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A Two-Step Strategy for Structure–Activity Relationship Studies of N-Methylated Aβ42 C-Terminal Fragments as Aβ42 Toxicity Inhibitors

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Neurotoxic Aβ42 oligomers are believed to be the main cause of Alzheimer's disease. Previously, we found that the C-terminal fragments (CTFs), Aβ(30–42) and Aβ(31–42) were the most potent inhibitors of Aβ42 oligomerization and toxicity in a series of Aβ(*x*–42) peptides (*x* = 28–39). Therefore, we chose these peptides as leads for further development. These CTFs are short (12–13 amino acids) hydrophobic peptides with limited aqueous solubility. Our first attempt to attach hydrophilic groups to the N terminus resulted in toxic peptides. Therefore, we next incorporated *N*-methyl amino acids, which are known to increase the solubility of such peptides by disrupting the β-sheet formation. Focusing on Aβ(31–42), we used a two-step *N*-methyl amino acid substitution strategy to study the structural factors controlling inhibition of Aβ42-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 an *N*-Me amino acid was an effective way to increase both the aqueous solubility and the inhibitory activity of Aβ(31–42). In particular, *N*-Me amino acid substitution at position 9 or 11 increased the inhibitory activity relative to the parent peptide. The data suggest that inhibition of Aβ42 toxicity by short peptides is highly structure-specific, providing a basis for the design of new peptidomimetic inhibitors with improved activity, physicochemical properties, and metabolic stability.

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 (Aβ), a small protein that exists primarily as 40- or 42-residue polypeptides (Aβ40 and Aβ42, respectively). Aβ42 has been shown to be more neurotoxic than Aβ40,[2] and it follows a different pathway of oligomerization.[3,4] Aβ42 is more prone to form high-order oligomers than Aβ40, and this tendency correlates with structural stabilization of the C terminus of Aβ42 mediated by the presence of isoleucine (I) 41 and alanine (A) 42.[3,5–7] Although, the mechanism underlying AD pathology is still unclear, mounting evidence supports a central role for Aβ oligomers, particularly those of Aβ42, in causing the cognitive impairment seen in AD patients.[8,9]

In view of the critical role of the C-terminal region of Aβ42 in self-assembly, previously, we prepared C-terminal fragments (CTFs) of the general formula Aβ(*x*–42), where *x* is 28 to 39, and tested them as inhibitors of Aβ42 assembly and toxicity.[10] Of the 12 CTFs tested, Aβ(31–42) was the strongest inhibitor of Aβ42-induced toxicity, in assays evaluating both synaptic activity and cell death.[10] It was found to inhibit Aβ42-induced neurotoxicity in differentiated rat pheochromocytoma (PC-12) cells with IC_{50} values of 14 ± 2 and 20 ± 4 μM in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release assays, respectively. In addition, Aβ(31–42) rescued mouse primary hippocampal neurons from Aβ42-induced inhibition of miniature excitatory postsynaptic current frequency.[10] The second most potent inhibitor was Aβ(30–42). A mechanistic investigation showed that both analogues inhibited Aβ42 hexamer formation (Aβ(31–42) $IC_{50} = 23 \pm 4$ μM; Aβ(30–42) $IC_{50} = 0.24 \pm 0.03$ μM) as determined by photo-induced cross-linking of unmodified proteins,[10] and suppressed formation of larger assemblies with a hydrodynamic radius (R_H) of 20–60 nm detected by dynamic light scattering (DLS).[10,11]

Low solubility is a general issue when working with hydrophobic peptides derived from Aβ. Different strategies have been investigated for overcoming difficulties related to low solubility of hydrophobic peptides. For example, Fülöp et al.

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introduced an N-terminal arginine (R) residue to Aβ(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 (*N*-Me) amino acids increased the solubility of Aβ(16–22) substantially.[16,17] Using hydrophilic appendages offers a large degree of versatility, exploring different peptidic and nonpeptidic 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 relationships (SARs), we first synthesized several analogues containing hydrophilic appendages of Aβ(30–42) and Aβ(31–42). The parent peptides had previously been found to have low aqueous solubility.[19] Based on the results of the initial screening of these analogues, we changed direction and continued to systematically explore Aβ(31–42) derivatives containing single *N*-Me amino acid substitutions, and evaluated their toxicity and inhibitory activity in cell viability assays.

