Specific aromatic foldamers potently inhibit spontaneous and seeded Aβ42 and Aβ43 fibril assembly

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INTRODUCTION

Protein misfolding can be fatal [1,2]. Proteins misfold from soluble species into highly stable, cross-β amyloid fibrils in Alzheimer’s disease (AD) and several other neurodegenerative diseases [1,2]. One strategy to combat these disorders is to develop inhibitors that prevent Aβ misfolding into protofibril-like conformers. Here, we design specific aromatic foldamers, synthetic polymers with an aromatic salicylamide (Sal) or 3-amino benzoic acid (Benz) backbone, short length (four repetitive units), basic arginine (Arg), lysine (Lys) or citrulline (Cit) side chains, and various N- and C-terminal groups that prevent spontaneous and seeded Aβ fibrillation. Ac-Sal-(Lys-Sal)-CONH\(_2\) and Sal-(Lys-Sal)-CONH\(_2\) selectively inhibited Aβ42 fibrillation, but were ineffective against Aβ43, an overlooked species that is highly neurotoxic and frequently deposited in AD brains. By contrast, (Arg-Benz)-CONH\(_2\) and (Arg-Sal)-(Cit-Sal)-CONH\(_2\) prevented spontaneous and seeded Aβ42 and Aβ43 fibrillation. Importantly, (Arg-Sal)-(Cit-Sal)-CONH\(_2\) inhibited formation of toxic Aβ42 and Aβ43 oligomers and proteotoxicity. None of these foldamers inhibited Sup35 prionogenesis, but Sal-(Lys-Sal)-CONH\(_2\) delayed aggregation of fused in sarcoma (FUS), an RNA-binding protein with a prion-like domain connected with amyotrophic lateral sclerosis and frontotemporal dementia. We establish that inhibitors of Aβ42 fibrillation do not necessarily inhibit Aβ43 fibrillation. Moreover, (Arg-Sal)-(Cit-Sal)-CONH\(_2\) inhibits formation of toxic Aβ conformers and seeding activity, properties that could have therapeutic utility.

Key words: Alzheimer’s disease, amyloid, Aβ42 (amyloid-β 42), Aβ43 (amyloid-β 43), foldamer, protein misfolding.
rapidly grow via their self-templating ends, which convert Aβ conformers into the cross-β conformation [20,28]. When coupled to fibril fragmentation, this ‘seeding’ activity enables Aβ fibrils to become self-propagating agents that transmit pathology and disease [1,29–31]. Aβ fibrils also provide catalytic surfaces for ‘secondary’ nucleation events distinct from fibril elongation [32–34]. Here, lateral Aβ fibril surfaces convert Aβ monomers into toxic oligomers [32–34]. Thus, formation of toxic oligomers and fibrils is intimately linked [32–34]. These secondary nucleation events also explain Aβ assembly kinetics [32–34]. Aβ forms different cross-β fibril structures termed ‘strains’, which can differ in toxicity and cause distinct brain pathology [35–38]. Aβ fibrils are usually less toxic than pre-amyloid oligomers [21,39]. However, Aβ fibrils also display toxicity [6,35,36,39]. A key challenge is to manipulate Aβ assembly in a manner that depopulates toxic conformers [7]. Agents that inhibit seeded assembly hold promise for preventing the spread of Aβ pathology in AD.

Numerous potential inhibitors of Aβ misfolding have been explored, including small molecules, peptides, molecular chaperones, protein disaggregases and antibodies [3,6,39–45]. In the present study, we explore a different strategy by pursuing foldamers; non-biological discrete chain molecules that lack a canonical peptide backbone but can fold into specific structures [46]. Foldamers have been utilized as antimicrobial agents and molecular scaffolds [47–50]. Peptides containing non-natural amino acids, similar to foldamers, have been useful for understanding the misfolding of various amyloidogenic peptides [42,51–53]. Foldamers have several advantageous properties that could make them a valuable class of amyloid inhibitors. Due to their semi-rigid backbone, foldamers can assume an organized conformation at low entropic cost with relatively few monomeric units [50,54]. Compared with α peptides, foldamers have greater thermodynamic stability and resist proteases. Furthermore, foldamers of varying lengths with diverse side chains and 3D shapes can be synthesized. These features enable foldamer design for interaction with diverse biological targets [47–50,55]. In the present study, we explore aromatic foldamers as antagonists of Aβ42 and Aβ43 amyloidogenesis.

