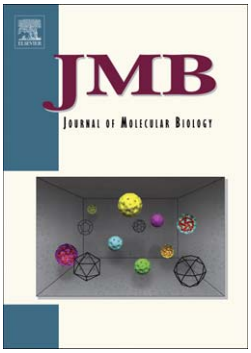

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Title:

New Strategy for the Generation of Specific D-peptide Amyloid Inhibitors

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Abbreviations:

CD: Circular Dichroism; EM: Electron Microscopy; DMSO: Dimethylsulfoxide

Running Title:

New Strategy for Inhibition of Amyloid Formation

Summary

The conversion of a soluble protein into β-sheet-rich oligomeric structures and further fiber formation is a critical step in the pathogenesis of the group of human diseases known as amyloidoses. Drugs that interfere with this process may thus be able to prevent or/and cure these diseases. Recent results have shown that short amino acid stretches can provide most of the driving force needed to trigger amyloid formation of a protein. These evidences suggest that compounds that specifically bind to peptides synthesized upon the sequence of such amyloidogenic protein stretches might be also able to inhibit amyloid formation of the corresponding full-length protein and likely amyloid-induced cytotoxicity as well. Here, we present a general strategy to obtain D-peptides that specifically interact with protein amyloid stretches. The screening of a D-peptide combinatorial library for inhibitors of an amyloidogenic peptide designed *de novo* has allowed us to extract a set of empirical rules for the design of D-peptide inhibitors of any 6-residue amyloidogenic stretch. D-peptides generated on these bases prevent amyloid formation and disassemble preformed fibrils of different amyloid hexapeptides identified in human amyloid proteins. In addition, they are also specific for their target sequence. The D-peptide designed here for the Alzheimer's A$\beta_{1\text{-}42}$ peptide not only inhibits and disassembles amyloid material, but it does also reduce A$\beta_{1\text{-}42}$ amyloid-induced cytotoxicity in cell culture.

Introduction

It is accepted that protein misfolding and subsequent amyloid fibril formation are directly linked to the pathogenesis of a group of human disorders known as amyloidoses [1]. Compounds able to interfere with these processes are thus expected to prevent and/or cure these diseases [2; 3]. Several approaches have been reported for the identification of antiamyloid agents [4; 5; 6]. However, a general strategy to obtain specific inhibitors of amyloid formation has not been identified yet.

Due to the lack of detailed structural information on amyloid fibrils and their precursors, structure-based design of antiamyloid compounds has been addressed so far only upon the native structure of the corresponding protein [7]. Compounds with antiamyloid activity have been typically discovered by screening libraries of organic molecules [8; 9]. Peptide-based inhibitors have been designed by using more rational principles, such as exploiting the self-recognition ability of amyloid sequences. First, short fragments capable of binding to the parent amyloid protein have been identified by different experimental techniques [10]. Second, key residues of these fragments have been chemically modified (N-methylation and/or covalent link to organic molecules) or mutated into Pro or α-aminoisobutyric acid (β-sheet breaker strategy) with the aim of disrupting the hydrogen bond network that propagates the amyloid fibril, while keeping binding properties [11; 12; 13; 14; 15] . The addition of a disrupting domain composed of charged residues to these fragments have also led to effective inhibitors of amyloid formation of different amyloid proteins[16; 17]. The drug-likeness of such peptide inhibitors has been optimized [18; 19; 20], the generated compounds shown to effectively reduce amyloid burden [18; 20], or to prevent amyloid deposition in animal models [20; 21], and entered phase I clinical trials in healthy volunteers [22].

D-peptides have been also identified as inhibitors of amyloid formation and cytotoxicity of the $A\beta_{1-42}$ amyloid peptide [23; 24; 25; 26]. In fact, D-peptides containing the key amyloidogenic motif of the $A\beta_{1-42}$ amyloid peptide, KLVFFA ($A\beta_{16-21}$), have been shown to be more effective at inhibiting fibrillogenesis than the respective L-peptides [24]. An additional therapeutic benefit of D- respect to L-peptides is that the formers are less protease sensitive [13; 14]. Good antiamyloid compounds could be thus in principle generated by simply synthesizing the D-peptide analogue of the protein stretch, which drives amyloid formation. We believe however that there may be some room for improvement of this strategy, as D-peptides may exist that bind to a given amyloid protein better than the D-version of any self-recognition motif within that protein [23; 25; 26]. Moreover, the D-version of an amyloid fragment is as amyloidogenic as the natural L-peptide [27; 28], which for

some highly amyloidogenic sequences may result in a very low effective concentration of inhibitor in solution or eventually in cell toxicity.

We have recently demonstrated that short amino acid stretches matching an *amyloid sequence pattern* [29] seem to provide most of the driving force needed to trigger amyloid formation of a protein, the so-called *amyloid stretch hypothesis* [30]. This hypothesis is also in agreement with results reported by other laboratories [31; 32]. Furthermore, we have experimental evidence that amyloid aggregates of these stretches are as toxic as those formed by the whole protein[33]. These results suggest that compounds that specifically bind to peptides synthesized upon the sequence of such amyloidogenic protein stretches might be also able to prevent and/or revert amyloid formation of the full-length protein and likely amyloid-induced cytotoxicity. Here, we present a general strategy to obtain D-peptides that specifically interact with protein amyloid stretches, thereby inhibiting amyloid formation and cell toxicity. This method is based on the combination of the results of screening a combinatorial D-peptide library for inhibitors of an amyloidogenic peptide with prior knowledge on the sequence determinants of amyloid fiber formation. D-peptides generated on these bases prevent amyloid formation and disassemble preformed fibrils of amyloid peptides and proteins. More importantly, they notably reduce amyloid-induced cytotoxicity of the $A\beta_{1-42}$ peptide in cell culture.

