Article

Structure-Based Design and Optimization of Multitarget-Directed 2H-Chromen-2-one Derivatives as Potent Inhibitors of Monoamine Oxidase B and Cholinesterases

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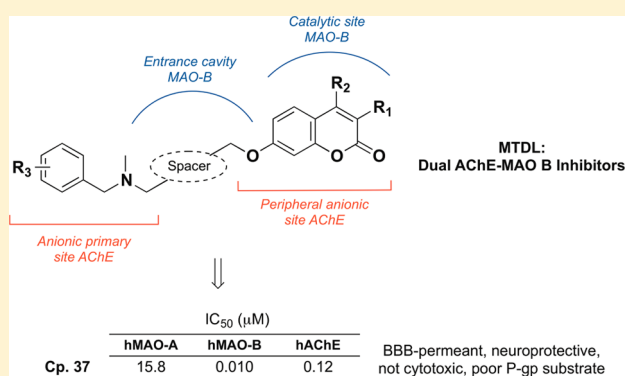
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S Supporting Information

ABSTRACT: The multifactorial nature of Alzheimer's disease calls for the development of multitarget agents addressing key pathogenic processes. To this end, by following a docking-assisted hybridization strategy, a number of aminocoumarins were designed, prepared, and tested as monoamine oxidases (MAOs) and acetyl- and butyryl-cholinesterase (AChE and BChE) inhibitors. Highly flexible N-benzyl-N-alkyloxy coumarins **2−12** showed good inhibitory activities at MAO-B, AChE, and BChE but low selectivity. More rigid inhibitors, bearing meta- and para-xylyl linkers, displayed good inhibitory activities and high MAO-B selectivity. Compounds **21**, **24**, **31**, **37**, and **39**, the last two featuring an improved hydrophilic/lipophilic balance, exhibited excellent activity profiles with nanomolar inhibitory potency toward hMAO-B, high hMAO-B over hMAO-A selectivity and submicromolar potency at hAChE. Cell-based assays of BBB permeation, neurotoxicity, and neuroprotection supported the potential of compound **37** as a BBB-permeant neuroprotective agent against H_2O_2-induced oxidative stress with poor interaction as P-gp substrate and very low cytotoxicity.

	IC$_{50}$ (μM)		
	hMAO-A	hMAO-B	hAChE
Cp. 37	15.8	0.010	0.12

MTDL: Dual AChE-MAO B Inhibitors

BBB-permeant, neuroprotective, not cytotoxic, poor P-gp substrate

INTRODUCTION

Neurodegenerative diseases (NDs) are widely investigated pathologies because of the low efficacy of current therapies[1,2] and severe functional impairments for daily life activities, resulting in high familiar, social, and financial costs of patient care.[3] Despite the huge efforts in private and public research settings, most clinical trials of potential drug candidates for NDs, and for Alzheimer's disease (AD) in particular,[4,5] failed. As a result, valuable disease-modifying therapies for NDs are still missing.

AD and Parkinson's disease (PD) are the most widespread and severe NDs. AD is the result of a progressive loss of neurons in basal forebrain regions associated with abnormal accumulation of beta amyloid protein (Aβ) in neuronal plaques and hyperphosphorylated tau protein in neurofibrillary tangles.[6] Neuronal degeneration in AD is triggered and maintained by low molecular weight Aβ oligomers and by reactive oxygen species (ROS), produced by oxidative degradation of neuro-

transmitters and xenobiotics.[7] The brain regions mostly affected by neuronal loss are essentially made of cholinergic neurons, so that restoring physiological acetylcholine levels has been considered a viable therapy in AD, as claimed by the so-called cholinergic hypothesis.[8] To date, drugs approved for AD therapy are the cholinesterase (ChE) inhibitors rivastigmine, galant-amine, and donepezil[9,10] (Chart 1). The NMDA partial antagonist memantine has also been approved for the symptomatic treatment of AD for its contrasting effects over glutamate excitotoxicity.[11]

Acetylcholinesterase (AChE, EC 3.1.1.7), the key enzyme targeted in the palliative therapy of AD, is present in both central and peripheral nervous system and in muscular motor plaques and is responsible for the enzymatic cleavage of neurotransmitter acetylcholine (ACh). The other ChE,

Chart 1. Chemical Structures of Reference AChE and MAO Inhibitors

Rasagiline   Clorgyline   Pargyline

Donepezil   NW-1772
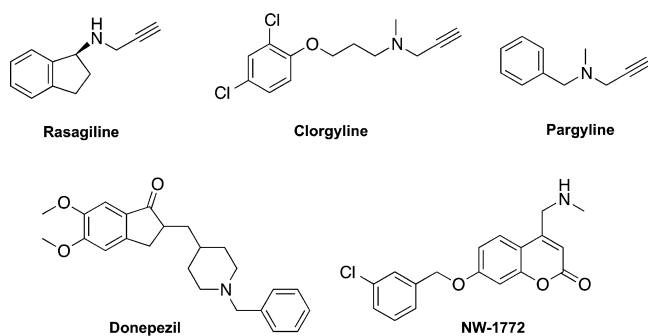
butyrylcholinesterase (BChE, EC 3.1.1.8), present in brain and peripheral tissues, but prevalently in serum, is up-regulated in advanced AD and may play a role in the maintenance and progression of the disease.[12]

The catalytic cleavage of ACh involves a tight cooperation of three amino acids, the so-called catalytic triad (Ser-His-Glu) and aromatic amino acid residues responsible for cation−π interactions.[13] The catalytic bottom cleft represents one of the binding sites for substrates and inhibitors, along with the peripheral anionic binding site constituted by a larger region lined chiefly by aromatic amino acids. This structural arrangement accounts for the strong binding interactions observed for inhibitors such as decamethonium and donepezil, able to interact with both catalytic (CAS) and peripheral (PAS) anionic binding sites of AChE and, therefore, called dual binding site (DBS) inhibitors.[14] As binding interactions at the PAS reduces the aggregation of Aβ peptide(s) leading to amyloid oligomers, DBS inhibitors are endowed with dual inhibitory activity on AChE and Aβ aggregation.[15]

The availability of X-ray crystallographic coordinates of many AChE−inhibitor complexes has allowed the identification of key interactions for high ligand binding affinity[16] and has enabled the target-based design of potent and selective AChE inhibitors (AChEIs). In this context, potent AChE inhibition by coumarin derivatives,[17,18] homo- and heterobis-quaternary ammonium salts,[19,20] 2-quinolones,[21] and other heterocyclic compounds[22−24] have been reported by our group.

In the therapy of PD, a key target enzyme is represented by monoamine oxidase (MAO; amine-oxygen oxidoreductase, EC 1.4.3.4), a FAD-dependent enzyme, responsible for oxidative deamination of amine neurotransmitters, including dopamine that is depleted in PD, and exogenous amines.[25,26] Two isoforms of MAO, namely MAO-A and MAO-B, have been characterized in terms of amino acid sequence, tissue distribution, and selectivity toward substrates and inhibitors.[27,28] Selective MAO-A inhibitors (MAO-AIs: e.g., clorgyline and moclobemide) are used in the treatment of depression, while selective MAO-BIs, i.e., rasagiline and selegiline, are employed as adjuvant or alternative drugs to L-DOPA in PD therapy.[29,30]

The resolution of the X-ray crystal structures of both human MAO-A[31,32] and MAO-B[33−35] bound to several inhibitors has newly spurred the research in the field of MAO inhibition, given to its therapeutic potential in neurological disorders, including AD, where selective MAO-BIs may play a role.[29,36]

Growing evidence in the past few years has outlined the multifactorial etiopathogenesis of AD, PD, and other NDs. Actually, neurodegeneration is a complex pathological event resulting from the imbalance and deregulation of multiple biochemical pathways, ultimately depending on transcriptional and epigenetic modulations and on environmental factors.[37] As a consequence, holistic, multifaceted pathologies are currently tackled by a polypharmacological approach based on multi-targeted therapy.[29,38,39] The paradigm "one drug−one target" has nowadays evolved into a more challenging "one drug−more targets" approach, provided that a good balance among potencies and efficacy toward selected targets, and optimal ADME-T properties, can be achieved.[40−42]

■ MULTITARGET-LIGAND DESIGN: RATIONALE AND METHODS

The experience achieved by our group in the field of ligand- and target-based design of potent, selective, and reversible MAO[43−50] and ChE inhibitors,[17−24] prompted us to further explore the challenging field of multitargeted ligand design by addressing compounds with dual MAO and AChE inhibition. The earliest application of this approach by us was reported nearly 15 years ago, when we discovered coumarin derivatives endowed with good and moderate inhibition against MAO-B and AChE, respectively.[51] Additional data have been later presented in an international meeting.[52]

Since then, many authors have described the potential therapeutic application of multimodal MAO-Is displaying additional activities, such as AChE inhibition,[53−59] metal ion chelation,[60] antioxidant,[61,62] and neuroprotective activities.[63] Further studies suggested the combination of MAO and ChE inhibition in the same molecule as a promising strategy in the treatment of AD.[64]

Starting from our previous findings,[51] we designed, synthesized, and tested a new series of suitably substituted coumarin derivatives with the aim of discovering multipotent compounds with different bioactivity profiles toward MAO-B and AChE. While MAO-B selectivity was an important goal of our study to avoid unwanted side effects arising from the intestinal MAO-A inhibition and the consequent hypertensive effect coming from tyramine-rich food (tyramine is indeed metabolized by intestinal MAO-A),[65] the lack of AChE over BChE selectivity was not deemed as important[66] due to a likely pathogenic role of BChE in advanced AD.[67]

The molecular framework of our new multitarget ligands was built following a hybridization strategy. Starting from known ligands of the two enzymes, simple pharmacophore motifs were selected and joined in a unique molecular entity. As molecular flexibility plays a key role in accommodating ligands inside the AChE narrow gorge, a small series of hybrids was designed by connecting the coumarin core of 7-(3-chlorobenzyloxy)-3-methylcoumarin (MC 1095)[45] to the N-benzylaminomethyl group characterizing many AChE inhibitors (e.g., donepezil) through a flexible spacer (Figure 1). The 2H-chromen-2-one ring of such compound was chosen as the moiety able to efficiently fit the MAO-B enzymatic cleft by facing the isoalloxazine ring of FAD. In the additional series of more rigid hybrids, to improve AChE inhibitory potency of 7-benzyloxycoumarins,[44] we approached a "designing in" strategy[29,39,42] by adding in a suitable position of the coumarin ring a protonatable basic moiety that might bind CAS through π−cation interactions (Figure 2). Taking into account that, as recently reported,[68,69] the steric hindrance of substituents placed at position 4 of the coumarin ring exerts a negative impact on MAO affinity, the basic head was anchored to the 7-benzyloxy substituent. This rational hybrid design was
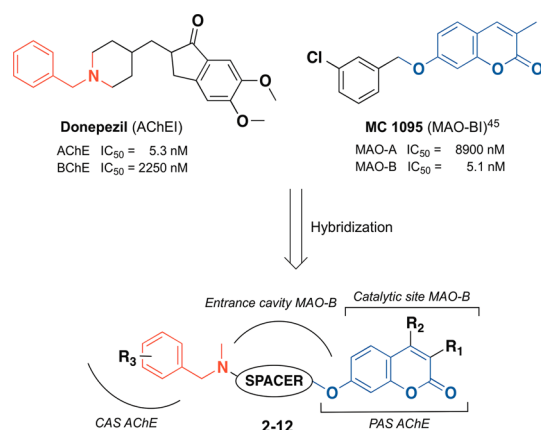
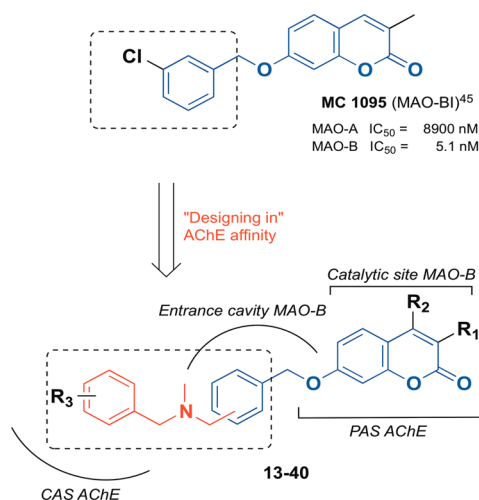**Figure 1.** Hybridization strategy for flexible multitarget MAO-B/AChE inhibitors 2−12.