Figure 1 Overview of aromatic foldamer structure

The core foldamer structure is shown in the dashed box, which can be decorated with different moieties at X-, R-, Y- and Z-positions indicated on the periphery. Foldamers possess an aromatic Sal or Benz backbone (Y = OMe or H), Arg, Lys or Cit side chains (R = Arg, Lys or Cit), short length (two to four repetitive units) and various N- (X = NH2 or Ac) and C- (Z = NH2, OH, OMe or β-Ala) terminal groups.

MATERIALS AND METHODS

Generation of soluble and fibrillar Aβ42 and Aβ43

To produce monomeric Aβ42, synthetic lyophilized Aβ42 or Aβ43 (W.M. Keck Facility, Yale University) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma) at 2 mg/ml. HFIP was removed by drying in a speed vacuum for 30 min. The resulting peptide film was dissolved in DMSO to 1 mM. Aβ42 or Aβ43 fibrils for seeding experiments were prepared by diluting monomerized Aβ42 or Aβ43 in KHMD (150 mM KCl, 40 mM Hepes–KOH pH 7.4, 20 mM MgCl2 and 1 mM DTT) to 10 μM. This solution was incubated at 37 °C for 3–5 days with agitation (700 r.p.m.) in an Eppendorf Thermomixer. For seeding experiments, preformed fibrils were briefly sonicated or vortex-mixed prior to use. We also prepared Aβ42 or Aβ43 using a protocol that avoids DMSO. Thus, Aβ42 or Aβ43 was dissolved in HFIP followed by evaporation of the solvent to dryness [56]. Dry peptide films were dissolved in a minimal volume of 60 mM NaOH followed by dilution with deionized water and sonication for 1 min using a bath sonicator. Peptides were diluted to 0.2 mM by adding an equal volume of 20 mM sodium phosphate buffer (PB, Sigma), pH 8 plus 0.2 mM EDTA (PBE). Samples were centrifuged at 16000 g for 3 min and subjected to Superdex 75 gel filtration in PBE to remove residual solvent.

Foldamers

Foldamers (Lys-Sal)4-CONH2, (Arg-Benz)4-CONH2, (Lys-Sal)4-COMe, (Lys-Sal)4-COOH, (Lys-Sal)4-COβ-Ala, Ac-(Lys-Sal)4-CONH2, Sal-(Lys-Sal)4-CONH2 and Ac-Sal-(Lys-Sal)4-CONH2, where Sal is salicylamide and Benz is 3-amino benzoic acid) were from PolyMedix and were dissolved in TBS (50 mM Tris/HCl pH 7.4, 150 mM NaCl) to obtain concentrated stock solutions. Foldamers (Cit-Sal)4-CONH2, (Arg-Sal)-(Cit-Sal)-(Arg-Sal)-CONH2, (Arg-Sal)-(Cit-Sal)-(Arg-Sal)-CONH2, (Cit-Sal)-(Arg-Sal)-(Cit-Sal)-(Ar-Sal)-(Cit-Sal)-(Arg-Sal)-(Cit-Sal)-(Arg-Sal)-(Cit-Sal)-(Arg-Sal)-CONH2 were also from PolyMedix. These foldamers were dissolved in 1:1 TBS/DMSO to obtain
Specific aromatic foldamers antagonize Aβ amyloidogenesis

Figure 2 Nomenclature and structure of aromatic foldamers

Three-letter amino acid nomenclature is used to indicate the side chain (Lys, Arg or Cit) and the Sal or Benz backbone is indicated. N- (Ac) and C- (NH₂, OH, OMe or β-Ala) terminal groups are also indicated. Foldamers that inhibit spontaneous Aβ42 and Aβ43 fibrillization, (Arg-Benz)₄-CONH₂ and (Arg-Sal)₃-(Cit-Sal)-CONH₂, are boxed in black. Foldamers that inhibit spontaneous Aβ42 fibrillization but not spontaneous Aβ43 fibrillization, Sal-(Lys-Sal)₃-CONH₂ and Ac-Sal-(Lys-Sal)₃-CONH₂, are boxed in grey.

Concentrated stocks. Subsequent dilutions were made from these stocks to appropriate concentrations in KHMD or PBE.

