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FULL PAPER

7-Amidocoumarins as multitarget agents against neurodegenerative diseases: substitution pattern modulation

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Abstract: This study explores the potential of 7-amidocoumarins as multitarget agents against Parkinson's and Alzheimer's diseases, by modulating the substitution patterns within the scaffold. Sixteen compounds were synthesized *via* 7-amino-4-methylcoumarin acylation, and *in vitro* evaluation of the molecules against *h*MAO-A, *h*MAO-B, *h*AChE, *h*BuChE and *h*BACE1 was performed. Five compounds turned out to be potent and selective *h*MAO-B inhibitors in the nanomolar range, six displayed inhibitory activity of *h*MAO-A in the low micromolar range, one showed *h*AChE inhibitory activity and another one *h*BACE1 inhibitory activity. MAO-B reversibility profile of 7-(4'-chlorobenzamido)-4-methylcoumarin (**10**) was investigated, being this compound a reversible inhibitor. Neurotoxicity on motor cortex neurons and neuroprotection against H_2O_2 were also studied, corroborating the safety profile of these molecules. Finally, theoretical ADME properties were also calculated, showing these molecules as good candidates for the optimization of a lead compound. Results suggest that by modulating the substitution pattern at position 7 of the scaffold, selective or multitarget molecules can be achieved.

Introduction

Neurological disorders have been reported as the major cause of disability-adjusted life-years, and the second leading cause of deaths worldwide (9.0 million), in 2016.[1] The main responsible for these number was stroke (~42%), followed by Alzheimer's and other dementias (~10.4%).[2,3] Neurodegenerative diseases are becoming increasingly prevalent with the aging of the worldwide population.[4] Within the context of increased longevity, rising numbers of people with dementia and surviving strokes is adding further pressure on already stretched health and social care services.[5]

Alzheimer's disease is the most common cause of dementia (60-80% of the cases), involving memory loss and deterioration of other cognitive abilities, interfering with patients' and caregivers' daily life.[6] Despite the advances in the medical sciences, the incidence of this disease is increasing rapidly and has been estimated to currently affect 66 million people worldwide and to increase to 115 million by 2050.[7]

The second most prevalent neurodegenerative disease is Parkinson's disease. Dorsey *et al.* reported that the total population affected by Parkinson's disease at age 50 in the 5 most populous nations in Western Europe and in the 10 most populous nations in the world were between 4.1 and 4.6 million in 2005[8] and estimated that these numbers will be doubled by 2030.[9]

Understanding some of the molecular changes associated to these ubiquitous and widespread diseases, has stimulated efforts to identify targets and develop drugs that especially interact with key enzymes involved in their development. Knowledge of biochemical processes brings the opportunity to provide treatments that are potentially less toxic and more effective than previous therapeutic approaches. As multifactorial diseases, multitarget approaches may be interesting solutions.[10]

Coumarins are a versatile family of compounds and a privileged scaffold in Medicinal Chemistry.[11] Their multitarget profile, especially regarding neurodegenerative disease, has already been described by several groups working in the field.[12-15] For more than ten years, our research group has been reporting differently substituted coumarins as monoamine oxidase (MAO),[16-20] acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitors, as well as neuroprotective agents (Figure 1A-D).[21-26] Furthermore, 7-substituted coumarins have been raised as potent multitarget molecules.[27-31] Based on this expertise, a new series of 7-amidocoumarins (Figure 1E) has been synthetized and studied against different targets. Besides the above-mentioned enzymes, beta-secretase 1 (BACE1) was also included in this study. Substitution patterns' modulation allowed to obtain selective compounds against one single target, as well as multitarget drugs with different profiles, with potential against either Alzheimer's or Parkinson's diseases.

