3-Amidocoumarins as Potential Multifunctional Agents Against Neurodegenerative Diseases

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Abstract: The present work provides an overview about the potentiality of differently substituted 3-amidocoumarins in the neurodegenerative diseases. The inhibitory activity of MAOs, as well as the reversibility of the inhibition, the neuroprotective effects on neuronal cells against H₂O₂, the antioxidant effect measured by DPPH free radical scavenging of 3-amidocoumarins, and the cross through blood-brain barrier for the most potent derivatives, have been evaluated. Many of these derivatives proved to be capable of selectively inhibiting the MAO-B isoenzyme. Substitution at position 4 with a hydroxyl group leads to a loss of activity of the 3-benzamidocoumarins against this
enzyme, but favours their neuroprotective activity against H₂O₂. Regarding the 3-heteroarylamide derivatives, it was the nature of the heterocycle that determined its neuroprotective effects. Although none of the studied derivatives violated the theoretical Lipinski's rules, the parallel artificial membrane permeation assay showed that not every compound could efficiently cross the blood-brain barrier. However, this new scaffold presented, in general, desirable properties for the development of potential drug candidates against neurodegenerative diseases.

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). Their chronic course produces a gradual but steady deterioration whose last step is death. Because of this, as the disease progresses, it is eroding the quality of life of patients. Therefore, the development of effective neuroprotective therapies that slow down or stop disease's progression in the earliest stages is one of the main goals of the researchers in this area.

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 or to enhancement of iron deposition in the SNpc. The failure of normal cellular processes that occur in relation to the aging are also believed to contribute to the increased vulnerability of dopaminergic (DA) neurons. Although the precise mechanism corresponding to ROS generation related to PD is still unknown, the major sources of oxidative stress generated by the DA neurons are DA metabolism, mitochondrial dysfunction, and neuroinflammation.

Metabolism of DA by monoamine oxidase (MAO) yields hydrogen peroxide (H₂O₂), an oxygen radical that leads to cytotoxicity through peroxidation of lipid membranes. Selegiline and rasagiline, two selective MAO-B inhibitors are currently used to retard the symptoms in PD because they increase the dopamine levels and may exert neuroprotective effects. Inhibition of MAO-B reduces oxygen radical generation although new neuroprotective functions independently of inhibiting MAO activity have been reported for these drugs. 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 substantia nigra. Accordingly, antioxidants
that scavenge free radicals and reactive species such as tocopherol and ascorbate may have beneficial therapeutic effects in PD by preventing the onset of apoptotic cell death and 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 the current therapies.

Since the beginning of medicine, the nature has played a key role as source of inspiration in the development of drugs with important biological activities. Coumarins (2H-1-benzopyran-2-one), common metabolites in plants (also detected in microorganisms and animal sources),\[12\] have shown great interest, in particular due to several pharmacological activities that they may display.\[13\] The pharmacological and biochemical properties, and therapeutic applications of simple coumarins depend on the various substitutions of the scaffold.\[13\] Of thousands of different coumarins currently existent, some natural or obtained by chemical synthesis have been evaluated in many different pharmacological targets of great interest in the field of medicinal chemistry.\[14\] Therefore, there are coumarins as antioxidants and anti-inflammatories,\[15\] neuroprotective agents,\[16,17\] antidepressants,\[18\] anticonvulsants,\[19\] antibacterials,\[20\] antivirals,\[21\] anticancer agents,\[22,23\] anticoagulants,\[24\] antihypertensives\[25\] and enzyme inhibitors,\[26-33\] among others. More recently it has been found that simple coumarins, usually substituted at positions 3, 4, 6, 7 or 8 have properties such as MAO inhibitors, which makes 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 by exhibiting activity as inhibitors of cholinesterases (ChE), both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). The planarity and aromaticity of these derivatives proved to be essential for the activity. The substitution at these positions of the coumarin ring also allowed interesting derivatives resulting $\beta$-secretase 1 (BACE-1) inhibitors.\[26,29\] All these properties encouraged our group to study differently substituted coumarins as lead compounds with potential interest for ND’s therapeutics. Therefore, our research group has been working, in recent years, in the study of 3-substituted coumarins with activity on different targets involved in ND. The introduction of an amide group as a linker between the coumarin skeleton and a phenyl at position 3 has allowed the obtaining of coumarins with dual activity as MAO and ChE inhibitors.\[29\] The introduction of hydroxyl groups in the molecule enhanced their antioxidant properties.\[34\]
Taking into account the background of our research group (Figure 1), the present work provides an overview about the potential of differently substituted 3-amidocoumarins as inhibitors of MAO, antioxidants and neuroprotective agents. The influence on the activity of the different nature of the amides introduced at position 3 and the introduction of a hydroxyl group at position 4 of the substituted 3-amidocoumarins, is herein studied.

