Discovery and Optimization of 3-Thiophenylcoumarins as Novel Agents Against Parkinson's Disease: Synthesis, *in vitro* and *in vivo* Studies

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Abstract

Monoamine oxidase B (MAO-B) inhibitors are still receiving great attention as promising therapeutic agents for central nervous system disorders. This study explores,

for the first time, the potential of 3-thiophenylcoumarins as *in vitro* and *in vivo* agents against Parkinson´s disease. Twelve compounds were synthesized via Perkin-Oglialoro reaction, and *in vitro* evaluation of six hydroxylated molecules was performed. MAO-A and MAO-B inhibition, DPPH scavenging and inhibition of ROS formation, neurotoxicity on motor cortex neurons and neuroprotection against H_2O_2, were studied. *In vivo* effect on locomotor activity using the open field test was also evaluated for the best candidate [3-(4'-bromothiophen-2'-yl)-7-hydroxycoumarin, **5**], a potent, selective and reversible MAO-B inhibitor (IC_{50} = 140 nM). This compound proved to have a slightly better *in vivo* profile than selegiline, one of the currently treatments for Parkinson's disease, in reserpinized mice pretreated with levodopa and benserazide. Results suggested that, comparing positions 7 and 8, substitution at position 7 of the coumarin scaffold is better for the enzymatic inhibition. However, the presence of a catechol at positions 7 and 8 exponentially increases the antioxidant potential and the neuroprotective properties. Finally, all the molecules present good theoretical physicochemical properties that make them excellent candidates for the optimization of a lead compound.

Keywords. 3-Thiophenylcoumarins, Parkinson's disease, monoamine oxidase B inhibitors, DPPH scavengers, neuroprotectors, Open field test.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative progressive disorder characterized by the presence of Lewy bodies in the brain of the patients and the loss of dopaminergic neurons, particularly in the substantia nigra *pars compacta* and their subsequent innervation of striatum [1]. Its clinical symptoms are well-known and are used to establish the diagnostic: muscle tremors, rigidity, bradykinesia and loss of balance,

among other motor and non-motor symptoms [2]. However, these pathognomonic well manifested symptoms appear only when the disease is in an advanced stage. Therefore, this pathology has no cure, as the treatments are exclusively symptomatic and may only slow down the manifestation/progression of the disease. The number of cases worldwide is higher than 6 million [3], and it is estimated by 2040 to grow over 12 million [4]. Based on this scenario, it is extremely relevant to work on this field to clarify the characteristics of PD and contribute with a new potential pharmacological treatment.

Levels of monoamine oxidase B (MAO-B) are increased in aged human brains, highlighting the importance of this isoform in metabolizing endogenous amines, including dopamine, in cases of neurodegeneration, as in PD [5]. Selegiline and rasagiline are MAO-B potent and selective inhibitors which can be used in PD as monotherapy or adjuvant therapy with levodopa (LD). They present a propargyl moiety on their structures that irreversibly binds and inactivates this enzyme. Being irreversible inhibitors is being pointed as a disadvantage of their therapeutic profile. Despite that, these are two molecules in the first line for the treatment of the disease [6]. In particular, rasagiline combines the MAO-B inhibitory activity with a high level of neuroprotection, preventing neuronal damage and secondary injuries by controlling oxidative stress, mitochondrial dysfunction, neuroinflammation, protein misfolding, disruptive autophagy and apoptosis [7]. Neuroprotection is currently leading the therapeutic strategy to slow PD progression [8,9]. Three years ago (march of 2017), Food and Drug Administration (FDA) approved the safinamide for the treatment of PD [10]. This new drug is a potent and reversible MAO-B inhibitor, with a more adequate pharmacological profile comparing to the previously available drugs.

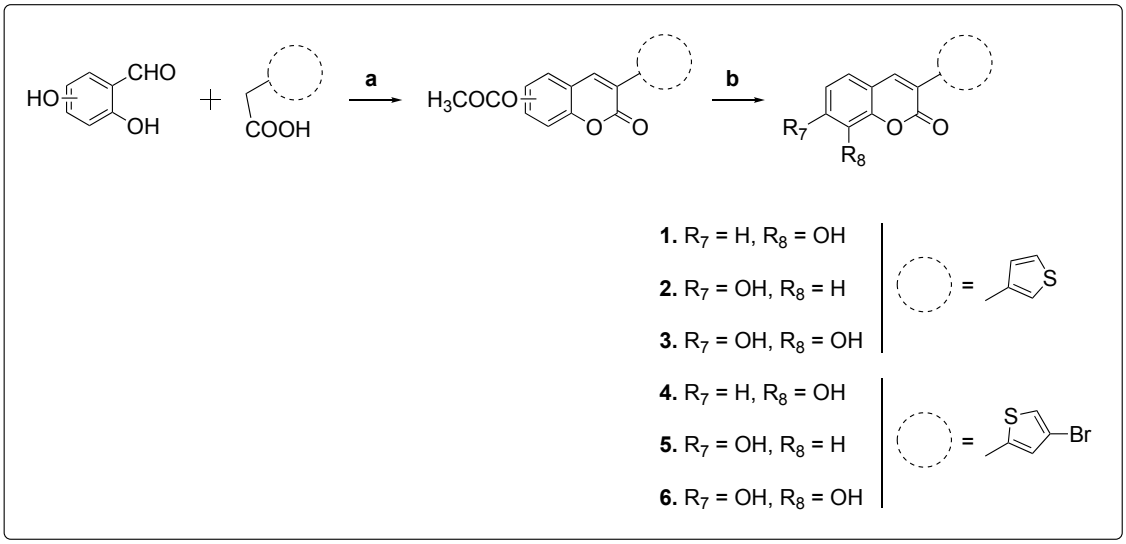
In the last years, our group has been describing 3-arylcoumarins displaying potent and selective MAO-B inhibitory activity, some of them being reversible or partially reversible inhibitors of this enzyme [11,12,13,14,15,16,17,18]. In particular, we found out that molecules bearing functional groups at position 8 of the coumarin scaffold are very potent MAO-B inhibitors [14,15]. Also, the presence of hydroxyl groups [12,16,18], and in particular catechols [19], may be responsible for an exponential increase in the antioxidant capacity and, indirectly, in the neuroprotective effects. Finally, the selection of the ring at position 3 of the coumarin scaffold was based on a common strategy in drug design: the replacement of an aryl ring by an heteroaryl one. For its characteristics (aromaticity and presence of a sulfur with the electron pairs significantly delocalized in the pi electron system), the thiophene appeared as the ideal candidate. Based on this, in the current work, a step further in the study of 3-arylcoumarins as antiparkinsonians leads to the discovery of 3-thiophenylcoumarins as good MAO-B inhibitors, presenting neuroprotective properties, being also promising *in vivo* candidates for hit-to-lead optimization.

2. Results and Discussion

2.1 Chemistry

Compounds **1-6** were synthesized via a two-step Perkin–Oglialoro reaction, according to Scheme 1. Different commercially available *ortho*-hydroxybenzaldehydes and thiophenylacetic acids were condensed in the presence of potassium acetate (CH_3CO_2K), in acetic anhydride (Ac_2O), under reflux, for 16 hours, to afford the acetoxy-3-thiophenylcoumarins. This step involves sequential acetylation of the hydroxyl groups and pyrone ring closure in a one-pot reaction. Further on, acetoxy derivatives were hydrolyzed in the presence of aqueous hydrochloric acid (HCl) and

methanol (MeOH), under reflux, for 3 hours, to afford the corresponding hydroxy-3-thiophenylcoumarins **1-6**. All the synthetic details and compounds characterization are included in the experimental section.



