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Communication

A Strategy for Designing Inhibitors of β-Amyloid Toxicity*

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β-Amyloid peptide is the major protein component of Alzheimer's plaques. When aggregated into amyloid fibrils, the peptide is toxic to neuronal cells. Here, an approach to the design of inhibitors of β-amyloid toxicity is described; in this strategy, a recognition element, which interacts specifically with β-amyloid, is combined with a disrupting element, which alters β-amyloid aggregation pathways. The synthesis, biophysical characterization, and biological activity of such an inhibitor is reported. This prototype inhibitor is composed of residues 15–25 of β-amyloid peptide, designed to function as the recognition element, linked to an oligolysine disrupting element. The inhibitor does not alter the apparent secondary structure of β-amyloid nor prevent its aggregation; rather, it causes changes in aggregation kinetics and higher order structural characteristics of the aggregate. Evidence for these effects includes changes in fibril morphology and a reduction in thioflavin T fluorescence. In addition to its influence on the physical properties of β-amyloid aggregates, the inhibitor completely blocks β-amyloid toxicity to PC-12 cells. Together, these data suggest that this general strategy for design of β-amyloid toxicity inhibitors is effective. Significantly, these results demonstrate that complete disruption of amyloid fibril formation is not necessary for abrogation of toxicity.

β-Amyloid peptide (Aβ)[1] is the major protein component of senile plaques and cerebrovascular amyloid deposits from Alzheimer's disease patients (1, 2). The deposition of Aβ in the form of amyloid fibrils is believed by many to be causally linked to the disease (3). Aβ is toxic to cultured neuronal cells, and this toxicity has been linked to the aggregational and/or conformational status of the peptide (4–6). Under physiological conditions, Aβ readily aggregates into fibrils with a cross-β-sheet conformation. Coincident with the conversion of monomeric Aβ to fibrillar Aβ is a transition from random coil to β-sheet (7). Several features of Aβ affect the facility of this transition. The peptide is amphiphilic, with a hydrophilic N terminus and hydrophobic C terminus; the length of the latter affects the rate of aggregate formation (8). In addition, a short hydrophobic stretch at residues 17–21 appears to be critical in the formation of fibrillar structure (9, 10), with charged residues adjacent to this region also contributing to fibril formation (10, 11). Aggregation likely proceeds via formation of a "nucleus" to initiate fibril formation followed by fibril elongation (8, 12, 13).

One strategy for developing lead candidates for drugs to treat Alzheimer's disease patients is to screen for small molecules that disrupt Aβ aggregation and thereby, presumably, interfere with its toxicity. Sulfonated dyes such as Congo red and related sulfonate anions reportedly disrupt Aβ aggregation and reduce Aβ toxicity (14, 15). The cationic surfactant hexadecyl-*N*-methylpiperidinium bromide inhibits Aβ fibril formation, possibly by binding to a site on Aβ necessary for Aβ self-assembly (16). β-Cyclodextrin, which has an affinity for hydrophobic groups, partially reduces Aβ toxicity (17).

An alternative approach is to use a fragment of Aβ to block Aβ fibril formation. Recently, it was reported that a pentapeptide KLVFF, containing the 16–20 sequence of full-length Aβ, binds to and disrupts fibril formation (18). An octapeptide, QKLVTTAE, with substitutions for the two Phe residues at positions 19 and 20, inhibited fibril formation at a 10-fold molar excess; this result was attributed to weak interactions between the octapeptide and monomeric Aβ (19). In both cases, fibril inhibition was assessed by electron microscopy. Effects of the peptide fragments on Aβ toxicity were not reported.

