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Articles

Potential Lead for an Alzheimer Drug: A Peptide That Blocks Intermolecular Interaction and Amyloid β Protein-Induced Cytotoxicity

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A peptide chAβ30−16 (15-mer; CTFVRTHIFCKEHQF) was designed to bind to a region encompassing the entire polymerization-related (^{16}KLVFF^{20}) and part of the polymerization and toxicity-related (^{25}-GSNKGAIIGLM^{35}) regions of amyloid β-protein, Aβ_{1-42} by a hydropathic complementary approach. This peptide efficiently binds to Aβ and blocks intermolecular interaction and the formation of Aβ aggregates. In addition, the peptide neutralizes the cell toxicity of Aβ fibrils. The chAβ30−16 peptide or its derivatives may be a starting point for the future development of drugs that prevent the neurotoxicity and deposition of Aβ in the brain of Alzheimer's disease.

Introduction

Alzheimer's disease (AD[a]) is the most common form of progressive senile dementia and primarily affects individuals over the age of 65. The disease leads to the inevitable destruction of neurons and ultimately death within 7−10 years. It is characterized by the presence of extracellular amyloid plaques and intraneuronal neurofibrillary tangles in the brain. Amyloid plaques are found as insoluble deposits between neurons, which are accumulated in the cortex and hippocampus of the brain. The amyloid plaques in the brain of AD patients are primarily composed of β-amyloid protein (Aβ), 39−43 amino acid long peptides derived from the amyloid precursor protein by proteolytic cleavage with β- and γ-secretases.[1] Both Aβ_{1-40} and Aβ_{1-42} are components of the deposits of amyloid plaques found in the brain tissue of AD patients. Aβ_{1-42} is believed to play a more important role in the early stage of fibril formation, exhibiting a greater tendency to form plaques than Aβ_{1-40}.[2] It was reported that Aβ itself is not toxic, but it becomes toxic to neuronal cells once it is aggregated into amyloid fibrils. However, recent studies have shown that soluble intermediates (oligomers and protofibrills) in the fibrillogenesis pathway are also toxic to cultured cells. To be toxic, Aβ must be in an organized, self-assembled state. This suggests that the prevention of Aβ polymerization may inhibit neuronal degeneration. Because Aβ peptides are self-assembling, it may be possible to prevent Aβ polymerization specifically by using a short peptide fragment binding to the Aβ peptide.[3]

The exploitation of hydropathic complementarity (HC) of amino acids based on the genetic code of the noncoding strand is one of the simple ways of designing peptides that interact specifically with a given amino acid sequence of peptides or proteins.[4−7] According to the HC hypothesis, peptides that are predicted from the codons read from the noncoding strand of a gene either in the 5′ → 3′ or in the 3′ → 5′ directions tend to bind to the protein derived from the coding strand. This concept has been successfully used for the development of novel antagonists to hormones, for the determination of a putative binding site for fibronectin, for the identification of interactive determinants on idiotypic-anti-idiotypic antibodies, and others.[8−14]

Two characteristic regions in the Aβ sequence that were previously investigated might be exploited as targets for developing anti-Aβ peptides: the sequence *KLVFF*, corresponding to residues 16−20 of Aβ and the sequence *GSNKGAIIGLM*, corresponding to residues 25−35 of Aβ. These two regions appear to be important for Aβ aggregation and toxicity. Many studies have shown that Aβ(16−20) is important in the early steps of Aβ misfolding and aggregation,[15,16] and as a peptide, the second region is more rapidly toxic and causes more oxidative damage than the parent Aβ peptide.[17,18] In particular, both regions promote the formation of low molecular weight oligomers and protofibrils of Aβ. The peptides alone are also toxic.[19] Therefore, it will be of interest to investigate if complementary peptides for either regions would interfere with Aβ aggregation and toxicity.

In this study, we found that a complementary peptide targeted to a region encompassing those two selected sequences of Aβ binds to and blocks the self-aggregation of Aβ and also neutralizes Aβ-induced cytotoxicity.

