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Development of a Proteolytically Stable Retro-Inverso Peptide Inhibitor of β-Amyloid Oligomerization as a Potential Novel Treatment for Alzheimer's Disease†

Mark Taylor,‡ Susan Moore,‡ Jennifer Mayes,‡ Edward Parkin,‡ Marten Beeg,§ Mara Canovi,§ Marco Gobbi,§ David M. A. Mann,∥ and David Allsop*,‡

‡*Division of Biomedical and Life Sciences, School of Health and Medicine, Lancaster University, Lancaster LA1 4YQ, U.K.,* §*Department of Biochemistry and Molecular Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy, and* ∥*Clinical Neurosciences Research Group, University of Manchester, Hope Hospital, Salford M6 8HD, U.K.*

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ABSTRACT: The formation of β-amyloid (Aβ) deposits in the brain is likely to be a seminal step in the development of Alzheimer's disease. Recent studies support the hypothesis that Aβ soluble oligomers are toxic to cells and have potent effects on memory and learning. Inhibiting the early stages of Aβ aggregation could, therefore, provide a novel approach to treating the underlying cause of AD. We have designed a retro-inverso peptide (RI-OR2, H_2N-r←G←k←l←v←f←f←G←r-Ac), based on a previously described inhibitor of Aβ oligomer formation (OR2, H_2N-R-G-K-L-V-F-F-G-R-NH_2). Unlike OR2, RI-OR2 was highly stable to proteolysis and completely resisted breakdown in human plasma and brain extracts. RI-OR2 blocked the formation of Aβ oligomers and fibrils from extensively deseeded preparations of Aβ(1−40) and Aβ(1−42), as assessed by thioflavin T binding, an immunoassay method for Aβ oligomers, SDS−PAGE separation of stable oligomers, and atomic force microscopy, and was more effective against Aβ(1−42) than Aβ(1−40). In surface plasmon resonance experiments, RI-OR2 was shown to bind to immobilized Aβ(1−42) monomers and fibrils, with an apparent K_d of 9−12 μM, and also acted as an inhibitor of Aβ(1−42) fibril extension. In two different cell toxicity assays, RI-OR2 significantly reversed the toxicity of Aβ(1−42) toward cultured SH-SY5Y neuroblastoma cells. Thus, RI-OR2 represents a strong candidate for further development as a novel treatment for Alzheimer's disease.

There is substantial evidence from molecular genetics, transgenic animal studies, and aggregation/toxicity studies to suggest that the conversion of the β-amyloid (Aβ)[1] peptide from soluble monomers to aggregated forms in the brain is a key event in the pathogenesis of Alzheimer's disease (AD). It seems increasingly likely that early "soluble oligomers" are responsible for the reported toxic effects of Aβ, rather than fully formed amyloid fibres, and these small oligomers could be one of the causes of neurodegeneration in vivo (*1*−*9*). They have also been reported to have potent effects on synaptic plasticity, learning, and memory in animal models (*2*, *3*, *7*). Inhibition of toxic Aβ oligomer formation is therefore a potential therapeutic target for AD (*10*).

Considerable progress has already been made in discovering inhibitors of Aβ aggregation and toxicity (*11*, *12*). One of the strategies employed has been the use of peptide-based inhibitors (*13*). These have been focused generally on the internal Aβ(16−22) sequence, KLVFFAE, because this region has been reported to be primarily responsible for the self-association and aggregation of the peptide (*14*, *15*). Soto and co-workers have designed "β-sheet breaker peptides" by incorporating proline residues into this peptide sequence (*16*, *17*). Another strategy has used N-methylated peptides (*18*, *19*), which act by binding to Aβ through one hydrogen-bonding face while blocking the propagation of the hydrogen bond array of the β-sheet with the other non-hydrogen-bonding face. Murphy and co-workers (*20*) have reported a different strategy, based on residues 15−25 of Aβ linked to an oligolysine disrupting element. However, these latter inhibitors appear not to prevent Aβ aggregation but cause a change in aggregation kinetics and higher-order structural characteristics of the aggregate (*20*). Some further modifications of these peptide inhibitors have included the incorporation of solubilizing (charged) residues (*21*), the use of D-amino acids to improve stability (*21*, *22*), multivalent peptides for increased potency (*23*), and the attachment of targeting sequences to enhance cell and blood−brain barrier permeability (*21*). It should be noted that these past studies have generally utilized techniques such as turbidity, thioflavin-T binding, sedimentation, and Congo red binding to test for inhibitors of Aβ aggregation. These methods can identify only compounds that are capable of inhibiting the formation of Aβ fibrils, so their effects on toxic oligomer formation are often not clearly established.

A peptide-based inhibitor, named OR2, with the amino acid sequence RGKLVFFGR-NH_2, was shown previously to inhibit the formation of early oligomeric forms of Aβ (*24*). This peptide, with an amidated C-terminus, was a better inhibitor of Aβ

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*To whom correspondence should be addressed: Division of Biomedical and Life Sciences, School of Health and Medicine, Lancaster University, Lancaster LA1 4YQ, U.K. Telephone: 44-1524-592122. Fax: 44-1524-593192. E-mail: d.allsop@lancaster.ac.uk.

[1]Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; AFM, atomic force microscopy; BSA, bovine serum albumin; ByBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; ECL, enhanced chemiluminescence; HFIP, hexafluoro-2-propanol; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PB, phosphate buffer; PBS, phosphate-buffered saline; SDS−PAGE, polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate; SPR, surface plasmon resonance; TFA, trifluoroacetic acid; Th-T, thioflavin T.

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OR1 H_2N-R→G→K→L→V→F→F→G→R-COOH

OR2 H_2N-R→G→K→L→V→F→F→G→R-NH_2

RI-OR2 H_2N-r←G←k←l←v←f←f←G←r-Ac

FIGURE 1: Structures of OR1, OR2, and RI-OR2 (retro-inverso OR2). L-Amino acids are in uppercase and D-amino acids in lowercase, with the direction of peptide bonds indicated by arrows. There are no separate enantiomers of glycine, which is represented in uppercase.

oligomer formation and associated Aβ toxicity than the same peptide with a conventional C-terminus [called OR1 (see Figure 1)] (*24*). These OR1 and OR2 peptides were designed from the central region of Aβ (KLVFF, residues 16−20), which, as noted above, is part of the binding region responsible for its self-association (*14*, *15*). However, to aid the solubility of these peptide inhibitors, and at the same time to prevent them from self-aggregating and so acting as a "seed" to promote Aβ aggregation, an additional cationic Arg was added at their N- and C-termini, in each case via Gly as a spacer (*24*). The intention of placing these Gly residues as spacers between Arg and the KLVFF binding sequence was to facilitate the interaction between the inhibitor peptides and native Aβ (*24*). A similar strategy has been employed for the development of successful inhibitors of α-synuclein aggregation, as a potential novel treatment for Parkinson's disease and related disorders (*25*). Although OR2 was shown to be an effective inhibitor of the formation of Aβ oligomers (*24*), there are many potential sites for proteolytic attack on this peptide, so it is unlikely itself to be a viable drug candidate. To attempt to avoid this problem but retain the antiaggregational properties of this peptide, we have now designed a "retro-inverso" version (*26*, *27*) of OR2 (designated RI-OR2). This is a more sophisticated approach than simply replacing some or all of the L-amino acids with D-amino acids (*21*, *22*). In a retro-inverso peptide, all of the natural L-amino acids are replaced with the D-enantiomer, but the peptide bonds are also reversed (*26*, *27*). In theory, this should maintain a three-dimensional shape similar to that of the "parent" peptide molecule and so help to retain biological activity. Here we report the results of Aβ aggregation, binding, and toxicity studies with this new RI-OR2 peptide inhibitor, as well as studies of the stability of the peptide inhibitor to proteolytic degradation.

EXPERIMENTAL PROCEDURES

Aβ Peptides. Recombinant Aβ(1−40) and Aβ(1−42) peptides (Ultrapure) were used for all of the experiments described below, except for the binding experiments involving the surface plasmon resonance (SPR) technique. The recombinant peptides were purchased from rPeptide Co. (Bogart, GA) and were already treated with hexafluoro-2-propanol (HFIP). Unless indicated otherwise, prior to use, the peptides were twice deseeded again in HFIP and dried using centrifugal evaporation before being dissolved in 10 mM phosphate buffer (PB) (pH 7.4) and sonicated for 4 × 30 s at 12 μm amplitude using an MSE sonicator.

