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Detection of interactions of the β-amyloid peptide with small molecules employing transferred NOEs

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The interaction of pineal hormone melatonin, the histological dye thioflavin T, and the olive tree polyphenol oleuropein, with the 28 amino acid residue *N*-terminal fragment of the β-amyloid peptide (β-AP) of Alzheimer's disease, [β-AP(1-28)], was detected in solution through the observation of transferred NOEs (trNOEs) in 1D and 2D NOE spectroscopy (NOESY) experiments. The trNOE method is applied for the first time in the detection of interactions of soluble β-AP(1-28) with small molecules and may provide a means of screening for the identification of possible inhibitors of the formation of neurotoxic β-AP assemblies.

Supporting information may be found in the online version of this article

Keywords: β-amyloid peptide; melatonin; thioflavin T; oleuropein; transferred NOE

Introduction

The progressive aggregation of β-amyloid peptide (β-AP) leading to the formation of extracellular fibrillar deposits in selected areas of the brain is widely considered to be fundamental to the development of the neurodegenerative pathology that characterizes Alzheimer's disease (AD) [1]. Inhibition of the fibrillization process of β-AP consequently emerged as an attractive therapeutic strategy against AD [2–4]. A diverse range of small organic compounds has been identified as inhibitors of β-AP fibrillization through the application of a variety of physicochemical and imaging techniques as well as toxicity assays [5–8]. β-AP fibrillization is a complex process occurring via multiple pathways that involve several morphologically and immunologically distinct β-AP aggregated species, including oligomers, protofibrils, and fibrils of variable structure [9,10]. Recent evidence indicates that soluble β-AP oligomers may represent the primary toxic species in AD and attention has shifted to the identification of inhibitors of β-AP oligomerization [11–13] as possible therapeutic agents against AD. β-AP oligomers are kinetically unstable intermediates of multiple types and sizes, the structural and functional characteristics of which are not well understood; as a result, the available screening assays for inhibition of β-AP oligomerization are qualitative, mainly employing SDS-PAGE analysis, and oligomer-specific antibodies [14]. As is the case for the inhibitors of β-AP fibrillization, the structure of the compounds identified as inhibitors of β-AP oligomerization is diverse [8,10,15] suggesting that they may bind to different sites within amyloid or even to different amyloid formations. This lack of structural similarity makes inferring conclusions from structure/activity relationships difficult and hampers rational drug design, necessitating the use of screening methods for the identification of ligands against β-AP assembly.

NMR screening techniques are being applied to quickly identify ligands that exhibit affinity for biologically important macromolecules in rational drug design [16–18]. Exchange-based methods like transferred NOEs (trNOEs) [19,20] are used in the detection of binding activity of small molecules taking advantage of the fact that when a relatively small molecule binds to a macromolecule, its NOE signal changes from positive to negative. The change in the NOE sign can be detected in the peaks of the free ligand provided that the ligand is in medium to fast exchange between the free and bound states (generally corresponding to K_D in the μM to mM range). Especially in the case of β-AP with its complex assembly process the details of which remain obscure, the trNOE experiment may reveal the existence of interaction with a small molecule in a quick, straightforward way and without the need of identifying the exact aggregation state of β-AP in solution.

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Abbreviations used: *SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NMR, nuclear magnetic resonance; DMSO-d_6, completely deuterated dimethyl sulfoxide; NOE, nuclear overhauser enhancement; K_D, dissociation constant; 1D, one-dimensional; 2D, two-dimensional; NOESY, nuclear Overhauser enhancement spectroscopy; HPLC, high-performance liquid chromatography; CD, circular dichroism; ESI, electrospray ionization.*