Results and Discussion

Chemistry

The described derivatives were efficiently synthesized according to the protocol outlined in Scheme 1. Coumarins 1-17 were prepared starting from the 3-aminocoumarin, commercially available, or from the 3-amino-4-hydroxycoumarin, which was prepared through the reduction of the commercially available 3-nitro-4-
hydroxycoumarin, in ethanol, with Pd/C as catalyst, in H₂ atmosphere, for 5 hours, with a yield of 90%. An acylation of the 3-aminocoumarins with the conveniently substituted acid chloride, using pyridine in dichloromethane, from 0 °C to room temperature, afforded the differently 3-substituted coumarins (1-17) in yields between 80 and 90%. The reaction conditions and chemical characterization of the new compounds are detailed in the experimental section.

Scheme 1. Reagents and conditions: (a) H₂, EtOH, Pd/C, r.t., 5 h; (b) pyridine, dichloromethane, 0 °C to r.t., overnight.

Pharmacology
MAO in vitro inhibition

The biological evaluation of the test drugs on hMAO activity was investigated by measuring their effects on the production of H₂O₂ 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. The production of H₂O₂ catalysed 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 H₂O₂ 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, in the experiments and under the experimental conditions, hMAO-A displayed a Michaelis constant ($K_m$) equal to $457.17 \pm 38.62 \mu M$ and a maximum reaction velocity ($V_{max}$) in the control group of $185.67 \pm 12.06 \text{ (nmol p-tyramine/min)/mg protein}$, whereas hMAO-B showed a $K_m$ of $220.33 \pm 32.80 \mu M$ and $V_{max}$ of $24.32 \pm 1.97 \text{ (nmol p-tyramine/min)/mg protein}$ ($n = 5$). Most tested compounds concentration-dependently inhibited this enzymatic control activity. The experimental IC$_{50}$ results are expressed in Table 1.

**Table 1.** In vitro hMAO-A and hMAO-B inhibitory activity of the synthesized derivatives 1-17 and reference compound.[a]

<table>
<thead>
<tr>
<th>Comp.</th>
<th>IC$_{50}$ hMAO-A (µM)</th>
<th>IC$_{50}$ hMAO-B (µM)</th>
<th>S. I.[b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>*</td>
<td>0.76 ± 0.05</td>
<td>&gt; 131.6[d]</td>
</tr>
<tr>
<td>2</td>
<td>*</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>*</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>*</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>*</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>*</td>
<td>36.91 ± 2.48</td>
<td>&gt; 2.7[d]</td>
</tr>
<tr>
<td>7</td>
<td>*</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>*</td>
<td>19.00 ± 1.27</td>
<td>&gt; 5.3[d]</td>
</tr>
<tr>
<td>9</td>
<td>*</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>*</td>
<td>**</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>*</td>
<td>**</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>*</td>
<td>2.27 ± 0.15</td>
<td>&gt; 44.1[g]</td>
</tr>
<tr>
<td>13</td>
<td>*</td>
<td>15.50 ± 1.04</td>
<td>&gt; 6.5[d]</td>
</tr>
<tr>
<td>14</td>
<td>*</td>
<td>21.11 ± 1.42</td>
<td>&gt; 4.7[d]</td>
</tr>
<tr>
<td>15</td>
<td>*</td>
<td>*</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>*</td>
<td>49.96 ± 3.35</td>
<td>&gt; 2.0[d]</td>
</tr>
<tr>
<td>17</td>
<td>*</td>
<td>22.47 ± 1.51</td>
<td>&gt; 4.5[d]</td>
</tr>
<tr>
<td>Selegiline</td>
<td>67.25 ± 1.02[c]</td>
<td>0.019 ± 0.001[^3]</td>
<td>3,539</td>
</tr>
<tr>
<td>Rasagiline</td>
<td>16.44±0.85</td>
<td>0.069 ± 0.004</td>
<td>238</td>
</tr>
<tr>
<td>Isatin</td>
<td>*</td>
<td>33.07 ± 1.47</td>
<td>&gt; 3.0</td>
</tr>
</tbody>
</table>

