantamine and Lazabemide. Most of these drugs are cholinesterase inhibitors. They inhibit the action of a specific enzyme, acetylcholinesterase, which breaks down acetylcholine. Acetylcholine is a neurotransmitter involved in nerve cell communication. These drugs do not provide a cure for AD; neither do they slow the progression of AD. These drugs are only designed to alleviate the cognitive symptoms, and seldom can one drug treat all of the symptoms effectively. These drugs are aimed at making the patient

more comfortable. The challenge remains of developing a new drug that is designed to cure, or at least slow the progress of the disease.

[013] Blocking A β aggregation and preventing amyloid fibril formation of A β forms the basis for an approach to Alzheimer's drug design based on the hypothesis that A β , in its nonaggregated form, is not toxic to the cells. The fact that A β is also produced in normal people indicates that there may be certain functions associated with it (Haass et al., 1992; Van Gool et al., 1992; Seubert et al., 1992). Therefore, preventing A β from forming amyloid fibrils and then plaques is also a very attractive approach.

[014] The initial work on this topic was done by Hilbich et al (Hilbich et al., 1992). In that paper, they tried to find the critical region of A β involved in amyloid fibril formation by substituting the hydrophobic amino acids in A β by more hydrophilic amino acids. Their results showed that the well-preserved hydrophobic core around residue 17-20 of A β , LVFF, is crucial for the formation of β -sheet structure and the amyloid properties of A β . The A β^{1-40} analogues, where the amino acids in 17-20 are substituted by more hydrophilic amino acids, are able to bind to full sequence of A β^{1-40} and inhibit the fibril formation in vitro. Therefore, these analogues were suggested as therapeutic reagents for AD.

[015] Tjernberg et al (Tjernberg et al., 1996) first synthesized the 31 possible 10-mers corresponding to amino acids 1-10 up to 31-40 of the A β^{1-40} molecule on a cellulose membrane matrix. The A β fragments capable of binding full length A β were identified by radioligand binding. A region located in the central part of A β (A β^{9-18} to A β^{13-22}) displayed prominent binding to A β . Being located in the center of the binding region, A β^{11-20} was selected for further studies of the structural requirements for binding. This peptide, as well as N- and C-terminally truncated fragments, were synthesized and tested. It was found that the shortest peptide still displaying consistent high A β binding capacity had the sequence KLVFF (corresponding to A β^{16-20}). This result agreed with Hilbich et al. This peptide was studied by microscopy and was found to be able to inhibit fibril formation in vitro.

[016] Having shown that the short peptide KLVFF can bind to A β and disrupt the fibril formation, Tjernberg et al (Tjernberg et al., 1997) showed that peptide KLVFF binds stereospecifically to the homologous sequence in A β (i.e. A β^{16-20}). Also in this paper they presented molecular modeling and suggested that association of the two homologous sequences leads to the formation of an atypical anti-parallel β -sheet structure stabilized primarily by interaction between the Lys, Leu and Phe residues.

[017] Based on the results given by these two papers, Ghanta et al (Ghanta et al., 1996) presented an approach to the design of inhibitors of β -Amyloid toxicity. In that strategy, a recognition element, which interacts specifically with β -Amyloid, is combined with a disrupting element, which alters β -Amyloid aggregation pathways. They synthesized a peptide composed of residues 15-25 of β -Amyloid peptide, designed as the recognition element, linked to an oligolysine disrupting element. This inhibitor does not alter the apparent secondary structure of β -Amyloid nor prevent its aggregation; rather, it causes changes in aggregation kinetics and higher order structural characteristics of the aggregate. In addition to its influence on the physical properties of β -Amyloid aggregates, the inhibitor completely blocks β -Amyloid toxicity to

PC-12 cells. All these results suggest that complete disruption of amyloid fibril formation is not necessary for abrogation of toxicity.

[018] Many peptide fragments that are homologous to the β -Amyloid peptide were synthesized and tested. They can only block the β -Amyloid aggregation, but they can't disaggregate or break the preformed β -Amyloid fibril. Claudio Soto and colleagues (Soto et al., 1998) designed small peptides to interfere with the development of beta sheet structures based on the observation that the β -Amyloid aggregates have beta sheet structure. The so-called 'beta sheet breaker', which is five-residue peptide with partial homology to the β -Amyloid peptide, was shown to be capable of preventing β -Amyloid fibril formation and disassembling preformed fibrils in vitro when 20 folds of inhibitor peptide was used. However, specific binding to plaque was not shown.

SUMMARY OF THE INVENTION

[019] The present invention comprises a peptide conjugate that can bind the amyloid fibril tightly and specifically and therefore prevent the beta amyloid peptide from forming fibril.

[020] Accordingly, it is a first object of the invention to provide a chemical compound capable of diagnosis and treating Alzheimer's disease in humans comprised of an amino acid conjugate.

[021] It is another object of the invention to provide chemical compound that includes an amino acid conjugate that is capable of plaque recognition and of inhibiting the formation of amyloid fibrils.

[022] It is another object of the invention to provide a chemical composition including several amino acid conjugate capable of passing through the Blood Brain barrier and an optional reporting element that allows for detection and imaging of the binding of the conjugate.

[023] It is still another object of the invention to provide a method for the use of a chemical compound according to the invention for the diagnosis and/or treatment of Alzheimer's disease in a human such as by formulating the chemical compound according to accepted pharmaceutical practice, administering the compound to a human.

BRIEF DESCRIPTION OF THE FIGURES

[024] Figure 1 is a schematic drawing that illustrates the role of APP in Alzheimer's Disease.

[025] Figure 2 is an illustration of the mechanism for blocking fibril formation.

[026] Figure 3 is a full sequence listing of APP770, the β -amyloid peptide.

[027] Figure 4 is a bar graph showing the aggregation of A β [1-40] with and without inhibitors.

[028] Figure 5a is a plot of the kinetics of β -amyloid fibril formation as indicated by reduction of fluorescence in fluorescamine assay..

[029] Figure 5b is a plot of the kinetics of β -amyloid fibril formation as quantified by centrifugation followed by fluorescamine assay.

[030] Figure 5c is a plot of the kinetics of β -amyloid fibril formation quantified by ThT assay.

[031] Figure 6 is a summary of a ThT assay ($R50/I_{max}$).

DETAILED DESCRIPTION OF THE INVENTION

[032] The invention is a peptide conjugate that can efficiently prevent amyloid fibril formation. Figure 1 illustrates the accepted mechanism of pathogenesis of Alzheimer's disease beginning with the conversion of amyloid precursor protein (APP) to β -amyloid peptide and the subsequent formation of fibrils and ultimately senile plaques.

[033] Design of peptides and conjugates

[034] Structural information of A β in fibrils

[035] The design of the peptides should be based on the structural information of the β amyloid peptide in fibril. However, because the amyloid peptide is non-crystalline and easy to aggregate, there is no direct structural information on this peptide in physiological conditions. Talafous et al (Talafous et al., 1994) determined the NMR structure of β -amyloid peptide fragment 1-28. Because of the existence of the long hydrophobic region from residue 29 to the end of C-terminus, the structure of the fibril forming fragment 1-42 might be quite different from the structure of this fragment. Shao et al (Shao et al., 1999) determined the structure of β -amyloid peptide by NMR in sodium dodecyl sulfate (SDS) solution. Since SDS is a strong denaturing reagent, this structural information may not be applicable to the native peptide.

[036] Another way to study the structure of this peptide is by molecular modeling. From the primary structure of β amyloid peptide, there are two hydrophobic regions: Leu17-Ala21 and residue 28 to the end of the C-terminus. The first hydrophobic core has an alpha-helix structure stabilized by intramolecular hydrogen bonding. The second hydrophobic core has a beta-sheet structure, and is able to form long-range, non-covalent, mainly hydrophobic interactions with other beta-sheets of the β -amyloid peptide. The beta-strands run in an anti-parallel direction (Mager et al., 1998). The molecular modeling done by Tjernberg et al (Tjernberg et al., 1997) showed how the peptide KLVFF interacts with β -amyloid peptide. Their molecular modeling suggests that association of the two homologous sequences lead to the formation a typical anti-parallel β -sheet structure stabilized primarily by the interaction between the Lys, Leu and the second (C-terminal) Phe.

[037] Even though we don't have a clear picture on how the A β is assembled and arranged in the amyloid fibrils, some pieces of information are mostly accepted. The A β is arranged in anti-parallel β -sheet structure inside amyloid fibrils with the β -strand running perpendicular to the fibril axis. The C-terminus of A β (28-42) always exists as β -strand while the N-terminus can exist as α -helix or random coil in solution. During the fibril formation process, the N-terminus has a conformation change from α -helix or random coil to β -strand. Amyloid fibrils are composed of five or six protofilaments arranged around a hollow center, each protofilament having a diameter of around 25-30 Å (Serpell 2000b). The full sequence listing of β -amyloid peptide is shown in Figure 3.

[038] Designing peptides

[039] Even though it is impossible to have rational drug design due to the lack of structural information of A β in fibril on atomic level, drug design based on the principle of blocking the amyloid fibril formation process is still possible. As shown in Figure 2, considering amyloid fibril formation as a protein misfolding problem, we can design a peptide inhibitor that can either stabilize the A β monomer, dimer or small oligomer, or destabilize the interaction that ultimately results in the fibril formation.

[040] Some published results showed that the peptide sequence KLVFF, which is a homologous fragment in the middle of A β [1-40], can bind to the full sequence of A β [1-40] and prevent the amyloid fibril formation (Tjernberg et al.,1996). We will start with this peptide, try to optimize it by improving its binding affinity and its capacity to prevent the amyloid fibril formation.

[041] To study the structure-function of A β ¹⁶⁻²⁰, we first will compare the peptide KLVFF with amide C-terminal and free acid terminal. We will also investigate the effect of the positive charge on the N-terminus.

[042] In vivo stability of peptide drugs has been big concern because they are subject to protease digestion and there are a lot of proteases in the body. In order to improve the in vivo stability of the peptide, we will try to make them protease resistant by converting them into peptides made of non-natural amino acids, that is D-amino acids. Retro-inverso peptides have the close configuration of its parent peptide (Goodman & Chorev 1979; Pallai et al., 1983). So the retro-inverso peptides fflvk and fflvkk will be synthesized and evaluated for their inhibitory effect in preventing fibril formation. In peptide fflvkk, we put an extra d-Lys at the C-terminal amino acid investigating the effect of positive charge.

[043] To investigate the possible arrangement of the A β 16-20 in the fibrils and to improve the binding to fibrils, dimer peptides will also be synthesized. Two dimer peptides, so called tandem dimer and cyclic dimer, will be synthesized.

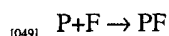
[044] Designing peptide conjugates

[045] In order to improve the binding of our peptide inhibitor to the amyloid fibrils and therefore prevent the fibril formation more efficiently, we can put multiple copies of peptide on a single conjugate molecule. In order to study the effect of length of the spacer between peptides, we will use three different peptide carriers for attaching the peptide. The carriers differ from each other in the length of the functional group for attaching the peptide. We will synthesize two PEG based carriers, that is, PEG₃₄₀₀-aspartic acid copolymer and PEG₂₈₂-aspartic acid copolymer. The primary amine on the aspartic acid will be used for attaching the peptides. The third peptide carrier is a peptide chain, OGOGOGOGOGO, where O is ornithine and G is glycine. We use glycine because it has the least steric hindrance on its side chain and might be more flexible. The side chain of the ornithine will be used for attaching the peptide units. Comparing to the PEG-aspartic acid copolymer, this peptide carrier has the shortest spacer between the primary amine functional group, which is used to attach the peptide. We will put the peptide on these peptide carriers and evaluate them. We will compare these conjugates with the single peptide and also compare these conjugates with each other to study the effect of spacer length.

