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Branched KLVFF Tetramers Strongly Potentiate Inhibition of β-Amyloid Aggregation

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The key pathogenic event in the onset of Alzheimer's disease (AD) is the aggregation of β-amyloid (Aβ) peptides into toxic aggregates. Molecules that interfere with this process might act as therapeutic agents for the treatment of AD. The amino acid residues 16–20 (KLVFF) are known to be essential for the aggregation of Aβ. In this study, we have used a first-generation dendrimer as a scaffold for the multivalent display of the KLVFF peptide. The effect of four KLVFF peptides attached to the dendrimer (K_4) on Aβ aggregation was compared to the effect of monomeric KLVFF (K_1). Our data show that K_4 very effectively inhibits the aggregation of low-molecular-weight and protofibrillar $Aβ_{1–42}$ into fibrils, in a concentration-dependent manner, and much more potently than K_1. Moreover, we show that K_4 can lead to the disassembly of existing aggregates. Our data lead us to propose that conjugates that bear multiple copies of KLVFF might be useful as therapeutic agents for the treatment of Alzheimer's disease.

Introduction

One of the most prominent neuropathological hallmarks in the brains of Alzheimer's disease (AD) patients is the deposition of senile plaques. These plaques are extracellular lesions that are primarily composed of β-amyloid (Aβ). Aβ is a 40- or 42-amino-acid peptide that is derived from the amyloid precursor protein (APP) by proteolytic processing. Genetic and neuropathological studies provide strong evidence for a central role of Aβ in the pathogenesis of AD.[1–4]

Aβ is a highly fibrillogenic peptide that is prone to aggregate into 6 to 10 nm diameter fibrils with the characteristic cross-β structure.[5–7] Initial studies have shown that the aggregation of Aβ into fibrils is a prerequisite for its toxicity.[8–10] Later studies demonstrated the neurotoxicity of different prefibrillar intermediates in the Aβ aggregation pathway.[11–17] These different aggregation states might mediate different toxic effects at different stages of the disease.[18] Protofibrils (PF)[19] for example, are intermediates on the pathway towards mature fibrils. PF are short (10–200 nm) soluble curvilinear structures that have several characteristics of mature fibrils (high β-sheet content, and a fibril-like structure). Like fibrils, PFs have also been shown to induce neurotoxicity in culture, and acute electrophysiological changes.[11] The exact nature of the Aβ species that causes toxicity and eventually neurodegeneration in vivo is not known, but these studies do indicate that Aβ must be in an aggregated state to be toxic. This suggests that the prevention of Aβ aggregation might inhibit Aβ neurotoxicity.

Previous studies on the aggregation process of Aβ identified the critical region that is involved in amyloid fibril formation.[20,21] The hydrophobic core at residues 16–20 of Aβ, KLVFF, are crucial for the formation of β-sheet structures by Aβ. It was also shown that KLVFF binds to the homologous sequence in Aβ and prevents its aggregation into amyloid fibrils.[22,23] This KLVFF sequence has served as a lead compound for the development of inhibitory agents that are aimed at preventing Aβ aggregation in vivo.[24,25] KLVFF has also been shown to reduce amyloid deposits in vivo.[26]

The use of multiple simultaneous interactions to enhance the affinity and specificity of binding, known as multivalency, is an important concept in biology. In the past, different types of scaffold molecules have been used to obtain multivalent structures, such as colloidal gold particles[13] and poly(ethylene glycol) (PEG).[25] A disadvantage of these approaches is the large size of the particles (PEG) and the lack of control over

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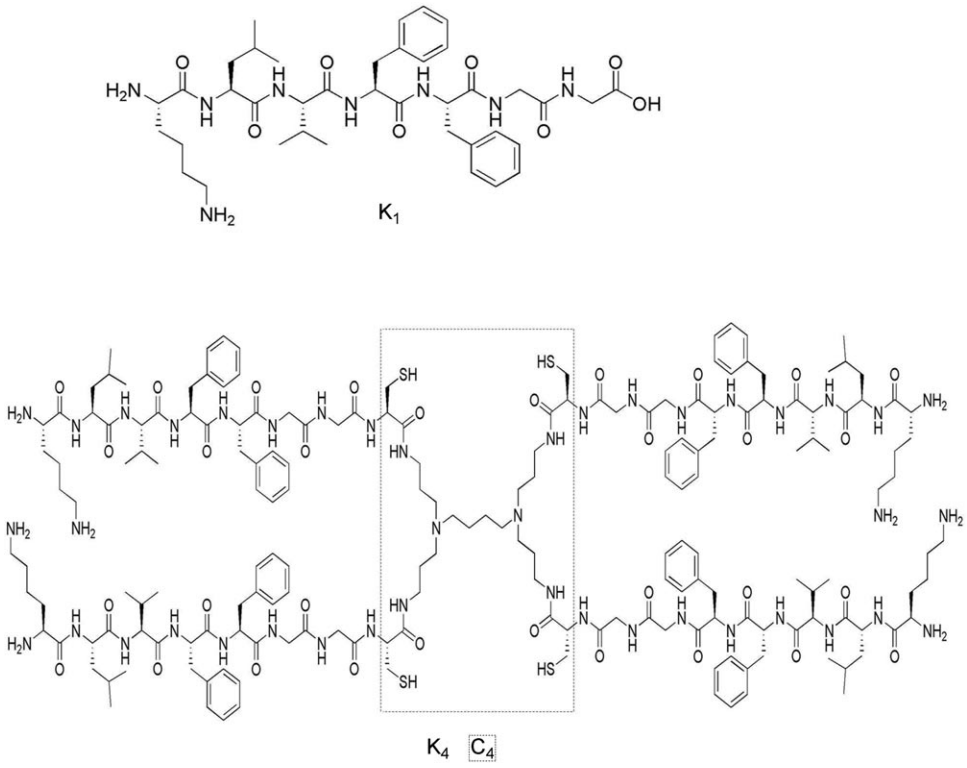
the number of peptides attached (colloidal gold). These disadvantages are circumvented when using dendrimers. Dendrimers are multivalent macromolecules with regular, highly branched structures and dimensions that resemble those of small proteins; they have been used in several different biological applications.[27] In this study we have synthesized a first-generation KLVFF dendrimer by using native chemical ligation,[28] thereby generating a small, well-defined molecule that is capable of making multivalent interactions. We have investigated the effect of dendritic KLVFF on the aggregation of low molecular weight (LMW) $A\beta_{1-42}$ as well as its effects on preformed aggregates.

