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A two-step strategy for SAR studies of *N*-methylated A β 42 C-terminal fragments as A β 42 toxicity inhibitors

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Abstract

Neurotoxic A β 42 oligomers are believed to be the main cause of Alzheimer's disease. Previously, we found that the C-terminal fragments (CTFs), A β (30–42) and A β (31–42) were the most potent inhibitors of A β 42 oligomerization and toxicity in a series of A β (x–42) peptides, (x=28–39). Therefore, we chose these peptides as leads for further development. These CTFs are 12/13-amino-acid long, hydrophobic peptides with limited aqueous solubility. Our first attempt to attach hydrophilic groups to the N-terminus resulted in toxic peptides. Therefore, next we incorporated N-methyl amino acids, which are known to increase the solubility of such peptides by disrupting β -sheet formation. Focusing on A β (31–42), we used a two-step N-methyl (N-Me) amino acid substitution strategy to study the structural factors controlling inhibition of A β 42-induced toxicity. First, each residue was substituted by N-Me-alanine (N-Me-A). In the next step, in positions where substitution produced a significant effect, we restored the original side-chain. This strategy allowed exploring the role of both side-chain structure and N-Me substitution in inhibitory activity. We found that the introduction of N-Me amino acid was an effective way to increase both the aqueous solubility and the inhibitory activity of A β (31–42). In particular, N-Me amino acid substitution at positions 9 or 11 increased the inhibitory activity relative to the parent peptide. The data suggest that inhibition of A β 42 toxicity by short-peptides is highly structure-specific, providing basis for the design of new peptidomimetic inhibitors with improved activity, physicochemical properties, and metabolic stability.

Keywords

Alzheimer's disease; amyloid β -protein; C-terminal fragment; *N*-methyl amino acid; structure-activity relationships

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder. AD is characterized by progressive memory and cognitive impairment and cerebral accumulation of extracellular amyloid plaques and intraneuronal neurofibrillary tangles.^[1] The major component of amyloid plaques is amyloid β -protein (A β), a small protein that exists

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primarily as 40- or 42-residue polypeptides (A β 40 and A β 42, respectively). A β 42 has been shown to be more neurotoxic than A β 40,^[2] and to follow a different pathway of oligomerization.^[3, 4] A β 42 is more prone to form high-order oligomers than A β 40 and this tendency correlates with structural stabilization of the C-terminus of A β 42 mediated by the presence of I41 and A42.^[3, 5-7] Although, the mechanism underlying AD pathology still is not clear, mounting evidence supports a central role for A β oligomers, particularly those of A β 42, in causing the cognitive impairment in AD.^[8, 9]

In view of the critical role of the C-terminal region of A β 42 in self-assembly, previously, we prepared C-terminal fragments (CTFs) of the general formula A β (x-42), x = 28-39, and tested them as inhibitors of A β 42 assembly and toxicity.^[10] Of the 12 CTFs tested, A β (31-42), was the strongest inhibitor of A β 42-induced toxicity, in both assays of synaptic activity and cell death.^[10] It was found to inhibit A β 42-induced neurotoxicity in differentiated rat pheochromocytoma (PC-12) cells with half-maximal inhibition (IC₅₀) values of 14 $\pm$ 2 and 20 $\pm$ 4 μ M in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release assays, respectively. In addition, A β (31-42) rescued mouse primary hippocampal neurons from A β 42-induced inhibition of miniature excitatory postsynaptic current frequency.^[10] The second most potent inhibitor was A β (30-42). Mechanistic investigation showed that both analogues inhibited A β 42 hexamer formation (IC₅₀ = 23 $\pm$ 4 μ M for A β (31-42) and IC₅₀ = 0.24 $\pm$ 0.03 μ M for A β (30-42)) as determined by photo-induced cross-linking of unmodified proteins,^[10] and suppressed formation of larger assemblies with hydrodynamic radius (R_H) = 20-60 nm detected by dynamic light scattering.^[10, 11]

