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A Multimeric Quinacrine Conjugate as a Potential Inhibitor of Alzheimer's β-Amyloid Fibril Formation

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Amyloid formation and accumulation of the amyloid β-peptide (Aβ) in the brain is associated with Alzheimer's disease (AD) pathogenesis. Therefore, among the therapeutic approaches in development to fight the disease, the direct inhibition of the Aβ self-assembly process is currently widely investigated and is one of the most promising approaches. In this study we investigated the potential of a multimeric display of quinacrine derivatives, as compared to the monomer quinacrine, as a design principal for a novel class of inhibitors against Aβ fibril formation. The presented multimeric conjugate exhibits a cluster of four quinacrine derivatives on a rigid cyclopeptidic scaffold. Herein is reported the synthesis of the conjugate, together with the in vitro inhibitory evaluation of $A\beta_{1-40}$ fibrils using the thioflavin T fluorescence assay, and imaging with atomic force microscopy. Our data show that the multimeric compound inhibits $A\beta_{1-40}$ fibril formation with an IC_{50} value of 20 ± 10 μM, which contrasts with the non-active monomeric analogue. This work suggests that assembling multiple copies of acridine moieties to a central scaffold, for multiple interactions, is a promising strategy for the engineering of inhibitors against Aβ fibril formation.

Introduction

Alzheimer's disease (AD) is characterized by the accumulation of insoluble fibrillar aggregates of amyloid β-peptide (Aβ) and the degeneration and death of neurons in brain regions that are concerned with learning and memory processes.[1] Abnormal protein deposition is also a shared characteristic of other age-related neurodegenerative diseases, such as Parkinsons's and Huntington's diseases[2–4] and of the prion diseases.[5,6]

In AD two peptides have been identified in the fibrillar deposition in the brain, the amyloid β-peptides $A\beta_{1-40}$ and $A\beta_{1-42}$.[1] Normally, Aβ exists as a random coil, but through formation of intermediate oligomers misfolds into protofibrils, and further aggregation of the protofibrils forms amyloid.[7,8] It is widely accepted that the formation of fibrils is implicated in the neurotoxicity associated with AD, but still there is much discussion on which aggregation state of Aβ is toxic and on the toxicity mechanisms.[9–13]

Several approaches aimed at blocking the neurotoxic activity of Aβ are presently pursued: such as reduction of amyloid production by inhibiting the enzymes beta- and gamma-secretase, which produce Aβ,[14] stimulation of the immune system to remove Aβ from the brain by immunizing with Aβ fragments,[15] and direct inhibition of the Aβ self-assembly process.[16,17] Especially the identification of inhibitors of protein deposition or fibril formation should provide drugs with neuroprotective effects for the suppression or prevention of the transition from monomeric to oligomeric and polymeric Aβ species. It may also be a critical step towards a better understanding of the role of fibrils in neurodegenerative processes and disease progression.

The search for potent drugs such as amyloid aggregation inhibitors led to the discovery of compounds inhibiting specifically and efficiently amyloid aggregation in vitro. Molecules issued from random screening as well as short amyloid peptide sequences mimicking and inhibiting amyloid aggregation are the two main classes of inhibitors. In the past few years, non-peptidic amyloid aggregation inhibitors have been identified in the literature and is an important approach in the development of therapeutic agents.[18–21] This approach was initially based on the finding that the small dye, Congo red, interacts specifically with amyloid fibrils and inhibits their formation.[22] Molecules such as melatonin, furansulfonic acid derivatives, small polyphenol molecules, and the well-known antibiotic Rifampacin, anthracyclones, benzofuran derivatives have been reported.[23] Although a structure–activity relationship is not easy to distinguish from all these works, many of the inhibitors are either "flat molecules bearing a lipophilic bi- or tri-(hetero)-cyclic scaffold and having a basic nitrogen on one of these rings",[23] or have a polyphenolic structure.[24]

Unexpectedly, only a few works have investigated the acridine derivatives as β-amyloid inhibitors although these flat heterocyclic scaffolds should display an ideal drug-like profile. The first acridine cores reported to inhibit or interact with the β-amyloid polymerization process in AD are diaryl heterocyclic compounds and 9-acridone derivatives.[25,26] More recently, the search for radioactive probes for imaging the amyloid-β peptide in vivo and for the detection of AD allowed the identifica-

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tion of a fluorinated acridine orange analogue with high affinity for Aβ aggregates.[27,28]

On the other hand, acridine compounds including quinacrine have been more extensively studied for the treatment of the prion diseases, and effective inhibitory activities on PrP^{Sc} replication in vitro have been reported.[29–34] Although the sequence of the prion protein and of the Aβ peptides are quite different, the mechanism of their aggregation may be similar, and compounds that are able to interact with the prion protein could also be active on β-amyloid peptides. In support of this, some well-known Aβ binding compounds such as tetracycline and thioflavin T are reported to exhibit binding to prion peptides similar to that observed with quinacrine.[35] Moreover, as both diseases lead to aberrantly folded proteins, rich in β-sheet structure that have a high tendency to form aggregates, the inhibitor binding might be more conformation-dependent rather than sequence-dependent. Such conformation-dependent interaction is reported for polyphenols which do not interact with the amyloidogenic monomer but rather with amyloidogenic structures.[24,36] From this, it appeared interesting to study the quinacrine core and its ability to interact with Aβ and to inhibit the amyloid polymerization process by analogy with its antiprion effect. To date, such effects on Aβ fibril formation has not been investigated.

Further, the search for new Aβ peptide aggregation inhibitors is hampered as the interactions and mechanism of inhibition is not well understood. Common to most known inhibitors is the presence of two substituted aromatic groups separated by a short spacer, such as curcumin and NDGA.[24,36] From this common feature, it is believed that these aromatic compounds interact with hydrophobic and aromatic amino acids of the misfolding peptides, such as phenylalanine 19 and 20 in Aβ that are important for fibril formation. As fibril formation proceeds through hydrophobic interactions to form intermediate oligomers with undefined structure, molecules that can interact with these different states on the fibril-folding pathway may prove to be important drug candidates against AD. When designing compounds as potential inhibitors, we postulated that a multimeric arrangement of aromatic residues would be capable of multiple simultaneous interactions with these different states and thus lead to more potent inhibitors because of multimeric effects.[37] Further, we expected that the multimeric arrangement of aromatic residues would be more potent if they were directionally constructed as a cluster by the aid of a well-defined and rigid scaffold.

Multivalency is a well known and efficient process used by nature to enhance the affinity of binding in biological phenomena such as recognition.[37] Recently, May et al. showed that the multivalency strategy may be beneficial to enhance the antiprion activity of acridines using covalent dimers.[29] Their conclusion that "this strategy may be generally applicable to other diseases of protein conformation, in which species along the pathway to the aggregated endpoint are characterized by high protein concentrations" supports our inhibitory multimeric approach in Aβ peptide aggregation. It was only recently (during the revision of this manuscript) that a multimeric approach was investigated in the field of Aβ peptide aggregation inhibitors.[38] This research showed that branched KLVFF tetramers are more potent inhibitors of β-amyloid aggregation compared to the monomeric KLVFF peptide. Moreover as we had in our research group a convenient and proved cyclopeptidic tool allowing the multimeric presentation of ligands, this encouraged us to investigate its multimeric strategy.

In light of the above, we first focused our investigations on the quinacrine derivative **7** as an inhibitor of the Aβ polymerization process by analogy with its antiprion effect. Simultaneously, we prepared its multimeric form **6**, exhibiting a cluster of acridines on a cyclopeptidic core for enhanced inhibitory properties by multiple interactions (Scheme 1). These compounds were investigated in vitro as potential inhibitors of Aβ fibril formation. Their design, synthesis, and the inhibitory results, using fluorescence spectroscopy with thioflavin T (ThT) and atomic force microscopy (AFM), are reported in detail in this paper.

Results

Design of the fibril inhibitor candidates

In this first approach, we have chosen the quinacrine derivative, compound **7** (Scheme 1), as a starting point for the design and synthesis of a potential amyloid inhibitor. Thereafter we prepared a mutimeric version, compound **6**, which displays four copies of the quinacrine derivative covalently attached to a well-defined and rigid scaffold (Scheme 1). We postulated that a covalent multimeric assembly of the acridines could be a more potent inhibitor because of the well-known local concentration effect.[37] Indeed, this multimeric arrangement is capable of interacting through multiple molecular contacts with one Aβ monomer or several monomers aggregated in oligomers or fibrils, thus enhancing the inhibition process. This multivalency approach has been first validated on the prion disease in a recent study where bis-acridines showed increased antiprion activity, around tenfold in vitro, compared to the corresponding monomeric compounds.[29] In our conjugate, the conformational restriction imparted by the scaffold may also be beneficial for the interaction of the acridine cores to Aβ peptides by minimizing the entropically conformational rearrangement before binding as noted for the bis-acridines on the prion protein. We also prepared as a reference compound the monomeric analogue **8** that bears only one copy of the quinacrine derivative on the scaffold (Scheme 1). This compound was designed to determine whether the multimeric arrangement of compound **6** increases the affinity towards Aβ.