Scheme 1. Synthetic methodology by a Perkin-Oglialoro reaction. Conditions and reagents: a) CH_3CO_2K, Ac_2O, reflux, 16 h; b) HCl, MeOH, reflux, 3 h.

2.2 Inhibitory activity on *h*MAO-A and *h*MAO-B

The *in vitro* effects of the synthetized compounds **1-6** on *h*MAO activity were studied using an Amplex® Red MAO assay kit [18]. The MAO isoforms were prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for *h*MAO-A or *h*MAO-B. The results of *h*MAO-A and *h*MAO-B inhibition are shown in Table 1.

Table 1. IC_{50} values of the new compounds **1-6** and reference inhibitors activity on recombinant *h*MAO isoforms expressed in baculovirus infected BTI insect cells (BTI-TN-5B1-4).

Compounds	IC_{50} *h*MAO-A (μM)	IC_{50} *h*MAO-B (μM)	S.I. [a]
1	17.64 ± 1.18	**	< 0.18 [b]
2	7.62 ± 0.51	8.50 ± 0.57	0.90
3	1.32 ± 0.09	4.75 ± 0.32	0.28
4	6.21 ± 0.42	**	< 0.06 [b]
5	9.16 ± 0.61	0.14 ± 0.01	65.43
6	6.08 ± 0.41	21.75 ± 1.46	0.28
Selegiline	68.73 ± 4.21	0.017 ± 0.002	4043
Iproniazid	6.56 ± 0.76	7.54 ± 0.36	0.87

Each IC_{50} value is the mean ± S.E.M. from five experiments (n = 5). ** At 100 μM the compound inhibits enzymatic activity by approximately 45-50% [a] S.I.: *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).

The effect on the MAO inhibitory activity of the substitution with hydroxyl groups at positions 7 and/or 8 of the 3-thiophenylcoumarin derivatives was studied, as well as the presence of a halogen (bromine) at position 4 of the thiophen-2-yl ring. Analyzing the results from Table 1, the presence of a hydroxyl group at position 8 of the coumarin resulted in MAO-A inhibitors (compounds **1** and **4**), while a substitution at position 7 (**2** and **5**) or at both positions 7 and 8 (**3** and **6**), originated derivatives with activity on both isoforms of the enzyme. In fact, compounds **2**, **3** and **6** have a similar profile to iproniazid, a non-selective and irreversible MAO inhibitor, with both MAO-A and MAO-B activities in the low micromolar range. In the case of 3-(4-bromothiophene-2'-yl)coumarins with a single hydroxyl group at position 7 or 8 (**5** and **4**, respectively), the presence of a bromine atom at position 4 of the thiophen-2-yl ring, and the presence of the sulfur at position 2 instead of 3 of the thiophenyl ring, led to an increase in the MAO inhibitory selectivity. The 3-(4'-bromothiophene-2'-yl)-7-hydroxycoumarin (**5**) is, therefore, the most potent and selective MAO-B inhibitor within the studied series (IC_{50} = 144 nM and S.I. = 65.43).

2.3 Reversibility profile of *h*MAO-B inhibition

The type of inhibition exerted by the 3-(4'-bromothiophene-2'-yl)-7-hydroxycoumarin (**5**) on *h*MAO-B isoform was evaluated by using a dilution method [20]. The selected compound is the most potent and selective within the studied series, bearing a hydroxyl substituent at position 7 of the coumarin scaffold. 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 **5** and the reference inhibitors (selegiline and isatin).

Compound	Slope (ΔUF/t) [%][a]
5	63.75 ± 4.28
Selegiline	12.73 ± 1.85
Isatin	88.63 ± 5.90

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

As seen in Table 2, the 3-(4'-bromothiophene-2'-yl)-7-hydroxycoumarin (**5**) proved to be a reversible *h*MAO-B inhibitor. Its degree of reversibility is slightly lower than that displayed by isatin, a reversible MAO-B inhibitor used as a reference. However, compound **5** presents a much more convenient reversibility profile than selegiline, which is being used in the therapy of PD. This result is aligned with previous data from our group, demonstrating that the synthesis of differently substituted 3-arylcoumarins may lead to reversible compounds [11,12,13]. More recently, our group has described another coumarin derivative bearing a pent-2-yn-1-amine group at position 7, that proved to behave as a partially reversible MAO-B inhibitor [21].

2.4 DPPH scavenging activity

Since molecules presenting radical scavenging properties proved to be neuroprotective agents for patients with PD, a free radical scavenging assay was also performed. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical molecule widely used to measure the free radical scavenging capacity. The percentage of DPPH scavenging of the studied compounds **1**-**6** and vitamin C, an effective scavenger against oxygen and nitrogen oxide species used as positive control, are represented in Figure 1.

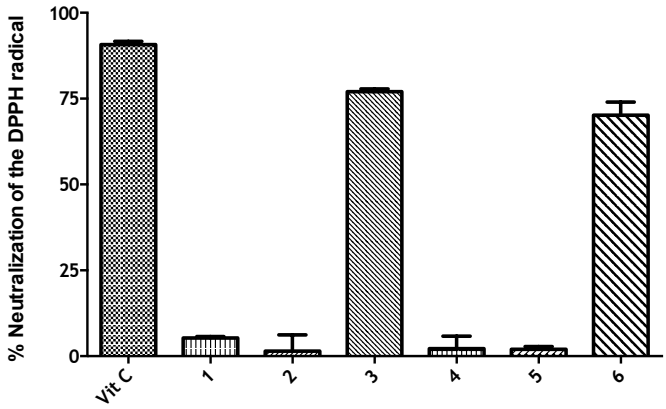


Figure 1. Percent of neutralization of the DPPH radical by the 3-thiophenylcoumarins and vitamin C, at 100 μM. Each value of % is the mean ± S.E.M. from three experiments (n = 3).

As described for other 3-phenylcoumarins [19], the 3-thiophenylcoumarins that present hydroxyl groups in contiguous positions of the coumarin scaffold (catechols), as 7,8-dihydroxy-3-(thiophen-3'-yl)coumarin (**3**) and 3-(4'-bromothiophen-2'-yl)-7,8-dihydroxycoumarin (**6**), are the ones presenting the highest activity, comparable to vitamin C (Figure 1). The EC_{50} values for these derivatives were calculated and appear in Table 3.

Table 3. EC_{50} values for compounds **3** and **6**, and vitamin C.

Compound	EC_{50} (μM)
3	9.20 ± 0.62
6	5.82 ± 0.39
Vitamin C	5.02 ± 0.34

Each EC_{50} value is the mean ± S.E.M. from three experiments (n = 3).

2.5 Neurotoxicity

Neurotoxic profile of compounds **1**-**6** was evaluated on rat motor cortex neurons. Neuronal primary cultures were obtained from embryos of 19 days pregnant albino rats (Rattus novergicus) of the Wistar-Kyoto strain [22]. After treatment and an incubation period of 24 hours, monitorization of cell viability was performed by using the dimethylthiazolyldipenyltetrazolium bromide method (MTT).

Figure 2 shows the results obtained by studying the possible neurotoxic effect of 3-thiophenylcoumarins **1**-**6**, at a concentration of 10 μM, in rat motor cortex neurons. At this concentration, only the 8-hydroxy-3-(thiophen-3'-yl)coumarin (**1**) presented a slightly but statistically significant decrease in the cell viability.