Figure 2. Hybridization strategy for rigid molecules as multitarget MAO-B/AChE inhibitors 13−40.

supported by prospective docking studies based on X-ray crystal structures of the two target enzymes. As suggested by docking calculations, the protonatable head may establish $\pi-$ cation interactions at the CAS of AChE, while the coumarin moiety may interact with PAS.[17] Such a pose resembled that of donepezil in the PDB complexes (entries: 1EVE and 4EY7). As for the binding to MAO-B, docking studies revealed that the coumarin nucleus can be accommodated in the catalytic region in proximity of FAD, thus matching the pose experimentally observed in the X-ray crystal structure of MAO-B selective coumarin inhibitor NW-1772 (Chart 1).[49] The spacer tethering the two main pharmacophore features, namely the coumarin nucleus and the protonatable amine moiety, was examined also in terms of a reduced flexibility, by synthesizing the *meta*- and *para*-xylyl derivatives listed in Table 2, to determine the optimal distance between the two key binding moieties in both target enzymes.

Docking studies suggested that both polymethylene and xylyl linkers overlaid, at least in part, with the 7-benzyloxy group of NW-1772 that faces the MAO-B entrance cavity (acting as B/A structural determinants for selectivity) and keeps the basic moiety close to the aromatic region more proximal to the solvent.

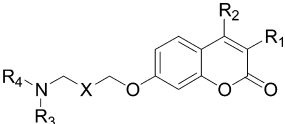The newly synthesized coumarin derivatives 2−40, 42, and 43 were tested in vitro for their inhibition of rat MAO-A and MAO-B (rMAOs), electric eel AChE (eeAChE), and equine serum BChE (esBChE). The quinolone isoster 45 was also synthesized and tested, taking into account our previous findings on AChE inhibitors,[21] with the aim of retaining activity toward the target enzymes while possibly improving the pharmacokinetics properties (i.e., lowering lipophilicity and increasing metabolic stability). The most potent inhibitors at rMAO-B and eeAChE were also assayed on the human isoforms of the two MAOs and ChEs. The inhibition data of nonhuman and human enzymes are reported in Tables 1−3 and 4, respectively. Finally, compound 37, which showed outstanding activities on human enzymes together with well-

**Table 1. MAO and ChE Inhibition Data of Coumarin Derivatives 2−12**

compd	X	R$_1$	R$_2$	MAO-A[a]	MAO-B[a]	AChE[b]	BChE[c]
				IC$_{50}$, μM (or inhibition % at 10 μM)
2[d]	−(CH$_2$)$_3$−	Me	H	(35%)	0.33	1.3	7.8
3[d]		Et	H	7.4	0.98	0.36	4.0
4	−(CH$_2$)$_4$−	Me	H	6.6	1.1	0.49	3.7
5[d]	−(CH$_2$)$_5$−	H	H	2.2	0.50	0.94	1.8
6		Me	H	4.2	0.98	0.55	2.8
7[d]		Me	3-CN	5.3	2.5	2.3	4.1
8[d]		Me	3-Cl	(42%)	8.9	0.29	2.6
9[d]		Me	4-CN	(48%)	3.3	2.8	4.3
10[d]		Et	H	9.5	7.8	0.75	0.94
11	−(CH$_2$)$_6$−	Me	H	0.51	1.7	0.095	0.67
12[d]		Et	H	2.1	0.72	0.32	0.49

[a]From rat brain. [b]From electric eel. [c]From equine serum. Values are mean of two/three independent experiments; SEM < 10%. [d]Hydrochloride salt.

Table 2. MAO and ChE Inhibition Data of Coumarin Derivatives 13–40

compd	X	R1	R2	R3	R4	MAO-A[a]	MAO-B[a]	AChE[b]	BChE[c]
13		H	H	Me	Me	10	3.2	(38%)	5.3
14[d]		H	H	Et	Et	(43%)	(35%)	1.7	6.8
15	(meta-phenyl)	H	H	Me	Bn	(8%)	2.0	2.3	1.0
16[d]		H	H	Me	3-CNBn	(2%)	3.7	4.2	2.8
17[d]		H	H	Me	3-MeOBn	(14%)	2.5	6.6	0.88
18[d]		H	H	Me	3-ClBn	(17%)	3.4	5.7	0.31
19		H	H	Me	Me	(17%)	3.7	7.1	0.24
20		H	H	Et	Et	(9%)	(44%)	2.9	3.4
21		H	H	Me	Bn	(7%)	0.85	0.75	11
22[d]		Me	H	Me	Bn	(0%)	2.4	0.26	1.1
23[d]		H	Me	Me	Bn	(10%)	4.1	0.18	1.0
24[d]		Me	Me	Me	Bn	(0%)	1.2	0.10	0.69
25[d]	(para-phenyl)	H	H	Me	3-CNBn	(5%)	2.8	7.4	4.1
26[d]		H	H	Me	3-MeOBn	(20%)	2.9	4.6	1.4
27[d]		H	H	Me	3-ClBn	(12%)	2.7	0.59	1.2
28[d]		Me	H	Me	3-ClBn	(0%)	4.5	1.3	(29%)
29[d]		H	Me	Me	3-ClBn	(0%)	5.0	0.12	(47%)
30[d]		Me	Me	Me	3-ClBn	(7%)	3.4	0.66	(48%)
31[d]		H	H	Me	4-CNBn	(6%)	0.27	5.6	4.4
32[d]		Me	H	Me	4-CNBn	(15%)	1.7	7.0	(16%)
33[d]		H	Me	Me	4-CNBn	(10%)	0.41	3.3	(29%)
34[d]		Me	Me	Me	4-CNBn	(0%)	2.9	4.2	(18%)
35[d]		CN	H	Me	Bn	(0%)	(41%)	0.20	5.5
36[d]	(para-phenyl)	CN	H	Me	4-CNBn	(0%)	0.99	(42%)	(14%)
37[d]		H	CH2OH	Me	Bn	(0%)	0.41	0.42	1.1
38		H	CH2OH	H	Bn	(19%)	0.53	0.44	0.57
39[d]		H	CH2OH	Me	3-ClBn	(0%)	0.24	0.25	0.63
40[d]		H	CH2OH	Me	4-CNBn	(0%)	0.035	6.3	(23%)

[a]From rat brain. [b]From electric eel. [c]From equine serum. Values are mean of two/three independent experiments; SEM < 10%. [d]Hydrochloride salt.

balanced solubility/lipophilicity properties, was also investigated as potential CNS-permeant neuroprotective agent in vitro.

■ CHEMISTRY

Compounds 2–40 were prepared from bromo-coumarin intermediates 1a–k through microwave-assisted reaction with appropriate benzylamines, in the presence of anhydrous potassium carbonate and a catalytic amount of potassium iodide, in anhydrous acetonitrile. Bromides 1a–k were, in turn, prepared from 7-hydroxycoumarin derivatives and commercial dibromides, as depicted in Scheme 1. Synthesis of analogues 42 and 43 (Scheme 2) started from the reduction of 4-(bromomethyl)phenylacetic acid with BH₃·SMe₂ complex to obtain alcohol 41a that was then coupled with 7-hydroxycoumarin. CBr₄/PPh₃-mediated bromination of 41b afforded 41c that underwent a final microwave-assisted nucleophilic substitution with the appropriate benzylamine, yielding the desired coumarins 42–43. As illustrated in Scheme 3, 7-hydroxy-2-quinolone 44b was prepared from the condensation of trans-cinnamic acid chloride with m-anisidine, followed by an intramolecular Friedel–Crafts acylation/dearylation[70] reaction in refluxing chlorobenzene in the presence of aluminum chloride as Lewis acid. Sequential regioselective alkylation of phenolic-OH with α,α′-dibromo-p-xylene and reaction of intermediate 44c with benzylmethylamine in the usual conditions gave final compound 45.

Analytical and spectroscopic data of tested compounds 2–40, 42, 43, and 45 are reported in Table 6 and Supporting Information.
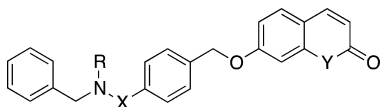
■ BIOLOGICAL ASSAYS

All compounds were tested for their inhibitory activities on rat MAOs (rMAOs), electric eel AChE (eeAChE), and equine serum BChE (esBChE) enzymes. For MAO inhibition test, the protocol using mitochondrial rMAO-A and -B obtained from rat brain homogenates was used as previously described.[45] As for eeAChE and esBChE, the well-known Ellman's spectrophotometric test[71] was used to determine both IC₅₀s and inhibition kinetics. Results are reported in Tables 1–3 as IC₅₀ (μM) or, for poorly active compounds, as percentage of inhibition at 10 μM. Inhibition kinetics plots are depicted in Figure 3.

Compounds 2, 4, 5, 11, 12, 21-24, 29, 31-34, 37, 39, 40, and 45 were also tested on human isoenzymes of MAOs (hMAOs) and/or ChEs (hChEs). While hChEs were tested through the Ellman's method, the assays for hMAOs were carried out with a fluorescence-based method using kynuramine as a nonselective substrate of hMAO-A and hMAO-B.[72] Results of inhibition tests on hMAOs and hChEs are reported in Table 4.

Apical to basolateral (AP-BL) and basolateral to apical (BL-AP) apparent permeability (P_{app}) of compound 37 was

Table 3. Inhibition Data of Derivatives 42, 43, and 45

compd	R	X	Y	MAO-A[a]	MAO-B[a]	AChE[b]	BChE[c]
42[d]	Me	−(CH₂)₂−	O	(12%)	4.1	0.85	0.52
43[d]	H	−(CH₂)₂−	O	1.7	(45%)	4.7	0.64
45[d]	Me	−CH₂−	NH	(10%)	(29%)	0.49	1.7

[a]From rat brain. [b]From electric eel. [c]From equine serum. Values are mean of two/three independent experiments; SEM < 10%. [d]Hydrochloride salt.

Table 4. Inhibition Data on hMAOs, hAChE, and hBChE

| compd | IC$_{50}$, μM (or inhibition % at 10 μM) $\pm$ SEM | | | |
	hMAO-A[a]	hMAO-B[a]	hAChE[b]	hBChE[c]
2[d]	(17 $\pm$ 6%)	0.134 $\pm$ 0.018	1.0 $\pm$ 0.1	nt
4	0.48 $\pm$ 0.04	0.096 $\pm$ 0.006	0.79 $\pm$ 0.01	nt
5[d]	0.39 $\pm$ 0.05	0.029 $\pm$ 0.006	0.30 $\pm$ 0.01	0.95 $\pm$ 0.02
11	0.191 $\pm$ 0.053	0.321 $\pm$ 0.038	0.34 $\pm$ 0.03	0.95 $\pm$ 0.05
12[d]	0.115 $\pm$ 0.015	0.018 $\pm$ 0.003	0.44 $\pm$ 0.06	nt
21	(0%)	0.041 $\pm$ 0.008	0.95 $\pm$ 0.08	0.89 $\pm$ 0.03
22[d]	nt	nt	0.50 $\pm$ 0.03	nt
23[d]	nt	nt	0.91 $\pm$ 0.07	nt
24[d]	4.3 $\pm$ 0.1	0.053 $\pm$ 0.001	0.45 $\pm$ 0.03	(46 $\pm$ 4%)
29[d]	nt	nt	1.0 $\pm$ 0.2	nt
31[d]	(0%)	0.045 $\pm$ 0.013	6.0 $\pm$ 0.5	nt
32[d]	(11 $\pm$ 3%)	0.017 $\pm$ 0.004	nt	nt
33[d]	(39 $\pm$ 2%)	0.039 $\pm$ 0.001	8.5 $\pm$ 0.8	nt
34[d]	(31 $\pm$ 4%)	0.024 $\pm$ 0.003	3.9 $\pm$ 0.5	nt
37[d]	15.8 $\pm$ 2	0.010 $\pm$ 0.002	0.12 $\pm$ 0.01	9.3 $\pm$ 0.7
39[d]	13.0 $\pm$ 2	0.024 $\pm$ 0.004	0.33 $\pm$ 0.03	(14 $\pm$ 3%)
40[d]	4.48 $\pm$ 0.4	0.0057 $\pm$ 0.0008	nt	nt
45[d]	(14 $\pm$ 3%)[e]	4.5 $\pm$ 0.2	1.5 $\pm$ 0.1	nt
Donepezil	(0%)	(0%)	0.015 $\pm$ 0.003	4.8 $\pm$ 0.6
Clorgyline	0.0049	11.0	nt	nt
Pargyline	4.10	0.13	nt	nt

[a]Human recombinant MAOs on Supersomes. [b]Human recombinant AChE. [c]Human serum BChE. [d]Hydrochloride salt. [e]Determined at 4 μM concentration. nt, not tested.

