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Multipotent drugs with cholinergic and neuroprotective properties for the treatment of Alzheimer and neuronal vascular diseases. I. Synthesis, biological assessment, and molecular modeling of simple and readily available 2-aminopyridine-, and 2-chloropyridine-3,5-dicarbonitriles

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ABSTRACT

The synthesis, molecular modeling, and pharmacological analysis of new multipotent simple, and readily available 2-aminopyridine-3,5-dicarbonitriles (**3**–**20**), and 2-chloropyridine-3,5-dicarbonitriles (**21**–**28**), prepared from 2-amino-6-chloropyridine-3,5-dicarbonitrile (**1**) and 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile (**2**) is described. The biological evaluation showed that some of these molecules were modest inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), in the micromolar range. The 2-amino (**3**, **4**), and 2-chloro derivatives **21**–**23**, **25**, **26** were AChE selective inhibitors, whereas 2-amino derivatives **5**, **14** proved to be selective for BuChE. Only inhibitor **24** was equipotent for both cholinesterases. Kinetic studies on compound **23** showed that this compound is a mixed-type inhibitor of AChE showing a K_i of 6.33 μM. No clear SAR can be obtained form these data, but apparently, compounds bearing small groups such as the *N*,*N*′-dimethylamino or the pyrrolidino, regardless of the presence of a 2-amino, or 6-chloro substituent in the pyridine ring, preferentially inhibit AChE. Molecular modeling on inhibitors **4**, **5**, **22**, and **23** has been carried out to give a better insight into the binding mode on the catalytic active site (CAS), and peripheral anionic site (PAS) of AChE. The most important differences in the observed binding relay on the modifications of the group at C2, as the amino group forms two hydrogen bonds that direct the binding mode, while in the case of compounds with a chlorine atom, this is not possible. The neuroprotective profile of these molecules has been investigated. In the LDH test, only compounds **26**, **3**, **22**, and **24** showed neuroprotection with values in the range 37.8–31.6% in SH-SY5Y neuroblastoma cells stressed with a mixture of oligomycin-A/rotenone, but in the MTT test only compound **17** (32.9%) showed a similar profile. Consequently, these compounds can be considered as attractive multipotent therapeutic molecules on two key pharmacological receptors playing key roles in the progress of Alzheimer, that is, cholinergic dysfunction and oxidative stress, and neuronal vascular diseases.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative process characterized by a progressive loss of cognitive abilities, such as memory, language skills, disorientation, attention, and depression.[1] Although the etiology of AD is still poorly understood, several factors such as amyloid-β (Aβ)[2] deposits, τ-protein aggregation, oxidative stress or low levels of acetylcholine[3] are thought to play significant roles in the pathology of the disease.[4] In spite of the enormous research effort, an efficient strategy for designing new drugs for the treatment of AD is still lacking.

The cholinergic theory[1] suggests that the selective loss of cholinergic neurons in AD results in a deficit of acetylcholine (ACh) in specific regions of the brain that mediate learning and memory functions.[5] Consequently, three acetylcholinesterase (AChE) inhibitors have been approved for commercial use. Thus, donepezil, rivastigmine, and galanthamine are known to improve AD symptoms

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by inhibiting AChE, that is, the enzyme responsible for the hydrolysis of ACh, thereby raising the levels of ACh in the synaptic cleft.[6] Recently, a renewed interest for AChE inhibitors has been stimulated by the potential role of AChE in accelerating the formation of amyloid fibrils in the brain and forming stable complexes with Aβ.[7] This role involves the peripheral anionic binding site (PAS) of AChE, as noted by the fact that propidium iodide, a potent AChEI agent binding specifically to the PAS, affects Aβ aggregation in vitro, whereas other catalytic active site (CAS) inhibitors such as tacrine have not a similar effect.[8]

The multifactorial nature of AD supports new therapeutic strategies. The most current innovative therapeutic approach is based on the 'one molecule, multiple targets' paradigm.[9–13] Thus, the multipotent approach[14] includes novel tacrine–melatonin hybrids,[15] dual inhibitors of AChE and monoamine oxidase[16] or serotonin transporters,[17] potent cholinesterase inhibitors with antioxidant and neuroprotective properties,[18] gallamine–tacrine hybrids binding at cholinesterases and M_2 muscarinic receptors,[19] NO-donor–tacrine hybrids as hepatoprotective drugs focusing on AD[20a] or fluorescent tacrine–coumarin hybrids.[20b]

On the other hand, it is known that Ca^{2+} overload is the main factor that triggers the processes leading to cell death. Thus, it has been shown that calcium dysfunction, involved in the pathogenesis of AD, augments Aβ formation and τ hyperphosphorylation.[21,22] Moreover, calcium entry through L-type Ca^{2+} channels (Cav 1.1–1.4) causes both calcium overload and mitochondrial disruption, which leads to the activation of the apoptotic cascade and cell death.[23] In fact, nimodipine, a neuronal L-type Ca^{2+} channel blocker, protects neurons from death evoked by focal cerebral ischemia.[24] Since 1,4-dihydropyridines (DHPs) are compounds that selectively block L-type voltage-dependent Ca^{2+} channels (VDCCs), hybrid molecules that combine an AChEI and a DHP, such as tacrine and nimodipine (Chart 1), might represent a promising approach to the treatment of AD. Support to this therapeutic strategy comes from the fact that bis(7)-tacrine attenuates Aβ-elicited neuronal apoptosis by regulating L-type calcium channels.[25] Besides inhibition of AChE and blockade of VDCCs, compounds able to prevent the oxidative stress might have increased therapeutical value, since oxidative damage precedes the appearance of other pathological hallmarks of AD.[26–28]

Based on these precedents, some years ago we have embarked in a project targeted to the synthesis of multipotent molecules able to interact with the cholinergic system, showing also L-type Ca^{2+} channel antagonism effect. As a result, we have reported the chemistry and pharmacology of *tacripyrines* (Chart 1),[29] and identified a new lead compound such as '*p*-methoxytacripyrine' (**RL2/101**) (Chart 1) as a potent and selective AChE versus BuChE and mixed-type inhibitor, binding preferentially at the PAS of AChE. Accordingly, **RL2/101** interferes with the pro-aggregation Aβ effect of human AChE (hAChE), and is a mild inhibitor of self-aggregation of Aβ42. In addition, **RL2/101** is a potent Ca^{2+}antagonist that permeates the blood–brain barrier (BBB) and displays neuroprotective and antioxidant properties.[29a] The racemate has been resolved, and the in vitro pharmacology of the enantiomers as well as the in vivo animal models are now being investigated.[29b]

In this context, and in order to validate the proposed binding mode,[29a] and to simplify the structure of the lead compound (**RL2/101**), we have now carried out the synthesis, the biological evaluation, and molecular modeling of a number of 2-aminopyridine-3,5-dicarbonitriles (**I**), and 2-chloropyridine-3,5-dicarbonitriles (**II**) (Chart 2), as two new families of very simple, extremely easy available molecules, from which we have identified compounds showing moderate, but selective AChE versus BuChE inhibitory activity, and a significant neuroprotective profile. In this report we describe these new results.

2. Results and discussion

2.1. Chemistry

The synthesis of 2-aminopyridine-3,5-dicarbonitrile (**I**), and 2-chloropyridine-3,5-dicarbonitrile (**II**) type of compounds has been carried out starting from the readily available 2-amino-6-chloropyridine-3,5-dicarbonitrile (**1**)[30] and 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile (**2**).[31] Compounds **1** and **2** are very well functionalized for the synthesis of molecules of type **I** and **II**, based on presumably simple aromatic nucleophilic substitution for incorporating new substituents at C6, followed by transformation of the amino into the chloro group (Chart 2).

The reaction of compounds **1** and **2** with several selected commercially available amines (dimethylamine, pyrrolidine, piperidine, 7-azabicyclo[2.2.1]heptane,[32] 4-hydroxypiperidine, ethanolamine, propargylamine, 4-benzylpiperidine, and benzylamine), gave the expected C6 substituted 2-aminopyridine-3,5-dicarbonitriles (**3**, **4**,[33] **5**,[33] **6–11**, **12**,[34] **13–15**) (Table 1).

Similarly, the reaction of 2-amino-6-chloropyridine-3,5-dicarbonitrile (**1**)[30] with sodium methoxide in methanol gave the known compound **16**[35] (Table 2). For the synthesis of compounds **17** and **18**, 2-amino-6-methoxy-pyridine-3,5-dicarbonitrile (**16**) was reacted with Na/EtOH, and 3-phenylpropanol with Na in DMF, respectively (see Section 4).

2-Amino-4-phenyl-6-(phenylthio)pyridine-3,5-dicarbonitrile (**19**) and 2-amino-6-mercapto-4-phenylpyridine-3,5-dicarbonitrile (**20**) (Chart 3) were prepared as described in the literature.[37]

The C6 substituted 2-aminopyridine-3,5-dicarbonitriles have been transformed into the corresponding new 2-chloropyridine-3,5-dicarbonitriles (**21–28**) (Table 3) by Sandmeyer reaction[38] using the conditions reported by Doyle.[39]

The new compounds have been conveniently characterized by their analytical and NMR spectroscopic data (see Section 4).

