Amyloid β-sheet mimics that antagonize protein aggregation and reduce amyloid toxicity

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The amyloid protein aggregation associated with diseases such as Alzheimer’s, Parkinson’s and type II diabetes (among many others) features a bewildering variety of β-sheet-rich structures in transition from native proteins to ordered oligomers and fibres. The variation in the amino-acid sequences of the β-structures presents a challenge to developing a model system of β-sheets for the study of various amyloid aggregates. Here, we introduce a family of robust β-sheet macrocycles that can serve as a platform to display a variety of heptapeptide sequences from different amyloid proteins. We have tailored these amyloid β-sheet mimics (ABSMs) to antagonize the aggregation of various amyloid proteins, thereby reducing the toxicity of amyloid aggregates. We describe the structures and inhibitory properties of ABSMs containing amyloidogenic peptides from the amyloid-β peptide associated with Alzheimer’s disease, β2-microglobulin associated with dialysis-related amyloidosis, α-synuclein associated with Parkinson’s disease, islet amyloid polypeptide associated with type II diabetes, human and yeast prion proteins, and Tau, which forms neurofibrillary tangles.

Amyloid aggregation is associated with many intractable protein aggregation diseases, notably including Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, type II diabetes and prion diseases1–3. Amyloid fibrils with characteristic highly ordered cross-β structures are the ultimate products of amyloid aggregation. More than 30 proteins have been linked to amyloidogenesis, and they demonstrate enormous variations in relation to their sequences and polymorphic fibril structures4–6. The fibril formation of a given polypeptide, however, greatly depends on its specific residue order7,8. Crystallographic structures of amyloid-like fibrils formed by amyloidogenic peptide fragments suggest that the formation of highly ordered parallel or antiparallel β-sheets and a steric zipper interface between β-sheets are two essential elements for amyloid fibril formation9,10.

Amyloid fibrils are the most visible evidence of pathology, but soluble oligomers are proving to be more important in amyloid toxicity11,12. Although there is an increasing level of evidence showing that these transient, unstable structures are rich in β-sheets, their dynamic and polymorphic properties make amyloid oligomers difficult to study at the atomic level13–15. Additional tools are needed to study amyloid oligomers and aggregation and to shed light on controlling these processes.

β-Sheet mimics that can display amyloid β-strands provide a means with which to study amyloid oligomers and aggregation. We previously introduced 42-membered ring macrocyclic β-sheets containing pentapeptide fragments from amyloid-β peptide (Aβ) and tau protein (Tau) to mimic amyloid-like β-sheets and shed light on the structures of transient amyloid oligomers16,17. We have also used these macrocyclic β-sheets to inhibit aggregation of the peptide Ac-VQIVYK-NH2 (AcPHf6), derived from Tau, to provide insights into the aggregation process18.

The development of a robust chemical model of β-sheets that can tolerate a variety of amino-acid sequences has been challenging, because amyloidogenic sequences vary enormously and because folding of β-sheet mimics largely depends on the amino-acid sequence1,19. In this Article, we introduce a new class of β-sheet macrocycles that can tolerate a wide range of amino-acid sequences from amyloid proteins and still fold into β-sheet structures. We call these macrocycles amyloid β-sheet mimics (ABSMs).

ABSM 1 is a 54-membered ring, comprising a heptapeptide β-strand (the upper strand), one Hao unit flanked by two dipeptides (the lower strand) and two δ-linked ornithine (δOrn) turns (Fig. 1a). The ‘upper’ β-strand of ABSM 1 incorporates different heptapeptide fragments from Aβ, Tau, yeast Sup35 prion protein (Sup35), human prion protein (hPrP), human β2-microglobulin (hβ2M), human α-synuclein (hαSyn) and human islet amyloid protein (h2-microglobulin) (Fig. 1b).