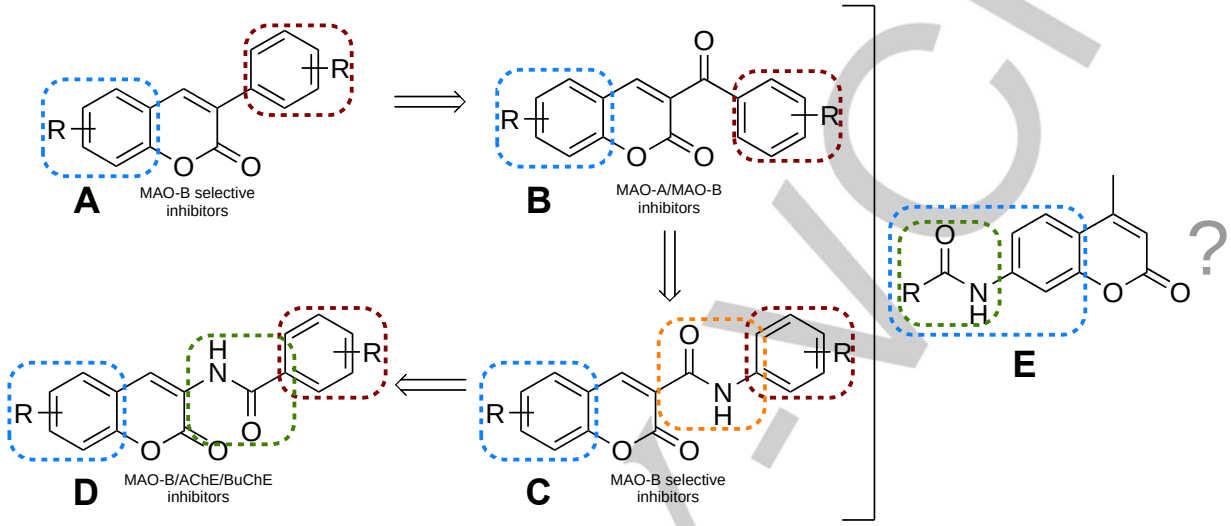
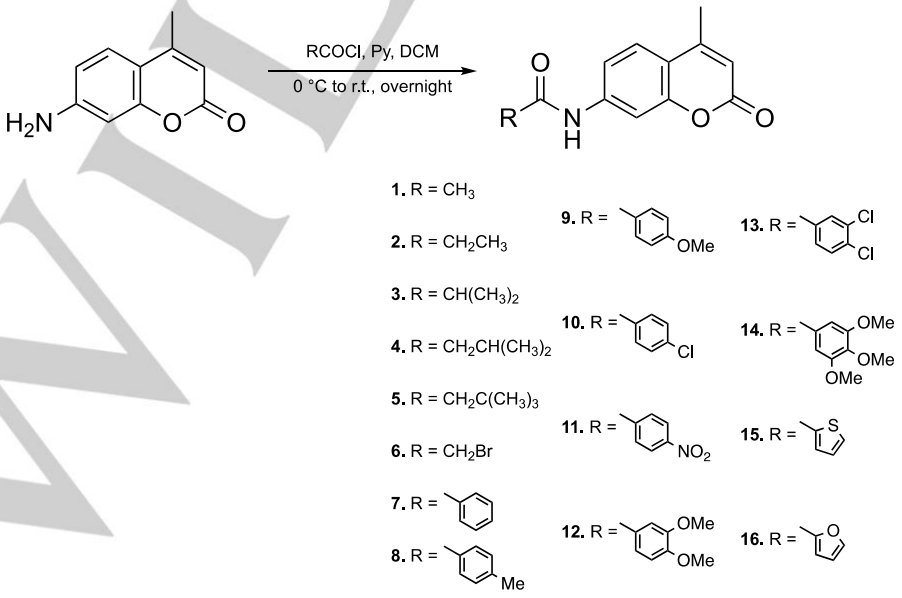


Figure 1. Rational design of the selection of the studied scaffold. **A.** 3-Arylcoumarin scaffold; **B.** 3-Benzoylcoumarin scaffold; **C.** 3-Carboxamidocoumarin scaffold; **D.** 3-Amidocoumarin scaffold and **E.** 7-Amidocoumarin scaffold.

Results and Discussion

Chemistry

The described derivatives **1**-**16** were efficiently synthesized according to the protocol outlined in Scheme 1. Starting from the commercially available 7-amino-4-methylcoumarin, an acylation reaction in the presence of the conveniently substituted acid chloride, using pyridine in dichloromethane, from 0 °C to room temperature, overnight, afforded the desired 7-amido-4-methylcoumarins (**1**-**16**) in yields between 80–90%. The reaction mixtures were purified by flash chromatography, using a mixture of *n*-hexane and ethyl acetate (9:1), as detailed in the experimental section.



Scheme 1. Synthetic methodology to afford the studied 7-amido-4-methylcoumarins (**1**-**16**).

The substituents at position 7 were selected taking into account their nature, size and volume, directing related to their steric hindrance. In the case of the 7-benzamido-4-methylcoumarins (**7**-**14**), the substituents present in the aromatic ring at position 7 of the coumarin scaffold were selected taking into account the different quadrants of the Craig diagram (methyl *vs* nitro and chlorine *vs* methoxy). Finally, two heterocycles were also selected to complete the series (thiophene and furan rings, corresponding to compounds **15** and **16**).

Enzymatic activity inhibition (*h*MAO-A, *h*MAO-B, *h*AChE, *h*BuChE and *h*BACE1)

The *in vitro* effects of compounds **1**-**16** on *h*MAO activity were studied using an Amplex® Red MAO assay kit and the recombinant *h*MAO-A or *h*MAO-B isoforms.[16] Selegiline and iproniazid were used as reference controls, and IC_{50} values and SI are organized in Table 1. The *in vitro* effects of compounds **1**-**16** on *h*AChE and *h*BuChE activities were determined by the Ellman method, using human recombinant AChE expressed in HEK 293 cells or BuChE isolated from human serum.[22,32] Tacrine and eserine were used as reference controls, and all the results are organized in Table 1. To study the possible effects of our compounds and the reference inhibitor OM-99 on the enzymatic activity of *h*BACE1, a method based on the TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) technique was used. The IC_{50} values are also presented in Table 1.[33]

Table 1. IC_{50} values of compounds **1**-**16** and reference inhibitors activity on recombinant *h*MAO isoforms expressed in baculovirus infected BTI insect cells (BTI-TN-5B1-4), recombinant *h*AChE expressed in HEK 293 cells or *h*BuChE isolated from human serum, and purified baculovirus-expressed *h*BACE1.

Comp.	IC_{50} *h*MAO-A (µM)	IC_{50} *h*MAO-B (µM)	SI [a]	IC_{50} *h*AChE (µM)	IC_{50} *h*BuChE (µM)	IC_{50} *h*BACE1 (µM)
1	25.69 ± 1.72	>100	<0.26 [b]	>100	>100	n.d.
2	96.65 ± 6.53	**	0.97 [b]	>100	>100	>100
3	24.50 ± 1.64	11.55 ± 0.78	2.12	>100	>100	n.d.
4	45.86 ± 10.25	0.44 ± 0.03	104.23	>100	>100	n.d.
5	>100	0.25 ± 0.02	>400.0 [b]	>100	>100	n.d.
6	78.16 ± 5.28	>100	<0.78 [b]	3.78 ± 0.25	>100	n.d.
7	>100	0.36 ± 0.02	>278 [b]	>100	>100	>100
8	>100	0.90 ± 0.06	>111.36 [b]	>100	>100	n.d.
9	>100	>100	-	>100	>100	n.d.