Low solubility is a general issue when working with hydrophobic peptides derived from A β . Different strategies have been investigated for overcoming difficulties related to low solubility of hydrophobic peptides. For example, Fülöp et al. introduced an *N*-terminal arginine (R) residue to A β (31-34), which was used as a fibrillogenesis inhibitor and showed increased aqueous solubility.^[12] Other hydrophilic moieties used for the same purpose include polyethylene glycol (PEG),^[13] carbohydrates,^[14] and betaine.^[15] Taking a different approach, Gordon et al. reported that introduction of *N*-methyl amino acids increased the solubility of A β (16-22) substantially.^[16, 17] Using hydrophilic appendages offers a large degree of versatility, exploring different peptidic and non-peptidic moieties, which can be either charged or neutral. On the other hand, an advantage of *N*-methylation relative to hydrophilic conjugates is that the molecular weight increase is kept to a minimum. Though *N*-methylation actually increases the overall hydrophobicity of the resulting derivative, aqueous solubility typically is increased due to prevention of β -sheet formation, particularly of amyloidogenic sequences.^[18]

Here, to explore structure-activity relationship (SAR), first we synthesized several analogues containing hydrophilic appendages of A β (30-42) and A β (31-42). The parent peptides previously had been found to have low aqueous solubility.^[19] Based on the results of first screening of these analogues, we changed direction and continued to explore systematically A β (31-42) derivatives containing single *N*-Me amino-acid substitutions, and evaluated their toxicity and inhibitory activity in cell viability assays.

Results and Discussion

Attachment of hydrophilic appendages to CTFs

Our initial approach was to attach different hydrophilic moieties to the *N*-terminus of A β (30-42) or A β (31-42) in an attempt to improve their aqueous solubility. Several appendages were explored, including neutral and negatively charged amino acids, and PEG (Table 1). We did not use positively charged residues because A β (28-42), which contains an

N-terminal K and was the only positively charged peptide in our original CTF series, was highly toxic.^[10] Unfortunately, we found that the new analogues also gained toxicity upon addition of the hydrophilic appendages, regardless of the chemical nature of the hydrophilic moiety (Figure 1). Therefore, we did not continue to explore this direction and instead, focused our efforts on a systematic study of *N*-methylated analogues of A β (31–42). We chose to focus on A β (31–42) because it was the strongest inhibitor of toxicity found in the original series.^[10]

***N*-methyl scanning of A β (31–42)**

Alanine (A)-scanning is a common method for studying side-chain function in bioactive peptides,^[20] because A is the smallest chiral amino acid. However, because A β (31–42) is a hydrophobic peptide with limited aqueous solubility,^[19] we suspected that analogues containing single A substitutions might be hard to synthesize and purify, similar to the parent peptide,^[21] and biophysical and biological evaluation of these peptides might be difficult. Therefore, we devised a two-step strategy, in which the first step achieves both a systematic structural study and increasing the aqueous solubility by substituting each residue by *N*-Me-A, and the second step distinguishes between the effects of side chain reduction and *N*-methylation by reintroducing the side chain in positions showing substantial effects on activity, while keeping the *N*-Me moiety in that position.

Synthesis of *N*-methylated A β (31–42) analogues—We introduced *N*-Me-A in each position along the A β (31–42) sequence (Table 2) using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) for introduction of *N*-Me-A itself and the following residue.^[22] This protocol allowed successful synthesis of 9 out of the 12 *N*-Me-A-containing derivatives. However, using this general protocol, we did not obtain correct products for [*N*-Me-A8]A β (31–42), [*N*-Me-A11]A β (31–42), and [*N*-Me-A12]A β (31–42), necessitating special steps for these analogues. Synthesis of [*N*-Me-A8]A β (31–42) led to products containing deletions of G7 or of both G7 and *N*-Me-A8. These products likely resulted either from low yield of *N*-Me-A8 coupling to V9 or from formation of a diketopiperazine (DKP) side product, which is a common problem when P, G, or *N*-alkylated amino acids are in the C-terminal dipeptide sequence or their combinations are in the middle of the sequence,^[22] particularly when benzyl alcohol-based solid supports are used.^[23] The reaction is both base- and acid-catalyzed and thus may occur during coupling, deprotection, and/or cleavage from the solid-support. Taking these considerations into account, we increased the coupling time and performed double coupling for G7 and *N*-Me-A8. We also reduced the deprotection time to 1–2 min, and reduced the cleavage time to 1 hour. Using these modifications, we obtained [*N*-Me-A8]A β (31–42) successfully.

