Amyloid Inhibitors Hot Paper

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Rational Design and Identification of a Non-Peptidic Aggregation Inhibitor of Amyloid-β Based on a Pharmacophore Motif Obtained from *cyclo*[-Lys-Leu-Val-Phe-Phe-]**

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Abstract: *Inhibition of pathogenic protein aggregation may be an important and straightforward therapeutic strategy for curing amyloid diseases. Small-molecule aggregation inhibitors of Alzheimer's amyloid-β (Aβ) are extremely scarce, however, and are mainly restricted to dye- and polyphenol-type compounds that lack drug-likeness. Based on the structure-activity relationship of cyclic Aβ16–20 (cyclo-[KLVFF]), we identified unique pharmacophore motifs comprising side-chains of Leu², Val³, Phe⁴, and Phe⁵ residues without involvement of the backbone amide bonds to inhibit Aβ aggregation. This finding allowed us to design non-peptidic, small-molecule aggregation inhibitors that possess potent activity. These molecules are the first successful non-peptidic, small-molecule aggregation inhibitors of amyloids based on rational molecular design.*

Protein aggregation is intimately related to several human diseases, including currently intractable neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease. More than 20 proteins have been identified to aggregate into so-called amyloid fibrils containing extensive β-sheet structures, and species generated in the aggregation processes (that is, oligomers, protofibrils, and fibrils) contribute to disease development.[1] Specifically, AD is an age-related neurodegenerative disorder, and affected individuals exhibit progressive memory loss and cognitive impairment.[2] Thirty million people worldwide are estimated to have the disease, and the number is predicted to increase to 106 million by 2050.[3] Although the precise etiology of AD remains unclear, the aggregation of 40- and 42-residue amyloid-β peptides (designated Aβ1–40 and Aβ1–42, respectively), produced by proteolytic processing of amyloid precursor protein, is critically involved in AD.[2] Both oligomers[4] and fibrils[5] of Aβ are neurotoxic, and Aβ1–42 is far more aggregative and toxic than Aβ1–40.[6]

Aggregation inhibitors of Aβ are therefore candidate drugs for the treatment (or prevention) of AD.[7] To date, a number of natural products possessing inhibitory activities against Aβ aggregation have been identified.[8] Most of those compounds are dye- or polyphenol-derivatives and, because these compounds bind non-selectively to a wide range of biomolecules, there is high potential for various side-effects. Structural optimization to enhance Aβ specificity is very difficult, however, owing to both the unknown three-dimensional structures of Aβ aggregates (oligomers and protofibrils) and the lability of Aβ conformation during aggregation.

Fragment peptides of Aβ bind to full-length Aβ and inhibit its aggregation.[9–12] An Aβ sequence-based approach will be advantageous in logical potentiation of aggregation inhibitors. KLVFF peptide (**1**) corresponding to the Aβ16–20 fragment, a region that plays a critical role in generating Aβ fibrils by forming a core β-strand structure,[13] was intensively derivatized.[14–20] Use of this approach led to the identification of an analogue of **1**, D-[chGly-Tyr-chGly-chGly-mLeu]-NH_2 (ch = cyclohexyl, m = *N*-methyl), that is more than 30 times more potent than **1**.[20] To date, however, all of the reported analogues resulting from this approach are peptidic molecules, and thus have inherent disadvantages, such as poor bioavailability and a high propensity to aggregate into deposits. To the best of our knowledge, no non-peptidic, small-molecule aggregation inhibitors of amyloids have yet been rationally designed from the lead peptide. Here, we report the first non-peptidic small molecules with potent Aβ aggregation inhibitory activities based on rational derivatization.

To develop non-peptidic inhibitors, the contribution of backbone amide moieties to Aβ binding affinity must be reduced relative to that of the side-chain functional groups. We first synthesized **3** (Figure 1), a cyclic analogue of **1**, to

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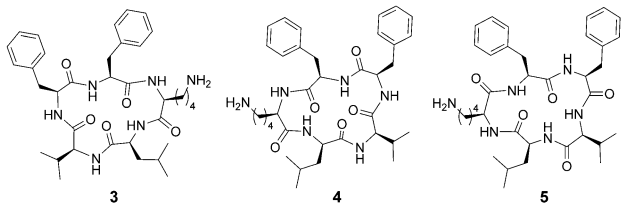

Figure 1. Structures of **3**, **4**, and **5**.