10	>100	0.31 ± 0.02	>322.58 [b]	**	**	n.d.
11	>100	>100	-	>100	>100	n.d.
12	>100	**	-	>100	>100	n.d.
13	***	***	-	***	***	***
14	***	***	-	***	***	***
15	**	1.59 ± 0.11	62.89 [b]	>100	>100	34.49 ± 2.31
16	30.14 ± 2.03	5.01 ± 0.34	6.02	>100	>100	>100
Selegiline	68.73 ± 4.21	0.017 ± 0.002	4,043	n.d.	n.d.	n.d.
Iproniazid	6.56 ± 0.76	7.54 ± 0.36	0.87	n.d.	n.d.	n.d.
Tacrine	n.d.	n.d.	n.d.	0.45 ± 0.03	0.15 ± 0.01	n.d.
Eserine	n.d.	n.d.	n.d.	0.12 ± 0.01	0.15 ± 0.01	n.d.
OM-99	n.d.	n.d.	n.d.	n.d.	n.d.	0.08 ± 0.002

Each IC_{50} value is the mean ± S.E.M. from three experiments (n = 3). ** At 100 μM inhibits enzymatic activity by approximately 45-50%. *** The compound proved to be insoluble at the tested conditions. [a] SI: *h*MAO-B selectivity index = IC_{50} (*h*MAO-A)/IC_{50} (*h*MAO-B). [b] Values obtained under the assumption that the corresponding IC_{50} against *h*MAO-A or *h*MAO-B is the highest concentration tested (100 μM). Highest concentration tested: 100 μM; at higher concentration, the compounds precipitate. n.d.: non determined.

Five of the studied compounds (**4**, **5**, **7**, **8** and **10**) display MAO-B activity in the nanomolar range. The first two derivatives, 7-isobutylacetamido-4-methylcoumarin (**4**) and 7-*tert*-butylacetamido-4-methylcoumarin (**5**), present small side chains attached to the amide at position 7. Although, both substitutions on those side chains are quite bulky. In fact, an increase in the number of carbon atoms of the alkyl substituent attached to the amide, leads to an increase in the MAO-B inhibitory activity: methyl < ethyl < isopropyl < *tert*-butyl. 7-Benzamido-4-methylcoumarin (**7**), 4-methyl-7-(4'-methylbenzamido)coumarin (**8**) and 7-(4'-chlorobenzamido)-4-methylcoumarin (**10**) present an aromatic group attached to the amide at position 7. The first one, without substituents, and the other two, with a *para*-methyl (electron donor group) and a *para*-chlorine (electron acceptor atom), respectively.

All these substituents seem to be acceptable for this activity. However, a *para*-methoxy or *para*-nitro substituent leads to inactive derivatives **9** and **11**. Finally, the increase in the number of substituents attached to the aromatic ring leads to the insoluble compounds **13** and **14**.

The exchange of the benzene ring with thiophene (**15**) or furan (**16**) rings, decreased MAO-B inhibitory activity to the low micromolar range (IC_{50} = 1.59 and 5.01 μM, respectively). Furthermore, the furan ring also decreased the selectivity. Compound **16** has activity on both isoforms, although it is more potent on MAO-B.

Six of the studied compounds present MAO-A inhibitory activity in the micromolar range (**1**-**4**, **6** and **16**). As observed, most of them present small lateral chains attached to the amide at position 7 of the coumarin scaffold. From these molecules, 7-acetamido-4-methylcoumarin (**1**), 4-methyl-7-propionamidocoumarin (**2**) and 7-(2'-bromoacetamido)-4-methylcoumarin (**6**) proved to be selective against this isoform whereas 7-isopropylacetamido-4-methylcoumarin (**3**) inhibits both MAO isoforms in the same concentration range.

From the studied series, none of the compounds displays significant inhibitory activity against BuChE. Regarding AChE inhibition, compound **6** proved to inhibit this enzyme in the low micromolar range. Therefore, this compound can be a promising dual target molecule, presenting activity against both MAO-A and AChE. Finally, and to push further the multitarget potential of these molecules, BACE1 inhibitory activity was evaluated for a selection of compounds. From those, 4-methyl-7-(2'-thiophenamido)coumarin (**15**) displays activity in the micromolar range IC_{50} = 34.49 μM). This is a very interesting achievement, since no other coumarin studied by our group ever presented activity against this target. Compound **15** displays also MAO-B inhibitory activity in the low micromolar range, being, therefore, a candidate to explore this dual target activity.

Reversibility profile of the best *h*MAO-B inhibitor

The type of inhibition exerted by the 7-(4'-chlorobenzamido)-4-methylcoumarin (**10**) on *h*MAO-B isoform was evaluated by using a dilution method.[34] The selected compound is the most potent and selective 7-benzamido-4-methylcoumarin within the studied series. Selegiline and isatin were used as controls, and the results are shown in Table 2.

Table 2. Reversibility results for the *h*MAO-B inhibition of compound **10** and the reference inhibitors (selegiline and isatin).

