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Novel Tacrine−8-Hydroxyquinoline Hybrids as Multifunctional Agents for the Treatment of Alzheimer's Disease, with Neuroprotective, Cholinergic, Antioxidant, and Copper-Complexing Properties

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Tacrine and PBT2 (an 8-hydroxyquinoline derivative) are well-known drugs that inhibit cholinesterases and decrease β-amyloid (Aβ) levels by complexation of redox-active metals, respectively. In this work, novel tacrine−8-hydroxyquinoline hybrids have been designed, synthesized, and evaluated as potential multifunctional drugs for the treatment of Alzheimer's disease. At nano- and subnanomolar concentrations they inhibit human acetyl- and butyrylcholinesterase (AChE and BuChE), being more potent than tacrine. They also displace propidium iodide from the peripheral anionic site of AChE and thus could be able to inhibit Aβ aggregation promoted by AChE. They show better antioxidant properties than Trolox, the aromatic portion of vitamin E responsible for radical capture, and display neuroprotective properties against mitochondrial free radicals. In addition, they selectively complex Cu(II), show low cell toxicity, and could be able to penetrate the CNS, according to an in vitro blood−brain barrier model.

Introduction

Alzheimer's disease (AD[a]) is clinically characterized by a progressive memory loss and other cognitive impairments. Although the etiology of AD is not completely known, deposits of aberrant proteins, namely, β-amyloid (Aβ) and τ-protein, oxidative stress, dyshomeostasis of biometals, and low levels of acetylcholine (ACh) seem to play significant roles.[1] These facts strongly suggest that AD is a multifaceted illness against which several research strategies have been developed, including cholinergic[2] and noncholinergic approaches.[3]

The current therapeutic options for the treatment of AD are acetylcholinesterase inhibitors (AChEIs), which increase neurotransmission at cholinergic synapses in the brain and reduce temporarily the cognitive deficit,[4,5] and the *N*-methyl-D-aspartate receptor antagonist memantine.[6] The AChEIs rivastigmine and galantamine are used for the treatment of mild-to-moderate AD, and memantine was approved for the management of moderate-to-severe AD. The AChEI donepezil is presently applied for treating mild, moderate, and severe AD patients in the United States.[7]

In addition to its important role in cholinergic neurotransmission, AChE also participates in other functions related to neuronal development, differentiation, adhesion, and Aβ processing. Biochemical studies have indicated that AChE promotes amyloid fibril formation by interaction through the peripheral anionic site of the enzyme (PAS), giving stable AChE−Aβ complexes, which are more toxic than single Aβ peptides.[8] The fact that AChE accelerates Aβ aggregation and that this effect is sensitive to PAS blockers has led to the development of dual inhibitors of both catalytic active site (CAS) and PAS. These compounds are promising disease-modifying AD drug candidates because they can simultaneously improve cognition and slow the rate of Aβ-elicited neurodegeneration.[9] The interest in these dual-site inhibitors has recently increased; in fact, NP-61 is an example of this type of compound that is currently in phase I clinical trials for AD.[10,11]

Butyrylcholinesterase (BuChE) is an enzyme also involved in cholinergic neurotransmission, which has received an increasing attention in the past years.[12] In healthy brains, AChE hydrolyzes about 80% of acetylcholine while BuChE plays a secondary role. However, as AD progresses, the activity of AChE decreases while that of BuChE rises in an attempt to modulate ACh levels in cholinergic neurons. Consequently, both enzymes are useful therapeutic targets for AD. In fact, recent clinical trials have demonstrated that patients treated with rivastigmine, an inhibitor of both AChE and BuChE, showed minor cortical atrophic changes

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[a]Abbreviations: Aβ, β-amyloid peptide; ACh, acetylcholine; AChE, acetylcholinesterase; AChEI, acetylcholinesterase inhibitor; AD, Alzheimer's disease; BBB, blood−brain barrier; BuChE, butyrylcholinesterase; CAS, catalytic active site; CNS, central nervous system; hAChE, human acetylcholinesterase; hBuChE, human butyrylcholinesterase; ORAC-FL, oxygen-radical absorbance capacity by fluorescence; PAMPA-BBB, parallel artificial membrane permeation assay for the blood−brain barrier permeation; LDH, lactate dehydrogenenase; PAS, peripheral anionic site; PBT2, 8-hydroxyquinoline derivative in clinical trials for Alzheimer's disease; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; SD, standard deviation.

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and attenuated loss of brain volume.[13,14] These findings are consistent with the hypothesis that inhibition of both enzymes may have neuroprotective and disease-modifying effects.[15]

Tacrine (**1**), the first drug approved for the treatment of AD, is a potent inhibitor of both AChE and butyrylcholinesterase (BuChE) that suffers from therapy limiting side effects, mainly liver toxicity.[16] Cytotoxicity studies using the human liver cell line HepG2 showed that a therapeutic blood concentration of tacrine induces ROS production stimulation and glutathione depletion, pointing out that oxidative stress might be involved in tacrine hepatotoxicity.[17] It has been also demonstrated that tacrine-induced oxidative stress can be prevented by treating hepatocytes with a free radical scavenger, such as anethole dithiolethione [5-(4-methoxyphenyl)-3*H*-1,2-dithiole-3-thione][18] or vitamin E.[19] Thus, tacrine derivatives endowed with additional antioxidant properties might be beneficial by reducing toxicity. Indeed, in the past years several tacrine−antioxidant hybrids have been reported, such as lipocrine that protects cells against oxidative stress[20] and NO-donor−tacrine hybrids that showed hepatoprotective properties.[21]

During aging, the endogenous antioxidant protection system progressively decays and may be further diminished in AD. Certainly, an increasing body of evidence supports the early involvement of oxidative stress in the pathogenesis and progression of AD.[22,23] Recent research has demonstrated that oxidative damage is an event that precedes the appearance of other pathological hallmarks of the disease, namely, amyloid plaques and neurofibrillary tangles.[24,25] Thus, drugs that specifically scavenge oxygen radicals could be useful for either the prevention or the treatment of AD.[26,27]

There is also evidence that suggests a central role of biometals such as copper, zinc, and iron in many critical aspects of AD. In amyloid plaques, elevated concentrations of Cu and Zn have been detected by spectroscopic studies,[28] and in in vitro experiments these metals are able to bind to Aβ, thus promoting its aggregation.[29] Redox-active metal ions like Cu and Fe contribute to the production of reactive oxygen species (ROS) and oxidative stress.[30] Therefore, modulation of such biometals in the brain has been proposed as a potential therapeutic strategy for the treatment of AD,[31,32] and several metal chelators such as desferrioxamine,[33] D-penicillamine,[34] and clioquinol (**2**, PBT1, 5-iodo-6-chloro-8-hydroxyquinoline)[35] have been studied in clinical trials. More recently PBT2, another 8-hydroxyquinoline derivative, has entered in phase IIb clinical tests for AD showing encouraging results.[36,37] PBT2 inhibits the redox-dependent formation of toxic soluble oligomers of Aβ, prevents deposition of Aβ as amyloid plaques, and promotes clearance by mobilizing and neutralizing Aβ from existing deposits. In addition, PBT2 does not contain iodine and is therefore not capable of forming the diiodo impurity that has been associated with clioquinol toxicity.[38,39]

The multifaceted condition of AD has encouraged active research in the development of multifunctional drugs with two or more complementary biological activities, since they may represent an important advance in the treatment of the disease.[40,41] Continuing with our research on various heterocyclic families with potential application in the AD field,[42−45] in the past years we reported the synthesis of multifunctional compounds that combine neuroprotective and dual inhibition of CAS and PAS of AChE.[46−48] More recently, we described a tacrine−melatonin hybrid that is able to reduce amyloid burden and behavioral deficits in a mouse model of Alzheimer's disease.[49]

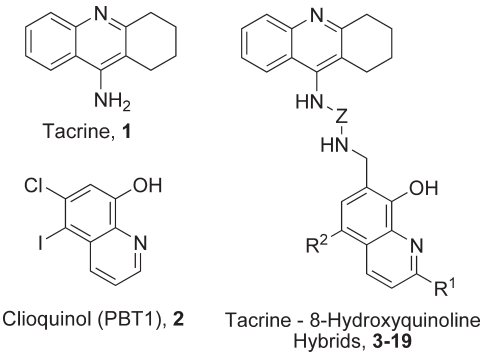


Figure 1. Structures of tacrine (**1**), clioquinol (**2**), and tacrine−8-hydroxyquinoline hybrids (**3**−**19**).

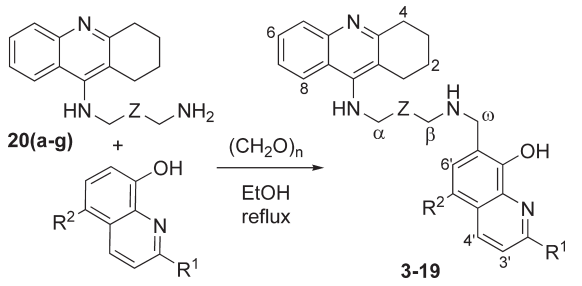
At present, our work is focused on the design of new multiactive neuroprotectants with antioxidant, metal-binding properties, and dual inhibition of AChE and BuChE in a single small molecule. Tacrine−8-hydroxyquinoline hybrids (**3**−**19**) were designed by using moieties with well-known properties for each biological activity: tacrine for the inhibition of cholinesterases through its binding to the CAS; PBT2 for its metal-chelating, neuroprotective, and antioxidant properties,[50] as well as for its potential interaction with the PAS due to its aromatic character (Figure 1). Regarding the possible structural modifications on the quinolin-8-ol fragment, we planned to use commercially available products, namely, 8-hydroxyquinoline and its 2-methyl and 5-chloro derivatives, with electron-donating or electron-withdrawing effects. Since the AChE-CAS is located at the bottom of a deep gorge, we considered connecting tacrine and quinoline fragments by alkylenediamine tethers of different lengths (from 6 to 12 carbons) or by a triamine skeleton such as 3,3′-diamino-*N*-methyldipropylamine. These flexible linkers could be lodged by the enzyme cavity, allowing simultaneous interaction between the heteroaromatic fragments and both the CAS and PAS of AChE. Furthermore, the triamine fragment could establish additional contacts between the protonated central tertiary amine and the aromatic residues of the enzyme mild-gorge through cation−π interactions[51]

In this paper, we describe the synthesis of new tacrine−8-hydroxyquinoline hybrids and their biological evaluation that includes inhibition of AChE and BuChE, displacement of propidium iodide from AChE, as a preliminary method to test their potential inhibition of Aβ aggregation, oxygen-radical absorbance capacity (ORAC), metal-chelating properties of redox-active metals, in vitro CNS penetration, and cell viability. We also studied the neuroprotective effects of these derivatives against death induced by mitochondrial oxidative stress triggered by rotenone in human neuroblastoma cells.