[a] Each IC$_{50}$ value is the mean ± S.E.M. from five experiments ($n = 5$). [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$_{50}$ obtained against MAO-B as determined by ANOVA/Dunnett’s. [d] Values obtained
under the assumption that the corresponding IC$_{50}$ against either MAO-A or MAO-B is major than 100 µM. * Inactive at 100 µM (highest concentration tested). ** 100 µM inhibits enzymatic activity by approximately 50-55%. At higher concentrations the compounds precipitate.
As shown in Table 1, many of the studied compounds displayed selective inhibitory activity against hMAO-B in the micromolar range, being the compound 1 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, in general, the introduction of a hydroxyl group at position 4 of the coumarin scaffold resulted in a loss of activity against hMAO-B, lacking this activity the derivatives 2-5, 7 and 9. In fact, from this series, only compound 6 presented hMAO-B inhibitory activity (IC$_{50}$ = 36.91 µM). Identical response was observed when the 3-substituent is a heteroarylamide group, with hydroxyl group at position 4. In general, it was produced a decrease (in the case of the thiophenyl derivative) or loss of hMAO activity (in the case of the furanyl and pyridyl derivatives). Moreover, the nature of the heterocycle determined the activity, and the compounds having a thiophene ring on their structure (compounds 12 and 13) proved to be active against hMAO-B (IC$_{50}$ = 2.27 µM and IC$_{50}$ = 15.50 µM, respectively), whereas those with a furan ring on it (compounds 10 and 11) lack this activity. Compounds bearing a pyridine ring on their structure (compounds 14 and 15), follow the general rule of the hydroxylated compounds. Compound 14, without hydroxyl group at position 4, proved to display activity against hMAO-B (IC$_{50}$ = 21.11 µM) while compound 15, with a hydroxyl group at position 4, did not. For derivatives with the amide group at position 3 linked to a cyclohexane group (compounds 16 and 17) it was observed that the introduction of the hydroxyl at position 4 improved the activity against hMAO-B (IC$_{50}$ = 49.96 µM and IC$_{50}$ = 22.47 µM, respectively). This proved to be the only case in which the introduction of a hydroxyl group at position 4 has led to a slightly improvement of the activity.

**Reversibility assay**

Reversibility experiments were performed to evaluate the type of inhibition of 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 are standards.$^{[44,45]}$
Table 2. Reversibility results of hMAO-B inhibition studied for derivatives 1, 12, 14 and 17, and reference inhibitors.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Slope (AUF/t) [%]&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41.511 ± 2.785</td>
</tr>
<tr>
<td>12</td>
<td>63.036 ± 4.229</td>
</tr>
<tr>
<td>14</td>
<td>38.429 ± 2.578</td>
</tr>
<tr>
<td>17</td>
<td>67.759 ± 4.545</td>
</tr>
<tr>
<td>Selegiline</td>
<td>3.208 ± 0.212</td>
</tr>
<tr>
<td>Isatin</td>
<td>88.634 ± 5.946</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage values represent the mean ± SEM of three experiments (n = 3) relative to control; data show recovery of hMAO-B activity after dilution.

hMAO-B inhibition was proved to be reversible in the presence of compounds 1, 12, 14 and 17, being their degree of reversibility lower than that described for isatin (reversible reference compound).

**Neuronal survival**

Compounds 1-17 were studied by *in vitro* test with the goal of evaluating their neuroprotective potential in addition to their MAO activity and assessed their effect on the oxidative stress. Firstly, in order to discard a possible cytotoxic effect of compounds 1-17 against rat cortical neurons, the cell viability was assessed at 24 h after treatment with 100 µM concentration of the new compounds. MTT test, which determines the cells dehydrogenase enzyme activity, was used. Hence, cells that are metabolically impaired reduce less MTT than “healthy cells”. When compared to the control (1% DMSO) only compounds 10 and 11 induced a significant reduction in viability at 100 µM post-treatment, while the rest of the compounds were void of any cytotoxicity (graphic 1).
Graphic 1. Cytotoxicity of compounds 1-17 (100 µM) on cortical neurons treated. Results are expressed as mean ± S.E.M from at least 5 experiments. * P <0.05, ** P <0.001 versus the corresponding viability obtained in the control group treated with DMSO.