Compound	Slope (ΔUF/t) [%][a]
10	59.30 ± 3.98
Selegiline	12.73 ± 1.85
Isatin	88.63 ± 5.90

[a] Values represent the mean ± S.E.M. of n = 2 experiments, relative to the control; data show recovery of *h*MAO-B activity after dilution.

Compound **10** proved to be a reversible *h*MAO-B inhibitor, according to the results presented in Table 2. Comparing to isatin, a reversible MAO-B inhibitor used as reference, its degree of reversibility is slightly lower. However, compound **10** presents a better reversibility profile than selegiline, a non-reversible inhibitor used in therapeutics. Some 3-amidocoumarins already reported by the group were described as reversible *h*MAO-B inhibitors.[23] Recently, a coumarin derivative bearing a pent-2-yn-1-amine group at position 7, the same position under study in this project, was reported as a partially reversible MAO-B inhibitor.[26]

Cellular studies

Neurotoxicity profile

Neurotoxic activity of compounds **1**-**16** was measured on rat motor cortex neurons, obtained from embryos of 19 days pregnant albino rats (Rattus novergicus) of the Wistar-Kyoto strain.[35] After treatment and an incubation period of 24 hours, monitorization of cell viability was performed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (MTT). The percentage (%) of cell viability after incubation with the 7-amidocoumarins **1**-**16**, at 10 μM concentration, is represented in Figure 2.

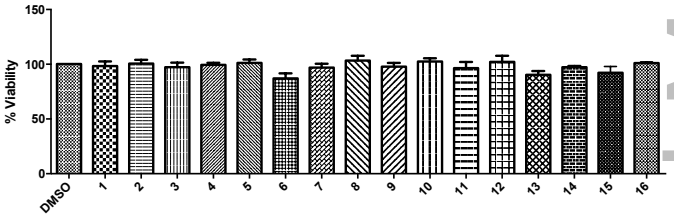


Figure 2. Evaluation of the neurotoxic effect of the 7-amidocoumarins **1**-**16** on rat motor cortex neurons. Cells were incubated with the compounds at 10 μM, dissolved in DMSO (1%). The results are expressed as % of viability against the control group (treated with DMSO 1%) in at least 5 experiments ± S.E.M.

As observed in Figure 2, none of the studied derivatives showed toxicity against the cells used for this study.

Neuroprotective activity

Together with the enzymatic inhibition, neuroprotective effect on neuronal populations affected during the progression of neurodegenerative diseases is desirable. The neuroprotective effect of the 7-amidocoumarins **1**-**16** against H_2O_2, in the cellular model previously described, was then evaluated (Figure 3). Cells were treated with H_2O_2 (100 μM) previously to the treatment with 7-amidocoumarins **1**-**16**. Monitorization of cell viability was performed after 24 hours incubation by using the MTT method.

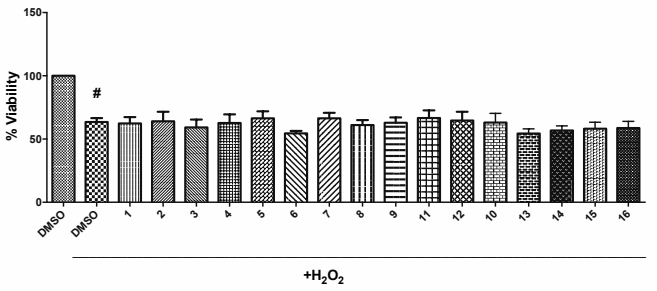


Figure 3. Neuroprotective effect of the 7-amidocoumarins **1**-**16** on rat motor cortex neurons. The cells were incubated with the molecules at 10 µM, dissolved in DMSO (1%), in the presence of H_2O_2 (100 µM). The results are expressed as % of viability against the control group (treated with DMSO 1%) in at least 5 experiments ± S.E.M. #*P*<0.0001, against the group treated with only DMSO.

As can be seen in Figure 3, all the derivatives, at the studied concentration, lack neuroprotective activity on the neurons of a primary culture of the motor cortex neurons. This can be explained by the lack of hydroxyl groups on their structures, as we observed in some previous projects from our group.

ADME properties

In order to assess the possible *in vivo* activity of these compounds, theoretical physicochemical properties of the 7-amidocoumarins **1**-**16** were calculated. These parameters are excellent indicators of the capacity to cross cellular membranes and therefore of their ADME (absorption, distribution, metabolism and excretion) properties. Molinspiration cheminformatics software was used to calculate the octanol/water partition coefficient (LogP), the polar surface area (TPSA), the number of atoms and molecular weight (MW), the number of H-bond acceptors (ON) and H-bond donors (OHNH), as well as the volume (V) and the number of rotatable links (rotb). The "CBligand-BBB predictor" program was used to theoretically predicted the probability to cross through the blood brain barrier (BBB) of the studied molecules. All the results, together with the prediction of the violations of Lipinski rules (n viol), are included in Table 3.

Table 3. Molecular properties of 7-amidocoumarins **1**-**16** calculated using the Molinspiration software and theoretical prediction of their passage through BBB using the CBLigand-BBB software.

