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β-Amyloid aggregation induced by human acetylcholinesterase: inhibition studies

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Abstract

The aggregation of β-amyloid (1–40) (Aβ) induced by human recombinant acetylcholinesterase (HuAChE) was studied by means of circular dichroism (CD) and by thioflavin T fluorescence spectroscopy. Aβ was incubated alone and with HuAChE. The kinetic of fibrils formation was followed for 48 hr. The increasing β-conformation content induced by HuAChE, preliminary to the formation of Aβ fibrils, was determined by circular dichroism. This phenomenon was found to be related to the thioflavin T emission of fluorescence at 490 nm. Incubation experiments were performed in the presence of known AChE inhibitors (physostigmine, edrophonium, decamethonium, propidium) and drugs used for Alzheimer's disease (AD) (tacrine, donepezil), to test their capability of preventing the HuAChE-induced Aβ aggregation. The non-competitive or mixed mode of AChE inhibition was confirmed to be an essential feature. At 100 μM propidium, decamethonium, donepezil and physostigmine were found to inhibit the HuAChE-induced Aβ aggregation by 82, 25, 22 and 30%, respectively.

Keywords: β-Amyloid (1–40); Human recombinant acetylcholinesterase; Fibrillogenesis; Alzheimer's disease; Acetylcholinesterase inhibitors; Mechanism of action

1. Introduction

AChE has become the target for the development of new drugs for AD, the most common form of dementia in adults, since a cholinergic deficit in the central nervous system has been associated with this disease [1]. The significant deficits in presynaptic cholinergic markers observed in brains of AD patients have led to formulation of the cholinergic hypothesis of AD [2]. To date, only few inhibitors of AChE, such as tacrine, donepezil and rivastigmine are used for the clinical treatment of AD symptoms by increasing acetylcholine (ACh) content in the synapse [3]. In fact, AChE inhibitors blocking the ACh hydrolysis promote the cholinergic function, thus improving the cognitive deficit.

However, another non-cholinergic role of AChE in the AD has been discovered: some evidences suggest that AChE may play a key role in the development of the senile plaques, by accelerating Aβ deposition [4,5].

One of the most relevant neuropathological characteristics of AD brain are the senile plaques which are formed by a core of Aβ fibrils [6–8]. The main polypeptide component is a 28–43-residue peptide fragment (Aβ) of a 695-amino acid precursor (amyloid precursor protein, or APP) which is a membrane associated, receptor-like molecule. The Aβ is derived from a portion of the highly hydrophobic predicted trans-membrane spanning domain of this protein. The mechanism by which this peptide is generated in the disease state is still of ongoing interest [9].

It has been shown that AChE forms a stable complex with senile plaque components through its peripheral anionic site [4]. This site is located close to the rim of the active gorge of the enzyme and might be involved in the acceleration of fibril formation [10]. In the mid-1960s the extracellular amyloid plaques and intraneuronal neurofibrillary tangles ultrastructures were described with the first electron microscopic analysis of the brain lesions of AD [7]. Recently, it has been observed that, in AD, brain

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Abbreviations: Aβ, β-amyloid peptide (1–40); AChE, acetylcholinesterase; HuAChE, human recombinant acetylcholinesterase; CD, circular dichroism; AD, Alzheimer's disease; ACh, acetylcholine; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent); HFIP, 1,1,1,3,3,3-hexafluoro2-propanol; TFE, 2,2,2-trifluoroethanol.

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cortical AChE activity is associated predominantly with the amyloid core of mature senile plaques [6]. Therefore, AChE might have a crucial role in amyloid deposition at an early stage of the disease. Inestrosa *et al*. published a fluorometric method based on thioflavin T to demonstrate that AChE promotes formation of amyloid filaments and tested some AChE inhibitors [4].

Synthetic Aβ is widely used to study fibril formation [11,12] and its starting conformation and assembly state of Aβ appears to be crucial in order to obtain consistent data. In this respect, with the aim of validating the fluorometric study on HuAChE-induced Aβ aggregation, we have carried out a parallel circular dichroism (CD) study to determine the Aβ conformational status in solution with HuAChE. Therefore, in order to check if the HuAChE-induced Aβ fibril formation is accompanied by a peptide conformation change from a soluble form to one capable of assembling into aggregates, CD was used to follow the conformation transition associated with the fibril formation.

In view of the development of new AChE inhibitors as drugs capable not only of reducing the symptoms of AD, but also of preventing or delaying the degeneration of cholinergic neurons, the capacity of known AChE inhibitors to block the amyloidogenic effect of HuAChE has been here tested and assessed.

Compounds characterised by different potencies and mechanisms of action, such as tacrine, physostigmine, donepezil, propidium, decamethonium, edrophonium (Fig. 1) were tested for their potential anti-aggregating effect. Even if they are already well-known inhibitors, their potency was tested on the human recombinant enzyme used in this study for the aggregation experiments. Then, their anti-aggregating property was evaluated to expand their activity profile and reveal potential additive pharmacological effects which may reinforce their therapeutic application, besides their capacity of increasing ACh levels.

Another crucial aim of this work was to explore the potential relationship between potency of AChE inhibition, anti-aggregating effect and binding site of the tested compounds, in order to begin to underline the structural requirements essential for a mixed pharmacological activity.