Results and Discussion

D-peptide Inhibitors of Amyloid Formation of the Model Peptide STVIIE

Library design and screening. Amyloid formation is stereospecific [27; 28], which suggests that a D-peptide able to interact with any intermediate of this process will break the chirality of the L-species and impair their growth. On this assumption, here we have extensively explored the D-amino acid sequence space with the aim of identifying molecules that specifically inhibit the self-assembly of an amyloid hexapeptide designed *de novo* in our laboratory, STVIIE [34]. To this end, we have screened a D-peptide library with six combinatorialized positions for molecules that specifically inhibit or reverse the self-association process of STVIIE. The library was synthesized in the so-called positional scanning format, i.e., a position is fixed (o) and the other five positions (x) contain close to an equimolar mixture of all natural amino acids in their D-version (see Table 1 and *Experimental Procedures*): GG-$\mathbf{o_1}x_2x_3x_4x_5x_6$-GcG,…, GG-$x_1x_2x_3x_4x_5\mathbf{o_6}$-GcG . Cys was excluded to avoid disulphide bond formation. Hence, the library consists of 114 peptide mixtures, each mixture containing 19^5 (~2,476,099) individual undecapeptides. This implies that the concentration of a particular defined sequence will be at most in the subnanomolar range in an inhibition assay containing 1mM peptide mixture. Therefore, mixtures exhibiting strong inhibitory properties most likely do so because of compatibility of the known fixed amino acid with many different amino acids combinations.

Our inhibition assays were designed to screen the library not only for prevention or reversion of the early species primarily responsible for amyloid cytotoxicity [35; 36; 37; 38], but also for molecules that disassemble mature fibers, as these may contribute to disease through different mechanisms [3; 39]. Inhibition assays were performed by using as starting point a heterogeneous mixture of amyloid species (monomers, nuclei, filaments and fibrils) prepared by sonication of mature fibrils (see *Experimental Procedures*). D-peptide mixtures were thus allowed to interact with different species likely present in the fibrillogenesis process of STVIIE.

D-peptide mixtures were incubated with such preparations of STVIIE at stoichiometric ratio during 1 week and alone as control samples (c = 500 μM). The inhibitory effect of the peptide mixture on STVIIE amyloid formation was assessed semi-quantitatively by estimating the changes in β-sheet population by Circular Dichroism (CD) and in amyloid material by Electron Microscopy (EM) as

compared to a reference solution containing plain buffer instead of D-peptide mixture stock solution. Although higher throughput and more quantitative estimations can be achieved by Thioflavine T and/or Congo Red binding [10], these assays could not be applied to reliably screen the library because dye binding to our hexapeptide model system displayed too weak signals (data not shown). CD and EM seem to provide reliable results for our system as analysis of the inhibition experiments of 1/3 of the mixtures indicated a very good correlation between both techniques, which supports their use for the purpose of this work (see Supplementary Figure 2 and Supplementary Table 1). Deconvolution of the complete library was performed only by CD, as it is a much less time-consuming technique than EM.

The inhibitory effect of the D-peptide mixtures as shown in Table 1 was established by calculating first the ratio **R** between the CD β-sheet content of a given inhibition assay (STVIIE + D-peptide mixture) and the reference solution for that assay (STVIIE + plain buffer): **R**= $\beta_{assay}/\beta_{reference}$; where β_{assay} and $\beta_{reference}$ are the ratio between the ellipticity at 217 nm (Θ_{217}), β-sheet minimum, and the ellipticity at 202 nm (Θ_{202}), isodichroic point of the random coil→β-sheet transition of STVIIE at 500 μM ($\Theta_{217}/\Theta_{202}$), for the assay and the reference solutions, respectively. Comparison to a reference prepared from the same STVIIE stock solution was mandatory because amyloid formation and thus β-sheet population are very sensitive to small changes in concentration or/and peptide batch. The ratio $\Theta_{217}/\Theta_{202}$ is concentration-independent [40] and it had been previously used to estimate changes in β-sheet content of amyloidogenic peptides upon point mutations [29]. For the shake of clarity **R** ratios were grouped into 5 intervals (see *Experimental Procedures* for a detailed explanation), and the inhibitory effect of D-peptide mixtures accordingly ranked as follows:∅ (no effect) < + (low) < ++ (medium) < +++ (good) < ++++ (very good). For the EM analysis two replicate grids of every sample were carefully examined, several pictures of randomly selected areas taken, and the amyloid material observed visually quantified.

According to the number of amino acids that exhibited a good (+++) or very good inhibitory effect (++++), the positions of the library can be classified into two groups: a group of restrictive positions (#4 > #2 = #5) where only 1 to 3 amino acids displayed a good performance, and a group of promiscuous positions (#1 > #3 = #6) where up to 6 amino acids exhibited a good to very good effect. #4 is the most restrictive position as only the mixture with the highly flexible Gly fixed at #4 was very good. Interestingly, none of the 19 amino acids tested showed good inhibitory effect at all six positions. The large percentage of inactive mixtures highlights that inhibition and/or reversion of

amyloid formation by D-peptides relies not only on the stereochemistry of the residues, but also on the specific nature of their side-chains.