In this work, the application of the trNOE method on the detection of interactions of β-AP(1–28), consisting of the N-terminal 28 residues of the native β-AP(1–40), was tested with the pineal hormone melatonin (Figure 1). Melatonin is known from the literature to interact with soluble β-AP [21–23] and, therefore, served as the positive control for the method. Sucrose, known from the literature not to affect the aggregation of β-AP [24], served as the negative control. The method was subsequently extended to include the dye thioflavin T (ThT) and the antioxidant olive tree metabolite oleuropein (Figures 2 and 3) and this is the first time that interaction between these two and β-AP in solution is detected by NMR. For the experiments, the 1D gradient enhanced NOESY was employed which, compared to 2D techniques, drastically reduces the requirements in acquisition time, a factor that becomes important for the low concentration solutions usually employed in the case of aggregation prone peptides like β-AP. In all cases, however, the results were confirmed through the use of the 2D NOESY experiment.

Materials and Methods

Peptide Synthesis

The β-AP(1–28) peptide, comprising the 28 *N*-terminal amino acids of the full-length β-AP(1–40) sequence, was synthesized by the Fmoc strategy [25] and its purity was evaluated to >95% by reverse-phase HPLC and mass spectrometry.

Reagents

Melatonin, ThT, and sucrose were purchased from Aldrich. Oleuropein was isolated from olive leafs [26] and was a donation from the laboratory of Pharmacognosy, University of Athens.

Sample Preparation

Samples of β-AP(1–28) were freshly prepared by dissolving the proper amount of the peptide in D_2O to a concentration of approximately 0.18 mM. Stock solutions of ThT, oleuropein, and sucrose were prepared in D_2O and of melatonin in CD_3OD. Stock solutions of melatonin, ThT, and oleuropein were also prepared in DMSO-d_6 in order to investigate the use of DMSO as a solvent for the preparation of stock solutions of hydrophobic organic compounds.

β-AP(1–28):ligand solutions of different molar ratios were prepared by adding aliquots of the ligand stock solutions in freshly prepared D_2O solutions of the β-AP(1–28). The pH of the solutions was adjusted to 3.9 ($\pm$0.2) by the addition of microliter quantities of NaOD or DCl. The 1D spectra of the solutions remained unchanged over the course of the experiments.

The intensities of trNOEs were investigated at β-AP(1–28):ligand molar ratios of 1 : 1, 1 : 5, 1 : 12, and 1 : 20 and the ratio of 1 : 5 was found to be optimal.

NMR Experiments

All NMR experiments were performed on a Bruker Avance DRX-500 spectrometer, operating at 500.133 MHz for ^{1}H, equipped with a *z*-gradient 5 mm broadband inverse (BBI) probe using standard pulse sequences and phase cycling. The 90 degree high power pulse for ^{1}H was calibrated at 8.8 µs for attenuation level of 3 dB. 1D NOESY spectra were recorded at 300 K using the following pulse sequence:

$$270^\circ(x, \text{sel}) - G_1 - 90^\circ(x) - t_m - G_m - 90^\circ(x) - t_2 - 180^\circ(x) - G_2 - \text{acq}$$

[27,28] (experiment name: *selnogs* in the Bruker library) with a Gaussian-shaped pulse of 80 ms (270 degree) truncated by 5% and

