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New multipotent tetracyclic tacrines with neuroprotective activity

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Abstract—The synthesis and the biological evaluation (neuroprotection, voltage dependent calcium channel blockade, AChE/BuChE inhibitory activity and propidium binding) of new multipotent tetracyclic tacrine analogues (**5**–**13**) are described. Compounds **7**, **8** and **11** showed a significant neuroprotective effect on neuroblastoma cells subjected to Ca^{2+} overload or free radical induced toxicity. These compounds are modest AChE inhibitors [the best inhibitor (**11**) is 50-fold less potent than tacrine], but proved to be very selective, as for most of them no BuChE inhibition was observed. In addition, the propidium displacement experiments showed that these compounds bind AChE to the peripheral anionic site (PAS) of AChE and, consequently, are potential agents that can prevent the aggregation of β-amyloid. Overall, compound **8** is a modest and selective AChE inhibitor, but an efficient neuroprotective agent against 70 mM K^+ and 60 μM H_2O_2. Based on these results, some of these molecules can be considered as lead candidates for the further development of anti-Alzheimer drugs.

1. Introduction

Alzheimer's disease (AD)[1] is the most frequent of the primary degenerative dementias. This disease is thought to be rising dramatically during the next years due to increased longevity of the population. Hallmarks of AD are different histo-pathological alterations like marked atrophy of the cerebral cortex with loss of cortical and subcortical neurons, formation of senile plaques by accumulation of the amyloid β (Aβ) peptide and neurofibrillary tangles composed by pairs of helical filaments of hyperphosphorylated τ protein and neuritic degeneration.

The neuropsychiatric symptoms associated with AD are related to the reduction in the number of functional neuronal nicotinic receptors (nAChR)[2,3] which correlates with the severity of the disease at the time of death.[4] During the past decade, cholinesterase inhibition has become the most widely studied and effective clinical approach to treat AD.[5] Cholinesterase inhibitors (AChEI), such as donepezil, rivastigmine and galantamine, have been approved by the FDA and EMEA for treating the symptoms of AD.[6] On the other hand, the multifactorial pathogenesis of AD suggests that drug treatments with two or more mechanisms of action, acting in a complementary manner, could be more efficacious to patients suffering the disease. In fact, galantamine, a drug currently used to treat AD, is a mild inhibitor of AChE that sensitizes the nicotinic receptor to ACh[7] and prevents cell death induced by amyloid β (Aβ) or thapsigargin.[8,9]

Tacrine, a potent and reversible AChE inhibitor,[10] was the first drug approved in the USA for the palliative treatment of AD; however, it exhibited side effects like hepatotoxicity.[11] Current research is focused on developing new AChE inhibitors with improved activity and reduced adverse side effects. The strategy leading to multipotent anti-Alzheimer drugs has given excellent results and has paved the way for future and more successful developments.[12]

In this context, and based on the fact that a sustained elevation of the cytosolic concentration of Ca^{2+} ions,

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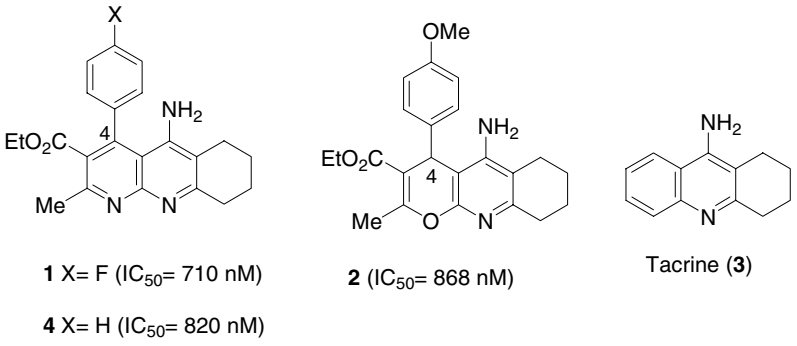


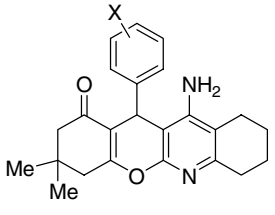
Figure 1.

leading to Ca^{2+} overload, seems to play a crucial role in neuronal degeneration and death,[13] we embarked on a long-term research project aimed at the synthesis of a series of multipotent compounds designed to target AChE and neuronal Ca^{2+} modulation.[14] As a result, we have synthesized and evaluated a number of hybrid compounds, such as **1** and **2** (Fig. 1), which combine the tetrahydroaminoquinoline moiety present in tacrine (**3**) (Fig. 1) with a pyridine or a 4*H*-pyran bearing a substitution pattern similar to that found in the isosteric 1,4-dihydropyridines, well-known calcium channel blockers.[14] Compounds **1** and **2** were less potent as AChEIs than tacrine (IC_{50} = 180 nM, using *Tc*AChE),[14b,14c] but they blocked voltage-dependent Ca^{2+} channels (38% and 50% inhibition, respectively).[14f] Furthermore, compounds **1** and **2** also blocked Ca^{2+} uptake induced by nicotinic stimulation (63% and 90% inhibition, respectively) in bovine chromaffin cells, more efficiently than tacrine (17% inhibition of Ca^{2+} channels and 55% inhibition of the Ca^{2+} uptake induced by nicotinic stimulation).[14f]

Molecular modelling studies, carried out by Prof. Luque and associates, suggested that binding of these AChE inhibitors to the catalytic centre of the enzyme might be hampered by unfavourable steric interactions, mainly due to the aromatic ring moiety installed at C-4; consequently, it was proposed that these tacrine analogues might interact with Trp279 binding at the PAS of the enzyme.[14e] We have confirmed this hypothesis in our laboratory as shown by the propidium displacement experiments. Therefore, these drugs have the ability to bind to both the active and peripheral sites of AChE, and could prevent the aggregation of Aβ[8,9] besides increasing cholinergic neurotransmission.

While Ca^{2+} overload plays a crucial role in causing cell death, a mild and sustained elevation of the cytosolic concentration of Ca^{2+} as a result of moderate chronic depolarization, has been shown to increase the survival of different neuronal types.[15–17] Our group has developed compound **4** (Fig. 1), as a novel compound that combines both an AChE inhibitory activity and a moderate Ca^{2+} promotor action, which leads to protection against a variety of toxic stimuli such as Ca^{2+} overload induced by veratridine, endoplasmic reticulum stress elicited by thapsigargin and free radical generation by hydrogen peroxide or Aβ in bovine chromaffin or human neuroblastoma cells;[14f] these properties make of compound **4** an interesting candidate with potential therapeutic effect in Alzheimer's disease. The neuroprotective effect of compound **4** is related to a mild and sustained increase in the cytosolic levels of Ca^{2+} which, in turn, has been shown to induce the expression of proteins implicated in cell survival such as those of the anti-apoptotic Bcl-2 family.

Based on these previous results, and continuing with our current project directed to the development of new anti-Alzheimer multipotent drugs, in this paper we describe the synthesis and pharmacological study of the new tetracyclic tacrine derivatives **5**–**13** (Fig. 2). Being structurally similar to compounds **1**, **2** or **4**, the synthesis and biological evaluation of compounds **5**–**13** is the first step on our projected structure activity relationship (SAR) analysis, directed to define the key structural and functional elements that might improve the pharmacological profile of these molecules. The selection of compounds **5**–**13** was mainly based on the presumed ability of these molecules to bind the PAS of AChE, as these substrates retain the basic core present on molecules **1** or **2**. Comparing with the lead compounds, we hypothesized that the incorporation of a fourth cyclohexane ring, fused at the 4*H*-pyran heterocyclic moiety, should afford more rigid molecules, giving possibly stronger π–π interactions between the *pyridine* nucleus present in compounds **5**–**13** with Trp 279 in the PAS of AChE. In order to evaluate the effect of the aromatic substituents we have located electron-withdrawing (F, CF_3, and NO_2) and electron-donating (Me, OMe) groups at different available positions (C-2, C-3, and C-4) in the aromatic ring.



