Designing Peptide Inhibitors for Oligomerization and Toxicity of Alzheimer's β-Amyloid Peptide†

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ABSTRACT: Convergent biochemical and genetic evidence suggests that the formation of β-amyloid (Aβ) deposits in the brain is an important and, probably, seminal step in the development of Alzheimer's disease (AD). Recent studies support the hypothesis that Aβ soluble oligomers are the pathogenic species that prompt the disease. Inhibiting Aβ self-oligomerization could, therefore, provide a novel approach to treating the underlying cause of AD. Here, we designed potential peptide-based aggregation inhibitors containing Aβ amino acid sequences (KLVFF) from part of the binding region responsible for Aβ self-association (residues 16−20), with RG-/-GR residues added at their N- and C-terminal ends to aid solubility. Two such peptides (RGKLVFFGR, named OR1, and RGKLVFFGR-NH_2, named OR2) were effective inhibitors of Aβ fibril formation, but only one of these peptides (OR2) inhibited Aβ oligomer formation. Interestingly, this same OR2 peptide was the only effective inhibitor of Aβ toxicity toward human neuroblastoma SH-SY5Y cells. Our data support the idea that Aβ oligomers are responsible for the cytotoxic effects of Aβ and identify a potential peptide inhibitor for further development as a novel therapy for AD.

Alzheimer's disease (AD)[1] is the most common cause of dementia in the elderly. One of the defining neuropathological features of AD is the occurrence of senile plaques containing insoluble, aggregated proteins, the main constituent of which is β-amyloid protein (Aβ). Aβ is a 39−43-residue peptide that plays a central role in disease progression and is derived from the proteolytic processing of the much larger amyloid precursor protein (for reviews, see refs *1* and *2*). Mutations in the genes encoding the amyloid precursor protein and the presenilins segregate with some forms of autosomal dominant familial AD. These mutations elevate the secretion of Aβ, especially the longer peptide Aβ42 (*1*, *2*). There is now substantial evidence from molecular genetics, transgenic animal studies, and aggregation/toxicity studies with synthetic Aβ (and various synthetic peptide fragments derived from it) to suggest that the conversion of this peptide from soluble monomers to aggregated, insoluble forms in the brain is a key event in the pathogenesis of AD (*3*−*5*). It seems increasingly likely that early soluble oligomers are actually the toxic species responsible for neurodegeneration and neuronal cell death (*6*−*18*), and the inhibition of Aβ oligomerization is an attractive therapeutic target for AD. Tjernberg et al. reported that the central region (amino acid residues 16−20) of Aβ is responsible for its self-association and aggregation (*19*). Recently, it has been demonstrated that modified synthetic peptides based on the sequence of this central region of Aβ are able to prevent its conversion to β-sheet-rich aggregated structures (*20*−*24*). However, these reported inhibitors were designed to block the formation of the late aggregates rather than the early aggregates of Aβ, and therefore, they were only able to partially reverse the toxicity of Aβ aggregates. The aim of the work reported here was to design short synthetic peptides that are capable of inhibiting the early aggregation of Aβ into toxic oligomeric species.

Recently, we successfully designed peptide inhibitors that can block the oligomerization and toxicity of the α-synuclein protein associated with Parkinson's disease (PD). Premised on the fact that Aβ has obvious parallels with the aggregation of α-synuclein, in this study, peptide inhibitors of Aβ aggregation were developed based on the same strategy that we have described for the design of α-synuclein inhibitors (*25*). The peptides were tested for their ability to inhibit the formation of Aβ oligomers and fibrils. Finally, the effect of the peptide inhibitors on the toxicity of Aβ toward human neuroblastoma SH-SY5Y cells was also investigated and correlated with their effects on Aβ oligomerization.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. All fluorenylmethoxycarbonyl-protected amino acids, fluorenylmethoxycarbonyl PAL-PS resin, Fmoc-

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[1] Abbreviations: Aβ, β-amyloid protein; AD, Alzheimer's disease; DMEM, Dulbecco's modified eagle medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; TEM, transmission electron microscopy.

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L-Val-PEG-PS resin, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridine-1-ylmethylene]-hexafluorophosphate *N*-oxide, di-isopropylethylamine, dimethylforamide, *N*-methylpyrrolidone piperidine, and dichloromethane, were purchased from PE Applied Biosystems (Cheshire, U.K.).

Peptides were synthesized at a 0.2 mmol scale using Milligen 9050 peptide synthesizer (PE Applied Biosystems Ltd. Cheshire, U.K.), using standard fluorenylmethoxycarbonyl chemistry as described recently (*26*). In brief, a 4-fold excess of amino acid was used, and coupling was achieved using *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridine-1-ylmethylene]hexafluorophosphate *N*-oxide. Cleavage of the peptides was carried out over a 2 h incubation at room temperature with a mixture of thioanisole, ethanedithiol, triisopropylsilane, water, and trifluoroacetic acid (2:1:1:1: 95 v/v/v/v/v).

The peptide purity was ascertained by reversed phase HPLC using a C4 reversed phase 250 mm × 4.6 mm column (Phenomenex, Torrance, CA). The peptide identity was verified using a Finnigan LCQ ion trap mass spectrometer.

Preparation of Aβ Solutions. Aβ40 was dissolved in sterilized distilled water, and then a 50% volume sterilized in 20 mM phosphate-buffered saline (PBS) [150 mM NaCl (pH 7.4)] was added to give a 100 μM concentration of Aβ40. Aβ42 (1.3 mg) was dissolved in 10 mM NaOH (32 μL), and sterilized distilled water was added, followed by a 50% volume of sterilized 2X PBS (pH 7.4) to obtain a 100 μM concentration of Aβ42. These solutions were aged either alone or with the peptide inhibitors at various molar ratios of Aβ/peptide inhibitor. The samples were placed in Parafilm-sealed, 1.5 mL polypropylene tubes and were incubated at 37 °C. Samples were collected at various points, and thioflavin T (Th-T) assays were carried out immediately, while the rest of the samples were stored at −80 °C until they were tested by the ELISA assay for Aβ oligomers.