[046] In the description and Examples that follow conjugates according to the invention will be synthesized and evaluated.

[047] Determination of Dissociation Constant for the Interaction of Peptide and β -Amyloid Fibril

[048] The Scatchard Plot was used to determine the dissociation constant (K_d) when the concentration of one of the two interacting substances can not be determined. For the interaction of peptide (P) and β -Amyloid fibril (F):



[050] The K_d is given by

[051] $K_d = [P] * [F] / [PF] \quad (1)$

[052] It is hard to measure the concentration of a solid fibril, [F], but the concentration of the unbound peptide is easy to determine. Assuming that the initial concentration of the fibril is $[F]_0$

[053] $[F]_0 = [F] + [PF] \quad (2)$

[054] $[F] = [F]_0 - [PF] \quad (3)$

[055] Plug equation (3) into (1):

[056] $K_d * [PF] = [P] * ([F]_0 - [PF]) \quad (4)$

[057] Rearrange equation (4), we get:

[058] $[PF] / [P] = -[PF] / K_d + [F]_0 / K_d \quad (5)$

[059] So if we plot $[PF] / [P]$ against $[PF]$, both of which can be measured, we will get a straight line. The absolute value of the slope of the line is equal to the K_d .

[060] Even though the Scatchard plot is a good way to determine the binding constant, it is not the simplest way to do. An alternative way to confirm the binding constant measured by the Scatchard plot. is needed For doing the Scatchard plot, the compound to be tested must be labeled either by fluorescence or radioactive isotopes. But for some compounds, there is no easy way to do this. Competition inhibition assay is easy way to compare the binding constant of two compounds.

[061] In a typical binding competition assay, we will use fixed amount of hot peptide and various amount of test reagent to competitively bind to fixed concentration of fibrils. When the amount of test reagent is very low, increasing the amount of test reagent has little effect on the amount of hot peptide bound on the fibrils. When the amount of test reagent is very big, increasing the amount of test reagent won't have much effect on the amount of hot peptide bound on the fibrils. So the plot of the amount of bound hot peptide against the concentration of test reagent will have two plateaus with a sharp transition. The concentration of the test reagent corresponding to the half-way of the transition is the dissociation constant of the binding of the test reagent to the fibrils.

[062] Thioflavin T (ThT) a kind of fluorescence reagent that can bind rapidly amyloid fibrils. Upon binding to amyloid fibrils, ThT changes to a distinct excitation maximum at 450 nm and enhanced emission at 482 nm, as opposed to its original excitation at 385 nm and emission at 445 nm of the free dye. This change depends on the aggregate states. The monomeric and dimeric amyloid peptide won't interact

with ThT and can't give the fluorescence signal. The guanidine dissociation of the aggregated amyloid fibrils can destroy the signal. Therefore, the fluorescence signal depends only on the amount of amyloid fibrils, not on the amount of the free peptide. So we can use the fluorescence signal of the ThT at the new excitation and emission wavelength to quantitate the amount of fibrils formed in a solution. By measuring the amount of fibril, we can evaluate the efficacy of a testing compound in preventing the amyloid fibril formation.

EXAMPLE

[063] Materials and Instrumentation

[064] Beta amyloid peptide [1-40] was obtained from Quality Controlled Biochemicals (Hopkinton, MA). PAL™ resin, peptide synthesis grade N, N-dimethylformamide (DMF) and 20% Piperidine in DMF were obtained from PerSeptive Biosystems (Framingham, MA). Benzotriazol-1-oxytris (dimethyl amino)-phosphonium hexafluorophosphate (BOP) and 1-hydroxybenzotriazol hydrate (Hobt) were obtained from Advanced ChemTech (Louisville, KY). Polypropylene column for the peptide synthesis was obtained from BioRad Laboratories (Melville, NY). Anisole, thioanisole, ethanedithiol (EDT), thioflavin T (ThT), triethylamine (TEA), diisopropylethyl amine (DIEA), 1,3-Diisopropylcarbodiimide (DIPC), 4-(Dimethyl amino)-pyridine (DMAP), p-toluenesulfonic acid monohydrate (PTSA) were obtained from Aldrich Chemicals (Milwaukee, WI). Most Fmoc amino acids were obtained from BACHEM (King of Prussia, PA) except specially indicated. Branched PEG with amino functional group (2K Da, 3 arms; 10 KDa, 3 arms; 10 KDa, 8arms; 20 KDa, 8 arms), α , ω -diamino-Poly(ethylene glycol) (NH₂-PEG-NH₂, MW 3,400 Da and MW 282 Da) and Fluorescein-PEG-NHS (MW 5000 Da) were obtained from Shearwater Polymers, Inc. (Huntsville, AL). Sulfosuccinimidyl-6-(biotinamido) hexanoate (EZ-Link™ Sulfo-NHS-LC-Biotin), N-succinimidyl-3-(2-Pyridyldithio) Propionate (SPDP) and dimethyl sulfoxide (DMSO) were obtained from Pierce (Rockford, IL). Ethyl ether, dichloromethane (DCM), methanol, ethanol, isopropanol, 1-butanol, acetonitrile, potassium phosphate monobasic, and potassium chloride were from Fisher Scientific (Springfield, NJ). Glacial acetic acid, concentrated hydrochloric acid and phosphoric acid were from J. T. Baker (Phillipsburg, NJ). Sodium azide, sodium borate, sodium chloride, potassium chloride, sodium carbonate, sodium bicarbonate, sodium phosphate monobasic, potassium phosphate monobasic and sodium phosphate dibasic were from EM Science (Gibbstown, NJ). Radio-isotope acetic anhydride was obtained from New England Nuclear (NEN) life sciences (Boston, MA). Fluorescamine was from Roche Diagnostic (Nutley, NJ). Bovine serum albumin (BSA), acetic anhydride, ninhydrin, and trifluoroacetic acid (TFA) were obtained from Sigma Chemicals (St. Louis, MO). MicroCon™ microconcentrators (3 KDa, 10 KDa, 50 KDa, MW cutoff) were from Amicon, Inc., (Beverly, MA).

[065] Amino acid analysis was performed on a PE Biosystems 420 Amino Acid Analyzer. Mass spectrometry was performed on matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometry equipped with a PE Biosystems DE-PRO Mass Spectrometer. Fluorescence intensity was measured on a CytoFluor™ II fluorescence multi-well plate reader (PerSeptive Biosystems, Framingham,

MA) using FALCON microtiter plates. Size exclusion chromatography was performed on a Bio-sil TSK-250 SEC column (Bio-Rad #125-0062) with a Bio-sil 250 Guard Column (Bio-Rad# 125-0073).

[066] Experimental procedures

[067] Fluorescamine Assay (Udenfriend, et al., 1972)

[068] Fluorescamine (15mg) was added to 30 ml acetonitrile to give a clear light yellow solution. To each well of the 96-well plates, standard solutions containing primary amine of different concentration or samples were added. Then 0.2 M borate buffer was added to make the final volume 150 μ L. Then quickly 50 μ L of fluorescamine solution was added and mixed immediately by pipetting up and down several times. The fluorescence intensity was measured at excitation wavelength 390 nm and emission wavelength 475 nm on CytoFluor™ II fluorescence multi-well plate reader.

[069] Kinetics of fibril formation from A β 1-40

[070] Beta Amyloid peptide (from Quality Control Biochemicals) was dissolved in DMSO to give a clear solution of 10 mg/ml. The solution was centrifuged at 12,000 rpm for 10 min to remove the possible fibril formed in the storage period. At each time point, 5 μ L of such solution was mixed with 95 μ L of 100 mM phosphate buffer pH7.4 and shaken constantly to allow fibrils to form. The amount of fibril formed was quantitated by the following methods:

[071] (1). Quantitation by fluorescence reduction. To each well of the 96 well plate, 50 μ L of each sample was mixed with 100 μ L of 0.2 M borate buffer pH 9.0. Then to each well, 50 μ L fluorescamine solution was added and mixed immediately. The fluorescence was measured at excitation wavelength of 390 nm and emission wavelength of 475 nm.

[072] (2). Quantitation by centrifugation. The samples were centrifuged at 12,000 rpm for 10 min. Then the supernatant (non-fibril peptide) was removed and kept for fluorescamine assay. The pellet was washed with PBS three times. Then the pellet (fibril peptide) was dissolved in 50 μ L of acetonitrile. Then to each sample, 100 μ L of 0.2 M borate buffer was added and the fluorescamine assay was performed as described above.

[073] (3). Quantitation by Thioflavin assay. To each sample, 500 μ L of 25 μ M ThT in phosphate buffer pH6.0 was added and vortexed briefly. The samples was let stand at room temperature for 30 min. Then the fluorescence intensity was measured at excitation wavelength of 450 nm and emission wavelength of 485 nm.

[074] In another experiment, fluorescein labeled A β [1-40] was used to study whether the inhibitors can prevent the A β aggregation process. In that experiment, 20 μ L fluorescein-A β [1-40] (10 mg/ml in DMSO) was mixed with 80 μ L A β [1-40] (10 mg/ml in DMSO). Then 5 μ L of such mixed solution was diluted into 95 μ L PBS or PBS containing 5 equivalents of inhibitors to give a 20 $\times$ dilution. After 2 hour or 24 hours of incubation at room temperature, the solution was spun at 12,000 rpm for 10min. The supernatants were transferred to microtiter plate for fluorescence measurement. The pellets were washed with PBS once and

then redissolved in 50 μ L acetonitrile and 50 μ L PBS. The fluorescence of the dissolved pellet was measured at excitation wavelength of 485 nm and emission wavelength of 520 nm.

[075] **Binding Assay**

[076] Preform fibrils in vitro

[077] The dissociation constant (K_d) for the peptide alone and the PEG conjugate with the fibrils was determined by Scatchard plot. Beta amyloid peptide[1-40] (1mg, MW 4331) (from Quality Control Biochemicals) was dissolved in 1 ml PBS with 0.05% sodium azide to form a clear solution (0.5 mg/ml). The solution was shaken for 3 days to allow fibrils to form. The preformed fibrils were checked visually by eyes and aliquot of the fibril suspension was checked by ThT assay.

[078] Separation method validation

[079] Ultrafiltration method (100 KDa MW cutoff MicroconTM, Amicon, Beverly, MA) was used to separate the free peptide or conjugate from those bound on the preformed fibrils. This separation method was validated properly first. The peptide or conjugate solution, properly labeled either with fluorescence reagent or tritium isotope, were placed in the centricon filter unit (100 KDa MWCO) and spun for 5 min at 5000 rpm. The permeate and retentate were checked either by fluorescence reading or scintillation counter, depending on the labeling, to make sure that the small peptide can go through the filter unit freely without enrichment effect on the top during the separation process. In the same way, the preformed fibril solution was placed in the filter unit and spun for 5 min at 5000 rpm. The permeate was checked by ThT assay to make sure the fibrils won't go through the filter membrane.

[080] Assay procedure

[081] The peptides or conjugates to be tested were properly labeled with either fluorescence reagent or radioactive isotope for easy quantitation. In a typical binding assay, the total volume will be 400 μ L. In each Centricon filter unit, 50 μ L of 0.5 mg/ml preformed fibril solution and 50 μ L of peptide or conjugate solutions of various concentrations, typically starting from 0.1 mM were added. PBS solution was used to adjust the total volume to 400 μ L. After incubation at room temperature with gentle shaking, some of the original mixture was taken and the fluorescence or the radioactive counts was measured to determine total peptide concentration. Then the mixture was spun for 5 min at 5000 rpm at room temperature. The fluorescence or the radioactive counts of the ultrafiltered permeate was measured to determine the free (unbound) peptide concentration. Then the ratio of bound concentration/free concentration was plotted against the bound concentration, which was calculated from the amount of reagent added in each tube. The K_d was calculated from the inverse slope of the plot.