5-13 X= H, F, CF_3, Me, NO_2, OMe

Figure 2.

Finally, the ready availability of 5,5-dimethyl-1,3.cyclohexadione ('dimedone'), the starting material for the presumed synthetic sequence, was an added bonus of interest in order to develop a simple and good yielding approach to the desired target molecules.

2. Results and discussion

2.1. Chemistry

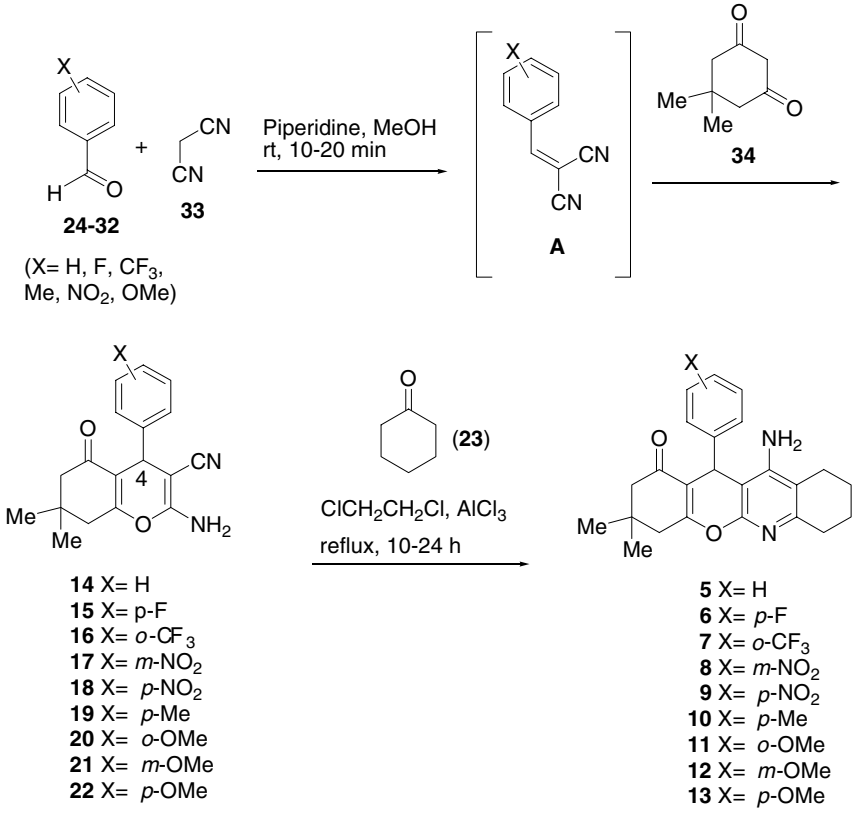
According to the standard methodology for the synthesis of tacrines,[14] the new tetracyclic derivatives could also be easily prepared by the $AlCl_3$ promoted Friedländer reaction[18] between the corresponding, known and polyfunctionalized 4*H*-chromenes[19] **14**–**22** and cyclohexanone (**23**) (Scheme 1). The 4*H*-chromenes **14**–**22** are well-described intermediates,[20] which were obtained uneventfully from commercially and readily available aldehydes **24**, **25** and **27**–**32**, respectively (Scheme 1). Only 4*H*-chromene **16**, with a trifluoromethylphenyl group attached to carbon C-4, has not been reported before, but we were able to synthesize it in a 'one-pot' reaction protocol starting from aldehyde **26**, after a piperidine catalyzed Knoevenagel reaction with malonitrile (**33**), giving the corresponding, not isolated, arylidenemalononitrile intermediate (**A**), followed by reaction with dimedone (**34**)[20a] (Scheme 1) (see Section 4). As expected, intermediates **14**–**22**, on treatment with cyclohexanone (**23**), in the presence of aluminium trichloride, in 1,2-dicholorethane as solvent, at reflux, afforded the target molecules **5**–**13** in good yields (53–96%) (see Section 4). These products gave satisfactory analytical and spectroscopic data in good agreement with their structures. Specific NMR experiments (^1H, ^{13}C, DEPT, ^1H–^1H COSY, HMQC and HMBC) allowed us to assign the corresponding signals to the respective protons and carbons.

With these compounds in hand, we tested them in several pharmacological assays.

2.2. Pharmacology

AChE inhibition by the currently used drugs in the treatment of AD improves the symptoms,[21] but does not delay the neuronal loss that is characteristic of this illness. As mentioned in Section 1, treatment strategies with drugs holding more than one mechanism of action, acting in a complementary manner, might be more adequate on patients suffering the disease. One of these possible mechanisms might be neuroprotection. Recently, several preclinical studies have shown that clinical useful AChEI, such as galantamine[22–24] and donepezil,[25,26] display neuroprotective properties.

Therefore, we decided to assess the potential neuroprotective effect of the new compounds on SH-SY5Y cells exposed during 24 h to a medium with a depolarizing concentration of KCl (70 mM) which induces a Ca^{2+} overload and the consequent cell death. Drugs, at the concentration of 0.3 μM, were administered 24 h before the incubation of cells with high K^+ (70 mM; hypertonic)



Scheme 1.

and maintained during the entire experiment. Thereafter, release of lactic dehydrogenase (LDH) was measured as a parameter of cell death, since this enzyme is released to the extracellular medium as a consequence of cell death. Basal release of LDH by the cells was subtracted from the values obtained after incubation with 70 mM K^+ in the presence or absence of the compounds.

Compounds **5**–**13** and their synthetic precursors 2-amino-4-aryl-3-cyano-5,6,7,8-tetrahydro-7,7-dimethyl-5-oxo-4*H*-chromenes (**14**–**22**) were tested. The results are shown in Table 1. All compounds showed a statistically significant neuroprotective effect at the assayed concentration. Note that tacrine (**3**), also investigated for comparative purposes, showed the poorest (13%) protection. Compounds **5**–**13** exhibited protection values in the range 35–44.8%, the most potent being compounds **5** and **12**. It is worth mentioning that precursors **14**–**22** evidenced average higher values than compounds **5**–**13**; in the range 32.5–61.6%, the most potent was compound **17**, bearing a 3-nitrophenylgroup at C-4. Since products **14**–**22** are inactive towards AChE, we conclude that the anticholinesterase activity may not display a clear relationship with the neuroprotective action.

Oxidative damage also appears to play an important role in the progressive neuronal death characteristic in AD.[27] Thus, we considered convenient to study the possible neuroprotective effect of our compounds on SH-SY5Y neuroblastoma cells exposed to 60 μM H_2O_2 for 24 h.

Compounds **5**–**13** and their synthetic precursors 2-amino-4-aryl-3-cyano-5,6,7,8-tetrahydro-7,7-dimethyl-5-oxo-4*H*-chromenes (**14**–**22**) were evaluated. Results are shown in Table 2. Fourteen of the total compounds studied manifested a statistically significant neuroprotective effect against H_2O_2 toxicity, the most potent of them were compounds **12**, **19** and **6** with a 61.3, 55.9 and 54.2% protection, respectively. In general, for the protection observed against free radicals generated from H_2O_2, we have observed that the trend was reversed; compounds **5**–**13** were more efficient than their synthetic precursors **14**–**22**, all of them showing a good neuroprotective profile.

In summary, from these assays we conclude that our new molecules (**5**–**13**) are quite promising from the neuroprotection point of view.

Since a significant cytoprotection was achieved with all compounds against Ca^{2+} overload induced by 70 mM K^+ (Table 1), next we decided to study if these compounds were blocking voltage-dependent Ca^{2+} channels (VDCC) and could therefore explain why they were preventing excess Ca^{2+} entry into the cells.