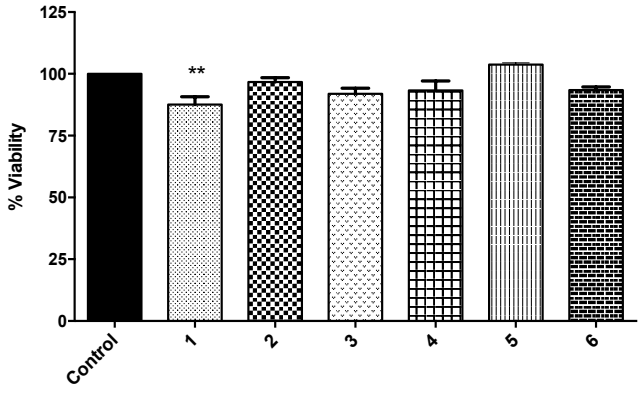


Figure 2. Evaluation of the possible neurotoxic effect of 3-thiophenylcoumarins **1**-**6** on rat motor cortex neurons. The cells were incubated with the molecules at 10 μM, dissolved in DMSO (1%). The results are expressed as % of viability against the control group (treated with DMSO 1%) in at least 5 experiments ± S.E.M. ** $P<0.005$.

2.6 Neuroprotective activity

In addition to the enzymatic inhibition and the capacity of neutralizing free radicals, neuroprotective effect on neuronal populations affected during the establishment and evolution of PD is highly desirable in an antiparkinsonian drug.

The potential neuroprotective effect of these derivatives against H_2O_2, in the cellular model previously described, was evaluated (Figure 3). For the neuroprotective study, after treatment with compounds **1**-**6**, neuronal cells were also treated with a reference neurotoxic agent, H_2O_2 (100 μM). After an incubation period of 24 hours, monitorization of cell viability was performed by using the MTT method.

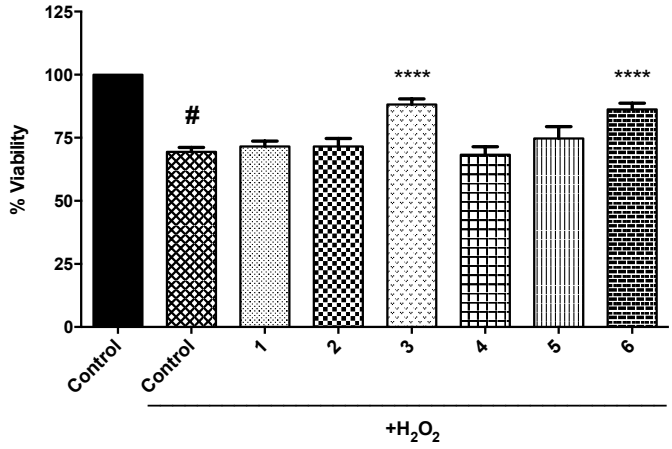


Figure 3. Neuroprotective effect of 3-thiophenylcoumarins **1**-**6** on rat motor cortex neurons. The cells were incubated with the molecules at 10 μM, dissolved in DMSO (1%), in the presence of H_2O_2 (100 μM). The results are expressed as % of viability against the control group (treated with DMSO 1%) in at least 5 experiments ± S.E.M. ****P<0.0001, $^{\#}P$<0.0001, against the group treated with only DMSO.

7,8-Dihydroxy-3-(thiophen-3'-yl)coumarin (**3**) and 3-(4'-bromothiophen-2'-yl)-7,8-dihydroxycoumarin (**6**), both with two hydroxyl groups at positions 7 and 8 of the coumarin scaffold, displayed neuroprotective activity against H_2O_2 in rat motor cortex neurons. The other derivatives within the series, which lack activity, have only one

hydroxyl group at position 7 or 8 of the coumarin scaffold. This chemical feature seems to play an important role in the neuroprotection, that can be related with the excellent profile of these catechols as DPPH scavengers.

2.7 Inhibition of ROS formation

For a deeper understanding of the neuroprotection exercised by these coumarin derivatives bearing a catechol group, compound **6** was selected to evaluate its inhibitory activity against reactive oxygen species (ROS) formation induced by H_2O_2 (100 μM) in cells SH-SY5Y using the DCFDA assay (conversion of 2',7'-dichlorofluorescin diacetate to the fluorescence dye 2',7'-dichlorofluorescein). The SH-SY5Y cell line derives from human neuroblastoma and represents a good model of human immature neurons, capable of cell differentiation. The results of the assay are represented in the Figure 4.

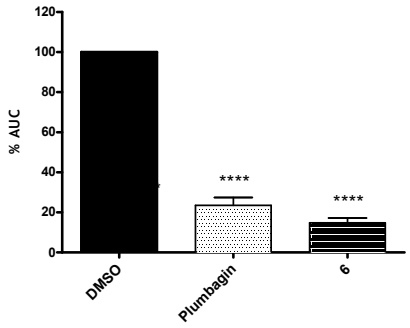


Figure 4. Effects of compound **6** and plumbagin (negative control) at 10 μM on the ROS production by SH-SY5Y cells treated with H_2O_2 (100 μM). AUC: area under the curve. Results are expressed as the mean of 3 experiments ± S.E.M., standardized as % of control (cells treated with the vehicle, DMSO). **** P<0.0001.

Compound **6** demonstrated considerable effects against ROS formation, comparable to the effect of plumbagin, a reference antioxidant agent.

2.8 Physicochemical properties

The physicochemical properties of the synthetized derivatives are a very good indicator of their capacity to cross through cellular membranes and predictors of their ADME (absorption, distribution, metabolism and excretion) parameters. In this study, the Molinspiration Cheminformatics software (https://www.molinspiration.com/) was used to calculate the physicochemical properties of the coumarin derivatives. 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) were calculated. The passage through the blood brain barrier (BBB) was also theoretically predicted using the "CBligand-BBB predictor" program (https://www.cbligand.org/BBB/). The results are included in Table 4, as well as the prediction of the violations of Lipinski rules (n viol). These results together give an idea of the potential bioavailability and the capacity of passing through the blood brain barrier (BBB) of these compounds.

Table 4. Molecular properties of 3-thiophenylcoumarin derivatives **1-6** 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	3.06	50.44	17	244.27	3	1	1	198.73	0	+
2	2.82	50.44	17	244.27	3	1	1	198.73	0	+
3	2.56	70.67	18	260.27	4	2	1	206.74	0	-

4	3.99	50.44	18	323.17	3	1	1	216.61	0	+
5	3.76	50.44	18	323.17	3	1	1	216.61	0	+
6	3.49	70.67	19	339.17	4	2	1	224.63	0	-

Theoretically, all the 3-thiophenylcoumarins have the suitable physicochemical properties to display good bioavailability, and none of them violate the Lipinski rule of five (Table 4). Compound **4** presents the highest value of LogP within the series, combined with a low TPSA. Although all the compounds have physicochemical properties that theoretically are suitable for passaging through membranes, theoretical prediction indicates that compounds **3** and **6**, both with two hydroxyl substituents on the scaffold, with TPSA value greater than 70, are not theoretically able to cross the BBB.

2.9 *In vivo* study: Open field test

In view of the above-described results and considering the *in vitro* activity as MAO-B inhibitors, the most active compound [3-(4'-bromothiophen-2'-yl)-7-hydroxycoumarin, **5**] was selected for the *in vivo* studies using the open field test (OFT). The studies were performed with both non-reserpinized and reserpinized mice. For non-reserpinized mice, the possible modification on the motor activity caused by the selegiline or compound **5**, in individual mode, was evaluated.

Reserpine blocks H^+-coupled vesicular monoamine transporters (VMAT2) preventing proper storage of recently synthesized cytoplasmatic dopamine or the synaptic recaptured dopamine in the neuronal vesicles. This causes the depletion of the dopaminergic nerve endings and, among them, those of the nigrostriatal pathway, leading to hypokinesia in experimental animals. Therefore, it is used as a good model of PD [23].

