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3-Amidocoumarins as Potential Multifunctional Agents against Neurodegenerative Diseases

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Monoamine oxidase (MAO) generates reactive oxygen species (ROS), which cause neuronal cell death, causing neurodegeneration. Agents that are able to concurrently inhibit MAO and scavenge free radicals represent promising multifunctional neuroprotective agents that could be used to delay or slow the progression of neurodegenerative diseases. In this work, variously substituted 3-amidocoumarins are described that exert neuroprotection in vitro against hydrogen peroxide in rat cortical neurons, as well as antioxidant activity in a 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical scavenging assay. Selective and reversible inhibitors of the MAO-B isoform were identified. Interestingly, in the case of the 3-benzamidocoumarins, substitution at position 4 with a hydroxy group abolishes MAO-B activity, but the compounds remain active in the neuroprotection model. Further evaluation of 3-heteroarylamide derivatives indicates that it is the nature of the heterocycle that determines the neuroprotective effects. Evaluation in a parallel artificial membrane permeability assay (PAMPA) highlighted the need to further improve the blood–brain barrier permeability of this compound class. However, the compounds described herein adhere to Lipinski's rule of five, suggesting that this novel scaffold has desirable properties for the development of potential drug candidates.

Introduction

Neurodegenerative diseases (ND) are characterized by a decrease in the number of cells of certain neuronal populations, and are clinically reflected by the appearance of specific symptoms, such as modification in the control and coordination of movement in Parkinson's disease (PD) or alterations in the language and memory processes in Alzheimer's disease (AD).[1] Their chronic course produces a gradual but steady deterioration, the final step of which is death.[2] Because of this, as the disease progresses, it erodes the quality of life for patients.[3] Therefore, the development of effective neuroprotective therapies that slow down or stop the disease's progression at the earliest stages is one of the main goals of researchers in this area.[4]

At the cellular level, PD is related to excess production of reactive oxygen species (ROS), to alterations in catecholamine metabolism, to modifications in mitochondrial electron transporter chain (METC) function, and to enhancement of iron deposition in the substantia nigra pars compacta (SNpc).[5] The failure of normal cellular processes that occur in relation to aging are also believed to contribute to the increased vulnerability of dopaminergic neurons.[6] Although the precise mechanism corresponding to ROS generation related to PD is still unknown, the major sources of oxidative stress generated by the dopaminergic neurons are dopamine metabolism, mitochondrial dysfunction, and neuroinflammation.[7]

Metabolism of dopamine by monoamine oxidase (MAO) yields hydrogen peroxide, an oxygen radical that leads to cytotoxicity through the peroxidation of lipid membranes. Selegiline and rasagiline, two selective MAO-B inhibitors, are currently used to retard the symptoms in PD because they increase dopamine levels and may exert neuroprotective effects. Inhibition of MAO-B decreases oxygen radical generation, although new neuroprotective functions independent of MAO-inhibitory activity have been reported for these drugs.[8] The occurrence of oxidative stress in PD patients is supported by postmortem studies and by preclinical studies showing the ability of oxidizing toxins to induce cell death in the SN.[9] Accordingly, antioxidants such as tocopherol and ascorbate that scavenge free radicals and other reactive species may have beneficial therapeutic effects in PD by preventing the onset of apoptosis and

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neuronal degeneration of the dopaminergic nigrostriatal pathway.[10] Nonetheless, all approved PD pharmacotherapies have limited efficacy, do not prevent the progression of the disease, and are associated with adverse motor and non-motor side effects.[11] Accordingly, there is an urgent need to develop novel therapies that are superior to current therapies.

Throughout the history of medicine, nature has played a key role as source of inspiration in the development of drugs with important biological activities. Coumarins (2*H*-1-benzopyran-2-one), common metabolites in plants that have also been detected in microorganisms and animal sources,[12] have been the focus of much interest owing to several pharmacological activities they display.[13] The specific pharmacological and biochemical properties and therapeutic applications of simple coumarins depend on the substituents present on the core scaffold.[13] Among the thousands of various coumarins present, some natural and synthetic versions have been evaluated against a wide range of pharmacological targets of particular interest in medicinal chemistry.[14] Coumarins have been found to act as antioxidants and anti-inflammatory agents,[15] neuroprotective agents,[16,17] antidepressants,[18] anticonvulsants,[19] antibacterials,[20] antivirals,[21] anticancer agents,[22,23] anticoagulants,[24] anti-hypertensives,[25] and enzyme inhibitors,[26–33] among other activities. More recently it was found that simple coumarins, usually substituted at positions 3, 4, 6, 7, or 8, have MAO inhibitory activities, making them an interesting option in the search for new drugs for the treatment of ND.[27–33] In recent years, simple coumarins substituted at positions 3 or 4 have been described to exhibit activity as inhibitors of cholinesterases (ChE), both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). The planarity and aromaticity of these derivatives proved to be essential for activity. Substitution at positions 3 and 4 of the coumarin ring also afforded derivatives found to be β-secretase 1 (BACE-1) inhibitors.[26,29] All these properties encouraged us to study variously substituted coumarins as lead compounds with potential capacities as therapeutics to treat ND. In recent years our research group has been studying 3-substituted coumarins with activity at different targets involved in ND. The introduction of an amide group as a linker between the coumarin skeleton and a phenyl group at position 3 yielded coumarins with dual activity as MAO and ChE inhibitors.[29] The introduction of hydroxy groups enhanced the antioxidant properties of these compounds.[34]

Taking into account the background of our research group (Figure 1)[28–33] and the knowledge that MAO activity generates ROS that cause neuronal cell death, the present work provides an overview on the potential of variously substituted 3-amidocoumarins as multifunctional agents. They are able to selectively inhibit MAO-B, scavenge free radicals and protect neuronal cells from H_2O_2-mediated damage, representing a promising multifunctional scaffold for the development of neuroprotective agents that could delay or slow the progression of ND. Herein we describe our studies of the activities of 3-amidocoumarins with amides introduced at position 3 and a hydroxy group at position 4.