Compd	LogP	TPSA	n atoms	MW	n ON	n OHNH	n rotb	V	n viol	BBB (±)
1	1.59	59.31	16	217.22	4	1	1	193.10	0	-
2	2.42	59.31	17	231.25	4	1	2	209.90	0	+
3	2.67	59.31	18	245.28	4	1	2	226.48	0	-
4	3.20	59.31	19	259.31	4	1	3	243.29	0	-
5	3.52	59.31	20	273.33	4	1	3	259.52	0	-
6	2.28	59.31	17	296.12	4	1	2	211.22	0	+
7	3.36	59.31	21	279.30	4	1	2	247.94	0	+
8	3.71	59.31	22	293.32	4	1	2	264.50	0	+
9	3.31	68.54	23	309.32	5	1	3	273.49	0	-
10	3.94	59.31	22	313.74	4	1	2	261.48	0	+
11	3.22	105.13	24	324.29	7	1	3	271.28	0	-
12	2.90	77.78	25	339.35	6	1	4	299.03	0	-

13	4.54	59.31	23	348.19	4	1	2	275.01	0	+
14	2.89	87.01	27	369.37	7	1	5	324.58	0	-
15	3.16	59.31	20	285.32	4	1	2	238.65	0	+
16	2.51	72.45	20	269.26	5	1	2	229.51	0	-
Selegiline	2.26	3.24	13	173.26	1	0	3	185.84	0	+
Tacrine	3.05	38.92	15	198.27	2	2	0	191.53	0	+
Eserine	1.94	44.81	20	275.35	5	1	2	261.48	0	+

Theoretically, all the 7-amidocoumarins **1**-**16** possess the desirable physicochemical properties for a good bioavailability, and none of the compounds violate the Lipinski rule of five (Table 3). 4-Methyl-7-(4'-methylbenzamido)coumarin (**8**) has the second highest value of LogP within the series, combined with a low TPSA. 7-Benzamido-4-methylcoumarin (**7**), 4-methyl-7-(4'-methylbenzamido)coumarin (**8**) and 7-(4'-chlorophenylbenzamido)-4-methylcoumarin (**10**), three of the MAO-B selective inhibitors (IC_{50} in the nanomolar range), present good ADME properties, as well as the theoretical ability to cross the BBB. Also, 4-methyl-7-(2'-thiophenamido)coumarin (**15**), the dual MAO-B/BACE1 inhibitor (IC_{50} in the low micromolar range), present both good ADME properties and theoretical ability to cross the BBB. Finally, the dual MAO-A/AChE inhibitor 7-(2'-bromoacetamido)-4-methylcoumarin (**6**, IC_{50} in the low micromolar range), also present good ADME properties and theoretical ability to cross the BBB.

Analyzing the data, it can be observed that, in general, the 7-benzamidocoumarins (**7**-**16**) present better profiles than the 7-alkylamidocoumarin (**1**-**6**). From these molecules (**1**-**6**), four molecules seem to have problems in crossing the BBB. The molecules with an aromatic ring attached to the 7-amide group (**7**-**16**) seem to be more likely to cross the BBB, as half of the studied compounds proved to have the right structures to cross this barrier.

Conclusion

This report describes the MAO-A, MAO-B, AChE, BuChE and BACE1 inhibitory activities of 7-amidocoumarins, observing that the nature of the substituents is decisive for their activity and selectivity. 7-Isobutylacetamido-4-methylcoumarin (**4**), 7-*tert*-butylacetamido-4-methylcoumarin (**5**), 7-benzamido-4-methylcoumarin (**7**), 4-methyl-7-(4'-methylbenzamido)coumarin (**8**) and 7-(4'-chlorobenzamido)-4-methylcoumarin (**10**) are MAO-B selective inhibitors, presenting IC_{50} in the nanomolar range. Compound **10** proved to be a reversible MAO-B inhibitor, presenting all the theoretical properties to have good bioavailability and cross the BBB. 4-Methyl-7-(2'-thiophenamido)coumarin (**15**) is a dual MAO-B/BACE1 inhibitor, with activities in the low micromolar range. Finally, 7-(2'-bromoacetamido)-4-methylcoumarin (**6**) is a dual MAO-A/AChE inhibitor, presenting IC_{50} in the low micromolar range. All these results together help to understand that, by modulating the substitution pattern at position 7 of the 7-amidocoumarins, it is possible to design molecules with different profiles against Parkinson's and Alzheimer's diseases.

Experimental Section

Chemistry

General information. All reagents were purchased from Sigma-Aldrich and used without further purification. All solvents were commercially available grade. All reactions were carried out under argon atmosphere, unless otherwise mentioned. Reaction mixtures were purified by flash column chromatography using Silica Gel high purity grade (Merck grade 9385 pore size 60Å, 230-400 mesh particle size). Reaction mixtures were analysed by analytical thin-layer chromatography (TLC) using plates precoated with silica gel (Merck 60 F254, 0.25 mm). Visualization was accomplished with UV light (254 nm), ninhydrin or potassium permanganate ($KMnO_4$). $_1$H NMR and $_{13}$C NMR spectra were recorded on a Bruker AMX spectrometer at 250 and 75.47 MHz in the stated solvents ($CDCl_3$ or DMSO-d_6) using tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in parts per million (ppm) on the δ scale from an internal standard (NMR descriptions: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet). Mass spectroscopy was performed using a Hewlett-Packard 5988A spectrometer. This system is an automated service utilizing electron impact (EI) ionization. Elemental analyses were performed using a Perkin-Elmer 240B microanalyzer and were within (0.4% of calculated

values in all cases). The purity of compounds was assessed by HPLC and was found to be higher than 99%.