For the SPR experiments only, Aβ(1−42) was synthesized first of all as the depsi-peptide molecule described by Taniguchi et al. (*28*). The depsi-peptide is much more soluble than the native peptide, thus avoiding the need of vortexing for its dissolution, and also has a much lower propensity to aggregate, thereby preventing the spontaneous formation of seeds in solution. The native Aβ(1−42) peptide was then obtained from the depsi-peptide by a "switching" procedure involving a change in pH (*28*). The Aβ(1−42) peptide solution obtained immediately after switching is seed-free as shown in carefully conducted previous work (*28*, *29*). To prepare Aβ(1−42) fibrils, the switched peptide solution was diluted with water to 100 μM, acidified to pH 2.0 with 1 M HCl, and left to incubate for 24 h at 37 °C. The presence of amyloid fibrils was confirmed by atomic force microscopy (AFM) (*29*) (see also Figure S2C of the Supporting Information).

Peptide Inhibitors. Inhibitory peptides OR2 and RI-OR2 were made by Cambridge Peptides (Birmingham, U.K.). OR2 was made on a conventional automated amino acid synthesizer, whereas RI-OR2 was made by manual Fmoc synthesis, using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (ByBOP) as a coupling agent, on Rink amide MBHA resin. After purification by reverse-phase high-performance liquid chromatography (HPLC), the peptides were >95% pure.

Stability of Inhibitors to Proteolysis. The stability of the inhibitors in plasma and brain extracts was assessed by addition of 10 μL of filtered (0.22 μm filter) human blood plasma (50 mg/mL) or postmortem human brain tissue extract (5 mg/mL) to 100 μL of filtered inhibitor (1 mM) in PBS. The brain tissue extract was prepared by macerating a small (∼50 μg) piece of frozen brain frontal cortex in 100 μL of sodium acetate buffer (pH 7.4). The inhibitor, in plasma or brain extract, was incubated at 37 °C for 24 h, loaded onto a Jupiter C18 reversed-phase HPLC column (Phenomenex) attached to a Dionex HPLC system, and separated using a gradient from 0 to 60% acetonitrile, containing 0.1% trifluoroacetic acid, over 30 min. Analysis of peptide recovery was performed with Chromeleon software. The absorbance was measured at 220 nm.

The stability of inhibitors to individual proteases was assessed following incubation of a 1 mM solution of inhibitor and 0.1 mg/mL purified protease for 24 h at 37 °C in 10 mM PB (pH 7.4) (except in the case of pepsin, where the pH was lowered to 4). Each solution (30 μL) was loaded onto a Jupiter C18 reversed-phase HPLC column, and the peptide was eluted and analyzed as described above.

Thioflavin T Assays. Th-T assays were conducted in 96-well clear-bottom microtiter plates (NUNC) by incubating the Aβ peptides (20 μM) at 25 °C in the continuous presence of Th-T (10 μM) in 10 mM PB (pH 7.4). Inhibitor, when present, was at molar ratios of 5:1, 2:1, 1:1, 1:2, and 1:5 relative to Aβ, and the total volume of the solution in each well was 100 μL. The plates were shaken and read every 10 min (λ_{ex} = 442 nm, and λ_{em} = 483 nm) in a BioTek Synergy plate reader.

Immunoassay for Oligomeric Aβ. This sandwich immunoassay follows the method described previously by Moore et al. (*30*). Briefly, 96-well ELISA plates (Maxisorb) were coated with mouse monoclonal antibody 6E10 diluted 1:1000 in assay buffer [Tris-buffered saline (TBS) (pH 7.4) containing 0.05% γ-globulins and 0.005% Tween 20]. The incubated samples of peptide, with or without inhibitor (20 μM Aβ in 10 mM PB and inhibitor:Aβ molar ratios of 2:1, 1:1, and 1:2), were diluted to 1 μM Aβ and incubated, in triplicate, in the 96-well plates for 1 h at 37 °C. The plates were washed with 10 mM phosphate-buffered saline (PBS), containing 0.5% Tween 20 (PBS-T). Following this, 100 μL of TBS containing a 1:1000 dilution of biotinylated 6E10 was added to each of the 96 wells and incubated for 1 h at 37 °C. After the samples had been washed further, europium-linked streptavidin was added at 1:500 dilution in StrepE buffer (TBS containing 20 μM DTPA, 0.5% bovine serum albumin, and 0.05% γ-globulins), again incubated

for 1 h, and washed. Enhancer solution was added, and the plates were read using the time-resolved fluorescence setting for europium on a Wallac Victor 2 plate reader.

Detection of Oligomers by SDS−PAGE and Immunoblotting. For these experiments, Aβ(1−42) was deseeded to a monomeric form, based on a modification of the method described by Manzoni et al. (*31*). The peptide (1 mg) was dissolved in 1 mL of trifluoroacetic acid (TFA) containing 45 μL of thioanisole in a glass container. This was incubated for 1 h at room temperature and sonicated (4 × 30 s) in an ice−water bath every 15 min. The liquid was evaporated using a stream of nitrogen gas, and the peptide was redissolved in 1 mL of HFIP. This was sonicated (4 × 30 s in an ice−water bath) after incubation for 10 min at room temperature. The HFIP was removed by centrifugal evaporation and the step repeated. Following this, the protein was again dissolved in HFIP, sonicated, and then centrifuged at 15700*g* at room temperature to pellet any aggregated peptide. The liquid was decanted before being divided into convenient aliquots and the HFIP removed by centrifugal evaporation. Dried peptide was stored at −20 °C.

To assess the ability of RI-OR2 to inhibit the formation of small oligomers of Aβ(1−42), we followed a slightly modified method of Chafekar et al. (*23*). Deseeded Aβ(1−42) was dissolved at a concentration of 5 μM in 10 mM PB (pH 7.4) containing 0, 5, or 25 μM RI-OR2. A sample was taken (time zero), and then the solutions were incubated at 37 °C for 4 h. Each solution was mixed with Novex tricine SDS sample buffer (Invitrogen) and loaded, without boiling, onto a Novex 10−20% tricine gel (Invitrogen). The gel was run for 1 h at 100 V and then protein transferred by Western blot onto a nitrocellulose membrane. The membrane was probed using mouse monoclonal antibody 6E10 at 1:10000 in 5% dried skimmed milk powder in PBS with 0.5% Tween. After any unbound antibody had been removed by washing with PBS Tween, the membrane was probed with a 1:5000 dilution of HRP-linked rabbit anti-mouse antibody (Sigma) in 5% milk powder as described above. Following another washing step, as before, bands were visualized using the Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate and detected using Amersham Hyperfilm ECL.

Atomic Force Microscopy. Atomic force microscopy (AFM) was conducted on a Digital Instruments Multimode Scanning Probe Microscope using tapping mode. Aβ(1−42), preincubated for 24 h at 25 °C in 10 mM PB (pH 7.4) with and without inhibitor (at a 1:1 molar ratio), was spotted onto freshly cleaved mica sheets and allowed to dry. Three scans were performed for each condition.

Cell Toxicity Studies. SHSY-5Y cells were maintained in Ham's F12 and EMEM medium mixed at a 1:1 ratio containing 2 mM glutamine, 1% nonessential amino acids, and 15% fetal calf serum supplemented with a 500 μg/mL penicillin/streptomycin solution. Cells were transferred to a sterile 96-well plate with approximately 25000 cells per well and allowed to acclimatize for 48 h. The Ham's F12/EMEM medium was removed by suction and replaced with Optimem medium (100 μL/well) containing either no Aβ or Aβ(1−42) (10 μM, preincubated on its own for 24 h in PB), with and without the OR2 and RI-OR2 inhibitors (10 μM). The cells were left for 24 h and then assessed. The toxicity of the inhibitors was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation (MTS) Assay (Promega) and CytoTox-ONE Homogeneous Membrane Integrity (LDH) Assay (Promega).