Neuroblastoma cells previously loaded with the fluorescence dye Fluo-4 were incubated in the presence of the

Table 1. Cell viability expressed as reduction in the increase of LDH released in the presence of 70 mM K^+[a]

Compound (0.3 μM)	X	LDH release (% vehicle)	% protection
Vehicle		100	—
Tacrine (**3**)		90.4 ± 4.1	13.4 ± 7.2
Galantamine		75.8 ± 2.1	34.80
14	H	63.2 ± 4.3	50.3 ± 2.2
15	4-F	65.5 ± 1.9	48.9 ± 2.1
16	2-CF_3	76.3 ± 2.8	32.5 ± 3.3
17	3-NO_2	57 ± 1.8	61.6 ± 3
18	4-NO_2	67.4 ± 1.2	47.3 ± 3.2
19	4-Me	62.4 ± 1.4	55.6 ± 4.7
20	2-OMe	65.7 ± 2.6	47.3 ± 2.5
21	3-OMe	65.1 ± 1.8	49.5 ± 2.6
22	4-OMe	64 ± 4.2	49.9 ± 5.5
5	H	63.7 ± 2,34	44.8 ± 4,5
6	4-F	65.2 ± 3.5	42.3 ± 6.6
7	2-CF_3	69.1 ± 2.8	40.0 ± 3.3
8	3-NO_2	70.5 ± 3.12	36.4 ± 5.7
9	4-NO_2	68.5 ± 2.9	39.4 ± 5.8
10	4-Me	70.3 ± 1.5	38.8 ± 1.7
11	2-OMe	70 ± 2.4	38.9 ± 3.9
12	3-OMe	65,7 ± 1.8	43.1 ± 4.3
13	4-OMe	75.6 ± 1.7	35 ± 2

[a] Data are expressed as means ± SEM of at least three different cultures in quadruplicate. LDH released was calculated for each individual experiment considering 100% the extracellular LDH released in the presence of vehicle with respect to the total. To calculate % protection, LDH release was normalized as follows: in each individual triplicate experiment, LDH release obtained in non-treated cells (basal) was subtracted from the LDH released upon 70 mM K^+ treatment and normalized to 100% and that value was subtracted from 100.

Table 2. Cell viability expressed as reduction in the increase of LDH released in the presence of 60 μM H_2O_2[a]

Compound (0.3 μM)	X	LDH release (% vehicle)	% protection
Vehicle		100	—
Tacrine (**3**)		98.0 ± 1.80	0.8 ± 4.1
Galantamine		89.9 ± 2.3	14.90
14	H	79.6 ± 2.6	28.2 ± 3.8
15	4-F	75.5 ± 2.2	34.9 ± 2.9
16	2-CF_3	87.5 ± 3.7	26.2 ± 2.8
17	3-NO_2	66.5 ± 2.6	49 ± 3.9
18	4-NO_2	64.3 ± 1,5	53.4 ± 3.9
19	4-Me	56.2 ± 1.3	55.9 ± 1.1
20	2-OMe	82.8 ± 3.1	27.5 ± 3.5
21	3-OMe	83.9 ± 3.6	27.3 ± 3.5
22	4-OMe	77.7 ± 2.6	31.7 ± 3
5	H	69.0 ± 8.2	39.8 ± 2.1
6	4-F	57,87 ± 1.57	54.2 ± 2.0
7	2-CF_3	85.6 ± 4.1	19.4 ± 3.2
8	3-NO_2	67.7 ± 1.5	41.4 ± 1.8
9	4-NO_2	65.5 ± 1.4	43.8 ± 1.9
10	4-Me	87.4 ± 2.9	15.7 ± 2.9
11	2-OMe	65.6 ± 2.6	44.8 ± 2.3
12	3-OMe	52.2 ± 1.9	61.3 ± 2.14
13	4-OMe	83.8 ± 2.5	20.9 ± 2.9

[a] Data are expressed as means ± SEM of at least three different cultures in quadruplicate. LDH released was calculated for each individual experiment considering 100% the extracellular LDH released in the presence of vehicle with respect to the total. To calculate % protection, LDH release was normalized as follows: in each individual triplicate experiment, LDH release obtained in non-treated cells (basal) was subtracted from the LDH released upon H_2O_2 treatment and normalized to 100% and that value was subtracted from 100.

Table 3. Effect of the different compounds on the $[Ca^{2+}]_c$ increase elicited by 70 mM K^+ in SH-SY5Y cells (% inhibition with respect to a control without any drug)[a]

Compound (0.3 μM)	X	% inhibition
14	H	24.6 ± 4.2
15	4-F	18.5 ± 5.6
16	2-CF_3	32.7 ± 3.0
17	3-NO_2	31.5 ± 4.8
18	4-NO_2	21.3 ± 4.8
19	4-Me	28.7 ± 7.0
20	2-OMe	27.3 ± 3.8
21	3-OMe	33.2 ± 5.6
22	4-OMe	31.2 ± 4.0
5	H	35.0 ± 3,5
6	4-F	36,5 ± 4.0
7	2-CF_3	43.1 ± 4.8
8	3-NO_2	30.0 ± 4.0
9	4-NO_2	32.0 ± 3.0
10	4-Me	25.5 ± 4.0
11	2-OMe	31.9 ± 4.5
12	3-OMe	43,5 ± 4.0
13	4-OMe	32.5 ± 5.0

All compounds were tested at the concentration of 0.3 μM.

[a] Data are expressed as means ± SEM of at least three different cultures in quadruplicate.

compounds for 10 min and then stimulated with a concentrated solution of KCl so that the final concentration in the medium was 70 mM K^+. Changes in fluorescence as a consequence of the $[Ca^{2+}]_c$ increase elicited by high K^+ concentrations were measured in a fluorescence plate reader FluoStar Optima (BMG).

Compounds **5**–**13** and their synthetic precursors 2-amino-4-aryl-3-cyano-5,6,7,8-tetrahydro-7,7-dimethyl-5-oxo-4*H*-chromenes (**14**–**22**) were tested. The results are shown in Table 3. All compounds induced a statistically significant inhibition of the $[Ca^{2+}]_c$ increase elicited by 70 mM K^+ in neuroblastoma cells, with values ranging from 18% to 43%, the most potent being compounds **12** (43.5%) and **7** (43.1%), showing similar values to the one observed for compound **2** (50%) (Fig. 1). These results clearly point out that our selection of compounds was correct, and that the new molecules still block Ca^{2+} channels efficiently. These results suggest that our compounds could be blocking VDCC and thus preventing Ca^{2+} overload in neuroblastoma cells.

Finally, and in view of these results, compounds **5**–**13** have been evaluated as possible AChEI according to standard methodology[28] (see Section 4).

In Table 4 we present the IC_{50} values corresponding to those compounds. As shown and as expected, all the compounds inhibit AChE, but comparing with tacrine (**3**) (IC_{50} 0.14 μM), the newly synthesized compounds manifest lower activity, the most active being compound **11** (IC_{50} 7.0 μM) (around 50-fold less active) which has an electron-donating group (OMe) at C-2. From the SAR results, in general, compounds (**10**–**13**) with electron-donating groups (Me, OMe) are more active than those with electron-withdrawing functionalities (F, CF_3 and NO_2) (**6**–**9**). Note, however, that compound **8** with an electron-withdrawing group (NO_2) at C-3 is the second most active AChE inhibitor in this series of compounds. Despite the electronic properties of the aromatic substituent, the order of the inhibitory activity increases on going from substituents at position C-4 to C-2, the most active being those with these groups at C-2 position. Also, a good direct correlation was found between the electron-donating power of the substituent and the activity of the compound (compare compound **10** with a Me group at C-4, and compound **13** with a OMe group at C-4). The same trend is observed for the electron-withdrawing groups (compare compound **6** with **7**, bearing a fluorine at C-4, and trifluoromethyl at C-2, respectively).

