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Selection of peptides binding to the amyloid b-protein reveals potential inhibitors of amyloid formation

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Abbreviations: *AD = Alzheimer's disease; TTR = transthyretin; FAD = Familial Alzheimer's disease; PBS = phosphate buffered saline; AβPP = amyloid beta-protein precursor; Aβ = amyloid-beta peptide*

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by extracellular amyloid plaques, cerebrovascular amyloid deposits, intracellular neurofibrillary tangles, and neuronal loss. Amyloid deposits are composed of insoluble fibers of a 39–43 amino acid peptide named the amyloid β-protein (Aβ). Neuropathological and genetic studies provide strong evidence of a key role for Aβ amyloidosis in the pathogenesis of AD. Therefore, an obvious pharmacological target for treatment of AD is the inhibition of amyloid growth and/or inhibition of amyloid function. We took an unbiased approach to generate new inhibitors of amyloid formation by screening a FliTrx^{TM} combinatorial peptide library for Aβ binding peptides and identified four groups of peptides with different Aβ binding motifs. In addition, we designed and examined peptides mimicking the Aβ binding domain of transthyretin (TTR). Our results showed that Aβ binding peptides selected from FliTrx^{TM} peptide library and from TTR-peptide analogs are capable of inhibiting Aβ aggregation and Aβ deposition in vitro. These properties demonstrate that binding of selected peptides to the amyloid β-protein may provide potent therapeutic compounds for the treatment AD.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by deposition of extracellular amyloid plaques, cerebrovascular amyloid deposition, intracellular neurofibrillary tangle formation, and neuronal loss [1]. Amyloid plaques are composed of insoluble fibers of a 39–43 amino acid peptide named the amyloid β-protein (Aβ), which is a proteolytic fragment of a large transmembrane protein, the amyloid precursor protein (AβPP) [1]. However, a soluble form of this peptide was detected at nanomolar concentrations in normal human plasma, cerebrospinal fluid, and in the conditioned media of neuronal and non-neuronal cell cultures [2–4]. These findings indicate that Aβ is a product of the normal metabolism of AβPP. Synthetic Aβ is also soluble at physiological concentrations, but appears to spontaneously aggregate at higher concentrations to form fibrils that are morphologically similar to those found in AD brain [5].

Neuropathological and genetic studies provide strong evidence of a key role for Aβ formation, aggregation and deposition in the pathogenesis of AD. Indeed, fibrillar Aβ has a toxic effect in neuronal cultures in vitro [1,6] and Aβ deposits are associated with a loss of synaptic density and loss of dendrites in the brains of transgenic mice overexpressing the V717F mutant of AβPP [7].

Furthermore, mutations in AβPP or in the presenilins that are associated with familial AD (FAD), resulted in overproduction of Aβ or in the increased content of highly amyloidogenic species of Aβ: $A\beta_{1-42}$ and $A\beta_{1-43}$ [8]. In addition to being a major genetic risk factor for late onset AD, the apoE4 protein product of the APOE4 gene strongly associates with enhanced vascular amyloid and

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plaque amyloid deposits of Aβ in the brains of AD patients [9].

Different strategies have been proposed to inhibit or prevent Aβ amyloid formation in AD [10–12]. Because Aβ is generated by cleavage of AβPP through the concerted actions of beta and gamma secretase (β- and γ-secretase), specific inhibition of these endoproteolytic enzymes are important targets for therapeutic intervention [1,13]. Alternatively, Shenk et al. [10] reported that immunization with an Aβ antigen leads to an immune response that inhibits the spontaneous formation of amyloid plaques in AβPP V717F transgenic mice. Moreover, passive immunization with peripherally administered antibodies against Aβ had similar reducing effects on Aβ deposition in this mouse model of Alzheimer disease [14]. Although the biological consequences of such immune system manipulations on the brain are not known, these results clearly indicate the possibility that vaccination with Aβ antigens may protect against the amyloid-mediated contributions to AD.

Another therapeutic strategy is based on the identification of biological molecules or chemical compounds that accelerate Aβ clearance or inhibit Aβ amyloid growth [11,15–17]. Historically, Aβ binding proteins were reported as the first putative inhibitors of Aβ aggregation in vitro [18–20]. Other studies demonstrated that transthyretin (TTR) and apolipoprotein E modulated Aβ amyloid formation in cellular systems and in transgenic animals [21–23]. Several reports described different chemical compounds that blocked Aβ aggregation or dissolved amyloid fibrils [24–27]. McLaurin et al. [28] showed that naturally occurring inositol stereoisomers interacted with Aβ in vitro and stabilized small Aβ aggregates. A similar effect has been described for short synthetic peptides, homologous to the central region of Aβ, which blocked amyloid formation [12,29–32]. Pappolla et al. [33] reported that melatonin, a hormone that crosses the blood-brain barrier, is also capable of blocking Aβ fibril formation.

Several reports show that peptide inhibitors of amyloid formation mainly derive from Aβ fragments which bind to the central part of the Aβ monomer and inhibit the elongation step of subsequent Aβ binding to itself, resulting in inhibition of Aβ polymerization [29,31,32]. However, numerous studies suggest that the actual mechanism for Aβ amyloid assembly may involve the interaction of the growing fibril with discrete intermediates, where the formation of the intermediates nor their association with growing fibrils depend upon reproducible elongation rates or nucleation rates [34–38]. Inhibition of the interactions required for these coalescing processes could also have the same net-effect on amyloid growth as direct inhibition of self-Aβ polymerization into fibrils. Therefore, the specificity of Aβ fragments needed to be used as unique inhibitors of the formation of amyloid fibrils that grow by the repetitive accretion of Aβ monomers may not effectively inhibit fibrils formed by coalescing processes. For these reasons, we have investigated non-Aβ peptides as potentially important amyloid fibril inhibitors. Here we describe Aβ binding peptides from a combinatorial FliTrx^{TM} peptide library and peptides mimicking the Aβ binding domain of TTR [39,40] that inhibit Aβ aggregation and Aβ deposition in vitro.