The scaffold used in this study to present the heterocyclic quinacrine molecules in a multimeric and well-defined spatial orientation is a backbone-cyclized decapeptide. The two prolyl-glycine sequences, as β type-II turn inducers, constrain the peptide backbone conformation into an antiparallel β-sheet.[39] As a result the cyclic peptide displays an "upper-face" with four residues (here and commonly lysine) and a "lower-face" with two residues (arginine residues here), see Scheme 1.

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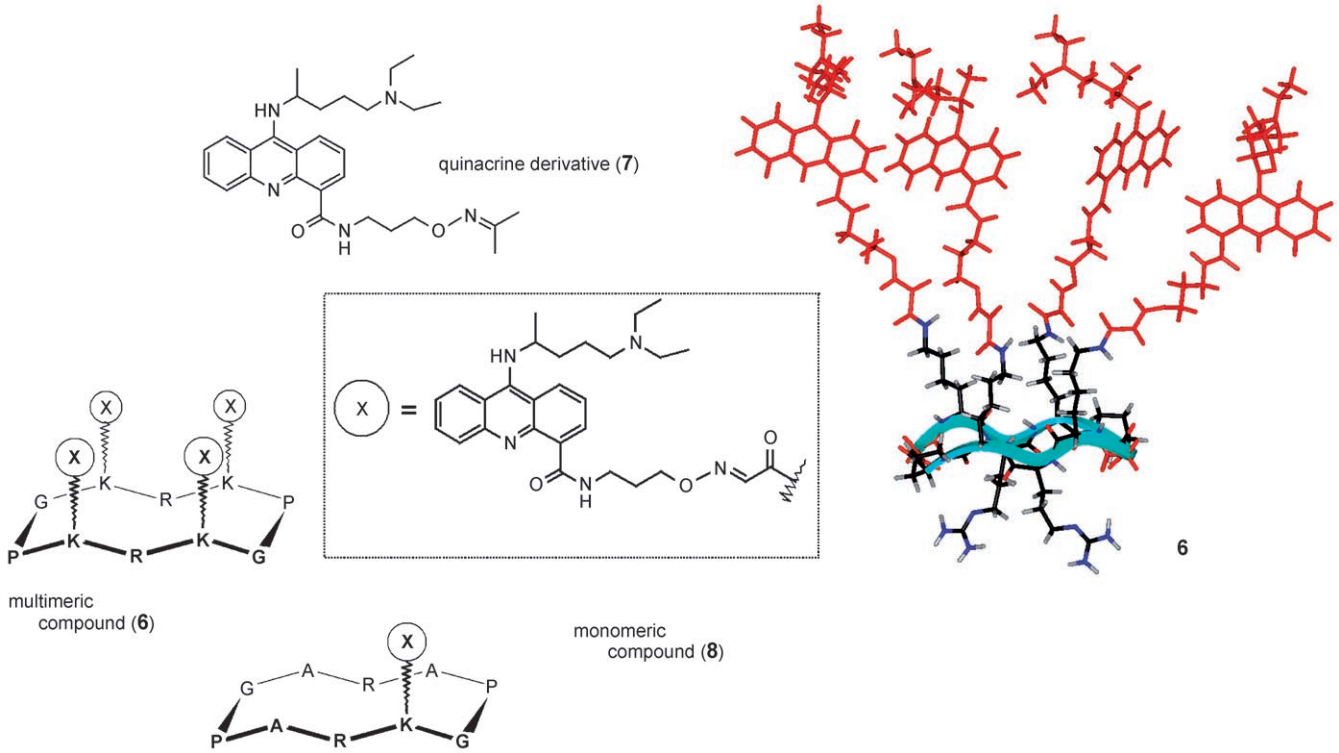

Scheme 1. Left, schematic representation of the inhibitor candidates. Right, molecular model of the multimeric quinacrine compound **6**. The quinacrine derivatives and oxime linkages are in red, the cyclic peptide scaffold with four functionalized lysines is represented as a turquoise ribbon and below the scaffold can be seen the two arginine side chains for increased solubility (Insight II).

These kind of templates, first proposed by Mutter and coworkers as structure inducing devices,[40,41] has found useful applications in the past as protein mimics (for a recent review see Singh et al.[42]). It has been used in our laboratory for the construction of vectors for neovasculature targeting in tumor therapy,[43] for multivalent presentation of carbohydrates,[44,45] or more recently as a scaffold to construct a β-amyloid peptide model.[46] Taking advantage of the synthetic versatility of this platform, we designed our candidate to display, via lysine side chains of the upper face, the clustering of the acridine cores and on the lower face, arginine side chains, Scheme 1. We placed the arginine residues to improve the poor solubility of the acridine molecules that may usually limit their in vivo use. Further, in future designs this lower face can be used to adjust the pharmacokinetic properties, the cytotoxic effects, or the membrane breaching properties by addition of other functions. This functionalization possibility is an important feature of this scaffold that allows, for example, the attachment of cell penetrating peptides (CPP)[47,48] for transport across the blood brain barrier (BBB).[49]

In our quinacrine analogue (Scheme 1), we have only kept the aliphatic side chain found in quinacrine at the nitrogen, position 9. This choice is based on studies with potent antiprion quinacrine derivatives, which revealed that a side chain on the nitrogen at this position is essential for antiprion activity,[32] unlike the chlorine and methyloxy substituent usually found on the quinacrine core.[31] At the 4 position of the aromatic core, we introduced a spacer arm allowing its grafting to the central cyclic scaffold. This linker may be beneficial to improve the conformational flexibility of the quinacrine cores on the scaffold surface, to easily reach a bioactive conformation.

Synthesis of the fibril inhibitor candidates

As chemoselective ligation by oxime bond formation is a convenient strategy widely used to assemble peptides[50–52] and improved in our laboratory[43,44] to conjugate biomolecules to cyclodecapeptidic scaffolds, its use has been found advantageous in our study. This method allows the ligation, without any coupling reagent in aqueous solution, between unprotected fragments, where one holds an oxyamine function and the other an aldehyde or ketone function. For the synthesis of our inhibitor, candidate **6** required a peptidic scaffold bearing glyoxylyl aldehyde functions on the upper lysine side chains, and a quinacrine analogue displaying the oxyamine group as the suitable complementary function (Scheme 2). For this purpose, the quinacrine derivative **4** was functionalized in the 4 position by an aminooxy spacer. Quinacrine **4** and the scaffold **5** were both synthesized separately and combined together using the oxime bond ligation.

The key cyclopeptidic scaffold **5** (Scheme 2), with the two arginine residues and the four glyoxylyl aldehyde functionalized lysine side-chains, was prepared using standard Fmoc/*t*Bu strategy from the linear peptide presenting Boc and Pmc at the lysine and arginine side chains respectively. Cyclization of the scaffold and introduction of the four glyoxyl-aldehyde

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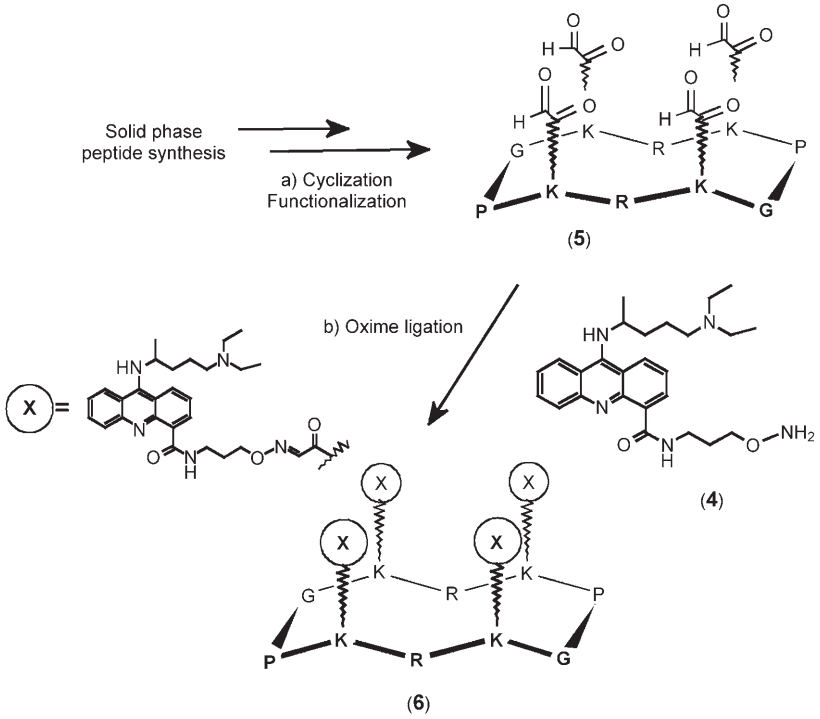
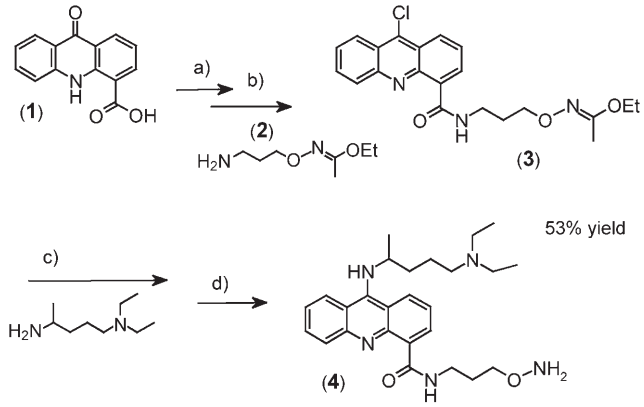


