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Design and synthesis of new trehalose-conjugated pentapeptides as inhibitors of Aβ(1–42) fibrillogenesis and toxicity‡

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Aggregation of the amyloid Aβ peptide and its accumulation into insoluble deposits (plaques) are believed to be the main cause of neuronal dysfunction associated with Alzheimer's disease (AD); small molecules that can interfere with the Aβ amyloid fibril formation are therefore of interest for a potential therapeutic strategy. Three new trehalose-conjugated peptides of the well known β-sheet breaker peptide iAβ5p, were synthesized. The disaccharide was covalently attached to different sites of the LPFFD peptide chain, i.e. at the N-terminus, C-terminus or at the Asp side chain. CD spectroscopy in different solvents was used to assess changes in the peptide conformation of these compounds. The effects of these glycopeptides on the self-assembly and morphology of Aβ aggregates were investigated by ThT fluorescence assay and dynamic Scanning Force Microscopy, respectively. All the synthesized compounds were tested as inhibitors of Aβ toxicity toward pure cultures of rat cortical neurons.

Keywords: β-sheet breaker peptides; amyloid-β; trehalose; SFM; neuronal cultures; thioflavin T

Introduction

Many neurodegenerative diseases are associated with the β-sheet aggregation of misfolded proteins and their pathological deposition in specific brain areas. In Alzheimer's disease (AD) the Amyloid -β (Aβ) peptide aggregation and fibril formation is considered the major contributing factor to neurodegeneration and dementia [1].

Although, initial studies have shown that the aggregation of Aβ into fibrils is a prerequisite for its toxicity [2–4], recent *in vitro* observations have demonstrated that small, soluble and diffusible oligomeric Aβ species are also capable of initiating pathogenic events [5]. This fact has motivated several researchers to postulate that Aβ oligomeric intermediates, rather than fully formed fibrils, are the predominant toxic species [6,7].

Targeting the initial formation of amyloid assemblies could be, therefore, a preferred approach to therapeutic intervention aimed at inhibiting Aβ neurotoxicity. One approach that is gathering interest is to synthesize short peptides that correspond to a self-recognition element of the native amyloid sequence but containing key modifications, so that the peptides bind to the parent protein and prevent further aggregation [8].

Studies on the aggregation process of Aβ identified the critical region of the peptide that is involved in amyloid fibril formation. This has been mapped within the hydrophobic core at the residues 16–20 (KLVFF) of Aβ [9]. Designed peptides based on this self-recognition motif bind to the homologous sequence of Aβ, thereby preventing its self-association. Despite the good *in vitro* activity found for some of these compounds, their therapeutic usefulness is questioned due to their tendency to self-aggregate, and to the risk of being incorporated into amyloid fibril. Therefore, a series of modified synthetic peptides or peptidomimetics, based on the sequence of this central region of Aβ, were designed to overcome these limitations. Such modifications include adding of bulky groups, charged sequences or polyethylene glycol to their termini, N-methylation, replacing a backbone amide by an ester and replacing a residue with proline [10–14].

A five-residue peptide (LPFFD), referred to as iAβ5p, has been reported to inhibit Aβ fibril formation and Aβ-induced neuronal death, as well as to significantly reduce Aβ deposition *in vivo*

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[15,16]. The main disadvantage of this peptide is its low biological stability [17].

Besides these peptide-based aggregation inhibitors, various small molecules have been reported to interfere with amyloid fibril formation including a group of naturally occurring inositole stereoisomers and simple disaccharides such as trehalose [18,19]. Trehalose was shown to be capable of inhibiting aggregation of Aβ(1–40) and reducing its cytotoxicity [19].

We hypothesized that the conjugation of trehalose with the pentapeptide LPFFD, migth result in new compounds with higher affinity for Aβ, acting as more effective inhibitors of Aβ aggregation. Moreover, it is expected that trehalose conjugation would confer to these systems higher stability toward proteolytic degradation.

In this paper, we report the synthesis and the spectroscopic characterization of three new trehalose conjugates with the LPFFD peptide (Scheme 1). All the synthesized compounds were tested as inhibitors of Aβ's fibrillogenesis and Aβ's toxicity toward pure culture of rat cortical neurons. The effects of these glycopeptides on the self-aggregation and morphology of Aβ aggregates were investigated by Scanning Force Microscopy (SFM).

Materials and Methods

Chemicals

Amyloid β-protein, Aβ(1–42), was obtained from Bachem (Switzerland). All N-fluorenylmethoxycarbonyl (Fmoc)-protected amino-acids, Novasyn TGR and Fmoc-Asp(Wang resin LL)-OAll were obtained from Novabiochem (Switzerland); 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and N-hydroxybenzotriazole (HOBT) were purchased from INBIOS (Italy); piperidine, pyridine, sodium azide, *N*,*N*-diisopropylethylamine (DIEA), triisopropylsilane (TIS), trifluoroacetic acid (TFA), D-(+)-trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) dihydrate (Th), trifluoroethanol (TFE), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and acetic anhydride were Sigma/Aldrich (Germany) products; *N*,*N*-dimethylformamide (DMF, peptide synthesis grade), acetonitrile, methanol, dichloromethane and *N*-methylpyrrolidone were obtained from Labscan (Ireland). All other chemicals were of the highest available grade and were used without further purification.

