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Design and biological activity of β-sheet breaker peptide conjugates

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ABSTRACT

The sequence LPFFD ($iA\beta_5$) prevents amyloid-β peptide (Aβ) fibrillogenesis and neurotoxicity, hallmarks of Alzheimer's disease (AD), as previously demonstrated. In this study $iA\beta_5$ was covalently linked to poly(ethylene glycol) (PEG) and the activity of conjugates was assessed and compared to the activity of the peptide alone by *in vitro* studies. The conjugates were characterized by MALDI-TOF. Competition binding assays established that conjugates retained the ability to bind Aβ with similar strength as $iA\beta_5$. Transmission electron microscopy analysis showed that $iA\beta_5$ conjugates inhibited amyloid fibril formation, which is in agreement with binding properties observed for the conjugates towards Aβ. The conjugates were also able to prevent amyloid-induced cell death, as evaluated by activation of caspase 3. These results demonstrated that the biological activity of $iA\beta_5$ is not affected by the pegylation process.

Specific peptides to prevent amyloid-β peptide (Aβ) aggregation have been designed based on the well-known self-recognition Aβ motif and structural requirements for Aβ fibrillogenesis [1,2]. It was proposed that neurodegeneration in Alzheimer's disease (AD) may be caused by deposition of Aβ in plaques in brain tissue [3]. Previous studies clearly showed that a significant proportion of Aβ aggregation is driven by hydrophobic sequences, the internal domain comprising residues 17–21 and the C-terminal region (residues 29–42) [4–6]. Based on those studies the sequence 17–21 was modified aiming the inhibition of Aβ fibrillogenesis. The amino acid valine, a key residue for β-sheet formation, was replaced by proline, an amino acid thermodynamically unable to fit in the β-sheet structure and a charged residue was introduced at the C-terminal part to increase solubility [7,8]. This sequence has been showed to prevent amyloid formation *in vitro* and *in vivo* and amyloid neurotoxicity [7,9]. The peptide approach allows that highly specific compounds can be produced with usually no toxicity. However, these molecules are predisposed for enzymatic degradation, resulting in very short half-life in the blood stream. Short peptides usually do not circulate more than a few minutes in blood [10]. They generally show as well poor bioavailability in tissues and organs, preventing their usefulness as therapeutic agents [8]. Coupling proteins to polyethylene glycol (PEG) components reduces their immunogenicity, increases their molecular weight, and as a result prolongs their half-life *in vivo* [11]. These advantages reason the use of PEG for preparing polymer–protein conjugates. Pegylation may, however, cause a loss of the protein biological activity.

According to the amyloid hypothesis, accumulation of Aβ in the brain is the primary influence driving AD pathogenesis [3]. Aggregation of Aβ is preceded by the formation of nonfibrillar species, the protofibrils, which are by their turn preceded by oligomeric species [12]. Several lines of evidence have converged recently to demonstrate that soluble oligomers of Aβ may be responsible for synaptic dysfunction in the brains of AD patients [3]. Moreover, their pathogenic relevance is supported by the finding that oligomer formation is increased by expression of AD, causing mutations in APP [13]. A possible therapeutic strategy proposed for AD is based on preventing oligomerization of Aβ [3]. The hydrophobic domain KLVFF of Aβ is fundamental for Aβ protein–protein interaction [14]. A peptide containing this fragment was able to bind to full length Aβ and prevent its assembly into amyloid fibrils. However, this peptide has ability to aggregate and being incorporated in amyloid fibrils. The cationic pentapeptide amide RVVIA, which is based on the C-terminal sequence VVIA of Aβ (1–42) ($A\beta_{42}$), interferes with fibril formation [15]. A 15-mer peptide inhibits $A\beta_{42}$ intermolecular interaction and aggregation [16]. These sequences are usually very unstable in blood and proteolytically degraded quickly [17]. Chemical modification of the peptide may reduce dramatically its activity or result in toxic species. The aim of this work was to covalently link LPFFD ($iA\beta_5$) to PEG derivatives

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and to study *in vitro* activity of its conjugates in amyloid aggregation process.