For reserpinized mice, their potentiation in the effect generated by the levodopa and benserazide (LD:B) combination, was studied. If these compounds act as *in vivo* MAO-B inhibitors at the administered dose, inhibiting the metabolism of dopamine, the residence time of this neurotransmitter in the striatal synaptic space must increase and subsequent dopamine receptor activation occur. Consequently, locomotor activity, velocity of movements and percentage of time in movement should increase as well. Therefore, these were the parameters under study.

2.9.1 OFT in non-reserpinized mice

As can be seen in Figure 5, the administration of 10 mg/Kg of selegiline to non-reserpinized mice increased the locomotor activity in a statistically significant manner (17,303 cm ± 1,161, $P<0.05$, Figure 5A) *versus* the corresponding control group (10,114 cm ± 678.46, Figure 5A) of animals treated with the vehicle (carboxymethylcellulose sodium, CMCNa 1%, Figure 5A). Selegiline also increased the velocity in a statistically significant manner (4.81 cm/s ± 0.32, $P<0.05$, Figure 5B), comparing to the control group (2.81 cm/s ± 0.19, Figure 5B). For the percentage of time in movement, although no statistically significant differences were observed, selegiline showed a tendency to increase it (57.68% ± 3.87, Figure 5C), comparing to the control (34.44% ± 2.31, Figure 5C). Bibliographic data presenting results of the impact of selegiline in locomotor activity of non-reserpinized mice is contradictory, showing both decrease or increase on locomotor activity, that can be attributed to differences in the studied dosages. At the studied dose, the results obtained herein can be explained due to the metabolization of selegiline to amphetamine derivatives, as previously described by Engberg and co-workers [24].

Similarly, a dose of 10 mg/kg of compound **5** was evaluated in non-reserpinized mice. Although a tendency to increase the locomotor activity (14,331 cm ± 961.34), the velocity (3.98 cm/s ± 0.27) and percentage of time in movement (52.43% ± 3.52) of mice treated with compound **5** can be appreciated, the differences, comparing to the control group, were not statistically significant, as shown in Figure 5.

Therefore, compound **5** was administered at a higher dose (100 mg/kg). At this dose, no statistically significant differences were observed, comparing to the control group. Also, in contrast with selegiline, no clear tendency to increase any of the evaluated parameters was observed in non-reserpinized mice.

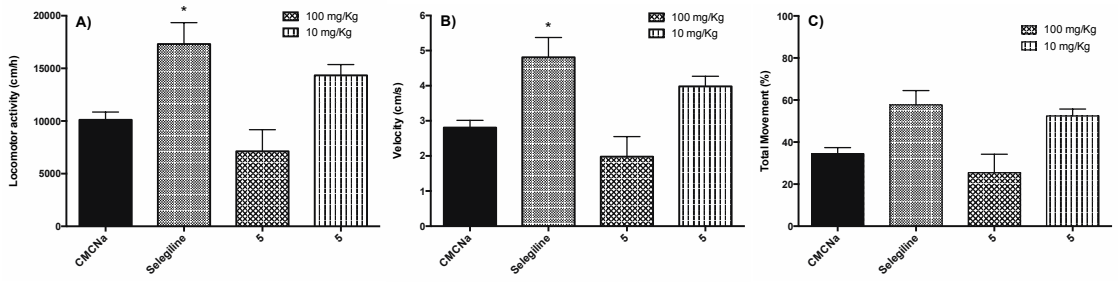


Figure 5. OFT results for non-reserpinized mice. Selegiline (10 mg/kg) and compound **5** (10 or 100 mg/Kg). **(A)** Locomotor activity in centimeters (cm) for one hour, **(B)** velocity in centimeters per second (cm/s) and **(C)** percentage of time in movement (%).

Once the study was completed, the non-reserpinized mice were kept under observation for five days, after which they were sacrificed. During these days, no mice died, nor visible physical signs of toxicity were observed.

2.9.2 OFT in reserpinized mice

After the first stage of the study, OFT was performed for reserpinized mice. The locomotor activity for the vehicle treated group (CMCNa + CMCNa) was very low *versus* the similar group of non-reserpinized mice.

In this study, the locomotor activity was not increased neither for the animals treated with CMCNa + LD:B, nor for those treated with selegiline + CMCNa, comparing to the vehicle. The difference in the locomotor effects caused exclusively by selegiline, in unpretreated mice (hypermotility) *versus* reserpinized mice (no effect), can be explained by the possible enzymatic inhibition caused by reserpine [25]. However, the combination of selegiline (10 mg/Kg) with LD:B (100:25 mg/Kg) modified the locomotor activity in a statistically significant manner (6,848 cm ± 459.37, $P<0.001$, Figure 6A) compared to the control group (1,703 cm ± 114.24, Figure 6A).

As for the reference drug (selegiline), the administration of compound **5** at 100 mg/Kg, combined with LD:B, increased the locomotor activity in a statistically significant manner (9,201 cm ± 617.21, $P<0.0001$, Figure 6A) comparing to the control group.

In the same way as for the locomotor activity, for animals treated with CMCNa + LD:B and those treated with selegiline + CMCNa, no changes in their average velocity were experienced when compared to those animals treated with the vehicle (Figure 6B). However, the combination of selegiline or compound **5** (100 mg/Kg) with LD:B, increased the velocity of the animals in a statistically significant manner (1.77 cm/s ± 0.12, $P<0.0001$ and 2.56 cm/s ± 0.17, $P<0.0001$, respectively), comparing to the control (0.47 cm/s ± 0.03).

Similarly to both above-mentioned parameters, treatment with only selegiline or LD:B failed in modifying the percentage of time in movement of the animals, comparing to

the control (Figure 6C). However, selegiline combined with LD:B, increased the percentage of time in movement of the animals in a statistically significant manner (25.78% ± 1.73, P<0.0001, Figure 6C), comparing to the control (4.30% ± 0.29, Figure 6C). Also, compound **5**, administered at 100 mg/Kg, combined with LD:B, showed a statistically significant increase in the time in movement (37.04% ± 2.48, P<0.0001) *versus* the control group (Figure 6C).

It should be noted that, in these conditions, the locomotor activity, velocity and percentage of total movement proved to be even higher for compound **5** than for selegiline (Figure 6A-C).

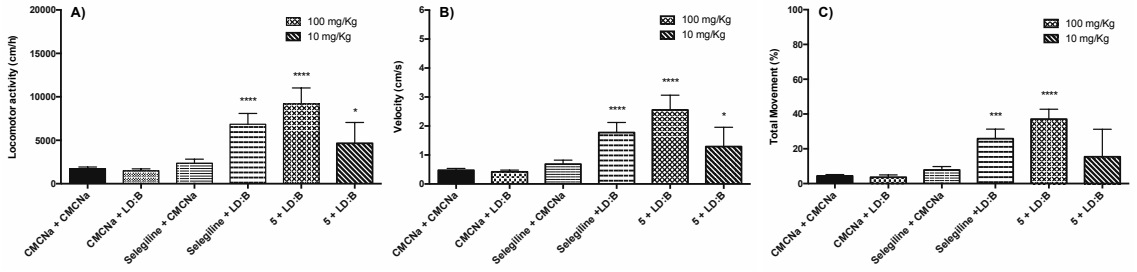


Figure 6. OFT results for reserpinized mice. Selegiline (10 mg/kg) and compound **5** (100 or 10 mg/kg). **(A)** Locomotor activity (cm), **(B)** velocity (cm/s) and **(C)** percentage of time in movement (%).

Compound **5** was also administered at a lower dose (10 mg/kg), in combination with LD:B. As observed in Figure 6, at this dose it also produced a statistically significant increase in the locomotor activity (4,655 cm ± 312.35, P<0.05) and in the velocity of the animals (1.29 cm/s ± 0.09, P<0.05) *versus* the control group. However, when analyzing the percentage of time in movement of the animals, after treatment with compound **5** (10 mg/Kg) and LD:B, it was not observed a statistically significant

increase. In this case, only a tendency to increase this parameter (12.98% ± 0.87), comparing to the control group, was observed (Figure 6C).