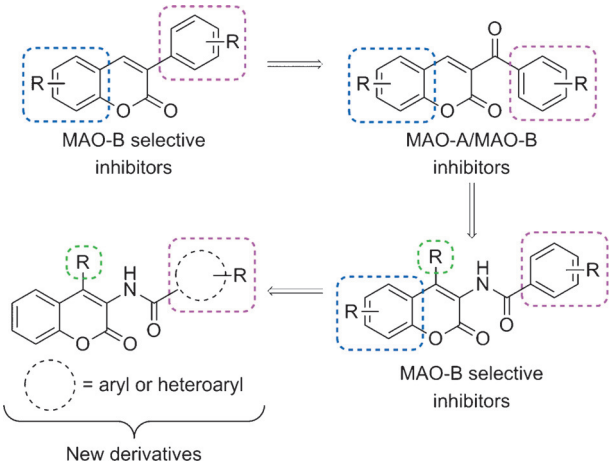


Figure 1. Rationale behind the design of the compounds studied in this work.

Results and Discussion

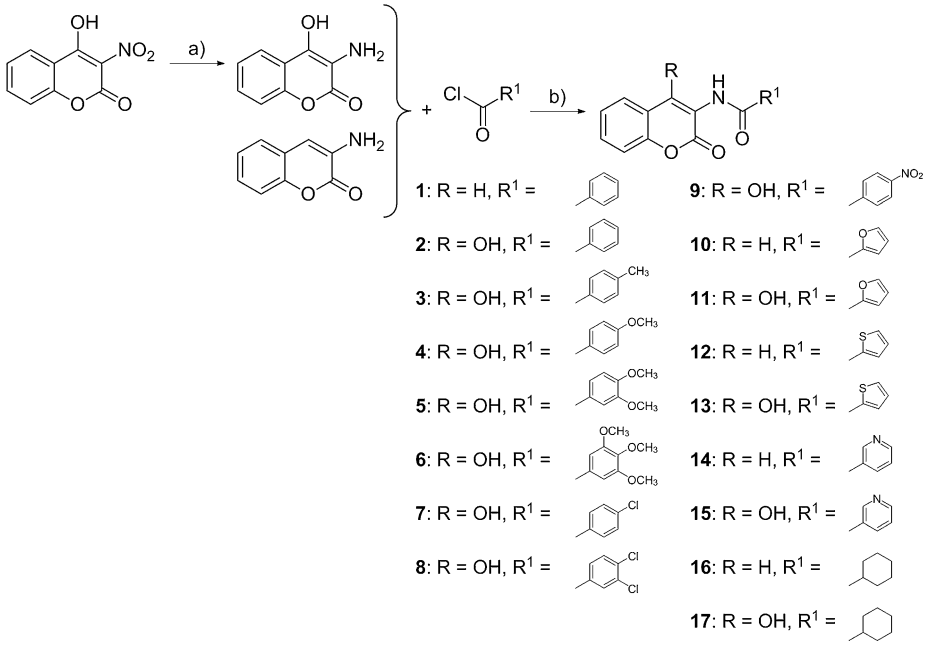
Chemistry

The studied derivatives were efficiently synthesized according to the protocol outlined in Scheme 1. Coumarins **1**–**17** were prepared by starting from commercially available 3-aminocoumarin, or from 3-amino-4-hydroxycoumarin, which was prepared by reduction of the commercially available 3-nitro-4-hydroxycoumarin in ethanol, using palladium on charcoal as a catalyst, under an atmosphere of H_2 for 5 h, with a yield of 90%.[35,36] Acylation of the 3-aminocoumarins with the conveniently substituted acid chloride, using pyridine in dichloromethane, from 0 °C to room temperature, afforded the variously 3-substituted coumarins **1**–**17** in yields between 80 and 90%.[32,37–42] The reaction conditions and characterization of the new compounds are detailed in the Experimental Section below.

Pharmacology

MAO in vitro inhibition

Biological evaluation of the test drugs on human MAO (hMAO) activity was investigated by measuring their effects on the production of H_2O_2 from *p*-tyramine (a common substrate for hMAO-A and hMAO-B), using the Amplex Red MAO assay kit and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for hMAO-A or hMAO-B.[43] The production of H_2O_2 catalyzed by the two MAO isoforms can be detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a non-fluorescent and highly sensitive probe that reacts with H_2O_2 in the presence of horseradish peroxidase to produce a fluorescent product: resorufin. New compounds and reference inhibitors were unable to react directly with the Amplex Red reagent, which indicates that these drugs do not interfere with the measurements. On the other hand, under the experimental conditions, hMAO-A displayed a Michaelis–



Scheme 1. *Reagents and conditions*: a) H_2, EtOH, Pd/C, RT, 5 h; b) pyridine, CH_2Cl_2, 0 °C→RT, overnight.

Menten constant (K_M) equal to 457.17 ± 38.62 μM and a maximum reaction velocity (V_{max}) in the control group of 185.67 ± 12.06 (nmol *p*-tyramine min^{-1}) (mg protein)$^{-1}$, whereas hMAO-B showed a K_M value of 220.33 ± 32.80 μM and a V_{max} of 24.32 ± 1.97 (nmol *p*-tyramine min^{-1}) (mg protein)$^{-1}$ ($n = 5$). Most compounds tested were found to inhibit this enzymatic control activity in a concentration-dependent manner. The experimental IC_{50} results are listed in Table 1.