Then, the neuroprotective effect of compounds 1-17 was assessed in cultured rat cortical neurons exposed to H₂O₂. Neurons incubated or not with compounds 1-17 (100 µM), were exposed to H₂O₂ (30 µM) at the same time point and incubated for 24 hours. The toxin treatment group in the cell viability assays showed a significant difference in production of toxicity compared to those treated only with DMSO.

The results obtained by studying the possible neuroprotective effects of our molecules against the effects of H₂O₂ in the cells are shown in graphic 2.

From the 3-benzamidocoumarins 1-9, the most promising results against the effects of H₂O₂ (graphic 2) corresponded, in general, to the derivatives presenting an hydroxyl group at position 4 of the coumarin scaffold and a single substituent in para position of the benzamide at position 3 (compounds 3, 7 and 9). In the case of 3-heteroarylamido- and 3-cyclohexanecarboxamidocoumarins 10-17 it was shown that the derivatives having a nicotinamide group at position 3 of the coumarin scaffold (compounds 14 and 15) exerted a statistically significant neuroprotection, whereas when the pyridine ring was substituted for a cyclohexane, thiophene or furan group, the compounds proved to be inactive against H₂O₂. Under these conditions, rasagiline (5µM) 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.
Graphic 2. Neuroprotective effects of compounds 1-17 (100 µM) and rasagiline (5 µM) on cortical neurons treated with H₂O₂. Results are expressed as mean ± S.E.M from at least 5 experiments. # P <0.05 versus the group treated only with DMSO. * P <0.05, ** P <0.001 versus the corresponding viability obtained in the control group treated with H₂O₂ in addition to DMSO.

Neuroprotection exerted by these compounds is concentration dependent. Therefore a decrease in the neuroprotection is observed when cultured rat cortical neurons were exposed to H₂O₂ but treated with compounds 1-17 at 10 µM concentration. No significant differences were found for neurons treated with any compounds and exposed to H₂O₂ (Table 3).
Table 3. Viability of the cells (expressed in %) treated with compounds 1-17 (at 10 µM and 100 µM).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Viability (%) at 10 µM</th>
<th>Viability (%) at 100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO + H₂O₂</td>
<td>72.21 ± 1.71</td>
<td>78.29 ± 1.68</td>
</tr>
<tr>
<td>1</td>
<td>73.33 ± 3.06&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>79.10 ± 1.00&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>67.96 ± 2.12&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>73.65 ± 3.33&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>63.80 ± 2.57&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>104.41 ± 6.26&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>69.69 ± 3.77&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>85.88 ± 6.70&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>71.73 ± 4.34&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>83.16 ± 5.86&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>78.49 ± 6.00&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>76.33 ± 2.27&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>79.43 ± 6.54&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>102.40 ± 3.76&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>71.40 ± 7.32&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>99.05 ± 5.20&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>72.24 ± 3.96&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>101.89 ± 0.75&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>72.44 ± 6.40&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>83.61 ± 2.75&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>70.61 ± 7.28&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>83.10 ± 3.85&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>68.19 ± 3.90&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>91.31 ± 5.26&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>71.27 ± 4.74&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>86.25 ± 3.26&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>67.88 ± 6.32&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>100.60 ± 10.56&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>73.62 ± 6.44&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>106.07 ± 6.26&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>72.90 ± 0.67&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>84.86 ± 6.27&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>74.20 ± 3.57&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>80.65 ± 2.98&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>ns</sup> no significant differences were found with the control group (DMSO and H₂O₂). * P <0.05, ** P <0.001 versus the corresponding viability obtained in the control group treated with H₂O₂ in addition to DMSO.