## Scheme 1. Synthesis of Compounds 2−40[a]
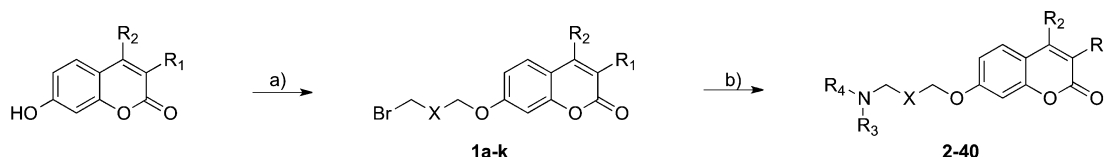
[a]Reagents and conditions: (a) suitable dibromo-derivative (1,3-dibromopropane for **1a**, 1,4-dibromobutane for **1b**, 1,5-dibromopentane for **1c**, 1,6-dibromohexane for **1d**, α,α'-dibromo-*m*-xylene for **1e**, α,α'-dibromo-*p*-xylene for **1f–k**), K$_2$CO$_3$, dry acetonitrile, 30 min, 130 °C, MW; (b) substituted benzylamine, K$_2$CO$_3$, KI (cat.) (for compounds **2−12**), dry acetonitrile, 30 min, 130 °C, MW.

Scheme 2. Synthesis of Compounds 42−43[a]
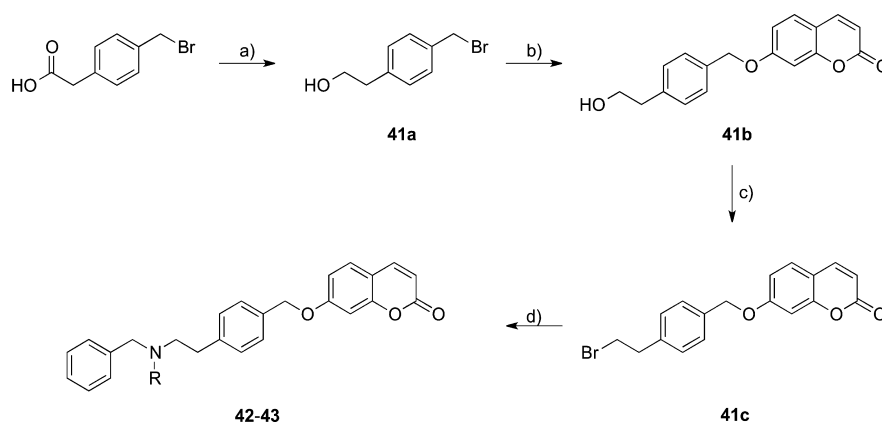
[a]Reagents and conditions: (a) BH$_3$·SMe$_2$, THF, 0 °C to room temperature, 4 h; (b) 7-hydroxycoumarin, K$_2$CO$_3$, dry acetonitrile, 130 °C, 30 min, MW; (c) CBr$_4$, PPh$_3$, dry dichloromethane, 0 °C to room temperature, 4 h; (d) benzylamine or *N*-benzylmethylamine, K$_2$CO$_3$, dry acetonitrile, KI (cat.), 130 °C, 30 min, MW.

measured using Madin−Darby canine kidney (MDCK) cells, retrovirally transfected with the human MDR1 cDNA (MDCKII-MDR1). P_{app} and efflux ratio (ER) were calculated and reported in Table 5.

Cytotoxicity of compound **37** was evaluated in human neuroblastoma cell line SH-SY5Y through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) viability assay (Figure 8).[73] The same cell-based

Scheme 3. Synthesis of compounds 45[a]
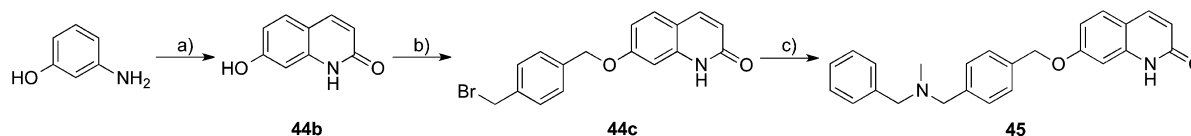
[a]Reagents and conditions: (a) (i) cinnamoyl chloride, dry dichloromethane, 4 h, reflux, (ii) AlCl$_3$, chlorobenzene, 8 h, reflux; (b) α,α'-dibromo-p-xylene, K$_2$CO$_3$, dry acetonitrile, 130 °C, 30 min, MW; (c) N-methybenzylamine, K$_2$CO$_3$, dry acetonitrile, 130 °C, 30 min, MW.
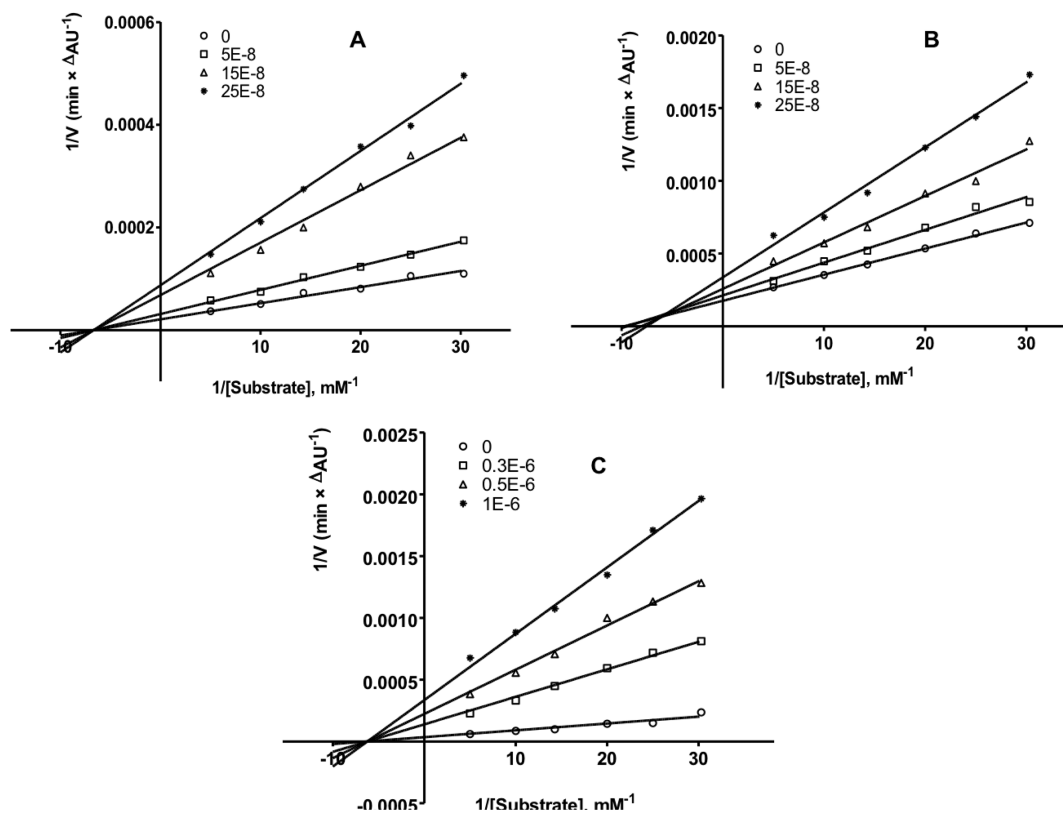

Figure 3. Lineweaver–Burk plots of inhibition kinetics of compounds **11** (A), **24** (B), and **37** (C). Reciprocals of enzyme activity (eeAChE) vs reciprocals of substrate (S-acetylthiocholine) concentration in the presence of different concentrations (0–250 nM) of inhibitor. Concentrations used for inhibitors are reported in inserts.

Table 5. Bidirectional Transport across MDCKII-MDR1 Cells of Compound 37

compd	P_{app}, AP-BL (cm/s)	P_{app}, BL-AP (cm/s)	ER[a] P_{app}BL-AP/P_{app}AP-BL
37	1.91×10^{-5}	3.38×10^{-5}	1.77
Diazepam	1.56×10^{-5}	1.23×10^{-5}	0.79
FD-4	1.13×10^{-6}	2.68×10^{-7}	0.23

[a]Efflux ratio (ER) was calculated using the following equation: ER = P_{app}, BL-AP/P_{app}, AP-BL, where P_{app}, BL-AP is the apparent permeability of basal-to-apical transport, and P_{app}, AP-BL is the apparent permeability of apical-to-basal transport. An efflux ratio greater than 2 indicates that a test compound is likely to be a substrate for P-gp transport.

method was used to for preliminarily investigate the cytoprotective effects of compound **37** against cell death induced by H$_2$O$_2$ (Figure 9).

■ RESULTS AND DISCUSSION

For the sake of clarity, structure–activity and structure–selectivity relationships (SAR and SSR, respectively) will be discussed first separately for the different classes of compounds reported in Tables 1–3, then a comparison will be made among the inhibition data from rMAOs and eeAChE with the corresponding data from hMAOs, hAChE and to a lesser extent hBChE (Table 4).

SARs and SSRs of N-Benzyl-N-alkyloxy Coumarin Derivatives (Table 1). Compounds **2–12** were designed to investigate the effects on inhibition potency and selectivity at the target enzymes of the length of the polymethylene linker and of the N-substitution at the NH-benzyl moiety with methyl and ethyl groups. A few substituents were also introduced on the benzyl ring of compound **6**, bearing a pentamethylene linker, to extend the knowledge of SARs and SSRs.

The inhibition data in Table 1 showed that, with a few exceptions, the designed compounds display from low- to submicromolar potencies against rMAO-B and eeAChE, whereas potencies against rMAO-A and esBChE were slightly lower. As the result, promising dual MAO-B and AChE inhibitors were discovered with limited MAO-B over MAO-A and AChE over BChE selectivity.