Th-T Assay. Aβ fibril formation was monitored by a Th-T binding assay. Th-T is a fluorescent dye that interacts with fibrils containing a β-sheet structure. The process was accompanied by a characteristic increase in fluorescence intensity in the vicinity of 480 mm. A 10 μL sample of each peptide solution was diluted into 190 μL of Th-T in PBS. Fluorescence was measured in 96-well black plates (Nunc, Roskilde, Denmark) using a Victor^2 1420 (PerkinElmer, Waltham, MA) microplate reader, with excitation at 440 nm and emission at 490 nm. To allow for background fluorescence, the fluorescence intensity of a blank PBS solution was subtracted from all readings.

ELISA to Measure Aβ Oligomers. This assay depends on the use of the same anti-Aβ monoclonal antibody for capture and detection (6E10 and biotinylated 6E10, respectively) in a sandwich format (*13*, *25*). A 96-well microtiter plate (Maxisorb plates; Nunc) was coated with 100 μL/well Aβ mouse monoclonal IgG antibody 6E10 (Signet Pathology Systems Inc., Dedham, MA), which recognizes amino acid residues 6−10 of Aβ. 6E10 was diluted (1 μg/mL) in 200 mM $NaHCO_3$, pH 9.6. The plate was then covered with a plate sealer and stored at 4 °C overnight. The next day, the plate was washed 4 times with 20 mM PBS, pH 7.4 containing 0.05% Tween 20 (PBST). Blocking solution was then added to the plate (200 μL/well), and after a 2 h incubation at 37 °C, the plate was washed 4 times with PBST. Aβ with or without inhibitor samples diluted in PBS to a final Aβ concentration of 1 μM was added (100 μL/well), and the plate was incubated at 37 °C. At the end of the 2 h incubation period, the plate was washed 4 times with PBST and biotinylated 6E10 (diluted in blocking buffer, 1 μg/mL) was added to the plate (100 μL/well). The plates were incubated for 2 h at 37 °C and then washed 4 times with PBST, prior to the addition of 100 μL/well ExtrAvidin-alkaline phosphatase (Sigma-Aldrich Company Ltd., Dorset, U.K.) diluted 3:5000 in blocking buffer, and incubated for 1 h at 37 °C. The wells were then washed 4 times with PBST, before adding the enzyme substrate Yellow "pNPP" (Sigma-Aldrich Company Ltd., Dorset, U.K.) (100 μL/well), and the color was left to develop for 30 min at room temperature. Absorbance values, at 405 nm, were determined using a Victor^2 1420 (Wallac) microplate reader.

Size Exclusion Chromatography. At various time points, aliquots (100 μL) of incubated solutions of Aβ at 460 μM in PBS alone or with the peptide inhibitors (at 460 μM) were loaded on a Superdex 75 gel filtration column (10 mm × 270 mm) in 0.1 M Tris-HCl (pH 7.4) with a flow rate of 0.5 mL/min. Elution was monitored at 215 nm.

Transmission Electron Microscopy (TEM). Electron micrographs were produced from aged Aβ solutions with or without inhibitors. The samples were deposited on Formvar coated 400 mesh copper grids, fixed with 0.5% (v/v) glutaraldehyde, negatively stained with uranyl acetate, and examined on a JEOL JEM-1010 transmission electron microscope.

Tissue Culture of SH-SY5Y Human Neuroblastoma Cells. SH-SY5Y cells (European Collection of Cell Cultures, Porton Down, U.K.) were routinely cultured in Dulbecco's MEM/Nutrient Mix F-12 (1:1) (Gibco BRL, Rockville, MD) containing 1% penicillin-streptomycin, 15% fetal calf serum, 1% minimal essential medium amino acid supplement, and 2 mM freshly prepared glutamine (Gibco BRL, Rockville, MD) and maintained at 37 °C in a humidified incubator with 5% CO_2/95% room air. Cells were used for a maximum of 20 passages.

Measurement of Cell Viability. The effect of Aβ peptides on cell viability was assessed by measuring cellular redox activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich Company Ltd., Dorset, U.K.) as previously described (*27*). SH-SY5Y cells were plated at a density of 15 000 cells/well in 96-well plates in 100 μL of fresh medium. After 24 h, the medium was replaced with 200 μL of OPTI-MEM (Gibco BRL) serum-free medium and freshly prepared or aged solutions of Aβ alone or with inhibitors that were diluted in OPTI-MEM to give the required concentration of peptides. Cells were incubated at 37 °C in 5% CO_2 for 24 h. A total of 20 μL of stock MTT (6 mg/mL) was added to each well, and the plate was incubated at 37 °C for 4.5 h. The medium-MTT solution was removed, cell lysis buffer (100 μL per well; 15% (w/v) SDS/50% (v/v) *N*,*N*-dimethylformamide, pH 4.7) was added, and the plate was incubated overnight at 37 °C. Absorbance values at 570 nm were determined with an automatic plate reader.

RESULTS

Designing Peptides as Inhibitors of Aβ Oligomerization. Tjernberg and co-workers demonstrated that the central

region of Aβ (residues 16−20) is the main binding region responsible for the self-association and consequent aggregation of Aβ (*19*). Our strategy to design compounds to inhibit Aβ aggregation was based on small peptides from this binding region (which should interact specifically with Aβ monomeric molecules and interrupt their self-aggregation) linked to further solubilizing amino acid residues. The hydrophobic residues 16−20 of Aβ (KLVFF) were selected as the most appropriate region for designing our peptide inhibitors. However, solubility is a major problem with hydrophobic peptides, which have a tendency to aggregate from solution and so can act as seeds for further aggregation. Therefore, it was essential to design peptides that are soluble and do not aggregate or have any tendency to self-oligomerize. In the case of peptide inhibitors of α-synuclein aggregation, we overcame this problem by adopting a strategy involving the addition of a cationic Arg as a solubilizing residue at both the N- and the C-terminus of the hydrophobic peptides (*25*). However, the addition of Arg could decrease the interaction between the peptides and the monomeric Aβ molecules, as the native sequence for the binding region of Aβ does not contain any Arg residues. This potential problem was overcome by the addition of Gly as a spacer. Gly is also a hydrophilic residue and is the most conformationally unrestrained amino acid and generally breaks secondary structure. By placing Gly residues as spacers between Arg and the binding residues, we hoped to prevent Arg from participating in the interaction between the peptide and the native sequence of Aβ. On the basis of the previous criteria, RG-/-GR residues were added at the N- and C-terminal ends, respectively, of the KLVFF peptide, giving rise to peptide inhibitor OR1. The second peptide inhibitor to be synthesized (OR2) consisted of the amino acids RGKLVFFGR with an amide group at its C-terminus, to render the peptide less charged. Since the OR peptides were designed from the region of Aβ40 covering amino acid residues 16−20 (KLVFF), the unmodified fragment of Aβ40 corresponding to residues 16−20 (KLVFF-NH_2) was also included as a control in our studies on Aβ40 aggregation.