[082] **Thioflavin Test** (LeVine III, 1993)

[083] The beta amyloid peptide [1-40] (1 mg, MW 4331) was dissolved in 100 μ L of HPLC grade DMSO to give a 10 mg/ml clear stock solution. The solution was spun for 10 min at 12,000 rpm to remove potential fibril formed in the storage process. In a typical ThT assay, the test reagent (peptide or peptide conjugate) was diluted serially into PBSA (PBS solution with 0.05% sodium azide). Then 95 μ L of such

diluted test reagent solution was mixed with 5 μL of beta amyloid peptide solution in an 1.5 ml Eppendorf tube. The mixtures were shaken at room temperature for two days. Then in each tube 500 μL of 12 μM Thioflavin T solution in phosphate buffer pH 6.0 was added. The tubes were vortexed briefly. The mixture was let stand at room temperature for 30 min. Then the fluorescence was measured at $\lambda_{\text{ex}} = 450 \text{ nm}$ and $\lambda_{\text{em}} = 482 \text{ nm}$. The fluorescence was plotted against the molar ratio of the test reagents to the $\text{A}\beta[1-40]$.

[084] Binding competition assay

[085] Binding competition assay was used to estimate the dissociation constant of the binding of peptides or conjugates to the preformed fibrils. In a typical competition assay, the total volume was fixed at 400 μL . In a 500 μL centricon filter unit (50 KDa MWCO), 100 μL of 0.1 mg/ml preformed fibrils was added. Then 100 μL hot peptide $\text{Ac}^*\text{-KLVFF}$ (1 μM in PBS) was added. The competitor peptides or conjugates to be tested were diluted in PBS in series into different concentration typically ranging from μM to 10^{-10}M . Then 100 μL of such diluted competitor solution was added into the filter unit. PBS was used to adjust the total assay volume to 400 μL . The mixture was shaken gently for 2 hours to allow for equilibrium. Then the filter was spun at 5000 rpm for 5 min. The filtrate and the membrane were counted in a 5 ml vial with 3 ml scintillation fluid using LKB liquid scintillation counter.

[086] Interference of BSA on the binding

[087] In order to study the interference of BSA on the binding of our inhibitors to the fibrils, we did two experiments. Binding competition assay was used to evaluate the effect of BSA on the binding of tandem dimer peptide to the preformed fibrils in presence of 0.1 mg/ml and 1 mg/ml BSA. In such experiment, exactly the same procedure was followed as described in section I.3.2.5 except that BSA solution was added to the systems. The final concentration of BSA in the systems was 1 mg/ml and 0.1 mg/ml. As a control, assay without BSA was also performed.

[088] In another experiment, we did the binding of conjugate $\text{PEG}_{10\text{kDa}}\text{-(biotin)}_2\text{-(ckffvlk)}_6$ to the fibrils in presence of 10 mg/ml BSA. In such assay, centricon filter (50 KDa MWCO) were used to separate bound and unbound conjugate. The total assay volume was 150 μL . In each centricon filter unit, 50 μL of 0.05 mg/ml (diluted from 0.5 mg/ml) preformed fibrils was added. Then 50 μL of 2 $\times$ serially diluted radiolabeled conjugate was added. The starting concentration of the conjugate was 0.45 nM. Then either 30 mg/ml of BSA or PBS was added to the filter unit to adjust the total volume to 150 μL . After incubation overnight, the filter was spun for 10 mins at 12000 rpm and the conjugate bound to fibrils on the membrane was counted by LKB liquid scintillation counter.

[089] Evaluation of binding of inhibitor to monomer $\text{A}\beta[1-40]$

[090] ELISA plate pre-coated with streptavidin was bought from Pierce (Rockford, IL) and used for immobilizing the peptides or conjugates with biotin attached to be tested. The plate was washed with PBS three times. Then fixed amount of peptides or peptide-PEG conjugates with biotin attached was added to

plate. The plate was shaken gently for 2 hours at room temperature to allow the binding of biotin to the streptavidin on the plate. The plate was washed carefully with PBS 4 times. The plate was tapped dry on paper towel between each wash. Then 200 μ L of fluorescein-A β [1-40] (Quality Controlled Biochemicals) of different concentration was added to each well. The plate was shaken at 100 rpm at room temperature for 2 hours. The fluorescence on the plate was read first directly on a CytoFluor™ II fluorescence multi-well plate reader at excitation wavelength of 485 nm and emission wavelength of 530 nm. This fluorescence was used to determine the total concentration of the fluorescein-A β [1-40]. Then the supernatant was transferred into another 96-well polystyrene plate and the fluorescence was measured to determine the concentration of the free fluorescein-A β [1-40]. The plate was then washed gently with PBS 3 times. The fluorescence on the plate was then again read to determine the concentration of bound fluorescein-A β [1-40]. The data was analyzed using Scatchard plot to determine the binding constant.

[091] Binding of peptide ffvkl to tandem dimer peptide

[092] The binding of peptide ffvkl to biotinylated tandem dimer peptide was evaluated in a similar way as described in I.3.2.6. Radiolabelled peptide ffvkl was used in this case instead of using fluorescence.

[093] Peptide preparation

[094] Peptide synthesis

[095] All peptides were manually synthesized with a carboxyamide C-terminus by the standard solid phase peptide synthesis using Fmoc chemistry. In a typical peptide synthesis, we start with 0.441 g (0.15 mmol) PAL-PS resin (from PerSeptive Biosystems). The resin was put in a polypropylene column with cap and stopper and 5 ml of DMF was added to the column to swell the resin. The DMF was drained by vacuum. In each deprotection step, the Fmoc group on the resin was removed by treatment with 20% (v/v) piperidine in DMF (15 min twice with constant shaking). In each coupling step, we used 3.5 equivalents of BOP/HOBT and 3 equivalents of amino acid, and 1% DIEA was added to keep the reaction basic. The coupling reaction was done in 2 hours with constant shaking. After each coupling/deprotection, the resin was washed with DMF three times, with methanol twice and then with DMF three times. At each coupling and at each deprotection step, the Kaiser test (Kaiser et al., 1970) was performed to ensure the coupling and deprotection steps are complete. In short, a small portion of the resin was taken to a small glass tube and equal volume of Kaiser test reagents (Solution 1: 0.2 mM KCN in pyridine; Solution 2: 4 mg/ml of phenol; Solution 3: 5% ninhydrin solution in butanol) were added. The tube was kept in 110°C on a heating block for 2 min. The appearance of blue color for Kaiser test after coupling steps means the coupling reaction was not complete. The less blue color for the Kaiser test after deprotection step indicates the deprotection step was not complete. In either case, the coupling or the deprotection step was repeated until the step passes the Kaiser test. The deprotection/coupling steps were repeated until the sequence was complete.

[096] After finishing the assembly of the peptide sequence, the Fmoc on the N-terminus was removed, the N-terminus was capped with acetyl group in some cases. The acetylation step was done with 5% (v/v) acetic anhydride in DMF with 2% DIEA for 2 hours at room temperature. The resin was washed with 5 ml

DCM twice and dried under air. The peptides were cleaved from the resin with TFA. The cleavage cocktail contains TFA/thioanisole/EDT/anisole in volume ratio of 90:5:3:2. Typically, the cleavage reaction was done in 2 hours with 1 ml of such cleavage cocktail at room temperature. The cleavage reaction mixture was filtered through glass wool and washed with TFA. All filtrates were combined and evaporated under argon to reduce the volume to about 0.5 ml. Then the solution was precipitated by adding dropwise into 8 ml ice-cold ethyl ether and spun at 5000 rpm for 5 min. The pellet was washed with ice-cold ether three times and dried.

[097] Peptide purification

[098] The crude peptides were dissolved in 0.1% TFA and purified by reverse phase HPLC on a Vydac (Separation Group, Hesperia, CA) C18 peptide column (218TP1022, 2.2X25 cm, 10 μ m, 300Å pore size). Mobile phase A was 0.1% TFA in milliQ water. Mobile phase B contained 50% acetonitrile, 40% isopropanol and 10% mobile phase A. The gradient used was: 0-5 min, 100% A; 5-20 min, 100% A to 80% B; 20-40 min, 80% B to 100% B; 40-45 min, 100% B. The flow rate used was 2 ml/min.

[099] Peptide characterization by Mass Spectroscopy

[0100] The purified peptides were characterized by Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectroscopy (MALDI-TOF MS). Matrix solution was prepared fresh each time by dissolving 10 mg α -cyano-hydroxyl-cinnamic acid in 500 μ L acetonitrile and 500 μ L 3% TFA. The solution was spun at 12000 rpm for 10 min to remove undissolved materials. 20 μ L of matrix solution was mixed with 1 μ L of HPLC peptide fraction. 1 μ L of such solution was spotted on the sample plate, dried in the air. The peptide samples were analyzed on a DE-PRO mass spectrometer. (PE Biosystems).

[0101] Peptide characterization by Amino Acid Analysis

[0102] Amino acid analysis of the purified peptides or peptide conjugates was done at Louisiana State University Medical Center Core Labs on a PE Biosystems 420 amino acid analyzer with automatic hydrolysis. Sarcosine (2 nmol) in 10% methanol was added to each aliquot of sample as an internal standard. Samples were dried in the speed vacuum, and inserted into a ceramic rack in a desiccator. Constant boiling HCl (2 ml) was mixed with 100 μ L of 2-mercaptoethanol and 100 mg of phenol in a beaker, and the beaker was set into the desiccator. The desiccator was evacuated to 3 mmHg pressure, refilled with argon and re-evacuated. Samples were hydrolyzed at 110 °C overnight, then 200 μ L of diluent buffer was applied to each vial to dissolve the sample. Hydrolysates (100 μ L) were injected into a sulfonated polystyrene cation-exchange column, and eluted by a step gradient of citrate-based buffer. Post-column derivatization was with ninhydrin in a solvent mixed with sulfolane at 130 °C. The detector was set at 580 nm. All peak areas were adjusted to a constant amount of the internal standard, and concentrations were calculated.

[0103] Quantitation of peptide concentration by UV

[0104] All the peptide sequence in this study contains phenylalanine residues. The phenylalanine has maximum absorbance at 254 nm. The phe standard solution was prepared by weighing phe accurately and dissolving in distilled water. The absorbance of the standard solutions and samples were measured at 254 nm. The standard curve was performed each time and used for calculating the peptide concentration.

[0105] Preparation of PEG-peptide conjugates**[0106] Synthesis of peptide-PEG-fluorescence conjugate**

[0107] In the case of the peptide-PEG-fluorescein conjugate, after assembling the peptide and removing the Fmoc in the last step, the resin was then coupled with fluorescein-PEG₅₀₀₀-NHS (Shearwater Polymers) in 1:1 DMF/100 mM NaHCO₃ buffer pH 8.5. After coupling, the resin was washed thoroughly with DMF, acetic acid and methylene chloride, and dried. The conjugate was then cleaved from the resin and precipitated with cold ether. The crude conjugate product was dried, redissolved in distilled water and purified by reverse phase HPLC. Radiolabel peptide

[0108] The peptide was radiolabeled by tritium acetic anhydride. After removing the Fmoc in the last step of peptide synthesis, a small portion of the resin was let to react with tritium acetic anhydride in DMF. The reaction was chased with cold acetic anhydride. After washing steps, the radiolabelled peptide was cleaved from the resin following exactly the same way as the cold peptide.