Interestingly, compounds **3, 5, 6,** and **12**, characterized by linkers of different length, displayed submicromolar affinities at

Table 6. Analytical Data of Compounds 2−40, 42, 43, and 45

compd	melting point, °C (CC or cryst solvent)[a]	anal. calcd %			anal. found %		
		C	H	N	C	H	N
2[b]	201−3 (CC)	68.12	6.76	3.61	68.10	6.86	3.73
3[b]	183−5 (CC)	68.73	7.02	3.48	68.43	6.75	3.28
4	38−9 (CC)	75.59	7.45	3.83	75.78	7.55	3.61
5[b]	238−40 (CC)	68.73	7.02	3.48	68.40	6.87	3.58
6	66−7 (CC)	75.96	7.70	3.69	75.58	7.45	3.80
7[b]	247−9 (CC)	68.09	6.63	6.35	67.73	6.62	6.09
8[b]	204−6 (CC)	64.00	6.49	3.11	63.94	6.35	2.98
9[b]	201−3 (CC)	68.09	6.63	6.35	67.70	6.53	6.37
10[b]	193−4 (CC)	69.83	7.50	3.26	69.55	7.37	3.31
11	54−5 (CC)	76.30	7.94	3.56	75.99	7.88	3.70
12[b]	171−3 (CC)	70.33	7.72	3.15	70.51	8.03	3.48
13	181−2 (EtOH)	73.77	6.19	4.53	73.42	6.36	4.24
14[b]	225−7 (EtOH)	67.46	6.47	3.75	67.27	6.50	3.79
15	156−7 (CC)	71.67	6.01	3.63	71.29	5.78	3.31
16[b]	132−4 (EtOH)	69.87	5.19	6.27	69.51	5.54	6.03
17[b]	121−3 (EtOH)	69.10	5.80	3.10	68.81	5.64	3.31
18[b]	131−3 (EtOH)	65.80	5.08	3.07	65.55	5.29	3.31
19	108−9 (EtOH/Et₂O)	73.77	6.19	4.53	73.88	6.41	4.35
20	167−8 (EtOH/Et₂O)	74.75	6.87	4.15	74.93	6.47	4.01
21	106−7 (EtOH)	71.67	6.01	3.63	71.44	5.89	3.55
22[b]	>250 (CC)	71.63	6.01	3.21	71.31	5.95	3.26
23[b]	>250 (CC)	71.63	6.01	3.21	71.37	6.06	3.34
24[b]	226−8 (CC)	72.07	6.27	3.11	71.77	6.23	3.14
25[b]	129−31 dec. (EtOH)	69.87	5.19	6.27	69.64	5.33	6.40
26[b]	128−30 (EtOH)	69.10	5.80	3.10	69.34	5.51	3.23
27[b]	124−6 (CC)	65.80	5.08	3.07	65.97	5.35	3.19
28[b]	>250 (CC)	66.39	5.36	2.98	66.04	5.29	2.80
29[b]	247−50 dec (CC)	66.39	5.36	2.98	65.99	5.39	2.80
30[b]	236−8 (CC)	66.95	5.62	2.89	67.31	5.80	3.22
31[b]	147−9 (CC)	69.87	5.19	6.27	70.12	5.29	6.37
32[b]	242−4 (CC)	70.35	5.47	6.08	69.99	5.49	6.10
33[b]	224−6 (CC)	70.35	5.47	6.08	70.72	5.59	6.12
34[b]	225−7 (CC)	70.80	5.73	5.90	70.55	5.97	5.76
35[b]	155−7 dec. (CC)	69.87	5.19	6.27	69.46	5.55	5.92
36[b]	183−5 dec. (CC)	68.72	4.70	8.90	68.88	4.79	8.59
37[b]	235−6 dec. (CC)	69.10	5.80	3.10	69.15	5.71	3.43
38	109 dec, 130−2 (EtOH)	74.80	5.77	3.49	74.44	5.69	3.47
39[b]	231−36 (CC)	64.20	5.18	2.88	63.80	5.13	2.89
40[b]	150−2 (CC)	67.99	5.28	5.87	67.61	5.29	5.56
42[b]	207−9 (CC)	71.63	6.01	3.21	71.81	5.91	3.01
43[b]	>250 (CC)	71.17	5.73	3.32	71.27	5.47	3.37
45[b]	154−5[c] (CC)	71.33	5.99	6.65	71.61	6.04	6.32

[a]CC: column chromatography (see Supporting Information for details). [b]Hydrochloride salt. [c]Free base.

both rMAO-B and eeAChE with a limited selectivity over rMAO-A and esBChE, respectively. The gradual elongation of the linker in the *N*-Me derivative **2**, affording compounds **4**, **6**, and **11**, provided a consistent increase of potency toward eeAChE (from 1.3 μM of **2** to 0.095 μM of **11**) and esBChE (from 7.8 μM of **2** to 0.67 μM of **11**), whereas an opposite effect was observed with rMAO-B (from 0.33 μM of **2** to 1.7 μM of **11**). Therefore, in this series of compounds, the goal of optimizing both MAO-B and AChE activities could not be achieved by varying the length of the linker. However, moving from the *N*-methyl derivative **11** to the *N*-ethyl homologue **12** good activities at the three target enzymes, rMAO-B, eeAChE, and esBChE, were obtained but along with a very low MAO-B over MAO-A selectivity (SI = 3; SI is the selectivity index calculated as the ratio IC_{50} MAO-A/IC_{50} MAO-B). Compound

11 was endowed with the highest eeAChE inhibitory potency within the whole examined series (IC_{50} = 0.095 μM) and, surprisingly, the highest rMAO-A inhibitory potency (IC_{50} = 0.51 μM) leading to a reversal of rMAO selectivity (SI = 0.3). Kinetics of eeAChE inhibition resulted in a mixed-type mechanism, as expected from putative dual binding site inhibitors (Figure 3A), with K_i equal to 0.080 ± 0.002 μM.

Notably, compounds with the longest linker **11** and **12** displayed the highest inhibitory potency on rMAO-A (IC_{50} = 0.51 and 2.1 μM, respectively) and ChEs (IC_{50} = 0.095 and 0.32 μM, respectively, at AChE, and 0.67 and 0.49 μM, respectively, at BChE). No clear relationship emerged from the comparison of inhibition potency of *N*-methyl and *N*-ethyl derivatives.

The introduction in compound **6** of a cyano group in position *meta* and *para* (**7** and **9**, respectively) and of a chlorine at position *meta* (compound **8**) did diminish the inhibitory potency toward all the tested enzymes except for compound **8** at both ChEs.

SARs and SSRs of *meta*- and *para-N,N*-Dialkylamino-methyl-7-benzyloxycoumarin Derivatives (Table 2). Compounds **13–40** were designed to investigate the effects on inhibition potency and selectivity at the three target enzymes of the *N,N*-dialkylaminomethyl substituents at the *meta* and *para* positions of the 7-benzyloxy moiety and of substituents at position 3 and 4 of the coumarin ring, having in mind our previous findings,[68,69] which highlighted the steric requirements for this region in binding MAO-B enzymatic cleft. A number of substituents were also introduced on the *N*-benzyl ring in R₄ to extend the study of SARs and SSRs.

It is worth underlining that all the examined compounds shared a *meta*- or *para*-xylyl linker joining the oxygen at position 7 of coumarin with the basic *N,N*-dialkylamino substituents. Both xylyl linkers have a lower conformational flexibility compared to the polymethylene linkers of compounds **2–12**, and this might result in a higher (iso)enzyme selectivity. Indeed, this was the case for rMAOs, as most compounds achieved micromolar to submicromolar rMAO-B inhibitory potency whereas an inhibition lower than 20% at 10 μM was generally displayed at rMAO-A. The same effect on selectivity was not observed for the ChEs even though most compounds appeared slightly more AChE-selective. Inhibition potency on eeAChE reached the submicromolar level in 11 cases, the most potent inhibitor being compound **24** (IC$_{50}$ = 0.10 μM), while on esBChE only six compounds exhibited a submicromolar activity, with compound **19** showing the highest potency (IC$_{50}$ = 0.24 μM).

As the *N,N*-dimethylaminomethyl derivative **13**, the lead compound of the *meta*-xylyl series, showed very low inhibitory effect on rMAO-A and eeAChE (IC$_{50}$ = 10 μM and 38% inhibition at 10 μM, respectively) and moderate activity on rMAO-B and esBChE (IC$_{50}$ = 3.2 and 5.3 μM, respectively), its *N,N*-diethylamino **14** and *N*-methyl-*N*-benzylamino **15** congeners were synthesized. Improved potencies were definitely gained with compound **15** that showed an interesting profile of inhibition with low micromolar potencies rMAO-B, eeAChE, and esBChE (IC$_{50}$ = 2.0, 2.3, and 1.0 μM, respectively) and very weak activity at rMAO-A (8% inhibition at 10 μM). The introduction of cyano, methoxy, and chloro substituents at the *meta* position of the phenyl ring of the *N*-benzylamino moiety, leading to compounds **16**, **17** and **18**, respectively, resulted in lower inhibitory potencies on rMAO-B and eeAChE, whereas a 3-fold increased inhibition was observed for the chloro derivative **18** on esBChE (IC$_{50}$ = 0.31 vs 1.0 μM).

The *para-N,N*-dimethylamino- and *N,N*-diethylamino-methyl derivatives **19** and **20** displayed lower activity against rMAO-A, similar activity against rMAO-B, and higher activity against esBChE when compared to the corresponding *meta*-substituted isomers **13** and **14**. Better results were obtained with the *para-N*-methyl, *N*-benzylaminomethyl derivative **21** that achieved submicromolar potency toward rMAO-B and eeAChE (IC$_{50}$ = 0.85 and 0.75 μM, respectively) and a significantly lower activity against rMAO-A and esBChE (7% at 10 μM and IC$_{50}$ = 11 μM, respectively).

Methyl substituents were introduced at the positions 3 and 4 of the coumarin ring to give the monomethyl derivatives **22** and **23** and the dimethyl derivative **24**. Compared to the lead compound **21**, the rMAO-B activity decreased while a significant increase of the inhibitory potency on eeAChE (IC$_{50}$ = 0.26, 0.18, and 0.10 μM) and esBChE (IC$_{50}$ = 1.1, 1.0, and 0.69 μM) was observed for compounds **22**, **23**, and **24**, respectively. It is worth noting that these very simple structural changes led to the most potent eeAChE inhibitors of the whole series of compounds and, even more interestingly, for compound **24** an impressive combination of high inhibitory activities toward rMAO-B, eeAChE, and esBChE and high rMAO-B-selectivity was revealed (IC$_{50}$ = 1.2, 0.10, and 0.69 μM at rMAO-B, eeAChE, and esBChE, respectively, and 0% inhibition at 10 μM at rMAO-A). As for open chain derivative **11**, also compound **24** displayed mixed-mode kinetics for eeAChE inhibition, with K_i equal to 0.22 ± 0.03 μM (Figure 3B).

As done for lead compound **15**, the isomeric compound **21** was modified by introducing cyano, methoxy, and chloro substituents at position *meta*, and the cyano group at position *para* as well, in the aromatic ring of the *N*-benzylamino moiety. *Meta*-substituted derivatives **25–27** proved to be less potent inhibitors than the lead compound **21** on rMAO-B. *Meta*-chloro derivative **27** maintained, however, an attractive inhibition profile (12% inhibition at 10 μM at rMAO-A; IC$_{50}$ = 2.7, 0.59, and 1.2 μM, at rMAO-B, eeAChE, and esBChE, respectively). Therefore, compound **27** was slightly modified by introducing methyl substituents at the positions 3 and 4 to afford the monomethyl derivatives **28** and **29** and the dimethyl derivative **30**. Compared to **27**, rMAO-B, rMAO-A, and esBChE activities considerably decreased, while the potency toward AChE was maintained, or even improved, in particular for the 4-methylderivative **29** (IC$_{50}$ = 0.12 vs 0.59 μM). Compared to **21**, 4-cyanobenzylamino derivative **31** displayed lower activities toward rMAO-A, eeAChE, and esBChE and a 3-fold increase of rMAO-B activity. The introduction in compound **31** of methyl substituents at the positions 3 and 4, to give the monomethyl derivatives **32** and **33** and the dimethyl derivative **34**, generally diminished the activity on almost all enzymatic targets.

