3-(4-{[Benzyl(methyl)amino]methyl}-phenyl)-6,7-dimethoxy-2*H*-2-chromenone (AP2238) Inhibits Both Acetylcholinesterase and Acetylcholinesterase-Induced *β*-Amyloid Aggregation: A Dual Function Lead for Alzheimer's Disease Therapy[§]

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Abstract: In recent years, the investigation of acetylcholinesterase (AChE) inhibitors has gained further interest, because the involvement of the peripheral site of the enzyme in the *β*-amyloid (A*β*) aggregation process has been disclosed. We present here, for the first time, a direct evidence of the A*β* antiaggregating action of an AChE inhibitor (AP2238) purposely designed to bind at both the catalytic and the peripheral sites of the human enzyme.

Introduction. Alzheimer's disease (AD) is a progressive, chronic, neurodegenerative disorder, characterized by loss of cognitive ability, severe behavioral abnormalities, and ultimately death. Treatments of AD are most likely to succeed if they are based on an understanding of the complex biology of the disease and its effects on cognition. At this regard, A*β* is considered to be critical for inducing the pathology, as its accumulation may result in a cascade of biochemical events leading to neuronal dysfunction.[1] Therapeutic approaches may be directed to decreasing A*β* production or aggregation, or increasing A*β* removal. Alternatively, more distal pathways may be targeted, by either modulating downstream events possibly due to the presence of A*β* fibrils, such as free radical toxicity, inflammation, cell membrane damage, calcium dishomeostasis, and excitotoxicity, or blocking the cellular response to injury through inhibition of neuronal apoptosis.[2] However, AChE inhibition is currently the most widely studied and developed approach for treating the symptoms of AD, and tacrine, donepezil, galantamine, and rivastigmine are the AChE inhibitors in clinical use.[3]

Recently, it has been pointed out that AChE is also responsible for several noncatalytic actions.[4] Among these, the proaggregating activity toward A*β* is intriguing, as some evidences suggest that the enzyme may play a key role in the development of the AD plaques by accelerating A*β* deposition.[5] Likely, AChE interacts with A*β* and promotes amyloid fibril formation through a pool of amino acids located in proximity of the peripheral anionic binding site (PAS) of the enzyme.[6] Moreover, it has been shown that molecules able to interact either exclusively with PAS or with both catalytic and peripheral AChE binding sites (i.e., dual inhibitors) can prevent the proaggregating activity of AChE toward A*β*.[5,7] Therefore, peripheral and dual binding AChE inhibitors might represent a new therapeutic option, as these compounds should be able, at least in principle, both to contrast the cognitive deficiency and to avoid A*β* aggregation.

AChE inhibitors targeted at the peripheral or both sites have already been reported,[8] but their effects on the A*β* proaggregating activity has never been experimentally determined. In this paper, we report design, synthesis, and in vitro biological properties of a compound that represents a novel class of AChE inhibitors endowed with the ability to partially block the action of AChE on A*β*. To this aim, we first designed the new inhibitors following a computational approach based on docking simulations carried out on the structure of human AChE (HuAChE). Then, we selected and synthesized 3-(4-{[benzyl(methyl)amino]methyl}phenyl)-6,7-dimethoxy-2*H*-2-chromenone (AP2238, **4**, Scheme 1) through a fast and convenient method. Finally, we tested on the isolated enzyme the ability of **4** to inhibit both the catalytic and the A*β* proaggregating actions of AChE. The results of the in vitro assays compared to the corresponding data of the leading AD drug, donepezil, reveal that **4** can be considered as the protoype of a new class of compounds endowed with an AChE inhibitory profile favorable for a therapeutic application in the treatment of AD.