Scheme 2. Strategy for the assembly of the multimeric compound **6**: a) see ref. [45], b) CH_3CN/AcONa buffer 0.1 M pH 4, 1:1, 15 h, 57%.

functionalized lysine side chains were performed as previously described by Grigalevicius et al.[45]

The preparation of the quinacrine analogue **4** was based on a previous synthetic work on dibasic 9-aminoquinacrine-4-carboxamides by Atwell et al.[53] Starting from the acridone **1** (Scheme 3), bearing a carboxylic acid group in the 4 position,



Scheme 3. Synthesis of the quinacrine analogue **4**. a) $SOCl_2$, 80 °C, 2 h; b) CH_2Cl_2, DIEA, **2**; c) 2-amino-5-diethylaminopentane, MeOH, 70 °C, 3 days; d) HCl(g), CH_2Cl_2.

allowed us to introduce an appropriate spacer arm as an aminooxypropylamine. The aminooxy part of this linker **2** was protected with an *N*-hydroxyacetimidate and was incorporated on the quinacrine core after activation of the acridone **1** with thionyl chloride. The 9-chloroquinacrine-4-carboxamide **3** was easily obtained in this way, in CH_2Cl_2 buffered with DIEA. Treatment of **3** with the commercially available 2-amino-5-diethylaminopentane, in methanol at 70 °C for three days, produced the protected 9-aminoquinacrine intermediate with the quinacrine side chain at the 9 position. Finally acidic treatment with HCl (g) gave the oxyamine-functionalized quinacrine **4** with 53% of overall yield from acridone **1**.

The aminooxylated quinacrine **4** was then incorporated (Scheme 2) to the upper face of the scaffold **5** in acetonitrile with sodium acetate buffer following the typical chemoselective ligation procedure previously described.[45] The reaction was monitored by analytical RP-HPLC and was complete within 15 h. The conjugate **6** was isolated in 57% yield after semipreparative RP-HPLC purification (Figure 1 A) and was characterized by MS-ESI (Figure 1 B). The reference compounds, quinacrine derivative **7** and monomeric conjugate **8** (Scheme 1), were prepared by the oxime ligation of the acridine core **4** with respectively, acetone or a scaffold bearing only one glyoxylyl aldehyde function on the upper face.

In vitro inhibition studies of amyloid β-fibril formation by quinacrine compounds

Initially we examined the effects of the monomeric quinacrine analogue **7** and its multimeric version **6** on the inhibition of $A\beta_{1-40}$ fibril formation by following the specific binding of thioflavin T (ThT) to amyloid fibrils. The ThT assay is the simplest way to identify amyloid structures, and to follow their formation and disaggregation in the search for inhibitor compounds. ThT is highly specific and sensitive for the characteristic cross-β sheet structure of amyloid fibrils and its binding is easily followed by fluorescence spectroscopy because of a large excitation spectral red shift, which allows selective excitation of bound ThT.[54] The ThT assay can be performed by studying aliquots of amyloid fibril mixtures added to a ThT solution or by studying, in situ, ThT added to the fibril forming mixtures, as it has been shown to have little effect on the assembly process.[55,56]

Multimeric 6 dose dependently inhibits Aβ fibril formation

The inhibition studies of $A\beta_{1-40}$ fibril formation were performed in the presence of 10 μM ThT, in stagnant solution buffered with HPO_4^{2-} (50 mM), pH 7.4 and NaCl (100 mM) at 37 °C. We

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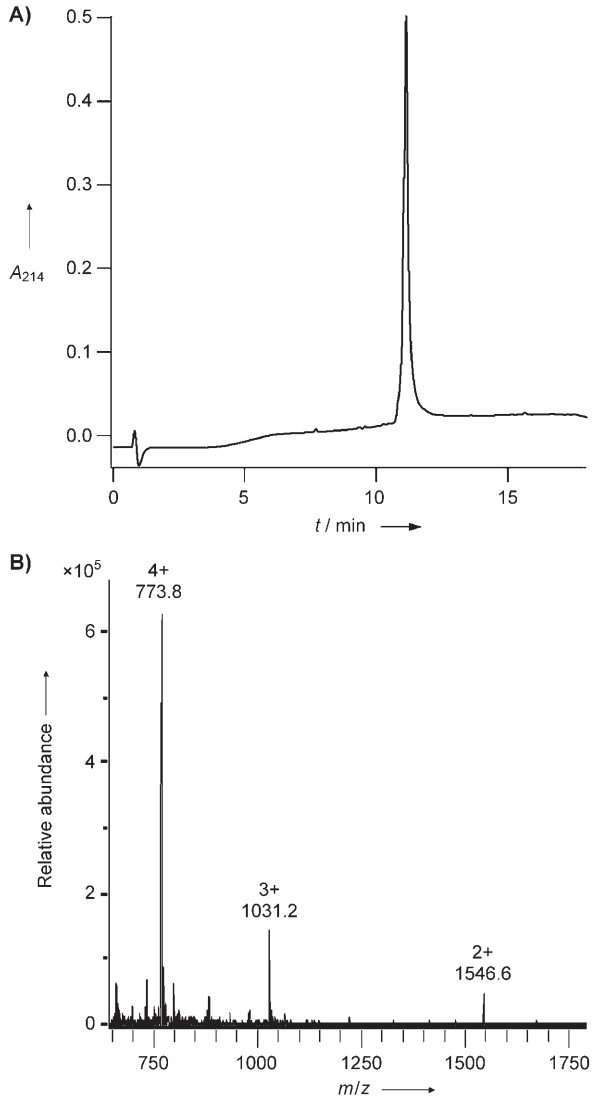

Figure 1. A) RP-HPLC profile of purified compound **6** at 214 nm. (Nucleosil 120 Å 3 μm C_{18} particles, 30×4 mm; 1.3 mL min^{-1}; linear gradient 95:5 C:D to 40:60 in 15 min; solvent D: 0.1% TFA and 9.9% H_2O in acetonitrile and solvent C: 0.1% TFA in water). B) Electrospray mass spectrum of compound **6**, the spectrum shows multiple charged species. The calculated weight is 3091.9 Da, found 3091.2 Da $[M+H]^+$.

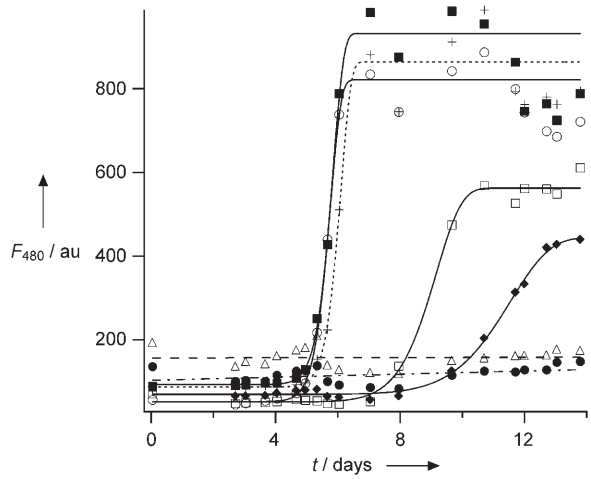

Figure 2. Inhibition studies of $A\beta_{1-40}$ (50 μM) fibril formation with the fourfold quinacrine compound **6**. Concentrations of **6** are: 0 μM (–■–), 0.1 (····+····), 1 μM (–○–), 10 μM (–□–), 20 μM (–◇–), 50 μM (·–·●·–·) and 100 μM (-----△-----). Kinetics were monitored by ThT fluorescence at 480 nm with excitation at 440 nm. The data obtained with 0 to 20 μM of **6** are fitted with extended-exponential functions. The data are representative of three independent experiments.

co-incubated $A\beta_{1-40}$ with 0, 0.1, 1.0, 10, 20, 50, and 100 μM of the multimeric compound **6** and studied for a period of 14 days the change in ThT fluorescence. In Figure 2, the results for **6** show complete inhibition with concentrations higher than 50 μM. When $A\beta_{1-40}$ was incubated with 20 μM or less of compound **6**, fibrils were formed as seen by the sigmoidal increase of the ThT fluorescence. A sigmoidal curve is consistent with the nucleation-dependent polymerization model.[7,8] From the above results an IC_{50} value for the fourfold quinacrine compound **6** is estimated to 20±10 μM (see the Supporting Information). In Figure 2 we can also see an increase in the lag phase of fibril formation with 10 and 20 μM of **6** in a dose dependant manner as compared to the sample without inhibitor, this indicates an inhibitory effect on oligomer formation during the nucleation phase. The decay of fluorescence signal for the low inhibitor concentrations at the end of the study is probably due to instability of the assay such as precipitation of amyloid.