As shown in Table 1, many of the studied compounds displayed selective inhibitory activity against hMAO-B in the micromolar range, with compound **1** being the most active derivative of the series ($IC_{50} = 0.76$ μM). Regarding the 3-benzamidocoumarins and comparing with previously published results,[29] it was observed that the introduction of a hydroxy group at position 4 of the coumarin scaffold generally resulted in a loss of activity against hMAO-B, with derivatives **2**–**5**, **7**, and **9** lacking this activity. In fact, from this series, only compound **6** presented hMAO-B inhibitory activity ($IC_{50} = 36.91$ μM). An identical response was observed with the 3-substituent as a heteroarylamide group, with a hydroxy group at position 4. In general, a decrease in (in the case of the thiophenyl derivative) or loss of hMAO activity (in the case of the furanyl and pyridyl derivatives) was observed. Moreover, the nature of the heterocycle determined the activity, and compounds with a thiophene ring in their structure (**12** and **13**) proved to be active against hMAO-B (IC_{50}: 2.27 and 15.50 μM, respectively), whereas those with a furan ring (**10** and **11**) lack such activity. Compounds bearing a pyridine ring (**14** and **15**) follow the general trend observed for the hydroxylated compounds. Compound **14**, without a hydroxy group at position 4, displayed activity against hMAO-B ($IC_{50} = 21.11$ μM), whereas compound **15**, with a hydroxy group at position 4, did not. For derivatives with an amide function at position 3 linked to a cyclohexane group (compounds **16** and **17**), the introduction of a hydroxy group at position 4 was found to improve activity against hMAO-B (IC_{50}: 49.96 and 22.47 μM, respectively). This proved to be the only case in which the introduction of a hydroxy group at position 4 led to a slight improvement in activity.

Table 1. In vitro hMAO-A and hMAO-B inhibitory activity of the synthesized derivatives **1**–**17** and reference compounds.

Compd	IC_{50} [μM][a]		SI[b]
	hMAO-A	hMAO-B	
1	inact.[e]	0.76 ± 0.05	> 131.6[d]
6	inact.[e]	36.91 ± 2.48	> 2.7[d]
8	inact.[e]	19.00 ± 1.27	> 5.3[d]
10	inact.[e]	~100[f]	–
11	inact.[e]	~100[f]	–
12	inact.[e]	2.27 ± 0.15	> 44.1[d]
13	inact.[e]	15.50 ± 1.04	> 6.5[d]
14	inact.[e]	21.11 ± 1.42	> 4.7[d]
16	inact.[e]	49.96 ± 3.35	> 2.0[d]
17	inact.[e]	22.47 ± 1.51	> 4.5[d]
selegiline	67.25 ± 1.02[c]	0.019 ± 0.001[3]	3539
rasagiline	16.44 ± 0.85	0.069 ± 0.004	238
isatin	inact.[e]	33.07 ± 1.47	> 3.0

[a] Values are the mean ± SEM of $n = 5$ experiments; compounds **2**–**5**, **7**, **9**, and **15** proved to be inactive toward both MAO-A and MAO-B at the highest concentration tested (100 μM). [b] Selectivity index: MAO-B selectivity ratios [IC_{50}(MAO-A)]/[IC_{50}(MAO-B)] for inhibitory effects of both new compounds and reference inhibitors. [c] $p < 0.01$ regarding the corresponding IC_{50} value obtained against MAO-B, as determined by ANOVA/Dunnett's. [d] Values obtained under the assumption that the corresponding IC_{50} value against MAO-A is > 100 μM. [e] Inactive at 100 μM (highest concentration tested). [f] 100 μM inhibits enzymatic activity by ~50–55%; at higher concentrations the compounds precipitate.

Reversibility

Reversibility experiments were performed to evaluate the type of inhibition effected by derivatives **1**, **12**, **14**, and **17** (Table 2). These compounds were selected based on their structure and activity against hMAO-B. An effective dilution method was used, and selegiline (irreversible inhibitor) and isatin (reversible inhibitor) were taken as standards.[44,45] hMAO-B inhibition was observed to be reversible in the presence of all four of these compounds with their degree of reversibility being lower than that described for isatin (reversible reference compound).

Table 2. Reversibility results of hMAO-B inhibition studied for derivatives **1**, **12**, **14** and **17**, and reference inhibitors.

Compd	Slope (AUF/*t*) [%][a]
1	41.51 ± 2.79
12	63.04 ± 4.23
14	38.43 ± 2.58
17	67.76 ± 4.55
selegiline	3.21 ± 0.21
isatin	88.63 ± 5.94

[a] Values represent the mean ± SEM of $n = 3$ experiments relative to control; data show recovery of hMAO-B activity after dilution.

Neuronal survival

Compounds **1**–**17** were studied in vitro to evaluate their neuroprotective potential along with their MAO activity and to assess their effect on oxidative stress. First, to discard any possible cytotoxic effects of compounds **1**–**17** against rat cortical neurons, cell viability was assessed after 24 h treatment with the new compounds, each at 100 μM. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which detects cellular dehydrogenase activity, was used. Cells that are metabolically impaired are less able to reduce MTT than healthy cells. In comparison with control (1% DMSO), only compounds **10** and **11** were found to induce a significant decrease in cell viability at 100 μM post-treatment, whereas the rest of the test compounds were devoid of any cytotoxic activity (Figure 2A).

The neuroprotective effects of compounds **1**–**17** were then assessed in cultured rat cortical neurons exposed to H_2O_2. Neurons incubated with or without compounds **1**–**17** (100 μM) were exposed to H_2O_2 (30 μM) at the same time point and incubated for 24 h. The toxin-treatment group in the cell viability assays showed a significant difference in the production of toxicity relative to those treated with DMSO alone. The results obtained by studying the possible neuroprotective effects of our new compounds against the effects of H_2O_2 in the cells are shown in Figure 2B.

From the 3-benzamidocoumarins **1**–**9**, the most promising results against the effects of H_2O_2 (Figure 2B) corresponded, in general, to derivatives with a hydroxy group at position 4 of the coumarin scaffold and a single substituent at the *para* position of the benzamide at position 3 (compounds **3**, **7**, and **9**). In the case of 3-heteroarylamido- and 3-cyclohexanecarboxamidocoumarins **10**–**17**, we observed that derivatives with a nicotinamide group at position 3 (compounds **14** and **15**) exerted a statistically significant level of neuroprotection, whereas compounds were found to be inactive against H_2O_2 if the pyridine ring was substituted for cyclohexane, thiophene, or furan. Under these conditions, rasagiline (5 μM) did not display significant neuroprotection. In view of these results it is possible that other mechanisms beyond MAO inhibition may be involved in the neuroprotective activity of these derivatives.

