A rational design to create hybrid beta-sheet breaker peptides to inhibit aggregation and toxicity of amyloid-β

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Supporting information

General experimental

Unless stated otherwise all chemicals were obtained from commercial sources and used without further purification. If no further details are given the reaction was performed under ambient atmosphere and temperature. Dichloromethane was dried over an activated alumina column using an MBraun SPS800 solvent purification system. The water used was deionised using a Labconco Water Pro PS purification system. If no further details are given, purification of peptide was carried out on a Gilson semipreparative RP-HPLC system at a flow rate of 4 mL/min, using a Dr. A. Maisch C_{18} column, with a linear gradient of 5% to 100% acetonitrile (0.1% TFA) in 60 minutes. The peptides were characterized by electrospray MS and analytical RP-HPLC (flow rate 0.4 ml/min; same gradient as semipreparative RP-HPLC). The solvent system used both for analytical and preparative HPLC was: deionised water and acetonitrile.

Mass spectrometry (MS)

Mass analyses were performed using electrospray ionization on a LCQ Advantage Max from Thermo Finnigan.

Synthesis

General procedure for pentapeptide 4-nitrobenzoate formation (2) by solid phase synthesis

The LPFFD peptide **(1)** was synthesized using standard 9-fluorenylmethyl carbamate (Fmoc) peptide synthesis on a Breipohl resin[1;2]. Peptide coupling was achieved using 3 equiv of Fmoc amino acid and dicyclohexylcarbodiimide (DCC, 3.3 equiv) with *N*-hydroxy benzotriazole (HOBt, 3.6 equiv) in DMF. The resin was swollen in DMF for 20 min prior to use. The Fmoc group was removed using piperidine in DMF (20% v/v, three times, 6 min). After coupling of the final amino acid, the Fmoc-protected peptide on the resin was washed thoroughly with DMF, dichloromethane and methanol. The resin was dried in vacuum and was divided in batches.

The dry resin was swollen for 30 min in DMF. Subsequently, the Fmoc-protecting group was removed using piperidine in DMF (20% v/v). The resin was washed well with DMF and dichloromethane. Three equivalents of p-nitrophenylchloroformate was dissolved in dichloromethane and 3 equivalents of N,N-diisopropylethylamine (DIPEA) was added. The solution was added to the resin and the resin was agitated for 1 h. The resin was washed with dichloromethane and DMF and used for further synthesis.

General procedure for alcohol addition by urea functionality (3a-3d)

After washing pentapeptide 4-nitrobenzoate (**2**) with dichloromethane and DMF, 5 equivalents of 3-amino-1-propanol, 3-amino-1,2-propanediol or 4-amino-1-butanol was dissolved in DMF together with 3 equivalents of N,N-diisopropylethylamine (DIPEA) and added to the resin. The resin was agitated for 18 h. The resin was washed thoroughly with DMF and methanol and dried *in vacuo*. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/water/1,2-ethanedithiol/triisopropylsilan (92.5/2.5/2.5/2.5, v/v) for 2 h. After co-evaporation with heptane, the peptides were lyophilized from acetic acid. The peptides were purified using HPLC and characterized by HPLC and electrospray MS.

The main peak of **3a** eluted at R_t of 28.2 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{51}N_7O_9$ [M+H$^+$] 738.8, found 738.3. The main peak of **3b** eluted at R_t of 27.2 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{51}N_7O_{10}$ [M+H$^+$] 754.8, found 754.5. The main peak of **3c** eluted at R_t of 27.2 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{51}N_7O_{10}$ [M+H$^+$] 754.8, found 754.5. The main peak of **3d** eluted at R_t of 29.1 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{38}H_{53}N_7O_9$ [M+H$^+$] 752.9, found 752.5.

General procedure for alcohol addition by carbamate functionality (5a-5c)

After washing compound 2 with dichloromethane and DMF, 5 equivalents of 1,3-propanediol or glycerol was dissolved in DMF together with 1.3 equivalents of 1,8-diazabicycloundec-7-ene (DBU) and added to the resin. The resin was agitated for 18 h. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/water/1,2-ethanedithiol/triisopropylsilan (92.5/2.5/2.5/2.5, v/v) for 2 h. After co-evaporation with heptane, the peptides were lyophilized from acetic acid. The peptides were purified using HPLC and characterized by HPLC and electrospray mass spectrometry. The main peak of **5a** eluted at R_t of 29.8 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{50}N_6O_{10}$ [M+H$^+$] 739.8, found 739.4. The main peak of **5b** eluted at R_t of 27.8 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{50}N_6O_{11}$ [M+H$^+$] 755.8, found 755.5. The main peak of **5c** eluted at R_t of 27.8 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{50}N_6O_{11}$ [M+H$^+$] 755.8, found 755.5.