[0109] Synthesis of PEG-aspartic acid copolymer

[0110] Boc-aspartic acid (23.3mg, MW233, 0.1 mmol) and 340mg H₂N-PEG-NH₂ (MW3400, 0.1 mmol) were dissolved in 3ml CH₂Cl₂. A small amount of p-toluenesulfonic acid (6 mg, 0.03 mmol) and 4-(dimethylamino)-pyridine (4 mg, 0.03 mmol) were added as catalysts. The coupling reagent 1,3-diisopropyl-carbodiimide (63mg, 0.5mmol) was then added. The reaction was kept in argon for two days. The reaction was monitored by SEC-HPLC. A small portion (20 µL) of the reaction mixture was taken out, dried by blowing with argon, redissolved in 50 mM TEAA buffer and loaded on HPLC SEC column. We stopped the reaction when the mixture gave a peak at 8.5 min, indicating that a copolymer of molecular weight 20,000 Da had formed. A rotary evaporator was used to reduce the volume to about 1 ml. The reaction mixture was then added dropwise into 20 ml ice-cold ethyl ether to precipitate the polymer. The polymer was washed with ice-cold ethyl ether three times and then taken to dryness under vacuum.

[0111] The polymer was dissolved in water, dialyzed against 3×1000 ml water for three days using dialysis tubing (Spectro/Por®, 12000-14000 dalton molecular weight cutoff). The polymer solution was then lyophilized in speed vacuum. The dried polymer was stored in freezer at -20°C.

[0112] The Boc protecting group on the copolymer was removed by treatment with 50% (v/v) TFA in methylene chloride for 2 hours. The polymer was precipitated in ice cold ethyl ether and washed with ethyl ether three times. The polymer was taken to dryness under vacuum and used without further purification.

[0113] In a similar way, we copolymerized Boc-aspartic acid with H₂N-PEG₂₈₂-NH₂. The reaction was done in exactly the same way, except that in the purification step we used SEC-HPLC to purify the polymer instead of the dialysis tubing.

[0114] Characterization of PEG-Aspartic acid copolymer by SEC-HPLC

[0115] The purified PEG-aspartic acid copolymer was characterized by SEC-HPLC using Bio-sil TSK-250 SEC column (Bio-Rad #125-0062) with a Bio-sil 250 Guard Column (Bio-Rad# 125-0073). Protein standard was used to estimate the molecular weight of the copolymer. The mobile phase contains 20 mM citrate, 100 mM NaCl pH 7.0. Protein standard (Bio-Rad, lot # 151-1901, 0.7 mg) was dissolved in 1ml

elution buffer. Then 300 μ L of such protein standard solution was injected into 500 μ L loop. The calculated amount of protein standard = 210 μ g. No gradient was used for this HPLC.

[0116] Synthesis of HOOC-PEG₆₀₀-(N-Fmoc)-KLVFF

[0117] In order to make this conjugate, in the last cycle of coupling reaction, we used Fmoc-Lys(mtt)-COOH instead of Fmoc-Lys(Boc)-COOH for the easy removal of the Mtt group. In the last step of peptide synthesis, with the Fmoc group still on the peptide, the mtt protecting group on the side chain of lysine was removed with 1% TFA. This is done by putting the resin in the polypropylene column, adding 1% TFA in DCM from the top constantly while there is slow dripping from the bottom of the column. The reaction was done at room temperature for 1 hour. The resin was then washed thoroughly with methanol and DMF. The amino group was coupled with 10 equivalent Poly(ethylene glycol) bis(carboxymethyl) ether (MW 600) to convert the amino functional group to the carboxylic acid group. The resin was washed thoroughly with DMF three times, followed by methanol three times, 10% (v/v) acetic acid in methylene chloride twice and then DMF three times.

[0118] The conjugate HOOC-PEG₆₀₀-(N-Fmoc)-KLVFF was cleaved from the resin, purified by reverse phase HPLC.

[0119] Synthesis of (PEG₃₄₀₀-D)_n-(PEG₆₀₀-KLVFF)_m

[0120] This conjugates was made by coupling HOOC-PEG₆₀₀-(N-Fmoc)-KLVFF with PEG-aspartic acid copolymer. This reaction was done in solution phase. Because PEG has the property of absorbing water readily in the air, both materials are dried in the vacuum overnight. PEG-aspartic acid copolymer (80 mg, MW 20 KDa, containing 0.0235 mmol free primary amine) was dissolved in about 5 ml DCM. The conjugate HOOC-PEG₆₀₀-(N-Fmoc)-KLVFF (12 mg, 0.011 mmol) was added into this solution. A small amount of p-toluenesulfonic acid (2 mg, 0.01 mmol) and 4-(dimethylamino)-pyridine (1.3 mg, 0.01 mmol) were added as catalysts. The coupling reagent 1,3-diisopropyl-carbodiimide (19 mg, 0.15 mmol) was then added. The reaction was kept in argon overnight at room temperature with constant stirring. The amount of peptide put on each polymer was monitored by the fluorescence decrease in the fluorescamine assay described above. The fluorescamine assay measures the amount of primary amine present, which is directly proportional to the fluorescence intensity. The conjugate was precipitated by cold ethyl ether, taken to dryness and purified by SEC-HPLC column.

[0121] The conjugate (PEG₃₄₀₀-D)_n-[PEG₆₀₀-(N-Fmoc)-KLVFF]_m was split into two halves and one half was saved for labeling with FITC. The other part was treated with 20% piperidine in DMF directly to remove the Fmoc group on Lys. The conjugate was precipitated with cold ethyl ether and purified by HPLC SEC column.

[0122] Label (PEG₃₄₀₀-D)_n-(PEG₆₀₀-KLVFF)_m with fluorescein

[0123] The PEG-peptide conjugate was further labeled with fluorescein by reacting the conjugate (PEG₃₄₀₀-D)_n-[PEG₆₀₀-(N-Fmoc)-KLVFF]_m with Fluorescein Isothiocyanate (FITC). FITC is an amino-reactive probe that reacts under alkaline conditions with primary amines to form a stable, highly fluorescent derivative. The conjugate (PEG₃₄₀₀-D)_n-[PEG₆₀₀-(N-Fmoc)-KLVFF]_m (50 mg, containing 0.011 mmol primary amine) was dissolved in 0.5 M NaHCO₃ buffer pH 9.5. Then 5 mg of FITC was dissolved in

10 μ L of DMF first and added in the conjugate solution. The reaction was done at room temperature for 2 hours with constant shaking. The product was purified by SEC column using 50 mM TEAA buffer as mobile phase. The product was dried under speed vacuum. The Fmoc group on Lys was finally removed by treating with 20% piperidine in DMF for 30 min at room temperature with vigorous shaking. The mixture was again precipitated with ice-cold ethyl ether, dried, redissolved in TEAA buffer and purified by HPLC SEC column.

[0124] Synthesize HOOC-PEG₆₀₀-(ϵ -Boc-K)-(ϵ -Boc-K)-LVFF

[0125] The peptide (ϵ -Boc)-K-(ϵ -Boc)-KLVFF was synthesized by the standard solid phase synthesis using the Fmoc chemistry described before. With the peptide still on the resin and the Fmoc on the N-terminus removed, 10 equivalent of PEG₆₀₀-(COOH)₂ was dissolved in DMF containing 2% DIEA and added to the polypropylene column containing the resin. Then 5 equivalents of BOP and Hobt were added as coupling reagents. The coupling reaction was done at room temperature for 2 hours with constant shaking. The resin was then washed with DMF, methanol and then DCM, just like the procedure in peptide synthesis. The peptide PEG conjugate was cleaved from the resin using the same procedure as the cleavage for the peptide cleavage. The Boc protecting groups on the side chain of Lys were also removed during the cleavage process. The conjugate HOOC-PEG₆₀₀-KKLVFF was further purified by reverse phase HPLC.

[0126] The side chain of the Lys was further protected by Boc anhydride. In a 25 ml flask, the conjugate HOOC-PEG₆₀₀-KKLVFF was dissolved in 5 ml of 0.1 N NaOH, then 5 equivalents of Boc anhydride was added into the solution. The reaction was done at room temperature overnight with constant stirring. Since the reaction was done at aqueous phase, the Kaiser test can't be done easily. So we used fluorescamine assay to monitor the reaction.

[0127] The volume of the reaction was reduced by speed vacuum to about 1 ml. Then 0.5 ml acetonitrile was added to help with the solubility. The reaction was further purified by Bio-Rad G-25 prepacked desalt column using 50 mM TEAA as mobile phase.

[0128] Synthesize HOOC-CH₂-CH₂-CO-(ϵ -Boc-K)-(ϵ -Boc-K)-LVFF

[0129] This compound was synthesized following almost exactly the same procedure as the synthesis of HOOC-PEG₆₀₀-(ϵ -Boc-K)-(ϵ -Boc-K)-LVFF described above except that in the last step on the resin, we used succinic anhydride instead of HOOC-PEG₆₀₀-COOH. And in the last step of purification, reverse phase HPLC was used to purify this compound instead of SEC column.

[0130] Synthesize Orn-(Gly-Orn)₅-(PEG₆₀₀-KKLVFF)_m

[0131] Peptide Orn-(Gly-Orn)₅ (O₆G₅ in short) was synthesized manually using the standard solid phase peptide synthesis described above. The peptide O6G5 was purified by reverse phase HPLC peptide column. The gradient for purifying this peptide was much slower than the gradient described above because the polarity of this peptide is much higher than the other peptides. The following condition were used for purifying this peptide. Mobile phase A contains 0.1% TFA in milliQ water. Mobile phase B contains 20% acetonitrile, 10% isopropanol and 70% buffer A. The gradient used was: 0-5 min, 100% A to 30% B; 5-40 min, 30% B to 70% B; 40-42 min, 70% B to 100% B; 42-45 min, 100% B. The flow rate used was 4 ml/min.

[0132] O₆G₅ peptide belt (5 mg, MW 986 Da, 5 μ mol) was coupled with HOOC-PEG₆₀₀-(ϵ -Boc-K)-(ϵ -Boc-K)-LVFF (24 mg, MW 1583 Da, 15 μ mol) in 2 ml DMF containing 2% DIEA with BOP (20 mg, MW 442, 45 μ mol) and Hobt (6 mg, MW 135 Da, 45 μ mol) as coupling reagents. The reaction was done at room temperature overnight. The reaction mixture was precipitated by ice-cold ethyl ether. The crude conjugate was dissolved in 50 mM TEAA pH6.0 and purified by Sepadex G-10 column.

[0133] The Boc groups on the side chain of Lys were finally removed by treating with 50% TFA in methylene chloride for 2 hours. The conjugate was again precipitated with ice-cold ethyl ether and washed with ice-cold ether 4 times. The conjugate was taken to dryness and used without further purification.

[0134] Synthesize (PEG₃₄₀₀-D)_n-(CO-CH₂-CH₂-CO-KKL VFF)_m

[0135] PEG-aspartic acid copolymer (PEG₃₄₀₀-D)_n was coupled with HOOC-CH₂-CH₂-CO-(ϵ -Boc-K)-(ϵ -Boc-K)-LVFF in DMF using BOP/Hobt as coupling reagents. The reaction was monitored by fluorescamine assay. The conjugate was purified by HPLC SEC column. The Boc protecting group was removed by treating with 50% TFA in DCM for 2 hours. The conjugate was precipitated with ice-cold ethyl ether and washed with cold ethyl ether 4 times. The conjugate was taken to dryness and used without further purification.

[0136] Synthesize (PEG₂₈₂-D)_n-(PEG₆₀₀-KKLVFF)_m

[0137] This conjugate was synthesized following almost exactly the same procedure as described above for synthesizing Orn-(Gly-Orn)₃-(PEG₆₀₀-KKLVFF)_m. Instead of using O₆G₅ peptide belt, the PEG282-aspartic acid copolymer was used for attaching the peptide. The relative ratio for all the reactions remained the same as the procedure above. The absolute amounts for the reagents were adjusted according to their molecular weight.