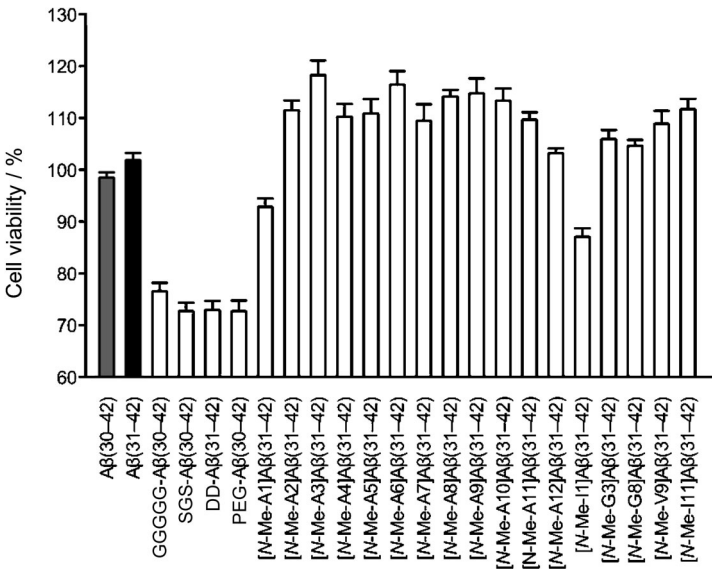

Figure 1. Effect of Aβ(30–42) and Aβ(31–42) derivatives on neuronal cells. Aβ(30–42) (■), Aβ(31–42) (■) and derivatives (□) at 50 μM were incubated with differentiated PC-12 cells for 24 h, and cell viability was measured using the MTT assay. The data are shown as the mean ± SEM of at least three independent experiments with six replicates per data point ($n \geq 18$).

Results and Discussion

Attachment of hydrophilic appendages to CTFs

Our initial approach was to attach different hydrophilic moieties to the N terminus of Aβ(30–42) or Aβ(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 Aβ(28–42), which contains an N-terminal lysine (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 in this direction and, instead, focused our efforts on a systematic study of N-methylated analogues of Aβ(31–42). We chose to focus on Aβ(31–42) because it was the strongest inhibitor of toxicity found in the original series.[10]

Table 1. N-Terminal modifications of Aβ(30–42) and Aβ(31–42).

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

N-Methyl-alanine scanning of Aβ(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 Aβ(31–42) is a hydrophobic peptide with limited aqueous solubility,[19] we suspected that analogues containing single A substitutions might be difficult to synthesize and purify, similar to the parent peptide,[21] and biophysical and biological evaluation of these peptides might be demanding. Therefore, we devised a two-step strategy, in which the first step achieves both a systematic structural study and an increase in aqueous solubility by substituting each residue by *N*-Me-A. 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 Aβ(31–42) analogues

We introduced *N*-Me-A in each position along the Aβ(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 nine out of the twelve *N*-Me-A-containing derivatives. However, using this general protocol, we did not obtain correct products for [*N*-Me-A8]Aβ(31–42), [*N*-Me-A11]Aβ(31–42) or [*N*-Me-A12]Aβ(31–42), necessitating special steps for these analogues. Synthesis of [*N*-Me-

Table 2. Sequences, masses, and IC_{50} values of Aβ(31–42) and derivatives.