Results and Discussion

Chemistry. Scheme 1 depicts the general procedure for the synthesis of tacrine−8-hydroxyquinoline hybrids **3**−**19**. Following described methods, N^1-(1,2,3,4-tetrahydroacridin-9-yl)alkane-1,*n*-diamines **20a**−**f** and N^1-(3-aminopropyl)-N^1-methyl-N^3-(1,2,3,4-tetrahydroacridin-9-yl)propane-1,3-diamine **20g** were obtained in good yields (75−85%).[52,53] Then the Mannich aminomethylation of different commercially available quinolines (8-hydroxyquinoline, 2-methyl-8-hydroxyquinoline, or 5-chloro-8-hydroxyquinoline) with **20a**−**g** in the presence of paraformaldehyde would yield the

Scheme 1. Synthesis of Tacrine−8-Hydroxyquinoline Hybrids **3**−**19**



desired hybrids. However, a preliminary experiment that was carried out by refluxing **20a**, 8-hydroxyquinoline, and paraformaldehyde in ethanol for 10 h yielded a complex mixture, where the wanted product was not found. Since it is known that the Mannich aminomethylation comprises two steps, namely, the formation of an iminic derivative from the amine and the formaldehyde and then the reaction of the above intermediate with a compound containing an activated hydrogen atom, we modified the order of reactants addition. First, aminoalkyltacrine **20a**−**g** was refluxed with 3 equiv of paraformaldehyde in ethanol for 6 h to favor the formation of the imine intermediate, which was not isolated but was detected by mass spectrometry. Then the corresponding 8-hydroxyquinoline was added and the mixture stirred at room temperature for an additional 18 h, obtaining **3**−**19** that were purified by column chromatography using either silica gel or reversed-phase cartridges (see Experimental Section for further details).

Although the Mannich reaction of the 8-hydroxyquinoline ring could occur at two different carbons, namely, C5′ and C7′, in these experimental conditions a main isomer could be detected and then isolated. In each of the hybrids **3**−**19** the position of aminoalkylation was unequivocally established by NMR using a combination of two-dimensional experiments. The corresponding HMQC (heteronuclear multiple quantum correlation) and HMBC (heteronuclear multiple bond correlation) diagrams allowed unambiguous assignment of all protons and carbons of each hybrid. The fact that the ω-methylenic protons (singlet at 4.10−4.80 ppm) showed HMBC correlations with C6′, C7′, and C8′ of the quinoline framework pointed out that the reaction took place in the ortho position with respect to the phenol group (Table 1).

Biology. Cholinergic and Antioxidant Activities. Inhibition of AChE and BuChE was determined by following the Ellman method, using tacrine as reference.[54] Because of their lower cost and their high degree of sequence identity to the human enzymes,[55] proteins of animal origin were initially used: AChE from bovine erythrocytes and BuChE from horse serum.

All tacrine−PBT2 hybrids **3**−**19** were evaluated toward bovine AChE, showing to be potent inhibitors of this enzyme with IC_{50} ranging from the submicromolar to the nanomolar concentration (Table 2). Molecules containing an unsubstituted 8-hydroxyquinoline fragment and a methylene tether of 7−10 carbons showed the best AChE inhibitory activities, with derivative **6** showing an interesting IC_{50} = 20 nM. Both the presence of an additional substituent in the quinoline ring or the insertion of a tertiary amine in the tether afforded compounds that were less potent than their respective counterparts.

To achieve potent dual inhibitors of AChE and BuChE, the most active inhibitors of bovine AChE showing IC_{50} < 100 nM were then tested against equine BuChE, and the results are also gathered in Table 2. The inhibitory concentrations obtained were almost the same order of magnitude as AChE IC_{50} values, pointing out that tested tacrine−PBT2 hybrids are potent inhibitors of both AChE and BuChE of animal origin.

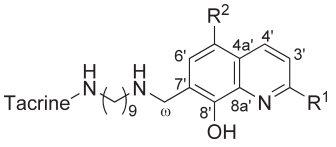
A selection of the most active compounds was then evaluated as inhibitors of human cholinesterases and as free radical scavengers. Hybrids **6** (8-hydroxyquinoline), **11** (2-methyl-8-hydroxyquinoline), and **16** (5-chloro-8-hydroxyquinoline), covering the different structural features in the quinoline fragment, were selected for these experiments (Table 3). All tested derivatives showed IC_{50} values in the nano- and subnanomolar range (0.5−5.5 nM). Hybrid **11** was the best hAChE inhibitor of the series, showing 700-fold greater potency than tacrine. All hybrids inhibited human AChE (hAChE) 4- to 150-fold more efficiently than the bovine enzyme. Since a higher degree of similarity between both enzymes was detected in the CAS region than in the PAS,[55] the superior affinity of tacrine−PBT2 hybrids toward hAChE could be due to a better fit between the 8-hydroxyquinoline fragment and the hAChE−PAS region.

Selected hybrids also inhibited human BuChE (hBuChE) with IC_{50} values ranging from 6.5 to 55 nM. Compound **6**, derived from unsubstituted 8-hydroxyquinoline, was shown to be a potent dual inhibitor of human AChE and BuChE. Introduction of a substituent in the quinolin-8-ol fragment gave hybrids **11** and **16** with a modest selectivity toward hAChE (Table 3). Since inhibition of both enzymes may have neuroprotective and disease-modifying effects,[13,14] the biological profile of hybrid **6** could be of interest for the potential treatment of AD.

The antioxidant activities of tacrine−PBT2 hybrids **6**, **11**, and **16** were evaluated by following the well-established ORAC-FL method (oxygen radical absorbance capacity by fluorescence)[56,57] that was recently applied by us to other compounds.[47,48] Peroxyl radicals were thermally generated from 2,2-azobis(amidinopropane) dihydrochloride and reacted with fluorescein to form nonfluorescent products at 520 nm. The antioxidant capacity of selected compounds (**6**, **11**, and **16**), tacrine (**1**), and 8-hydroxyquinoline was determined by their competition with fluorescein in the radical capture, using a fluorescence microplate reader. Trolox, a vitamin E analogue, was used as a standard, and the results were expressed as Trolox equivalents (μmol of Trolox/μmol of tested compound) in a relative scale where ORAC (Trolox) = 1. Tacrine showed negligible radical-capture ability, whereas tested hybrids and 8-hydroxyquinoline exhibited potent peroxyl radical absorbance capacities (Table 3). Hybrid **6** was 3.3-fold more potent than the vitamin E analogue, similar to the ORAC value of 8-hydroxyquinoline. As expected, the introduction of an electron-donating group in the quinolin-8-ol fragment decreased the radical capture capacity (hybrid **11**, ORAC = 2.6), whereas the presence of an electron-withdrawing substituent increased this property (hybrid **16**, ORAC = 4.7).

Propidium Displacement Assay as a Probe of the Inhibition of Aβ Aggregation. As mentioned in the Introduction, AChE has shown noncholinergic functions related to cell adhesion and neural differentiation. This enzyme also interacts with Aβ oligomers and fibrils, promoting their aggregation into amyloid plaques.[58] The key interaction between AChE and

Table 1. Assignment of ^{1}H and ^{13}C NMR Chemical Shifts (δ, ppm) of the Quinoline Fragment for Derivatives **6**, **12**, and **16**, Established by HSQC and HMBC Experiments



6, $R^1 = R^2 = H$
12, $R^1 = CH_3$; $R^2 = H$
16, $R^1 = H$; $R^2 = Cl$

		connected carbons by	
compd	proton	HSCQ	HMBC
6	8.82 (H2′)	148.6 (C2′)	139.0 (C8a′); 135.7 (C4′)
	7.34 (H3′)	121.2 (C3′)	127.6 (C4a′)
	8.07 (H4′)	135.7 (C4′)	127.6 (C4a′); 139.0 (C8a′)
	7.23 (H5′)	117.2 (C5′)	127.6 (C4a′); 139.0 (C8a′); 119.5 (C7′)
	7.26 (H6′)	127.8 (C6′)	127.6 (C4a′); 117.2 (C5′); 152.7 (C8′)
	4.13 (ω)	51.2 (ω)	119.5 (C7′); 152.8 (C8′); 127.8 (C6′)
12	7.19 (H3′)	122.2 (C3′)	126.1 (C4a′)
	7.93 (H4′)	135.8 (C4′)	126.1 (C4a′); 138.4 (C8a′)
	7.12 (H5′)	116.9 (C5′)	126.1 (C4a′); 138.4 (C8a′)
	7.12 (H6′)	126.7 (C6′)	126.1 (C4a′); 151.8 (C8′); 116.9 (C5′); 120.1 (C7′)
	4.10 (ω)	51.2 (ω)	120.1 (C7′); 151.8 (C8′); 126.7 (C6′)
	2.70 (CH_3)	25.1 (CH_3)	157.3 (C2′)
16	9.14 (H2′)	151.7 (C2′)	140.2 (C8a'); 134.2 (C4′); 124.8 (C3′)
	7.89 (H3′)	124.8 (C3′)	128.4 (C4a′); 151.7 (C2′)
	8.74 (H4′)	134.2 (C4′)	140.2 (C8a′); 121.5 (C5′)
	7.85 (H6′)	129.9 (C6′)	128.4 (C4a′); 121.5 (C5′); 153.1 (C8′)
	4.60 (ω)	46.8 (ω)	114.8 (C7′); 129.9 (C6′); 153.1 (C8′)

Table 2. Inhibition of Mammalian AChE and BuChE by Tacrine−8-Hydroxyquinoline Hybrids **3**−**19**[a]

				$IC_{50} \pm SD$ (nM)[b]	
compd	R^1	R^2	Z	AChE[c]	BuChE[d]
3·2HCl	H	H	$(CH_2)_4$	200 ± 10	nd
4·2HCl	H	H	$(CH_2)_5$	25 ± 1	150 ± 7
5·2HCl	H	H	$(CH_2)_6$	90 ± 4	30 ± 1
6·2HCl	H	H	$(CH_2)_7$	20 ± 1	5.0 ± 0.2
7·2HCl	H	H	$(CH_2)_8$	50 ± 2	65 ± 3
8·2HCl	H	H	$(CH_2)_{10}$	250 ± 12	nd
9·2HCl	H	H	$(CH_2)_2NCH_3(CH_2)_2$	80 ± 4	50 ± 2
10·2HCl	CH_3	H	$(CH_2)_5$	200 ± 10	nd
11·2HCl	CH_3	H	$(CH_2)_6$	75 ± 3	2.0 ± 0.1
12·2HCl	CH_3	H	$(CH_2)_7$	100 ± 5	nd
13·2HCl	CH_3	H	$(CH_2)_8$	70 ± 3	7.0 ± 0.3
14·2HCl	CH_3	H	$(CH_2)_2NCH_3(CH_2)_2$	200 ± 10	nd
15·2HCl	H	Cl	$(CH_2)_6$	150 ± 7	nd
16·2HCl	H	Cl	$(CH_2)_7$	85 ± 0.4	6.5 ± 0.3
17·2HCl	H	Cl	$(CH_2)_8$	100 ± 5	nd
18·2HCl	H	Cl	$(CH_2)_{10}$	400 ± 20	nd
19·2HCl	H	Cl	$(CH_2)_2NCH_3(CH_2)_2$	90 ± 4	30 ± 1
1				40 ± 2	10 ± 0.4

[a] Compounds were tested as hydrochlorides. [b] Results are the mean of three independent experiments ($n = 3$) ± SD. [c] AChE (EC 3.1.1.7) from bovine erythrocytes. [d] BuChE (EC 3.1.1.8) from horse serum. nd: not determined.