Our data show that the coupling of KLVFF to a dendrimeric scaffold potentiates its inhibitory effect on $A\beta_{1-42}$ aggregation as well as on the disassembly of pre-existing aggregates. Thus, our studies indicate that a dendrimer that contains multiple copies of KLVFF might have therapeutic potential in Alzheimer's disease.

Results

Synthesis and characterization of fibrillogenesis inhibitor compounds

The peptide–dendrimer complexes in this study were synthesized by using native chemical ligation. Four equivalents of the MPAL thioester of KLVFFGG (K_1) were ligated to one equivalent of the cysteine dendrimer C_4 (see the dotted box in Scheme 1); this resulted in the formation of the $[KLVFFFGG]_4$ dendrimer (K_4). The chemical structures of K_1 and K_4 are shown in Scheme 1. The identity and purity of the peptides were verified by MALDI-TOF (see Figure S1)



Scheme 1. Chemical structures of the interfering peptides. Chemical structures of monomeric KLVFFGG (K_1) and dendritic KLVFFGG (K_4). The dotted box shows the structure of the cystein dendrimer (C_4).

Aggregation properties of the KLVFF compounds

Before assessing the performance of K_1 and K_4 as inhbitors of $A\beta_{1-42}$ aggregation, we first studied the possible self-aggregation of these KLVFF peptides. Neither K_1 nor K_4 showed Thioflavin T (ThT) binding after incubation for 24 h at 37 °C. (Figure 1 A). Increasing the aggregation time of either compound up to 5 days did not lead to an increase in ThT fluorescence (not shown). Morphological analysis by AFM showed the absence of fibrillar aggregates in the K_4 sample; this confirms the results from the ThT assay (Figure 1 B). Only globular structures, with a diameter of 20–80 nm and height of 10–15 nm were observed after aggregation at 37 °C for 24 h (Figure 1 B). These data indicate that the dendritic KLVFF compound does not form fibrils or other structures with high β-sheet content.

Dendritic KLVFF is a more potent inhibitor of LMW Aβ aggregation than monomeric KLVFF

LMW Aβ was prepared as described in the Experimental Section. Electron microscopy (EM) analysis showed, as expected for LMW Aβ, only very few oligomer-like structures at $t = 0$ (Figure 2 B, upper left). LMW $A\beta_{1-42}$ was aggregated for 24 h in the presence of K_1 or K_4 in different ratios. In order to directly compare the effects of K_1 and K_4 on Aβ aggregation, the ratios are expressed per mole of KLVFF peptide subunit. ThT analysis showed that K_4 inhibits $A\beta_{1-42}$ aggregation in a concentration-dependent manner; the strongest inhibition occurred at the highest concentration used (Figure 2 A). A 1:4 ratio of Aβ/K (in K_4) inhibits the relative change of ThT fluorescence (Rel.ΔThT) by more than 90%, whereas the same ratio of K_1 inhibits Rel.ΔThT by only 25%. Moreover, a much lower ratio of 1:0.2 for K_4 also resulted in an inhibition of 25%; this indicates that K_4 inhibits $A\beta_{1-42}$ aggregation to the same extent as K_1 at a 20-fold lower concentration of the peptide subunit (and at 80-fold lower concentration per conjugate molecule). K_4 is thus a much more potent inhibitor of $A\beta_{1-42}$ aggregation than K_1. Half-maximal inhibition of LMW Aβ aggregation in assays that were performed both at 20 μM and 10 μM occurs at a comparable

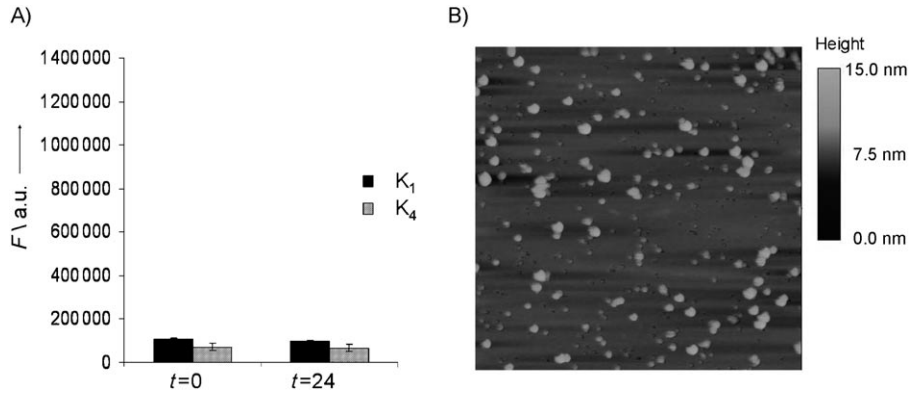


Figure 1. KLVFF derivatives do not self aggregate. A) KLVFF derivatives were aggregated for 24 h at 37 °C and the β-sheet content was measured by using a ThT assay. Shown is a representative graph of a ThT assay of the K_1/K_4 peptides aggregated at $t=0$ and 24 h. B) The morphology of the novel dendritic KLVFF was analyzed by AFM. A representative AFM micrograph (image size is 2000×2000 nm) at $t=24$ h is shown.

Aβ/K (K_4) ratio of approximately 1:0.5, which corresponds to a concentration of 10 μM Aβ and 1.25 μM K_4.