Synthetic methodology. The 7-amino-4-methylcoumarin (commercially available, 1.0 mmol) was dissolved in CH_2Cl_2 (9 mL). Pyridine (1.1 mmol) was then added, and the mixture was cooled to 0 °C. The corresponding acyl chloride (1.1 mmol) was added dropwise at this temperature, and the mixture was stirred overnight at room temperature. The batch was evaporated and purified by column chromatography (hexane/EtOAc, 9:1) to give the desired compound **1**-**16**.

7-Acetamido-4-methylcoumarin (1).[36]

4-Methyl-7-propionamidocoumarin (2).[37]

7-Isopropylacetamido-4-methylcoumarin (3). ^{1}H NMR ($CDCl_3$) δ: 1.27 (s, 3H, CH_3), 1.30 (s, 3H, CH_3), 2.44 (s, 3H, CH_3), 2.65 (q, 1H, CH, *J*=6.8), 6.22 (s, 1H, H-3), 7.55 (d, 1H, H-5, *J*=8.7), 7.67 (d, 1H, H-8, *J*=2.2), 7.86 (dd, 1H, H-6, *J*=8.7, 2.2), 8.06 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 18.5, 19.4, 20.6, 36.5, 106.7, 113.0, 115.7, 121.4, 125.2, 139.0, 145.2, 152.1, 162.1, 175.8. MS *m/z* (%): 245 (M_+, 76). Ana. Elem. Calc. for $C_{14}H_{15}NO_3$: C, 68.56; H, 6.16. Found: C, 68.52; H, 6.15.

7-Isobutylacetamido-4-methylcoumarin (4). ^{1}H NMR (DMSO-*d*$_6$) δ: 0.88 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 2.06 (q, 1H, CH, *J*=7.3), 2.20 (d, 2H, CH_2, *J*=7.3), 2.35 (s, 3H, CH_3), 6.20 (s, 1H, H-3), 7.43 (dd, 1H, H-6, *J*=8.7, 2.0), 7.64 (d, 1H, H-5, *J*=8.7), 7.73 (d, 1H, H-8, *J*=2.0), 10.25 (s, 1H, NH). ^{13}C NMR (DMSO-*d*$_6$) δ: 18.1, 22.4, 25.6, 45.8, 105.5, 112.2, 114.9, 115.2, 126.0, 142.7, 153.2, 153.8, 160.2, 171.5. MS *m/z* (%): 259 (M_+, 87). Ana. Elem. Calc. for $C_{15}H_{17}NO_3$: C, 69.48; H, 6.61. Found: C, 69.51; H, 6.58.

7-*tert*-Butylacetamido-4-methylcoumarin (5). ^{1}H NMR ($CDCl_3$) δ: 1.12 (s, 9H, 3xCH_3), 2.30 (s, 2H, CH_2), 2.43 (s, 3H, CH_3), 7.55 (d, 1H, H-5, *J*=8.7), 7.61 (d, 1H, H-8, *J*=2.2), 7.77 (dd, 1H, H-6, *J*=8.7, 2.2), 8.09 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 24.8. 29.6, 31.0, 43.0, 109.9, 112.5, 119.4, 119.7, 125.1, 143.3, 157.2, 161.0, 170.5, 174.0. MS *m/z* (%): 273 (M_+, 91). Ana. Elem. Calc. for $C_{16}H_{19}NO_3$: C, 70.31; H, 7.01. Found: C, 70.28; H, 7.04.

7-(2'-Bromoacetamido)-4-methylcoumarin (6).[38]

7-Benzamido-4-methylcoumarin (7).[39]

4-Methyl-7-(4'-methylbenzamido)coumarin (8).[39]

7-(4'-Methoxybenzamido)-4-methylcoumarin (9).[38]

7-(4'-Chlorobenzamido)-4-methylcoumarin (10).[39]

4-Methyl-7-(4'-nitrobenzamido)coumarin (11).[39]

7-(3',4'-Dimethoxybenzamido)-4-methylcoumarin (12). ^{1}H NMR (DMSO-*d*$_6$) δ: 2.40 (s, 3H, CH_3), 3.84 (s, 6H, 2xOCH_3), 6.25 (s, 1H, H-3), 7.09 (d, 1H, H-5, *J*=8.5), 7.54 (d, 1H, H-8, *J*=1.9), 7.65 (dd, 1H, H-6, *J*=8.5, 1.9), 7.72-7.75 (m, 2H, H-5', H-6'), 7.93 (s, 1H, H-2'), 10.46 (s, 1H, NH). ^{13}C NMR (DMSO-*d*$_6$) δ: 18.2, 55.8, 55.9, 111.0, 111.3, 112.5, 115.3, 116.3, 121.5, 125.8, 126.5, 140.0, 148.5, 153.3, 153.7, 154.6, 160.3, 165.6. MS *m/z* (%): 339 (M_+, 73). Ana. Elem. Calc. for $C_{19}H_{17}NO_5$: C, 67.25; H, 5.05. Found: C, 67.23; H, 5.08.

7-(3',4'-Dichlorophenylbenzamido)-4-methylcoumarin (13). ^{1}H NMR (DMSO-*d*$_6$) δ: 2.48 (s, 3H, CH_3), 6.27 (s, 1H, H-3), 7.75-7.83 (m, 2H, H-5, H-6), 7.91-7.98 (m, 2H, H-5', H-6'), 8.25 (d, 1H, H-8, *J*=1.8), 8.88 (s, 1H, H-2'), 10.77 (s, 1H, NH). ^{13}C NMR (DMSO-*d*$_6$) δ: 19.5, 111.9, 112.5, 115.2, 118.3, 124.3, 128.2, 128.9, 130.3, 133.5, 133.7, 136.8, 137.3, 152.7, 154.3, 160.8, 164.7. MS *m/z* (%): 349 (M_+, 17), 347 (M_+, 30). Ana. Elem. Calc. for $C_{17}H_{11}Cl_2NO_3$: C, 58.64; H, 3.18. Found: C, 58.62; H, 3.22.