Code	Sequence	Mass (calcd)	Mass (found)	Yield [%]	Solubility [μM]	IC_{50}[a,c] [μM]	IC_{50}[b,c] [μM]
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-IGLMVGGVVIA	1113.4	1113.5	13.8	8 ± 1	n.d.	n.d.
[*N*-Me-A2]Aβ(31–42)	I-(*N*-Me)A-GLMVGGVVIA	1113.4	1112.1	10.8	101 ± 18	n.d.	n.d.
[*N*-Me-A3]Aβ(31–42)	II-(*N*-Me)A-LMVGGVVIA	1169.5	1169.5	11.4	102 ± 16	18 ± 1	34 ± 3
[*N*-Me-A4]Aβ(31–42)	IIG-(*N*-Me)A-MVGGVVIA	1113.4	1113.6	9.9	127 ± 37	n.d.	n.d.
[*N*-Me-A5]Aβ(31–42)	IIGL-(*N*-Me)A-VGGVVIA	1096.4	1095.6	4.4	105 ± 20	n.d.	n.d.
[*N*-Me-A6]Aβ(31–42)	IIGLM-(*N*-Me)A-GGVVIA	1127.4	1127.5	6.9	87 ± 18	n.d.	n.d.
[*N*-Me-A7]Aβ(31–42)	IIGLMV-(*N*-Me)A-GVVIA	1170.4	1169.7	20.5	88 ± 14	n.d.	n.d.
[*N*-Me-A8]Aβ(31–42)	IIGLMVG-(*N*-Me)A-VVIA	1169.5	1169.8	5.7	41 ± 12	12 ± 1	14 ± 1
[*N*-Me-A9]Aβ(31–42)	IIGLMVGG-(*N*-Me)A-VIA	1127.4	1125.3	9.2	115 ± 21	6 ± 1	7 ± 1
[*N*-Me-A10]Aβ(31–42)	IIGLMVGGV-(*N*-Me)A-IA	1127.4	1127.1	7.4	121 ± 28	n.d.	n.d.
[*N*-Me-A11]Aβ(31–42)	IIGLMVGGVV-(*N*-Me)A-A	1113.4	1112.8	7.5	86 ± 22	10 ± 1	37 ± 3
[*N*-Me-A12]Aβ(31–42)	IIGLMVGGVVI-(*N*-Me)A	1155.5	1155.6	2.7	28 ± 13	n.d.	n.d.
[*N*-Me-I1]Aβ(31–42)	(*N*-Me)I-IGLMVGGVVIA	1155.5	1155	2.1	7 ± 1	n.d.	n.d.
[*N*-Me-G3]Aβ(31–42)	II-(*N*-Me)G-LMVGGVVIA	1155.5	1155.6	6.2	118 ± 11	26 ± 1	–[d]
[*N*-Me-G8]Aβ(31–42)	IIGLMVG-(*N*-Me)G-VVIA	1155.5	1155	1.3	18 ± 2	28 ± 1	71 ± 20
[*N*-Me-V9]Aβ(31–42)	IIGLMVGG-(*N*-Me)V-VIA	1155.5	1155	6.2	136 ± 18	6 ± 1	67 ± 8
[*N*-Me-I11]Aβ(31–42)	IIGLMVGGVV-(*N*-Me)I-A	1155.5	1155	3.5	132 ± 34	13 ± 1	49 ± 3

[a] IC_{50} values obtained from the MTT assay. [b] IC_{50} values obtained from the LDH assay. [c] At least three independent experiments with five replicates ($n \geq 15$) were performed. The results are the mean ± SEM. Not determined (n.d.). [d] No inhibition.

A8]Aβ(31–42) led to products containing deletions of G7 or of both G7 and *N*-Me-A8. These products likely resulted either from a low yield for the *N*-Me-A8 coupling to V9 or from formation of a diketopiperzaine (DKP) side product, which is a common problem when proline (P), glycine (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 reaction time to 1–2 min and reduced the cleavage reaction time to 1 hour. Using these modifications, we obtained [*N*-Me-A8]Aβ(31–42) successfully.

For [*N*-Me-A11]Aβ(31–42) and [*N*-Me-A12]Aβ(31–42), due to the proximity of the *N*-Me amino acid to C-terminal carboxyl group, DKP formation was predicted to occur easily during the synthesis on NovaSyn TGA resin (an alcohol-based solid support).[23] To avoid this side reaction, we used the highly hindered chlorotrityl (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 reaction times, resulting in the successful synthesis of these two analogues.