EM analysis was performed to study the morphology of the aggregates that form in the presence and absence of K_1 and K_4. Aggregation of LMW $A\beta_{1-42}$ in the absence of inhibitors showed the formation of fibrillar structures (Figure 2B, upper right). When LMW $A\beta_{1-42}$ aggregation is strongly inhibited (>90%) by K_4 (Aβ/K = 1:4), only small nonfibrillar structures are detected (Figure 2B, lower right), but when the same ratio for K_1, which only results in 25% aggregation inhibition, was used, fibrillar structures were still observed (Figure 2B, compare lower left to lower right). EM analysis thus confirmed the results of the ThT assay.

To show that the effect of K_4 on $A\beta_{1-42}$ aggregation is specifically caused by the KLVFF sequence, a similar co-aggregation experiment was performed on LMW $A\beta_{1-42}$ with a dendrimer that contained four peptides, each with five non-Aβ-related amino acids (GRGDS) with a GG spacer (G_4). G_4 had no inhibitory effect on $A\beta_{1-42}$ aggregation even at the highest ratio used (1:4). This result shows that the effect caused by K_4 is mediated by the Aβ-derived sequence KLVFF.

Dendritic KLVFF disassembles mature $A\beta_{1-42}$ fibrils

To test whether the KLVFF derivatives can also cause the disassembly of mature $A\beta_{1-42}$ fibrils (fAβ), fAβ was incubated with K_1 and K_4 for 24 hours in different ratios. Figure 3A shows that K_4 disassembles fAβ in a concentration-dependent manner. A significant effect of K_4 on fibril disassembly of 20% was already observed at an Aβ/K ratio of 1:0.2. Increasing the concentration of K_4 up to an Aβ/K ratio of 1:16 increased the disassembly to 65%. An Aβ/K (in K_1) ratio of 1:16 caused a fibril breakdown of only 25%. Thus, K_4 breaks down the preformed $A\beta_{1-42}$ fibrils much more potently. Disassembly of mature fibrils by 50% by K_4 is observed at an Aβ/K ratio of 1:2.7; this corresponds to concentrations of 10 μM Aβ and 6.75 μM K_4. The specificity of the effect of K_4 was again shown by the small effect that the G_4 dendrimer had on fibril disassembly (10% at an Aβ/G (in G_4) ratio of 1:16). As an additional control for the specificity of the effect of K_4, we also studied the effect of the dendrimer itself (C_4), because previous studies have shown that dendritic structures are capable of breaking down preformed fibrillar aggregates by themselves. C_4 reduced ThT binding only by 10% at an $A\beta/C_4$ ratio of 1:16. This indicates that the observed effect by K_4 is sequence specific. EM analysis of fibrillar Aβ preparations, confirmed the fibrillar nature of the starting material (Figure 3B, upper left). EM analysis of the $A\beta/K_1$ co-aggregate (Aβ/K-ratio 1:16) shows many fibrillar structures; this confirms that K_1 has only a minor effect on fibril disassembly (Figure 3B, upper right). In contrast, at the same Aβ/K (in K_4) ratio no fibrillar structures were detected. Instead, globular, more oligomer-like structures were observed (Figure 3B, lower left). The starting level of ThT binding was only 35%, which indicates that fewer fibrillar structures might be have been present. With $A\beta/G_4$ co-aggregates no differences with Aβ alone were observed (Figure 3B, lower right). Together these results show the increased potency of K_4 on fibril disassembly compared to K_1.

Purification and characterization of protofibrillar $A\beta_{1-42}$

To study the effect of the inhibitory agents on preformed protofibrils (PF), we prepared highly enriched PF Aβ aggregates as described in the Experimental Section. The PF fraction was separated from the LMW forms of $A\beta_{1-42}$ by size-exclusion chromatography (SEC), which showed two separate peaks in the chromatogram (Figure 4A).

To study the kinetics of $A\beta_{1-42}$ aggregation, PF and LMW $A\beta_{1-42}$ preparations were aggregated for 24 h at 37 °C. LMW $A\beta_{1-42}$ shows little ThT fluorescence at $t=0$. In contrast, PF $A\beta_{1-42}$ had a fivefold higher ThT fluorescence; this indicates that PF $A\beta_{1-42}$ contains more β sheets than LMW $A\beta_{1-42}$ at $t=0$. After 24 h of aggregation, LMW $A\beta_{1-42}$ and PF $A\beta_{1-42}$ both reached a similar level of ThT fluorescence (Figure 4B). To confirm the structure of the PF preparation, EM analysis was performed. Short curvilinear structures, which are characteristic for protofibrils, were observed (Figure 4C, left). Incubation of protofibrils for 24 h at 37 °C resulted in the formation of fibrils (Figure 4B, right); this confirmed that protofibrils are intermediates on the pathway to fibrils.

Dendritic KLVFF interferes in protofibril-to-fibril conversion by disassembling protofibrils

To study the effect of the K_4 peptide dendrimer on PF aggregates, SEC-purified PF $A\beta_{1-42}$ was aggregated for 24 h in presence or absence of K_1 and K_4 in different ratios. ThT analysis showed that K_4 interferes in the protofibril-to-fibril conversion

