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Nontoxic and Neuroprotective β-Naphthotacrines for Alzheimer's Disease

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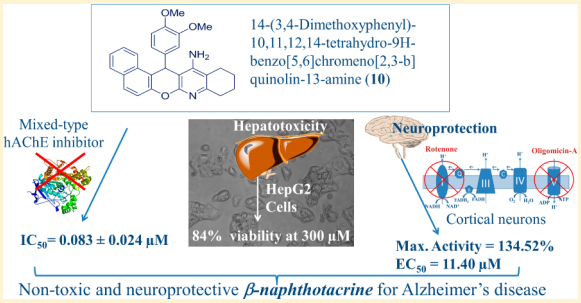
Supporting Information

ABSTRACT: The synthesis, toxicity, neuroprotection, and human acetylcholinesterase (hAChE)/ human butyrylcholinesterase (hBuChE) inhibition properties of *β-naphthotacrines* **1**−**14** as new drugs for Alzheimer's disease (AD) potential treatment, are reported. *β-Naphthotacrines* **1**−**14** showed lower toxicity than tacrine; moreover, at the highest concentration assayed (300 μM) compounds **7**, **10** and **11** displayed 2.25−2.01-fold higher cell viability than tacrine in HepG2 cells. A neuroprotective effect was observed for compounds **10** and **11** in a neuronal cortical culture exposed to a combination of oligomycin A/rotenone. An efficient and selective inhibition of hAChE, was only observed for the *β-naphthotacrines* bearing electron-donating substituents at the aromatic ring, *β-naphthotacrine* **10** being the most potent (hAChE: IC_{50} = 0.083 ± 0.024 μM). Kinetic inhibition analysis clearly demonstrated that *β-naphthotacrine* **10** behaves as a mixed-type inhibitor (K_{i2}= 0.72 ± 0.06 μM) at high substrate concentrations (0.5−10 μM), while at low concentrations (0.01−0.1 μM) it behaves as a hAChE competitive inhibitor (K_{i1}= 0.007 ± 0.001 μM). These findings identified *β-naphthotacrine* **10** as a potent and selective hAChE inhibitor in a nanomolar range, with toxicity lower than that of tacrine both in human hepatocytes and rat cortical neurons, with a potent neuroprotective activity and, consequently, an attractive multipotent active molecule of potential application in AD treatment.



INTRODUCTION

Alzheimer's disease (AD) is the most prominent form of dementia in the world affecting about 6% of the population aged over 65 and whose incidence increases with age.[1] Despite huge efforts and numerous successes in the investigation of AD pathophysiology, the disease is still incurable.[2] AD is clinically characterized by memory impairment and progressive deficit in different cognitive domains related to a pronounced cholinergic system degradation and to an alteration in other neurotransmitter systems.[3] The cholinergic hypothesis of AD[4] asserts that declination of acetylcholine (ACh) levels leads to cognitive and memory deficits; therefore, sustaining or recovering cholinergic function is supposed to be clinically beneficial.[5] ACh can be degraded by two types of cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Indeed, nowadays AD therapy is bolstered mainly by AChE inhibitors (AChEI), such as tacrine, rivastigmine, donepezil and galanthamine, which are able to increase the ACh level in the cholinergic synapses.[6] Unfortunately, instead of curing or preventing the neurodegeneration, AChEI only enable a palliative treatment,[7] and their clinical effectiveness is still under debate.[8] Taking into account AD multifactorial nature, the traditional approach of single-target molecules generally offers only limited and transient benefits. Multitarget-directed ligand strategy (MTDL)[9] has been recently applied to AChEIs[10] research, which means that the focus has been on pharmacophores with other properties besides cholinesterase inhibition.

In this complex scenario, tacrine (Table 1), the most potent AChEI clinically effective,[11] was approved for clinical use by the United States Federal Drug Agency (FDA) in 1993. However, tacrine exhibited hepatotoxicity via elevation of serum alanine aminotransferase levels and thus showed limited clinical application. In consequence, tacrine was withdrawn from the

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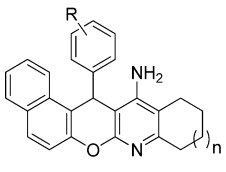
Table 1. In Vitro Toxicity of Tacrine, and the *β-Naphthotacrines* 1−14 in HepG2 Cells[a]



	R	n		R	n
1	H	1	**8**	4'-OH,3'-OMe	1
2	4'-Me	1	**9**	2',4'-di-OMe	1
3	4'-OH	1	**10**	3',4'-di-OMe	1
4	4'-OMe	1	**11**	3',4',5'-tri-OMe	1
5	3'-OMe	1	**12**	4'-NO_2	1
6	2'-OMe	1	**13**	2',6'-di-Cl	1
7	4'-OH,3'-OMe	0	**14**	3',4'-di-Cl	1