Table 3. Inhibition of Human Cholinesterases and Oxygen Radical Absorbance Capacity (ORAC, Trolox Equivalents) by Selected Hybrids **6**, **11**, and **16**[a]

	IC_{50} (nM)			
compd	hAChE[b]	hBuChE[c]	hAChE selectivity[d]	ORAC[e]
6·2HCl	5.5 ± 0.2	20 ± 1.0	3.6	3.3 ± 0.01
11·2HCl	0.50 ± 0.02	6.5 ± 0.3	13	2.6 ± 0.01
16·2HCl	1.0 ± 0.05	55 ± 2	55	4.7 ± 0.01
1	350 ± 10	40 ± 2	0.1	< 0.01
8-hydroxyquinoline	> 100	> 100		2.9 + 0.06

[a] Results are the mean of three independent experiments ($n = 3$) ± SD. [b] AChE (EC 3.1.1.7) from human erythrocytes. [c] BuChE (EC 3.1.1.8) from human serum. [d] Selectivity for hAChE = IC_{50}(hBuChE)/IC_{50}(hAChE). [e] Data are expressed as μmol of Trolox equivalents/μmol of tested compound.

Aβ seems to be located at the PAS, since selective CAS inhibitors do not decrease Aβ aggregation, whereas the PAS-specific ligand propidium is able to abolish fibril formation.[59]

The experimental affinity of selected compounds for the PAS was studied by displacement of propidium iodide that shows a 10-fold fluorescence enhancement when bound to AChE.[60] At 0.3 μM hybrids **6**, **11**, and **16** showed a propidium displacement of 22%, 19%, and 27%, respectively. The AChE inhibitor donepezil at 0.3 μM, used as positive control, gave a propidium displacement of 19%. These results pointed out that the new compounds could bind to the AChE-PAS and could therefore inhibit Aβ fibril formation promoted by this enzyme.[61]

In Vitro Blood−Brain Barrier Permeation Assay. Since the first requirement for successful CNS drugs is to reach their therapeutic targets, screening for the blood−brain barrier (BBB) penetration is of great importance. To explore whether selected tacrine−PBT2 derivatives would be able to penetrate into the brain, we used a parallel artificial membrane permeation assay for blood−brain barrier (PAMPA-BBB). This simple and rapid model, described by Di et al.[62] and recently applied by us to different compounds,[46−48,63−66] has the advantage of predicting passive BBB permeation with high success. The in vitro permeabilities (P_e) of the above selected tacrine−PBT2 hybrids (**6**, **11**, and **16**) and 15 commercial drugs through a lipid extract of porcine brain were determined using PBS/EtOH (70:30). Assay validation was made by comparing the experimental permeability with the reported values of these commercial drugs that gave a good lineal correlation, $P_e(exptl) = 1.24P_e(bibl) + 1.98$ ($R^2 = 0.93$). From this equation and taking into

Table 4. Permeability Results from the PAMPA-BBB Assay for Selected Tacrine−PBT2 Hybrids (P_e, 10^{-6} cm s^{-1}) with their Predictive Penetration into the CNS

compd	P_e (10^{-6} cm s^{-1})[a]	prediction
6·2HCl	17.4 ± 0.3	CNS+
11·2HCl	14.1 ± 0.2	CNS+
16·2HCl	10.0 ± 0.8	CNS+

[a] PBS/EtOH (70:30) was used as solvent, and data are the mean of three independent experiments ± SD.

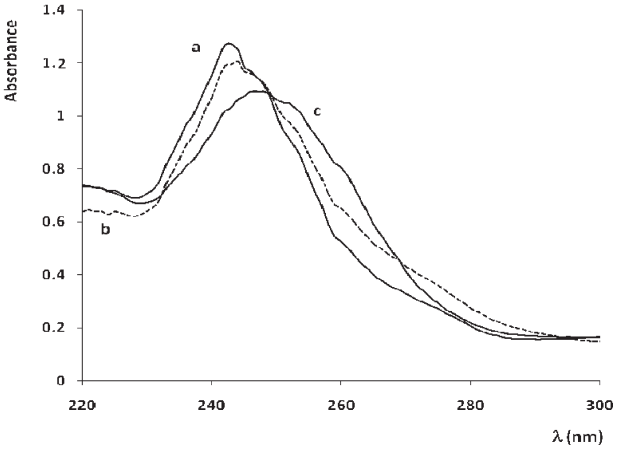


Figure 2. (a) UV spectrum of compound **6** (22.5 μM). (b) Spectrum of a mixture of **6** (22.5 μM) and $Fe_2(SO_4)_3$ (4.2 μM). (c) Spectrum of a mixture of **6** (22.5 μM) and $CuSO_4$ (4.2 μM). All solutions were made using Tris buffer, pH 7.4.

account the limits established by Di et al. for BBB permeation,[62] we found that molecules with a permeability of $> 7.0 \times 10^{-6}$ cm s^{-1} would be able to cross the BBB by passive permeation. All tested tacrine−clioquinol hybrids showed permeability values over the above limit, as the known CNS drugs used in the assay validation, pointing out that molecules herein described would cross the BBB by passive diffusion (Table 4).

On the basis of its previous biological results and its structural simplicity, hybrid **6** was chosen as an illustrative compound of the series for further tests, which included metal-chelating properties, cell viability, and neuroprotection against mitochondrial free radicals.

Metal-Chelating Properties of Compound 6. The complexation ability of hybrid **6** toward biometals such as Fe(III) and Cu(II) was studied by UV−vis spectrometry. Upon the addition of $CuSO_4$ the maximum absorption at 242 nm suffered a bathochromic shift at 248 nm, pointing out the formation of complex **6**−Cu(II). To establish the time required for the complexation process, the spectrum of the mixture **6** plus $CuSO_4$ was recorded at different times, observing that the curve did not change after the first minute. When the same experiment was performed using compound **6** and $Fe_2(SO_4)_3$, no significant differences were observed in the UV spectrum after 30 min, indicating that this hybrid did not appreciably complex Fe(III) (Figure 2).

The observed selectivity of the hybrid tacrine−8-hydroxyquinoline **6** toward Cu(II) compared to Fe(III) may have interesting therapeutic applications. Several studies have shown that Cu contributes more to the formation of senile plaques than iron does because of its superior affinity for $A\beta_{1-42}$.[67,68] In addition, the complex $A\beta_{1-42}$−Cu(II) produces more reactive oxygen species, because of its higher reduction potential (500 mV), compared with the corresponding

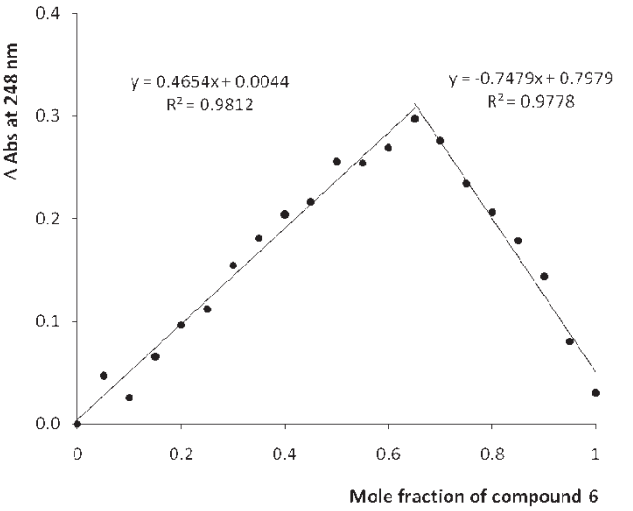


Figure 3. Determination of the stoichiometry of complex **6**−Cu (II) by Job's method.

complex with Fe(III).[69] Therefore, the selective complexation of copper by the hybrid tacrine−PBT2 **6** could simultaneously stop the formation of amyloid plaques and relieve oxidative stress.

To determine the stoichiometry of the complex **6**−Cu(II), the technique of continuous variations (also called Job's method) was used[70,71] by preparing solutions of compound **6** and $CuSO_4$ so that the sum of concentrations of both species was constant in all samples, but the proportions of both components varied between 0 and 100%. The UV spectra were recorded and the absorbance change at 248 nm was plotted versus the mole fraction of compound **6**, giving two straight lines whose equations were calculated. The equations gave a solution at a mole fraction of 0.65 for compound **6**, revealing a 2:1 stoichiometry for complex **6**−Cu(II) (Figure 3).

Cell Viability and Neuroprotection against Mitochondrial Free Radicals of Hybrid 6. To examine the potential cytotoxic effects of hybrid **6**, the human neuroblastoma cell line SH-SY5Y was exposed to this compound at 3 μM for 24 h, and then the cell viability was evaluated by measuring lactate dehydrogenenase (LDH) release. Under these experimental conditions hybrid **6** showed negligible cell death.

Conditions of oxidative stress in neuroblastoma cells were simulated by using rotenone as toxic insult. Rotenone is a specific inhibitor of complex **I** of the electron transport chain, which induces apoptosis by enhancing the generation of mitochondrial ROS.[72] The endogenous antioxidant enzyme catalase was employed as a reference. Hybrid **6** was tested at 3 μM, showing an interesting 30% of neuroprotection, a value equal to the endogenous enzyme.

Conclusions

In summary, we have developed new tacrine−PBT2 hybrids that display interesting in vitro biological activities for the treatment of Alzheimer's disease: cholinergic, antioxidant, copper-complexing, and neuroprotective properties. They are potent inhibitors of human AChE and BuChE with IC_{50} values in the nano- and subnanomolar ranges, being more potent than the parent fragment tacrine. They show better antioxidant properties than Trolox, the aromatic portion of vitamin E and that responsible for radical capture. They displace propidium from the PAS of the AChE and thus could

diminish Aβ aggregation promoted by AChE. In human neuroblastoma cells they show protective properties against damage caused by mitochondrial free radicals. In addition, they selectively complex Cu(II) and are able to penetrate the CNS to reach their cerebral targets.