Foldamers (Lys-Sal)₂-CONH₂, Ac-(Lys-Sal)₂-CONH₂, Sal-(Lys-Sal)₂-CONH₂, (Lys-Sal)₃-CONH₂ and Ac-(Lys-Sal)₃-CONH₂ were synthesized at room temperature on a 100 μmol scale using rink amide resin (GemScript Corporation, 0.6 mmol/g substitution) for support of alternating α- (Bachem) and aromatic amino acids. Resin was swelled in 100% dimethylformamide (DMF, Fisher Scientific) for 1 h, followed by a 30 min deprotection using 5% piperazine (Sigma–Aldrich) in DMF. The first residue was coupled to the resin using 3 equiv. of amino acid, 2.8 equiv. of 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU, GL Biosciences) activator and 7.5 equiv. of di-isopropylethylamine (DIEA, CHEM-IMPEX International), shaking for 1 h at room temperature. The resin was washed three times each with DMF, dichloromethane (DCM, Fisher Scientific) and DMF. This step was followed by deprotection (as above). Coupling and deprotection steps were cycled for the remaining residues in each respective peptide sequence. After deprotection of the
Figure 3  (Arg-Benz)$_2$-CONH$_2$, (Arg-Sal)$_3$-(Cit-Sal)-CONH$_2$, Sal-(Lys-Sal)$_3$-CONH$_2$ and Ac-Sal-(Lys-Sal)$_3$-CONH$_2$ inhibit spontaneous Aβ42 fibrillization

(A) Aβ42 (5 μM) was incubated with agitation for 8 h at 25°C plus or minus the indicated foldamer (10 μM). Aβ42 fibrillization was assessed by ThT fluorescence. Values represent means ± S.E.M. (n = 3–6). A one-way ANOVA with the post-hoc Dunnett’s multiple comparisons test was used to compare Aβ42 alone to each Aβ42 plus foldamer condition (* denotes P < 0.05). Foldamers that selectively inhibit Aβ42 fibrillization are indicated by grey bars and foldamers that inhibit Aβ42 and Aβ43 fibrillization are indicated by black bars. (B) Aβ42 (5 μM) was incubated with agitation for 4 h at 25°C in the absence or presence of the indicated foldamer (10 μM). Aβ42 fibrillization was assessed by EM. Scale bar, 500 nm.
Specific aromatic foldamers antagonize A\(\beta\) amyloidogenesis

Figure 4 Effect of inhibitory foldamers on spontaneous A\(\beta\)42 fibrillization kinetics

(A–D) A\(\beta\)42 (5 \(\mu\)M) was incubated with agitation for 0–8 h at 25°C in the absence (open circles) or presence of 5 \(\mu\)M (filled triangles), 10 \(\mu\)M (filled squares) or 20 \(\mu\)M (filled circles) (Arg-Benz)\(_4\)-CONH\(_2\) (A), (Arg-Sal)\(_3\)-(Cit-Sal)-CONH\(_2\) (B), Sal-(Lys-Sal)\(_3\)-CONH\(_2\) (C) or Ac-Sal-(Lys-Sal)\(_3\)-CONH\(_2\) (D). A\(\beta\)42 fibrillization was assessed by ThT fluorescence. Values represent means \(\pm\) S.E.M. (n = 3).

Spontaneous and seeded A\(\beta\)42, A\(\beta\)43 and N-terminal and middle domain of Sup35 (NM) fibrillization

For spontaneous fibrillization, soluble A\(\beta\)42 or A\(\beta\)43 (1 mM) in DMSO was diluted to 5 \(\mu\)M in KHMD containing 25 \(\mu\)M thioflavin-T (ThT) plus or minus foldamer (0–20 \(\mu\)M). For seeded fibrillization, preformed A\(\beta\)42 or A\(\beta\)43 fibrils (10 \(\mu\)M monomer) were added at a final concentration of 0.1 \(\mu\)M (monomer). Alternatively, A\(\beta\)42 or A\(\beta\)43 were prepared using just HFIP and were assembled at 5 \(\mu\)M in PBE containing 25 \(\mu\)M ThT plus or minus foldamer (20 \(\mu\)M). NM was purified as described [57]. NM (5 \(\mu\)M) was assembled in KHMD containing 25 \(\mu\)M ThT plus or minus foldamer (20 \(\mu\)M). For seeded fibrillization, preformed NM fibrils (5 \(\mu\)M monomer) were added at a final concentration of 0.1 \(\mu\)M (monomer). Reactions were conducted in 96-well plates and incubated at 25°C in a TECAN Safire II plate reader (Tecan USA) for up to 8 h with agitation. ThT fluorescence was measured at the indicated times. The excitation wavelength was 450 nm (5 nm bandwidth) and the emission wavelength was 482 nm (10 nm bandwidth). ThT fluorescence values reported are arbitrary and are normalized to the final assembly time point of the A\(\beta\) alone condition.

FUS aggregation

GST–TEV–FUS was purified as described [58]. Aggregation was initiated by addition of tobacco etch virus (TEV) protease to GST–TEV–FUS (5 \(\mu\)M) plus or minus foldamer (20 \(\mu\)M) in assembly buffer (50 mM Tris/HCl pH 8, 0.2 M trehalose and 20 mM glutathione). Aggregation was for 0–90 min at 25°C without agitation in a 96-well plate and was assessed by turbidity (absorbance at 395 nm) using a TECAN Infinite M1000 plate reader [58]. No aggregation occurred unless TEV protease was added to separate GST from FUS [58]. SDS/PAGE and Coomassie staining revealed that foldamers did not inhibit cleavage of GST–TEV–FUS by TEV.

