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Brief Article

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Coumarin *versus* chromone monoamine oxidase B inhibitors: Quo vadis?

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KEYWORDS: benzopyrone; coumarin; chromone; monoamine oxidase B; mechanism; docking simulation

ABSTRACT: Due to the lack of significant disease-modifying drugs for neurodegenerative disorders, a pressing need for new chemical entities endowed with IMAO-B still exists. Within this framework, and for the first time, a study was performed to compare coumarin- and chomone-3-phenylcarboxamide scaffolds. Compounds **10a** and **10b** were the most potent, selective and reversible non-competitive IMAO-B. The benzopyrone sp^2 oxygen atom was found to be position independent and a productive contributor for the ligand–enzyme complex stability.

INTRODUCTION

According to epidemiological studies, Parkinson's (PD) and Alzheimer's diseases (AD) are the most prevalent neurodegenerative disorders, representing a heavy burden on the patient, family, caregivers and society. Unfortunately, the available therapies are only palliative, which makes the design and development of new disease-modifying drugs an unmet clinical need.[1,2] Given the establishment of dopaminergic loss as a pathological hallmark of PD, the therapeutic strategies until now have been focused on boosting the levels of dopamine in the brain. Monoamine oxidase B (MAO-B), a flavin monoamine oxidase located in the outer mitochondrial membrane, is a pharmacological target for the treatment of PD, since its inhibition enhances DA levels.[3] Selective MAO-B inhibitors (IMAO-B) are generally a valid asset in early PD therapy in combination with L-DOPA. The first selective and irreversible IMAO-B introduced in the market were selegiline (L-deprenyl) and rasagiline. Safinamide, a reversible IMAO-B, was recently introduced. However, due to the lack of significant disease modifying properties of these drugs, a pressing need for new chemical entities endowed with selective and reversible IMAO-B still exists. Heterocycles play a key role in the discovery of new pharmacologically active compounds.[4] Benzopyrones, namely coumarin (α-benzopyrone) and chromone (γ-benzopyrone) (Figure **1A**), are currently considered as valid templates for the design and development of new chemical entities.[5–7]

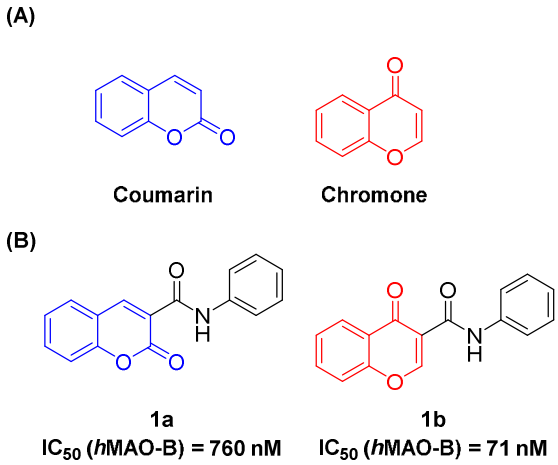


Figure 1A. Coumarin (2H-benzopyran-2-one) and chromone (4H-benzopyran-4-one) scaffolds; **B**. Coumarin-3-phenylcarboxamide (**1a**) and chromone-3-phenylcarboxamide (**1b**) IMAO-B.[8,9]

Coumarins and chromones are ubiquitous in nature, and have relevant pharmacological activities such as anti-inflammatory, antioxidant, cardioprotective and antimicrobial properties.[10–12] Our research group has shown that coumarin-3-phenylcarboxamide (**1a**) and chromone-3-phenylcarboxamide (**1b**) are privileged structures for the rational discovery and development of new IMAO-B.[8,9]