Surface Plasmon Resonance Experiments. These experiments were conducted using a Bio-Rad ProteOn XPR36 SPR machine (*32*). Aβ(1−42) monomers or fibrils (see Aβ peptides), with bovine serum albumin (BSA) as a control, were immobilized in parallel-flow channels of the same GLC sensor chip (Biorad) using amine coupling chemistry. Briefly, after surface activation, the monomeric or fibrillar peptide preparations were diluted to 10 μM in acetate buffer (pH 4.0) and then injected for 5 min at a flow rate of 30 μL/min. Any remaining activated groups were blocked with ethanolamine (pH 8.0). The final immobilization levels were similar in each case, with both being ∼2500 resonance units (1 RU = 1 pg of protein/mm^2). An empty "reference" surface was prepared in parallel using the same immobilization procedure, but without addition of the peptide. Both Aβ(1−42) monomers and fibrils immobilized on the sensor chip bound 6E10 antibody, whereas fibrils bound only Congo red (M. Gobbi, personal communication). Sensorgrams were then obtained via injection of three different concentrations of RI-OR2 (3, 10, and 30 μM), as well as the vehicle (PBS with 0.005% Tween 20), over the immobilized ligands or control surfaces, in parallel, at the same time. In a second set of experiments, further sensorgrams were obtained via injection of Aβ(1−42) monomers (3 μM), RI-OR2 alone (30 μM), or a combination of the two (preincubated for 15 min before injection) into the flow chambers.

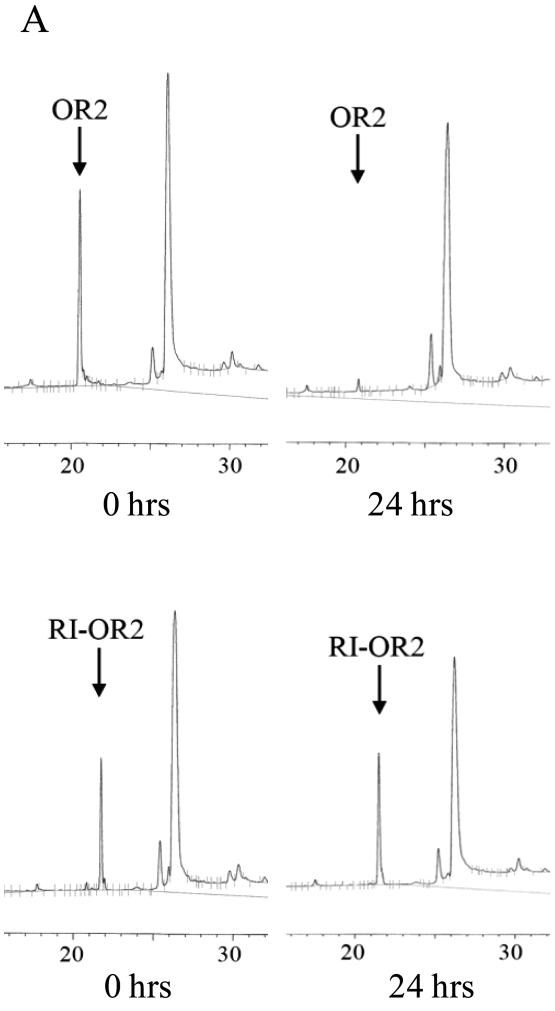
RESULTS

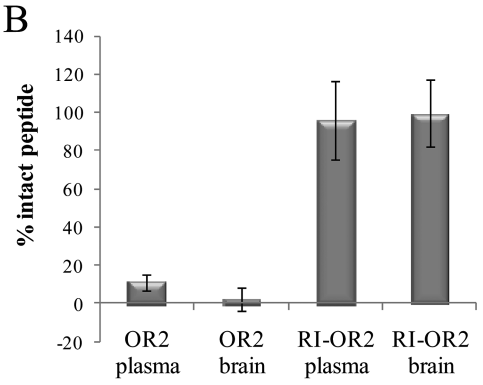
Stability of Inhibitors to Proteolytic Degradation. The stability of the inhibitors to protease action was assessed by incubating them in the presence of either a human blood plasma or brain extract, followed by HPLC analysis to quantify the amount of intact peptide remaining in solution. After an incubation period of 24 h, OR2 was almost completely degraded by either the human plasma or the brain extract, whereas the amount of RI-OR2 remained at (or close to) 100% (Figure 2A,B).

Studies with individual proteolytic enzymes showed that OR2 was sensitive to degradation by elastase, cathepsin G, kallikrein, thrombin, trypsin, and chymotrypsin. In contrast, RI-OR2 was completely resistant to attack by these proteases (Figure 2C).

Thioflavin T Assay of Inhibition of Aggregation. The aggregation of Aβ(1−40) and Aβ(1−42) into Th-T positive amyloid fibrils was first of all monitored by incubation of each peptide (20 μM) in the continuous presence of Th-T (10 μM) in a 96-well microtiter plate, with shaking and then measurement of fluorescence every 10 min, for periods of up to 48 h. Panels A and B of Figure 3 show data for the first 22 h, for each peptide. Aβ(1−40) exhibited a lag time (corresponding to the nucleation phase) of ∼16 h, before a period of rapid fibril formation, which was complete after 18−20 h, whereas Aβ(1−42) exhibited virtually no lag period and achieved maximal fluorescence after only 2−3 h. RI-OR2, at an equimolar concentration to Aβ, almost completely eliminated the development of Th-T fluorescence over these time periods, for both Aβ(1−40) and Aβ(1−42). The data presented in Figure 3A,B are from a single experiment but are representative of three separate experiments. After 22 h in the presence of OR2, approximately 40% of control Th-T fluorescence (after subtraction of background) was reached for Aβ-(1−40), compared to < 10% for RI-OR2. Corresponding figures for Aβ(1−42) were approximately 80% (OR2) and < 10% (RI-OR2) of control fluorescence. Thus, under these experimental conditions, RI-OR2 was a more effective inhibitor of Aβ aggregation than OR2.

The data presented in panels C and D of Figure 3 show inhibition of aggregation of 20 μM Aβ(1−40) and Aβ(1−42)





C

Protease	OR2	RI-OR2
None	✓	✓
Elastase	×	✓
Cathepsin G	×	✓
Kallikrein	×	✓
Thrombin	×	✓
Plasmin	✓	✓
Factor Xa	✓	✓
Trypsin	×	✓
Chymotrypsin	×	✓
ADAM 10	✓	✓

FIGURE 2: Relative resistance of OR2 and RI-OR2 to proteolysis. (A) HPLC traces (absorbance at 220 nm and elution time in minutes) for OR2 and RI-OR2 after incubation in plasma for 0 and 24 h. (B) Percentage recovery following a 24 h incubation of the peptide inhibitors in plasma and brain extracts. (C) Their susceptibility to individual proteases (√, stable; ×, degraded). Results in panel B are means ± the standard deviation for three separate repeats.

under experimental conditions similar to those described above, but with different inhibitor:Aβ molar ratios ranging from 1:5 to 5:1, at a fixed time point for Aβ incubation [Aβ(1−40) at 22 h and Aβ(1−42) at 2 h]. Both OR2 and RI-OR2 were effective inhibitors, with RI-OR2 showing the most convincing concentration-dependent trend. In this assay, OR2 appeared to be a better inhibitor than RI-OR2 at the lowest concentration tested, with 69% inhibition for OR2 and 33% for RI-OR2 [against Aβ(1−40)], and 98% inhibition for OR2 and 78% for RI-OR2 [against Aβ(1−42)] for an inhibitor:Aβ molar ratio of 1:5. However, at a 1:1 ratio, RI-OR2 was the better inhibitor, in agreement with the data shown in panels A and B of Figure 3. Interestingly, both inhibitors were more effective against Aβ-(1−42) than Aβ(1−40). The inhibition of the fluorescence signal in Figure 3 was not due to the inhibitors interfering with the binding of Th-T to Aβ, because in control experiments in which the Aβ peptides were preincubated on their own before addition of inhibitor and Th-T, there was no effect on the fluorescence signal (data not shown).