We report a new strategy for generating molecules that interfere with Aβ toxicity. This approach relies on two features of Aβ: it is a self-recognizing peptide, and its toxicity depends on its adopting a specific conformational and/or aggregational state. Therefore, attachment of a short fragment of Aβ, which can specifically recognize the full-length peptide, to a disrupting element, which functions to alter Aβ self-assembly, may afford a new molecule capable of ameliorating the toxicity of Aβ. To test the feasibility of this approach, we synthesized such a hybrid compound. We found that this inhibitor not only alters Aβ self-assembly, but also blocks Aβ toxicity *in vitro*.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—A peptide homologous to the first 39 residues of Aβ, β-(1–39), was purchased from AnaSpec, Inc. (San Jose, CA) and was described previously (12). Two "recognition" peptides, containing recognition elements only, and two "hybrid" peptides, combining recognition with disrupting elements, were synthesized; sequences are shown in Fig. 1. Peptides homologous to residues 18–25 and 16–25 of Aβ, R1, and R2, respectively, were synthesized using standard solid-phase methods. The peptides were purified by reverse-phase HPLC on a Vydac C-18 column using a 20–100% linear gradient of acetonitrile/water with 0.1% TFA as the mobile phase. Analysis by mass spectrometry using a VG AutoSpec M (LSIMS) afforded masses of 1124.5 (theoretical 1124.4) and 883.3 (theoretical 883.0), respectively. Two hybrid peptides containing a sequence homologous to residues 15–25 of Aβ linked via glycine spacers to a lysine hexamer at either the N or C terminus (H1 and H2, respectively) were synthesized for us by AnaSpec. These peptides were purified by reverse-phase HPLC, and their purity was estimated to be >96%. LSIMS mass

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[1] The abbreviations used are: Aβ, β-amyloid peptide; ACN, acetonitrile; HPLC, high pressure liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PBSA, PBS with azide; TFA, trifluoroacetic acid; ThT, thioflavin T.

disrupting	recognition	disrupting	peptide designation
	VFFAEDVG		R1
	KLVFFAEDVG		R2
*KKKKKK*GG	**QKLVFFAEDVG**		H1
G	**QKLVFFAEDVG**	Ga*KKKKKK*	H2

FIG. 1. **Peptide sequences tested for effects on physical and toxicity characteristics of Aβ.** Recognition elements which are homologous to a section of Aβ are shown in *bold*; "disrupting" elements are *italicized*. The *small a* in H2 indicates an aminocaproate linker. Sequences are designated R1, R2 (recognition peptides 1 and 2, respectively), H1 and H2 (hybrid peptides 1 and 2, respectively).

spectroscopy of H1 revealed a molecular mass of 2136.1 (theoretical: 2136.6). Analysis of H2 suggested it was a mixture with the major component having the expected molecular mass of 2190.3 (theoretical: 2192.7) and a minor component with molecular mass 2062.6, likely corresponding to a des-Gln contaminant that was not resolvable by HPLC. β-(25–35) was purchased from Sigma. Peptides were stored in lyophilized form at −70 °C.

Circular Dichroism Spectroscopy—β-(1–39), H2, or a mixture of β-(1–39) and H2 (1:2 molar ratio) were dissolved in 0.01 M phosphate buffer, pH 7.2. The final concentration was 0.5 mg/ml for each peptide. The solution was passed through a 0.45-μm Millipore filter to remove dust and then degassed. Circular dichroism spectra in the far UV (190–240 nm) were obtained using a modified Cary Model 60 spectropolarimeter (On-line Instrument Systems, Bogart, CA) and analyzed for secondary structure as described (20).

Dynamic Light Scattering—β-(1–39) was dissolved in 35% ACN, 0.1% TFA, then diluted 20-fold into PBSA or into PBSA containing H2. R1 and R2 were dissolved in the same solvent, then mixed with β-(1–39) stock solution and diluted 20-fold into PBSA. PBSA was double-filtered through a 0.22-μm filter prior to use. pH was adjusted to 7.4 with 0.5 N NaOH, then the sample was immediately filtered through a 0.45-μm filter (Millipore) directly into a clean light scattering cuvette. In all cases, β-(1–39) concentration was 0.5 mg/ml. R1 and R2 were added at a 1:1 molar ratio, H2 was added at a 1:2 (Aβ/H2) molar ratio. Autocorrelation functions at 90° scattering angle were collected as described previously (22). Data were fit using the method of cumulants to derive an apparent hydrodynamic radius.