[0138] Synthesize PEG_{10k8a}-(biotin)₂-(cfffvlk)₆

[0139] Branched PEG (MW 10 KDa) with 8 amino functional groups (denoted as PEG_{10k8a}) was bought from ShearWater Polymers (Huntsville, AL). PEG_{10k8a} (50 mg, 5 μ mol) was dissolved in 2 ml of 0.2 M borate buffer pH 8.5 in a 4.5 ml tube. A small portion (20 μ L) of this solution was taken and saved as control for the fluorescamine assay. Into this solution, Sulfo-NHS-LC-biotin (8 mg, MW 556 Da, 15 μ mol) was added. The reaction was done at room temperature overnight with constant shaking. The amount of biotin put on each PEG molecules was quantified by fluorescamine assay as described above. The reaction mixture was purified by centricon filter (3 KDa MWCO) by washing through the filter unit with 3 volumes of 0.2 M borate buffer pH8.5. The solution on the top of filter unit was transferred to a 1.5 ml Eppendorf tube and used directly for the next step reaction.

[0140] SPDP was used to link the amine group to the peptide with free thiol group. In the next step, SPDP was put on the PEG-biotin conjugate. SPDP (14 mg, MW 312, 45 μ mol) was dissolved in 40 μ L of DMSO and added to the Eppendorf tube containing the purified PEG-biotin conjugate. The reaction was monitored by fluorescamine assay and was done in 4 hours as indicated by the disappearance of the fluorescence in the fluorescamine assay. The reaction mixture was purified by HPLC SEC column using 50 mM NaAc-HAc pH5.2 buffer. The volume of the fraction from the HPLC column was reduced by speed vacuum to about 1 ml and used directly for the next step reaction.

[0141] Retro-inverso peptide ckffvlk was synthesized and purified according the procedure described above. This peptide was made fresh just before using because the thiol group on cys can be easily oxidized in the air. About 30 mg peptide ckffvlk (MW 885, 34 μ mol) was dissolved in 100 μ L of 50 mM NaAc-Hac pH 5.0 buffer and added to tube containing PEG_{10k3a}-(biotin)₂-(TP)₆ just purified from HPLC SEC column. The reaction was monitored by the release of TP group which at a distinct maximum absorbance at 343 nm. The unreacted peptide was further purified by HPLC SEC column.

[0142] Synthesize tandem dimer peptide and cyclic dimer peptide

[0143] These two peptides are synthesized used the same procedure described in the peptide synthesis section. In the first cycle of peptide synthesis, N-Fmoc-(ϵ -mtt)-Lys was put on the resin first. After removing the Fmoc group, Fmoc- β -Ala was added on the N-terminal as a spacer. Then the mtt group on the side chain of Lys was removed by 1% TFA in DCM and the Fmoc group was removed by 20% piperidine in DMF. Then the amino acids was added onto both side chain and N-terminal of Lys in parallel in the later synthesis cycle. The synthesis was repeated until the complete of the desired sequence.

[0144] For the cyclic peptide, in the last step of synthesis, succinic acid was used to cyclize the peptide.

Results and discussion

[0145] Fluorescamine assay

[0146] Fluorescamine assay (Udenfriend et al., 1972) is a very sensitive assay for quantifying the amount of primary amine existing in a system. It is widely applied in quantifying the concentration of peptide and protein. Since the fluorescamine derivatives of primary amine-containing compounds can be detected with high sensitivity, it is also widely used to make the derivatives for enhancing the detection (Carretero et al., 1999; Grant & Patabhi, 2001).

[0147] Fluorescamine was extensively used in quantifying the peptide concentration and quantifying the extent of the coupling reaction between primary amine and carboxylic group. The fluorescamine assay has very good linearity in the range of 10^{-5} to 10^{-4} M, which is the typical working concentration of primary amine, even though it has been reported that the sensitivity of this assay can be as low as nM.

[0148] There was a little difference between the standard curves of Ala and diamino-PEG. This small difference could be caused by the inaccuracy of the molecular weight of diamino-PEG. We prepared the standard solution of diamino-PEG based on the information that the molecular weight of diamino-PEG is 3400 Da from the company. There might be some inaccuracy on this molecular weight because it is very hard to determine the molecular weight accurately.

[0149] The other possibility of this difference might be caused by the interference of PEG on the fluorescence intensity. Our results showed that this fluorescamine assay has very little interference from other materials. Small amount of PEG has no interference on this assay. We also showed that small amount of BOP, Hobt and DMF has no interference on this assay. So this assay can be used to monitor the coupling

reaction between the primary amine and carboxylic acid directly in presence of small amount of BOP, Hobt and DMF when they were diluted into the 0.2 M borate assay buffer.

[0150] Kinetics of fibril formation

[0151] There are a lot of research groups trying to deduce the fibril formation pathway by studying the fibril formation kinetics (Lomakin et al., 1996; Elser et al., 1996; Hasegawa et al., 1999). Quantitative kinetic studies of β -amyloid fibril formation have been complicated by the fact that it is sensitive to variations in the method of preparation of the initial A β stock solution. The kinetics of amyloid fibril formation in vitro from synthetic peptide can vary dramatically for peptide from different company. A lot of different methods have been used to quantify the amount of fibril formed in kinetic study. Some methods, such as turbidity (Jarret, et al., 1992) and ThT assay (Hasegawa et al., 1999). But these methods provide no information about the size of fibrils. Microscopic methods are very good in providing the fibril size but they are not appropriate for real time kinetic study. Quasielastic light scattering (QLS) (Lomakin et al., 1996) seems to be a good way to study the fibril formation kinetics.

[0152] The objective of the kinetic study is just to get the proper time for fibril formation and for evaluating our peptide inhibitors.

[0153] We used three approaches to study the fibril formation in vitro. Our first approach used fluorescamine assay directly. When the beta amyloid peptide aggregates, the primary amines on Lys side chains are buried inside and therefore can not react with fluorescamine. There are two Lysines in beta amyloid peptide; Thus, including the N-terminal primary amine, there are three primary amines present in beta amyloid peptide [1-40]. The N-terminal primary amine is not buried inside when the fibril is formed while the primary amine groups on the side chains of lysines are buried inside. So we are expecting that fluorescence of the Fluorescamine assay to decrease to 1/3 of its original level. Our result fits this very well (Figure 5a). In our second approach (Figure 5b), we spun down the fibril that had formed and redissolved the pellet. This approach should be more reflective of the physical state of the aggregate. And this approach also showed kinetics very similar to the first approach, measuring either increase of the peptide aggregate in the pellet or decrease of free peptide in solution with time. Our third approach, quantification by ThT assay (Figure 5c) also showed very similar results as the first approaches.

[0154] From our kinetic study, in our experimental conditions, with 0.5 mg/ml Beta Amyloid peptide in PBS, both Lys side chains got buried in 12-15 hours. Then fibrils were formed in 18-20 hours under constant shaking at room temperature. Therefore, 24 hours might be long enough to ensure the completeness of fibril formation when making fibrils in vitro. While this might be true, some scanning tunneling microscopy (STM) (Shivji et al., 1995) showed that even though the fibrils can form in 24 hours, the fibril size still increase after 24 hours. To make our experimental results consistent, for most of our experiments, we chose 3 days to let the fibrils to form into a relatively consistent size.

[0155] Peptide preparation

[0156] All the peptides were synthesized manually on PALTM-PS resin by standard Fmoc chemistry as described in previous section. The peptide sequences we have synthesized typically range from 5 aa to 10 aa. Even though the manual peptide synthesis is a little time-consuming, it did give us good overall yield. In manual synthesis, we did Kaiser test each coupling/deprotecting step to make sure that each coupling and deprotecting step is complete. And these steps are repeated if the Kaiser test showed that the step was not complete. When the peptide was cleaved from resin, RP-HPLC showed that the crude peptide product typically had more than 80% purity for most of the peptides synthesized. We also did MALDI-TOF mass spectroscopy for some of the crude peptides. The peaks from impurities are often negligible comparing to the main peptide peak.

[0157] The structures of all of the peptides, after cleavage from the resin and purification, were confirmed by mass spectroscopy.

[0158] The peptide concentration was mostly quantified by phenylalanine standard, especially when we were working with small scale of peptides or working with radio-labeled peptide, in which cases it is not practical to quantify the peptide concentration by weight. All of our peptide inhibitors contain two phenylalanine residues in the peptide sequences. And phenylalanine is the only residue containing aromatic ring. The UV absorbance at 254 nm from other residues is negligible. This method for quantifying peptide concentration by UV was compared with the concentration determined by weight when we were working with relatively large scale peptide. The results from both methods agree well.

[0159] Fluorescamine assay is another way for determining the peptide concentration. Ala standard had been used as the standard curve as described in fluorescamine assay section. This method also showed that UV absorbance is a valid method for quantifying peptide concentration.

[0160] Peptide conjugate preparation

[0161] Characterize of PEG-Aspartic copolymer by SEC-HPLC

[0162] The molecular weight of the synthesized PEG-aspartic acid copolymer was determined by HPLC SEC column using protein standard to calibrate.. We can see that this column gave very good separation for molecules of MW from 1350 Da to 670,000 Da. The elution times were plotted against the logarithm of MW and gave good linearity in this MW range.

[0163] The molecular weight for PEG₃₄₀₀-Asp copolymer was determined to be 20128 Da in Dr. Joachim Kohn's lab using PEG standard. Comparing to PEG molecule, protein is folded into more compact structure and therefore its behavior on SEC looks smaller than PEG molecule of the same molecular weight. The apparent molecular weight of PEG on SEC using protein standard is usually 3 times of its real molecular weight based on our observation of PEG of known molecular weight. The molecular weight of the PEG₂₈₂-Asp copolymer was determined using the protein standard above and the empirical factor of 3 was used to adjust the difference between protein and PEG. The molecular weight of the PEG₂₈₂-Asp copolymer was determined to be 3200 Da. Therefore, there are about 5.9 primary amine attaching sites on PEG₃₄₀₀-Asp copolymer and 11.3 attaching sites on PEG₂₈₂-Asp copolymer.

[0164] Determine the number of copies of peptides on conjugates

[0165] The number of copies of peptides on the peptide conjugates were calculated based on the fluorescamine assay and the number of attaching sites of the peptide carrier. The percentage reduction of fluorescence in fluorescamine assay was used to estimate the percentage of sites reacted with the peptides. The results were summarized in Table I.4.2.

[0166] Scatchard plot

[0167] Binding to preformed fibrils

[0168] Binding of peptides and peptide-PEG conjugates to preformed fibrils was measured by Scatchard plot. Peptide KLVFF is the natural sequence of residues 16-20 from β -Amyloid peptide. Peptide ffvlk is a retro-inverso peptide. We call it retro-inverso peptide because it is made of all D-amino acids and its sequence is the reverse of the natural sequence. Peptide ffvlkk is the retro-inverso peptide with one extra lysine. We use that extra lysine for two purposes: to improve the water solubility and to study the effect of the positive charge on the binding of the peptide to fibrils. Peptide FLKVF is a control peptide whose sequence is scrambled and the lysine with positive charge is put in the middle. From the Scatchard plots, we found that the binding to preformed fibril is sequence specific binding and the control peptide has much lower binding affinity to preformed fibrils. We also found that the retro-inverso peptides ffvlk and ffvlkk both have slightly higher binding constants with β -amyloid fibrils than does the natural KLVFF peptide. Not only are the binding constants 3-5 times higher, but the peptides are also made of all D-amino acids and are resistant to protease digestion in vivo. Therefore, these retro-inverso peptides have a great advantage over the regular peptide KLVFF as candidate antagonists for amyloid polymerization.