As seen in Figure 2, the inhibition study of **6** at 100 μM shows some background fluorescence. This background is due to the fluorescence from the quinacrine moieties and was not observed at 20 μM or lower concentrations of **6**. However, monomeric quinacrine **7** displayed a strong fluorescence in the ThT fluorescence region, which prevented its study of fibril inhibition by the ThT assay (Supporting Information). The reason we were able to study the multimeric **6** and not the monomeric compound **7** is most probably due to self-quenching of the quinacrine fluorescence, in compound **6**, as the four quinacrines form a cluster on the cyclic-decapeptide scaffold.

Confirmation of multimeric 6 inhibition of Aβ fibril formation by AFM

The inhibition of fibril formation by the fourfold quinacrine compound **6** was confirmed by visualization with atomic force microscopy (AFM). When analyzing the AFM image of the $A\beta_{1-40}$ mixture co-incubated with **6** (100 μM) after 14 days (Figure 3 A), we see that no amyloid fibrils have been formed. This can be compared with the control mixture of $A\beta_{1-40}$ without inhibitor where a number of long protofilaments can be seen (Figure 3 B). These protofilaments have lengths to about 4 μm and the heights of the fibrils are 4 nm.

Multimeric 6 has enhanced inhibitory activity than the monomer 7 and the monomer conjugate 8

As compound **7** has a strong fluorescence in the same range as thioflavin T, it was essential to evaluate the extent of fibril formation by visualizing with AFM. To compare results be-

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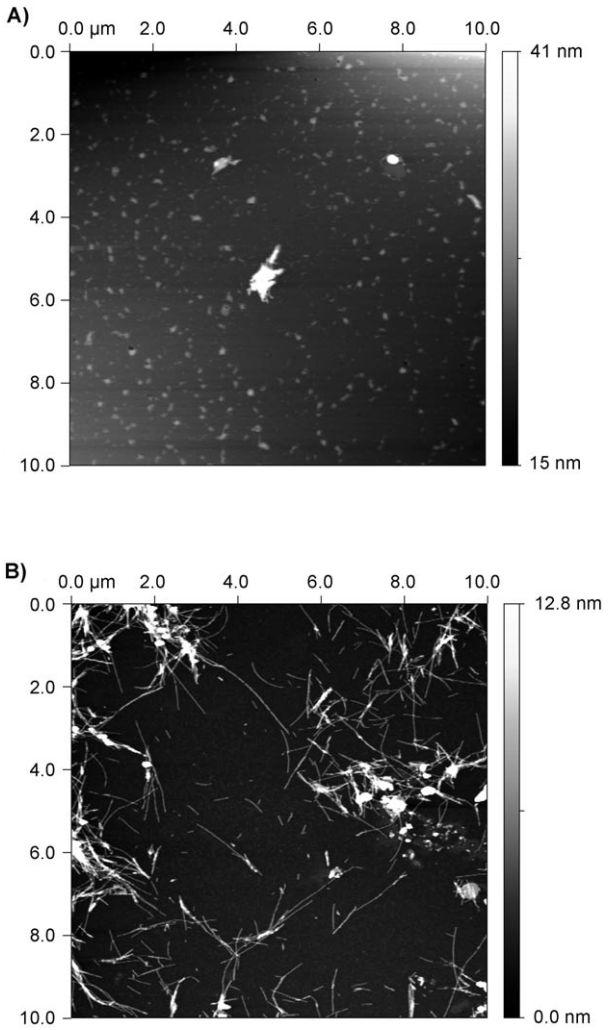

Figure 3. AFM images (height data) of inhibition studies of $A\beta_{1-40}$ obtained at the end of the aggregation reaction (14 days; scan size 10 µm). A) Co-incubation with the fourfold quinacrine compound **6** (100 µM). No fibrils are observed. B) With no inhibitor compound long protofilaments are observed with a height of 4 nm. The *Z* scale is placed to the right of the images, colors from black to white correspond to the *Z* range (height).

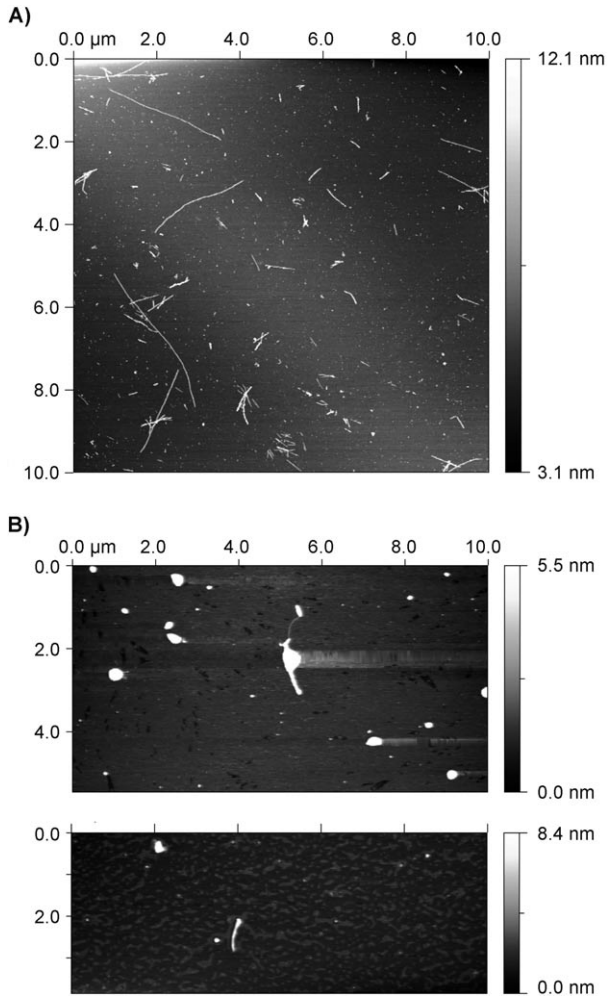

Figure 4. AFM image (height data) of protofilaments formed from $A\beta_{1-40}$ co-incubated with the monomeric quinacrine **7** at A) 100 µM and B) 400 µM. The filaments have a height of 4 nm (scan size 10 µm).

tween the multimeric **6** (100 µM) and monomer **7**, we have studied **7** at 100 µM as well as at a fourfold higher concentration (400 µM) as there are four quinacrine units on the conjugate **6**. Figure 4A is an AFM image of the $A\beta_{1-40}$ mixture, which has been co-incubated with **7** (100 µM) for 14 days. Here can be seen that this compound has no inhibitory effect on $A\beta_{1-40}$ fibril formation, as number of long protofilaments can be detected, also with lengths to about 4 µm and heights of 4 nm (as in the control study, Figure 3B). At the fourfold concentration of **7** (400 µM), fibrils were observed but to a lesser extent (Figure 4B). We cannot determine exactly an IC_{50} value for **7**, because of background fluorescence, but from the AFM study an IC_{50} value can be estimated to be much larger than 100 µM. Thus the fourfold quinacrine conjugate **6** is a much more potent inhibitor of $A\beta_{1-40}$ aggregation than the monomer **7**.

As another control experiment we have prepared a decapeptide with one quinacrine moiety, compound **8**, compared to four to prove multimeric effects. The inhibitory effect of compound **8** was determined by AFM as it also has a strong fluorescence in the ThT region. With 100 µM of this compound we found no inhibitory activity in the inhibition study of $A\beta_{1-40}$ fibril formation, thus an IC_{50} value can be estimated to be >100 µM (Figure 5).

To determine if the cyclic decapeptide scaffold of our multimeric compound alone induces antiamyloidogenic activity, we prepared a scaffold without quinacrine moieties as a control compound. This control **9** contains acetylated lysine residues instead of lysinyl-quinacrine functions. In $A\beta_{1-40}$ fibril formation studies followed by ThT fluorescence, this control **9** showed, at 100 µM, a very weak inhibition effect (Supporting Information).

