Fluorescent amyloid β-peptide ligand derivatives as potential diagnostic tools for Alzheimer's disease*

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Abstract: Aβ-peptide ligands based on a *cis*-glycofused benzopyran structure have been fluorescently labeled using coumarine derivatives. Among the synthesized compounds, two conserved their binding ability to β-amyloid peptides, as shown by NMR experiments. Moreover, exploiting its fluorescent property, it was demonstrated that one of such compounds was able to cross an in vitro model of blood–brain barrier (BBB) and to stain Aβ-deposits.

Keywords: Alzheimer's disease; Aβ-peptide ligands; fluorescent derivatives; glycofused compounds.

INTRODUCTION

Among the neurodegenerative diseases, Alzheimer's disease (AD) is the most common and the principal cause of dementia in the elderly population [1–5]. One of the key pathological features of the disease is the abnormal production of β-amyloid peptides (Aβ) and their subsequent accumulation as aggregates in the forms of oligomers, fibrils, and finally plaques, which induce neurodegeneration [6]. In our previous work [7], we identified *cis*-glycofused benzopyran compounds as new Aβ-peptide ligands. These compounds maintained the aromatic feature, which is present in a wide range of small molecules able to interact with Aβ-peptide and which seems to be crucial for the binding ability [8–10], and, at the same time, possesses a glycofused entity that confers them ideal water solubility properties. Moreover, the glycidic moiety assures further possible derivatizations, such as conjugation to other molecular entities (nanoparticles, polymeric supports, etc.), which may be exploited as useful features for diagnostic and therapeutic purposes. The binding ability of these compounds to Aβ1-42 peptide, demonstrated by NMR experiments, represents a fundamental but not unique feature for the develop-

ment of a potential diagnostic/therapeutic tool. In order to be useful, such compounds should perform their action within the brain, therefore they have to be able to cross the blood–brain barrier (BBB). The BBB is formed by the complex tight junctions between the endothelial cells of the brain capillaries and their low endocytic activity. This results in the capillary wall that behaves as a continuous lipid bilayer and prevents the passage of polar and lipid insoluble substances. Unlike most of the tissue, the BBB prevents the paracellular passage, thus the small molecule should pass exploiting a transcellular mechanism. It is, therefore, the major obstacle to drugs that may combat diseases affecting the central nervous system (CNS) [11]. Only a few compounds with the correct hydro-/lipophilic balance have a greater chance to overcome the BBB through a diffusion mechanism.

The main mechanisms allowing the transport of drugs across the BBB account for (I) passive diffusion for small lipophilic drugs which may enter by penetrating the luminal and abluminal membranes, (II) active carrier-mediated, (III) receptor-mediated transport, or (IV) via adsorption-mediated transcytosis, for nondiffusible molecules. The net uptake of a drug by the brain via the BBB depends on the overall difference between the uptake and efflux processes. The uptake is controlled by several factors, including the systemic disposition of the drug and the properties of endothelial cells. The permeability of endothelial cells and their capacity to metabolize drugs actively control the amounts of drug crossing the BBB. Permeability is controlled by several properties of the endothelial cells. There is no paracellular movement of drugs because of the tight junctions linking the endothelial cells, but small lipophilic drugs (<600 Da) may enter the brain by penetrating the lipid membrane of the endothelial cells. The passive diffusion of a drug depends on its blood/brain concentration gradient and its lipid solubility, but it is inversely related to its degree of ionization and its molecular weight. Factors other than lipophilicity and molecular weight also modulate the transport of a drug across the BBB. Reducing the relative number of polar groups increases the transfer of a drug across the BBB [11].