Solubility of *N*-Me-A-substituted Aβ(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 μM 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 *N*-Me-A-substituted analogues of Aβ(31–42) had increased solubility relative to that of Aβ(31–42)—25 ± 4 μM, except for [*N*-Me-A1]Aβ(31–42), whose solubility was 8 ± 1 μM. The low solubility of [*N*-Me-A1]Aβ(31–42) can be explained by the increase in hydrophobicity, similar to all other analogues, but without disruption of the β-hairpin structure of Aβ(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]Aβ(31–42), this is likely due to a similar reason as in the case of [*N*-Me-A1]Aβ(31–42), i.e., the *N*-Me group is too far from the β-strands to disrupt their association. According to the structure of Aβ(31–42), calculated based on ion mobility mass spectrometry data, position 8 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 three- to five-fold, suggesting effective disruption of the β-hairpin structure.

Inhibition of Aβ42-induced neurotoxicity by *N*-Me-A-substituted Aβ(31–42) analogues

As an initial step before testing inhibitory activity, we checked whether the Aβ(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]Aβ(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 (Figure 1).[25]

Next, we screened the N-methylated Aβ(31–42) derivatives for inhibition of Aβ42-induced neurotoxicity in single-dose experiments. Differentiated PC-12 cells were incubated with Aβ42 (5 μM) for 24 h in the absence or presence of a tenfold excess of each derivative, and cell viability was assessed using the MTT assay (Figure 2). [*N*-Me-A3]Aβ(31–42), [*N*-Me-A8]Aβ(31–42), [*N*-Me-A9]Aβ(31–42), and [*N*-Me-A11]Aβ(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.

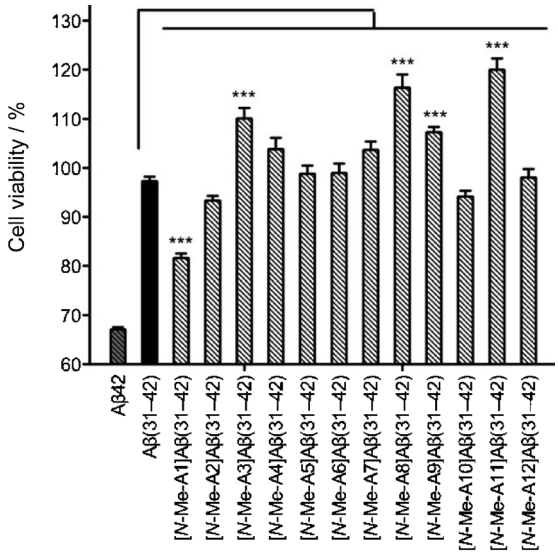

Figure 2. Inhibitory activity of *N*-Me-A-substituted Aβ(31–42) analogues. Aβ42 (5 μM, ■), and mixtures of Aβ42:Aβ(31–42) (■) and derivatives (□ striped) at a 1:10 concentration ratio were incubated with differentiated PC-12 cells for 24 h, and cell viability was measured using the MTT assay. The data are shown as the mean ± SEM of at least three independent experiments with six replicates per data point ($n \geq 18$). Statistical significance was calculated and compared with Aβ(31–42) using ANOVA followed by Dennett's multiple-comparison tests (*** $p < 0.001$).

To further evaluate the analogues, which were found to significantly increase inhibitory activity in the initial screen, we examined each peptide in both the MTT and LDH dose–response assays (Figure 3a and 3b, respectively). 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 Aβ(31–42) analogues yielded dose-dependent inhibition of Aβ42-induced toxicity (Figure 3). The IC_{50} values obtained are summarized in Table 2. [*N*-Me-A3]Aβ(31–42) had similar inhibitory activity to Aβ(31–42), whereas the other three derivatives showed increased inhibitory activity. [*N*-Me-A9]Aβ(31–42) was the most potent analogue, yielding protection from Aβ42-induced toxicity with an IC_{50} value of 6 ± 1 μM in the MTT assay (threefold improvement relative to the parent peptide) and 7 ± 1 μM in the LDH assay (sixfold 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*-Me version of these residues (Table 2). These included analogues substituted both at the four positions that yielded a significant increase in inhibitory activity (positions 3, 8, 9, and 11) and in the single position that caused increased toxicity (position 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]Aβ(31–42), [*N*-Me-G8]Aβ(31–42), [*N*-Me-V9]Aβ(31–42), and [*N*-Me-I11]Aβ(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 for the difficult synthesis of *N*-Me-A analogues described above were also necessary for the successful synthesis of these three analogues. In contrast, [*N*-Me-G3]Aβ(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 peptides with high solubility (> 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]Aβ(31–42) was toxic, similarly to [*N*-Me-A1]Aβ(31–42) (Figure 1).