Figure 1 | Design of ABSM 1. a, Representation of ABSM 1 illustrating the upper β-strand (recognition β-strand), the δ-linked ornithine (δOrn) turn unit and the Hao amino-acid blocker unit. b, Representation of ABSM 1 recognizing and blocking amyloid aggregation through β-sheet interactions.
polypeptide (hIAPP). Hao is a tripeptide β-strand mimic that not only serves as a template for intramolecular hydrogen bonding, but also minimizes the exposed hydrogen-bonding functionality of the ‘lower’ strand. This structural design of Hao helps prevent ABSMs 1 from aggregating in solution to form an infinite network of β-sheets; instead, ABSMs 1 dimerize and then further self-assemble into oligomers. The ‘upper’ and ‘lower’ strands of ABSM 1 are connected by two Orn β-turn mimics.

We envisioned that ABSM 1 would fold well because it is conformationally constrained by cyclization and has a Hao template to promote intramolecular hydrogen bonding and two Orn β-turn mimics to promote turn formation. We also envisioned that four pairs of side chains (R1–R11, R2–R19, R8–R9 and R7–R8) would provide stabilizing transannular interactions. We anticipated that the flexibility of the dipeptides flanking Hao in the ‘lower’ strand would better accommodate the flatness of the Hao template and thus minimize the kinks in the β-strands that we had previously observed in 42-membered ring macrocycles.

We designed ABSMs 1 to display exposed heptapeptide β-strands so that these β-strands can recognize and bind their parent amyloid proteins (Fig. 1b). We envisioned recognition between ABSMs 1 and their parent amyloid proteins to take place through the β-sheet interactions observed in amyloid aggregation. Here, we present structural studies of these ABSMs 1 and describe their effect upon amyloid aggregation and toxicity.

Results

Design of ABSMs 1. To test the folding of ABSMs 1, we selected 16 amyloidogenic heptapeptide β-strands from seven β-sheet-rich amyloid proteins for positions 1–7 in the ‘upper’ strands (Table 1). ABSMs 1a–g contain heptapeptide sequences from two important hydrophobic and fibril-forming regions of Apβ associated with Alzheimer’s disease, residues 16–23 and 29–40 (refs 5,22). ABSMs 1a–d and f contain native heptapeptide sequences, while ABSMs 1e and 1g are G33F and G37F mutants, in which the aromatic residue across from Hao promotes better folding. ABSM 1h contains residues 7–13 from Sup35, which is associated with dialysis-related amyloidosis. ABSMs 1i and 1o contain residues 69–75 and 75–81 from hPrP, which is associated with Parkinson’s disease. ABSMs 1p and 1q contain residues 11–17 and 26–32 from hIAPP, associated with type II diabetes. We chose polar and hydrophobic residues at positions 8–11 in the ‘lower’ strands of ABSM 1 to promote solubility in water and to increase hydrophobic residues that favour β-sheet formation.

Synthesis of ABSMs 1. ABSMs 1 were prepared by synthesizing the corresponding protected linear peptides, followed by solution-phase cyclization and deprotection. The protected linear peptide precursors were synthesized on 2-chlorotrityl chloride resin by conventional Fmoc-based solid-phase peptide synthesis. Macrocyclization was typically performed using HCTU and N,N-diisopropylethylamine in DMF at a concentration of ~0.5 mM. The ABSMs 1 were isolated in ~20–30% overall yield after high-performance liquid chromatographic purification and lyophilization. Each synthesis produces tens of milligrams of ABSMs 1 as fluffy white solids (for details, see Supplementary Information).