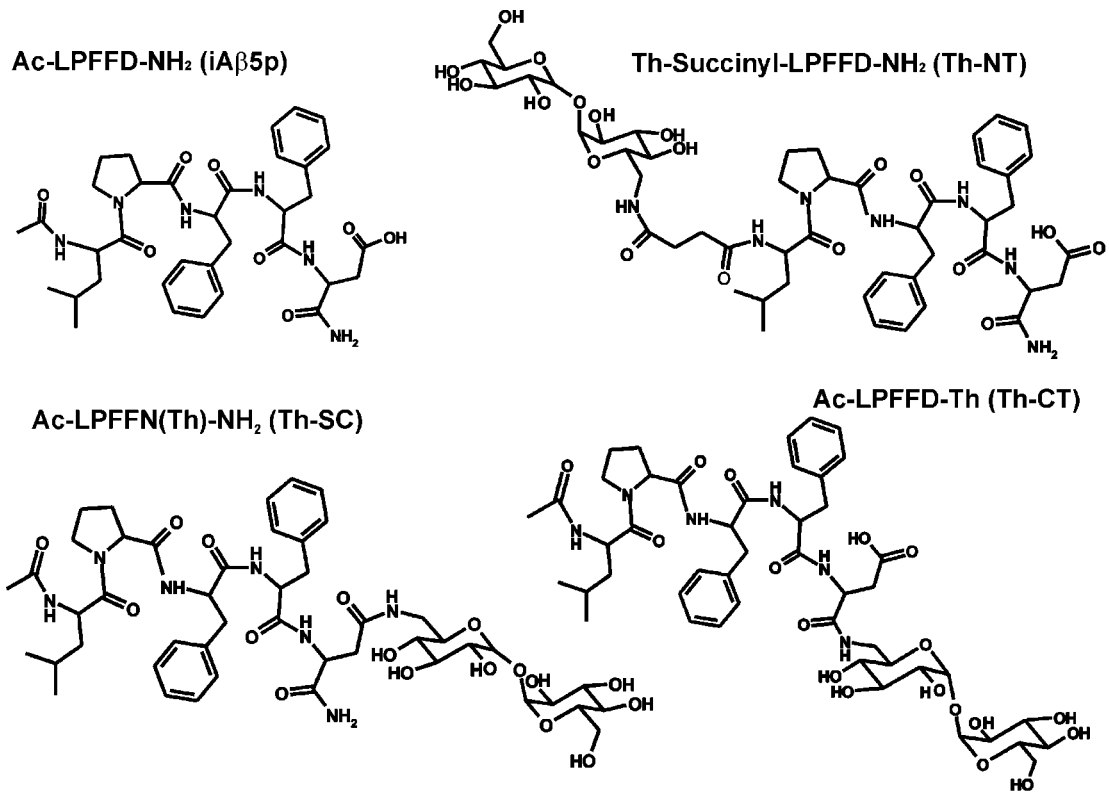
HPLC and Mass Spectroscopy

Analytical RP-HPLC analyses were performed using a Waters 1525 instrument, equipped with a Waters 2996 photodiode array, using a Vydac C_{18} (300 Å pore size, 5 µm particle size 250 × 4.6 mm) column at a flow rate of 1 ml/min. Preparative RP-HPLC was carried out by means of Varian PrepStar 200 model SD-1 chromatography system equipped with a Prostar photodiode array detector on a Vydac C_{18} 250 × 22 mm (300 Å pore size, 10–15 µm particle size) column at flow rate of 10 ml/min. Detection in both cases was at 222 nm. HPLC eluents were A: 0.1% TFA/water and B: 0.1% TFA/acetonitrile.

The ESI-MS measurements were performed on a Finnigan LCQ-Duo ion trap electrospray mass spectrometer.

Synthesis of 6-Amino-6-Deoxy-Trehalose (Th-NH_2)

The 6-amino-6-deoxy-trehalose was synthesized as reported elsewhere [20]. Briefly, trehalose was tosylated in pyridine with the *p*-toluenesulfonyl chloride and purified by reverse phase chromatography on LiChroprep (40–63 µm) using a linear



Scheme 1. Representative structure of the synthesized compounds.

gradient of methanol in water. The pure 6-tosylated product was dissolved in DMF and treated with sodium azide to obtain the azido derivative. The 6-deoxy-6-azido-trehalose was purified by reverse phase chromatography and then the azido fuction was reduced to amino group with triphenyl posphine. The obtained product was purified by precipitation in freshly distilled acetone. All intermediates were characterized by ESI-MS and TLC.

6-O-(p-Toluen-sulfonyl)-trehalose. ESI-MS [obsd: m/z $(M + Na)^+$ 519.1; $(2M + Na)^+$ 1014.8; calcd for $C_{19}H_{28}O_{13}S$: 496.12]; TLC: $R_f = 0.70$ ($PrOH/H_2O/AcOEt/NH_3$, 5 : 3 : 1 : 1.5).

6-Azido-6-deoxy-trehalose. ESI-MS [obsd: m/z $(M+Na)^+$ 390.0; $(2M + Na)^+$ 756.9; calcd for $C_{12}H_{21}N_3O_{10}$: 367.12]. TLC: $R_f = 0.42$ ($PrOH/H_2O/AcOEt/NH_3$, 5 : 3 : 3 : 1).

6-Amino-6-deoxy-trehalose (Th-NH_2). ESI-MS [obsd: m/z $(M + H)^+$ 342.2; calcd for $C_{12}H_{23}NO_{10}$: 341.13]; TLC: $R_f = 0.25$ ($PrOH/H_2O/AcOEt/NH_3$, 5 : 3 : 1 : 2).

Peptide Synthesis

Peptides were assembled using the microwave-assisted solid phase peptide synthesis strategy on a Liberty Peptide Synthesiser. All Fmoc-amino acids were introduced according to the TBTU/HOBT/DIEA activation method. All syntheses were carried out under a 4-fold excess of amino acid. Removal of Fmoc protection during synthesis was achieved by means of 20% piperidine solution in DMF. The following instrumental conditions were used for each coupling cycle: microwave power 25 Watts, reaction temperature 75 °C, coupling time 300 s. The instrumental conditions used for the deprotection cycles were: microwave power 25 Watts, reaction temperature 75 °C, deprotection time 180 s. The acetylation was carried using a 6% acetic anhydride in DMF containing 5% DIEA.