facilitate the identification of pharmacophores by stabilizing the active conformation.[21] Aggregation inhibitory activities were evaluated using a thioflavin-T (ThT) dye assay whose fluorescence intensity increases according to the extent of Aβ aggregation of β-sheet structures (Table 1).[22] The aggregation inhibitory activity of a head-to-tail cyclic peptide **3** (designated *cyclo*-[KLVFF]) was significantly higher than that of **1**. Namely, when **3** was co-incubated with Aβ1–42 in a 3:1 ratio at pH 7.4 and 37 °C for 3 h, the fluorescence intensity was 53 % compared to the control without inhibitor (100 %), whereas **1** decreased the intensity only slightly to 97 % under the identical experimental conditions. Inhibition by **3** was maintained at least for 12 h, and was concentration-dependent (Supporting Information, Figure S1). Furthermore, the greater activity of **3** compared to **1** to inhibit Aβ fibril formation was also confirmed using atomic force microscopy (Supporting Information, Figure S2).

Table 1: Structure–activity relationship studies of cyclic KLVFF.

Compound	Structure	ThT fluorescence intensity [%][a]
1	KLVFF	97
2	klvff (= D-[KLVFF])	69[b]
3	*cyclo*-[KLVFF]	53[b]
4	*cyclo*-[klvff]	50[c]
5	*cyclo*-[FFVLK]	49
6	FFVLK	99[d]
7	*cyclo*-[alvff]	51
8	*cyclo*-[kavff]	72
9	*cyclo*-[klaff]	75
10	*cyclo*-[klvaf]	99
11	*cyclo*-[klvfa]	97
12	*cyclo*-[Klvff]	75
13	*cyclo*-[kLvff]	99
14	*cyclo*-[klVff]	82
15	*cyclo*-[klvFf]	88
16	*cyclo*-[klvfF]	88

[a] Relative fluorescence intensity of Aβ1–42 (10 μM) + inhibitor (30 μM) vs. Aβ1–42 (10 μM) alone incubated for 3 h. All data shown are mean values of at least three independent experiments. [b] $p < 0.01$ vs. **1**, [c] $p < 0.05$ vs. **2**, [d] $p < 0.01$ vs. **2** (Tukey's multiple comparisons among **1**–**6**).

Interestingly, the inhibitory activity of the enantiomer of **3**, *cyclo*-D-[KLVFF] (designated *cyclo*-[klvff], **4**, Figure 1) was similar to that of **3** (50 %; Table 1; Supporting Information, Figure S3a). This finding is in sharp contrast to the results observed for the corresponding linear peptides **1** and **2**. Side-chains of the Leu[2], Val[3], Phe[4], and Phe[5] residues could be overlaid between **3** and **4** by reversing the backbone amide bonds (Supporting Information, Figure S4). Therefore, these side-chains likely substantially contribute to the activity of the cyclic peptides. Indeed, a retro-inverso derivative (that is, chirality of Cα and the order of the amino acid sequence are reversed)[23] of **4**, *cyclo*-[FFVLK] (**5**, Figure 1), completely retained the inhibitory activity (Table 1; Supporting Information, Figure S3). All five side-chains of **4** can be overlaid with those of **5** despite reversing the direction of the amide bonds. This finding strongly suggests that the side-chains, rather than the backbone amide functionalities, have a critical role in the inhibitory activity of **4**. In contrast, a retro-inverso peptide of linear **2**, L-[FFVLK] (**6**), exhibited no inhibitory activity, suggesting that the backbone amide bonds of **2** predominantly contribute to the activity in the case of linear peptide inhibitors.

Having identified unique properties of cyclic peptide **4**, we next examined the effect of the side-chain of each amino acid residue on Aβ aggregation inhibitory activity by conducting an Ala-scan (**7**–**11**, Table 1). The inhibitory activity of **7** (Lys[1]→Ala) was fully retained, suggesting that the side-chain of Lys[1] in **4** has no significant effect on the activity. In contrast, the inhibitory activity of **10** (Phe[4]→Ala) and **11** (Phe[5]→Ala) was completely lost, highlighting the importance of the two phenyl groups. The side-chains of Leu[2] and Val[3] moderately contributed to the activity of **4**, based on the results of **8** (Leu[2]→Ala) and **9** (Val[3]→Ala). Moreover, substitution of the D-Leu[2], D-Val[3], D-Phe[4], and D-Phe[5] of **4** with the corresponding L-amino acids significantly decreased the inhibitory activity (**13**–**16**, Table 1), whereas moderate activity (75 %) was observed following substitution of the D-Lys[1] of **4** with L-Lys[1] (**12**). Overall, these studies allowed us to identify a pharmacophore that comprises the side-chains of Leu[2], Val[3], Phe[4], and Phe[5] residues rather than the backbone amide functionalities, to exert the inhibitory activity of cyclic peptides **3** and **4**.