X-ray crystallographic studies of ABSM 1r. X-ray crystallography of ABSM 1r validated the design of ABSMs 1 (Fig. 2). ABSM 1r is a homologue of ABSM 1d, with the Tyr residue in the ‘lower’ strand replaced with 4-bromophenylalanine for crystallographic phase determination. ABSM 1r adopts a β-sheet structure in which the

| Table 1 | Amino acid sequences and key NOEs of ABSMs 1a–q. |
|---|---|---|---|---|---|---|---|---|---|
| Sequence | R1–R7 | R6–R3 | OrnHα–S | 2–10 | 4–Haoβ | 6–9 | OrnHα–S | Folding |
| 1a | Aβ22 | KLFFAE | KLIE | S | S | S | S | S | Good |
| 1b | Aβ22 | LVFPAE | KLIE | S | S | S | S | S | Good |
| 1c | Aβ29–35 | GAIILM | KFYK | S | S | S | S | S | Good |
| 1d | Aβ30–36 | AIIILMV | KFYK | S | S | S | S | S | Good |
| 1e | Aβ30–36, G33F | AIIILMV | KFYK | S | S | S | S | S | Good |
| 1f | Aβ34–40 | LMVGVV | KFYK | S | S | W* | S | S | Moderate |
| 1g | Aβ34–40, G37F | LMVGVV | KFYK | S | S | S | S | S | Good |
| 1h | Sup35–13 | GQQNNQY | KFYK | W | — | — | — | W | Poor |
| 1i | hPrP16–22 | AAAGAVV | KFYK | W | — | — | — | W | Poor |
| 1j | Tau20–31 | SVQVVK | EFYK | S | S | S | S | S | Good |
| 1k | hB2M56–66 | FYLLYT | KNSA | S | S | S | S | S | Good |
| 1l | hB2M56–69 | YLTTYE | FKV5 | W | — | — | — | W | Poor |
| 1m | hB2M63–69 | YLTTYE | KKV5 | S | S | — | — | S | Good |
| 1n | hB2Syn79–85 | AVTGYV | KFYK | S | S | S | S | S | Good |
| 1o | hB2Syn81–91 | TAVANT | KFYK | S | S | S | S | S | Good |
| 1p | hIAPP11–17 | RLANFL | KFYK | S | S | S | S | S | Good |
| 1q | hIAPP17–26 | ILSTN | KFYK | S | S | S | S | S | Good |
| 1r | Aβ22 | AIIILMV | KFFK | S | S | S | S | S | Good |

*S, strong NOE; W, weak NOE. †NOE not observed due to overlap of proton resonances. ‡NOE not observed. §NOE not observable due to overlap with HOD.
‘upper’ and ‘lower’ strands are intramolecularly hydrogen-bonded to form eight hydrogen bonds (Fig. 2a). The two Orn residues of ABSM 1r fold into β-turn-like conformations, Hao mimics a tripeptide β-strand, and the ‘upper’ strand displays an exposed heptapeptide β-sheet edge.

ABSM 1r forms a dimer in the crystal lattice in which the two recognition β-strands come together in an antiparallel β-sheet fashion (Fig. 2b). The β-strands of the dimerization interface are shifted out of register, forming only six hydrogen bonds instead of the eight that would form through in-register contact.

The dimers stack in the crystal lattice, with hydrophobic contacts between the layers of the stack. The Ile, Leu and Val at positions 3, 5 and 7 on the ‘top’ layer of the dimer pack together in one set of hydrophobic contacts ‘above’ the dimer, while the Met and Phe at positions 6 and 9 on the ‘bottom’ face of the dimer pack together in another set of hydrophobic contacts ‘below’ the dimer (Fig. 2c,d). The hydrophobic contacts between the dimer layers appear to be important in the crystallization and supramolecular assembly of ABSM 1r and may explain the formation of the out-of-register interface within the dimer.