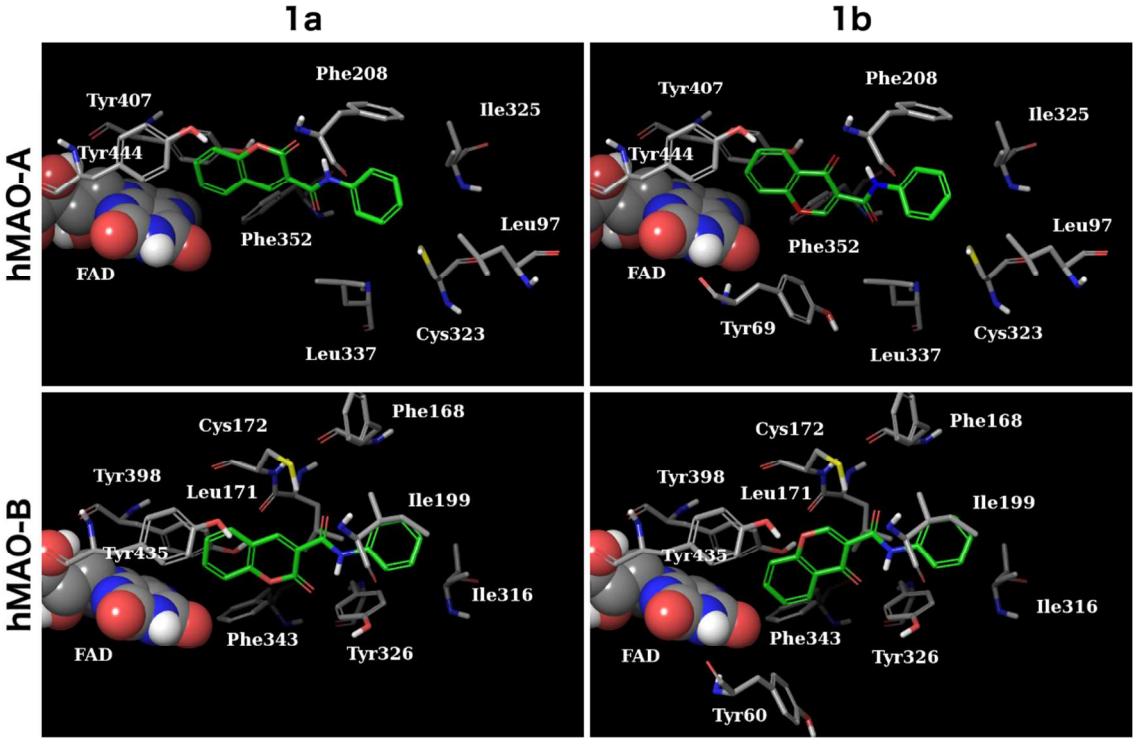


Figure 2. Best docking poses of compounds **1a** and **1b** into the hMAOs active sites. The most relevant interacting residues are depicted in polytube CPK colored, FAD cofactor is shown as spacefill and ligands as green carbons polytube.

Focusing on the lead optimization, and guided by the data obtained so far, we performed a comprehensive structure-activity relationship (SAR) study between the two structural isomers. Firstly, target recognition of compounds **1a** and **1b** (Figure **1B**) was investigated at the molecular level by means of docking simulations. Then, the benzopyrane-3-phenylcarboxamide scaffolds were derivatized by introducing substituents on the benzopyrane and exocyclic aromatic nucleus. A small library of new coumarin (**8a-21a**) (Scheme **1A**) and chromone (**8b-21b**) (Scheme **1B**) derivatives was synthesized and screened for MAO inhibition. The druglike properties, kinetics, and mechanism of enzymatic inhibition were evaluated for the best IMAO-B. Pan assay interference properties (PAINS) of the compounds under study were theoretically investigated.