Inhibitor Design. To obtain an inhibitor able to bind at both the catalytic and the peripheral sites of AChE, we designed a molecule composed by two moieties optimal for the binding at each site and linked by an appropriate spacer. The two selected moieties were a benzylamino group and a coumarin (2*H*-2-chromenone) heterocycle. The first one is present in many potent AChE inhibitors,[9] and its binding capability at the catalytic center of AChE is demonstrated by the X-ray crystallographic studies of the AChE/donepezil and AChE/galantamine complexes.[10,11] The coumarin ring is an heterocyclic moiety we and others demonstrated to be compatible with a high anti-AChE potency,[12,13] and its choice was also favored by the optimal synthetic accessibility. As regards the spacer, we reasoned that a linking chain bearing a phenyl ring might have the chance to favorably interact with some of the numerous aromatic residues lining the wall of the AChE gorge.

Different combinations of these structural fragments were modeled, and the docking of the resulting molecules into the HuAChE gorge was studied by means of the DOCK 4.1 software.[14] Atomic partial charges of the compounds, whose geometries were optimized at PM3 semiempirical level,[15] were calculated by carrying out single point ab initio calculations at HF/6-31G(d) level using Gaussian98 package (Gaussian98, Gaussian, Inc., Pittsburgh PA, 1994) and then by applying the restrained electrostatic potential (RESP) procedure.[16] The all-atom Amber force field[17] was used, and the energy estimation was carried out without using the grid approach, to properly represent the *π*-cation interaction. Actually, this enabled us to account also for the

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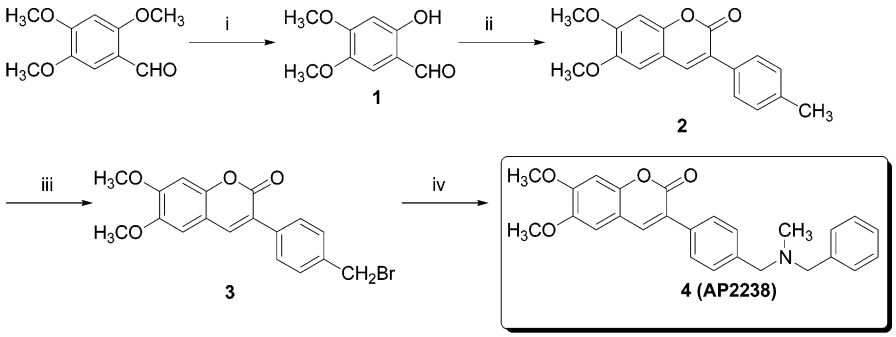
Scheme 1. Synthesis of Compound **4** (AP2238)[a]



[a] Reagents: (i) BCl_3, CH_2Cl_2, −78 °C; (ii) *p*-tolylacetic acid sodium salt, Ac_2O, 180 °C; (iii) *N*-bromosuccinimide, $(PhCOO)_2$, CCl_4, reflux; (iv) *N*-benzyl-*N*-methylamine, toluene, reflux. Analytical data: mp 230 °C (MeOH), ^{1}H NMR ($CDCl_3$) δ: 2.41 (s, 3H), 3.68 (s, 2H), 3.69 (s, 2H), 3.93 (s, 3H), 3.95 (s, 3H), 6.84 (d, 2H), 7.19−7.48 (m, 7H), 7.63 (d, 2H), 7.77 (s, 1H). ESMS *m/z* 416 (M + 1).

multipole/ion contributions, which are fundamental when modeling π-cation interactions by means of classical force field-based calculations.[18]

The best results of the docking simulations (Figure 1a) were obtained with compound **4**. In the lowest energy conformation of the ligand/enzyme complex, the following interactions could be detected between **4** and some HuAChE residues: (i) the benzyl group of **4** interacts by means of a $\pi-\pi$ stacking with the indole ring of Trp86; (ii) the spacer phenyl ring can establish both a $\pi-\pi$ interaction with the phenol ring of Tyr341 and an OH-π interaction[19] with the hydroxyl group of Tyr124; (iii) the protonated ammonium group of **4** is involved in a π-cation interaction with the phenol ring of Tyr337; (iv) the carbonyl group of the coumarin moiety and eventually the endocyclic oxygen can establish H-bond interactions with the backbone amide group of Phe295; (v) the aromatic moiety of the coumarin ring interacts with Trp286, by means of a $\pi-\pi$ stacking. Indeed, **4** seemed to achieve the desired property. It contacted the catalytic site of AChE and apparently it reached the PAS of the enzyme (Trp286 and surrounding residues) better than donepezil (Figure 1b).