Electron microscopy

Reactions were adhered on to 300-mesh-formvar carbon-coated EM grids overnight before being negatively stained with 2% uranyl acetate for 2 min and rinsed with milli-Q distilled water. Micrographs were acquired using a JEOL 1010 TEM (Jeol USA).
Tracking A11-reactive Aβ42 or Aβ43 conformers

The oligomer-specific A11 antibody was used to detect toxic Aβ42 or Aβ43 oligomers by ELISA as described [21]. Foldamers did not cross-react with A11.

Toxicity assays

SH-SY5Y human neuroblastoma cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) plus 10 mM Hepes, 10% FBS, 4 mM glutamine, penicillin (200 units/ml) and streptomycin (200 μg/ml) in 5% CO₂ at 37°C. Cells were differentiated in serum-free DMEM with N2 supplement and 10 μM all-trans-retinoic acid before use. Cells were plated at (10000 cells/well) in 96-well plates and grown overnight. Medium was removed and Aβ and cells were incubated for 24 h at 37°C. Toxicity was assessed using an MTT kit (Tox-1; Sigma) or via lactate dehydrogenase (LDH) release using the CytoTox-ONETM kit (Promega). Toxicity values were normalized to the buffer control without Aβ.

RESULTS AND DISCUSSION

Rationale and foldamer design

As potential inhibitors of Aβ42 and Aβ43 amyloidogenesis, we explored aromatic foldamers (Figures 1 and 2). Some of these foldamers were originally synthesized as inhibitors of heparin and are rich in aromatic and positively charged groups [55]. They possess an aromatic salicylamide (Sal) or 3-amino benzoic acid (Benz) backbone (Figure 1; Y = OMe or H), lysine (Lys), arginine (Arg) or citrulline (Cit) side chains (Figure 1; R = Lys, Arg or Cit), short length (two to four repetitive units) (Figure 1) and various N- (Figure 1; X = NH₂ or COMe [Ac]) and C- (Figure 1; Z = NH₂, OH, OMe or β-Ala) terminal groups. We selected this design for four reasons. First, the aromatic backbone is similar to ones employed by Nowick et al. [42,51–53] in protein aggregation inhibitors. Secondly, interactions between aromatic residues within short amyloidogenic peptides mediate molecular recognition during fibrillization [59]. Moreover, polyphenols such as (-)-epigallocatechin-3-gallate (EGCG) inhibit amyloidogenesis and prevent cytotoxicity [57,59–61]. Thus, the aromatic foldamer spine might antagonize aromatic interactions critical for fibrillization. Thirdly, the aromatic foldamers investigated are approximately the same length (two to four repetitive units) as steric zipper hexapeptides that form amyloid [19]. Finally, basic side chains, particularly arginine exert hydrotropic effects and prevent protein aggregation [62].

Foldamer inhibition screen

We tested 18 aromatic foldamers (Figure 2) for inhibition of spontaneous (i.e. in the absence of preformed fibrils) Aβ42 fibrillation. The majority of foldamers did not significantly inhibit Aβ42 fibrillation (Figure 3A). However, (Arg-Benz)4-CONH₂, (Arg-Sal)3-(Cit-Sal)-(Arg-Sal)-CONH₂ and Ac-Sal-(Lys-Sal)-CONH₂ were strong inhibitors (Figure 2 boxed in black or grey; Figures 3A and 3B; Figures 4A–4D). (Arg-Sal)3-(Cit-Sal)-CONH₂ was the most potent with an IC₅₀ of ~1.6 μM. Several important foldamer properties emerge for inhibition of Aβ42 fibrillation. First, a foldamer must have a backbone with at least four aromatic units to antagonize Aβ42 fibrillation. Thus, (Lys-Sal)2-CONH₂, (Lys-Sal)3-CONH₂, (Lys-Sal)4-COMe, (Lys-Sal)4-COOH and (Lys-Sal)4-COβ-Ala were strong inhibitors (Figure 2). Secondly, foldamers with more than three lysine or citrulline side chains were ineffective, encompassing: (Lys-Sal)₄-CONH₂, (Cit-Sal)₄-CONH₂, (Lys-Sal)₄-COMe, (Lys-Sal)₄-COOH and (Lys-Sal)₄-COβ-Ala (Figures 2 and 3A). By contrast, Sal-(Lys-Sal)₃-CONH₂ and Ac-Sal-(Lys-Sal)-CONH₂, which possess three lysine side chains and four aromatic backbone units, were potent inhibitors (Figures 2 and 3A). Thirdly, foldamers with three or more consecutive Arg side chains were effective inhibitors. Thus, (Arg-Benz)₄-CONH₂ and (Arg-Sal)₃-(Cit-Sal)-CONH₂ were potent inhibitors, whereas (Arg-Sal)₃-(Cit-Sal)-(Arg-Sal)-CONH₂, (Cit-Sal)₃-(Arg-Sal)-(Cit-Sal)-CONH₂, (Cit-Sal)₃-(Arg-Sal)-(Cit-Sal)-CONH₂ and (Arg-Sal-Cit-Sal)₂-CONH₂ were ineffective (Figures 2 and 3A).