Effect of the OR Peptides on Aβ Fibril Formation. To investigate the effect of OR1 and OR2 peptides on Aβ40 aggregation, it was first necessary to identify the best conditions (i.e., Aβ40 concentration and time of incubation) for the aggregation of this batch of Aβ40 peptide. Incubation of Aβ40 solutions at 25, 50, and 100 μM for 12 days at 37 °C in PBS (pH 7.4) led to aggregation of the peptide and the formation of amyloid fibrils, as confirmed by negative stain electron microscopy. Amyloid fibril formation by Aβ40 was monitored over 12 days by the Th-T binding assay (Figure 1). It is clear that the best Aβ40 concentration is 100 μM and that 12 days of incubation resulted in full aggregation of this batch of peptide, as shown by the Th-T results (Figure 1).

Prior to investigating the effects of the OR1 (RGKLVFF-GR) and OR2 (RGKLVFFGR-NH_2) peptides on Aβ40 aggregation, the peptides were first tested alone to determine if they had any tendency to self-aggregate. All of the peptide solutions were aged at 100 μM in PBS (pH 7.4) for 12 days at 37 °C. A Th-T binding assay was carried out on the peptide samples every day to assess the presence of any amyloid aggregates. Under these conditions, none of the peptides showed any tendency to aggregate. However, the KLVFF-NH_2 peptide did aggregate when it was incubated at high concentration (5 mg/mL) as revealed by the Th-T assay. The effects of the OR peptides on Aβ40 aggregation over 12 days were then investigated using the Th-T assay.

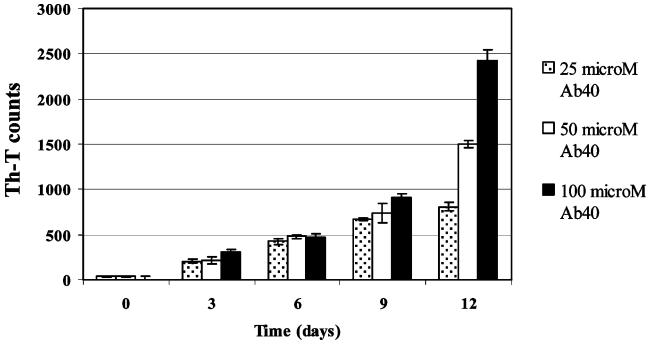

FIGURE 1: Effect of concentration and time of incubation on Aβ40 aggregation. Aβ40 samples were incubated for 12 days at 37 °C. Aβ40 aggregation was measured by a Th-T binding assay (see Experimental Procedures for details). The assays were performed in triplicate, and mean ± standard deviations are shown.

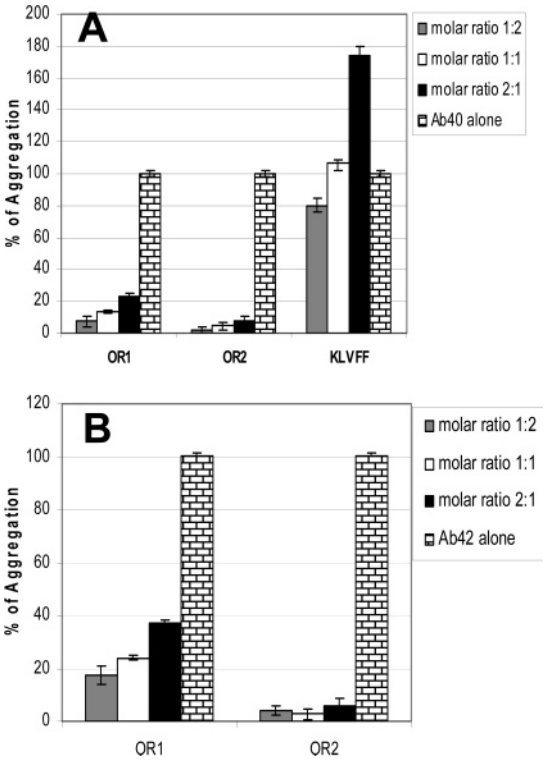

FIGURE 2: Effect of the peptide inhibitors on Aβ fibrillogenesis. Samples of Aβ40 (A) were incubated for 12 days and Aβ42 (B) for 2 days at 37 °C (see Experimental Procedures for details) in the presence of various concentrations of peptides. The amyloid fibril formation was then measured by the Th-T binding assay. Data were collected and presented as a percentage of the signal obtained from Aβ incubated in the absence of the peptide inhibitors. The assays were performed in triplicate, and mean ± standard deviations are shown.

When the Aβ40 solution was incubated with the OR2 peptide at 1:2; 1:1, and 2:1 (Aβ40/peptide) molar ratios with a constant Aβ40 concentration of 100 μM, complete inhibition of amyloid fibril formation was achieved (Figure 2A). OR1 also showed complete inhibition at 1:2 and 1:1, but to a less extent at 2:1, on Aβ40 fibril formation (Figure 2A). Under the same conditions, the KLVFF-NH_2 peptide showed no inhibition of Aβ40 fibrillogenesis (Figure 2A), and at