***Screening Validation*.** Previous studies support the premise that combination of the best performing amino acid at each position of the library may have a synergistic effect resulting in the best inhibitor possible for a given amyloid sequence [23; 26; 41]. 1620 D-peptide sequences can be generated by combining amino acids ranked as good (+++) and very good (++++) in Table 1. Given the moderate throughput of our technical set up, only two good performing amino acids of different nature (aromatic *vs*. aliphatic) were selected at each position. Only amino acids with non-ionizable side chains were considered to avoid changes of ionization state that might prevent binding to the target sequence at physiological pH (shadowed results in Table 1).

Although a smaller set of 64 defined sequences can be now constructed, only a subset of 32 peptides was synthesized to validate the screening and test our assumptions (Supplementary Table 2). This subset was carefully selected to allow for rationalization of the effect of point mutations and different amino acid combinations. At position #3, however, most molecules (28 out 32) contain a ser instead of a tyr. In sequences dominated by hydrophobic residues, here we decided to favor ser respect to tyr to increase the solubility of our peptide set.

In order to validate the results of the screening, the effect of these 32 molecules was evaluated in two different types of assays: 1.) Assays whose starting point was amyloid mixture preparations of STVIIE as described above for consistency with the conditions under which the library was screened; and 2.) Assays with mature fibrils of STVIIE to test for pure fiber disassembly. Comparison of results from these two assays should shed some light onto which amyloid species are preferentially targeted by each sequence. No effect on mature fibrils samples, but on mixtures, would indicate a preference to bind smaller species likely present at the early stages of the amyloid process; good effect vs. mature fibrils and low or no effect vs. mixtures would indicate a preference for binding fibers and disassemble them; good effect in both assays would imply that the corresponding molecule is effective both at early and later stages of the process. This question may be presumably of relevance for the eventual treatment of disorders related to amyloid formation.

About 2/3 of the molecules exhibited a good or very good effect in one or both types of assays, which suggests that the use of heterogenous mixtures of amyloid species generated by sonication of fibrils was a good approach to screen the library versus all stages of the process at once.

Extraction of Empirical Rules. Analysis of the sequences of the D-peptides as a function of their inhibitory effect in these assays suggests that the location of aromatic residues within the sequence modulates the efficacy and the type of inhibitory activity exerted by the D-peptide (Supplementary Table 2). We observed that for peptides with identical residues at positions 1, 3, 4, and 6, the inhibitory efficacy correlated with the nature of the residues at positions 2 and 5 as follows: a2r5 (**13**, v**i**sw**f**a) >> r2a5 (**5**, v**f**sw**l**a) ≥ r2r5 (**7**, v**f**sw**f**a) ≥ a2a5 (**11**, v**i**sw**l**a), where "a" stands for aliphatic and "r" for aromatic residue, and numbers indicate peptide sequence positions. Regarding positions #1 and #6, an aromatic residue at #1 (r1) seems to exert the same effect as an aromatic at #6 (r6), **27** (r1, **y**iswla) *vs*. **12** (r6, viswl**f**), or a better one **25** (r1, **y**isgfa) *vs*. **10** (r6, visgf**f**) depending on the composition at positions #4 and #5. Molecules that combine two aromatic residues at #1 and #6 (r1r6) are also better than peptides containing only one aromatic at either terminus irrespective of the composition at other positions: **26** (r1r6, **y**isgf**f**) *vs*. **25** (r1, **y**isgfa) & **10** (r6, visgf**f**). Therefore, for peptides with identical residues at positions 2 and 5, the efficacy varied upon the presence or absence of aromatic residues at positions #1 & #6 according to the following rule: r1r6 >> r1 ≥ r6. Due to the low sequence variability chosen at #3 and #4, no rules can be inferred for these positions. Nevertheless and despite these limitations, some preferences or trends were observed that deserve to be commented. It seems that a single aromatic residue at #3 (r3) does not lead to any good inhibitory effect (**15**, vi**y**gla). It does, however, if combined with r1 (**31**, r1r3, **y**i**y**gla) but not in combination with r6 (r3r6, **16**, vi**y**gl**f**). r1r3 seems to be as good as r1r3r6 (32, **y**i**y**gl**f**), which means that aromatics at this position might be omitted in favor of more polar residues with the consequent impact in solubility. At position #4, as long as one makes the right choice for the adjacent positions, it seems that good inhibitors can be obtained both with trp and Gly

Different amino acid combinations also seem to modulate the type of inhibitoty activity exerted. For example, the introduction of aromatic residues on the edges of the inactive molecule **11** (viswla) yields two molecules, **12** (viswl**f**) and **27** (**y**iswla), with very good activity against both sonicated and mature fibers (Supplementary Table 2). Nonetheless, mutation of **11** according to the a2r5 rule generates molecule **13** (v**i**sw**f**a) that seems to be active preferentially against early stages (sonicated samples). Interestingly, the introduction of an aromatic residue at position #1 of **13** (**29**, **y**iswfa) seems to make stronger the preference towards early stages. The peptide set tested thus illustrates that aromatic residues do not only modulate the magnitude of the inhibitory effect but also the species targeted in the inhibition reaction. The study of a more extensive set of peptides could

allow the identification of rules for the design of antiamyloid molecules that selectively target different species of the amyloid pathway.