Methods

Isolation of Aβ-binding clones from the FliTrx^{TM} random peptide library

A$\beta_{1–42}$ (US peptide) was diluted in deionized H_2O, sonicated for 15 s, filtered through Millex GV 0.22 mm filters (Millipore) and immediately added into a 60-mm tissue culture plate (Nunc) at a concentration of 50 μg/ml for 2 h at room temperature. After washing, plates were blocked with IMC medium containing 100 μg/ml ampicillin, 1% nonfat dry milk, 150 mM NaCl, 1% α-methyl mannoside. Screening of an amplified dodecapeptide FliTrx^{TM} random peptide library (1.77×10^8 primary clones, Invitrogen) was performed as recommended by the manufacturer (Invitrogen, Manual 160304). We panned 10^{10} bacterial clones in the first round and after five rounds of panning with immobilized A$\beta_{1–42}$, we picked 50 random clones that bound immobilized Aβ and sequenced them with the FliTrx^{TM} forward sequencing primer 5′ ATTCACCTGACTGACGAC 3′.

E. coli attachment assay

One hundred microliters of stock solution of A$\beta_{1–42}$ in deionized H_2O was sonicated for 15 s and filtered through Millex GV 0.22 mm filters (Millipore). Diluted A$\beta_{1–42}$ was coated directly onto polyvinyl 96-well plates (Costar) at a concentration of 20 ng/well in PBS (150 mM NaCl/10 mM Na phosphate buffer, pH 7.4) for an overnight at 4°C. To obtain fibrillar Aβ, 100 μM stock solution of A$\beta_{1–42}$ was incubated for 36 h at 37°C in PBS with separation of Aβ fibrils from Aβ-monomers and Aβ-oligomers by centrifugation through a 20% sucrose pad (15,000 × g, 10 min). Fibril formation in the pellet was monitored by electron microscopy. Fibrillar Aβ was coated on 96-well plates (Costar) at a concentration 100 ng/well. The wells were blocked with PBS containing 2% bovine serum albumin for 3 h at room temperature. Selected

E. coli clones (10^4 colony forming units/well) were added to the wells in PBS containing 2% bovine serum albumin and incubated for 1 h at room temperature in orbital shaker. After extensive washing, bacterial cells were detached by mechanical shearing and plated in different dilutions.

Aβ Binding assay

Polyvinyl 96-well plates (Costar) were coated with synthetic peptides (100 μg/ml) (Biosynthesis) in PBS buffer overnight at 4°C. After aspirating excess peptide solution, wells were blocked with PBS containing 2% bovine serum albumin and 1% gelatin for 3 h at room temperature. One hundred microliters of $A\beta_{1-42}$ (100 μM) in the same blocking solution was added to each of the wells. Following 2 h incubation at room temperature, the wells were washed five times with PBS. Peptide-bound $A\beta_{1-42}$ was detected by incubating with the monoclonal anti- Aβ antibody 4G8 (Senetek, 1:1000 dilution, 2 h, room temperature) followed by washing the plate 5x with PBS, and then adding a peroxidase-conjugated goat anti-mouse antibody (Sigma, 1:3000 dilution) and incubating for 1 h. After washing away non-bound secondary antibody-peroxidase conjugate, peroxidase-catalyzed color development was initiated using color developer (Bio-Rad) and quantified at 450 nm in a microplate reader. The relative amount of $A\beta_{1-42}$ bound in the wells was calculated as being the difference in optical density between the experimental and control wells where no synthetic peptide was added.

Congo red based aggregation assay

One hundred microliters of $A\beta_{1-42}$ (US peptide) in deionized H_2O was sonicated for 15 s, filtered through Millex GV 0.22 mm filters (Millipore), and incubated for 24 h at 37°C in PBS alone or with synthetic peptides (1:2 molar ratio $A\beta_{1-42}$/synthetic peptide). To control for self-aggregation, 50 μM of each binding peptide in deionized H_2O was sonicated for 15 s, filtered through Millex GV 0.22 mm filters (Millipore), and incubated for 24 h at 37°C in PBS. Fibril formation was monitored by electron microscopy. After incubation, 150 μl of PBS and 2 μl of Congo Red (5 μM) were added to each sample followed by additional incubation at room temperature in the dark for 10 min. The microplate was spun at 3000 rpm for 40 min, supernatant was discarded and absorbance of red pellets was measured in a spectrophotometer at 490 nm. The percent of absorbance was over a range from 0%, where Congo Red only was added to a microplate well, to 100% where Congo Red and Aβ peptide in buffer was added to a microplate well. Percent of absorbance was determined by dividing the average absorbance of duplicate samples to the average absorbance of quadruplicate wells containing Congo Red plus Aβ and multiplying times 100%. Self-aggregation of binding peptides was not observed under these conditions except for very weak self-aggregation of peptide 7-sh, which was less than 10% of the signal generated from aggregation of Aβ alone.

Electron microscopy

Samples containing 100 μM $A\beta_{1-42}$ were incubated for 36 h at 37°C in PBS alone or with synthetic peptides (1:2 molar ratio Aβ/synthetic peptide). After incubation, samples were sonicated for 20 s, applied to carbon-coated copper grids, negatively stained with uranyl acetate, and visualized with JEOL 200c× electron microscope operating at 80 kV. Samples were examined and photographed at magnification of 60,000.