DPPH scavenging

Under normal conditions, free radicals are rapidly neutralized in the mitochondria, but over the years, this neutralization is no longer effective and dysfunction and even cell death occurs. We studied the DPPH (1,1-diphenyl-2-picyrylhydrazyl) radical scavenging activity of those compounds showing best neuroprotective effects, in cells treated with H₂O₂ (compounds 3, 7, 9, 14 and 15). As it can be seen in graphic 3, most of the studied compounds exerted free radical scavenging, being compounds 3 and 7 (100 µM) the most active derivatives, which showed a scavenging activity slightly higher
than 50%. These are also two of the best compounds of the series of 3-benzamidocoumarins as neuroprotective agents against H$_2$O$_2$. By contrast, for the series of 3-nicotinamidocoumarins, compounds 14 and 15, only when a hydroxyl group is present at position 4 (compound 15) a free radical scavenging activity by approximately 20% was observed. Therefore, the presence of a hydroxyl group, in this specific case at position 4, seems to be critical for neutralization of free radicals by these derivatives.

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

**In vitro blood-brain barrier permeation assay**

A basic condition of any compound to act on neurodegenerative processes is to penetrate into the brain, that is, to be able of crossing the blood-brain barrier (BBB). To examine the capability of our compounds to pass this barrier, we select compounds 3, 12, 14 and 15, some of the most active derivatives as MAO inhibitors and/or neuroprotective agents, and used a parallel artificial membrane model (PAMPA). This is a fairly easy and successful method to predict the passive central nervous system (CNS) permeation, which had been previously optimized in order to be applied to investigational compounds with limited aqueous solubility. Experimental results are represented in table 4.
Table 4. In vitro evaluation of the CNS penetration "experimental permeability (Pe 10^{-6} cm s^{-1})" using the PAMPA methodology. Results are expressed as the mean of the experimental prediction through at least 3 independent experiments ± SD.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Pe Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>7.4 ± 0.01 SNC -</td>
</tr>
<tr>
<td>12</td>
<td>44.2 ± 2.1 SNC +</td>
</tr>
<tr>
<td>14</td>
<td>20.2 ± 0.4 SNC +</td>
</tr>
<tr>
<td>15</td>
<td>9.3 ± 0.1 SNC +/−</td>
</tr>
<tr>
<td>Verapamil</td>
<td>14.8 ± 0.1 SNC +</td>
</tr>
</tbody>
</table>

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

Theoretical evaluation of ADME-related physicochemical/structural parameters

In order to better understand the overall properties and the drug-like characteristics of compounds 1-17, the lipophilicity (expressed as the octanol/water partition coefficient and herein called logP), and the theoretical prediction of other ADME properties (molecular weight, TPSA, number of hydrogen donors and acceptors, and volume) were carried out with Molinspiration calculation software and are presented in the supplementary data.\(^{[50,51]}\)
From the data presented in the supplementary data, it is significant that all the described derivatives possess logP values compatible with those required to cross membranes. Although TPSA (described to be a predictive indicator of membrane penetration) of selegiline is too different of those found to the studied compounds, all values were found to be in the desirable range. In addition, it can be observed that no violations of Lipinski’s rule (molecular weight, logP, number of hydrogen donors and acceptors) were found. The studied compounds, as MAO inhibitors and neuroprotective agents, have to pass different membranes and reach the CNS. The obtained information supports the potential of these derivatives as potential drug candidates. The theoretical information obtained is partially in accordance to the experimental in vitro BBB permeation assay. The combination of both results can help to better understand the drug likeness of this series of compounds.