For [*N*-Me-A11]A β (31–42) and [*N*-Me-A12]A β (31–42), due to the proximity of the *N*-methyl amino acid to C-terminal carboxyl group, DKP formation was predicted to happen easily during the synthesis on NovaSyn TGA resin (alcohol-based solid support).^[23] To avoid this side reaction, we used the highly hindered chlorotriyl (Cl-Trt) resin, which had been reported to be an effective way to reduce DKP formation.^[23] We also used double coupling and reduced deprotection and cleavage times, resulting in successful synthesis of these two analogues.

Solubility of *N*-Me-A-substituted A β (31–42) analogues—To determine the solubility of *N*-Me-A-containing analogues, we used a simple filtration assay.^[19] Briefly, lyophilized peptides were dissolved or suspended in 10 mM sodium phosphate at 200 μ M nominal concentration, sonicated for 1 min, and filtered through a 20-nm pore-size filter to

remove insoluble material. Following this treatment, the actual concentrations were determined by amino acid analysis (AAA) and are shown in Table 2.

Most of *N*-Me-A substituted analogues of A β (31–42) had increased solubility relatively to that of A β (31–42), $25 \pm 4 \mu\text{M}$, except for [*N*-Me-A1]A β (31–42), whose solubility was $8 \pm 1 \mu\text{M}$. The low solubility of [*N*-Me-A1]A β (31–42) may be explained by the increase in hydrophobicity, similar to all other analogues, but without disruption the β -hairpin structure of A β (31–42),^[24] because the methylation is at the *N*-terminus. *N*-Me-A substitution in positions 8 and 12 also resulted in peptides with relatively low solubility (Table 2) suggesting that *N*-methylation in these positions did not effectively disrupt the β -hairpin structure. In the case of [*N*-Me-A12]A β (31–42), this likely is due to a similar reason as in the case of [*N*-Me-A1]A β (31–42), i.e., the *N*-Me group is too far from the β -strands to disrupt their association. For [*N*-Me-A8]A β (31–42), according to the structure of A β (31–42) calculated based on ion-mobility–mass-spectrometry data, this position is located within a β -turn^[24] where *N*-methylation is unlikely to disrupt the β -hairpin structure. *N*-Me-A substitution in other positions increased the solubility 3–5 fold, suggesting effective disruption of the β -hairpin structure.

Inhibition of A β 42-induced neurotoxicity by *N*-Me-A-substituted A β (31–42) analogues

As an initial step before testing inhibitory activity, we checked whether the A β (31–42) analogues were toxic themselves. The peptides were dissolved in a small amount of 60 mM NaOH, diluted to 50 μM with cell culture media, and added to differentiated PC-12 cells. Most of the analogues, with the exception of [*N*-Me-A1]A β (31–42), showed no toxicity to the cells and even moderately increased cell viability relative to cells incubated with media alone, as assessed by the MTT assay^[25] (Figure 1).

Next, we screened the *N*-methylated A β (31–42) derivatives for inhibition of A β 42-induced neurotoxicity in single-dose experiments. Differentiated PC-12 cells were incubated with A β 42 (5 μM) for 24 h in the absence or presence of 10-fold excess of each derivative, and cell viability was assessed using the MTT assay (Figure 2). [*N*-Me-A3]A β (31–42), [*N*-Me-A8]A β (31–42), [*N*-Me-A9]A β (31–42), and [*N*-Me-A11]A β (31–42) showed significantly higher inhibitory activity than the parent peptide, whereas *N*-Me-A substitution in positions 2, 4–7, 10, and 12 yielded peptides with similar activity to the parent peptide.

To evaluate further the analogues found to increase inhibitory activity significantly in the initial screen, we examined each one in dose-response assays in both the MTT (Figure 3a) and LDH (Figure 3b) assays. We used both assays because they address different aspects of cell toxicity — The MTT assay measures mitochondrial activity of viable cells, whereas the LDH assay detects membrane integrity as a direct measurement of cell death.^[26]

Similarly to the parent peptide, the *N*-methylated A β (31–42) analogues yielded dose-dependent inhibition of A β 42-induced toxicity (Figure 3). The IC₅₀ values obtained are summarized in Table 2. [*N*-Me-A3]A β (31–42) had similar inhibitory activity to A β (31–42), whereas the other 3 derivatives showed increased inhibitory activity. [*N*-Me-A9]A β (31–42) was the most potent analogue, yielding protection from A β 42-induced toxicity with IC₅₀ = $6 \pm 1 \mu\text{M}$ in the MTT assay (3-fold improvement relative to the parent peptide) and $7 \pm 1 \mu\text{M}$ in the LDH assay (6-fold improvement).

