The invention relates to rapid methods for determining formation of Aβ amyloid and screening compounds which inhibit formation of Aβ amyloid in vitro, as well as kits for carrying out the present methods. Such an agent used in vivo may prevent, ameliorate or reverse the symptoms of Alzheimer’s disease and Aβ amyloidotic disorders related to Alzheimer’s disease, Down’s syndrome, and Guamanian amyotrophic lateral sclerosis/Parkinson’s dementia complex. The process described in this invention involves the rapid induction of Aβ amyloid by a heavy metal cation capable of binding to a polypeptide comprising at least amino acids 6 to 28 of Aβ, such as zinc to form amyloid and determination of formation of tinctorial Aβ amyloid. Moreover, a method of determining effectiveness of a candidate anti-amyloidotic agent for prevention or treatment of Aβ amyloidosis is described which uses cell cultures which express at least a human Aβ peptide.
Figure 1a

$K_a = 107 \text{ nM}$

$K_a = 5.2 \mu M$

BOUND Zn (II) (μM)

BOUND Zn/(II) FREE
FIG. 3a
FIG. 3b

EDTA

Cu²⁺

Zn²⁺

REMAINDER IN SUPERNATANT (\%)

0 5 10 15 20
FIG. 4a
$K_a = 3.8 \mu M$

**FIG. 5**

BOUND Zn$^{2+}$ (µM)

BOUND Zn (II)/FREE

0.2 0.18 0.16 0.14 0.12 0.1 0.08 0.06 0.04 0.02
IN VITRO SYSTEM FOR DETERMINING FORMATION OF ABETA AMYLOID

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a divisional application of U.S. application Ser. No. 08/294,819, filed Aug. 26, 1994 (now allowed), which disclosure is incorporated by reference herein in entirety.

STATEMENT REGARDING FEDERALLY-SPONSORED RESEARCH AND DEVELOPMENT

[0002] Statement under MPEP 310. The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grants Nos. RO1 NS3048-03 and RO1 AG11899-01 awarded by The National Institutes of Health (NIH).

BACKGROUND OF THE INVENTION

[0003] 1. Field of the Invention

[0004] The present invention is directed to various assays for detection of Ab amyloid, screening candidate agents for their ability to prevent or reverse the formation of Ab amyloid in vitro, as well as kits which are used in the present methods.

[0005] 2. Background Art


[0007] Soluble Ab is secreted in cell cultures and is found as a 40-residue peptide (Ab40) in the cerebrospinal fluid (CSF) (Shoji et al., Science 258:126-129 (1992); Seubert et al., Nature 359:325-327 (1992); Haas et al., Nature 359:322-325 (1992)), but is not found at elevated levels in sporadic AD cases (M. Shojo et al., Science 258:126 (1992); P. Seubert et al., Nature 359:325 (1992)). Physiological factors which can induce the aggregation of soluble Ab are of interest in determining the cause of Ab amyloid formation. Synthetic Ab40 remains soluble at concentrations up to 16 mg/ml in neutral phosphate buffer (Tomskis & Murphy, Arch. Biochem. Biophys. 294:630-638 (1992)), indicating that overproduction of soluble Ab cannot sufficiently explain Ab precipitation. Hence, biochemical mechanisms which promote Ab amyloid formation in sporadic cases would appear to be relevant to the pathogenesis of AD. Furthermore, soluble Ab in cerebrospinal fluid is not increased in AD cases (Shoji et al., Science 258:126-129 (1992)), indicating that other pathogenetic mechanisms are likely to be involved.

[0008] In recent years, the study of Ab peptide has led to making cell lines that express or overexpress Ab or its precursor protein, APP or increased amounts of its more amyloidogenic Ab42 form. See N. Suzuki et al., Science 265:1336-1340 (1994); X-D Gao et al., Science 283:516 (1993); F. S. Esch et al., Science 284:1122-1124 (1990). Moreover, monoclonal antibodies to Ab peptide have been generated (see, e.g. U.S. Pat. No. 5,231,000, issued Jul. 27, 1993). These monoclonal antibodies are useful as reagents for use in detecting presence of Ab amyloid.

BRIEF SUMMARY OF THE INVENTION

[0009] The process described in this invention involves the rapid induction of Ab amyloid by a heavy metal cation such as zinc to form amyloid. In a preferred embodiment of the invention, the proportion of an Ab40 solution which remains filterable after incubation with zinc is assayed and the effects of candidate pharmacological agents on the filtrate are measured to determine their ability to maintain the solubility of Ab in physiological solution and thus prevent Ab amyloid formation.

[0010] A method for the in vitro induction of Ab amyloid has been previously described (J. T. Jarrett et al., Biochem. J. 32:4693-4697 (1993)). However, this method has many disadvantages, such as a requirement for high concentrations of peptide and prolonged incubation periods (days) with results that are qualitative rather than quantitative. In contrast, some of the major advantages of the present invention are that the technique is reliable, rapid (can be carried out in minutes), is easily quantifiable, and is achieved with low micromolar concentrations of peptide.

[0011] Hence, the present invention relates to an in vitro method for the rapid screening of candidate reagents which are likely to be effective in preventing or reversing the formation of amyloid deposits in vivo which are characteristic of Alzheimer's disease and related pathological conditions. Promising candidate reagents which are selected through one of the in vitro methods of, N. Suzuki et al., Science 265:1336-1340 (1993), may then be tested for their effectiveness in vivo in patients which are suffering from Alzheimer's disease or who are at risk for developing Alzheimer's disease.

[0012] One aspect of the invention relates to a rapid analytical method for detection of Ab amyloid formation in a biological fluid which comprises:

[0013] (a) preparing a first set of reaction mixtures comprising neat biological fluid from a control human subject, and serial dilutions of the same made in aqueous buffer or physiological solution;
(b) preparing a second set of reaction mixtures comprising neat biological fluid from a human patient suspected of amyloidosis, and serial dilutions of the same made in aqueous buffer or physiological solution;

(c) adding an equal amount of Aβ peptide comprising at least amino acids 6 to 28 of AB to each serial dilution sample;

(d) contacting each of the first and the second set of reaction mixtures with an amount greater than 300 nM of a heavy metal cation capable of binding to an Aβ peptide comprising at least amino acids 6 to 28 of Aβ;

(e) centrifuging each of the first and the second sets of reaction mixtures to give a first and a second set of pellets, respectively; and

(f) comparing the amount of amyloid in the first and the second set of pellets and thereby detecting excessive Aβ amyloid formation in the biological fluid from the human patient suspected of amyloidosis.

A second aspect of the invention relates to a method for determining whether a compound inhibits the formation of Aβ amyloid which comprises:

(a) pre-filtering an aqueous buffer solution of Aβ peptide, which comprises at least the region in the Aβ peptide from amino acid number 6 to 28 to give a first filtrate;

(b) measuring the amount of AD peptide in the first filtrate obtained in step (a);

(c) contacting the first filtrate obtained in step (a) with a heavy metal cation capable of binding to the peptide comprising at least amino acids 6 to 28 of Aβ to give a reaction mixture;

(d) contacting the reaction mixture obtained in step (c) with a candidate anti-amyloidotic agent;

(e) filtering the reaction mixture obtained in step (d) to give a second filtrate; and

(f) comparing the amount of Aβ peptide in the second filtrate with the amount of Aβ peptide in the first filtrate, thereby determining whether the candidate compound inhibits formation of Aβ amyloid.

A third aspect of the invention relates to a method for determining whether a compound inhibits formation of Aβ amyloid which comprises:

(a) assembling a first and a second reaction mixture, wherein each reaction mixture comprises an equal amount of a pre-filtered Aβ peptide solution, which comprises at least the region in the Aβ peptide from amino acid number 6 to 28, and an aqueous buffer or physiological solution;

(b) contacting each of the first and the second reaction mixtures with an equal amount of a candidate anti-amyloidotic agent;

(c) contacting the first reaction mixture with a heavy metal cation capable of binding to the peptide comprising at least amino acids 6 to 28 of Aβ;

(d) contacting the second reaction mixture with EDTA; and

(e) comparing the amount of amyloid formed in the first reaction mixture with that in the second reaction mixture, thereby determining whether the candidate compound inhibits the formation of Aβ amyloid.

A fourth aspect of the invention relates to a method for determining whether a compound inhibits formation of Aβ amyloid which comprises:

(a) assembling a first and a second reaction mixture, wherein each reaction mixture comprises an equal amount of a pre-filtered Aβ peptide solution, which contains at least the region in the Aβ peptide from amino acid number 6 to 28, and an aqueous buffer or physiological solution;

(b) contacting each of the first and the second reaction mixtures with an equal amount of a candidate anti-amyloidotic agent;

(c) contacting only the first reaction mixture with a heavy metal cation capable of binding to the peptide comprising at least amino acids 6 to 28 of Aβ; and

(d) comparing the amount of amyloid formed in the first reaction mixture with that in the second reaction mixture, thereby determining whether the compound inhibits formation of Aβ amyloid.

A fifth aspect of the invention relates to a method for determining whether a compound inhibits formation of Aβ amyloid which comprises:

(a) establishing a first and a second cell culture comprising a cell line which expresses at least a human Aβ peptide comprising at least the region of the Aβ peptide from amino acid number 6 to 28;

(b) contacting equal concentrations of zinc to each cell culture;

(c) contacting the first cell culture with the candidate agent, and contacting the second cell culture with a heavy metal chelating agent; and

(d) comparing the amount of amyloid and zinc-induced Aβ aggregates in each cell culture, thereby determining effectiveness of the candidate anti-amyloidotic agent.

A sixth aspect of the invention relates to a method for determining whether a compound inhibits formation of Aβ amyloid which comprises:

(a) establishing a first and a second cell culture comprising a cell line which expresses at least a human AD peptide comprising at least the region of the Aβ peptide from amino acid number 6 to 28;

(b) contacting the first cell culture with zinc to give a first reaction mixture;

(c) contacting the first reaction mixture with zinc to give a second reaction mixture; and

(d) comparing the amount of amyloid and zinc-induced Aβ aggregates in each cell culture, thereby determining effectiveness of the candidate anti-amyloidotic agent.
A seventh aspect of the invention relates to a kit for determining whether a compound inhibits formation of Aβ amyloid which comprises a carrier means being compartmentalized to receive in close confinement therein one or more container means wherein

(a) the first container means contains a peptide comprising at least the region of the Aβ peptide from amino acid number 6 to 28; and

(b) a second container means contains a heavy metal cation.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1a, 1b, 1c, 1d and 1e. Analyses of 65Zn²⁺ binding to Aβ. Values shown are means ± S.D., n = 3. FIG. 1a. Scatchard plot. Aliquots of Aβ were incubated (60 min) with 65Zn²⁺ in the presence of varying concentrations of unlabelled Zn²⁺ (0.01-50 μM total). The proportion of 65Zn²⁺ binding to immobilized peptide (1.0 nmol) described two binding curves as shown. The high-affinity binding curve has been corrected by subtracting the low-affinity component, and the low-affinity curve has had the high-affinity component subtracted. FIG. 1b. Bar graph showing the specificity of the Zn²⁺ binding site for metals. Aβ was incubated (60 min) with 65Zn²⁺ (157 nM, 138,000 cpm) and competing unlabelled metal ions (50 μM total). FIG. 1c. Bar graph showing 65Zn²⁺ (74 nM, 104,000 cpm) binding to negative (aprotinin, insulin a-chain, reverse peptide 40-1) and positive (bovine serum albumin (BSA)) control proteins and Aβ fragments (identified by their residue numbers within the Aβ sequence, gln11 refers to Aβ25-35, where residue 11 is glutamine). Percent binding of total counts 65Zn²⁺/min added is corrected for the amounts (in nanomoles) of peptides adhering to the membrane. FIG. 1d. As for FIG. 1c, with Aβ(1-25) peptide substituting for Aβ(1-40). 157 nM 65Zn²⁺ (138,000 cpm) is used in this experiment to probe immobilized peptide (1.6 nmol). FIG. 1e. Graph showing the pH dependence of 65Zn²⁺ binding to Aβ(1-35).

FIGS. 2a, 2b and 2c. Effect of Zn²⁺ and other metals on Aβ polymerization using G50 gel filtration chromatography. Results shown are indicative of n=3 experiments where 55 μg of Aβ is applied to the column and eluted in 15 ml, monitored by 254 nm absorbance.

FIGS. 2a. A graph showing the chromatogram of Aβ in the presence of EDTA, 50 μM, Zn²⁺, 0.4 μM; Zn²⁺, 25 μM; and Cu²⁺, 25 μM. The elution points of molecular mass standards and relative assignments of Aβ peak elutions are indicated. Mass standards were blue dextran (2x10⁵ kDa, Vₕ, =void volume), BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa). The mass of AD is 4.3 kDa.

FIGS. 2b. Bar graph showing the relative amounts (estimated from areas under the curve) of soluble AD eluted as monomer, dimer, or polymer in the presence of various metal ions (25 μM), varying concentrations of Zn²⁺ or Cu²⁺, (the likelihood of Tris chelation is indicated by upper limit estimates), and EDTA. Data for experiments performed in the presence of copper were taken from 214 nm readings and corrected for comparison.