Preparation of $C_5H_{10}O_4$-LPFFD-OH (5d)

The LPFFD peptide was synthesized using standard 9-fluorenylmethyl carbamate (Fmoc) peptide synthesis on a 2-chlorotrityl chloride resin[3;4]. The resin was swollen in dichloromethane for 20 min and 5 equivalents of DIPEA and 3 equivalents of Fmoc-protected aspartic acid (OBz protected) were added and the mixture was agitated for 30 minutes. After washing with dichloromethane, 5 equivalents of DIPEA and 3 equivalents of methanol were added and the mixture was agitated for 30 minutes to cap possible unreacted sites on the resin. The resin was washed thoroughly with dichloromethane and DMF. The Fmoc group was removed using piperidine in DMF (20% v/v, three times, 6 min). Further peptide coupling was achieved using 3 equiv of Fmoc amino acid and dicyclohexylcarbodiimide (DCC, 3.3 equiv) with *N*-hydroxybenzotriazole (HOBt, 3.6 equiv) in DMF. After coupling of the final amino acid, the Fmoc-protected peptide on the resin was washed thoroughly with DMF and methanol. The resin was dried in vacuum and was divided in batches.

The dry resin was swollen for 30 min in DMF. Subsequently, the Fmoc-protecting group was removed using piperidine in DMF (20% v/v). The resin was washed well with DMF and dichloromethane. Three equivalents of p-nitrophenylchloroformate was dissolved in dichloromethane and 3 equivalents of DIPEA was added. The solution was added to the resin and the resin was agitated for 1 h. After washing the resin with dichloromethane and DMF, 5 equivalents of 2-hydroxymethyl-1,3-propanediol was dissolved in DMF together with 1.3 equivalents of DBU and added to the resin. The resin was agitated for 18 h. The peptides were cleaved from the resin by treatment with dichloromethane/1,1,1-trifluoroethane/acetic acid (3/1/1, v/v) for 1 h. After removal of the solvents under reduced pressure, the peptides were lyophilized from acetic acid.

The peptides were dissolved in methanol until a concentration of 0.1 N was reached. After removal of air from the solution, 5% Pd/C was a added to the stirred mixture and the benzylester of the peptides was hydrogenated at ordinary pressure (balloon) and temperature for 18 h. The reaction mixture was filtered using Celite and the filtrate was concentrated under reduced pressure. The peptides were lyophilized from acetic acid. The

peptides were purified using HPLC and characterized by electrospray mass spectrometry. The main peak of **5d** (L-proline) eluted at R_t of 25.7 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for [M+H$^+$] 770.8, found 770.4. The main peak of **5d'** (D-proline) eluted at R_t of 26.4 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for [M+H$^+$] 770.8, found 770.5.

General procedure for sulfonation of alcohols (4a-4d and 6a-6d)

The alcohol groups of the peptides were sulfonated according to a modified procedure from Lee et al[5]. In short, the peptides were dissolved in DMF (1,0 ml) and 10 equivalents of sulfur trioxide-trimethylamine complex was added to the solvent in 5 steps in 3 h. The mixture was stirred at 50 ºC under nitrogen overnight. The reaction flask was cooled down to room temperature, the reaction was quenched with saturated aqueous $NaHCO_3$ (2 ml) and the mixture was kept stirring for another 16 h. The solvent was coevaporated with ethanol under reduced pressure, and a mixed solvent of dichloromethane and methanol (1/1, 10 ml) was added to the residue. The mixture was filtered over Celite and the filtrate was concentrated under reduced pressure to give a syrup. After desalting the residue by column chromatography on Sephadex G-15 using deionised water, the sulfonated peptides were purified and analysed using C_{18} RP-HPLC. The solvent system used both for analytical and preparative HPLC was: deionised water 0.1 M $AcONH_4$ and acetonitrile[6-8], linear gradient from 5% to 75% acetonitril in 30 minutes.