N-methyl substitutions with restoration of the original side chain

As the second step in our SAR strategy, to determine the contribution of the *N*-methylation versus the side-chain change to the inhibitory activity, we synthesized analogues containing substitution of the original residue in positions found to affect biological activity significantly, by the *N*-methyl version of these residues (Table 2). These included analogues

substituted both at the four positions that yielded a significant increase in inhibitory activity (3, 8, 9, and 11) and in the single position that caused increased toxicity (1).

The synthesis of the analogues containing *N*-methylation at positions 1, 9, and 11 was challenging because the original side-chains in these positions are β -substituted (I1, V9, and I11) causing substantial steric hindrance. Accordingly, we found that [*N*-Me-I1] $\text{A}\beta$ (31–42), [*N*-Me-G8] $\text{A}\beta$ (31–42), [*N*-Me-V9] $\text{A}\beta$ (31–42), and [*N*-Me-I11] $\text{A}\beta$ (31–42) were unstable during overnight storage either at 4°C under Ar or under vacuum due to the presence of residual trifluoroacetic acid (TFA) following cleavage from the solid support. To prevent the degradation of these analogues, we neutralized the crude peptide immediately after cleavage using *N,N*-diisopropylethylamine (DIPEA). The conditions described above for the difficult *N*-Me-A analogues also were necessary for successful synthesis of these three analogues. In contrast, [*N*-Me-G3] $\text{A}\beta$ (31–42) was successfully synthesized using the general protocol described above and did not require DIPEA neutralization.

Filtration experiments showed that, similar to the *N*-Me-A analogues, the analogues substituted at positions 1 and 8 had low solubility, whereas substitutions at positions 3, 9, and 11 yielded high solubility (above 100 μM) (Table 2). Cell viability assessment using the MTT assay revealed that *N*-methylation at positions 3, 8, 9 or 11 did not cause toxicity, whereas [*N*-Me-I1] $\text{A}\beta$ (31–42) was toxic, similarly to [*N*-Me-A1] $\text{A}\beta$ (31–42) (Figure 1).

Interestingly, dose-response evaluation of these analogues by the MTT (Figure 3c) and LDH assays (Figure 3d) showed that restoring the original side-chain did not improve the inhibitory activity but rather decreased it in most cases (Table 2). In particular, [*N*-Me-G8] $\text{A}\beta$ (31–42) showed 2.3- and 5.0-fold decrease in activity relative to [*N*-Me-A8] $\text{A}\beta$ (31–42), respectively, whereas [*N*-Me-G3] $\text{A}\beta$ (31–42) and [*N*-Me-I11] $\text{A}\beta$ (31–42) showed a smaller decrease in activity, 1.4- and 1.3-fold, respectively, in the MTT assay. For [*N*-Me-I11] $\text{A}\beta$ (31–42) a similar result was obtained in the LDH assay, whereas [*N*-Me-G3] $\text{A}\beta$ (31–42) showed no inhibition in LDH assay. [*N*-Me-V9] $\text{A}\beta$ (31–42) yielded similar activity to [*N*-Me-A9] $\text{A}\beta$ (31–42) in the MTT assay, whereas in the LDH assay, its inhibitory activity decreased by 9.6-fold.

Linear regression analysis showed that the changes in activity of all the analogues for which dose-response experiments have been performed did not correlate with the change in solubility of these peptides (MTT IC_{50} vs. solubility $-r^2 = 0.250$, $p = 0.221$; LDH IC_{50} vs. solubility $-r^2 = 0.003$, $p = 0.901$).

Our new, two-step *N*-methyl amino acid substitution strategy, allowed systematic SAR study of $\text{A}\beta$ (31–42) as an inhibitor of $\text{A}\beta$ 42-induced toxicity. [*N*-Me-I1] $\text{A}\beta$ (31–42) was found to be as toxic as [*N*-Me-A1] $\text{A}\beta$ (31–42), suggesting that the toxicity was caused by the introduction of the *N*-Me group rather than the side chain change and might correlate with the increase in both basicity and hydrophobicity associated with conversion of the *N*-terminal primary amine into a secondary amine.