Ac-LPFFD-NH_2 (iAβ5p)

This peptide was assembled using a Novasyn TGR resin (substitution 0.18 mmol/g). After the completion of the coupling cycles, the peptidyl resin Ac-LPFFD-TGR was treated with a mixture of TFA/TIS/H_2O (95/2.5/2.5 v/v) for 1 h at room temperature to give the peptide Ac-LPFFD-NH_2. The solution containing the free peptide was filtered off from the resin and concentrated *in vacuo* at 30 °C. The peptide was precipitated with cold freshly distilled diethyl ether, then filtered and dried under vacuum. The resulting crude peptide was purified by RP-HPLC and characterized by ESI-MS. Yield: 80%. HPLC [from 0 to 5 min isocratic elution with 100% A, then linear gradient from 0% to 30% B in 15 min, finally isocratic elution with 30% B; $R_t = 28.37$ min]. ESI-MS [obsd: m/z $(M + H)^+$ 679.3; $(M + Na)^+$ 701.5; calcd for $C_{35}H_{46}N_6O_8$: 678.33].

Ac-LPFFN(Th)-NH_2 (Th-SC)

The Th-NH_2 was covalently bonded to the β-carboxy group of Asp by adding 50 mg (0.074 mmol) of Ac-LPFFD-NH_2 to a DMF (2 ml) solution containing Th-NH_2 (0.88 mmol), HOBt (0.088 mmol), TBTU (0.088 mmol) and DIEA (0.088 mmol). After 1 h stirring at room temperature, the solvent was evaporated under *vacuo* and the product precipitated with cold diethyl ether. The trehalose-conjugated peptide, Ac-LPFFN(Th)-NH_2, was purified by RP-HPLC and characterized by ESI-MS. Yield: 65%. HPLC [from 0 to 5 min isocratic elution with 100% A, then linear gradient from 0% to 30% B in 15 min, finally isocratic elution with 30% B; $R_t = 26.4$ min]. ESI-MS [obsd: m/z $(M + H)^+$ 1002.4; $(M + Na)^+$ 1024.5; calcd for $C_{47}H_{67}N_7O_{17}$: 1001.456].

Th-Succinyl-LPFFD-NH_2 (Th-NT)

For this derivative, a two-step synthesis was performed. First, a solution of succinic anhydride (0.04 mmol) and DIEA (0.04 mmol) in DMF (2 ml) was added to the pre-swollen peptidyl resin NH_2-LPFFD-TGR (0.20 mg, 0.032 mmol). The mixture was stirred for 1.5 h and then the resin was filtered and washed 5× with 20 ml DMF. The trehalose moiety was covalently linked to the succinyl-LPFFD-TGR by adding the peptidyl resin to a DMF (3 ml) solution of Th-NH_2, HOBt, TBTU and DIPEA (0.04 mmol for all compounds). The conjugated peptide Th-NT was cleaved from the resin with a TFA/TIS/H_2O (95/2.5/2.5 v/v) mixture and precipitated with freshly distilled diethyl ether. The derivative was purified by RP-HPLC and characterized by ESI-MS. Yield: 78%. HPLC [from 0 to 5 min isocratic elution with 100% A, then linear gradient from 0% to 30% B in 15 min, finally isocratic elution with 30% B; $R_t = 26.4$ min]. ESI-MS [obsd: m/z $(M + H)^+$ 1060.5; $(M + Na)^+$ 1082.7; $(M + K)^+$ 1098.5; calcd for $C_{49}H_{69}N_7O_{19}$: 1059.464].

Ac-LPFFD-Th (Th-CT)

For the synthesis of this derivative the Fmoc-Asp(Wang LL)-OAll resin (0.36 mmol/g) was employed. After the completion of the synthesis of Ac-LPFFD(Wang LL)-OAll precursor, the dried peptidyl resin (0.7 g, 0.25 mmol) was treated with 10 ml of a $CHCl_3$/AcOH/NMM (37 : 2:1) mixture containing 0.75 mmol of $Pd(PPh_3)_4$, to selectively remove the allyl protecting group according to the literature procedure [21]. The resulting peptidyl resin with the free α-carboxy group of Asp was allowed to react with Th-NH_2 (0.33 mmol) in the presence of HOBt (0.33 mmol), TBTU (0.33 mmol) and DIPEA (0.33 mmol) in DMF (10 ml) solution. The obtained trehalose-conjugated peptidyl resin was then treated with the TFA/TIS/H_2O (95/2.5/2.5 v/v) mixture, the solution was filtered, concentrated under vaccum and the product precipitated with cold freshly distilled diethyl ether. The obtained crude trehalose-peptide was purified by RP-HPLC and characterized by ESI-MS. Yield: 75%. HPLC [from 0 to 5 min isocratic elution with 100% A, then linear gradient from 0% to 35% B in 20 min, finally isocratic elution with 35% B; $R_t = 28.0$ min]. ESI-MS [obsd: m/z $(M+Na)^+$ 1025.6; $(M+K)^+$ 1041.1; calcd for $C_{47}H_{66}N_6O_{18}$: 1002.44].

CD Spectroscopy

CD measurements were performed at 25 °C under a constant flow of nitrogen on a JASCO model J-810 spectropolarimeter. The CD spectra of the synthesized compounds were recorded in the far-UV region (190–260 nm) using a 1 mm path length cell. The spectra represent the average of 10 scans. All derivatives (0.2 mM) were solubilized in pure water and in water/TFE solution at pH 7.4.

In vitro Assay of Peptides Stability

The biological stability of the conjugated peptides was evaluated in 10% of rat brain homogenate and the amount of the intact peptide was determined by RP-HPLC analysis. Peptides samples were prepared at a concentration of 1 mM in phosphate-buffered saline (PBS). 600 µl of each peptide solution were dissolved in 2.4 ml of 10% rat brain homogenate (in PBS). The solutions were incubated at 37 °C and aliquots of 300 µl were taken at 0, 5, 10, 20, 30, 40, 60 and 80 min. The bulk of brain proteins were precipitated with cold methanol (700 µl) for 2 h at −30 °C. The precipitate was pelleted by centrifugation at 10 000 × g for 10 minutes at 4 °C. The supernatant was concentrated 5 times and analysed by RP-HPLC using a linear gradient from 0% to 35% solvent B in 20 min.