Radioiodination

$A\beta_{1-40}$ was labeled with ^{125}I using the chloramine T procedure. In brief, 1 μl of a 1 mM peptide solution in water (1 nmole) was mixed with 10 μl of 1 M potassium phosphate buffer, pH 7.4. Two microliters of $Na^{125}I$ (0.2 mCi, Amersham,) and 10 μl chloramine T (1 mg/ml in phosphate buffer) were mixed with peptide solution and incubated for 30s. The reaction was stopped by adding 10 μl of 1 M $Na_2S_2O_3$ (Merck). Labeled peptides were purified by HPLC and stored at −20°C in a solution containing 20% acetonitrile, 0.1% trifluoroacetic acid and 0.5% 2-mercaptoethanol.

Deposition of ^{125}I-Aβ onto synthaloid

For the analysis of Aβ deposits, we used the method described by Esler et al. (1997) [27]. Briefly, 200 μM stock solution of $A\beta_{1-42}$ (US peptide) in deionized H_2O was sonicated for 15 s and filtered through Millex GV 0.22 mm filters (Millipore). One hundred microliters of stock solution of $A\beta_{1-42}$ was incubated for 36 h at 37°C in PBS and Aβ fibrils separated from Aβ-monomers and Aβ-oligomers by centrifugation through a 20% sucrose pad (15,000 × g, 10 min). Fibril formation was monitored by electron microscopy. To produce synthetic amyloid (synthaloid), aggregated Aβ in PBS was diluted in 0.1% gelatin (57°C) to yield concentrations of 1–10 μg Aβ peptide/ml. Aβ/gelatin suspension was aliquoted to 96-well plates (Dynatech laboratories 001-010-2401) at 1 μg Aβ per well. Plates containing Aβ/gelatin were dried overnight at 57°C. Synthaloid (in 96-well plate) was preincubated with buffer (50 mM Tris-HCl, 0,1% BSA, pH 7.5) for 1 h. The preincubation buffer was

removed and ^{125}I-Aβ (10 pM) in Tris-HCl buffer was added to each well in the presence or absence of tested peptides. Following incubation (4 h), the Aβ solution was removed and each well was washed with buffer (5×3 min). The wells were capped and separated, and the amount of ^{125}I-Aβ deposited was quantified by γ-counting. The amount of ^{125}I-Aβ bound to the prepared control wells (gelatin solution without Aβ aggregates) was measured as a background control and subtracted from sample values bound to Aβ/gelatin-synthaloid to give a corrected number of counts bound per well.

Aβ accumulation in smooth muscle cells

Cultures of smooth muscle cells were obtained from leptomeningeal blood vessel walls of old dogs as previously described [21]. Briefly, vascular smooth muscle cells were isolated by enzymatic digestion from dissected leptomeningeal vessels. Vessels were digested in 0.1% collagenase type 1A (Sigma) in D-MEM (BioWhittaker) for 30 min at 37°C to remove connective tissue. Subsequent digestion in 0.1% collagenase and 0.025% elastase (Sigma) in D-MEM for 1 h at 37°C yielded isolated cells. Cells were transferred to 75 cm^2 flasks (Falcon) in HEPES-buffered D-MEM with 10% fetal bovine serum (FBS). Cultures used for experiments were between four and eight passages, and contained at least 95% myocytes. Aβ-immunoreactive material was detected in formalin-fixed cultured cells by the immunoperoxidase method with mouse monoclonal anti-Aβ antibody 4G8. The specificity of the reaction was confirmed by preabsorbtion with synthetic Aβ. The percentage of cells with multiple (at least 10) intracytoplasmic Aβ-immunoreactive granules was evaluated in 200–500 cells per experimental condition. Synthetic peptides were obtained from US peptide and Biosynthesis and plasma TTR was from Calbiochem.

Results

Selection of peptides binding to Aβ_{1-42} from FliTrx^{TM} display peptide library

In an effort to identify peptides binding to Aβ_{1-42}, we undertook the screening of the FliTrx^{TM} random peptide library (Invitrogen), which displays 12-amino acid long peptides on the surface of E. coli inserted into the major bacterial flagellar protein, FliC. E. coli clones expressing Aβ_{1-42}–binding peptides were affinity purified using immobilized synthetic Aβ_{1-42} in five rounds of panning. Fifty bacterial clones were randomly picked after the fifth panning and the insert of each clone was sequenced. Table I lists the amino acid sequences of four groups of peptides that yielded consensus-binding motifs.

As seen in Table I, Group 1 clones exposed a consensus sequence best described as "++Hy+(Hy/+)", where + is a positively charged amino acid (most frequently arginine) and Hy is a hydrophobic amino acid. Consensus sequence of Group 2 clones represents the motif "++XX+", where + is a positively charged amino acid and X is a hydrophobic or polar amino acid. Group 3 clones revealed the consensus sequence "(D/E)LVHy" (Hy is a hydrophobic amino acid, preferentially leucine). Group 4 clones displayed the consensus sequence "+LVLF", where + is arginine or lysine). Groups 1 and 2 are enriched by positively charged amino acids.

Table I. Aβ binding peptides selected from FliTrx^{TM} display peptide library.

ID#	
	Group 1
1	R A R P **R K V R P** T A T
2	**R R T H P** W V L N V G A
3	G R P L D G **K R A R V** L
4	**K K V R R** L C N I P F G
5	G L R M R **R K I R R** V P (3)
6	L K **R H N R R** Q T I Y E
CS	**+ + Hy + (Hy/+)**
	Group 2
7	N G **R H V L R** P K V Q A (2)
8	V **R H V L P** K V Q A P V (3)
9	I H H **R R G V T** L K R Q
10	W E R **G K T F R** G R A L
11	G E V R **R K S E K** M R F
12	T R A **R R L A R** Q D S V
13	R **N R I G R** I R S C A V
14	V A **R R S G K** S R I G H
15	C C **H K L T R** L R N D F
CS	**+ + X X +**
	Group 3
16	E Q C **D L V L G** G Y Y A
17	V R A R K G **D L P R L** L
18	G Y L I G **E L V L R** A A
19	S L D **E I L A V** I F G L
20	R K E A **E N V P L** V A K
21	S L R T G R **D L V P L** A (2)
22	R R K **D I V V W** V C L N
23	G G **D L V L A** V Y N A A
24	V P G T L N K **D V V L L**
CS	**(D/E) L V X Hy**
	Group 4
25	Y L K D Y R **R L V L F** G
26	K E G V G **K R V A F** N H
27	A A **R L V L F** G I L Y G (2)
28	V C Y **I L S L F** G H N A
CS	**+ L V L F**

Residues within the peptides that match the suggested consensus are shown in bold typeface. The numbers of identified clones encoding the same peptide are shown in parentheses.