RESULTS AND DISCUSSION

The first studies on the ligand-target recognition were performed with compounds **1a** and **1b** (Figure **2**). The results clearly indicated that **1a** and **1b** interact almost equally with both MAOs (Supporting information, Table S1). The docking geometries graphical examination showed that **1a** and **1b** interacted with the active site of *h*MAO-A by positioning the benzopyrone moiety towards the FAD cofactor, whereas the anilide ring was located at the entrance gorge. The benzopyrone and exocyclic aromatic rings played a main role in the stabilization of the complexes. In fact, the benzopyrone cores of **1a** and **1b** were involved in stacking to Tyr444 and Phe208 side chains, respectively. Other productive interactions included weak hydrophobic contacts with active site residues. Notably, both inhibitors highlighted unfavorable electrostatic repulsion between the benzopyrone ring and Asn181 side chain. On the other hand, the most stable **1a** and **1b** poses into the hMAO-B active site were similar to those previously reported, while head-tail orientations were observed in less stabilised poses only. A remarkable stabilizing contribution was due to a hydrogen bond (HB) between the ligands' oxygen atoms and the Cys172 side chain. For both compounds, the best poses involved the anilide sp^2 oxygen whereas in less stable complexes such an interaction occurred by means of HB acceptor atoms in the benzopyrone ring. Since the other complexes stabilizing contribution were equivalent to the *h*MAO-A ones, and taking into account that *h*MAO-B Cys172 is replaced by Asn181 in *h*MAO-A, we could attribute the experimentally observed *h*MAO-B selectivity to the ligands-Cys172 hydrogen bonding (Figure **2**). Additionally, the **1a** and **1b** binding modes analysis confirmed that no relevant differences can be observed between coumarin and chromone scaffolds with respect to the recognition of the same target, and this was particularly evident as these structures played a key role in isoform selectivity.

The role of the benzopyrane-3-phenylcarboxamide scaffolds was then investigated by the introduction of substituents on the benzopyrane ring and on the exocyclic aromatic nucleus. The rational design strategy (Figure **3**) was mainly focused on the study of a) the effect of different substituents (R_1 = CH_3; OCH_3) on the benzopyrone ring; b) the effect of different electron donating or withdrawing substituents (R_2 = H, CH_3, Cl, OH) located at *meta* and *para* positions (the most relevant positions found in previous studies[13]) on the exocyclic aromatic ring, and on c) the assessment of the impact of the position of the carbonyl group on the benzopyrone isomeric structures on MAO activity.

Both systems preserve the carboxamide spacer located at 3-position of the pyrone ring (Figure **3**).

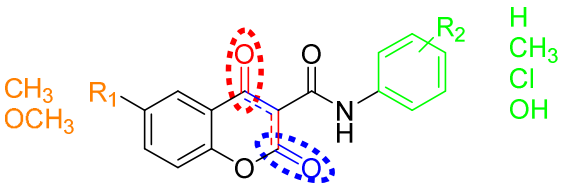


Figure 3. Rational design strategy followed in this work

The coumarin derivatives (Scheme **1A**) were synthesized starting from the appropriate salicylaldehyde to yield coumarin-3-carboxylates (**4a** or **5a**), which after hydrolysis yielded the corresponding coumarin carboxylic acids **6a** and **7a**.[14] The coumarin-3-carboxamide derivatives **8a-21a** were synthesized via EDC-induced coupling of **6a** and **7a** with the appropriate phenylamines (see supporting information).[15]

The chromone-based compounds (Scheme **1B**) were synthesized starting from the appropriate acetophenone to yield chromon-3-carbaldehydes (**4b** or **5b**).[16] Afterwards, the oxidation of the formyl group with sodium chlorite yielded the chromone carboxylic acids **6b** and **7b**.[17] The chromone-3-carboxamide derivatives **8b-21b** were obtained through a reaction that encompassed the *in situ* generation of an acyl chloride intermediate, and the subsequent addition of the appropriate phenylamine.[13]

The *h*MAO-A and -B *in vitro* inhibition (IC_{50}) and selectivity, expressed as SI ([IC_{50} (MAO-A)]/[IC_{50} (MAO-B)]), of the compounds under study and standard drugs (clorgyline for MAO-A and (R)-(−)-deprenyl, rasagiline, safinamide for MAO-B), are shown in Table **1.**