In conclusion, it is expected that tacrine−PBT2 hybrids would increase patient cognition (by increasing levels of acetylcholine), protect neurons from oxidative stress (by capturing free radicals and by complexing copper), and reduce the formation of senile plaques (due to their interaction with the PAS of AChE and their complexing properties). Such biological properties, along with their ability to reach therapeutic targets in CNS, highlight these tacrine−PBT2 hybrids as very interesting multifunctional prototypes in the search for new disease-modifying drugs useful in the treatment of Alzheimer's disease.

Experimental Section

Chemistry. General Methods. Reagents were purchased from common commercial suppliers and were used without further purification. Solvents were purified and dried by standard procedures. Chromatographic separations were performed either on silica gel (Kielgel 60 Merck of 230−400 mesh) or C18 reversed-phase (Sep-Pak Vac C18 cartridges). Compounds were detected with UV light (λ = 254 nm). HPLC analyses were performed on Waters 6000 equipment at a flow rate of 1.0 mL/min, with a UV detector (λ = 214−274 nm), and using a Delta Pak C_{18} 5 μm, 300 Å column.

Melting points (uncorrected) were determined with a Reichert-Jung Thermovar apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded in CD_3OD or $CDCl_3$ solutions using a Varian XL-300 spectrometer. Chemical shifts are reported in δ scale (ppm) relative to internal Me_4Si. *J* values are given in hertz, and spin multiplicities are expressed as s (singlet), d (doublet), t (triplet), quint (quintuplet), or m (multiplet). Mass spectra (MS) were obtained by electron spray ionization (ESI) in positive mode using a Hewlett-Packard MSD 1100 spectrometer. Elemental analyses were carried out in a Perkin-Elmer 240C equipment in the Centro de Química Orgánica Manuel Lora-Tamayo (CSIC), and the results are within ±0.4% of the theoretical values.

General Procedure for the Synthesis of Tacrine−PBT2 Hybrids 3−19. A mixture of corresponding N^1-(1,2,3,4-tetrahydroacridin-9-yl)alkane-1,*n*-diamine **20a**−**f**[52] or N^1-(3-aminopropyl)-N^1-methyl-N^3-(1,2,3,4-tetrahydroacridin-9-yl)propane-1,3-diamine **20g**[53] (1.0 mmol) and paraformaldehyde (3.0 mmol) in ethanol (20 mL) was heated at 90 °C for 6 h. After that, the reaction mixture was cooled to room temperature, and then a solution of 8-hydroxyquinoline derivative (1.0 mmol) in ethanol (2 mL) was added dropwise. The reaction mixture was stirred at room temperature for an additional 18 h, and the solvent was removed under reduced pressure. The residue was dissolved in dichloromethane (50 mL), washed with aqueous 10% solution of NaOH (3 × 50 mL), brine (3 × 50 mL), and H_2O (3 × 30 mL), dried over Na_2SO_4, and evaporated to dryness, yielding an oil that was purified employing one of the following methods.

Method A involved flash chromatography on silica gel column using as eluent EtOAc/CH_3OH/NH_4OH of increasing polarity. The corresponding tacrine−PBT2 compound was obtained as a syrup and identified by ^{1}H NMR, ^{13}C NMR, and MS. Then the treatment of the previous syrup with HCl (g) in toluene yielded the dihydrochloride derivative as a pure solid that was collected by filtration and used for obtaining combustion analysis results and the biological activities.

In method B, the crude oil was treated with aqueous HCl (10%) and evaporated to dryness. Then it was purified by reverse phase chromatography employing a C18 Sep-Park Vac 35 cm^3 (10 g) column using H_2O/CH_3OH/CF_3CO_2H as eluent. Following method B, tacrine−PBT2 derivatives were obtained as dihydrochlorides that were used for both structural elucidation and biological activities.

7-{[6-(1,2,3,4-Tetrahydroacridin-9-ylamino)hexylamino]methyl}-quinolin-8-ol Dihydrochloride (3·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)hexane-1,6-diamine (50 mg, 0.17 mmol), $(CH_2O)_n$ (16 mg, 0.50 mmol), and 8-hydroxyquinoline (24.6 mg, 0.17 mml). Purification involved method A, EtOAc/CH_3OH/NH_4OH (from 5:1:0.2 to 3:1:0.5). **3**: yellow oil (34.5 mg, 45%). R_f = 0.3 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS *m*/*z* 455 $[M + H]^+$. ^{1}H NMR ($CDCl_3$): δ = 8.81 (dd, 1H, *J* = 5.0 Hz, *J* = 1.6 Hz, H2′), 8.06 (dd, 1H, *J* = 8.3 Hz, *J* = 1.6 Hz, H4′), 7.95 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, H8), 7.91 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, H5), 7.53 (dt, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, H6), 7.34 (dd, 1H, *J* = 8.3 Hz, *J* = 5.0 Hz, H3′), 7.33 (dt, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, H7), 7.24 (d, 2H, *J* = 8.1 Hz, H5′,6′), 4.12 (s, 2H, ω), 3.48 (t, 2H, *J* = 7.0 Hz, α), 3.06 (m, 2H, H4), 2.68 (t, 2H, *J* = 7.0 Hz, β), 2.64 (m, 2H, H1), 1.88 (m, 4H, H2,3), 1.65 (m, 4H), 1.38 (m, 4H). ^{13}C NMR ($CDCl_3$): δ = 157.6, 152.6, 151.2, 148.6, 146.5, 139.0, 135.7, 128.7, 128.1 (2C), 127.8, 123.7, 122.9, 121.2, 119.6, 119.1, 117.2, 115.2, 51.2, 48.7, 49.3, 33.3, 31.6, 29.5, 26.9, 26.7, 24.6, 22.8, 22.5. **3a**·2HCl: yellow solid (mp 152−154 °C). Purity: 99% (by HPLC). Anal. ($C_{29}H_{34}N_4O$·2HCl) C, H, N.

7-{[7-(1,2,3,4-Tetrahydroacridin-9-ylamino)heptylamino]methyl}-quinolin-8-ol Dihydrochloride (4·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)heptane-1,7-diamine (100 mg, 0.31 mmol), $(CH_2O)_n$ (26.5 mg, 0.88 mmol), and 8-hydroxyquinoline (43 mg, 0.29 mmol). Purification involved method A, EtOAc/CH_3OH/NH_4OH (from 5:1:0.1 to 5:1:0.5). **4**: yellow oil (54.9 mg, 38%). R_f = 0.4 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS *m*/*z* 469 $[M + H]^+$. ^{1}H NMR ($CDCl_3$): δ = 8.86 (dd, 1H, *J* = 4.1 Hz, *J* = 1,6 Hz, H2′), 8.09 (dd, 1H, *J* = 8.3 Hz, *J* = 1.6 Hz, H4′), 7.95 (d, 1H, *J* = 8.3 Hz, H8), 7.85 (d, 1H, *J* = 8.3 Hz, H5), 7.55 (t, 1H, *J* = 8.3 Hz, H6), 7.40 (dd, 1H, *J* = 8.3 Hz, *J* = 4.1 Hz, H3′), 7.35 (t, 1H, *J* = 8.3 Hz, H7), 7.27 (d, 2H, *J* = 8.1 Hz, H5′,6′), 4.16 (s, 2H, ω), 3.47 (t, 2H, *J* = 7.1 Hz, α), 3.07 (m, 2H, H4), 2.73 (t, 2H, *J* = 7.1 Hz, β), 2.70 (m, 2H, H1), 1.93 (m, 4H, H2,3), 1.60 (m, 4H), 1.35 (m, 6H). ^{13}C NMR ($CDCl_3$): δ = 157.9, 152.9, 151.5, 148.6, 146.6, 139.4, 135.7, 128.6, 128.2, 127.9, 127.7, 123.8, 122.9, 121.2, 119.7, 118.8, 117.2, 115.3, 51.4, 49.4, 48.9, 33.5, 31.6, 29.5, 29.1, 27.0, 26.7, 24.6, 22.9, 22.5. **4**·2HCl: yellow solid (mp 156−158 °C). Purity: 98% (by HPLC). Anal. ($C_{30}H_{36}N_4O$·2HCl·2H_2O) C, H, N.

7-{[8-(1,2,3,4-Tetrahydroacridin-9-ylamino)octylamino]methyl}-quinolin-8-ol Dihydrochloride (5·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)octane-1,8-diamine (100 mg, 0.31 mmol), $(CH_2O)_n$ (27.6 mg, 0.92 mmol), and 8-hydroxyquinoline (45 mg, 0.31 mmol). Purification involved method A, EtOAc/CH_3OH/NH_4OH (from 9:1:0 to 5:1:0.3). **5**: yellow oil (34 mg, 23%). R_f = 0.4 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS *m*/*z* 483 $[M + H]^+$. ^{1}H NMR ($CDCl_3$): δ = 8.74 (dd, 1H, *J* = 4.1 Hz, *J* = 1.0 Hz, H2′), 8.00 (dd, 1H, *J* = 8.3 Hz, *J* = 1.0 Hz, H4′), 7.91 (d, 1H, *J* = 8.0 Hz, H8), 7.87 (d, 1H, *J* = 8.0 Hz, H5), 7.47 (t, 1H, *J* = 8.0 Hz, H6), 7.31 (dd, 1H, *J* = 8.3 Hz, *J* = 4.1 Hz, H3′), 7.27 (t, 1H, *J* = 8.0 Hz, H7), 7.20 (d, 2H, *J* = 8.1 Hz, H5′,6′), 4.07 (s, 2H, ω), 3.44 (t, 2H, *J* = 7.2 Hz, α), 3.00 (m, 2H, H4), 2.65 (t, 2H, *J* = 7.2 Hz, β), 2.60 (m, 2H, H1), 1.81 (m, 4H, H2,3), 1.56 (quint, 2H, *J* = 7.2 Hz), 1.47 (quint, 2H, *J* = 7.2 Hz), 1.22 (m, 8H). ^{13}C NMR ($CDCl_3$): δ = 157.3, 152.8, 151.4, 148.5, 146.1, 139.1, 135.6, 128.7, 128.2, 127.7, 127.4, 123.7, 123.0, 121.2, 119.5, 118.8, 117.1, 114.9, 51.2, 49.2, 48.7, 33.1, 31.4, 29.4, 29.2, 29.1, 26.9, 26.7, 24.5, 22.8, 22.4. **5**·2HCl: yellow solid (mp 123−125 °C). Purity: 98% (by HPLC). Anal. ($C_{31}H_{38}N_4O$·2HCl·2H_2O) C, H, N.