Results and Discussion

Design of Peptides. Regions of the nucleotide sequence of a noncoding strand of Aβ_{1-42} cDNA were read in the 5′ to 3′ or the 3′ to 5′ directions to derive codons of HC peptides as shown in Figure 1 and Table 1. The regions known to be involved in aggregation and cytotoxicity, (^{16}*KLVFF*^{20}) and (^{25}*GSNKGAIIGLM*^{35}), were the focus of our study. Stop codons were encountered in several places in the noncoding strand (one in the 5′ to 3′ direction, and two in the 3′ to 5′ direction; see Figure 1). The most frequent amino acid derived from the remaining degenerate codons for the same amino acid of the coding strand was utilized for the synthesis of the HC peptides: for example, the anti-sense RNA of the *Ser*^{26} codon

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[a] Abbreviations: AD, Alzheimer's disease; HC, hydropathic complementarity.

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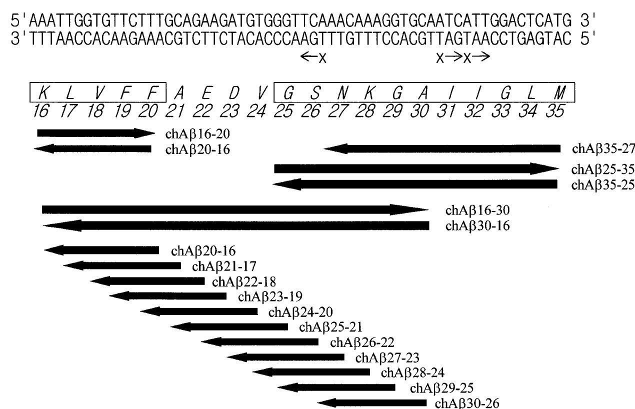

Figure 1. Design of hydropathic complementary (HC) peptides. Shown are the Aβ cDNA sequence corresponding to the region of Aβ from 16 to 35 amino acids (coding and noncoding strands) and the amino acid sequence. The two rectangular boxes surrounding the Aβ amino acids denote the polymerization- and toxicity-related regions of Aβ. The heavy arrows indicate the region and direction of the noncoding strand read for the deduction of codes for the HC peptides. The three x's underneath the DNA sequence indicate stop codons encountered when the noncoding strand is read in either the $5' \rightarrow 3'$ or the $3' \rightarrow 5'$ directions.

(TCA) will generate a stop codon when read in the 5′ to 3′ direction. In this case, we predicted anti-sense codons from degenerate codon sequences for *Ser* and chose a more frequent amino acid. Thus, *Arg* was selected in place of the stop codon.

Binding Ability and Neutralizing Activity of Peptides. We studied whether the toxic effect of Aβ fibrils would be reduced in the presence of HC peptides against the two 16−20 and 25−35 regions.

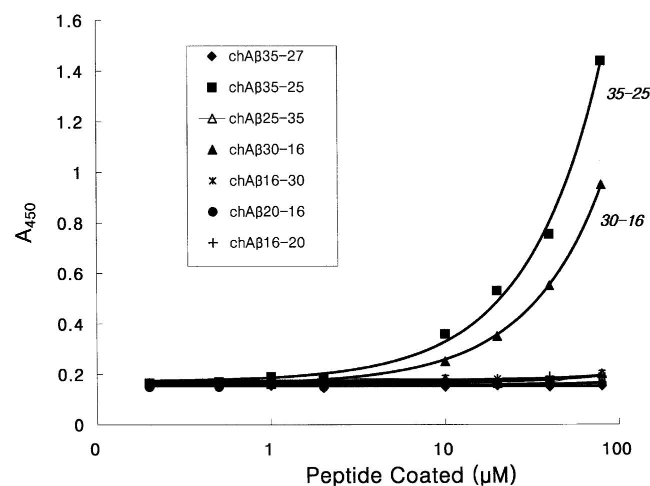

Figure 2. Binding of various HC peptides to Aβ. The chAβ peptides were coated on a plastic microtiter plate. After blocking nonspecific binding sites, biotin-labeled Aβ_{1-42} was added. After 3 h of incubation at room temperature, the bound Aβ_{1-42} was determined as described in the Experimental Section.