Neuroprotection exerted by these compounds is concentration dependent. Therefore, a decrease in neuroprotection is observed when cultured rat cortical neurons were exposed to H_2O_2 but treated with compounds **1**–**17** at 10 μM. No significant differences were found for neurons treated with any compounds and exposed to H_2O_2 (Table 3).

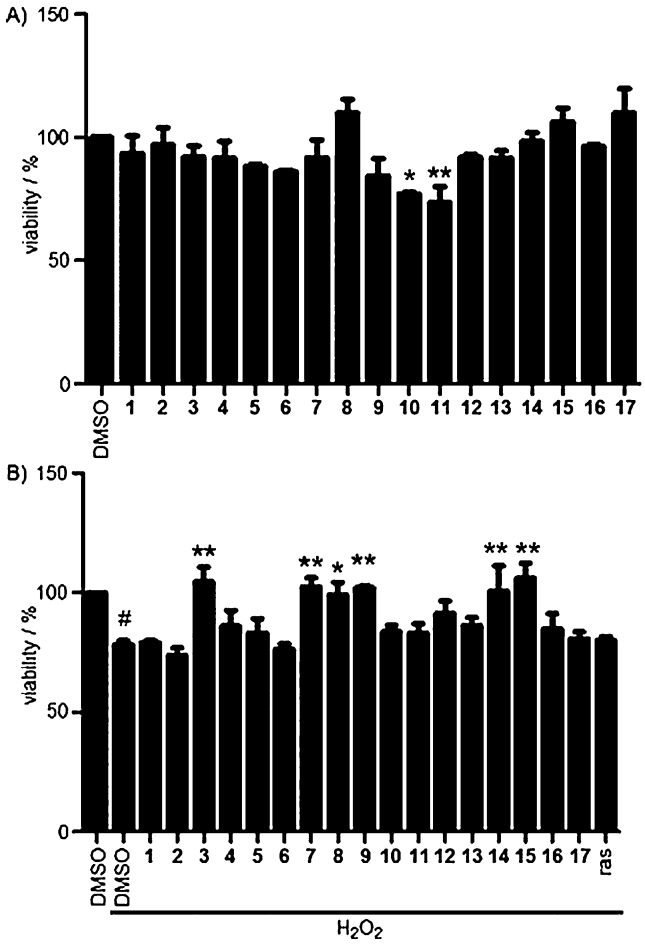


Figure 2. A) Cytotoxicity of compounds **1**–**17** (100 μM) against cortical neurons, and B) neuroprotective effects of compounds **1**–**17** (100 μM) and rasagiline (ras; 5 μM) on cortical neurons treated with H_2O_2 and percentage of living cells in cultures exposed to DMSO/H_2O_2 relative to cells exposed to DMSO alone. Results are expressed as the mean ± SEM of at least five independent experiments; $^{*}p < 0.05$, $^{**}p < 0.001$ versus the corresponding viability obtained in the control group treated with DMSO (panel A) or H_2O_2 in addition to DMSO (panel B); $^{\#}p < 0.001$ versus the group treated only with DMSO.

DPPH· scavenging

Under normal conditions, free radicals are rapidly neutralized in the mitochondria; over time, however, this neutralization becomes less effective, and dysfunction and even cell death occurs. We studied the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·) scavenging activity of those compounds showing the best neuroprotective effects in cells treated with H_2O_2 (compounds **3**, **7**, **9**, **14**, and **15**). As can be seen in Figure 3, most of the studied compounds were able to scavenge free radicals, with compounds **3** and **7** (100 μM) being the most active, showing scavenging activity slightly higher than 50%. These are also two of the best compounds in the series of 3-benzamidocoumarins as neuroprotective agents against H_2O_2. In

Table 3. Cell viability after treatment with compounds **1**–**17** at 10 and 100 μM.

Compd	Viability [%]	
	10 μM	100 μM
$DMSO + H_2O_2$	72.2 ± 1.7	78.3 ± 1.7
1	73.3 ± 3.1[c]	79.1 ± 1.0[c]
2	68.0 ± 2.1[c]	73.6 ± 3.3[c]
3	63.8 ± 2.6[c]	104.4 ± 6.3[b]
4	69.7 ± 3.8[c]	85.9 ± 6.7[c]
5	71.7 ± 4.3[c]	83.2 ± 5.9[c]
6	78.5 ± 6.0[c]	76.3 ± 2.3[c]
7	79.4 ± 6.5[c]	102.4 ± 3.8[b]
8	71.4 ± 7.3[c]	99.1 ± 5.2[a]
9	72.2 ± 4.0[c]	101.9 ± 0.8[b]
10	72.4 ± 6.4[c]	83.6 ± 2.8[c]
11	70.6 ± 7.3[c]	83.1 ± 3.9[c]
12	68.2 ± 3.9[c]	91.3 ± 5.3[c]
13	71.3 ± 4.7[c]	86.3 ± 3.3[c]
14	67.9 ± 6.4[c]	100.7 ± 10.6[b]
15	73.6 ± 6.4[c]	106.1 ± 6.3[b]
16	72.9 ± 0.7[c]	84.9 ± 6.3[c]
17	74.2 ± 3.6[c]	80.7 ± 3.0[c]

[a] $p < 0.05$, [b] $p < 0.001$ versus the corresponding viability obtained in the control group treated with H_2O_2 + DMSO. [c] No significant differences observed with the control group ($DMSO + H_2O_2$).