In this work we designed and synthesized the fluorescent derivatives **1**, **2**, **3**, **4** (Fig. 1), in order to perform BBB passage studies. Moreover, the presence of the fluorophore allowed us to study the ligands' ability to stain Aβ deposits in Tg CRND8 mice. The choice of the fluorophore was done in order to modulate the hydrophilic properties of the molecule, maintaining the molecular weight low enough for a diffusion mechanism. For this reason, we selected coumarine derivatives **7**, **11**, and **18** (Fig. 2). We exploited the primary hydroxyl group of the glycidic moiety to perform the linkage, since, as expected, previous binding studies confirmed that the sugar moiety was not or was very poorly involved in the binding with the Aβ-peptide [7].
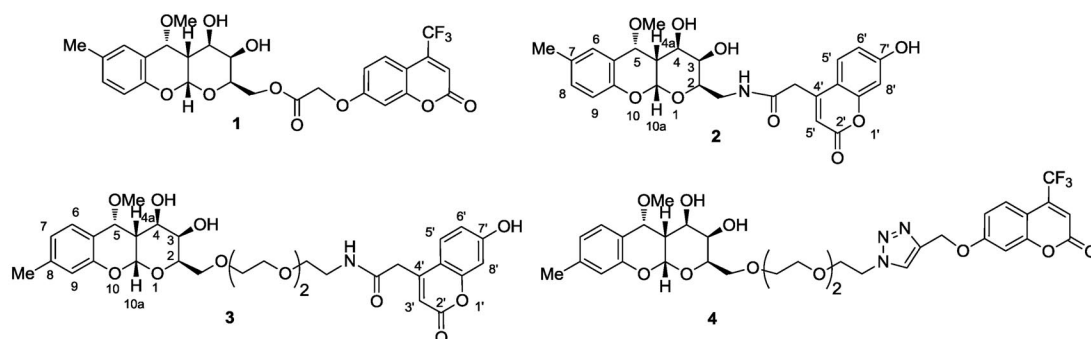
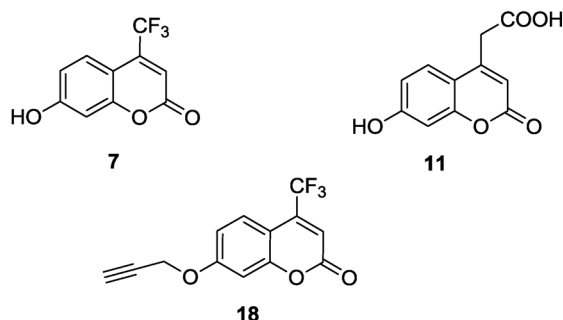

Fig. 1 Fluorescent derivatives of Aβ-peptide ligands.

Fig. 2 Coumarine derivatives.

SYNTHESIS AND NMR BINDING STUDIES

Our first approach was focused on the direct conjugation of the fluorophore to the Aβ-peptide ligand. Among the previously [7] identified ligands, compounds bearing the methyl substituent at position C7 and/or C8 of the aromatic ring showed the best binding properties, thus these compounds were used in the present work. For the synthesis of compound **1** (Scheme 1), ligand **5** was regioselectively acylated with bromoacetylbromide at low temperature (–45 °C), affording compound **6**, which was reacted with coumarine derivative **7** in basic conditions (CsCO$_3$) to afford the final product with a 20 %, not optimized, overall yield. For the synthesis of compound **2** (Scheme 1), we first converted the primary hydroxyl to the corresponding amine **10**, by a tosylation (TsCl, Py) followed by a nucleophilic azide substitution (NaN$_3$, DMF) and final reduction (H$_2$, Pd-Lindlar, MeOH). Compound **10** was then coupled with coumarine derivative **11**, using standard coupling conditions (DIC, HBTU, DIPEA, DMF dry), affording the final product **2**.
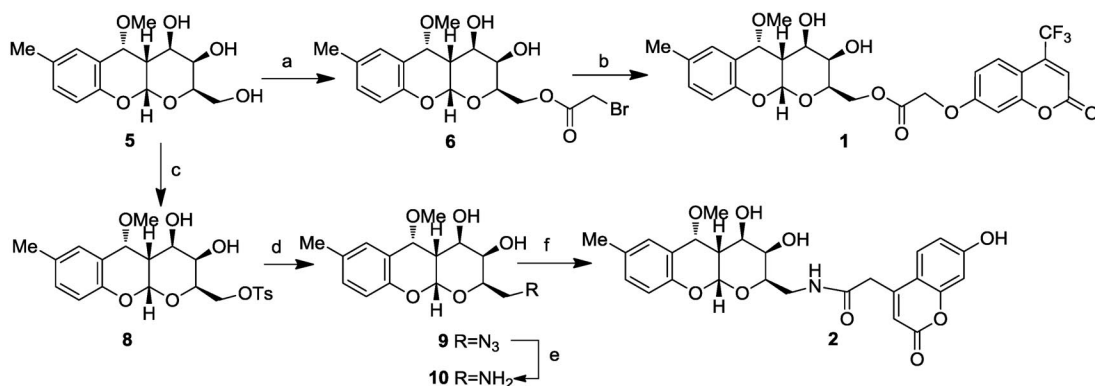
Scheme 1 Reagents and conditions: (a) BrCH$_2$COBr, dry DMF, sym-collidine, –45 °C, 40 min, 54 %; (b) **7**, CsCO$_3$, dry DMF, r.t., 1 h, 37 %; (c) TsCl, DMAP, dry Py, 0 °C r.t., 12 h, 98 %; (d) NaN$_3$, dry DMF, 100 °C, 12 h, 60 %; (e) H$_2$, Pd-Lindlar, MeOH, r.t., 1 h; (f) **11**, DIC, HBTU, DIPEA, dry DMF, r.t., 47 % (over two steps).