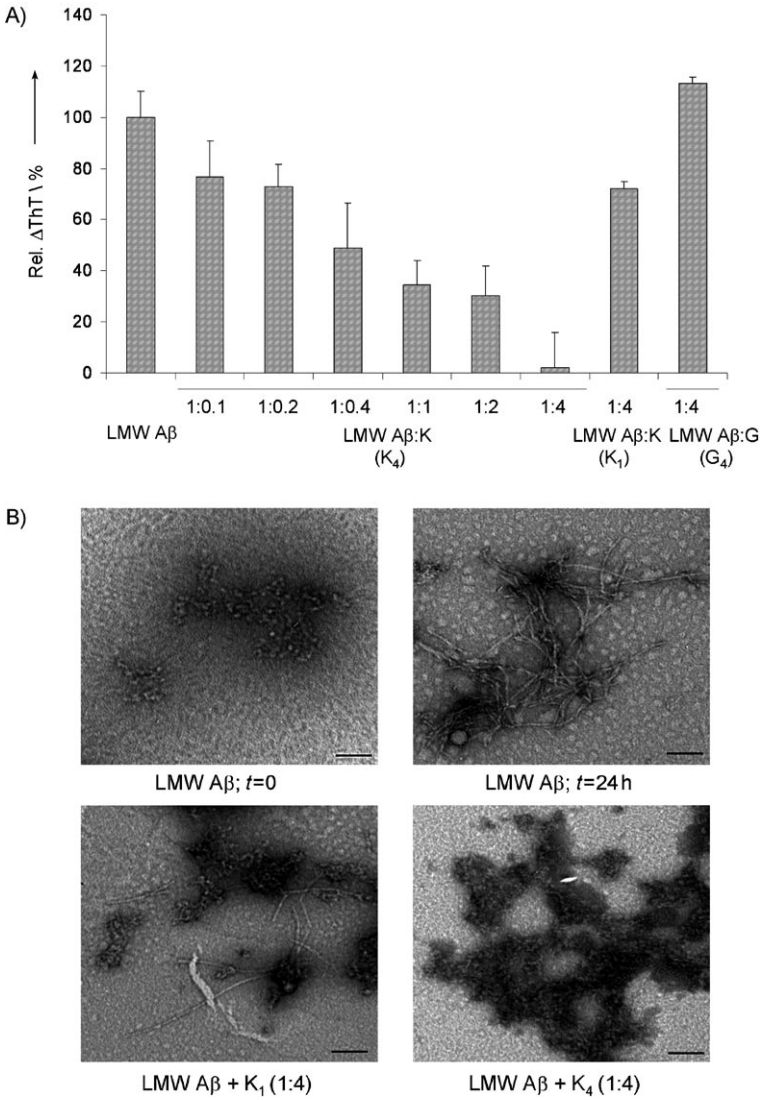


Figure 2. K_4 is a potent inhibitor of LMW Aβ aggregation. A) LMW Aβ was co-incubated with K_1, K_4 and G_4 in the indicated ratios for 24 h. The β-sheet content was analyzed by the ThT assay. Shown is a graph of a ThT assay that was performed on the aggregates ($n = 6$–9 from 2–3 independent experiments; mean ± S.D.). The increase in ThT fluorescence, without inhibitors, was set to 100% (Rel.ΔThT). Inhibitory effects of the KLVFF compounds on LMW Aβ aggregation were all relative to this control. Ratios are expressed per KLVFF peptide subunit. B) The morphology of the (co-)aggregates was analyzed by EM. Shown are representative EM pictures of the co-aggregates of LMW Aβ without inhibitors at $t = 0$ (upper left) and $t = 24$ h (upper right) and at $t = 24$ with K_1 (lower left) or K_4 (lower right). Aβ to peptide ratios are indicated in the figure. Scale bar: 100 nm.

of $A\beta_{1-42}$ in a concentration-dependent manner (Figure 5A). A 1:1 ratio of Aβ/K (in K_4) limits the relative change of the ThT fluorescence (Rel.ΔThT) to only 35% of the normally observed increase. At Aβ/K (in K_4) ratios of 1:2 and higher the Rel.ΔThT of $A\beta_{1-42}$ after 24 h is actually decreased; this indicates the disassembly of pre-existing PF. Increasing the K_4 concentration up to an Aβ/K ratio of 1:16 caused further disassembly of PF of up to 70%. A 1:8 ratio of Aβ/K (in K_1) limits the conversion of PF to fibril to 55%, whereas the same ratio of Aβ/K (in K_4) results in the breakdown of the pre-existing PF by 55%. EM analysis of the morphology of the PF Aβ/K_4 co-aggregates shows the absence of fibrils and disappearance of PF in the presence of high concentrations of K_4. Instead, a rearrangement of PF $A\beta_{1-42}$ into amorphous aggregates (Figure 5B; right) is observed. This corroborates the results from the ThT assay because amorphous aggregates do not show ThT fluorescence. In the presence of K_1, fibrillar structures were still observed (Figure 5B; left).

Discussion

One of the key pathogenic factors in AD is the Aβ peptide. Aβ aggregates into fibrils via several intermediate aggregation species. Different aggregated species might exert different toxic effects,[18] for example, our own previous work showed the induction of endoplasmic reticulum stress with oligomeric, but not with fibrillar Aβ aggregates.[29] Nevertheless, plaques in AD patients consist mainly of fibrillar Aβ aggregates. Fibrillar Aβ has been shown to be toxic in culture, and might also be responsible for the neuronal loss that is observed in AD patients. In any case, it is clear that aggregation of Aβ is a prerequisite for toxicity, therefore the prevention of Aβ aggregation might inhibit Aβ-induced neurotoxicity. To this end, we investigated intervention in $A\beta_{1-42}$ aggregation by using a multivalent KLVFF variant. We successfully produced a dendritic variant of KLVFF by using native chemical ligation. Like the monomeric K_1, the dendritic K_4 shows no self-aggregation, not even after prolonged incubation at 37 °C, which is important for downstream applications that require a soluble agent.

In this study the effect of the branched KLVFF structure (K_4) was compared to the monomeric KLVFF (K_1). Because different Aβ species are known to contribute to AD pathogenesis, intervention of Aβ aggregation was studied at different aggregation stages of Aβ: the first onset of aggregation (LMW), the aggregation of intermediate species (PF) and the aggregated mature fibrils (fAβ). To prevent Aβ from reaching an aggregated state, we intervened at the first onset of aggregation by starting from the LMW state. K_4 was found to be highly effective in preventing LMW $A\beta_{1-42}$ aggregation, and to be much more potent than K_1, as indicated by both the amount of fluorescence reduction at the same 1:4 ratio for K_1 (30%) and K_4 (90%) as well as the 20-fold lower concentration of K_4 (per peptide subunit) that was needed for a similar inhibition as K_1. Previously, a branched hexameric retro-inverso FFVLK was shown to increase the potency of $A\beta_{1-40}$ aggregation inhibition.[25] The multivalent effect that is reported in this study was threefold of a hexamer compared to a dimeric conjugate; this indicates that the K_4 conjugate from our study gives a stronger multivalent effect. However, a direct comparison of the data is difficult because another study showed that a KLVFF analogue is a more potent inhibitor of $A\beta_{1-40}$ than of $A\beta_{1-42}$ aggregation,[30] and because vastly different Aβ and conjugate concentrations were used.