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Aβ(1–42) Sample Preparation

Monomeric Aβ(1–42) was prepared using a Zagorsky modified procedure [22,23]. The Aβ(1–42) lyophilized peptide was dissolved in TFA (1 mg/ml) and sonicated in a water bath sonicator for 10 min. Then the TFA was evaporated under a gentle stream of argon and 1 ml HFIP was added to the peptide. After 1 h incubation at 37 °C, the peptide solution was dried under a stream of argon, and then solubilized again by adding 2 ml of HFIP. Finally, HFIP was removed by argon streaming followed by further drying in a lyophilizer for 1 h.

The dried peptide was first dissolved in 2 mM of freshly prepared NaOH at a concentration of 1 mM, then the appropriate volume of 20 mM PBS buffer was added to obtain a final peptide concentration of 200 μM. Samples containing Aβ(1–42) 100 μM in PBS without and with 5-fold or 20-fold molar excess of each candidate inhibitor peptide were incubated for 4 days at 37 °C.

As concerns the aggregation studies carried out in NEM buffer (25 mM at 7.4 pH), a 200 μM stock solution of Aβ(1–42) was prepared by adding the appropriate volume of buffer solution after the lyophilization. 100 μM Aβ(1–42) samples were incubated for 96 h at 37 °C in the absence or in the presence of a 5-fold molar excess of each synthesized compound.

For the treatment of neuronal cultures, lyophilised Aβ(1–42) samples (2.5 mM) were solubilised in sterile double-distilled water.

Thioflavin T (ThT) Fluorescence Measurements

Fluorescence emission spectra of ThT undergo a red shift and an increased fluorescence emission intensity upon incorporation into β-sheet amyloid structures [24,25]. Aliquots (60 μl) of Aβ(1–42) from incubated samples in different buffers were added to a ThT solution. Final solutions resulted in 10 μM ThT and contained 3 μM Aβ(1–42). The measurements were carried out using a Perkin Elmer LS55 spectrofluorimeter.

Fluorescence emission spectra were monitored from 450 to 600 nm in a 1 cm light path quartz cell. An excitation wavelength of 440 nm was used. Excitation and emission bandwidths were set to 5 and 8 nm respectively. The ThT spectra were corrected by subtraction of the background solvent spectrum obtained under identical experimental conditions. The experiments were performed in triplicate. For time dependent measurements, the ThT fluorescence assay was performed on Aβ(1–42) samples at concentration of 100 μM either in the absence or in the presence of 5-fold molar excess of the Th-CT peptide. Excitation and emission wavelength were 440 and 480 nm respectively and the bandwidth were set 5/5 nm. A programmable refrigerated circulating bath (dulabo F25HE) was used to perform the assay at 37 °C.

Pure Cultures of Cortical Neurons and Treatments

The use of animals was in accordance with the Institutional Guidelines. Pure neuronal cultures were obtained from E15 rat embryos according to a well established method that allows the growth of a more than 99% pure neuronal population [26]. Cortical cells were dissected, mechanically dissociated, and seeded on medium consisting of DMEM/Ham's F12 (1 : 1) supplemented with the following components: 10 mg/ml bovine serum albumin, 10 μg/ml insulin, 100 μg/ml transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, 2 mM glutamine, 6 mg/ml glucose, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cortical cells were plated on 24-well plates precoated with 0.1 mg/ml poly-D-lysine and incubated at 37 °C with 5% CO_2 in a humidified atmosphere. Cytosine-D-arabinofuranoside (10 μM) was added to the cultures 18 h after plating to avoid the proliferation of non-neuronal elements and was kept for 3 days before medium replacement. Aβ(1–42) (25 μM) was applied to mature neuronal cultures at 7 days *in vitro*, either in the absence or in the presence of all synthesized peptides (125 μM) for 4 days. All the experiments were performed in the presence of the glutamate receptor antagonists MK-801 (10 μM) and DNQX (30 μM), to avoid the potentiation of endogenous glutamate toxicity. In some experiments, to overcome the potential degradation of the peptides in the culture media, candidate inhibitors were added twice.

To assess cell viability, cells were incubated with 3-[(4,5-dimethylthiazol-2-yl)-2,5- diphenyl] tetrazolium bromide (MTT; 0.9 mg/ml final concentration; Sigma) for 2 h at 37 °C. A solubilization solution containing 20% sodium dodecyl sulfate (SDS) was then added for 1 h, and formazan production was evaluated in a plate reader (absorbance = 560 nm).

Scanning Force Microscopy

SFM imaging was performed by a Multimode/Nanoscope IIIA (Digital Instrument, Santa Barbara, CA). Immediately prior to SFM imaging, freshly prepared and incubated samples were used without any further dilution. 8 μl of each sample was applied to the mica surface, left for 5 min and then rinsed twice with double-distilled water. Samples were then dried by a nitrogen stream for 1 min and imaged immediately. In order to avoid sample damage, the instrument was set in dynamic mode working in the net tip-sample attractive regime, as previously described [27]. Commercial available etched silicon probes (Digital) were used. 512 × 512 points were collected for each image by maintaining the scan rate at about 1 Hz.