In order to verify the influence of the fluorophore on the binding properties to Aβ-peptides, saturation transfer difference (STD) NMR experiments were carried out on both compounds.

Unfortunately, compound **1** resulted as chemically unstable, as it is hydrolyzed immediately after dissolution in aqueous buffer (data not shown), probably because of the presence of the ether linkage in α position to the ester group.

Conversely, compound **2** resulted as stable to hydrolysis and its interaction with Aβ1–42 was investigated by STD NMR experiments [12].

NMR binding studies were carried out employing the same methodology previously described [7,9,10]. In particular, compound **2**'s ability to interact with Aβ1–42 oligomers was assessed by STD NMR spectroscopy. STD NMR experiments were performed using a ligand:peptide 10:1 mixture dissolved in deuterated PBS, pH 7.4, 37 °C. The mixture was analyzed irradiating the sample at –1.0 ppm to achieve the selective saturation of some aliphatic resonances of Aβ oligomers. In general, the presence of NMR signals of the test molecule in the STD spectrum is a clear demonstration of the existence of interaction. Conversely, the absence of compound resonances in the STD spectrum indicates that the molecule is not an Aβ ligand. Different NMR resonances of compound **2** appeared in the STD spectrum recorded in the presence of Aβ oligomers (Fig. 3), thus showing its ability to recognize and bind Aβ1–42.
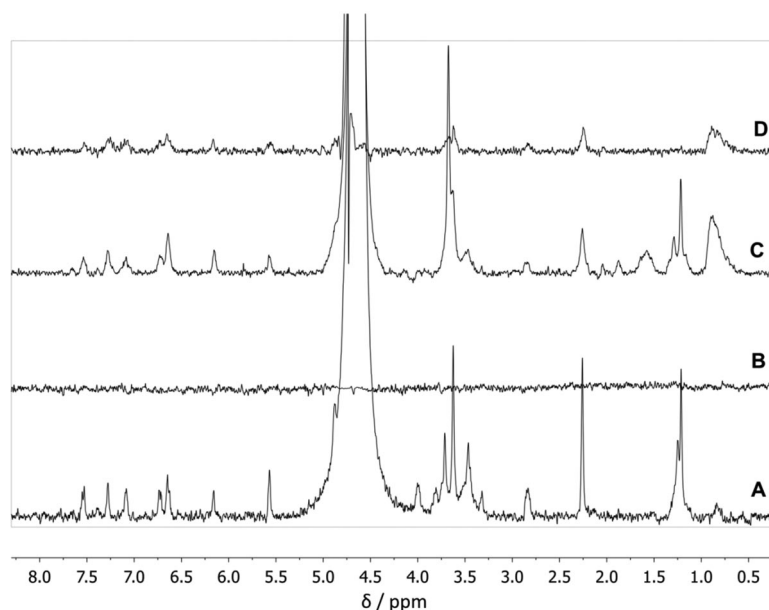

Fig. 3 (**A**) ^{1}H NMR spectrum of the compound **2** (0.5 mM) in PBS, pH 7.4, 37° C; (**B**) blank STD NMR spectrum of compound **2** acquired with a saturation time of 3.0 s and 2304 scans; (**C**) ^{1}H NMR spectrum of the mixture containing the peptide Aβ1–42 (50 μM) and compound **2** (0.5 mM) in PBS, pH = 7.4, 37 °C; (**D**) STD NMR spectrum acquired on the same mixture with a saturation time of 3.0 s and 2304 scans.