Based on the above findings, we designed a non-peptidic small molecule inhibitor **17** (Figure 2a). Compound **17** contains an isopentyl carboxamide (Leu[2] side-chain mimic), a benzyl (Phe side-chain mimic), and a phenoxy (Phe side-chain mimic) groups arranged at the 2, 4, and 6-positions of a pyridine ring core, respectively.[24] To avoid structural complexity of the molecule, we adopted three of the four pharmacophore moieties. Molecular modeling studies suggest that the isopentyl group and the two phenyl groups of **17** can be overlaid to those of **4** (Figure 2b; Supporting Information, Figure S5).

Although the inhibitory activity of **17** was slightly lower than that of the corresponding cyclic peptide **9** (Val[3]→Ala), **17** exhibited dose-dependent inhibitory activity of Aβ aggregation in the ThT fluorescence assay (Figure 2c and Table 2, ThT intensity of **9**: 58 %; **17**: 71 %, at Aβ: inhibitor = 10 μM: 50 μM). The activity of **17** was maintained at least for 12 h (Supporting Information, Figure S6).[25] When any of the three functional moieties of **17** was removed, the activity was significantly reduced (Supporting Information, Figure S7). The concentration-dependent activity of **17** was further confirmed using the sedimentation assay, in which quantities of Aβ1–42 in the supernatants after centrifugal separations