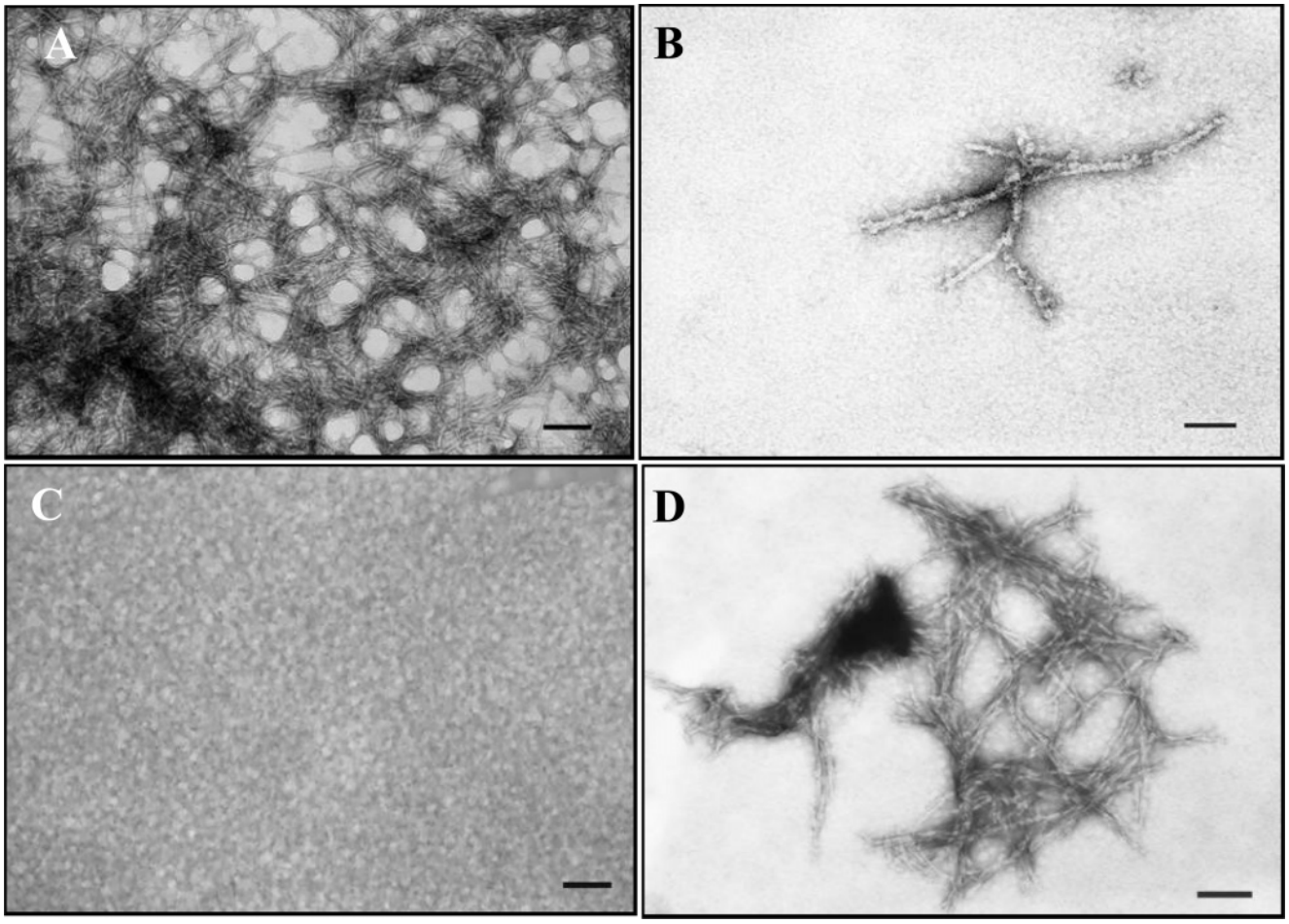

FIGURE 3: Electron microscopic examination of the effect of OR and control peptides on Aβ40 fibril formation. Aβ40 samples at 100 μM were incubated alone or in the presence of peptides at a 1:2 molar ratio (Aβ40/peptide) for 12 days at 37 °C (see Experimental Procedures for details). The figure shows negatively stained samples: (A) Aβ40 alone; (B) Aβ40 incubated with OR1, (C) Aβ40 incubated with OR2, and (D) Aβ40 incubated with KLVFF-NH_2. Scale bar = 100 nm.

higher concentrations, this peptide even seemed to accelerate the rate of Aβ40 aggregation (Figure 2A). The inhibitors were then tested against Aβ42 aggregation (at 100 μM in PBS (pH 7.4) over 2 days of incubation at 37 °C) to determine their effect on this more amyloidogenic peptide. A complete inhibition of amyloid fibril formation by the OR2 peptide was observed at molar ratios of 1:2; 1:1, and 2:1 (Aβ42/peptide) (Figure 2B). However, OR1 was less effective on Aβ42 aggregation and showed inhibition of Aβ42 aggregation by 82, 76, and 63% at molar ratios of 1:2; 1:1, and 2:1, respectively (Figure 2B). Again, the KLVFF-NH_2 peptide showed no effect on Aβ42 fibril formation at any concentrations tested (data not shown). Thus, only the designed OR peptides had the potential to act as inhibitors of Aβ40 and Aβ42 aggregation.

Effect of the OR Peptides on Aβ Fibril Morphology. We used electron microscopy to examine negatively stained samples of Aβ solutions that were prepared under the same conditions as those used for the Th-T binding assay and either aged alone or with OR peptides. The Aβ40 solution incubated alone showed the classic appearance of amyloid fibrils, revealed as a dense network of fibrils averaging 10 nm in diameter and extending for several hundred nanometers in length (Figure 3A). The ultrastructural appearance of these fibrils was similar to that reported previously (*3*, *4*). In contrast, when Aβ40 was incubated with peptides OR1 or OR2, inhibition of fibril formation was achieved, especially with OR2. As shown in Figure 3B, in the presence of the OR1 peptide, there were only very few fine, truncated fibrils, whereas in the presence of OR2, there were no fibrils at all (Figure 3C). Similar results were also obtained when Aβ42 solutions were incubated in the presence of OR peptides. No fibrils were detected in the samples containing OR2, whereas samples containing OR1 had a thin scattering of irregular aggregates and truncated fibrils (data not shown). The appearance and distribution of the fibrils obtained in the presence of KLVFF-NH_2 appeared to be very similar to those seen with Aβ40 alone (Figure 3D) or Aβ42 alone (data not shown). These results are in agreement with the Th-T data and confirm that only the OR peptides were able to inhibit Aβ40 and Aβ42 fibril formation and not the KLVFF-NH_2 peptide.