	viability (%) HepG2 cells					
cmpd	1 μM[e]	3 μM[e]	10 μM[e]	30 μM[e]	100 μM[e]	300 μM[e]
1	100.2 ± 0.46[ns]	87.6 ± 2.03[d]	84.5 ± 1.03[c]	75.4 ± 3.84[b]	67.5 ± 1.81[b]	52.9 ± 2.58[b]
2	96.4 ± 0.75[ns]	93.9 ± 2.12[ns]	83.7 ± 0.99[b]	86.2 ± 1.02[c]	71.2 ± 0.23[b]	70.1 ± 0.18[b]
3	100.2 ± 0.83[ns]	99.6 ± 0.26[ns]	93.2 ± 1.65[ns]	71.5 ± 4.98[c]	67.7 ± 4.97[b]	64.9 ± 4.92[b]
4	80.8 ± 1.26[b]	76.3 ± 0.61[b]	82.6 ± 0.27[b]	80.1 ± 0.07[b]	82.7 ± 0.92[b]	65.8 ± 0.77[b]
5	97.2 ± 1.06[ns]	93.7 ± 0.40[ns]	84.9 ± 0.87[b]	85.3 ± 0.87[b]	82.3 ± 1.4[b]1	79.1 ± 0.20[b]
6	99.9 ± 0.50[ns]	74.4 ± 0.26[c]	73.0 ± 0.41[b]	71.8 ± 0.52[b]	74.3 ± 1.58[b]	65.8 ± 0.09[b]
7	100.1 ± 1.13[ns]	97.6 ± 0.92[ns]	96.2 ± 1.20[ns]	90.1 ± 0.33[d]	84.5 ± 1.20[c]	85.1 ± 1.46[c]
8	98.2 ± 0.98[ns]	94.3 ± 1.28[ns]	96.8 ± 0.91[ns]	97.0 ± 0.93[ns]	92.2 ± 0.58[d]	74.9 ± 0.43[b]
9	99.5 ± 1.37[ns]	97.1 ± 1.09[ns]	86 ± 0.44[c]	80.9 ± 1.67[b]	74.9 ± 2.78[b]	36 ± 2.60[b]
10	99.2 ± 0.41[ns]	93.6 ± 0.47[ns]	91.5 ± 2.14[d]	90.1 ± 0.30[c]	81.8 ± 0.1[b]0	84.1 ± 0.37[b]
11	99.1 ± 0.39[ns]	97.3 ± 0.11[ns]	98.0 ± 1.36[ns]	96.7 ± 1.11[ns]	95.1 ± 0.57[ns]	90.9 ± 0.68[c]
12	100.3 ± 1.42[ns]	98.4 ± 0.65[ns]	97.8 ± 0.56[ns]	97.5 ± 0.32[ns]	90.9 ± 0.37[c]	83.1 ± 0.76[b]
13	100 ± 1.20[ns]	99.7 ± 1.08[ns]	98.3 ± 1.68[ns]	93.9 ± 0.62[ns]	79.8 ± 2.04[b]	61.5 ± 1.13[b]
14	100.1 ± 0.79[ns]	96.8 ± 0.61[nse]	97.2 ± 1.77[nse]	96.1 ± 0.74[ns]	92.2 ± 0.38[ns]	77.5 ± 4.61[b]
tacrine	93.4 ± 4.69[ns]	90 ± 2.95[ns]	88.7 ± 3.42[ns]	81.6 ± 4.88[d]	64.3 ± 4.54[b]	40 ± 2.20[b]

[a]Cell viability was measured as MTT reduction, and data were normalized as % control. Data are expressed as the means ± SEM of triplicate of at least three different cultures. All compounds were assayed at increasing concentrations (1−300 μM). Comparisons between drugs and control group were performed by one-way ANOVA followed by the Newman−Keuls posthoc test. [b] $P \leq 0.001$. [c] $P \leq 0.01$. [d] $P \leq 0.05$. [e] ns = not significant, with respect to control group.

pharmaceutical market shortly after its approval.[12] For this reason tacrine is not considered as a gold standard for AD drug discovery. In fact, although new AChEIs continue to be under investigation, recent efforts are focused on the development of target molecules in AD's underlying pathogenic mechanisms, such as the amyloid cascade hypothesis,[13] oxidative stress, and free radical generation.[14] These new approaches and the fact that today most of the funding agencies declare limited interest in cholinesterase inhibitor programs make AChEI drug discovery less relevant from a medicinal chemistry perspective.