Material and methods

Polymer conjugates. The conjugates were prepared as follows:

a) Solid-phase supported strategy – direct synthesis of the conjugate was performed applying a resin comprising a cleavable PEG spacer ($DP_{n,PEG} \approx 73$). Automated stepwise amino acid attachment was applied, following standard Fmoc-protocols. TentaGel PEG Attached Peptide resin (loading: 0.24 mmol/g; Mn = 3200, PDI = 1.06 [GPC (THF, calibrated against linear PEG3200 standards, PSS, Germany)] was purchased from Rapp, Polymere GmbH. Fmoc-amino acid derivatives (Fmoc-Phe OH, Fmoc-Pro OH, Fmoc-Leu OH, Fmoc-Asp OH) were used as received from IRIS Biotech GmbH. This conjugate will be referred as iAβ_5-PEG3200.

b) N-terminal α-amine modification by activated PEG – The β-sheet breaker peptide [iAβ_5, Mw 637.73, Bachem] was conjugated to activated PEG of Mw 5000 (succinimidyl propionate, Sigma Aldrich Co) by dissolving the peptide (1 mg/mL) and the polymer in a final 10-fold molar excess over peptide in sodium phosphate buffer (10 mM, pH 7.4). The reaction was conducted at 25 °C for 120 min, quenched by adding glycine to a final molar ratio of 50:1 (glycine:PEG). The pegylation reaction was purified by Sephadex G-25 size-exclusion chromatography using phosphate buffer as elution solvent. This conjugate will be referred as iAβ_5-PEG5000.

Production of oligomeric and fibrillar Aβ42. Aβ_{42} (Genscript) was dissolved at a concentration of 1 mg/mL in 100% HFIP and kept at room temperature for 1–2 h. The solvent was evaporated under a gentle stream of nitrogen gas and the peptide was resuspended in 100% DMSO at a concentration of 2 mM. Aβ_{42} was diluted to 100 μM in F′12 medium and incubated at 4 °C for 48 h or at 37 °C for 6 days to obtain oligomers and fibrils, respectively.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS). Measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perceptive Biosystems, Inc., Framingham, MA, USA) at an acceleration voltage of 20 kV. Samples were dissolved in 0.1% TFA in acetonitrile–water (1:1, v/v) at a concentration of 0.1 mg/mL. One microliter of the analyte solution was mixed with 1 μL of alpha-cyano-4-hydroxycinnamic acid matrix solution consisting of 10 mg of matrix dissolved in 1 mL of 0.1% TFA in acetonitrile–water (1:1, v/v). From the resulting mixture 1 μL was applied to the sample plate. Samples were air-dried at ambient temperature (25 °C). Each spectrum obtained was the mean of 250 laser shots.

Binding assays. Aβ_{42} was iodinated with $Na^{125}I$ (NEN) using the Iodogen (Sigma) method following the supplier instructions. The reaction mixture was subsequently desalted by Sephadex G25 gel filtration. 96-well plates (Maxisorp, Nunc) were coated with iAβ_5 and pegylated iAβ_5 (5 μg/well) in coating buffer (0.1 M bicarbonate/carbonate buffer, pH 9.6) and incubated overnight at 4 °C. Unoccupied sites were blocked by incubation with 5% non-fat dried milk in PBS for 2 h at 37 °C. A constant amount of ^{125}I-labeled Aβ_{42} (66 nM) was added to each well of coated plates with 0-, 1-, 10- and 100-fold molar excess of unlabeled Aβ_{42} in binding buffer (0.1% skim milk in minimal essential medium-MEM [Gibco, Gaithersburg, Maryland]) for 2 h at 37 °C with gentle shaking. Binding was determined after five washes in ice-cold PBS with 0.05% Tween 20 (0.2 mL/wash). Then, 100 μL elution buffer (NaCl 0.1 M containing 1% nonidet P40) was added for 10 min at 37 °C, and the contents of the wells were aspirated and counted in a gamma spectrometer. Each assay was performed in quadruplicate and repeated three times.

Transmission electron microscopy (TEM). Aβ_{42}/DMSO was added to the control (Ham's F-12), iAβ_5 (5 mg/mL) or conjugates (iAβ_5 - PEG3200 32 mg/mL, iAβ_5-PEG5000 48 mg/mL) to a Aβ_{42} final concentration of 100 μM (pH 7.4). The samples were incubated for 6 days at 37 °C. Aliquots of 5 μl of Aβ_{42} assay were adsorbed for 2 min to glow discharged carbon-formvar-coated grids. Grids were washed with ultrapure water three times and negatively stained with 1% filtered uranyl acetate solution for visualization by TEM (Zeiss microscope operated at 60 kV).