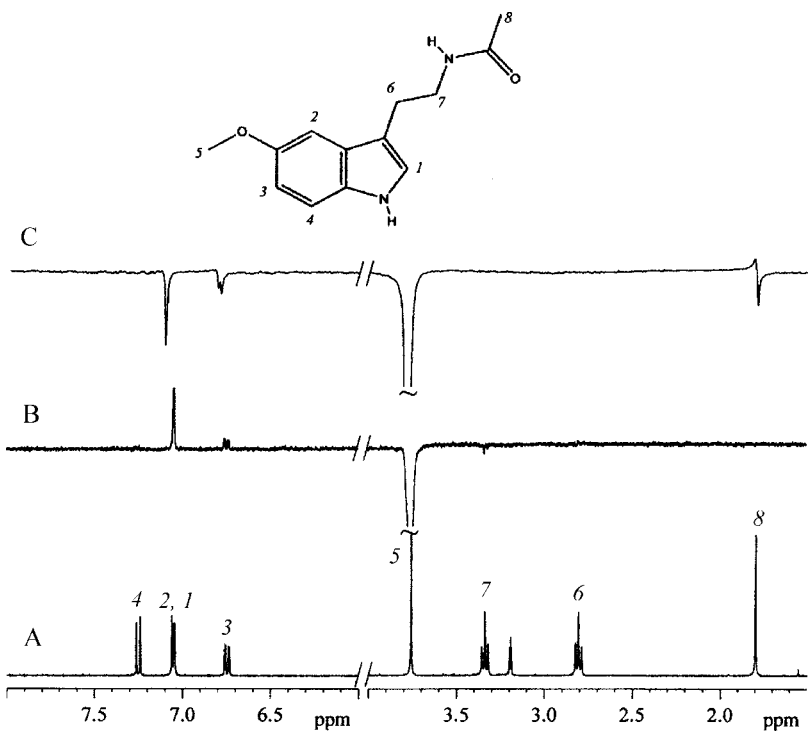

Figure 1. (A) ^{1}H NMR spectrum of melatonin in D_2O; (B) 1D NOESY spectrum of the same sample with t_m 150 ms; and (C) 1D NOESY spectrum of a 1 : 5 β-AP(1–28):melatonin solution with t_m 150 ms. The numbering of protons is shown on the structure of melatonin.

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2k points, and the defocusing (G_1), purged (G_m), and refocusing (G_2) gradients having a ratio of 30 : 50 : 30. The recycling delay was set to 2 s and the mixing times (t_m) used were 150 and 800 ms. The number of scans was 12k for 1 : 1 solutions, and 1k for the rest of the experiments – the sign of the NOE peak, however, was visible quite early in the experiment. 2D NOESY [29,30] were recorded with the same t_m as the 1D spectra, with 2k points in t_2 dimension, 256 t_1 increments, and 128 transients in the phase-sensitive mode using time-proportional phase incrementation (TPPI) [31]. Suppression of the residual water peak was achieved either through the excitation sculpting pulse sequence [32] or by presaturation.

Data sets were processed using the XWIN-NMR versus 2.6 software of Bruker. 1D NOESY spectra were processed using a line broadening (lb) value of 2. In 2D NOESY spectra, t_1 data were zero-filled to 512 data points and Fourier transform was performed after applying a $\pi/4$ phase-shifted squared sine-bell function in both dimensions.

The possibility that the trNOEs generated are due to background contribution arising from the peptide is ruled out because no peptide protons generate NOE signals at the chemical shifts where the intramolecular NOEs of the small molecules appear (Supporting Information, Figure S1).

Results

Melatonin

The 1D ^{1}H NMR spectrum of a melatonin solution in D_2O (prepared from a stock solution in CD_3OD) at pH 4.0 is shown in Figure 1(A). In this sample, the selective excitation of the H-5 protons of the methoxy group with a t_m of 150 ms, generated in the 1D NOESY spectrum positive NOE signals for the two aromatic H-2 and H-3 protons (Figure 1(B)). As seen in Figure 1(C), in the presence of β-AP(1–28) at a ratio of β-AP(1–28):melatonin 1 : 5 and at the same t_m, these NOE signals became negative. The change in the sign of the NOEs can only be explained as trNOEs generated by the interaction of β-AP(1–28) with melatonin. The pH of the β-AP(1–28)/melatonin solution was adjusted to 3.9 since this pH is suitable for studies of the β-AP(1–28) peptide [33,34] and for interaction of melatonin with β-AP according to mass spectrometry studies [23]. 2D NOESY spectra recorded with the same t_m were in complete agreement with the 1D NOESY observations.

When stock solutions of melatonin were prepared in DMSO-d_6 no interaction could be detected even when DMSO-d_6 was present in the very small concentration of 1% in the interaction solution.