Immunoassay of Inhibition of Aggregation. When aggregation was monitored using a sandwich immunoassay system, which detects early oligomers with much higher sensitivity than the Th-T method (see Discussion), both OR2 and RI-OR2 demonstrated clear inhibition of formation of Aβ(1−40) and Aβ(1−42) oligomers (Figure 4). Indeed, at an inhibitor:Aβ molar ratio of 1:1, both inhibitors almost completely blocked the development of an immunoassay signal in solutions of Aβ(1−42) incubated for periods of up to 24 h. This was not due to the inhibitors interfering with the binding of the 6E10 monoclonal antibody to Aβ, because in control experiments, where the Aβ peptides were preincubated on their own before addition of the inhibitors, no diminution of the immunoassay signal was seen (data not shown). Again, the inhibitors seemed to have more potent effects on Aβ(1−42) than Aβ(1−40) aggregation, and RI-OR2 appeared to be a slightly better inhibitor than OR2 (Figure 4).

SDS−PAGE of Inhibition of Aggregation. Incubation of 5 μM Aβ(1−42) at 37 °C for 4 h in PB resulted in the formation of SDS-stable oligomers, with apparent molecular masses of ~11 and ~13 kDa, and a much more intense band at 35−50 kDa, whereas only the monomer was detected at zero incubation time (Figure 5, lanes 1 and 2). The formation of all of the bands corresponding to oligomers was totally eliminated by the presence, during peptide incubation, of a 1:1 or 5:1 molar ratio of RI-OR2 to Aβ (Figure 5, lanes 3 and 4).

Atomic Force Microsopy of Inhibition of Aggregation. AFM with samples of Aβ(1−42) incubated in the presence or absence of an equimolar concentration of RI-OR2 showed a

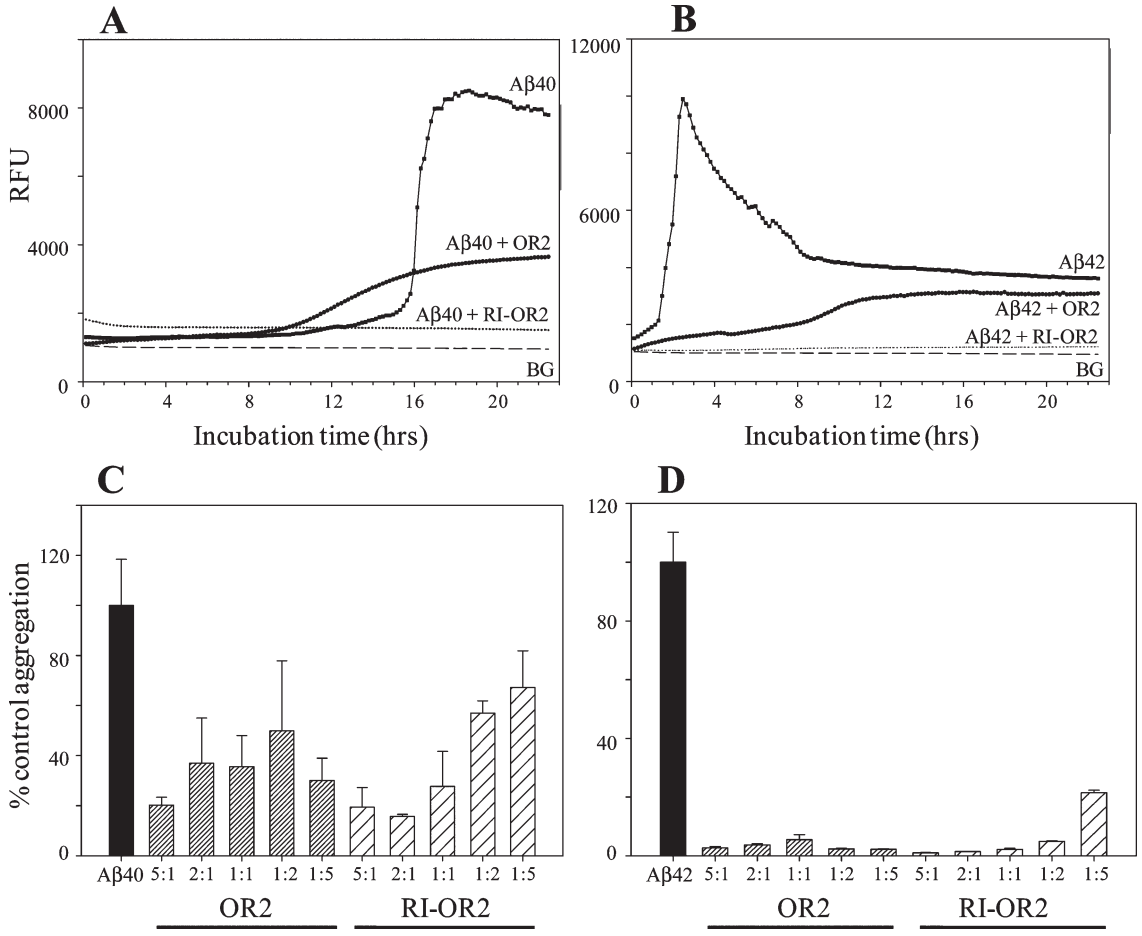


FIGURE 3: Effects of OR2 and RI-OR2 on Aβ fibril formation, as monitored by the Th-T assay. Results in panels A and B were obtained by incubation of an equimolar concentration of OR2 or RI-OR2 with 20 μM Aβ(1−40) and Aβ(1−42), respectively, in the presence of Th-T. Results in panels C and D show the effects of different concentrations of OR2 and RI-OR2 on the aggregation of Aβ(1−40) (at 22 h) and Aβ(1−42) (at 2 h), relative to noninhibited controls (solid bars). Results are means ± the standard deviation (n = 3), and inhibitor:Aβ molar ratios are given.

dramatic inhibition of amyloid fibril formation (Figure 6). In the absence of inhibitor, numerous amyloid fibrils with a twisted ropelike appearance, similar in ultrastructural appearance and dimensions to those described in many previous studies, were seen (Figure 6A), whereas these structures were totally absent when RI-OR2 was present (Figure 6B). Furthermore, in the presence of RI-OR2, no structures resembling ADDLs, oligomers, or protofibrils were found.

Inhibition of Cell Toxicity. SH-SY5Y human neuroblastoma cells exposed to preaggregated Aβ(1−42) exhibited evidence of membrane damage [in the LDH membrane integrity assay (Figure 7A)] and loss of cell viability [in the MTS cell viability assay (Figure 7B)], although this effect just failed to reach statistical significance in the former case (p = 0.055) and was statistically significant only in the latter case (p = 0.0023). Both OR2 and RI-OR2, when present at an equimolar concentration to Aβ (10 μM), rescued the cells from the toxic effects of preaggregated Aβ(1−42). This protective effect was statistically significant for both OR2 and RI-OR2, in both types of cell toxicity assays (see Figure 7 for details).

Surface Plasmon Resonance Experiments. In these experiments, RI-OR2 exhibited clear dose-dependent binding to both immobilized Aβ(1−42) monomers and fibrils (see Figure 8A,B), whereas binding to the reference surface (no ligand) or to BSA was negligible (data not shown). The small nonspecific signal observed in the case of the immobilized BSA was subtracted from the binding data presented in Figure 8. Further analysis of these data, conducted considering the maximum binding attained at plateau (equilibrium) with the different RI-OR2 concentrations, resulted in apparent K_d values of 9 and 12 μM for binding of RI-OR2 to Aβ(1−42) monomers and fibrils, respectively. The binding of RI-OR2 to both forms of Aβ(1−42) was characterized by a very fast association rate, and a very fast dissociation rate, with k_{on} values of 7180 ± 200 and 4740 ± 175 M^{-1} s^{-1} and k_{off} values of 0.06 ± 0.001 and 0.05 ± 0.001 s^{-1} for monomers and fibrils, respectively. Using this "kinetic analysis", the K_d estimates were 8.8 and 9.5 μM for the binding of RI-OR2 to monomers and fibrils, respectively. The K_d values obtained by these two different methods were, therefore, in close agreement, giving strong support to the reliability of these estimates.