7-{[9-(1,2,3,4-Tetrahydroacridin-9-ylamino)nonylamino]methyl}-quinolin-8-ol Dihydrochloride (6·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)nonane-1,9-diamine (100 mg, 0.29 mmol), $(CH_2O)_n$ (26.5 mg, 0.88 mmol), and 8-hydroxyquinoline (43 mg, 0.29 mmol). Purification involved method A, EtOAc/CH_3OH/NH_4OH (from 5:1 to 5:1:0.5). **6**: yellow oil (54.9 mg, 38%). R_f = 0.3 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS *m*/*z* 497 $[M + H]^+$. ^{1}H NMR ($CDCl_3$): δ = 8.82 (dd, 1H, *J* = 4.3 Hz, *J* = 1.2 Hz, H2′), 8.07 (dd, 1H, *J* = 8.3 Hz, *J* = 1.2 Hz, H4′), 7.97 (dd, 1H, *J* = 8.0 Hz, *J* = 1.2 Hz, H8), 7.96 (dd, 1H, *J* = 8.0 Hz, *J* = 1.2 Hz, H5), 7.58 (dt, 1H, *J* = 8.0 Hz, *J* = 1.2 Hz, H6), 7.36 (dt, 1H,

J = 8.0 Hz, J = 1.2 Hz, H7), 7.34 (dd, 1H, J = 8.3 Hz, J = 1.2 Hz, H3′), 7.26 (d, 1H, J = 8.1 Hz, H6′), 7.23 (d, 1H, J = 8.1 Hz, H5′), 4.13 (s, 2H, ω), 3.51 (t, 2H, J = 7.2 Hz, α), 3.07 (m, 2H, H4), 2.70 (t, 2H, J = 7.2 Hz, β), 2.67 (m, 2H, H1), 1.99 (m, 4H, H2,3), 1.64 (quint, 2H, J = 7.2 Hz), 1.54 (quint, 2H, J = 7.2 Hz), 1.40 (m, 10H). ^{13}C NMR ($CDCl_3$): δ = 157.4, 152.7, 151.4, 148.6, 146.6, 139.0, 135.7, 128.7, 127.9, 127.8, 127.6, 123.7, 123.0, 121.2, 119.5, 118.9, 117.2, 115.0, 51.2, 49.2, 48.9, 33.1, 31.6, 29.6, 29.3 (2C), 29.1, 27.1, 26.8, 24.5, 22.8, 22.4. **6**·2HCl: yellow solid (mp 135−137 °C). Purity: 99% (by HPLC). Anal. ($C_{32}H_{40}N_4O$·2HCl·H_2O) C, H, N.

7-{[10-(1,2,3,4-Tetrahydroacridin-9-ylamino)decylamino]methyl}quinolin-8-ol Dihydrochloride (7·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)decane-1,10-diamine (120 mg, 0.34 mmol), $(CH_2O)_n$ (30.6 mg, 1.02 mmol), and 8-hydroxyquinoline (49 mg, 0.34 mmol). Purification involved method B, H_2O/CH_3OH/CF_3CO_2H (from 60:40:0.05 to 40:60:0.05). **7**·2HCl: yellow solid (33.0 mg, 20%), mp 145−147 °C. R_f = 0.4 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS m/z 511 $[M + H]^+$. ^{1}H NMR (CD_3OD): δ = 9.32 (dd, 1H, J = 5.1 Hz, J = 1.4 Hz, H2′), 9.27 (dd, 1H, J = 8.4 Hz, J = 1.4 Hz, H4′), 8.59 (dd, 1H, J = 8.4 Hz, J = 1.0 Hz, H8), 8.27 (dd, 1H, J = 8.4 Hz, J = 5.1 Hz, H3′), 8.16 (d, 1H, J = 8.6 Hz, H6′), 8.08 (d, 1H, J = 8.6 Hz, H5′), 8.04 (ddd, 1H, J = 8.4 Hz, J = 7.0 Hz, J = 1.0 Hz, H6), 7.97 (dd, 1H, J = 8.4 Hz, J = 1.0 Hz, H5), 7.78 (ddd, 1H, J = 8.4 Hz, J = 7.0 Hz, J = 1.0 Hz, H7), 4.76 (s, 2H, ω), 4.15 (t, 2H, J = 7.2 Hz, α), 3.37 (t, 2H, J = 7.2 Hz, β), 3.21 (m, 2H, H4), 2.90 (m, 2H, H1), 2.15 (m, 4H, H2,3), 2.00 (m, 4H), 1.60 (m, 12H). ^{13}C NMR (CD_3OD): δ = 158.0, 151.6, 149.0, 146.8, 146.3, 139.7, 134.1, 133.3, 133.2, 131.9, 126.5, 126.3, 124.2, 121.7, 121.5, 120.9, 117.0, 112.8, 49.2, 49.0, 46.8, 31.5, 30.5, 30.4, 30.3, 30.2, 29.3, 27.7, 27.6, 27.2, 24.9, 22.9, 21.8. Purity: 98% (by HPLC). Anal. ($C_{33}H_{42}N_4O$·2HCl) C, H, N.

7-{[12-(1,2,3,4-Tetrahydroacridin-9-ylamino)dodecylamino]methyl}quinolin-8-ol Dihydrochloride (8·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)dodecane-1,12-diamine (138 mg, 0.37 mmol), $(CH_2O)_n$ (34 mg, 1.12 mmol), and 8-hydroxyquinoline (54 mg, 0.34 mmol). Purification involved method B, H_2O/CH_3OH/CF_3CO_2H (from 70:30:0.05 to 35:65:0.05). **8**·2HCl: yellow solid (48 mg, 22%), mp 138−140 °C. R_f = 0.4 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS m/z 539 $[M + H]^+$. ^{1}H NMR (CD_3OD): δ = 8.82 (dd, 1H, J = 4.5 Hz, J = 1.2 Hz, H2′), 8.06 (dd, 1H, J = 8.2 Hz, J = 1.2 Hz, H4′), 7.94 (d, 1H, J = 8.1 Hz, H8), 7.92 (d, 1H, J = 8.1 Hz, H5), 7.53 (t, 1H, J = 8.1 Hz, H6), 7.34 (dd, 1H, J = 8.2 Hz, J = 4.5 Hz, H3′), 7.31 (t, 1H, J = 8.1 Hz, H7), 7.25 (d, 2H, J = 8.2 Hz, H5′,6′), 4.12 (s, 2H, ω), 3.48 (t, 2H, J = 7.2 Hz, α), 2.76 (t, 2H, J = 7.2 Hz, β), 3.05 (m, 2H, H4), 2.67 (m, 2H, H1), 1.89 (m, 4H, H2,3), 1.64 (quint, 2H, J = 7.2 Hz), 1.54 (quint, 4H, J = 7.2 Hz), 1.30 (m, 14H). ^{13}C NMR (CD_3OD): δ = 158.0, 152.5, 151.4, 148.9, 147.0, 139.3, 135.9, 128.8, 128.3 (2C), 128.0, 123.9, 123.1, 121.4, 120.0, 118.8, 117.5, 115.0, 51.5, 49.7, 49.2, 33.7, 31.9, 29.9, 29.7 (3C), 29.5 (2C), 27.4, 27.2 (2C), 24.8, 23.2, 22.8. Purity: 99% (by HPLC). Anal. ($C_{35}H_{46}N_4O$·2HCl·H_2O) C, H, N.

7-{{3-{Methyl[3-(1,2,3,4-tetrahydroacridin-9-ylamino)propyl]amino]propylamino}methyl}quinolin-8-ol Dihydrochloride (9·2HCl). Reagents were N^1-(3-aminopropyl)-N^1-methyl-N^3-(1,2,3,4-tetrahydroacridin-9-yl)propane-1,3-diamine (90 mg, 0.28 mmol), $(CH_2O)_n$ (25 mg, 0.83 mmol), and 8-hydroxyquinoline (41 mg, 0.28 mmol). Purification involved method B, H_2O/CH_3OH/CF_3CO_2H (from 70:30:0.05 to 35:65:0.05). **9**·3HCl: yellow solid (33 mg, 25%), mp 187−189 °C. R_f = 0.2 (CH_2Cl_2/CH_3OH/NH_4OH, 5:1:0.2). ESI-MS m/z 484 $[M + H]^+$. ^{1}H NMR (CD_3OD): δ = 9.00 (dd, 1H, J = 4.1 Hz, J = 1.4 Hz, H2′), 8.59 (dd, 1H, J = 8.5 Hz, J = 1.2 Hz, H8), 8.42 (dd, 1H, J = 8.3 Hz, J = 1.4 Hz, H4′), 8.04 (ddd, 1H, J = 8.5 Hz, J = 6.8 Hz, J = 1.2 Hz, H6), 7.90 (dd, 1H, J = 8.5 Hz, J = 1.2 Hz, H5), 7.78 (d, 1H, J = 8.3 Hz, H6′), 7.77 (ddd, 1H, J = 8.5 Hz, J = 6.8 Hz, J = 1.2 Hz, H7), 7.75 (dd, 1H, J = 8.3 Hz, J = 1.4 Hz, H3′), 7.60 (d, 1H, J = 8.4 Hz, H5′), 4.64 (s, 2H, ω), 4.24 (t, 2H, J = 7.0 Hz, α), 3.41 (m, 6H), 3.20 (m, 2H, H4), 2.91 (m, 2H, H1), 3.01 (s, 3H, CH_3), 2.45 (q, 4H, J = 7.0 Hz), 2.13 (m, 4H, H2,3). ^{13}C NMR (CD_3OD): δ = 157.7, 154.3, 151.9, 150.0, 139.7, 139.5, 137.3, 134.0, 130.8, 130.1, 126.7, 126.4, 123.7, 120.1, 118.9, 117.1, 114.2, 113.2, 55.0, 54.8, 49.9, 47.7, 46.3, 46.0, 29.4, 26.9, 25.3, 22.9, 22.5, 21.7. Purity: 100% (by HPLC). Anal. ($C_{30}H_{37}N_5O$·2HCl·H_2O) C, H, N.