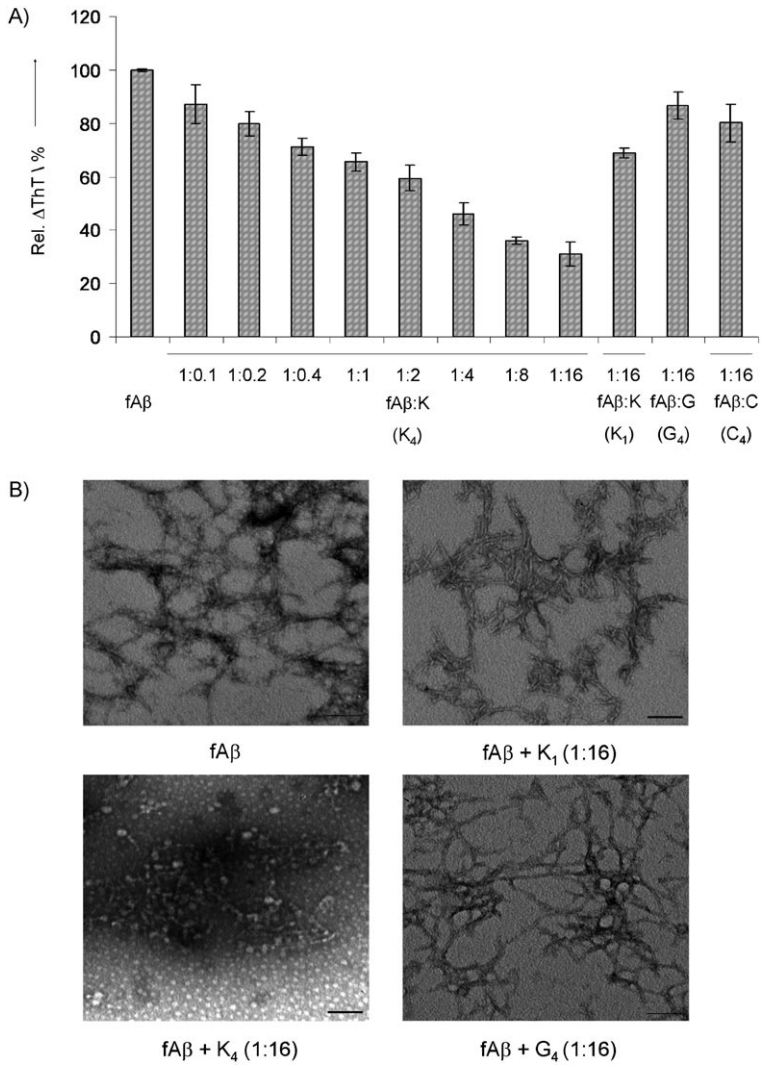


Figure 3. K_4 disassembles mature Aβ fibrils more potently then K_1. A) Fibrillar Aβ was co-incubated with different concentrations of K_1, K_4, G_4 and C_4 for 24 h. The β-sheet content was analyzed by the ThT assay. Shown is a graph of a ThT assay that was performed on the aggregates ($n = 6$ from two independent experiments; mean ± S.D.). The ThT fluorescence of fibrillar Aβ without inhibitors was set to 100%. The inhibitory effect of the compounds was compared to this control. Ratios are expressed per KLVFF peptide subunit. B) The morphology of the (co-)aggregates was analyzed by EM. Shown are representative EM pictures of (co-)aggregates of fibrillar Aβ alone (upper left), in presence of K_1 (upper right), K_4 (lower left) and G_4 (lower right). Aβ/peptide ratios are indicated in the figure. Scale bar: 100 nm.

Another possibility for aggregation intervention is the disassembly of preformed fAβ. Previous studies have shown that KLVFF analogues are capable of disassembling preformed fibrils.[30,31] Our study also shows that K_4 disassembles fAβ in a concentration-dependent manner and in a more potent way than K_1; this was indicated by both the amount of ThT fluorescence reduction at the same 1:16 Aβ/K ratio for K_1 (25%) and K_4 (65%), as well as the 40-fold lower concentration of K_4 (per peptide subunit) that was needed to induce similar inhibition to K_1. It should be noted however, that even the highest concentration of K_4 did not cause a complete breakdown of the fibrils; about 35% of potentially toxic amyloid fibrils remained in solution. This might be a drawback for the potential use of K_4 as a therapeutic agent in fibril breakdown. In contrast, the previously reported hexameric PEG–FFVLK conjugate was not capable of inducing fibril disassembly.[25] This might be due to the use of the PEG scaffold, which is relatively large (10 kDa) compared to the first generation dendrimer (729 Da).

Because PF are intermediate species on the pathway to fibrillization, they are an excellent experimental model to study both aggregation inhibition and the breakdown of pre-exisiting aggregates in one experimental setup. In addition, PF have been shown to have diverse toxic effects in vitro.[11] An early onset variant of AD, caused by the "Arctic" mutation in APP is shown to result in accelerated production of PF;[32] this indicates the relevance of PF for AD pathogenesis. We show that K_4 also has a more potent inhibitory effect than K_1 for the aggregation from PF to $fA\beta_{1-42}$. This is best indicated by the fact that an Aβ/K ratio (for K_1) of 1:8 results in inhibition of the PF-to-fAβ conversion by 55% whereas the same ratio for K_4 results in PF breakdown of 55%. ThT analysis shows a concentration-dependent effect of K_4 on PF aggregation; lower concentrations inhibit the further aggregation of fibrils, and higher K_4 concentrations cause increased PF disassembly. We also show that K_4 rearranges PF $A\beta_{1-42}$ into amorphous aggregates. This is different from the disassembly of preformed fibrils into globular more oligomer-like structures, and it suggests that there is a different mechanism for the disassembly of protofibrils by K_4.