In the next set of SPR experiments, the effect of RI-OR2 on the binding of Aβ(1−42) monomers to preformed fibrils was determined. Sensorgrams were obtained via injection of Aβ(1−42) monomers (3 μM), RI-OR2 (30 μM) alone, or a combination of the two over the same immobilized ligand surfaces (monomer, fibrils) and control surfaces (no ligand, BSA) as described above. The Aβ(1−42) monomers did not exhibit any significant binding to BSA, to the empty chamber (data not shown for both), or to immobilized Aβ(1−42) monomers (Figure 8C), but they did exhibit clear binding to Aβ fibrils (Figure 8D). Since there was no binding of Aβ monomers to immobilized monomers, no relevant information was obtained for the effects of RI-OR2 on this interaction (Figure 8C). However, the binding of Aβ monomers to fibrils was clearly affected by RI-OR2 (Figure 8D). Note, in

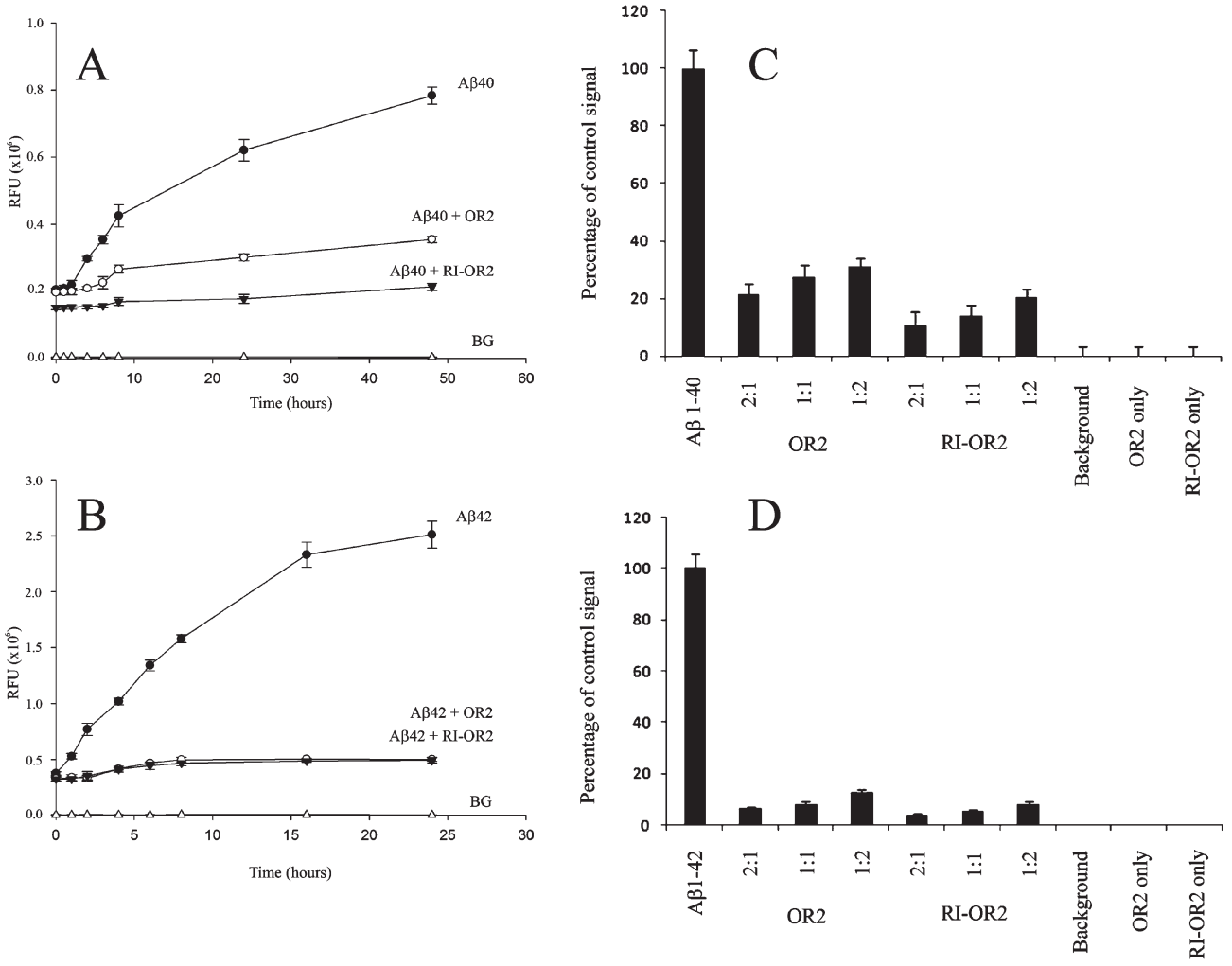


FIGURE 4: Effects of OR2 and RI-OR2 on formation of Aβ oligomers, as determined by an immunoassay. Results in panels A and B were obtained by incubation of an equimolar concentration of OR2 or RI-OR2 with 20 μM Aβ(1−40) and Aβ(1−42), respectively. Results in panels C and D show the effects of different concentrations of OR2 and RI-OR2 on the aggregation of Aβ(1−40) (at 48 h) and Aβ(1−42) (at 24 h), relative to noninhibited controls. Results are means ± the standard deviation (n = 3), and inhibitor:Aβ molar ratios are given.

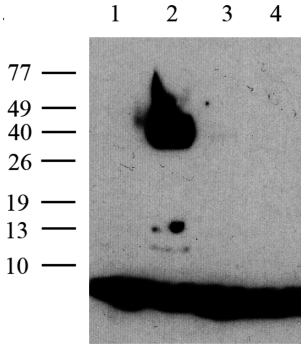


FIGURE 5: Effects of RI-OR2 on Aβ oligomer formation, as determined by SDS−PAGE and immunoblotting: lane 1, Aβ(1−42) at zero incubation time (monomer only); lane 2, Aβ(1−42) after a 4 h incubation (oligomers present); lane 3, Aβ(1−42) incubated for 4 h in the presence of RI-OR2, at a 1:1 molar ratio; lane 4, Aβ(1−42) incubated for 4 h in the presence of RI-OR2, at a 5:1 molar ratio (inhibitor:Aβ). Molecular mass markers (kilodaltons) are at the left.

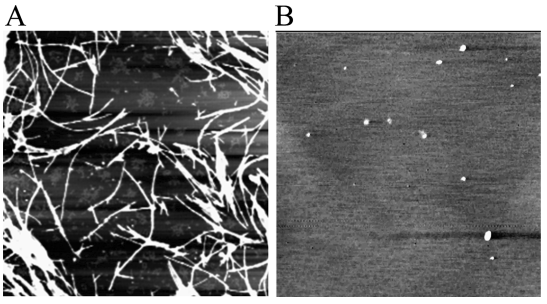


FIGURE 6: AFM images of 20 μM Aβ(1−42) incubated for 24 h at 25 °C in (A) the absence of inhibitor and (B) the presence of an equimolar concentration of RI-OR2. The area in each case is 5 μm × 5 μm.

particular, that the magnitude of the residual binding signal observed after the injection of the mixture of Aβ(1−42) and RI-OR2 was much lower than that observed after the injection of Aβ(1−42) alone, suggesting that RI-OR2 impairs the binding of Aβ monomers to fibrils (i.e., it inhibits fibril elongation) (Figure 8D). The inhibitory effect of RI-OR2 can be better appreciated in Figure 9, in which the signal observed with RI-OR2 alone was subtracted from the signal observed for injection of the mixture Aβ(1−42) and RI-OR2 to yield the net binding of Aβ(1−42) monomers in the presence of RI-OR2 (blue line) (see also the Discussion). Figure 9 shows that the presence of RI-OR2 markedly impaired the binding of Aβ(1−42) monomers (with or without being in a complex with RI-OR2) to Aβ(1−42) fibrils.