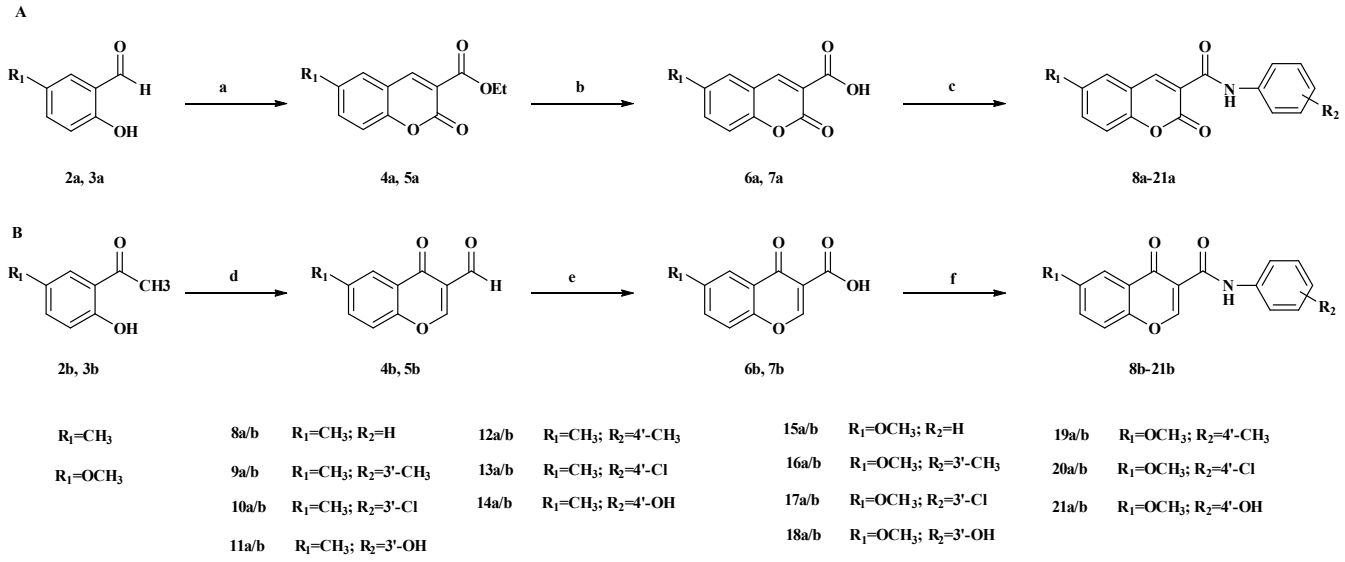
Following the previous studies on coumarin-[18] and chromone-based[13] compounds as MAO inhibitors, and to understand the effect of substituents located at similar positions on the isomeric scaffolds, a SAR study was performed.

The introduction of 6-methyl or 6-methoxy substituents at the benzopyrone-3-phenylcarboxamide scaffolds generally resulted in potent and selective IMAO-B (Table **1**). Nevertheless, 6-methylcoumarin derivatives (**8a-14a**) displayed a higher potency than the 6-methoxy analogues (**15a-21a**). With the exception of compound **8b** (no substituents on the chromone exocyclic ring), the introduction of a 6-methyl (compounds **8b-14b**) or a 6-methoxy (compounds **15a-21a**) substituent on the chromone scaffold had no noteworthy influence on MAO-B inhibitory activity.

For both series, a higher potency was observed for derivatives bearing *meta* substituents on the exocyclic ring. Interestingly, the most active compounds of both series were compounds **10a** (IC_{50} = 5.07 nM), **10b** (IC_{50} = 4.2 nM) and **17b** (IC_{50} = 3.94 nM), all bearing a *m*-chlorine substituent. The introduction of *para* substituents resulted in a decrease of activity, apart from compound **19a** (IC_{50} = 19.43 nM), bearing a *p*-methyl group when compared to compound **16a** (IC_{50} = 47.24 nM) with a *m*-methyl group. The presence of a hydroxyl group either in *meta* or *para* position resulted in a decrease of activity. Although the benzopyrone carbonyl group is important for MAO-B inhibition, its position on the pyrone ring did not seem relevant (see supporting information). All compounds under study were selective towards MAO-B, since no significant activity was found towards MAO-A (cut-off 10 μM, Table **1**).

The interaction with the active sites of *h*MAO-A and *h*MAO–B for coumarin and chromone-based derivatives **8-21** was then evaluated by means of docking simulation (Supporting information).