The final structural modifications of lead compound **21** were aimed at reducing molecular lipophilicity while maintaining good inhibitory activities on the target enzymes. One approach aimed at the modulation of the pK_b of the basic head, through the elongation of an arm of the linker that allowed a higher distance between the two electron withdrawing phenyl rings from the basic nitrogen, resulting in lower pK_b and log D. This structural modification increased the distance between the two key binding moieties of **21**, that is, the coumarin ring and the *N*-benzyl group, giving rise to the more flexible *N*-benzyl, *N*-phenethyl analogue **42** (Table 3). In addition, *N*-demethylated derivative **43** was synthesized to test the effect of having a more hydrophilic basic moiety endowed with HB-donor ability. Compared to compound **21**, the homologue **42** showed a similar activity toward eeAChE (IC$_{50}$ = 0.85 vs 0.75 μM) and rMAO-A (12% vs 7%), a much higher activity at esBChE (IC$_{50}$ = 0.52 vs 11 μM), and a decreased activity at rMAO-B (IC$_{50}$ = 4.1 vs 0.85 μM). To our surprise, the *N*-demethylated congener **43** reversed MAO selectivity, being the activity on rMAO-A higher than that on rMAO-B (IC$_{50}$ = 1.7 μM and 45% inhibition at 10 μM, respectively). The inhibition potency on eeAChE worsened (IC$_{50}$ = 4.7 vs 0.85 μM), while conversely the potency against esBChE remained nearly unchanged (IC$_{50}$ = 0.64 vs 0.52 μM). An additional less lipophilic analogue of **21**, namely **45** in Table 3, was prepared through bioisosteric
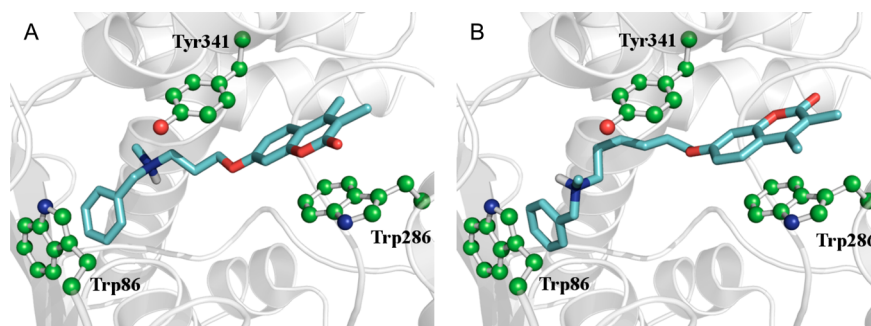
Figure 4. Docking poses of inhibitor **2** (A) and **11** (B) into human AChE binding site (PDB code: 4EY7). Inhibitors are represented in sticks, while relevant amino acid residues are in ball-and-sticks, colored according to the atom code (C atoms in cyan and green for inhibitors and amino acid residues, respectively). Docking scores are equal to −98.2 and −106.2 kJ/mol for inhibitor **2** and **11**, respectively.

replacement of the 2H-chromen-2-one with the 2-quinolone as successfully done in the past for a dual binding site AChE inhibitor.[21] Actually, the activity toward both cholinesterases improved (IC$_{50}$ = 0.49 vs 0.75 μM and 1.7 vs 11 μM at eeAChE and esBChE, respectively) and the low activity on rMAO-A was maintained but, unexpectedly, the activity on rMAO-B dramatically decreased (29% inhibition at 10 μM vs IC$_{50}$ = 0.85 μM).

As the homologation of the basic head and the isosteric replacement yielded unsatisfying results, compound **21** was modified by introducing polar groups on the coumarin core. The insertion of a polar cyano group on the coumarin ring at position 3 (comp. **35**) was highly detrimental for rMAO-B activity (41% at 10 μM vs IC$_{50}$ = 0.85 μM), while a 3- and 2-fold potency increase was observed against eeAChE (IC$_{50}$ = 0.20 vs 0.75 μM) and esBChE (IC$_{50}$ = 5.5 vs 11 μM), respectively. Better results on rMAO-B but much worse toward both ChEs came from the introduction of a second polar substituent, a *para*-cyano group on the N-benzyl ring (comp. **36**).

Guided by docking simulations and a 3D-QSAR model recently developed from a large series of 4,7-disubstituted coumarins,[69] a different, more polar substituent, that is the hydroxymethyl group, was introduced at position 4 of the coumarin core. Very satisfactorily, inhibitor **37** showed good inhibitory activities with the three target enzymes (IC$_{50}$ = 0.41, 0.42, and 1.1 μM at rMAO-B, eeAChE, and esBChE, respectively) and no activity on rMAO-A (0% inhibition at 10 μM). As for compounds **11** and **24**, also **37** showed a mixed-type inhibition mode (Figure 3C), with a K_i equal to 0.10 ± 0.01 μM. The N-demethylated derivative of **37**, that is compound **38**, was also prepared and tested. Its inhibitory potencies and selectivity profile (IC$_{50}$ = 0.53, 0.44, and 0.57 μM at rMAO-B, eeAChE, and esBChE, respectively, and 19% inhibition at rMAO-A at 10 μM) were very good and compared well with those of the N-methyl analogue.

As previous SAR/3D-QSAR[45] and current docking studies (data not shown) suggested favorable interactions of a halogen atom at the *meta* position of a benzyl ring at AChE and MAO-B binding sites, the *meta*-chloro derivative **39** was synthesized and tested. Its excellent inhibitory activities on the three target enzymes (IC$_{50}$ = 0.24, 0.25, and 0.63 μM for rMAO-B, eeAChE, and esBChE, respectively, and 0% inhibition toward rMAO-A at 10 μM) proved the goodness of our molecular design. Indeed, compound **39** attained the pursued combination of strong and well-balanced inhibitory potencies and an excellent rMAO-B over rMAO-A selectivity.

The introduction of a *para*-cyano substituent in the N-benzyl ring of **37** led to **40**, which showed an exquisite rMAO-B activity and selectivity (rMAO-B, IC$_{50}$ = 0.035 μM; rMAO-A, 0% inhibition at 10 μM) but also a dramatic drop of activity at eeAChE (IC$_{50}$ = 6.3 μM) and esBChE (23% inhibition at 10 μM), indeed resulting as the most potent MAO-B inhibitor of the whole set of compounds herein investigated.

Inhibitory Activities on Human Enzymes. The good biological profiles of many inhibitors toward MAO and ChE enzymes, prompted us to extend our inhibition assays to the corresponding human enzymes. On the basis of our previous studies,[72] significant changes of inhibitory activities were expected for hMAOs (generally an improvement, especially at hMAO-B), whereas more limited differences were awaited for hAChE inhibition. However, to gain further support to these general predictions and select compounds with high activity on hMAO-B and hAChE, a prospective docking study was performed on the series of inhibitors listed in Table 4. Inhibition activities on human enzymes were not assessed for compounds exhibiting low potency against the corresponding rMAO and eeAChE enzymes.

Prospective docking simulations were carried out using GOLD Suite v5.2[74] on the X-ray crystal structures of AChE and MAO-B available from the PDB. For AChE studies, the X-ray crystal structures of *Torpedo californica* AChE (TcAChE) (PDB entry: 1EVE) and human recombinant AChE (hAChE) (PDB entry: 4EY7), both complexed with donepezil, were used. The careful inspection of the two crystal structures revealed that PAS and CAS binding sites of AChE are at about 18 Å distance (measured between the Cα carbon atoms of Trp86 and Trp286 of hAChE). Distances between the two putative binding moieties close to such a value might ensure efficient interactions at both PAS and CAS. On this basis, we conceived predictive docking studies aimed at forecasting the optimal length of the polymethylene spacer joining the pharmacophore features, ensuring strong AChE binding. In this respect, the in silico studies were focused on two compounds (**2** and **11** of Table 1), bridging the protonatable basic tail and the coumarin head with a short (trimethylene) or long (hexamethylene) linear linker, respectively. Interestingly, docking simulations returned similar poses for the two compounds, which were both able to engage steady $\pi−\pi$ stacking interactions with the aromatic residues of Trp86 and Trp286. The compound bearing the longer linear linker showed a higher score (−106.2 kJ/mol for hAChE and −96.3 kJ/mol for TcAChE) compared to that bearing the shorter linker (score: −98.2 kJ/mol for hAChE and −88.6 kJ/mol for TcAChE). For clarity, a zoomed
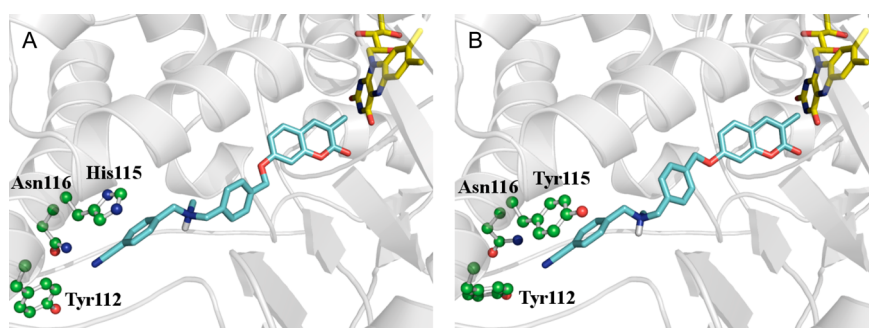
Figure 5. Docking poses of inhibitor **32** into human (PDB code: 2V60) (A) and rat MAO-B (homology model) (B) binding sites. Inhibitors and FAD cofactor are represented in sticks, while relevant amino acid residues are in ball-and-sticks, colored according to the atom code (C atoms in cyan, yellow, and green for inhibitors, cofactor, and amino acid residues, respectively). Docking scores are equal to −88.21 and −80.22 kJ/mol for inhibitor **32** into human and rat isoforms, respectively.
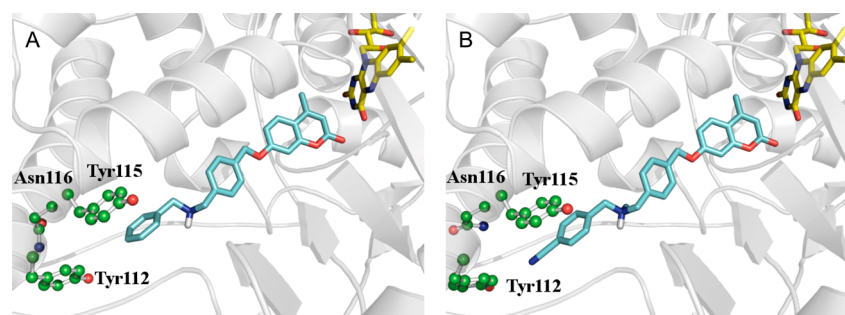

Figure 6. Docking poses of inhibitor **23** (A) and **33** (B) into rat MAO-B binding site (homology model). Inhibitors and FAD cofactor are represented in sticks, while relevant amino acid residues in ball-and-sticks, colored according to the atom code (C atoms in cyan, yellow, and green for inhibitors, cofactor, and amino acid residues, respectively).
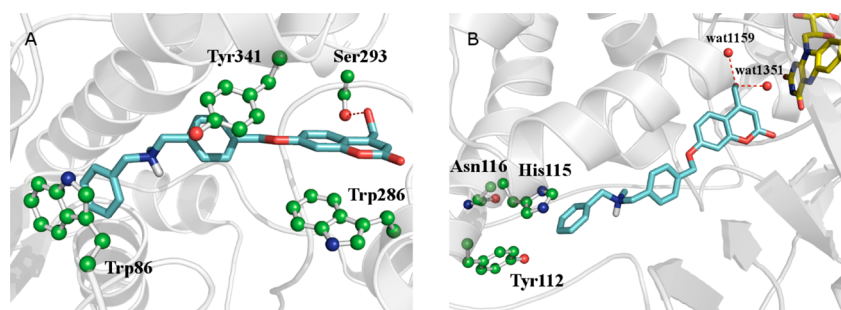

Figure 7. Docking poses of inhibitor **37** into (A) hAChE (PDB code: 4EY7) and (B) hMAO-B (PDB code: 2V60) binding sites. Inhibitors are represented in sticks, while relevant amino acid residues in ball-and-sticks, colored according to the atom code (C atoms in cyan and green for inhibitors and amino acid residues, respectively). Structural water molecules (that are HOH1159 and HOH1351 according to the numbering of 2V60) are represented as red balls. Hydrogen bonds are represented as red dashed lines. Docking scores are equal to −109.38 and −95.95 kJ/mol for inhibitor **37** into hAChE and hMAO-B, respectively.

in view of top-scored solutions is shown in Figure 4. Compounds **2** and **11** actually proved effective in inhibiting AChE, in good agreement with the docking scores.

For MAO-B studies, the X-ray crystal structure of hMAO-B (PDB entry: 2V60) and a homology model for the rMAO-B were used. We performed a number of exploratory docking runs to assess whether the decoration of the well-known 7-benzyloxycoumarin with a protonatable amino tail (mimicking the basic head of donepezil) could be suitable in biasing also MAO-B. To this end, we docked three probe compounds (**23**, **32**, and **33** in Table 2) into the binding site of MAO-B and observed that the ad hoc incorporated benzylamine fragment was located in a peripheral region surrounded by Tyr112, His115 (mutated to Tyr115 in rMAO-B), and Asn116. As shown in Figure 5, the residue at position 115 is critical in

establishing π−π stacking interactions with the terminal benzyl ring of **32**, although the scores at hMAO-B were consistently higher than those at rMAO-B. These interspecies differences were even more pronounced in enzyme inhibition potency of **32**, showing two log units higher activity at hMAO-B compared to rMAO-B (IC$_{50}$ = 0.017 vs 1.7 μM). We hypothesized that such scoring and biological differences in hMAO-B and rMAO-B can rely on a likely greater strength of the π−π stacking interactions engaged by the imidazole ring of His115 compared to that of the phenyl ring of Tyr115.[75]

The potential benefit of introducing a cyano group at the *para* position of the benzylamine was also challenged by docking compounds **23** and **33**. Actually, we did not observe any appreciable difference in the scores; however, the presence of the cyano group increased the rMAO-B inhibition

approximately 10-fold (**23**, IC_{50} = 4.1 μM vs **33**, IC_{50} = 0.41 μM). We tentatively explained this experimental result by visually inspecting the docking poses of **23** and **33**. As shown in Figure 6, the cyano group could form HBs (even water-mediated) with Asn116 or Tyr112.