General Strategy for the Generation of D-Peptide Inhibitors of Amyloid Formation

The generic *β*-structure shared by all amyloid species suggests that the key elements of the amyloid aggregation process may be common to all amyloid proteins, which in turn implies that molecules that disrupt this structural motif should share some similarities as well. Based on our observation that the particular location of aromatic residues is critical to inhibition, we propose a novel strategy to design *de novo* D-peptide inhibitors of any given protein amyloid stretch. We summarize this strategy as follows: First, inhibitors are generated by using the D-peptide analogue of the stretch identified with the *amyloid pattern* [29] as a starting point. Second, mutations at positions #1, #2, #5, and #6 are performed with the rules outlined above as guidance: a2r5 >> r2a5 $\geq$ r2r5 $\gtrsim$ a2a5, and r1r6 >> r1 $\gtrsim$ r6. At these positions the amino acid of the parent D-amino acid will be kept if its nature coincides with that indicated by the rules. If the residue at position #1,#2, #5 and/or 6 residues do not correspond to the amino acid type suggested by the rules, they willl be mutated into the corresponding aliphatic (#1 val, #2 ile, #5 leu, & #6 ala) or aromatic (#1 tyr, #2 phe, #5 phe & #6 phe) residue selected from the deconvolution of the positional scanning D-library (shadowed results in Table 1). Third, as no rules could be extracted for positions #3 and #4, amino acids at these positions will be kept identical to the parent peptide.

***Experimental Validation*.** This new design strategy was experimentally tested by synthesizing inhibitors of 6-residue peptides bearing the sequence of amyloidogenic stretches identified with the *amyloid pattern* in two relevant proteins involved in Alzheimer's disease: Tau and $A\beta_{1-42}$ The stretch identified with the amyloid pattern for Tau corresponds to residues 590-595 (KVQIIN, $Tau_{590-595}$) and for $A\beta_{1-42}$ to residues 16-21 (KLVFFA, $A\beta_{16-21}$) [29, 33]. The amyloid hexamer$Tau_{590-595}$ was used to validate the rules or sequence trends presented above and both amyloid peptides, $Tau_{590-595}$ & $A\beta_{16-21}$, to investigate whether or not rules are additive (molecules designed upon combining all the rules, so-called in this work "superinhibitors"; Table 2). For comparison purposes, we also synthesized the D-peptide klvffa (D-$A\beta_{16-21}$), which is the D-version of the Aβ sequence fragment comprising residues 16 to 21. klvffa has been shown somewhere else to successfully block $A\beta_{1-42}$ polymerization and cytotoxicity [24]. Two different experiments were performed to test the designed D-peptides: 1.) t=0

experiments using as starting point monomeric preparations of the target amyloid hexapeptide to test for inhibition; and 2.) Mature fibrils experiments to test for fiber disassembly.

As shown in Table 2 and Supplementary Figure 3, there is a very good correlation between the extracted empirical rules and the results obtained in disassembling experiments (mature fibrils). No conclusions can be drawn, however, for rules r6 (kvqii**f**) and a2a5 (D-$Tau_{590-595}$, k**v**qi**i**n) as these peptides aggregate under the conditions used in the assays. Regarding prevention of amyloid formation (t=0 experiments), only molecules carrying at least an aromatic residue at position #1 exhibited arresting effect. Not even the best combination of amino acids at positions 2 and 5 (a2r5) was effective without the aromatic residue at position 1. Remarkably, the combination of the rules leads to "superinhibitor" D-peptides, S-$Tau_{590-595}$ (**yv**qi**ff**) and S-$A\beta_{16-21}$ (**yl**vf**ff**), of exceptional inhibitory properties at the two stages of the amyloid fibril formation process tested (Figure 1). S-$A\beta_{16-21}$ disassembled mature fibrils of the hexapeptide KLVFFA ($A\beta_{16-21}$) as effectively as the previously reported D-peptide inhibitor, D-$A\beta_{16-21}$, did, but it was more effective at preventing amyloid material formation (Table 2 and Figure 1).

Sequence Specificity. We also evaluated whether or not peptides designed by using this strategy are specific for the target stretches on which they are based. In order to investigate this question, we carried out cross-inhibition experiments: the inhibitor S-$Tau_{590-595}$ was tested *vs*. the amyloidogenic peptide $A\beta_{16-21}$, and the superinhibitor S-$A\beta_{16-21}$ *vs*. the amyloid hexapeptide$Tau_{590-595}$. As shown in Figure 1a and Supplementary Table 3, S-$Tau_{590-595}$ has almost no effect in the amyloid formation process of $A\beta_{16-21}$. Similar conclusions can be drawn from the cross-inhibition experiments *vs*. $Tau_{590-595}$ (Figure 1b).

Inhibition of $A\beta_{1-42}$ Amyloid-Induced Cytotoxicity in PC12 Cell Culture

Amyloid formation and derived cytotoxicity relies on the establishment of stabilizing contacts between self-complementary stretches of a protein [30; 32; 42]. We propose that molecules shown to specifically interact with peptides synthesized upon the sequence of these stretches may be also able to bind to the corresponding protein fragments, thereby preventing or disrupting this undesired process and its related noxious effects. In order to validate this hypothesis, we assessed whether or not the D-peptide designed for $A\beta_{16-21}$, S-$A\beta_{16-21}$, was also effective *vs*. the full-length amyloid peptide