Electron Microscopy—Solutions of β-(1–39) alone (0.5 mg/ml) or with 1 mg/ml H2 were prepared as described above and aged at room temperature for approximately 1 week. A drop of the peptide solution was placed on a Pioloform coated 300-mesh electron microscope copper grid. After blotting, a drop of 1% ammonium molybdate was placed on the grid while being held with fine forceps. The grid was blotted on filter paper and allowed to dry before observing the specimen in a JEOL (Peabody, MA) 100 CX electron microscope at 60 kV.

Cellular Toxicity—Solutions of R1 and H2 were prepared by direct dissolution of lyophilized peptide into sterile PBS (with antibiotics). β-(1–39) was dissolved in 0.1% TFA (10 mg/ml), then diluted 20-fold into sterile PBS (with antibiotics), PBS containing R1 (1:1 molar ratio), or PBS containing H2 (1:2 or 1:4 Aβ/H2 molar ratio). The samples were incubated at room temperature for 7 days, then diluted 5-fold with medium and incubated for 1 day. Solutions of β-(25–35) alone and with H2 were prepared in a similar manner. As a control, PBS was diluted 5:1 with medium and added to some wells. Preliminary experiments showed that the trace amount of TFA that would be present after dilution of the β-(1–39) solution did not affect assay results. PC12 cells (ATCC) were cultured in medium containing 85% RPMI 1640, 5% fetal bovine serum, 10% heat-inactivated horse serum, 3.6 mM L-glutamine, and antibiotics (penicillin and streptomycin), all from Life Technologies, Inc. The cells were harvested from tissue culture flasks with brief exposure to 0.05% trypsin, 0.53 mM EDTA and aspirated three times with a 22-gauge needle to break up clumps. Approximately 10,000 cells/well were plated onto 96-well tissue culture plates with 100 μl of medium added. After 1 day, medium was removed, and the peptide solutions were added to the plates. The plates were incubated for another 24 h. Toxicity was assessed using the MTT assay (23). Briefly, 10 μl of 5 mg/ml MTT solution was added to each well, and the plates were incubated for 4 h at 37 °C. At this point, 100 μl of dimethylformamide and SDS were added and plates were incubated overnight at 37 °C to solubilize formazan products. Absorbance at 570 nm was measured with a microplate reader with background subtraction.

ThT Fluorescence Spectroscopy—Samples used for cellular toxicity assays were tested for ThT fluorescence after 1-week aging and prior to dilution into cell culture medium. ThT (Sigma) was dissolved at 100 μM in PBSA. Forty μl of peptide solution and 40 μl of ThT stock solution were mixed with 920 μl of PBSA and briefly vortexed. Fluorescence emission spectra were taken using a Model M-3 Alphascan (Photon Technology International, South Brunswick, NJ) spectrofluorimeter, with excitation at 450 nm. An increase in fluorescence with a maximum at 482 nm is indicative of amyloid (21). Fluorescence intensity for diluted ThT in the absence of peptide was determined and subtracted from sample intensities.

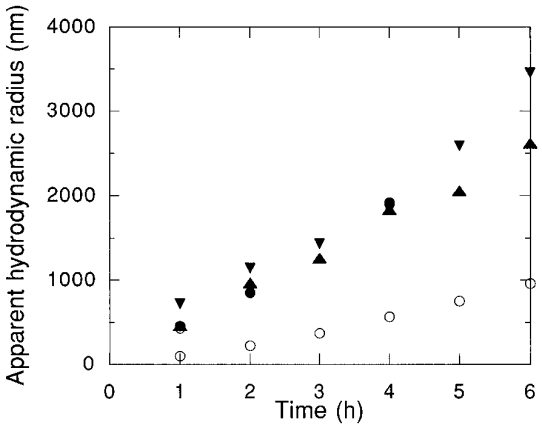

FIG. 2. **Aggregation kinetics of Aβ-related peptides mixed with Aβ.** The apparent hydrodynamic radius (measured at 90° scattering angle) as a function of time is plotted for β-(1–39) alone (*open circles*) or mixed with (*solid symbols*) R1 (*triangles*), R2 (*inverted triangles*), or H2 (*circles*).