Inhibition of Aβ Oligomerization (Early Aggregates). The peptides were also tested for their effect on Aβ oligomerization and the formation of early aggregates. The same fresh and aged samples of Aβ solutions that were used for Th-T and the TEM assays and were incubated alone, or with the inhibitor peptides, were tested by a novel ELISA developed by our laboratory for detecting Aβ oligomers (Figure 4). The ELISA for oligomeric Aβ is a sandwich ELISA, in which oligomeric Aβ is captured by an anti-Aβ antibody, namely, Aβ mouse monoclonal antibody (6E10) and detected by the biotinylated form of the same antibody. The biotinylated 6E10 antibody was subsequently detected by Extr-

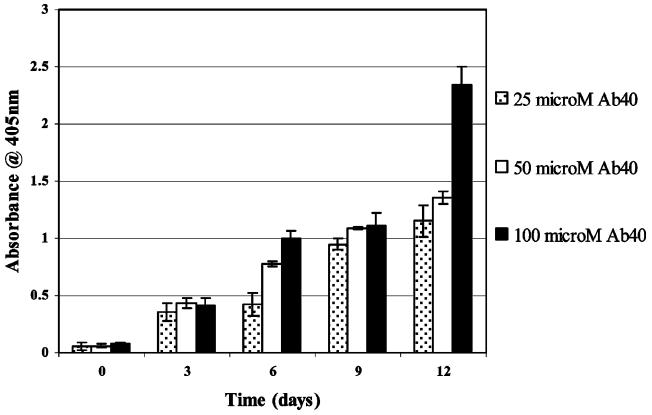

FIGURE 4: Effect of the concentration and incubation on Aβ40 oligomerization. Solutions of Aβ40 in PBS incubated at 37 °C at 25, 50, and 100 μM were diluted to 1 μM and transferred to a microtiter plate coated with immobilized 6E10. Additional epitopes formed by oligomerization of Aβ40 during the preincubation step were measured by the subsequent binding of biotinylated 6E10. Measurements were taken in triplicate, and the results show the mean ± standard deviation for each point.

Avidin alkaline phosphatase, followed by a colorimetric enzyme substrate. Monomeric Aβ cannot produce a signal in this assay as the primary antibody occupies the only 6E10 antibody binding site (i.e., amino acid residues 6−10 of Aβ sequence), and therefore, the biotinylated 6E10 antibody cannot subsequently bind. However, in the case of oligomeric forms of Aβ, there are multiple 6E10 antibody binding sites available, permitting both capture by the primary antibody and detection by the biotinylated secondary antibody (Figure 4).

Unlike OR2, which inhibited both late and early aggregates (as demonstrated by TEM and Th-T and ELISA, respectively) (Figure 5A,D), OR1 had some effect on the formation of Aβ40 oligomers only when it was present at higher concentrations, but it did not have any effect at lower concentrations (Figure 5B,D). In contrast, KLVFF-NH_2 did not affect the Aβ40 oligomerization at any concentration tested (Figure 5C,D).

The oligomeric ELISA showed that Aβ42 peptide forms soluble oligomers immediately in fresh solution (Figure 5E), in agreement with our previous reported results (*13*). Interestingly, when OR2 was added to freshly prepared Aβ42 solution at a molar ratio of 1:2, an immediate reverse (~53%) of Aβ42 oligomers was observed (Figure 5E), and this effect was concentration dependent (Figure 5E). When Aβ42 solutions were aged in the presence of OR2 at different molar ratios, OR2 was also able to inhibit the formation of Aβ42 oligomers, and its effect was again concentration dependent (Figure 5E). In contrast, OR1 had a slight effect on the oligomers when added to fresh solutions or incubated solutions of Aβ42 (Figure 5E). The KLVFF-NH_2 peptide had no affect on Aβ42 oligomerization at any concentrations tested (data not shown).

Furthermore, we also investigated the effects of OR peptides on Aβ40 oligomerization as detected by size exclusion chromatography. When we loaded 100 μL of Aβ40 solution, at 100 μM concentration, onto a gel filtration column, only a very small peak was detectable, and therefore, we had to use a higher concentration of Aβ40 (at 460 μM) for the peak to be large enough to monitor accurately. Using this higher concentration of Aβ40 solution allowed us to investigate the effects of the peptide inhibitors on its oligomerization by the size exclusion assay. Since the oligomerization and fibril formation of Aβ are concentration dependent, this explains as to why the Aβ40 solution (at 460 μM) oligomerized within 24 h of incubation, whereas at 100 μM, it took 12 days for the Aβ solution to aggregate.

The freshly prepared Aβ40 solution produced a single chromatographic peak with a retention time (35 min) equivalent to a monomer or dimer (Figure 6). The area of this peak declined by ~15% over 24 h. Protofibrils formed from Aβ40 were detected as a peak at 19.4 min elution time that appeared after 24 h of incubation (Figure 6) and increased at 48 h incubation time. However, after 6 days of incubation, the protofibril peak height decreased (data not shown). For Aβ40, this initial formation and subsequent decline in the protofibril peak area were consistent with the protofibrils being an intermediate on the pathway to the formation of Aβ40 insoluble fibrils. In contrast, the Aβ40 solution incubated in the presence of OR2 showed only a monomer/dimer peak that did not change as the incubation was prolonged, and no higher molecular weight peak was detected, consistent with the absence of oligomers (Figure 6). However, OR1 showed no effect on the peak area of protofibrils (Figure 6) when this was co-incubated with the Aβ40 solution. These findings confirm the results obtained by the oligomeric ELISA assay (Figures 5).

Effect of the OR Peptides on Aβ40 Toxicity. SH-SY5Y human neuroblastoma cells were treated with fresh and aged Aβ solutions at four different concentrations ranging from 3.125 −to 25 μM, and the MTT assay was employed to assess toxicity. Aged Aβ solutions inhibited the reduction of MTT in a dose-dependent fashion (Figure 7B). As the MTT reduction is directly proportional to the number of surviving cells (*28*), it becomes clear that the higher the Aβ concentration, the less cells survived. It should also be also noted that the pre-aggregated Aβ40 solutions, especially at low concentrations, had a more severe effect on the cells than the freshly prepared solutions (Figure 7A,B).

The effects of OR1, OR2, and KLVFF-NH_2 peptides on Aβ40 toxicity were then tested, and solutions of Aβ40 at 100 μM were prepared in the presence of 100 μM OR1, OR2, or KLVFF-NH_2. Half of each solution was aged, and the other half was frozen immediately at −80 °C and was tested as a fresh solution in the toxicity assay. Solutions of all the peptides alone were also prepared and used as controls.