FIGS. 2c. Bar graph showing the effects of pre-blocking the chromatography column with BSA upon the recovery of Aβ species in the presence of zinc (25 μM), copper (25 μM), or chelator.

FIGS. 3a and 3b. Aβ binding to kaolin (aluminum silicate) effects of zinc (25 μM), copper (25 μM), and EDTA (50 μM).

FIG. 3a. Bar graph showing the concentration (by 214 nm absorbance) of Aβ remaining in supernatant after incubation with 10 μg of G50 Sephadex.

FIG. 3b. Bar graph showing the concentration (by 214 nm absorbance) of Aβ remaining in supernatant after incubation with 10 μg of kaolin, expressed as percent of the starting absorbance.

FIGS. 4a and 4b. Effect of Zn²⁺ upon Aβ resistance to tryptic digestion.

FIG. 4a. A blot of tryptic digests of Aβ (13.9 μg) after incubation with increasing concentrations of zinc (lane labels, in micromolar), stained by Coomassie Blue. Digestion products of 3.6 kDa (Aβ9-40), and 2.1 kDa (Aβ17-40), as well as undigested Aβ(1-40) (4.3 kDa), are indicated on the left. The migration of the low molecular size markers (STD) are indicated (in kilodaltons) on the right.

FIG. 4b. A bar graph showing 65Zn²⁺ binding to Aβ tryptic digestion products. The blot in a was incubated with 65Zn²⁺, the visible bands excised, and the bound counts for each band determined. These data are typical of n=3 replicated experiments.

FIGS. 5. Scatchard analysis of 65Zn²⁺ binding to rat Aβ(1-40). Dissolved peptides (1.2 nmol) were dot-blotted onto 0.20 1 PVDF membrane (Pierce) and competition analysis performed as described in Example 1 (FIG. 1). Rat Aβ(1-35) and human Aβ(1-40) were synthesized by solid-phase Fmoc chemistry. Purification by reverse-phase HPLC and amino acid sequence confirmed the synthesis. The regression line indicates a Kₘ of 3.8 μM. Stoichiometry of binding is 1:1. Although the data points for the Scatchard curve are slightly suggestive of a biphasic curve, a biphasic iteration yields association constants of 2 and 9 μM, which does not justify an interpretation of physiologically separate binding sites.

FIGS. 6a, 6b, 6c and 6d. Effect of zinc upon human, 125I-human and rat Aβ(1-40) aggregation into >0.2 μm particles. Stock human and rat Aβ(1-40) peptide solutions (16 μM) in water were pre-filtered (Spin-X, Costar, 0.2μm cellulose acetate, 700 g), brought to 100 mM NaCl, 20 mM Tris-HCl, pH 7.4 (buffer 1) i EDTA (50 μM) or metal chloride salts, incubated (50 minutes, 37° C) and then filtered again (700 g, 4 minutes). The fraction of the Aβ(1-40) in the filtrate was calculated by the ratio of the filtrate OD₂₅₄ (the response of the OD₂₅₄, titrated against human and rat Aβ(1-40) concentrations (up to 20 μM in the buffers used in these experiments), was determined to be linear) relative to the OD₂₅₄ of the unfiltered sample. All data points are in triplicate, unless indicated.

FIGS. 6a. A graph showing the proportions of Aβ(1-40), incubated +Zn²⁺ (25 μM) or EDTA (50 μM) and then filtered through 0.2 μm, titrated against peptide concentration.

FIGS. 6b. A graph showing the proportion of Aβ(1-40) (1.6 AM) filtered through 0.2 μm, titrated against Zn²⁺ concentration. 125I-human Aβ(1-40) [125I-human Aβ(1-40)] was prepared according to the method in Mantyh et al., J Neurochem. 61:1171 (1993) (15,000 CPM, the kind gift of Dr. John Maggio, Harvard Medical School) was added to unlabelled Aβ(1-40) (1.6 μM) as a tracer, incubated and filtered as...
The CPM in the filtrate and retained on the excised filter were measured by a β-counter.

[0065] FIG. 6c. A bar graph showing the proportion of $\beta_{1-40}$ (1.6 μM) filtered through 0.2μ following incubation with various metal ions (3 μM). The atomic number of the metal species is indicated.

[0066] FIG. 6d. A graph showing the effects of $\text{Zn}^{2+}$ (25 μM) or EDTA (50 μM) upon kinetics of human $\beta_{1-40}$ aggregation measured by 0.2μ filtration. Data points are in duplicate.

[0067] FIGS. 7a, 7b, 7c and 7d. Size estimation of zinc-induced $\beta_{1-40}$ aggregates.

[0068] FIGS. 7a and 7b. Bar graphs showing the proportion of $\beta_{1-40}$ (1.6 μM) in 100 mM NaCl, 20 mM Tris-HCl, pH 7.4 (buffer 1), incubated $\text{Zn}^{2+}$ (25 μM) or EDTA (50 μM) and then filtered through filters of indicated pore sizes (Durapore filters, Ultrafree-MC, Millipore) were used for this study. Therefore, there is a slight discrepancy between the values obtained with the 0.22μ filters in this study compared to values obtained in FIG. 6 using 0.2μ Costar filters.

[0069] FIG. 7c. A bar graph showing $\text{ZnCl}_2$ (130,000 CPM, 74 nM) used as a tracer of the assembly of the zinc-induced aggregates of human $\beta_{1-40}$ produced in FIGS. 7a and 7b. By determining the amounts of $\beta_{1-40}$ and $\text{Zn}$ in the filtrate, the quantities retarded by the filters could be determined, and the stoichiometry of the zinc $\beta$ assemblies estimated.

[0070] FIG. 7d. Bar graph. Following this procedure, the filters, containing $\text{Zn}$ $\beta$ aggregates, were washed with buffer 1 (100 mM NaCl, 20 mM Tris-HCl, pH 7.4)+EDTA (50 μM×300 μl, 700 g, 4 minutes). The amounts of zinc-precipitated $\beta_{1-40}$ resolubilized in the filtrate fraction were determined by OD$_{214}$, and expressed as a percentage of the amount originally retained by the respective filters. $\text{Zn}$ released into the filtrate was measured by γ-counting.

[0071] FIGS. 8a and 8b. Zinc-induced tectorial amyloid formation.

[0072] FIG. 8a. Zinc-induced human $\beta_{1-40}$ precipitate stained with Congo Red. The particle diameter is 40μ. $\beta_{1-40}$ (200 μg/25 μM in buffer 1 (100 mM NaCl, 20 mM Tris-HCl, pH 7.4)) was incubated (30 minutes, 37° C) in the presence of 25 μM $\text{Zn}^{2+}$. The mixture was then centrifuged (16,000 g×15 minutes), the pellet washed in buffer 1 (100 mM NaCl, 20 mM Tris-HCl, pH 7.4)+EDTA (50 μM), pelleted again and resuspended in Congo Red (1% in 50% ethanol, 5 minutes). Unbound dye was removed, the pellet washed with buffer 1 (100 mM NaCl, 20 mM Tris-HCl, pH 7.4) and mounted for microscopy.

[0073] FIG. 8b. The same aggregate visualized under polarized light, manifesting green birefringence. The experiment was repeated with EDTA (50 μM) substituted for $\text{Zn}^{2+}$ and yielded no visible material.

[0074] FIG. 9. A graph showing the effect of zinc and copper upon human, $\beta_{1-40}$-human and rat $\beta_{1-40}$ aggregation into >0.2μ particles. Stock human and rat $\beta_{1-40}$ peptide solutions (16 μM) in water were pre-filtered (Spin-X, Costar, 0.2μ cellulose acetate, 700 g), brought to 100 mM NaCl, 20 mM Tris-HCl, pH 7.4 (buffer 1)+EDTA (50 μM) or metal chloride salts, incubated (30 minutes, 37° C) and then filtered again (700 g, 4 minutes). The fraction of the $\beta_{1-40}$ in the filtrate was calculated by the ratio of the filtrate OD$_{214}$ (the response of the OD$_{214}$, titrated against human and rat $\beta_{1-40}$ concentrations (up to 20 μM in the buffers used in these experiments), was determined to be linear) relative to the OD$_{214}$ of the unfiltered sample. All data points are in triplicate, unless indicated. (FIG. 9). The graph shows the proportions of $\beta_{1-40}$ incubated $\text{Zn}^{2+}$ (25 μM) or Cu$^{2+}$ or EDTA (50 μM) and then filtered through 0.2 μ, titrated against peptide concentration.

[0075] FIG. 10. The amino acid sequence of human $\beta$ peptide (SEQ ID NO: 1). The amino acid sequence of human $\beta$ peptide is depicted and amino acid positions are numbered.

[0076] Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DETAILED DESCRIPTION OF THE INVENTION

[0077] $\beta_{1-40}$, a major component of Alzheimer’s disease cerebral amyloid, is present in the CSF and remains relatively soluble at high concentrations (≤3.7 mM). Thus, physiological factors which induce $\beta$ amyloid formation provide valuable clues to the pathogenesis of the disease. It has been discovered that human $\beta$ specifically and saturably binds zinc. Concentrations of zinc above 300 nM rapidly destabilize human $\beta_{1-40}$ solutions, inducing tectorial amyloid formation. Meanwhile, rat $\beta_{1-40}$ binds zinc less avidly and is immune to these effects, perhaps explaining the scarcity with which these animals form cerebral $\beta$ amyloid. Collectively, these data suggest a potentially critical role for cerebral zinc metabolism in the neuropathogenesis of Alzheimer’s disease.

[0078] Further, it has been observed that abnormalities of zinc homeostasis occur in AD and DS patients. It has now been shown that $\beta$ specifically and saturably binds zinc, manifesting high-affinity binding (K$_{d}$=107 nM) compatible with normal CSF zinc levels, and low-affinity binding (K$_{d}$=5.2 μM). Cerebral zinc homeostasis, which has been reported to be abnormal in AD (D. Wenstrup, W. R. Markesbery, Brain Res. 533:125 (1990); J. Constantinidou, Encephal. 16:231 (1990); J. M. Corrigan, G. P. Reynolds, J. H. Ward, Biomutat. 6:149 (1993); C. O. Hershley et al., Neurology 33:1350 (1983)) may be important for the metabolic fate of $\beta$ since increased concentrations of zinc promote the peptide’s adhesiveness and resistance to proteolytic digestion. Moreover, oral zinc supplementation has recently been shown to have an acutely adverse effect on cognition in AD subjects, but not age-matched controls indicating that environmental or nutritional zinc exposure may be a contributing factor to AD pathophysiology.

[0079] The present findings have indicated that $\beta$ strongly and specifically binds zinc in a pH dependent manner. In the brain milieu, these metal ions are present in sufficient concentration to exert these effects on binding and solubility. A decrease in $\beta$ solubility occurs in the presence of concentrations of zinc as low as 0.3 μM. Occupation of the zinc binding site on $\beta$ increases the resistance of the peptide to tryptic digestion at the ε-secretase site. ε-Secretase is an, as yet, unidentified protease which has been observed to cleave the precursor molecule of $\beta$, the Amy-
loid Protein Precursor (APP) within the Aβ domain, rendering Aβ incapable of accumulating. Hence, occupation of the zinc binding site on Aβ will increase the biological half-life of the peptide and so increase its availability for deposition.

Hence, pharmacological agents which prevent binding of zinc to its binding sites on Aβ or which prevent amyloid formation after Aβ has contacted excess zinc, i.e. greater than 300 nM, could be used in the treatment of Alzheimer’s disease, Down’s syndrome, and GALS/PDC by preventing and/or reversing the Aβ depositions. There currently exists no means for rapidly screening candidate anti-amyloidotic agents with therapeutic potential in these diseases. The present invention provides such means and methods for screening such candidate pharmaceutical agents.

The effects of candidate anti-amyloidotic pharmacological agents upon zinc-induced Aβ amyloid formation may be rapidly screened by the present filtration assay. The zinc-induced Aβ aggregation reaction is performed in the presence of the candidate agent, with and without (a) Aβ1-40 and (b) filtration conditions performed while varying the concentration of the drug, the zinc, and the peptide (whose concentration is brought to physiological levels by the use of 3H-Aβ). The assay may also be performed in the presence of human CSF, to bring any anti-amyloidotic effect observed to a closer in vitro approximation of the actual physiological situation.

Stock human and rat Aβ1-40 peptide solutions (16 μM) in water were pre-filtered (Spin-X, Costar, 0.2 μm cellulose acetate, 700 g), brought to 100 mM NaCl, 20 mM Tris-HCl, pH 7.4+zinc chloride (0.3 to 25 μM, sampling interval between these concentration limits), and incubated with and without (a) candidate anti-amyloidotic agent, incubated (30 minutes, 37°C) and then filtered again (700 g, 4 minutes). The fraction of the Aβ1-40 in the filtrate was calculated by the ratio of the filtrate OD234, relative to the OD234 of the unfiltered sample. The response of the OD234, titrated against human Aβ1-40 concentrations (up to 20 μM in the buffers used in these experiments) has been determined to be linear. The effect of the candidate anti-amyloidotic agent can be compared to the proportion of peptide that is filtered when the incubation is performed in the presence of EDTA (50 μM) instead of zinc.