STD experiments evidenced the binding ability of compound **2**, even if its STD signals were rather low in intensity, probably due to solubility problems in physiological conditions. The poor quality of STD spectrum prevented the obtainment of a detailed epitope mapping and the acquisition of further NMR interaction experiments. In order to overcome the limitations encountered for derivatives **1** and **2**, we planned to introduce a triethylene spacer between the ligand and the fluorophore. This should increase both water solubility and chemical stability, avoiding the liable α-oxy ester group. For the preparation of this second set of derivatives, we used Aβ peptide ligand **12** (Scheme 2), which in our previous work showed the same binding properties of compound **5**, but a more straightforward preparation from commercially available reagents. Secondary hydroxyls were protected with an isopropylidene group (DMP, CH$_3$CN, CSA), and the free primary hydroxyl was reacted with triethylene derivative **14** [14] (NaH, DMF), to afford intermediate compound **15**. Removal of the protecting group
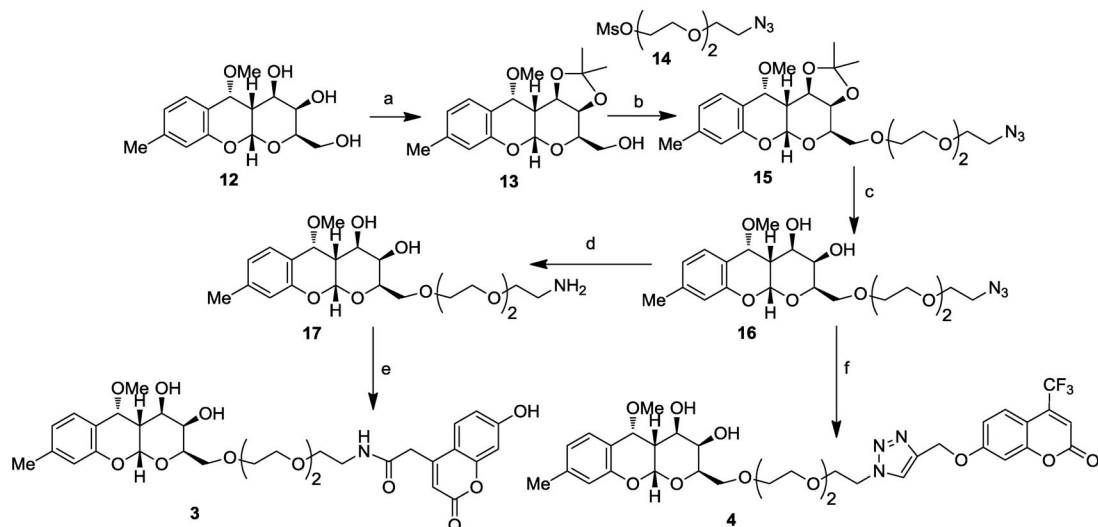
Scheme 2 Reagents and conditions: (a) DMP, CSA, dry CH$_3$CN, r.t., 1 h, 60 %; (b) **14**, NaH 60 %, dry DMF, 100 °C, 12 h, 75 %; (c) *p*-TsOH, H$_2$O:CH$_3$CN (0.5/1.5:v/v), r.t., 30 min, 90 %; (d) H$_2$, Pd-Lindlar, MeOH, r.t., 1 h, 90 %; (e) **11**, DIC, HBTU, DIPEA, dry DMF, r.t., 12 h, 20 %; (f) **18**, CuSO$_4$5H$_2$O, sodium ascorbate, *t*-BuOH:THF (1/1:v/v), r.t., 12 h, 55 %.

[*p*-TsOH, H$_2$O:CH$_3$CN (0.5/1.5:v/v)], afforded derivative **16** bearing an azido functionality at the end of the triethylene moiety. For the synthesis of compound **3**, the azido group was reduced (H$_2$, Pd-Lindlar) and the resulting amine **17** was coupled with coumarine derivative **11** (DIC, HBTU, DIPEA, DMF), while for the preparation of compound **4**, the azido group was exploited in a chemoselective click cycloaddition reaction [CuSO$_4$5H$_2$O, sodium ascorbate, *t*-BuOH:THF(1/1:v/v)] with the alkyne coumarine derivative **18**, obtained from **7** through propargylation of phenolic OH (see supplementary information).

As expected, the solubility of compound **3** in water allowed us to perform both STD and trans-ferred nuclear Overhauser spectroscopy (trNOESY) [9,10,12b,c] NMR experiments. Both of the tech-niques were applied on a sample containing Aβ1–42 and compound **3** dissolved in deuterated PBS, pH 7.4, 25 °C at the final concentrations of 80 μM and 0.5 mM, respectively. Figure 4 reports the STD spec-tra recorded with five different saturation times (**D**, 3.0 s; **E**, 2.0 s; **F**, 1.2 s; **G**, 0.7 s; **H**, 0.3 s).