Both OR1 and OR2 inhibited the toxicity of freshly prepared Aβ40, as in the presence of 25 μM of each of these peptides the percentages of viable cells were 70 and 82%, respectively, whereas in the presence of 25 μM Aβ40 alone, only 50% of the cells survived (Figure 7A). KLVFF-NH_2, on the other hand, did not have any inhibitory effect on the toxicity of Aβ40 but actually promoted it (Figure 7A). In the presence of 25 μM Aβ40 and 25 μM KLVFF-NH_2, less than 40% of the cells survived, as compared to approximately 50% survival when the cells were treated with Aβ40 alone (Figure 7A). However, if the Aβ40 solutions were preincubated in the presence of OR2 prior to their addition to the cell culture medium, the ability of the cells to metabolize MTT was dramatically restored to 80−100% of the controls without OR2 (Figure 7B), whereas OR1 showed only a

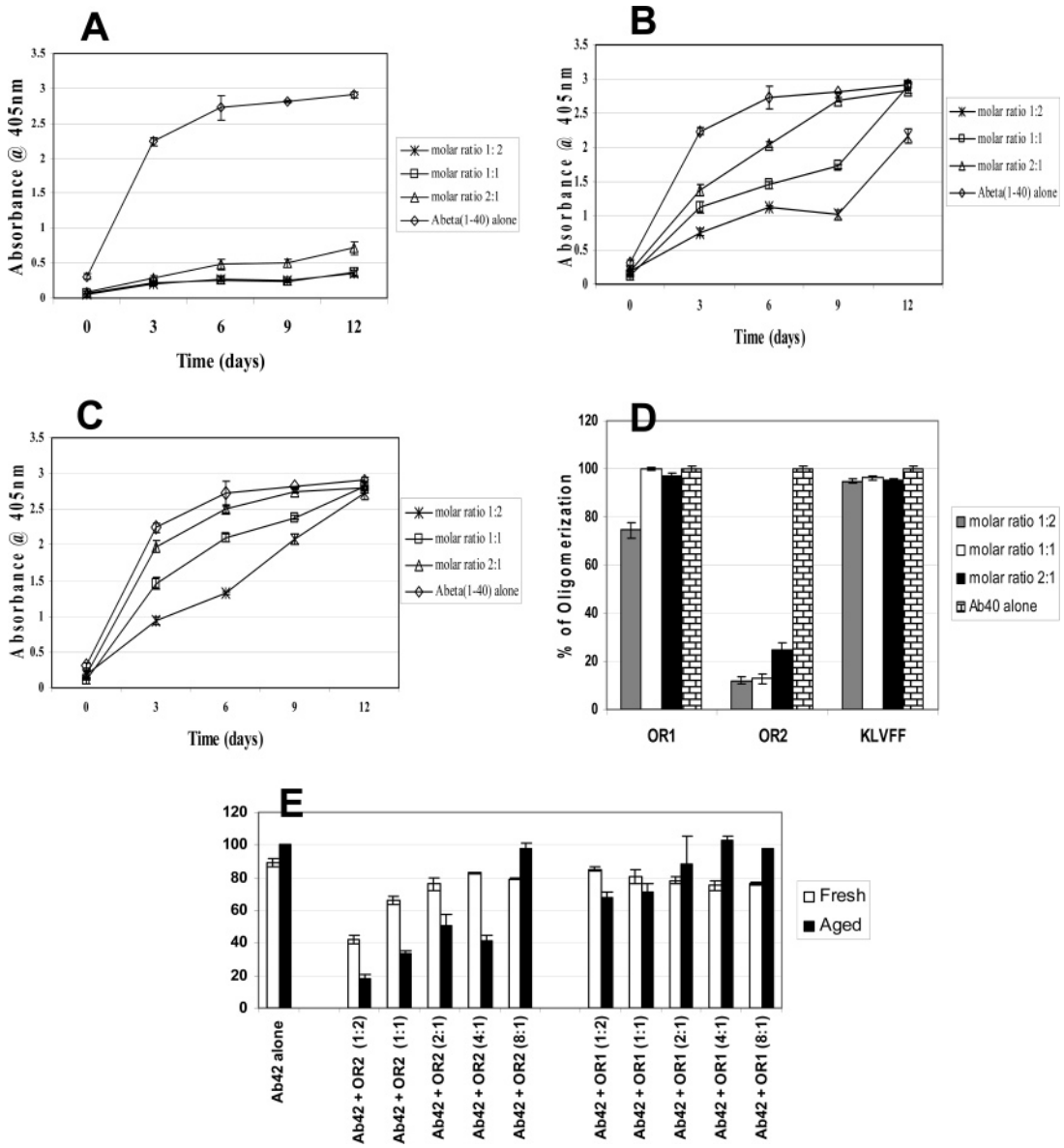

FIGURE 5: Effect of OR and control peptides on Aβ40 oligomerization. Aβ40 samples were incubated for 12 days at 37 °C in the presence of various concentrations of peptides, at the indicated molar ratios of Aβ40/peptide, and Aβ40 oligomerization was measured by ELISA (see Experimental Procedures for details). The effect of OR2 (A), OR1 (B), and KLVFF-NH_2 (C) on Aβ40 oligomerization was monitored over 12 days by ELISA. In panel D, data were collected after 12 days from Aβ40 samples, and in panel E, data were collected after 2 days from Aβ42 samples incubated with OR and control peptides and presented as a percentage of the signal obtained from the Aβ sample incubated in the absence of inhibitors. The assays were performed in triplicate, and mean ± standard deviations are shown.

partial effect on Aβ40 toxicity (Figure 7B). However, KLVFF-NH_2 did not have any inhibitory effect on the toxicity of aged Aβ40, but again, it enhanced the toxicity of Aβ40 (Figure 7). Finally, we tested Aβ42 solutions aged at 100 μM in the presence of 100 μM OR1, OR2, or KLVFF-NH_2. The solutions were then diluted in the culture media at four different concentrations ranging between 3.125 −and 25 μM and then their toxicity was tested on SH-SY5Y cells using the MTT assay. Only OR2 showed dramatic inhibition of Aβ42 toxicity between 72 and 100% as compared to control aged Aβ42 alone (Figure 7C), whereas OR1 (Figure 7C) or KLVFF-NH_2 (data not shown) showed no inhibition on Aβ42 toxicity. Our results clearly show that the OR2 peptide, which inhibits Aβ oligomer formation, also significantly reduced the toxicity of Aβ.