3. Conclusion

This report describes the MAO inhibitory activity of 3-thiophenylcoumarin derivatives substituted at positions 7 and/or 8 of the coumarin with hydroxyl groups, observing that the position of the substituents is decisive for their activity and selectivity. 8-Hydroxy-3-(thiophene-3'-yl)coumarin (**1**) is a selective MAO-A inhibitor, while substitutions at position 7 and simultaneously positions 7 and 8, lead to a loss of selectivity. The introduction of a bromine atom at position 4 of the 2-thiophenyl ring gave the most potent *h*MAO-B inhibitor within the series [3-(4'-bromothiophen-2'-yl)-7-hydroxycoumarin, **5**], with higher activity than other 3-thiophenylcoumarins previously described. Also, compound **5**, at doses of 100 and 10 mg/Kg, enhanced the effect produced by the administration of LD:B in reserpinized mice (locomotor activity, velocity and percentage of time in movement). It should be noted that, at a dose of 100 mg/Kg, the motor activity proved to be even higher than for selegiline, the reference compound. 3-Thiophenylcoumarins that have two hydroxyl groups at positions 7 and 8 of the coumarin scaffold (**3** and **6**) also proved to be excellent neuroprotective agents. This highlights the importance of the presence of a catechol group for this activity. Both compounds showed a potent DPPH scavenging activity. Additionally, compound **6** inhibited ROS formation. These properties may be responsible for their neuroprotective activity. As final thoughts, by modulating chemical substituents and their position on the scaffold, it is possible to have selective *h*MAO inhibitors, neuroprotectors or multitarget compounds, all of them with great potential to present the desirable bioavailability properties.

4. Experimental Section

4.1 Chemistry: Synthesis of the new compounds

4.1.1 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 analyzed 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 ($KMnO_4$). ^1H 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).

4.1.2 General procedure for the synthesis of acetoxy-3-thiophenylcoumarins

Compounds were synthesized under anhydrous conditions, using a material previously dried at 60 °C for at least 12 h and at 300 °C for a few minutes immediately before use. A solution containing anhydrous CH_3CO_2K (2.94 mmol), the corresponding

thiophenylacetic acid (1.67 mmol) and the corresponding hydroxysalicylaldehyde (1.67 mmol), in Ac_2O (1.2 mL), was refluxed for 16 h. The reaction mixture was cooled, neutralized with 10% aqueous $NaHCO_3$, and extracted with EtOAc (3 x 30 mL). The organic layers were combined, washed with water, dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure. The product was purified by recrystallization in EtOH and dried in vacuum to afford the desired compound [26,27].

4.1.3 General procedure for the synthesis of hydroxy-3-thiophenylcoumarins (1-6)

Compounds **1-6** were obtained by hydrolysis of their acetoxylated counterparts, respectively. The appropriate acetoxy-3-thiophenylcoumarin, mixed with 2 N aqueous HCl and MeOH, was refluxed for 3 h. The resulting reaction mixture was cooled in an ice-bath and the reaction product and the obtained solid was filtered, washed with cold distilled water, and dried under vacuum, to afford the desired compounds (**1-6**) [26,27].

8-Acetoxy-3-(thiophen-3'-yl)coumarin (precursor of 1). Yield 79%. ^{1}H NMR ($CDCl_3$) δ: 2.42 (s, 3H, CH_3), 7.23-7.26 (m, 2H, H-6, H-7), 7.35-7.39 (m, 2H, H-5, H-5'), 7.48 (dd, 1H, *J*=5.1, 1.3, H-4'), 7.89 (s, 1H, H-4), 8.15 (dd, 1H, *J*=2.9, 1.3, H-2'). ^{13}C NMR ($CDCl_3$) δ: 20.6, 120.4, 120.7, 124.1, 124.2, 124.4, 125.1, 125.7, 126.1, 126.3, 134.0, 134.3, 136.6, 158,7, 168.6. MS *m/z* (%): 286.0 (M^+, 28). Anal. Elem. Calcd. for $C_{15}H_{10}O_4S$: C, 62.93; H, 3.52. Found: C, 62.90; H, 3.48.

8-Hydroxy-3-(thiophen-3'-yl)coumarin (1). Yield 88%. ^{1}H NMR (DMSO-d_6) δ: 7.04-7.17 (m, 3H, H-5', H-6, H-7), 7.62-7.71 (m, 2H, H-2', H-4'), 8.24 (dd, 1H, *J*=7.5, 1.6, H-5), 8.43 (s, 1H, H-4), 10.25 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) δ: 117.9, 118.0, 118.5, 118.6, 124.6, 124.8, 125.7, 126.4, 127.0, 139.0, 142.9, 144.5, 153.1. MS *m/z* (%):

244.0 (M^+, 100). Anal. Elem. Calcd. for $C_{13}H_8O_3S$: C, 63.92; H, 3.30. Found: C, 63.91; H, 3.28.

7-Acetoxy-3-(thiophen-3'-yl)coumarin (precursor of 2). Yield 78%. ^{1}H NMR ($CDCl_3$) δ: 2.30 (s, 3H, CH_3), 7.04-7.09 (m, 2H, H-6, H-8) 7.35-7.49 (m, 3H, H-4', H-5, H-5'), 7.8 (s, 1H, H-2'), 8.14 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 21.0, 100.0, 109.6, 109.7, 118.4, 125.7, 126.0, 126.1, 128.3, 130.6, 134.0, 136.5, 139.8, 162.3, 168.4. MS *m/z* (%): 285.9 (M^+, 27). Anal. Elem. Calc. for $C_{15}H_{10}O_4S$: C, 62.93; H, 3.52. Found: C, 62.88; H, 3.49.

7-Hydroxy-3-(thiophen-3'-yl)coumarin (2). Yield 92%. ^{1}H NMR (DMSO-d_6) δ: 6.76 (bs, 1H, *J*=1.6, H-2'), 6.83 (dd, 1H, *J*=8.5, 2.5, H-6), 7.58 (d, 1H, *J*=8.5, H-5), 7.61-7.66 (m, 2H, H-4', H-5'), 8.14 (d, 1H, *J*=2.5, H-8), 8.39 (s, 1H, H-4), 10.62 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) δ: 101.8, 111.9, 113.6, 113.7, 117.0, 124.3, 126.2, 126.8, 129.9, 139.2, 154.5, 161.3. MS *m/z* (%): 244.0 (M^+, 100). Anal. Elem. Calcd. for $C_{13}H_8O_3S$: C, 63.92; H, 3.30. Found: C, 63.90; H, 3.27.

7,8-Diacetoxy-3-(thiophen-3'-yl)coumarin (precursor of 3). Yield 83%. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.35 (s, 3H, CH_3), 2.44 (s, 3H, CH_3), 7.13 (d, 1H, *J*=8.6, H-6), 7.39-7.51 (m, 3H, H-4', H-5, H-5'), 7.90 (s, 1H, H-2'), 8.17 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 20.2, 20.6, 118.3, 119.0, 122.3, 124.7, 125.8, 126.0, 126.3, 129.8, 133.8, 136.3, 144.6, 145,6, 158.6, 167.3, 167.8. MS *m/z* (%): 343.9 (M^+, 45). Anal. Elem. Calcd. for $C_{17}H_{12}O_6S$: C, 59.30; H, 3.51. Found: C, 59.32; H, 3.54.