Thioflavin T

The 1D ^{1}H NMR spectrum of a ThT solution in D_2O, at pH 4.1 is shown in Figure 2(A). 1D NOESY spectra of the plain ThT solution were acquired by selective excitation of the H-7/H-8 doublet at 6.80 ppm with a t_m of 150 ms. As can be seen in Figure 2(B), weak NOEs were generated with the H-6/H-9 protons at 7.68 ppm and the *N*,*N*-dimethyl H-10 protons at 2.97 ppm. In the presence of β-AP(1–28) at a ratio of β-AP(1–28):ThT 1 : 5 and at pH 3.8 the selective excitation of the ThT H-7/H-8 resonance with the same t_m gave rise to negative NOEs for the H-6/H-9 and H-10 *N*,*N*-dimethyl protons (Figure 2(C)). As in the case of melatonin, these negative

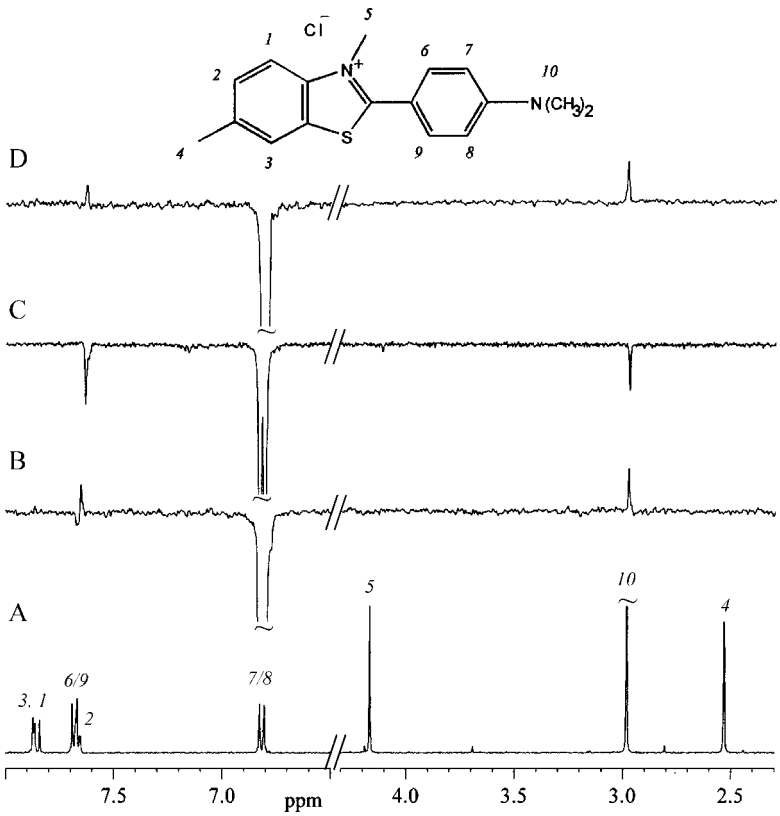

Figure 2. (A) ^{1}H NMR spectrum of ThT in D_2O; (B) 1D NOESY spectrum of the same sample with t_m 150 ms; (C) 1D NOESY spectrum of a 1 : 5 β-AP(1–28):ThT solution with t_m 150; and (D) 800 ms. The numbering of protons is shown on the structure of ThT.