STD spectra clearly demonstrated compound **3**'s ability to recognize and bind Aβ oligomers. The existence of interaction was also supported by the broadening of molecule **3** resonances when the com-pound was dissolved in the presence of the peptide (compare spectra **3A** and **3C**). This broadening reflects a decrease in proton relaxation times due to the formation of a receptor-ligand complex.

To map the ligand binding epitope, the STD spectrum acquired with a saturation time of 0.3 s was analyzed to minimize the effect of relaxation on STD intensities [13]. Figure 5 reports schematically the fractional STD effect for some ligand protons (or groups of protons), calculated as $(I_0 - I)/I_0 \times 100$, where I is the intensity of the monitored signal in the STD spectrum and I_0 is the intensity of the same signal in a reference spectrum. The region of the ligand presenting the higher fractional STD effect (value around 40 %), is the aromatic ring of the tricycline, thus resulting in the ligand structural moiety mainly involved in the interaction with Aβ, this achievement is in agreement with data obtained for the nonfunctionalized ligand [7]. Also, the coumarine moiety participates in the interactions, but its contri-bution is less significant.
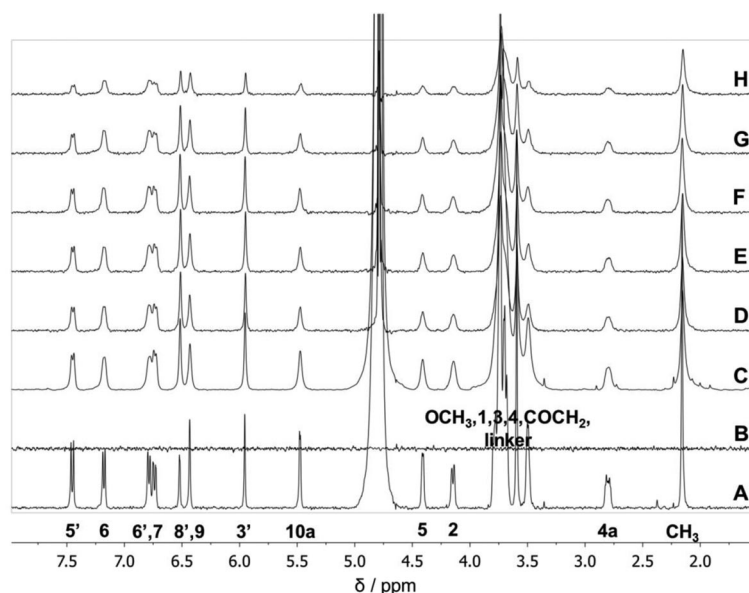
Fig. 4 (**A**) ¹H NMR spectrum of a solution of compound **3** (0.5 mM); (**B**) blank STD NMR spectrum of the same solution acquired with 3.0 s of saturation time; (**C**) ¹H NMR spectrum of the mixture containing Aβ1–42 (80 μM) and compound **3** (0.5 mM) in PBS, pH = 7.5, 25 °C; (**D–H**) STD NMR spectra of the same mixture acquired with different saturation times. (**D**, 3.0 s; **E**, 2.0 s; **F**, 1.2 s; **G**, 0.7 s; **H**, 0.3 s).
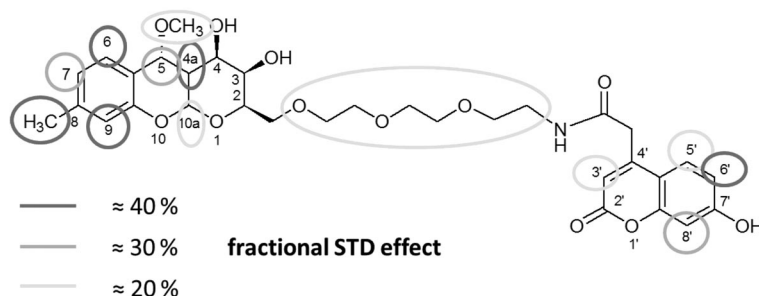

Fig. 5 Fractional STD effects calculated for different protons of compound **3**.

The binding was further assessed by trNOESY experiments. A blank NOESY spectrum of compound **3** was recorded in the absence of the Aβ1–42 peptide (Fig. 6A). An inversion of the sign of its NOESY cross-peaks was observed between both spectra, passing from positive (light gray color), in the absence of Aβ1–42 peptide (Fig. 6A), to negative (dark gray color) in the presence of Aβ1–42 peptide (Fig. 6B). This change is due to an increase of the effective rotational motion correlation time of the molecule in the presence of the Aβ oligomers, which demonstrated the existence of a binding between the small molecule and Aβ aggregates [10,12b].