In spite of this and because of the high potency of tacrine, this structure has been widely and successfully used in medicinal chemistry for application in hybrid or multitarget compounds.[15,16] In order to combine tacrine's AChE inhibition with other pharmacological properties, tacrine's structure was covalently connected to other pharmacophores,[17] such as CB1 receptor antagonists and an M1 agonist.[18,19] Very well-known tacrine derivatives include the following: (a) **dimer bis(7)-tacrine**,[20] which exhibited a 1000-fold higher AChE inhibition potency, a better pharmacological profile consisting in the inhibition of the AChE-induced Aβ aggregation through the interaction with its peripheral binding site (PAS),[21] and in neuroprotective effects related to the interaction with β-secretase enzyme and NMDA and $GABA_A$ receptors;[22] (b) **cystamine−tacrine dimer**, endowed with a lower toxicity in comparison to that of **bis(7)tacrine**, able to inhibit AChE/BuChE, self- and AChE-induced Aβ aggregation in the same range of the reference compound, and exerting a neuroprotective action on SH-SY5Y cell line against H_2O_2-induced oxidative injury;[23] (c) **tacrine−ferulic acid hybrids** as potent cholinesterase inhibitor (ChEIs) which can block the PAS;[24] (d) **tacrine−organic nitrates**,[25,26] and (e) **tacrine−silibinin codrug** which showed high AChE and BuChE inhibition, neuroprotective effects, no hepatotoxicity in vitro and in vivo, but with the same in vivo pro-cognitive effects as tacrine, being superior to the physical mixture of tacrine and silibinin in all these regards.[27] Particularly interesting among all studied tacrines, **7-MEOTA** (9-amino-7-methoxy-1,2,3,4-tetrahydroacridine), a Czech cholinergic drug first synthesized by Patocka et al.,[28] is a potent, centrally active ChEI free of the serious side effects related to tacrine.[12]

On the basis of these precedents, having worked previously on this topic[15,16] and looking for nontoxic, neuroprotective tacrines as a MTDL strategy for AD, here we report the biological evaluation of several racemic *β-naphthotacrines* (**I**) (Figure 1),

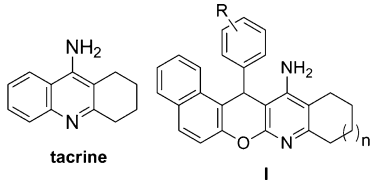


Figure 1. Structures of tacrine and of *β-naphthotacrines* (I).[29]

such as 14-aryl-10,11,12,14-tetrahydro-9*H*-benzo[5,6]-chromeno[2,3-*b*]quinolin-13-amines **1−14** (Table 1).[29] On the basis of this work, we identified *β-naphthotacrine* **10** {14-(3,4-dimethoxyphenyl)-10,11,12,14-tetrahydro-9*H*-benzo[5,6]-chromeno[2,3-*b*]quinolin-13-amine} (Table 1) as a potent and selective human AChEI (hAChEI), neuroprotector in a nanomolar range and nontoxic in human HepG2 cells and, consequently, a promising molecule in AD treatment.

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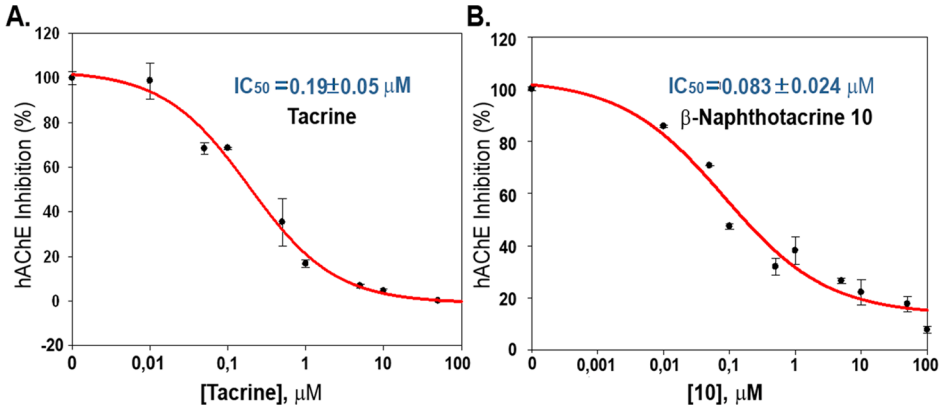

Figure 2. IC_{50} (μM) calculations for hAChE of tacrine (A) and *β-naphthotacrine* **10** (B). IC_{50} values were calculated from hAChE inhibition data, at 10 nM to 100 μM compound concentrations. Fittings were performed by a nonlinear regression analysis by using the function $f1 = \min + (\max - \min)/(1 + (x/IC_{50})(\text{Hillslope})$. Values represent the means ± SEM of three different experiments performed in triplicate.

RESULTS AND DISCUSSION

Chemistry. The synthesis of known racemic 14-aryl-10,11,12,14-tetrahydro-9*H*-benzo[5,6]chromeno[2,3-*b*]-quinolin-13-amines **2**–**8**, and **10**–**12** (Table 1) has been carried out as previously reported.[29] New *β-naphthotacrines* **1**, **9**, **13**, and **14** have been synthesized by Friedländer-type reactions of 3-amino-1-aryl-1*H*-benzo[*f*]chromene-2-carbonitriles **19**–**22**, prepared from the corresponding 2-arylidenemalononitriles **15**–**18**, with selected cycloalkanones (see Supporting Information). We have obtained these compounds by incorporating different substituents in the aromatic ring at C14, taking into account their electronic properties, the number of groups, and the position at the aromatic nucleus. All the new compounds showed analytical and spectroscopic data in good agreement with their structures.