DISCUSSION

Accumulating evidence supports the hypothesis that Aβ fibrillogenesis is a seminal pathogenetic event in AD (*29*−*32*). This suggests that inhibition of Aβ aggregation may be a viable strategy for therapeutic intervention in AD. However, recent studies on amyloid proteins including Aβ support the idea that nonfibrillar aggregates or soluble oligomers are the pathogenic components that drive neurodegeneration and neuronal cell death, rather than mature amyloid fibrils (*6*−*18*). Therefore, inhibiting and/or reversing the early stages of Aβ oligomerization is an attractive therapeutic approach for targeting the underlying disease progression of AD (*29*−*32*). Considering the importance of soluble oligomeric forms of Aβ in AD pathogenesis, it is now clear that drug development in this area should focus on inhibitors of the

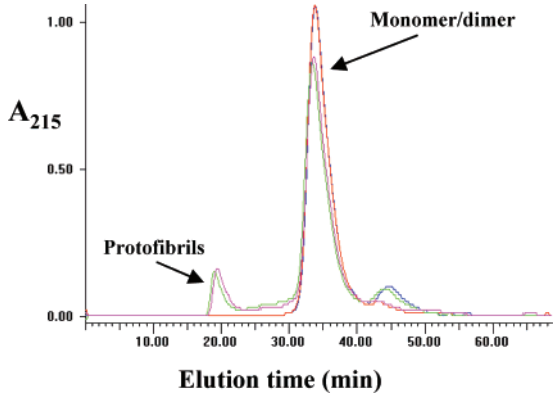

FIGURE 6: Size exclusion chromatography of Aβ40. A freshly prepared solution of Aβ40 (460 μM) was prepared alone or with 460 μM peptide inhibitor in PBS (pH 7.4) buffer. The solutions were fractionated on a Superdex 75 column after incubation for 24 h at 37 °C. Fresh Aβ40 alone (red), aged Aβ40 with OR1 (green), and aged Aβ40 with OR2 (blue) are shown. The gel-included peak elutes at ~30.5 min, while the gel-excluded peak elutes at ~19.4 min.

oligomerization of Aβ rather than inhibitors of fibril formation (*18*).

Aβ self-aggregation is driven by hydrophobic−hydrophobic interactions and is accompanied by conformational transition from the non-pathogenic random coil to the pathogenic β-sheet. Considerable progress has already been made in designing and discovering inhibitors of Aβ aggregation and toxicity (*19*−*24*). Several strategies have been employed to design peptide inhibitors of Aβ aggregation and toxicity. All of the peptides designed in these studies are based on the Aβ (*16*−*22*) fragment, which is responsible for the self-aggregation of Aβ (*20*−*24*). Soto and co-workers designed peptides, termed β-sheet breaker peptides, by incorporating proline residues into the peptide sequence (*20*). These β-sheet breaker peptides can bind to soluble Aβ and prevent its conversion into toxic aggregates. The second strategy reported recently uses *N*-methylamino acids of the Aβ (*16*−*22*) sequence (*23*, *24*). The methylated peptides appear to act by binding to Aβ through one hydrogen bonding face and simultaneously blocking the propagation of the hydrogen bond array of the β-sheet with a non-hydrogen bonding face. Recently, Murphy and co-workers reported another strategy for designing peptides to inhibit Aβ toxicity (*22*). These peptide inhibitors were composed of residues 15−25 of Aβ, designed to function as the recognition element, linked to an oligolysine disrupting element. Interestingly, these inhibitors neither alter the apparent secondary structure of Aβ nor prevent its aggregation; rather, they cause a change in aggregation kinetics and the higher-order structural characteristics of the aggregate. However, most of the past studies have utilized techniques such as turbidity, TEM, Th-T, sedimentation, and Congo red binding assays to screen for inhibitors of Aβ aggregation. Although these methods have been useful for high-throughput screening, they can only identify the compounds that are capable of inhibiting the formation of Aβ fibrils.

To identify inhibitors of Aβ oligomerization as a potential new drug for AD, we made some new peptides and tested their effects on Aβ oligomerization and toxicity. These peptides were designed from the region of Aβ (KLVFF, residues 16−20) that is responsible for its self-association (*19*, *33*). The addition of RG-/-GR to the N- and C-terminal ends of this peptide gave rise to inhibitor OR1. The second peptide inhibitor, OR2, also contained the RG-/-GR amino acids but had an amide group at its C-terminus to render it less charged. The KLVFF peptide alone, containing an amide group at its C-terminus, was also synthesized and used as a control in our study. This attempt to design potential inhibitors of Aβ aggregation follows the same rationale as that used successfully for peptide inhibitors of α-synuclein aggregation, which were based on the hydrophobic binding sequence of α-synuclein (residues 69−72) linked to a solubilizing component (RG-/-GR) (*25*).

On the basis of Th-T results and TEM micrographs, OR1 and OR2 inhibited the formation of Aβ late aggregates, while the unmodified KLVFF-NH_2 fragment had no inhibitory effect at any concentration tested (Figures 2 and 3). However, using the ELISA that detects Aβ soluble oligomers, the results for the inhibition of Aβ oligomerization revealed that OR2 (Figures 5 and 6) had the best inhibitory effect, with OR1 (Figures 5 and 6) having a slight blocking effect at higher concentrations. On the other hand, the unmodified KLVFF-NH_2 had no effect at all (Figures 5 and 6). It is likely that OR2 inhibits oligomeric formation by binding to the monomeric Aβ molecule, thereby blocking the formation of early soluble aggregates, as shown by size exclusion chromatography and oligomer specific ELISA.

Comparing OR2 (i.e., RGKLVFFGR-NH_2), which is a good inhibitor of Aβ40 oligomerization, to OR1 (i.e., RGKLVFFGR), the difference between the two peptides is that OR1 lacks the amide (-NH_2) group at the C-terminus. As the peptide inhibitors arose from an internal Aβ sequence (i.e., they did not arise from the natural N- or C-terminus of the protein), it was thought best to block the end of the peptide by having the C-terminus made as an amide. By using this design, the introduction of a charged group at a site where the native peptide does not contain any charged groups was avoided. Blocking the C-terminus with the amide group could be beneficial, as the peptides would then be more likely to behave like, or be recognized, as if they were a part of the whole protein from which the sequence was chosen. According to our results, the blocking of the C-terminus with the amide group has proven to be beneficial, as OR2 is overall a much better inhibitor than OR1.