The main peak of **4a** eluted at R_t of 23.0 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{50}N_7O_{12}SNa$ [M-Na$^+$] 816.9, found 816.5. The main peak of **4b** eluted at R_t of 19.5 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{50}N_7O_{12}S_2Na_2$ [M-2.Na$^+$ + H$^+$] 913.0, found 912.9. The main peak of **4c** eluted at R_t of 20.5 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{50}N_7O_{12}S_2Na_2$ [M-2.Na$^+$ + H$^+$] 913.0, found 912.9. The

main peak of **4d** eluted at R_t of 23.3 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{38}H_{52}N_7O_{12}SNa$ [M-Na^+] 830.9, found 830.5.

The main peak of **6a** eluted at R_t of 23.6 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{49}N_6O_{13}SNa$ [M-Na^+] 817.9, found 817.5. The main peak of **6b** eluted at R_t of 19.6 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{48}N_6O_{17}S_2Na_2$ [M-2.Na^+ + H^+] 913.9, found 913.3. The main peak of **6c** eluted at R_t of 20.6 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for $C_{37}H_{48}N_6O_{17}S_2Na_2$ [M-2.Na^+ + H^+] 913.9, found 913.3. The main peak of **6d** (L-proline) eluted at R_t of 18.5 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for [(M-2.Na^+)/2] 463.9, found 463.5. The main peak of **6d'** (D-proline) eluted at R_t of 18.5 minutes of the analytical chromatogram, was confirmed by electrospray mass spectrometry: m/z calculated for [(M-2.Na^+)/2] 463.9, found 463.5.

Aggregation assay

To study the effect of the hybrid ligands on β-sheet formation of Aβ in the first 48 hours of incubation, we analyzed the formation of β-pleated sheets using thioflavin T (Th-T). The assay is based on the fluorescence emission of Th-T. Th-t binds to Aβ, producing a fluorescent signal that is proportional to the amount of fibrils formed[9]. $Aβ_{1-42}$ was incubated either with or without hybrid ligand in a molar ratio of 1:20 ($Aβ_{42}$:hybrid ligand) in 50 mM Tris-HCl buffer with 150 mM NaCl (pH 7.4) at a concentration of 50 μM for 48 hours. At different time points, 20 μl samples were taken and mixed with 50 mM glycine (pH 9.2), 3 μM Th-T in a final volume of 1 ml. Fluorescence was measured in duplicate with excitation at 450 nm and emission at 482 nm using a Perkin-Elmer luminescence spectrometer.

Cell culture

Human brain pericytes (HBP) and human leptomeningeal smooth muscle cells (HLSMC) were isolated and characterized as described previously[10-12]. Cells were maintained in Eagle's minimal essential medium (EMEM; Bio Whittaker Europe, Verviers, Belgium) supplemented with 10% human serum (Gemini Bio-Products, Calabasas, CA, USA), 20% fetal calf serum (Life Technologies, Rockville, USA), 0.1% basic fibroblast growth factor and 2% gentamycin at 37ºC and 5%-CO_2. Cell passages 3-15 were used for the experiments. For degeneration studies, cells were incubated in an eight-well chamber slide (Nunc, Roskilde, Denmark) with EMEM and 0.1% BSA (serum-free medium) supplemented with 10.0 μM wild-type $A\beta_{1-42}$ for 6 days[13;14]. Control cells incubated with the serum-free medium alone demonstrated normal morphology. Cells were co-incubated with the hybrid ligands in a molar ratio of 1:5 ($A\beta_{42}$:hybrid ligand) for 6 days. Cell viability was quantified using a fluorescent Live/Dead ® Viability/Cytotoxicity Kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA) and analyzed using a Leica fluorescence microscope. The percentage of dead cells was determined from at least four counts per well (approximately 800 cells per count), and the experiments were performed in duplicate. Each experiment was repeated at least two times.

Statistical analysis

Statistical analysis was performed using ANOVA with Bonferroni's post hoc analyses for multiple comparisons using SPSS 14.0 for Windows (SPSS inc., Chicago, IL).