Pharmacology. *Hepatotoxicity of β-Naphthotacrines.* We first investigated, as the most critical and crucial point, hepatotoxicity of *β-naphthotacrines* **1**–**14** on HepG2 cells in a concentration range (1, 3, 10, 30, 100, and 300 μM) as previously described by Denizot and Lang[30] (see Supporting Information). HepG2 cells are considered to be a reasonable model to study in vitro xenobiotic metabolism and liver toxicity. In particular, HepG2 cells are able to activate and detoxify xenobiotics, and they are employed to study drug mechanisms of action.[31] Cytotoxicity of compounds **1**–**14** was determined using the MTT assay. These compounds were well tolerated after a 24 h incubation period in human HepG2 cells compared with tacrine, as shown in Table 1. The hepatotoxicity of tacrine is associated with increased levels of intracellular reactive oxygen species (ROS) and decreased levels of antioxidant defenses.[32] As shown in Table 1, tacrine decreases the number of cells even at the lowest concentration (30 μM), being more hepatotoxic gradually at rising doses. It is worth mentioning that *β-naphthotacrines* **11**, **7**, and **10**, in this order, at the highest concentration used (100 and 300 μM) showed a reduced hepatotoxicity compared with tacrine. Thus, these compounds exhibited a wide therapeutic safety range.

From a structure–activity relationship (SAR) perspective, it seems that electron-donating substituted *β-naphthotacrines* are less toxic, in general, than electron-withdrawing substituted ones. An additive effect also operates, as *β-naphthotacrine* **11**, substituted with three methoxy groups, is less toxic than *β-naphthotacrines* **7** or **10**, substituted with two electron-donating groups, such as OH-OMe or di-OMe, respectively. In addition, *β-naphthotacrines* **7** and **10** were also less toxic than the nonsubstituted *β-naphthotacrine* **1** or *β-naphthotacrines* **2**–**6**, substituted with only one Me, OH, or OMe group. In this case the position of the substituents in the aromatic ring at C-14 is also important as shown by comparing compound **9** (toxic) with **10** (less toxic), where one of the two methoxy groups moves from C2′ to C3′. All these effects are also in good agreement with the low toxicity found in 7-MEOTA.[28] The case of *β-naphthotacrine* **12** bearing a nitro substituent, an electron-withdrawing group at C4′ in the aromatic ring, is an exception to this general trend, as it shows a viability value of 83% at 300 μM concentration (see Table 1). Finally, it is also clear that the substitution of a fused benzene ring by a benzochromane motif in tacrine, coupled to a correct selection of the substituents at defined and synthetically available aromatic positions, produces new tacrines devoid of the toxic effects present in the reference compound.

Cholinesterase Inhibition. On the basis of these promising results that enhanced interest in them and in potential therapeutic application, *β-naphthotacrines* **1**–**14** were evaluated as inhibitors of hAChE and hBuChE, according to Ellman's protocol.[33] The IC_{50} values for hAChE were measured at concentrations between 0.01 and 100 μM. An example of IC_{50} calculation for *β-naphthotacrine* **10**, compared with that for tacrine, is represented in Figure 2. The remaining IC_{50} values for the tested compounds and tacrine are shown in Table 2.

β-naphthotacrines **1**–**14** were selective hAChE inhibitors since they produced poor or no inhibition of hBuChE activity. In fact, only compounds **5**, **6** and **9** had an $IC_{50} < 100$ μM, which is very high as compared with tacrine. With the exception of *β-naphthotacrines* **12**–**14**, bearing electron-withdrawing substituents at the aromatic ring at C-14, the electron-donating substituted *β-naphthotacrines* **1**–**11** were potent hAChE inhibitors, from 0.048 to 4.48 μM. The most potent, and insignificantly different from tacrine, were *β-naphthotacrine* **4** ($IC_{50} = 0.048 \pm 0.016$ μM), bearing a hydroxyl group at C4′, and *β-naphthotacrine* **10** ($IC_{50} = 0.083 \pm 0.024$ μM), bearing two methoxy groups at C3′ and C4′.

Comparing C′4-substituted *β-naphthotacrines* **2**–**4** IC_{50}, we notice that inhibition potency increases gradually from methyl to hydroxyl up to methoxy electron-donating substituents. With the same methoxy substituent at different positions, we notice that AChE inhibition potency was higher for *β-naphthotacrine* **6** (methoxy at C2′) than for *β-naphthotacrine* **5** (methoxy at C3′), which showed higher inhibition potency than *β-naphthotacrine* **4** (methoxy at C4′). Once again the substituent position in the

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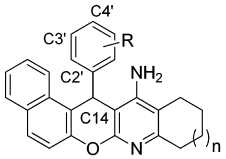
Table 2. IC_{50} (μM) Values for the Inhibition of hAChE and hBuChE by Tacrine and Racemic *β-Naphthotacrines* 1−14[a]