$A\beta_{1-42}$ and compared its performance with the D-peptide klvffa (D-$A\beta_{16-21}$) known to inhibit $A\beta_{1-42}$ amyloid formation and toxicity [24] . First, we evaluated its effectiveness to block the formation of amyloid material and toxic species starting from the monomeric state of $A\beta_{1-42}$ (t=0 experiments). Both S-$A\beta_{16-21}$ (ylvfff) and our benchmark D-$A\beta_{16-21}$ (klvffa) strongly interfered with the formation of amyloid material and proportionally decreased $A\beta_{1-42}$ amyloid-induced cytotoxicity in PC12 cell culture in a dosage dependent manner (Figure 2). Nonetheless, only S-$A\beta_{16-21}$ was able to disassemble preformed $A\beta_{1-42}$ fibers. Addition of S-$A\beta_{16-21}$ to samples of $A\beta_{1-42}$ mature fibers drastically reduced the amount of amyloid material *in vitro* through all the concentration range tested, correlating with an almost complete protection against amyloid-induced cell death even at 10-times molar defect of S-$A\beta_{16-21}$ (Figure 2). In contrast, the benchmark peptide D-$A\beta_{16-21}$ has no evident effect in the *in vitro* test and its protection against $A\beta_{1-42}$ toxicity showed very pronounced dosage dependence. Cell survival in samples incubated with D-$A\beta_{16-21}$ dropped from 92.3 ± 2.6% to 75.8 ± 5.7%, similar to the control, upon a 5-fold reduction of the D-peptide concentration (from ratio $A\beta_{1-42}$:D-$A\beta_{16-21}$ 2:1 to 10:1). Therefore, these results suggests that by using our general strategy we have designed a molecule that is as effective as a previously reported D-peptide at interfering with early stages of $A\beta_{1-42}$ amyloid formation process, but with the additional feature that at substoichiometric concentrations, it is able to dissolve preformed fibrils and generate species that are not toxic in PC12 cell culture.

Conclusions

Here, we have demonstrated that short amyloid stretches within a protein can be used as target motifs for amyloid inhibiton. We have also extracted a set of empirical rules for the generation of D-peptide specifically targeted against those amyloid stretches. These results suggest new opportunities for the search of antiamyloid compounds. First, amyloid proteins can be scanned for 6-residue stretches that match the *amyloid sequence pattern* [29]. Other prediction tools [43; 44; 45] and experimental methods [10] can be also used to search for amyloidogenic sequences within proteins; second, peptides based on these protein amyloid stretches can be utilized to screen for inhibitory molecules. This methodology presents the clear advantage that amyloidogenic hexapeptides are cheaper and easier-to-handle than amyloidogenic proteins. Alternatively, lead D-peptide inhibitors can be directly generated by applying our general strategy. Peptides designed upon this empirical approach are sequence specific, which minimizes the possibilities of interaction with other proteins; inhibit amyloid formation not only of the amyloid stretch on which they are based, but also of the

corresponding full-length protein; and more importantly, prevent and reverse amyloid-induced cytotoxicity of a relevant amyloid protein, $A\beta_{1-42}$.

For the $A\beta_{1-42}$ peptide some other fragments able to form amyloid fibrils *in vitro* have been identified [46] or predicted *in silico* [43; 44].Our results show that targeting only one amyloid stretch is sufficient to inhibit and reverse amyloid fibril formation and cytotoxicity of the full-length $A\beta_{1-42}$. It is not clear, however, whether or not this will apply to all polypeptides, specially to larger proteins such as the human prion or the Apolipoprotein among others [29] containing several amyloid stretches . The design of D-peptide inhibitors with our methodology for every one of the amyloid stretches identified for a given protein might help to shed light onto this question.

A detailed study of the interactions that lead to the inhibition of amyloid formation by these D-peptides could serve as the basis for the design of compounds with better pharmacological profiles than D-peptides. Molecular dynamics simulations using the model peptide STVIIE are indeed shedding light onto this question (unpublished results). Recent structural models of the cross-β structure of amyloid peptides at atomic detail should also pave the way for rational design exercises [42; 46].

Experimental Procedures

Materials

Peptides were supplied by Diverdrugs (Spain) or Thermo Electron Company (Germany). Typically, purity higher than 90% was required. A positional D-peptide library of general scaffold GG-$\mathbf{o_1}x_2x_3x_4x_5x_6$-GcG,…, GG-$x_1x_2x_3x_4x_5\mathbf{o_6}$-GcG was synthesized by coupling a mixture of 18 D-amino acids and Gly (D-Cys omitted) with the relative ratio suitability adjusted to yield close to equimolar incorporation at "x" positions and a defined residue at "o" positions. The flanking residues GG and GcG were originally introduced for cross-linking experiments related to a different project. Mass spectra were analyzed for 10% of the vials. No peaks out of the range were observed. The amino acid composition was checked for 5% of the vials. The general scaffold of the 32 defined sequences built upon the deconvolution of the positional D-library is Ac-GG-xxxxxx-GG-Am. D-peptides designed on the bases of the empirical rules lacked flanking residues and presented protected termini (Ac-xxxxxx-Am). All the D-peptides used in this study have protected termini. $A\beta_{1-42}$ peptide was purchased from Bachem (Switzerland).

Sample Preparation

D-peptide mixture solutions. Each vial of the D-peptide library was dissolved in 200 μL of distilled water. 5% DMSO (v/v) was added to the vials showing solubility problems. The approximate concentration of each vial was calculated by UV-spectroscopy (ε_{220} = 1961 $cm^{-1}mM^{-1}$) and 1mM aliquots were prepared in fresh and filtered buffered solution 20 mM glycine/HCl pH 2.6.

D-peptide solutions. D-peptides were dissolved in 20 mM glycine/HCl pH 2.6 buffer, sonicated for 15 minutes in a Sonorex water bath at room temperature to disrupt any possible preformed aggregated material, centrifuged (10 minutes at 16,100xg) and filtered using an 0.02 μm Anatop filter (Whatman). Molecules with solubility problems were dissolved in pure DMSO.

Fibril samples. Stock solutions of all the amyloidogenic hexapeptides were prepared as previously reported [34]. Samples were usually incubated for one week at room temperature and buffered solution

20 mM Glycine/HCl pH 2.6 in separate syliconized vials. $A\beta_{1-42}$ peptide was disaggregated following the protocol described somewhere else [47]. Detailed conditions for every sequence can be found in Supplementary Experimental Procedures.