\[ \text{Inhibition of amyloid aggregation by ABSMs 1.} \]

\[ \text{Thioflavin T (ThT) fluorescence assays and transmission electron microscopy (TEM) studies showed that the ABSMs containing amyloidogenic sequences can inhibit the aggregation of amyloid proteins. We studied the inhibition of } \beta\text{-amyloid } (\beta\text{-A}) \text{ aggregation by ABSM 1a, the inhibition of } \alpha\text{-synuclein (hSyn}_{1-100}) \text{ aggregation by ABSM 1m and the inhibition of truncated human } \alpha\text{-synuclein (hSyn}_{1-100}) \text{ aggregation by ABSM 1o.} \]

\[ \text{ThT fluorescence assays show that ABSMs 1a, 1m and 1o effectively delay aggregation of their parent proteins at sub-stoichiometric concentrations in a dose-dependent manner (Fig. 3a–d). At 0.2 equiv., ABSM 1a delays } \beta\text{-A} \text{ aggregation by 280% and 350%, respectively, and at 0.5 equiv. by 430 and 600% (Fig. 3a,b). Although ThT fluorescence assays show that } \beta\text{ aggregation exhibits comparable lag times at 0.5 and 1.0 equiv. of ABSM} \]

\[ \text{Figure 2 | X-ray crystallographic structure of ABSM 1r, which contains the heptapeptide sequence AllGLMV (Aβ}_{30-36}).} \]

\[ \text{a. The monomer. b,c. The dimer (top view, b, side view, c). d. Stacked layers of dimer in the crystal lattice. Note that the view in b is perpendicular to the β-sheet (top view), whereas the view in c and d is 90° away, parallel to the β-sheet (side view), and shows the hydrophobic contacts. Some side chains in c and d have been omitted for clarity.} \]
Figure 3 | Effect of ABSMs on inhibition of Aβ_{40}, Aβ_{42}, hβ_{2M} and hooSyn_{1–100} aggregation monitored by thioflavin T fluorescence assays and TEM.

a. Lag time of Aβ_{40} (20 μM) aggregation in the absence and presence of ABSM 1a, b. Lag time of Aβ_{42} (20 μM) aggregation in the absence and presence of ABSM 1a, c. Lag time of hβ_{2M} (30 μM) aggregation in the absence and presence of ABSM 1m, d. Lag time of hooSyn_{1–100} (50 μM) aggregation in the absence and presence of ABSM 1o, e. TEM images of Aβ_{40} (20 μM) after incubation for 6 h without ABSM 1a (top) and incubation for 6 h with 1.0 equiv. of ABSM 1a (bottom), f. TEM images of Aβ_{42} (20 μM) after incubation for 7 h without ABSM 1a (top) and incubation for 7 h with 1.0 equiv. of ABSM 1a (bottom), g. TEM of hβ_{2M} (30 μM) after incubation for 2 h without ABSM 1m (top) and incubation for 2 h with 1.0 equiv. of ABSM 1m (bottom), h. TEM of hooSyn_{1–100} (50 μM) after incubation for 72 h without ABSM 1o (top) and incubation for 72 h with 1.0 equiv. of ABSM 1o (bottom). Error bars correspond to the standard deviation of four or more sets of experiments. For experimental details, see the Supplementary Information. *hooSyn_{1–100} aggregation exhibits longer lag times with 0.5 and 1.0 equiv. of ABSM 1o than with 0.2 equiv., with some runs showing complete suppression of aggregation and other runs showing typical sigmoidal curves (for details see Supplementary Fig. S4.)

1a, the growth phases of the aggregation are much slower at 1.0 equiv. than at 0.5 equiv. (for details, see Supplementary Figs S1 and S2). ABSM 1m delays hβ_{2M} aggregation by 160% at 0.2 and 0.5 equiv. and by 340% at 1.0 equiv. (Fig. 3c). ABSM 1o delays hooSyn_{1–100} aggregation by 150% at 0.2 equiv. (Fig. 3d). Although hooSyn_{1–100} aggregation exhibits longer lag times with 0.5 and 1.0 equiv. of ABSM 1o than with 0.2 equiv., some runs showed complete suppression of aggregation, and yet other runs showed typical sigmoidal curves. Because of this scatter in the data, precise lag times are not reported (asterisk in Fig. 3d; for details see Supplementary Fig. S4.)

TEM studies of samples taken directly from the ThT assays show that Aβ, hβ_{2M} and hooSyn_{1–100} form fibrils without ABSMs and do not form fibrils with ABSMs (1.0 equiv.) during the delayed lag time (Fig. 3e–h).