	R	n		R	n
1	H	1	**8**	4′-OH,3′-OMe	1
2	4′-Me	1	**9**	2′,4′-di-OMe	1
3	4′-OH	1	**10**	3′,4′-di-OMe	1
4	4′-OMe	1	**11**	3′,4′,5′-tri-OMe	1
5	3′-OMe	1	**12**	4′-NO_2	1
6	2′-OMe	1	**13**	2′,6′-di-Cl	1
7	4′-OH,3′-OMe	0	**14**	3′,4′-di-Cl	1

cmpd	R	n^b	hAChE[c] (μM)	hBuChE (μM)
1	H	1	0.24 ± 0.07[ns]	>100
2	4′-Me	1	4.48 ± 1.56[f]	>100
3	4′-OH	1	0.98 ± 0.08[d]	>100
4	4′-OMe	1	0.048 ± 0.016[ns]	>100
5	3′-OMe	1	0.47 ± 0.23[ns]	∼60
6	2′-OMe	1	1.46 ± 0.21[e]	∼50
7	4′-OH,3′-OMe	0	4.42 ± 1.28[f]	>100
8	4′-OH,3′-OMe	1	1.54 ± 0.22[e]	>100
9	2′,4′-di-OMe	1	2.94 ± 0.11[f]	∼30
10	3′,4′-di-OMe	1	0.083 ± 0.024[ns]	>100
11	3′,4′,5′-tri-OMe	1	3.14 ± 1.62[f]	>100
12	4′-NO_2	1	11.87 ± 0.53[f]	>100
13	2′,6′-di-Cl	1	59.41 ± 2.18[f]	>100
14	3′,4′-di-Cl	1	33.54 ± 1.23[f]	>100
tacrine			0.19 ± 0.05	0.04 ± 0.002

[a]Values are expressed as mean ± standard error of the mean of at least three different experiments in triplicate. [b]nd = not determined. n = 0, cyclopentil; n = 1, cyclohexyl. Statistical comparisons between IC_{50}'s for drugs and that for tacrine were performed by one-way ANOVA followed by the Holm−Sidak posthoc test. [c]ns = not significant. [d]P < 0.05. [e]P < 0.01. [f]P < 0.001.

aromatic ring at C14 is critical for AChE inhibition. Thus, not surprisingly, compound **10**, a dimethoxy derivative with these groups at C3′/C4′ positions, is much more potent than **9**, a dimethoxy derivative with these groups at C2′/C4′ positions. The effect of the cycloalkane size is also clearly favoring, as in the case of tacrine, the six-membered vs the five-membered ring when comparing the IC_{50} for *β-naphthotacrines* **8** and **7**. To gain further insight into this family of compounds' mechanism of action on hAChE, a kinetic study was carried out for nontoxic and potent hAChEI *β-naphthotacrine* **10**. Graphical analysis of the reciprocal Lineweaver−Burk plots (Figure 3A) showed both increased slopes (decreased V_{max}) and intercepts (higher K_m) at increasing concentrations of the inhibitor. This pattern indicates a mixed-type inhibition. Replots of the slope vs concentration of compound **10** gave an estimate of the inhibition constant, K_i = 0.35 ± 0.04 μM (Figure 3B).

When we analyzed statistically the K_m and V_{max} modifications at different concentrations of *β-naphthotacrine* **10**, we observed that at low concentrations (from 0.01 to 0.1 μM) V_{max} does not significantly change, but K_m does (Table 3), whereas at high concentrations, both V_{max} and K_m change in a statistically significant way (Table 3). These results mean that *β-naphthotacrine* **10** behaves as a competitive inhibitor at low concentrations and as a mixed-type inhibitor at higher concentrations.

In Figure 4 we show the Lineweaver−Burk kinetic analysis of *β-naphthotacrine* **10** hAChE inhibition. This analysis gives two inhibition constants (K_i): K_{i_1} = 0.007 ± 0.001 μM and K_{i_2} = 0.72 ± 0.06 μM, at low and high concentrations, respectively. These results suggest that, depending on the inhibitor concentration, *β-naphthotacrine* **10** is able to bind to different sites of hAChE.

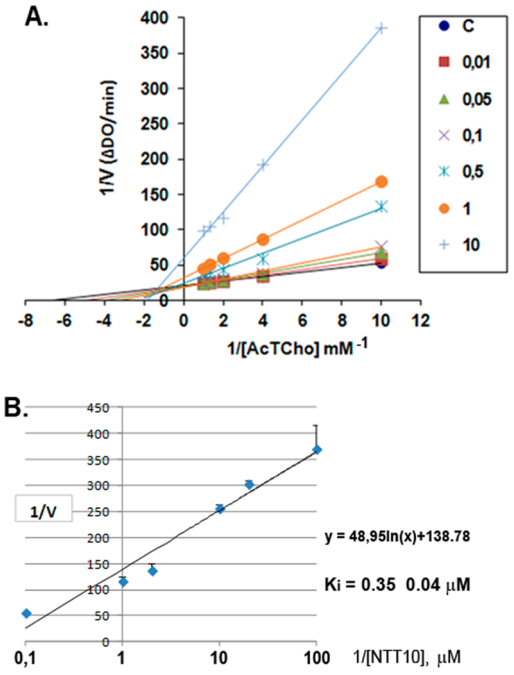


Figure 3. Kinetics of inhibition of hAChE hydrolysis of acetylthiocholine (ATCh) by *β-naphthotacrine* **10**. (A) Lineweaver−Burk reciprocal plots of initial velocity and substrate concentrations (0.01−10 μM) are presented. Lines were derived from a weighted least-squares analysis of data. (B) K_i calculation for *β-naphthotacrine* **10** (P < 0.001 Anova, n = 6).