Interestingly, dose–response evaluation of these analogues by the MTT (Figure 3c) and LDH (Figure 3d) assays 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]Aβ(31–42) showed 2.3- and 5.0-fold decrease in activity relative to [*N*-Me-A8]Aβ(31–42), in the MTT and LDH assays, respectively, whereas [*N*-Me-G3]Aβ(31–42) and [*N*-Me-I11]Aβ(31–42) showed a smaller decrease in activity of 1.4- and 1.3-fold, respectively, in the MTT assay. For [*N*-Me-I11]Aβ(31–42), a similar result was obtained in the LDH assay, whereas [*N*-Me-G3]Aβ(31–42) showed no inhibition in LDH assay. [*N*-Me-V9]Aβ(31–42) exhibited a similar activity to [*N*-Me-A9]Aβ(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*-Me amino acid substitution strategy allowed a systematic SAR study of Aβ(31–42) analogues as inhib-

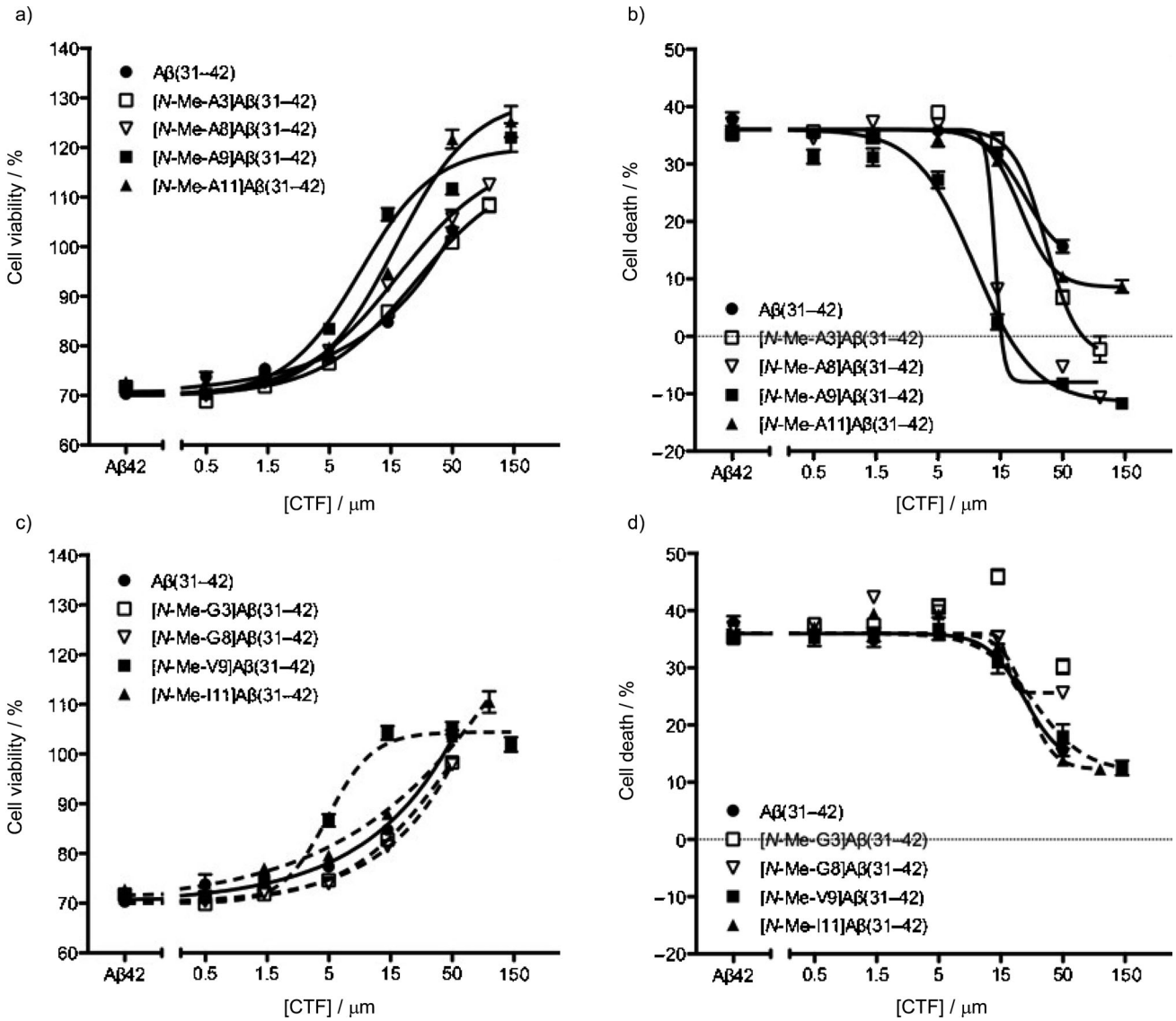

Figure 3. Inhibition of Aβ42-induced toxicity by Aβ(31–42) C-terminal fragments (CTF). Aβ42 (5 μM) in the absence or presence of Aβ(31–42) derivatives in 1:0.1, 1:0.3, 1:1, 1:3, and 1:10 (and in some cases 1:20 and 1:30) concentration ratios were added to differentiated PC-12 cells. a) and c) Cell viability was determined using the MTT assay. b) and d) Cell death was measured using the LDH assay. The data are shown as the mean ± SEM and are representative of at least three independent experiments with five replicates per data point ($n \geq 15$). Dose–response curves were obtained by sigmoidal fitting (variable slope) using Prism 5.0c (GraphPad, La Jolla, CA, USA).

itors of Aβ42-induced toxicity. [*N*-Me-I1]Aβ(31–42) was found to be as toxic as [*N*-Me-A1]Aβ(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.

A summary of the SAR findings

1. The activity of Aβ(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 NH_2 group was important for maintaining the nontoxic nature of Aβ(31–42) whereas an N-terminal $NH(CH_3)$ group induced toxicity.
3. An *N*-Me-A substitution at positions 3 and 8 increased inhibitory activity of these peptides relative to the analogous *N*-Me-G peptides.
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 peptides with better inhibition than those with 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 to reflect more efficient binding to Aβ42 and/or disruption of particular toxic structures.