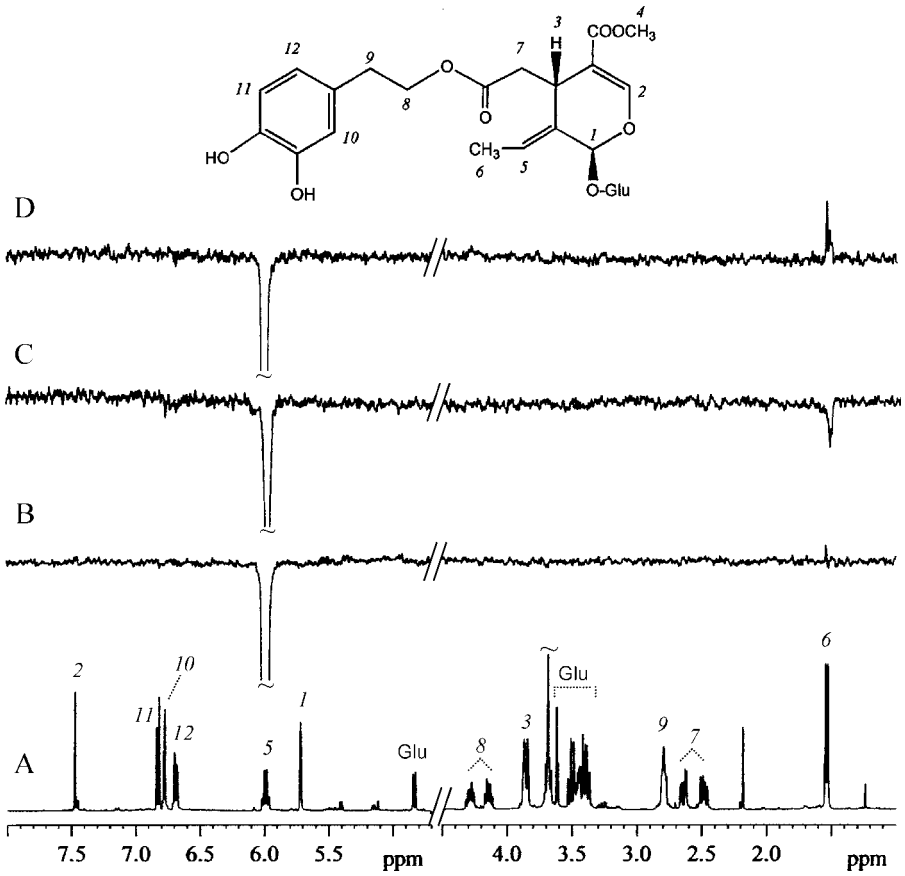

Figure 3. (A) ^{1}H NMR spectrum of oleuropein in D_2O; (B) 1D NOESY spectrum of the same sample with t_m 150 ms; (C) 1D NOESY spectrum of 1 : 5 β-AP(1–28):oleuropein solution with t_m 150; and (D) 800 ms. The numbering of protons is shown on the structure of oleuropein.

NOEs suggest that ThT interacts with β-AP(1–28). As expected from the nature of trNOEs, at the higher t_m of 800 ms the observed NOEs become positive as they are generated by the free ligand in solution (Figure 2(D)).

As in the case of melatonin, the 2D NOESY spectra gave the same results, while no interaction could be observed when the ThT stock solution was prepared in DMSO-d_6.

Oleuropein

The 1D ^{1}H NMR spectrum of oleuropein in D_2O is shown in Figure 3(A). The selective excitation of the H-5 resonance at 5.99 ppm with a t_m of 150 ms, gives rise to a very weak positive NOE signal on the neighboring H-6 protons of the methyl group at 1.54 ppm (Figure 3(B)). This positive peak changed sign when the experiment was conducted in the presence of β-AP(1–28) at a 1 : 5 β-AP(1–28):oleuropein molar ratio indicating that interaction of oleuropein with the peptide is taking place (Figure 3(C)). 1D NOESY spectra recorded with a higher t_m of 800 ms were identical to the spectra of plain oleuropein under the same conditions (Figure 3(D)).

As in the case of melatonin and ThT, the 2D NOESY spectra gave the same results, while no interaction could be observed when the oleuropein stock solution was prepared in DMSO-d_6.

Sucrose

The 1D ^{1}H NMR spectrum of a D_2O solution of sucrose is shown in Figure 4(A). The selective excitation of the H-1 proton at 5.36 ppm with t_m 150 ms resulted in a positive NOE signal for H-11 at 3.63 ppm (Figure 4(B)). The peak remained positive in the presence of β-AP(1–28) in a molar ratio β-AP(1–28):sucrose 1 : 5 (Figure 4(C)), as expected by the lack of interaction of the two molecules in solution.