Candidate anti-amyloidotic agents will be broad-ranging but can be classified as follows:

Agents which modify the availability of zinc for interaction with Aβ;

They include chelating agents such as desferrioxamine, but also include amino acids histidine and cysteine which bind free zinc, and are thought to be involved in bringing zinc from the plasma across the blood-brain barrier (BBB). These agents include all classes of specific zinc chelating agents, and combinations of non-specific chelating agents capable of chelating zinc such as EDTA (Edetic acid, N,N’,L,2-Ethene diyl bis[N-carboxymethylglycine] or (ethylenedinitriilo) tetraacetic acid, entry 3490 in Merck Index 10th edition) and all salts of EDTA, and/or thiotic acid [myo-Inositol hexakis(dihydrogen phosphate), entry 7269 in the Merck Index 10th edition] and phytate salts.

Solvants:

dimethyl sulfoxide has been proposed as a treatment for some forms of systemic amyloidosis; ethanol; glycine (an amino acid which has solvent properties).

Copper:

In higher concentrations, copper prevents Aβ1-40 adhering to glass and stabilizes a soluble Aβ dimer. Its effects on zinc-induced Aβ aggregation may be competitive.

Lithium Carbonate:

Lithium bromide has been used to maintain Aβ synthetic peptides in solution (Halverston et al., Biochem. 29:2639-2644 (1990)). This observation invites the speculation that lithium salts, per se, may inhibit AP aggregation. Lithium carbonate is a neuroleptic medication used in the treatment of bipolar affective disorder, where its systemic therapeutic levels are kept at 1 mM. If lithium carbonate at 1 mM has an inhibitory effect on Aβ aggregation, it would be a reasonable candidate as a therapeutic agent for AD and related pathological conditions.

Miscellaneous:

Because there is no precedent for an effective anti-amyloidotic pharmaceutical, it is reasonable to scrutinize try out compounds which may have access to the brain compartment for their ability to inhibit zinc-induced Aβ aggregation. These compounds include dye compounds, heparin, heparin sulfate, and anti-oxidants, e.g., ascorbate, trolox and tocopherols.

Definitions

Aβ peptide is also known in the art as AP, P protein, P-A4 and A4.

Amyloid as is commonly known in the art, and as is intended in the present specification, is a form of aggregated protein.

Similarly, Aβ Amyloid is an aggregated Aβ peptide. It is found in the brains of patients afflicted with AD and DS and may accumulate following head injuries and in GALS/PDC.

Tinctorial amyloid is referred to amyloid that in addition to being insoluble in aqueous buffer can be stained with Congo Red, and has positive birefringence in polarized light.

Anti-amyloidotic agent refers to a compound that inhibits formation of amyloid.

Zinc-induced Aβ aggregates are, like tinctorial amyloid, insoluble in aqueous buffer and stain with Congo Red. However, unlike tinctorial amyloid, they do not manifest positive birefringence in polarized light.

Aβ amyloidosis, as is commonly known in the art and intended in the present specification, refers to the pathogenic condition in humans and other animals which is characterized by formation of Aβ amyloid in neural tissue such as brain.

Pre-filtering and pre-filtered as used in the present specification means passing a solution, e.g. Aβ peptide in aqueous solution, through a porous membrane by any method, e.g. centrifugation, drip-through by gravitational force, or by application of any form of pressure, such as gaseous pressure.

Physiological solution as used in the present specification means a solution which comprises compounds at
physiological pH, about 7.4, which closely represents a bodily or biological fluid, such as CSF, blood, plasma, et cetera.

[0104] Heavy metal chelating agent refers to any agent, e.g., compound or molecule, which chelates heavy metals, i.e., renders the heavy metal incapable of reacting and/or binding other agents, e.g., compound or molecule. Examples of such heavy metal chelating agents are EDTA or Desferrioxamine.

[0105] In the present invention, the heavy metal salts are of any heavy metal or any transition metal, in any form, soluble or insoluble.

[0106] In the present specification, unless otherwise indicated, zinc means salts of zinc, i.e., Zn\(^{2+}\) in any form, soluble or insoluble.

[0107] Biological fluid means fluid obtained from a person or animal which is produced by said person or animal. Examples of biological fluids include but are not limited to cerebrospinal fluid (CSF), blood, serum, and plasma. In the present invention, biological fluid includes whole or any fraction of such fluids derived by purification by any means, e.g., by ultrafiltration or chromatography.

[0108] Neat sample of a biological fluid means that the biological fluid has not been altered, by for example, dilution.

[0109] Control human subject refers to a healthy person who is not afflicted with amyloidosis.

[0110] In the rapid analytical method for detection of A\(\beta\) amyloid, a biological fluid, such as CSF, serum or plasma, of a human patient who is suspected of being afflicted with amyloidosis is titrated in a serial dilution. Similarly, a control sample (biological fluid from a healthy person who is not afflicted with amyloidosis) is titrated by serial dilution. Dilutions may range from a neat (undiluted) sample up to greater than 1:10,000. It is expected that a sample from a person afflicted with amyloidosis would have a lower titre because these patients suffer from a condition which makes them significantly more prone to forming amyloid. Next, an equal amount of AD peptide in aqueous buffer or physiologcal solution is added to each sample. Then, the samples are contacted with large (greater than 300 nM), preferably 25 \(\mu\)M, of a heavy metal cation capable of binding to the peptide comprising at least amino acids 6 to 28 of A\(\beta\) peptide. The preferred heavy metal of the present invention is zinc. Hence, the A\(\beta\) peptides will form A\(\beta\) amyloid in the presence of the heavy metal cations. The A\(\beta\) amyloid can then be collected by pelleting them through centrifugation. Finally, the pellets are stained using an Amyloid-staining dye, such as Congo Red, and the pellets are observed under microscope and quantified (if desired) using a grid. Since, the biological fluid of a patient with amyloidosis (as compared with a healthy person) already has a greater propensity for formation of amyloid, and his/her biological fluid, e.g., CSF, already contains amyloid, therefore, it is expected that the amount of titrational AD amyloid in the pellets obtained from an afflicted patient be higher than the healthy control samples.

[0111] Alternatively, after the samples are titrated in serial dilutions, an equal amount of a titrated A\(\beta\) peptide, comprising at least amino acids 6 to 28 of A\(\beta\), is added to each sample. The samples can be as before centrifuged and the counts per minute determined in each pellet. Preferably, however, the samples are filtered and the CPM of the filters are determined by scintillation counter.

[0112] In the present methods for determining whether a compound inhibits formation of A\(\beta\) amyloid, the amount of soluble or precipitated A\(\beta\) peptide remaining in the reaction after exposure to zinc- or heavy metal-induced is measured and compared with the initial amount of the soluble peptide that was added to the reaction mixture, hence, demonstrating that the candidate reagent is able to inhibit formation of amyloid. Conversely, the amount of A\(\beta\) amyloid formed in the zinc- or heavy metal-induced reaction is measured and compared with the control reaction mixture containing a chelator of heavy metal cations capable of binding to the peptide comprising at least amino acids 6 to 28 of A\(\beta\), such as EDTA or Desferrioxamine, to determine whether the candidate reagent can inhibit formation of amyloid.

[0113] Further, the reaction mixtures can be filtered in order to measure and compare the amounts of the peptide or the amyloid as described above.

[0114] Conversely, the reaction mixtures can be centrifuged, the pellet stained, with for example Congo Red, and observed under a microscope to detect formation of amyloid. Moreover, the amount of amyloid formed can be quantified by using a grid.

[0115] In the present invention, the A\(\beta\) peptide may be comprised of any sequence of the A\(\beta\) peptide as long as it contains at least the amino acids corresponding to positions 6 through 28 of A\(\beta\) peptide which comprise the binding site for zinc, the most preferred heavy metal cation capable of binding to a polypeptide comprising at least amino acids 6 to 28 of A\(\beta\). The preferred embodiments of the invention make use of peptides A\(\beta\)\(_{6-28}\), A\(\beta\)\(_{14-40}\), A\(\beta\)\(_{1-41}\), A\(\beta\)\(_{1-42}\), and A\(\beta\)\(_{1-43}\). The most preferred embodiment of the invention makes use of A\(\beta\)\(_{1-40}\). However, any of the A\(\beta\) peptides which comprises at least amino acids 6 to 28 of A\(\beta\) may be employed according to the present invention. The sequence of A\(\beta\) peptide, including amino acids 6 to 28, is found in C. Hilbich et al., J. Mol. Biol. 228:460-473 (1992).

[0116] In the present method, the A\(\beta\) peptide is detected by using optical spectrophotometry. This is possible because a direct correlation exists between concentration of the peptide and OD\(_{214}\) measurements. Although the preferred wave length for the OD measurements is about 214, the measurements may be carried out for the purpose of the present invention at wave lengths from about 190 to about 440. Preferred wave lengths are, however, from about 208 to about 280.

[0117] Further, the A\(\beta\) peptide may be detected by radio-labeling the peptide and measuring the counts per minute (CPM) of the filtrates and/or the pellets. A preferred radio-labeled A\(\beta\) peptide in the present invention is \(^{3}H\)-A\(\beta\). Other radio-labels which can be used in the present invention are \(^{14}C\) and \(^{35}S\).

[0118] Conversely, one can detect the formation of A\(\beta\) amyloid formed in the reaction, using non-specific protein stains, e.g., Coomassie Blue (Bush et al., J. Biol. Chem. 269(16):12152-12158 (1994), or antibodies specific for A\(\beta\) amyloid (see, e.g. U.S. Pat. No. 5,231,000, issued Jul 27, 1993). Hence, by measuring the level of A\(\beta\) amyloid for-
mation in the reaction according to the present methods, it can be determined whether the candidate agent is effective for inhibiting formation of Aβ amyloid. The level of Aβ amyloid may be quantitated by measuring the amount of Aβ in the solubilized or precipitated fraction following centrifugation, or in the filtrate following filtration by ELISA (Suzuki ref.) or by Western Blot.

[0119] Other heavy metal cations capable of binding to a polypeptide comprising at least amino acids 6 to 28 of Aβ which may be used in the practice of the invention include metallochloride salts, preferably of zinc, copper, or mercury.

[0120] The most preferred embodiment of the invention, however, makes use of zinc chloride.

[0121] The pH of the various reaction mixtures are preferably close to neutral (about 7.4). The pH, therefore, may range from about 6.8 to about 8, preferably from about 7 to about 7.8, and most preferably about 7.4.

[0122] Buffers which can be used in the methods of the present invention include, but are not limited to, Tris-chloride and Tris-base, MOPS, HEPES, bicarbonate, Krebs, and Tyrode's. The concentration of the buffers may be about 10 mM and about 500 mM. However, considering that these buffers chelate zinc, the concentration of the buffers should be kept as low as possible without compromising the results.

[0123] The filters used in the present invention have a pore size which allows passage of Aβ peptides, e.g., from about 0.2 to about 60 microns; preferably from about 0.2 to about 8 microns; and most preferably from about 0.2 to about 0.65 microns. In a preferred embodiment of the invention, a 0.2 micron filter is used. Aβ peptide monomer has a molecular mass of 4.3 kDa. Hence, filters which can retain particles greater than 4.3 kDa, e.g., 4.4 kDa, may be used to practice the present invention. Aβ peptide and fragments can form dimers and polymers. Based on the size of the Aβ peptide used in the present invention, persons of ordinary skill in the art will be able to choose a filter with appropriate pore size so that it allows passage of Aβ peptides and prevents passage of most of all of the amyloid and Aβ aggregates induced by heavy metal cations, such as zinc-induced Aβ aggregate.

[0124] Further, any amyloid-staining dye may be used in the methods of the present invention to facilitate the determination of the deposit of amyloid and Aβ aggregates induced by heavy metal cations, such as zinc-induced Aβ aggregate, in solution. Such dyes include but are not limited to congo red, bilirubin S and bilirubin T. The concentration of such dyes may range from about 0.1% (weight/volume) to about 50% (weight/volume). In fact the upper limit of the dye concentration is limited to solubility limit of the dye in solution.

[0125] The present invention permits use of very low concentrations of Aβ peptide, e.g., from about 0.1 nM to 3.7 mM, i.e., the limit of solubility. A preferred embodiment of the invention employs about 0.8 mM concentration of Aβ peptide, a concentration of the peptide which is the lowest detected by optical density. The lowest concentrations reported previously (J. T. Jarrett et al., Biochem. 32:4693-4697 (1993)) were 20 micromolar for Aβ1-40, and 2 micromolar for Aβ1-42. Therefore, an advantage of the present invention is that very low concentrations of the peptide may be used due to the high sensitivity of the assay of the present invention.