Restoring the side-chain in positions 3, 8, 9, or 11 had a relatively weak effect on the inhibitory activity (Table 2), suggesting that the main cause for the increased inhibitory activity of these analogues was the introduction of the *N*-Me group, rather than the side-chain substitution.

To summarize the SAR findings

1. The activity of $\text{A}\beta$ (31–42) was not sensitive to *N*-methylation at positions 2, 4, 5, 6, 7, 10, or 12, whereas *N*-Me-A substitution at positions 3, 8, 9, and 11 increased the inhibitory activity.

2. An *N*-terminal –NH₂ group was important for maintaining Aβ(31–42) non-toxic whereas an *N*-terminal –NH-CH₃ group induced toxicity.
3. *N*-Me-A substitution in positions 3 and 8 increased inhibitory activity relative to *N*-Me-G.
4. *N*-methylation at position 9 provided the strongest increase in inhibitory activity. *N*-Me-A provided stronger inhibition in the LDH assay, whereas in the MTT assay, we did not find a difference between the *N*-Me-A- and *N*-Me-V-containing analogues.
5. *N*-methylation at position 11 increased the inhibitory activity and the small side-chain of A yielded better inhibition than the bulky hydrophobic side-chain of I.
6. *N*-methylation of residues previously shown to be in a β-strand conformation in Aβ(31–42) increased the solubility of Aβ(31–42) substantially. *N*-methylation in the turn region or at the C-terminal residue was less effective in increasing solubility. *N*-methylation at the *N*-terminal residue decreased the solubility.
7. The changes in inhibitory activity observed relative to the parent peptide were not merely a reflection of better solubility of certain analogues, but rather likely reflect more efficient binding to Aβ42 and/or disruption of particular toxic structures.

Thanks to the increased solubility of most of the *N*-methylated analogues, their synthesis was facilitated relative to that of the parent peptide. Though in some cases protocol modifications were needed to overcome facile DKP formation, those were relatively simple and purification of the products by RP-HPLC was straightforward, in contrast to Aβ(31–42).^[21] There was no obvious pattern predicting which residue would be difficult to add as an *N*-methyl amino acid. Previously, it was reported that although bulky, hydrophobic *N*-methylated amino acids might pose challenges in coupling to a growing peptide, in some cases even sterically unhindered *N*-methylated amino acids gave poor yields.^[18] We found this to be the case in the synthesis of [*N*-Me-A8]Aβ(31–42) and [*N*-Me-G8]Aβ(31–42), both of which were obtained in low yield.

The mechanisms by which introduction of *N*-methylated amino acids prevents aggregation of amyloidogenic peptides and proteins were summarized in a review by Sciarretta et al.^[18] Replacement of an amide proton by a methyl group breaks hydrogen-bonds among individual β-strands. In addition, the methyl group is larger than the amide proton and prevents the close approach of the peptide chains by steric hindrance. The same reasons likely improve peptide solubility upon introduction of *N*-methyl amino acids. The replacement of an amide proton by a methyl group may break hydrogen bonds inside hydrophobic clusters or organized structures, allowing water molecules to insert between polypeptide chains and interact with the peptide backbone. *N*-Me groups may disrupt association of both intramolecular and intermolecular β-strands. In the case of Aβ(31–42), if disruption happens intramolecularly, the β-hairpin structure is destabilized and no β-sheets form. In contrast, if the *N*-Me groups project outward, the β-hairpin conformation may still form but the *N*-Me group would interfere with intermolecular β-sheet formation. Either way, self-association of the *N*-Me-substituted peptides and their association with full-length Aβ42 are attenuated resulting in increased aqueous solubility and inhibition of toxicity. Interestingly, Gordon et al. found that *N*-methylated Aβ(16–20) analogues were highly soluble in both aqueous and organic solutions, suggesting that *N*-methylated peptides might be able to pass spontaneously through cell membranes, an important property for drug delivery, diagnostics, and inhibitory activity.^[17]

Previously, a coil-turn structure of certain Aβ42 CTFs, including Aβ(31–42), was found to correlate with the degree of inhibition of Aβ42-induced toxicity, whereas a β-strand/β-turn

conformation did not.^[19, 24] We predicted that introduction of *N*-methylated amino acids would shift the equilibrium from β -strand/ β -turn toward coil-turn, facilitating association of the *N*-methylated analogues with A β 42 that promote formation of non-toxic assembly. The data suggest that these predictions were correct and imply that inhibition is achieved through specific interaction between particular analogues and A β 42.