The compounds **5**–**13** were also assayed to determine its activity on butyrylcholinesterase, following Ellman's method.[29] Only two of them, compounds **7** and **11**, inhibited butyrylcholinesterase with IC_{50}s of 2.2 and 8.7 μM, respectively. For the rest of the compounds the maximal concentration assayed, 100 μM, did not produce an inhibition sufficient to calculate an IC_{50}.

Comparing with the parent compound **2** (Fig. 1), the corresponding new tetracyclic analogue **13** is 50 fold less active, suggesting that, at least for a 4-OMe substituent at the aromatic ring, the newly incorporated saturated cyclohexane ring is deleterious for the AChE inhibitory activity. We have also investigated the AChE/BuChE inhibitory activity of precursors 2-amino-4-aryl-3-cyano-5,6,7,8-tetrahydro-7,7-dimethyl-5-oxo-4*H*-chromenes **14**–**22**, but no significant activity was observed. In summary, compounds **5**–**13** are AChE inhibitors revealing modest potency but, with the exception of compounds **7** and **11**, high selectivity towards AChE, as they were, in general, devoid of any inhibitory activity regarding BuChE.

As stated in Section 1, one of the hallmarks of AD lies in the formation of senile plaques by accumulation of the Aβ peptide. Different studies have proven that AChE binds to Aβ inducing the formation of fibrils[30] and the PAS of the enzyme is involved in the adhesion function.[31] Ligands, like propidium iodide, that bind to the PAS are known to block Aβ aggregation.[31a] Thus, we were interested in investigating whether some of our compounds (**5**–**13**) might be able to bind to the PAS, a fact that could make them good candidates for the development of new anti Aβ drugs.

A propidium competition assay was accomplished for those compounds which inhibited AChE with an $IC_{50} < 100$ μM at the concentration of 0.3 μM. Propidium, a selective ligand for the PAS of AChE, exhibits an increase in the fluorescence upon binding to that site.[32] A decrease in propidium fluorescence in the presence of the compounds could be interpreted as a displacement of propidium from the PAS.

As shown in Table 4, at the concentration of 0.3 μM, compounds **5**–**13** showed consistent and significant ability to displace propidium from the PAS of AChE. These results clearly confirm our initial hypothesis regarding

Table 4. Inhibition of AChE (IC_{50} in μM), and displacement of propidium iodide from the peripheral anionic site, measured as the inhibition of the fluorescence of propidium bound to AChE, by the new compounds **5**–**13**

Compound	X	AChE inhibition IC_{50} (μM)	Propidium displacement (% inhibition at 0.3 μM[a])
Tacrine (**3**)	—	0.14	3.9 ± 1.8
5	H	48.6	17.4 ± 2.6
6	4-F	69.7	40 ± 4.1
7	2-CF_3	17.8	38.4 ± 3.9
8	3-NO_2	11.9	23.9 ± 3.4
9	4-NO_2	94.1	22.3 ± 3.8
10	4-Me	51.6	35.4 ± 3.6
11	2-OMe	7.0	32.1 ± 3.9
12	3-OMe	21.5	28.2 ± 2.7
13	4-OMe	40.5	39 ± 4.7

[a] Data are expressed as means ± SEM of at least three different experiments in quadruplicate.

the site where these inhibitors are interacting with AChE. First of all, the most active compound was the fluorinated derivative **6**, with a 40% inhibition. Both compounds **6** (69.7 μM) and **10** (51.6 μM) showed higher IC_{50} for AChE than compound **7** (Table 1). Thus, compounds **6** and **10** are almost 10- and 7-fold, respectively, less active than inhibitor **11**. Although the ability to displace propidium did not follow the increasing order of AChE inhibitory activity,[33] two of the most potent AChE inhibitors (**11** and **7**) showed high inhibition values, around 32.1% and 38.4%, respectively. In summary, these results reveal that compounds **5**–**13** are weaker and more selective AChE inhibitors than the parent compounds **1**, **2** or **4** (Fig. 1) but, interestingly, they still bind significantly the enzyme at the PAS, confirming that the proposed structural modification of compounds **1**, **2** or **4** leading to the new substrates was a correct choice.

3. Conclusions

To summarize, we have reported the synthesis and biological evaluation including the neuroprotective properties, voltage-dependent calcium channel blockade, AChE/BuChE inhibitory activities and propidium binding to the AChE PAS of new tetracyclic tacrines (**5**–**13**). Regarding AChE inhibition, none of the assayed compounds manifested higher activity than tacrine (IC_{50} 0.14 mM), showing just moderate activity, the most potent compound (**7**) being 50-fold less active than tacrine. The majority of these compounds are extremely selective for AChE as they were devoid of any inhibitory activity regarding BuChE. From SAR, we have observed that compounds **5**–**13**, bearing electron-donating substituents in the aromatic ring, are more active than those substituted with electron-withdrawing groups; the activity increases from carbon 4 to carbon 3 and to carbon 2 in the aromatic nucleus. In addition, the stronger this electronic property was, the more active the compounds were.

In our opinion, the main interest of these new compounds lies in their neuroprotective capacity against Ca^{2+} overload or free radical generation. In addition, their moderate activity as AChE inhibitors would cause a more selective interaction with the peripheral site of the enzyme. In fact, our new compounds displace propidium from the PAS with values around 40% for compounds **6** or **10**. This could be an indication of a possible antiagregant effect of Aβ.

A good candidate for further pharmacological studies could be compound **8** that has the following pharmacological profile: (1) IC_{50} to block AChE 11.9 μM; (2) displacement of propidium from the AChE peripheral site, 24% at 0.3 μM; (3) neuroprotection against Ca^{2+} overload, 36% at 0.3 μM; (4) neuroprotection against H_2O_2, 41% at 0.3 μM; and (5) blockade of Ca^{2+} entry through Ca^{2+} channels, 30% at 0.3 μM. Full concentration-response curves against those parameters can allow the prediction of EC_{50} values of around 1–10 μM, not far away from the AChE IC_{50} value. Hence, a nice possibility is that compound **8** (or some related compounds of those reported here) could have a polyfunctional pharmacological profile that causes: (i) an improvement of cognitive symptoms by inhibiting AChE and improving cholinergic neurotransmission; (ii) a neuroprotective effect against Ca^{2+} overloading and free radical generation, thus delaying apoptosis and neuronal death and (iii) a prevention of Aβ deposition and the formation of senile plaques, by acting on the AChE peripheric site. These are qualities to be expected to benefit Alzheimer disease patients by improving cognition and delaying the progression of the disease.

4. Experimental

4.1. General methods

Reactions were monitored by TLC using precoated silica gel aluminium plates containing a fluorescent indicator (Merck, 5539). Detection was done by UV (254 nm) followed by charring with sulfuric–acetic acid spray, 1% aqueous potassium permanganate solution or 0.5% phosphomolybdic acid in 95% EtOH. Anhydrous Na_2SO_4 was used to dry organic solutions during work-ups and the removal of solvents was carried out under vacuum with a rotary evaporator. Flash chromatography was performed using silica gel 60 (230–400 mesh, Merck). Melting points were determined on a Kofler block and are uncorrected. IR spectra were obtained on a Perkin-Elmer Spectrum One spectrophotometer. ^1H NMR spectra were recorded with a Varian VXR-200S spectrometer, using tetramethylsilane as internal standard and ^{13}C NMR spectra were recorded with a Bruker WP-200-SY. All the assignments for protons and carbons were in agreement with 2D COSY, gHSQC, gHMBC and 1D NOESY spectra. Values with (*) can be interchanged. Elemental analyses were conducted on a Carlo Erba EA 1108 apparatus.