Table 3. Kinetics of the hAChE Inhibition for *β-Naphthotacrine* 10 (K_m's and V_{max}'s)

[I_{10}] μM	V_{max}(ΔDO/min)	P <	K_m (mM)	P <
control[a]	0.047 ± 0.003	–	0.145 ± 0.014	–
0.01	0.052 ± 0.003	ns[b]	0.205 ± 0.003	*d*
0.05	0.053 ± 0.002	ns[b]	0.254 ± 0.006	*d*
0.1	0.054 ± 0.004	ns[b]	0.305 ± 0.016	*d*
0.5	0.042 ± 0.002	*c*	0.445 ± 0.03	*d*
1	0.029 ± 0.001	*d*	0.456 ± 0.008	*d*
10	0.016 ± 0.001	*d*	0.537 ± 0.014	*d*

[a]Statistical comparisons were carried out against the control (one way ANOVA; n = 6). [b]ns = not significant. [c]P < 0.05. [d]P < 0.001.

Thus, at low concentrations, *β-naphthotacrine* **10** acts as a competitive inhibitor, binding the enzyme at positions closely located to the catalytic active site (CAS), while at high concentrations, **10** acts as a mixed-type inhibitor, suggesting that *β-naphthotacrine* **10** is possibly binding simultaneously to the CAS and to the PAS of hAChE.[33]

Neuroprotection Studies. Finally, we investigated the neuroprotection profile of *β-naphthotacrines* **2**−**8**, and **10**−**12** on primary cortical neurons treated with oligomycin-A (10 μM) and rotenone (30 μM) (Olig/Rot), two mitochondrial respiratory chain inhibitors, which block complex V and I, respectively, as previously described[34] by using the XTT (2,3-*bis*(2-methoxi-4-nitro-5-sulfophenyl)-2*H*-tetrazolyl-5-carboxyanilide) assay to determine neuronal viability (see Supporting Information).

β-Naphthotacrines **2**−**8** and **10**−**12** were added 15 min before Olig/Rot, at concentrations between 0.1 μM and 100 μM, using tacrine as a reference compound. In Table 4, the highest neuroprotective effects and the EC_{50} values are shown. These values clearly demonstrate that after 24 h, the decrease in cellular viability [63.15 ± 2.75% (mean ± SEM; n = 15, p < 0.001 vs

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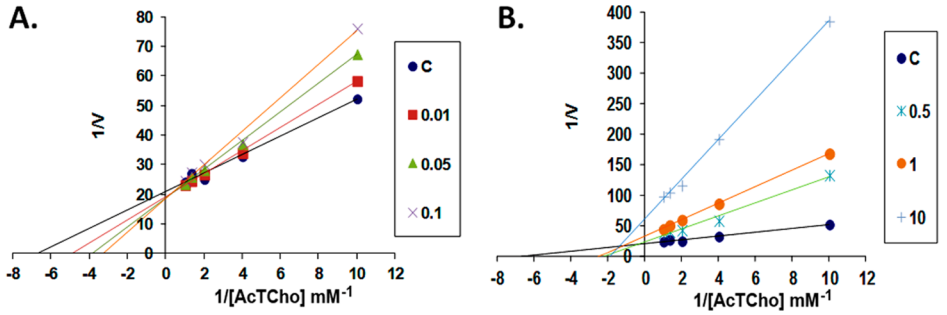
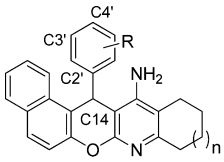


Figure 4. Lineweaver–Burk representation of the kinetics of the hAChE inhibition for *β-naphthotacrine* **10**, at low concentrations (0.01–0.1 μM) (A) and at high concentrations (0.5–10 μM) (B).

Table 4. Neuroprotective Effects of *β-Naphthotacrines* 2–8, 10–12, and Tacrine on the Decrease in Cellular Viability Induced by Mitochondrial Respiratory Chain Blockers Olig/Rot in Primary Cultures of Cortical Neurons[a]



	R	n		R	n
2	4'-Me	1	**8**	4'-OH,3'-OMe	1
3	4'-OH	1	**10**	3',4'-di-OMe	1
4	4'-OMe	1	**11**	3',4',5'-tri-OMe	1
5	3'-OMe	1	**12**	4'-NO_2	1
6	2'-OMe	1	**13**	2',6'-di-Cl	1
7	4'-OH,3'-OMe	0	**14**	3',4'-di-Cl	1

cmpd	structure (R)	maximal activity (%)	EC_{50} (μM)
tacrine	–	64.34 ± 17.60	2.29 ± 1.50
2	4′-Me, *n* = 1	50.02 ± 8.16	23.73 ± 5.49
3	4′-OH, *n* = 1	65.50 ± 6.21	5.30 ± 0.88
4	4′-OMe, *n* = 1	55.66 ± 1.41	24.79 ± 7.01
5	3′-OMe, *n* = 1	77.24 ± 4.82[b]	15.89 ± 3.45
6	2′-OMe, *n* = 1	94.58 ± 4.82[c]	4.95 ± 2.50
7	4′–OH-3′-OMe, *n* = 0	73.33 ± 4.82[b]	11.41 ± 3.40
8	4-OH,3-OMe, *n* = 1	77.28 ± 4.82[b]	10.81 ± 2.70
10	3′,4′-di-OMe, *n* = 1	134.52 ± 21.35[c]	11.40 ± 2.30
11	3′,4′,5′-tri-OMe, *n* = 1	107.60 ± 15.36[c]	10.09 ± 1.23
12	4′-NO_2, *n* = 1	no neuroprotector	–