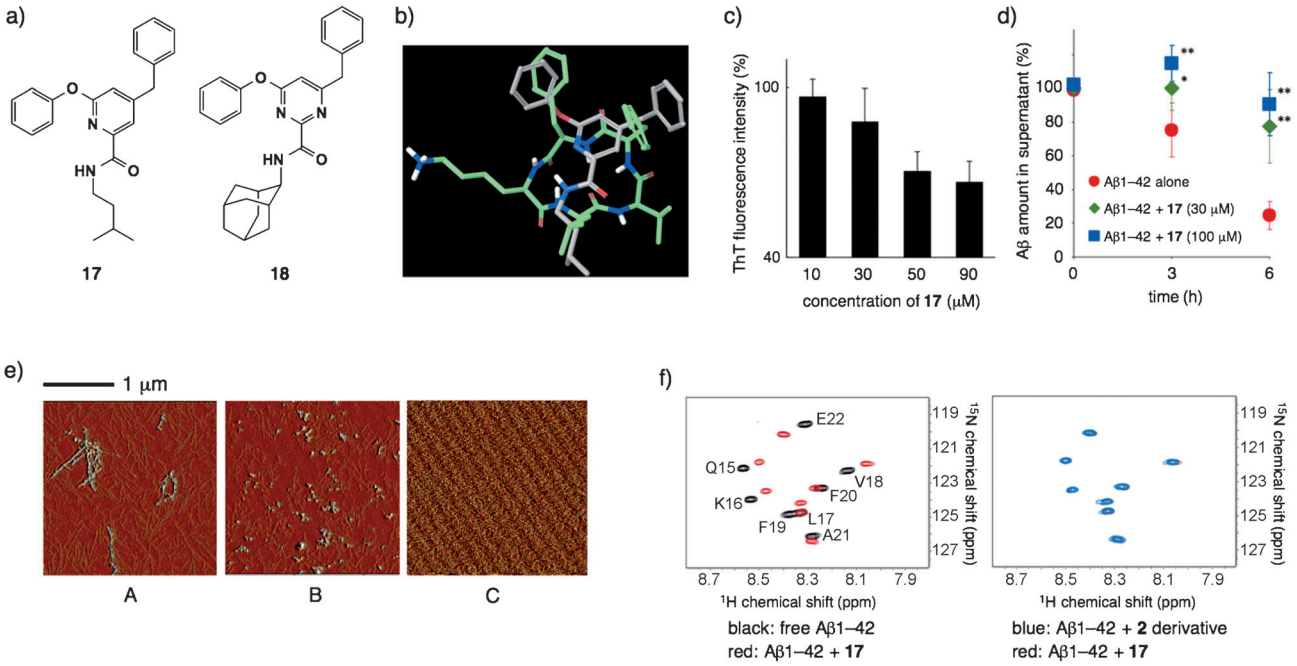

Figure 2. a) Chemical structures of **17** and **18**. b) Overlaid structures of **4** (green) and **17** (gray). c) Relative ThT fluorescence intensity to that of Aβ1–42 alone (10 μM) incubated in the presence of **17** for 3 h ($n=3$, mean ± S.D.). d) Sedimentation assay (red: Aβ1–42 alone (10 μM), green: Aβ1–42 + **17** (10 μM + 30 μM), blue: Aβ1–42 + **17** (10 μM + 100 μM)), Mean ± S.D., $n=3$–5, $^{**}p<0.01$ vs. Aβ1–42 alone, $^{*}p<0.05$ vs. Aβ1–42 alone (Student's *t*-test). e) Atomic force microscopy images of Aβ1–42 alone (10 μM, A), Aβ1–42 + **17** (10 μM each, B), and Aβ1–42 + **17** (10 μM + 30 μM, C). Incubation time = 6 h; f) Overlaid two-dimensional ^{15}N–^{1}H heteronuclear single quantum coherence spectra for the labeled Aβ1–42 and derivatives. Left panel shows spectra for Aβ1–42 alone and with **17**. Right panel shows spectra for Aβ1–42 with **17** and Aβ1–42 with a derivative of **2**.[27]

Table 2: Activities of **4**, **9**, **17**, and **18**.

Compound	ThT fluorescence intensity [%][a]
4	39 ± 1.4
9	58 ± 5.9
17	71 ± 9.4
18	47 ± 4.6[b]

[a] Relative fluorescence intensity to that of Aβ1–42 alone (10 μM) incubated in the presence of each inhibitor (50 μM) for 3 h. Mean ± S.D. values, $n=3$. [b] $p<0.01$ vs **17** (Student's *t*-test).

increased depending on the amount of **17** (Figure 2d). Moreover, an experiment using SDS-PAGE suggested that **17** attenuated the formation of large oligomers of Aβ1–42 (Supporting Information, Figure S8); the intensity of a large oligomer-derived band (a broad smear between 100 and 200 kDa) decreased by the presence of **17**. Atomic force microscopy analysis also indicated that the formation of large oligomers and fibrils of Aβ1–42 was diminished in proportion to the amount of **17** present (Figure 2e). Thus, various evaluations clearly support the notion that non-peptidic small molecule **17** attenuated the aggregation of Aβ1–42.

To gain insight into the interaction site of **17** with Aβ1–42, 2D NMR ^{15}N–^{1}H heteronuclear single quantum coherence of the Aβ1–42 sample (15–22 residues labeled with ^{15}N) was measured (Figure 2f). The N–H cross-peaks, which are highly sensitive to perturbations by the nearby environment, in the 15–22 residues of Aβ1–42 markedly shifted in the presence of **17** (Figure 2f, left panel). Thus, the local conformation of the backbone N–H bonds in the region of Lys^{16}-Leu^{17}-Val^{18}-Phe^{19}-Phe^{20} was changed by binding with **17**. In the presence of a derivative of the original linear peptidic inhibitor D-KLVFF (**2**), the shift patterns of the N–H cross peaks were very similar to those in the presence of **17** (Figure 2f, right panel). Therefore, **17** appears to interact with the same region of Aβ1–42 as **2**.

Finally, we demonstrated that **17** could be used as a lead compound for developing more-potent small-molecule aggregation inhibitors. Based on the structure optimization studies of **17**, we identified **18** (pyridine → pyrimidine and isopentyl → 2-adamantyl) with increased inhibitory activity to the extent higher than that of the original control peptide **9** (Table 2; ThT intensity of **18**: 47%; Supporting Information, Figure S6). This result is promising for further intensive structural optimization.

In conclusion, we identified pharmacophore motifs for Aβ aggregation inhibitors based on structure–activity relationship studies using *cyclo*-[KLVFF] (**3**) as a lead molecule. The identified pharmacophores include the side-chains of Leu^{2}, Val^{3}, Phe^{4}, and Phe^{5} residues, but not the backbone amide functionalities. Based on this information, we developed non-peptidic small-molecule aggregation inhibitors **17** and **18** possessing significant activity comparable to corresponding cyclic peptides **3**, **4**, and **9**. To the best of our knowledge, this is the first rational design of non-peptidic, small-molecule aggregation inhibitors of amyloids starting from peptidic

inhibitors. Further optimization of aggregation inhibitors with more potent activity and preferred drug-likeness is ongoing in our group.

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