Our study shows that K_4 is much more potent than K_1 and thus has a multivalent inhibitory effect on all of the aggregation species that were studied. The effect of K_4 on the inhibition of LMW aggregation compared to the breakdown of pre-formed fibrillar aggregates, however, occurs with different kinetics as indicated by the concentrations of K_4 that are needed for half-maximal inhibition. The half-maximal inhibition for aggregation of 10 μM Aβ is attained with 1.25 μM K_4. To reach half-maximal disassembly of 10 μM fibrils, however, a more than fivefold higher K_4 concentration of 6.75 μM is required. This suggests that K_4 exerts its effect predominantly on LMW Aβ. Because fAβ is in equilibrium with LMW species, this suggests that the strong effect of K_4 on LMW Aβ will shift this equilibrium between fAβ and LMW towards the LMW state, and thus cause a disassembly of existing fibrillar aggregates.

Previous studies have shown an inhibitory effect of nonfunctionalized amine-terminated polyamine dendrimers on amyloid formation by $A\beta_{1-28}$ and prion protein (PrP) fragments.[33–36] Aβ aggregation was inhibited by third-generation amine-terminated polyamine dendrimers, and the inhibition increased with increasing dendrimer concentration and generation. To show that the multivalent effect of K_4 was specifically caused by the KLVFF sequence and not by the dendritic structure itself, we performed similar aggregation assays with a dendrimer that consisted of 4 unrelated pentapeptides (G_4). Co-incubation of G_4 and $A\beta_{1-42}$ caused only a small inhibition of LMW $A\beta_{1-42}$ ag-

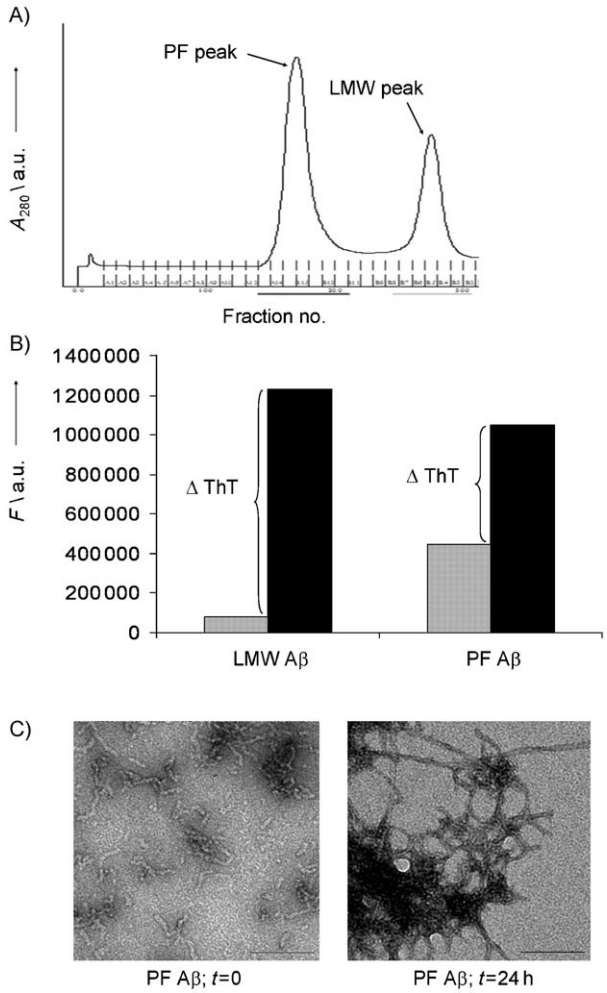


Figure 4. Purification and aggregation kinetics of PF Aβ. A) Protofibrillar (PF) Aβ was prepared as described in the Experimental Section and was purified by size exclusion chromatography (SEC) by using a calibrated Superdex 75 10/30 HR column. Size exclusion of the Aβ preparation results in separation of LMW Aβ species from PF Aβ. Shown is a representative size exclusion chromatogram. B) LMW and PF fractions were aggregated for 24 h at 37 °C. The β-sheet content was analyzed by the ThT assay at $t=0$ (●) and 24 h (●). Shown is a representative graph from a ThT assay on PF and LMW Aβ. The bracket in the graph indicates the change in ThT fluorescence after 24 h (ΔThT) C) The morphology of PF Aβ before and after the aggregation was studied by EM analysis. Shown are representative electron micrographs of PF Aβ at $t=0$ (left) and 24 h (right). Scale bar: 100 nm