[0126] Similarly, very low concentrations of the heavy metal cation capable of binding to a polypeptide comprising at least amino acids 6 to 28 of Aβ may be employed, e.g., from about 200 nM and up to the limit of solubility of the heavy metal cation. The most preferred heavy metal cation, zinc, may be used in the present invention at a concentration as low as about 300 nM. The lowest reported concentrations used (P. W. Mantyh et al., J. Neurochem. 61:1 171 (1993)) was 1 mM, i.e., three orders of magnitude higher than the concentration which may be used in the present invention. One of ordinary skill in the art can easily optimize the concentration of the heavy metal cation with no more than routine experimentation.

[0127] The present invention may be practiced at temperatures ranging from about 1 degree centigrade to about 99 degrees centigrade. The preferred temperature range is from about 4 degrees centigrade to about 40 degrees centigrade. The most preferred temperature for the practice of the present invention is about 37 degrees centigrade, i.e., human body temperature.

[0128] The aggregation of Aβ peptide occurs at near-instantaneous rate. Hence, results may be obtained by the present methods substantially immediately upon contacting the heavy metal and Aβ peptide. However, if desired, the reaction may be allowed to proceed longer. In a preferred embodiment of the invention, the reaction is carried out for about 30 minutes.

[0129] The invention may also be carried out in the presence of biological fluids, such as CSF, to closely simulate actual physiological conditions. The biological fluid may be added directly into the reaction mixtures or may be diluted several fold. Dilutions may range from about 1:10,000 to about 1:1 fold. The preferred biological fluid in the present invention, i.e., CSF, may be used directly or diluted from about 1:1,000 to about 1:5 fold.

[0130] The assay of the present invention is ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement therein one or more container means, such as vials, tubes, and the like, each of said container means comprising one of the separate elements of the assay to be used in the method. For example, there may be provided a container means containing standard solutions of the Aβ peptide or lyophilized Aβ peptide and a container means containing a standard solution or varying amounts of a heavy metal cation capable of binding to the peptide comprising at least amino acids 6 to 28 of Aβ peptide, in any form, i.e., in solution or dried, soluble or insoluble, in addition to further carrier means containing varying amounts and/or concentrations of reagents used in the present methods, e.g., standard solutions or varying amounts of chelators of heavy metal cations in any form, in solution or dried. Standard solutions of Aβ peptide preferably have concentrations above about 10 μM, more preferably from about 10 to about 25 μM or if the peptide is provided in its lyophilized form, it is provided in an amount which can be solubilized to said concentrations by adding an aqueous buffer or physiological solution. Standard solutions of heavy metal cations preferably have concentrations above 300 nM, more preferably about 25 μM. The standard solutions of analytes may be used to prepare control and test reaction mixtures for comparison,
according to the methods of the present invention for determining whether a compound inhibits formation of \( \beta \)-amyloid.

[0131] One \( \text{Zn}^{2+} \)-binding site in the APP ectodomain has already been described (Bush et al., *J. Biol. Chem.*, 268:16109-16112 (1993)). The possibility of additional zinc binding sites on APP was investigated. The \( \text{A}^\beta_{1-40} \) structure possesses 3 histidines and several negatively charged residues, structural features that support \( \text{Zn}^{2+} \) binding. These studies show that \( \text{A}^\beta \) binds zinc in a saturable and specific manner. Moreover, it is demonstrated that physiological concentrations of \( \text{Zn}^{2+} \) increase the resistance of the peptide to proteolytic catabolism and promote \( \text{A}^\beta \) precipitation by aluminosilicate. Based on these findings, it has been discovered that excessive zinc concentrations accelerate \( \text{A}^\beta \) deposition in AD and related pathological conditions.

[0132] Further, the effects of physiological concentrations of zinc upon the stability of synthetic human \( \text{A}^\beta_{1-40} \) in solution were studied, using the rat/mouse species of the peptide ("rat \( \text{A}^\beta \)") for comparison. Soluble \( \text{A}^\beta_{1-40} \) is produced by rat neuronal tissue (C. Haass and D. J. Selkoe, personal communication), however, \( \text{A}^\beta \) amyloid deposition is not a feature of aged rat brains (D. W. Vaughan and A. Peters, *J. Neuropathol. Exp. Neurol.* 40:472 (1981)). \( \beta \)-amyloidogenesis occurs in other aged mammals possessing the human \( \text{A}^\beta \) sequence, which is strongly conserved in all reported animal species, except rat and mouse (E. M. Johnstone, M. O. Chaney, F. H. Norris, R. Pascual, S. P. Little, *Mol. Brain Res.* 10:299 (1991)). The rat/mouse \( \text{A}^\beta \) substitutions (Arg→Gly, Tyr→Phe and His→Arg at positions 5, 10 and 13, respectively [B. D. Shivers et al., *EMBO J.* 7:1365 (1988)]) appear to cause a specific change in the peptide's physicochemical properties sufficient to confer upon the peptide its relative immunity to amyloid formation. Since zinc binding to human \( \text{A}^\beta_{1-40} \) is histidine-mediated; rat \( \text{A}^\beta \) therefore may be expected to manifest altered zinc binding properties.

[0133] The binding affinity of zinc to rat \( \text{A}^\beta_{1-40} \) was studied in a \( \text{Zn}^{2+} \)-based competitive assay system as described in Example 1 (FIG. 1), to measure the \( K_a \) of zinc binding to human \( \text{A}^\beta_{1-40} \). In contrast to human \( \text{A}^\beta_{1-40} \) the Scatchard analysis of zinc binding to rat \( \text{A}^\beta_{1-40} \) reveals only one binding association (\( K_a=3.8 \mu M \)), with 1:1 stoichiometry (FIG. 5).

[0134] It was observed that the recovery of human \( \text{A}^\beta_{1-40} \) in filtration chromatography is dramatically reduced in the presence of zinc, due, in part, to increased adsorption of \( \text{A}^\beta \). To determine whether the aggregation of human \( \text{A}^\beta_{1-40} \) is also enhanced in the presence of zinc, the peptide was incubated with various concentrations for 30 minutes with \( \text{Zn}^{2+} \) (25 \( \mu M \)) or EDTA and then filtered the solutions through 0.2\( \mu M \)-filters. Zinc caused up to 80% of the available peptide to aggregate into \( \text{A}^\beta \) particles (FIG. 6a). Incubation of \( \text{A}^\beta_{1-40} \) solutions in the filter devices, without actual filtration, indicated that there was no specific loss of peptide to the plastic or membrane surfaces.) There appears to be a shallow negative log-linear relationship between human \( \text{A}^\beta \) peptide concentration and the proportion of filterable peptide in 25 \( \mu M \) \( \text{Zn}^{2+} \), but even at the lowest concentration tested (0.8 \( \mu M \)) >70% of the human \( \text{A}^\beta_{1-40} \) solution aggregated. In contrast, the effect of \( \text{Zn}^{2+} \) on rat \( \text{A}^\beta_{1-40} \) was unremarkable, with no aggregation of a 0.8 \( \mu M \) peptide solution detected under the same conditions, and only 25% aggregation of a 4 \( \mu M \) peptide solution. Meanwhile, in the presence of EDTA, human and rat \( \text{A}^\beta_{1-40} \) solutions behaved indistinguishably, with no detectable aggregation observed at 0.8 \( \mu M \), and ~15% aggregation at higher peptide concentrations.

[0135] Next, the formation of \( \text{A}^\beta \) particles was titrated against increasing zinc concentrations (FIG. 6b), and a shallow response curve for human \( \text{A}^\beta_{1-40} \) (1.6 \( \mu M \)) was observed until the zinc concentration reached 300 nM, corresponding to the saturation of high-affinity binding. At zinc concentrations above 300 nM, corresponding to low-affinity binding, human \( \text{A}^\beta_{1-40} \) dramatically aggregates. In contrast, rat \( \text{A}^\beta_{1-40} \) remains stable in the presence of up to 10 FM zinc, and only at 25 \( \mu M \) zinc was aggregation observed.

[0136] To determine the effects of zinc on \( \text{A}^\beta_{1-40} \) at physiological peptide concentrations requires an assay more sensitive than spectroscopy. (Human \( \text{A}^\beta_{1-40} \) at 0.8 \( \mu M \) in buffer 1 corresponds to 0.090 absorbance units at 214 nm. Aggregation studies of peptides at lower starting concentrations would involve readings at the limits of sensitivity.) Thus, the effects of zinc on \( \text{A}^\beta_{1-40} \) used as a tracer in the presence of unlabeled peptide was characterized. Unlike its unlabeled precursor, \( \text{A}^\beta_{1-40} \) (at 1.6 \( \mu M \) total peptide) remained stable in the presence of increasing zinc concentrations, indicating that \( \text{A}^\beta_{1-40} \) is not a suitable tracer (FIG. 6b). The tracer is iodinated on the tyrosine residue at position 10, which is a phenylalanine in the rat peptide. Thus, the tyrosine residue may be critical to the stability of the human peptide. This data may also explain why a recent report required relatively high concentrations of \( \text{Zn}^{2+} \) (1 mM) to precipitate \( \text{Zn}^{2+} \)-human \( \text{A}^\beta_{1-40} \) in centrifugation studies (P. W. Mantyh et al., *J. Neurochem.* 61:1171 (1993)). Extrapolating the curve in FIG. 6a to 0.6 nM currently provides the best estimate of the effect of zinc upon physiological \( \text{A}^\beta \) concentrations (M. Shoji et al., *Science* 258:126 (1992); P. Seubert et al., *Nature* 359:325 (1992)), and indicates that 25% of the peptide would aggregate into >0.2\( \mu M \) particles under these conditions. The specific vulnerability of human \( \text{A}^\beta_{1-40} \) for \( \text{Zn}^{2+} \) is indicated by the observation that \( \text{Zn}^{2+} \) is the only one of several metal ions tested on an equimolar basis, including \( \text{Al}^{3+} \), to induce significant aggregation of human \( \text{A}^\beta_{1-40} \) in this system (FIG. 6c).

[0137] Next, the kinetics of the assembly of zinc-induced human \( \text{A}^\beta_{1-40} \) aggregates (FIG. 6d) was investigated. (In order to achieve time point measurements of less than 1 minute, the procedure was modified so that samples were centrifuged at 2500 g, allowing the sample volume to be completely filtered in 40 seconds.) The data obtained indicate that following the addition of stock \( \text{A}^\beta_{1-40} \) in water (15.9 \( \mu M \), pH 5.6) to \( \text{Zn}^{2+} \) (25 \( \mu M \)) in saline buffer (pH 7.4) there is a near-instantaneous aggregation of the peptide (1.6 \( \mu M \) final concentration) into filterable particles with two phases observed over two hours. The initial phase is rapid, with a half-maximal assembly rate of ~0.4 \( \mu M \) min. The steady state of the second phase is achieved within about 2 minutes, whereupon particle assembly proceeds at a rate of 3.2 \( \text{nm} \) min. with no evidence of saturation within 2 hours. At this rate, the available peptide is exhausted within five hours of initiation. Although the addition of EDTA buffer caused the near-instantaneous aggregation of 20% of the 1.6 \( \mu M \) \( \text{A}^\beta_{1-40} \) solution into >0.2\( \mu M \) particles, no further particle assem-
bly was observed over the time course of the experiment. In comparison, human Aβ40 (20 μM in PBS, pH 7.4) has been reported to be stable for 10 days (J. T. Jarrett, E. P. Berger, P. T. Lansbury, *Biochemistry* 32:4093 (1993)), and seeding the solution with Aβ1-42 (2 μM), the more amyloidogenic Aβ species, induced aggregation of this solution which was half-maximal only after 4-5 days. Thus, the results presented here represent a major advance among attempts to induce amyloid formation in vitro using the wild-type form of the main species of secreted Aβ (Aβ1-42).

[0138] To estimate the size of the AD aggregates formed in the presence of zinc, Aβ1-40 (1.6 μM) was incubated with Zn2+ (25 μM) or EDTA and then passed through filters with various pore sizes (FI. GS. 7a and 7b). Following incubation in EDTA, human Aβ1-42 assembled into populations of heterogeneous particle sizes, >0.1 μ: 47%, >0.22 μ: 40%, >0.65 μ: 32%. The comparable proportions of filtered rat Aβ1-42 particles were, >0.1 μ: 36%, >0.22 μ: 27%, >0.65 μ: 25%. Upon incubation with Zn2+ (25 μM), the proportion of >0.65 μ rat peptide particles increased only slightly, however the proportion of >0.65 μ human peptide particles dramatically increased, recruiting 82% of the available peptide. Interestingly, the proportions of <0.1 μ and >0.22 μ particles formed from the human Aβ40 also increased by 90 and 55%, respectively, following incubation with Zn2+, however, the same reaction induced only a 20% and 30% increase, respectively, in the amounts of these particles assembled from rat peptide. Remarkably, only 4% of the human Aβ40 incubated with Zn2+ remains in solution following 0.1 μ filtration. Collectively, these data indicate that the human species of Aβ1-40 differs from the rat species both in the extent and size of zinc-induced particle formation.