Mixture of All Ligands

In order to further establish the application of the trNOE method as a screening tool for the detection of interactions with the β-AP peptide, 2D NOESY experiments were conducted in a solution of β-AP(1–28) containing all four compounds studied earlier. Aliquots from the stock solutions of melatonin, ThT, oleuropein, and sucrose were added in the solution of β-AP(1–28) in D_2O to achieve a 1 : 3 molar concentration ratio of the peptide relative to each compound. The pH of the solution was adjusted to 3.9. The 2D spectrum acquired with a mixing time of 150 ms was in complete agreement with the 1D NOESY spectra, showing positive cross peaks for sucrose and negative cross peaks for melatonin, ThT, and oleuropein (Figure 5). Interaction of β-AP(1–28) with the ligands was still present at the lower β-AP(1–28) concentration of 0.03 mM and peptide : ligand ratio 1 : 20 (Supporting Information, Figure S2).

The effect of DMSO on the already established interaction of the three ligands with β-AP(1–28) was also examined (Supporting Information, Figure S3).

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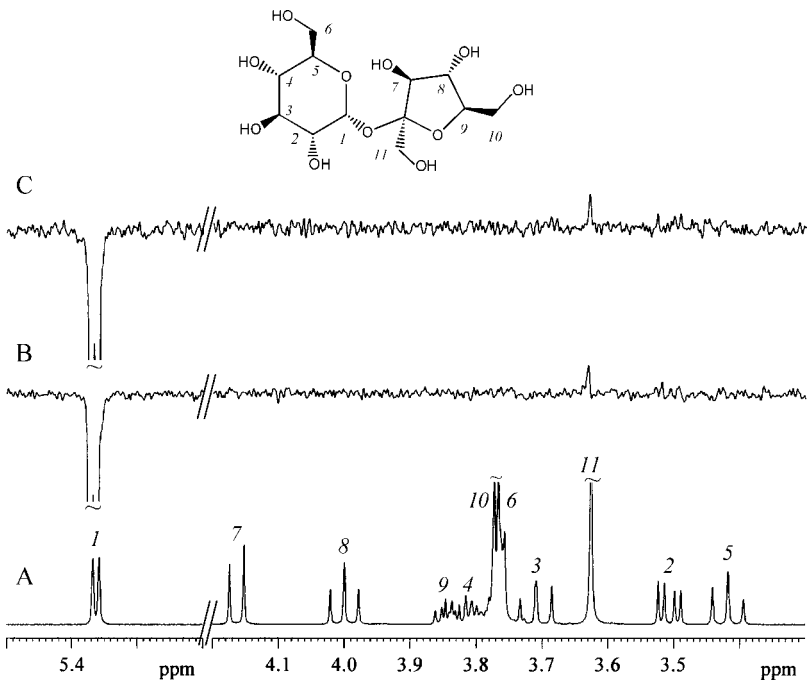

Figure 4. (A) ^{1}H NMR spectrum of sucrose in D_2O; (B) 1D NOESY spectrum of the same sample with t_m 150 ms; and (C) 1D NOESY spectrum of a 1 : 5 β-AP(1–28):sucrose solution with t_m 150 ms. The numbering of protons is shown on the structure of sucrose.

Discussion

In the present work, trNOEs, a well-established method in the study of protein-ligand interactions, are successfully applied for the first time in the detection of binding activity of small organic molecules to soluble β-AP. Interestingly, the detected trNOEs indicate the presence of interaction even without any visible changes in the NMR spectra (chemical shifts, linewidths, intermolecular NOEs; Supporting Information, Figures S4–S6).

The synthetic β-AP(1–28) employed in this study is often used as a structural model for the full-length β-AP(1–42) because it assembles *in vitro* in a manner similar to that observed *in situ* [35–38]. The pH of the β-AP(1–28):ligand solutions was adjusted to 3.9 ± 0.2 because aggregation and precipitation of β-AP(1–28) from solution becomes rapid at pHs higher than 4 [39,40] and the study aims at the detection of interaction at the initial stages of β-AP(1–28) assembly where a possible inhibitory action is expected to be more effective.