Chemistry. The synthesis of the new inhibitor was obtained in a rather straightforward way, owing to the good accessibility of the coumarin nucleus. The target compound was synthesized by standard procedures (Scheme 1) and was characterized by ^{1}H NMR, mass spectra, and elemental analysis. Partial demethylation of 2,4,5-trimethoxybenzaldehyde with BCl_3 gave 2-hydroxy-4,5-dimethoxybenzaldehyde (**1**),[20] whose condensation with the sodium salt of *p*-tolylacetic acid in the presence of acetic anhydride afforded 6,7-dimethoxy-3-(4-methylphenyl)-2*H*-2-chromenone (**2**). This tolyl intermediate was treated with *N*-bromosuccinimide and a catalytic amount of benzoyl peroxide to give 3-[4-(bromomethyl)phenyl]-6,7-dimethoxy-2*H*-2-chromenone (**3**). The bromo derivative **3** was condensed with *N*-benzyl-*N*-methylamine to afford the final compound.

Biology. The biological profile of both **4** and donepezil against HuAChE (catalytic and Aβ proaggregating actions) and butyrylcholinesterase BuChE was determined.

The inhibitory potency of **4** against recombinant human AChE and isolated serum BuChE was evaluated by studying the hydrolysis of acetylthiocholine (ATCh) following the method of Ellman[21] as reported in detail elsewhere.[22] The graphical analysis of steady-state HuAChE inhibition data for **4** in comparison to donepezil is shown in Figure 2, whereas estimates of K_i and IC_{50} values are reported in Table 1 along with the corresponding values for donepezil. From the kinetic analysis, we concluded that both compounds caused a mixed type of inhibition, implying binding at both the catalytic and the peripheral sites of AChE. This was already known for donepezil,[23] while for **4** it was an indirect confirmation of the inhibitor/enzyme interaction hypothesis involving both AChE sites described above.

The IC_{50} values for inhibition of both cholinesterases were determined for **4**, allowing an estimation of the eventual selectivity of the compound. From the data shown in Table 1, a factor of 3 log units appears to favor AChE with respect to BuChE inhibition.

The ability of **4** to inhibit the proaggregating action of AChE toward Aβ was assessed through a thioflavin T-based fluorometric assay early reported by Inestrosa[5] and recently validated through circular dichroism.[7] The results are reported in Table 1. Although **4** shows a lower degree of antiaggregating potency with respect to the exclusive PAS ligand propidium (82% inhibition[7]), it is still able to significantly prevent the AChE-induced Aβ aggregation. This result is striking, and again it confirms the design hypothesis pointing to AChE dual-site inhibitors. Remarkably, we previously reported a similar (but lower) effect for donepezil,[7] that was shown by X-ray crystallographic analysis to contact both the catalytic and the peripheral AChE binding sites.[10]

Discussion. Despite their far from optimal pharmacological profile, AChE inhibitors are at the moment the only drugs available for the clinical treatment of AD. Therefore, besides the exploration of alternative approaches targeting early events in the neurotoxic cascade, it still seems worthwhile to try to improve this class of drugs, for which a vast amount of pharmacological evidence and expertise exists. A suitable strategy to achieve this goal might be that of simultaneously targeting another system involved in the pathogenesis of the disease in addition to the cholinergic one. At this regard, two ways are open: either to associate to an AChE inhibitor a drug able to control the clinical symptoms of AD through a different mechanism, or to combine into a single compound the ability of acting at both the AChE and another target.[24] Recently appeared examples of the latter approach[13,25,26] consisted of multipotent compounds able, at least in principle, to provide