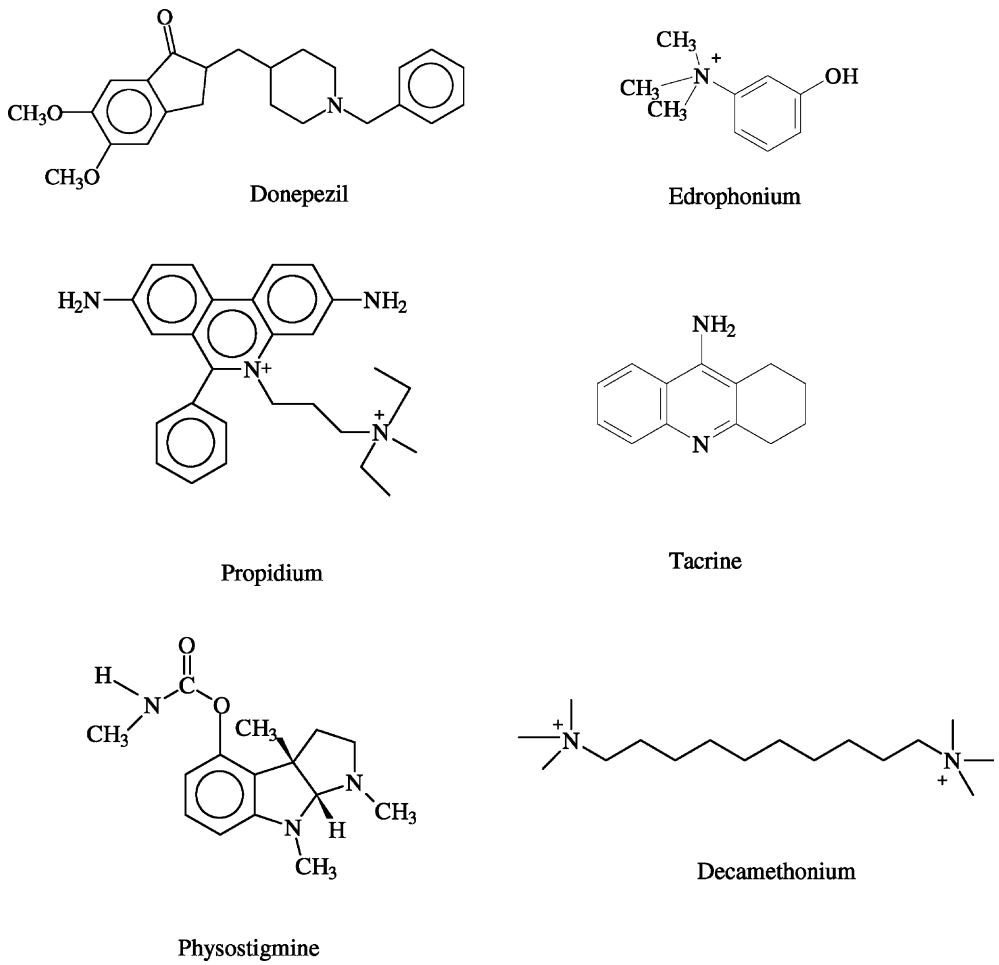

Fig. 1. Structures of some AChE inhibitors.

2. Experimental

2.1. Materials

Thioflavin T (Basic Yellow 1), HuAChE lyophilised powder, *S*-acetylthiocholine iodide, DTNB, HFIP, Triton X-100 and TFE were from Sigma. Buffers and other chemicals were of analytical grade. Tested inhibitors (propidium iodide, tacrine hydrochloride, physostigmine, decamethonium bromide, edrophonium chloride) were purchased from Sigma-Aldrich.

Absolute DMSO over molecular sieves was from Fluka. Donepezil was a kind gift from Pfizer. Water was deionised and doubly distilled. Aβ, supplied as trifluoroacetate salt, was purchased from Bachem AG.

Aβ (2 mg mL^{-1}) was dissolved in HFIP, lyophilised and used in this form after dilution for aggregation experiments.

2.2. CD studies

For CD measurements a CD Jasco J-810 Spectropolarimeter was used. Direct CD spectra for Aβ and Aβ plus AChE were recorded in the spectral range 185–260 nm using a 1 mm pathlength cell and a mountable cell (0.1 mm) at room temperature. Spectra were recorded at 0.5 nm intervals. $\Delta\varepsilon$ values are expressed on a per amide basis. Baseline was performed with sodium phosphate buffer 0.215 M (pH 8.0). In co-incubation experiments, HuAChE CD spectrum was subtracted to the mixture spectrum to obtain Aβ CD spectrum.

Stock solutions of Aβ were prepared by dissolving the lyophilised peptide, from HFIP solution, with the minimum amount of 10 mM sodium hydroxide to titrate the solution to pH 8–9. After sonicating for 2 min, aliquots of 0.215 M sodium phosphate buffer were added to create an Aβ aqueous stock solution (230 μM) in 0.170 M sodium phosphate buffer (pH 8.0). For co-incubation experiments, a buffer solution containing HuAChE was added to obtain the same final Aβ solution in the presence of HuAChE 2.3 μM (molar ratio Aβ:AChE 100:1) used in the thioflavin T fluorescence experiments.

The Aβ and Aβ plus AChE solutions were sonicated and incubated for various times (0–48 hr). Fluorometric (thioflavin T assay) and CD analyses were then performed at selected times.

CD measurements on Aβ samples were also carried out in H_2O and in a mixture consisting of 4.3 mM sodium hydroxide TFE solution:4.3 mM sodium hydroxide water solution:0.05 M sodium phosphate buffer pH 7.4 (45:11:44, v/v/v) mixture.