Results

Conformational Features of the Investigated Peptides

In order to verify possible difference in the conformational preferences among the three-trehalose conjugated peptide, a series of CD experiments were carried out in different solvents. The CD spectra of the Ac-LPFFD-NH_2 (iAβ5p) were also acquired for comparison under the same experimental conditions. All the peptides exhibited CD profiles with strong negative ellipticity below 200 nm in aqueous solution, suggesting that an unordered conformation is adopted (see Figure 1(A)). When the CD spectra were recorded in the presence of TFE, which provides a more hydrophobic environment, an evident negative band, centered around 216 nm, became observable. In addition, the development of a positive band at 195 nm is observed along the CD profiles of the examined compounds (see Figure 1(B)). It is clear that in 50% aqueous TFE all the peptides adopt a more structured conformation that, based on the observed CD profiles, could be attributed to an extended β-conformation [28]. In any case, trehalose conjugation does not significantly modify the conformational features of the peptide chain, only a slight effect in facilitating structuring of the peptide chain depending on its position along the peptide sequence can be observed.

Stability of the Conjugates and iAβ5p in Rat brain Homogenate

The stability of the peptide conjugates, and iAβ5p, was determined by incubating them at 37 °C in 10% rat brain homogenate, followed

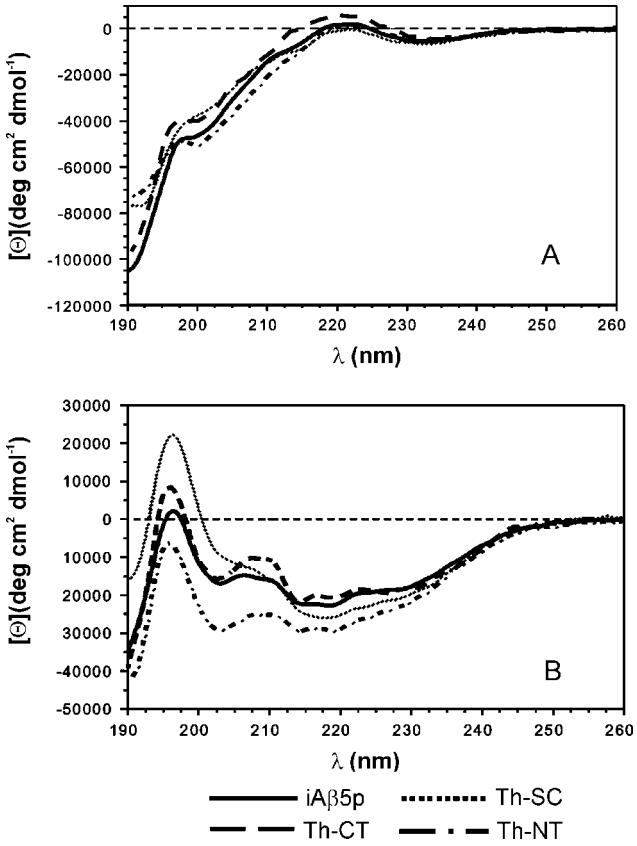

Figure 1. CD spectra of the studied compounds (0.2 mM) in water solution at pH 7.4 (A) and in 50% of TFE in water at pH 7.4 (B).

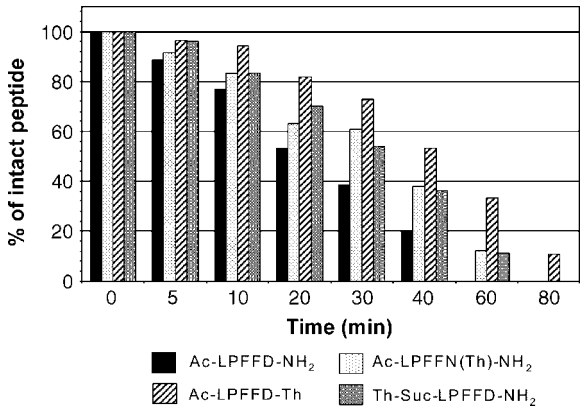

Figure 2. Stability of the peptide derivatives in 10% of rat brain homogenate. All peptides were incubated at a final concentration of 0.25 mM at 37 °C. Aliquots were taken at different times. Bar represent the percentage of the intact peptide as determined by HPLC analysis.

by HPLC analysis at different time intervals. In Figure 2, the decrease of the peak area of each peptide over the time is reported. The data are expressed as the percentage of intact peptide detected in the incubated samples and referred to the peptide solution at the known initial concentration (100% of intact peptide) in PBS. All the conjugated peptides exhibited higher stability in this medium compared to the unmodified Ac-LPFFD-NH_2 that showed a $t_{1/2} < 20$ min under these experimental conditions. Accordingly, the trehalose conjugation provides stability toward enzymatic degradation, with the Th-CT derivative being the most stable compound (Figure 2).

Inhibition of Aβ(1–42) Aggregation

We studied whether the trehalose-conjugated peptides could inhibit aggregation of Aβ(1–42) using the thioflavin T assay. The measurements were done in two different buffers, i.e. PBS and NEM at pH 7.4. The iAβ5p derivative was also comparatively assayed using the same experimental conditions.

Aβ(1–42) samples were prepared as described in the experimental section and were incubated alone or in the presence of 5- or 20-fold molar excess of each compound in 20 mM PBS for 4 days at 37 °C. Identical incubation time and temperature were employed in 25 mM NEM (pH 7.4) but with 5-fold molar excess of the screened compounds. The inhibitory effect of the various peptides on Aβ fibrillogenesis is presented in Figure 3. The ThT results obtained in PBS (Figure 3(A)) show that all the investigated compounds have negligible effects at 5-fold molar excess. In particular both iAβ5p and Th-NT induced Aβ(1–42) aggregation as