2.2. Pharmacology and computational chemistry

2.2.1. AChE/BuChE inhibitory activity

2-Amino-3,5-dicyanopyridines (**3–20**) and 2-chloro-3,5-dicyanopyridines (**21–28**) were evaluated as inhibitors of AChE from

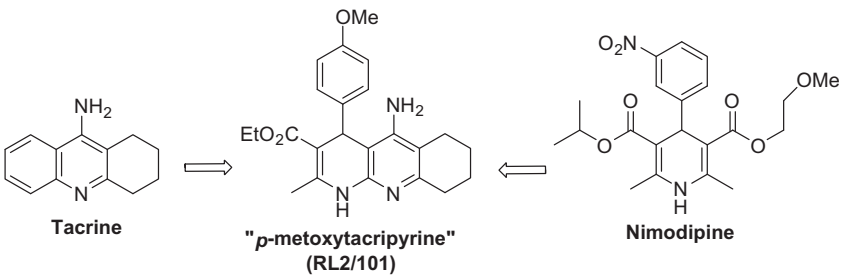


Chart 1. Tacripyridines as hybrid molecules based on the juxtaposition of tacrine and nimodipine.

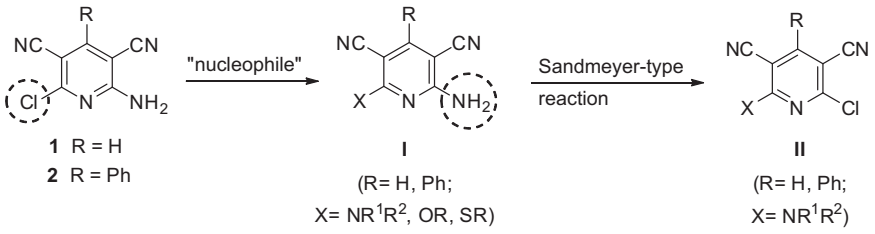
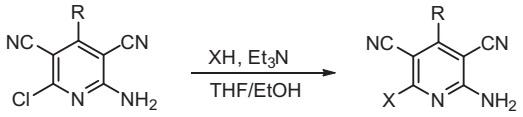


Chart 2. The target 2-aminopyridine-(**I**), and 2-chloropyridine-3,5-dicarbonitriles (**II**).

Table 1
Synthesis of 6-N-substituted 2-aminopyridine-3,5-carbonitriles (**3**–**18**)



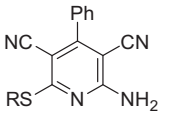


Chart 3.

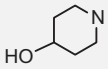
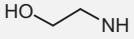
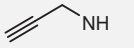
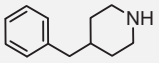
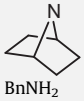
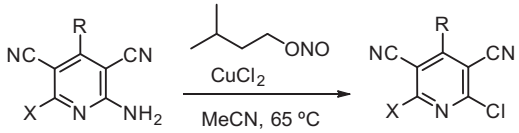
Entry	Product[(ref)]	R	X	Yield (%)
a	**3**	H	Me_2N	98
b	**4**[33]	H		95
c	**5**[33]	H		98
d	**6**	H		90
e	**7**	H		99
f	**8**	H		99
g	**9**	H		99
h	**10**	H		97
i	**11**	Ph	Me_2N	75
j	**12**[34]	Ph		62
k	**13**[34]	Ph		97
l	**14**	Ph		16
m	**15**	Ph	$BnNH_2$	91

Table 3
Synthesis of 6-N-substituted 2-chloropyridine-3,5-dicarbonitriles (**21**–**28**)

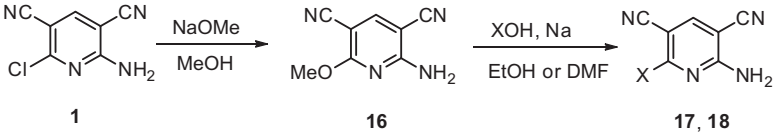


Entry	Product	R	X	Yield (%)
a	**21**	H	Me_2N	56
b	**22**	H		84
c	**23**	H		72
d	**24**	H		66
e	**25**	Ph	Me_2N	67
f	**26**	Ph		64
g	**27**	Ph		60
h	**28**	Ph		50

Electrophorus electricus (Ee), and BuChE from equine serum, according to Ellman's protocol.[40a]

From the IC_{50} data shown in Tables 4 and 5, we conclude that most compounds in these series were inactive, the 2-amino-3,5-dicyanopyridines were in general less potent than the 2-chloro-3,5-dicyanopyridines, and between those compounds being active for AChE or BuChE inhibition, that is, **3**–**5**, **14**; **21**–**23**, **25**, **26**, a

Table 2
Synthesis of 6-O-substituted 2-aminopyridine-3,5-dicarbonitriles (**16**–**18**)



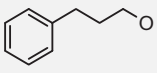
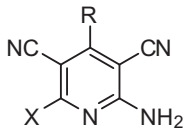
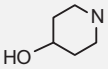
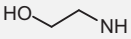
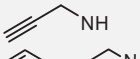
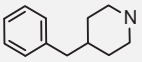
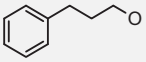
Entry	Product	X	Yield (%)
a	**16**[35]	MeO	96
b	**17**[36]	EtO	91
c	**18**		76

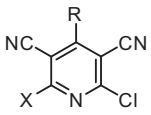
Table 4
Inhibition of AChE from *Electrophorus electricus* (EeAChE) and equine serum butyrylcholinesterase (eqBuCEh) by 2-aminopyridine-3,5-dicarbonitriles (**3**–**20**)[a]



Entry	Product	R	X	IC_{50} (μM) EeAChE	IC_{50} (μM) eqBuChE
a	**3**	H	Me_2N	**22.7 ± 0.9**	>100
b	**4**	H		**3.5 ± 0.2**	>100
c	**5**	H		>100	**26 ± 1**
d	**6**	H		>100	>100
e	**7**	H		>100	>100
f	**8**	H		>100	>100
g	**9**	H		>100	>100
h	**10**	H		>100	>100
i	**11**	Ph	Me_2N	>100	>100
j	**12**	Ph		>100	>100
k	**13**	Ph		>100	>100
l	**14**	Ph		>100	**3.8 ± 0.5**
m	**15**	Ph	$BnNH_2$	>100	>100
n	**16**	H	MeO	>100	>100
o	**17**	H	EtO	>100	>100
p	**18**	H		>100	>100
q	**19**	Ph	PhS	>100	>100
r	**20**	Ph	HS	>100	>100

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

Table 5
Inhibition of AChE from *Electrophorus electricus* (EeAChE) and equine serum butyrylcholinesterase (eqBuCEh) by 2-chloropyridine-3,5-dicarbonitriles (**21**–**28**)[a]



Entry	Product	R	X	IC_{50} (μM) EeAChE	IC_{50} (μM) eqBuChE
a	**21**	H	Me_2N	**5.0 ± 0.8**	>100
b	**22**	H		**0.8 ± 0.1**	>100
c	**23**	H		**9.5 ± 0.4**	>100
d	**24**	H		**75 ± 3**	**24 ± 2**
e	**25**	Ph	Me_2N	**10 ± 2**	>100
f	**26**	Ph		**5.6 ± 0.9**	>100
g	**27**	Ph		>100	>100
h	**28**	Ph		>100	>100

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

complete selectivity was observed regarding one of the cholinesterases. The 2-amino-**3**, **4**, and 2-chloro-derivatives **21**–**23**, **25**, **26** were AChE selective inhibitors, whereas 2-amino derivatives **5**, **14** proved to be selective for BuChE. Compound **24** (Table 5) was the only inhibitor that was equipotent for AChE/BuChE. When active, they were modest cholinesterase inhibitors, in the micromolar range.

From these results, several structure–activity relationships (SAR) can be established as follows. In the 2-amino-3,5-dicyanopyridine family (Table 4), and for those bearing an hydrogen as the substituent at C4, only compounds **3**, **4**, and **5** were active, with **4** being the most potent inhibitor, showing also a complete selectivity for AChE inhibition (IC_{50} for AChE 3.5 μM, and >100 μM for BuChE). The incorporation of a second 'fused-five-membered ring system' into the active N-substituted pyrrolidine derivative **4**, or the addition of a hydroxyl group (or a phenylmethyl chain) at C4′ in **5**, resulted in inactive molecules such as **6**, **7**, and **10**, respectively (Table 4). We have also observed that the N-substitution produced also inactive molecules (see compounds **8** and **9** in Table 4). Curiously, the incorporation of a phenyl group at C4 in inhibitors **3**, **4**, and **5** dramatically reduced the inhibition, as none of the resulting molecules **11**–**13** was active; conversely, the inactive molecule **6** becomes now the moderate and specific BuChE inhibitor **14** (Table 4). Finally, the N-benzylated derivative **15**, the oxygenated **16**–**18**, or the sulfur-containing molecules **19**, **20** lacked any cholinergic activity (Table 4).

For the 2-chloro-3,5-dicyanopyridines, most of the compounds were active, and completely selective for AChE (Table 5). Compound **22** was the most potent inhibitor with a *N*-pyrrolidine nucleus at C6 (IC_{50}: 0.8 μM). Only products **27** and **28**, bearing a phenyl at C4, and piperidine or a 7-azabiclo[2.2.1]heptane substituent at C6, showed no inhibition at all.