2.3. Thioflavin T-based fluorometric assay

For the thioflavin T-based fluorometric assay, analyses were performed with a Jasco Spectrofluorimeter FP-750 using a 3 mL quartz cell. Fluorescence was monitored at 446 nm (λ_{exc}) and 490 nm (λ_{em}) with excitation and emission slits of 2 nm bandwidth. The fluorescence emission spectrum was recorded between 450 and 600 nm, with excitation at 446 nm. To determine amyloid fibril formation, the thioflavin T fluorescence method was performed [13–15]. After incubation, the solutions containing Aβ, or Aβ plus AChE, or Aβ plus AChE in the presence of inhibitors were added to 50 mM glycine–NaOH buffer (pH 8.5) containing 1.5 μM thioflavin T in a final volume of 2.0 mL. Fluorescence was monitored with excitation at 446 nm and emission at 490 nm. A time scan of fluorescence was performed and the intensity values reached at the plateau (around 300 s) were averaged after subtracting the background fluorescence from 1.5 μM thioflavin T and AChE.

2.4. Time course of AChE-induced Aβ aggregation

Aβ, lyophilised from 2 mg mL^{-1} HFIP solution, was dissolved in DMSO to obtain a 2.3 mM Aβ solution. Aliquots (2 μL) of Aβ in DMSO were then incubated for different times (0–48 hr) at room temperature in 0.215 M sodium phosphate buffer (pH 8.0) at a final Aβ concentration of 230 μM. For co-incubation experiments, aliquots (16 μL) of HuAChE dissolved in the same buffer were added to obtain HuAChE final concentration 2.30 μM. The final volume of each assay was 20 μL. Each assay was run in duplicate. To quantify amyloid fibril formation the thioflavin T fluorescence method was used [13–15] (as above described).

In order to verify if the HuAChE-induced aggregation was a function of AChE concentration, aliquots (2 μL) of Aβ in DMSO (final Aβ concentration, 230 μM) were incubated with increasing AChE concentration ranging from 0.29 to 2.30 μM and the fluorescence values were detected after 48 hr of incubation.

2.5. Assay of AChE-induced Aβ aggregation in the presence of AChE inhibitors

Aliquots of 2 μL Aβ peptide, lyophilised from 2 mg mL^{-1} HFIP solution and dissolved in DMSO, were incubated for 48 hr at room temperature in 0.215 M sodium phosphate buffer (pH 8.0) at a final concentration of 230 μM. For co-incubation experiments aliquots (16 μL) of HuAChE (final concentration 2.30 μM, Aβ/AChE molar ratio 100:1) and HuAChE in the presence of 2 μL of the tested inhibitors in 0.215 M sodium phosphate buffer pH 8.0 solution (final inhibitor concentration ranging between 10 and 250 μM) were added.

Blanks containing Aβ, HuAChE, and Aβ plus inhibitors at various concentrations in 0.215 M sodium phosphate buffer (pH 8.0) were prepared. The final volume of each vial was 20 μL. Each assay was run in duplicate. To quantify amyloid fibril formation, the thioflavin T

fluorescence method was then applied [13–15] (as above described).

The fluorescence intensities were compared and the percent inhibition due to the presence of test compounds was calculated. The percent inhibition of the HuAChE-induced aggregation due to the presence of increasing test compound concentration was calculated by the following expression: $100 - (IF_i/IF_o \times 100)$ where IF_i and IF_o are the fluorescence intensities obtained for Aβ plus HuAChE in the presence and in the absence of inhibitor, respectively. Inhibition curves were obtained for each compound by plotting the percentage inhibition versus the logarithm of inhibitor concentration in the assay sample. The linear regression parameters were determined and the IC_{50} extrapolated, when possible (GraphPad Prism 3.0 GraphPad Software Inc.).

2.6. Inhibition of acetylcholinesterase

The method of Ellman *et al*. was followed [16]. The concentration of 0.037 M acetylthiocholine iodide solution was prepared in water. 0.01 M DTNB was dissolved in potassium phosphate buffer (pH 7.0) containing 0.15% (w/v) sodium bicarbonate. HuAChE stock solution was prepared by dissolving 1000 U in 0.1 M phosphate buffer (pH 8.0) containing Triton X-100 0.1%. The enzyme was dilute before use, in order to reach an activity ranging between 0.130 and 0.100 AU min^{-1} in the final assay conditions. Stock solutions of the tested compounds (1 mM) were prepared in water. Five different concentrations of each compound were used in order to obtain inhibition of HuAChE activity ranging between 20 and 80%.

The assay solution consisted of a 0.1 M phosphate buffer (pH 8.0), with the addition of 340 μM DTNB, HuAChE and 550 μM acetylthiocholine iodide. The final assay volume was 1 mL. Test compounds were added to the assay solution and pre-incubated with the enzyme for 20 min, before the addition of the substrate.

Initial rate assays were performed at 37° with a Jasco V-530 double beam Spectrophotometer: the rate of increase in the absorbance at 412 nm was followed for 5 min. Assays were run with a blank containing all components except HuAChE in order to account for non-enzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analysed in triplicate. The percent inhibition of the enzyme activity due to the presence of increasing test compound concentration was calculated by the following expression: $100 - (v_i/v_o \times 100)$ where v_i is the initial rate calculated in the presence of inhibitor and v_o is the enzyme activity. Inhibition curves were obtained for each compound by plotting the percentage inhibition versus the logarithm of inhibitor concentration in the assay solution. The linear regression parameters were determined for each curve and the IC_{50} extrapolated. The computer program used to analyse these data was GraphPad Prism 3.0 (GraphPad Software Inc.).