4.2. General method for the synthesis of 2-amino-4-aryl-3-cyano-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromenes

To a solution of the corresponding aldehyde (1 equiv) in dry methanol (1 mL/mmol), under argon, malonodinit-

rile (1 equiv) and a catalytic amount of piperidine were added. The mixture was stirred at rt for 10–20 min. Then, 5,5-dimethyl-1,3-cyclohexanedione (1 equiv) and a catalytic amount of piperidine were added. The mixture was stirred at rt for 30 min–14 h, and the precipitated solid was isolated by filtration, washed with cold methanol, dried and recrystallized from mixtures of dichloromethane/hexane.

4.3. 2-Amino-3-cyano-4-[2-(trifluoromethyl)phenyl]-5,6,7,8-tetrahydro-7,7-dimethyl-5-oxo-4*H*-chromene (16)

Following Section 4.1 for the synthesis of 4*H*-chromenes, 3-trifluoromethylbenzaldehyde (1 g, 5.74 mmol) was reacted with malonodinitrile (416 mg, 6.31 mmol), 5,5-dimethyl-1,3-cyclohexanedione (824 mg, 6.31 mmol), piperidine (15 drops) and methanol (30 mL); after 45 min, compound **16** (2.05 g, 99%) was obtained: mp 218–220 °C; IR (KBr) *v* 3481, 3328, 3202, 2964, 2199, 1665, 1595, 1366, 1251, 1156 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.61 (t, $J_{4'-3'}$ = 7.9 Hz, 1H, H4′), 7.57 (d, $J_{6'-5'}$ = 7.7 Hz, 1H, H6′), 7.37 (t, $J_{5'-6'}$ = 7.7 Hz, 1H, H5′), 7.27 (d, $J_{3'-4'}$ = 7.9 Hz, 1H, H3′), 7.04 (br s, 2H, NH_2), 4.55 (s, 1H, H4), 2.50 (br s, 2H, H8), 2.14 [AB, *J* = 15.9 Hz, 2H, H6], 1.03 [s, 3H, CH_3(C7)], 0.95 [s, 3H, CH_3(C7)]; ^{13}C NMR (DMSO-d_6, 75 MHz) δ 195.6 (C5), 163.3 (C2), 158.4 (C8a), 144.57/144.55/144.54/144.52 ($J_{C1'-F}$ = 1.2 Hz, C1′), 133.1 (C5′), 129.9/126.3/122.6/119.0 (J_{C-F} = 275.3 Hz, CF_3), 129.7 (C6′), 127.1 (C4′), 127.1/126.7/ 126.3/125.9 ($J_{C2'-F}$ = 29.7 Hz, C2′), 125.7/125.6 / 125.5 / 125.4 ($J_{C3'-F}$ = 5.8 Hz, C3′), 119.2 (C≡N), 112.8 (C4a), 58.4 (C3), 50.3 (C6), 40.1 (C8), 32.1 (C7), 31.6 (C4), 28.6 [C*H*$_3$(C7)], 27.2 [C*H*$_3$(C7)]; MS (APIES+) *m*/*z*: $[M + 1]^+$ 363.3 $[M + Na]^+$ 385.2; $[2M+Na]^+$ 747.5. Anal. Calcd for $C_{19}H_{17}N_2O_2F_3$: C, 62.98; H, 4.73; N, 7.73. Found: C, 62.82; H, 4.76; N, 7.48.

4.4. General method for the Friedländer reaction

Aluminium chloride (1.2–1.7 equiv) was suspended in dry 1,2-dichloroethane (10 mL) at rt under argon. The corresponding 4*H*-benzopyran (1 equiv) and cyclohexanone (1.2–1.7 equiv) were added. The reaction mixture was heated under reflux (10–24 h). When the reaction was over (TLC analysis), a mixture of THF/H_2O (1:1) was added at rt. An aqueous solution of sodium hydroxide (10%) was added dropwise to the mixture until the aqueous solution was basic. After stirring for 30 min, the mixture was extracted three times with dichloromethane. The organic layer was washed with brine, dried over anhydrous sodium sulfate, filtered and the solvent was evaporated. The resultant solid was purified by silica gel flash chromatography using methanol/dichloromethane mixtures as eluent to give pure compounds.

4.5. 11-Amino–2,3,4,7,8,9,10,12-octahydro-3,3-dimethyl-12-phenyl-1*H*-chromeno[2,3-*b*]quinolin-1-one (5)

Following Section 4.4, from compound **14** (250 mg, 0.85 mmol), $AlCl_3$ (170.67 mg, 1.28 mmol), $ClCH_2CH_2Cl$ (5 mL), cyclohexanone (125.1 mg, 1.28 mmol), after 5 h, product **5** (300 mg, 90%) was obtained: mp 301°C; IR (KBr) *v* 3430, 3355, 3246, 3028, 2934, 2862, 1640, 1453, 1371, 1229 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.29 (m, 2 H, 2× H2′), 7.17 (m, 2H, 2× H3′), 7.07 (m, 1H, H4′), 5.63 (s, 2H, NH_2), 4.95 (s, 1H, H12), 2.55-2.49 (m, 4H, 2H10, 2H7), 2.29 (m, 1H, H4A), 2.26 (d, *J* = 16.1 Hz, 1H, H2A), 2.16 (m, 1H, H4B), 2.05 (d, *J* = 16.1 Hz, 1H, H2B), 1.65 (br s, 4H, 2H9, 2H8), 1.02 [s, 3H, CH_3(C3)], 0.87 [s, 3H, CH_3(C3)]; ^{13}C NMR (DMSO-d_6, 75 MHz) δ 195.6 (C1), 164.7 (C4a), 153.8 (C5a), 152.1 (C11*), 151.3 (C6a*), 144.2 (C1′), 128.0 (2× C2′), 127.8 (2× C3′), 126.1 (C4′), 113.6 (C10a), 113.1 (C12a), 99.1 (C11a), 50.0 (C2), 44.3 (C4), 32.8 (C12), 31.9 (C3), 31.6 (C7), 28.7 [*C*H$_3$(C3)], 26.4 [*C*H$_3$(C3)], 22.9 (C10), 22.2 (C9), 21.9 (C8); MS (APCI+) *m*/*z*: $[M+1]^+$ 375.3; $[M+Na]^+$ 397.3; $[2M+Na]^+$ 771.3. Anal. Calcd for $C_{24}H_{26}N_2O_2$: C, 76.98; H, 7.00; N, 7.48. Found: C, 76.34; H, 7.00; N, 7.23.

4.6. 11-Amino-12-(4-fluorophenyl)-2,3,4,7,8,9,10,12-octahydro-3,3-dimethyl-1*H*-chromeno[2,3-*b*]quinolin-1-one (6)