Conclusions

In this study, a general and efficient synthesis of a new series of 3-amidocoumarins was developed, using an amidation reaction as key step. Determination of hMAO isoform activity was carried out, and many of the compounds exhibited selectivity for the hMAO-B isoenzyme with activity in the range of nanomolar (compound 1) or micromolar concentrations (compounds 6, 8, 12-14, 16 and 17). Neuroprotective effects against H2O2 were also studied. For the series of 3-benzamidocoumarins, the most promising results in cells treated with H2O2 corresponded, in general, to the derivatives that present a substituent in para position of the benzamide ring, additionally to a hydroxyl group at position 4 of the coumarin scaffold (compounds 3, 7 and 9). For the series of 3-heteroarylamidocoumarins, derivatives having a nicotinamide group at position 3 of the coumarin scaffold (compounds 14 and 15) exerted the most remarkable neuroprotection. Most of the selected derivatives exerting neuroprotection showed also DPPH scavenging activity (excepting compound 14, with no hydroxyl groups on its structure). Additionally, prediction of BBB partitioning through a PAMPA assay showed the potential of this type of compounds to cross the BBB and act in the CNS. From the four studied compounds, those without a hydroxyl group at position 4 proved to have better facility to cross the BBB (compounds 12 and 14). Compound 15 proved to partially be 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 to possess the desire drug ability properties. The results encourage us to further explore the potential 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 DMSO-$d_6$ 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 Perkin Elmer 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 F254). Organic solutions were dried over anhydrous Na$_2$SO$_4$. 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 ethanol 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 the completion of the reaction, the mixture was filtered to eliminate the catalyst. The obtained crude product was then purified by FC (hexane/ethyl acetate, 9:1) to give the desired coumarin, in a yield of 90%.

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 dichloromethane (9 mL). Then, pyridine (1.1 mmol) was added and the mixture was cooled to 0 °C. Differently substituted acid chloride (1.1 mmol) was added drop-wise at this temperature, and the mixture was stirred overnight at room temperature. The batch was evaporated and purified by column chromatography (hexane/ethyl acetate, 9:1) to give the desired compounds 1-17.

$N$-(4-Hydroxycoumarin-3-yl)-4'-methylbenzamide (3) Yield: 83%. Mp: 210-211 °C. $^1$H NMR (DMSO-$d_6$) δ (ppm), J (Hz): 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 (s, 1H, OH). $^{13}$C NMR (DMSO-$d_6$) δ (ppm): 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. DEPT (DMSO-$d_6$) δ (ppm): 21.0, 116.2, 123.7, 124.3, 128.1, 128.8, 132.4. MS m/z (%): 296 (6), 295 (M+, 29), 119 (100), 91 (30), 65 (10). Ana. Elem. Calc. for C$_{17}$H$_{13}$NO$_4$: C, 69.15; H, 4.44. Found: C, 69.12; H, 4.42.

**N-(4-Hydroxycoumarin-3-yl)-3',4'-dimethoxybenzamide (5)** Yield: 88%. Mp: 247-248 °C. $^1$H NMR (DMSO-$d_6$) δ (ppm), $J$ (Hz): 3.82 (s, 6H, (CH$_3$)$_2$), 7.07 (d, 1H, H-5', J=8.3), 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, 1H, H-5, J=7.8), 9.44 (s, 1H, NH), 12.20 (s, 1H, OH). $^{13}$C NMR (DMSO-$d_6$) δ (ppm): 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 (DMSO-$d_6$) δ (ppm): 56.3, 111.5, 112.0, 116.9, 122.3, 124.3, 125.0, 133.0. MS m/z (%): 342 (6), 341 (M+, 15), 323 (6), 165 (100), 121 (6), 92 (7), 77 (9). Ana. Elem. Calc. for C$_{19}$H$_{15}$NO$_6$: C, 63.34; H, 4.43. Found: C, 63.31; H, 4.41.

**N-(4-Hydroxycoumarin-3-yl)-3',4'-dichlorobenzamide (8)** Yield: 86%. Mp: 284-285 °C. $^1$H NMR (DMSO-$d_6$) δ (ppm), $J$ (Hz): 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 (s, 1H, OH). $^{13}$C NMR (DMSO-$d_6$) δ (ppm): 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. DEPT (DMSO-$d_6$) δ (ppm): 116.5, 123.9, 124.0, 124.5, 128.4, 130.1, 130.8. 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). Ana. Elem. Calc. for C$_{18}$H$_{13}$Cl$_2$NO$_4$: C, 54.88; H, 2.59. Found: C, 54.85; H, 2.55.