The trNOE method was initially tested on the detection of the interaction of β-AP(1–28) with the pineal hormone melatonin. A possible link between melatonin and AD has been established in the literature based on the facts that melatonin is cytoprotective against β-AP toxicity [39], it intervenes with fibril formation *in vitro* [21], and its levels decrease in ageing and even more in AD patients [40]. In view of a possible role of melatonin as a preventive or therapeutic agent for AD, its interaction with β-AP(1–40) was studied by NMR and CD experiments [21] and by ESI mass spectrometry [22,23].

Under the conditions of the trNOE experiment, interaction of melatonin with β-AP(1–28) became evident by the inversion of the sign of the NOE signal observed on melatonin protons after selective excitation of their neighbors. This change in NOE sign denotes a change in the correlation time of melatonin generated by its association with β-AP(1–28) and confirms the existing literature evidence on the formation of a noncovalent weak complex between β-AP(1–28) and melatonin.

The fact that the well-established interaction of melatonin with β-AP(1–40) could be detected with trNOEs employing the synthetic β-AP(1–28) fragment, prompted us to apply the method on two interesting compounds linked in the literature with AD, ThT and oleuropein.

The benzothiazole dye ThT is a histological amyloid stain [41] extensively used in the *post-mortem* visualization of amyloid plaques in the brains of AD patients. ThT associates rapidly with amyloid fibrils, isolated from tissues [42] or produced *in vitro* from synthetic β-AP(1–40) and β-AP(1–28) [43], and displays a notable hypochromic shift in its fluorescence spectrum. This property has found application in the development of a fluorometric assay for monitoring the course of amyloid fibril assembly, as well as for identifying agents that might inhibit this process [44]. The enhancement of fluorescence correlates with the formation of sedimentable aggregates of β-AP; however, it is reported that nonsedimentable β-AP retains the ability to fluorescence in the presence of ThT [43], possibly due to the interaction of ThT with a soluble oligomeric assembly. These data combined with a recent report that ThT inhibited the interaction of β-AP with the amyloid-binding alcohol dehydrogenase [45] led to the trNOE study where it was clearly demonstrated through the inversion of the sign of the NOEs that interaction of ThT with β-AP is taking place in solution. The possibility that the change in the sign of NOEs is due to association of ThT with fibrils in solution is ruled out since measurements were done in freshly prepared solutions of β-AP(1–28) at a pH where aggregation is slow and by employing the short duration 1D NOESY sequence.

This is the first time that interaction of ThT with soluble β-AP is demonstrated by NMR. Even though ThT does not interfere with the fibrillization process of β-AP the detected interaction may be related with the inhibitory activity of ThT on the oligomerization process of β-AP that has been recently documented in the