Following Section 4.4, from compound **15** (250 mg, 0.80 mmol), $AlCl_3$ (159.6 mg, 1.2 mmol), $ClCH_2CH_2Cl$ (5 mL), cyclohexanone (117.6 mg, 1.2 mmol), after 6.5 h, product **6** (308 mg, 91%) was obtained: mp 313 °C; IR (KBr) *v* 3418, 3355, 3246, 2939, 2869, 1639, 1601, 1567, 1371, 1226, 1205, 1168 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.30 (t, *J* = 8.7 Hz, 2H, 2× H2′], 7.00 (t, *J* = 8.7 Hz, 2H, 2× H3′), 5.66 (s, 2H, NH_2), 4.98 (s, 1H, H12), 2.55–2.50 (m, 4H, 2H10, 2H7), 2.29 (m, 1H, H4A), 2.26 (d, *J* = 16.1 Hz, 1H, H2A), 2.16 (m, 1H, H4B), 2.05 (d, *J* = 16.1 Hz, 1H, H2B), 1.65 (br s, 4H, 2H9, 2H8), 1.02 [s, 3H, CH_3(C3)], 0.86 [s, 3H, CH_3(C3)]; ^{13}C NMR (DMSO-d_6, 75 MHz) δ 195.6 (C1), 164.7 (C4a), 162.1/159.8 ($J_{C4'-F}$ = 172.5 Hz, C4′), 153.8 (C5a), 152.2 (C11*), 151.3 (C6a*), 140.3 (C1′), 129.8/129.7 ($J_{C2',C6'-F}$ = 8.2 Hz, 2× C2′), 114.6/114.3 ($J_{C3',5'-F}$ = 21.0 Hz, 2× C3′), 113.4 (C12a), 113.2 (C10a), 98.8 (C11a), 50.4 (C2), 40.7 (C4), 32.0 (C12), 31.9 (C3), 31.8 (C7), 28.6 [*C*H$_3$(C3)], 26.4 [*C*H$_3$(C3)], 22.9 (C10), 22.2 (C9), 22.0 (C8); MS (APCI+) *m*/*z*: $[M+1]^+$ 393.2; $[2M+1]^+$ 807.5. Anal. Calcd for $C_{24}H_{25}N_2O_2$: C, 73.45; H, 6.42; N, 7.14. Found: C, 73.37; H, 6.40; N, 7.18.

4.7. 11-Amino-12-(2-trifluoromethylphenyl)-2,3,4,7,8,9,10,12-octahydro-3,3-dimethyl-1*H*-chromeno[2,3-*b*]quinolin-1-one (7)

Following Section 4.4, from compound**16** (250 mg, 0.71 mmol), $AlCl_3$ (141.64 mg, 1.06 mmol), $ClCH_2$-CH_2Cl (5 mL), cyclohexanone (104.4 mg, 1.06 mmol)], after 6 h, product **7** (287 mg, 91%) was obtained: mp 171–172°C; IR (KBr) *v* 3500, 3429, 3246, 2939, 2869, 1649, 1447, 1368, 1305, 1232 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.19 (m, 2H, H3′, H6′), 6.39 (m, 2H, H4′, H5′), 5.58 (s, 1H, H12), 5.18 (s, 2H, NH_2), 2.70-2.52 (m, 4H, 2H4, 2H7), 2.38 (m, 1H, H4A), 2.30 (d, *J* = 16.1 Hz, 1H, H2A), 2.27 (m, 1H, H4B), 2.07 (d, *J* = 16.1 Hz, 1H, H2B), 1.71 (br s, 4H, 2H9, 2H8), 1.04 [s, 3H, C*H*$_3$(C3)], 0.91 [s, 3H, C*H*$_3$(C3)]; ^{13}C NMR (DMSO-d_6, 75 MHz) δ 195.3 (C1), 164.4 (C4a), 153.5

(C5a), 152.9 (C6a), 151.3 (C11), 142.23/142.20/142.18/142.16 ($J_{C1'-F}$ = 1.7 Hz, C1′), 133.6 (C6′), 133.0/129.5/126.0/123.5 (J_{C-F} = 265.1 Hz, CF_3), 131.72/131.70/131.69/131.67 ($J_{C4'-F}$ = 1.3 Hz, C4′), 128.2 (C5′), 127.2/126.8/126.4/126.0 ($J_{C2'-F}$ = 29.6 Hz, C2′), 126.88/126.80/126.74/126.67 ($J_{C3'-F}$ = 5.0 Hz, C3′), 113.8 (C12a), 113.0 (C10a), 99.1 (C11a), 49.9 (C2), 40.4 (C4), 31.8 (C12), 31.2 (C3), 30.8 (C7), 28.5 [*C*H$_3$(C3)], 26.4 [*C*H$_3$(C3)], 22.8 (C10), 22.1 (C9), 21.9 (C8); MS (APIES+) *m/z*: $[M + 1]^+$ 443.1 $[M + Na]^+$ 466.1; $[2M+Na]^+$ 907.2. Anal. Calcd for $C_{25}H_{25}N_2O_2F_3$: C, 67.86; H, 5.69; N, 6.33. Found: C, 67.77; H, 5.36; N, 6.21.

4.8. 11-Amino-2,3,4,7,8,9,10,12-octahydro-3,3-dimethyl-12-(3-nitrophenyl)-1*H*-chromeno[2,3-*b*]quinolin-1-one (8)

Following Section 4.4, from compound **17** (250 mg, 0.74 mmol), $AlCl_3$ (146.3 mg, 1.1 mmol), $ClCH_2CH_2Cl$ (5 mL), cyclohexanone (108.7 mg, 1.11 mmol), after 7 h, product **8** (167.2 mg, 53%) was obtained: mp 293–295°C; IR (KBr) *v* 3428, 3246, 2934, 2869, 1637, 1528, 1371, 1205 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.35 (s, 1 H, H2′), 7.96 (d, *J* = 7.9 Hz, 1H, H4′), 7.64 (d, *J* = 7.9 Hz,1H, H6′), 7.49 (t, *J* = 7.9 Hz,1H, H5′), 5.82 (s, 2H, NH_2), 5.19 (s, 1H, H12), 2.71–2.48 (m, 4H, 2H10, 2H7), 2.30 (m, 1H, H4A), 2.15 (m, 1H, H4B), 2.32 (d, *J* = 16.0 Hz, 1H, H2A), 2.05 (d, *J* = 16.1 Hz, 1H, H2B), 1.66 (br s, 4H, 2H9, 2H8), 1.03 [s, 3H, CH_3(C3), 0.85 [s, 3H, CH_3(C3)]; ^{13}C NMR (DMSO-d_6, 75 MHz) δ 195.6 (C1), 165.3 (C4a), 153.6 (C5a), 152.6 (C1′), 151.5 (C11), 147.1 (C6a), 146.4 (C3′), 134.4 (C6′), 129.5 (C5′), 122.9 (C2′), 121.3 (C4′), 113.3 (C10a), 112.7 (C12a), 97.9 (C11a), 49.9 (C2), 40.4 (C4), 32.5 (C12), 31.9 (2 C, C3, C7), 28.6 [*C*H$_3$(C3)], 26.2 [*C*H$_3$(C3)], 22.9 (C10), 22.1 (C9)*, 21.9 (C8)*; MS (APCI+) *m/z*: $[M+1]^+$ 420.3; $[M+Na]^+$ 442.2; $[2M+Na]^+$ 861.5. Anal. Calcd for $C_{24}H_{25}N_3O_4$: C, 68.72; H, 6.01; N, 10.02. Found: C, 68.96; H, 6.22; N, 9.94.