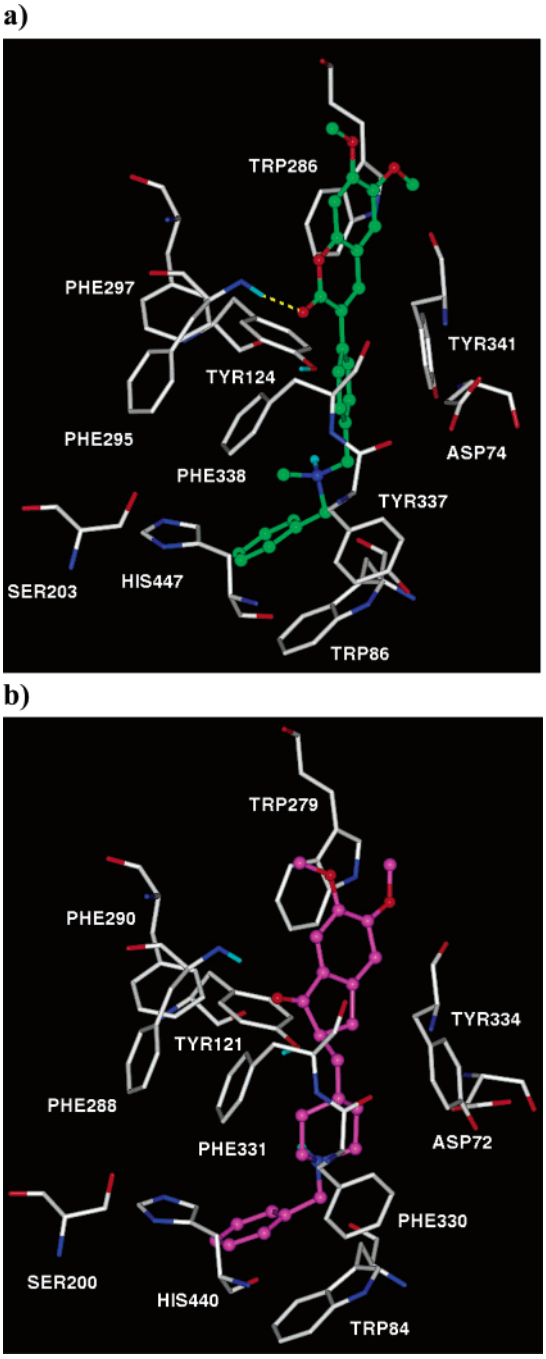


Figure 1. (a) Docking model of **4** (carbon atoms are green) within the HuAChE gorge. The H-bond between the carbonyl of the coumarin moiety of **4** and the hydrogen (cyan) of the Phe295 backbone NH is shown as a yellow dotted line. The hydrogen (cyan) of the hydroxyl group of Tyr124, which can establish OH−π interaction with a phenyl ring of **4**, is also shown. (b) The X-ray structure of donepezil (carbon atoms are magenta) within the *Torpedo californica* AChE gorge (PDB code: 1eve) is reported for comparison: **4** seems to contact the peripheral Trp better than donepezil.

a synergistic effect toward different molecular and biochemical targets of AD.

As one of the central events in the AD pathogenesis is the Aβ fibrillization, an intervention against this phenomenon seems crucial. Interestingly, research efforts pioneered by Inestrosa[5] and recently reappraised by us[7] have pointed out a direct link between AChE and Aβ aggregation: AChE was shown to exert an Aβ proaggregating action mediated by its peripheral binding site.[6] Following this line, articles started appearing in the literature reporting the design and synthesis of putative dual-binding AChE inhibitors.[8]