All compounds, excluding **10a** with respect to *h*MAO-A, were able to bind both MAO isoforms. Even if a linear correlation between docking score and experimental data was not observed (Supporting information, Table S2), the theoretical descriptor GScore clearly indicated that the complexes between the studied molecules and *h*MAO-B are more stable than the corresponding *h*MAO-A ones. Afterwards, molecular modeling studies were focused into the role of R1 and R2 substituents. Regarding *h*MAO-A, the most stable docking poses suggested a modest role of methyl and methoxy substituents at R1, since they didn't significantly alter the ligand binding modes (see supporting information, Figures S1-S24). In *h*MAO-B, the best poses of **8a** and **8b** reported a quite similar interaction profile when compared to **1a** and **1b**. The most evident difference concerned the opposite configuration of the global minimum **8a** complex with respect to the **1a** one. However, such a change was not observed in higher energy poses. Even the most stable docking models of **15a** matched with the non-substituted compounds. In particular, the positioning of the coumarin scaffold overlapped with **1a**. Furthermore, the methoxy group of **15a** accepted one HB by Tyr188, which led to a greater complex stabilization and enhanced theoretical affinity. Such an interaction could also be addressed to **15b**, even if its complex geometry limited the HB energy contribution. The presence of a methoxy group at R1 allowed **15b** to establish one HB between the benzopyrone carbonyl oxygen and the target's Cys172. The anilide moiety was similarly located when compared to the previously reported for **1b**. Subsequently, we investigated the role of the different chemical nature of substituents at R2 and their position (see supporting information, Figures S25-S40). The top poses for coumarins and chromones with R2 = *m*-methyl, *m*-hydroxyl or *m*-chlorine (**9-11**, **16-18**) showed the benzopyrone moiety facing towards the FAD cofactor, except for **11a**, which remarked the same binding mode of **8a**. Theses derivatives were mainly involved in stacking interactions to Tyr398 and in HB to Cys172. Additionally, regarding *h*MAO-B, no differences were found among higher affinity poses of R2 *para* derivatives with respect to the corresponding *meta* analogues. Since its chlorine substituent was directed toward the FAD cofactor, **17b** represented the only exception to the previous observation.



Scheme 1. A- Synthetic strategy followed for the synthesis of the coumarin derivatives **8a-21a** Reagents and conditions: (a) diethyl malonate, EtOH, piperidine, reflux, overnight; (b) NaOH (0.5% aq./EtOH), reflux, 4h (c) EDC, DMAP, DCM, appropriate phenylamine, 0º to r.t., 4h; **B-** Synthetic strategy followed for the synthesis of chromone-3-phenylcarboxamide **8b-21b**. Reagents and conditions: (d) $POCl_3$, DMF, -10ºC, 15h; (e) H_3NSO_3, $NaClO_2$, 0ºC, 12h (f) $POCl_3$, DMF, appropriate phenylamine, r.t., 1-5h.

Subsequently, the kinetics and binding affinities of the most promising compounds - coumarin **10a** (6-methyl, *m*-chloro) and chromone **10b** (6-methyl; *m*-chloro) - were evaluated on hMAO-B. From the Lineweaver-Burk plots (Figure **4**), we observed that both compounds operated by a non-competitive inhibition mechanism (see supporting information). The enzyme binding affinities, determined as inhibition constants (*Ki*), were calculated from the corresponding Dixon plots. Coumarin **10a** (Figure **4A**) and chromone **10b** (Figure **4B**) displayed *Ki* values of 1.17 and 2.69 nM, respectively. The estimated *Ki* values correlated well with the experimental data (IC_{50}): coumarin **10a** (IC_{50} = 5.07 nM) and chromone **10b** (IC_{50} = 4.20 nM) displayed slightly different IC_{50} and *Ki* values, but both within the low nanomolar range.