[0139] The stoichiometry of zinc:human Aβ in these aggregates is at least 1:1 (FI. GC), but increases to 1.3:1 with the smaller (0.1 μ) pore size filters. Because the stoichiometries for high- and low-affinity Zn:A binding are 1:1 and 2:1 respectively, these data indicate that formation of >0.65 μ Aβ aggregates is mediated by high-affinity zinc interaction, whereas low-affinity zinc interaction most likely contributes to the formation of smaller (<0.22 μ) aggregates. Interestingly, when the retained aggregates are washed with EDTA, only 22% of the peptide is recovered from >0.65 μ aggregates, although the complexed zinc (using 65Zn as tracer) is completely recovered (FI. 7d). This indicates that zinc-induced Aβ aggregation is largely irreversible by chelation. The amount of >0.22 μ peptide resolubilized by EDTA treatment is 7% greater, which may reflect the increased contribution of low-affinity zinc binding to the smaller, chelation-reversible, Aβ particle formation.

[0140] Sedimentation of zinc-induced Aβ particles by centrifugation resulted in an abundant precipitate of human Aβ1-40, which stained with Congo Red (FI. 8c) and manifested green birefringence under polarized light (FI. 8d), meeting the criteria for titinctorial amyloid formation. However, following incubation with Zn2+ under the same conditions, the rat peptide formed significantly fewer and smaller particles, with minimal birefringence. No rat Aβ amyloid was induced by Zn2+ concentrations of less than 10 μM, whereas, by titinctorial criteria, human amyloid was induced by Zn2+ concentrations as low as 3 μM. In neither case was Congo Red-stained material detected following incubation with EDTA-containing buffer.

[0141] Taken together, these data indicate that soluble human Aβ1-40 has a dramatically greater propensity than rat Aβ1-40 to form amyloid in the presence of physiological zinc concentrations. The tinctorial amyloid aggregates are frequently as large as the amorphous amyloid plaque cores purified from AD brain tissue (C. I. Masters et al., Proc. Natl. Acad. Sci. USA 82:4245 (1985)). Meanwhile, the small degree (10-20%) of >0.2 μ Aβ1-40 particle aggregate observed following the incubation of Aβ1-40 with EDTA probably reflects the relatively slow aggregation which occurs in the presence of neutral pH (S. Tomski and R. M. Murphy, *Arch. Biochem. Biophys.* 294:630 (1992)) and NaCl (H. Hilbich, B. Kisters-Wolle, J. Reed, C. I. Masters, K. Beyreuther, *J. Mol. Biol.* 218:149 (1991)). Hence, the specific vulnerability of human Aβ to zinc-induced amyloid formation is a promising explanation for aspects of the pathology of AD and related pathological conditions.

12:189 (1975) scores, after four days of zinc supplementation. This represented a deterioration which, in the ordinary course of the disease, would only be expected after two to four years (Galasko et al., JAGS 30:932 (1991)). Plasma APP levels also rose significantly in response to zinc in both the AD and the control groups. All changes were rapidly reversible following cessation of the four day supplementation. Collectively, these reports indicate that there may be an abnormality in the uptake or distribution of zinc in the AD brain. Pervasive abnormalities of zinc metabolism, and premature AD pathology, are also common clinical complications of Down’s syndrome (C. Franceschi et al., J. Ment. Defic. Res. 32:169 (1988); B. Rumble et al., N. Engl. J. Med. 320:1446 (1989)).

[0143] The data presented here indicate that stability in the presence of physiological concentrations of zinc clearly differentiates the propensity of human and rat Aβ1-40 peptide species to form amyloid. The rapid induction of tinctorial human Aβ amyloid, under physiologically relevant conditions, at peptide concentrations more than an order of magnitude lower than the lowest levels achieved previously for Aβ1-40 aggregation (in order to achieve time point measurements of less than 1 minute, the procedure was modified so that samples were centrifuged at 2500 g, allowing the sample volume to be completely filtered in 40 seconds), and within two minutes of incubation, establishes a novel assay system for the study of Aβ amyloidosis. More importantly, these findings can have profound implications for the potential role of zinc in Alzheimer-associated neuropathogenesis.

[0144] The following examples are provided by way of illustration to further describe certain preferred embodiments of the invention, and are not intended to be limiting of the present invention, unless specified.

EXAMPLES

[0145] Experimental Procedures

[0146] Unless otherwise indicated, the following experimental procedures, materials, and reagents were used in the present invention:

[0147] Reagents—Precautions taken to avoid zinc contamination included using analytical-grade reagents, electrophoresis-grade Tris-HCl (Bio-Rad), and highly deionized water. Aβ1-40 was synthesized by the Biopolymers Laboratory, MIT. Aβ1-40 (reverse peptide) was purchased from Bachem (Torrance, Calif.). Other reagents were from Sigma. Aβ1-40 and Aβ1-42 results were replicated with peptides from Bachem and Sigma. AD, 40 results were also replicable with peptide synthesized by W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University. 65Zn was purchased from Amersham Corp.

[0148] 65Zn⁺ Binding Studies—Dissolved peptides (1.2 nMol, unless otherwise stated) were dot-blotted onto 0.2-um polyvinylidene difluoride membrane (Pierce Chemical Co.), washed twice with eluting buffer (200 μl×100 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4), then five times with blocking buffer (200 μl×100 mM NaCl, 20 mM Tris-HCl, 1 mM NaCl, pH 7.4), and then incubated (60 min, 20°C) with 65Zn (unless otherwise stated 130,000 cpm, 74 mM 65ZnCl₂ in 200 μl of blocking buffer competing metal ion chloride). The dot-blot was then washed with blocking buffer (5×200 μl), the dot excised, placed in a test tube, and assayed by γ-counting (11% efficiency). The equilibration volume for stoichiometry estimates was regarded as 6×200 μl. The 214 nm UV absorbance of the unbound flow-through was assayed to determine the total amount of peptide remaining bound onto the membrane. Peptide stock concentrations were confirmed by amino acid analysis. To alter the pH, the 65Zn incubation was carried out in the presence of 100 mM buffer: MOPS (pH 6.5-7.0), MES (pH 5.0-6.0), acetate (pH 3.5-4.5). The dot-blot apparatus was washed with detergent and EDTA (50 mM) then rinsed and silica-concentrated between use.

[0149] Aβ Chromatography—Aβ (55 μg) was incubated with metal salt solution or EDTA in siliconized 1.5-ml plastic reaction vessels in 100 mM NaCl, 20 mM Tris-HCl, pH 7.4 (“TBS,” 100 μl, 1 h, 37°C). Aβ was stored in aliquots of 0.52 mg/ml in water at -20°C, then kept in 4°C when thawed. Reagents were mixed without vortex mixing. The incubated Aβ was directly applied to a G50 SF (Pharmacia, Uppsala, Sweden) column (Bio-Rad Econo-Column, 30×0.7 cm) pre-equilibrated with metal salt solution or EDTA (50 μM) in TBS at 20°C C. and eluted at 8 ml/h (Wiz peristaltic pump, Isco, Lincoln, Nebr.), Absorbance was measured at 254 and 214 nm (Type 6 optical unit, Isco). The amount of Aβ eluting at various peaks was estimated from the area under the curve. This was possible because the relationship of UV absorbance was determined to be linear over the range of Aβ dilutions used in these studies, indicating that absorbance is proportional to the amount of peptide present despite polymerization state (see below). The maximum recovery of Aβ occurs in the presence of EDTA. Because the sample eluted in a volume of approximately 15 ml, the average concentration of the peptide on the column was 0.8 μM.

[0150] To study the effects of protein blocking upon adsorption of Aβ to the chromatography column, a Sephadex G50 SF column which had been characterized previously for Aβ behavior was eluted with 3% bovine serum albumin (BSA) in TBS (50 ml) and equilibrated with 0.1% BSA-containing buffer, subsequent to repeating the Aβ experiments.

[0151] Spectroscopic Assay—Measurements were performed on a Hewlett-Packard 8452A diode array spectrophotometer using a 1-cm path length quartz cuvette. Concentration versus absorbance curves were performed at 214 nm, 254 nm, 280 nm, and full spectrum. 214 nm readings were 50-fold more sensitive in detecting the peptide than 254 nm readings, whereas the 280 nm readings of low micromolar Aβ solutions were below sensitivity limits and hence could not be used in these studies. The standard curves generated were linear at concentrations below 0.1 mg/ml. In addition, the effects of Cu²⁺, Zn²⁺, EDTA, and TBS upon absorbance were examined. At concentrations below 0.1 mg/ml, adjusting the peptide in water to TBS caused -15% quenching. Cu²⁺, Zn²⁺, and EDTA-containing Aβ solutions were studied for artificial absorbance over the linear range of the 214 nm absorbance curve. 1 mM EDTA caused 60% quenching, hence 50 μM EDTA was employed, contributing a similar degree of quenching to that observed with Cu²⁺ and Zn²⁺.

[0152] Aβ Binding to Kaolin (Aluminum Silicate)—Kaolin suspension was prepared in high performance liquid chromatography water (Fisher), defined, and adjusted to
50% (v/v). Aβ (40 μg) was incubated in siliconized reaction vessels with either kaolin or Sephadex G50 SF (10 μl, 50% (v/v)) in Cu²⁺, Zn²⁺, or EDTA (100 μl in TBS, 5 min, room temperature). The suspension was then pelleted (1500g, 3 min) and the supernatant removed and diluted 20-fold with water to bring the UV absorbance readings into the linear range. Samples were assayed at 214 nm before and after incubation with kaolin or Sephadex.

[0153] Tryptic Digestion of Aβ: Aβ (13.9 μg) was incubated with Zn²⁺ (12 μl in blocking buffer, 1 h, 37°C) and then digested with trypsin (12 ng, 3 h, 37°C). The reaction was stopped by adding SDS sample buffer containing p-nitrophenylmethanesulfonyl fluoride (1 mM), boiling the samples (5 min), and applying the samples to Tris/Tricine gel electrophoresis and transfer. The blot was washed with EDTA, Coomassie-stained, incubated with ²⁰²H, individual bands were excised, assayed for ²⁰²H binding, and N-terminal sequenced to confirm the identity of the digestion products. The effects of Zn²⁺ (up to 100 μM in TBS) on the activity of trypsin, itself, were assayed by assay of Z-Arg-amidomethylcoumarin (Sigma) fluorescent cleavage product and determined to be negligible. It was found that 200 μM Zn²⁺, however, inhibited tryptic activity by 12%.

Example 1

Analyses of ⁶⁵Zn²⁺ binding to Aβ

[0154] Aliquots of Aβ were incubated (60 min) with ⁶⁵Zn²⁺ in the presence of varying concentrations of unlabeled Zn²⁺(0.01-50 μM total). The proportion of ⁶⁵Zn²⁺ binding to immobilized peptide (1.0 nmol) described two binding curves as shown in FIG. 1a (Scatchard plot). Values shown are means ± SD, n=3. The high-affinity binding curve has been corrected by subtracting the low-affinity component, and the low-affinity curve has had the high-affinity component subtracted. (FIG. 1b) depicts specificity of the Zn²⁺ binding site for various metals. Aβ was incubated (60 min) with ⁶⁵Zn²⁺ (157 mM, 138,000 cpm) and competing unlabeled metal ions (25 μM total). (FIG. 1c) depicts ⁶⁵Zn²⁺ (74 mM, 104,000 cpm) binding to negative (aprotinin, insulin α-chain, reverse peptide 40-1) and positive (bovine serum albumin (BSA)) control proteins and AD fragments (identified by their residue numbers within the Aβ sequence, gln11 refers to Aβ1-28 where residue 11 is glutamine). Percent binding of total binding ⁶⁵Zn²⁺/min added is corrected for the amounts (in nanomoles) of peptides adhering to the membrane. (FIG. 1d) depicts as for 1a, with Aβ1-28 peptide substituting for Aβ1-40. 157 nM ⁶⁵Zn²⁺ (138,000 cpm) is used in this experiment to probe immobilized peptide (1.6 nmol). (FIG. 1e) depicts pH dependence of ⁶⁵Zn²⁺ binding to Aβ1-40.

Example 2

Effect of Zn²⁺ and Other Metals on Aβ
Polymerization Using G50 gel Filtration Chromatography

[0155] Results shown are indicative of n=3 experiments where 55 μg of Aβ is applied to the column and eluted in 15 ml. monitored by 254 nm absorbance. (FIG. 2a) chromatogram of Aβ in the presence of EDTA, 50 μM, Zn²⁺, 0.4 μM; Zn²⁺, 25 μM; and Cu²⁺, 25 μM. The elution points of molecular mass standards and relative assignments of Aβ peak elutions are indicated. Mass standards were blue dextran (2x10⁶ kDa), α-Lactalbumin (14.3 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa), and aprotinin (6.5 kDa). The mass of Aβ is 4.3 kDa. (FIG. 2b) depicts relative amounts (estimated from areas under the curve) of soluble Aβ eluted as monomer, dimer, or polymer in the presence of various metal ions (25 μM), varying concentrations of Zn²⁺ or Cu²⁺ (the likelihood of Tris chelation is indicated by upper limit estimates), and EDTA. Data for experiments performed in the presence of copper were taken from 214 nm readings and corrected for comparison. (FIG. 2c) depicts effects of pre-blocking the chromatography column with BSA upon the recovery of Aβ species in the presence of Zn (25 μM), copper (25 μM), or chelator.