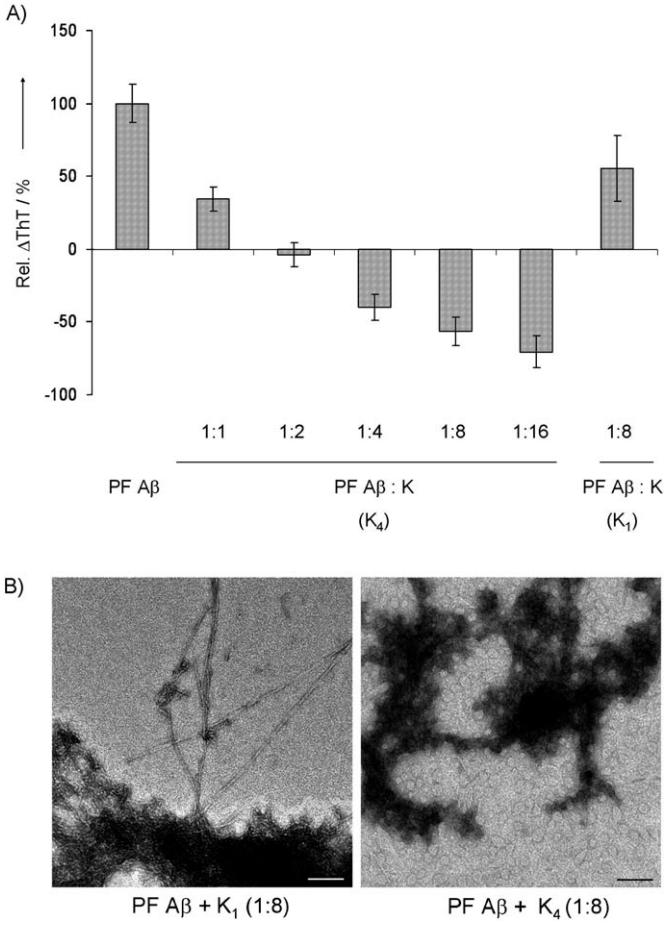


Figure 5. K_4 dissassembles protofibrillar Aβ. A) Protofibrillar Aβ was co-incubated with different concentrations of K_1 and K_4 for 24 h and the β-sheet content was analyzed by the ThT assay. Shown is a graph of a ThT assay that was performed on the aggregates ($n=5$ from two independent experiments; mean ± S.D.). The increase of ThT fluorescence without inhibitors, was set to 100 % (Rel.ΔThT). Ratios are expressed per KLVFF peptide subunit. B) The morphology of the co-aggregates was analyzed by EM. Shown are representative EM pictures of co-aggregates of PF Aβ in the presence of K_1 (left) or K_4 (right). The Aβ-to-peptide ratios are indicated in the figure. Scale bar: 100 nm.

gregation, and had no effect on fibril breakdown in any of the Aβ/G_4 ratios that were studied. The cysteine-dendrimer (C_4) showed only a minor effect on fibril breakdown; this also suggests that it is unlikely that the amine-terminated PPI dendrimers would have an effect on Aβ aggregation. This result clearly shows that the more potent effect of K_4 on $Aβ_{1-42}$ aggregation is sequence specific. The potency of a multivalent KLVFF derivative might be enhanced by using a higher generation dendrimer, but this is likely to also enhance sequence-unspecific effects, as observed with the third and higher generation amine-terminated polyamine dendrimers.[34,35] In addition, with increasing generation, the hydrophobicity will increase, as well as its size and shape, thereby loosing the advantage of a soluble small-size inhibitor.

Although we can not fully exclude the possibility that the strong inhibiton caused by K_4 is caused by steric hindrance by the attachment of any structure to KLVFF, the strongly increased potency of K_4 relative to K_1 suggests a multivalent effect. This multivalent effect might also apply for other potent KLVFF-derived aggregation inhibitors. Delivery across the blood–brain barrier (BBB) might be important for a therapeutic agent for AD. However, a study in mice showed that a monoclonal antibody against Aβ can still be effective when applied in the periphery.[37] Injection of this antibody was shown to produce a "sink" effect by drawing soluble Aβ from the brain into the bloodstream. This could then potentially halt or even reverse plaque formation. Whether dendritic KLVFF can work in a similar manner awaits further study. In addition, modifications can facilitate passage across the BBB. An analogue of the KLVFF peptide that was protected against proteolytic degradation, and that had increased BBB permeability has been successfully used in a rat model of brain amyloidosis.[26] It was

shown to prevent Aβ-induced spatial memory impairments, and caused a partial reduction of amyloid deposits. Our data suggest that dendritic derivatives of KLVFF might further increase its efficacy. In addition, several small-molecule inhibitors have also been shown to inhibit amyloid aggregation,[38] and their efficacy might also be further increased via multivalent display on dendrimers. Dendrimers might thus be a valuable tool that can increase the therapeutic efficacy of many aggregation inhibitors.

The small size and controlled synthesis of dendrimeric structures is a major advantage when creating multivalent molecules. In this study, we have employed this advantage to create a small, multivalent and sequence-specific inhibitor of Aβ aggregation. The effect of K_4 on the inhibition of fibril formation as well as fibril disassembly suggests that many of the steps of Aβ fibrillogenesis are reversible. Because K_4 acts on all different aggregation species, it has potential for use as a therapeutic agent.

Experimental Section

Synthesis of peptide–dendrimer conjugate: In this study native chemical ligation was used for the generation of peptide dendrimers.[28] Standard *tert*-butyloxycarbonyl (*t*-Boc)-mediated solid-phase peptide synthesis was used to make the KLVFFGG peptide. The two glycine residues (GG) were included to provide a spacer between the KLVFF sequence and the dendrimer structure. The KLVFFGG peptides were functionalized with a C-terminal mercaptopropionic acid leucine (MPAL) thioester, as described in Hackeng et al.[39] Functionalization of the amine-terminated poly(propylene imine) dendrimers (kindly provided by DSM (Geleen, The Netherlands)) with cysteine residues, was done as described in van Baal et al.[28]

Four equivalents of peptide thioester were ligated to one equivalent of the cysteine dendrimer by using standard ligation conditions (Tris buffer pH 7.5, 6 M guanidine, 2% (*v/v*) thiophenol, 2% (*v/v*) benzylmercaptan, 37 °C), with stirring for 1 h at 37 °C. This resulted in the formation of $[KLVFFGG]_4$ dendrimer. The ligation product was further purified by using reversed-phase HPLC as described in van Baal et al.[28] The cysteine dendrimer is referred to as C_4, KLVFFGG is referred to as K_1 and the $[KLVFFGG]_4$ dendrimer is referred to as K_4. As an additional control in the aggregation experiments, we used a dendrimer with 4 copies of the unrelated pentapeptide GRGDS with GG as a spacer (G_4).[28] All peptides and peptide dendrimers were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis, by using α-cyano-4-hydrocinnamic acid as a matrix. The MS data of the K_1 and K_4 structures are shown in the supplementary data. The MS data of G_4 as well as C_4 have been shown previously in van Baal et al.[28]