The comparison between 2-amino-3,5-dicyanopyridines (Table 4) and 2-chloro-3,5-dicyanopyridines (Table 5) seems appropriate. For those bearing an hydrogen at C4, compounds **21** (C6: NMe_2) and **22** (C6: pyrrolidine) are more potent at AChE than their analogous **3** and **4**. Similarly, for those with a phenyl ring at C4, compounds **25** (C6: NMe_2) and **26** (C6: pyrrolidine) are active, while their analogues **11** and **12** were not. Now, compounds **27** and **13** are inactive, as well as compound **28**, while its analogue **14** was active and selective for BuChE.

To sum up, no clear SAR can be obtained from these data, but apparently, compounds bearing small groups such as the *N*,*N*′-dimethylamino or the pyrrolidino, regardless of the presence of a 2-amino, or 6-chloro substituent in the pyridine ring, do inhibit AChE.

Consequently, to find possible answers to this puzzling situation, we have carried out the kinetics of the inhibition for the active compound **23**, and docking analyses on the active molecules using the automated program Autodock (see below).

2.2.2. Kinetic study of AChE inhibition by compound 23

The mechanism of inhibition of EeAChE was investigated with compound **23** (Table 5), a moderate inhibitor of EeAChE (Table 5). The inhibitory constant, K_i (the inhibition constant for competitive inhibition) was determined from the analysis of Lineweaver–Burk plots, which show both increasing slopes (lower V_{max}) and intercepts (higher K_m) with increasing inhibitor concentration, indicating mixed-type inhibition. The graphical analysis of steady-state inhibition data for inhibitor **23** is shown in Chart 4. A K_i of 6.33 μM was estimated from the plots of the slope versus the concentration of **23**. A K_I, defined as the inhibition constant for the noncompetitive inhibition, of 17.2 μM was determined from replotting intercepts of Lineweaver–Burk versus concentration of inhibitor. As this value was very close to that found for K_i, we propose that compound **23** would show a mixed-type inhibition with a slight preference for the binding to the PAS of AChE. For comparative purposes, note that a competitive inhibitor such as galantha-

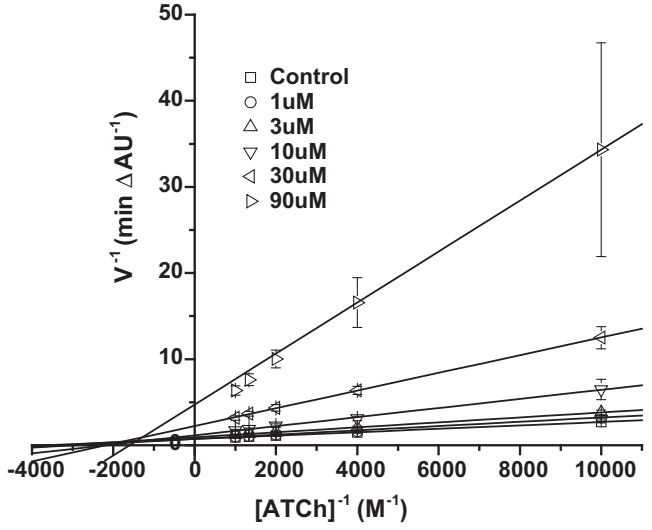


Chart 4. Steady-state inhibition of acetylcholinesterase (AChE) hydrolysis of acetylthiocholine (ATCh) by compound **23**. Lineweaver–Burk reciprocal plots of initial velocity and substrate concentrations are presented. Lines were derived from a weighted least-squares analysis of data points.

mine has K_i of 428 nM,[40b] and a K_I infinite, while for propidium iodide, a cholinergic agent binding specifically at PAS, the values are K_i = 8.6 μM, and K_I = 8.9 μM.[40c]

2.2.3. Molecular modeling

In order to carry out the docking analyses, an area of EeAChE that included both CAS and PAS was selected in order to evaluate the relative binding at both active sites. Each docking analysis gave 100 solutions. Consequently, from the resulting values we can conclude that preferential binding will be observed for the active site with the most populated cluster. In Table 6 we show the energy score and the cluster populations for the docking solutions, at CAS and PAS, for compounds **4** (R = H; R_1 = NH_2; XH = pyrrolidine), **5** (R = H; R_1 = NH_2; XH = piperidine), **23** (R = H; R_1 = Cl; XH = piperidine), and **22** (R = H; R_1 = Cl; XH = pyrrolidine).

As shown from these data, the population cluster for inhibitors **4**, **23** and **22** were the highest in the CAS, with values equal to 99, 94, and 93, respectively.

Thus, we have selected compound **4** as a model, and in Figure 1 we show its putative binding in the CAS and PAS. In the CAS, the aromatic ring establishes π-stacking interactions with Trp86 and Tyr337, whereas the amino group forms two hydrogen bonds with Asp74 and Tyr341. This orientation allows the pyrrolidine ring to be well accommodated in the lipophilic pocket formed by Trp439, Met443, Pro446, and Tyr449 (Fig. 1).

In the case of inhibitor **5**, bearing a piperidine ring the steric interactions are more important and likely prevent a better binding at the CAS.

The ligand in most populated clusters for compound **8** shows a hydrogen bond between the hydroxyl group and the pyridinic nitrogen in a pseudo-seven membered cycle, that prevents an easy accommodation in the hydrophobic pocket.

In the PAS, inhibitor **4** forms two hydrogen bonds with Asp74 and Tyr72, the aromatic ring laying between the indole ring of Trp286, and the phenol of Tyr341. The pyrrolidine ring points to the inside gorge of the enzyme, while the nitrile groups are oriented towards the outside part of enzyme, and should be solvated (Fig. 1).

For inhibitor **23**, bearing a chlorine atom at C2, and a piperidine ring at C6, in the CAS, the absence of the amino group results in a new binding mode based on stereoelectronic interactions. Accordingly, no interactions are now observed with Asp74 and Tyr341, but π-stacking interactions with Trp86 and Tyr337 are still possible, allowing the piperidine ring to be accommodated out of the hydrophobic pockets where the steric interactions are less demanding (Fig. 2).

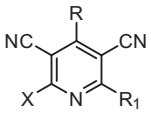
In the PAS, the aromatic ring is located between Trp286 y Tyr341, establishing stabilizing π-stacking interactions, whereas the piperidine ring points to the bottom of the AChE gorge. However, and comparing with inhibitor **4**, the absence of an amino group, being substituted by a chlorine atom in **23**, allows the Cl atom to be oriented to the outer side of the enzyme showing stabilizing interactions by solvation (Fig. 3).

To sum up, the most important differences in the binding in the active sites relay on the modifications of the group at C2: the amino group forms two hydrogen bonds that direct the binding mode, while in the case of compounds with a chlorine atom, this is not possible.

2.2.4. Neuroprotective effect of compounds 3–28 against rotenone/oligomycin-A-induced death of human neuroblastoma SH-SY5Y cells

The mixture of rotenone plus oligomycin-A (rot/olig) blocks mitochondrial electron transport chain complexes I and V, respectively,[41] inducing cell death by oxidative stress. The neuroprotective effect of compounds **3–28** against this toxic stimulus was

Table 6
The energy scores and cluster populations for the docking solutions, at the CAS and PAS sites of acetylcholinesterase (AChE), for compounds **4**, **5**, **22** and **23**



Product	Catalytic site (CAS)		Peripheral anionic site (PAS)	
	Score (kcal/mol)	Population of cluster (100)	Score (kcal/mol)	Population of cluster (100)
4	−3.84	99	−3.50	1
5	−3.60	79	−3.84	21
23	−4.82	94	−4.29	5
22	−4.55	93	−4.11	5

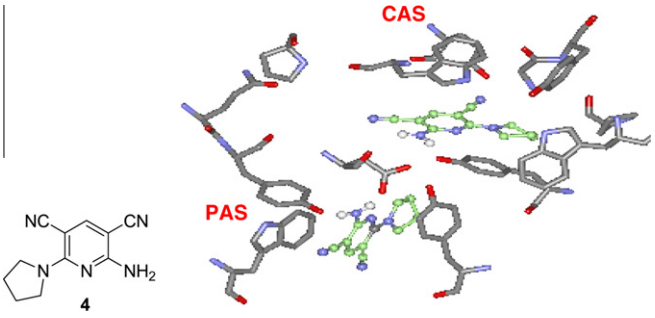


Figure 1. Binding of inhibitor **4** in the CAS and PAS of AChE. The ligand appears as balls and sticks, with the carbon atoms in green.

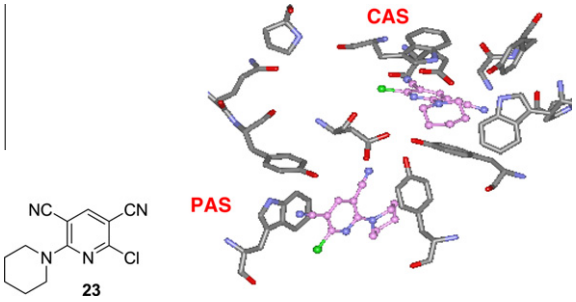


Figure 2. Binding of inhibitor **23** in the CAS and PAS of AChE. The ligand appears as balls and sticks, with the carbon atoms in pink.