Example 3

Aβ Binding to Kaolin (Aluminum Silicate): Effects of Zinc (25 μM), Copper (25 μM), and EDTA (50 μM)

[0156] (FIG. 3a) depicts concentration (by 214 nm absorbance) of Aβ remaining in supernatant after incubation with 10 mg of G50 Sephadex. (FIG. 3b) depicts concentration (by 214 nm absorbance) of Aβ remaining in supernatant after incubation with 10 mg of kaolin, expressed as percent of the starting absorbance.

Example 4

Effect of Zn²⁺ upon Aβ Resistance to Tryptic Digestion

[0157] (FIG. 4a) depicts a blot of tryptic digests of Aβ (13.9 μg) after incubation with increasing concentrations of zinc (lane labels, in micromolar), stained by Coomassie Blue. Digestion products of 3.6 kDa (Aβ1-40), and 2.1 kDa (Aβ1-7), as well as undigested Aβ1-40 (4.3 kDa), are indicated on the left. The migration of the low molecular size markers (STD) are indicated (in kilodaltons) on the right. (FIG. 4b) depicts ⁶⁵Zn²⁺ binding to Aβ tryptic digestion products. The blot in 4a was incubated with ⁶⁵Zn²⁺, the visible bands excised, and the bound counts for each band determined. These data are typical of n=3 replicated experiments.

[0158] To determine whether Aβ binds zinc, a synthetic peptide representing secreted Aβ1-40 was incubated with ⁶⁵Zn²⁺. Rapid binding (60% of ⁶⁵Zn²⁺, at 1 min), which plateaued at 1 h, was observed. Scatchard analysis of ⁶⁵Zn²⁺ binding describes two saturable binding curves, a high affinity curve (K<sub>b</sub>,<sub>off</sub> at 107 nM), and a lower affinity curve (K<sub>b</sub>,<sub>off</sub> at 52.5 μM) (FIG. 1a). The affinity constant estimates might be skewed by assuming that the Tris buffer does not bind zinc. In fact, Tris-HCl binds zinc and copper with stability constants of 4.0 and 2.6, respectively (Dawson et al., Data for Biochemical Research, Oxford University Press (1986)). Incubating Aβ in the presence of higher concentrations of Tris (150 and 500 mM) abolishes ⁶⁵Zn²⁺ binding to Aβ (~50% and ~95%, respectively), indicating that Tris-induced Zn²⁺ chelation cannot be excluded. Our calculated affinity constants are therefore upper limit estimates.

[0159] ⁶⁵Zn²⁺ binding is very specific, with Zn²⁺ being the only unlabeled metal ion tested that is capable of competing off the label (FIG. 1b). To determine the specific region of Aβ involved in zinc binding and to validate the dot-blot
The reverse sequence (40-1) control peptide only binds 50% of $B_{\text{max}}$ compared with $A_{\beta1-40}$ (FIG. 1c), indicating that zinc binding is not merely a consequence of the presence of favorable residues. $A_{\beta1-28}$ bound 30% of $B_{\text{max}}$, indicating that the carboxyl terminus plays an important role in promoting zinc binding. Glutamate substitution for the glutamate at position 11 of $A_{\beta1-28}$, in accordance with the Down’s syndrome AD sequence reported by Glennner and Wong, Biochem. Biophys. Res. Commun. 120:885-890 (1984), does not interfere with $^{65}Zn^{2+}$ binding. The Scatchard plot of $^{65}Zn^{2+}$ binding to $A_{\beta1-28}$ reveals similar lower affinity ($K_c<15 \mu M$) and higher affinity ($K_c<334 \mu M$) binding associations (FIG. 1d) to those of $A_{\beta1-40}$ but overall the $A_{\beta1-28}$ peptide binds zinc less avidly. Although the $A_{\beta1-28}$ peptide clearly binds zinc, peptides overlapping this region (1-17 and 12-28) do not individually bind zinc. Additionally, a peptide covering a region of the carboxyl terminus (25-35) is also unable to bind zinc (FIG. 1c).

The calculated stoichiometry of high-affinity $Zn^{2+}$ binding to $A_{\beta1}$, derived from the x-intercepts on the Scatchard plots (FIGS. 1u and 1d), is 0.7:1 ($A_{\beta1-40}$) and 1:4 ($A_{\beta1-28}$). For low-affinity binding, the $Zn^{2+}$: $A_{\beta}$ ratio is 2.5:1 ($A_{\beta1-40}$) and 4:1 ($A_{\beta1-28}$).

$^{65}Zn^{2+}$ binding of sequenced tryptic digestion products of $A_{\beta1}$ (FIG. 4b) indicates that the 6-40 fragment binds zinc, but that the other visible digestion fragment 17-40 (FIG. 4b), representing the post-secretase (Esch et al., Science 248:1122-1124 (1990); Sisodia et al., Science 248:492-495 (1990)) carboxy-terminal product, does not bind zinc. The contribution of histidines (residues 6, 13, and 14) to $Zn^{2+}$ binding is indicated by the deterioration of binding with lower pH (3% of $B_{\text{max}}$ at pH 6.0, FIG. 1e). Taken together, these data indicate that zinc coordination requires the contiguous sequence between residues 6 and 28, a region containing all 3 histidine residues, but optimal zinc binding also requires the presence of the carboxy-terminal domain.

Next, it was tested whether zinc binding could affect $A_{\beta1}$ conformation as assayed by migration upon gel filtration chromatography. Major $A_{\beta1}$ species believed to correspond to monomeric, dimeric, and polymeric forms were observed (FIG. 2a). Total concentrations of $Zn^{2+}$ as low as 0.4 $\mu$M decrease recoverable $A_{\beta1}$ eluting from the column when compared with the elution profile obtained in the presence of EDTA and other metals (FIGS. 2a and 2b). At 25 $\mu$M total $Zn^{2+}$, <20% of the $A_{\beta1}$ elutes. This deficit mainly affects the high order polymer and dimeric species which apparently do not enter the gel. Meanwhile, the relative amount of monomeric $A_{\beta1}$ is preserved. A systematic assessment of several metals indicates that the reduction of $A_{\beta1}$ recoverable by chromatography is most sensitive to $Zn^{2+}$, with related transition metals Co$^{2+}$, Ni$^{2+}$, and Fe$^{2+}$ (at 25 $\mu$M) displaying similar effects on chromatography to those obtained with only 10 $\mu$M $Zn^{2+}$ (FIG. 2b). Other transition metals, heavy metals, and $A_{\beta1}$ (25 $\mu$M) have partial effects on $A_{\beta}$ solubility comparable with 3 $\mu$M total $Zn^{2+}$. Meanwhile, Ba$^{2+}$, Ag$^{+}$, Mg$^{2+}$, and Cu$^{2+}$ (25 $\mu$M) have the least effect on $A_{\beta1}$ compared with the EDTA profile, although 40% less total peptide appears to elute. Pb$^{2+}$ (25 $\mu$M) most strongly promotes the elution of the monomeric peptide, abolishing high order polymers; overall recovery is similar to that obtained with 0.4 $\mu$M total $Zn^{2+}$. In making comparisons of the effects of these metal ions, it is again important to consider the differential metal ion chelating effects of Tris mentioned earlier.

A dramatic increase in $A_{\beta1}$ dimerization is observed with Cu$^{2+}$ (25 $\mu$M total). This metal also induces exaggerated $A_{\beta1}$ absorbance (4-fold) at 254 nm when compared with 214 nm absorbance and induces the monomeric species to apparently fluoresce at 254 nm causing negative readings (FIG. 2a) which are proportionally positive at 214 nm (FIG. 2b). A higher concentration of Cu$^{2+}$ (80 $\mu$M total) promotes increased recovery of $A_{\beta1}$ indicating that the presence of Cu$^{2+}$ favors solubility in this system.

The metal ions which most favored $A_{\beta1}$ solubility (Mg$^{2+}$, 25 $\mu$M and total Cu$^{2+}$, 25 $\mu$M) were tested for their ability to stabilize $A_{\beta1}$ in a soluble state in the presence of 25 $\mu$M total $Zn^{2+}$. These combinations neither rescue nor worsen $Zn^{2+}$-induced loss of $A_{\beta}$ recovery (FIG. 2b). Overall, these data suggest that $Zn^{2+}$ binding reduces the recovery of $A_{\beta1}$ whereas a chelating agent attenuates this effect.

To determine whether the zinc-induced loss of $A_{\beta1}$ during chromatography occurs because of $A_{\beta1}$ precipitation onto a surface of the apparatus, it was attempted to block the effect. Pretreating the column with 3% BSA as an adsorption blocker significantly decreases the amounts of $A_{\beta}$ recovered from the column, indicating that the peptide precipitates onto a column component (FIG. 2c). Blocking the column results in a 200% increase in the recovery of $A_{\beta}$ in the presence of $Zn^{2+}$ (25 $\mu$M total), a 75% increase in recovery in the presence of Cu$^{2+}$ (25 $\mu$M total), but only a 10% increase in the presence of EDTA (50 $\mu$M). This confirms that precipitation onto the column is most specifically accelerated by zinc.

To determine the part of the column onto which $A_{\beta1}$ was precipitating, $A_{\beta1}$ solutions were incubated with various column components and assayed $A_{\beta1}$ concentrations by UV absorption before and after the incubation. Replicating the chromatography experimental conditions, $A_{\beta1}$ (100 $\mu$M in equilibration buffer) was incubated for 1 h in plastic reaction vessels with or without the presence of Sephadex. Loss to the plastic accounts for <5% of the observed precipitation, to siliconized plastic <1%, and binding to Sephadex <1%. Hence, $A_{\beta1}$ precipitates are unlikely to be adsorbing to the Sephadex or plastic support. However, similar incubations in borosilicate glass test tubes result in 20% adsorption, which increase to 35% in the presence of zinc (25 $\mu$M).

The glass in the Bio-Rad Econo Columns is made of 7740 Pyrex (Corning, Park Ridge, Ill.) and is composed of SiO$_2$, 80.6%; B$_2$O$_3$, 13.0%; Na$_2$O, 4.0%; and Al$_2$O$_3$, 2.3%. Because of reports associating aluminosilicates with ß-amyloid deposition (Masters et al., EMBO J. 4:2757-2763 (1985a); Candy et al., Lancet 1:354-357 (1986)), experiments were carried out to test whether $A_{\beta1}$ binds to aluminum silicate. Rapid and extensive binding of $A_{\beta1}$ to kaolin, an insoluble hydrated aluminum silicate was observed. Moreover, incubation of $A_{\beta1}$ (0.4 mg/ml) with Sephadex (5%, v/v) in the presence of zinc, copper, or EDTA causes only small changes in solubility which may be attributed to binding to the plastic in the reaction vessels (FIG. 3a). Incubation of $A_{\beta1}$ (0.4 mg/ml) with kaolin (5%, v/v, 5 min, room temperature), causes precipitation of up to 87% of the peptide.
present. This precipitation is greatest in the presence of zinc (25 \mu M) where the amount of A\beta recovered from the zinc incubation supernatant is nearly half of the amount recovered from the EDTA incubation supernatant (FIG. 3b). The effect of copper (25 \mu M) upon kaolin-induced A\beta precipitation is similar to the effect of EDTA (FIG. 3b). The binding of A\beta to kaolin is not reversible to subsequent treatment with 10 mM EDTA, but can be eluted by 2 M NaOH.

To further test whether zinc induces irreversible precipitation of A\beta in the absence of kaolin, A\beta incubated with Zn^{2+} (200 \mu M, 1-24 h, 20^\circ C) was subjected to SDS Tris/Tricine gel electrophoresis. The monomeric species was the major band detected on Coomassie-stained gels and migrated identically to unincubated A\beta, indicating that zinc does not induce equimolar or SDS-resistant polymerization of A\beta.

Since the APP secretase site at Lys-16 (Esch et al., Science 248:1122-1124 (1990); Sisodia et al., Science 248:492-495 (1990)) in A\beta is within the obligatory zinc binding region, the ability of Zn^{2+} to protect A\beta from secretase-type cleavage by trypsin, a serine-protease whose activity was found to be unaffected by zinc, was next tested. Amino-terminal sequence on A\beta tryptic digestion products transferred to polyvinylidene difluoride membrane following SDS-polyacrylamide gel electrophoresis indicated two detectable fragments corresponding to residues 6-40 and 17-40 (FIG. 4a). The predicted tryptic cleavage product representing residues 29-40 did not appear on the blot and may not be retained by the polyvinylidene difluoride membrane during transfer and treatment. Digestion is inhibited by the presence of increasing concentrations of Zn^{2+}. At 200 \mu M, Zn^{2+} causes complete inhibition of A\beta hydrolysis; however, at this zinc level, tryptic activity is also slightly inhibited. Probing the blot with ssZn^{2+} confirmed the zinc binding identity of the peptide fragments and facilitated quantification of the hydrolysis of the zinc binding site (FIG. 4b). The rate of digestion of A\beta_{1-40} and the A\beta_{1-40} fragment is inhibited by the presence of zinc, whereas the digestion of the A\beta_{1-40} fragment is not inhibited by increasing zinc concentrations. Hence, only the peptides possessing the intact zinc binding domain of A\beta (residues 6-28), and therefore capable of binding Zn^{2+} (FIG. 4b), have their rates of digestion inhibited by zinc in this experiment. These data indicate that secretase-type cleavage of A\beta can be inhibited by Zn^{2+} binding to the A\beta substrate.