Select small molecules that inhibit Aβ42 fibrillation also disassemble Aβ42 fibrils [4,57,60]. However, even when present in 4-fold molar excess, (Arg-Benz)-CONH₂, (Arg-Sal)-(Cit-Sal)-CONH₂, Sal-(Lys-Sal)-CONH₂ and Ac-Sal-(Lys-Sal)-CONH₂ did not disassemble Aβ42 fibrils after 24 h (results not shown). Thus, these foldamers do not reverse Aβ42 fibrillation.

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Foldamers that inhibit Aβ42 fibrillization do not inhibit NM fibrillization

Next, we assessed foldamer specificity by testing whether they inhibited amyloidogenesis of the prion domain, NM, of yeast Sup35 [63]. (Arg-Benz)₄-CONH₂, (Arg-Sal)₃-(Cit-Sal)-CONH₂, Sal-(Lys-Sal)₃-CONH₂ and Ac-Sal-(Lys-Sal)₃-CONH₂ did not inhibit NM fibrillization (Figure 5A). In the presence of (Arg-Benz)₄-CONH₂, NM formed fibrils that exhibited greater ThT fluorescence (Figure 5A). EM revealed that purely NM fibrils formed in the presence or absence of (Arg-Benz)₄-CONH₂ and sedimentation analysis revealed that equal quantities of NM formed fibrils (results not shown). Thus, (Arg-Benz)₄-CONH₂ does not stimulate NM fibrillization. Rather, we suggest that NM accesses a different prion strain in the presence of (Arg-Benz)₄-CONH₂. NM accesses different prion strains in the presence of certain small molecules, such as EGCG [57,63]. None of these foldamers inhibited seeded NM fibrillization (results not shown). Thus, these foldamers are not generic inhibitors of amyloidogenesis.

Sal-(Lys-Sal)₃-CONH₂ delays FUS aggregation

To further test specificity, we assessed inhibition of aggregation of FUS, an RNA-binding protein with a prion-like domain, which is connected with amyotrophic lateral sclerosis and frontotemporal dementia [1,58,64].
(Arg-Benz)$_4$-CONH$_2$, (Arg-Sal)$_3$-(Cit-Sal)-CONH$_2$, and Ac-Sal-(Lys-Sal)$_3$-CONH$_2$ did not inhibit FUS aggregation (Figure 5B). Interestingly, Sal-(Lys-Sal)$_3$-CONH$_2$ delayed FUS aggregation (Figure 5B). Sal-(Lys-Sal)$_3$-CONH$_2$ could serve as a lead foldamer to be optimized against FUS misfolding.

(Arg-Sal)$_3$-(Cit-Sal)-CONH$_2$ and Ac-Sal-(Lys-Sal)$_3$-CONH$_2$ inhibit seeded Aβ42 fibrillization

(Arg-Benz)$_4$-CONH$_2$, and Sal-(Lys-Sal)$_3$-CONH$_2$ only inhibited seeded Aβ42 fibrillation when present at a 4-fold molar excess over Aβ42 (Figures 6A and 6C, filled circles; Figure 6E). Even at this high concentration, some fibrillation occurred in the presence of (Arg-Benz)$_4$-CONH$_2$ (Figure 6A, filled circles) but was very limited by Sal-(Lys-Sal)$_3$-CONH$_2$ (Figure 6C, filled circles). Thus, (Arg-Benz)$_4$-CONH$_2$ and Sal-(Lys-Sal)$_3$-CONH$_2$ are more potent inhibitors of spontaneous Aβ42 fibrillation (Figures 4A and 4C) than seeded Aβ42 fibrillation (Figures 6A and 6C). (Arg-Benz)$_4$-CONH$_2$ and Sal-(Lys-Sal)$_3$-CONH$_2$ likely preferentially inhibit the rearrangement of Aβ42 oligomers into fibril-nucleating species [22]. Once Aβ42 fibrils have formed,
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Figure 8 Foldamers (Arg-Benz)₄-CONH₂ and (Arg-Sal)₃-(Cit-Sal)-CONH₂ inhibit seeded Aβ₄₃ fibrillization