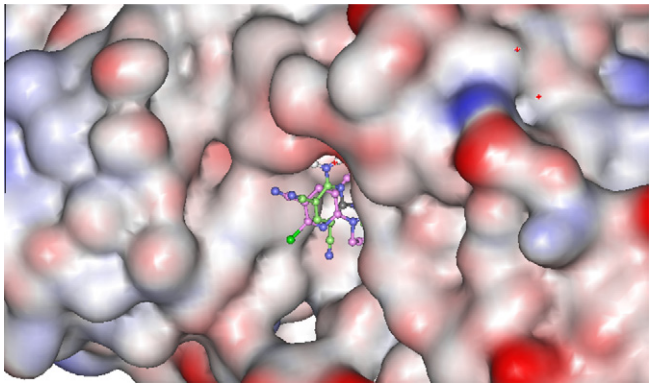
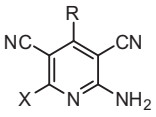
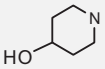
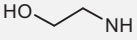
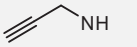
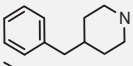
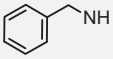
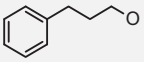


Figure 3. Superposition of the preferred ligand orientation for inhibitor **4** (X = NH_2), and **23** (X = Cl) in the PAS, showing the part of the ligand that points to the outer site of enzyme.

evaluated with the methods of LDH release[42] and MTT metabolic reduction[43] methods, at the concentration of 1 μM, in SH-SY5Y neuroblastoma cells, exposed to 30 μM rotenone plus 10 μM oligomycin-A for 24 h. As shown in Tables 7 and 8, the neuroprotective

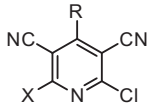
Table 7
Neuroprotection induced by compounds **3**–**20** (1 μM) in human neuroblastoma cells stressed with a mixture of rotenone/oligomycin-A[a]



Product	R	X	% Protection (at 1 μM)	
			LDH	MTT
3	H	Me_2N	36.2	12.9
4	H		nd	20.1
5	H		14.8	10.2
6	H		nd	0
7	H		15.1	3
8	H		3	21.4
9	H		nd	17.7
10	H		nd	19.1
11	Ph		nd	0
12	Ph		13.1	11.1
13	Ph		10.7	2.7
14	Ph		nd	0.7
15	Ph		nd	10.6
16	H	MeO	nd	11
17	H	EtO	10.9	32.9
18	H		nd	0
19	Ph		3.84	11.2
20	Ph	SH	14.1	5.8

* $p < 0.05$. nd = not determined.
[a] Each experiment has been repeated three times. The results are the averaged values.

Table 8
Neuroprotection induced by compounds **21**–**28** (1 μM) in human neuroblastoma cells stressed with the combination of rotenone/oligomycin-A[a]



Product	R	X	% Protection (at 1 μM)	
			LDH	MTT
21	H	Me_2N	nd	6
22	H		32.0	7.5
23	H		nd	14.7
24	H		31.6	7.5
25	Ph	Me_2N	nd	0
26	Ph		37.7	7.5
27	Ph		nd	0
28	Ph		nd	12.9

* p <0.05. nd = not determined.
[a] Each experiment has been repeated three times. The results are the averaged values.

profile of the target molecules ranged from moderate to low for both LDH and MTT determinations. In the LDH test, compounds **26**, **3**, **22**, and **24** were the ones that showed more neuroprotection with values in the range 32–38%. In the MTT test only compound **17** (32.9%) showed a similar profile. This difference could be due to the fact that LDH is measuring necrotic cell death, while MTT is measuring mitochondrial damage and apoptotic cell death. Regarding the families of compounds, it seems again, as observed for the cholinergic inhibition, that the 2-chloropyridine-3,5-dicarbonitriles are more potent than the 2-aminopyridine-3,5-dicarbonitriles. In particular, the selective AChE inhibitors **3**, **22**, **24**, and **26** showed also the highest neuroprotection values in the LDH experiment, whereas the selective BuChE inhibitor **5** showed a modest neuroprotective profile. Conversely, the inactive cholinergic compound **18** showed modest activity in the MTT assay (32.9%) and low activity in the LDH test (10.9%).

3. Conclusions

In this work we have synthesized a series of simple, and readily available 2-aminopyridine-3,5-dicarbonitriles (**3**–**22**), and 2-chloropyridine-3,5-dicarbonitriles (**21**–**28**) derived from 2-amino-6-chloropyridine-3,5-dicarbonitrile (**1**) and 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile (**2**). The biological evaluation showed that some of these molecules were modest AChE/BuChE inhibitors, in the micromolar range; however, some compounds were highly selective for AChE (i.e., 2-amino derivatives **3**, **4**; 2-chloro derivatives **21**–**23**, **25**, **26**), and for BuChE (2-amino derivatives **5**, **14**); no inhibitor caused inhibition of both cholinesterases with IC_{50} of less than 50 μM. Kinetic studies on compound **23** proved that this is a mixed-type inhibitor showing a K_i of 6.33 μM. No clear SAR can be obtained form these data, but it seems that compounds bearing small groups such as the *N*,*N*′-dimethylamino or the pyrrolidino, regardless of the presence of a 2-amino, or 6-chloro substituent in the pyridine ring, preferentially inhibit AChE. Docking analysis on selected inhibitors **4**, **5**, **22**, and **23** have been carried out to give a better insight into the binding mode of the active molecules in the CAS/PAS active sites of AChE. The most important differences in binding to active sites relay on the modifications of the group at C2, as the amino group forms two hydrogen bonds that direct the binding mode, while in the case of compounds with a chlorine atom, this is not possible. Cholinergic inhibitors **3**, **17**, **22**, **24**, and **26** showed also the highest neuroprotection values in the LDH/MTT tests for the rotenone/oligomycin-A assay, affording neuroprotection around 30%. Consequently, these compounds might be further developed to attractive multipotent molecules acting on two key pharmacological mechanisms having a relevant role in the pathogenesis of Alzheimer's disease, that is, cholinergic dysfunction and oxidative stress, and neuronal vascular diseases.

4. Experimental part

4.1. General methods

Melting points were determined on a Koffler apparatus, and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at 300, 400, or 500 MHz and at 75, 100 or 125 MHz, respectively, using solvent peaks [$CDCl_3$: 7.27 (*D*), 77.2 (*C*) ppm; D_2O: 4.60 ppm and DMSO-d_6: 2.49 (*D*), 40 (*C*) ppm] as internal reference. The assignment of chemical shifts is based on standard NMR experiments (^{1}H, ^{13}C-DEPT, ^{1}H, ^{1}H-COSY, gHSQC, gHMBC). Mass spectra were recorded on a GC/MS spectrometer with an API-ES ionization source. Elemental analyses were performed at CNQO (CSIC, Spain). TLC were performed on Silica F254 and detection by UV light at 254 nm or by charring with either ninhydrin, anisaldehyde or phosphomolybdic-H_2SO_4 dyeing reagents. Anhydrous solvents were used in all experiments. Column chromatography was performed on Silica Gel 60 (230 mesh).

4.1.1. General procedure for the synthesis of 6-N-substituted 2-amino-3,5-dicyanopyridines

The corresponding 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1** or **2**) was suspended in a 3:1 mixture of THF/EtOH (15 mL/mmol of starting pyridine). An excess of the secondary amine (see Table 1, entries a–m) was then added, followed by triethylamine (1.0 equiv). The mixture was then heated under reflux. After cooling, the solvent was removed in vacuum and the resulting crude submitted to flash column chromatography, to give compounds **3–15**.

4.1.1.1. 2-Amino-6-dimethylamino-pyridine-3,5-dicarbonitrile (3). Following the general procedure, reaction of 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1**) (0.4 g, 1.685 mmol), with dimethylamine (33% in ethanol) (0.8 mL) in THF/EtOH (10:5, 15 mL), after 1 h, gave product **3** (0.305 g, 98%) as a white solid: R_f = 0.52 (CH_2C_2/AcOEt, 10:1, v/v); mp 170–172 °C; IR (KBr) *v* 3408, 3350, 3240, 3037, 2212, 1666, 1600, 1549, 1509, 1484, 1423, 1403, 1286 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 (s, 1H, CH), 5.28 (s, 2H, NH_2), 3.30 (s, 6H, 2 × CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.1 (C6), 159.0 (C2), 149.9 (C4), 118.9, 116.5 (2 × CN), 81.3, 80.3 (C3, C5), 40.3 (2 × CH_3); MS (EI) *m/z* (%): 172 (61) [M−CH_3]$^+$, 187 (100) [M]$^+$. Anal. Calcd for $C_9H_9N_5$: C, 57.74; H, 4.85; N, 37.41. Found: C, 57.61; H, 4.80; N, 37.24.

4.1.1.2. 2-Amino-6-(pyrrolidin-1′-yl)pyridine-3,5-dicarbonitrile (4). Following the general procedure, reaction of 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1**) (357 mg, 2 mmol) with pyrrolidine (0.2 mL, 2.4 mmol, 1.2 equiv) and NEt_3 (0.28 mL, 2 mmol, 1 equiv) in THF/EtOH (3:1, 30 mL), after 90 min, gave the known compound **4**[33] (402 mg, 95%).

4.1.1.3. 2-Amino-6-(piperidin-1′-yl)pyridine-3,5-dicarbonitrile (5). Following the general procedure, reaction of 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1**) (0.3 g, 1.685 mmol) with piperidine (0.33 mL, 3.37 mmol) in THF/ EtOH (10:5, 15 mL), after 90 min, gave compound **5**[33] (0.375 g, 98%).