Calculations

The structure of the $A\beta_{42}$ fibrils used for the molecular modeling experiment was based on the NMR structure deposed in RCSB Protein Data Bank under the ID number 2BEG[15]. The missing residues 1-16 were modelled using YASARA module LOOP MODELLING (www.yasara.com). Atoms of these flexible residues were never constrained. Two kinds of molecular modeling experiments were performed. First, to determine the alignment of the

hybrid ligands with the Aβ fibrils, the synthesized peptides were superimposed on residues LVFF of the chain E. In the next step the chain E was deleted and the interactions of a peptide with A-D molecules of $Aβ_{42}$ were further optimized using geometry minimization procedure as implemented in Yasara software. During the optimization and molecular dynamics simulations the positions of the backbone atoms of Aβ (residues 17-42) were constrained. The energy minimization and the molecular dynamics were performed using Amber99 force field. The molecular dynamics simulations carried for 2.5 ns in water at 300K gave an insight into the nature of interaction between the Aβ protein and proposed ligands as well as the flexibility of the ligands.

Second, to determine if the hybrid ligands could be incorporated into the β-sheet structure of aggregated Aβ, synthesized peptides were superimposed on residues LVFF of the chain C. After deletion of chain C were the molecular modeling experiments performed as described above.

Figure legends

Fig. S1: Alignment of **4a** to the $A\beta_{42}$ protein. A nice β-pleated sheet can be observed when aligning several $A\beta_{42}$ proteins. According to the obtained model a spacer of three carbon atoms should be sufficient to target His13 and/or His14 in the $A\beta_{42}$ protein with a sulfate group of the hybrid ligand. Therefore, our synthetic compounds could simultaneously target the binding domains of GAGs (binding to Aβ amino acids 13-16) and of pentapeptides (binding to Aβ amino acids 16-22).

Fig. S2: Snapshot of molecular dynamics simulations of **6b** aligned to the $A\beta_{42}$ protein. Although both phenylalanines of the hybrid ligand adopt the same orientation as the phenylalanines of the $A\beta_{42}$ protein, it is difficult to target the histidines in the $A\beta_{42}$ protein with a non-peptide side-chain of the hybrid ligand. The side-chain bends in such a way that the sulfate groups rather interact with Lys15 of the $A\beta_{42}$ protein backbone than with His13 or His14.

Fig. S3: Snapshot of molecular dynamics simulations of **6d'** (yellow) aligned into the β-sheet structure of aggregated $A\beta_{42}$ protein. The ligands can be incorporated into the β-sheet structure of aggregated Aβ, as was observed during these molecular dynamics calculations. The ligand connects/binds the two parts of the lower sheet (green) with on overage six hydrogen bonds and the two parts of the upper sheet (red) with on average two hydrogen bonds.

Results

Table 1: Overview biological effects of the hybrid ligands on Aβ aggregation and Aβ-mediated cytotoxicity

	Effect on $A\beta_{42}$ Aggregation (average ± standard deviation)*	Effect on $A\beta_{42}$ Toxicity (average ± standard deviation)*
3a	82.7 ± 5.8	96.1 ± 12.6
4a	83.0 ± 4.9	95.9 ± 12.6
3b	98.7 ±14.7	92.5 ± 11.4
4b	127.3 ± 3.7	92.0 ± 10.3
3c	98.7 ± 14.7	96.6 ± 12.0
4c	119.5 ± 7.3	92.0 ± 10.3
3d	100.0 ± 12.7	99.6 ± 9.9
4d	100.5 ± 8.0	96.5 ± 8.6
5a	92.9 ± 6.9	93.0 ± 14.7
6a	107.1 ± 21.5	106.7 ± 19.2
5b	100.0 ± 28.8	91.6 ± 9.2
6b	96.3 ± 13.1	95.9 ± 9.6
5c	97.0 ± 12.6	89.7 ± 17.3
6c	95.1 ± 3.6	91.4 ± 17.9
5d	76.4 ± 23.3	81.1 ± 28.3
6d	90.1 ± 28.6	86.3 ± 23.2
5d'	87.9 ± 10.4	94.8 ± 19.4
6d'	94.7 ± 6.7	93.8 ± 13.0
7	51.9 ± 16.3	51.8 ± 15.3

* $A\beta_{42}$ aggregation and toxicity is set to 100%; molar ratio 1:20 ($A\beta_{42}$:hybrid ligand) in aggregation assay and 1:5 in viability assay

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