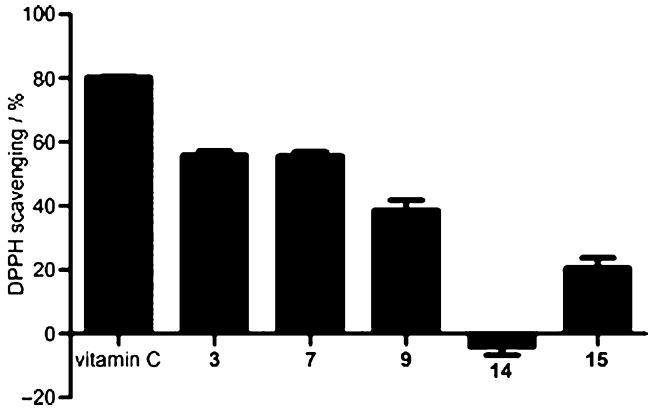


Figure 3. DPPH· scavenging activity of coumarin derivatives **3**, **7**, **9**, **14**, **15**, and vitamin C (positive control).

contrast, for the series of 3-nicotinamidocoumarins, compounds **14** and **15**, only if a hydroxy group is present at position 4 (compound **15**) is free radical scavenging activity observed (~20%). Therefore, the presence of a hydroxy group, in this specific case at position 4, seems to be crucial for neutralization of free radicals by these derivatives.

In vitro blood–brain barrier permeation

A fundamental requirement for any compound to act on neurodegenerative processes is access to brain tissue, that is, to be able to cross the blood–brain barrier (BBB). To examine the capacity of our compounds to pass this barrier, we selected compounds **3**, **12**, **14**, and **15**, some of the most active MAO inhibitors and/or neuroprotective agents, for use in a parallel artificial membrane permeation assay (PAMPA).[46] This is a relatively easy and straightforward method to predict the passive central nervous system (CNS) permeation, which had been previously optimized for application to investigated compounds with limited aqueous solubility.[47–49] Experimental PAMPA results are listed in Table 4.

Table 4. In vitro evaluation of CNS penetration (experimental permeability, P_e) using PAMPA methodology.

Compd	P_e [10^{-6} cm s^{-1}][a]	Prediction
3	7.4 ± 0.01	CNS−
12	44.2 ± 2.1	CNS+
14	20.2 ± 0.4	CNS+
15	9.3 ± 0.1	CNS+/−
verapamil	14.8 ± 0.1	CNS+

[a] Results are the mean of the experimental prediction ± SD of at least three independent experiments.

The capacity of the compounds to pass through a lipid extract of porcine brain were determined using a 70:30 mixture of phosphate-buffered saline solution and ethanol (PBS/EtOH). In each experiment 10 commercial drugs were also evaluated for assay validation. A graphical representation of experimental permeability versus reported values of such well-known drugs gave a linear correlation: $P_e(\text{expt.}) = 0.72\,P_e(\text{lit.}) + 6.70$ ($R^2 = 0.80$). From this equation and taking into account the described limits for BBB permeation, we established that compounds with permeability values $> 9.6 \times 10^{-6}$ cm s^{-1} could penetrate into the CNS by passive diffusion (CNS+), whereas products with $P_e < 8.1 \times 10^{-6}$ cm s^{-1} could not enter (CNS−). Between these values, the CNS permeation was considered to be uncertain (CNS+/−). Therefore, from the selected compounds, compounds **12** and **14** (P_e: 44.2×10^{-6} and 20.2×10^{-6} cm s^{-1}, respectively) would be able to cross the BBB and reach their therapeutic targets. In addition, both compounds showed higher P_e values than verapamil ($P_e = 14.8 \times 10^{-6}$ cm s^{-1}), which is generally used as a standard of high permeability. In the case of compound **15**, passage through the BBB is unlikely ($P_e = 9.3 \times 10^{-6}$ cm s^{-1}).

Theoretical evaluation of ADME-related physicochemical/structural parameters

To better understand the overall properties and the drug-like characteristics of compounds **1**–**17**, the calculated lipophilicity (expressed as the octanol/water partition coefficient, or clog *P*) and theoretical predictions of other ADME properties (molecular weight, topological polar surface area (TPSA), number of hydrogen bond donors and acceptors, and molecular volume) were carried out with Molinspiration software, and are presented in the Supporting Information.[50,51] It is significant that all the described derivatives possess clog *P* values compatible with those required to cross membranes. Although the TPSA (described to be a predictive indicator of membrane penetration) of selegiline differs greatly from the TPSA values determined for the studied compounds, all other predicted ADME

values were found to fall in the desirable range. In addition, we observed no violations of Lipinski's rules (re. molecular weight, log *P*, number of hydrogen bond donors and acceptors). The studied compounds, as MAO inhibitors and neuroprotective agents, must pass various membranes to reach the CNS. The obtained information supports the potential of these derivatives as viable drug candidates. The theoretical information obtained is partially in accordance with results of the experimental in vitro BBB permeation assay. The combination of both experimental results and calculated values can aid in improving our understanding of the drug likeness of this compound series.

Conclusions

In this study, a general and efficient synthesis of a new series of 3-amidocoumarins was developed, using an amidation reaction as a key step. Determination of hMAO isoform activity was carried out, and many of the compounds exhibited selectivity for the hMAO-B isoform with activity in the nanomolar (compound **1**) or micromolar ranges (compounds **6**, **8**, **12**–**14**, **16**, and **17**). Neuroprotective effects against H_2O_2 were also studied. For the series of 3-benzamidocoumarins, the most promising results in cells treated with H_2O_2 generally corresponded to derivatives with a substituent at the *para* position of the benzamide ring, in addition to a hydroxy group at position 4 of the coumarin scaffold (compounds **3**, **7**, and **9**). For the series of 3-heteroarylamidocoumarins, derivatives with a nicotinamide group at position 3 of the coumarin scaffold (compounds **14** and **15**) exerted the most notable neuroprotection. Most of the selected derivatives exerting neuroprotection also showed DPPH· scavenging activity (with the exception of compound **14**, with no hydroxy groups on its structure). Additionally, the prediction of BBB accessibility by PAMPA showed the potential of this compound class to cross the BBB and to thus exert activity in the CNS. From the four compounds studied in the PAMPA, those without a hydroxy group at position 4 proved to have a greater capacity to cross the BBB (compounds **12** and **14**). Compound **15** was found partially able to cross biological barriers. In addition, prediction of ADME-related physicochemical/structural parameters provided a preliminary indication of the potential of this family of compounds as suitable molecules for further development. These results encourage us to further explore the potential of members of this chemical family as potential drug candidates for the treatment of Parkinson's disease.