7,8-Dihydroxy-3-(thiophen-3'-yl)coumarin (3). Yield 90%. ^{1}H NMR ($CDCl_3$) δ: 6.85 (d, 1H, *J*=8.4, H-6), 7.09 (d, 1H, *J*=8.4, H-5), 7.63 (dd, 1H, *J*=4.9, 2.0, H-5'), 7.67 (dd, 1H, *J*=4.9, 1.1, H-4'), 8.13-8.16 (m, 1H, H-2'), 8.36 (s, 1H, H-4), 9.44 (s, 1H, OH),

10.17 (s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ: 112.7, 113.1, 117.0, 119.2, 124.3, 126.2, 126.9, 132.0, 135.2, 139.8, 149.6, 159.8, 162.8. MS *m/z* (%): 260.0 (M^+, 100). Anal. Elem. Calcd. for $C_{13}H_8O_4S$: C, 59.99; H, 3.10. Found: C, 60.01; H, 3.13.

8-Acetoxy-3-(4'-bromothiophen-2'-yl)coumarin (precursor of 4). Yield 84%. ^{1}H NMR ($CDCl_3$) δ: 2.44 (s, 3H, CH_3), 7.25-7.34 (m, 3H, H-5', H-6, H-7), 7.38-7.46 (m, 1H, H-5), 7.67 (d, 1H, *J*=1.3, H-3'), 7.98 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 25.5, 114.5, 124.6, 125.4, 130.1, 130.6, 131.2, 131.9, 133.6, 141.6, 141.9, 142.2, 149.0, 163.0, 173.5. MS *m/z* (%): 365.8 (M^+, 25), 363.8 (M^+, 24). Anal. Elem. Calcd. for $C_{15}H_9BrO_4S$: C, 49.33; H, 2.48. Found: C, 49.35; H, 2.50.

3-(4'-Bromothiophen-2'-yl)-8-hydroxycoumarin (4). Yield 88%. ^{1}H NMR (DMSO-d_6) δ: 7.11-7.16 (m, 3H, H-5, H-6, H-7), 7.79 (s, 1H, H-5'), 7.88 (s, 1H, H-3'), 8.66 (s, 1H, H-4), 10.37 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) δ: 109.5, 118.5, 118.8, 119.0, 120.2, 125.2, 126.6, 128.1, 137.1, 137.9, 141.1, 144.6, 161.2. MS *m/z* (%): 323.8 (M^+, 100), 321.8 (M^+, 97). Anal. Elem. Calcd. for $C_{13}H_7BrO_3S$: C, 48.32; H, 2.18. Found: C, 48.31; H, 2.16.

7-Acetoxy-3-(4'-bromothiophen-2'-yl)coumarin (precursor of 5). Yield 55%. ^{1}H NMR ($CDCl_3$) δ: 2.35 (s, 3H, CH_3), 7.08-7.11 (m, 1H, H-5), 7.16 (d, 1H, *J*=0.7, H-8), 7.32 (d, 1H, *J*=1.2, H-5'), 7.55 (dd, 1H, *J*=8.4, 0.7, H-6), 7.67 (d, 1H, *J*=1.2, H-3'), 7.97 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 21.1, 109.5, 110.1, 117.2, 119.5, 123.4, 126.2, 126.6, 128.2, 129.6, 136.9, 146. 0, 153.1, 158.9, 168.9. MS *m/z* (%): 365.8 (M^+, 20), 363.8 (M^+, 19). Anal. Elem. Calcd. for $C_{15}H_9BrO_4S$: C, 49.33; H, 2.48. Found: C, 49.34; H, 2.43.

3-(4'-Bromothiophen-2'-yl)-7-hydroxycoumarin (5). Yield 90%. ^{1}H NMR (DMSO-d_6) δ: 6.79 (d, 1H, *J*=2.1, H-8), 6.87 (dd, 1H, *J*=8.5, 2.1, H-6), 7.59 (d, 1H, *J*=8.5, H-5), 7.72 (d, 1H, *J*=1.2, H-5'), 7.77 (d, 1H, *J*=1.2, H-3'), 8.62 (s, 1H, H-4), 10.78 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) δ: 99.8, 102.1, 109.3, 111.7, 114.2, 114.8, 125.3, 126.9, 130.3, 137.7, 138.3, 154.5, 161.9. MS *m/z* (%): 323.9 (M^+, 100), 321.9 (M^+, 97). Anal. Elem. Calcd. for $C_{13}H_7BrO_3S$: C, 48.32; H, 2.18. Found: C, 48.34; H, 2.20.

7,8-Diacetoxy-3-(4'-bromothiophen-2'-yl)coumarin (precursor of 6). Yield 62%. ^{1}H NMR ($CDCl_3$) δ: 2.33 (s, 3H, CH_3), 2.42 (s, 3H, CH_3) 7.15 (d, 1H, *J*=8.7, H-6), 7.33 (d, 1H, *J*=1.3, H-5'), 7.42 (d, 1H, *J*=8.7, H-5), 7.65 (d, 1H, *J*=1.3, H-3'), 8.0 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$) δ: 20.1, 20.5, 101.5, 109.6, 118.2, 119.1, 120.1, 126.1, 126.9, 128.6, 136.7, 137.0, 145.0, 157.9, 158.0, 167.6, 168.2. MS *m/z* (%): 423.8 (M^+, 10), 421.8 (M^+, 9). Anal. Elem. Calcd. for $C_{17}H_{11}BrO_6S$: C, 48.24; H, 2.62. Found: C, 48.26; H, 2.58.

3-(4'-Bromothiophen-2'-yl)-7,8-dihydroxycoumarin (6). Yield 90%. ^{1}H NMR (250 MHz, DMSO-d_6) δ: 6.86 (d, 1H, *J*=8.4, H-6), 7.09 (d, 1H, *J*=8.4, H-5), 7.70 (s, 1H, H-5'), 7.77 (s, 1H, H-3'), 8.57 (s, 1H, H-4), 9.52 (s, 1H, OH), 10.35 (s, 1H, OH). ^{13}C NMR (75.5 MHz, DMSO-d_6) δ: 109.3, 112.5, 113.5, 114.5, 119.6, 125.4, 126.9, 132.1, 132.2, 137.8, 138.9, 150.4, 159.4. MS *m/z* (%): 339.9 (M^+, 100), 337.9 (M^+, 98). Anal. Elem. Calcd. for $C_{13}H_7BrO_4S$: C, 46.04; H, 2.08. Found: C, 46.01; H, 2.11.

4.2 Determination of MAO isoforms *in vitro* activity

The effects of the 3-thiophenylcoumarins on *h*MAO enzymatic activity were evaluated by a fluorimetric method following the experimental protocol previously described by us [**Error! Bookmark not defined.**]. 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 (excitation, 545 nm, 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. 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.

4.3 Reversibility

To evaluate whether the 3-(4'-bromothiophene-2'-yl)-7-hydroxycoumarin (**5**) is a reversible or irreversible *h*MAO-B inhibitor, a dilution method was used [28]. 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.

4.4. 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. Neurobasal medium was supplemented with 2% B-27 to obtain cortex neuronal cultures. Cells were seeded in 96-well plates at a density of 200,000 cells/mL. 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 3-thiophenylcoumarins at 10 μM to evaluate the cytotoxicity and additionally with H_2O_2 (100 μM) in order to evaluate their neuroprotective effect. After incubation for 24 h under the conditions above-described, cell viability was evaluated using MTT as follows: 0.5 mg/mL MTT was added to each well and incubation was performed at 37 °C, for 2 h. Medium was removed, and

formazan salt formed was dissolved in DMSO. The colorimetric determination was performed at 540 nm.