Together AFM and ThT studies revealed an induced inhibitory effect with our multimeric conjugate **6** with an IC_{50} value of 20 ± 10 µM, where as the monomeric compounds **7** and **8** at 100 µM showed no inhibitory effect. To compare the inhibitory activity with other reported inhibitors of Aβ fibril formation using our assay conditions, we included tetracycline in our study (Supporting Information). Tetracycline has a reported IC_{50} value of 50 µM in a inhibition study of 220 µM of $A\beta_{1-42}$.[20]

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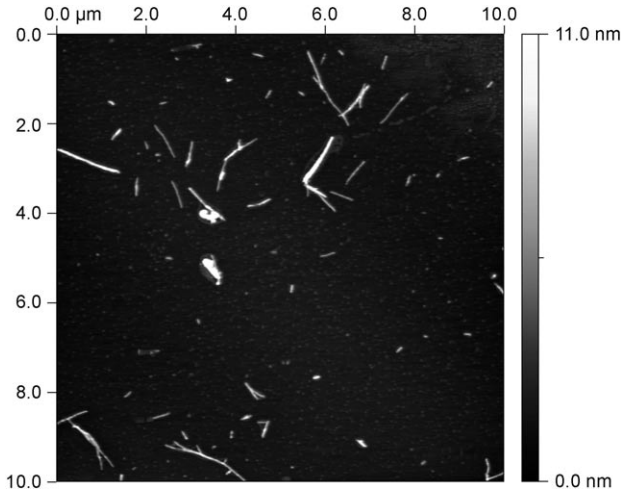

Figure 5. AFM image (height data) of protofilaments formed from $A\beta_{1-40}$ co-incubated with the monomeric conjugate **8** at 100 µM.

With our experimental conditions, using $A\beta_{1-40}$, we have determined an IC_{50} value of 30 µM for tetracycline this is comparable to that previously reported and to our conjugate **6**.

Multimeric 6 inhibits fibril growth on preformed fibril seeds

The inhibitory effect of **6** on $A\beta_{1-40}$ fibril formation was also studied by the ThT assay in the presence of preformed $A\beta_{1-40}$ fibrils as seeds. The kinetics revealed exponential-like formation of fibrils with a strongly reduced lag phase (Figure 6). Incubating $A\beta_{1-40}$ with increasing concentrations of **6** showed a dose dependent decrease of the final fluorescence level. No formation of fibrils was observed with 100 µM of **6** in the ThT kinetic data and this was confirmed by AFM imaging (data not shown). From this study an apparent IC_{50} value of 2 ± 1 µM for compound **6** was determined.

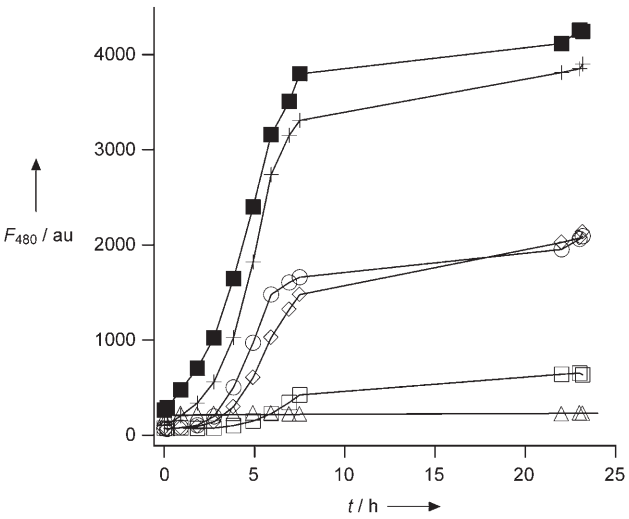

Figure 6. Inhibition studies of $A\beta_{1-40}$ (50 µM) fibril formation with the fourfold quinacrine compound **6**, in the presence of sonicated preformed $A\beta_{1-40}$ fibrils (2.5 µM). Concentrations of **6** are: 0 µM (■), 0.1 µM (+), 1 µM (○), 2 µM (◇), 10 µM (□) and 100 µM (△). The data are representative of two independent experiments.

Seeding experiments were also performed with the control compounds **7** and **8**, the decapeptide scaffold **9** with acetylated lysines and tetracycline. The effects of compounds **7** and **8** were determined by visualizing with AFM. In the presence of 400 µM of **7**, formation of snake-like fibrils was observed, and normal fibril growth was seen with 100 µM of **8**, showing that these monomeric forms of quinacrine do not inhibit fibril growth (Supporting Information). Thus, again the specificity of multimeric **6** on fibril inhibition was shown as monomers **7** and **8** show no inhibitory activity. The decapeptide scaffold **9** and tetracycline were followed by the ThT assay in the seeding study. The control **9** showed weak inhibitory activity at 100 µM. For tetracycline we determined an IC_{50} value of 80 µM, we have not found a previous report of a seeding study with tetracycline (Supporting Information).

Finally, we studied the ability of compound **6** to disaggregate preformed amyloid fibrils. In the studied concentration range from 0 µM to 100 µM, no decrease of the fluorescence was observed in the ThT assay. Fibrils were also observed by AFM imaging after co-incubating for eight days with 100 µM of the quinacrine compound **6** (data not shown). Thus, compound **6** does not markedly dissociate preformed amyloid fibrils in the studied concentration range. With the control scaffold with four *N*-acetylated lysine residues **9**, we observed no inhibitory activity in the destabilization study followed by ThT fluorescence (Supporting Information). Disaggregation studies were however not performed on monomeric compounds **7** and **8**. Indeed, as no inhibition was observed in experiments with or without fibril seeds, it is unlikely that they could disaggregate fibrils.

Discussion

The aggregation and formation of fibrils from Aβ is widely accepted to be a key factor in the development of AD. The cytotoxic mechanisms, however, are still not well understood and the identity of the aggregation state that inserts toxicity remains unclear. The toxicity has been proposed to arise from unstructured assemblies during the nucleation phase, fibrils, amyloid, or combinations of these. For this reason different therapeutic approaches are pursued that target different stages in the accumulation of Aβ and formation of amyloid. Important strategies include, stimulating the immune system to remove Aβ from the brain,[15] direct inhibition of the Aβ self-assembly process,[16,17] and reduction of Aβ production.[14]

However, strategies that preferentially target $A\beta_{1-40}$ production may actually worsen the disease course in AD as recently pointed out by McGowan and co-workers.[57] They have crossed transgenic mice that selectively express high levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ and thereafter compared the cerebrovascular Aβ deposition in the bitransgenic and singly transgenic littermates. From their studies they could see that levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ had opposing effects on Aβ deposition, where $A\beta_{1-40}$ inhibited amyloid deposition and $A\beta_{1-42}$ promoted it.

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They also observed that increasing levels of $A\beta_{1-40}$ in the bitransgenic mice protected them from premature-death, which was observed in the $A\beta_{1-42}$ transgenic mice.

For these reasons, the development of drugs that inhibit Aβ aggregation seems to be a more promising therapeutic approach in the prevention and control of AD compared to the strategies that preferentially target $A\beta_{1-40}$ production. Following this approach, we have investigated in this report the potential of a multimeric quinacrine conjugate **6** as a lead inhibitor compound against fibril formation associated with AD. Our drug candidate exhibits a directional and well-defined cluster of four quinacrines that was easily prepared by the oxime-ligation strategy. We performed in vitro analysis of the inhibitory effects of this compound, where we monitored the formation of $A\beta_{1-40}$ fibrils directly using fluorescence spectroscopy with ThT. We showed herein that the quinacrine compound **6** displays an antiamyloidogenic effect and dose dependently increased the lag phase of fibril formation. This lag phase increase indicates an inhibitory effect during the nucleation phase, such as inhibition of oligomer formation. Complete inhibition of fibril formation was observed at 50 μM of compound **6** by the ThT assay and this was confirmed by imaging with AFM where no fibrils were observed. From this study we have estimated the IC_{50} value to 20 ± 10 μM.

To evaluate the multimeric inhibitory effects of the fourfold quinacrine compound **6** we compared **6** with the monomeric quinacrine derivative **7** on the inhibition of $A\beta_{1-40}$ fibril formation. As a result of strong fluorescence from the monomeric quinacrine, we were only able to study fibril formation by AFM. We found no observable decrease in the amount or change of the fibril structure with a 100 μM concentration of **7** as compared to the control without inhibitor. In a comparison of monomer **7** and the fourfold quinacrine compound **6** we have to consider the fact that compound **6** holds four times more of the quinacrine moiety than **7**. For this reason we have studied the inhibition effects of **7** at 400 μM by AFM. The formation of fibrils also observed at this concentration indicates that the multimeric presentation of **6** enhances its inhibitory activity. Further, the results from the control experiment performed with the monomeric conjugate **8**, strengthen the evidence of multimeric effects of the inhibitor compound **6**. Indeed, with 100 μM of **8** we found no inhibitory activity in the inhibition study of $A\beta_{1-40}$ fibril formation. The control compound **9**, which is the scaffold alone, was studied to see if it induces inhibitor activity. With this compound at 100 μM, only a slight decrease of the ThT fluorescence was observed. Together these results demonstrate that the induced inhibitory activity of compound **6** arises from the fourfold cluster of quinacrines that are attached to the decapeptide scaffold.