Initially, the HC peptides for the Aβ 16−20 region (chAβ20−16 and chAβ16−20) and the HC peptides for the Aβ 25−35 region (chAβ35−25, chAβ25−35 and chAβ35−27) were synthesized and tested for their ability to bind to Aβ_{1-42} and neutralize the cell toxicity of Aβ fibrils. Among the peptides tested, chAβ35−25 alone showed a significant binding ability at a high concentration (10^{-5}−10^{-4} M) (Figure 2). Interestingly, chAβ35−27, which is only two residues shorter than chAβ35−25 (missing residues 25 and 26), showed no binding. Unfortunately, the chAβ35−25 peptide exhibits no neutralizing activity

Table 1. Amino Acid Sequence and Analytical Data for the HC Peptides Investigated in This Study[a]

Name	Sequence (N-term to C-term)	HPLC k' (system 1)	% Purity	HPLC k' (system 2)	% Purity	Mass Spectral Analysis, m/z (M + 1)
chAβ16-20	*FNHKK*	3.39	98.40	4.24	99.39	673.8
chAβ25-35	*PSLFPRYYPEY*	8.79	95.45	12.15	95.29	1431.4
chAβ35-25	*HESNDCTFVRT*	6.26	95.15	9.48	95.66	1309.5
chAβ35-27	*HESNDCTFV*	8.06	98.80	12.84	97.51	1052.0
chAβ16-30	*FNHKKRLLHPSLFPR*	7.82	99.52	12.24	99.53	1890.7
chAβ30-16	*CTFVRTHIFCKEHQF*	8.52	96.03	12.35	98.41	1897.7
chAβ30-26	*CTFVR*	6.83	94.77	12.18	93.37	625.7
chAβ29-25	*TFVRT*	5.20	99.09	8.39	97.64	623.8
chAβ28-24	*FVRTH*	3.86	96.51	5.38	97.16	659.8
chAβ27-23	*VRTHI*	4.84	98.02	8.01	95.18	625.8
chAβ26-22	*RTHIF*	6.72	97.48	10.85	96.33	673.8
chAβ25-21	*THIFC*	8.20	95.43	13.08	95.69	620.7
chAβ24-20	*HIFCK*	6.99	98.72	12.40	99.75	647.6
chAβ23-19	*IFCKE*	6.92	98.01	11.78	99.24	639.7
chAβ22-18	*FCKEH*	4.79	97.00	7.08	96.70	663.7
chAβ21-17	*CKEHQ*	0.07	95.34	0.10	95.75	644.0
chAβ20-16	*KEHQF*	4.58	98.81	6.55	98.42	688.7

[a] The parts that are underlined in the sequence indicate where the stop codons were encountered during the reading of the noncoding strand of Aβ DNA sequence shown in Figure 1. HPLC $k' = $ ((peptide retention time) − (solvent retention time))/(solvent retention time). Gradient system 1: the gradient was started at 0% B in A to 40% B in A in 13 min and then to 100% B in A in 4 min (A: 0.1% trifluoroacetic acid/water and B: acetonitrile). Gradient system 2: the gradient was started at 0% C in A to 40% C in A in 13 min and then to 100% C in A in 4 min (C: methanol). A narrow bore Vydac C18 column (Vydac 218TP5215) was used with a flow rate of 0.4 mL/min. The elution of the peptide was determined at 215 nm.