4.9. 11-Amino-2,3,4,7,8,9,10,12-octahydro-3,3-dimethyl-12-(4-nitrophenyl)-1*H*-chromeno[2,3-*b*]quinolin-1-one (9)

Following Section 4.4, from compound **18** (250 mg, 0.74 mmol), $AlCl_3$ (146.03 mg, 0.11 mmol), $ClCH_2CH_2Cl$ (5 mL), cyclohexanone (108.78 mg, 1.11 mmol), after 5 h, product **9** (215 mg, 69%) was obtained: mp 323°C; IR (KBr) *v* 3390, 3227, 2936, 2869, 1639, 1517, 1371, 1345, 1227 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.87 (d, *J* = 8.6 Hz, 2H, H3′, H5′), 7,35 (d, *J* = 8.6 Hz, 2H, H2′, H6′), 5.53 (s, 2H, NH_2), 4.95 (s, 1H, H12), 2.42–2.30 (m, 4H, 2H10, 2H7), 2.07 (m, 1H, H4A), 1.92 (m, 1H, H4B), 2.07 (d, *J* = 16.1 Hz, 1H, H2A), 1.83 (d, *J* = 16.1 Hz, 1H, H2B), 1.44 (br s, 4H, 2H9, 2H8), 0.81 [s, 3H, CH_3(C3)], 0.63 [s, 3H, CH_3(C3)]; ^{13}C NMR (DMSO-d_6, 75 MHz) δ 195.5 (C1), 165.3 (C4a), 153.7 (C5a)*, 152.6 (C1′)*, 151.8 (C11)**, 151.5 (C6a)**, 145.8 (C4′), 129.3 (2 C, C2′, C6′), 123.0 (2 C, C3′, C5′), 113.4 (C10a), 112.5 (C12a), 97.8 (C11a), 49.9 (C2), 40.3 (C4), 32.8 (C12), 31.9 (C3), 31.8 (C7), 28.6 [*C*H$_3$(C3)], 26.4 [*C*H$_3$(C3)], 22.9 (C10), 22.1 (C9), 21.9 (C8); MS (APCI+) *m/z*: $[M+1]^+$ 420.3. Anal. Calcd for $C_{24}H_{25}N_3O_4$: C, 68.72; H, 6.01; N, 10.02. Found:C, 68.58; H, 6.12; N, 10.10.

4.10. 11-Amino–2,3,4,7,8,9,10,12-octahydro-3,3-dimethyl-12-(4-methylphenyl)-1*H*-chromeno[2,3-*b*]quinolin-1-one (10)

Following Section 4.4, from compound**19** (250 mg, 0.84 mmol), $AlCl_3$ (211.15 mg, 1.26 mmol), $ClCH_2CH_2Cl$ (5 mL), cyclohexanone (196.15 mg, 1.26 mmol)], after 8 h, product **10** (246 mg, 91%) was obtained: mp 310°C; IR (KBr) *v* 3417, 3244, 2936, 2869, 1638, 1568, 1454, 1372, 1228 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.16 (d, *J* = 7.9 Hz, 2H, H2′), 6.72 (d, *J* = 7.9 Hz, 1H, H3′), 5.57 (s, 2H, NH_2), 4.88 (s, 1H, H6), 2.60 (br s, 4H, H4, H8), 2.17 [s, 3H, CH_3(C4′)], 2.42–2.30 (m, 4H, 2H10, 2H7), 2.07 (m, 1H, H4A), 1.92 (m, 1H, H4B), 2.07 (d, *J* = 16.1 Hz, 1H, H2A), 1.83 (d, *J* = 16.1 Hz, 1H, H2B), 1.44 (br s, 4H, 2H9, 2H8), 1.02 [s, 3H, CH_3(C3)], 0.87 [s, 3H, CH_3(C3)]; ^{13}C NMR (DMSO-d_6, 75 MHz) δ 195.9 (C1), 164.9 (C4a), 154.1 (C5a), 152.3 (C6a), 151.7 (C11), 141.6 (C1′), 135.5 (C4′), 128.8 (2 C, C2′), 128.2 (2 C, C3′), 114.1 (C12a), 113.5 (C10a), 99.6 (C11a), 50,5 (C2), 40,6 (C4), 32,8 (C12), 32,3 (C3), 29,1 (C7), 28,7 [*C*H$_3$(C3)], 26,8 [*C*H$_3$(C3)], 23.3 (C10), 22.6 (C9), 22.4 (C8), 20.9 [*C*H$_3$(C4′)]; MS (APCI+) *m/z*: $[M+1]^+$ 389.2; $[M+Na]^+$ 411.2; $[2M+1]^+$ 799.5. Anal. Calcd for $C_{25}H_{28}N_2O_2$: C, 77.29; H, 7.26; N, 7.21. Found: C, 77.02; H, 7.25; N, 6.69.

4.11. 11-Amino–2,3,4,7,8,9,10,12-octahydro-12-(2-methoxyphenyl)-3,3-dimethyl-1*H*-chromeno[2,3-*b*]quinolin-1-one (11)

Following Section 4.4, from compound **20** (250 mg, 0.77 mmol), [$AlCl_3$ (151.70 mg, 1.14 mmol), $ClCH_2CH_2Cl$ (5 mL), cyclohexanone (112.50 mg, 1.14 mmol)], after 5 h, product **11** (291 mg, 94%) was obtained: mp 145°C; IR (KBr) *v* 3442, 3381, 2934, 2869, 1650, 1490, 1453, 1372, 1241, 1173 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.09 (d, *J* = 8,3 Hz, 1H, H3′), 7.07 (t, *J* = 7,2 Hz, 1H, H6′), 6.97 (d, *J* = 8.3 Hz, 1H, H4′), 6.65 (t, *J* = 7.2 Hz, 1H, H5′), 5.54 (s, 2H, NH_2), 5.03 (s, 1H, H12), 3.86 (s, 3H, CH_3O), 2.42–2.30 (m, 4 H, 2H10, 2H7), 2.07 (m, 1H, H4A), 1.92 (m, 1H, H4B), 2.07 (d, *J* = 16.1 Hz, 1H, H2A), 1,83 (d, *J* = 16,1 Hz, 1H, H2B), 1,44 (br s, 4H, 2 H9, 2H8), 1,04 [s, 3H, CH_3(C3)], 0.93 [s, 3H, CH_3(C3)]; ^{13}C NMR (DMSO-d_6, 75 MHz) δ 195.7 (C1), 165.8 (C4a), 155.8 (C2′), 154.0 (C5a), 152.2 (C6a), 151.7 (C11), 132.5 (C1′), 129.8 (C6′), 128.1 (C3′), 121.5 (C5′), 113.2 (C10a), 112.9 (C12a), 112.2 (C4′), 99.4 (C11a), 56.6 (CH_3O), 50.4 (C2), 40.7 (C4), 32.2 (C12), 31.6 (C3), 29.2 (C7), 27.5 [*C*H$_3$(C3)], 26.7 [*C*H$_3$(C3)], 23.2 (C10), 22.6 (C9), 22.3 (C8); MS (APCI+) *m/z*: $[M+1]^+$ 405.1; $[M+Na]^+$ 427.1; $[2M+1]^+$ 831.2. Anal. Calcd for $C_{25}H_{28}N_2O_3$: C, 74.23; H, 6.98; N, 6.93. Found: C, 74.53; H, 7.10; N, 7.02.

4.12. 11-Amino-12-(3-methoxyphenyl)-3,3-dimethyl-2,3,4,7,8,9,10,12-octahydro-1*H*-chromeno[2,3-*b*]quinolin-1-one (12)

Following Section 4.4, from compound **21** (200 mg, 0.64 mmol), $AlCl_3$ (127.28 mg, 0.96 mmol), $ClCH_2CH_2Cl$ (5 mL), cyclohexanone (93.81 mg, 0.96 mmol), after 8 h, product **12** (246 mg, 96%) was obtained: mp 245°C; IR

(KBr) ν 3420, 3347, 3243, 2938, 2833, 1636, 1453, 1372, 1229, 1171 cm^{-1}; ^1H NMR (DMSO-d_6,300 MHz) δ 7.09 (t, $J = 7.8$ Hz, 1H, H5′), 6.97 (s, 1H, H2′), 6.77 (d, $J = 7.8$ Hz, 1H, H4′), 6.65 (d, $J = 7{,}8$ Hz, 1H, H6′), 5.65 (s, 2H, NH_2), 4.93 (s, 1H, H12), 3.66 (s, 3H, CH_3O), 2.42–2.30 (m, 4H, 2H10, 2H7), 2.07 (m, 1H, H4A), 1.92 (m, 1H, H4B), 2.07 (d, $J = 16.1$ Hz, 1H, H2A), 1.83 (d, $J = 16.1$ Hz, 1H, H2B), 1.44 (br s, 4H, 2H9, 2H8), 1.03 [s, 3H, CH_3(C3)], 0.89 [s, 3H, CH_3(C3)]; ^{13}C NMR (DMSO, 75 MHz) δ 195.5 (C1), 164.8 (C4a), 158.7 (C3′), 153.8 (C5a), 152.1 (C11*), 151.3 (C6a*), 145.7 (C1′), 128.9 (C5′), 120.0 (C4′), 114.0 (C2′), 113.4 (C10a), 113.1 (C12a), 110.8 (C6′), 99.0 (C11a), 54.9 (CH_3O), 50.0 (C2), 40.3 (C4), 32.8 (C12), 31.9 (C3), 31.8 (C7), 28.7 [CH_3(C3)], 26.3 [CH_3(C3)], 22.9 (C10), 22.2 (C9), 22.0 (C8); MS (APCI+) m/z: $[M+1]^+$ 405.2; $[2M+1]^+$ 831.7. Anal. Calcd for $C_{25}H_{28}N_2O_3$: C, 74.23; H, 6.98; N, 6.93. Found: C, 74.31; H, 6.56; N, 6.93.