The above data indicate that soluble A\beta_{1-40} possesses high and low affinity zinc binding affinities. The zinc binding site on A\beta maps to residues 6-28, with possibly conformational- and histidine-dependent properties. The affinity constants for zinc binding indicate that both binding associations are within physiological zinc concentrations, but that occupancy of the low affinity binding site may be associated with accelerated precipitation of A\beta by aluminum silicate (kaolin). Occupancy of the high affinity site appears to have little effect on A\beta precipitation and is very highly specific, although the data cannot exclude the possibility of specific binding sites for alternative metals elsewhere on A\beta. Copper is a strong conformational interaction (Methylation and Fluorescence) with A\beta indicates that it may also directly interact with the peptide and may have a role in preventing A\beta precipitation onto aluminum silicate.

Extracellular zinc may play a role in the physiology of APP function by modifying its adhesiveness to extracellular matrix elements (Bush et al., J. Biol. Chem. 268:16100-16112 (1993)). This is important because APP may play a role in cell adhesiveness (Shives et al., EMBO J. 7:1365-1370 (1988)) and neurite outgrowth (Millward et al., Neuron 9:129-137 (1992)). The physiological function of the A\beta-zinc interaction remains unclear, however, increased resistance of A\beta to proteolytic cleavage in the presence of zinc would increase the peptide’s biological half-life, and increased adhesiveness may also promote its binding to extracellular matrix elements. It has been reported recently that A\beta promotes neurite outgrowth by complexing with laminin and fibronectin in the extracellular matrix (Koo et al., Proc. Natl. Acad. Sci. USA 90:4748-4752 (1993)). Hence, both APP and A\beta interact with the extracellular matrix to modulate cell adhesion. The possibility that zinc is a local environmental cofactor modulating this interaction merits further investigation.

APP is highly abundant in platelets and brain (Bush et al., J. Biol. Chem. 265:15977-15983 (1990)) where zinc is also highly concentrated (Baker et al., Thromb. Haemostasis 39:360-365 (1978); Frederickson, C. J., Int. Rev. Neurobiol. 31: 145-328 (1989)). Although APP is concentrated in vesicles in both of these tissues (Bush et al., J. Biol. Chem. 265:15977-15983 (1990); Schubert et al., Brain Res. 563:184-194 (1991)), and zinc is actively taken up (Wolf et al., Neurosci. Lett. 51:277-280 (1984)) and stored in synaptic vesicles in nerve terminals throughout the telencephalon (Perez-Clausell and Danscher, Brain Res. 337:91-98 (1985), the co-localization of APP with zinc in these vesicles has yet to be demonstrated. Vescular zinc storage is thought to play a role in stabilizing functional molecules such as NGF and insulin as insoluble intravesicular precipitates (Frederickson et al., J. Histochem. Cytotech. 35:579-583 (1987)). Zinc may similarly play a role in stabilizing APP and A\beta.

The interaction between A\beta and zinc may be compared with that of insulin, a peptide whose zinc binding properties are well characterized. Like AD, insulin exhibits histidine-dependent high affinity (Kd=150 \mu M) and low-affinity (Kd=1.2 \mu M) zinc binding with stoichiometries of 1:1 (insulin:zinc) and 1:2, respectively (Goldman and Carpenter, Biochemistry 13:4556-4574 (1974)). Additionally, metal-free insulin exhibits a pH-dependent polymerization pattern consisting of monomer, dimer, tetramer, hexamer, and higher aggregation states, in dynamic equilibrium. At neutral pH, zinc and other divalent metal ions shift the equilibrium toward the higher aggregation states. At stoichiometric ratios of Zn^{2+}:insulin in excess of 0.33, the peptide precipitates (Fredericq, E., Arch. Biochem. Biophys. 65:218-228 (1956)), reminiscent of zinc’s effects upon A\beta observed in the current studies.

Choi and co-workers (Weiss et al., Nature 338:212 (1989)) have proposed that this trans-synaptic movement of zinc may have a normal signaling function and may be involved in long term potentiation. The hippocampus is the region of the brain that both contains the highest zinc concentrations (Frederickson et al., Brain Res. 273:335-339 (1983)) and is most severely and consistently affected by the pathological lesions of Alzheimer’s disease (Hyman et al., Ann. Neurol. 20:472-481 (1986)). One of the prominent neurochemical deficits in Alzheimer’s disease is cholinergic deafferentation of the hippocampus, which has been shown to raise the concentration of zinc in this region (Stewart et al., Brain Res. 290:43-51 (1984)).

[0176] The rapid zinc-accelerated precipitation of $\beta$-amyloid (a-kinin) is significant because of the candidacy of aluminum as a pathogenic agent in AD (Perl and Brody, Science 208:297-299 (1980)). Recent reports of $\text{Zn}^{2+}$- and $\text{Al}^{3+}$-induced sedimentation of $\beta$-amyloid (Manthit et al., J. Neurochem. 61:1171-1174 (1993)), and the nucleation of $\beta$-amyloid precipitation by aluminumosilicate (Candy et al., Biochem. Soc. Trans. 21:535 (Abstract) (1992)) also support these observations.


[0178] These results indicate that abnormally high zinc concentrations increase $\beta$-amyloid resistance to secretase-type cleavage and also accelerate $\beta$-amyloid precipitation onto aluminumosilicates. Zinc-induced accumulation of $\beta$-amyloid in the neuropil may, in turn, invoke a glial inflammatory response, free radical attack, and oxidative cross-linking to form an, ultimately, “mature” amyloid. Collectively, these findings support the biochemical rationale for the chelation approach in the therapy of Alzheimer’s disease (Crapper McLaughlan et al., Lancet 337:1304-1308 (1991)), since reduction of cerebral concentrations of both aluminum and zinc could potentially decelerate the precipitation of $\beta$-amyloid.

Example 5

Scatchard Analysis of $^{65}$Zn binding to rat $\beta_{1-40}$

[0179] Dissolved peptides (1.2 nmol) were dot-blotted onto 0.20 μm PVDF membrane (Pierce) and competition analysis performed as described in Example 1 to measure the $K_a$ of zinc binding to human $\beta_{1-40}$ (FIG. 1).

[0180] In the present invention, rat $\beta_{1-40}$ and human $\beta_{1-40}$ were synthesized by solid-phase Fmoc chemistry. Purification by reverse-phase HPLC and amino acid sequencing confirmed the synthesis. The tabulated results are presented in FIG. 5. The regression line indicates a $K_a$ of 3.8 μM. Stoichiometry of binding is 1:1. Although the data points for the Scatchard curve are slightly suggestive of a biphasic curve, a biphasic iteration yields association constants of 2 and 9 μM, which does not justifiably an interpretation of physiologically separate binding sites.

Example 6

Effect of Zinc Upon Human, $^{125}$I-Human and Rat $\beta_{1-40}$ Aggregation into >0.2 μm Particles

[0181] Stock human and rat $\beta_{1-40}$ peptide solutions (16 μM) in water were prefiltered (Spin-X, Costar, 0.2 μm cellulose acetate, 700 g), brought to 100 mM NaCl, 20 mM Tris-HCl, pH 7.4 (buffer 1)±EDTA (50 μM) or metal chloride salts, incubated (30 minutes, 37°C,) and then filtered again (700 g, 4 minutes). The fraction of the $\beta_{1-40}$ in the filtrate was calculated by the ratio of the filtrate OD (the response of the OD) against rat $\beta_{1-40}$ concentrations (up to 20 μM in the buffers used in these experiments), which was determined to be linear relative to the OD of the unfiltered sample. The results are tabulated in FIG. 6. All data points are in triplicate, unless indicated. (FIG. 6a) Rat $\beta_{1-40}$ incubated ±Zn$^{2+}$ (25 μM) or EDTA (50 μM) and then filtered through 0.2 μL, titrated against peptide concentration. (FIG. 6b) Rat $\beta_{1-40}$ (1.6 μM) filtered through 0.2 μL, titrated against Zn$^{2+}$ concentration. $^{125}$I-human $\beta_{1-40}$ ($^{125}$I-human $\beta_{1-40}$ was prepared according to the method in J. E. Maggio, J. Neurochem. 69:1091-1097 (1993)). Into the $\beta_{1-40}$ in the filtrate and retained on the excised filter were measured with a γ-counter. (FIG. 6c) Rat $\beta_{1-40}$ (1.6 μM) filtered through 0.2 μL, following incubation with various metal ions (3 μM). The atomic number of the metal species is indicated. (FIG. 6d) Effects of Zn$^{2+}$ (25 μM) or EDTA (50 μM) upon kinetics of human $\beta_{1-40}$ aggregation measured by 0.2 μL filtration. Data points are in duplicate.

Example 7

Size Estimation of Zinc-Induced $\beta$-Amyloid Aggregates

[0183] (FGS 7a and 7b) Rat $\beta_{1-40}$ (1.6 μM in buffer 1 (100 mM NaCl, 20 mM Tris-HCl, pH 7.4)) was incubated ±Zn$^{2+}$ (25 μM) or EDTA (50 μM) and was then...
filtered through filters of indicated pore sizes (Durapore filters (Ultratrace-MC, Millipore) were used for this study, hence there is a slight discrepancy between the values obtained with the 0.22 μm filters in this study compared to values obtained in FIG. 2 using 0.2 μm Costar filters). (FIG. 7c) 65ZnCl2 (130,000 CPM, 74 nM) was used as a tracer of the assembly of the zinc-induced aggregates of human Aβ1-40 produced in FIG. 3a. By determining the amounts of Aβ1-40 and 65Zn in the filtrate, the quantities retarded by the filters could be determined, and the stoichiometry of the zinc: Aβ assemblies estimated. (FIG. 7d) Following this procedure, the filters, retaining Zn: Aβ assembled, were washed with buffer 1 (100 mM NaCl, 20 mM Tris-HCl, pH 7.4) and EDTA (50 μM, 300 μl, 700 g, 4 minutes). The amounts of zinc-precipitated Aβ1-40 resolubilized in the filtrate fraction were determined by OD253 and expressed as a percentage of the amount originally retained by the respective filters. 65Zn released into the filtrate was measured by y-counting.

Example 8

[0185] Zinc-Induced Tinctorial Amyloid Formation

[0186] (FIG. 8a) depicts Zinc-induced human Aβ1-40 precipitate stained with Congo Red. The particle diameter is 40μ. Aβ1-40 (200 μl, 25 μM in buffer 1 (100 mM NaCl, 20 mM Tris-HCl, pH 7.4)) was incubated (30 minutes, 37°C) in the presence of 25 μM Zn2+. The mixture was then centrifuged (16,000 g, 15 minutes), the pellet washed in buffer 1 (100 mM NaCl, 20 mM Tris-HCl, pH 7.4) and EDTA (50 μM), pelleted again and resuspended in Congo Red (1% in 50% ethanol, 5 minutes). Unbound dye was removed, the pellet washed with buffer 1 (100 mM NaCl, 20 mM Tris-HCl, pH 7.4) and mounted for microscopy. (FIG. 8b) The same aggregate visualized under polarized light, manifesting green birefringence. The experiment was repeated with EDTA (50 μM) substituted for Zn2+ and yielded no visible material.

Example 9

Effect of Zinc and Copper upon Human, 125I-human and rat Aβ1-40 aggregation into >0.2μ particles

[0187] Stock human and rat Aβ1-40 peptide solutions (16 μM) in water were pre-filtered (Spin-X, Costar, 0.2μ cellu-lose acetate, 700 g), brought to 100 mM NaCl, 20 mM Tris-HCl, pH 7.4 (buffer 1) and EDTA (50 μM) or metal chlor-ide salts, incubated (30 minutes, 37°C) and then filtered again (700 g, 4 minutes). The fraction of the Aβ1-40 in the filtrate was calculated by the ratio of the filtrate OD253 (the response of the OD253, titrated against human and rat Aβ1-40 concentrations (up to 20 μM in the buffers used in these experiments), was determined to be linear) relative to the OD253 of the unfiltered sample. All data points are in triplicate, unless indicated. (FIG. 9) A graph showing the proportions of Aβ1-40, incubated Zn2+ (25 μM) or Cu2+ EDTA (50 μM) and then filtered through 0.2μ, titrated against peptide concentration.

Example 10

Effect of Zinc upon Aβ Produced in Cell Culture

[0188] A cell culture, preferably mammalian cell culture, expressing, preferably overexpressing, human APP is estab-

lished according to well-known methods in the art, e.g. N. Suzuki et al., Science 264:1336–1340 (1994); X-D Cai et al., Science 259:514–516 (1993); F. E. Schartner et al., Science 248:1122–1124 (1990). Next, zinc is added to the culture medium to final concentration from about 200 nM to about 5 μM. Then the cell cultures, containing zinc, are incubated from about 15 minutes to as long as they can survive in the culture. Preferably, the cells are incubated for 3 to 4 days. While fresh media may be added to the cultures, no spent medium should be taken out since it contains amyloid or zinc-induced Aβ aggregates.