2-Methyl-7-{[7-(1,2,3,4-tetrahydroacridin-9-ylamino)heptylamino]methyl}quinolin-8-ol Dihydrochloride (10·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)heptane-1,7-diamine (90 mg, 0.29 mmol), $(CH_2O)_n$ (26.0 mg, 0.87 mmol), and 2-methyl-8-hydroxyquinoline (46.0 mg, 0.29 mmol). Purification involved method A, EtOAc/CH_3OH/NH_4OH (6:1:0 to 5:1:0.5). **10**: yellow oil (43.3 mg, 33%). R_f = 0.6 (EtOAc/CH_3OH/NH_4-OH, 5:1:0.5). ESI-MS m/z 483 $[M + H]^+$. ^{1}H NMR ($CDCl_3$): δ = 7.92 (dd, 1H, J = 8.5 Hz, J = 1.0 Hz, H8), 7.91 (d, 1H, J = 8.5 Hz, H4′), 7.86 (dd, 1H, J = 8.5 Hz, J = 1.0 Hz, H5), 7.50 (ddd, 1H, J = 8.5 Hz, J = 7.0 Hz, J = 1.0 Hz, H6), 7.30 (ddd, 1H, J = 8.5 Hz, J = 7.0 Hz, J = 1.0 Hz, H7), 7.19 (d, 1H, J = 8.5 Hz, H3′), 7.18 (d, 2H, J = 8.5 Hz, H5′,6′), 4.10 (s, 2H, ω), 3.44 (t, 2H, J = 7.2 Hz, α), 3.02 (m, 2H, H4), 2.76 (s, 3H), 2.70 (m, 4H, H1, β), 1.89 (m, 4H, H2,3), 1.60 (quint, 2H, J = 7.2 Hz), 1.50 (quint, 2H, J = 7.2 Hz), 1.25 (m, 6H). ^{13}C NMR ($CDCl_3$): δ = 158.0, 157.4, 151.8, 150.9, 147.0, 138.4, 135.8, 128.9, 128.5, 128.1, 126.8, 123.6, 122.8, 122.1, 119.9, 119.8, 116.9, 115.7, 51.1, 49.4, 48.9, 33.7, 31.6, 29.6, 29.1, 27.0, 26.7, 25.3, 24.6, 22.9, 22.6. **10**·2HCl: yellow solid (mp 157−159 °C). Purity: 99% (by HPLC). Anal. ($C_{31}H_{38}N_4O$·2HCl·$3H_2O$) C, H, N.

2-Methyl-7-{[8-(1,2,3,4-tetrahydroacridin-9-ylamino)octylamino]methyl}quinolin-8-ol Dihydrochloride (11·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)octane-1,8-diamine (100 mg, 0.31 mmol), $(CH_2O)_n$ (28 mg, 0.92 mmol), and 2-methyl-8-hydroxyquinoline (50.0 mg, 0.31 mmol). Purification involved method A, EtOAc/CH_3OH/NH_4OH (from 9:1:0 to 7:1:0.2). **11**: yellow oil (54.0 mg, 35%). R_f = 0.6 (EtOAc/CH_3-OH/NH_4OH, 5:1:0.5). ESI-MS m/z 497 $[M + H]^+$. ^{1}H NMR ($CDCl_3$): δ = 7.92 (dd, 1H, J = 8.5 Hz, J = 1.0 Hz, H8), 7.91 (d, 1H, J = 8.5 Hz, H4′), 7.86 (dd, 1H, J = 8.5 Hz, J = 1.0 Hz, H5), 7.50 (ddd, 1H, J = 8.5 Hz, J = 6.9 Hz, J = 1.0 Hz, H6), 7.30 (ddd, 1H, J = 8.5 Hz, J = 6.9 Hz, J = 1.0 Hz, H7), 7.19 (d, 1H, J = 8.5 Hz, H3′), 7.17 (d, 2H, J = 8.5 Hz, H5′,6′), 4.10 (s, 2H, ω), 3.44 (t, 2H, J = 7.2 Hz, α), 3.02 (m, 2H, H4), 2.70 (m, 4H, H1, β), 2.70 (s, 3H), 1.89 (m, 4H, H2,3), 1.60 (quint, 2H, J = 7.2 Hz), 1.50 (quint, 2H, J = 7.2 Hz), 1.25 (m, 8H). ^{13}C NMR ($CDCl_3$): δ = 158.0, 157.4, 151.8, 150.9, 147.0, 138.4, 135.8, 128.9, 128.5, 128.1, 126.8, 123.6, 122.8, 122.1, 119.9, 119.8, 116.9, 115.7, 51.1, 49.4, 48.9, 33.7, 31.6, 29.6 (2C), 29.1, 27.0, 26.7, 25.3, 24.6, 22.9, 22,6. **11**·2HCl: yellow solid (mp 146−148 °C). Purity: 98% (by HPLC). Anal. ($C_{32}H_{40}N_4O$·2HCl·H_2O) C, H, N.

2-Methyl-7-{[9-(1,2,3,4-tetrahydroacridin-9-ylamino)nonylamino]methyl}quinolin-8-ol Dihydrochloride (12·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)nonane-1,9-diamine (100 mg, 0.31 mmol), $(CH_2O)_n$ (28 mg, 0.92 mmol), and 2-methyl-8-hydroxyquinoline (50 mg, 0.31 mmol). Purification involved method A, EtOAc/CH_3OH/NH_4OH (from 8:1 to 5:1:0.5). **12**: yellow oil (54 mg, 35%). R_f = 0.6 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS m/z 511 $[M + H]^+$. ^{1}H NMR ($CDCl_3$): δ = 7.93 (dd, 1H, J = 8.5 Hz, J = 1.0 Hz, H8), 7.91 (d, 1H, J = 8.5 Hz, H4′), 7.86 (dd, 1H, J = 8.5 Hz, J = 1.0 Hz, H5), 7.50 (ddd, 1H, J = 8.5 Hz, J = 6.9 Hz, J = 1.0 Hz, H6), 7.29 (ddd, 1H, J = 8.5 Hz, J = 6.9 Hz, J = 1.0 Hz, H7), 7.19 (d, 1H, J = 8.5 Hz, H3′), 7.12 (d, 2H, J = 8.5 Hz, H5′,6′), 4.10 (s, 2H, ω), 3.43 (t, 2H, J = 7.2 Hz, α), 3.03 (m, 2H, H4), 2.70 (s, 3H), 2.67 (m, 4H, H1, β), 1.89 (m, 4H, H2,3), 1.60 (quint, 2H, J = 7.2 Hz), 1.50 (quint, 2H, J = 7.2 Hz), 1.28 (m, 10H). ^{13}C NMR ($CDCl_3$): δ = 158.3, 157.3, 151.8, 150.7, 147.4, 138.4, 135.8, 128.6, 128.1, 126.7, 126.1, 123.4, 122.8, 122.2, 122.0, 120.1, 116.9, 115.7, 51.2, 49.4, 48.9, 33.9, 31.7, 29.7, 29.4, 29.3, 29.2, 27.0, 26.8, 25.1, 24.7, 22.9, 22.7. **12**·2HCl: yellow solid (mp 138−140 °C). Purity: 99% (by HPLC). Anal. ($C_{33}H_{42}N_4O$·2HCl·H_2O) C, H, N.

2-Methyl-7-{[10-(1,2,3,4-tetrahydroacridin-9-ylamino)decylamino]methyl}quinolin-8-ol Dihydrochloride (13·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)decane-1,10-diamine (70 mg, 0.20 mmol), $(CH_2O)_n$ (18 mg, 0.6 mmol), and 2-methyl-8-hydroxyquinoline (31.5 mg, 0.20 mmol). Purification involved

method A, EtOAc/CH_3OH/NH_4OH (from 8:1 to 5:1:0.3). **13**: yellow oil (54.0 mg, 35%). R_f = 0.6 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS m/z 524 $[M + H]^+$. ^1H NMR ($CDCl_3$): δ = 7.87 (d, 2H, J = 8.5 Hz, H8, H4′), 7.82 (d, 1H, J = 8.5 Hz, H5), 7.50 (t, 1H, J = 8.5 Hz, H6), 7.30 (t, 1H, J = 8.5 Hz, H7), 7.18 (d, 1H, J = 8.5 Hz, H3′), 7.13 (d, 2H, J = 8.8 Hz, H5′,6′), 4.10 (s, 2H, ω), 3.44 (t, 2H, J = 7.2 Hz, α), 3.02 (m, 2H, H4), 2.70 (m, 4H, H1, β), 2.66 (s, 3H), 1.89 (m, 4H, H2,3), 1.60 (quint, 2H, J = 7.2 Hz), 1.50 (quint, 2H, J = 7.2 Hz), 1.29 (m, 12H). ^{13}C NMR ($CDCl_3$): δ = 158.0, 157.4, 150.9, 151.8, 147.2, 138.4, 135.8, 128.9, 128.5, 128.3, 126.8, 123.6, 122.8, 122.1, 120.0, 119.8, 116.9, 115.7, 51.2, 49.5, 49.0, 33.8, 31.7, 29.7, 29.4, 29.3, 29.2, 27.1, 26.8, 25.8, 25.3, 24.6, 22.9, 22.6. **13**·2HCl: yellow solid (mp 131−133 °C). Purity: 98% (by HPLC). Anal. ($C_{34}H_{44}N_4O$·2HCl·H_2O) C, H, N.

2-Methyl-7-{{3-{methyl[3-(1,2,3,4-tetrahydroacridin-9-ylamino)propyl]amino}propylamino}methyl}quinolin-8-ol Dihydrochloride (14·2HCl). Reagents were N^1-(3-aminopropyl)-N^1-methyl-N^3-(1,2,3,4-tetrahydroacridin-9-yl)propane-1,3-diamine (90 mg, 0.28 mmol), $(CH_2O)_n$ (25.0 mg, 0.83 mmol), and 2-methyl-8-hydroxyquinoline (44 mg, 0.28 mmol). Purification involved method B, H_2O/CH_3OH/CF_3CO_2H (from 70:30:0.05 to 35:65:0.05). **14**·2HCl: yellow solid (38 mg, 27%), mp 178−180 °C. R_f = 0.3 (CH_2Cl_2/CH_3OH/NH_4OH, 4:1:0.1). ESI-MS m/z 498 $[M + H]^+$. ^1H NMR (CD_3OD): δ = 8.64 (d, 1H, J = 8.5 Hz, H4′), 8.37 (dd, 1H, J = 8.3 Hz, J = 1.4 Hz, H8), 8.03 (ddd, 1H, J = 8.3 Hz, J = 6.8 Hz, J = 1.4 Hz, H6), 7.98 (dd, 1H, J = 8.3 Hz, J = 1.4 Hz, H5), 7.81 (ddd, 1H, J = 8.3 Hz, J = 6.8 Hz, J = 1.4 Hz, H7), 7.71 (d, 1H, J = 8.5 Hz, H6′), 7.66 (d, 1H, J = 8.5 Hz, H3′), 760 (d, 1H, J = 8.5 Hz, H5′), 4.66 (s, 2H, ω), 4.28 (t, 2H, J = 7.1 Hz, α), 3.53 (m, 4H), 3.42 (t, 2H, J = 7.8 Hz), 3.22 (m, 2H, H4), 3.13 (s, 3H, CH_3), 2.97 (m, 2H, H1), 2.92 (s, 3H, CH_3), 2.52 (quint, 4H, J = 7.5 Hz, β), 2,16 (m, 4H, H2,3). ^{13}C NMR (CD_3OD): δ = 159.7, 158.1, 153.1, 152.4, 139.7, 138.8, 137.8, 134.2, 129.3, 129.2, 126.9, 126.5, 124.9, 120.3, 119.4, 117.4, 114.1, 113.6, 54.9, 54.4, 47.5, 46.5, 45.5, 40.7, 29.5, 26.6, 25.5, 25.1, 23.1, 22.4, 21.9. Purity: 100% (by HPLC). Anal. ($C_{31}H_{39}N_5O$·2HCl·$2H_2O$) C, H, N.