RESULTS AND DISCUSSION

We hypothesized that the toxic effects of Aβ might be diminished in the presence of molecules possessing an Aβ recognition element and an amyloid disrupting unit. In the peptides reported here, a short fragment of the self-recognizing peptide Aβ was chosen to mediate specific binding to full-length Aβ. The disrupting element employed, a short stretch of lysine residues, was selected because it was reasoned that attachment of polar hydrophilic groups to the recognition element might prevent elongation of the β-structures formed in amyloid fibrils.

An important concern in the choice of the recognition element is that it should interact specifically with Aβ but not with itself. In designing a recognition element, we focused on regions of Aβ that are believed to be essential to fibril formation. The C-terminal sequence plays an important role in the formation and stability of Aβ-amyloid fibrils (8). This sequence, however, is highly hydrophobic and aggregates rapidly (24); thus, it would not be a suitable recognition element. Several lines of evidence show that the interior sequence 17–23 is crucial to formation of cross-β fibrils (7, 9, 10, 25, 26). Because this region has been implicated in Aβ self-recognition, we chose the sequence 15–25 as our recognition element. Attachment of polar, hydrophilic functionality to the recognition element was envisioned to afford a molecule that could bind to Aβ but would not form fibrils. To test this hypothesis, we chose to add a sequence of lysine residues to the Aβ binding sequence.

To examine the structural and functional properties of the synthetic peptides, we employed a variety of biophysical measurements including light scattering, circular dichroism spectroscopy, thioflavin T (ThT) fluorescence, and electron microscopy. For each analytical method, we compared the features of the recognition peptides alone (*R1* and *R2*, Fig. 1), the hybrid peptides alone (*H1* and *H2*, Fig. 1), and β-(1–39) alone or in the presence of recognition or hybrid peptides. These measurements were then correlated with cellular toxicity studies.

The aggregation properties of the peptides H1, H2, and β-(1–39) were assessed by light scattering. These experiments identified dramatic differences in the behavior of H1 and H2 and

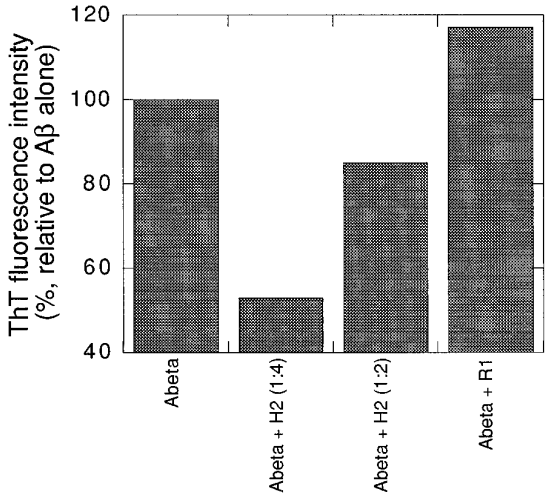

FIG. 3. **ThT fluorescence of Aβ-related peptides mixed with Aβ.** ThT fluorescence intensity was measured for β-(1–39) alone or mixed with R1 or H2 after 1-week incubation at room temperature. Samples were diluted 25-fold just prior to measurement. Results shown are from a single representative experiment; at least three similar experiments were run.

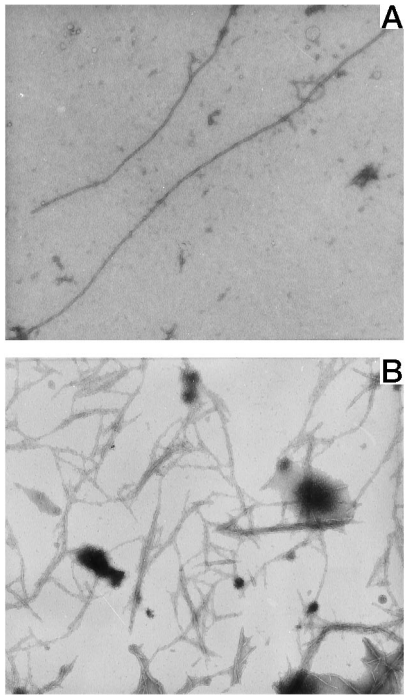

FIG. 4. **Electron micrographs of β-(1–39) (*A*) alone and β-(1–39) with H2 (*B*).** Magnification is × 72,500.