Aβ has been shown to cross-interact with different amyloidogenic proteins containing similar primary sequences. To investigate cross-interaction of Aβ with ABSMs, we compared the interaction of Aβ with ABSM 1a to that with ABSM 1m, which has a closely homologous sequence, and to that with ABSM 1o, which does not (Supplementary Fig. S5). ThT fluorescence assays show that ABSM 1m inhibits Aβ aggregation, like ABSM 1a, whereas ABSM 1o has little or no inhibitory effect (Supplementary Fig. S5). This result suggests that structurally homologous ABSMs can not only interact with their parent amyloid proteins but can also cross-interact with different amyloid proteins.

To further investigate the effect of sequence on inhibition, we compared the interaction of Aβ with ABSM 1a to that of ABSMs 1b, 1c, 1d and 1f with Aβ_{40}. ThT fluorescence assays show that ABSM 1b is effective against Aβ_{40} aggregation, whereas ABSMs 1c, 1d and 1f cause little or no inhibition (Supplementary Fig. S6). The inhibition of Aβ aggregation by both ABSMs 1a and 1b indicates that the central hydrophobic sequence Aβ_{17–21} is
mixture were used directly in cell viability assays. These assays showed that the Aβ40 and Aβ42 preincubated without ABSM 1a kill 42% and 46% of the PC-12 cells, respectively, relative to controls in which the cells are incubated in only phosphate-buffered saline (PBS) buffer solutions (Fig. 4).

Cell viability assays further established that preincubation of Aβ with ABSM 1a rescues the cells in a dose-dependent manner. Preincubation of Aβ40 and Aβ42 with 0.2 equiv. of ABSM 1a reduces the death of PC-12 to 29% and 38%, respectively, while preincubation with 1.0 equiv. reduces cell death to 27% and 30% and preincubation with 5 equiv. reduces cell death to 14% and 6%. The rescue of these neuron-like cells by ABSM 1a suggests that ABSMs may reduce the production of toxic amyloid oligomers or bind the oligomers and reduce their toxicity.

Discussion

ABSMs provide a unique tool with which to elucidate the process of amyloid aggregation. Although many of the details of amyloid aggregation remain unclear, nucleation-dependent polymerization, where seeding to form a β-structured nucleus is the rate-determining step, is widely accepted1,22. Based on nucleation-dependent polymerization, we propose a model for the potent inhibition of Aβ aggregation by ABSM 1a. In this model, ABSM 1a binds early β-structured oligomers and blocks Aβ nucleation (Fig. 5a). Without ABSM 1a, the unstructured monomer forms β-structured oligomers, which, in the rate-determining step, go on to form a β-structured nucleus that ultimately assembles to form cross-β fibrils. The solid line in Fig. 5a illustrates this pathway. ABSM 1a creates a new aggregation pathway for the early β-structured oligomers. In this pathway, ABSM 1a binds the β-structured oligomers to form Aβ-oligomer-ABSM–ABSM–1a complexes and blocks the Aβ oligomer-to-nucleus transition. The dashed line in Fig. 5a illustrates this pathway.