Consensus sequences consist of two groups of positively charged amino acids divided by one hydrophobic or polar amino acids in Group 1 or by two hydrophobic or polar amino acids in Group 2. Identification of Aβ binders with a high content of positively charged amino acids is not an unexpected result. It should be noted that the presence of hydrophobic or polar amino acids are important for interaction of peptides with Aβ. However, 8-mer synthetic peptides containing just positively charged amino acids (arginine or lysine) failed to bind Aβ (data not shown).

To determine the affinity of selected clones to monomeric versus fibrillar Aβ, we performed an additional panning with low concentrations of monomeric or fibrillar Aβ_{1-42} (100 ng /well, Figure 1). As seen in Figure 1A, each of the selected clones bound to immobilized monomeric Aβ_{1-42}. However, binding strength had significant variations between clones in each group. In Group 1, clones expressing peptides KKVRRLCNIPFG, GLRMRRKIRRVP, and LKRHNRRQTIYE revealed the strongest binding while clones expressing peptides RARPRKVRPTAT, RRTHPWVLNVGA, and GRPLDGKRARVL bound poorly. Analysis of consensus sequences of these peptides shows that substitution of the last arginine for any hydrophobic amino acid in the core motif: ++Hy+(Hy/+), significantly decreased binding to monomeric Aβ_{1-42}. In Group 2, very low binding was detected for clones expressing peptides IHHRRGVTLKRQ, WERGKTFRGRAL, and RNRIGRIRSCAV. However, in this group we did not observe a strong correlation between amino acid residues in the consensus sequence and binding strength. Substitution of any hydrophobic amino acid with arginine in the consensus motif: D/ELVHy of Group 3 resulted in a significant reduction of binding. In Group 4, the strongest binding was observed for two different clones with a consensus sequence of RLVLF. Flanking residues did not change the binding, but substitutions of the first arginine or the second leucine in the consensus sequence blocked binding of clones to Aβ.

Surprising results were obtained for binding of selected clones to fibrillar Aβ_{1-42} (Figure 1B). Most clones exhibited very low binding to fibrillar Aβ_{1-42} when compared to binding of monomeric Aβ. Only two related clones from Group 2 which expressed the sequences NGRHVLRPKVQA and VRHVLPKVQAPV were found to bind to be preformed Aβ fibrils.

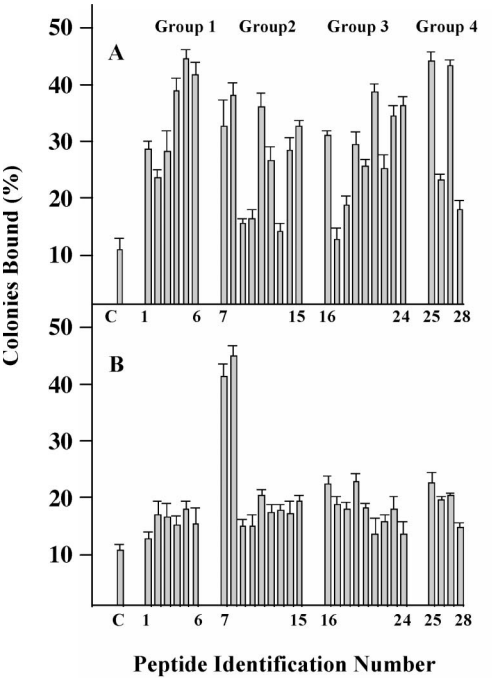

Figure 1. Binding strength of bacterial expression clones selected from the FliTrx^{TM} random peptide library. Panel A: Relative binding of selected E. coli clones to monomeric Aβ. Panel B: Relative binding of selected E. coli clones to fibrillar Aβ. Selected E. coli clones (10^4 colony/well) were added individually to the wells coated with monomeric or fibrillar Aβ_{1-42}. To obtain fibrillar Aβ, 100 μM stock solution of Aβ_{1-42} was incubated for 36 h at 37°C in PBS and Aβ fibrils separated from Aβ-monomers and Aβ-oligomers by centrifugation through the 20% sucrose pad (15,000 × g, 10 min). Fibril formation was monitored by electron microscopy. The wells were blocked with PBS containing 2% bovine serum albumin for 3 h at room temperature. Selected E. coli clones (10^4 colony/well) were then added to the wells in PBS containing 2% bovine serum albumin and incubated for 1 h at room temperature with orbital shaking. After extensive washing, bacterial cells were detached by mechanical shearing and plated in different dilutions. Colonies were counted using a ChemiDoc System (Bio-Rad). The data represent means ± SE from triplicate wells. Control binding of non-specific E. coli clones to the well coated with monomeric or fibrillar Aβ_{1-42} is labeled with a "C". Each peptide number coincides with peptide identification numbers in Table I.

Aβ binding peptides inhibit Aβ aggregation

To verify Aβ-binding motifs of peptides from selected expression clones, we synthesized shortened peptides which correspond to the consensus sequence motif found in the full sequence expressed in each clone for all Aβ-binders selected from FliTrx^{TM} library. These synthetic peptides are listed in Table II.