Due to the increased solubility of most of the N-methylated analogues, their synthesis was more facile relative to that of the parent peptide. Though in some cases protocol modifications were needed to overcome DKP formation, the changes 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 to predict which residue would be difficult to add as an *N*-Me amino acid. Previously, it was reported that bulky, hydrophobic N-methylated amino acids might pose challenges in coupling to a growing peptide, and 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*-Me amino acids. The replacement of an amide proton by a methyl group could 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 could disrupt association of both intramolecular and intermolecular β-strands. In the case of Aβ(31–42), if disruption occurs 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 can 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 Aβ42 that promote formation of a nontoxic assembly. The data suggest that these predictions were correct and imply that inhibition is achieved through specific interaction between particular analogues and Aβ42.

Conclusions

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

Experimental Section

Reagents: 9-Fluorenylmethoxycarbonyl (FMOC)-protected amino acids, Fmoc-A-NovaSyn TGA resin (0.22 mmole g^{-1}), H-A-2-Cl-Trt resin (0.77 mmole g^{-1}) and 2-Cl-(Trt)-Cl resin (1.3 mmole g^{-1}) were purchased from Novabiochem (Gibbstown, NJ, USA). Wang resin and all other reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA) and were of the highest purity available. All commercially available solvents and reagents were used without further purification. High-purity water (18.2 MΩ) was obtained using a Milli-Q system (Millipore, Bedford, MA, USA).

Peptide synthesis: Synthesis, purification, and characterization of Aβ42 were carried out as described previously.[27] Peptides were purified using reverse-phase high-performance liquid chromatography (RP-HPLC), and characterized by mass spectrometry (MS) and amino acid analysis (AAA).