[0169] By putting two copies of antagonist peptides on one single PEG-peptide conjugate molecule, we improved the dissociation constant K_d to 10^{-8} M, which is 100 fold better than single peptide. The tandem dimer peptide gave the K_d of the same magnitude as the PEG-peptide conjugate. We have expected that the tandem dimer peptide might have better binding affinity to preformed fibrils than the PEG-peptide conjugate for two reasons. Firstly, the tandem dimer peptide was made of retro-inverso peptide while in the PEG-peptide conjugate the peptides were made of L-amino acids. Previously we had showed that the retro-inverso peptide has slightly better binding to preformed fibrils than the peptide made of all L-amino acids. Secondly, in the peptide-PEG conjugate, the two peptides are separated by long PEG chain. While the dimer peptide has two copies of peptide linked together more closely and may have better synergistic effect. While all these reasoning might be true, the result showed that the binding of tandem dimer peptide is slightly worse than the PEG-peptide conjugate. This result showed that the peptide unit in the dimer peptide is probably not arranged in the space correctly. Because the two peptide units are linked so closely, the steric hindrance might prevent them from arranging into the correct configuration and binding to the preformed fibril simultaneously. Our thioflavin T assay for the cyclic peptide, which will be showed below, also confirmed this reasoning.

[0170] By putting more copies of peptides on the PEG-peptide conjugate, we expected to make the conjugate bind to the preformed fibrils even more tightly and therefore inhibit the fibril formation using lower inhibitor concentrations. The conjugate $\text{PEG}_{10k8s}-(\text{biotin})_2-(\text{ckffvlk})_6$ was made for this purpose. The

PEG used in this conjugate was branched PEG of MW 10 KDa with 8 amino functional groups. The dissociation constant K_d for binding of this conjugate to fibrils was determined to be $1.1 \times 10^{-10} M$, which is 4 orders of magnitude better than the single peptide.

[0171] Binding to monomer A β [1-40]

[0172] The binding of tandem dimer peptide and conjugate PEG_{10kDa}-(biotin)₂-(ckffvlk)₆ to monomer A β [1-40] was done by immobilizing them to the ELISA plate and measuring the binding of fluorescein-A β [1-40] as described in the method section. The dissociation constant has been determined to be $1.7 \mu M$ for tandem dimer peptide and $0.155 \mu M$ for the PEG-peptide conjugate with 6 copies of peptide. Both of them showed much lower binding affinity to monomeric A β [1-40]. In monomeric A β [1-40], not like the preformed fibrils, there will be only one binding site. Therefore, increasing the number of copies of peptide won't improve the binding constant.

[0173] Binding of peptide ffvlk to biotinylated tandem dimer peptide

[0174] The binding of peptide ffvlk to biotinylated tandem dimer peptide was done by immobilizing the dimer peptide to the ELISA plate and measuring the binding to radio-labelled peptide ffvlk to the tandem dimer peptide as described in the method section. The Scatchard plot showed dissociation constant K_d of $1.8 \mu M$. Comparing to the K_d for binding of ffvlk to preformed fibrils, which is $0.5 \mu M$, even though this binding is a little weaker, these two K_d are in the same magnitude. This result showed that the peptide sequence ffvlk has similar binding affinity to itself to its binding to preformed fibrils. This result is consistent to the result that peptide sequence KLVFF is the contact site when forming fibrils (Hilbich et al., 1992).

[0175] **Thioflavin T (ThT) assay**

[0176] While the Scatchard plot is a good way to evaluate a peptide or conjugate by determining the K_d of their binding to the preformed fibrils, it didn't provide information whether the test compound can prevent the fibril formation. In Scatchard plot, all the tested compounds have to be labeled by fluorescence reagents or radio-active isotope.

[0177] The benzothiazole dye ThT is a classical amyloid stain for senile plaques containing beta amyloid peptide in Alzheimer's disease brain. ThT binds rapidly and specifically to the anti-parallel beta-sheet fibrils formed from synthetic beta (1-40) peptide, but does not bind to monomer or oligomeric intermediates. ThT is a useful probe for the fibrillar state of beta amyloid fibrils as it is amyloid-specific and reports only fibrillar species (LeVine III, 1997).

[0178] It is noteworthy to state here that the β -amyloid peptide in the β -amyloid fibrils in senile plaque is arranged in a highly ordered, condensed anti-parallel β -sheet structure. The neuronal toxicity of the amyloid fibrils is linked to this structural characteristics. In order to differentiate from the amorphous aggregate that is not neuronal toxic, we will call the fibrillar aggregate with highly ordered, condensed anti-parallel β -sheet structure associated with neuronal toxicity "amyloid fibrils" while the non-toxic amorphous aggregate from A β "aggregate". Completely preventing the aggregation of β -amyloid peptide is

not necessary in order to block its toxicity. It has been shown that the ThT fluorescence in ThT assay is correlated to the aggregation morphology and its cell toxicity, inhibitors that can reduce the fluorescence in ThT assay can also change the fibril morphology and blocking the cell toxicity efficiently (Ghanta et al., 1996). Therefore, ThT assay is an efficiently way in evaluating the inhibitor in preventing the toxicity of amyloid fibrils.

[0179] We use two parameters to evaluate the efficiency of an inhibitor in ThT assay, one is R50 and the other one is I_{max}. R50 is the ratio of inhibitor peptide to the A β 40 where the inhibitor inhibits the ThT fluorescence by 50% comparing to the fluorescence without inhibitor in the ThT assay. I_{max} is the maximal percentage inhibition versus no inhibitor. All the ThT assay results are summarized in Table I.4.4 and are explained below in details. From this table we note that the absolute values of R50 and I_{max} for the same inhibitor have very high variation from experiment to experiment. This high variation is probably caused by the difference of the initial state of the A β in stock solution. It has been shown that the A β peptide from different vendors showed quite different kinetics in fibril formation (Hasegawa et al., 1999). Therefore, it is possible that A β peptide from different batches can have high variation. The storage time may also affect the A β state and cause the variation even the same batch of peptide was used to perform the experiment. Nevertheless, the result from the same experiment should be comparable.

[0180] Protease-resistant peptide

[0181] One important drawback of peptide drug is its instability in vivo. There are a lot of protease in the body that can digest the peptide before the drug can be absorbed and perform its pharmacological effect. Therefore, we tried to make the peptide protease resistant by using all D-amino acids, which are non-natural.

[0182] Two peptides that contains all D-amino acids have been synthesized, klvff and klvffk (By convention, the lower-case letter denotes D-amino acid). These two peptide have the same sequence as the A β ¹⁶⁻²⁰ while contains all D-amino acid. The inhibitory effect in preventing fibrils formation was tested by ThT assay and compared with the peptide of L-amino acid, KLVFF showed that peptides klvff and klvffk have almost no effect in preventing amyloid fibrils formation, while peptide KLVFF and fvlkk have good inhibitory effect in preventing fibrils formation. Peptide fvlkk will be described below. This result showed that the binding of KLVFF to A β [1-40] is not only sequence specific but also steric specific.

[0183] The retro-inverso peptides fvlk and fvlkk, as described in Scatchard plot section, were also tested for their inhibitory effect in preventing fibril formation. Our result showed that both retro-inverso peptides can inhibit the fibril formation in vitro. Peptide fvlkk not only binds to the fibril more tightly from the Scatchard plot, but also prevents fibril formation from the monomer peptide more efficiently than does peptide KLVFF. Although the difference of inhibitory effect between the two retro-inverso peptides and peptide KLVFF seems to be insignificant, the property that these two peptides are resistant to protease digestion make them much favorable compared to peptide KLVFF.

[0184] Positive charge on N-terminus is important for peptide function

[0185] When we acetylated the N-terminus to eliminate the positive charge on the N-terminal Phe of fvlkk, the Thioflavin T test showed that the peptide lost its capacity for preventing fibril formation

dramatically. We further put a Lys on the N-terminus followed by N-terminal acetylation. The resulting peptide Ac-k-ffvlkk has similar ability to prevent fibril formation compared to the unmodified peptide ffvlkk. This result showed that the positive charge near the N-terminus is very important in preventing the amyloid fibril formation.

[0186] The amide on C-terminal is better than free acid

[0187] The peptide KLVFF with free carboxylic acid C-terminal was purchased from Bachem. The ThT assay for this peptide was done and compared with that with amide C-terminal to study the charge effect in the C-terminal. The inhibitory effect of KLVFF with amide C-terminal is significantly better than that with free acid C-terminal.

[0188] Conjugates with multiple copies of peptides have better inhibitory effect

[0189] In previous section, we have showed that by putting multiple copies of peptides on a single conjugate molecule, we significantly increased the binding affinity of the inhibitors to preformed fibrils. Here, we also tested their ability in preventing fibril formation. The conjugate containing two copies of peptide is much more effective in preventing fibrils formation as indicated by the amount of fluorescence reduction in ThT assay. This result may seem to be surprising since we are testing the inhibitory effect of a conjugate in preventing fibril formation from monomer A β [1-40]. There should be only one binding site on the monomer A β [1-40]. Therefore increasing the number of copies of peptides on the conjugate shouldn't have any effect in preventing the fibrils formation. While this might be true, there are a lot of intermediate states in the fibril formation process. In the beginning of fibril formation process, several monomer A β [1-40] aggregate into the intermediate state. Therefore, our inhibitor conjugate actually prevents the fibril formation by binding to the intermediate and therefore change the pathway of fibril formation. The intermediate consists of several monomer A β [1-40]. Therefore, increasing the binding constant of conjugate to the preformed fibrils can also improve its ability in preventing fibril formation.

[0190] Based on the result above, we synthesized some other conjugates, which contain two copies of peptide but separated by spacer of different length. Only ThT assay was performed to evaluate these conjugates. The result is shown in Figure 6. Firstly, we can see that all the conjugates have significantly better effect than the peptide alone in preventing fibril formation. Secondly, we compared the three conjugates, in which the peptides are separated by spacer of different length. We can see that the OG-peptide conjugate, on which the peptides are separated by the shortest spacer, is the best in preventing fibril formation. The other two conjugates have no significant difference in their capacity of preventing fibril formation.

[0191] ThT assay for tandem dimer peptide and cyclic dimer peptide

[0192] It can be seen that both tandem dimer peptide and cyclic dimer peptide are more efficient in preventing the fibril formation than single peptide. Also, we can see that tandem dimer peptide is better than cyclic dimer peptide, in which the conformation of the peptide is constrained. This result showed that the conformation adopted by the cyclic dimer peptide is not the correct conformation for binding to the A β [1-40] intermediate. It is widely accepted that in the amyloid fibrils, the A β aggregates by forming anti-parallel β -sheet structure. But we have no idea how the fragment A β ¹⁶⁻²⁰, ie, KLVFF is arranged in the

fibrils or in the intermediate when forming fibrils. In the cyclic dimer peptide, the two peptide chains are parallel to each other and this conformation is not good for the inhibition for fibril formation as showed by the ThT assay comparing to the tandem dimer peptide. Therefore, we can suggest from this result that in the fibril intermediate, the KLVFF probably also arranged in an anti-parallel conformation. In the tandem dimer peptide, there is no conformational constrain. To two peptide chains are more flexible and can adopt more preferable conformation for binding to the fibril intermediate.

[0193] ThT assay for PEG_{10kDa}-(biotin)₂-(peptide)₆

[0194] The ThT assay was also done for the conjugate PEG_{10kDa}-(biotin)₂-(peptide)₆ and compared with the tandem dimer peptide. The conjugate PEG_{10kDa}-(biotin)₂-(peptide)₆ has 100 fold better binding constant than tandem dimer peptide based on the Scatchard plot, it also has better effect in preventing the fibril formation.

[0195] None of the peptides or conjugates can break preformed fibrils

[0196] These experiments were done to test if the peptides or conjugates can break the preformed fibrils. The preformed fibrils were incubated with peptides or conjugates of different ratio for 3 days at room temperature. Then the ThT assay was done to quantify the amount of fibrils remained after treating with different amount of test reagents. Our result showed that none of the peptides or peptide conjugates can break the preformed fibrils according to the ThT assay.