Inhibition Assays

General procedure. D-peptides and D-mixtures were incubated alone under the same conditions of the inhibition assays to check for aggregation. Detailed conditions for every assay can be found in Supplementary Experimental Procedures.

Heterogenous mixtures of amyloid species of STVIIE. Samples of mature fibers of STVIIE (c = 1 mM) were sonicated during 6-8 hours (Sonorex water bath). Ice was added periodically to avoid overheating. After sonication, it was always checked by EM that a heterogeneous mixture of species (nuclei, filaments and fibrils) was produced (Figure S1). Such samples were diluted to 500 μM and inhibitor solution (peptide mixture or defined sequence) was added to reach ratio STVIIE:D-peptide 1:1.

Inhibition assays (t=0). Monomeric solutions were aliquoted in independent syliconized vials and mixed with the D-peptides solutions at the desired ratio. Detailed conditions for every sequence can be found in Supplementary Experimental Procedures. Inhibition assays were incubated 1 week at room temperature, except for $A\beta_{1-42}$ whose inhibition assays were incubated for 2 days at 37 °C.

Disassembling assays. Mature fibrils were diluted to reach the same concentration used in t=0 assays (Supplementary Experimental Procedures) and mixed with the corresponding D-mixture or D-defined sequences at the ratio required. Samples were incubated 1 week at room temperature, except for $A\beta_{1-42}$ whose inhibition assays were incubated for 4 days at 37 °C.

Far-UV Circular Dichroism

Far UV CD spectra were acquired as described before [34]. To compare the β-sheet content of solutions of STVIIE incubated with different D-mixtures or D-peptides, the ratio between the ellipticity at 217

nm (Θ_{217}), β-sheet minimum, and the ellipticity at 202 nm (Θ_{202}), isodichroic point of the random coil→ β-sheet transition of STVIIE at 500 μM ($\Theta_{217}/\Theta_{208}$), was calculated. This ratio is concentration-independent [40; 48]. The contribution of the D-peptide mixture or D-peptide solution incubated alone was always substracted from the spectrum of the inhibition assay (amyloid peptide + inhibitor). The inhibitory effect was estimated by comparing the β-sheet content of the products of the inhibition reaction to the content of the reference solution: $R = \beta_{assay}/\beta_{reference}$. According to this ratio, the inhibitory ranking was established as follows: $R \in (-10, -1]$ (very good effect, ++++); $R \in (\infty, -10]$ (good effect, +++); $R \in (\infty, +10)$ (medium effect, ++); $R \in (0, +10]$ (low effect, +); and $R \in [0, +1]$ (no effect, ∅).

Electron Microscopy

EM measurements were carried out as described before. The inhibitory effect of the D-molecule as compared to a reference sample was assessed by visual inspection of the grids. Two grids were always taken for every sample. The estimation was based on an overall assessment of the amount and morphology of material in the whole grid. Typically, at least 5 pictures of random fields in the grid were taken to give a representative overview of the sample. In the cases where a big reduction of material was observed, the second grid was also checked as a confirmation. All experiments were carried out at least twice to confirm the observed trends.

Cytotoxicity Assays

Aliquots (10 μL) of the products of the different inhibition reactions and the reference solution (fibrils or monomeric peptide incubated without D-peptide) were added to 100 μL of PC12 cells. Usual incubation time was 24 hours. Afterwards, medium containing the inhibition samples was removed and cells were grown in fresh medium until reaching 24 hours of overall incubation. PC12 cell toxicity was evaluated by using a commercial MTT kit (Roche).

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Figure Legends

Figure 1. Inhibitory effect and sequence specificity of *de novo* designed D-peptide amyloid inhibitors

Target amyloid sequences: a) $A\beta_{16-21}$ (KLVFFA) and b) $Tau_{590-595}$ (KVQIIN). Panel distribution: Electron micrographs of the effect of the D-peptides S-Aβ (ylvfff) and S-Tau (yvqiff) against their respective target sequences are shown in the first row. The second row illustrates the result of the cross-inhibition experiments (assays to test for sequence specificity). The first column shows electron micrographs of the reference solution or blank of the target amyloid sequence incubated without D-peptide. The second and third columns present the result of the incubation of the D-peptides with mature fibrils or with monomeric peptide (t=0). Amyloid peptide: inhibitor D-peptide ratio used in the experiments: $Tau_{590-595}$:D-peptide 2:1 [c ($Tau_{590-595}$)= 350 μM, 20 mM Gly/HCl pH 2.6] and $A\beta_{16-21}$:D-peptide 5:1 [c ($A\beta_{16-21}$) = 1.5 mM, 20 mM Gly/HCl pH 2.6]. The inhibitory ranking of the samples can be found in Supplementary Table 3. Scale bar, 500 nm.

Figure 2. Prevention and reversal of $A\beta_{1-42}$ amyloid formation and cytotoxicity by the *de novo* designed peptide S-$A\beta_{16-21}$**.**

a) *In vitro* test: Effect of S-$A\beta_{16-21}$ and the benchmark peptide D-$A\beta_{16-21}$ as assessed by EM. Different ratios $A\beta_{1-42}$:D-peptide with monomeric $A\beta_{1-42}$ (t=0) and mature $A\beta_{1-42}$ fibrils have been analyzed (see Table). Inhibitory effect increases as follows: ∅ (no effect) < + (low) < ++ (medium) < +++ (good) < ++++ (very good). The electron micrographs show the results of the incubation of S-$A\beta_{16-21}$ and D-$A\beta_{16-21}$ with mature fibrils at ratio $A\beta_{1-42}$: D-peptide 2:1. A complete set of electron micrographs can be found in Supplementary Figure S4.

b) Cytotoxicity of the reaction products of the inhibition (t=0) and disassembling (mature) experiments in PC12 cells. Cell viability was measured by using the MTT reduction test (see Experimental Section). $A\beta_{1-42}$ peptide concentration used in these assays was 100 μM in 20 mM Na_2HPO_4/NaH_2PO_4 buffer.