General protocol for synthesis of N-methylated Aβ(31–42) derivatives: Aβ(31–42) and derivatives were synthesized using a Discover microwave-assisted synthesis system (CEM, Matthews, NC, USA). 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 20% piperidine (or 4-methylpiperidine) in DMF (5 mL) for 20 min at RT. After washing with DMF (3×3 mL), a mixed solution of Fmoc-AA-OH (0.3 mmol), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 0.3 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 0.6 mmol) in DMF (4 mL) was added to the reaction vessel. Fmoc-*N*-Me-A-OH and the following amino acid were coupled using 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as an 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, USA) was applied to detect remaining free amino

groups. The coupling reaction efficacy 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 (3×3 mL) and then with CH_2Cl_2, dried under vacuum, and the peptide was cleaved using a 95:2.5:2.5 (*v*/*v*/*v*) mixture of trifluoroacetic acid (TFA)/1,2-ethanedithiol/H_2O for 1.5 h at RT. The cleavage solution was collected, and its volume was reduced to 1–2 mL using a gentle stream of high-purity N_2. Peptides were precipitated by addition of cold Et_2O, purified by RP-HPLC, and characterized by MS and AAA. The purity of all peptides was higher than 95%, as determined by analytical RP-HPLC. The peptide sequences, calculated masses, and observed masses are listed in Table 2.

Synthetic procedure for [*N*-Me-A12]Aβ(31–42): 2-Cl-(Trt)-Cl resin (1.3 mmol g^{-1},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 DMF (3 mL). The mixture was shaken for 1 h at RT. Capping of excess reactive groups on the resin was achieved using a 17:2:1 (*v*/*v*/*v*) mixture of CH_2Cl_2/MeOH/DIPEA. The resin was washed with CH_2Cl_2 (3×3 mL), DMF (3×3 mL), and CH_2Cl_2 (3×3 mL). 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 an activation reagent and with double coupling. The general protocols described above were followed for coupling and deprotection of other amino acids, and cleavage from the resin.

Synthetic procedure for [*N*-Me-I11]Aβ(31–42): H-A-2-Cl-Trt resin (0.77 mmol g^{-1}, 0.3 g) was swollen in DMF and filtered. Fmoc-*N*-Me-I-OH and Fmoc-V-OH were coupled with HATU as an activation reagent and with double coupling. The general protocols described above were followed for coupling and deprotection of other amino acids. The peptide was cleaved from the resin using a 92:6:2 (*v*/*v*/*v*) mixture of TFA/thioanisole/triisopropylsiliane for 1 h. The solution was collected, and its volume reduced to 1–2 mL using a gentle stream of high-purity N_2. The peptide was precipitated by addition of cold Et_2O and collected by centrifugation. After the Et_2O was removed, the peptide was neutralized with DIPEA and washed with cold Et_2O (2×10 mL). The crude peptide was immediately dissolved in H_2O, frozen, lyophilized, and then purified using RP-HPLC. The fractions containing the pure peptide were frozen immediately and re-lyophilized.

Solubility: A solubility study was carried out 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 NaH_2PO_4/Na_2HPO_4 (pH 7.4) to a nominal concentration of 200 μM. 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, USA). Three to five replicates were measured for each peptide. The actual peptide concentrations were determined by AAA and the results are presented as the mean ± standard error of the mean (SEM).

Cell viability assays: The methods for evaluation of the biological activity of the CTFs themselves and their inhibition of Aβ42-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^{-1}) for 48 h. The cells then were incubated with solutions of Aβ42 alone at 5 μM, Aβ(31–42) analogues alone at 50 μM, or Aβ42/Aβ(31–42) analogue mixtures at a concentration ratio of 1:10, for 24 h. For initial screening of the new analogues, cell viability was determined by the MTT assay using a CellTiter 96 kit (Promega, Fitchburg, WI, USA). Negative controls included NaOH at the same concentration as in the peptide solutions and media alone. A positive control was 1 μM 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, Aβ42 alone and Aβ42:Aβ(31–42) analogue mixtures at concentration ratios of 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) were used. Cell viability was measured using both the MTT assay and the LDH-release assay (CytoTox-ONE Homogenous Membrane Integrity Assay kit, Promega, Fitchburg, WI, USA). At least three independent experiments with five replicates ($n \geq 15$) were performed. The results were averaged and presented as the mean ± SEM.

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