7-(3',4',5'-Trimethoxybenzamido)-4-methylcoumarin (14). ^{1}H NMR (DMSO-*d*$_6$) δ: 2.41 (s, 3H, CH_3), 3.73 (s, 3H, OCH_3), 3.87 (s, 36H, 2xOCH_3), 6.27 (s, 1H, H-3), 7.30 (s, 2H, H-2', H-6'), 7.73-7.75 (m, 2H, H-5, H-6), 7.93 (d, 1H, H-8, *J*=1.1), 10.54 (s, 1H, NH). ^{13}C NMR (DMSO-*d*$_6$) δ: 18.2, 56.3, 60.3, 105.7, 106.9, 112.6, 115.5, 116.5, 125.9, 127.0, 129.6, 142.7, 152.8, 153.3, 153.7, 160.0, 165.6. MS *m/z* (%): 369 (M_+, 54). Ana. Elem. Calc. for $C_{20}H_{19}NO_6$: C, 65.03; H, 5.19. Found: C, 65.06; H, 5.13.

4-Methyl-7-(2'-thiophenamido)coumarin (15). ^{1}H NMR (DMSO-*d*$_6$) δ: 2.39 (s, 3H, CH_3), 6.26 (s, 1H, H-3), 7.24 (t, 1H, H-4', *J*=3.8), 7.71-7.74 (m, 2H, H-5, H-6), 7.89-7.91 (m, 2H, H-8, H-3'), 8.12 (d, 1H. H-5', *J*=3.8), 10.65 (s, 1H, NH). ^{13}C NMR (DMSO-*d*$_6$) δ: 18.2, 106.7, 112.6, 115.5, 116.2, 125.9, 128.4, 130.1, 132.9, 139.5, 142.4, 153.2, 153.7, 160.2, 160.5. MS *m/z* (%): 285 (M_+, 61). Ana. Elem. Calc. for $C_{15}H_{11}NO_3S$: C, 65.15; H, 3.89. Found: C, 63.15; H, 3.92.

7-(2'-Furanamido)-4-methylcoumarin (16). ^{1}H NMR (DMSO-*d*$_6$) δ: 2.38 (s, 3H, CH_3), 6.25 (s, 1H, H-3), 7.22 (t, 1H, H-4', *J*=3.5), 7.45 (d, 1H, H-5', *J*=3.5), 7.72-7.78 (m, 2H, H-5, H-6), 7.89-7.97 (m, 2H, H-8, H-3'), 10.62 (s, 1H, NH). ^{13}C NMR (DMSO-*d*$_6$) δ: 18.1, 106.7, 112.6, 115.5, 115.9, 116.2, 125.9, 142.2, 146.4, 147.1, 153.2, 153.6, 156.6, 160.2. MS *m/z* (%): 269 (M_+, 46). Ana. Elem. Calc. for $C_{15}H_{11}NO_4$: C, 66.91; H, 4.12. Found: C, 66.93; H, 4.16.

Pharmacology

Determination of MAO isoforms *in vitro* activity. The effects of the 7-amidocoumarins (**1**-**16**) on *h*MAO enzymatic activity were evaluated by a fluorimetric method following the experimental protocol previously described by us.[16] Briefly, 50 µL of sodium phosphate buffer (0.05 M, pH 7.4) containing the test molecules (new compounds or reference inhibitors) in different concentrations and adequate amounts of recombinant *h*MAO-A or *h*MAO-B [adjusted to obtain in our experimental conditions the same reaction velocity (*h*MAO-A: 1.1 µg protein; specific activity: 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min/mg protein; *h*MAO-B: 7.5 µg protein; specific activity: 22 nmol of *p*-tyramine transformed/min/mg protein)] were incubated for 10 min at 37 °C

in a flat-black bottom 96-well microtest plate, placed in the dark fluorimeter chamber. After this incubation period, the reaction was started by adding 50 µL of the mixture containing (final concentrations) 200 µM of the Amplex® Red reagent, 1 U/mL of horseradish peroxidase and 1 mM of *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin, was quantified at 37 °C in a multidetection microplate fluorescence reader (Fluo-star Optima™, BMG LABTECH, Offenburg, Germany) based on the fluorescence generated (λ excitation, 545 nm, λ emission, 590 nm) over a 10 min period, in which the fluorescence increased linearly. Control experiments were carried out simultaneously by replacing the tested molecules with appropriate dilutions of the vehicles (DMSO ≤ 0.5 %). In addition, the possible capacity of these molecules to modify the fluorescence generated in the reaction mixture, due to non-enzymatic inhibition (i.e. for directly reacting with Amplex® Red reagent), was determined by adding these molecules to solutions containing only the Amplex® Red reagent in sodium phosphate buffer. The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of the background activity, which was determined from wells containing all components except the *h*MAO isoforms, which were replaced by sodium phosphate buffer solution.