Table 1. Deconvolution by circular dichroism[a] of a positional scanning D-peptide library[b] for inhibitors of STVIIE amyloid fiber formation

	O_1-$X_2X_3X_4X_5X_6$	X_1-**O_2**$X_3X_4X_5X_6$	X_1X_2-**O_3**$X_4X_5X_6$	$X_1X_2X_3$-**O_4**X_5X_6	$X_1X_2X_3X_4$-**O_5**X_6	$X_1X_2X_3X_4X_5$-**O_6**
gly	+++	∅	+	**++++**	∅	∅
ala	∅	+	+	++	∅	**++++**
val	**++++**	∅	++++	∅	∅	∅
ile	∅	**+++**	∅	∅	∅	∅
leu	∅	∅	∅	∅	**++++**	+
met	∅	∅	∅	+	∅	++++
ser	+++	∅	**++++**	+	+	∅
thr	+	∅	+	∅	∅	∅
tyr	**+++**	∅	**++++**	∅	∅	∅
trp	+	∅	+	**++**	∅	∅
phe	∅	**++++**	+	∅	**++++**	**+++**
asn	+	∅	++++	∅	+	∅
asp	∅	+	+	∅	∅	+++
gln	∅	∅	+++	+	∅	∅
glu	∅	+	∅	∅	++++	++++
lys	∅	∅	+	∅	∅	++++
arg	++++	∅	++++	∅	∅	∅
his	∅	++++	∅	∅	∅	∅
pro	∅	∅	+	∅	∅	∅

[a] CD ranking: the lower the β-sheet population in solution upon incubation with the peptide mixture, the better the inhibitory effect. Inhibitory effect increases as follows: ∅ (no effect) < + (low) < ++ (medium) < +++ (good) < ++++ (very good). A detailed explanation of the ranking method can be found in Experimental Procedures and Supplementary Material. The correlation between CD and EM ranking for a set of samples is shown in Supplementary Figure 1.

[b] General scaffold of the library: GG- x_1-$x_2x_3x_4x_5x_6$-GcG.

[c] Shadowed results indicate amino acids selected to construct defined D-peptide molecules.

[d] Experiments were carried out at equimolar ratio STVIIE:D-mixture [c = 500 μM, 20 mM Gly/HCl pH 2.6] .

Table 2. Experimental validation of the general strategy for the design of D-peptide amyloid inhibitors

Target amyloid stretch	**Empirical design rule**[a]	**D-peptide**[b]	**t=0**[c]	**MATURE**[c]
KVQIIN[e] ($Tau_{590-595}$)	a2a5	k-**v**-q-i-**i**-n (D-$Tau_{590-595}$)	_ [d]	_[d]
	r2r5	k-**f-**q-i-**f-**n	∅	+/∅
	r2a5	k-**f-**q-i-**i-**n	∅	+/∅
	a2r5	k-**v-**q-i-**f-**n	∅	+++
	r1	**y-**v-q-i-i-n	++	+++
	r6	k-v-q-i-i-**f**	_ [d]	_ [d]
	r1r6	**y-**v-q-i-i-**f**	++	+++
	r1a2r5r6	**y-v-**q-i-**f-f** (S-$Tau_{590-595}$)	++++	++++
KLVFFA[e]	r1a2r5r6	**y-l-**v-f-**f-f** (S-$A\beta_{16-21}$)	++++	++++
	a2r5	k-**l-**v-f-**f-**a (D-$A\beta_{16-21}$)	+	++++