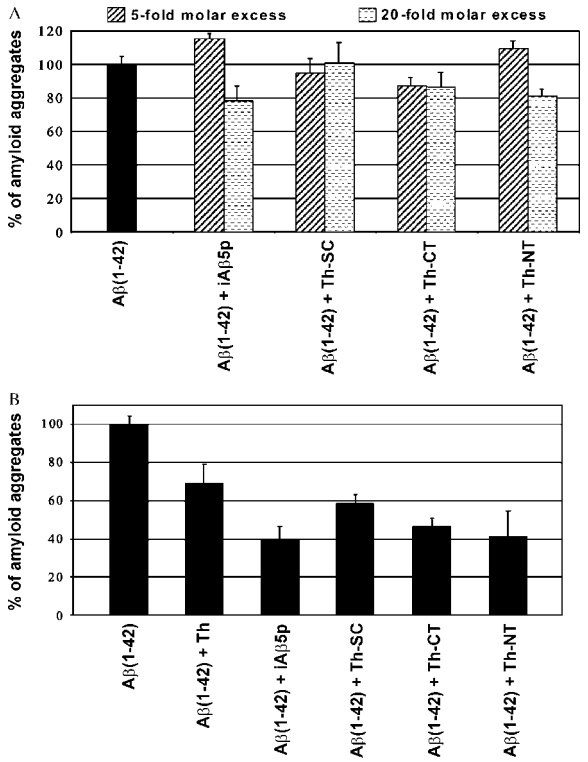

Figure 3. Thiflavin T (ThT) fluorescence emission at 485 nm of samples containing 100 μM Aβ(1–42) incubated in the absence or in the presence of the candidate inhibitors in PBS (A) or NEM (B) buffers.

shown by the higher fluorescence observed with respect to the control alone. At 20-fold molar excess the iAβ5p, Th-CT and Th-NT derivatives revealed to have some inhibitory effect resulting in 20% reduction of the fluorescence. Overall the results indicate that these peptides are not potent inhibitors in PBS buffer. A quite different behaviour was observed in NEM buffer: here a 5-fold molar excess was sufficient to observe a clear inhibitory effect (see Figure 3(B)). It is noteworthy that the iAβ5p, Th-CT and Th-NT conjugates reduced amyloid formation by Aβ(1–42) to about 40% whereas trehalose itself revealed to be scarcely active. Control ThT experiments carried out on conjugate peptides and

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iAβ5p indicated that they are not able to form amyloid fibrils (not shown).

Finally, the kinetics of Aβ(1–42) fibrils formation and the effect of the Th-CT derivative on the aggregation process were monitored by ThT fluorescence in NEM buffer. The results are shown in Figure 4 and indicate that in the presence of 5-fold excess of Th-CT the lag-phase period, preceding the Aβ(1–42) peptide chain assembly, was more than doubled compared to that one observed for the Aβ(1–42) alone. Moreover, the total intensity of the ThT fluorescence showed about 50% reduction when monitored in the presence of the Th-CT derivative; after 90 min co-incubation the fluorescence intensity is of the same order of magnitude as that measured after 4 days incubation.

The above findings were confirmed by dynamic SFM. Figure 5 shows a top view SFM image of a typical Aβ fibrils plaque together with a 3D view in a zoomed region. By adding iAβ5p, TH-CT and TH-NT conjugates an inhibition of fibril formation was observed in NEM buffer, whereas it was only a scarce effect in PBS. In particular, Figure 6 reports the SFM images of the control sample together with those resulting from the addition of the above conjugates. The presence of a mixture of structures including globular systems (3–6 nm tall; 20–50 nm wide) and linear aggregates (3–6 nm tall; up to hundreds of nanometers long) formed by the association of globular systems was observed on all the samples. A statistical analysis was performed in order to understand the effect of the above conjugates on the percentage of Aβ proteins leading to elongated structures as reported in Figure 7. The relative histogram was obtained by assuming the aggregation mechanism proposed by Blackley *et al*. [29] who showed the formation of structures very similar to those here observed. Accordingly, these authors pointed toward a fibrillization mechanism based on the association of globular oligomeric intermediates [29] followed by an elongation phase (fibrillization). The globular structures here observed show an average spacing distance inside the linear aggregate of 12 nm. This distance has been considered as the dimension of a building block unit to evaluate the amount of proteins within each structure. Thus, the number of building units within each structure was estimated and normalized in terms of protein percentage in the dimensional ranges of the histograms. On this respect it has to be noted that globular units of these dimensions are expected to be formed by the association of few monomers as elsewhere reported [30].

Our findings show that in the control sample about 50% protein is involved in the formation of linear aggregates longer than 100 nm. As a result of the co-incubation with Th-NT and Th-CT inhibitors, about half of the protein is no longer involved in the formation of such aggregates being mainly present under the form of low-weight globular systems. The effect of the above two conjugates is comparable to that of the iAβ5p peptide reported as reference. On the other hand, only minor effects have been found by using the Th-SC conjugate.

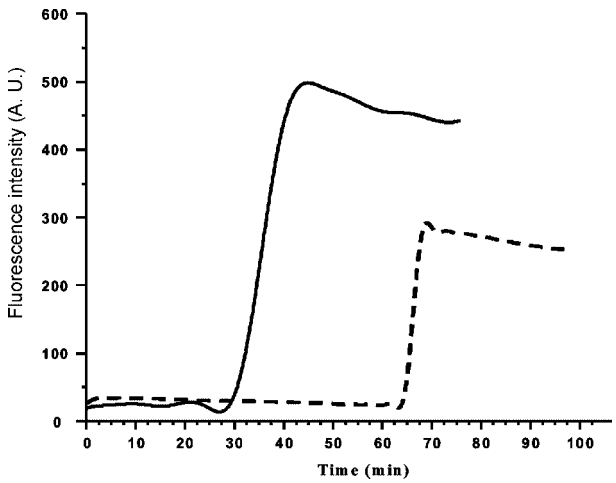

Figure 4. ThT fluorescence assay for the effect of Th-CT peptide on the Aβ(1–42) aggregation. The time dependence of ThT fluorescence intensity of the peptide solutions, incubated at 37 °C, was monitored. Solid line for the Aβ(1–42) and dashed line for Aβ(1–42) in the presence of Th-CT inhibitor.