indicated that mixing H2, R1, or R2 with Aβ affects the kinetics of Aβ aggregation. H1 aggregated rapidly and precipitated in PBS. In contrast, H2 remained soluble and did not scatter light (100-milliwatt argon ion laser incident beam, detected by photomultiplier tube at 90° scattering angle), indicating that there was no significant aggregation. Consequently, the location of the poly(Lys) sequence was found to be a critical feature in the properties of these hybrid peptides. Although H2 did not form large aggregates by itself, the peptide had dramatic effects on the aggregation properties of β-(1–39). The rate of increase in size of the aggregates, as measured by dynamic light scattering, was much greater for the H2/β-(1–39) mixture than for β-(1–39) alone (Fig. 2). Sequences lacking the lysine hexamer, R1 and R2, also increased the aggregation rate of β-(1–39) (Fig. 2). This change in aggregation kinetics could result from an

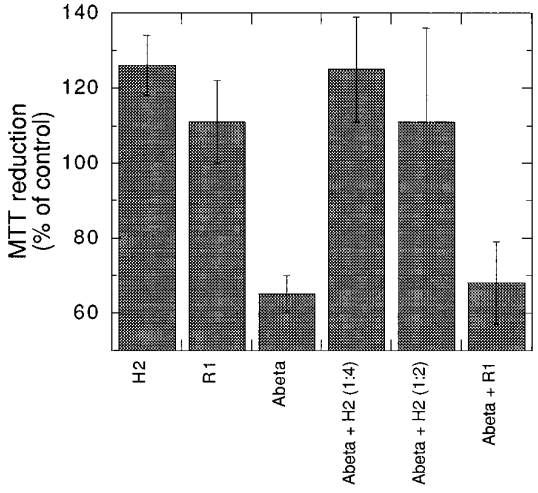

FIG. 5. **Toxicity of Aβ-related peptides mixed with Aβ.** Toxicity was measured by MTT reduction in PC-12 cells relative to control (PBS). Solutions of β-(1–39) alone or with Aβ-related peptides were prepared as described under "Experimental Procedures." Results shown are the mean ± S.D. of two to three replicates, with 2–5 determinations per experiment. Final concentration of β-(1–39) in all wells is 0.1 mg/ml (24 μM).

increase in the rate of fibril formation and/or a change in fibril morphology (*e.g.* increased fibril length, fibril stiffness, or fibril-fibril entanglement). Although the light scattering data indicated that the hybrid peptide affected fibril assembly, no specific contribution due to the poly(Lys) sequence of H2 could be identified.

To determine whether changes in assembly correlated with secondary structural features, circular dichroism spectra of β-(1–39) and H2 in phosphate buffer (0.01 M, pH 7.4) were collected and quantitatively analyzed. The spectra indicated that β-(1–39) was composed of 65% β-sheet and 30% random coil, in accord with previous measurements (20). The secondary structure of H2 alone was found to be essentially the same (64% β-sheet, 36% random coil, data not shown). No significant alterations in circular dichroism spectra were detected for samples in which H2 was added to β-(1–39) (not shown). In contrast to the light scattering studies, these measurements did not identify differences between samples containing only β-(1–39) and those containing both β-(1–39) and H2.

The effect of the recognition and hybrid peptides on β-(1–39) properties was further assessed by ThT fluorescence measurements. Positive fluorescence was taken as indicative of the presence of amyloid structure (21). None of the peptides in Fig. 1 caused a detectable increase in ThT fluorescence (data not shown). Combined with the light scattering data, this shows that the placement of the lysine hexamer on the N-terminus of the Aβ fragment β-(15–25), as in H1, leads to aggregation but not amyloid fibril formation. β-(1–39) caused ThT to fluoresce, as expected. When R1 was mixed with β-(1–39), a small but reproducible increase in ThT fluorescence relative to β-(1–39) alone (Fig. 3) was observed. In contrast, the ThT fluorescence intensity of β-(1–39) in the presence of H2 was reduced by about a factor of 2 (Fig. 3). Because R2 was not soluble in PBS, the assay could not be repeated in a like manner for that peptide; however, a parallel experiment was conducted by dissolution of R1 or R2 into 35% ACN/TFA followed by mixing with β-(1–39) and dilution into PBS. Using this protocol, the ThT fluorescence of R1 or R2 mixed with β-(1–39) was greater than that of β-(1–39) alone (data not shown). These data provided an indication that H2 was interfering with fibrillogenesis of β-(1–39), and that the decrease in ThT fluorescence required the oligolysine element.