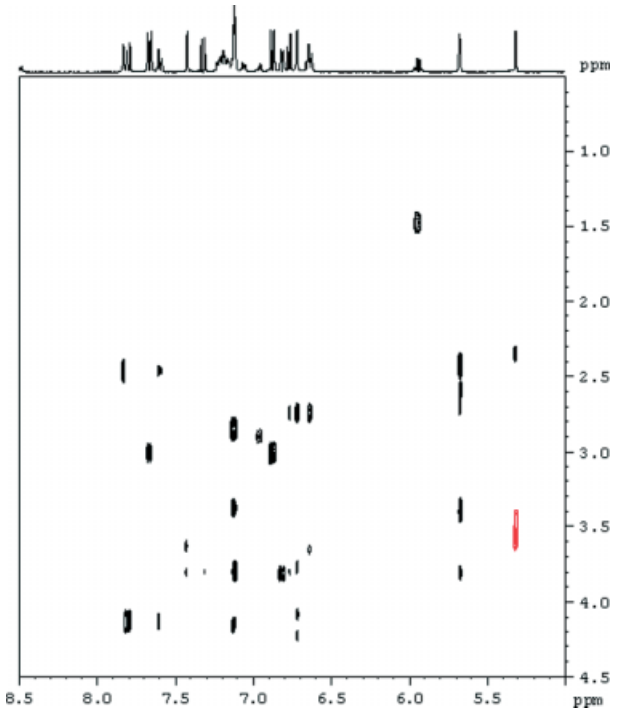

Figure 5. 2D NOESY spectrum (t_m 150 ms, region f_2 = 8.50–5.00 ppm, f_1 = 4.50–0.50 ppm) of a D_2O solution of β-AP(1–28) in the presence of melatonin, ThT, oleuropein, and sucrose. A molar ratio of 1 : 3 of β-AP(1–28) to each compound was employed resulting in a final ratio of 1 : 12 of β-AP(1–28) to all compounds present. Negative trNOE cross peaks (black) are detected for melatonin, ThT and oleuropein indicating their interaction with β-AP(1–28), while positive (red) cross peaks are observed for sucrose in accordance with lack of interaction with β-AP(1–28).

literature [10]. According to this report, β-oligomers are not obligate intermediates in the fibril formation pathway, and the β-oligomerization and fibrillization pathways are independent and distinct. If oligomers are the primary toxic species in AD [11–13], then ThT may serve as a base for the design of potential AD therapeutics.

The third compound tested for interaction with β-AP(1–28) through trNOEs is oleuropein, one of the major phenolic compounds of olive tree with strong antioxidant properties [46]. Formation of a noncovalent complex of high binding energy between oleuropein and β-AP(1–40) has been detected by ESI mass spectrometry [47] and its ability to interact with β-AP(1–28) in aqueous solution was revealed by the generated trNOEs in this study. The interaction of oleuropein with β-AP is of significant value due to the presence of oleuropein in olive oil, the main component of mediterranean diet. The neuroprotective impact of naturally extracted phenolic compounds on the incidence and progress of age-related disorders [48], and the demonstrated ability of oleuropein to associate with β-AP, makes worth the further investigation of a possible role of oleuropein as an agent against AD.

Overall, it was shown that the trNOE method can be successfully applied to detect interactions of soluble β-AP(1–28) with small organic molecules as possible modulators of the β-AP(1–28) assembly process provided that the interaction is not very strong (interactions with K_Ds in the nM range would be missed). Testing of a single candidate can be efficiently carried out with the 1D experiment, while for the screening of a mixture the 2D NOESY is the method of choice. The detection of trNOEs for melatonin, ThT, and oleuropein places these ligands in the low-affinity range and characterizes their interaction with β-AP(1–28) under the specific experimental conditions as weak with $K_D > 10^{-6}$ M.

The exact nature of the interacting species of β-AP(1–28) remains obscure. Evidence in the literature suggests that fresh solutions of β-AP of different lengths contain mixtures of monomers and small size oligomers in fast exchange [49–51]. The NMR data of the β-AP(1–28) solution in this study do not contradict, in principle, the literature results showing no chemical shift change or line broadening upon dilution and a diffusion coefficient of 1.66×10^{-10} (Supporting Information Figures S7 and S8). So interaction may be taking place with a monomer or an oligomer, both species being important for the development of anti-aggregation agents. The lack of interaction of melatonin, ThT, and oleuropein with β-AP(1–28) in the presence of DMSO, appears to favor soluble oligomeric structures as the interacting species, since according to a recent report [10], in the presence of 1% DMSO oligomers of β-AP are not detected. However, it should be emphasized that further research is required before a definite conclusion can be reached on such a complicated and much investigated subject as the nature of the self-association of β-AP and its interactions. We are proceeding in this direction with the detailed investigation of the interaction of oleuropein with the full-length β-AP(1–40) with a variety of biophysical techniques [52].

Supporting information

Supporting information may be found in the online version of this article.

Acknowledgements

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