Effects on Aβ(1–42)-Induced Neuronal Toxicity

All experiments were done using a preparation of un-aggregated Aβ(1–42) to assure a homogenous starting material. Aβ(1–42) was solved in double-distilled water and immediately used.

Primary cortical neurons were treated with un-aggregated Aβ(1–42) at a final concentration of 25 μM for 4 days. This concentration produced about a 60% of neuronal death as assessed by MTT assay. All investigated peptides *per se* were devoid of toxicity when applied to the cultures for 4 days up to a concentration of 250 μM (data not shown). In order to evaluate if the synthesized peptide derivatives were able to prevent Aβ toxicity, each peptide (125 μM) was added together with 25 μM Aβ(1–42) and kept for 4 days. A single addition of the peptide derivatives was unable to prevent significantly Aβ toxicity (data not shown), however, all the glycopeptides protected against Aβ toxicity when added twice (i.e. at the time of Aβ addition and 48 h later), as reported in Figure 8.

Discussion

Intensive efforts are presently addressed to the development of therapeutic agents for amyloidogenic diseases, such as AD. One approach to this issue is to find compounds that bind Aβ because these might interfere with its aggregation and toxicity. A number of low molecular weight molecules have been shown to be capable of inhibiting Aβ aggregation including small β-sheet breaker peptides and osmolytes such as cyclohexanoles [31], mannosylglycerates [18] and threalose [19]. Tjernberg and co-workers reported for the first time the ability of the KLVFF peptide, corresponding to Aβ(16–20) sequence, to inhibit the Aβ fibrillogenesis [9]. Unfortunately, this peptide fragment showed also propensity to self-aggregation and the challenge for the subsequent studies was to modify the Aβ(16–20) sequence in order to obtain new compounds with higher solubility at the same time maintaining the antifibrillogenic properties. Soto and co-workers made steps further toward this issue by replacing the valine residue with proline and introducing an aspartic acid residue at the C-Terminus to afford the well known, beta-sheet breaker peptide iAβ5p [15]. This peptide was reported to inhibit Aβ fibril formation and Aβ-induced neuronal death as well as to significantly reduce Aβ deposition *in vivo* [15,16]. β-Sheet breaker peptides are thought to interfere with Aβ aggregation by covering the 'hot spots' responsible for Aβ fibrillation through a mechanism essentially driven by hydrophobic interactions [9,15]. On the other hand, inhibition of Aβ fibrillogenesis by osmolytes is hypothesized to occur through an alteration of the arrangement of the water molecules surrounding the peptide chains, thus making aggregation energetically less favourable [19].

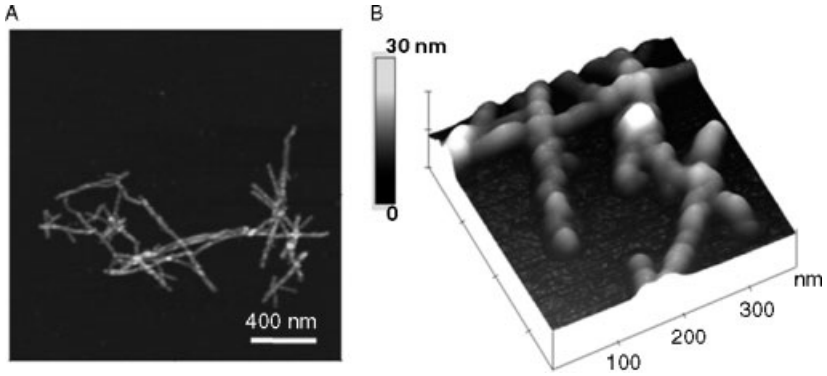

Figure 5. SFM imaging of Aβ fibrils obtained in PBS: (A) top view; (B) 3D view in a zoomed region. Z-scale is 30 nm both in (A) and in (B).

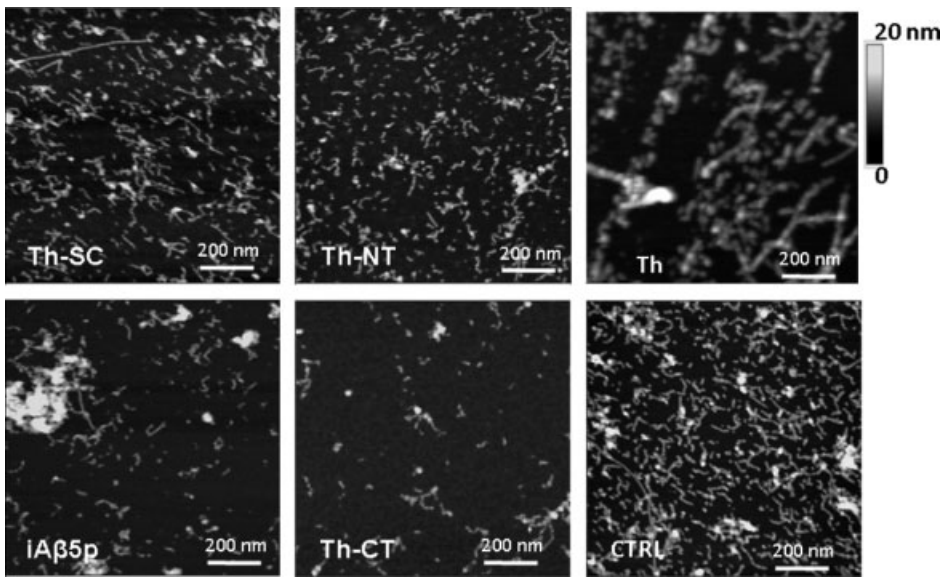

Figure 6. SFM imaging of Aβ samples co-incubated with different conjugates/inhibitors including the iAβ5p one reported for comparison. The control sample is reported on the right.