[0197] **Binding competition assay**

[0198] Binding competition assay was used to validate the binding constant determined by Scatchard plot described above. Comparing to the Scatchard plot, this competition assay is easier to do. For doing Scatchard plot, we have to label the reagent to be tested and some reagent is not easy to label. Some reagents may change their binding property after labelling. For example, the cyclic peptide we synthesized doesn't have an easy way to label. The binding competition assay turned out to be an easy way to compare the relative binding constant.

[0199] The dissociation constant K_d for peptide KLVFF is about 10^{-6} M and the K_d for tandem dimer peptide is about 10^{-8} M, which are consistent with the results from Scatchard plot. The binding for the cyclic dimer peptide is a little weaker than the tandem dimer peptide and the K_d is about 5×10^{-8} M.

[0200] **Interference of BSA on binding**

[0201] In order to study the specificity of binding of tandem dimer peptide to the preformed fibrils. We did the binding competition assay of tandem dimer peptide the preformed fibrils in presence of 0.1 mg/ml of BSA and 1 mg/ml BSA and compared with the result without BSA. The result showed that 0.1 mg/ml of BSA had no effect on the binding and 1 mg/ml of BSA had very little effect on the binding. This result showed that the binding of the tandem dimer peptide to the preformed fibrils is specific.

[0202] Comparing to the binding without BSA, the conjugate maintained 41% binding in presence of 10 mg/ml BSA. Even though the interference from BSA is significant, there was big difference between the concentration of BSA and fibrils in the assay system. In the assay system, the concentration of fibrils was

only 0.017 mg/ml, which is only 0.17% of the BSA concentration. Considering the difference of BSA and fibril concentration, 41% of binding of the conjugate still showed that the binding is specific.

[0203] A β aggregation rate with and without inhibitors

[0204] In our ThT assay section, we have shown that most of our inhibitors can prevent amyloid fibril formation efficiently. In order to investigate the possible mechanism of the fibril formation process, we try to determine if the inhibitors can prevent the aggregation of A β from monomeric A β . Our result showed that either tandem dimer peptide or the PEG_{10kDa}-(biotin)₂-(peptide)₆ conjugate can not prevent the aggregation process. On the contrary, both inhibitors can increase the rate of A β aggregation. After 2 hour of incubation in PBS at room temperature, without inhibitors all the A β stays in supernatant and there is negligible amount of A β in the pellet. After 2 hours, with inhibitors all the A β goes to the pellet and there is very little amount of A β staying in the supernatant.

[0205] A summary of the ThT assay is shown in Figure 6. In the ThT assay, we have shown that both tandem dimer peptide and PEG_{10kDa}-(biotin)₂-(peptide)₆ are effective in preventing amyloid fibril formation. Therefore, the aggregates formed from A β when the inhibitors were present have decreased fluorescence in ThT assay and must have structure different from amyloid fibrils, probably amorphous and loose. Since the aggregates have low fluorescence in ThT assay, they must be non-toxic to neuronal cells (LeVine 1999). Therefore, our inhibitors prevent A β peptide from amyloid fibril formation by changing its folding pathway and aggregating kinetics. In other word, our inhibitors just precipitate out the A β peptide. Since the aggregates formed with our inhibitors present are non-toxic and probably have amorphous, loose structure, body clearance is possible. To justify this argument, a similar observation was reported by Ghanta et al (Ghanta et al., 1996). In that paper, they showed that their inhibitor can completely block the A β toxicity to PC-12 cells and can reduce ThT fluorescence in ThT assay even though their inhibitor can not prevent the A β from aggregating. Their EM study showed that the morphology of the aggregate formed with inhibitor present is quite different from amyloid fibrils.

CLAIMS:

1. A chemical compound for use in diagnosing or treating patients with Alzheimer's disease, said compound comprising multiple copies of a plaque-recognition peptide comprising the sequence phenylalanine-phenylalanine-valine-leucine-lysine (acronym=ffvlk), all amino acids being the D-isomer.
2. The compound of claim 1 further comprising a reporter element.
3. A chemical compound for use in diagnosing or treating patients with Alzheimer's disease, said compound comprising:
 - a) multiple copies of a plaque-recognition peptide comprising the sequence phenylalanine-phenylalanine-valine-leucine-lysine, all amino acids being the D-isomer; and
 - b) a plaque disruptor element.
4. A chemical compound for use in diagnosing or treating patients with Alzheimer's disease, said compound comprising:
 - a) multiple copies of a plaque-recognition peptide comprising the sequence phenylalanine-phenylalanine-valine-leucine-lysine, all amino acids being in the D-isomer;
 - b) a reporter element; and
 - c) a plaque disruptor element.
5. A chemical compound for use in diagnosing or treating patients with Alzheimer's disease, said compound comprising:
 - a) multiple copies of a plaque-recognition peptide comprising the sequence phenylalanine-phenylalanine-valine-leucine-lysine, all amino acids being the D-isomer; and
 - b) one or more copies of a ligand for a transporter that can cross the blood brain barrier; and
 - c) a reporter element.
6. A chemical compound for use in diagnosing or treating patients with Alzheimer's disease, said compound comprising:
 - a) multiple copies of a peptide comprising the sequence phenylalanine-phenylalanine-valine-leucine-lysine, all amino acids being in the D-isomer;
 - b) one or more copies of a ligand for a transporter that can cross the blood brain barrier; and
 - c) a plaque disruptor element.

7. A chemical compound for use in diagnosing or treating patients with Alzheimer's disease, said compound comprising multiple copies of a peptide comprising the sequence phenylalanine-phenylalanine-valine-leucine-lysine (acronym=ffvlk), all amino acids being the D-isomer and being optionally joined by a link selected from the group consisting of additional amino acids and a polymer.
8. A chemical compound for use in diagnosing or treating patients with Alzheimer's disease, said compound comprising about 3 to about 8 copies of a peptide, covalently linked into a single molecule, comprising:
 - a) the sequence phenylalanine-phenylalanine-valine-leucine-lysine, all amino acids being the D-isomer; and
 - b) at least one copy of noncovalently bound Ab1-40 or an analog thereof.
9. The chemical compound of claim 1 in which the peptide ffvlk preserves at least one positive charge at the N-terminus at pH 7 when it is incorporated into a conjugate comprising a transporter ligand and a reporter or disruptor element.
10. The chemical compound of claim 9 in which the transporter ligand is a vitamin selected from the group consisting of biotin and folate.
11. The chemical compound of claim 10 in which the vitamin is attached to the peptide.
12. The chemical compound of claim 11 in which the vitamin-peptide is biotin-kffvlk.
13. The chemical compound of claim 11 in which the vitamin-peptide is kffvlk-biotin.
14. The chemical compound of claim 9 in which the transporter ligand is glucose or an analog of glucose that binds the glucose transporter.
15. The chemical compound of claim 1 in which the multiple copies of the plaque recognition peptide cause the chemical compound to have a dissociation constant of 1000nM or lower (i.e. stronger binding) as measured by Scatchard plot using preformed fibrils made from bA1-40.
16. The chemical compound of claim 1 in which the plaque recognition peptide is structurally altered by addition or substitution of amino acids without significantly changing the value of the dissociation constant with preformed fibrils.
17. The chemical compound of claim 2 in which the reporter element comprises a radio isotope.

18. The chemical compound of claim 17 in which the radio isotope is Tc-99m or any other metal commonly used for radio imaging appended by a chelator or any other metal binding group.
19. The chemical compound of claim 2 in which the reporter element comprises a moiety for which there exists a method for detection that is applicable to human medical practice.
20. The chemical compound of claim 3 in which the disruptor element is a β -sheet breaker.
21. The chemical compound of claim 3 in which the disruptor element is an immune system simulator.
22. The chemical compound of claim 21 in which the immune system stimulator is the peptide, N-formyl-Met-Leu-PheOH, or an analog thereof, having chemoattractant activity for phagocytic cells.
23. The use of a chemical compound according to claim 1 for the diagnosis of Alzheimer's disease in mammals.
24. The use of a chemical compound formulated according to claim 3 for the treatment of Alzheimer's disease in mammals.
25. The use of claim 23 wherein the mode of administration is selected from the group consisting of oral administration and administration by injection.
26. The use of claim 23 wherein the mammal is a human.
27. The use of claim 24 wherein the mammal is a human.

[001]