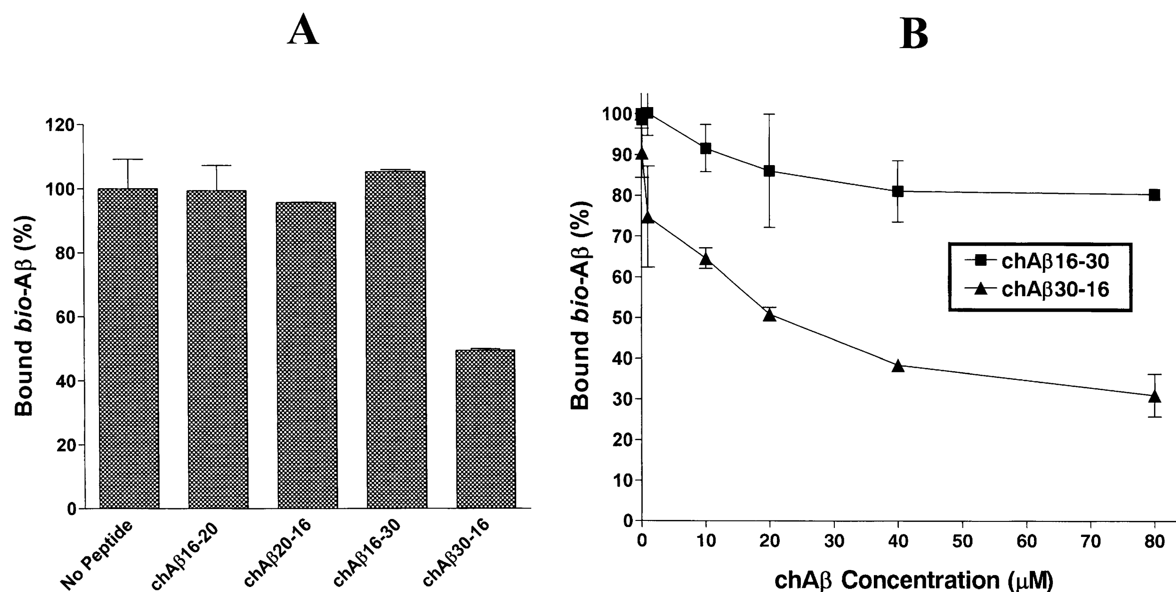

Figure 3. Inhibition of $A\beta - A\beta$ interaction (A). Inhibition of $A\beta - A\beta$ interaction by various HC peptides; (B) concentration-dependent inhibition of $A\beta - A\beta$ interaction by chAb30−16. $A\beta_{1-42}$ (40 nM) was coated on a 96-well plastic microtiter plate, and 20 nM biotinylated $A\beta_{1-42}$ and 20 μM HC peptide were added to each well. The plate was incubated for the $A\beta - A\beta$ interaction (3 h at room temperature). The bound $A\beta_{1-42}$ was determined as described in the Experimental Section.