3. Results

3.1. Initial conformation of Aβ

The study of the Aβ aggregation phenomenon induced by HuAChE was carried out by a CD analysis performed in parallel to the fluorescence assay with thioflavin T. The experimental conditions were optimised so that the monitored fluorescence signal was selectively related to the Aβ aggregation induced by HuAChE and not to spontaneous Aβ aggregation. Then, some AChE inhibitors were tested for their capability of blocking the HuAChE-induced Aβ aggregation.

As it has been shown [17,18], Aβ can adopt two different conformational states in solution, depending on the secondary structure adopted by the N-terminal domain. One species is highly amyloidogenic (Aβ-ha) and has a predominantly anti-parallel β-sheet structure, whilst the other (Aβ-na) is poorly amyloidogenic and contains mainly random coil or an α-helix structures. The experimental conditions are essential in determining the conformation and the solubility of Aβ, which in turn affects the rate and the extent of amyloid fibril formation [17]. In the absence of HuAChE, in aqueous solution, the Aβ-ha form can produce a higher thioflavin T fluorescence signal than that obtained with Aβ-na. Following incubation with HuAChE, enhancement of amyloid formation was higher with Aβ-na, whereas no significant effect was observed with (Aβ-ha). Therefore, one critical issue in monitoring HuAChE-induced aggregation was to start with an Aβ solution mainly random coil or α-helix structure.

Even if the oligopeptide or protein amino acid sequence usually gives the propensity to a prevailing secondary structure [19], however the conformation is significantly affected by the environment, i.e. the nature of the solvent [20]. As an example, in TFE solution we found that Aβ showed high α-helix content, and very low β-sheet and random structures (Fig. 2a). On the contrary, in water Aβ showed a quite high propensity to assume β-structure (Fig. 2b). As the storing conditions of the solid state peptide were found to significantly affect its conformation, a method was then validated for the preparation of the starting material with a high non-amyloidogenic conformation content. In fact, in aggregation studies many unreliable results may occur due to different starting conformations and assembly states of Aβ. A homogeneous conformation of the starting material was obtained by lyophilising the Aβ samples from a non-amyloidogenic solvent and resolubilising the peptide in the appropriate solvent before the aggregation experiments. HFIP resulted the ideal solvent for this purpose allowing the (Aβ-na) conformation to be the prevalent one, which was also

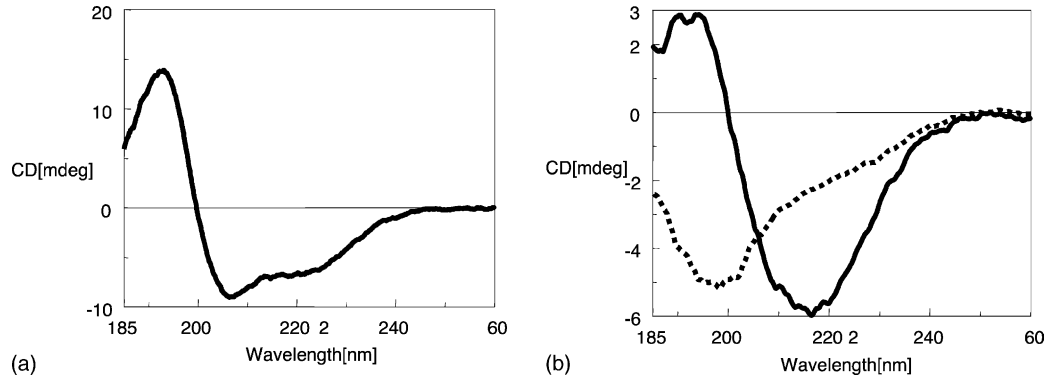

Fig. 2. (a) CD spectra of Aβ (trifluoroacetate salt) (105 μM) in TFE–NaOH buffer, 0.1 mm cell; (b) CD spectra of Aβ (46 μM) in H_2O, 1 mm cell, fresh solution $t = 0$ (—) and after 24 hr (▬).

maintained after lyophilisation, as checked by CD measurements on freshly prepared Aβ solutions (Fig. 3).

3.2. CD studies

CD studies were essential in determining a relation between fluorescence intensity and Aβ conformation with and without HuAChE.

The time-dependent HuAChE-induced Aβ conformational change (random and α- to β-sheet) was followed by CD spectra registration. At varying times, CD spectra were obtained on Aβ alone and Aβ incubated with HuAChE, after subtraction of the HuAChE CD spectrum (Fig. 3a). In principle, the observed β form increase could arise from structural changes of both HuAChE and Aβ. However, as the structural change upon binding of a peripheral binding site ligand like propidium resulted almost negligible for Torpedo AChE [21], we assumed that the Aβ-bound HuAChE CD spectrum could be the same as that of the free HuAChE, therefore not affecting the CD analysis of the Aβ–enzyme complex. Thus, the CD contribution of HuAChE was considered constant in the absence and in the presence of Aβ.