[a]Data are expressed as mean ± SEM of at least three experiments, each one performed in triplicate, performed in at least three different cortical neuron cultures. *n* = 0 cyclopentil, *n* = 1 cyclohexyl. Statistical comparisons with tacrine were performed by one-way ANOVA followed by the Holm–Sidak post-hoc test. [b] $P < 0.05$. [c] $P < 0.001$.

100% control, one way ANOVA test)] induced by Olig/Rot was totally or partially reverted by *β-naphthotacrines* **2–8**, **10–12**, and tacrine, in a concentration-dependent manner, these effects being statistically significant. As shown in Table 4, at 50 μM, only compounds **10** and **11** were able to reach 100% cellular viability after 24 h.

Due to the fact that the neuroprotective effects of some of these *β-naphthotacrines* decrease at ≥25–50 μM, we evaluated the basal neurotoxicity. In contrast to the toxic effects shown by tacrine (>25–60% at 25 μM; Figure 5), *β-naphthotacrines* **1–11** and **13** and **14** proved to be non-neurotoxic between 1 and 50 μM; in addition, *β-naphthotacrine* **10** was neuroprotective between 10 and 50 μM, but this effect decreased mostly at the highest (100 μM) concentration analyzed (Figure 5).

From the SAR analysis only *β-naphthotacrine* **12**, bearing the nitro electron-withdrawing group at C4′, showed a significant neurotoxic effect of 30–50% at concentrations between 5 and 100 μM, whereas the other *β-naphthotacrines* bearing electron-donor substituents in the aromatic ring at C-14, and particularly, compounds **10** and **11**, showed a very good non-neurotoxic profile.

CONCLUSIONS

Although the AD therapeutics is focused on immunization procedures against A*β* deposition in senile plaques, antiphosphorylating agents to halt neurofibrillary tangle formation and *γ*- and *β*-secretase inhibitors, the results reported with tacrine-related compounds should not be neglected.[8] Thus, this may be the moment to revise the *old-fashioned therapeutic strategies*. In this arena, and not surprisingly, some researchers have clearly banked on tacrine. The present work clearly shows that tacrine analogues such as the *β*-naphthotacrines reported here and, particularly, those bearing electro-donor groups at aromatic positions deserve attention in the search for new drugs for AD.

In this work we have reported the synthesis and pharmacological analysis (including toxicity, neuroprotection, and hAChE and hBuChE inhibition properties) of racemic *β-naphthotacrines* **1–14**, bearing the 14-aryl-10,11,12,14-tetrahydro-9*H*-benzo[5,6]chromeno[2,3-*b*]quinolin-13-amine heterocyclic ring skeleton (**I**), as new drugs for the potential treatment of AD. *β-Naphthotacrines* **1–14** have been prepared in high yield by a Friedländer-type reaction of 3-amino-1-phenyl-1*H*-benzo[*f*]-chromene-2-carbonitriles, **19–22**, with selected cyclohexanone or cyclopentanone. Hepatotoxicity analyses on compounds **1–14** showed that the *β-naphthotacrines* are nontoxic derivatives, and at the highest concentration assayed (300 μM) the HepG2 cell viabilities in the presence of compounds **11**, **7**, and **10**, in this order, were 2.25–2.01-fold higher compared with that in the presence of tacrine. Efficient and selective inhibition of hAChE, in a micromolar range, was only observed for the *β-naphthotacrines* bearing electron-donating substituents at the aromatic ring. One of the most potent was *β-naphthotacrine* **10** (hAChE: IC_{50} = 0.083 ± 0.024 μM). Kinetic inhibition analysis clearly demonstrated that *β-naphthotacrine* **10** behaves as a mixed-type (K_{i_2} = 0.72 ± 0.06 μM) at high concentrations, while at low concentrations it is a hAChE competitive inhibitor (K_{i_1} = 0.007 ± 0.001 μM). On the other hand, strong neuroprotection effect was observed for compounds **10** and **11** in the Olig/Rot assay. From this work we have identified *β-naphthotacrine* **10** as a potent and selective hAChE inhibitor in a nanomolar range, nontoxic in human HepG2 cells with a strong neuroprotective activity and no neurotoxic effects. Consequently, *β-naphthotacrine* **10** is an attractive, multipotent molecule, anti-AD candidate. Studies are now in progress to resolve this racemic mixture and separately analyze each enantiomer in the same pharmacological tests.