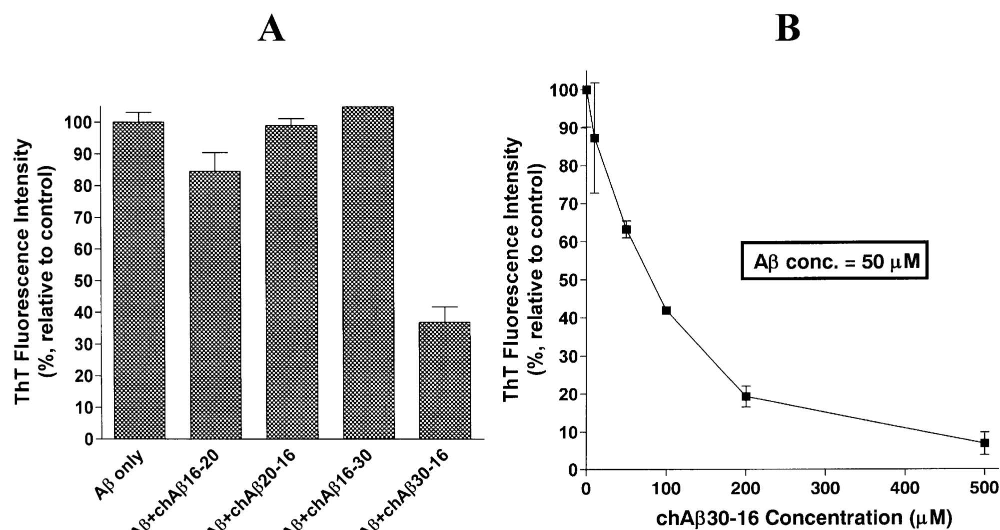

Figure 4. Inhibiton of $A\beta$ aggregation. (A) Inhibition of $A\beta$ aggregation by various HC peptides; (B) concentration-dependent inhibition of $A\beta$ aggregation by chAβ30−16. The $A\beta$ aggregation mixture was assembled in PBS buffer with 50 μM $A\beta$ alone or in the presence of 100 μM HC peptides as described in the Experimental Section. After 24 h incubation at 37 °C, ThT was added to the aggregation mixture, and fluorescence intensity was determined as described in the Experimental Section.

at 10^{-4} M (see below). Next, two peptides (chAβ30−16 and chAβ16−30) spanning the 16−20 region and the 25−35 region were synthesized and tested for binding ability and neutralizing activity. The chAβ30−16 peptide showed significant binding to $A\beta_{1-42}$ and effectively neutralized cell toxicity caused by $A\beta$ fibrils beginning at molar peptide/$A\beta_{1-42}$ ratios of 10 (see below; Figure 5). However, chAβ16−30 showed no binding to $A\beta$ (Figure 2).

Effect of Peptides on Intermolecular Interaction of Aβ. The effect of HC peptides on the intermolecular interaction of $A\beta$ was investigated by employing an assay measuring the binding of biotin labeled $A\beta$ to stationary $A\beta$. This experiment identified dramatic differences in the behavior of the chAβ30−16 peptide from the others (chAβ16−20, chAβ20−16, and chAβ16−30 peptides) (Figure 3A). When the same molar concentration (peptide: $A\beta_{1-42}$ = 1000:1) of each peptide was compared, only the chAβ30−16 peptide showed significant inhibition of the $A\beta_{1-42}$ to $A\beta_{1-42}$ interaction (50%). And the inhibition of the intermolecular interaction of $A\beta$ by chAβ30−16 was concentration-dependent (Figure 3B). These results show that chAβ30−16 peptide specifically binds to $A\beta_{1-42}$.

Effect of Peptides on the Self-Aggregation of $A\beta_{1-42}$. The evidence that the HC peptide blocks the self-aggregation of $A\beta_{1-42}$ was obtained with a thioflavin T (ThT) assay that is based on the selective affinity of ThT for fibrils.[20] Peptide chAβ30−16, at only a 2:1 molar ratio with $A\beta$, caused a 50% reduction of ThT fluorescence intensity (Figure 4A). Peptides chAβ20−16 and chAβ16−30 had no effect on the aggregation of $A\beta$. A small decrease in ThT fluorescence intensity was observed when the chAβ16−20 peptide was added to the aggregation reaction. The inhibition of $A\beta_{1-42}$ aggregation by the chAβ30−16 peptide was dose-dependent (Figure 4B). The chAβ30−16 peptide seems to be a more effective inhibitor of $A\beta$ aggregation than the intermolecular interaction of $A\beta$.