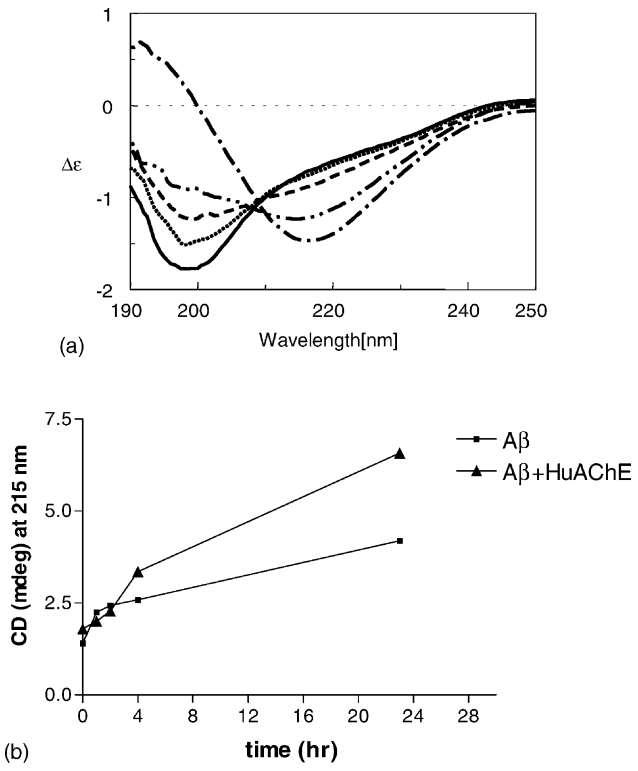

Fig. 3. (a) Time-dependent difference CD spectra of Aβ (230 μM in 0.170 M sodium phosphate buffer) samples incubated with HuAChE (2.3 μM). Fresh solution $t = 0$ (—), and after 0.5 (· · ·), 2 (- - -), 4 (- · · -), and 23 hr (– · –). (b) Time-dependent CD signal intensity at 215 nm (negative band) for Aβ and Aβ incubated with HuAChE. Experimental condition as in (a).

DMSO was not used for CD experiments being not transparent at low UV wavelenght range; thus Aβ, after lyophilisation from HFIP solution, was directly dissolved in water with sodium hydroxide [22] and then the pH 8.0 was adjusted by adding a suitable volume of 0.215 M pH 8.0 sodium phosphate buffer to obtain a 0.17 M buffer solution.

The increasing β-conformation content (positive CD band at about 195 nm and negative CD band at 215 nm) determines a shift in the random coil Aβ spectrum showing a negative CD band at 215 nm, whose intensity was found to increase with time of incubation (Fig. 3b). The data are reported up to 23 hr of incubation, and represent the HuAChE-induced random versus β-conformation change, preliminary to the Aβ fibrils formation. Data were collected also after 48 hr of incubation (not shown): the CD spectrum suggested a higher β-structure content, and its lower intensity profile indicated peptide precipitation. The solubility loss of the peptide was also supported by a concomitant UV absorption decrease.

By analysing the same samples with the fluorometric method, this conformational change phenomenon was found to be related to the thioflavin T emission of fluorescence at 490 nm. With the increase of the negative CD 215 nm band, the fluorescence intensity with thioflavin increased as well, up to a stable plateau value (Fig. 4). However, in the conditions for CD spectra registration, with a more diluted incubation buffer (0.17 M) and Aβ directly solubilised in water, the kinetic of HuAChE-induced aggregation was found slower and also a more pronounced Aβ self-aggregation was observed than in the

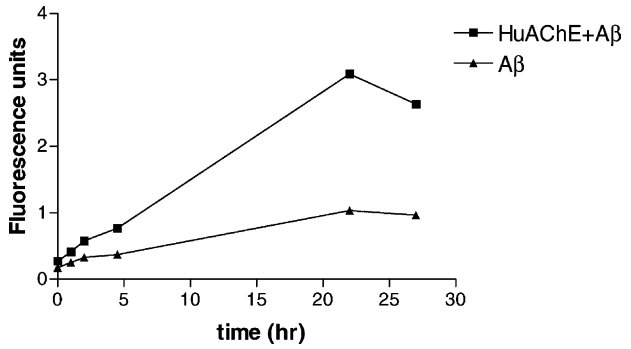

Fig. 4. Time-dependent thioflavin T fluorescence intensity increase on the same samples as in Fig. 3a and b.

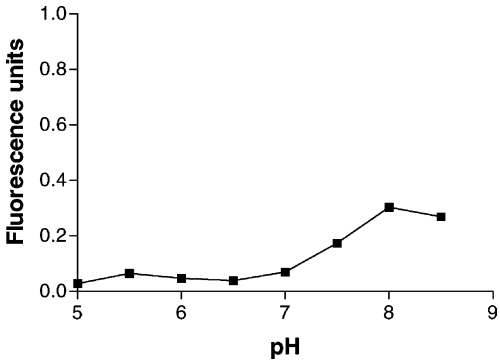

Fig. 6. pH dependence of Aβ (230 μM) self-aggregation in sodium phosphate buffer 0.215 M. Thioflavin T assay (λ_{exc} = 446 nm; λ_{em} = 490 nm), after incubation for 48 hr.

conditions used by the thioflavin T-based fluorometric assay (Aβ in DMSO) (Fig. 5). Anyhow, these results allow to support a connection between the fluorescence data (Fig. 4) and the Aβ conformation assessed by CD analysis (Fig. 3).