The solubility of compound **4** in aqueous buffer was very poor. In particular, NMR binding experiments were performed on a sample containing the test molecule at a concentration of 0.5 mM in PBS, pH = 7.5, 25 °C, to which 5 % of d_6-DMSO was added to promote its dissolution. Aβ1–42 peptide was added at a final concentration of 80 μM. In these conditions, we obtained an STD spectrum of low quality (Fig. 7A-4), but sufficient to assess the existence of interaction with Aβ1–42 peptide. The binding was further supported by the significant broadening of compound **4** ¹H resonances, observed when the
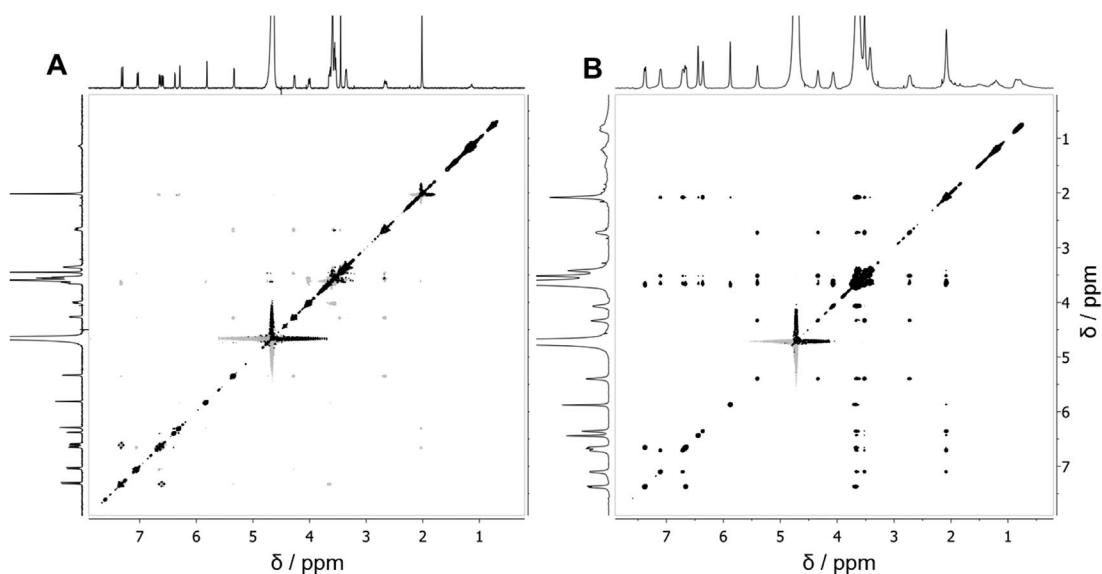
Fig. 6 (**A**) 2D-NOESY spectrum of compound **3** dissolved in deuterated PBS, pH 7.5, 25 °C, mixing time 0.9 s; (**B**) trNOESY of mixture containing Aβ1–42 (80 μM) and compound 3 (0.5 mM) dissolved in deuterated PBS, pH 7.5, 25 °C, mixing time 0.3 s. Positive cross-peaks are light gray, negative ones dark gray.
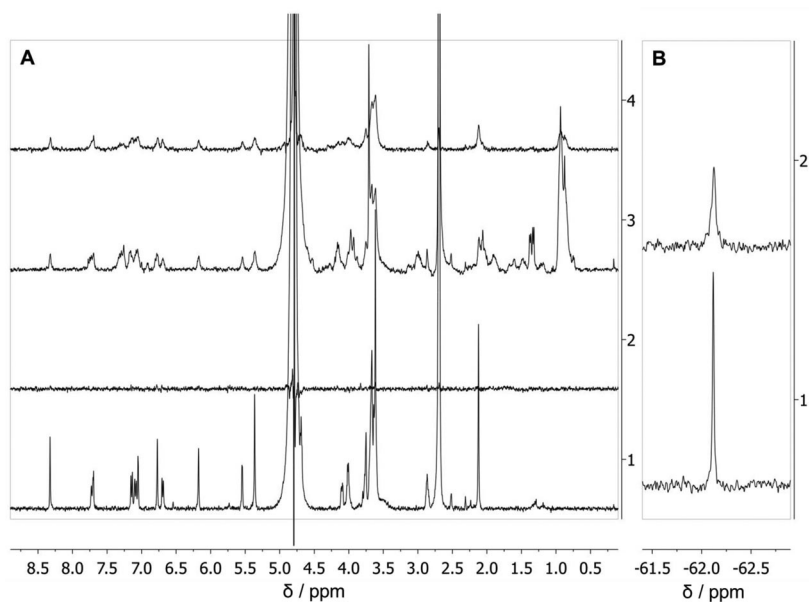