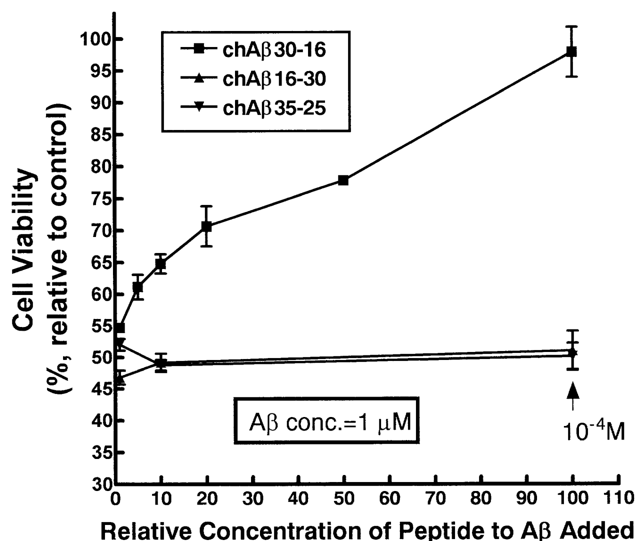

Figure 5. Protection against Aβ-induced cytotoxicity by the chAβ30−16 peptide. $A\beta_{1-42}$ was incubated at 37 °C for 24 h in an aggregation reaction mixture. An aliquot was added to PC-12 cells in culture to a final concentration of 1 μM. The treated cells were incubated for 24 h at 37 °C, and cell viability was determined by MTT assay as described in the Experimental Section.

Protective Effect of the chAβ30−16 Peptide against Aβ-Induced Cytotoxicity. Finally, the possible protective effects of the chAβ30−16 peptide against $A\beta_{1-42}$-induced neurotoxicity were investigated with PC-12 cells. The cells were treated with $A\beta_{1-42}$ that was preincubated in the absence or presence of chAβ30−16 peptide for 24 h. Cell viability was determined by using the MTT reduction assay. This assay relies on the reduction of MTT by mitochondrial dehydrogenases. The chAβ30−16 peptide displayed a marked protection against the well-known toxicity of Aβ fibrils (Figure 5). Aβ at 1 μM caused a 50% reduction of cell viability. Peptide chAβ30−16 induced an increase in cell viability beginning at 10 μM in a dose-dependent manner reaching complete protection from Aβ-induced toxicity at 100 μM. It was also notable that the chAβ30−16 peptide alone had no detectable toxic effects on PC-12 cells at concentrations up to 100 μM (data not shown).

Localization of the Binding Ability and Neutralizing Activity within a Pentapeptide Region of the chAβ30−16 Peptide. Next, the chAβ30−16 peptide (15-mer) was subdivided into a series of pentapeptides to determine whether binding ability and neutralizing activity could be localized within a five amino acid-region (Figure 1 and Table 1, for the sequence and position of the peptides in the series). Among them, only chAβ30−26 showed significant binding ability to $A\beta_{1-42}$ (data not shown). But when the cell viability assay was carried out, none of the series of pentapeptides including chAβ30−26 showed any protective effect from the toxicity of Aβ fibrils (data not shown). These results demonstrated that binding ability and neutralization activity could not be confined to a short pentapeptide length.

In this study, we used the HC approach for the development of peptides, that can bind to Aβ and neutralize the cell toxicity of β-amyloid, the major protein component of senile plaques in AD. From the results of several peptides targeted to the two regions responsible for the polymerization and toxicity of Aβ, we found that effective binding to one region does not necessarily lead to neutralization activity. Furthermore, neutralization activity could not be localized within a short pentapeptide length, although some had significant binding activity. This implies that for full neutralizing activity both of the critical regions, namely, 16−20 and 25−35 regions of Aβ, are required.

Our peptide, chAβ30−16, initially targeted to Aβ(16−20), could not only block the self-aggregation of Aβ (Figure 4A and B) but also inhibit the intermolecular interaction of monomeric Aβ (Figure 3A and B). The intermolecular interaction of Aβ would also be necessary for Aβ oligomerization. The peptide contains two cysteines, but the presence or absence of the reducing agent dithiothreitol had no noticeable effect on the inhibitory activity of the peptide for Aβ intermolecular interaction as well as for the aggregation of Aβ (data not shown). This suggests that the monomeric form of chAβ30−16 is the active form.