3.3. Fluorometric studies

A number of studies with synthetic Aβ have shown that this peptide aggregates *in vitro* giving amyloid cross-β-fibrils similar to the filaments found in the brains of AD patients [11,12,23].

The *in vitro* Aβ aggregation can be evaluated by a method based on the fluorescence emission by using thioflavin T. Thioflavin T binds specifically to amyloid fibrils giving rise to an intense specific emission band (λ_{max} = 490 nm) in its fluorescent spectrum [13–15]. Therefore, we applied this assay to find the optimal conditions, in terms of pH, buffer type and concentration, to monitor the HuAChE-induced Aβ aggregation, whilst minimising the Aβ spontaneous aggregation. Then, the kinetic of Aβ fibril formation was followed to assess the optimal time for routine aggregation studies and for testing inhibitors.

The Aβ sample preparation was different from the CD experiments. Aβ, after lyophilisation from HFIP solution, was dissolved in DMSO. Aliquots (2 μL) of Aβ in DMSO were incubated alone and with HuAChE (molar ratio 100:1) in 0.215 M sodium phosphate buffer (pH 8.0). These conditions were found more favourable than those previously reported [4] in order to minimise the peptide self-aggregation. The kinetic of fibrils formation was followed for 48 hr, studying the effect of HuAChE on the aggregation of Aβ peptide. It was found that the induced process is time dependent: after 8 hr, the aggregation of Aβ incubated with HuAChE in 0.215 M sodium phosphate buffer reached a plateau, and was 10 times higher than the aggregation of Aβ alone in the same conditions (Fig. 5). Under the proposed experimental conditions, Aβ alone showed a very poor aggregation which was found to be pH-dependent, with a maximum extent at pH 8.0 (Fig. 6). Anyhow, as it is shown in Fig. 5, at this pH the Aβ self-aggregation fluorescence response is still much lower (10 times) than that obtained in the presence of HuAChE. The pH value was fixed at 8.0 because at this pH AChE can perform its best activity and is in its best conformation for ligand binding [16].

At 48 hr, in the experiment of HuAChE-induced Aβ aggregation the fluorescence intensity was found to be related to the HuAChE concentration used at constant Aβ (Fig. 7). The fluorescence signal was also found to be

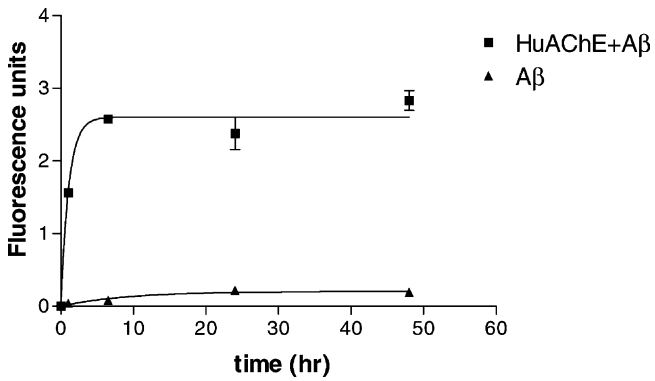

Fig. 5. Time-dependent fluorescence increase of Aβ samples (lyophilised and dissolved in DMSO) incubated in 0.215 M sodium phosphate buffer pH 8.0 with and without HuAChE (molar ratio Aβ:HuAChE 100:1). Thioflavin T assay (λ_{exc} = 446 nm; λ_{em} = 490 nm).

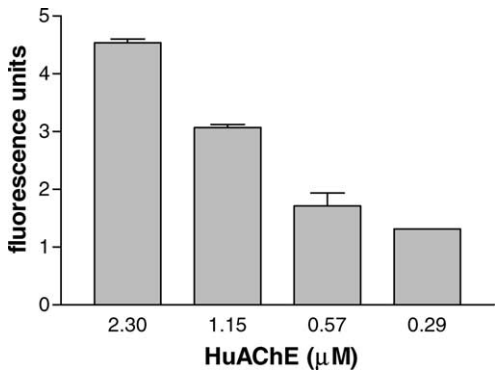

Fig. 7. Fluorescence emission of Aβ (230 μM) samples incubated for 48 hr with increasing HuAChE concentrations. Thioflavin T assay (λ_{exc} = 446 nm; λ_{em} = 490 nm).

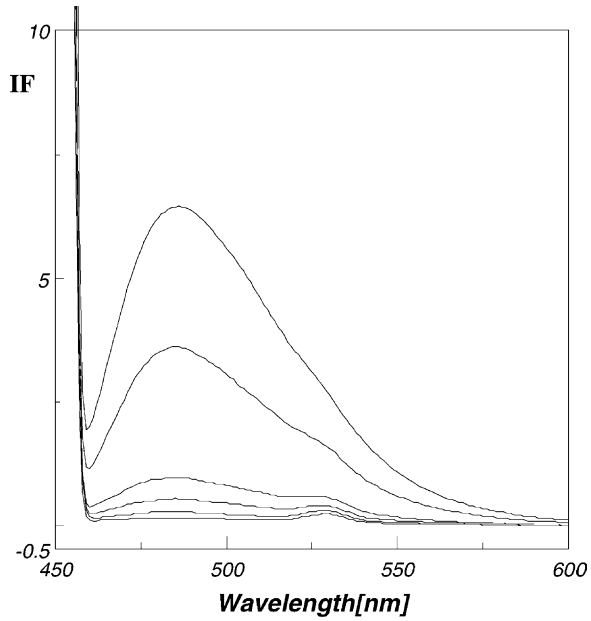

Fig. 8. Thioflavin T emission fluorescence spectra (range 450–600 nm) ($\lambda_{exc} = 446$ nm) (48 hr incubation) of HuAChE (2.30 μM) induced Aβ aggregation samples (Aβ concentration range 10–230 μM).

proportional to the amount of amyloid fibril formed (Fig. 8), as previously described [15].