(A-D) Aβ₄₃ (5 µM) was incubated with agitation for 0–2 h at 25 °C without (open squares) or with Aβ₄₃ fibril seed (0.1 µM monomer) in the absence (open circles) or presence of 5 µM (filled triangles), 10 µM (filled squares) or 20 µM (filled circles) (Arg-Benz)₄-CONH₂ (A), (Arg-Sal)₃-(Cit-Sal)-CONH₂ (B), Sal-(Lys-Sal)₃-CONH₂ (C) or Ac-Sal-(Lys-Sal)₃-CONH₂ (D). Aβ₄₃ fibrillation was assessed by ThT fluorescence. Values represent means ± S.E.M. (n = 3). (E) Aβ₄₃ (5 µM) plus Aβ₄₃ fibril seed (0.1 µM monomer) was incubated with agitation for 4 h at 25 °C plus or minus the indicated foldamer (10 µM). Aβ₄₂ fibrillation was assessed by EM. Scale bar, 500 nm.

It is unknown whether inhibitors that target Aβ₄₂ will also be active against Aβ₄₃. In the absence of foldamer, Aβ₄₃ fibrillation assembled more rapidly than Aβ₄₂ (Figures 4A–4D, and 7A–7D). Sal-(Lys-Sal)₃-CONH₂ and Ac-Sal-(Lys-Sal)₃-CONH₂ did not block spontaneous Aβ₄₃ fibrillation (Figures 7C–7E). Indeed, Sal-(Lys-Sal)₃-CONH₂ enabled Aβ₄₃
fibrils to form that exhibited higher ThT fluorescence (Figures 7C and 7E) and sedimentation analysis revealed that equal quantities of Aβ43 formed fibrils (results not shown). Thus, Sal-(Lys-Sal)4-CONH2 does not stimulate Aβ43 fibrillation. Rather, Aβ43 may access a different amyloid strain in the presence of Sal-(Lys-Sal)4-CONH2. These findings suggest that potent inhibitors of spontaneous Aβ42 fibrillation may not inhibit spontaneous Aβ43 fibrillation. By contrast, (Arg-Benz)4-CONH2 and (Arg-Sal)4-(Cit-Sal)-CONH2 blocked spontaneous Aβ43 fibrillation (Figures 7A, 7B and E). In both cases, small oligomers were the major species (Figure 7E). The IC50 of (Arg-Sal)4-(Cit-Sal)-CONH2 was ∼3.1 μM (Figures 7A and 7B).

(Arg-Benz)4-CONH2 and (Arg-Sal)4-(Cit-Sal)-CONH2 inhibited seeded Aβ43 fibrillation

Aβ43 fibrils eliminated the lag phase of Aβ43 assembly (Figures 8A–8D, compare open squares and open circles). Sal-(Lys-Sal)4-CONH2 and Ac-Sal-(Lys-Sal)4-CONH2 did not inhibit seeded Aβ43 fibrillation (Figures 8C–8E). Sal-(Lys-Sal)4-CONH2 enabled Aβ43 to access fibrillar forms that generated a higher ThT fluorescence signal, perhaps indicative of a distinct Aβ43 amyloid strain (Figure 8C). By contrast, (Arg-Benz)4-CONH2 and (Arg-Sal)4-(Cit-Sal)-CONH2 blocked seeded Aβ43 fibrillation (Figures 8A, 8B and 8E). The IC50 of (Arg-Sal)4-(Cit-Sal)-CONH2 against seeded Aβ43 fibrillation was ∼1.7 μM.

Foldamers inhibit Aβ42 and Aβ43 fibrillation under different assembly conditions

Next, we established that foldamers inhibited spontaneous and seeded Aβ42 and Aβ43 fibrillation under different assembly conditions, which might support formation of different amyloid strains. Thus, we avoided DMSO in Aβ preparation and assembled in a higher pH buffer. Under these conditions, a negative control foldamer, (Cit-Sal)4-CONH2 (Figure 2), had no effect (Figure 9). By contrast, (Arg-Benz)4-CONH2, (Arg-Sal)4-(Cit-Sal)-CONH2, Sal-(Lys-Sal)3-CONH2 and Ac-Sal-(Lys-Sal)3-CONH2 inhibited spontaneous and seeded Aβ42 fibrillation (Figure 9), whereas only (Arg-Benz)4-CONH2 and (Arg-Sal)4-(Cit-Sal)-CONH2 inhibited spontaneous and seeded Aβ43 fibrillation (Figure 9).