Caspase 3 assay. Human neuroblastoma cells (SH-SY5Y cell line) were propagated in 25 cm^2 flasks and maintained at 37 °C in a humidified atmosphere of 95% and 5% CO_2. Cells were grown in Ham's F′12:MEM (Invitrogen) in a proportion of 1:1 supplemented with 2 mM glutamine, 1% of non essencial aminoacids (Sigma), 15% FBS.

Aβ_{42}/DMSO was added to the control (Ham's F-12 medium) or to iAβ_5 alone (4 mg/mL) or conjugated (iAβ_5-PEG3200 22 mg/mL, iAβ_5-PEG5000 32 mg/mL) to a final Aβ concentration of 100 μM. The samples were incubated at 4 °C for 48 h. Toxicity of the amyloid aggregates was evaluated by activation of caspase 3, measured using the CaspACE colorimetric 96-well plate assay system (Promega, Madison, WI, USA), following the manufacturer's instructions. Briefly, confluent cells were exposed for 48 h to 10 μM Aβ (either alone or treated with iAβ_5 or iAβ_5 conjugates). Subsequently, each well was trypsinized and the cell pellet was lysed in 100 μL hypotonic lysis buffer (Promega); 40 μL of each cell lysate was used in duplicate to determine caspase 3 activation. The remaining cell lysate was used to measure total cellular protein concentration with the Bio-Rad protein assay kit, using bovine serum albumin as standard. Values shown are the mean of quadruplicates of three independent experiments. Student's *t*-test statistical analysis was used to determine statistical significance between cells exposed to assay media and cells exposed to Aβ aggregates.

Results

Conjugates

The strategy to improve the half-life time of iAβ_5 (LPFFD) in the blood stream involved anchoring the peptide to PEG. The iAβ_5 contains only one potential site for conjugation with PEG, i.e., the primary amine of the N-terminus (Leucine). This avoids the heterogeneity of the product, since PEG will attach to a site-specific amino acid. The iAβ_5-PEG3200 conjugate was directly synthesized by applying a resin comprising a cleavable PEG3200 spacer. The conjugate iAβ_5-PEG5000 was prepared by N-terminal α-amine modification by activated PEG compounds. Fig. 1 shows a typical MALDI-TOF-MS result of pegylated iAβ_5. The polydispersity of PEG is reflected into the polydispersity of the conjugate. A polydispersivity value (*M*w/*Mn*) of at least 1.01 has been described for low molecular weight polymer (3–5 kDa).[18] The spectra exhibit a single homologous series with a repetitive mass unit of about 44 Da, which could be assigned to the monomer unit of PEG. It can be seen in Fig. 1 that there is a clear mass shift of approximately 600 Da units, which corresponds to the Mw of iAβ_5, in the overall spectrum of the conjugate when compared to that of PEG alone, indicating pegylated products.

Binding of conjugates to Aβ42 peptide

The interaction of Aβ_{42} with iAβ_5 and conjugates was studied by competition binding assays using ^{125}I-labeled Aβ_{42}. The β-sheet breaker peptide and its conjugates were adsorbed to the plate

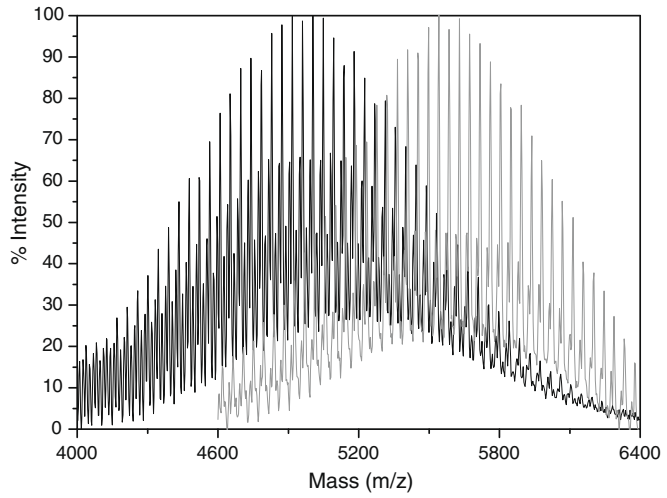

Fig. 1. MALDI-TOF-MS spectra of PEG5000 (black) and iAβ$_5$-PEG5000 conjugate (gray).