All shortened consensus peptides were analyzed for their ability to bind Aβ and to inhibit Aβ aggregation and fibril formation (Figure 2). Anti-amyloidogenic potential of shortened consensus peptides was compared with a previously described inhibitor of amyloid formation, the peptide KLVFF (Aβ_{16-20} fragment) which spans residues 16–20 of the Aβ protein [29]. The Aβ-binding domain of TTR, including residues 30–60 of the TTR monomer: VHVFRKAADDTWEPFASGKTSESGELHGLTT (TTR$_{30-60}$), was tested and its shortened counterpart including residues 37–43,

ADDTWEP ($TTR_{37–43}$) [39], was also tested. To confirm the specificity of Aβ binding to the TTR fragments, we synthesized mutated TTR peptides ($TTR_{30–60}$ M and $TTR_{37–43}$ M), which had glutamate at position 42 mutated to a glycine. This mutation within the Aβ-binding domain of TTR inhibits binding to Aβ. As shown in Figure 2A, $A\beta_{1–42}$ bound to all immobilized shortened consensus peptides with the single exception to peptide sh-9 (HRRGVTLK). We also observed strong binding of $A\beta_{1–42}$ to KLVFF ($A\beta_{16–20}$ fragment) and TTR-derived peptides $TTR_{30–60}$ and $TTR_{37–43}$. Mutated TTR peptides $TTR_{30–60}$ M and $TTR_{37–43}$ M revealed decreased binding to $A\beta_{1–42}$ relative to their non-mutated counterparts.

Table II. Amino acid sequences of synthetic peptides corresponding to Aβ-binding motifs selected from FliTrx^{TM} peptide library.

ID#	Sequence	ID#	Sequence
4-sh	KKVRR	11-sh	RRKSEK
5-sh	RRKIRR	14-sh	RRSGKSR
6-sh	RRHNRR	21-sh	DLVPL
7-sh	RHVLRPK	23-sh	DLVLA
8-sh	RHVLPKVQA	24-sh	DVVLL
9-sh	HRRGVTLK	25-sh	RLVLF
		26-sh	KRVAF

"-sh" designates a shortened peptide. Each sh-peptide corresponds to the suggested consensus peptide sequence within the same number peptide in Table I.

Comparisons between the binding strength of synthetic peptides and their ability to inhibit $A\beta_{1–42}$ aggregation showed that not all Aβ-binding peptides are inhibitors of Aβ aggregation (Figure 2B). At a 2: 1 molar ratio of Aβ/synthetic peptide, no or very low inhibition of aggregation activity was found for the Aβ-binders: 4-sh (KKVRR), 14-sh (RRSGKSR), 24-sh (DVVLL), 26-sh (KRVAF), $TTR_{37–43}$, and for non-Aβ-binder, peptide sh-9 (HRRGVTLK). The highest inhibition of $A\beta_{1–42}$ aggregation was observed for 5-sh (RKIRR), 7-sh (RHVLRPK), 8-sh (RHVLPKVQA), 21-sh (DLVPL), KLVFF, and $TTR_{30–60}$. As expected from Figure 1, only peptides 7-sh (RHVLRPK) and 8-sh (RHVLPKVQA) bound fibrillar Aβ.

Morphology of amyloid fibrils formed in the absence of synthetic peptide inhibitors is characterized by numerous, massive, fibrillar clusters connected by long and branched fibrils (Figure 3A). In the presence of synthetic peptides