To determine if the inhibitory effect of compound **6** was entirely due to an increase of the lag phase, we performed seeding experiments, where preformed fibrils were added to the $A\beta_{1-40}$ aggregation mixture. This procedure eliminates the processes during the lag phase, allowing us to follow only the exponential growth and inhibition of the $A\beta_{1-40}$ fibrils. In this study we observed a dose dependent decrease of the final fluorescence level. In the aggregation mixture with 100 μM of the fourfold quinacrine compound **6**, no formation of fibrils was observed in the ThT assay or by AFM imaging. From this study we determined an apparent IC_{50} value of 2 ± 1 μM for compound **6**. We conclude from the seeding experiment that compound **6** inhibits fibril growth and that inhibition is not only due to inhibition of oligomer formation during the lag phase. The apparent IC_{50} values obtained from the inhibition experiments, with and without preformed fibrils as seeds, are 2 ± 1 and 20 ± 10 μM, respectively. The difference of these two values may be due to differences of inhibitory mechanisms (see below), but also to the very nature of the assay as described by Findeis et al.[58] As mentioned by them, the assay is reliable for the identification of potent inhibitors of Aβ polymerization but accurate determination can be difficult.

The control compounds **7**–**9** were also studied with seeding experiments. The results from these studies correlate well with the studies that were performed without the addition of preformed fibrils and thus fibril formation was observed for all controls. Interestingly, in the presence of **7** at 400 μM formation of snake-like fibrils were observed, this may be due to the mere high concentration of compound **7** that changes the morphology of the forming fibrils (Supporting Information).

We further analyzed the inhibitory mechanisms of compound **6** with disaggregation studies. However, the disaggregation ability of compound **6** on preformed amyloid fibrils was not very strong, as we were able to observe fibrils by AFM imaging from a mixture of preformed fibrils and 100 μM of **6** after an incubation period of eight days.

Even though compound **6** was not able to disaggregate preformed $A\beta_{1-40}$ fibrils, it has from the unseeded and seeded experiment an IC_{50} value of 20 ± 10 μM and 2 ± 1 μM for $A\beta_{1-40}$ fibril formation and inhibition, respectively, and thus can be compared with other reported inhibitors. For example, the antiamyloidogenic activity of tetracycline was determined here, with our experimental conditions, to have an IC_{50} value of 30 μM which is similar to that previously reported (IC_{50} value of about 50 μM).[20] Another reported inhibitor is 2-amino-4-chlorophenol with an IC_{50} value of 10 μM.[59] Nordihydroguaiaretic acid (NDGA) is a polyphenolic natural product isolated from the creosote bush *Larrea tridentata*.[60] NDGA is expected to bind to free Aβ and has a reported IC_{50} value of 0.16 μM, it is also a potent oxygen radical scavenger.[61] The modified Aβ fragment cholyl-LVFFA is a β-sheet breaker peptide which uses the important recognition sequence LVFFA of the Aβ sequence to bind to the growing fibrils and disturb and dissociate them; it has a reported IC_{50} value of about 5 μM.[58] Rifampicin, a semisynthetic antibiotic drug also suggested to scavenge hydroxyl free radicals,[62] is an another reported inhibitor of Aβ fibril formation with an IC_{50} value of 6 μM.[63] From the above, our multimeric conjugate is highly competitive as an inhibitor against Aβ polymerization, also its ability to delay polymerization is of interest as was observed with the Aβ fragment LVFFA.[58]

When considering the antiamyloidogenic mechanisms of inhibitors, one should consider the peptide interactions that induce and stabilize the fibril structure. It is believed that hydrophobic interactions play an important role in Aβ aggregation. Also the aromatic residues are expected to contribute to

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the stability, specificity, and directionality of the fibrils cross-β sheet structure, by means of aromatic stacking and their rigid properties.[64] The two phenylalanines at positions 19 and 20 in the Aβ sequence have been determined to be essential for amyloid formation.[65] Thus, it is conceivable that potential inhibitors would have hydrophobic and aromatic components that could interfere with the hydrophobic and aromatic stacking interactions that are formed during the different stages of fibril formation. In addition to hydrophobic interactions, potential inhibitors would also be able to form hydrogen bonds with the Aβ backbone amides thus disturbing β-sheet formation.[24] As detailed information of known inhibitors and their interactions with fibrils are not available, with the above reasoning one can speculate on the mechanisms of fibril inhibition. Forloni et al. have proposed the antiamyloidogenic activity of tetracycline to be related to hydrophobic, aromatic, and specific hydrogen bond formation with residues of Aβ.[20] Felice et al. have suggested that some di- and tri-substituted aromatic compounds, such as 2-amino-4-chlorophenol, bind to the hydrophobic region of Aβ by hydrophobic and/or π-π interactions thus blocking associations between Aβ molecules which would lead to fibril formation.[59] Rifampicin is also known to bind to Aβ through hydrophobic interactions thus inhibiting its aggregation.[63]

Regarding the antiamyloidogenic mechanism of the current multimeric quinacrine compound **6**, the folding interactions during the nucleation phase (oligomer formation) as well as during the formation of fibrils should be taken into account. This is due to the fact that compound **6** induced a longer lag phase and also inhibited further fibril growth in the seeding study with preformed fibrils. The propensity of **6** to bind to specific regions of $A\beta_{1-40}$ may occur through a number of interactions as described above. The planar aromatic structure of the quinacrine cores together with multimeric interactions may bind Aβ through π-π interactions thus blocking associations between Aβ molecules and inhibiting fibril formation. In the same way, the aromatic core could bind Aβ molecules through hydrophobic interactions. Also in addition, the current compound could interact with Aβ through a number of polar interactions such as by the basic functions on the 9 position of the quinacrine, the oxime attachment to the scaffold, and the scaffold itself. The fact that there was no detectable inhibition with the monomeric compound **7** and especially with the monomeric conjugate **8** implies that the inhibitory mechanism of compound **6** is a result of the multiple quinacrines that are directionally displayed in a convenient manner on the central scaffold. As May et al. explained the improved activity of dimers of acridine as antiprion inhibitors compared to the corresponding monomers "Conformational restriction has been used extensively in drug design to pre-organize ligands into a bioactive conformation" and "a rigid scaffold can correctly position and orient key structure features without having to undergo an entropically costly conformational rearrangement before binding".[29]

It is also reasonable to consider that our multimeric compound could bind specifically to Aβ through a mechanism yet unknown, which leads to the observed antiamyloidogenic properties. Further NMR studies could be of interest to give answers about this mechanism and the interactions of this conjugate with free Aβ.

Other reports have shown binding of 9-aminoacridine compounds to β-sheet rich peptides.[35,66] Such as Phuan et al. that recently reported a chemical proteomic approach where they functionalized a Sepharose matrix with a bis-9-aminoacridine compound.[34] From binding studies with the prion protein from scrapie-infected cultured cells, termed PrP, they observed selectivity of the 9-aminoacridine matrix for the misfolded and β-sheet rich PrP^{Sc} as compared to the normally folded PrP^{C}. From experimental studies they conclude that this selectivity must be due to conformational and/or oligomeric differences between PrP^{C} and PrP^{Sc}. They suggest that the possible mechanism of action of antiprion 9-aminoacridine compounds on inhibiting PrP^{Sc} replication may occur by modification of templating epitopes for PrP^{C} conversion or by altering the stability of PrP^{Sc} oligomers. In view of this study, our quinacrine compound **6** could also interact with cross β-sheets formed during oligomerization.

It is interesting to note, as pointed out by Cohen and Kelly that fibril disaggregating compounds may shift the equilibrium of Aβ aggregation from fibrils to toxic oligomers.[16] Considering this, the inhibitory effect of our multimeric quinacrine compound on Aβ fibril formation, displays an interesting profile where preformed fibrils are not disaggregated but fibril growth is inhibited. Such an inhibiting profile is interesting for the treatment of AD patients, where fibrils are not transformed into toxic oligomers and further fibril growth is inhibited, facilitating free Aβ clearance by proteolytic degradation.

When considering the potential therapeutic use of our multimeric acridine compound **6** and especially its potential use as a drug against AD, two major questions arise. What about its cytotoxicity as a DNA intercalating agent and the ability of this high-molecular weight molecule to pass through the blood brain barrier (BBB)? In this preliminary study, these two parameters have not been evaluated and should be taken into account in further investigations. Nevertheless some comments about these are discussed below.

The cytotoxicity of 9-aminoacridine compounds,[29,33] potential inhibitors of prion replication, and of 9-aminoacridinecarboxamide derivatives[53] is undoubtedly dependent on their ability to interact with DNA. The cytotoxicity–structure relationship is not easy to evaluate. Not only do the substituents of the acridine heterocycle appear to be key factors in determining cytotoxicity but also the steric hindrance and the restricting conformational flexibility of the heterocycles as reported for bis-acridine intercalators.[67] In light of this, the rigid scaffold of our conjugate **6** could result in a lower cytotoxicity than the multimeric acridine version. Moreover the possibility of an easy chemical modulation of our assembly, especially on the lower face, could help to overcome the problem and to achieve a convenient balance between desirable bioactivity and cytotoxicity.