Experimental Section

Chemistry

Starting materials and reagents were obtained from commercial suppliers and were used without further purification (Sigma–Aldrich). Melting points (mp) are uncorrected and were determined with a Reichert Kofler thermopan or in capillary tubes in a Büchi 510 apparatus. ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorded with a Bruker AMX spectrometer using $CDCl_3$ or [D_6]DMSO as solvent. Chemical shifts (δ) are expressed in ppm using TMS as an internal standard. Coupling constants (*J*) are expressed in Hz. Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), td (triplet of doublets), and m (multiplet). Mass spectrometry was carried out with a Hewlett–Packard 5972 MSD spectrometer. Elemental analyses were performed with a PerkinElmer 240B microanalyzer and are within 0.4% of calculated values in all cases. Flash chromatography (FC) was performed on silica gel (Merck 60, 230–400 mesh); analytical TLC was performed on pre-coated silica gel plates (Merck 60 F_{254}). Organic solutions were dried over anhydrous Na_2SO_4. Concentration and evaporation of the solvent after reaction or extraction was carried out on a rotary evaporator (Büchi Rotavapor) operating at reduced pressure. The analytical results showed >95% purity for all compounds.

Preparation of the precursor 3-amino-4-hydroxycoumarin. The commercially available 4-hydroxy-3-nitrocoumarin (2.5 mmol) was dissolved in EtOH, and a catalytic amount of Pd/C was added to the mixture. The solution was stirred at room temperature under H_2 atmosphere for 5 h. After completion of the reaction, the mixture was filtered to remove the catalyst. The obtained crude product was then purified by FC (hexane/EtOAc, 9:1) to give the desired coumarin in 90% yield.

General procedure for the preparation of 3-amidocoumarins 1–17. The 3-aminocoumarin (commercially available) or the 3-amino-4-hydroxycoumarin (1 mmol) was dissolved in CH_2Cl_2 (9 mL). Pyridine (1.1 mmol) was then added, and the mixture was cooled to 0 °C. Variously substituted acid 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 compounds **1**–**17**.

***N*-(4-Hydroxycoumarin-3-yl)-4′-methylbenzamide (3)**: white solid (83% yield); R_f = 0.26 (9:1, hexane/EtOAc); mp: 210–211 °C; ^{1}H NMR ([D_6]DMSO): δ = 2.49 (s, 3H, CH_3), 7.31 (s, 1H, H-4), 7.37–7.44 (m, 4H, H-6, H-8, H-3′, H-5′) 7.64–7.70 (m, 1H, H-7), 7.89–7.92 (m, 3H, H5, H-2′, H-6′), 9.47 (s, 1H, NH), 12.13 ppm (s, 1H, OH); ^{13}C NMR ([D_6]DMSO): δ = 21.0, 113.1, 116.2, 116.3, 123.7, 124.3, 128.1, 128.8, 130.9, 132.4, 141.7, 151.6, 159.3, 160.4, 166.4 ppm; MS *m/z* (%): 296 (6), 295 ([M^+], 29), 119 (100), 91 (30), 65 (10); Anal. calcd for $C_{17}H_{13}NO_4$: C 69.15, H 4.44, N 4.74, O 21.67, found: C 69.12, H 4.42, N 4.77.

***N*-(4-Hydroxycoumarin-3-yl)-3′,4′-dimethoxybenzamide (5)**: white solid (88% yield); R_f = 0.25 (9:1, hexane/EtOAc); mp: 247–248 °C; ^{1}H NMR ([D_6]DMSO): δ = 3.82 (s, 6H, $(CH_3)_2$), 7.07 (d, *J* = 8.3 Hz, 1H, H-5′), 7.40–7.45 (m, 2H, H-6, H-8) 7.59–7.69 (m, 3H, H-2′, H-6′, H-7), 7.90 (d, *J* = 7.8 Hz, 1H, H-5), 9.44 (s, 1H, NH), 12.20 ppm (s, 1H, OH); ^{13}C NMR ([D_6]DMSO): δ = 56.3, 103.9, 111.5, 112.0, 116.9, 122.3, 124.3, 125.0, 126.6, 133.0, 148.8, 152.2, 152.4, 159.9, 161.1, 166.8; DEPT ^{13}C NMR ([D_6]DMSO): δ = 56.3, 111.5, 112.0, 116.9, 122.3, 124.3, 125.0, 133.0 ppm; MS *m/z* (%): 342 (6), 341 ([M^+], 15), 323 (6), 165 (100), 121 (6), 92 (7), 77 (9); Anal. calcd for $C_{18}H_{15}NO_6$: C 63.34, H 4.43, N 4.10, O 28.12, found: C 63.31, H 4.41, N 4.12.

***N*-(4-Hydroxycoumarin-3-yl)-3′,4′-dichlorobenzamide (8)**: white solid (86% yield); R_f = 0.22 (9:1, hexane/EtOAc); mp: 284–285 °C; ^{1}H NMR ([D_6]DMSO): δ = 7.37–7.44 (m, 2H, H-6, H-8), 7.64–7.70 (m, 1H, H-5′) 7.79–7.96 (m, 3H, H-6′, H-5, H-7), 8.24 (s, 1H, H-2′), 9.78 (s, 1H, NH), 12.24 ppm (s, 1H, OH); ^{13}C NMR ([D_6]DMSO): δ = 87.0, 116.5, 123.8, 123.9, 124.0, 124.5, 128.4, 130.1, 130.8, 131.3, 132.7, 134.5, 150.5, 151.5, 160.1, 163.2 ppm; MS *m/z* (%): 351 (54), 350 ([M^+], 15), 349 (84), 333 (22), 331 (34), 175 (100), 174 (94), 147 (24), 145 (36), 121 (19), 111 (14), 109 (14), 85 (17), 71 (19), 69 (14), 65

(14), 57 (19); Anal. calcd for $C_{16}H_9Cl_2NO_4$: C 54.88, H 2.59, Cl 20.25, N 4.00, O 18.28, found: C 54.85, H 2.55, Cl 20.28, N 3.99.