4.1.1.4. 2-Amino-6-(7′-azabicyclo[2.2.1]heptan-7′-yl)pyridine-3,5-dicarbonitrile (6). Following the general procedure, reaction of 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1**) (57 mg, 0.32 mmol) with 7-azabicyclo[2.2.1]heptane hydrochloride[32] (52 mg, 0.39 mmol) and NEt_3 (0.05 mL, 0.39 mmol, 1.2 equiv) in THF/EtOH (3:1, 4.8 mL), after 36 h, and flash column chromatography (40% hexane in DCM), gave compound **6** (69 mg, 90%) as a solid: mp 189–191 °C; IR (KBr) ν 3469, 3347, 2208, 1627, 1599, 1527, 1460, 1360, 1138 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.69 (s, 1H, H4), 5.39–5.18 (br s, 2H, NH_2), 5.04 (s, 2H, H1′, H4′), 1.93–1.70 (m, 4H, H2′, H3′, H5′, H6′), 1.58–1.44 (m, 4H, H2′, H3′, H5′, H6′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 160.0 (C6), 157.9 (C2), 148.9 (C4), 118.4, 116.5 (2 × CN), 83.1, 81.0 (C3, C5), 57.6 (C1′, C4′), 29.4 (C2′, C3′, C5′, C6′); MS (ES) m/z $[M+1]^+$ 240.2/241.2. Anal. Calcd for $C_{13}H_{13}N_5$: C, 65.25; H, 5.48; N, 29.27. Found: C, 64.98; H, 5.39; N, 29.04.

4.1.1.5. 2-Amino-6-(4-hydroxy-piperidin-1′-yl)pyridine-3,5-dicarbonitrile (7). Following the general procedure, reaction of 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1**) (0.3 g, 1.685 mmol) with 4-hydroxypiperidine (0.34 g, 3.371 mmol) in THF/EtOH (10:5, 15 mL), after 12 h, and flash column chromatography (CH_2C_2/MeOH, 8:1, v/v), gave compound **7** (0.405 g, 99%): R_f = 0.49 (CH_2C_2/MeOH, 8:1, v/v); mp 176–177 °C; IR (KBr) ν 3488, 3442, 3339, 3228, 2939, 2923, 2219, 2196, 1645, 1592, 1529, 1488, 1474, 1457 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.01 (s, CH), 7.38 (br, 2H, NH_2), 4.79 (d, J = 4.5 Hz, 1H, OH), 4.09 (m, 2H, CH_2), 3.75 (m, 1H, CH), 3.39 (m, 2H, CH_2), 1.80 (m, 2H, CH_2), 1.40 (m, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.4 (C6), 158.9 (C2), 150.5 (C4), 118.4, 116.4 (2 × CN), 79.7, 79.6 (C3, C5), 65.2 (CHpip), 44.2 (2 × CH_2 pip), 34.0 (2 × CH_2 pip); MS (IE) (%): 243.1 (100) $[M]^+$, 225 (40) $[M-H_2O]^+$. Anal. Calcd for $C_{12}H_{13}N_5O$: C, 59.25; H, 5.39; N, 28.79. Found: C, 59.27; H, 5.36; N, 28.57.

4.1.1.6. 2-Amino-6-(2-hydroxyethylamine)pyridine-3,5-dicarbonitrile (8). Following the general procedure, reaction of 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1**) (0.3 g, 1.685 mmol) with ethanolamine (0.2 mL, 3.371 mmol) in THF/EtOH (10:5, 15 mL), after 90 min, and flash column chromatography (CH_2Cl_2/MeOH, 8:1, v/v), gave compound **8** (0.338 g, 99%): R_f = 0.55 (CH_2C_2/MeOH, 8:1, v/v); mp 180–182 °C; IR (KBr) ν 3383, 3333, 3229, 2944, 2207, 1638, 1609, 1568, 1512, 1489 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 8.02 (s, 1H, CH), 7.33 (br s, 2H, NH_2), 7.23 (t, J = 5.26 Hz, 1H, NH), 4.72 (t, J = 5.26 Hz, 1H, OH), 3.5 (m, 2H, CH_2), 3.42 (m, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.7 (C6), 158.9 (C2), 147.9 (C4), 116.9, 116.8 (2 × CN), 79.5, 77.8 (C3, C5), 59.2 (CH_2), 43.1 (CH_2); MS (ES) m/z (%): 143.0 (3) $[M-OH(CH_2)_2NH]^+$, 186.0 (7) $[M-OH]^+$, 204.0 (100) $[M+H]^+$, 205 (14) $[M+2H]^+$, 266 (10) $[M+Na]^+$. To a solution of compound **8** (50 mg, 1.685 mmol) in MeOH (10 mL) was added dropwise a recently prepared solution of MeOH saturated with HCl. The mixture was stirred at rt overnight. The precipitate was filtered, washed with ether and dried to give compound **8**. HCl: mp 183–184 °C; ^1H NMR (300 MHz, DMSO-d_6) δ 8.022 (s, 1H, CH), 7.33 (br s, 2H, NH_2), 7.23 (m, 2H, Har), 4.36 (br s, 2H, OH+NH), 3.50 (t, J = 5.2 Hz, 2H, CH_2), 3.42 (t, J = 5.39 Hz, CH_2). Anal. Calcd for $C_9H_{10}ClN_5O$: C, 45.10; H, 4.21; Cl, 14.79; N, 29.22. Found: C, 44.98; H, 4.42, Cl, 14.40; N, 29.16.

4.1.1.7. 2-Amino-6-propargylamine-pyridine-3,5-dicarbonitrile (9). Following the general procedure, reaction of 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1**) (0.36 g, 2.022 mmol) with propargylamine (0.166 mL, 2.427 mmol) and triethylamine (0.3 mL) in EtOH/THF (5:10, 15 mL), after 1 h, gave product **9** (0.394 g, 99%) as a white solid: R_f = 0.21 (CH_2C_2/AcOEt, 10:1, v/v); mp 216–218 °C; IR (KBr) ν 3449, 3339, 3225, 2211, 1631, 1603, 1570, 1512, 1483 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.69 (s, 1H, CH), 5.61 (s, 1H, NH), 5.46 (s, 2H, NH_2), 4.26 (dd, J = 5.3, 2.5 Hz, 2H, CH_2), 2.29 (t, J = 2.5 Hz, 1H, C≡CH); ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.3 (C6), 158.9 (C2), 148.8 (C4), 117.4, 117.2 (2 × CN), 82.1, 80.2 (C3, C5), 79.3 (*C*≡CH), 73.3 (C≡*C*H), 30.4 (CH_2); MS (ES) m/z (%): 159 (75) $[M-CH_2C{\equiv}CH+H]^+$, 198 (100) $[M+H]^+$. Anal. Calcd for $C_{10}H_7N_5$: C, 60.91; H, 3.58; N, 35.51. Found: C, 60.68; H, 3.85; N, 35.42.

4.1.1.8. 2-Amino-6-(4-benzylpiperidin-1′-yl)pyridine-3,5-dicarbonitrile (10). Following the general procedure, reaction of 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1**) (0.3 g, 1.685 mmol) with 4-benzylpiperidine (0.6 mL, 3.371 mmol) in THF/EtOH (10:5, 15 mL), after 12 h, and flash column chromatography (CH_2C_2/AcOEt, 20:1, v/v), afforded compound **10** (0.515 g, 97%): R_f = 0.60 (CH_2C_2/AcOEt, 20:1, v/v); mp 107–109 °C; IR (KBr) ν 3439, 3341, 3227, 2930, 2844, 2214, 1599, 1529, 1494, 1456 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 8.1 (s, 1H, CH), 7.36 (br s, 2H, NH_2), 7.28 (m, 2H, Har), 7.18 (m, 3H, Har), 4.46 (d, J = 13.3 Hz, 2H, CH_2), 2.93 (dt, J = 13.2, 2.21 Hz, 2H, CH_2), 2.52 (d, J = 7.2 Hz, CH_2-Ph), 1.81 (m, 1H, CH), 1.63 (dd, J = 13.2, 2.79, 2H, CH_2), 1.2 (m, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 159.3 (C6), 159.0 (C2), 150.5 (C4), 139.9 (C), 129.0 (2 × CHph), 128.1 (2 × CHph), 125.8 (CHph), 118.4, 116.4 (2 × CN), 79.8, 79.7 (C3, C5), 46.9 (2 × CH_2pip), 42.0 (CHpip), 37.34 (CHpip), 31.6 (2 × CH_2pip); MS (ES+), m/z (%): 211 (61) $[M-Bn-NH_2]^+$; 224 (25) $[M-Bn]^+$; 317 (100) $[M]^+$. Anal. Calcd for $C_{19}H_{19}N_5$: C, 71.90; H, 6.03; N, 22.07. Found: C, 71.79; H, 6.16; N, 22.20.