(Arg-Sal)4-(Cit-Sal)-CONH2 antagonizes formation of A11-reactive Aβ42 and Aβ43 oligomers

Could foldamers inhibit the formation of toxic Aβ42 and Aβ43 oligomers? To assess toxic Aβ42 and Aβ43 oligomer formation, we employed the conformation-specific A11 antibody, which specifically recognizes preamyloid oligomers formed by multiple proteins, including Aβ42, but not monomers or fibrils [21]. We assessed formation of A11-reactive species at the start of spontaneous assembly (0 h), at the end of lag phase (0.5 h), and at the endpoint of fibrillation (4 h). In the absence of Aβ42 and Aβ43, no A11 immunoreactivity was observed (results not shown). For Aβ42 and Aβ43, A11-reactive conformers were scarce at the start of the reaction (Figure 10A, buffer controls, black bars), abundant at end of lag phase (Figure 10A, buffer controls, grey bars), and declined once fibrillation was complete (Figure 10A, buffer controls, white bars). Aβ43 exhibited greater A11-immunoreactivity than Aβ42 and appears more prone to accessing this toxic conformation (Figure 10A).

A negative control foldamer, (Cit-Sal)4-CONH2 (Figure 2), had no effect on the appearance and disappearance of A11-reactive Aβ42 and Aβ43 conformers (Figure 10A). (Arg-Benz)4-CONH2, Sal-(Lys-Sal)3-CONH2 and Ac-Sal-(Lys-Sal)3-CONH2 had no effect on the abundance of A11-reactive Aβ42 or Aβ43 oligomers after 0.5 h (Figure 10A, grey bars). Thus, these foldamers inhibit spontaneous Aβ42 or Aβ43 fibrillation without affecting the formation of A11-reactive conformers. Furthermore, after 4 h in the presence of (Arg-Benz)4-CONH2, Sal-(Lys-Sal)3-CONH2, and Ac-Sal-(Lys-Sal)3-CONH2, A11-reactive Aβ42 species remained at higher levels and did not decline as much as they did in the absence of foldamer (Figure 10A, white bars). Thus, (Arg-Benz)4-CONH2, Sal-(Lys-Sal)3-CONH2, and Ac-Sal-(Lys-Sal)3-CONH2 stabilize A11-reactive conformers. (Arg-Benz)4-CONH2, but not Sal-(Lys-Sal)3-CONH2 or Ac-Sal-(Lys-Sal)3-CONH2, had a similar effect on A11-reactive Aβ43 species (Figure 10A). By contrast, A11-reactive Aβ43 species declined more extensively after 4 h in the presence of Sal-(Lys-Sal)3-CONH2 or Ac-Sal-(Lys-Sal)3-CONH2 (Figure 10A, white bars), which do not inhibit spontaneous Aβ43 fibrillation (Figures 7C and 7D).

(Arg-Sal)4-(Cit-Sal)-CONH2 inhibits formation of toxic Aβ42 and Aβ43 conformers

Next, we evaluated the relative toxicity of Aβ42 and Aβ43 conformers formed in the absence or presence of foldamers. We applied Aβ42 and Aβ43 conformers to SH-SYSY neuroblastoma cells and assessed cell viability using MTT reduction and LDH release. Foldamers and buffer display little toxicity in the absence...
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Figure 10  (Arg-Sal)₃-(Cit-Sal)-CONH₂ inhibits formation of toxic Aβ₄₂ and Aβ₄₃ conformers