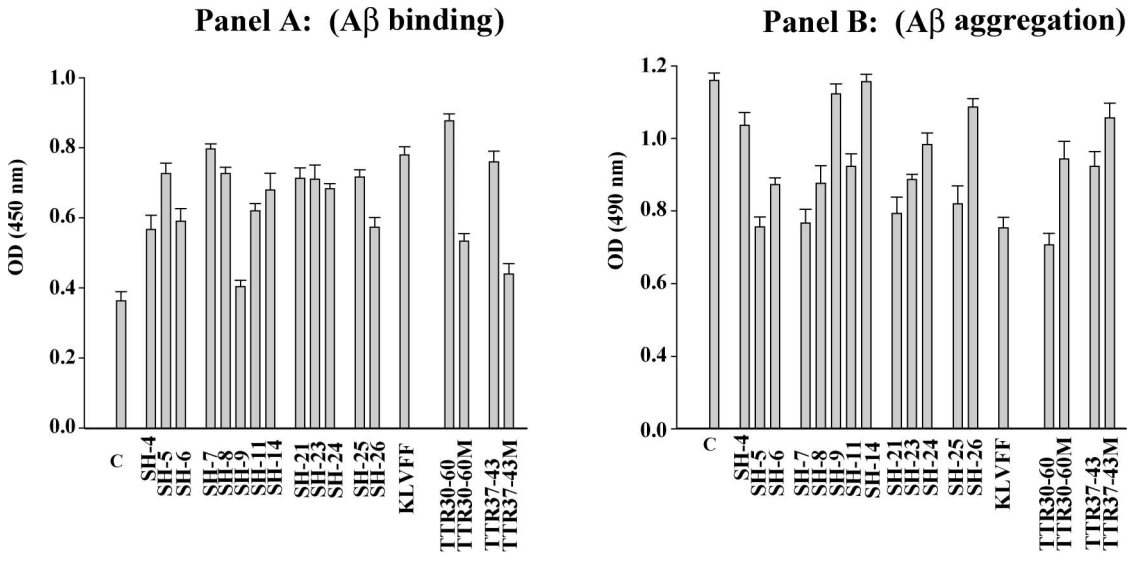

Figure 2. Aβ binding and inhibition of Aβ aggregation by selected synthetic peptides. Panel A: Binding of $A\beta_{1–42}$ to immobilized synthetic peptides. Polyvinyl 96-well plates (Costar) were coated with synthetic peptides (100 μg/ml, Biosynthesis) in PBS buffer overnight at 4°C. After aspirating the excess peptide solution, wells were blocked with PBS containing 2% bovine serum albumin and 1% gelatin for 3 h at room temperature. One hundred microliters of $A\beta_{1–42}$ (100 μM) in the same blocking solution was then added to the wells. Following 2 h incubation at room temperature, the wells were washed five times with PBS. Peptide-bound Aβ was detected using monoclonal anti-Aβ antibody 4G8 (Senetek, 1:1000 dilution). After washing the plate with PBS, a peroxidase-conjugated goat anti-mouse antibody (Sigma, 1:3000 dilution) was added and the plate was incubated for 1 h. Peroxidase-catalyzed color development was initiated using Color developer (Bio-Red) and quantified at 450 nm in a spectrophotometer. The relative amount of Aβ bound in the wells was calculated as being the difference in optical density between the experimental and control wells. Control binding of $A\beta_{1–42}$ to the blocked well in the absence of peptides is labeled with a "C". The data represent means $\pm$ SE from triplicate wells. Panel B: $A\beta_{1–42}$ aggregation in the presence of synthetic peptides. One hundred micrometers of $A\beta_{1–42}$ was incubated for 24 h at 37°C in PBS alone or with synthetic peptides (1:2 molar ratio of Aβ/peptide). Fibril formation was monitored by electron microscopy. After incubation, 150 μl of PBS and 2 μl of Congo Red (5 μM) were added to each sample followed by additional incubation at room temperature in the dark for 10 min. The microplate was spun at 3000 rpm for 40 min. Supernatant was discarded and absorbance of red pellets was measured at 490 nm. The percent of absorbance was determined by comparing the average of duplicate samples to the Congo Red blank. The data represent means $\pm$ SE from triplicate wells.

that inhibited Aβ aggregation (1:2 molar ratio Aβ/synthetic peptide), we mostly detected small fibrillar clusters, numerous short fibrils and pieces of fibrils (Figure 3C–3F). In the presence of synthetic peptides which did not inhibit Aβ aggregation in the Congo Red binding assay (Figure 2B), morphology of Aβ fibrils formed was indistinguishable from that formed in the absence of peptides.

Deposition of ^{125}I-Aβ onto synthaloid

Amyloid assembly is a complex process involving multiple phases and intermediates. The first rate-limiting step of the amyloid formation cascade is the nucleation-dependent formation of ''nucleation seeds'' [35,41,42]. Latter steps of the amyloid formation pathway lead to Aβ deposition and reflect a growth of amyloid fibrils upon pre-existing amyloid templates by addition of monomeric Aβ or oligomeric Aβ-intermediates [35,43].

To analyze the effects of selected peptides on amyloid growth, we examined Aβ depositions on preexisting synthetic amyloid templates (synthaloid) as described by Esler et al. [27]. Figure 4 demonstrates analysis of inhibitory activity of selected synthetic peptides on Aβ depositions onto amyloid template at physiological (nM) concentration of soluble ^{125}I-Aβ. As shown in Figures 2 and 4, the ability of Aβ binding peptides to inhibit additional Aβ depositions does not correlate with their potency to inhibit Aβ aggregation. For example, $TTR_{37–43}$ exhibited very low activity to inhibit Aβ self-aggregation, while significantly decreasing additional Aβ-deposition onto pre-formed amyloid fibrils. In contrast, peptides KLVFF, 21-sh (DLVPL), and

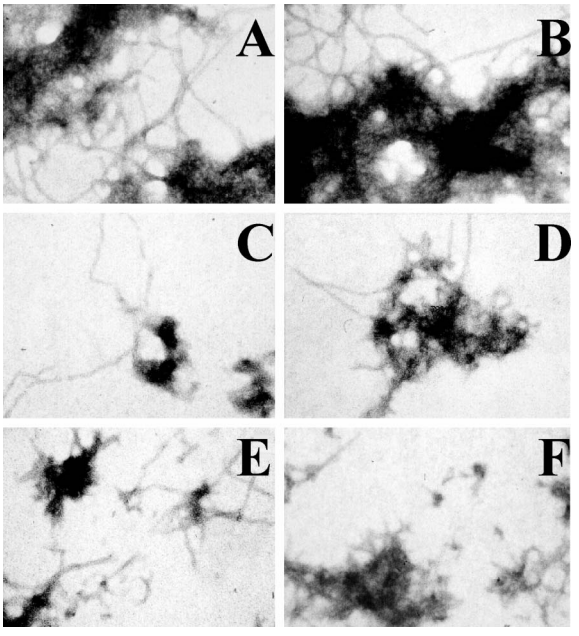

Figure 3. Electron micrographs of $A\beta_{1–42}$ fibrils formed in the presence of synthetic peptides. Panel A: Control sample of Aβ alone (no peptide); Panel B: Aβ plus sh-9 peptide; Panel C: Aβ plus $TTR_{30–60}$; Panel D: Aβ plus $TTR_{37–43}$; Panel E: Aβ plus 6-sh peptide; Panel F: Aβ plus 8-sh peptide. Samples containing 100 μM $A\beta_{1–42}$ were incubated for 36 h at 37°C in PBS alone or with synthetic peptides (2:1 molar ratio of Aβ/peptide). After incubation samples were sonicated for 20 s, applied to carbon-coated copper grids, negatively stained with uranyl acetate, and visualized with JEOL 200c× electron microscope at 80 kV. Samples were examined and photographed at a magnification of 60,000. Bar = 100 nm.