4.1.1.9. 2-Amino-6-dimethylamino-4-phenylpyridine-3,5-dicarbonitrile (11). Following the general procedure, reaction of 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile (**2**) (0.31 g, 1.217 mmol) with dimethylamine (33% in ethanol) (0.33 mL) in THF/EtOH (10:5, 15 mL), after 1 h, give product **11** (0.24 g, 75%) as a solid: R_f = 0.63 (CH_2C_2/AcOEt, 10:1, v/v); mp 251–253 °C; IR (KBr) ν 3473, 3320, 3221, 2210, 1624, 1586, 1570, 1550, 1515, 1491, 1420, 1400, 1228 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.43–7.54 (m, 5H, CH-ar), 735 (s, 2H, NH_2), 3.21 (s, 6H, 2 × CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 162.4 (C6), 160.6 (C2), 159.8 (C4), 135.9 (C), 130.3 (CH), 129.1 (2 × CH), 129.0 (2 × CH), 118.5, 116.8 (2 × CN), 81.0, 80.6 (C3, C5), 40.4 (2 × CH_3); MS (EI) m/z (%): 262 (110) $[M-H]^+$, 263 (50) $[M]^+$. Anal. Calcd for $C_{15}H_{13}N_5$: C, 68.42; H, 4.98; N, 26.60. Found: C, 68.19; H, 4.90; N, 26.31.

4.1.1.10. 2-Amino-6-(pyrrolidin-1′-yl)-4-phenylpyridine-3,5-dicarbonitrile (12). Following the general procedure, reaction of 6-amino-2-chloro-4-phenylpyridine-3,5-dicarbonitrile (**2**) (509 mg, 2 mmol) with pyrrolidine (0.2 mL, 2.4 mmol, 1.2 equiv) and NEt_3 (0.28 mL, 2 mmol, 1 equiv) in THF/EtOH (3:1, 30 mL), after 90 min, and flash column chromatography (100% DCM), gave known product **12**[34] (360 mg, 62%).

4.1.1.11. 2-Amino-6-(piperidin-1′-yl)-4-phenylpyridine-3,5-dicarbonitrile (13). Following the general procedure, reaction of 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile (**2**) (0.63 g, 2.48 mmol) with piperidine (0.37 mL, 3.72 mmol) in THF/ EtOH (20:10, 30 mL), after 2 h, gave product **13** (0.726 g, 97%) as a solid: mp 199–200 °C; IR (KBr) ν 3474, 3325, 3221, 2202, 1624, 1583,

1567, 1535, 1489, 1449, 1290, 1022 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.52–7.50 (m, 5H, Ph), 5.40 (br s, 2H, NH_2), 3.79 (m, 4H, 2 × CH_2, piperidine), 1.70 (m, 6H, 3 × CH_2, piperidine); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 162.1 (C6), 151.0 (C2), 159.3 (C4), 134.7 (C, Ph), 130.3 (CH, Ph), 128.6 (2 × CH, Ph), 128.5 (2 × CH, Ph), 117.6, 116.4 (2 CN), 83.4, 81.5 (C3, C5), 49.0 (2 × CH_2, piperidine), 25.8 (2 × CH_2, piperidine), 24.3 (CH_2, piperidine); MS (ES) *m/z* (%): 304 (100) $[M+H]^+$, 326 (79) $[M+Na]^+$, 629 (8) $[2M+Na]^+$. Anal. Calcd for $C_{18}H_{17}N_5$: C, 71.27; H, 5.65; N, 23.09. Found: C, 70.99; H, 5.54; N, 22.93.

4.1.1.12. 2-Amino-6-(7′-azabicyclo[2.2.1]heptan-7′-yl)-4-phenylpyridine-3,5-dicarbonitrile (14). Following the general procedure, reaction of 6-amino-2-chloro-4-phenylpyridine-3,5-dicarbonitrile (**2**) (82 mg, 0.32 mmol) with 7-azabicyclo[2.2.1]heptane hydrochloride[32] (52 mg, 0.39 mmol) and NEt_3 (0.05 mL, 0.39 mmol, 1.2 equiv) in THF/EtOH (3:1, 4.8 mL), after 36 h, and flash column chromatography (1:1 hexane/DCM), gave compound **14** (16 mg, 16%), as a solid: mp 184–186 °C; IR (KBr) *ν* 3432, 3337, 3233, 2210, 1637, 1557, 1523, 1497, 1451 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.61–7.42 (m, 5H, Ph), 5.45–5.27 (br s, 2H, NH_2), 5.08 (s, 2H, H1′, H4′), 1.97–1.79 (m, 4H, H2′, H3′, H5′, H6′), 1.60–1.48 (m, 4H, H2′, H3′, H5′, H6′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 161.7 (C6), 160.0 (C2), 158.9 (C4), 134.7 (C, Ph), 130.5, 128.9, 128.6 (CH, Ph), 117.8, 116.6 (2 × CN), 84.1, 82.2 (C3, C5), 57.9 (C1′, C4′), 29.3 (C2′, C3′, C5′, C6′); MS (ES) *m/z* $[M+1]^+$ 316.0/317.0, $[M+23]^+$ 338.0/339.0. Anal. Calcd for $C_{19}H_{17}N_5$: C, 72.36; H, 5.43; N, 22.21. Found: C, 72.08; H, 5.59; N, 21.99.

4.1.1.13. 2-Amino-6-benzylamino-4-phenylpyridine-3,5-dicarbonitrile (15). Following the general procedure, reaction of 2-amino-6-chloro-4-phenylpyridine-3,5-dicarbonitrile (**2**) (0.305 g, 1.2 mmol) with benzylamine (0.17 mL, 1.56 mmol) and triethylamine (0.5 mL, 3.6 mmol) in THF/EtOH (10:5, 15 mL), after 2 h, gave compound **15** (0.355 g, 91%) as solid: mp 180–182 °C; IR (KBr) *ν* 3463, 3338, 2203, 1625, 1587, 1563 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.52 (s, 5H, Ph), 7.39–7.31 (m, 5H, Ph), 5.94 (t, *J* = 5.3 Hz, H, NH), 5.50 (br s, 2H, NH_2), 4.69 (d, *J* = 5.69 Hz, 2H, CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 160.9 (C6), 159.3 (C2), 159.3 (C4), 137.4 (C, Bn), 134.1 (C, Ph), 130.5 (CH, Ph), 128.8 (CH, Ph), 128.8 (CH, Bn), 128.2 (CH, Ph), 127.8 (CH, Bn), 127.7 (CH, Bn), 116.4, 116.3 (2 × CN), 82.6, 80.7 (C3, C5), 45.3 (CH_2); MS (EI) *m/z* (%): 91 (69), 248 (4) $[M-Ph]^+$, 324 (100) $[M-H]^+$, 325 (73) $[M]^+$. Anal. Calcd for $C_{20}H_{15}N_5$: C, 73.83; H, 4.65; N, 21.52. Found: C, 73.59; H, 4.47; N, 21.80.

4.1.1.14. 2-Amino-6-methoxypyridine-3,5-dicarbonitrile (16). A mixture of 6-amino-2-chloropyridine-3,5-dicarbonitrile (**1**) (0.3 g, 1.685 mmol) and NaOMe (0.12 g, 2.191 mmol) in methanol (20 mL) was heated at reflux and stirred for 90 min. Then, the reaction was cooled and 10 mL of water was added. The precipitate was filtered, washed and dried to afford known compound **16**[35] (0.283 g, 96.5%).

4.1.1.15. 2-Amino-6-ethoxy-pyridine-3,5-dicarbonitrile (17). A mixture of 2-amino-6-methoxy-pyridine-3,5-dicarbonitrile (**16**) (0.4 g, 2.298 mmol), sodium (0.52 g, 22.98 mmol) in dry EtOH (25 mL) was stirred at reflux for 2 h. Then, cooled water was added and the precipitate was separated by filtration and washed with water. The yellow solid was purified by column chromatography (CH_2Cl_2/AcOEt, 20:1, v/v), and recrystallized from ethanol to afford known compound **17**[36] (0.393 g, 91%).

4.1.1.16. 2-Amino-6-(3-phenylpropoxy)pyridine-3,5-dicarbonitrile (18). To a solution of 2-amino-6-methoxy-pyridine-3,5-dicarbonitrile (**16**) (0.2 g, 1.149 mmol), 3-phenyl-propanol (0.313 g, 2.198 mmol), and sodium (79 mg, 3.448 mmol) in dry DMF (10 mL) was stirred at reflux for 2 h. Then, cooled water was added and the precipitate was separated by filtration and washed with water. The yellow solid was purified by column chromatography (CH_2Cl_2/AcOEt, 40:1, v/v), and recrystallized from ethanol to obtain compound **18** (0.227 g, 76%): R_f = 0.5 (CH_2C_2/AcOEt, 20:1, v/v); mp 140–142 °C; IR (KBr) *ν* 3433, 3329, 3231, 2219, 1657, 1607, 1544, 1476, 1452 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H, CH), 7.32–7.19 (m, 5H, CH_arom), 5.6 (s, 2H, NH_2), 4.35 (t, *J* = 6.5 Hz, 2H, CH_2), 2.78 (t, *J* = 7.7 Hz, 2H, CH_2), 2.12 (m, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.7 (C6), 160.5 (C2), 147.3 (CH), 140.7 (C, Ph), 128.4 (2 × CH, Ph), 128.4 (2 × CH, Ph), 126.0 (CH, Ph), 115.2, 114.4 (2 × CN), 86.2, 83 (C3, C5), 67.14 (CH_2), 31.71 (CH_2), 92.82 (CH_2); MS (IE), *m/z* (%): 91 (96), 118 (100), 160 (6) $[M-Ph(CH_2)_3]^+$; 278 (30) $[M]^+$. Anal. Calcd for $C_{16}H_{14}N_4O$: C, 69.05; H, 5.07; N, 20.13. Found: C, 69.20; H, 5.18; N, 20.42.