wells at a constant concentration of 5 μg/well. Unlabeled Aβ$_{42}$ was tested for its ability to compete with ^{125}I-labeled Aβ$_{42}$ for binding to the adsorbed iAβ$_5$ or pegylated iAβ$_5$. Unlabeled Aβ$_{42}$ at 1- and 10-molar excess (relative to ^{125}I-Aβ$_{42}$) was able to displace ^{125}I-Aβ$_{42}$ bound not only to iAβ$_5$ but also to its conjugates (Fig. 2), indicating that conjugation of iAβ$_5$ to PEG did not alter the ability of the sequence to interact with Aβ$_{42}$ peptide. Furthermore, binding affinity of the conjugates towards Aβ$_{42}$ is similar to the β-sheet breaker, as competition observed was of the same magnitude.

Impact on Aβ$_{42}$ fibrillization

The impact of pegylated iAβ$_5$ on Aβ fibrillogenesis was assessed by ultra-structural analysis of Aβ$_{42}$ incubated with iAβ$_5$ or conjugates at 37 °C for 6 days. The incubation of Aβ$_{42}$ (100 μM) resulted in amyloid-like, unbranched fibrils, as expected (Fig. 3a) [19]. The peptide co-incubated from the beginning with iAβ$_5$ alone (5 mg/mL) formed only small aggregates and amorphous material (Fig. 3b), as described by Soto et al. [7]. Visualization of Aβ$_{42}$ and iAβ$_5$-PEG3200 (32 mg/mL) samples by TEM revealed the presence of small aggregates and short fibrils in reduced number, indicating that this conjugate was able of inhibiting Aβ$_{42}$ fibrillogenesis at a significant extent (Fig. 3c). The conjugate iAβ$_5$-PEG5000 (48 mg/mL) inhibited Aβ$_{42}$ fibrillogenesis more strongly than iAβ$_5$-PEG3200 and at a similar extent as the iAβ$_5$ peptide, since only amorphous and small aggregates were observed (Fig. 3d). The results suggest that these conjugates did not only conserved the ability to bind Aβ$_{42}$ but also they did not affect the inhibitory properties of the β-sheet breaker.

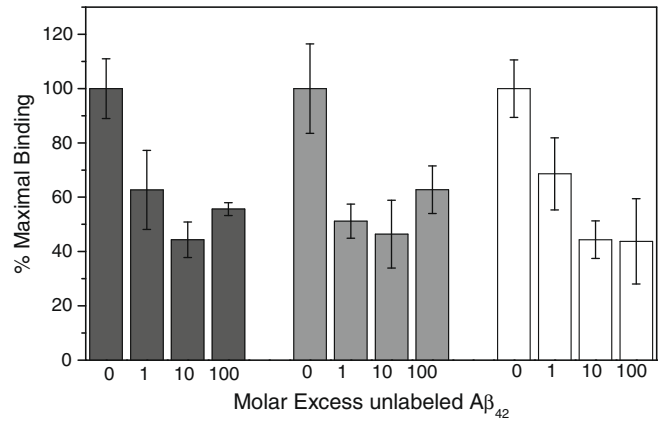

Fig. 2. Binding of ^{125}I-labeled Aβ$_{42}$ (66 nM) to iAβ$_5$ alone (dark gray), iAβ$_5$-PEG5000 (gray) and iAβ$_5$-PEG3200 (white): competition by unlabeled Aβ$_{42}$. ^{125}I-labeled Aβ$_{42}$ was allowed to bind to wells coated with iAβ$_5$ alone and its conjugates (5 μg/well) in the presence of increasing concentrations of unlabeled Aβ$_{42}$.