As for the ability to cross the blood brain barrier (BBB), it is an important issue that needs to be addressed for drugs that are intended to be active in the human brain. The penetration

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to the central nervous system is one of the major hurdles for compounds that are effective in vivo against AD. The in vivo potential of our multimeric compound lies in the fact that it can be further functionalized with targeting and membrane breaching properties. An example of a modified version of our quinacrine complex, would be to attach a cell penetrating peptide (CPP) such as Tat to the central scaffold.[47,48] Tat is a short 11 residue sequence, derived from the HIV transactivator of transcription,[49] which has been shown to be able to transport large proteins across the BBB. Conjugation of a CPP can occur on the opposite side of our scaffold that currently holds two arginine residues.

In conclusion, we show in this study that the designed multimeric acridine conjugate dose-dependently inhibits fibril formation from fresh $A\beta_{1-40}$, in vitro. We demonstrated that not only does our compound inhibit fibril formation, but that its antiamyloidogenic activity is due to the multimeric assembly of the four acridines. Even though the toxicity is unknown and the exact mechanism remains unclear, this multimeric compound should be considered as a lead for the development of a new class of well-defined inhibitors against amyloid fibril formation in Alzheimer's disease. Indeed, the possibility of chemical modulation of our assembly opens up the opportunity to further optimize its inhibitory properties for in vivo use as a therapeutic agent and as a tool to study fibril formation mechanisms.

Experimental Section

Materials: All chemical reagents, protected amino acids, chlorotrityl® resin, and solvents were purchased from Sigma Aldrich, Fluka, Acros, Carlo–Erba, or France Biochem S.A. and were used without further purification. Synthetic $A\beta_{1-40}$ was prepared as previously described.[68] The 9-oxoacridan-4-carboxylic acid **1** was obtained as described by Atwell et al.[53] The linker (Ethyl-*N*-hydroxyacetimidate)propylamine **2**[69] was built up by reaction of ethyl-*N*-hydroxyacetimidate with 3-bromopropylphtalimide followed by selective removal of the phtalimide.

Reversed-phase HPLC analyses were performed on Waters equipment using C_{18} columns. For the HPLC analyses of compounds **1**–**3**, a Waters μ-bondapack column 120 Å 10 μm, 300×3.9 mm^2 was used and operated at 2 mL min^{-1} using a linear A-B gradient of 0 to 100% B in 10 min (solvent A: phosphoric acid solution in water, pH 2.5; solvent B: MeOH/H_2O, 95/5, *v*/*v*). For HPLC analyses of compounds **4**–**7**, a Nucleosil column 120 Å 3 μm, 30×4 mm^2 was operated at 1.3 mL.min^{-1} with UV monitoring at 214 nm and 250 nm using a linear C-D gradient (5 to 60% D in 15 min run time; solvent C: H_2O containing 0.1% TFA; solvent D: CH_3CN containing 9.9% H_2O and 0.1% TFA). For HPLC purification of **4**–**7**, the preparative column, Delta-Pak™ 300 Å 15 μm, 200×25 mm^2 was operated at 22 mL min^{-1} with linear C-D gradient programs in 30 min run time. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC300 spectrometers. Mass spectra were obtained by electron spray ionization (MS-ESI) on a VG Platform II in the positive mode.

Synthesis of *N*-[propyl(ethyl-*N*-hydroxyacetimidate)]-9-chloroquinacrine-4-carboxamide 3: The 9-oxoacridan-4-carboxylic acid **1** (1.46 mmol) was chlorinated by $SOCl_2$ as previously described.[53] The obtained 9-chloroquinacrine-4-carbonyl chloride was then cooled to −10 °C under argon and to this was added in one portion an ice-cold solution of (Ethyl-*N*-hydroxyacetimidate)propylamine **2** (350 mg, 2.19 mmol) and diisopropylethylamine DIEA (381 μL, 2.19 mmol) in anhydrous CH_2Cl_2 (3 mL). After being stirred for 10 min at −10 °C, the reaction solution was warmed to room temperature and left for a further 2 h. The mixture was diluted with CH_2Cl_2 (10 mL), washed twice with 10% aqueous Na_2CO_3, and once with saturated aqueous NaCl. The organic layer was then dried on Na_2SO_4 and evaporated. The compound **3** was obtained as yellow brown oil in quantitative yield and used without further purification. The structure of **3** was confirmed by ^{1}H and ^{13}C NMR analyses (300 and 75.5 MHz, $CDCl_3$). HPLC: t_R = 9.7 min; MS-ESI: calcd 399.8, found 400.0 [M+H$^+$].

Synthesis of *N*-[propyloxyamine)]-9-amino(5-diethylaminopentane)quinacrine-4-carboxamide 4: The crude **3** was dissolved (1.46 mmol) in HPLC grade MeOH (3 mL) and the commercially available 2-amino-5-diethylaminopentane, was added in one portion. The mixture was heated at 70 °C under stirring until the disappearance of the starting material (seven days). The solvent was evaporated to get the *N*-[propyl(ethyl-*N*-hydroxyacetimidate]-9-amino(5-diethylaminopentane)quinacrine-4-carboxamide as a brown oil. This intermediate was dissolved in anhydrous CH_2Cl_2 and the mixture was cooled at 0 °C before adding gaseous HCl. No precipitation of the salt was observed. The solvent was evaporated under high vacuum to get a brown powder (630 mg, 72%). The crude was dissolved in solvent C and purified by RP-HPLC (5 to 60% D in 30 min, t_R = 12 min) to get 53% of pure compound **4** from the starting material **1**. HPLC: t_R = 7.7 min; ^{1}H NMR (300 MHz, D_2O): δ = 0.95 (t, 3H; CH_3), 0.98 (t, 3H; CH_3), 1.36 (m, 2H; CH_2), 1.53 (d, 3H; CH_3), 1.71 (m, 2H; CH_2), 2.00 (m, 2H; CH_2), 2.85 (m, 6H; 3CH_2), 3.47 (t, 2H; CH_2), 4.15 (t, 2H; CH_2), 4.40 (m, 1H; CH), 7.34–7.43 (m, 3H; H2, H5 et H7), 7.82 (dd, ^3J(H,H) = 7.4 Hz, ^4J(H,H) = 0.9 Hz, 1H; H6), 7.96 (d, ^3J(H,H) = 8.5 Hz, 1H; H1 or H8), 8.02 (dd, 1H; H8 or H1), 8.09 ppm (d, ^3J(H,H) = 8.4 Hz, 1H; H3); ^{13}C NMR (75.5 MHz, D_2O): δ = 8.2, 8.3, 20.3 (3CH_3), 20.5, 27.3, 33.7, 36.9, 47.4, 47.6, 51.0 (7CH_2), 55.8 (CH), 73.4 (CH_2), 110.8, 114.7, 118.6, 118.8 (4C_{aro}), 119.4 (CH_{aro}), 122.4 (C_{aro}), 122.9, 125.2, 135.0, 136.4 (4CH_{aro}), 158.3 (CO), 162.7 (q, CF_3), 169.0 ppm (CF_3COOH); MS-ESI: calcd 451.6, found 452.2 [M+H]$^+$.

Synthesis of glycoxyl-aldehyde functionalized cyclic-decapeptide scaffold 5: The cyclic scaffold was prepared following the previously reported strategy by Grigalevicius et al.[45] First the linear peptide, K(Boc)-R(Pmc)-K(Boc)-P-G- K(Boc)-R(Pmc)-K(Boc)P-G, was built up on chlorotrityl® resin (300 mg) with a loading of 0.6 mmol g^{-1}, by standard solid phase procedures. Coupling conditions were, *N*-Fmoc amino acids (1.5 equiv), PyBOP (benzotriazol-1-yloxy)tris (pyrrolidino)phosphonium hexafluorophosphate) (1.5 equiv), and DIEA (4 equiv) in DMF. *N*α-Fmoc removal was performed in piperidine solution (20% in DMF). The linear peptide was cleaved from the resin with a solution of 1%TFA in CH_2Cl_2. After evaporation of solvent, the crude was precipitated and washed in diethyl ether to get a white powder (88%). Thereafter, the quantitative cyclization was performed under high dilution for 3 h in acetonitrile with PyBOP as coupling reagent and DIEA to adjust the pH to 8–9. After solvent removal, the cyclic peptide was precipitated and washed in ether (quantitative yield). The removal of Boc and Pmc protecting groups from the lysine and arginine residues was achieved in TFA acidic conditions during 3 h (140 mg, 77%). The lysine side chains were thereafter coupled with protected serine residues, BocSer(*t*Bu)OH, using standard coupling conditions. The Boc and *t*Bu on the serine residues were removed with a solution of TFA/TIS/H_2O (95:2.5:2.5) for 2 h, then serine oxidation was carried out in water during 1 h using an excess of $NaIO_4$. The

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solution was diluted with water and purified by RP-HPLC (t_R = 10 min, 5 to 40% D in 30 min) to afford the glycoxyl-aldehyde scaffold **5** (69 mg, 32% for the three last steps). HPLC: t_R = 5.8 min; MS-ESI: calcd 1357.50, found 1357.5 $[M+H]^+$, 1427.4 $[M+4H_2O+H]^+$, 715.2 $[M+4H_2O+2H]^{2+}$.