Fig. 7 (**A-1**) ^{1}H NMR spectrum of the compound **4** (0.5 mM); (**A-2**) blank STD NMR spectrum of compound **4** acquired with a saturation time of 2.0 s and 5200 scans; (**A-3**) ^{1}H NMR spectrum of the mixture containing the peptide Aβ1–42 (80 μM) and compound **4** (0.5 mM); (**A-4**) STD NMR spectrum acquired on the same mixture with a saturation time of 2.0 s and 5200 scans. (**B**) ^{19}F spectra of compound **4** dissolved in the absence (**1**) or in the presence (**2**) of Aβ oligomers. Samples were dissolved in PBS, pH = 7.5, 25 °C, adding 5 % of d_6-DMSO.

molecule was dissolved in the presence of Aβ oligomers; this effect results from evidence of the comparison of the ^{1}H spectrum recorded in the absence (Fig. 7A-1), with the spectrum acquired in the presence of Aβ1–42 peptide (Fig. 7A-3). In addition, we could exploit the presence of the CF_3 substituent on the coumarine moiety, as a dramatic change also in its line width can be observed when the ^{19}F spectrum of the molecule alone (Fig. 7B-1) is compared with the corresponding spectrum acquired on the ligand:receptor mixture (Fig. 7B-2).

TRANSPORT EXPERIMENTS

Evaluation of drug transport to the brain in vitro has usually been carried out by studying the transport of individual molecules across endothelial cell monolayers. Currently available in vitro models for BBB allow one to evaluate quickly and in a reproducible way the predictive in vivo permeability of compounds and drugs under development.

The development of a cell culture system that mimics an in vivo BBB requires endothelial cells to be cultured on microporous supports. hCMEC/D3 cells (passages 25–35) were seeded on 12-well Transwell® inserts coated with type I collagen in a density of 5×10^4 cells/cm^2 and cultured with 0.5 and 1 mL of culture medium in the upper and in the lower chamber, respectively, as previously described [15]. Cells were treated with compound **3** when the *trans*-endothelial-electrical resistance (TEER) value was found to be highest.

The functional properties of monolayers were assessed by measuring the endothelial permeability of sucrose (between 0–180 min) as previously described [16]. 0.5 mL of 9.4 and 94 μM compound **3** were added to the upper chamber and incubated between 0 and 180 min. After these periods of incubation, the fluorescence in the upper and lower chambers was measured (λ_{ex} = 280 nm) to calculate the endothelial permeability (PE) across the cell monolayers, taking account of the passage of compound **3** through the filter without cells [16]. Each experiment was performed at least in triplicate. All the transport studies have been replicated in the presence of a paracellular marker, [^{14}C]-sucrose, in order to monitor potential toxic effects on the BBB exhibited by compound **3**.

The Aβ-peptide ligand compound **3** does not affect the tightness of the hCMEC/D3 cell monolayer, since the TEER value (data not shown) and the permeability of [^{14}C]-sucrose did not change, within the experimental error (<3 %), during hCMEC/D3 incubation with compound **3** (PE sucrose = $1.38 \pm 0.1 \times 10^{-3}$ cm/min; PE sucrose = $1.40 \pm 0.1 \times 10^{-3}$ in the presence of derivative **3**) (Fig. 8).
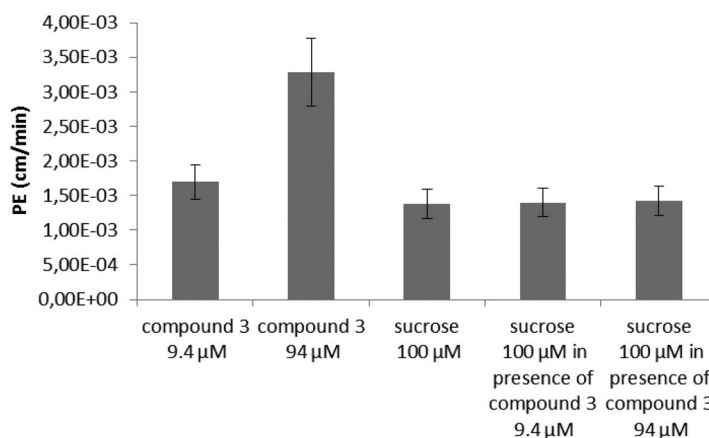