We figured out that combining compounds that bind Aβ by these two mechanisms would result in new amphiphilic molecules with potentiated antifibrillogenic activity. Our new threalose conjugated peptides display different amphiphilic profiles due to the different position of the hydrophilic trehalose moiety with respect to the hydrophobic peptide chain. We reasoned that this could facilitate the recognition of Aβ(1–42) which is also an amphiphilic molecule. The CD spectra recorded in a 50% solution of TFE, suggests that the peptides adopt a different conformation respect to that one observed in aqueous solution. As TFE is known to provide a hydrophobic environment that mimics the interior of proteins, it is expected that the peptides could change their conformation upon interaction with the hydrophobic region of Aβ(1–42). Our data do not directly deal with this issue, but a recent CD study carried out on the strictly related LPFFD-OH peptide suggested that this peptide adopts a mixture of extended and β-turn conformation in the presence of TFE or when associated with Aβ(1–42) [32]. Notably, our results demonstrate that trehalose conjugation confers enhanced stability in biological fluids. The fact that the trehalose-conjugated peptides are more stable than the underivatised iAβ5p parent peptide in rat brain homogenate could be of relevance in case of experiments employing the administration of similar compounds directly to cell cultures or even *in vivo*.

Based on the ThT results and SFM images obtained in NEM buffer, a clear inhibitory effect of Aβ(1–42) fibrillogenesis was observed at 5-fold molar excess by the investigated peptides. According to the mechanism proposed by Blackley *et al*., [29] it can be hypothesized that our trehalose-conjugated peptides not necessarily interfere with the elongation phase of the fibrils, but instead they act at an intermediate level where the globular oligomeric species start to assemble. Such an hypothesis is supported by the kinetic data obtained for the Th-CT derivatives, which show that the inhibitors could affect the nucleation phase of Aβ(1–42) aggregation process as evidenced by increasing the lag-phase period and by the overall decrease of the amount of fibrils formed.

Due to the fact that NEM is toxic to cell cultures, the assessment of the biological effects of all synthesized peptide derivatives was carried out using water-solubilized peptides. Under this experimental condition (i.e. direct addition to the cultures of monomeric Aβ in the presence of peptide derivatives) we could unmask a protective effect of the trehalose-conjugated peptides against Aβ(1–42) toxicity, which was not shared by the parent peptide iAβ5p. In this regard, it is tempting to speculate that the observed cytoprotective effect could be related to the enhanced

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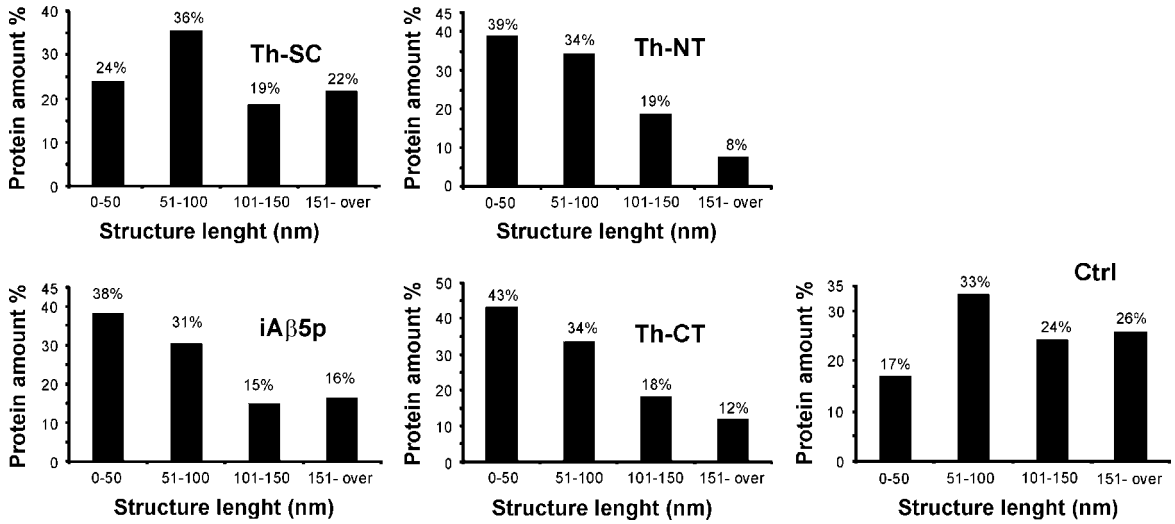

Figure 7. Histograms showing the percentage of Aβ proteins involved in the aggregation process in presence of the different conjugates/inhibitors. Data of the control sample are reported on the right.

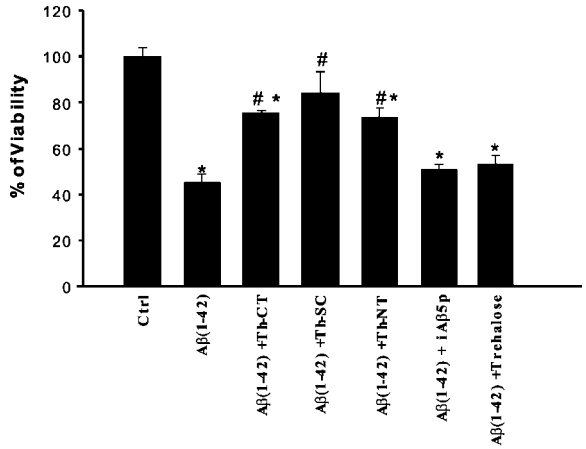

Figure 8. Effect of the studied peptides on the Aβ(1–42)-induced toxicity. All compounds were incubated in a 5-fold molar excess with respect to Aβ(1–42) for 4 days in the culture wells. Cell viability was measured by MTT assay and expressed as percentage of control (sham treated cell). Values are the means±SEM of five determinations from a representative experiment. * $p < 0.01$ vs Ctrl and # Aβ(1–42) alone, respectively. (one-way ANOVA + Fisher's LSD test).

stability of our conjugates towards proteolytic degradation in the biological environment.

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