Synthesis of the multimeric compound 6: The scaffold **5** (20 mg, 12.62 μmol included TFA salts) was dissolved in AcONa buffer (0.1 mM, pH 4) (3 mL). The oxyamine-quinacrine **4** (45 mg, 75.60 μmol) was dissolved in CH_3CN (3 mL) and added to the mixture and stirred at room temperature for 15 h. The reaction was followed by RP-HPLC. The product **6** was isolated after RP-HPLC purification (t_R = 18 min, 5 to 60% D in 30 min) as a yellow powder (24 mg, 57%). HPLC: t_R = 11.1 min; MS-ESI: calcd 3091.9, found 619.0 $[M+5H]^{5+}$, 773.8 $[M+4H]^{4+}$, 1031.4 $[M+3H]^{3+}$, 1546.6 $[M+2H]^{2+}$, 3091.2 $[M+H]^+$.

Synthesis of the compound 7: The oxyamine-compound **4** (38 mg, 0.068 mmol) was dissolved in 4 mL of acetone/solvent D (CH_3CN containing 9.9% H_2O and 0.1% TFA), 1/1. The solution was stirred at room temperature for 3 h and then evaporated. No precipitation of the compound was observed by adding ether, dichloromethane, or ethyl acetate. The crude was dissolved in solvent D and purified by RP-HPLC (5 to 60% D in 30 min) to get 27 mg of pure compound as a TFA salt (52%). HPLC: t_R = 9.2 min; ^{1}H NMR (300 MHz, D_2O): δ = 1.06 (t, 3H; CH_3), 1.10 (t, 3H; CH_3), 1.52 (m, 2H; CH_2), 1.66 (d, ^3J (H,H) = 6.3 Hz, 3H; CH_3), 1.81, 1.83 (2 s, 6H; 2CH_3), 1.89 (m, 2H; CH_2), 2.06 (m, 2H; CH_2), 2.92–3.05 (m, 6H; 3CH_2), 3.60 (t, 2H; CH_2), 4.19 (t, 2H; CH_2), 4.68 (m, 1H; CH), 7.50–7.59 (m, 2H; H2, H7), 7.68 (d, ^3J(H,H) = 8.4 Hz, 1H; H5), 7.96 (dd, ^3J-(H,H) = 7.4 Hz, 1H; H6), 8.17–8.21 (m, 2H; H1, H8), 8.33 ppm (d, ^3J-(H,H) = 8.3 Hz, 1H; H3); ^{13}C NMR (75.5 MHz, D_2O): δ = 8.3, 8.4, 15.6, 20.4 (4CH_3), 20.6 (CH_2), 21.1 (CH_3), 28.2, 33.8, 37.7, 47.6, 47.7, 51.1 (6CH_2), 55.7 (CH), 71.3 (CH_2), 118.7, 114.8 (2C_{aro}), 119.5 (CH_{aro}), 119.8 (C_{aro}), 123.0, 125.1, 135.0, 136.5 (4CH_{aro}), 158.9 (CO), 160.4 (CF_3COOH), 168.6 ppm (CF_3COOH); MS-ESI: calcd 491.7, found 492.2 $[M+H]^+$, 246.3 $[M+2H]^{2+}$.

Synthesis of the monomeric compound 8: Compound **8** was prepared as previously described for **6** by ligation of the oxyamine-acridine **4** and the cyclic scaffold c[K(COCHO)-R-A-P-G-A-R-A-P-G]. HPLC: t_R = 11.8 min; MS-ESI: calcd 1450.8, found 363.7 $[M+4H]^{4+}$, 484.7 $[M+3H]^{3+}$, 726.7 $[M+2H]^{2+}$, 1452.0 $[M+H]^+$.

Synthesis of the scaffold 9: The scaffold **9** c[K(Ac)-R-K(Ac)-P-G-K(Ac)-R-K(Ac)-P-G], was prepared by acetylation of the cyclopeptide c[K-R-K-P-G-K-R-K-P-G], intermediate in the synthesis of **5**, using a mixture of Ac_2O/Py/DMF, 1/2/7. After 30 min under stirring, the solvents were removed and compound **9** was precipitated and washed in ether. HPLC: t_R = 11.0 min; MS-ESI: calcd 1300.7, found 434.5 $[M+3H]^{3+}$, 651.4 $[M+2H]^{2+}$, 1301.7 $[M+H]^+$.

Preparation of $A\beta_{1-40}$ peptide stock solution: For aggregation assays, a stock solution of $A\beta_{1-40}$ was prepared as follows: $A\beta_{1-40}$ (2.7 mg) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (200 μL) to disassemble preformed aggregates, thereafter it was lyophilized. One mL of pure water was added to the lyophilized peptide and the solution was centrifuged at 12000 *g* to remove eventual aggregates. The stock solution was divided into portions and stored at −20 °C until use. The stock concentration of $A\beta_{1-40}$ was 500 μM.

Preparation of inhibitor stock solutions: Inhibitors were dissolved in DMSO. Stock solutions of 10 to 20 mM were first prepared, thereafter they were diluted with DMSO to the following concentrations 5 mM, 500 μM, 50 μM, and 5 μM. Final concentrations of DMSO in inhibition studies were 2%.

Aggregation measurement of $A\beta_{1-40}$: Aggregation of $A\beta_{1-40}$ was performed in 96-well black polypropylene microplates (Greiner). To each well an aliquot of the peptide stock solution was mixed into the aggregation buffer giving a final composition of $A\beta_{1-40}$ (50 μM) and ThT (10 μM) in sodium phosphate (50 mM) and NaCl (100 mM) pH 7.4. Thereafter 2 μL aliquots of the inhibitor compounds were added, giving the aggregation mixture a total volume 100 μL. Microplates were sealed with a plastic sheet and incubated in a Molecular Devices Spectra MAX Gemini XS microplate reader at 37 °C. Kinetic data were fitted with the stretched exponential function: $F(t) = F(\infty) - \Delta F \exp(-(kt)^n)$, where $F(t)$ is the fluorescence at time t, $F(\infty)$ is the fluorescence after complete fibril formation, ΔF is the difference in fluorescence between $t(0)$ and $t(\infty)$, k is the rate constant, and values larger than 1 for the parameter n indicate a sigmoidal transition with an initial lag-phase.[70]

Aggregation measurement with preformed fibrils: The seeding experiment was identical to the aggregation measurement of $A\beta_{1-40}$, with the addition of preformed fibrils (2.5 μM). The preformed fibrils of $A\beta_{1-40}$ were prepared from the aggregation measurement of $A\beta_{1-40}$ without inhibitor compounds (described above). Fibrils were sonicated for ten seconds immediately before use.

Fibril destabilization: The preformed fibrils of $A\beta_{1-40}$ were prepared as described above, by following the formation of fibrils with the ThT assay. The fibrils were mixed and sonicated (2 × 10 s) and left overnight before the destabilization study. The experimental conditions were identical to the aggregation measurement of $A\beta_{1-40}$.

Determination of IC_{50} values: The IC_{50} values were determined from the fluorescence values obtained at the end of the kinetic studies. Thereafter we plotted these values against Log of the inhibitor concentration. Fitting the data with a sigmoidal function in the Igor Pro software (WaveMetrics) the IC_{50} value was obtained at the fluorescence halfway point.

Fluorescence spectroscopy: Microplates were analyzed in a Molecular Devices Spectra MAX Gemini XS microplate spectrophotometer at 37 °C. Measurements of ThT binding were recorded once or twice daily using bandpass filters of 440 nm for excitation and 480 nm for emission and a cut-off filter of 475 nm was used.

Atomic force microscopy: An aliquot (3 μL), from each inhibition study was withdrawn at the end of the aggregation reaction and deposited onto freshly cleaved mica sheets for AFM inspection. The sample was incubated on mica for 2 min followed by three washes with water (5 μL) to gently remove the material not adsorbed to the substrate. The mica was dried in air for one hour. In-air AFM images were acquired in noncontact mode in a vibration insulated environment, using a PicoPlus microscope (Molecular Imaging), equipped with a PicoScan-3000 controller. For imaging, we used single beam aluminum-coated cantilevers (type NSC36/ALBS, μmasch) with Rc < 10 nm, 110–130 μm lengths and nominal spring constant 0.6 N m^{-1}. The drive frequency was between 200 and 400 kHz.

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