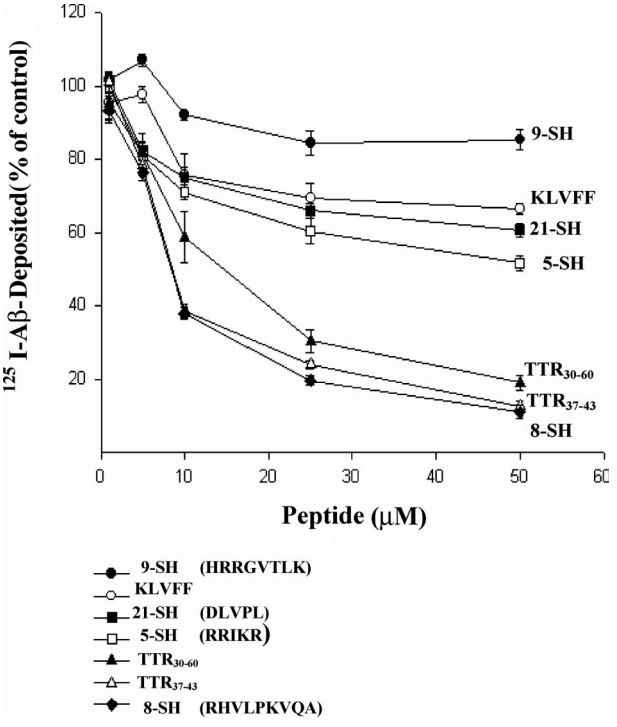

Figure 4. Inhibition of Aβ depositions onto amyloid template by synthetic peptides. All experiments were performed according to the modified procedure described by (Esler et al. 1999 [36]). Briefly, 200 μM stock solution of $A\beta_{1–42}$ (US peptide) in deionized H_2O was solicited for 15 s and filtered through Millex GV 0.22 mm filters (Millipore). One hundred microliters of stock solution of $A\beta_{1–42}$ was incubated for 36 h at 37°C in PBS with Aβ fibrils separated from Aβ-monomers and Aβ-oligomers by centrifugation through the 20% sucrose pad (15,000 × g, 10 min). Fibril formation was monitored by electron microscopy. The solutions of aggregated Aβ were aliquotted, frozen on dry ice, and stored at –20°C. To produce synthetic amyloid (synthaloid), aggregated Aβ in PBS was diluted in 0.1% gelatin (57°C) to yield concentrations of 1–10 μg Aβ peptide/ml. Aβ/gelatin suspension was aliquoted into 96-well plates (Dynatech laboratories 001-010-2401) at 1 μg Aβ per well. Plates were dried overnight at 57°C. Synthaloid in wells was preincubated with buffer (50 mM Tris-HCl, 0,1% BSA, pH 7.5) for 1 h. The preincubation buffer was removed, and ^{125}I-Aβ (10pM) in Tris-HCl buffer was added to each well in the presence or absence of tested peptides. Following incubation (4 h), the Aβ solution was removed and each well was washed with buffer (5x3 min). The wells were capped and separated, and the amount of ^{125}I-Aβ deposited was quantified by γ-counting. The amount of ^{125}I-Aβ bound to the control wells (gelatin solution without Aβ aggregates) was subtracted as background. The data represent means ± SE from triplicate wells.

5-sh (RRIKR), which effectively inhibited Aβ self-aggregation displayed low inhibitory activity when tested in this additional Aβ deposition onto pre-formed amyloid fibrils assay. These results support previous data [36] that inhibition of rate-limiting stages of Aβ self-aggregation and inhibition of Aβ depositions onto pre-formed fibrils are two distinguishable processes in the amyloid formation cascade. It should be noted that among all synthetic peptides tested, only three peptides (8-sh, $TTR_{37–43}$ and $TTR_{30–60}$) revealed significant inhibition of Aβ-depositions. Interestingly, only 8-sh (RHVLPK-VQA) peptide bound aggregated Aβ while $TTR_{37–43}$ and $TTR_{30–60}$ bound only soluble, monomeric forms of Aβ.

Effects of synthetic peptides on Aβ accumulation in smooth muscle cells

One potential drawback of the synthaloid assay is that labeling of Aβ with iodine leads to its modification and this may affect aggregation or binding of labeled Aβ to preformed fibrils. Therefore, we used an independent approach to analyze the ability of selected peptides to inhibit Aβ deposition. This method is based on well-described observations that Aβ accumulates in smooth muscle cells cultivated from leptomeningeal vessels of old dogs [44] and was previously used to evaluate the affects of apolipoprotein E and TTR on Aβ deposition in these cells [21,45].

Among those tested, only a few peptides displayed an inhibitory effect on Aβ accumulation in these cell cultures (See Figure 5). This observation may reflect differential stability or differential activity of peptides in cell cultures. Each peptide was added every 24 h for 3 days at 5, 10 or 20 μM. Treatment with a single dose of peptide (20 μM) produced only a small reduction in Aβ accumulation (less than 15%) for peptides 5-SH, $TTR_{37–43}$ and $TTR_{30–60}$. In Figure 5 we show experiments that were performed with cultures containing 16–40% of Aβ-positive cells. In cultures that were moderately ($>40\%$ of positive cells) or strongly ($>70\%$ of positive cells) engaged in amyloidogenesis, we observed significant inhibitory effect only for peptides 5-SH and $TTR_{30–60}$ (data not shown).

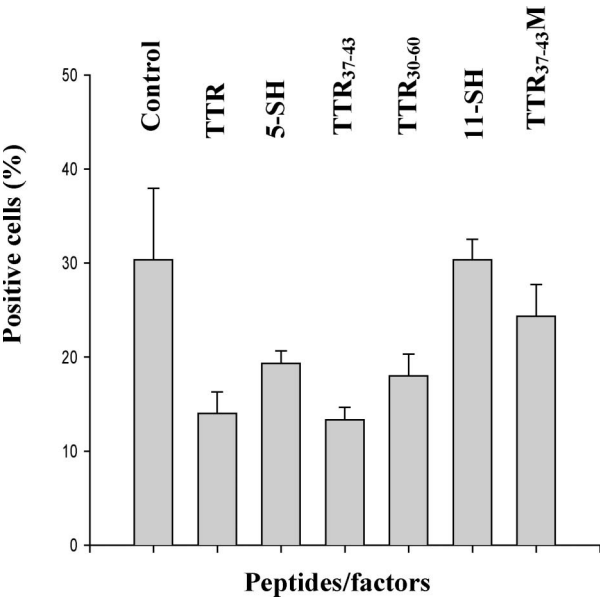

Figure 5. Effects of synthetic peptides on Aβ accumulation in smooth muscle cells. Cell cultures were obtained from leptomeningeal blood vessel walls of old dogs as previously described [21,44]. Aβ-immunoreactive material was detected in formalin-fixed cultured cells by the immunoperoxidase method with monoclonal anti-Aβ antibody 4G8. Each peptide was added every 24 h for 3 days at 5, 10 and 20 μM. TTR (4.5 μM) was added in the first day of culturing. The data represent means $\pm$ SE from seven independent experiments.