**N-(Coumarin-3-yl)furan-2-carboxamide (10)** Yield: 90%. Mp: 183-184 °C. $^1$H NMR (CDCl$_3$): 6.74 (dd, 1H, H-4', J=3.6, J=1.8), 7.34-7.58 (m, 4H, H-5, H-6, H-8, H-5'), 7.77 (td, 1H, H-7, J=8.0, J=1.4), 8.00 (dd, 1H, H-3', J=1.8, J=0.8), 8.58 (s, 1H, H-4), 9.26 (s, 1H, NH). $^{13}$C NMR (CDCl$_3$): 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. MS m/z (%): 256 (16), 255 (M+, 79), 227 (7), 132 (6), 95 (100), 77 (10). Anal. Elem.Calc. for C$_{14}$H$_9$NO$_5$: C, 62.00; H, 3.34. Found: C, 62.03; H, 3.31.

**N-(4-Hydroxycoumarin-3-yl)cyclohexanecarboxamide (17)** Yield: 91%. Mp: 199-200 °C. $^1$H NMR (CDCl$_3$) δ (ppm), $J$ (Hz): 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, 1H, H-7, J=7.8, J=1.7), 8.01 (dd, 1H, H-5, J=7.9, J=1.7), 8.28 (s, 1H, NH), 13.87 (s, 1H, OH). $^{13}$C NMR (CDCl$_3$) δ (ppm): 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. DEPT (DMSO-$d_6$) δ (ppm): 25.4, 25.7, 29.7, 45.5, 116.2,

**Pharmacological assays**

**Determination of MAO isoforms enzymatic activity**

The tested compounds were dissolved in DMSO (Sigma-Aldrich, Alcobendas, Madrid, Spain) to prepare 10 mM stock solutions, which were kept for storage at -20 °C. Percentage of DMSO used in the experiments was never higher than 1%. Selegiline and rasagiline, used as reference inhibitors, have been acquired from Sigma-Aldrich (Alcobendas, Madrid, Spain). Human recombinant MAO isoforms, used in the experiments, was purchased from Sigma-Aldrich (Alcobendas, Madrid, Spain). Resorufin sodium salt, p-tyramine hydrochloride, sodium phosphate buffer, horseradish peroxidase and Amplex® Red reagent has been supplied in the assay kit of Amplex® Red MAO Molecular Probes (Molecular Probes, Inc., Eugene, Oregon, USA).

Briefly, 0.1 mL of sodium phosphate buffer (0.05 M, pH 7.4) containing different concentrations of the test drugs (new compounds or reference inhibitors) in various concentrations and adequate amounts of recombinant hMAO-A or hMAO-B required and adjusted to obtain in our experimental conditions the same reaction velocity, i.e., to oxidize (in the control group) the same concentration of substrate: 165 pmol of p-tyramine/min (hMAO-A: 1.1 μg protein; specific activity: 150 nmol of p-tyramine oxidized to p-hydroxyphenylacetaldehyde/min/mg protein; hMAO-B: 7.5 μg protein; specific activity: 22 nmol of p-tyramine transformed/min/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 horseradish peroxidase and 1 mM p-tyramine. The production of H₂O₂ and, consequently, of resorufin was quantified at 37 °C in a multi detection microplate fluorescence reader (FLX800, BioTek Instruments, Inc., Winooski, VT, USA) based on the fluorescence generated (excitation, 545 nm, emission, 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., for directly reacting with 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 $V_{\text{max}}$), the corresponding enzymatic activity of both isoforms was evaluated (under the experimental conditions described above) in the presence of a number (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. In our experimental conditions, this background activity was practically negligible.

MAO activity of the test compounds and reference inhibitors is expressed as IC$_{50}$, e.g. the concentration of each drug required to produce a 50% decreased on control value activity isoforms MAO.

**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. A 100x 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 buffer pH 7.4 (PBS), Hanks buffer, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), $H_2O_2$ were acquired from Sigma-Aldrich, Química S.A., Alcobendas, Spain. 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 killed 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 the dissection of the brain. 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 cell/mL. Neuronal cultures were allowed to grow for 8-10 days keeping in an incubator (Form Direct Heat CO₂, Thermo Electron Corporation, Madrid, Spain) under saturated humidity at a partial pressure of 5% CO₂ 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 the Neuronal Survival**

Neuronal cultures were treated with the compounds in the study at 100 µM concentration (final DMSO concentration ≤1%) or with studied compounds and H₂O₂ (30 µM) over an incubation period of 24 hours. H₂O₂ was used as reference neurotoxic agent for neurons.