Impact on Aβ$_{42}$ toxicity

A cellular model of amyloid-induced cytotoxicity was used to screen the conjugates for *in vitro* activity. Toxicity of Aβ$_{42}$ in cell culture has been reported to be related to the formation of β-sheet-rich aggregates and has been used to screen diverse compounds to prevent amyloid neurotoxicity and consequent apoptosis and cell death [20]. Previous studies have demonstrated that soluble oligomers are the most toxic forms of Aβ$_{42}$ [21–24]. In this work caspase 3 activity was evaluated in SH-SY5Y cells incubated with Aβ$_{42}$ alone or pre-incubated with iAβ$_5$ or conjugates, in order to assess the biological activity of the conjugates. The results from this study indicated that both conjugates have activities similar to the unconjugated iAβ$_5$ in preventing amyloid-induced cell death (Fig. 4): considerable caspase 3 activation was observed in cells treated with Aβ$_{42}$, whereas cells treated with iAβ$_5$ did not show noteworthy caspase 3 activation, when compared to non-treated cells. Importantly, both conjugates were also able to abrogate Aβ$_{42}$ toxicity, since no significant caspase 3 activation was measured, indicating that the conjugation of iAβ$_5$ to PEG did not interfere with its anti-amyloidogenic properties.

Discussion

Peptides have immunogenicity, low stability and poor bioavailability. Chemical modification of peptides with therapeutic properties may have drawbacks such as activity loss and toxicity. β-Sheet breaker peptides have recently emerged as drugs for diseases characterized by amyloid fibril formation [1]. The peptides intended for AD treatment are based on β-sheet disrupting elements and the self-recognition motif of Aβ, the region implicated in early misfolding and protein–protein interaction [25]. The sequence LPFFD, which is based on the central residues 17–21 of Aβ, inhibits amyloid fibril formation and amyloid neurotoxicity [7]. The short peptide iAβ$_5$, due to its nature, has to be modified to overcome problems such as short half-life in the blood stream and high overall clearance rate [9]. One strategy to increase the stability of proteins is to covalently couple them to PEG, since this polymer has very low toxicity and is known to mask the protein, reducing its degradation by proteolytic enzymes [11]. Although conjugate properties depend on the nature of the protein, many pegylated proteins have reduced bioactivity [26,27].

In the present work, iAβ$_5$ was conjugated to PEG of different molecular weight and activity of pegylated peptide was assessed. Both conjugates bind to Aβ$_{42}$ in a dose-dependent and specific manner as observed and described for iAβ$_5$ alone [7]. It has been proposed that phenylalanines (positions 3 and 4) and leucine (position 1) are responsible for selective binding of iAβ$_5$ to Aβ [9]. Pegylation of iAβ$_5$ seems to not significantly change the ability of the sequence to bind to Aβ. PEG alone did not bind to Aβ (data not shown). Ultrastructural studies showed that pegylated iAβ$_5$ inhibits amyloid fibril formation *in vitro* in a similar extension as the unconjugated peptide. This is in agreement with binding properties observed for the conjugates towards Aβ. Previous work has demonstrated that pegylation of the central core amino acids of Aβ (residues 10–35) resulted in amyloid fibril formation [28]. Although PEG was not able of preventing amyloid formation, the fibrils had two distinct properties when compared to fibrils pro-