Aged solutions of Aβ alone were active in reducing MTT metabolism in SH-SY5Y cells (Figure 7B). When we investigated the effect of our peptide inhibitors on Aβ cytotoxicity, both OR peptides had the ability to prevent Aβ fibril formation as revealed by Th-T and electron microscopy, whereas OR2 was also shown to be a potent inhibitor of Aβ oligomerization. Interestingly, only OR2, which demonstrated complete inhibition for the early aggregates of Aβ (Figures 5 and 6), was able to protect the cells from Aβ toxicity (Figure 7). However, in the case of OR1, which showed only inhibition for the late aggregates, no neuroprotective effects were demonstrated (Figure 7). The results reported here provide clear evidence that the neuroprotective effects demonstrated by OR2 were due to the inhibition of formation of early aggregates rather than late aggregates of Aβ. This is consistent with previous reports showing that soluble nonfibrillar aggregates of Aβ are the toxic species (*6*−*18*), and it has been proposed that compounds that can block the formation of early aggregates of Aβ may serve as new drugs

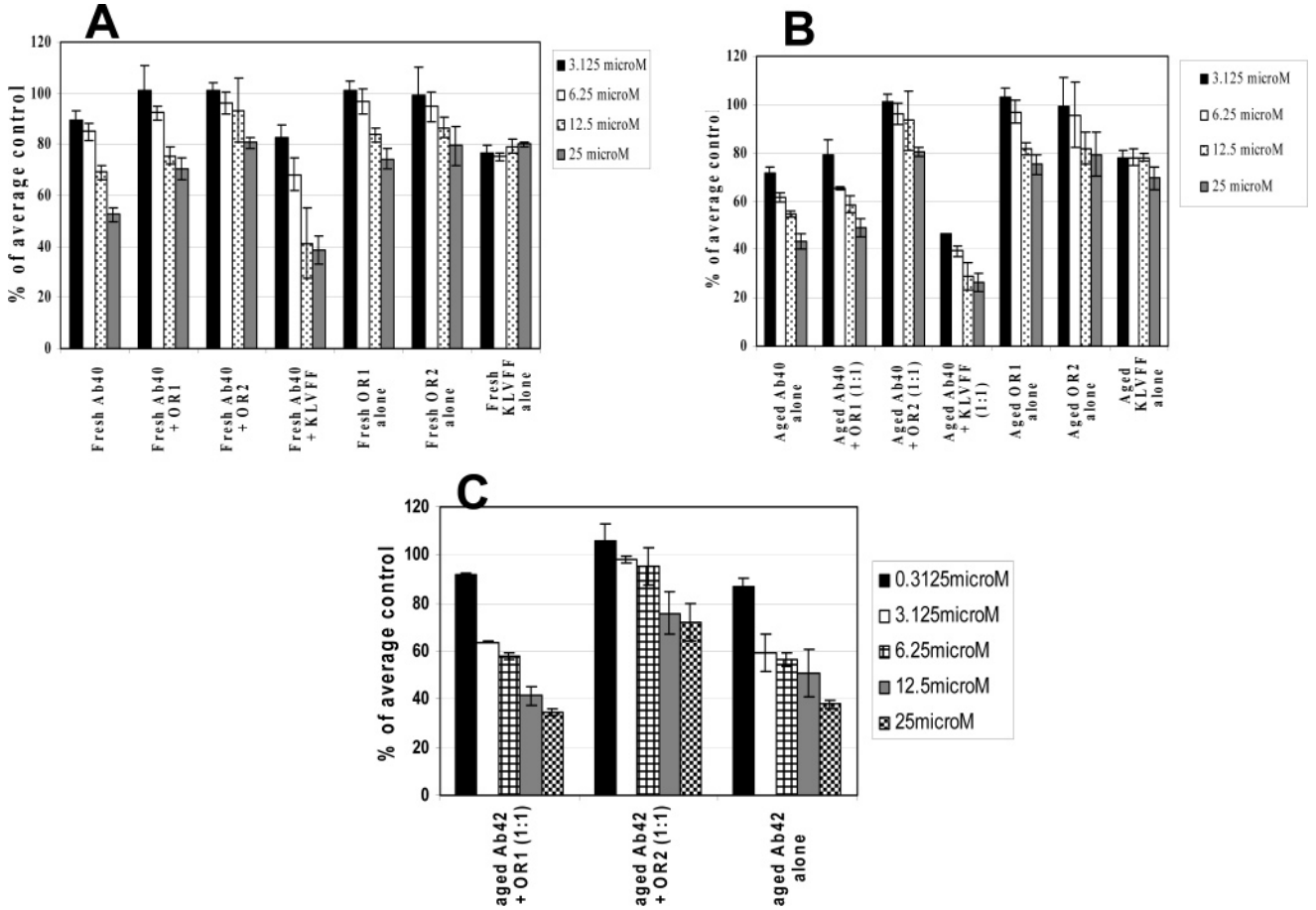

FIGURE 7: Effect of OR1, OR2, and KLVFF-NH_2 on the toxicity of fresh Aβ40 (A), aged Aβ40 (B), or aged Aβ42 solutions (C). Viable SH-SY5Y cells were detected using the MTT assay. The results are expressed as percentages of average control (i.e., untreated cells). The cells were treated with the peptides for 24 h prior to MTT addition (av of 6 wells ± SD).

for the treatment of AD (*29*−*32*). Since the OR2 peptide identified here is relatively small, it could represent the starting point for designing peptidomimetic molecules more suitable for chronic therapy, which may be used as new drugs for the treatment of AD.

In summary, this study provides a new strategy for designing peptide inhibitors of Aβ oligomerization and toxicity. Using several methods, we tested the effect of our inhibitors on the early and late stages of Aβ aggregation. Only the peptide that can inhibit the formation of early stages of Aβ aggregation was also capable of blocking its toxicity. Therefore, elucidation of the molecular events that initiate Aβ oligomerization is crucial to understand the pathology and to develop potential therapeutics for AD.

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