4.1.2. General procedure for the synthesis of 2-chloro-3,5-dicyanopyridines

The corresponding pyridine and $CuCl_2$ (1.5 equiv) were dissolved in dry MeCN under argon. *iso*-Amylnitrite (1.5 equiv) was added, and the mixture heated at 65 °C until TLC analysis showed complete reaction. The solution was acidified (2 N HCl) to pH 3 and extracted with DCM. After drying the organic layer with Na_2SO_4, the solvent was removed in vacuum and the crude product purified by flash column chromatography using hexane–DCM mixtures.

4.1.2.1. 2-Chloro-6-(dimethylamino)pyridine-3,5-dicarbonitrile (21). Following the general procedure, reaction of 2-amino-6-(dimethylamino)pyridine-3,5-dicarbonitrile (**3**) (0.187 g, 1 mmol) with $CuCl_2$ (0.2 g, 1.5 mmol) and *iso*-amylnitrite (0.2 mL, 1.5 mmol), in acetonitrile (8 mL), after 2 h, gave product **21** (0.115 g, 56%) as a solid: mp 155–157 °C; IR (KBr) *ν* 2226, 1599, 1559, 1494, 1424, 1407, 1280 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.91 (s, 1H, H-4), 3.41 (s, 6H, 2 × CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 157.1 (C6), 154.2 (C2), 149.8 (C4), 116.2, 114.4 (2 × CN), 96.5 (C5), 88.7 (C3), 40.4 (2 × CH_3); MS (EI) *m/z* (%) 177 (39), 191 (51) $[M-CH_3]^+$, 206 (37) $[M]^+$. Anal. Calcd for $C_9H_7ClN_4$: C, 52.31; H, 3.41; Cl, 17.16; N, 27.11. Found: C, 52.60; H, 3.54; Cl, 16.80; N, 27.02.

4.1.2.2. 2-Chloro-6-(pyrrolidin-1′-yl)pyridine-3,5-dicarbonitrile (22). Following the general procedure, 2-amino-6-(pyrrolidin-1′-yl)pyridine-3,5-dicarbonitrile (**4**) (43 mg, 0.2 mmol), with $CuCl_2$ (40.3 mg, 0.3 mmol, 1.5 equiv) and *iso*-amylnitrite (40.3 μL, 0.3 mmol, 1.5 equiv), in acetonitrile (1.6 mL), after 12 h, provided compound **22** [(39 mg, 84%; flash column chromatography (40% DCM in hexane)]: mp 168–170 °C; IR (KBr) *ν* 2223, 1596, 1550, 1486, 1409, 1335, 1276, 1063 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.88 (s, 1H, H4), 4.15–3.56 (br s, 4H, H2′, H5′), 2.06 (s, 4H, H3′, H4′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 155.2 (C6), 155.0 (C2), 149.6 (C4), 117.0, 115.0 (2 CN), 96.3, 89.1 (C3, C5), 50.0 (C2′, C5′), 29.9 (C3′, C4′); MS (ES) *m/z* $[M+1]^+$ 233.1/234.9. Anal. Calcd for $C_{11}H_9ClN_4$: C, 56.78; H, 3.90; Cl, 15.24; N, 24.08. Found: C, 56.50; H, 4.01; Cl, 15.27; N, 24.29.

4.1.2.3. 2-Chloro-6-(piperidin-1-yl)pyridine-3,5-dicarbonitrile (23). Following the general procedure, reaction of 2-amino-6-(piperidin-1-yl)pyridine-3,5-dicarbonitrile (**5**) (0.3 g, 1.32 mmol), with $CuCl_2$ (0.236 g, 1.98 mmol) and *iso*-amylnitrite (0.266 mL, 1.98 mmol), in acetonitrile (10 mL), after 12 h, gave compound **23** (0.234 g, 0.951 mmol, 72%): mp 113–114 °C; IR (KBr) *ν* 2926, 2227, 2218, 1714, 1599, 1544, 1486, 1406, 1336, 1251 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.88 (s, 1H, H4), 3.91 (m, 4H, 2 × CH_2), 1.71 (m, 6H, 3 × CH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.8 (C6), 154.4 (C2), 149.9 (C4), 116.2, 114.4 (2 × CN), 96.5, 89.3 (C3, C5), 48.6 (2 × CH_2), 25.8 (2 × CH_2), 23.9 (CH_2); MS (IE), *m/z*(%): 84 (47), 217

(100), 246 (79) $[M]^+$. Anal. Calcd for $C_{12}H_{11}ClN_4$: C, 58.42; H, 4.49; Cl, 14.37; N, 22.71. Found: C, 58.25; H, 4.37; Cl, 14.3; N, 22.76.

4.1.2.4. 2-Chloro-6-(7′-azabicyclo[2.2.1]heptan-7′-yl)pyridine-3,5-dicarbonitrile (24). Following the general procedure, 2-amino-6-(7′-azabicyclo[2.2.1]heptan-7′-yl)pyridine-3,5-dicarbonitrile (**6**) (47.9 mg, 0.2 mmol), with $CuCl_2$ (40.3 mg, 0.3 mmol, 1.5 equiv) and *iso*-amylnitrite (40.3 μL, 0.3 mmol, 1.5 equiv), in acetonitrile (1.6 mL), after 90 min, gave compound **24** [(34 mg, 0.131 mmol, 66%, flash column chromatography (30% DCM in hexane)], as a solid: mp 124–126 °C; IR (KBr) ν 3068, 2948, 2230, 1594, 1520, 1574, 1454, 1403, 1260, 1204, 1163 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.86 (m, 1H, H4), 5.18 (s, 2H, H1′, H4′), 2.01–1.77 (m, 4H, H2′, H3′, H5′, H6′), 1.73–1.53 (m, 4H, H2′, H3′, H5′, H6′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 155.6 (C6), 155.3 (C2), 149.3 (C4), 116.4, 114.8 (2 × CN), 97.4, 90.1 (C3, C5), 58.2 (C1′, C4′), 29.4 (C2′, C3′, C5′, C6′); MS (ES) m/z $[M+1]^+$ 259.0/261.0/260.0, $[M+23]^+$ 281.0/283.0/282.0. Anal. Calcd for $C_{13}H_{11}ClN_4$: C, 60.35; H, 4.29; Cl, 13.70; N, 21.66. Found: C, 60.18; H, 4.31; Cl, 13.17; N, 21.54.

4.1.2.5. 2-Chloro-6-(dimethylamino)-4-phenylpyridine-3,5-dicarbonitrile (25). Following the general procedure, reaction of 2-amino-6-(dimethylamino)-4-phenylpyridine-3,5-dicarbonitrile (**11**) (0.288 g, 1.095 mmol) with $CuCl_2$ (0.218 g, 1.642 mmol) and *iso*-amylnitrite (0.22 mL, 1.642 mmol), in acetonitrile (10 mL), after 12 h, gave product **25** (0.207 g, 67%) as a solid: mp 158–160 °C; IR (KBr) ν 2223, 1583, 1571, 1486, 1420, 1410, 1271, 1184 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.58–7.46 (m, 5H, Ph), 3.43 (s, 6H, 2 × CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 163.0 (C6), 158.4 (C2), 154.5 (C4), 133.4 (C, Ph), 130.8 (CH, Ph), 128.8 (2 × CH, Ph), 128.4 (2 × CH, Ph), 115.8, 114.5 (2 × CN), 97. (C3), 89.1 (C5), 40.9 (2 × CH_3); MS (EI) m/z (%) 281 (100) $[M-H]^+$, 282 (36) $[M]^+$. Anal. Calcd for $C_{15}H_{11}ClN_4$: C, 63.72; H, 3.92; Cl, 12.54; N, 19.82. Found: C, 63.55; H, 4.03; Cl, 12.42; N, 19.69.

4.1.2.6. 2-Chloro-6-(pyrrolidin-1′-yl)-4-phenylpyridine-3,5-dicarbonitrile (26). Following the general procedure, 2-amino-6-(pyrrolidin-1′-yl)-4-phenylpyridine-3,5-dicarbonitrile (**12**) (57.9 mg, 0.200 mmol), with $CuCl_2$ (40.3 mg, 0.3 mmol, 1.5 equiv) and *iso*-amylnitrite (40.3 μL, 0.3 mmol, 1.5 equiv), in acetonitrile (1.6 mL), after 17 h, provided compound **26** [(39.5 mg, 0.128 mmol, 64%; flash column chromatography (1:1 DCM/hexane)] as a solid: mp 210–212 °C; IR (KBr) ν 2223, 2211, 1580, 1562, 1535, 1482, 1454, 1335, 1265, 1248 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.63–7.39 (m, 5H, Ph), 4.08–3.76 (br s, 4H, H2′, H5′), 2.05 (s, 4H, H3′, H4′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 161.9 (C6), 155.0, 154.3 (C2, C4), 132.8 (C, Ph), 130.0, 128.1, 127.6 (CH, Ph), 115.4, 114.0 (2 × CN), 96.5, 88.3 (C3, C5), 49.3 (C2′, C5′), 28.8 (C3′, C4′); MS (ES) m/z $[M+1]^+$ 309.0/311.0/310.0, $[M+23]^+$ 331.0/333.0/332.0. Anal. Calcd for $C_{17}H_{13}ClN_4$: C, 66.13; H, 4.24; Cl, 11.48; N, 18.15. Found: C, 65.98; H, 4.36; Cl, 10.88; N, 18.01.