Additionally, time-dependent inhibition studies with compounds **10a** and **10b** were performed (see supporting information). After the first 15 minutes of incubation, a continuous decrease on enzymatic residual activity was observed for irreversible IMAO-B ((R)-(-)-Deprenyl and rasagiline, Figure **4A**), while an enhancement on enzymatic activity was observed across the analysis time for safinamide, a reversible IMAO-B (Figure **4A**). For the compounds **10a** and **10b** (Figure **4B**), the binding to the active site was observed in the first 15 minutes, followed by an increase on enzymatic activity over the following 60 minutes, pointing towards MAO-B reversible inhibition.[13]

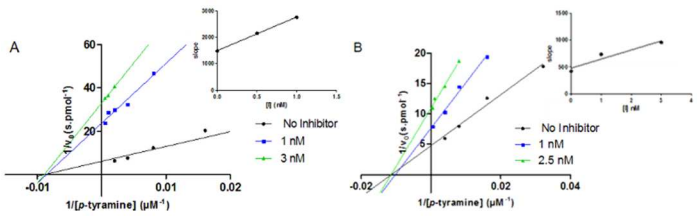


Figure 4. Kinetic studies on the mechanism of *h*MAO-B inhibition by (**A**) coumarin **10a** and (**B**) chromone **10b**. Dixon plots insets on the top right of each graphic.

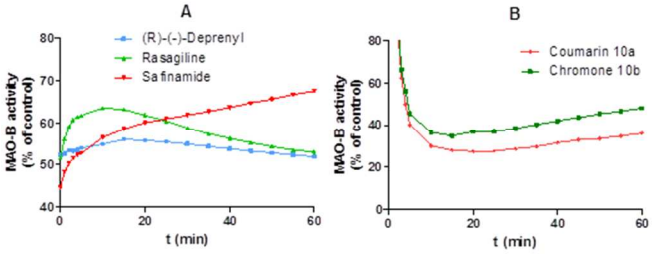


Figure 5. Time-dependent inhibition of recombinant human MAO-B by (**A**) standard drugs (R)-(−)-deprenyl (50 nM), safinamide (40 nM) and rasagiline (200 nM) and (**B**) test compounds **10a** (12.5 nM), and **10b** (12.5 nM). The residual activity was expressed as % of control. Data are the mean ± S.D. of three different experiments.

Table 1. MAO inhibitory activities of benzopyrone derivatives **8a**-**21a** and **8b**-**21b** and standard inhibitors.

Compound	IC_{50} (nM)		SI	Compound	IC_{50} (nM)		SI
	*h*MAO-A	*h*MAO-B			*h*MAO-A	*h*MAO-B	
8a	*	15.32 ± 1.02	>6527[a]	**8b**	*	21.35 ± 1.10	>4684[a]
9a	*	7.52 ± 1.05	>13300[a]	**9b**	*	17.10 ± 1.17	>5848[a]
10a	*	5.07 ± 1.25	>19724[a]	**10b**	*	4.20 ± 1.08	>23810[a]
11a	*	45.40 ± 1.30	>2203[a]	**11b**	*	78.22 ± 1.30	>1278[a]
12a	*	13.90 ± 1.30	>7194[a]	**12b**	*	151.6 ± 5.14	>660[a]
13a	*	11.08 ± 1.20	>9030[a]	**13b**	*	45.42 ± 2.32	>2202[a]
14a	*	621.70 ± 1.8	>161[a]	**14b**	*	512.6 ± 2.81	>195[a]
15a	*	5.95 ± 1.28	>16810[a]	**15b**	*	41.8 ± 2.2	>2392[a]
16a	*	47.24 ± 1.12	>2120[a]	**16b**	*	21.80 ± 1.21	>4590[a]
17a	*	9.03 ± 1.07	>11074[a]	**17b**	*	3.94 ± 1.08	>25381[a]
18a	*	228.6 ± 1.26	>447[a]	**18b**	*	113.5 ± 1.10	>881[a]
19a	*	19.43 ± 1.19	>5150[a]	**19b**	*	210.8 ± 8.1	>474[a]
20a	*	18.90 ± 1.01	>5291[a]	**20b**	*	10.31 ± 1.55	>9699[a]
21a	*	*	n.a.	**21b**	*	674.2 ± 1.72	>148[a]
Deprenyl	68730 ± 421	16.73 ± 1.48	4108	**Safinamide**	*	23.07 ± 2.07	>4335[a]
Rasagiline	52974 ± 742	49.66 ± 2.26	1067	**Clorgyline**	4.46 ± 0.32	*	>0.0446

(*) Inactive at 100 μM; n.a. non-applicable; SI: *h*MAO-B selectivity index = IC_{50}(*h*MAO-A)/IC_{50}(*h*MAO-B). [a] Values obtained under the assumption that the corresponding IC_{50} against MAO-A is 100 μM.