Fig. 8 PE values across the hCMEC/D3 monolayers of compound **3** (9.4 and 94 μM) and PE values of sucrose (100 μM) alone and in presence of compound **3**.

hCMEC/D3 cells, grown on transwell membrane inserts, were incubated with compound **3** on day 12, when the maximal TEER value was registered (68 ± 8 Ω cm^2). Transport of [^{14}C]-sucrose was measured, with PE values of $1.38 \pm 0.2 \times 10^{-3}$ cm/min in agreement with the values reported in literature [17]. Compound **3**, at different concentrations (9.4 and 94 µM), has been added in the upper compartment, and the amount in the lower compartment has been measured over time at 0, 60, and 180 min. The PE of compound **3** across the cell monolayers was $3.03 \pm 1.19 \times 10^{-3}$ cm/min and $1.69 \pm 0.59 \times 10^{-3}$ cm/min, and these values were strictly correlated to the administered amount within the upper compartment, respectively, 94 vs. 9.4 µM. The PE of compound **3** was closer to that of the transcellular marker propranolol, a reference point in terms of lypophilic compound, which can permeate the BBB [17]. Taking together these preliminary results might account for passive diffusion as the main mechanism allowing the transport of compound **3** across the BBB. Further experiments are needed to deeply investigate this issue.

STAINING

The ability of the compound **3** to bind amyloid deposits was tested in brain sections from Tg CRND8 mice. These animals carry a human APP with double mutations and accumulate Aβ deposits in brain parenchyma and at cerebrovascular level. Cryostatic sections of 20 µm were obtained from fresh tissue, mounted on gelatin-coated microscope slides and used for a staining assay. Solutions of EtOH:water 50:50 (v/v) of test compound were layered on tissue sections. Fluorescent sections were viewed using a fluoromicroscope equipped with FITC and DAPI filters. Thioflavin T (ThT) at 3 µM was used as a reference. Compound **3** was able to label amyloid plaques at a concentration of 6 µM, obtaining a result comparable to thioflavin T staining. These results suggested that compound **3** is able to recognize the β-pleated sheet structure of amyloid fibrils similarly to ThT and without being involved in a specific binding to tissue preparations (Fig. 9).
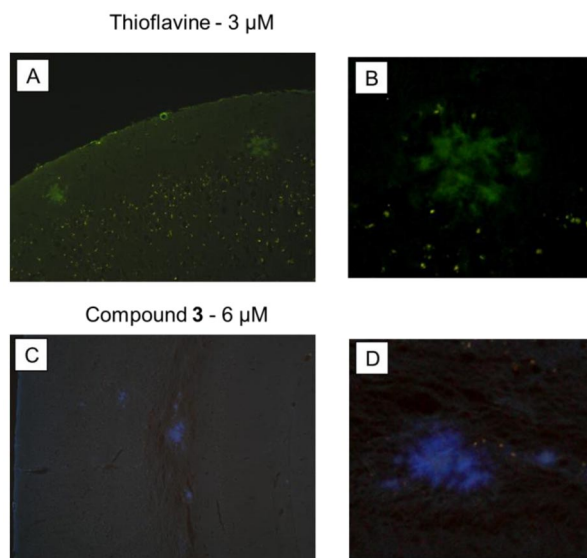

Fig. 9 Brain sections of Tg CRND8 mice incubated with ThT (A-B) at 3 µM and compound **3** (C-D) at 6 µM. Fluorescent sections were viewed using fluoromicroscope FITC for ThT staining and DAPI for compound staining.

CONCLUSION

In conclusion, in this work we demonstrate through NMR experiments the preserved ability of derivatives **3** and **4** to bind Aβ peptides; moreover, fluorescence measurements carried out with compound **3** indicate that its favorable physicalchemical properties, in terms of balanced hydro-/lipophilicity, allow these compounds to permeate through the BBB, most probably exploiting a diffusion mechanism, and to stain amyloid plaques. These properties, taken together, suggest that these compounds could be very promising candidates for possible future applications both in the therapy and diagnosis of AD-related disease.

SUPPLEMENTARY INFORMATION

Supplementary information is available online (http://dx.doi.org/10.1351/PAC-CON-12-11-07).

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