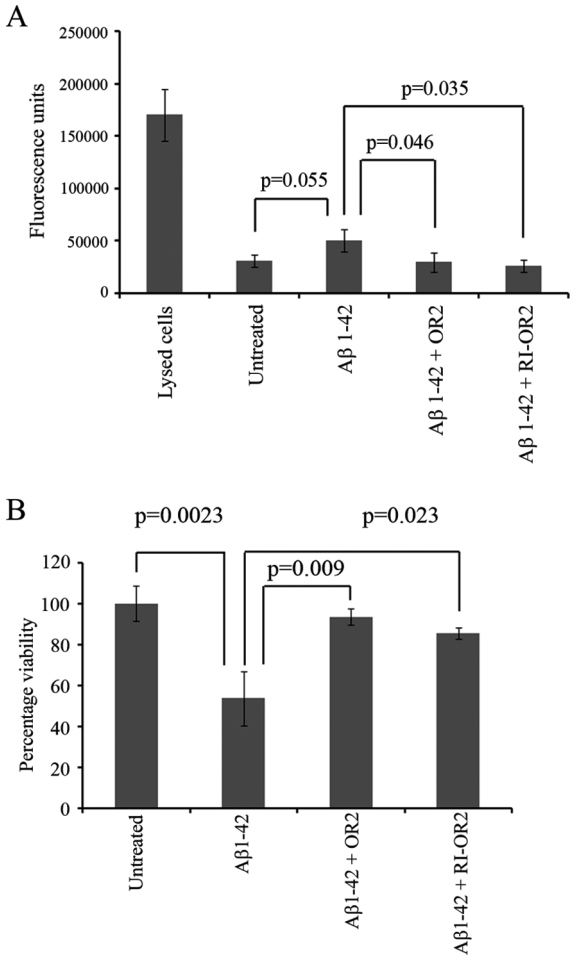


FIGURE 7: Protective effects of RI-OR2 on the toxicity of Aβ(1−42) toward cultured SH-SY5Y cells in (A) a membrane integrity (LDH) assay and (B) a cell viability (MTS) assay. Results are means ± the standard deviation (n = 3).

DISCUSSION

The "amyloid cascade hypothesis" originally proposed that the Aβ fibrils found in senile plaques in the brains of patients with AD are the neurotoxic agent responsible for initiating the events leading to neurofibrillary tangle pathology and neurodegenerative brain damage in this disease (*33*). However, this hypothesis has recently been revised, and "soluble oligomers" are now thought to be the main toxic form of Aβ (*1*−*9*). This shift in thinking has meant that efforts toward the development of antiaggregatory drugs should now be directed at the early stages of Aβ oligomer assembly, rather than the later stages of amyloid fibril formation (*10*). The 42-amino acid form of Aβ (Aβ42) is highly prone to toxic oligomer formation (*1*, *34*, *35*) and has also been linked strongly to the etiology of AD (*36*, *37*), suggesting that it is a particularly appropriate target for aggregation inhibitors.

Peptide OR2 (RGKLVFFGR-NH_2) has already been identified as an inhibitor of the formation in vitro of both Aβ oligomers and fibrils (*24*). The closely related peptide OR1 (RGKLVFFGR) also blocks Aβ fibril formation but is much less effective than OR2 in preventing the formation of Aβ oligomers (*24*), suggesting that it may act at a later point in the aggregation pathway than OR2. Remarkably, the only difference between these two peptides is that the carboxyl terminus of OR1 was replaced by the less charged amide group in OR2. In these peptides, KLVFF was designed as the binding region responsible for interacting with native Aβ, whereas the RG and GR flanking residues were intended to be solubilizing components. The simpler peptide, KLVFF-NH_2, without these flanking residues, does not inhibit Aβ aggregation, probably because it has a tendency to self-aggregate, unlike OR1 and OR2 (*24*).

Peptides are short-lived molecules in vivo, and to convert them into useful drugs, it is usually necessary to transform them into peptidomimetics. Many previous studies, with a variety of different peptide ligands, have shown that retro-inverso peptides

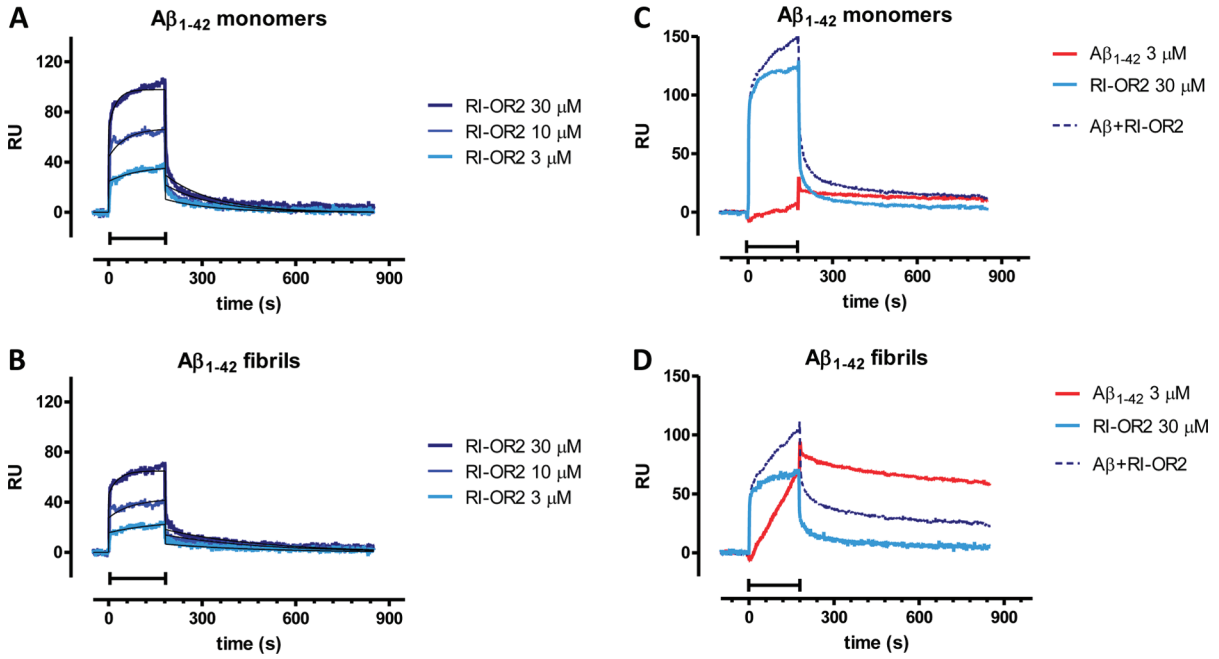


FIGURE 8: SPR sensorgrams showing dose-dependent binding of RI-OR2 to (A) Aβ(1−42) monomers and (B) Aβ(1−42) fibrils and the effects of RI-OR2 on the binding of Aβ(1−42) monomers to (C) Aβ(1−42) monomers and (D) Aβ(1−42) fibrils. The nonspecific binding obtained from a BSA-coated surface has been subtracted from all data.

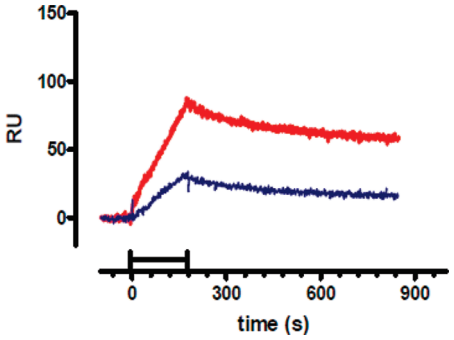


FIGURE 9: SPR sensorgrams showing the net binding of Aβ(1−42) monomers (3 μM) to immobilized Aβ(1−42) fibrils (fibril extension) in the absence (red line) or presence (blue line) of 30 μM RI-OR2. The latter curve was obtained via subtraction of the signal observed for injection of RI-OR2 alone from the signal observed for injection of the Aβ(1−42)/RI-OR2 mixture. See the Discussion for a detailed explanation.

can maintain a topology, a potency, and a selectivity similar to those of their native "parent" molecules. However, in general, they have a much improved bioavailability profile, following oral or intravenous administration, because of their greatly enhanced stability to proteolysis (*26*, *27*). Some retro-inverso peptides have also exhibited good BBB permeability (*38*) and enhanced membrane translocation properties (*39*). Hence, we have now designed a retro-inverso version of OR2 [called RI-OR2 (see Figure 1)], maintained the positioning of the solubilizing flanking residues, and mimicked the C-terminal amide group in OR2 with an N-terminal acetyl group in RI-OR2 (CH_3CONH is close in chemical properties to $CONH_2$, with both being uncharged and unreactive). In our stability experiments (Figure 2), RI-OR2 proved to be much more stable to proteolysis in a human brain extract and in blood plasma than OR2, and this was confirmed by studies with individual proteolytic enzymes. This represents a significant step forward in the development of a suitable druglike molecule for future bioavailability studies and clinical trials. In general, the results obtained with the individual proteases are in accord with those expected, based on their known specificities. Elastase, cathepsin G, kallikrein, thrombin, trypsin, and chymotrypsin should all cleave OR2, and this was borne out by the results. Plasmin has been shown to cut full-length Aβ at the Lys16−Leu17 bond (*40*) but did not accept the much smaller OR2 peptide as a substrate. As anticipated, Factor Xa did not cleave OR2. ADAM10 is a known α-secretase (*41*) and so will cleave full-length, membrane-bound APP at the Lys16−Leu17 bond and will also accept some smaller peptides spanning this region as suitable substrates (*42*) but does not accept Aβ as a substrate (E. Parkin, personal communication) and did not cleave OR2. None of these proteases had any effect on RI-OR2.