Overall, coumarin and chromone derivatives presented druglike properties similar to those of the standard IMAO-B safinamide (see supporting information, Table S1).[19] All compounds complied with the Lipinski's rule of five[20] and the clogP and tPSA values were within the ideal parameters for oral bioavailability. Furthermore, the blood (plasma)–brain partitioning (log BB) complied with the requirements for BBB permeability (log BB > -1).[19] In general, multiparameter score LLE[21] values ranged from 3 to 5, with the exception of chromone **15b** (LLE = 5.36) and coumarin **17a** (LLE = 5.08).

Taking into account the inhibition and selectivity observed experimentally with respect to MAO isoforms and theoretical results (see supporting information), we are confident that our compounds, in particular the most promising ones, cannot be considered PAINS.

CONCLUSIONS

Since coumarins and chromones are structural isomers, an innovative project was developed involving molecular modelling studies, synthesis of a small library of coumarin (**8a-21a**) and chromone (**8b-21b**) derivatives and screening toward MAO-A and MAO-B inhibition. From the current study, coumarin **10a** (IC_{50} = 5.07 nM) and chromones **10b** (IC_{50} = 4.2 nM) and **17b** (IC_{50} = 3.94 nM) were identified as the most potent IMAO-B. In both series, the presence of a *m*-chlorine substituent was favourable. Compounds **10a** and **10b** showed drug-like properties and were potent reversible and non-competitive IMAO-B. The data of molecular modeling indicated the existence of a Cys172 HB involving the carboxamide spacer located at position 3 of the pyrone ring. Considering that *h*MAO-B Cys172 is replaced by Asn181 in *h*MAO-A, the experimental *h*MAO-B selectivity data of compounds **10a** and **10b** could be addressed to this feature. Additionally, it was observed that the benzopyrone core, and in particular its sp^2 oxygen atom is a stabilizer of the complexes between our most promising inhibitors and the target. Overall, no relevant differences were observed between coumarin and chromone-3-phenylcarboxamide derivatives with respect to the recognition of the same target.

EXPERIMENTAL SECTION

Starting materials and reagents were obtained from commercial suppliers and were used without further purification (Sigma-Aldrich, Portugal). The purity of the final products (>97% purity) was verified by high-performance liquid chromatography (HPLC) equipped with a UV detector.

Synthesis of coumarin derivatives

Synthesis of ethyl 6-methylcoumarin-3-carboxylate **(4a),** ethyl 6-methoxycoumarin-3-carboxylate **(5a),** 6-methylcoumarin-3-carboxylic acid **(6a)** and 6-methoxycoumarin-3-carboxylic acid **(7a)**

A solution of 5-methylsalicylaldehyde (**2a**) or 5-methoxysalicylaldehyde (**3a**) (1 mmol) and diethyl malonate (1 mmol) in ethanol (10 mL) was stirred at room temperature for 5 min. Pirimidine (15µL) was then added and the reaction was maintained under reflux overnight. Afterwards, it was allowed to cool at room temperature and the resulting suspension was filtered off. The solid was washed with cold ethanol and diethyl ether and recrystallized from ethanol. Then, to a solution of coumpound **4a** or **5a** (2 mmol) in ethanol (25 mL), an aqueous solution of NaOH (0.5%, 5mL) was added. The mixture was kept under reflux for 1h. Afterwards, an aqueous solution of HCl (10%) was added dropwise until pH around 2. The resulting solid was filtered, washed with water and recrystallized from ethanol.