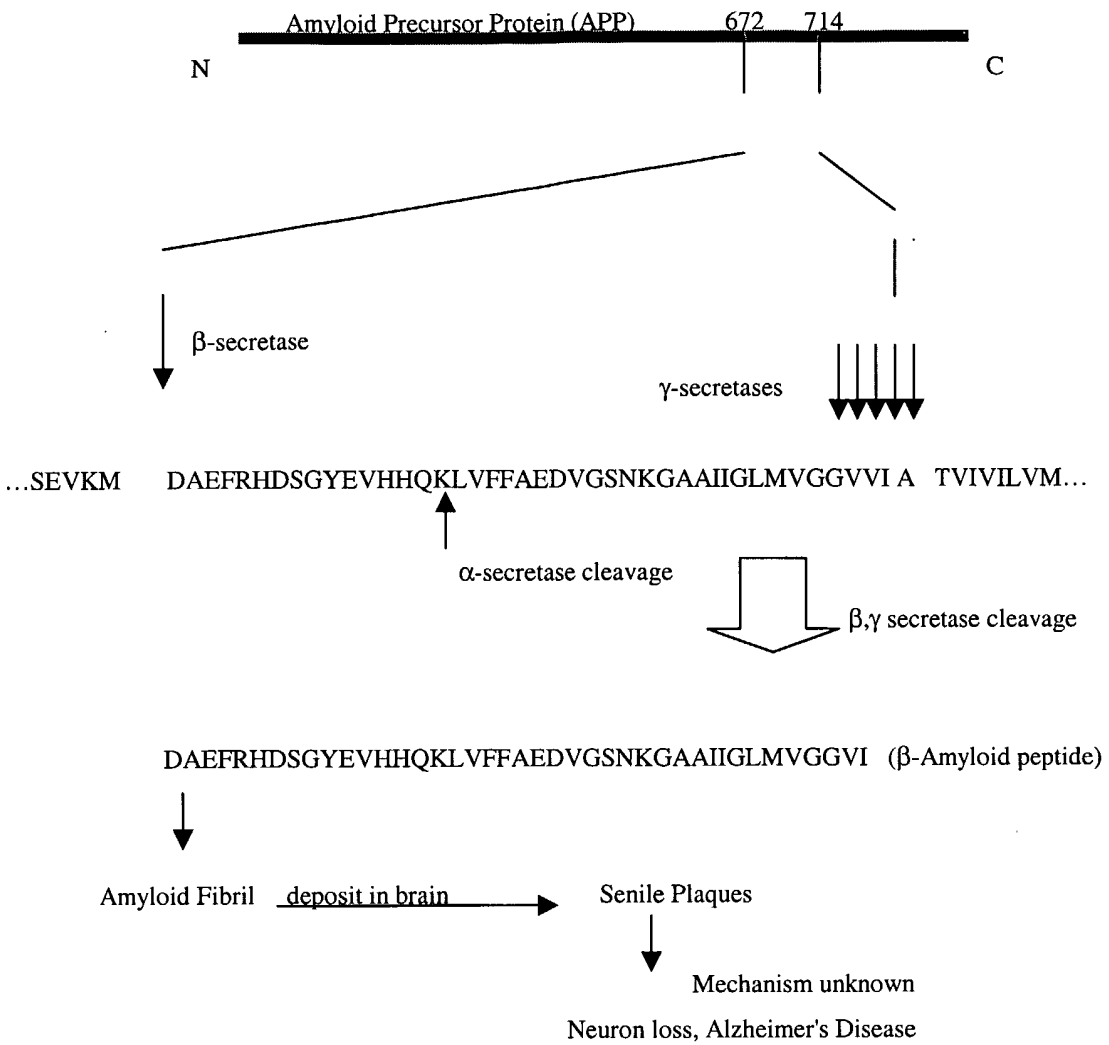


FIGURE 1

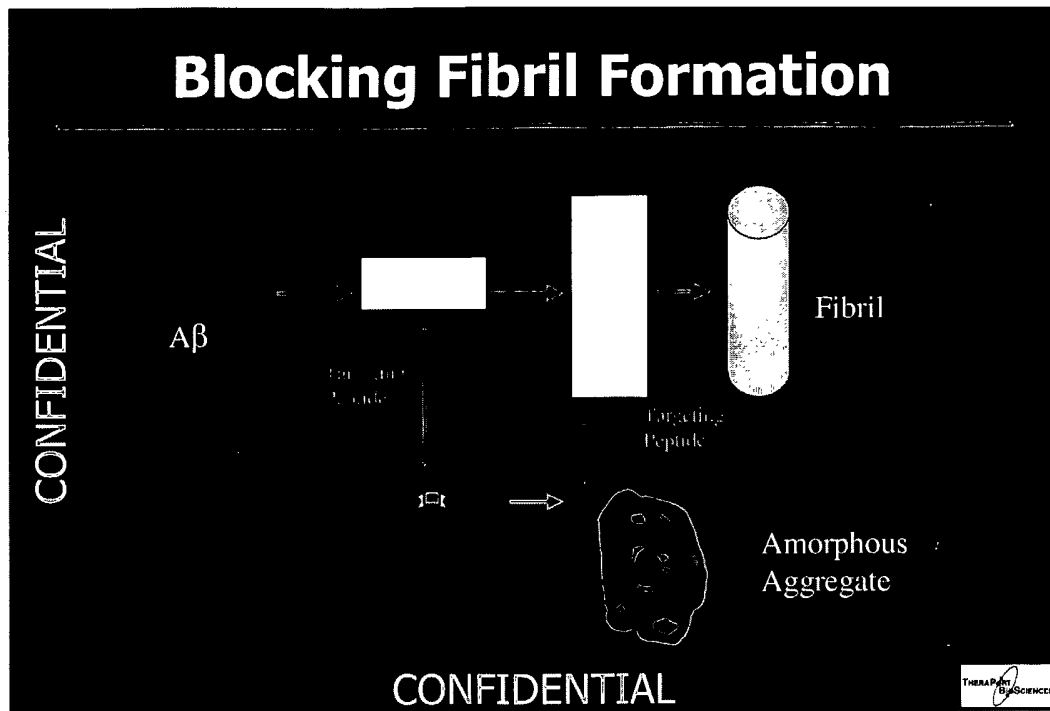


FIGURE 2

1	10	20	30	40	50	
MLPGLALLLL AAWTARALEV PTDGNAGLLA EPQIAMFCGR LNMHMHNVQNG						
51	60		70	80	90	100
KWSDSPSGTK TCIDTKEGIL QYCQEVYPEL QITNVVEANQ PVTIQNWCKR						
101	110		120	130	140	150
GRKQCKTHPH FVIPYRCLVG EFVSDALLVP DKCKFLHQER MDVCETHLHW						
151	160		170	180	190	200
HTVAKETCSE KSTNLHDYGM LLPCGIDKFR GVEFVCCPLA EESDNVDSAD						
201	210		220	230	240	250
AEEDDSVWVW GGADTDYADG SEDKVVEVAE EEEVAEVEEE EADDDDEDDED						
251	260		270	280	290	300
GDEVVEEAEE PYEEATERTT SIATTTTTTTT ESVEEVVREV CSEQAETGPC						
301	310		320	330	340	350
RAMISRWFYD VTEGKCAPFF YGGCGGNRRN FDTEEYCMAY CGSAMSQSL						
351	360		370	380	390	400
KTTQEPLARD PVKLPTTAAS TPDAVDKYLE TPGDENEHAH FQKAKERLEA						
401	410		420	430	440	450
KHRERMSQVM REWEEAERQA KNLPKADKKA VIQHFQEKVE SLEQEAAANER						
451	460		470	480	490	500
QQLVETHMAR VEAMLNDRRR LALENYITAL QAVPPRPRHV FNMLKKYVRA						
501	510	520	530	540	550	
EQKDRQHTLK HFEHVRMVDV KKAQIRSQV MTHLRVIYER MNQSLSLLYN						
551	560		570	580	590	600
VPAVAEEIQD EVDELLQKEQ NYSDDVLAMN ISEPRISYGN DALMPSLTET						
601	610		620	630	640	650
KTTVELLPVN GEFSLDDLQP WHSFGADSVF ANTENEVEPV DARPAADRGL						
651	660		670	680	690	700
TTRPGSGLTN IKTEEISEVK MDAEFRHDSG YEVHHQKLVF FAEDVGSNKG						
701	710		720	730	740	750
AIIGLMVGGV VIATVIVITL VMLKKKQYTS IHGVEVDA AVTPEERHLS						
751	760		770			
KMQQNGYENP TYKFFEQMQN						

FIGURE 3

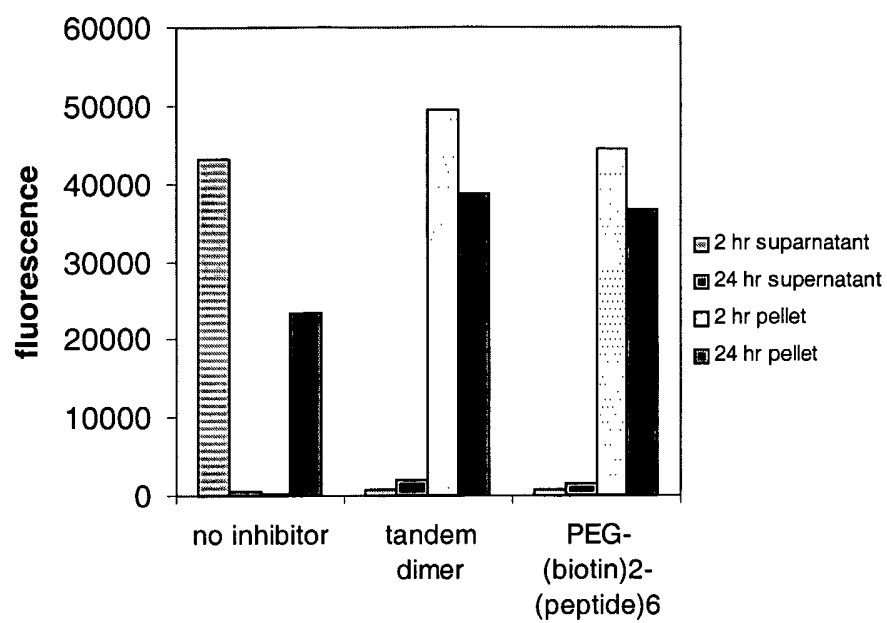
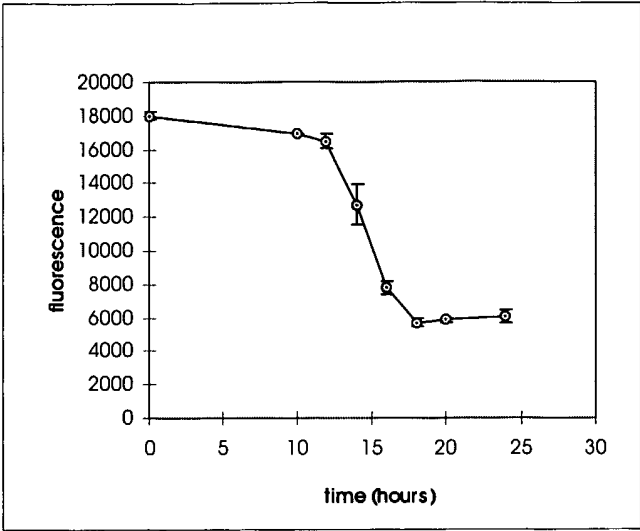
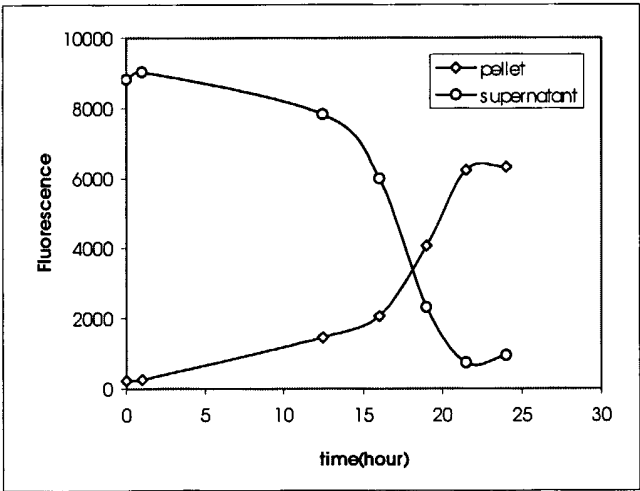


FIGURE 4

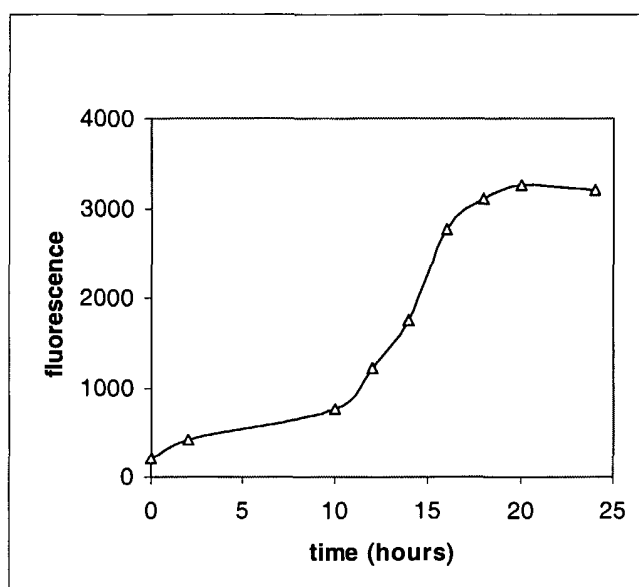
(A)



(B)



(C)



(A) Fibril formation as indicated by reduction of fluorescence in fluorescamine assay.

(B) Fibril quantified by centrifugation followed by fluorescamine assay.

(C) Fibril quantified by ThT assay.

FIGURE 5

Inhibitor	exp1	exp2	exp3	exp4	exp5	exp6	exp7	exp8
klvff	na/ 26							
klvffk	na/ 28							
KLVFF	1.45/ 68	3.1/ 60		1.7/ 60			0.92/ 58	2.1/ 60
ffvlkk ^a	0.54/ 78	1.9/ 62	1.0/ 70					
ffvlk ^a		4.27/ 60						
Ac-ffvlkk ^{a,b}			na/ 27					
Ac-kffvlkk ^{a,b}			1.0/ 70					
KLVFF-COOH ^c				na/ 45				
KKLVFF					6.0/ 56	2.75/ 55		
O ₆ G ₅ -(KKLVFF) _{2.6} ^d						0.56/ 77		
(PEG ₃₄₀₀ -D) ₆ - (KKLVFF) _{2.2} ^e					1.8/ 77	0.81/ 65		
(PEG ₂₈₂ -D) ₈ - (KKLVFF) ₂ ^e						0.75/ 68		
tandem dimer ^f							0.2/ 97	0.71/ 78
cyclic dimer ^f							0.52/ 87	
PEG _{10k8a} -biotin ₂ - peptide ₆								0.33/ 87

Notes: a.Retro-inverso peptide b. Ac = Acetyl c. Free acid, all others are C-amide d. O = Ornithine, G = Glycine e. D = aspartic acid in PEG-Asp copolymer f.

FIGURE 6