In our aggregation studies with RI-OR2, we took great care to extensively "deseed" the Aβ peptides, to start with a preparation as close to the monomer as possible. This allowed us to look at the effects of the inhibitors on the very early stages of oligomer formation and also allowed us to test the direct binding of RI-OR2 to the monomeric form of Aβ. In the case of the aggregation and cell toxicity experiments, this was achieved by deseeding the already HFIP-treated recombinant Aβ(1−40) and Aβ(1−42) peptides twice more with HFIP, or with TFA and thioanisole (*31*) followed by two treatments with HFIP. The Aβ(1−42) monomer used for the SPR binding studies was prepared using the "click peptide" approach described by Taniguchi et al. (*28*). This depsi-peptide is a water-soluble precursor of Aβ(1−42) with an ester bond between Gly25 and Ser26. The depsi-Aβ(1−42) peptide is stable under acidic conditions and retains a monomeric, random coil state. At neutral or basic pH, it rapidly converts to native monomeric Aβ(1−42) through an O-to-N intramolecular acyl migration (*28*). In this study, we induced the shift under basic conditions, to prevent any aggregation during formation of the native peptide (*29*).

The first test of RI-OR2, using the Th-T method, demonstrates that an equimolar concentration of RI-OR2 almost completely blocks the formation of β-pleated sheet fibrils during incubation of 20 μM solutions of deseeded Aβ(1−40) and Aβ(1−42), even for periods of up to 48 h, and is actually a better inhibitor than OR2 (Figure 3A,B). It should be noted that these experiments involved shaking the peptide solution every 10 min in the plate reader, which, in our experience, substantially increases the rate of aggregation. Therefore, these results suggest that RI-OR2 blocks, rather delays, the formation of amyloid fibrils [i.e., it does not act solely as a "kinetic" inhibitor (*43*)]. This is demonstrated most convincingly for Aβ(1−42), the aggregation of which would normally be complete in less than 3 h in this experiment. Although RI-OR2 was still active at substoichiometric concentrations relative to Aβ, with ~80% inhibition at a 1:5 inhibitor:Aβ(1−42) molar ratio, under these conditions OR2 appeared to be the better inhibitor (Figure 3B,C). In these experiments, both OR2 and RI-OR2 were consistently better at inhibiting Aβ(1−42) aggregation than Aβ(1−40) aggregation, which, as noted above, is a very desirable property.

Since the Th-T method cannot detect early oligomers directly [although there is some suggestion that it can, in fact, detect "protofibrils" (*44*) and the lag period prior to rapid fibril growth corresponds to seed formation and nucleation], we sought alternative methods for looking at the effects of RI-OR2 on oligomer formation. The first method employed was a novel sandwich immunoassay developed in our laboratory for detecting Aβ oligomers (*24*, *30*). Here, the oligomers are captured by mouse monoclonal antibody 6E10 and detected by a biotinylated form of the same antibody. Monomeric Aβ does not produce a signal in this assay, because the capture antibody occupies the only 6E10 epitope available, and so the biotinylated 6E10 antibody cannot subsequently bind. However, in the case of oligomers, multiple 6E10 epitopes are available, permitting both capture and detection. We have found that this is a very sensitive technique for detecting early stage oligomers and, in aggregation time course experiments, produces a clear signal well before the Th-T method, when only small oligomers can be seen by AFM (see the Supporting Information). Other researchers have also employed the "double-antibody" approach to detect oligomers, including 6E10−6E10 (*45*, *46*), 4G8−4G8 (*47*), and 82E1−82E1 or 3D6−3D6 (*48*). Using the 6E10−6E10 assay, we found that both OR2 and RI-OR2, at 2:1, 1:1, and 1:2 inhibitor:Aβ molar ratios, very substantially blocked the development of an immunoassay signal, even at the earliest time points where such a signal was detectable [1 h for Aβ(1−42)], suggesting inhibition of early oligomer formation (Figure 4). The inhibitors were designed to bind to their cognate region (KLVFF, residues 16−20) in native Aβ, which should not interfere with binding of the 6E10 monoclonal antibody to its epitope (residues 6−10 of Aβ), and this was confirmed in control experiments. Again the inhibitors were more effective against Aβ(1−42) than Aβ(1−40). The second method for detection of Aβ oligomers involved separation of the monomer and oligomers by SDS−PAGE, followed by immunodetection of the bands. Incubation of 5 μM Aβ(1−42) at

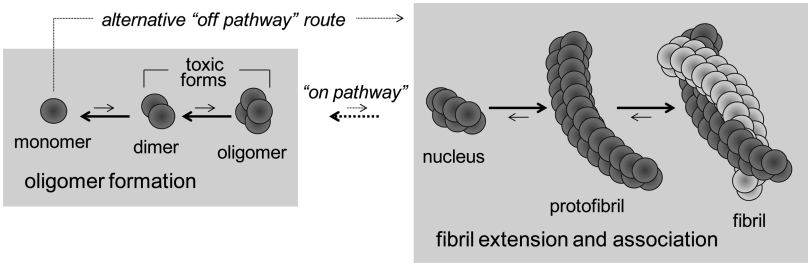


FIGURE 10: Scheme showing aggregation of monomeric Aβ into oligomers and amyloid fibrils. The toxic oligomers can be either on pathway or off pathway with respect to fiber formation. RI-OR2 inhibits both Aβ oligomerization and fibril extension.

37 °C for 4 h resulted in the formation of faint bands at 11 and 13 kDa, corresponding to SDS-stable, low-*n* oligomers (presumably dimers and trimers, or trimers and tetramers), together with a more intense band spanning the range of 35−50 kDa, which according to its molecular mass would appear to be a mixture of 9−12-mers. The formation of these oligomers cannot be due to an interaction between Aβ and SDS during electrophoresis (*49*, *50*), because they were not seen in the nonincubated (t = 0) control. The band at 35−50 kDa is particularly interesting because it could correspond to the 9−12-mer Aβ assembly, termed Aβ*56, which has been implicated as being responsible for memory impairment in a transgenic mouse model of AD (*51*). The presence of RI-OR2, at a 1:1 or 5:1 inhibitor:Aβ molar ratio, completely eliminated the formation of all of the oligomer bands described above, confirming that it blocks even the very early stages of oligomer formation. In AFM studies, an equimolar concentration of RI-OR2 completely prevented the formation of any structures resembling fibrils or smaller aggregates (ADDLs, oligomers, or protofibrils) from extensively deseeded preparations of Aβ(1−42) (Figure 6). OR2 was previously shown to block the formation of oligomers and fibrils from Aβ(1−40), using negative stain electron microscopy (*24*). These results support and complement the other data, and all of the results together, using a variety of different techniques, demonstrate convincingly that RI-OR2 blocks both oligomer and fibril formation.

In previous work, employing the MTT assay, OR2 was shown to be better at protecting SH-SY5Y cells from the toxic effects of Aβ than OR1 (for structures, see Figure 1) and the simple peptide KLVFF-NH_2 actually promoted Aβ-induced toxicity (*24*). This indicates that the presence of the C-terminal amide group and the flanking residues (RG- and -GR) in OR2 are required not only for an effective aggregation inhibitor but also for an inhibitor that can block the cytotoxicity of Aβ. Here, we have confirmed that OR2 protects against the toxic effects of preaggregated Aβ(1−42), in both MTS and LDH assays, and have shown that RI-OR2 shares this property (Figure 7).