4.5 DPPH radicals scavenging assay

The DPPH was dissolved in methanol (50 μM), and 99 μL of the solution were transferred to each well of a 96-well microplate. Compounds **1**-**6** or vitamin C, used as control, were added to each well at a final concentration of 100 μM. Solutions were incubated at room temperature for 30 min. The changes in color were read at 517 nm using a microplate reader (Fluo-star Optima™, BMG LABTECH, Offenburg, Germany). DPPH radical solution in methanol was used as control, whereas a mixture of methanol and sample served as blank. The scavenging activity percentage (AA%) was determined according to the equation described by Mensor *et al*. [29]: $AA\% = 100 - [(Abs_{sample} - Abs_{blank})x100/Abs_{control}]$

4.6 Inhibition of ROS formation

SH-SY5Y cells (ATCC® CRL-2266™, American Type Culture Collection, Virginia, US.) were cultured in Nutrient Mixture F-12 Ham (Ham's F12) and Minimum Essential Medium Eagle (EMEM) supplemented with 15% FBS, 1% L-Glutamine 1% NEAA and 100 U/mL penicillin-G and 100 μg/mL streptomycin and grown at 37 °C in humidified air containing 5% CO_2.

SH-SY5Y cells were grown at a density of $20x10^3$ cel/mL in flat-bottom, transparent 96-well black plates (Corning® 96 Well Black Polystyrene Microplate, Costar), treated with compound **6** or plumbagin (10 μM concentration, DMSO 1%) for 24 h under standard conditions (37 °C, 5% CO_2). After 24 h, the medium was removed, and the cells were washed with PBS. 100 μL of medium was added with the cDCFDA probe (5

μM) and the cells were again incubated for 30 min under standard conditions (37 °C, 5% CO_2). At the end of this time, the medium containing the probe was removed and the cells were washed with PBS. 100 μL of Hank's were added to each well and the cells were again treated with compound **6** or plumbagin (10 μM). Subsequently, H_2O_2 was added as a pro-oxidant agent and then, the increase in fluorescence produced during a period of 2 h was determined in 5 min intervals (λ excitation 485 nm, λ emission 520 nm) at 37 °C in a fluorescence reader plate (Fluo-star OptimaTM, BMG LABTECH) [30].

ROS were determined by calculating the area under the curve (AUC) obtained by representing the fluorescence generated (Y axis) *versus* time (X axis) for the different treatments. Each experiment was performed in duplicate. In the bar graph, the different treatment groups were represented on the abscissa axis and the percentage of AUC for each of the groups versus the AUC corresponding to the control (100%) on the ordinate axis. The results represent the mean ± standard error of the mean (S.E.M.) of at least 3 experiments (n=3). Statistically significant differences were calculated using the Dunnett's test.

4.7 *In vivo* studies

4.7.1 Animals

Swiss male mice with a weight of 25 ± 5 g were used. The stabling, handling and the different experimental techniques, were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of September 22, on the protection of animals used for scientific purposes, and the Royal Decree 53/2013 of February 1, which establishes the basic rules applicable to the protection of animals

used in experimentation and other scientific purposes, including teaching and the Guide for the care and use of animals Laboratory developed by AAALAC International (Association for Assessment and Accreditation of Laboratory Animal Care International, International Association for the Evaluation and Accreditation of Laboratory Animal Care). The experiments were carried out following the procedure informed with Registration Code 15007AE/09/INV MED 02/NER02/JAFG4, authorized by the Consellería do Medio Rural - Autonomous Government of Galicia (Xunta de Galicia). The mice were housed in standard Makrolon cages without any restriction on access to food and water, except during the experiment. Prior to conducting the experiments, the animals were acclimatized, for a minimum of 72 h, to the environmental conditions in a silent, thermostatted chamber (22 ± 1 °C), with a 12-h light/dark cycle (08:00 h–20:00 h) and with humidity controlled (45–65%). The experiments were always carried out every day at the same hour in order to avoid alterations due to the circadian cycles. The mice were used only once to avoid alterations in the response due to tolerance or learning phenomena.

4.7.2 Equipment

The evaluation of the motor activity in mice was performed using the Open Field Test (OFT). For the experiment, the animals were placed in a black square box (1x1x0.30 m^3), subdivided into 4 independent areas (0.50x0.50x0.30 m^3) where each animal was placed independently. The evaluation of the OFT was carried out from a room adjacent to the one containing the animal, using a video registration system. The behavior of the animals was captured with an analog camcorder (Sony DXC-107A, Sony Corporation, Japan) suspended on the ceiling. The camera is connected to an adapter (Sony CMA D2) that sends the signal to a monitor (Sony PVM-14M2E) and to two digitizing cards:

i) an internal one located in a PCI slot of the computer (Picolo frame graber, Euresys, Liege, Belgium); ii) an external one with USB connection (DVC-USB, Dazzle, USA). The direct signal of the Picolo card is used by the video-computerized animal observation system (EthoVision V. 3.1.16, Noldus Information Technology, Wageningen, The Netherlands). The EthoVision software locates the center of the animal, stores the data and allows further analysis.

4.7.3 Protocol

Reserpinized mice were used as a PD model. Non- reserpinized mice were used as a comparative model. A suspension of 1% carboxymethylcellulose sodium (CMCNa) was used as control, while selegiline was used as MAO-B drug baseline. Compound **5** was tested at doses of 10 and 100 mg/kg, in a suspension of CMCNa 1%. The minimum number of animals used in each group was 8 (n=8). Four independent groups ($n \geq 8$) of unpretreated mice, the vehicle (CMCNa 1%, ip), the reference drug (selegiline, 10 mg/kg, ip) or compound **5** (100 or 10 mg/kg, ip) in a volume/animal weight ratio of 10 mL/kg, were administered. The pharmacological PD model consisted in the administration of reserpine (1.25 mg/kg, ip) to the animals 22 h before the experiment. Afterwards, six independent groups were established: two groups were treated with the vehicle (CMCNa 1%, ip); two groups were treated with selegiline (10 mg/kg); two groups were treated with compound **5** (100 mg/kg or 10 mg/kg, ip). After 30 min, a mixture of LD:B (100:25 mg/kg, ip) was injected to four of the above mentioned groups (CMCNa, selegiline or **5**, at both doses). The other two groups were administered again with the vehicle. Half an hour after the last administration started the evaluation of the motor activity for a period of one hour. The parameters evaluated were: Locomotor activity (cm/h), velocity (cm/s) and percentage of time in movement (%). The graphical

representation and statistical analysis were performed using GraphPad Prism (V 6.0, San Diego, USA). Statistically significant differences were determined by one-way ANOVA followed by the Dunnett test.

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REFERENCES

[1] Poewe, W.; Seppi, K.; Tanner, C. M.; Halliday, G. M.; Brundin, P.; Volkmann, J.; Schrag, A.-E.; Lang, A. E. Parkinson disease, Nat. Rev. Dis. Prim. 3 (2017) 17013.

[2] Heinzel, S.; Berg, D.; Gasser, T.; Chen, H.; Yao, C.; Postuma, R. B. MDS Task Force on the Definition of Parkinson's Disease, Update of the MDS research criteria for prodromal Parkinson's disease, Mov. Disord. 34(10) (2019) 1464–1470.

[3] Bandres-Ciga, S.; Diez-Fairen, M.; Kim, J. J.; Singleton, A. B. Genetics of Parkinson's disease: An introspection of its journey towards precision medicine., Neurobiol. Dis. 137 (2020) 104782.

[4] Dorsey, E. R.; Sherer, T.; Okun, M. S.; Bloem, B. R. The emerging evidence of the Parkinson pandemic, J. Parkinson Dis. 8(s1) (2018) S3–S8.

[5] Finberg, J. P. M. The discovery and development of rasagiline as a new anti□Parkinson medication, J. Neural Transm. 127 (2020) 125–130.