***N*-(Coumarin-3-yl)furan-2-carboxamide (10)**: white solid (90% yield); $R_f = 0.42$ (9:1, hexane/EtOAc); mp: 183–184 °C; ^{1}H NMR ($CDCl_3$): $\delta = 6.74$ (dd, $J = 3.6$, 1.8 Hz, 1H, H-4′), 7.34–7.58 (m, 4H, H-5, H-6, H-8, H-5′), 7.77 (td, $J = 8.0$, 1.4 Hz, 1H, H-7), 8.00 (dd, $J = 1.8$, 0.8 hz, 1H, H-3′), 8.58 (s, 1H, H-4), 9.26 ppm (s, 1H, NH); ^{13}C NMR ($CDCl_3$): $\delta = 112.1$, 113.2, 115.7, 116.8, 122.9, 124.3, 126.2, 126.8, 127.7, 145.8, 146.5, 149.5, 155.3, 159.8 ppm; MS *m/z* (%): 256 (16), 255 ([M^+], 79), 227 (7), 132 (6), 95 (100), 77 (10); Anal. calcd for $C_{14}H_9NO_4$: C 65.88, H 3.55, N 5.49, O 25.07, found: C 65.87, H 3.56, N 5.52.

***N*-(4-Hydroxycoumarin-3-yl)cyclohexanecarboxamide (17)**: white solid (91% yield); $R_f = 0.27$ (9:1, hexane/EtOAc); mp: 199–200 °C; ^{1}H NMR ($CDCl_3$): $\delta = 1.17$–1.69 (m, 6H, $(CH_2)_3$), 1.75–2.06 (m, 4H, $(CH_2)_2$), 2.30–2.55 (m, 1H, CH), 7.30–7.40 (m, 2H, H-6, H-8), 7.56 (td, $J = 7.8$, 1.7 Hz, 1H, H-7), 8.01 (dd, $J = 7.9$, 1.7 Hz, 1H, H-5), 8.28 (s, 1H, NH), 13.87 ppm (s, 1H, OH); ^{13}C NMR ($CDCl_3$): $\delta = 25.4$, 25.7, 29.7, 45.5, 104.7, 116.2, 117.2, 124.4, 124.7, 131.6, 150.5, 152.8, 161.2, 177.6 ppm; MS *m/z* (%): 288 (5), 287 ([M^+], 25), 177 (54), 121 (18), 111 (22), 83 (100), 55 (43); Anal. calcd for $C_{16}H_{17}NO_4$: C 66.89, H 5.96, N 4.88, O 22.27, found: C 66.91, H 5.99, N 4.85.

Pharmacological assays

Activity against MAO isoforms: The tested compounds were dissolved in DMSO (Sigma–Aldrich, Alcobendas, Madrid, Spain) to prepare 10 mM stock solutions, which were kept in storage at −20 °C. Percentage of DMSO used in the experiments was never >1%. Selegiline and rasagiline, used as reference inhibitors, were acquired from Sigma–Aldrich (Alcobendas). Human recombinant MAO isoforms, used in the experiments, were also purchased from Sigma–Aldrich (Alcobendas). Resorufin sodium salt, *p*-tyramine hydrochloride, sodium phosphate buffer, horseradish peroxidase and Amplex Red reagent were supplied in the Amplex Red MAO assay kit (Molecular Probes Inc., Eugene, OR, USA). Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4) containing various concentrations of the test drugs (new compounds or reference inhibitors) and adequate amounts of recombinant hMAO-A or hMAO-B required and adjusted to obtain the same reaction velocity under our experimental conditions, i.e., to oxidize (in the control group) the same concentration of substrate: 165 pmol of *p*-tyramine per min (hMAO-A: 1.1 μg protein; specific activity: 150 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde per min per mg protein; hMAO-B: 7.5 μg protein; specific activity: 22 nmol of *p*-tyramine transformed per min per mg protein) were incubated for 15 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 (final concentrations) 200 μM Amplex Red reagent, 1 U mL^{-1} horseradish peroxidase, and 1 mM *p*-tyramine. The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in a multi-detection microplate fluorescence reader (FLX800, Bio-Tek Instruments Inc., Winooski, VT, USA) based on the fluorescence generated (λ_{ex} 545 nm, λ_{em} 590 nm) over a 15 min period, in which the fluorescence increased linearly.[43] Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the fluorescence generated in the reaction mixture due to non-enzymatic inhibition (e.g., by direct reaction with the Amplex Red reagent) was determined by adding these drugs to solutions containing only the Amplex Red reagent in a sodium phosphate buffer. To determine the kinetic parameters of hMAO-A and hMAO-B (K_M and V_{max}), the corresponding activity of both isoforms was evaluated (under the experimental conditions described above) in the presence of a wide range of *p*-tyramine concentrations. 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 hMAO isoforms, which were replaced by a sodium phosphate buffer solution. Under our experimental conditions, this background activity was practically negligible. MAO activity of the test compounds and reference inhibitors is expressed as IC_{50}, i.e., the concentration of each drug required to produce a 50% decrease in control value activity for MAO isoforms.

Determination of inhibition mode: To evaluate whether compounds **1**, **12**, **14**, and **17** are reversible or irreversible hMAO-B inhibitors, a dilution method was used.[44] A 100× concentration of the enzyme used in the above-described experiments was incubated with a concentration of inhibitor equivalent to 10-fold its IC_{50} value. After 30 min, the mixture was diluted 100-fold into reaction buffer containing Amplex Red reagent, horseradish peroxidase, and *p*-tyramine, and the reaction was monitored for 15 min. 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. Control tests were carried out by pre-incubating and diluting the enzyme in the absence of inhibitor.