Conclusion

In summary, a 15-mer peptide (chAβ30−16), a HC peptide, binds specifically to $A\beta_{1-42}$ and inhibits $A\beta_{1-42}$ intermolecular interaction and aggregation. These effects are accompanied by protection against Aβ-induced toxicity in PC-12 cells. These results raise the possibility that the chAβ30−16 peptide or its derivatives may be a starting point for the development of drugs that reduce neurotoxicity as well as for the prevention of the deposition of Aβ in AD.

Experimental Section

Peptide Synthesis. All of the chAβ peptides were synthesized and purified by HPLC at the A&PEP Research Institute (Yeongi-Gun, Chungnam, Korea) and PEPTRON, Inc. (Yuseong-Gu, Daejeon, Korea). The purified peptides were at least ≥95% pure as determined by HPLC in two diverse solvent systems and had the correct molecular mass (Peptide Library Support Facility of POSTECH, Korea; Table 1). $A\beta_{1-42}$, which is biotinylated at the *N*-terminus was purchased from American Peptide Company (Sunnyvale, CA; product #62-0-51).

Binding Assay. ELISA. The binding of HC peptides to $A\beta_{1-42}$ was determined by an ELISA assay. Test peptides (dissolved in water) were added to the wells of a 96-well plate (with Aβ already bound). After drying in air at room temperature, blocking solution (150 μL/well, 3% BSA (fraction V) in PBST (0.05% Tween-20 in 1× PBS)) was added, and the plate was incubated for 3 h at room temperature for blocking nonspecific binding sites. The blocking solution was decanted, and each well was rinsed three times with 150 μL of PBST. Biotinylated $A\beta_{1-42}$ (20 nM, 50 μL/well) was added and incubated for 3 h at room temperature. Each well was rinsed three times with 150 μL of PBST. Streptavidin-HRP (1/5000 dilution in 3% BSA/PBST; 50 μL/well) was added and incubated for 1 h at room temperature and then washed three times with 150 μL of PBST. SuperSignal ELISA Pico Working Solution (Pierce, Rockford, IL) (100 μL) was added to each well, and light intensity was determined in a luminometer at 425 nm.

Aβ Polymerization Conditions. $A\beta_{1-42}$ was solubilized in water to 1 mg/mL (222 μM) and stored at −80 °C. For aggregation studies, $A\beta_{1-42}$ was mixed on ice to a final concentration of 50 μM in 1× PBS. Then, it was incubated at 37 °C for 24 h as described previously.[21]

Thioflavin T (ThT) Assay. ThT in 0.5× PBS (10 μM, 90 μL) was added to 10 μL of the aggregation reaction mixture (50 μM $A\beta_{1-42}$ in 1× PBS) in a 384-well plate. After incubation for 30 min at room temperature, fluorescence was read at $Ex = 444$ nm and $Em = 485$ nm.[22−23]

Cell Viability Assay. PC-12 rat pheochromacytoma cells were grown on Primaria (Nunc, Naperville, IL) plates in a medium containing 85% RPMI-1640, 5% fetal bovine serum, and 10% horse serum at 37 °C in 5% CO_2. The cells were plated in 100 μL of media on a poly-D-lysine-coated 96-well plate at a density of 2×10^4 cells/well. An Aβ aggregation mixture plus peptide was dissolved in 98% RPMI-1640 medium, 1% horse serum, and 1% antibiotics and added to the cells. The treated cells were incubated for 24 h at 37 °C under 5% CO_2. Cell viability was determined using the MTT assay.[24] MTT (3-[4,5-dimethylthizaol-2-yl]-2,5-diphenyl tetrazolium bromide) reagent (10 μL) was added to each

well, and the plate was incubated at 37 °C for 4 h. Solubilization solution (10% SDS and 25% DMF; 100 μL) was added to each well, and the plate was incubated at room temperature for 4 h. The absorbance of the samples was measured at 570 nm.

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Supporting Information Available: The tracings of HPLC and mass spectral analysis for the peptides listed in Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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