Cell viability was determined to know the possible cytotoxicity of new compounds or their neuroprotective effects against a pro-oxidant (H₂O₂) agent, by reducing MTT to formazan by mitochondrias of viable cells. MTT (5 mg/mL in Hanks buffer) was added to each well to a final concentration of 10%.⁰⁵²

After incubating for two hours at 37 °C, the medium was removed and formazan crystals formed were suspended in 100 µL/well of DMSO. The production of formazan by viable cells was quantified at 37 °C in a reader absorbance (Fluo-star Optima, BMG LABTECH, Offenburg, Germany) by determining the absorbance (570 nm).⁰⁵³,⁵⁴

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.

**Determination of the neutralization of free radicals**

DMSO, DPPH, ascorbic acid and ethanol were acquired from Sigma-Aldrich Química S.A., Alcobendas, Spain.

DPPH radical scavenging activity of each compound was determined as previously described with minor modifications. The DPPH radical was dissolved in ethanol (100 µM) and 99 µL of the solutions were transferred to each well of a 96-well microplate. 1 µL of compounds 3, 7, 9, 14 and 15 (100 µM, final concentration) in ethanol was added to each well of a 96 well microplate and the mixtures were incubated at room
temperature for 30 min. Vit. C (100 μM) was used as a positive control in the experiments. The absorbance at 540 nm was measured using a microplate reader. The 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.\textsuperscript{[55-57]}

**In vitro blood-brain barrier permeation assay**

Hydrocortisone, desipramine, promazine, aldosterone, caffeine, ofloxacin, corticosterone, imipramine, testosterone, verapamil, piroxicam, lipid pig brain, phosphate buffered saline solution at pH 7.4 (PBS) and dodecane were purchased from Sigma-Aldrich Química S.A., Alcobendas, Spain and Acros, Madrid, Spain.

Prediction of the brain penetration was evaluated using a PAMPA-BBB assay, in a similar manner as previously described.\textsuperscript{[46-49]} Pipetting was performed with a semi-automatic 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 of PBS:ethanol (70:30) and the filter surface of the donor microplate was impregnated with 4 mL of PBL in dodecane (20 mg mL\textsuperscript{-1}). Compounds were dissolved in PBS: ethanol (70:30) at 10 μg mL\textsuperscript{-1}, filtered through a Millex filter, and then added to the donor wells (200 μL). The donor filter plate was carefully put on the acceptor plate to form a sandwich, which was left undisturbed for 240 min at 25 °C. After incubation, the donor plate is carefully removed and the concentration of compounds in the acceptor wells was determined by UV-Vis spectroscopy. Every sample is analyzed at five wavelengths, in four wells and at least in 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 absorption, distribution, metabolism and excretion properties**

The absorption, distribution, metabolism and excretion (ADME) properties of the studied compounds were calculated using the Molinspiration property programme. LogP was calculated using the methodology developed by Molinspiration as a sum of fragment-based contributions and correction factors.\textsuperscript{[50]} TPSA was calculated based on
the methodology published by Ertl et al. as a sum of fragment contributions.\textsuperscript{58} Oxygen and nitrogen-centred polar fragments were considered.\textsuperscript{50} PSA has been shown to be a very good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability and blood–brain barrier penetration. The method for calculation of molecule volume developed at Molinspiration is based on group contributions. These have been obtained by fitting the sum of fragment contributions to ‘real’ three dimensional (3D) volume for a training set of about 12,000, 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 mean of at least 3 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. The graphical representation, the statistical analysis and calculation of IC\textsubscript{50} values were performed using GraphPad Prism (V 4.03) software (San Diego, USA).

Acknowledgements

This work was supported in part by the University of Santiago de Compostela, the Xunta de Galicia (EM2014/016), the Spanish Ministry of Economy and Competitiveness (SAF2012-31035), the Portuguese Foundation for Science and Technology (FCT), and QREN (FCUP-ClQ-UP-NORTE-07-0124-FEDER-000065), Galician Plan of Research, Innovation and Growth 2011–2015 (Plan I2CED481B 2014/086-0) and FCT, POPH, and QREN (SFRH/BPD/95345/2013).

Keywords: Coumarin • Parkinson’s disease • Neuroprotection • MAO inhibition • PAMPA assays
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