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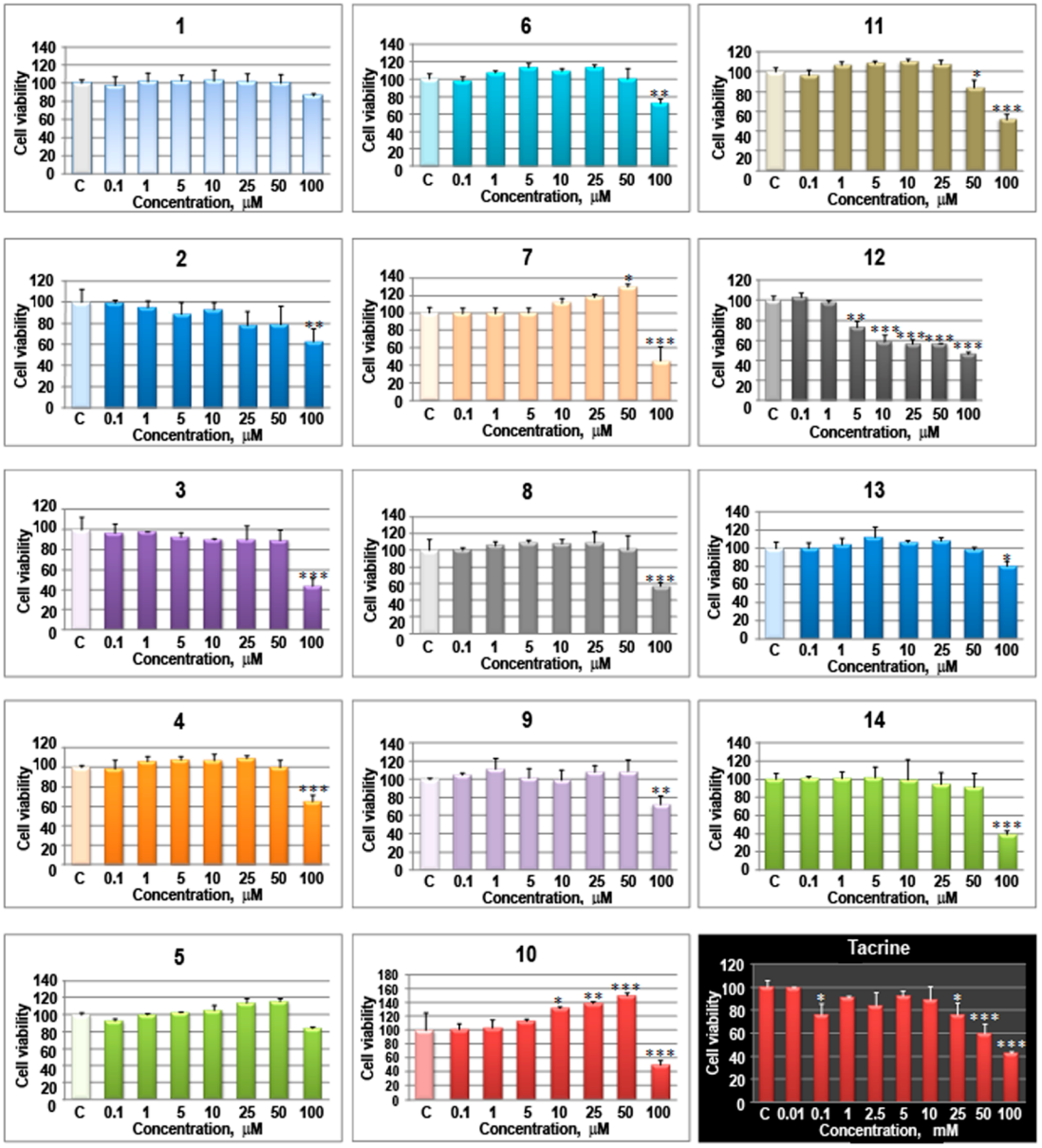

Figure 5. Neurotoxic effect of *β-naphthotacrines* **1**−**14** and tacrine on the cellular viability of cortical neurons in primary cultures represented by the % of cellular viability in the presence or absence (control; C) of the indicated *β-naphthotacrines* and tacrine concentrations. The values are the mean ± SEM of at least three independent experiments, each one carried out in triplicate, in different cell cultures. The statistical analysis shows the neurotoxic or neuroprotective effects against controls at * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (one way ANOVA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Preparation of compounds **1**, **9**, **13**, and **14**, and the pharmacological protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

hAChE, human acetylcholinesterase; hBuChE, human butyrylcholinesterase; ACh, acetylcholine; AD, Alzheimer disease; MTDL, multitarget-directed ligands strategy; AChEI, acetylcholinesterase inhibitor; PAS, peripheral anionic binding site; CAS, catalytic anionic site; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; XTT, (2,3-*bis*(2-methoxi-4-nitro-

5-sulfophenyl)-2*H*-tetrazolyl-5-carboxyanilide; ROS, reactive oxygen species; Olig/Rot, oligomycin-A/rotenone

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