3.4. AChE-induced Aβ aggregation: inhibition studies

A series of inhibitors with different potencies and mechanisms of action was chosen to explore the relation among HuAChE potency and site of action and fibrillogenesis inhibition.

Aβ (230 μM) was incubated at room temperature for 48 hr with and without HuAChE (2.3 μM) in the presence of various inhibitors, some of which (tacrine and donepezil) currently used as drugs in treatment of AD as they increase the cholinergic transmission [24]. Aggregation was monitored by the thioflavin T method as above described.

In Table 1, the fibrillogenesis inhibition of the tested compounds at 100 μM are reported, as well as their power of inhibiting HuAChE catalytic activity (potency expressed as IC_{50}) and their mechanisms of action [25–28]. AChE possesses an active site at which ACh is hydrolysed, as well as a peripheral anionic site, which is spatially distinct from the active site and where selective inhibitors can bind with high affinity and specificity. Hydrolysis of ACh proceeds through the formation of an acyl-enzyme intermediate, and inhibitors can affect steady state kinetics by association with the transient acyl intermediate, in addition to the free enzyme and enzyme–substrate complex [29,30]. As the association of AChE with Aβ and the formation of amyloid-like fibrils is supposed to occur through the interaction with the peripheral anionic site [4,6,10], inhibitors capable of binding to the acyl-enzyme and to the peripheral site were thought to be promising in blocking this interaction.

Accordingly, propidium, a purely non-competitive AChE inhibitor binding to the peripheral site [25,31], showing a relatively low HuAChE IC_{50}, was found to decrease significantly HuAChE-induced Aβ aggregation, as previously reported by Inestrosa *et al.* [4]. Its IC_{50} was found to be 12.6 ± 0.5 μM, and its inhibition at 100 μM was 82%. The effect of potassium iodide was also tested to exclude a potential fluorescence quenching effect by iodide in the thioflavin T assay (Fig. 9). As observed, potassium iodide does not inhibit the HuAChE-induced Aβ aggregation, whereas the inhibitory effect by propidium iodide is related to the compound concentration.

Similarly, the non-selective bis quaternary inhibitor decamethonium inhibited nearly 25% of HuAChE's effects on amyloid formation at 100 μM, and its IC_{50} resulted to be around 250 μM. In agreement, X-ray crystallography and molecular modelling showed that also decamethonium can establish favourable interactions simultaneously with the peripheral and active sites [25].

On the contrary, active site competitive inhibitors like edrophonium [31,32] did not show any effect on HuAChE-induced Aβ fibrillogenesis.

Donepezil is a potent mixed type competitive AChE inhibitor, highly selective for AChE and recently approved as drug for the treatment of AD [26,33–35]. Kinetic studies suggested a potential different site of interaction which could be the peripheral binding site [26]. Its aggregation inhibitory potency was found to be 22% at 100 μM concentration.

Tacrine, the first compound introduced on the market as an AD drug [33,36] and characterised by AChE inhibitory potency one order of magnitude inferior to donepezil, showed a very low anti-aggregating effect at 100 μM.

Physostigmine, a pseudo-irreversible inhibitor [37] which inhibits the enzyme by carbamoylation of the serine

Table 1
Inhibition of AChE-induced Aβ aggregation produced by the tested compounds at 100 μM concentration and IC_{50} against HuAChE

Compounds	Fibrillogenesis inhibition (%) (SD within 3%)	Inhibition type [25–28]	HuAChE IC_{50} (M)
Propidium	82	Non-competitive	$3.23 \pm 0.22 \times 10^{-5}$
Decamethonium	25	Mixed	$2.05 \pm 0.21 \times 10^{-5}$
Donepezil	22	Mixed	$2.31 \pm 0.48 \times 10^{-8}$
Tacrine	7	Mixed	$4.24 \pm 0.21 \times 10^{-7}$
Edrophonium	0	Competitive	$7.30 \pm 0.35 \times 10^{-6}$
Physostigmine	30	Pseudo-irreversible	$1.34 \pm 0.54 \times 10^{-8}$

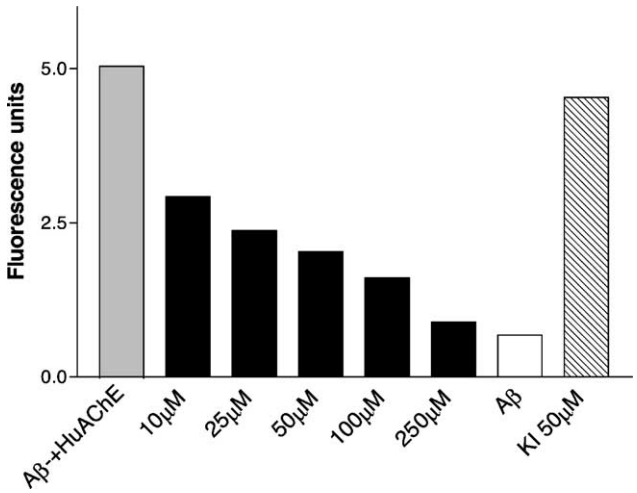

Fig. 9. Determination of propidium inhibition potency on HuAChE-induced Aβ aggregation. Fluorescence thioflavin T signal of Aβ (230 μM) in sodium phosphate buffer 0.215 M (pH 8.0) coincubated with HuAChE (2.3 μM) and with propidium iodide at increasing concentrations and with potassium iodide for 48 hr. The values are the mean of two independent measurements (SD within ±3%).