5-Chloro-7-{[8-(1,2,3,4-tetrahydroacridin-9-ylamino)octylamino]methyl}quinolin-8-ol Dihydrochloride (15·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)octane-1,8-diamine (100 mg, 0.31 mmol), $(CH_2O)_n$ (28 mg, 0.90 mmol), and 5-chloro-8-hydroxyquinoline (55 mg, 0.31 mmol). Purification involved method B, H_2O/CH_3OH/CF_3CO_2H (from 70:30:0.05 to 35:65:0.05). **15**·2HCl: yellow solid (37 mg, 23%), mp 145−147 °C. R_f = 0.4 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS m/z 517 $[M + H]^+$. ^1H NMR (CD_3OD): δ = 8.95 (dd, 1H, J = 4.2 Hz, J = 1.4 Hz, H2′), 8.60 (dd, 1H, J = 8.5 Hz, J = 1.4 Hz, H4′), 8.36 (dd, 1H, J = 8.5 Hz, J = 1.0 Hz, H8), 7.83 (ddd, 1H, J = 8.5 Hz, J = 7.0 Hz, J = 1.0 Hz, H6), 7.74 (dd, 1H, J = 8.5 Hz, J = 1.0 Hz, H5), 7.70 (dd, 1H, J = 8.5 Hz, J = 4.2 Hz, H3′), 7.68 (s, 1H, H6′), 7.56 (ddd, 1H, J = 8.5 Hz, J = 7.0 Hz, J = 1.0 Hz, H7), 4.41 (s, 2H, ω), 3.93 (t, 2H, J = 7.5 Hz, α), 3.08 (t, 2H, J = 7.5 Hz, β), 2.99 (m, 2H, H4), 2.67 (m, 2H, H1), 1.95 (m, 4H, H2,3), 1.82 (quint, 2H, J = 7.2 Hz), 1.73 (quint, 2H, J = 7.2 Hz), 1.41 (m, 8H). ^{13}C NMR (CD_3OD): δ = 158.2, 153.5, 151.8, 151.0, 140.4, 140.0, 134.3, 131.8, 129.8, 128.6, 126.6, 126.4, 124.9, 121.6, 120.3, 117.3, 114.8, 113.0, 49.1, 48.6, 46.9, 31.7, 30.2, 29.4, 28.7, 27.8, 27.6, 27.1, 24.9, 23.2, 22.0. Purity: 97% (by HPLC). Anal. ($C_{31}H_{37}ClN_4O$·2HCl) C, H, N.

5-Chloro-7-{[9-(1,2,3,4-tetrahydroacridin-9-ylamino)nonylamino]methyl}quinolin-8-ol Dihydrochloride (16·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)nonane-1,9-diamine (100 mg, 0.30 mmol), $(CH_2O)_n$ (27.0 mg, 0.88 mmol), and 5-chloro-8-hydroxyquinoline (53.0 mg, 0.29 mmol). Purification involved method B, H_2O/CH_3OH/CF_3CO_2H (from 70:30:0.05 to 35:65:0.05). **16**·2HCl: yellow solid (54.0 mg, 35%), mp 135−137 °C. R_f = 0.4 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS m/z 531 $[M + H]^+$. ^1H NMR (CD_3OD): δ = 9.14 (dd, 1H, J = 4.2 Hz, J = 1.5 Hz, H2′), 8.74 (dd, 1H, J = 8.6 Hz, J = 1.5 Hz, H4′), 8.56 (dd, 1H, J = 8.2 Hz, J = 1.0 Hz, H8), 8.03 (ddd, 1H, J = 8.2 Hz, J = 7.1 Hz, J = 1.0 Hz, H6), 7.94 (dd, 1H, J = 8.2 Hz, J = 1.0 Hz, H5), 7.89 (dd, 1H, J = 8.6 Hz, J = 4.2 Hz, H3′), 7.85 (s, 1H, H6′), 7.75 (ddd, 1H, J = 8.2 Hz, J = 7.1 Hz, J = 1.0 Hz, H7), 4.60 (s, 2H, ω), 4.12 (t, 2H, J = 7.3 Hz, α), 3.28 (t, 2H, J = 7.3 Hz, β), 3.19 (m, 2H, H4), 2.87 (m, 2H, H1), 2.14 (m, 4H, H2,3), 2.01 (quint, 2H, J = 7.1 Hz), 1.93 (quint, 2H, J = 7.1 Hz), 1.50 (m, 10H). ^{13}C NMR (CD_3OD): δ = 158.7, 153.1, 151.7 (2C), 140.2, 139.9, 134.2 (2C), 129.9, 128.4, 126.6, 126.4, 124.8, 121.5, 120.3, 117.1, 114.8, 112.9, 49.2, 48.5, 46.8, 31.6, 30.5, 30.3, 30.2, 29.4, 27.9, 27.7, 27.1, 24.9, 23.1, 21.9. Purity: 97% (by HPLC). Anal. ($C_{32}H_{39}ClN_4O$·2HCl·H_2O) C, H, N.

5-Chloro-7-{[10-(1,2,3,4-tetrahydroacridin-9-ylamino)decylamino]methyl}quinolin-8-ol Dihydrochloride (17·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)decane-1,10-diamine (100 mg, 0.29 mmol), $(CH_2O)_n$ (27.0 mg, 0.88 mmol), and 5-chloro-8-hydroxyquinoline (53.0 mg, 0.29 mmol). Purification involved method B, H_2O/CH_3OH/CF_3CO_2H (from 70:30:0.05 to 35:65:0.05). **17**·2HCl: yellow solid (54.0 mg, 35%), mp 140−142 °C. R_f = 0.4 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS m/z 545 $[M + H]^+$. ^1H NMR (CD_3OD): δ = 9.15 (dd, 1H, J = 4.3 Hz, J = 1.6 Hz, H2′), 8.78 (dd, 1H, J = 8.5 Hz, J = 1.6 Hz, H4′), 8.57 (dd, 1H, J = 8.5 Hz, J = 1.2 Hz, H8), 8.06 (ddd, 1H, J = 8.5 Hz, J = 7.2 Hz, J = 1.2 Hz, H6), 7.93 (dd, 1H, J = 8.5 Hz, J = 1.2 Hz, H5), 7.90 (dd, 1H, J = 8.5 Hz, J = 4.3 Hz, H3′), 7.89 (s, 1H, H6′), 7.73 (ddd, 1H, J = 8.5 Hz, J = 7.2 Hz, J = 1.2 Hz, H7), 4.61 (s, 2H, ω), 4.13 (t, 2H, J = 7.4 Hz, α), 3.28 (t, 2H, J = 7.4 Hz, β), 3.20 (m, 2H, H4), 2.88 (m, 2H, H1), 2.15 (m, 4H, H2,3), 2.04 (quint, 2H, J = 7.1 Hz), 1.85 (m, 2H), 1.55 (m, 12H). ^{13}C NMR (CD_3OD): δ = 158.3, 153.0, 151.5, 151.2, 140.1, 139.7, 134.1, 133.9, 129.7, 128.2, 126.3, 126.1, 124.6, 121.3, 119.9, 116.8, 114.6, 112.7, 49.3, 48.4, 46.7, 31.4, 30.6, 30.1, 30.0 (2C), 29.1, 27.9, 27.7, 27.1, 24.4, 22.9, 22.3. Purity: 99% (by HPLC). Anal. ($C_{33}H_{41}ClN_4O$·2HCl) C, H, N.

5-Chloro-7-{[12-(1,2,3,4-tetrahydroacridin-9-ylamino)dodecylamino]methyl}quinolin-8-ol Dihydrochloride (18·2HCl). Reagents were N^1-(1,2,3,4-tetrahydroacridin-9-yl)dodecane-1,12-diamine (110 mg, 0.29 mmol), $(CH_2O)_n$ (26.0 mg, 0.88 mmol), and 5-chloro-8-hydroxyquinoline (51.7 mg, 0.29 mmol). Purification involved method B, H_2O/CH_3OH/CF_3CO_2H (from 70:30:0.05 to 35:65:0.05). **18**·2HCl: yellow solid (32.0 mg, 20%), mp 123−125 °C. R_f = 0.3 (EtOAc/CH_3OH/NH_4OH, 5:1:0.5). ESI-MS m/z 573 $[M + H]^+$. ^1H NMR ($CDCl_3$): δ = 8.85 (dd, 1H, J = 4.0 Hz, J = 1.4 Hz, H2′), 8.43 (dd, 1H, J = 8.6 Hz, J = 1.4 Hz, H4′), 8.14 (dd, 1H, J = 8.3 Hz, J = 1.2 Hz, H5), 8.02 (dd, 1H, J = 8.3 Hz, J = 1.2 Hz, H8), 7.58 (ddd, 1H, J = 8.3 Hz, J = 7.0 Hz, J = 1.2 Hz, H6), 7.46 (dd, 1H, J = 8.6 Hz, J = 4.0 Hz, H3′), 7.40 (s, 1H, H6′), 7.34 (ddd, 1H, J = 8.3 Hz, J = 7.0 Hz, J = 1.2 Hz, H7), 4.11 (s, 2H, ω), 3.62 (t, 2H, J = 7.2 Hz, α), 3.15 (m, 2H, H4), 2.68 (t, 2H, J = 7.2 Hz, β), 2.63 (m, 2H, H1), 1.89 (m, 4H, H2,3), 1.65 (quint, 2H, J = 7.0 Hz), 1.54 (quint, 2H, J = 7.0 Hz), 1.30 (m, 16H). ^{13}C NMR ($CDCl_3$): δ = 155.7, 152.6, 152.2, 149.1, 144.6, 139.6, 132.8, 129.8, 127.4, 125.3, 124.1, 123.3, 121.9 (2C), 120.0, 119.7, 118.4, 115.0, 50.9, 49.2, 48.9, 31.8, 31.5, 29.6, 29.4 (5C), 29.2, 27.1, 26.8, 24.2, 22.6, 21.9. Purity: 96% (by HPLC). Anal. ($C_{35}H_{45}ClN_4O$·2HCl) C, H, N.