Neuroprotective study: DMSO, phosphate-buffered saline (PBS, pH 7.4), Hanks buffer, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and H_2O_2 were acquired from Sigma–Aldrich (Alcobendas). Poly-D-lysine, neurobasal medium, L-glutamine, B-27, and fetal bovine serum (FBS) were acquired from Gibco/Invitrogen S.A., Barcelona, Spain.

Primary culture of neurons and glia: Pregnant rats (19–20 days) were euthanized by CO_2 inhalation, and embryos were immediately extracted from the womb by caesarean section, and their brains were carefully dissected out. Meninges were removed, and a portion of motor cortex was isolated after dissection of the brain.[26] Fragments obtained from several embryos were subjected to mechanic disintegration. Neurobasal medium supplemented with 2% B-27 (for cortical neurons) was used to seed the cells in 96-well plates at a density of 100 000 cells per mL. Neuronal cultures were allowed to grow for 8–10 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. Experiments were conducted on female Wistar Kyoto (WKY) rats, obtained from the rat colony maintained at the animal facilities of our department. Rats were housed, cared for, and acclimatized (before the experiments). All experiments were carried out in accordance with European regulations on the protection of animals (Directive 2010/63/UE), the Spanish Real Decreto 53/2013 (1.February) and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the USA.

Determination of neuronal survival: Neuronal cultures were treated with the compounds in the study at 100 μM (final DMSO concentration ≤1%) or with studied compounds and H_2O_2 (30 μM) over an incubation period of 24 h. H_2O_2 was used as reference neurotoxic agent for neurons. Cell viability was determined to gauge the possible cytotoxicity of new compounds or their neuroprotective effects against a pro-oxidant (H_2O_2) agent, by reducing MTT to formazan via the mitochondria of viable cells. MTT (5 mg mL^{-1} in

Hanks buffer) was added to each well to a final concentration of 10%.[52] After incubating for 2 h at 37 °C, the medium was removed, and formazan crystals formed were suspended in DMSO (100 μL well^{-1}). The production of formazan by viable cells was quantified at 37 °C in an absorbance reader (Fluo-star Optima, BMG LABTECH, Offenburg, Germany) by measuring the absorbance at λ 570 nm).[53,54] In addition, the possible capacity of the above test drugs to modify the absorbance generated by reaction with MTT was determined by adding these drugs to solutions containing only MTT reagent in neurobasal medium.

Neutralization of free radicals: DMSO, DPPH·, L-ascorbic acid (vitamin C), and EtOH were acquired from Sigma–Aldrich (Alcobendas). The DPPH· radical scavenging activity of each compound was determined as previously described, with minor modifications. The DPPH· radical was dissolved in EtOH (100 μM) and 99 μL of the solutions were transferred to each well of a 96-well microplate. Compounds **3**, **7**, **9**, **14**, and **15** (1 μL, 100 μM, final concentration) in EtOH were added to each well of a 96-well microplate, and the mixtures were incubated at room temperature for 30 min. Vitamin C (100 μM) was used as a positive control in the experiments. The absorbance at 540 nm was measured using a microplate reader. The radical-scavenging activity of each compound was estimated by comparing the DPPH· absorbance value in the antioxidant–radical reaction mixture after subtraction of the background activity.[55–57]

In vitro blood–brain barrier permeation assay: Hydrocortisone, desipramine, promazine, aldosterone, caffeine, ofloxacin, corticosterone, imipramine, testosterone, verapamil, piroxicam, lipid pig brain, PBS (pH 7.4), and dodecane were purchased from Sigma–Aldrich (Alcobendas) and Acros (Madrid, Spain). Prediction of the brain penetration was evaluated using a PAMPA BBB assay, in a manner similar to that described previously.[46–49] Pipetting was performed with a semiautomatic pipetter (CyBi-SELMA), and UV reading with a microplate spectrophotometer (Multiskan Spectrum, Thermo Electron Co.). Millex filter units (PVDF membrane, diameter 25 mm, pore size 0.45 μm) were acquired from Millipore. The porcine brain lipid (PBL) was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μm), and the acceptor microplate was an indented 96-well plate, both from Millipore. The acceptor 96-well microplate was filled with 200 μL PBS/EtOH (70:30), and the filter surface of the donor microplate was impregnated with 4 mL PBL in dodecane (20 mg mL^{-1}). Compounds were dissolved in PBS/EtOH (70:30) at 10 μg mL^{-1}, filtered through a Millex filter, and then added to the donor wells (200 μL). The donor filter plate was carefully placed on the acceptor plate to form a sandwich, which was left undisturbed for 240 min at 25 °C. After incubation, the donor plate was carefully removed, and the concentration of compounds in the acceptor wells was determined by UV/Vis spectroscopy. Every sample was analyzed at five wavelengths, in four wells and in at least three independent runs, and the results are given as the mean ± standard deviation. In each experiment, 10 quality control standards of known BBB permeability were included to validate the analysis set.

Theoretical evaluation of ADME properties: The absorption, distribution, metabolism, and excretion (ADME) properties of the studied compounds were calculated using the Molinspiration software package. Log *P* values were calculated using the methodology developed by Molinspiration as a sum of fragment-based contributions and correction factors.[50] TPSA was calculated based on the methodology published by Ertl et al. as a sum of fragment contributions.[58] Oxygen and nitrogen-centered polar fragments were considered.[50] PSA has been shown to be a very good descriptor for characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability, and blood–brain barrier penetration. The method for calculation of molecular volume developed at Molinspiration is based on group contributions. These were obtained by fitting the sum of fragment contributions to 'real' three-dimensional (3D) volume for a training set of ~12000 mostly drug-like molecules; 3D molecular geometries for a training set were fully optimized by the semi-empirical AM1 method.

Statistics: Results are expressed as the mean of at least three experiments ± SEM or ± SD for blood–brain barrier permeation assay. Statistically significant differences between two measurements ($P < 0.05$, $P < 0.01$, or $P < 0.001$) were determined by analysis of variance (ANOVA) followed by the multiple comparison Dunnett's test. Graphical representation, statistical analysis, and calculation of IC_{50} values were performed with GraphPad Prism software (ver. 4.03, San Diego, CA, USA).

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