(A–C) Aβ₄₂ or Aβ₄₃ (5 μM) was incubated at 25°C with agitation for 0 h (black bars), 0.5 h (grey bars) or 4 h (white bars) in the absence or presence of 20 μM (Arg-Benz)₄-CONH₂, (Arg-Sal)₃-(Cit-Sal)-CONH₂, Sal-(Lys-Sal)₃-CONH₂, Ac-Sal-(Lys-Sal)₃-CONH₂ or (Cit-Sal)₄-CONH₂. At the indicated times, the amount of A11-reactive species present (A) or toxicity to SH-SY5Y neuroblastoma cells in culture was determined via MTT reduction (B) or LDH release (C). We also assessed the toxicity of buffer, (Arg-Benz)₄-CONH₂, (Arg-Sal)₃-(Cit-Sal)-CONH₂, Sal-(Lys-Sal)₃-CONH₂, Ac-Sal-(Lys-Sal)₃-CONH₂ or (Cit-Sal)₄-CONH₂ alone (B and C). Values represent means ± S.E.M. (n = 3). A one-way ANOVA with the post-hoc Dunnett’s multiple comparisons test was used to compare Aβ₄₂ plus buffer to each Aβ₄₂ plus foldamer condition (* denotes P < 0.05). Likewise, a one-way ANOVA with the post-hoc Dunnett’s multiple comparisons test was used to compare Aβ₄₃ plus buffer to each Aβ₄₃ plus foldamer condition (* denotes P < 0.05).
of Aβ (Figures 10B and 10C, far right). In the absence of foldamer, Aβ42 and Aβ43 exhibited little toxicity after 0 h (Figures 10B and 10C), consistent with reduced Aβ11 immunoreactivity at this time (Figure 10A). Aβ42 and Aβ43 were more toxic after 0.5 h of assembly than after 4 h (Figures 10B and 10C), indicating that conformers that accumulate at the end of lag phase are more toxic than mature fibrils. In the absence of foldamer, Aβ43 conformers were generally more toxic than Aββ42 conformers (Figures 10B and 10C). The negative control foldamer, (Cit-Sal)3-CONH2, had no effect on toxicity (Figures 10B and 10C). (Arg-Benz)4-CONH2, Sal-(Lys-Sal)3-CONH2 and Ac-Sal-(Lys-Sal)3-CONH2 had no effect on the toxicity of Aβ42 conformers after 0.5 h of assembly (Figures 10B and 10C, grey bars), but after 4 h of assembly the toxicity of Aβ42 conformers was enhanced (Figures 10B and 10C, white bars). Thus, (Arg-Benz)4-CONH2, Sal-(Lys-Sal)3-CONH2 and Ac-Sal-(Lys-Sal)3-CONH2 inhibited spontaneous Aβ42 fibrilization such that more toxic conformers are maintained (Figures 10A–C). For Aβ43, neither Sal-(Lys-Sal)3-CONH2 nor Ac-Sal-(Lys-Sal)3-CONH2 affected the toxicity of conformers after 0.5 h or 4 h (Figures 10B and 10C). However, as for Aβ42, (Arg-Benz)4-CONH2 had no effect on the toxicity of Aβ43 conformers after 0.5 h of assembly (Figures 10B and 10C, grey bars), but after 4 h the toxicity of Aβ43 conformers was enhanced (Figures 10B and 10C, white bars). Thus, (Arg-Benz)4-CONH2 inhibits spontaneous Aβ43 fibrilization in a manner that maintains toxic conformers (Figures 10A–10C).

(Arg-Sal)3-(Cit-Sal)-CONH2, which inhibited the formation of A11-reactive Aβ42 and Aβ43 conformers after 0.5 h (Figure 10A, grey bars), also partially reduced the toxicity of Aβ42 and Aβ43 conformers at this time (Figures 10B and 10C, grey bars) and at 4 h (Figures 10B and 10C, white bars). Although Aβ42 and Aβ43 conformers still conferred toxicity in comparison with buffer controls, (Arg-Sal)3-(Cit-Sal)-CONH2 was the only foldamer that antagonized Aβ42 and Aβ43 toxicity.

(Arg-Sal)3-(Cit-Sal)-CONH2 inhibits spontaneous and seeded Aβ42 and Aβ43 fibrilization and reduces accumulation of toxic Aβ42 and Aβ43 conformers. This combination of properties could have therapeutic potential for three reasons. First, (Arg-Sal)3-(Cit-Sal)-CONH2 antagonizes Aβ42 as well as Aβ43, which is an often overlooked but highly toxic Aβ species [13–16]. Secondly, (Arg-Sal)3-(Cit-Sal)-CONH2 inhibits the formation of toxic Aβ42 and Aβ43 conformers, which could reduce localized neurodegeneration [65]. Thirdly, (Arg-Sal)3-(Cit-Sal)-CONH2 inhibits seeded Aβ42 and Aβ43 assembly, which could prevent the spreading of Aβ pathology throughout the brain in AD [29–31]. Further studies are needed to assess the utility of (Arg-Sal)3-(Cit-Sal)-CONH2 against Aβ42 and Aβ43 assembly in the metazoan nervous system.

Future studies will reveal the mechanisms by which foldamers antagonize Aβ-fibrillization. Foldamers have amides oriented in a manner that maintains toxic conformers (Figures 10A–10C). Aromatic foldamers could be useful amyloidogenesis inhibitors for various disease-associated proteins. Indeed, another class of aromatic foldamers inhibits amylin fibrillation, which is connected to Type 2 diabetes [66]. Thus, foldamers await further development to antagonize protein misfolding in several settings.
Specific aromatic foldamers antagonize Aβ amyloidogenesis

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