Conclusion

Using a two-step *N*-methyl amino acid substitution strategy, we successfully increased both the aqueous solubility and the inhibitory activity of A β (31–42). This two-step strategy is applicable to SAR studies of other hydrophobic/amyloidogenic peptides where the parent peptide is characterized by low solubility, and may lead to development of therapeutic agents for Alzheimer's disease and other amyloid diseases

Experimental Section

Chemicals and Reagents

9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, Fmoc-Ala-NovaSyn TGA (0.22 mmole/g), H-Ala-2-Cl-Trt resin (0.77 mmole/g), 2-Cl-(Trt)-Cl resin (1.3 mmole/g) were purchased from Novabiochem (Gibbstown, NJ). Wang resin and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. All commercially available solvents and reagents were used without further purification. High purity water (18.2 M Ω) was obtained using a Milli-Q system (Millipore, Bedford, MA).

Peptide Synthesis

Synthesis, purification, and characterization of A β 42 were carried out as described previously,^[27] purified using reverse-phase high-performance liquid chromatography (RP-HPLC), and characterized by mass spectrometry (MS) and AAA.

General protocol for synthesis of *N*-methylated A β (31–42) derivatives—A β (31–42) and derivatives were synthesized using a Discover® microwave-assisted synthesis system (CEM, Matthews, NC). Fmoc-protected, pre-loaded NovaSyn TGA resin (0.1 mmol) was placed in a peptide synthesis vessel, swollen in *N,N*-dimethylformamide (DMF), and deprotected with 5 mL of 20% piperidine (or 4-methylpiperidine) in DMF for 20 min at room temperature. After washing with DMF thrice, a mixed solution of 0.3-mmol Fmoc-AA-OH, 0.3-mmol (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU), and 0.6-mmol DIPEA in 4 mL DMF was added to the reaction vessel. Fmoc-*N*-Me-A-OH and the following amino acid were coupled using HATU as activating reagent. The coupling reaction was performed using 40 W microwave energy for 8 min at 50°C. A 2,4,6-trinitrobenzenesulfonic acid (TNBS) color test kit (TCI AMERICA, Portland, OR) was applied to detect remaining free amino groups. The efficacy of coupling reaction was monitored by the formation of piperidine-dibenzofulvene (or 4-methylpiperidine-dibenzofulvene) using UV spectroscopy.^[28] After completion of the sequence, the resin was thoroughly washed with DMF and then with dichloromethane, dried under vacuum and the peptide was cleaved using a mixture of TFA:1,2-ethanedithiol:H₂O – 95:2.5:2.5 (v/v) for 1.5 hours at room temperature. The cleavage solution was collected and its volume was reduced to 1–2 mL with a gentle stream of high-purity nitrogen. Peptides were precipitated by addition of cold diethyl ether, purified by RP-HPLC, and characterized by MS and AAA. The purity of all peptides was higher than 95% determined by analytical RP-HPLC. The peptide sequences, calculated masses, and observed masses are listed in Table 2.

Synthetic procedure for [N-Me-A12]A β (31–42)—2-Cl-(Trt)-Cl resin (1.3 mmole/g, 0.5 g) was swollen in DMF and filtered. The first amino acid was attached by adding a mixture of Fmoc-N-Me-A-OH (0.4 mmol) and DIPEA (4 mmol) in 3 mL DMF. The mixture was shaken at RT for 1 hour. Capping excess reactive groups on the resin was achieved using a mixture of DCM:MeOH:DIPEA – 17:2:1 (v/v). The resin was washed with DCM (3 mL $\times$ 3), DMF (3 mL $\times$ 3), and DCM (3 mL $\times$ 3). The loading rate was tested using UV spectroscopy as described above. The following Fmoc-I-OH and Fmoc-V-OH were coupled with HATU as activation reagent and with double coupling. The coupling and deprotection of other amino acids, and cleavage followed the general protocol described above.