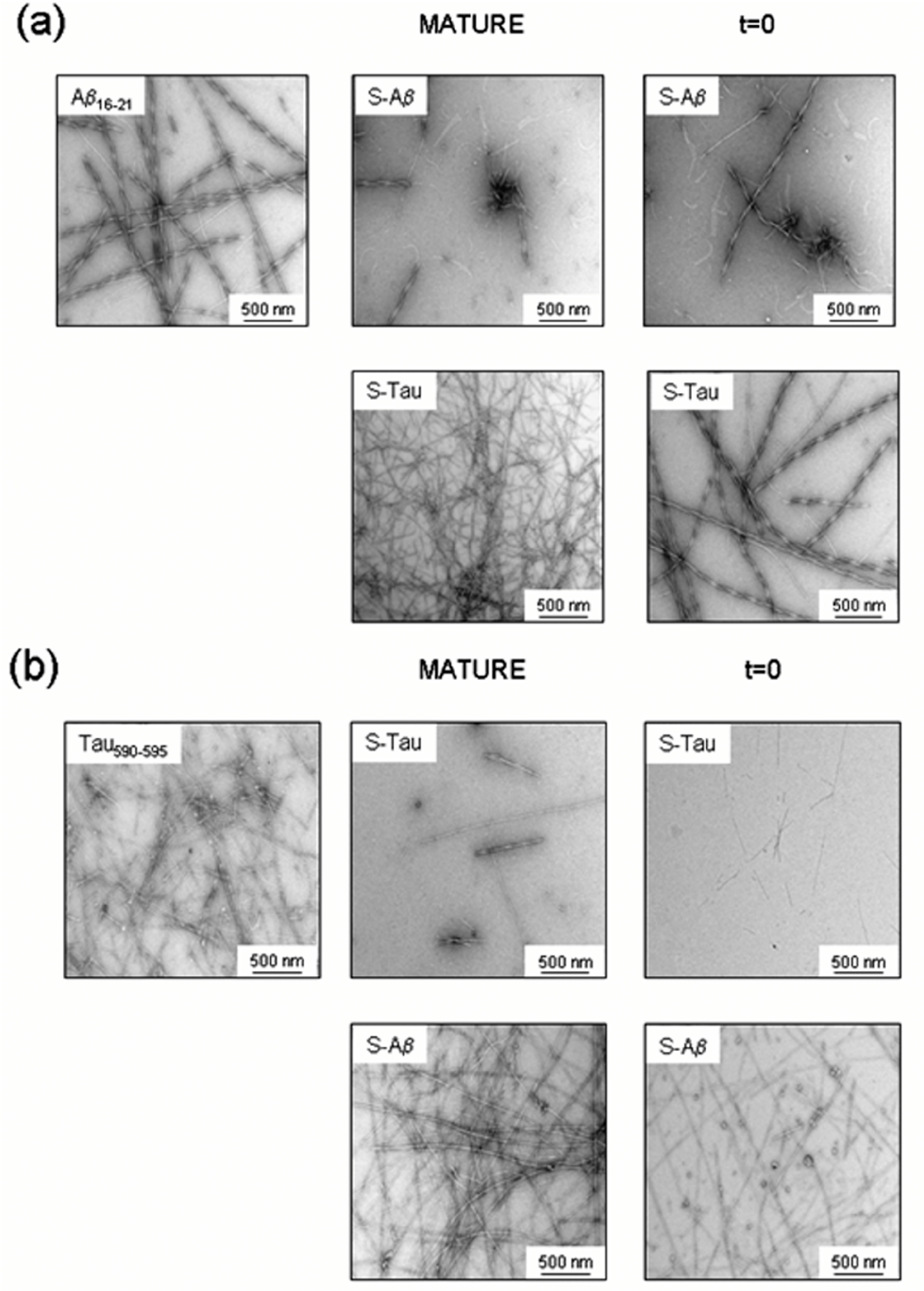
[a] "a" stands for residue of aliphatic nature, "r" for aromatic residue, and numbers indicate the position of the residue of the mentioned nature in the hexapeptide sequence.

[b] D-hexapeptides designed upon the strategy presented in the main body of the text. Mutations respect to the D-version of the target amyloid stretch are highlighted in bold. D-$Tau_{590-595}$ and D-$A\beta_{16-21}$ are used as benchmark or reference peptides as their sequences are the mirror image of the respective target amyloid peptides. S-$Tau_{590-595}$ and S-$A\beta_{16-21}$ have been designed upon combination of the most effective empirical rules (r1r6 and a2r5; *superinhibitors*). D-peptide termini protected.

[c] Disassembling (mature) and blocking (t=0) assay results as assessed by EM: ∅ (no effect) < + (low) < ++ (medium) < +++ (good) < ++++ (very good). A representative set of EM pictures can be found in Supplementary Figure 2 and 3.

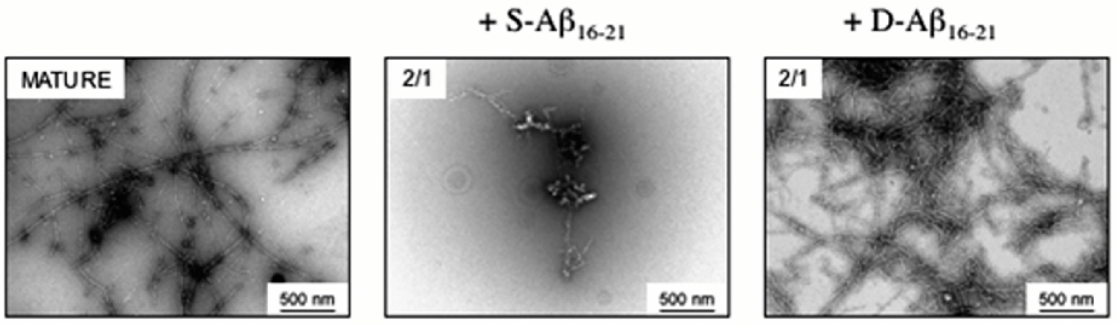
[d] The effect of D-$Tau_{590-595}$ and r6 peptide (kvqiif) could not be assessed as reference solutions containing these molecules aggregate under conditions used in these experiments.

[e] Experimental conditions: Equimolar ratio $Tau_{590-595}$:D-peptide [c ($Tau_{590-595}$) = 350 μM; 20 mM Gly/HCl pH 2.6]. $A\beta_{16-21}$: D-peptide ratio 5:1 [c ($A\beta_{16-21}$) = 1.5 mM; 20 mM Gly/HCl pH 2.6].


(a)
MATURE
t=0
Aβ16-21
S-Aβ
S-Aβ
S-Tau
S-Tau
500 nm
(b)
MATURE
t=0
Tau590-595
S-Tau
S-Tau
S-Aβ
S-Aβ
500 nm

(a)

RATIO ($A\beta_{1-42}$/D-peptide)	S-$A\beta_{16-21}$		D-$A\beta_{16-21}$	
	t=0	MATURE	t=0	MATURE
2/1	++++	++++	+++	∅*
5/1	+++	+++	++	∅*
10/1	++	++	∅	∅



(b)