Synthesis of coumarin derivatives (**8a-21a)**

To a solution of the appropriate coumarin-3-carboxylic acid (**6a** or **7a**, 1 mmol) in dichloromethane (DCM) (5 mL), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (1.10 mmol) and 4-dimethylaminopyridine (DMAP) (1.10 mmol) were added. The mixture was kept with a flux of argon at 0ºC for ten minutes. Shortly after, the aromatic amine (1 mmol) with the intended substitution pattern was added in small portions. The reaction mixture was stirred at room temperature. The solid obtained was filtered and purified by column chromatography (hexane/ethyl acetate 9:1) or by recrystallization from ethanol to give the desired products **8a-21a**.

Synthesis of chromone derivatives

Synthesis of 6-methylchromone-3-carbaldehyde **(4b),** 6-methoxychromone-3-carbaldehyde **(5b),** 6-methylchromone-3-carboxylic acid **(6b)** and 6-methoxychromone-3-carboxylic acid **(7b)**

A solution of 5-methyl-2-hydroxyacetophenone (**2b**), or 5′-methoxy-2′-hydroxyacetophenone (**3b**) (6 mmol), in anhydrous *N,N*-dimethylformamide (12 mL) was stirred at a temperature of -10ºC for 30 minutes. Phosphoryl chloride ($POCl_3$) (12 mmol) was added dropwise at a temperature below -10ºC during 1 hour. The mixture was stirred at room temperature for 15 h and poured into water (40 mL).[29] The product was filtered and washed with ethyl ether. Then, asolution of sodium chlorite ($NaClO_2$) (80 %, 32 mmol) in water (25 mL) was added dropwise to a mixture of 6-methyl-4-oxo-*4H*-chromene-3-carbaldehyde (**4b**) or 6-methoxy-4-oxo-*4H*-chromene-3-carbaldehyde (**5b**) (8 mmol) in dicloromethane (50 mL) and sulfamic acid (NH_2SO_3H) (40 mmol) in water (50 mL) at 0 ºC. After 15 hours, the reaction was extracted with dicloromethane.[30] The combined organic phases were dried over Na_2SO_4, filtered and evaporated. The product was finally washed with ethyl ether.

Synthesis of chromone derivatives (**8b-21b)**

$POCl_3$ (2.6 mmol) was added to a solution of the correspondent chromone-3-carboxylic acid (**6b** or **7b**, 2.6 mmol) in DMF (4 mL). The mixture was stirred at room temperature for 30 min for the *in situ* formation of the acyl chloride. Then, the aromatic amine with the desired aromatic pattern (for compounds **8b-21b**) was added. After 1-5 hours, the mixture was diluted with dichloromethane (20 mL), washed with H_2O (2 x 10 mL) and with saturated $NaHCO_3$ solution (2 x 10 mL). The organic phases were combined, dried, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography and/or crystallization.

Pharmacology. The evaluation of the monoamine oxidase inhibitory activity of the compounds under study on both *h*MAO isoforms was assessed following a previously described method.[13]

To evaluate the mechanism of *h*MAO-B inhibition of coumarin **10a** and chromone **10b**, substrate-dependent kinetic experiments and time-dependent reversibility studies were performed following a previously described method.[13] PAINS studies details were included in the supporting information.

ASSOCIATED CONTENT

Supporting Information. Chemistry: Detailed synthesis and compound characterization; Pharmacology: Detailed biological assays, inhibitory activity, kinetic and reversibility assays; Theoretical drug-like properties determination; Molecular modelling: Detailed procedures, docking scores and binding poses od all derivatives; Molecular formula strings.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ¥ Authors contribute equally.

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ABBREVIATIONS

AD, Alzheimer's Disease, BBB, Blood-Brain Barrier, FAD, Flavin Adenine Dinucleotide, HB, Hydrogen Bond; LLE, Lipophilic Ligand Efficiency; MAO, monoamine oxidase; ND, Neurodegenerative Diseases; PD, Parkinson's Disease; ROS, Reactive Oxygen Species; PAINS, Pan Assay Interference Properties.

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Table of contents graphic