To further examine the aggregates, electron micrographs were taken. β-(1–39) formed characteristic long, semiflexible fibrils (Fig. 4*A*). Mixing R2 with β-(1–39) had no obvious effects on fibril morphology (not shown). The addition of H2 to β-(1–39) did not prevent fibril formation, but it did appear to reduce the average length of fibrils and increase the extent of fibril entanglement, compared to β-(1–39) alone (Fig. 4*B*). Together with the other biophysical data, these results suggest that the recognition element alone increases aggregation kinetics and the mass fraction of amyloid fibrils but does not alter the aggregation pathway. The hybrid peptide does not prevent β-sheet formation or fibril initiation. Rather, it appears to bind to the growing Aβ fibril and disrupt fibril elongation processes. It has recently been suggested that inhibition of fibril growth processes may be a more feasible therapeutic target than inhibition of fibril initiation (27).

Given the changes in the physical properties of the aggregates formed by β-(1–39) in the presence of H2, we examined the ability of the hybrid peptide to modulate the cellular toxicity of aged β-(1–39) using an MTT reduction assay. Cell reducing activity is a measure of mitochondrial function; a decrease in ability to reduce MTT is an early indicator of Aβ-mediated toxicity (23). Freshly prepared solutions of β-(1–39) were not toxic to the cells (not shown), consistent with other reports in the literature (23). Neither H2 nor R1 alone was toxic. "Aged" β-(1–39) caused a decrease in cellular MTT reduction to ~60% of controls (Fig. 5). Incubating H2 with β-(1–39) during the aging process eliminated the β-(1–39)-mediated decrease in MTT reduction (Fig. 5). In contrast, R1 had no effect on the toxicity of β-(1–39). Thus, the inhibitory activity required the presence of the oligolysine disrupting element. Interestingly, the inhibition of Aβ toxicity by H2 did not require measurable conformational changes, the inhibition of aggregation kinetics, or the prevention of fibril formation. Instead, more subtle changes in the Aβ aggregation pathway caused by H2 apparently alter the toxicity of Aβ aggregates. ThT fluorescence was reduced by a factor of approximately 2, a result that suggests that ThT fluorescence may serve as an effective means of identifying inhibitors of Aβ toxicity. Complete inhibition of ThT fluorescence, however, was clearly not a prerequisite for complete inhibition of toxicity. It should be noted that Aβ is toxic at considerably lower concentrations (factor of ~100, data not shown) than those used in this work, concentrations at which ThT fluorescence would also be correspondingly lower.

H2 shares essentially no homology with β-(25–35), which is also toxic to neuronal cells. β-(25–35) alone caused a substantial increase in ThT fluorescence intensity; mixing H2 with β-(25–35) had neither a positive nor a negative effect on ThT fluorescence (data not shown). β-(25–35) decreased MTT reduction to 45% of control; co-incubation of H2 with β-(25–35) had no effect on the toxicity of β-(25–35). The selective effects of H2 on the physical properties and toxicity of β-(1–39) suggest that there is a specific interaction between the recognition element of the inhibitor and a homologous segment of Aβ.

Our results suggest that, by combining appropriate Aβ recognition elements with amyloid disrupting elements, Aβ aggregation pathways are altered and Aβ toxicity is inhibited. The combination of a recognition element with a disrupting element was essential for the prevention of toxicity; the recognition element alone altered some of the physical features of Aβ but did not alter its toxicity. Given the successful demonstration of this strategy, we envision H2 to be a prototype for the design of more effective inhibitors. For example, shorter peptide sequences, D-amino acid sequences, or organic peptidomimetics could serve as recognition elements in place of β-(15–25); a host of polar hydrophilic segments, either peptide- or non-peptide-based, could function as amyloid disrupting agents.

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