4.13. 11-Amino-2,3,4,7,8,9,10,12-octahydro-12-(4-methoxyphenyl)-3,3-dimethyl-1*H*-chromeno[2,3-*b*]quinolin-1-one (13)

Following Section 4.4, from compound**22** (250 mg, 0.77 mmol), $AlCl_3$ (151.62 mg, 1.14 mmol), $ClCH_2CH_2Cl$ (5 mL), cyclohexanone (112.50 mg, 1.14 mmol), after 7 h, product **13** (289 mg, 92%) was obtained: mp 291°C; IR (KBr) ν 3414, 3241, 2937, 2360, 1635, 1509,1455, 1372, 1295, 1228 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 7.19 (d, $J = 8.6$ Hz, 2H, H2′), 6.72 (d, $J = 8.6$ Hz, 1H, H3′), 5.58 (s, 2 H, NH_2), 4.88 (s, 1H, H6), 3.64 (s, 3H, CH_3O), 2.42–2.30 (m, 4H, 2H10, 2H7), 2.07 (m, 1H, H4A), 1.92 (m, 1H, H4B), 2.07 (d, $J =$ 16.1 Hz, 1H, H2A), 1.83 (d, $J = 16.1$ Hz, 1H, H2B), 1.44 (br s), 4H, 2H9, 2H8), 1.02 [s, 3H, CH_3(C3)], 0.88 [s, 3H, CH_3(C3)]; ^{13}C NMR (DMSO-d_6, 75 MHz) δ 195.9 (C1), 164.7 (C4a), 157.9 (C4′), 154.1 (C5a), 152.3 (C6a), 151.6 (C11), 136.6 (C1′), 129.3 (2 C, C2′), 114.1 (C12a), 113.6 (2 C, C3′), 113.5 (C10a), 99.7 (C11a), 55.2 (CH_3O), 50.4 (C2), 40.7 (C4), 32.3 (C12), 32.2 (C3), 29.4 (C7), 29.0 [CH_3(C3)], 26.8 [CH_3(C3)], 23.3 (C10), 22.6 (C9), 22.4 (C8); MS (APCI+) m/z: $[M+1]^+$ 405.1; $[M+Na]^+$ 427.0; $[2M+1]^+$ 831.2. Anal. Calcd for $C_{25}H_{28}N_2O_3$: C, 74.23; H, 6.98; N, 6.93. Found: C, 74.11; H, 6.98; N, 7.37.

4.14. Pharmacological studies

4.14.1. Culture of SH-SY5Y cells. SH-SY5Y cells, at passages between 3 and 16 after de-freezing, were maintained in a Dulbecco's modified Eagle's medium (DMEM) containing 15 non-essential amino-acids (NEAAs) and supplemented with 10% foetal calf serum (FCS), 1 mM glutamine, 50 U/mL penicillin and 50 μg/ml streptomycin (reagents from Gibco, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37°C in 5% CO_2/humidified air. Stock cultures were passaged 1:4 twice weekly. For assays, SH-SY5Y cells were sub-cultured in 24-well plates at a seeding density of 2×10^5 cells per well, or in 96-well plates at a seeding density of 8×10^4 cells per well. For the cytotoxicity experiments cells were treated with drugs before confluence, in DMEM free of serum.

4.14.2. Measurement of lactic dehydrogenase (LDH) activity. Extracellular and intracellular LDH activity was spectrophotometrically measured using a Cytotoxicity Cell Death kit (Roche-Boehringer. Mannheim, Germany) according to the manufacturer's indications. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity; released LDH was defined as the percentage of extracellular compared to total LDH activity.

4.14.3. Measurement of cytosolic Ca^{2+} concentrations. For these experiments, SH-SY5Y neuroblastoma cells were grown at confluence in 96-well black dishes. Cells were loaded with 4 μM fluo 4/AM for 1 h at 37°C in DMEM. Then cells were washed twice with Krebs-Hepes solution and kept at room temperature for 30 min before the beginning of the experiment. Fluorescence was measured in a fluorescence microplate reader (FLUOstar Optima, BMG, Germany). Wavelengths of excitation and emission were 485 and 520 nm, respectively.

4.14.4. Measurement of AChE activity. To assess the inhibitory activity of the compounds towards AChE, we followed the spectrophotometric method of Rappaport[28] using purified AChE from electric eel (*Torpedo californica*) and acetylcholine chloride (29.5 mM) as a substrate. The reaction took place in a final volume of 2.5 mL of an aqueous solution containing 0.78 U AChE and 1.9 mM *m*-nitrophenol to produce a yellow colour which is lost as a function of enzyme activity. Inhibition curves were made by incubating with the different compounds for 30 min; a sample without any compound was always present to determine the 100% of enzyme activity. After the 30 min incubation, the loss of yellow colour by *m*-nitrophenol was evaluated by measuring absorbance at 405 nm in a spectrophometric plate reader (iEMS Reader MF, Labsystems). The concentration of compound that produces 50% AChE activity inhibition (IC_{50}) was calculated by transforming the values of absorbance to Rappaport enzymatic activity units extrapolating from a calibration curve previously obtained. Data are means ± SEM of at least three different experiments in triplicate.

4.14.5. Measurement of BuChE activity. The inhibitory activity of the compounds towards BuChE was determined following the method of Ellman[29] using BuChE from human serum and butiryl thiocholine cloride (5 mM) as a substrate. The reaction took place in a final volume of 1 mL of a phosphate buffer solution at pH 7.2 containing 0.035 U BuChE and 0.25 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) which produces the yellow anion 5-thio-2-nitrobenzoic acid. Inhibition curves were made by incubating with the different compounds for 15 min; a sample without any compound was always present to determine the 100% of enzymatic activity. After the 15 min incubation period, the production of colour, as an indication of enzymatic activity, was evaluated by measuring absorbance at 412 nm in a spectrophotometric plate reader (iEMS Reader MF, Labsystems).

4.14.6. Measurement of propidium iodide displacement from the peripheral anionic site (PAS) of AChE. A solution of AChE from bovine erythrocyte at the concentration of 5 μM in 0.1 mM Tris buffer, pH 8, was used.

Aliquots of the compounds to get the final concentration of 0.3 μM were added, and the solutions were kept at room temperature for at least 6 h. After that time, the samples were incubated for 15 min with propidium at a final concentration of 20 μM and the fluorescence was measured in a fluorescence microplate reader (FLUOstar Optima, BMG, Germany). Wavelengths of excitation and emission were 485 and 620 nm, respectively.

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33. Studies directed to explain the apparent discrepancies observed between the results of this assay and the AChE inihibitory activity are in progress in our laboratory and will be reported in due course. We thank a reviewer for the addressing our attention to this point.