5-Chloro-7-{{3-{methyl[3-(1,2,3,4-tetrahydroacridin-9-ylamino)propyl]amino}propylamino}methyl}quinolin-8-ol Dihydrochloride (19·2HCl). Reagents were N^1-(3-aminopropyl)-N^1-methyl-N^3-(1,2,3,4-tetrahydroacridin-9-yl)propane-1,3-diamine (100 mg, 0.31 mmol), $(CH_2O)_n$ (28 mg, 0.93 mmol), and 5-chloro-8-hydroxyquinoline (55 mg, 0.31 mmol). Purification involved method B, H_2O/CH_3OH/CF_3CO_2H (from 70:30:0.05 to 35:65:0.05). **19**·2HCl: yellow solid (40 mg, 25%), mp 137−139 °C. R_f = 0.5 (CH_2Cl_2/CH_3OH/NH_4OH, 4:1:0.2). ESI-MS m/z 518 $[M + H]^+$. ^1H NMR (CD_3OD): δ = 9.00 (dd, 1H, J = 4.2 Hz, J = 1.5 Hz, H2′), 8.56 (dd, 1H, J = 8.6 Hz, J = 1.5 Hz, H4′), 8.46 (dd, 1H, J = 8.2 Hz, J = 1.5 Hz, H8), 7.98 (ddd, 1H, J = 8.2 Hz, J = 7.0 Hz, J = 1.5 Hz, H6), 7.91 (dd, 1H, J = 8.2 Hz, J = 1.5 Hz, H5), 7.84 (s, 1H, H6′), 7.76 (dd, 1H, J = 8.6 Hz, J = 4.2 Hz, H3′), 7.71 (ddd, 1H, J = 8.2 Hz, J = 7.0 Hz, J = 1.5 Hz, H7), 4.54 (s, 2H, ω), 4.14 (t, 2H, J = 7.1 Hz, α), 3.41 (t, 2H, J = 7.0 Hz), 3.18 (m, 2H, H4), 3.09 (m, 2H), 3.06 (m, 2H), 2.82 (m, 2H, H1), 2.74 (s, 3H), 2.26 (m, 4H), 2.13 (m, 4H, H2,3). ^{13}C NMR ($CDCl_3$): δ = 157.5, 154.1,

151.6, 150.4, 140.2, 139.6, 134.0, 133.9, 129.9, 128.0, 126.4 (2C), 124.3, 121.0, 120.1, 116.8, 115.5, 112.8, 56.7, 55.6, 47.8, 47.7, 47.3, 41.8, 29.3, 27.7, 25.0, 23.5, 22.9, 21.8. Purity: 98% (by HPLC). Anal. ($C_{30}H_{36}ClN_5O \cdot 2HCl \cdot 3H_2O$) C, H, N.

Biochemical Studies. Cholinesterase Inhibitory Activities. Acetylcholinesterase (AChE, EC 3.1.1.7) from bovine erythrocytes (0.25−1.0 unit/mg, lyophilized powder), AChE (EC 3.1.1.7) from human erythrocytes (minimal 500 units/mg protein in buffered aqueous solution), butyrylcholinesterase (BuChE, EC 3.1.1.8) from equine serum (10 units/mg protein, lyophilized powder), and BuChE (EC 3.1.1.8) from human serum (3 units/mg protein, lyophilized powder) were purchased from Sigma. Compounds were measured in 100 mM phosphate buffer, pH 8.0, at 30 °C, using acetylthiocholine and butyrylthiocholine (0.4 mM) as substrates, respectively. In both cases, 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB, Ellman's reagent, 0.2 mM) was used and the values of IC_{50} were calculated by UV spectroscopy from the absorbance changes at 412 nm.[54] Experiments were performed in triplicate.

Oxygen Radical Absorbance Capacity Assay. The ORAC-FL method of Ou et al.[56] partially modified by Dávalos et al.[57] was followed, using a Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) with 485-P excitation and 520-P emission filters. The equipment was controlled by Fluorostar Galaxy software (version 4.11-0) for fluorescence measurement. 2,2′-Azobis(amidinopropane) dihydrochloride (AAPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and fluorescein (FL) were purchased from Sigma-Aldrich. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. Antioxidant (20 μL) and FL (120 μL, 70 mM, final concentration) solutions were placed in a black 96-well microplate (96F untreat, Nunc). The mixture was preincubated for 15 min at 37 °C, and then AAPH solution (60 μL, 12 mM, final concentration) was added rapidly using a multichannel pipet. The microplate was immediately placed in the reader and the fluorescence recorded every minute for 80 min. The microplate was automatically shaken prior each reading. Samples were measured at eight different concentrations (0.1−1 μM). A blank (FL + AAPH in phosphate buffer) instead of the sample solution and eight calibration solutions using Trolox (1−8 μM) were also used in each assay. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample. Raw data were exported from the Fluostar Galaxy software to an Excel sheet for further calculations. Antioxidant curves (fluorescence vs time) were first normalized to the curve of the blank corresponding to the same assay, and the area under the fluorescence decay curve (AUC) was calculated. The net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all samples. ORAC-FL values were expressed as Trolox equivalents by using the standard curve calculated for each assay, where the ORAC-FL value of Trolox was taken as 1.

In Vitro Blood−Brain Barrier Penetration Assay. Prediction of the brain penetration was evaluated using a parallel artificial membrane permeation assay (PAMPA) in a similar manner as previously described.[62] Commercial drugs, phosphate buffer saline solution at pH 7.4 (PBS), and dodecane were purchased from Sigma, Aldrich, Acros, and Fluka. Millex filter units (polyvinylidene fluoride PVDF membrane, diameter 25 mm, pore size 0.45 μm) were acquired form Millipore. The porcine brain lipid was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μm), and the acceptor microplate was an indented 96-well plate, both from Millipore. The acceptor 96-well microplate was filled with 180 μL of PBS/ethanol (70:30), and the filter surface of the donor microplate was impregnated with 4 μL of porcine brain lipid in dodecane (20 mg mL^{-1}). Compounds were dissolved in PBS/ethanol (70:30) at 1 mg mL^{-1}, filtered through a Millex filter, and then added to the donor wells (180 μL). The donor filter plate was carefully put on the acceptor plate to form a sandwich, which was left undisturbed for 120 min at 25 °C. After incubation, the donor plate was carefully removed and the concentration of compounds in the acceptor wells was determined by UV spectroscopy. Every sample was analyzed at five wavelengths, in four wells, and at least in three independent runs, and the results are given as the mean ± standard deviation (SD). In each experiment, 20 quality control standards of known BBB permeability were included to validate the analysis set.

Measurement of Propidium Iodine Displacement from the Pheripheral Anionic Site (PAS) of AChE. A solution of AChE (EC 3.1.1.7) from bovine erythrocytes at 5 μM in 0.1 mM tris(hydroxymethyl)aminomethane buffer, pH 8.0, was used. Aliquots of compounds were added to give final concentrations of 0.3, 1.0, and 3 μM, and the solutions were kept at room temperature for at least 6 h. Afterward, the samples were incubated for 15 min with propidium iodide at a final concentration of 20 μM, and the fluorescence (λ_{ex} = 485 nm, λ_{em} = 620 nm) was measured in a fluorescence microplate reader (Fluostar Optima, BMG, Germany).

Culture of SH-SY5Y Cells and Studies of Cell Viability and Neuroprotection. SH-SY5Y cells, at passages between 3 and 16 after thawing, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15 nonessential amino acids and supplemented with 10% fetal calf serum, 1 mM glutamine, 50 U mL^{-1} penicillin, and 50 μg mL^{-1} streptomycin (reagents from GIBCO, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and were maintained at 37 °C in 5% CO_2/humidified air. Stock cultures were passaged 1:4 twice weekly. For assays, SHSY5Y cells were subcultured in 48-well plates at a seeding density of 10^5 cells per well. For the cytotoxicity/protection experiments, cells were treated with drugs before confluence in serum-free DMEM.

To study the potential cytotoxic effects of the compounds alone, cells were plated at a density of 10^5 cells per well at least 48 h before toxicity measurements. Cells were exposed for 24 h to the compound at various concentrations, and the quantitative assessment of cell death was made by measurement of the percentage of the intracelular enzyme lactate dehydrogenase (LDH) released to the extracelular medium (cytotoxicity detection kit, Roche). The quantity of LDH was evaluated in a microplate reader (Fluostar Optima, BMG, Germany) at 492 nm (λ_{ex}) and 620 nm (λ_{em}). Controls were taken as having 100% viability. To study the cytoprotective action of various compounds against cell death induced by 30 μM rotenone, drugs were given at t_0 and maintained for 24 h. The media were then replaced by fresh media still containing the drug plus the cytotoxic stimulus, and cells were left for an additional 24 h. Cell survival was assessed by measuring LDH activity.

Measurement of Lactic Dehydrogenase (LDH) Activity. Extracellular and intracellular LDH activity was measured by UV/vis using a cytotoxicity cell death kit (Roche-Boehringer, Mannheim, Germany) according to the manufacturer's protocol. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity, and released LDH was defined as the percentage of extracelular versus total LDH activity. Data were expressed as the mean ± SEM of at least three different cultures in quadruplicate. LDH released was calculated for each individual experiment, considering as 100% the extracellular LDH released by the vehicle with respect to the total. To determine percent protection, LDH release was normalized as follows: in each individual triplicate experiment, LDH release obtained in untreated cells (basal) was subtracted from the LDH released upon the toxic treatment and normalized to 100%, and that value was subtracted from 100.

Metal-Chelating Properties of Tacrine−PBT2 Hybrid 6. The complexing studies were made in Tris buffer (pH 7.4) at 298 K using a UV−vis spectrophotometer (ThermoMultiscan).

The absorption spectra of compound **6**, alone or in the presence of $CuSO_4$ or $Fe_2(SO_4)_3$, were recorded at room temperature in a 1 cm quartz cell.

The stoichiometry of the complex **6**−Cu^{2+} was determined by employing Job's method.[70,71] From separate solutions of hybrid **6** and $CuSO_4$ in Tris buffer, each of the same concentration (2.3×10^{-7} M), 21 solutions were obtained with the condition that the sum of concentrations of both species was a constant in all samples but the proportions of both components varied between 0 and 100%. The absorbance differences at 242 nm were plotted vs the mole fraction, showing a maximum at 0.7 that revealed a stoichiometry of 2:1 for complex **6**−Cu(II).

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Supporting Information Available: Elemental analysis results of new tacrine−PBT2 hybrids; experimental details for PAMPA-BBB assay and for metal-chelating ability studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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