For the sake of completeness, additional modeling studies were conducted on **37**, one of the most potent multitarget inhibitors of the series. **37** was first docked into both hAChE and TcAChE to assess the binding interactions resulting from the substitution of an aliphatic linker with an aromatic one and of the 4-H atom with a more polar 4-hydroxymethyl group. Beyond hydrophobic interactions already described for inhibitors **2** and **11** bearing an aliphatic linker, inhibitor **37** was engaged in a $\pi-\pi$ interaction involving the *para*-xylyl linker and the aromatic side chain of Tyr341 (hAChE numbering referred to PDB entry 4EY7).[17] In addition, a HB occurred between the 4-hydroxymethyl group and Ser293 of hAChE as shown in Figure 7A. The higher score of **37** toward hAChE (−109.38 kJ/mol) compared to **2** and **11** may be therefore ascribed to both $\pi-\pi$ and HB interactions (Figure 7A).

As reported in previous studies,[68,69] the introduction of polar groups at position 4 of the coumarin scaffold improved physicochemically relevant properties (e.g., aqueous solubility and lipophilicity) while maintaining good MAO-B inhibitory potency and selectivity. Thus, inhibitor **37** was docked also on hMAO-B, resulting in a binding pose similar to that of **23**, **32**, and **33** but with a higher score (−95.95 kJ/mol). Most likely the high hMAO-B inhibitory potency arose from the formation of multiple HBs between the hydroxyl group and structural water molecules[45,49,68] as illustrated in Figure 7B.

Inhibition data in Table 4 fulfilled our expectations and docking calculations. As far as the inhibitory activities on hMAO-B are concerned, a consistent increase of activity, from 2.5-fold for compound **2** up to 121-fold for **34**, was recorded, whereas the activities on hMAO-A remained low or were slightly incremented, from 2.7-fold for compound **11** to 18-fold for compound **12**. As the result, the hMAO-B over hMAO-A selectivity strongly raised. Actually, the SIs measured for compounds **37**, **39**, and **40** were 2210, 842, and 910, respectively. Because many other inhibitors activities on hMAO-A remained very low, the IC_{50} values were not measured and the hMAO selectivity could not be exactly assessed. Nevertheless, taking into account the low percentage of inhibition of hMAO-A at 10 μM concentration, high hMAO-B over hMAO-A selectivity can be confidently foreseen also for inhibitors **21**, **31**, and **32**.

It is worth noting that all the coumarin derivatives listed in Table 4 attained submicromolar inhibitory on hMAO-B. Coumarin derivatives **37** and **40**, bearing the 4-CH_2OH group, were the most potent inhibitors within the whole molecular series examined, with IC_{50} values in the low nanomolar range (10 and 5.7 nM, respectively). Interestingly, some similarities and striking differences emerged by comparing inhibitory activities at rat and human MAO enzymes. For instance, 4-CH_2OH-bearing coumarin derivatives **37**, **39**, and **40** resulted in the most active MAO-B inhibitors at both the human and rat enzymes, with compound **40** showing the highest activities (IC_{50} = 0.0057 and 0.035 μM, respectively). It is noteworthy that the inverted selectivity observed on rMAOs for compound **11**, bearing the longest polymethylene linker, was maintained also on hMAOs.

As anticipated in the Introduction, the selective inhibition of either AChE or BChE was not a primary aim of our work.

Nonetheless, a number of representative compounds, that is **5**, **11**, **21**, **24**, **37**, and **39**, were tested also on hBChE (Table 4). Despite an 89.4% of amino acid sequence identity, the human and equine enzymes displayed significant differences in the catalytic site and even more in the peripheral sequence. Notably, Gly277, Pro285, and Phe398 in human mutated to Asp, Leu, and Ile, respectively, in equine. The amino acid numbering refers to hBChE as reported in PDB. Therefore, different inhibition potencies at the two enzymes may be expected and this was indeed observed for compounds **24**, **39**, **21**, and **37** (46% and 14% at 10 μM and IC_{50} = 0.89 and 9.3 μM versus IC_{50} = 0.69, 0.63, 11, and 1.1 μM, respectively). In contrast, compounds **5** and **11** displayed submicromolar inhibition potencies on hBChE close to the ones observed on esBChE.

Appealing inhibition profiles resulted for compounds **5**, **37**, and **39**. The first displayed submicromolar potencies at all the four tested human enzymes, along with a significant, but limited, selectivity for hMAO-B (SI = 13.5) and no selectivity for either one of the two ChEs. The latter two showed high hMAO-B inhibitory potencies and selectivities (IC_{50} = 10 and 24 nM, and SI = 1580 and 542, respectively) and also a good hAChE inhibitory potency and selectivity (IC_{50} = 0.12 and 0.33 μM, and SI = 77.5 and ≫30, respectively).

Taking into account inhibition potencies, selectivity data, and physicochemical parameters, inhibitor **37** was selected for a preliminary evaluation of its ability to cross blood−brain barrier (BBB) and its cytotoxicity and cytoprotective properties.

■ BBB PERMEABILITY AND TOXICITY PROFILING OF COMPOUND 37

MDCK-MDR1 cell lines are known to express P-glycoprotein (P-gp), which plays an important role in the efflux transport of drugs from brain to blood. Thus, we were particularly interested to see if compound **37** was able to permeate by passive diffusion the BBB and to interact with P-gp as well. To this purpose, transport studies were performed on MDCKII-MDR1 cells which are characterized by high P-gp expression and represent a well-established in vitro model mimicking the BBB.[76,77] Transport studies were conducted in both apical-to-basal (AP-BL) and basal-to-apical (BL-AP) directions, and the results were reported in Table 5. Fluorescein isothiocyanate-dextran (FD4) and diazepam were used as paracellular and transcellular markers, respectively, of cell monolayers' integrity and as internal controls to verify tight junction integrity during the assay; the results for controls were within the expected values. Both apparent permeabilities (AP-BL, 1.91×10^{-5} cm·s^{-1}; BL-AP, 3.38×10^{-5} cm·s^{-1}) were comparable with those of diazepam, thus supporting a good BBB penetration. The efflux ratio (ER) equal to 1.77 detected for compound **37** disclosed no significant differences in P_{app} values between AP-to-BL and BL-to-AP direction. Because a value of ER greater than 2 indicates that a test compound is likely to be a substrate for P-gp transport, the measured value suggested that compound should be able to permeate the monolayer without significant interactions with such efflux system.

The effects of compound **37** on the viability of human neuroblastoma cell line SH-SY5Y were studied, using donepezil as reference compound. As shown in Figure 8, compound **37**, like donepezil, was not cytotoxic at the tested concentrations ranging from 0.1 to 50 μM after 24, 48, and 72 h of incubation. The neuroprotective capacity of **37** against oxidative stress was also evaluated using the same cell line and hydrogen peroxide
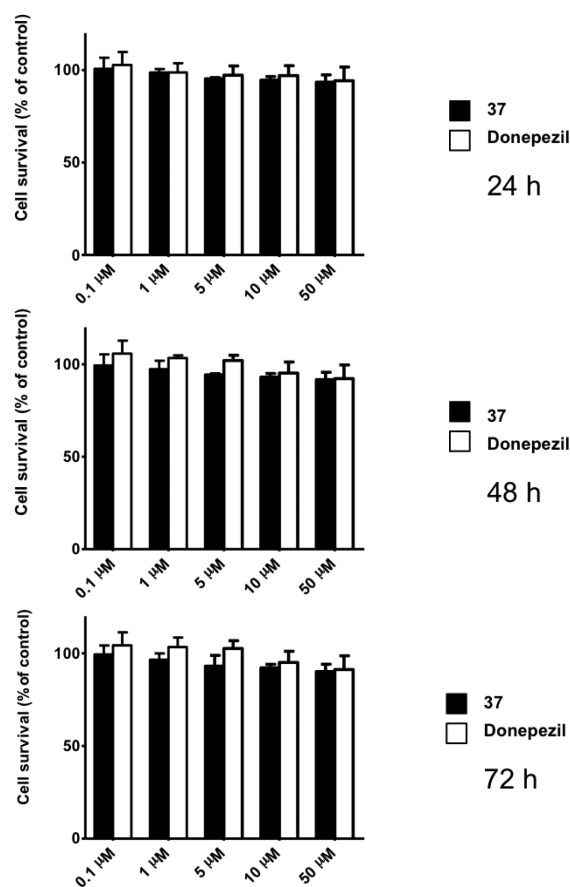
Figure 8. Viability of human neuroblastoma SH-SY5Y cells exposed to compound **37** at different concentrations and incubation times. SH-SY5Y cells were incubated with increasing concentrations (range 0.1–50 μM) of the test compounds for 24, 48, and 72 h. Untreated cells were used as control. Results are expressed as percentage of viable cells observed after treatment with compounds **37** and donepezil vs untreated control cells (100%) and shown as mean ± SD ($n = 3$).

701 (H$_2$O$_2$) for the generation of exogenous free radicals. Cells
702 were incubated with **37** at two nontoxic concentrations (1 and
703 10 μM) for 24 h, then, H$_2$O$_2$ (60 μM) was added and the cells
704 maintained for further 24 h. Cell death was determined using
705 the MTT assay. As shown in Figure 9, compound **37** at 10 μM
706 concentration prevented the H$_2$O$_2$-induced cell death (ca. 25%)
707 with a statistically significant effect ($P < 0.01$).
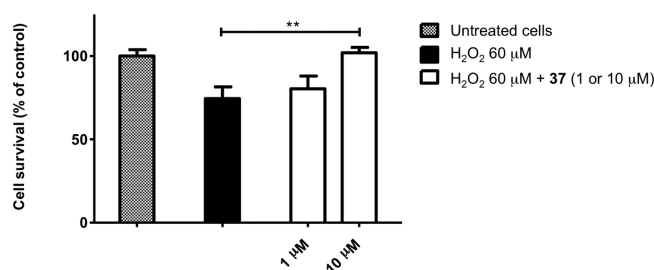

Figure 9. Percentage of cell survival in the human neuroblastoma cell line SH-SY5Y of compounds **37** in the following conditions: untreated cells (gray column), in the presence of H$_2$O$_2$ (60 μM) alone (black column), in the presence of both H$_2$O$_2$ (60 μM), and compound **37** (1 and 10 μM, white columns). Data represent means ± SD ($n = 3$); statistical significance was estimated using one-way ANOVA and Bonferroni post hoc test (** $P < 0.01$).