Reversibility. To evaluate whether the 7- (4'-chlorobenzamido)-4-methylcoumarin (**10**) is a reversible or irreversible *h*MAO-B inhibitor, a dilution method was used.[34] A 100X concentration of the enzyme used in the above described experiment was incubated with a concentration of inhibitor equivalent to 10-fold the IC_{50} value. After 30 min, the mixture was diluted 100-fold into a reaction buffer. Enzymatic activity was then evaluated as previously described. Reversible inhibitors show linear progress with a slope equal to ~91% of the slope of the control sample, whereas irreversible inhibition reaches only ~9% of this slope. A control test was carried out by pre-incubating and diluting the enzyme in the absence of inhibitor.

Determination of AChE and BuChE *in vitro* activity. Ellman´s method [32] was used to determine *in vitro* ChE activity. 0.01 U/mL human recombinant AChE expressed in HEK 293 cells or 0.0005 U/mL BuChE isolated from human serum were added to a 50 mM phosphate buffer solution (pH 7.2) containing different concentrations of compounds **1-16** or reference inhibitors. Mixture was preincubated at 37 ºC for 5 min followed by the addition of 5 mM acetylthiocholine or butyrylthiocholine and 0.25 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DNTB). The activity was measured by the absorbance increasing at λ 412 nm at 1 min intervals for 10 min at 37 ºC (Fluo-Star Optima™, BMG LABTECH, Offenburg, Germany). Control experiments were performed simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles (DMSO ≤ 0.5 %). The specific absorbance (used to obtain the final results) was calculated after subtraction of the background activity, which was determined in wells containing all components except the AChE or BuChE, which was replaced by a sodium phosphate buffer solution.

Determination of BACE1 *in vitro* activity. The assay kit used in the experiments was LanthaScreen TR-FRET BACE1 (Invitrogen). 30 µL of sodium acetate buffer (50 mM, pH 4.5) containing different concentrations of the compounds to be studied (or the reference inhibitor OM-99), the enzyme BACE1 (2.1 U/mL) and the substrate FL- BACE1-biotin (200 nM concentration) were incubated in 96-well black flat bottom plates (96-well Half Area Black Flat Bottom Polystyrene NBS Microplate, Corning®) at room temperature and protected from light for 60 min. Control experiments were simultaneously carried out, replacing the reference drugs or inhibitors with the appropriate dilutions of the vehicles (DMSO ≤ 0.5 %). After the incubation period, the reaction was stopped by adding 10 µL of the stop solution (Tb-anti-biotin antibody, 5 nM concentration). After mixing, the solution was kept 60 min at room temperature and protected from light. Once the incubation was finished, fluorescence intensity was measured using the TR-FRET technique (λ excitation 545, λ emission 585) in a multifunction plate reader (CLARIOstar® Plus, BMG LABTECH, Offenburg, Germany). The fluorescence emitted by the FL-BACE1-Biotin substrate was also determined at the same concentration used in the experiments in the absence of the enzyme and subtracted from the total fluorescence.

Cell viability. Embryos were extracted by caesarean section from pregnant Wistar Kyoto rats, which were euthanized by CO_2 inhalation. Brains were carefully dissected out and after removing meninges, a portion of motor cortex was isolated. Fragments obtained from several embryos were mechanically digested and cells were resuspended in neurobasal medium supplemented with 2% B-27. Cells were seeded in 96-well plates at a density of 200,000 cells/mL to obtain cortex neuronal cultures. Cultures were allowed to grow for 7-8 days in an incubator (Form Direct Heat CO_2, Thermo Electron Corporation, Madrid, Spain) under saturated humidity at a partial pressure of 5% CO_2 in air, at 37 °C. Once a dense neuronal network could be observed, motor cortex cultures were treated with the 7-amidocoumarins (**1**-**16**) at 10 µM to evaluate the cytotoxicity. In order to evaluate their neuroprotective effect cultures were previously treated with H_2O_2 (100 µM). After incubation for 24 h under the conditions above-described, cell viability was evaluated using MTT as follows: 10 µL of a solution containing 0.5 mg/mL MTT in Hank's was added to each well containing 100 µL of culture medium and incubation was performed at 37 °C, for 2 h. After incubation, medium was removed, and formazan salt formed was dissolved in DMSO. The colorimetric determination was performed at λ 540 nm.

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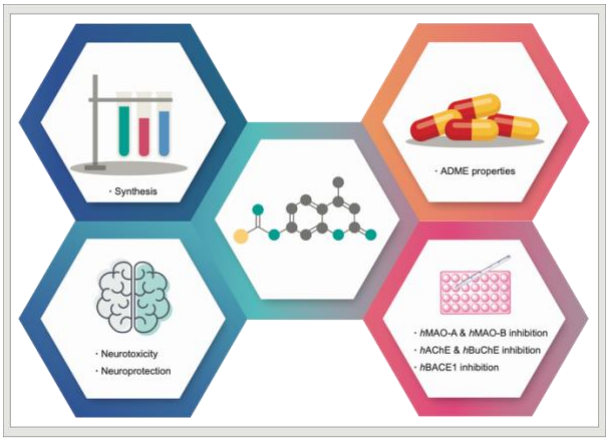

Alzheimer's and Parkinson's diseases are the most prevalent neurodegenerative disorders, and there is no cure for them. Therefore, it is urgent to develop new therapeutic solutions. 7-Amidocoumarins were synthetized and studied as MAO-A, MAO-B, AChE, BuChE and BACE1 inhibitors, and neuroprotective agents. All the studied compounds proved to be non-neurotoxic on rat motor cortex neurons, five turned out to be potent and selective *h*MAO-B inhibitors in the nanomolar range, six displayed *h*MAO-A inhibition in the low micromolar range, one showed AChE inhibitory activity and another one BACE-1 inhibitory activity.

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