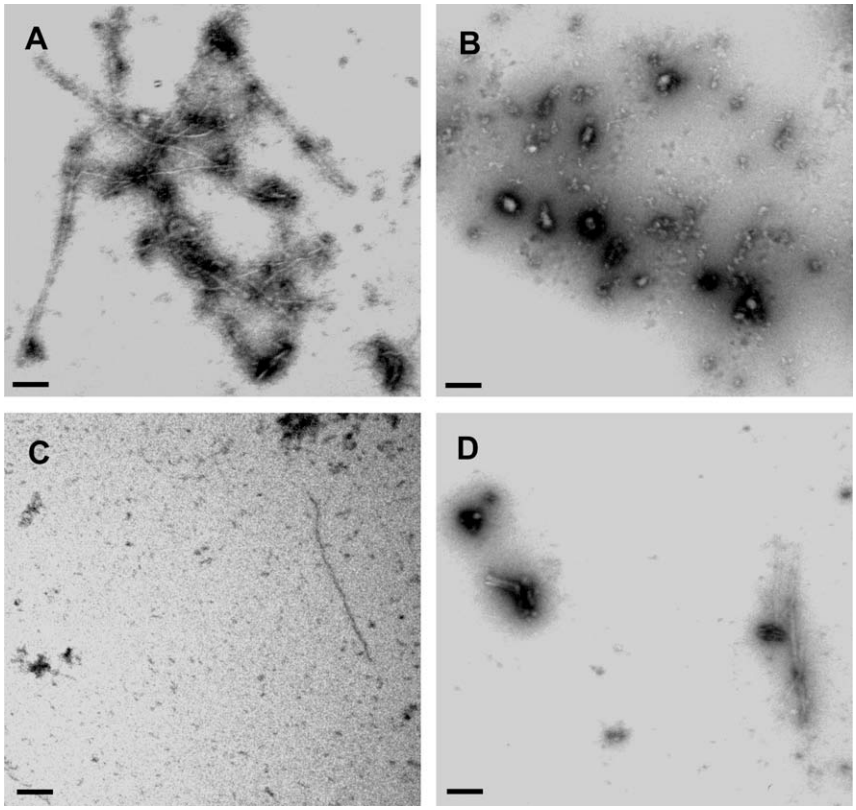

Fig. 3. TEM analysis of the effect of iAβ_5 conjugates on fibril formation. Samples were incubated for 6 days at 37 °C. (A) Aβ_{42} alone. (B) Aβ_{42} with iAβ_5 peptide. (C) Aβ_{42} with iAβ_5-PEG3200 conjugate. (D) Aβ_{42} with iAβ_5-PEG5000 conjugate. The scale bar is 200 nm.

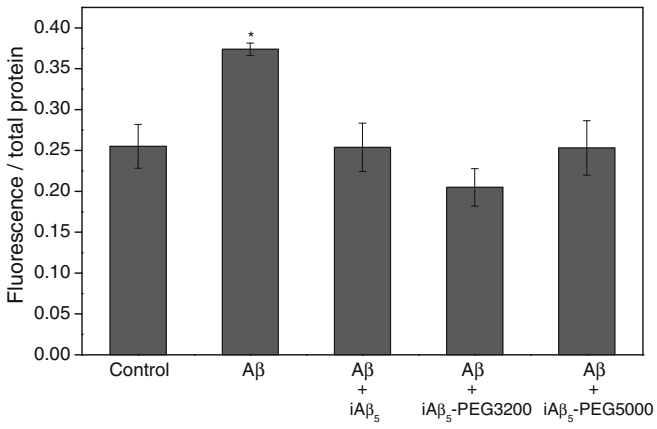

Fig. 4. Activation of caspase 3 in neuroblastoma cells exposed for 48 h to 10 μM of Aβ_{42} after incubation alone and with iAβ_5 or conjugates. *Significantly different from control ($P < 0.01$).

duced by unconjugated Aβ_{10-35}. The fibrils were separated and did not self-associate as opposed to unconjugated Aβ_{10-35} fibrils that readily self-associate laterally into bundles. Moreover PEG seemed to coat the surface of the fibril. PEG inhibited irreversible fibril–fibril association [28,29]. In addition, PEG has been described to diminish the interaction of Aβ peptide with amyloid plaques [29]. Radiolabeled Aβ was shown to promptly deposit at preexisting amyloid plaques in tissue sections of autopsy AD brain [30]. The conjugation of radiolabeled Aβ with PEG of Mw 3400 interfered with Aβ binding to amyloid plaques [29].

PEG alone is not able of inhibiting Aβ aggregation. In fact Schmuck et al. reported that tri(ethylene glycol) seems to accelerate fibril formation [31]. However PEG linkers can be effective for inhibit protein aggregation. It has been demonstrated that increasing PEG linker length of transthyretin (TTR) tethered inhibitors decreases TTR amyloidogenicity [32,33]. TTR is a homotetrameric protein that transports the small molecule hormone thyroxine (T4) [32]. Inhibition of TTR amyloid fibril formation can be achieved by small molecules that bind selectively to T4 binding sites in complex biological fluids, imposing stabilization on TTR [34,35]. The efficacy of the tethered inhibitors was highly dependent on the length of the linker [33].

The data presented here showed that pegylation of a fibrillogenesis inhibitor did not affect its activity. The β-sheet breaker peptide LPFFD inhibited amyloid fibril formation and cell toxicity by Aβ even when conjugated to PEG. Small differences are observed between the efficacy of conjugates with PEG3200 and PEG5000, indicating that low molecular weight polymers for 3–5 kDa are equally effective. Pegylation of β-sheet breaker peptides is a valuable strategy to improve their stability with no effect on their activity and will facilitate targeting the peptide. Previous studies have shown that placing a PEG linker between peptides and BBB drug-targeting systems enhances their receptor binding [36].

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