The SPR experiments were conducted to shed light on the mechanism of action of RI-OR2. The first set of SPR data (Figure 8A,B) demonstrates convincingly that RI-OR2 binds directly and rapidly (with a very fast association rate) to both Aβ(1−42) monomers and fibrils, with an apparent affinity (K_d) of 9−12 μM for both. A very rapid dissociation is seen, however, when the bound RI-OR2 is washed from the immobilized monomers and fibrils (Figure 8A,B). Similar SPR studies have shown that OR2 also binds to Aβ(1−42) monomers and fibrils (and also oligomers), but with a slightly lower affinity of 25−30 μM (Figure S2A of the Supporting Information). This suggests that the binding affinity of RI-OR2 for Aβ(1−42) is at least comparable if not better than that of OR2. The ability of RI-OR2 to bind directly to monomeric Aβ is an important property of the molecule and would suggest that this is the mechanism by which RI-OR2 inhibits early oligomer formation. Although the binding affinity is not particularly high, it is greater than the apparent affinity for the initial binding of Aβ(1−40) or Aβ(1−42) monomers to the growing end of an amyloid fibril (K_d = 123 or 43 μM, respectively) (*52*, *53*), and furthermore, it is not necessarily the case that a higher binding affinity equates to a better aggregation inhibitor. This is illustrated by studies with the phage-selected affibody protein, $A_{A\beta 3}$, which binds to monomeric Aβ(1−40) with high affinity (K_d = 17 nM) but, in the Th-T assay, is only effective at a 1:1 stoichiometry and, unlike RI-OR2, fails to inhibit very effectively even at a 1:2 inhibitor:Aβ molar ratio (*54*). What may be required for the best aggregation inhibitor is a molecule with moderate affinity and a fast exchange rate, so that multiple Aβ association events are affected in a short period of time by a single molecule of inhibitor. In contrast, a tight-binding inhibitor, with a slow exchange rate, might work only at a 1:1 stoichiometry.

Further SPR sensorgrams were obtained by injecting monomeric Aβ(1−42), RI-OR2 alone, or the combination of the two onto immobilized Aβ(1−42) monomers and fibrils (Figure 8C, D). As anticipated on the basis of previous work, the Aβ(1−42) monomers did not bind to themselves, but they did show clear binding to Aβ(1−42) fibrils, as demonstrated before for both Aβ(1−40) (*52*) and PrP82−146 (*53*). The strong binding between flowing monomers and immobilized fibrils is thought to be due to the presence of a seeding structure on the fibril end giving rise to a fibril elongation process, as evidenced by the lack of saturation, and the very stable binding (*52*, *53*). This seeding structure is not present in the monomers, so the binding between monomers is expected to have a much lower affinity (*52*, *53*). Figure 8D shows that weaker binding was measured after injection of the mixture of Aβ(1−42) and RI-OR2 (3 and 30 μM, respectively) onto immobilized fibrils than of Aβ(1−42) alone, suggesting that this retro-inverted peptide can also act as an inhibitor of fibril extension. Assuming that RI-OR2 and Aβ(1−42) monomers bind to each other with an affinity of 9 μM, reaching equilibrium in a few minutes (see above and Figure 8A), the preincubation (for 15 min) of 3 μM Aβ(1−42) with 30 μM RI-OR2 is expected to produce the following species: 2.2 μM Aβ(1−42)−RI-OR2 complex, 0.8 μM free Aβ(1−42), and 27.8 μM free RI-OR2. Accordingly, we could reasonably subtract the signal obtained with RI-OR2 alone (30 μM RI-OR2) from the signal obtained with the mixture of Aβ(1−42) and RI-OR2 (27.8 μM RI-OR2), to dissect the net binding of Aβ(1−42) in the presence of RI-OR2 (Figure 9, blue line). The weaker Aβ(1−42) binding observed under this latter condition could indicate either that, when complexed with RI-OR2, Aβ(1−42) does not bind to fibrils or that the elongation is prevented by the binding of RI-OR2 to immobilized fibrils. In either case, the ability of RI-OR2 to interfere with fibril elongation would be a desirable property of

the inhibitor, if fibril extension is associated with toxicity, as suggested by some authors (*55*).

Currently, it is not clear if the most toxic forms of Aβ (i.e., "low-*n*" oligomers) are on the main pathway leading to fibril growth ("on pathway") or if they are "off pathway" (see Figure 10). If they are on pathway, then inhibitors of oligomer formation would also be expected to block the formation of fibrils. However, inhibition of the later stages of fibril formation could then, in theory at least, lead to the stabilization of early toxic oligomers. A decrease in oligomer levels could also be achieved by compounds promoting the latest stage of the aggregation process, i.e., fibrillization (*56*). Thus, it is important to determine the mechanism of action of the inhibitor and identify which aggregation species are affected. Most previous studies of Aβ aggregation inhibitors have used methods (e.g., Th-T binding, Congo red binding, and turbidity) that detect mainly fibrils, so their effects on oligomer formation are seldom clear. One exception is a recent study by Necula et al. (*57*), who examined very carefully the effects of a range of different Aβ aggregation inhibitors and found that they fell into three distinct classes. Class I compounds inhibited oligomerization but not fibrillization; class II compounds inhibited assembly of Aβ into both oligomers and fibrils, and class III compounds inhibited fibrillization but not oligomerization. According to this system, RI-OR2 is a class II inhibitor that acts on both Aβ oligomer formation and amyloid fibril formation and extension. According to Necula et al. (*57*), this type of inhibitor appears to work by stabilizing a conformation of Aβ that does not support aggregation. RI-OR2 was designed to bind to its cognate region (KLVFF, residues 16−20) in native Aβ and interfere with Aβ self-association in this way, and we presume that this is the mechanism whereby RI-OR2 is able to block both oligomer and fibril formation. RI-OR2 was found to be a more effective inhibitor of Aβ(1−42) aggregation than of Aβ(1−40) aggregation. These two peptides are known to have slightly different conformations in their monomeric state, with Aβ(1−42) having a higher β-sheet and β-turn content than Aβ(1−40) (*35*). This results in their aggregation through distinctly different pathways (*35*). Thus, we suppose that RI-OR2 has an enhanced interaction with the conformation adopted by monomeric Aβ-(1−42) or that it interferes most effectively with the initial stages of the particular pathway involved in the assembly of Aβ(1−42) into toxic oliogomers. RI-OR2 is also active at substoichiometric concentrations relative to Aβ and is stable to proteolysis. As such, it represents a potential therapeutic agent for further development as a treatment for the underlying cause of neurodegeneration in AD. Further studies in animal models, including studies of bioavailability and brain penetration, will be required before this drug, or a suitable derivative thereof, is able to enter human clinical trials. To the best of our knowledge, no peptide-based antiaggregation inhibitor has been tested yet in any clinical trial.

SUPPORTING INFORMATION AVAILABLE

Additional data showing the sensitivity of the 6E10−6E10 double-antibody immunoassay and the binding of OR2 to immobilized Aβ(1−42) monomers, oligomers, and fibrils are available. For the immunoassay experiments, Aβ(1−40) was incubated at 25 and 50 μM in PB, at 37 °C, and samples were taken for the Th-T assay and the immunoassay. The Th-T data (Figure S1A,B) show a 10 h lag phase for 50 μM Aβ and a $>$12 h lag phase for 25 μM Aβ. In contrast, the immunoassay detects significant changes within the first 2 h, even at 25 μM Aβ. An AFM image taken after incubation at 25 μM Aβ for 4 h shows only very small oliogomers (Figure S1C). For the SPR experiments, Aβ monomers and fibrils were prepared as described above (see Experimental Procedures), whereas the oligomers were made by incubating the deseeded Aβ(1−42) peptide at 68 μM for 24 h at 4 °C in PBS. The presence of oligomers and fibrils was confirmed by AFM. These structures were then immobilized onto the SPR chip surface as described above (see Experimental Procedures). Sensorgrams were obtained via injection of four different concentrations of OR2 (3, 10, 30, and 90 μM) over the immobilized ligands. Figure S2 shows the dose-dependent binding of OR2 to these different forms of Aβ(1−42), after subtraction of nonspecific binding (empty reference cell). Further analysis of these data, conducted considering the maximum binding attained at plateau ("equilibrium analysis") with the different OR2 concentrations, resulted in apparent K_d values of 24, 27, and 29 μM for the binding of OR2 to Aβ(1−42) monomers, oligomers, and fibrils, respectively. This compares with the K_d values of 9 and 12 μM (i.e., of slightly higher affinity) obtained in a similar way for the binding of RI-OR2 to Aβ(1−42) monomers and fibrils. This material is available free of charge via the Internet at http://pubs.acs.org.

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