4.1.2.7. 2-Chloro-4-phenyl-6-(piperidin-1-yl)pyridine-3,5-dicarbonitrile (27). Following the general procedure, reaction of 2-amino-6-(piperidin-1′-yl)-4-phenylpyridine-3,5-dicarbonitrile (**13**) (0.315 mg, 1.038 mmol) with $CuCl_2$ (0.21 g, 1.578 mmol) and *iso*-amylnitrite (0.21 mL, 1.578 mmol), in acetonitrile (10 mL), after 12 h, gave product **27** (0.198 mg, 60%) as a solid: mp 113–114 °C; IR (KBr) ν 2937, 2862, 2219, 1584, 1566, 1484, 1471, 1260 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 1.75 (m, 6H), 3.92 (m, 6H), 7.47–7.56 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 163.0 (C6), 158.5 (C4), 154.8 (C2), 133.5 (C), 130.9, 128.9, 128.5 (CH-Ph), 115.7, 114.1 (2 × CN), 97.83 (C3), 90.1 (C5), 49.33 (C2′, C6′), 25.89 (C3′, C5′), 23.9 (C4′); MS (ES) m/z 323.3, $[M+23]^+$ 345.2. Anal. Calcd for $C_{18}H_{15}ClN_4$: C, 66.98; H, 4.68; Cl, 10.98; N, 17.36. Found: C, 67.12; H, 4.71; Cl, 11.20; N, 17.22.

4.1.2.8. 2-Chloro-6-(7′-azabicyclo[2.2.1]heptan-7′-yl)-4-phenylpyridine-3,5-dicarbonitrile (28). Following the general procedure, 2-amino-6-(7′-azabicyclo[2.2.1]heptan-7′-yl)-4-phenylpyridine-3,5-dicarbonitrile (**14**) (20.6 mg, 0.065 mmol) with $CuCl_2$ (13.1 mg, 0.098 mmol) and *iso*-amylnitrite (13.1 μL, 0.098 mmol), in acetonitrile (0.8 mL), after 6 h, afforded compound (**28**) (11 mg, 50%), after flash column chromatography (30% DCM in hexane), as a solid: mp 195–197 °C; IR (KBr) ν 2227, 1560, 1499, 1480 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.66–7.39 (m, 5H, Ph), 5.23 (s, 2H, H1′, H4′), 2.04–1.81 (m, 4H, H2′, H3′, H5′, H6′), 1.74–1.55 (m, 4H, H2′, H3′, H5′, H6′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 162.6 (C6), 156.1, 156.0 (C2, C4), 133.5 (C, Ph), 131.1, 129.1, 128.6 (CH, Ph), 116.2, 114.8 (2 × CN), 98.4, 90.6 (C3, C5), 58.4 (C1′, C4′), 29.4 (C2′, C3′, C5′, C6′); MS (ES) m/z $[M+1]^+$ 335.2/337.2/336.2. Anal. Calcd for $C_{19}H_{15}ClN_4$: C, 68.16; H, 4.52; Cl, 10.59; N, 16.73. Found: C, 67.90; H, 4.69; Cl, 10.34; N, 17.07.

4.2. Pharmacology

4.2.1. Measurements of AChE and BuChE inhibition

To assess the inhibitory activity of the compounds towards AChE and BuChE, we followed the spectrophotometric method of Ellman,[40a] using purified AChE from *Electrophorus electricus* (type V-S), human erythrocytes (buffered aqueous solution) or BuChE from equine serum (lyophilized powder) (Sigma–Aldrich, Madrid, Spain). The reaction took place in a final volume of 3 mL of a phosphate-buffered solution (0.1 M) at pH 8, containing 0.035 U of AChE or 0.05 U of BuChE, and 0.35 mM of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Sigma–Aldrich, Madrid, Spain). Inhibition curves were made by pre-incubating this mixture with at least nine concentrations of each compound for 10 min. A sample with no compound was always present to determine the 100% of enzyme activity. After this pre-incubation period, acetylthiocholine iodide (0.35 mM) or butyrylthiocholine iodide (0.5 mM) (Sigma–Aldrich, Madrid, Spain) were added, allowing an additional 15 min incubation period in which DTNB produces the yellow anion 5-thio-2-nitrobenzoic acid along with the enzymatic degradation of acetylthiocholine iodide or butyrylthiocholine iodide. Changes in absorbance were detected at 405 nm in a spectrophotometric plate reader (FluoStar OPTIMA, BMG Labtech). Compounds inhibiting AChE or BuChE activity would reduce the color generation; thus IC_{50} values were calculated as the concentration of compound that produces 50% AChE inhibition of the enzyme activity. Data are expressed as mean ± SEM of at least three different experiments in quadruplicate.

4.2.2. Kinetic analysis of AChE inhibition

To obtain estimates of the competitive inhibition constant K_i, reciprocal plots of $1/V$ versus $1/[S]$ were constructed at different concentrations of the substrate acetylthiocholine (0.1–1 mM) by using Ellman's method.[40a] The plots were assessed by a weighted least square analysis. Slopes of these reciprocal plots were then plotted against the concentration of compound **23** (range 0–90 μM) in a weighted analysis and K_i was determined as the intercept on the negative x-axis. For the determination of the noncompetitive inhibition constant (K_I), intercepts of the reciprocal plots were also plotted against the concentration of compound **23** (range 0–90 μM) in a weighted analysis and K_I was determined as the intercept on the negative x-axis. Data analysis was performed with Origin Pro 7.5 software (Origin Lab Corp.).

4.2.3. Quantification of viability of SH-SY5Y cells using the mitochondrial probe MTT

Cell death and neuroprotection afforded by synthesized compounds was studied in human neuroblastoma cell line, a kind gift from Dr. F. Valdivieso from the Centro de Biología Molecular

(Madrid, Spain). SH-SY5Y cells were maintained in a 1:1 mixture of F-12 Nutrient Mixture (Ham12) and Eagle's minimum essential medium (EMEM) (Sigma–Aldrich, Madrid, Spain) supplemented with 15 non-essential amino acids, 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (reagents from Invitrogen, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For assays, SH-SY5Y cells were subcultured in 48-well plates at a seeding density of 1×10^5 cells per well. Cells were treated with the drugs before confluence in EMEM with 1% FBS. Cells were used at a low passage number (<13). Cell viability, virtually the mitochondrial activity of living cells, was measured by quantitative colorimetric assay with MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma–Aldrich, Madrid, Spain), as described previously.[43] SH-SY5Y cells were seeded into 48-well culture plates and allowed to attach. MTT was added to all wells (5 mg/mL) and allowed to incubate, in the dark at 37 °C for 2 h followed by cell lysis and spectrophotometric measurement at 540 nm. The tetrazolium ring of MTT can be cleaved by active reductases in order to produce a precipitated formazan derivative. The formazan produced was dissolved by adding 200 μL DMSO, resulting in a colored compound whose optical density was measured in an ELISA reader at 540 nm. All MTT assays were performed in triplicate.

4.2.4. Measurement of cell death and neuroprotection taking as indicator of the release of lactate dehydrogenase (LDH)

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. At the end of the 24-h incubation period with oligomycin/rotenone, samples were collected to estimate extracellular LDH as indication of cell death.[42] Intracellular LDH activity was measured in the cells after incubation with 10% Triton X-100. LDH activity was measured at 490–620 nm, using a microplate reader (Labsystems iEMS reader MF; Labsystems, Helsinki, Finland). Total LDH (intracellular plus extracellular) was normalized to 100%; then, the amount of LDH released to the extracellular medium was expressed as percentage of this total. Data were normalized by subtracting basal LDH (cells not subjected to any treatment) to the different treatment groups in each individual experiment, and the result for rotenone/oligomycin-A group was normalized to 100% (percentage cell death).

4.2.5. Computational methods

Geometry of the ligand was optimized using GAUSSIAN03[44] and the B3LYP/6-31G* basis set. Partial atomic charges were then obtained using the RESP methodology.[45] As target, the X-ray crystallographic structure of the hAChE–fasciculin complex (PDB code 1B41)[46] was used. Fasciculin was removed from the structure, truncated residues were reconstructed, and missing residues were modeled. Docking was performed with the program AutoDock 4.0.[47] A box encompassing both catalytic and peripheral binding sites was defined for the exploration of possible binding modes. The Lamarckian genetic algorithm (LGA) implemented in AutoDock was then used to generate docked conformations of the ligand by randomly changing the overall orientation of the molecule as well as the torsion angles of all rotatable bonds. Default settings were used except for number of runs, population size, and maximum number of energy evaluations, which were fixed at 100, 100, and 250.000, respectively. Rapid intra- and intermolecular energy evaluation of each configuration was achieved by having the enzyme's atomic affinity potential for aliphatic and aromatic carbon, oxygen, nitrogen, and hydrogen atoms precalculated in a three-dimensional grid with a spacing of 0.375 Å. In a second round of docking experiments, we built smaller grids around both potential binding sites (0.200 Å). A distance-dependent dielectric function was used in the computation of electrostatic interactions. After docking, the solutions were clustered in groups with root mean square deviations less than 1.0 Å. The clusters were ranked by the lowest energy representative of each cluster.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.095. These data include MOL files and InChiKeys of the most important compounds described in this article.

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