[6] Laine, K.; Anttila, M.; Huupponen, R.; Maki-IkolaIkola, O.; Heinonen, E. Multiple-dose pharmacokinetics of selegiline and desmethylselegiline suggest saturable tissue binding, Clin. Neuropharmacol. 23(1) (2000) 22–27.

[7] Carrera, I.; Cacabelos, R. Current drugs and potential future neuroprotective compounds for Parkinson's, Dis. Curr. Neuropharmacol. 17 (2019) 295–306.

[8] Aguirre, P.; García-Beltrán, O.; Tapia, V.; Muñoz, Y.; Cassels, B. K.; Núñez, M. T. Neuroprotective effect of a new 7,8-dihydroxycoumarin-based Fe2+/Cu2+ chelator in cell and animal models of Parkinson's disease. ACS Chem. Neurosci. 8(1) (2017) 178–185.

[9] Elmabruk, A.; Das, B.; Yedlapudi, D.; Xu, L.; Antonio, T.; Reith, M. E. A.; Dutta, A. K. Design, synthesis, and pharmacological characterization of carbazole based dopamine agonists as potential symptomatic and neuroprotective therapeutic agents for Parkinson's disease. ACS Chem Neurosci. 10(1) (2019) 396–411.

[10] Blair, H. A.; Dhillon, S. Safinamide: A review in Parkinson's Disease, CNS Drugs. 31(2) (2017) 169–176.

[11] Matos, M. J.; Rodríguez-Enríquez, F.; Vilar, S.; Santana, L.; Uriarte, E.; Hripcsak, G.; Estrada, M.; Rodríguez-Franco, M. I.; Viña, D. Potent and selective MAO-B inhibitory activity: Amino- versus nitro-3-arylcoumarin derivatives, Bioorg. Med. Chem. Lett. 25 (2015) 642–648.

[12] Matos, M. J.; Vilar, S.; García-Morales, V.; Tatonetti, N. P.; Uriarte, E.; Santana, L.; Viña, D. Insights into functional and structural properties of 3-arylcoumarins as an interesting scaffold in MAO-B inhibition. ChemMedChem 9 (2014) 1488–1500.

[13] Ferino, G.; Cadoni, E.; Matos, M. J.; Quezada, E.; Uriarte, E.; Santana, L.; Vilar, S.; Tatonetti, N. P.; Yáñez, M.; Viña, D.; Picciau, C.; Serra, S.; Delogu, G. MAO inhibitory activity of 2-arylbenzofurans *versus* 3-arylcoumarins: Synthesis, *in vitro* study and docking calculations, ChemMedChem 8 (2013) 956–966.

[14] Viña, D.; Matos, M. J.; Ferino, G.; Cadoni, E.; Laguna, R.; Borges, F.; Uriarte, E.; Santana, L. 8-Substituted-3-arylcoumarins as potent and selective MAO-B inhibitors: Synthesis, pharmacological evaluation and docking studies, ChemMedChem 7 (2012) 464–470.

[15] Matos, M. J.; Viña, D.; Janeiro, P.; Borges, F.; Santana, L.; Uriarte, E. New halogenated 3-phenylcoumarins as potent and selective MAO-B inhibitors, Bioorg. Med. Chem. Lett. 20 (2010) 5157–5160.

[16] Matos, M. J.; Viña, D.; Picciau, C.; Orallo, F.; Santana, L.; Uriarte, E. Synthesis and evaluation of 6-methyl-3-phenylcoumarins as potent and selective MAO-B inhibitors, Bioorg. Med. Chem. Lett. 19 (2009) 5053–5055.

[17] Matos, M. J.; Viña, D.; Quezada, E.; Picciau, C.; Delogu, G.; Orallo, F.; Santana, L.; Uriarte, E. A new series of 3-phenylcoumarins as potent and selective MAO-B inhibitors, Bioorg. Med. Chem. Lett. 19 (2009) 3268–3270.

[18] Matos, M. J.; Terán, C.; Pérez-Castillo, Y.; Uriarte, E.; Santana, L.; Viña, D. Synthesis and study of a series of 3-arylcoumarins as potent and selective monoamine oxidase B inhibitors, J. Med. Chem. 54(20) (2011) 7127–7137.

[19] Matos, M. J.; Pérez-Cruz, F.; Vazquez-Rodriguez, S.; Uriarte, E.; Santana, L.; Borges, F.; Olea-Azar, C. Remarkable antioxidant properties of a series of hydroxy-3-arylcoumarins. Bioorg. Med. Chem. 21 (2013) 3900–3906.

[20] Robinson, S. J.; Petzer, J. P.; Petzer, A.; Bergh, J. J.; Lourens, A. C. Selected furanochalcones as inhibitors of monoamine oxidase. Bioorg. Med. Chem. Lett. 23(17) (2013) 4985–4989.

[21] Matos, M. J.; Herrera Ibatá, D. M.; Uriarte, E.; Viña, D. Coumarin-rasagiline hybrids as potent and selective hMAO-B inhibitors, antioxidants and neuroprotective agents, ChemMedChem 15 (2020) 532–538.

[22] Yáñez, M.; Matías-Giui, J.; Arranz-Tagarro; J.-A., Galán, L.; Viña, D.; Gómez-Pinedo, U.; Vela, A.; Guerrero, A.; Martínez-Vila, E.; García, A. G. The neuroprotection exerted by memntine, minocycline and litium, against neurotoxicity of CSF from patients with amyotrophic lateral sclerosis, is antagonized by riluzole. Neurodegner. Dis. 13 (2014) 171–179.

[23] Duty, S.; Jenner, P. Animal models of Parkinson's disease: A source of novel treatments and clues to the cause of the disease. Br. J. Pharmacol. 164(4) (2011) 1357–1391.

[24] Engberg, G.; Elebring, T.; Nissbrandt, H. Deprenyl (selegiline), a selective MAO-B inhibitor with active metabolites; effects on locomotor activity, dopaminergic neurotransmission and firing rate of nigral dopamine neurons. J. Pharmacol. Exp. Ther. 259 (1991) 841–847.

[25] Englund, G.; Lundquist, P.; Skogastierna, C.; Johansson, J.; Hoogstraate, J.; Afzelius, L.; Andersson, T. B.; Projean, D. Cytochrome P450 inhibitory properties of common efflux transporter inhibitors. Drug Metab. Dispos. 42 (2014) 441–447.

[26] Matos, M. J.; Varela, C.; Vilar, S.; Hripcsak, G.; Borges, F.; Santana, L.; Uriarte, E.; Fais, A.; Di Petrillo, A.; Pintus, F.; Era, B. Design and discovery of tyrosinase inhibitors based on a coumarin scaffold, RSC Adv. 5 (2015) 94227–94233.

[27] Pintus, F.; Matos, M. J.; Vilar, S.; Hripcsak, G.; Varela, C.; Uriarte, E.; Santana, L.; Borges, F.; Medda, R.; Di Petrillo, A.; Era, B.; Fais, A. New insights into highly potent tyrosinase inhibitors based on 3-heteroarylcoumarins: Anti-melanogenesis and antioxidant activities, and computational molecular modeling studies, Bioorg. Med. Chem. 25 (2017) 1687–1695.

[28] Robinson, S. J.; Petzer, J. P.; Petzer, A.; Bergh, J. J.; Lourens, A. C. Selected furanochalcones as inhibitors of monoamine oxidase, Bioorg. Med. Chem. Lett. 23(17) (2013) 4985–4989.

[29] Mensor, L. L.; Menezes, F. S.; Leitão, G. G.; Reis, A. S.; dos Santos, T. C.; Coube, C. S.; Leitão, S. G. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother. Res. 15 (2001) 127–130.

[30] Armstrong, D. Advanced protocols in oxidative stress III. Adv. Protoc. Oxidative Stress III. 594 (2014) 1–477.