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)† 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 hexacycl-N-methylpiridinium bromide inhibits Aβ fibril formation, possibly by binding to a site on Aβ necessary for Aβ self-assembly (16). β-Cyclolextine, which has an affinity for hydrophobic groups, partially reduces Aβ toxicity (17).

An alternative approach is to use a fragment of Aβ to block fibril formation. Recently, it was reported that a pentapeptide KLFFF, containing the 16–20 sequence of full-length Aβ, binds to and disrupts fibril formation (18). An octapeptide, KQLVTTAE, 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
Inhibitors of β-Amyloid Toxicity

**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 Aβ-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 Aβ (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 Aβ (1–39) were assessed by light scattering. These experiments identified dramatic differences in the behavior of H1 and H2 and...
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 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.
Inhibitors of β-Amyloid Toxicity

To further examine the aggregates, electron micrographs were taken. β-(1–39) formed characteristic long, semiflexible fibrils (Fig. 4A). 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. 4B). 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.

REFERENCES
1. Glenner, G. G., and Wong, C. W. (1984) Biochem. Biophys. Res. Commun. 120, 885–890
2. Wong, C. W., Quaranta, V., and Glenner, G. G. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 8729–8732
3. Joachim, C. L., and Seiloe, D. J. (1992) Alzheimer Dis. Assoc. Disord. 6, 7–34
4. Pike, D. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. (1983) J. Neurosci. 13, 1676–1687
5. Simmons, L. K., May, P. C., Tomaselli, K. J., Rydel, R. E., Fuson, K. S., Brigham, E. F., Wright, S., Lieberburg, I., Becker, G. W., Brems, D. N., and Li, W. (1994) Mol. Pharmacol. 45, 373–379
6. Ueda, K., Pakui, Y., and Kageyama, H. (1994) Brain Res. 639, 240–244
7. Terzi, E., Holzemann, G., and Seelig, J. (1995) J. Mol. Biol. 233, 643–642
8. Jarrett, J. T., Berger, E. P., and Lansbury, P. T., Jr. (1993) Biochemistry 32, 4693–4697
9. Hilibich, C., Kisters-Waite, B., Reed, J., Masters, C. L., and Beyreuther, K. (1992) J. Mol. Biol. 228, 460–473
10. Fraser, P. E., Nguyen, J. T., Surewicz, W. K., and Kirschner, D. A. (1991) Biophys. J. 60, 1190–1201
11. Fraser, P. E., McLachlan, D. R., Surewicz, W. K., Milzen, C. A., Snow, A. D., Nguyen, J. T., and Kirschner, D. A. (1994) J. Mol. Biol. 244, 64–73
12. Shen, C.-L., and Murphy, R. M. (1995) Biophys. J. 69, 640–651
13. Lomakin, A., Chung, D. S., Benecke, G. B., Kirschner, D. A., and Teplow, D. B. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 1125–1129
14. Pollack, S. J., Sadler, I. J. J., Hawtin, S. R., Tailor, V. J., and Shearman, M. S. (1995) Neurosci. Lett. 197, 211–214
15. Kauliesky, R., Lemieux, L. J., Fraser, P. E., Kong, X., Hultin, P. G., and Starek, W. A. (1996) Nat. Med. 1, 143–146
16. Wood, S. J., MacKenzie, L., Maleeff, B., Hurle, M. R., and Wetzel, R. (1996) J. Biol. Chem. 271, 4096–4092
17. Camilleri, P., Haskins, N. J., and Howlett, D. R. (1994) FEBS Lett. 341, 256–258
18. Tjernberg, L. O., Naslund, J., Lindqvist, F., Johansson, J., Karlstrom, A. R., Thyberg, J., Terenius, L., and Nordstedt, C. (1996) J. Biol. Chem. 271, 8545–8548
19. Hughes, S. R., Goyal, S., Sun, J. E., Gonzalez-DeWhitt, P., Fortes, M., Riedel, N. G., and Sahasrabadufe, S. R. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 2065–2070
20. Shen, C.-L., Scott, G. L., Merchant, F., and Murphy, R. M. (1995) Biophys. J. 65, 2383–2395
21. LeVine, H. (1993) Protein Sci. 2, 404–410
22. Shen, C.-L., Fitzgerald, M. C., and Murphy, R. M. (1994) Biophys. J. 65, 2383–2395
23. Shearman, M. S., Ragan, C. I., and Iverson, L. L. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1470–1474
24. Halverson, K., Fraser, P. E., Kirschner, D. A., and Lansbury, P. T., Jr. (1990) Biochemistry 29, 2639–2644
25. Wood, S. J., Wetzel, R., Martin, J. D., and Hurle, M. R. (1995) Biochemistry 34, 724–730
26. Lee, J. P., Stimson, R. R., Ghilardi, J. R., Manthy, P. W., Lu, Y.-A., Felix, A. M., Llanes, W., Behhin, A., Cummings, M., Van Crieckinge, M., Timms, W., and Maggio, J. E. (1995) Biochemistry 34, 5191–5200
27. Edler, W. P., Sisson, E. R., Ghilardi, J. R., Vinters, H. V., Lee, J. P., Manthy, P. W., and Maggio, J. E. (1996) Biochemistry 35, 749–757