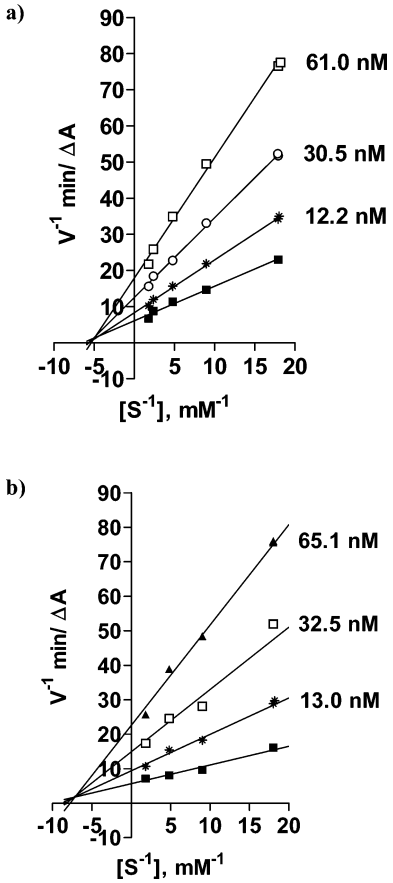


Figure 2. Steady-state inhibition by (a) **4** and (b) donepezil of AChE hydrolysis of ATCh. Reciprocal plots of initial velocity and substrate concentrations (20−550 μM) are reported. Lines were derived from a weighted least-squares analysis of data points.

Table 1. In Vitro Biological Data of AP2238 (**4**) and Donepezil

compound	K_i[a] (nM) AChE	IC_{50} (nM) AChE	IC_{50} (nM) BuChE	% inhibition of Aβ aggregation[b]
AP2238	21.7 ± 1.4	44.5 ± 6.5	48900 ± 3700	35 ± 1
donepezil	20.5 ± 3.3	23.1 ± 4.8[c]	7420 ± 390	22 ± 3[c]

[a] Estimates of the competitive inhibition constants (K_i) were obtained from replots of the slopes of the graphs of Figure 2 vs inhibitor concentration. [b] Inhibition of AChE-induced Aβ aggregation produced by the tested compound at 100 μM concentration. [c] Data obtained from ref 7.

In this letter, we report for the first time a direct evidence of the Aβ antiaggregating action of an AChE inhibitor purposely designed to bind at both the catalytic and the peripheral sites of the human enzyme. The ability of the compound **4** to contrast the Aβ aggregation is higher than that of other AChE inhibitors tested,[7] with the exception of propidium, which is a bis-quaternary ammonium PAS ligand in no way useful for the treatment of AD.

In addition, **4** shows a potency against HuAChE which is comparable to that of donepezil, and a quite high selectivity for AChE with respect to BuChE. This remarkable selectivity, which largely overcomes that shown by donepezil, suggests some considerations. Actually, a peculiar structural difference between the

two cholinesterases is the lack of the PAS (in particular of Trp286) in BuChE.[27] This seems to prevent the interaction of BuChE with Aβ, such that no role on Aβ aggregation can be played by this enzyme.[5] For instance, tacrine, a nonselective mixed-type AChE inhibitor, binds more tightly to BuChE and is almost inactive in inhibiting the AChE-promoted Aβ aggregation.[7] This inhibitor mostly interacts with AChE catalytic site,[28] as it likely does with BuChE. In contrast, propidium, a well-known AChE inhibitor binding exclusively at the PAS, is almost inactive against BuChE and strongly inhibits the AChE-induced Aβ aggregation.[7] The results presented in Table 1 confirm the phenomenological correlation between AChE selectivity and inhibition of the AChE-induced proaggregating action. It might be concluded that inhibitors of AChE able to strongly interact with the PAS show high AChE/BuChE selectivity and eventually an inhibitory action against AChE-induced Aβ aggregation. We might therefore advance that AChE/BuChE selectivity is key information when searching new potential inhibitors of AChE-induced Aβ aggregation.

In conclusion, here we present evidence of the in vitro Aβ antiaggregating action of an AChE inhibitor that was designed in such a way as to be able to contact simultaneously both the catalytic and the peripheral binding sites of the enzyme. This action combines with an elevated AChE inhibitory potency to provide a compound endowed with a potentially useful biological profile for the treatment of AD. By the way, we also showed how the integration of focused computational, synthetic and biochemical efforts led to the identification and in vitro characterization of a new lead compound bearing the desired biological properties.

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