Synthetic procedure for [N-Me-I11]A β (31–42)—H-Ala-2-Cl-Trt resin (0.77 mmole/g, 0.3 g) was swollen in DMF and filtered. Fmoc-N-Me-I-OH and Fmoc-V-OH were coupled with HATU as activation reagent and with double coupling. The coupling and deprotection of other amino acids followed the general protocol described above. The peptide was cleaved from the resin using TFA:thioanisole:triisopropylsilane – 92:6:2 (v/v) for 1 hour. The solution was collected and its volume reduced to 1–2 mL under a gentle stream of high-purity nitrogen. The peptide was precipitated by addition of cold diethyl ether, collected by centrifugation, the ether was removed, and the peptide neutralized with DIPEA and washed with cold ether. The crude peptide immediately was dissolved in water, frozen, lyophilized, and then purified using RP-HPLC. The fractions containing the pure peptide were frozen immediately and re-lyophilized.

Solubility

The solubility study was carried as described previously.^[19] Briefly, peptides were dissolved or suspended in 60 mM NaOH (10% of the final volume) and then diluted with 10 mM sodium phosphate, pH 7.4, to 200 μ M nominal concentration. The solution was sonicated for 1 min and then filtered through an Anotop 10 syringe filter with 20 nm pore size (Whatman, Florham Park, NJ). Three-to-five replicates were measured for each peptide. The actual peptide concentrations were determined by AAA and the results are presented as mean $\pm$ SEM.

Cell Viability Assays

The methods for evaluation of the biological activity of the CTFs themselves and their inhibition of A β 42-induced toxicity were described previously.^[10] Briefly, PC-12 cells were differentiated into a neuronal phenotype by incubation with nerve growth factor (50 ng/mL) for 48 h. The cells then were incubated with solutions of A β 42 alone at 5 μ M, A β (31–42) analogues alone at 50 μ M, or A β 42:A β (31–42)-analogue mixtures at 1:10 concentration ratio, respectively, for 24 h. For initial screening of the new analogues, cell viability was determined by the MTT assay using a CellTiter 96® kit (Promega, Madison, WI). Negative controls included NaOH at the same concentration as in the peptide solutions and media alone. A positive control was 1 μ M staurosporine for full kill, which was used to represent a 100% reduction in cell viability, based on which the percentage viability of all of the experimental conditions was calculated. Active analogues were characterized further for dose-dependent activity. In these experiments, A β 42 alone and A β 42:A β (31–42) analogue mixtures at 1:0.1, 1:0.3, 1:1, 1:3, and 1:10 (and in some cases 1:20 and 1:30 according to peptide solubility) concentration ratios were used. Cell viability was measured by both the MTT assay and the LDH-release assay (CytoTox-ONE Homogenous Membrane Integrity Assay kit (Promega)). At least three independent experiments with five replicates ($n = 15$) were performed. The results were averaged and presented as mean $\pm$ SEM.

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Figure 1. Effect of Aβ(30-42) and Aβ(31-42) derivatives on neuronal cells

A) Aβ(30-42) (grey), Aβ(31-42) (black), and derivatives at 50 μM were incubated with differentiated PC-12 cells for 24 h and cell viability was measured using the MTT assay. The data are shown as mean ± SEM of at least three independent experiments with 6 replicates per data point (n = 18).

Figure 2. Inhibitory activity of *N*-Me-A-substituted Aβ(31–42) analogues

Aβ42 (5 μM, grey diagonal stripes) and mixtures of Aβ42:Aβ(31–42) (black) and derivatives (white diagonal stripes) at a 1:10 concentration ratio were incubated with differentiated PC-12 cells for 24 h and cell viability was measured using the MTT assay. The data are shown as mean ± SEM of at least three independent experiments with 6 replicates per data point ($n = 18$). Statistical significance was calculated and compared with Aβ(31–42) using ANOVA followed by Dennett's multiple-comparison tests (*** $p < 0.001$).