Preparation and purification of different Amyloid-β_{1-42} species: Purified $A\beta_{1-42}$ peptide was purchased from California peptide (Napa, CA, USA) or Anaspec (San Jose, CA, USA). For the preparation of protofibrils, $A\beta_{1-42}$ was initially diluted in DMSO, then in water, and finally in Tris–HCl, pH 7.4, to give final concentrations of 5%, 25 mM and 1 mg mL^{-1} (222 μM) for DMSO, Tris and Aβ, respectively. Large, insoluble particles (fibrils) were removed by a 5 min centrifugation step at 13000 *g* at 4 °C. The supernatants were loaded onto a Superdex 75 HR 10/30 gel-filtration column (GE Healthcare Europe, Munich, Germany), and subsequently equilibrated with Tris–HCl (5 mM, pH 7.4), NaCl (75 mM) to separate PF from LMW species. The column was calibrated by using the following proteins as molecular mass standards: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and albumin (67 kDa). The protein was eluted at a flow rate of 0.5 mL min^{-1} and 0.5 mL fractions were collected. To assess which eluted fractions from the PF or LMW peaks had the highest $A\beta_{1-42}$ concentration, a sample of each of the fractions was run on a 16.5% SDS gel and proteins were visualized by silver staining. For each peak the most intense bands on the gels (which contained the most of the $A\beta_{1-42}$ peptide) were pooled and the concentration of the final mixture was determined by using a BCA protein assay (Pierce, Rockford, IL, USA), before the co-aggregation experiments were initiated. EM analysis was performed to confirm the protofibrillar structures.

To obtain LMW species, hexafluoroisopropanol (HFIP) was used as an initial solvent. $A\beta_{1-42}$ was dissolved in HFIP at a final concentration of 1 mg mL^{-1}, aliquoted, and allowed to evaporate by using a SpeedVac centrifuge. The resulting peptide film was stored at −80 °C until further use. For co-aggregation experiments the peptide film was dissolved in DMSO, and sonicated in a bath sonicator for 10 min. For LMW preparation, $A\beta_{1-42}$ was subsequently diluted in 5 mM Tris–HCl, pH 7.4, 75 mM NaCl buffer to a final concentration of 20 mM. EM analysis was performed to confirm the absence of fibrillar structures or other aggregates. This method has previously been shown to yield mainly monomeric and small soluble Aβ species.[16] HFIP-treated LMW Aβ species were also analyzed by SEC (not shown); they eluted from the column at approximately the same time as the SEC-purified LMW Aβ separated from protofibrils. SEC-purified LMW Aβ (not shown) as well as HFIP-treated Aβ (Figure 2B) showed very few structures on EM. These findings confirmed that LMW species that were obtained by either method were too small to be detected by EM as was previously reported.[11,16,19] Both LMW species also showed very low ThT binding. This indicated that they were similar structures. In addition, the same co-aggregation assays as with HFIP-treated LMW Aβ (at 20 μM, see below), were also performed with the SEC-purified LMW Aβ species at 10 μM (not shown) and showed similar results.

To obtain fibrillar $A\beta_{1-42}$ (fAβ) 10 mM HCl was added to Aβ/DMSO under continuous vortexing to bring the peptide to a final concentration of 100 μM followed by incubating at 37 °C for 24 h. The fibrillar $A\beta_{1-42}$ preparation was centrifuged (220000 *g*) in a Beckman tabletop ultracentrifuge for 30 min at room temperature and the pellet was resuspended in 4% DMSO/10 mM HCl (pH 4). The relative concentration of the fibrillar $A\beta_{1-42}$ preparations was determined by a Bradford protein assay (Biorad, Hercules, CA, USA).

Co-aggregation assay: For the fibril disaggregation experiments, the fibrillar samples were further diluted in 5 mM Tris–HCl, pH 7.4, 75 mM NaCl buffer to a final concentration of 10 μM prior to the addition of K_1 or K_4. For the protofibril aggregation experiments, isolated protofibril preparations were used at a final concentration of 10 μM in 5 mM Tris–HCl, pH 7.4, 75 mM NaCl in the presence or absence of the inhibitor peptides. Freshly prepared HFIP-treated LMW Aβ was used at a final concentration of 20 mM in the co-aggregation assays. The samples were first incubated at 4 °C for 1 h to allow binding of the aggregation inhibitors to $A\beta_{1-42}$. Subsequently, the samples were allowed to aggregate for 24 h at 37 °C. After 24 h samples were taken to perform a Thioflavin T assay and EM analysis.

Thioflavin T assay: A 100 μM aqueous solution of Thioflavin T (ThT) was prepared and filtered through a 0.2 μm filter. The Aβ aggregates were diluted to a final concentration of 5 μM into gly-

cine/NaOH (90 mM, pH 8.5) that contained 10 μM ThT. Fluorescence was measured in 96-well plates by using a Fluostar microplate reader (Fluostar Optima, BMG Labtech, Offenburg, Germany) at an excitation wavelength of 450 nm and emission at 485 nm. In the ThT graph of the co-aggregation experiments the relative change in fluorescence (Rel.ΔThT in %) was plotted against the molar ratio of $A\beta_{1-42}$/test sample. In order to directly compare the effects of K_4 and K_1 on Aβ aggregation, the ratios were expressed per mole of KLVFF peptide subunit, and *not* per mole of conjugate molecule. The change in ThT fluorescence of LMW or PF $A\beta_{1-42}$ after 24 h of aggregation in the absence of the interfering peptides was set at 100%. The other experimental conditions were all related to this control. For the aggregation assays with fAβ, the ThT fluorescence of fibrils in the absence of interfering peptides was set at 100%.

Electron microscopy: $A\beta_{1-42}$ preparations were adsorbed onto 300-mesh formvar/copper grids for 5 min and the excess fluid was filtered off. Subsequently, the samples were stained with 1% uranyl acetate for 5 min, the excess fluid was removed and the grids were analyzed with a Philips EM-420 or a Philips CM 100 transmission electron microscope operated at 100 kV.

Atomic force microscopy (AFM): A total of 10 μL of each sample was adsorbed onto a freshly cleaved mica surface and was left to dry at room temperature for 1 h. AFM images were obtained at room temperature with a Nanoscope III (Digital Instruments, Santa Barbara, CA) by using force–volume mode operating in liquid. Measurements were performed with a constant retraction speed of 355 $nm\,s^{-1}$. Calibrated standard triangular Si_3N_4 AFM cantilevers from Veeco (Santa Barbara, CA) were used with a 0.06 $N\,m^{-1}$ nominal spring constant.

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