hydroxyl in the active site, produced 30% reduction of the fluorescence signal associated to the amyloid aggregation at 100 μM. Its derivatives are promising candidates as drugs for AD [26,35]. Despite physostigmine covalent interaction occurs in the enzyme active site, the inhibition reaction of similar carbamates may form a transient enzyme-inhibitor intermediate at the AChE peripheral anionic and may follow the irreversible inactivation by a conformational change of the enzyme [36].

4. Discussion

Our results confirmed that HuAChE may constitute an important co-factor in Aβ fibrillogenesis, being able to induce a conformational change of Aβ in solution and thus having a direct role in fibril formation. The aggregation conditions proposed here in terms of pH, buffer concentration and starting conformation of the Aβ peptide were found suitable to monitor the specific interaction between HuAChE and Aβ, by reducing the Aβ self-aggregation component and confirmed the direct role of HuAChE in fibril formation (Fig. 5).

Concerning the physiological relevance of these *in vitro* studies, HuAChE concentration is around 40 U per 30 mg of assay solution, while AChE has been found to be present in the different areas of the central nervous system at concentrations ranging from 4 to 45 U per gram of tissue [38]. Although the HuAChE concentration is nearly 100 times higher than *in vivo*, however it was found to be necessary to maximise, speeding up and to measure a phenomenon which might require a longer time to be fully developed.

The Aβ sample preparation was found critical for monitoring this phenomenon. The starting Aβ conformation had to be random or α-helix to make possible the detection of the AChE conformational change. At this regard, the Aβ dissolution in HFIP was found essential to obtain a suitable homogeneous starting sample to selectively monitor the AChE interaction and its induced shift from α or random to β-sheets conformation, previous to fibril formation. This critical point was established through CD studies by registering time-dependent difference spectra of Aβ-HuAChE incubation samples (Fig. 3). The CD studies were found essential to define the Aβ sample preparation protocol for the incubation experiments and to validate the fluorometric analysis with thioflavin T. The thioflavin T fluorescence units on the same aggregated Aβ samples were correlated to β-CD band intensity increase (Figs. 3b and 4).

Our goal was also to understand if there is any link between the mechanism of action of AChE inhibitors, assessed by enzyme kinetic analysis and the determination of the stability constants of the AChE–inhibitor complex, their potency (HuAChE IC_{50}) and their Aβ aggregation inhibition capacity (Table 1).

The HuAChE-induced aggregation was found to be inhibited by peripheral anionic site ligands such as propidium (Fig. 9), and only partially inhibited by molecules binding both to the acyl-enzyme and to the peripheral site such as physostigmine and donepezil (Table 1).

Non-competitive or mixed-mode inhibitors could be the best candidates for inhibiting AChE-induced Aβ aggregation, according to their ability to bind to the peripheral site.

In this respect, the fact that tacrine, a mixed type inhibitor, did not show significant inhibitory activity against the HuAChE-induced Aβ aggregation might be attributed to tacrine's highest affinity with the active site than with the peripheral one. The lack of tacrine significant inhibition on HuAChE-induced aggregation might further justify its symptomatic action in clinical trials [39].

Furthermore, no correlation was found between the HuAChE inhibitory potencies of the tested compounds and their ability to preventing HuAChE-induced fibril formation.

Deeper studies need to be accomplished to have a more defined picture of the specific structural requirements and physico-chemical properties (like lipophilicity and molecular dimension) leading to a stronger interaction with the peripheral anionic site where Aβ interacts with high affinity [10]. In fact, there is only one additional example of inhibition of AChE-induced aggregation, from work on a monoclonal antibody directed against AChE that binds at the peripheral site of the enzyme [40].

5. Conclusions

Aβ fibrillogenesis studies were performed by means of thioflavin T fluorescence spectroscopy to investigate the potential to inhibit HuAChE-induced Aβ aggregation by

various AChE inhibitors. A parallel CD study was found useful to confirm the Aβ reversible α-helix or random coil to β-structure transition, followed by irreversible fibril formation in the presence of HuAChE. CD was also applied to identify the starting Aβ conformation capable of giving reproducible results in terms of induced conformational change.

The development of a new method to test the capability of AChE inhibitors to inhibit Aβ aggregation is of utmost importance to define the full therapeutic potential of already known compounds. As a future perspective, new AChE inhibitors, which we found to possess interesting potencies and selectivity profiles [25,27,28,41,42] will be submitted to this analysis and tested for their Aβ fibrillogenesis inhibition power.

The results obtained in this study suggest that together with the capacity of ameliorating cholinergic level in the central nervous system blocking the breakdown of ACh, AChE inhibitors, which strongly interact with the AChE peripheral binding site may additionally inhibit the amyloid fibrillogenesis induced by HuAChE. Therefore, AChE inhibitors that are also Aβ fibrillogenesis inhibitors might become drug candidates capable not only of reducing the symptoms of AD, but also of preventing the disease or slowing down the disease progression.

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