CONCLUSIONS

708
The most salient features emerging from the SARs and SSRs of 709 the two classes of compounds examined in this paper can be 710 summarized as follows. Highly flexible inhibitors of the *N*- 711 benzyl, *N*-alkyloxy coumarin series in Table 1 showed good 712 inhibitory activities at the target enzymes when bearing a penta- 713 or hexamethylene linkers but low selectivity (see compounds **6**, 714 **11**, and **12** in Table 1). The inhibitors bearing a more rigid 715 xylyl linker (Table 2) displayed good inhibitory potencies and 716 high rMAO-B over rMAO-A selectivity ratios. Compound **21**, a 717 more rigid analogue of **6**, exhibited good activities and 718 selectivity toward rMAO-B and eeAChE. *Meta*-chlorobenzyl 719 analogues (i.e., **27**, **35**, and **39**) showed an improved inhibition 720 at eeAChE and esBChE but a worse inhibition at rMAO-B, 721 whereas in contrast the *para*-cyanobenzyl analogues **31**, **33**, and 722 **36** showed better potencies toward rMAO-B and decreased 723 potencies toward eeAChE. The more polar and hydrophilic 4- 724 hydroxymethyl derivatives **37**–**39** displayed the most interest- 725 ing activity profiles with low submicromolar activity at the three 726 target enzymes rMAO-B, eeAChE, and esBChE and low or no 727 activity at rMAO-A. 728

More interesting results came from the assays of selected 729 inhibitors on human MAOs and AChE. In comparison with 730 nonhuman enzymes, a significant increase of inhibitory 731 activities was observed for hMAOs, more pronounced on 732 hMAO-B, with a consequent increase of the selectivity index. 733 More limited variations, generally a decrease of inhibitory 734 potency, were instead observed with hAChE and hBChE. The 735 most interesting inhibitors, e.g., **5**, **12**, **21**, and **37**–**39**, showed 736 excellent activity profiles with low nanomolar inhibitory 737 potency on hMAO-B, high MAO-B over MAO-A selectivity, 738 and submicromolar potency on hAChE. Kinetic inhibition data 739 and docking studies on selected compounds suggested a mixed- 740 type mechanism of inhibition and binding interactions at both 741 the CAS and PAS of AChE. Therefore, our compounds do 742 behave as dual binding site inhibitors and have the potential to 743 block another pathological mechanism of AD, that is, the 744 AChE-promoted A-beta aggregation taking place at the 745 PAS.[78,79] 746

Ultimately, our hybridization strategy proved successful in 747 designing and optimizing novel coumarin-containing com- 748 pounds targeting MAO-B and ChEs, two key enzymes involved 749 in AD, PD, and other neurodegenerative diseases, with well- 750 balanced inhibition potencies. Activity, selectivity, and phys- 751 icochemical properties were improved compared to other 752 ligands targeting the same enzymes described so far.[53−57] 753 Moreover, toxicity, neuroprotection, and transport data, as 754 preliminarily assessed using cell-based models, suggested that 755 the 4-hydroxymethyl coumarin derivative **37**, which resulted 756 the most promising inhibitor, is devoid of significant neuro- 757 toxicity, shows moderate neuroprotective effects against H$_2$O$_2$- 758 induced cell death, as well as a good BBB permeability profile 759 with limited P-gp affinity. 760

In conclusion, some of the multipotent inhibitors reported 761 herein, and compound **37** in particular, may be considered 762 promising leads for further preclinical studies in cognitive and 763 neurodegenerative disease models. 764

EXPERIMENTAL SECTION

765
Chemistry. Starting materials, reagents, and analytical grade 766 solvents were purchased from Sigma-Aldrich Europe. All reactions 767 were routinely checked by TLC using Merck Kieselgel 60 F254 768 aluminum plates and visualized by UV light. Microwave reactions were 769

performed in a Milestone MicroSynth apparatus, setting temperature and hold times, fixing maximum irradiation power to 500 W and heating ramp times to 2 min. The purity of all the intermediates was checked by ^{1}H NMR and ESI-MS. ESI-MS analyses were performed on an Agilent 1100 LC–MSD trap system VL. Flash chromatographic separations were performed on a Biotage SP1 purification system using flash cartridges prepacked with KP-Sil 32–63 μm, 60 Å silica. Elemental analyses were performed on a EuroEA 3000 analyzer only on the final compounds and are reported in Supporting Information. The measured values for C, H, and N agreed to within ±0.40% of the theoretical values. Melting points (MP) were taken on a Gallenkamp MFB 595010 M apparatus (open capillary method) and are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz on a Varian Mercury 300 instrument at ambient temperature in the specified deuterated solvent. Chemical shifts (δ) are quoted in parts per million (ppm) and are referenced to the residual solvent peak. The coupling constants J are given in hertz (Hz). The following abbreviations were used: s (singlet), d (doublet), t (triplet), dd (doublet of doublet), m (multiplet), brs (broad signal). Signals due to NH/OH protons were located by deuterium exchange with D_2O. Noncommercial 7-hydroxy-3-methylcoumarin,[45] 7-hydroxy-3,4-dimethylcoumarin,[44] 7-hydroxy-3-cyanocoumarin,[80] and 7-hydroxy-4-(hydroxymethyl)coumarin,[35] were prepared as referenced.

Analytical data of final compounds **2–40**, **42**, **43**, and **45** are reported in Table 6. Analytical details and spectroscopic data of all the intermediates **1a–k** and final compounds are available as Supporting Information.

General Procedure for the Synthesis of 7-(ω-Bromoalkyloxy)-3,4-dimethylcoumarins 1a–d, 7-(3-(bromomethyl)-benzyloxy)coumarin 1e, and 7-(4-(Bromomethyl)benzyloxy)-3,4-substituted Coumarins 1f–k. A Pyrex vessel was charged with a magnetic stirring bar, and then the appropriate 7-hydroxycoumarin derivative (5.0 mmol) and potassium carbonate (0.70 g, 5.0 mmol) were suspended in dry acetonitrile (20 mL). The suitable commercially available dibromo-derivative (1,ω-dibromoalkane or α,α′-dibromo-m-xylene or α,α′-dibromo-p-xylene, 25 mmol) was added. The reactor was placed in a microwave apparatus and irradiated at 130 °C for 30 min. After cooling to room temperature, the solid residue was filtered and washed with dichloromethane. The solution was concentrated to dryness, and the resulting crude was purified through flash chromatography (gradient eluent, different mixtures of ethyl acetate in n-hexane).

2-(4-(Bromomethyl)phenyl)ethanol (41a). (4-Bromomethyl)-phenylacetic acid (0.60 g, 2.6 mmol) was dissolved under magnetic stirring with 8 mL of anhydrous THF in a flame-dried round-bottomed flask kept to 0 °C. Borane·dimethyl sulfide (0.40 mL, 3.9 mmol) was then added dropwise and the mixture carefully cooled to room temperature and then left for additional 2 h. Water was cautiously added, and the organic solvent was evaporated. Aqueous layer was extracted with ethyl acetate; the organic layers were collected, dried over sodium sulfate, and evaporated to dryness to give the title product. Yield: 87%. ^{1}H NMR (DMSO-d_6) δ: 2.69 (t, J = 7.1 Hz, 2H), 3.57 (t, J = 7.1 Hz, 1H, exch D_2O), 3.79–3.87 (m, 2H), 4.66 (s, 2H), 7.18 (d, J = 7.7 Hz, 2H), 7.33 (d, J = 7.7 Hz, 2H).

7-{[4-(2-Hydroxyethyl)benzyl]oxy}-2H-chromen-2-one (41b). A Pyrex vessel was charged with a magnetic stirring bar, and then 7-hydroxycoumarin (0.32 g, 2.0 mmol) and potassium carbonate (0.28 g, 2.0 mmol) were suspended in dry acetone (12 mL). Bromide **41a** (0.43 g, 2.0 mmol) was added, and the reactor was placed in a microwave apparatus and irradiated at 130 °C for 30 min. After cooling to room temperature, the solid residue was filtered and washed with dichloromethane. The solution was concentrated to dryness, and the resulting oil was purified through flash chromatography (gradient eluent, methanol in dichloromethane 0% → 10%). Yield: 77%. ^{1}H NMR (DMSO-d_6) δ: 2.70 (t, J = 7.1 Hz, 2H), 3.56–3.61 (m, 2H), 4.59–4.63 (m, 1H, exch. D_2O), 5.15 (s, 2H), 6.27 (d, J = 9.6 Hz, 1H), 6.98–7.06 (m, 2H), 7.35 (d, J = 8.0 Hz, 2H), 7.22 (d, J = 8.0 Hz, 2H), 7.62 (d, J = 8.5 Hz, 1H), 7.97 (d, J = 9.6 Hz, 1H).

7-(4-(2-Bromoethyl)benzyloxy)-2H-chromen-2-one (41c). Intermediate **41b** (0.40 g, 1.4 mmol) was dissolved in 5.0 mL of anhydrous dichloromethane under stirring with carbon tetrabromide (0.49 g, 1.5 mmol). To this mixture, a solution of triphenylphosphine (0.43 g, 1.6 mmol) in 5.0 mL of anhydrous dichloromethane was dropped at 0 °C and the resulting solution was kept at room temperature for 4 h. Evaporation of the solvent and purification by flash chromatography (gradient eluent, ethyl acetate in n-hexane 0% → 60%) afforded bromide **41c**. Yield: 77% yield. ^{1}H NMR (DMSO-d_6) δ: 3.12 (t, J = 7.1 Hz, 2H), 3.72 (t, J = 7.1 Hz, 2H), 5.17 (s, 2H), 6.28 (d, J = 9.6 Hz, 1H), 7.00 (dd, J_1 = 8.5 Hz, J_2 = 2.5 Hz, 1H), 7.06 (d, J = 2.5 Hz, 1H), 7.29 (d, J = 8.0 Hz, 2H), 7.40 (d, J = 8.0 Hz, 2H), 7.62 (d, J = 8.5 Hz, 1H), 7.97 (d, J = 9.6 Hz, 1H).

(2E)-N-(3-Methoxyphenyl)-3-phenylacrylamide (44a). To a suspension of trans-cinnamic acid (0.89 g, 6.0 mmol) in dry dichloromethane (5.0 mL), thionyl chloride (5.0 mL) was added. The resulting reaction mixture was refluxed for 6 h and then concentrated to dryness. The oily residue was dissolved in dry dichloromethane (20 mL), and m-anisidine (0.67 mL, 6.0 mmol) was slowly added. After refluxing for 4 h, the solvent was evaporated under vacuum and the resulting crude was crystallized from hot ethanol. Yield: 70%. ^{1}H NMR (DMSO-d_6) δ: 3.73 (s, 3H), 6.61–6.66 (m, 1H), 6.81 (d, J = 15.7 Hz, 1H), 7.18–7.25 (m, 2H), 7.36–7.46 (m, 4H), 7.53–7.62 (m, 3H), 10.17 (s, 1H, exch, D_2O).

7-Hydroxy-2-quinolinone (44b).[70] Phenylacrylamide **44a** (0.63 g, 2.5 mmol) was dissolved in chlorobenzene (12 mL) in a flame-dried round-bottomed flask. AlCl$_3$ (1.3 g, 10 mmol) was added portionwise while cooling to 0 °C. The reaction mixture was refluxed for 8 h, then cooled and poured into crushed ice. The resulting precipitate was washed with chloroform followed by diethyl ether, thus yielding the desired intermediate. Yield: 67%. ^{1}H NMR (DMSO-d_6) δ: 6.19 (d, J = 9.3 Hz, 1H), 6.59–6.71 (m, 2H), 7.42 (d, J = 8.3 Hz, 1H), 7.72 (d, J = 9.3 Hz, 1H), 10.08 (s, 1H, exch. D_2O), 11.48 (s, 1H, exch. D_2O).

7-(4-(Bromomethyl)benzyloxy)-2H-quinolin-2-one (44c). In a Pyrex vessel charged with a magnetic and a Weflon stirring bar, phenol **44b** (0.24 g, 1.5 mmol) and potassium carbonate (0.21 g, 1.5 mmol) were suspended in dry acetone (10 mL). α,α′-Dibromo-p-xylene (1.2 g, 4.5 mmol) were added, and the reactor was placed in a microwave apparatus and irradiated at 130 °C for 30 min. After cooling to room temperature, the solid residue was filtered off and washed with dichloromethane. The solution was concentrated to dryness, and the resulting crude solid was purified through flash chromatography (gradient eluent, methanol in dichloromethane 0% → 10%). Yield: 49%. ^{1}H NMR (DMSO-d_6) δ: 4.71 (s, 2H), 5.13 (s, 2H), 6.28 (d, J = 9.3 Hz, 1H), 6.84–6.85 (m, 2H), 7.40–7.57 (m, 5H), 7.78 (d, J = 9.3 Hz, 1H), 11.60 (brs, 1H, exch. D_2O).