[0189] The media which can be used are isotonic or physiological media, at physiological pH (about 7.4). Preferably Tyrode’s buffer is used with calcium, magnesium, and potassium, as well as glucose. Any medium used must be devoid of cysteine, glutamate, aspartate, and histidine since these amino acids chelate zinc. Basically, any isotonic buffer or physiological medium which minimizes constituents which chelate zinc may be used. For example, Krebs Mammalian Ringer Solutions, in Data for Biochemical Research, 3rd Edition by Dawson et al., Oxford Science Publications, pp.446 (N.Y. 1986), and page 447 for Balanced Salt Solutions, provide recipes for making various useful media.

[0190] The constituents that should be left out are serum and the four amino acids mentioned above.

[0191] The cell culture should be incubated at about 37 degrees centigrade with air or O2/CO2 (the maximum concentration of CO2 is 5%).

[0192] Next, the cells and the medium are harvested together. A detergent such as Triton (at concentrations of about 1-2% v:v) is added and the mixture is incubated for about 3 minutes to overnight. Preferably, however, it is incubated for about 1 to 2 hours.

[0193] After incubation, the cell debris as well as amyloid and zinc-induced Aβ aggregates are pelleted by centrifuga-

tion. The pellet is suspended in pepsin (about 2%) or in any other peptidase, and it is incubated from about 1 hour to overnight to allow digestion of the cell debris.

[0194] Again, it is pelleted, washed with PBS or any other appropriate salt solution, stained with Congo Red, washed again, pelleted to remove any unbound Congo Red, and resuspended in aqueous solution. At this point, a sample can be visually inspected under a microscope. Further, it can be quantitated using a grid.

Example 11

Assay for Predicting the Effectiveness of Candidate Reagents in Cell Culture

[0195] The assay is set up in duplicate as described in Example 10. However, a candidate reagent is added to one of the two cell cultures and EDTA is added to the other cell culture. After the final step in Example 10, the amount of amyloid and zinc-induced Aβ aggregates are compared under the microscope. The probability and level of effectiveness of the candidate reagent is assessed based on the degree decrease in formation of amyloid and zinc-induced Aβ aggregates in the cell culture.
Example 12

Rapid Assay for Detection of Aβ Amyloid Formation in Biological Fluid

Cerebrospinal fluid (CSF) is obtained from a healthy human subject (control) and a human patient suspected of amyloidosis. Both samples of CSF are titrated by serial dilutions, e.g., neat, 1:2, 1:4, 1:6, ...; dilutions may be made up to 1:10,000.

To each of the samples, an equal amount of Aβ peptide in water is added to the final concentration of about 10 μM, preferably about 10 to about 25 μM.

What is claimed is:

I. A rapid analytical method for detection of Aβ amyloid formation in a biological fluid which comprises:

(a) preparing a first set of reaction mixtures comprising neat biological fluid from a control human subject, and serial dilutions of the same made in aqueous buffer or physiological solution;

(b) preparing a second set of reaction mixtures comprising neat biological fluid from a human patient suspected of amyloidosis, and serial dilutions of the same made in aqueous buffer or physiological solution;

(c) adding an equal amount of Aβ peptide comprising at least amino acids 6 to 28 of Aβ to each serial dilution sample;

(d) contacting each of the first and the second set of reaction mixtures with an amount greater than 300 nM of a heavy metal cation capable of binding to an AD peptide comprising at least amino acids 6 to 28 of Aβ;

(e) centrifuging each of the first and the second sets of reaction mixtures to give a first and a second set of pellets, respectively; and

(f) comparing the amount of amyloid in the first and the second set of pellets and thereby detecting excessive Aβ amyloid formation in the biological fluid from the human patient suspected of amyloidosis.
2. A rapid analytical method for detection of \(A\beta\) amyloid formation in a biological fluid as claimed in claim 1, wherein said biological fluid is CSF.

3. A rapid analytical method for detection of \(A\beta\) amyloid formation in a biological fluid as claimed in claim 2, wherein in step (c), said heavy metal cation capable of binding to an \(A\beta\) peptide comprising at least amino acids 6 to 28 of \(A\beta\) is zinc.

4. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid which comprises:

(a) pre-filtering an aqueous buffer solution of \(A\beta\) peptide, which comprises at least the region in the \(A\beta\) peptide from amino acid number 6 to 28 to give a first filtrate;

(b) measuring the amount of \(A\beta\) peptide in the first filtrate obtained in step (a);

(c) contacting the first filtrate obtained in step (a) with a heavy metal cation capable of binding to the peptide comprising at least amino acids 6 to 28 of \(A\beta\) to give a reaction mixture;

(d) contacting the reaction mixture obtained in step (c) with a candidate anti-amyloidotic agent;

(e) filtering the reaction mixture obtained in step (d) to give a second filtrate; and

(f) comparing the amount of \(A\beta\) peptide in the second filtrate with the amount of \(A\beta\) peptide in the first filtrate, thereby determining whether the candidate compound inhibits formation of \(A\beta\) amyloid.

5. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 4, wherein the heavy metal cation is selected from the group consisting of metalchloride salts of zinc, copper, and mercury.

6. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 4, wherein the heavy metal cation is zinc chloride.

7. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 6, wherein said \(A\beta\) peptide is selected from the group consisting of \(A\beta_{1-40}\), \(A\beta_{1-42}\), \(A\beta_{1-40}\), \(A\beta_{1-42}\), and \(A\beta_{1-43}\).

8. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 6, wherein said \(A\beta\) peptide is \(A\beta_{1-40}\).

9. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 6, wherein the pH of the reaction mixtures are between 6.8 to 7.8.

10. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 4, wherein the pH of the reaction mixtures are about 7.4.

11. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 4, wherein the concentration of the \(A\beta\) peptide is about 0.8 \(\mu\text{M}\).

12. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid which comprises:

(a) assembling a first and a second reaction mixture, wherein each reaction mixture comprises an equal amount of a pre-filtered \(A\beta\) peptide solution, which comprises at least the region in the \(A\beta\) peptide from amino acid number 6 to 28, and an aqueous buffer or physiological solution;

(b) contacting each of the first and the second reaction mixtures with an equal amount of a candidate anti-amyloidotic agent;

(c) contacting the first reaction mixture with a heavy metal cation capable of binding to the peptide comprising at least amino acids 6 to 28 of \(A\beta\);

(d) contacting the second reaction mixture with EDTA;

(e) comparing the amount of amyloid formed in the first reaction mixture with that in the second reaction mixture, thereby determining whether the candidate compound inhibits the formation of \(A\beta\) amyloid.

13. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 12, wherein the concentration of \(A\beta\) peptide in the reaction mixture is about 0.8 \(\mu\text{M}\).

14. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 12, wherein step (d) comprises the steps of:

(i) centrifuging the first and the second reaction mixtures, so that the soluble \(A\beta\) peptides are separated from the insoluble amyloid and a pellet is formed; and

(ii) comparing the amount of soluble \(A\beta\) peptide in the first reaction mixture with the soluble \(A\beta\) peptide in the second reaction mixture, thereby determining effectiveness of the candidate anti-amyloidotic agent.

15. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 12, wherein the pH of the reaction mixtures are about 6.8 to 7.8.

16. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 12, wherein the pH of the reaction mixtures are about 7.4.

17. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 14, wherein in step (ii), said pellets are stained with an amyloid-staining dye.

18. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 14, wherein said heavy metal cation is selected from the group consisting of salts of zinc, copper, and mercury.

19. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 14, wherein said heavy metal cation is a zinc salt.

20. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 12, wherein step (a), the \(A\beta\) peptide solution is prefiltered before assembling said first and second reaction mixtures; and wherein step (d) comprises the steps of:

(i) filtering the first and the second reaction mixtures, separately, and

(ii) comparing the amount of \(A\beta\) peptide in the filtrate, thereby determining effectiveness of the candidate anti-amyloidotic agent.

21. A method for determining whether a compound inhibits formation of \(A\beta\) amyloid as claimed in claim 20, wherein step (d)(ii) comprises the step of measuring the fraction of \(A\beta\) peptide in the filtrate by calculating ratio of the filtrate OD254 relative to the OD234 of the prefiltered \(A\beta\) peptide solution at step (a), thereby determining whether the compound inhibits formation of \(A\beta\) amyloid.
22. A method for determining whether a compound inhibits its formation of Ab amyloid as claimed in claim 21, wherein said Ab peptide is selected from the group consisting of Ab_{1-40}, Ab_{1-42}, Ab_{1-43}, and Ab_{1-40},

23. A method for determining whether a compound inhibits its formation of Ab amyloid as claimed in claim 21, wherein said Ab peptide is Ab_{1-40}.

24. A method for determining whether a compound inhibits its formation of Ab amyloid as claimed in claim 21, wherein all processing is done using filters with a pore size that allows passage of the soluble Ab peptide used in the reaction mixtures but does not allow passage of amyloid.

25. A method for determining whether a compound inhibits its formation of Ab amyloid which comprises:

(a) assembling a first and a second reaction mixture, wherein each reaction mixture comprises an equal amount of a prefiltered Ab peptide solution, which contains at least the region in the Ab peptide from amino acid number 6 to 28, and an aqueous buffer or physiological solution;

(b) contacting each of the first and the second reaction mixtures with an equal amount of a candidate anti-amyloidotic agent;

(c) contacting only the first reaction mixture with a heavy metal cation capable of binding to the peptide comprising at least amino acids 6 to 28 of Ab; and

(d) comparing the amount of amyloid formed in the first reaction mixture with that in the second reaction mixture, thereby determining whether the compound inhibits its formation of Ab amyloid.

26. A method for determining whether a compound inhibits its formation of Ab amyloid as claimed in claim 25, wherein the concentration of Ab peptide in the reaction mixture is about 0.8 μM.

27. A method for determining whether a compound inhibits its formation of Ab amyloid as claimed in claim 25, wherein step (d) comprises the steps of:

(i) filtering the first and the second reaction mixtures, separately, through filters with a pore size that allows passage of the soluble AP peptide used in the reaction mixtures but does not allow passage of amyloid;

(ii) comparing the amount of amyloid accumulated at step (i) on the filters, thereby determining effectiveness of the candidate anti-amyloidotic agent.

28. A method for determining whether a compound inhibits its formation of Ab amyloid as claimed in claim 12, wherein said physiological solution is CSF.

29. A method for determining whether a compound inhibits its formation of Ab amyloid as claimed in claim 25, wherein said physiological solution is CSF.

30. A method for determining whether a compound inhibits its formation of Ab amyloid which comprises:

(a) establishing a first and a second cell culture comprising a cell line which expresses at least a human AD peptide comprising at least the region of the AD peptide from amino acid number 6 to 28,

(b) contacting equal concentrations of zinc to each cell culture;

(c) contacting the first cell culture with the candidate agent, and contacting the second cell culture with a heavy metal chelating agent; and

(d) comparing the amount of amyloid and zinc-induced AP aggregates in each cell culture, thereby determining effectiveness of the candidate anti-amyloidotic agent.

31. A method for determining whether a compound inhibits its formation of Ab amyloid as claimed in claim 30, wherein said heavy metal chelating agent is EDTA or Deferoxamine.

32. A method for determining whether a compound inhibits its formation of Ab amyloid which comprises:

(a) establishing a first and a second cell culture comprising a cell line which expresses at least a human Ab peptide comprising at least the region of the Ab peptide from amino acid number 6 to 28;

(b) contacting the first cell culture with zinc to give a first reaction mixture;

(c) contacting the first reaction mixture and the second cell culture with the candidate agent;

(d) comparing the amount of amyloid and zinc-induced AO aggregates in each cell culture, thereby determining effectiveness of the candidate anti-amyloidotic agent.

33. A kit for determining whether a compound inhibits formation of Ab amyloid which comprises a carrier means being compartmentalized to receive in close confinement therein one or more container means wherein

(a) the first container means contains a peptide comprising at least the region of the Ab peptide from amino acid number 6 to 28; and

(b) a second container means contains a heavy metal cation.

34. The kit of claim 33, wherein said Ab peptide is present as a solution in an aqueous buffer or a physiological solution, at a concentration above about 10 μM.

35. The kit of claim 34, wherein said concentration is about 10 to about 25 μM.

36. The kit of claim 33, wherein said Ab peptide is present in lyophilized form.

37. The kit of claim 33, wherein said heavy metal cation is present as a metallochloride solution, at a concentration above about 300 nM.

38. The kit of claim 37, wherein said concentration is about 25 μM.

39. The kit of claim 37, wherein said heavy metal cation is zinc.

40. The kit of claim 38, wherein said heavy metal cation is zinc.

41. The kit of claim 33, further comprising

(c) one or more container means containing standard solutions of chelators of heavy metal cations.

42. The kit of claim 41, further comprising

(d) one or more container means containing standard solutions of amyloid-staining dyes.