Discussion

Numerous Aβ binding proteins, biological and chemical compounds have been described as putative inhibitors of Aβ fibril formation. For example, Aβ binding proteins interact and form complexes with a variety of biological macromolecules derived from different metabolic pathways and it is unlikely that Aβ would be a specific target for binding these proteins in vivo. Chemical compounds that specifically recognize β-pleated sheet conformations, however, may interact with other proteins or protein complexes with a high content of β-pleated sheet structures.

On the other hand, small synthetic peptides are known as highly specific biological ligands and possess enormous potential for selective treatment of many disease processes [46,47]. The additional benefit of synthetic peptides as putative inhibitors of Aβ amyloidosis is the opportunity to modify their amino acid sequence in such a way as to increase biological activity and stability. Moreover, a small peptide is easier to use as a template for designing non-peptide mimetics that are typically employed in drug therapy [48].

Recent strategies in the search for novel pharmacological-peptide-based drugs have focused on the generation of combinatorial peptide libraries, which represent a powerful technique for studying protein-protein interactions [46,49]. The main advantage of peptide libraries is that random screening selects peptides interacting with targeted molecules in the absence of any bias or knowledge of structural information. Another advantage of peptide libraries is that unbiased screening usually identifies groups of peptides with different binding motifs. Thus, peptide libraries provide the opportunity to find previously unknown binding epitopes and motifs.

Our screening of a FliTrx^{TM} random peptide library with immobilized Aβ_{1-42} identified four groups of peptides with different Aβ binding motifs (Table I). Groups 1 and 2 are enriched by positively charged amino acids. Consensus sequences consist of two groups of positively charged amino acids divided by one hydrophobic or polar amino acid residue in Group 1, or by two hydrophobic or polar amino acid residues in Group 2. In part, Groups 1 and 2 revealed similarity with the perlecan binding domain of Aβ (Aβ_{12-17} = VHHQKL) [50]. Thus, the high content of positively charged amino acid residues in Groups 1 and 2 is not an unexpected result. Solution structure of Aβ shows that this peptide is dipolar in nature and contains two demarcated regions of negative potentials [19], which most likely represent contact areas in the interaction with Aβ binding peptides. The consensus sequence of Group 3 consists of one negatively charged amino acid and four hydrophobic residues. This group represents a new class of Aβ binding peptides whose precise interaction with Aβ is not yet understood. Presently is not clear what region of Aβ binds peptides of this group. The consensus sequence of Group 4 is strikingly similar with an Aβ fragment (Aβ_{16-20} = KLVFF) that was shown to bind Aβ [29].

Surprising results were obtained for binding selected clones to fibrillar Aβ. Among all selected clones, only two related clones expressing peptides NGRHVLRPKVQA and VRHVLPKVQAPV bound Aβ fibrils. These clones have two consensus sequences: RHVL(R/P) and PKVQA. It is quite possible that first consensus sequence RHVL(R/P), which is common for Group 2 binding peptides, recognizes monomeric Aβ. In contrast, the highly hydrophobic motif PKVQA specifically recognizes cross-β-pleated sheet structure of Aβ fibrils.

In addition to peptides selected from the FliTrx^{TM} peptide library, we also examined synthetic peptides homologous to the Aβ-binding domain of TTR [39]. We and others previously demonstrated that TTR inhibited Aβ fibril formation in vitro and in vivo [19,21,22,45,51], but the inhibitory effects of TTR-derived peptides on Aβ deposition was unexplored. The studies presented here indicate that TTR fragments homologous to the Aβ binding domain of TTR can bind Aβ and inhibit Aβ aggregation in vitro.

Among all synthetic peptides tested, only three peptides (8-sh, TTR_{37-43} and TTR_{30-60}) significantly inhibited Aβ-deposition. Moreover, for peptides 5-SH, TTR_{37-43} and TTR_{30-60}, there was a good correlation between the ability to inhibit Aβ deposition onto synthetic amyloid templates and inhibition of Aβ accumulation in smooth muscle cells. The mechanism by which these peptides inhibit deposition of Aβ is not clear at this time. Given their effectiveness in these assays, we propose that these peptides may affect more then one step of Aβ assembly.

Certainly, we need to further determine the optimal sequence of all identified inhibitors. The results from full-length peptide may not be the same as for shortened peptides and need to be investigated. Although we found several inhibitors that preferentially inhibit amyloid growth, the mechanism of this inhibition is not clear. In this view, it will be interesting to study effect of found inhibitors on protofibril formation and protofibril interaction. Although protofibrils are transient structures and are in the equilibrium with low molecular weight Aβ (monomeric or dimeric), the mechanism of blocking protofibril formation and protofibril interaction may be quite different [37]. Recent results show that nordihydroguaiaretic acid does not disaggregate Aβ protofibrils, but does inhibit amyloid growth arising from direct protofibril association [52].

Finally, it should be noted that isolation of new Aβ inhibitors might be especially important for ultimately understanding the mechanism of amyloid formation. As we do not presently know whether each of the inhibitors we have identified here act via the same mechanism, having as many inhibitors as possible would permit a more thorough evaluation of those mechanisms that cause amyloid fibril formation.

Acknowledgments

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