General Procedure for the Synthesis of Final Compounds 2–40, 42, 43, and 45. Appropriate bromide **1a–k**, **41c**, or **44c** (0.50 mmol) were suspended under magnetic stirring in 4.0 mL of anhydrous acetonitrile in a Pyrex microwave reactor in the presence of potassium carbonate (0.64 g, 0.50 mmol) and a catalytic amount of potassium iodide (for compounds **2–12** and **42–43**). Benzylamine (2.5 mmol), suitable substituted N-methylbenzylamine[81,82] (0.75 mmol), or N-ethylbenzylamine (0.75 mmol) was added. The vessel was placed in a microwave apparatus and heated at 130 °C for 30 min. After cooling to room temperature, the reaction mixture was poured into ice-cold water (50 g) and extracted with dichloromethane (3 × 20 mL). The organic fractions were collected, dried over anhydrous sodium sulfate, and evaporated to dryness to give a residue that was crystallized from an appropriate solvent (as indicated in the Supporting Information) or purified by flash chromatography (gradient eluent: different mixtures of methanol in dichloromethane or ethyl acetate in n-hexane). Compounds **2**, **3**, **7–10**, and **12** were crystallized as hydrochlorides by treatment with HCl 1.25 N in ethanol. Compounds **5**, **14**, **16–18**, **22–37**, **39–40**, **42**, **43**, and **45** were transformed into the corresponding hydrochloride salts by treating the crude oil with HCl 4.0 N in 1,4-dioxane (commercially available) or by dissolving the solid crude in the minimum volume of 1,4-dioxane before adding HCl 4.0 N in 1,4-dioxane.

Rat and Human Monoamine Oxidase Inhibition Assays. rMAO inhibitory activity of compounds in Tables 1–3 was assessed using a continuous spectrophotometric assay,[83] monitoring the rate of

oxidation of the nonselective nonfluorescent MAO substrate kynuramine to 4-hydroxyquinoline. MAO-A and MAO-B activities in rat mitochondrial preparations were assayed using as the controls the selective and irreversible inhibitors clorgyline (250 nM) and (−)-L-deprenyl (250 nM), respectively. IC_{50} values were determined by nonlinear regression of MAO inhibition vs −log of the concentration plots, using the program Origin, version 6.0 (Microcal Software Inc., Northampton, MA).

Human monoamine oxidase inhibition assays were carried out with a fluorescence based method,[72] also using kynuramine as nonselective substrate of MAO-A and MAO-B. Briefly, reactions were performed in triplicate in black, flat-bottomed polystyrene 96-well microtiter plates (FluoroNunc/LumiNund, MaxiSorpTM surface, NUNC, Roskild, Denmark) containing potassium phosphate buffer (158 μL), an aqueous stock solution of kynuramine 0.5 mM (final kynuramine concentration corresponding to 50 μM), and DMSO solution of inhibitor in final concentrations ranging from 10^{-4} to 10^{-11} M. Samples were incubated at 37 °C, and then diluted human recombinant MAO-A and MAO-B (Supersomes; BD Gentest, Woburn, MA) were delivered to obtain final protein concentrations of 0.009 and 0.015 mg/mL, respectively. Incubation was carried out at 37 °C for 30 min, and then the reactions were stopped by addition of 75 μL of 2N NaOH.

Formation of 4-hydroxyquinoline was quantified with a 96-well microplate fluorescence reader (FLx 800, BioTek Instruments, Inc. Winoosli, USA) at excitation/emission wavelengths of 310/400 nm (20 nm slit width for excitation, 30 nm slit width for emission). Inhibitory activities (IC_{50}s) were determined by means of nonlinear regressions performed with GraphPad Prism 5.0 software. Results are the mean of at least two independent experiments.

Electric Eel, Equine Serum, and Human Cholinesterases Inhibition Assays. The spectrophotometric Ellman's test[71] for in vitro inhibition assay of AChE from electric eel (463 U/mg; Sigma) and BChE from equine serum (13 U/mg; Sigma) was followed as previously described.[24] The concentration of compound which determined 50% inhibition of the cholinesterase activity (IC_{50}) was calculated by nonlinear regression of the response/log(concentration) curve, using GraphPad Prism version 5. Kinetic studies were performed with the same test conditions, using six concentrations of substrate (from 0.033 to 0.2 mM) and four concentrations of inhibitor (0−0.25 μM). Apparent inhibition constants and kinetic parameters were calculated within the "Enzyme kinetics" module of Prism. Inhibition tests on human recombinant AChE (2770 U/mg; Sigma) and BChE from human serum (50 U/mg; Sigma) were run under the same experimental conditions used for eeAChE.

Bidirectional Transport Studies on MDCKII-MDR1 Monolayers. MDCKII-MDR1 cells were cultured in DMEM medium and seeded at a density of 100000 cell/cm^2 onto polyester 12-well Transwell inserts (pore size 0.4 μm, 12 mm diameter, apical volume 0.5 mL, basolateral volume 1.5 mL). MDCKII-MDR1 cell barrier function was verified prior to the described transport experiments by means of trans-epithelial electrical resistance (TEER) using an EVOM apparatus, and the measurement of the flux of fluorescein isothiocyanate−dextran (FD4, Sigma-Aldrich, Italy) (200 μg/mL) and diazepam (75 μM). The TEER was measured in growth media (DMEM) at room temperature and calculated as the measured resistance minus the resistance of an empty Transwell (blank without cells). Cell monolayers with TEER values 800 Ohm/cm^2 were used. Following the TEER measurements, the cells were equilibrated in transport medium in both the apical and basolateral chambers for 30 min at 37 °C. The composition of transport medium was as follows: 0.4 mM K$_2$HPO$_4$, 25 mM NaHCO$_3$, 3 mM KCl, 122 mM NaCl, and 10 mM glucose, pH = 7.4, and osmolarity 300 mOsm as determined by a freeze point based osmometer. At time 0, culture medium was aspirated from both the AP and BL chambers of each insert, and cell monolayers were washed three times (10 min per wash) with Dulbecco's Phosphate Buffered Saline (DPBS) pH = 7.4. Finally, a solution of compound diluted in transport medium was added to the apical or basolateral chamber. For AP-to-BL or BL-to-AP flux studies, the drug solution was added in the AP chamber or in the BL chamber,

respectively. Except for FD4, which was solubilized directly in the assay medium at a concentration of 200 μg/mL, the other compounds were first dissolved in DMSO and then diluted with the assay medium to a final concentration of 75 μM. Next, the tested solutions were added to the donor side (0.5 mL for the AP chamber and 1.5 mL for the BL chamber), and fresh assay medium was placed in the receiver compartment. The percentage of DMSO never exceeded 1% (v/v) in the samples. The transport experiments were carried out under cell culture conditions (37 °C, 5% CO$_2$, 95% humidity). After incubation time of 120 min, samples were removed from the apical and basolateral side of the monolayer and then stored until further analysis.

Quantitative analysis of compounds **37** and diazepam were performed through UV−visible (Vis) spectroscopy using a PerkinElmer double-beam UV−visible spectrophotometer Lambda Bio 20 (Milan, Italy), equipped with 10 mm path-length-matched quartz cells. Standard calibration curves were prepared at maximum absorption wavelength of each compound using PBS as solvent and were linear (r^2 = 0.999) over the range of tested concentration (from 5 to 100 μM). The FD4 samples were analyzed with a Victor3 fluorimeter (Wallac Victor3, 1420 multilabel counter, PerkinElmer) at excitation and emission wavelengths of 485 and 535 nm, respectively. Each compound was tested in triplicate, and the experiments were repeated three times.

The apparent permeability, in units of cm/s, was calculated using the following equation:

$$P_{app} = \left(\frac{V_A}{\text{area} \times \text{time}} \right) \times \left(\frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{initial}}} \right)$$

where "V_A" is the volume in the acceptor well, "area" is the surface area of the membrane, "time" is the total transport time, "$[\text{drug}]_{\text{acceptor}}$" is the concentration of the drug measured by UV-spectroscopy, and "$[\text{drug}]_{\text{initial}}$" is the initial drug concentration in the AP or BL chamber. Efflux ratio (ER) was calculated using the following equation: ER = P_{app}, BL-AP/P_{app}, AP-BL, where P_{app}, BL-AP is the apparent permeability of basal-to-apical transport, and P_{app}, AP-BL is the apparent permeability of apical-to-basal transport. An efflux ratio greater than 2 indicates that a test compound is likely to be a substrate for P-gp transport.

Cytotoxicity Assays. Human neuroblastoma cells SH-SY5Y were maintained at 37 °C in a humidified incubator containing 5% CO$_2$ in DMEM nutrient (Lonza) supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were dispensed into 96-well microtiter plates at a density of 10000 cells/well. Following overnight incubation, cells were treated with a range of compound concentrations (0.1−50 μM). Then the plates were incubated at 37 °C for 24, 48, and 72 h. An amount of 10 μL of 0.5% w/v MTT was further added to each wel,l and the plates were incubated for an additional 3 h at 37 °C. Finally the cells were lysed by addition of 100 μL of DMSO/EtOH 1:1 (v/v) solution. The absorbance at 570 nm was determined using a PerkinElmer 2030 multilabel reader Victor TM X3.

Neuroprotection against Oxidative Stress. Human neuroblastoma SH-SY5Y cells were dispensed into 96-well microtiter plates at a density of 10000 cells/well. Following overnight incubation, cells were treated with a range of compound concentrations (1 and 10 μM) at time zero and maintained for 24 h. Then, the media were replaced by fresh media still containing the drug plus the cytotoxic stimulus represented by 60 μM H$_2$O$_2$ that was left for an additional 24 h period. Thereafter, cell survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The putative cytotoxic effects of **37** and of H$_2$O$_2$ were studied by exposing the cells to the compound at the highest concentration used in the neuroprotection studies for 24 h. Each compound was tested in triplicate, and the experiments were repeated three times. Statistical significance was assigned to $p < 0.01$ and calculated using a one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc tests (GraphPad Prism vers. 5). Where indicated, standard error of the mean (SD) for data points has been calculated and the number of experiments is given (n).

Molecular Docking. GOLD (version 5.2), a genetic algorithm-based software, was used for the docking study. ChemPLP was selected as a fitness function. Automatic genetic algorithm parameter settings were used. All the crystal structures used in docking simulations were retrieved from the Protein Data Bank. In particular, the entry codes 1EVE and 4EY7 were downloaded for modeling hAChE and TcAChE, respectively; the entry code 2V60 was downloaded for hMAO-B, whereas a previously built homology modeling was used for rMAO-B.[47] The Protein Preparation Wizard available from Schroedinger[84] was used for the protein pretreatment in order to add missing hydrogen atoms, define the protonation states at pH equal to 7.4. and tautomers for histidine residues and to soft-minimize the whole structure. For each simulation, 10 conformations were generated for each inhibitor in a sphere of a 17 Å, using as references the centroids of ligand cocrystallized in 1EVE and 2V60. In MAO docking runs, the X-ray coordinates of 7-(3-chlorobenzyloxy)-4-carboxaldehyde-coumarin taken from 2V60 were used as a scaffold to constrain bias binding mode toward MAO-B. In addition, the side chains of Glu206, Tyr112, Asn116, and His115 (mutated to Tyr115 in the case of rMAO-B) were set be flexible. Finally, eight ordered water molecules were explicitly taken into account in docking runs, as elsewhere reported.[68]

ASSOCIATED CONTENT

ⓢ Supporting Information

Analytical and spectroscopic (^{1}H NMR) data for intermediates **1a−k** and final compounds **2−40**, **42**, **43**, and **45** (PDF); SMILES data (CSV). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00599.

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All authors contributed to the writing of the manuscript, gave approval to the final version of the manuscript and declared no conflict of interest.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

3D-QSAR, three-dimensional quantitative structure−activity relationships; Aβ, beta amyloid protein; ACh, acetylcholine; AChE, acetylcholinesterase; AChEI, AChE inhibitors; AD, Alzheimer's disease; BBB, blood−brain barrier; BChE, butyrylcholinesterase; CAS, catalytic anionic binding site; DBS, dual binding site; DMEM, Dulbecco's Modified Eagle Medium; ER, efflux ratio; ESI-MS, electrospray ionization mass spectrometry; FAD, flavin adenine dinucleotide; FD4, fluorescein isothiocyanate-dextran; MAO-A, monoamine oxidase A; MAO-B, monoamine oxidase B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ND, neurodegenerative disease; NMDA, N-methyl-D-aspartate; P_{app}, apparent permeability; P_{app} AP-BL, apparent permeability apical-to-basal; P_{app} BL-AP, apparent permeability basal-to-apical; PAS, peripheral anionic binding site; PD, Parkinson's disease; P-gp, P-glycoprotein; ROS, reactive oxygen species; SAR, structure−activity relationships; SSR, structure−selectivity relationships; TEER, trans-epithelial electrical resistance

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