It is significant that ABSM 1a substantially delays the aggregation of Aβ at sub-stoichiometric concentrations (as low as 1 μM), for example, 0.05 equiv. of ABSM 1a per equivalent of Aβ (Supplementary Fig. S2), while simple linear peptide fragments derived from Aβ generally show substantial inhibitory effects at stoichiometric or greater concentrations33,34. This observation suggests that ABSM 1a binds a larger oligomer, not the monomer.
or a smaller oligomer such as a dimer, trimer or tetramer. ABSM 1a binds the early β-structured oligomers more strongly than the unstructured monomers bind oligomers, because the recognition β-strand of ABSM 1a is preorganized. This preorganization therefore promotes the formation of Ββ-oligomer–ABSM–1a complexes. The complexation may occur through edge-to-edge interactions between the hydrogen-bonding edge of ABSM 1a and exposed hydrogen-bonding groups of the Ββ oligomers, and through face-to-face hydrophobic interactions between ABSM 1a and the hydrophobic surfaces of the Ββ-oligomers. These types of interactions should take place between the hydrophobic sequence AB17–21 of ABSM 1a and that of the Ββ oligomers, as observed in the amyloid-related oligomers containing the pentapeptide sequence LVFFA shown in Fig. 5b and the amyloid-like fibrils from the hexapeptide KLFFFA shown in Fig. 5c. Similar interactions should also occur in the interactions of other ABSMs with their parent amyloidogenic peptides and proteins. The stabilization of these complexes creates a higher energy barrier to formation of the Ββ-structured nucleus and thus delays or halts fibril formation. Therefore, ABSM 1a cannot sequester all of the equilibrating Ββ oligomers, the Ββ monomers and oligomers eventually succumb to thermodynamics and form Ββ fibrils.

The X-ray crystallographic structure of ABSM 1r may provide insights not only into the stabilization of the dimerization and higher-order supramolecular assembly of ABSMs, but also into the stabilization and structure of intermediates formed during amyloid aggregation. The hydrophobic contacts formed by the Ile, Leu and Val at positions 3, 5 and 7 of ABSM 1r are akin to the steric zipper of amyloid-like fibrils formed by fragments AB16–21, AB30–35, AB35–40 and AB37–42 (refs 10 and 35). Both the layered crystal structure of ABSM 1r and the amyloid-like fibrils are stabilized by hydrophobic contacts. These observations suggest that maximization of both hydrophobic contact and hydrogen bonding is key to stabilizing not only amyloid fibrils but also transient amyloid oligomers.

Conclusion

The ABSMs described herein provide a single platform with which to display a variety of amyloidogenic heptapeptide Ββ-strands and provide a rational design for inhibitors to control amyloid aggregation. X-ray crystallographic and 1H NMR studies validate that the design of ABSMs 1— including cyclicity, Hao template, two Ββ-turn mimics and paired side chains—promotes the formation of Ββ sheets in which the folding is largely independent of the amino-acid sequence. ABSMs 1 can be tailored to inhibit the aggregation of different amyloid proteins. The inhibition of Ββ, hβ-M and hOY1110aggregation by ABSMs indicates that ABSMs containing one hydrogen-bonding edge and one blocking edge are an effective design for inhibitors of amyloid aggregation. The ability of ABSMs 1a, 1m and 1o to inhibit amyloid aggregation and to detoxify amyloid aggregates suggests the potential for therapeutic applications in amyloid-related diseases.

Materials and methods

Synthetic AB16–21 was purchased from GL Biochem (Shanghai). AB16–21, hβ-M and hOY1110 were expressed in Escherichia coli (for details, see Supplementary Information). ABSMs 1 were synthesized as described above (for details, see Supplementary Information). 1H NMR, 2D TOCSY and ROEY experiments with ABSMs 1 were performed in D2O with DSA (4,4-dimethyl-4-silapentane-1-sil ammonium trifluoracetate) as an internal standard at 500 MHz and 298 K (for details, see Supplementary Information). Crystallization, data collection and structure determination for the ABSM 1r are described in the Supplementary Information. TḩT fluorescence assays and TEM studies of Ββ, hβ-M and hOY1110 aggregation with ABSMs 1a, 1m and 1o are described in the Supplementary Information. Cell viability assays to establish the toxicity of ABSMs 1a, 1m and 1o towards HeLa, HEK-293 and PC-12 cells are also described in the Supplementary Information.

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**Author contributions**


**Additional information**

Supplementary information and chemical compound information are available in the online version of the paper. Reprints and permission information is available online at http://www.nature.com/reprints. Correspondence and requests for materials should be addressed to D.E. and J.S.N.

**Competing financial interests**

The authors declare no competing financial interests.