Figure 3. Inhibition of Aβ42-induced toxicity by Aβ(31-42) derivatives

Five μM Aβ42 in the absence or presence of Aβ(31-42) derivatives in 1:0.1, 1:0.3, 1:1, 1:3, and 1:10 (and in some cases 1:20 and 1:30) concentration ratios, respectively, were added to differentiated PC-12 cells. a and c) Cell viability was determined using the MTT assay. b and d) Cell death was measured using the LDH assay. The data are shown as mean ± SEM and are representative of at least three independent experiments with five replicates per data point (n = 15). Dose-response curves were obtained by sigmoidal fitting (variable slope) using Prism, 5.0c (GraphPad, La Jolla, CA).

Table 1

N-terminal modifications of A β (30–42) and A β (31–42).

Peptide	Sequence
GGGGG-A β (30–42)	GGGGG -AIIGLMVGGVVIA
SGS-A β (30–42)	SGS -AIIGLMVGGVVIA
DD-A β (31–42)	DD -IIGLMVGGVVIA
PEG-A β (30–42)	PEG -AIIGLMVGGVVIA

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Table 2

Sequences, masses, and IC₅₀ values of Aβ(31–42) and derivatives.

Code	Sequence	Mass (Calculated)	Mass (Observed)	Yield	Solubility (μM)	IC ₅₀ (μM) (MTT)	IC ₅₀ (μM) (LDH)
Aβ(31–42)	IIGLMVGGVVIA	1141.7	1141.3	3% or 23%[21]	25 ± 4	18 ± 1	43 ± 2
[N-Me-A1]Aβ(31–42)	(N-Me)A-IGLMVGGWIA	1113.4	1113.5	13.8%	8 ± 1		
[N-Me-A2]Aβ(31–42)	I-(N-Me)A-GLMVGGWIA	1113.4	1112.1	10.8%	101 ± 18		
[N-Me-A3]Aβ(31–42)	II-(N-Me)A-LMVGGWIA	1169.5	1169.5	11.4%	102 ± 16	18 ± 1	34 ± 3
[N-Me-A4]Aβ(31–42)	II G-(N-Me)A-MVGGWIA	1113.4	1113.6	9.9%	127 ± 37		
[N-Me-A5]Aβ(31–42)	II GL-(N-Me)A-VGGWIA	1096.4	1095.6	4.4%	105 ± 20		
[N-Me-A6]Aβ(31–42)	II GLM-(N-Me)A-GGWIA	1127.4	1127.5	6.9%	87 ± 18		
[N-Me-A7]Aβ(31–42)	II GLMV-(N-Me)A-GWIA	1170.4	1169.7	20.5%	88 ± 14		
[N-Me-A8]Aβ(31–42)	II GLMV G-(N-Me)A-WIA	1169.5	1169.8	5.7%	41 ± 12	12 ± 1	14 ± 1
[N-Me-A9]Aβ(31–42)	II GLMVGG-(N-Me)A-VIA	1127.4	1125.3	9.2%	115 ± 21	6 ± 1	7 ± 1
[N-Me-A10]Aβ(31–42)	II GLMVGGV-(N-Me)A-1A	1127.4	1127.1	7.4%	121 ± 28		
[N-Me-A11]Aβ(31–42)	II GLMVGGW-(N-Me)A-A	1113.4	1112.8	7.5%	86 ± 22	10 ± 1	37 ± 3
[N-Me-A12]Aβ(31–42)	II GLMVGGWI-(N-Me)A	1155.5	1155.6	2.7%	28 ± 13		
[N-Me-I1]Aβ3(31–42)	(N-Me)I-IGLMVGGWIA	1155.5	1155	2.1%	7 ± 1		
[N-Me-G3]Aβ(31–42)	II-(N-Me)G-LMVGGWIA	1155.5	1155.6	6.2%	118 ± 11	26 ± 1	[a]
[N-Me-G8]Aβ(31–42)	II GLMV G-(N-Me)G-WIA	1155.5	1155	1.3%	18 ± 2	28 ± 1	71 ± 20
[N-Me-V9]Aβ(31–42)	II GLMVGG-(N-Me)V-VIA	1155.5	1155	6.2%	136 ± 18	6 ± 1	67 ± 8
[N-Me-I11]Aβ3(31–42)	II GLMVGGW-(N-Me)I-A	1155.5	1155	3.5%	132 ± 34	13 ± 1	49 ± 3

[a]No inhibition.